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Mitchell, Matthew David (2012) ***The role of chemical alarm cues in risk assessment and predator recognition in coral reef fishes.*** **PhD thesis, James Cook University.**

The role of chemical alarm cues in risk assessment and predator recognition in coral reef fishes

Thesis submitted by

Matthew David Mitchell BSc (Hons), MSc AppSci

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For the degree of Doctor of Philosophy

School of Marine and Tropical Biology

James Cook University

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Statement on the contribution of others

This thesis includes collaborative work with my supervisors Prof. Mark McCormick and Dr. Maud Ferrari, as well as Prof. Douglas Chivers and Dr. Peter Cowman. As part of these collaborations I was responsible for project design, data collection, analysis and interpretation of data. My co-authors provided technical assistance, intellectual guidance, editorial assistance and financial support. Dr Peter Cowman provided the phylogenetic data used in Chapter 2.

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General Abstract

Through the removal of individuals, predation shapes the distribution and abundance of prey communities. Consequently, how prey species detect and respond to predation threats within their environment will determine survival and future fitness. The mere presence of predators in an environment has a significant effect on the life histories of prey individuals. The use of visual, olfactory and auditory cues allows prey to detect and learn about predators and their associated risks within their environment in a way that allows the development of predator specific antipredator responses. In complex environments, such as coral reefs, olfactory cues are thought to be especially important as increased complexity reduces access to visual cues. There is evidence to suggest that olfactory cues such as chemical alarm cues maybe used by a wide range of coral reef fishes, however there is lack of information regarding the role such cues play in risk perception and predator recognition. This study therefore investigates how coral reef fishes use chemical alarm cues to assess risk and learn to recognise predators and the risk they represent.

To understand how fishes use chemical alarm cues to assess risk, there is a need to understand what cues they are able to detect. How juvenile coral reefs fishes use cues from heterospecific prey guild members is unclear but use of heterospecific cues should enhance risk assessment. **Chapter 2** tested if naïve juvenile fish have an innate recognition of heterospecific alarm cues and whether such recognition arises from phylogenetic conservation of chemical alarm cues. Naïve juvenile *Amphiprion percula* were tested to see if they displayed antipredator responses to chemical alarm cues from four closely related heterospecific species (family Pomacentridae), a distantly related sympatric species (*Asterropteryx semipunctatus*; family Gobidae) and a saltwater control. Juveniles displayed significant reductions in foraging rate when exposed to all four confamilial species and the intensity of the response was strongly correlated to the extent to which species were related to *A. percula*. These findings demonstrate that chemical alarm cues are conserved within the Pomacentridae family, as predicted by the phylogenetic relatedness hypothesis.

In the absence of innate predator recognition prey must learnt to recognise predators in an efficient manner, particularly when entering a novel environment. Predation pressure should therefore selectively promote mechanisms that enable the rapid identification of novel predators. **Chapter 3** tested the ability of a juvenile marine fish, lemon damselfish (*Pomacentrus moluccensis*), to simultaneously learn the identity of multiple previously unknown predators. Individuals were conditioned with a ‘cocktail’ of novel odours (from two predators and two non-predators) paired with either a conspecific alarm cue or a saltwater control and then tested the following day for recognition of the four odours individually and two novel odours (one predator and one non-predator). Individuals conditioned with the ‘cocktail’ and alarm cue responded to the individual ‘cocktail’ odours with an antipredator response but not the controls. These results demonstrate the ability to of juvenile fishes to process multiple sources of information regarding predator identities simultaneously and still recognise predators individually. The ability to rapidly assimilate information regarding predator identities should significantly enhance risk assessment and their chances of survival.

Learnt predator recognition, although potentially costly, provides animals with an adaptive mechanism to rapidly adjust to current levels of predation risk. Prey may reduce the costs associated with learning if they can use information learned about known predators to respond to cues from closely related predators with which they are unfamiliar. **Chapter 4** demonstrated that, in a community where the ability to predict the predatory status novel species is low (i.e. high diversity of closely related predators and non-predators), prey fish generalise predator recognition to novel congeneric species but not confamilial species. *P. moluccensis*, conditioned to recognise the odour of a predatory moon wrasse, *Thalassoma lunare*, as a risky stimulus subsequently displayed antipredator responses not only to *T. lunare* odour, but also to the congeneric *Thalassoma amblycephalum* and *Thalassoma hardwicke* odours. Recognition was not extended to species beyond the genus level. Our results showed that *P. moluccensis* could not distinguish between predators and non-predators when generalising predator recognition. The extent to which prey generalise predator recognition appear to depend on the ability to accurately predict predator identities based on an innate knowledge of the functional diversity within the community to which previous generations have been exposed.

In communities of high biodiversity, the ability to distinguish predators from non-predators is crucial for prey success. Coral reef fishes enter new environments apparently naïve to the identity of both predators and non-predators. The remarkable efficiency of learning using chemical alarm cues means that recognition mistakes may occur if prey inadvertently learn that a species is a predator when it is not. Latent inhibition is a means by which prey that are pre-exposed to an unknown species in the absence of negative reinforcement can learn that the unknown animal is likely not a threat. **Chapter 5** demonstrated that a common coral reef fish, *P. moluccensis*, can learn to recognize a predator as non-threatening through latent inhibition. Furthermore, we showed that we could override the latent inhibition effect by conditioning the prey to recognize the predator numerous times. These results highlight that prey fish are able to correct recognition mistakes by continually updating the information regarding the threat posed by other fishes in their vicinity.

Fishes are exposed to different suits of predators throughout ontogeny and they should therefore respond to and learn about predators using cues that are most relevant to their current situation. **Chapter 6** tested whether juvenile spiny chromis, *Acanthochromis polyacanthus*, could distinguish between chemical alarm cues originating from conspecifics of different ontogenetic stages and whether cue origin affected its efficacy when learning about predators. Juveniles displayed a significant antipredator response to juvenile chemical alarm cues and subsequently only learned to recognise the predator after being conditioned with juveniles alarm cues. Juveniles failed to respond and learn from chemical alarm cues from larger individuals. This demonstrates that prey are highly selective in how they use information about predation risks from conspecifics, responding to and learning from only those cues that are relevant to their current developmental stage.

This study demonstrates the importance of chemical alarm cues for risk assessment by coral reef fishes, particularly at a time when they are highly vulnerable to predation following settlement onto coral reefs. Through the detection of these cues prey fish are able to accurately assess risk, learn about predators in their local environment, and the level of threat that they pose and respond to fluctuations in risk as they change through time. Such flexible risk assessment confirms the predictions of theories on risk assessment by prey. Furthermore, this study provides an insight into the underlying cognitive processes that enable prey fish to make decisions regarding aspects of their life history that will ultimately determine if they survive to reproduce.

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Chapter 1: General Introduction

Predation is the dominant source of mortality for prey individuals and plays a major role in structuring communities and ecosystems (Begon et al. 1986; Lima and Dill 1990; Sih et al 2000). Even the mere presence of a predator (non-consumptive effects) can have significant impact on an individual's behavioural decisions, life history characteristics and even survival (Reyla 2001, 2003: Werner and Peacor 2003: Peckarsky et al. 2008). While there is a need to avoid predators, such tactics are costly as they reduce time and energy available for feeding, reproduction and may alter optimal habitat use (Horat and Semlitsch 1994; Lima 1998; Lind and Cresswell 2005; Brown and Chivers 2006). How individuals deal with predation risks has profound ramifications in determining the balance of processes that structure communities. Therefore, understanding the dynamics of predator-prey interactions is a key component in understanding the processes regulating communities and species diversity (Begon et al. 1986). While much pioneering work into the interplay of predators and their prey has been done in relatively simple systems such as freshwater lakes and ponds, there is a paucity of information regarding such interactions in complex systems, such as tropical coral reefs.

Given the potential cost of predation (i.e. death), prey have developed an elaborate array of defences including behavioural responses (Horat and Semlitsch 1994), morphological defences/deterrents (i.e. increased body depth and spines) (Brönmark and Miner 1992; Stankowhich 2011), crypsis (Broom and Ruxton 2005) and chemical defence (e.g. toxins; Hayes et al. 2009; Skelhorn and Rowe 2009). While these are all effective means of deterring predators, early detection and avoidance are seen as the most effective ways to avoid predation (Kelley and Magurran 2006, Chap 3; Ferrari et al. 2010). Early detection enhances the chance of survival, as it allows prey to assess the potential risks to which they are exposed and can respond accordingly without the need to directly encounter the predator (Lima and Dill 1990; Kelley and Magurran 2006).

Prey must also be able to adapt and respond to predation risk as it changes in its intensity and frequency through time and space (Sih et al. 2000). Those individuals that are better able to recognise risky situations and implement the appropriate antipredator response will enhance their chances of surviving to reproduce (Helfman 1989). Prey are able to assess predation risks within their local environment using a variety of senses, including visual, chemical, electrical and mechanical (including auditory and lateral line). Of these, visual and chemical cues are thought to be the primary senses used in aquatic environments to assess local risk and have consequently received the most attention (Brown and Chivers 2006, Chapter 4). Visual cues are both spatially and temporally reliable, providing accurate information about location (both direction and distance), behaviour and potential levels of risk of a predator. However, not only are visual cues high risk, as the predator must be within visual range for such information to be gained, but they can also be manipulated by the predator and are compromised at night and in highly turbid or complex habitats (Kats and Dill 1998). Conversely, damage-released chemical cues are thought to be more reliable as they are released involuntarily (Brown and Chivers 2006). These damage-released cues elicit an innate fright response in conspecifics and so the products released are commonly called chemical alarm cues. Given that chemical cues are carried over greater distances they are less risky but at the same time their dispersive ability means they are less accurate both spatially and temporally (Kats and Dill 1998). Clearly the value of visual and olfactory cues varies under different circumstances. Indeed, it has been shown that under different habitat conditions prey preferentially use different senses (Hartman and Abrahams 2000).

In freshwater systems, chemical alarm cues have been shown to play a central role in risk assessment and learned predator recognition (see below) for a diverse range of species from a variety of taxa (reviewed by Chivers and Smith 1998; Ferrari et al. 2010). Recently, chemical alarm cues have been shown to be used for risk assessment in two of the most diverse and abundant fish family’s in coral reefs: damselfishes and gobies (Table 1). However, there is a paucity of information regarding how fishes use such cues to assess risk, learn about predators and regulate predator-prey interactions. The need to address this gap in our knowledge of predator-prey interactions has recently been highlighted by findings that show increases in dissolved CO_2 in the shallow oceans resulting from climate change impairs the abilities reef fishes to use olfactory cues during settlement (Munday et al. 2009) and in risk perception (Munday et al. 2010; Ferrari et al. 2011a, b).

Chemical alarm cues and their prevalence in marine systems

Chemical alarm cues have received much attention over the last two decades and are thought to play a central role in the assessment of risk and facilitating learned recognition of novel predator (Chivers and Smith 1998; Wisenden et al. 2004; Brown and Chivers 2006). Found in the epidermis or in specialised cells within the epidermis of aquatic vertebrates (Carreau-Green et al. 2008; Ferrari et al. 2010; Mathuru et al. 2012) chemical alarm cues are released when the epidermis is damaged, typically during a predation event, and therefore provide a honest indication of a predation threat in the local vicinity (Chivers and Smith 1998; Wisenden et al. 2004). The detection of these cues elicits distinct short term antipredator responses in both conspecifics (Smith 1989; McCormick and Larson 2007) and heterospecific (Chivers and Smith 1994) prey guild members, which in turn enhances survival during subsequent predation events (Mirza and Chivers 2001; Ferrari et al. 2010; Lönnstedt et al 2012).

The potential importance of chemical alarm cues in risk assessment is highlighted by the diversity of species found to possess and respond to chemical alarm cues within both freshwater and marine environments, including sponges, sea anemones, flatworms, annelids, molluscs, arthropods, larval insects, crustaceans, echinoderms, amphibians and fishes (reviewed in Chivers and Smith 1998; Ferrari et al 2010). Most of these studies have focused on fishes and in particular freshwater species. Recently, there has been an increase in studies investigating the role of chemical alarm cues in risk perception by coral reef organisms including fishes and invertebrates and a number of species are now known to possess chemical alarm cues (Table 1). Studies have mainly focused on the damselfish family (Pomacentridae) (McCormick and Manassa 2008; Holmes and McCormick 2010, 2011; Ferrari et al. 2011; Lönnstedt and McCormick 2011a, b) but chemical alarm cues have also been report for gobies (Smith 1989; Smith et al. 1991; McCormick and Larson 2007) and some species of sea urchins and corals (Vadas and Elner 2003; Goddard 2006). That chemical alarm cues are used by both gobies and damselfish, two of the most abundant and diverse fish families found on coral reef, suggests that chemical alarm should play a significant role in predator-prey interactions within reef communities.

Table 1.1. Species currently known to respond to and learn using chemical alarm cues in coral reefs.

Species	**Response to conspecific chemical alarm cues**	**Learned predator recognition**
Fishes		
Lemon damsel, *Pomacentrus moluccensis*	Larson 2003	Bosiger et al. 2012
Ambon damsel, *P. amboinensis*	Larson 2003, Holmes and McCormick 2010	Holmes and McCormick 2010, Ferrari et al. 2012
Ward’s damsel, *P. wardi*	Lönnstedt et al. 2012	Lönnstedt et al. 2012
Nagasaki damsel, *P. nagasakiensis*	Ferrari et al. 2011a	
Whitetail damsel, *P. chrysurus*	Ferrari et al. 2011a	
Black-axil chromis, *C. atripectoralis*	Mitchell, unpublished	
Orange clownfish, *Amphiprion percula*	Mitchell, unpublished	
Cinnamon clownfish, *A. melanopus*	Mitchell, unpublished	
Spiny chromis, *Acanthochromis polyacanthus*	Leahy et al. 2011	Manassa and McCormick 2012

Starry goby, *Asterropteryx semipunctatus*	Smith 1989
Eye-bar goby, *Gnatholepis anjerensis*	Smith 1989
Echinoderms	
Sea egg, *Tripneustes ventricosus*	Vades and Elner 2003

The role of heterospecific alarm cues in risk assessment

All prey are under a selective pressure to sample all available information about predation risks in their environment in order to modify their antipredator behaviour to match their current level of risk (Helfman 1989; Lima and Dill 1990; Lima and Bednekoff 1999). Individuals that are able to detect and respond to alarm cues from heterospecific species that share a common predator will gain a fitness advantage (Chivers et al. 2002; Dalesman and Rundle 2010). The diversity of fishes on coral reefs is without equal in any other aquatic environment, encompassing several of the most specious fish families (Bellwood and Hughes 2001). For example, a single reef within the Great Barrier Reef can contain as many as 850 different species of fishes (Goldman and Tyler 1976). Individuals are surrounded by other species that are targeted by a similar predator community. There is therefore a considerable repository of public information available for prey fish to gain knowledge about local predation risks. Responses to heterospecific alarm cues in coral reef fishes have been demonstrated only for the starry goby, *Asterropteryx semipunctatus*, which responded to the chemical alarm cues the eye-bar goby, *Gnatholepis anjerensis* (Smith et al. 1991). However, *G. anjerensis* failed to respond to alarm cues from *A. semipunctatus* raising questions about the relevance of heterospecific cues to reef fishes.

Responses to heterospecific cues are thought to arise through one of two mechanisms. Firstly, fishes may have an innate recognition of alarm cues from closely related species (Mirza and Chivers

2005; Schoppner ans Relya 2005; 2009). There is considerable evidence to suggest that alarm cues are conserved within phylogenetic groups and that the intensity of antipredator responses are dependent on how closely related species are to one another (Dalesman et al. 2007). Species that are closely related to each other are likely to have similar ecology and are targeted by similar predators. Thus responding to closely related species will enhance chances of detecting a predator. While, chemical alarm cues appear to be conserved within the ostariophysan (Pfeiffer 1977; Brown et al. 2001) and salmonid (Mirza and Chivers 2001) families, evidence is inconclusive for a conserved cues within family Gobiidae (Smith et al. 1991) and the the *Etheostoma* genus of the Percidae family (Commens-Carson and Mathis 2007) . Alternatively, recognition of heterospecifics may be learned through experience. Studies have demonstrated that through co-habitation, prey species exposed to a common predator will learn to respond to the heterospecific species over time (Pollock and Chivers 2004). While the contrasting responses to heterospecific alarm cues by the starry goby (responded to heterospecific alarm cues) and the eye-bar goby (did not respond to heterspecific alarm cues) provides little evidence to suggest how responses to heterospecific cues may arise in reef fish (Smith et al. 1991), it is plausible that recognition of heterospecific cue will result from both phylogenetically conserved cues and from experience through co-habitation with prey guild members.

The role of chemical alarm cues in learning about predators

There is a growing body of evidence that suggests that coral reef fishes do not possess an innate recognition of predators (but see Dixson et al. 2010 and Vail and McCormick 2011 for exceptions) and that fishes need to learn about predators to survive (Karplus et al. 2006; Holmes and McCormick 2010; Lönnstedt et al. 2012). Intuitively it would makes sense that prey are able to recognise predators on their first encounter, given the potentially deadly consequences. Learning on the other hand allows individuals to recognise novel predators as they encounter them and modify antipredator responses to match spatial and temporal changes in predation risk and predator community composition (Lima and Dill 1990) and has been shown to significantly enhance survival in fishes both in freshwater environments (Chivers et al. 2003) and recently in coral reefs (McCormick and Holmes 2006; Lönnstedt et al. 2012). Learning therefore allows prey to balance costly antipredator responses with fitness promoting activities. Additionally, learning does not necessarily require the prey to endure a direct encounter with a predator, as they can learn to recognise predators remotely using a several distinct mechanisms based on associative learning (reviewed in Brown and Chivers 2006; Ferrari et al. 2010). The majority of studies have focused on how prey learn to recognise predators when they encounter the sight or odour of a novel predator paired with a chemical alarm cue from conspecifics or heterospecifics (Ferrari et al. 2010). Learning about predators has now been demonstrated for several species of coral reef fishes (Table 1).

Learning will play an important role throughout an individual's life, particularly during critical shifts in life history. For example, when juvenile fishes settle to the coral reefs at the end of their larval phase they are subject to intense levels of predation from a diverse array of predators (Almany and Webster 2006) and there will be a selective pressure to learn the identities of relevant predators rapidly in order to survive. Recent studies have demonstrated that fishes are capable of rapidly learning the identity of predators. Glowlight tetras, are able to learn multiple predators simultaneously from a single conditioning event (Dawish et al. 2005). Not only are fish able to learn multiple predators during a single conditioning event they have been show to be able to recognise multiple predators on their first encounter if they have been conditioned to recognise a closely related predator (Ferrari et al. 2007). Ferrari et al. (2007) demonstrated that fish are able to generalise recognition from one known predator to unknown predators based on similarities between the odours of the predators. Predator-naïve fathead minnows were conditioned to recognise lake trout as a predator and in subsequent tests recognised not only lake trout but also the novel odours of the closely related rainbow trout and brook trout. Recognition was not extended to northern pike or white sucker, demonstrating that responses were generalised only to closely related species with similar odours, rather than predators in general. Through learning mechanisms such as multispecies recognition and generalised predator recognition juveniles settling onto coral reef should be able to rapid gain information about the local predation threats and greatly enhance their chances of survival. However, whether such learning mechanisms occurs in reef fish species is unknown.

Even if coral reef fishes are able to rapidly acquire information regarding potential predators and their associated risk, not all of it will be relevant or of equal value. Individuals should not only learn to recognise predators but also the level of risk they pose. Studies in freshwater have shown that fishes can learn about predators in a threat sensitive way and that the concentration of chemical alarm cues determines the strength of response to learned predators, as a stronger concentration of cue indicated a more dangerous predator (Brown at al. 2001; Chivers et al. 2001; Ferrari et al. 2005). Coral reef fishes are known to display threat sensitive responses to the concentration of chemical alarm cues and also in response to chemical alarm cues from different ontogenetic stages (Lönnstedt and McCormick 2011). Additionally, while the concentration determines how prey learn about novel odours the novel odours themselves may not be relevant. At the time of settlement juveniles will not only be naïve to the odours of reef-associated predators but also to non-predators. Encountering any novel odour paired with a chemical alarm cue will result in the novel odour being labelled as a threat. The diversity of fishes on coral reefs is such that there is the distinct possibility of mislabelling a non-predator as a predator. Responding to non-threatening odours will be detrimental to an individual’s fitness. Benefits gained from learning predator identities will be negated if individuals do not possess mechanisms to prevent irrelevant cues from being perceived as a threat (Davey 1989). However such mistakes maybe prevented if an individual already has experience with the odour. If an individual has encountered the odour prior to encountering them in a predatory context mechanism such as latent inhibition (a mechanism whereby prior experience with an odour prevent associative learning; Settleworth 1998; Acquistapace et al. 2003; Ferrari and Chivers 2006) and learned irrelevance (when temporal mismatch between novel odour and alarm cue prevent learning; Hazlett 2003) will prevent irrelevant odours being learned as a risk and allow prey to distinguish predators from back ground odours.

Aims and thesis outline

The study will explore the role chemical alarm cues play in the dynamic nature of predator-prey interactions amongst settlement stage coral reef fishes. Initially in **Chapter 2**, I will examine what cues are available to reef fish by testing a common reef fish, *Amphirion percula*, for its responses to chemical alarm cues originating from a range of species from within the Pomacentridae family and whether relatedness determines the intensity of response.

The following chapters will address how species use chemical alarm cues to learn about predators in a way that balances the need to avoid being preyed upon with the need to feed, grow and ultimately reproduce. **Chapters 3 and 4** examine mechanisms by which juvenile reef fish can acquire recognition of predators in a rapid and efficient way. Specifically, **Chapter 3** assesses whether juvenile recruits are able to recognise individual predators from a single multispecies conditioning event. **Chapter 4** then investigates whether, after being taught to recognise one predator, individuals are able to generalise recognition of this predator to other closely related species. Once the mechanisms that allow rapid learning have been established the focus of the thesis then shifts to how individuals prevent learning irrelevant cues. **Chapter 5** investigates whether latent inhibition is a viable mechanism to prevent prey from learning non-predators. Alternatively, fish may reduce the chances of incorrectly identifying a novel fish by learning about predators only from other individuals that provide the most accurate information regarding current predation risks. **Chapter 6**, therefore, examines whether juveniles learn in a threat-sensitive manner from conspecifics that because of size differences belong to different prey guilds. Understanding the mechanisms that underlie predator-prey interactions provides valuable information for understanding the patterns of distribution and abundance of reef fishes and will ultimately contribute to the management and protection of coral reefs.

Chapter 2: Are chemical alarm cues conserved within the coral reef fish family Pomacentridae?

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Authors: M. D. Mitchell, P. F. Cowman and M. I. McCormick

2.1 Summary

Fishes are known to use chemical alarm cues from both conspecifics and heterospecifics to assess local predation risks and enhance predator detection. Yet it is unknown how recognition of heterospecific cues arises for coral reef fishes. Here, we test if naïve juvenile fish have an innate recognition of heterospecific alarm cues. We also examine if there is a relationship between the intensity of the antipredator response to these cues and the degree to which species are related to each other. Naïve juvenile anemone fish, *Amphiprion percula*, were tested to see if they displayed antipredator responses to chemical alarm cues from four closely related heterospecific species (family Pomacentridae), a distantly related sympatric species (*Asterropteryx semipunctatus*) and a saltwater (control). Juveniles displayed significant reductions in foraging rate when exposed to all four confamilial heterospecific species but they did not respond to the distantly related sympatric species or the saltwater control. There was also a strong relationship between the intensity of the antipredator response and the extent to which species were related, with responses weakening as species became more distantly related. These findings demonstrate that chemical alarm cues are conserved within the pomacentrid family, providing juveniles with an innate recognition of heterospecific alarm cues as predicted by the phylogenetic relatedness hypothesis.

2.2 Introduction

Accurate assessment of predation risk is vital to the success of any individual, as early detection of a predator enhances the chances of prey survival (Lima and Dill 1990: Mirza and Chivers 2001a). However, to be successful antipredator defences must be balanced with other fitness enhancing behaviours e.g. feeding and reproduction (Lima and Dill 1990). This leads to a selective pressure on individuals to acquire information about current predation risks within their environment, in order to modify their antipredator behaviour to reflect their current level of risk. Such a strategy should optimise the trade-off between predator avoidance and other fitness enhancing behaviours (Helfman 1989; Lima and Bednekoff 1999). Individuals that are also able to detect and respond to alarm cues from heterospecific species that share a common predator will also have a fitness advantage (Chivers et al. 2002; Dalesman and Rundle 2010). The use of heterospecific alarm signals in risk assessment is common across multiple taxa: birds (Fallow et al. 2011), mammals (Shriner 1998), freshwater fishes (Mathis and Smith 1993), amphibians (Schoeppner and Relyea 2005, 2009), insects (Huryn and Chivers 1999) and crustaceans (Hazlett and McLay 2005). Furthermore, information from heterospecific individuals may be more valuable than that from conspecifics, as heterospecific species may impose a lower competitive cost than a conspecific (Seppänen et al. 2007).

In aquatic systems, chemical cues along with visual cues are the primary sources of information for assessment of predation risk (Hartman and Abrahams 2000). Released from specialised cells in the epidermis, following mechanical damage during a predation event, chemical alarm cues provide early warning of potential danger for other individuals within the local area (Brown and Chivers 2005), enhancing chances of survival (Mathis and Smith 1993; Mirza and Chivers 2001b; Lönnstedt et al. 2012). The importance of chemical cues is highlighted by the simultaneous evolution of chemical alarm cues within most aquatic taxa found in both freshwater and marine environments (reviewed in Ferrari et al. 2010a). They are of particular importance in complex or turbid habitats where visual cues are reduced (Hartman and Abrahams 2000; Leahy et al. 2011). Unsurprisingly, prey also use chemical alarm cues derived from heterospecifics to gain information about local predation risks (Mathis and Smith 1993; Schoeppner and Relyea 2005; Mirza and Chivers 2003a).

Responses to heterospecific alarm cues may arise from one of two non-exclusive mechanisms: 1) Individuals may possess an innate recognition of alarm cues common to closely related species (the "phylogenetic relatedness hypothesis") (Schoeppner and Relyea 2005, 2009; Chivers and Mirza 2001); or 2) Individuals may acquire recognition of relevant alarm cues through experience (the "ecological coexistence hypothesis") Dalesman and Rundle 2007, Chivers and Mirza 2001; Dalesman et al. 2010). The phylogenetic relatedness hypothesis proposes that alarm cues are conserved within taxonomic groups and thus individuals are able to generalise the recognition of their own alarm cue to those of closely related heterospecific species, as the composition of both alarm cues should be similar, having been derived from a recent common ancestor (Schoeppner and Relyea 2005, 2009). Individuals should therefore display a stronger antipredator response to closely related species and a weaker response to species that are more distantly related, irrespective of whether the species are allopatric or sympatric ((Schoeppner and Relyea 2009; Mirza and Chivers 2001c; Sullivan 2003). Strong evidence supporting the phylogenetic conservation of alarm cues is provided for grey tree frog tadpoles, *Hyla versicolor* (Schoeppner and Relyea 2009) and Ostariophysan fishes (Brown et al. 2000).

In contrast, the ecological coexistence hypothesis suggests that responses to alarm cues from heterospecific species arise due to individuals co-existing with species that are part of the same prey guild (Schoeppner and Relyea 2009; Dalesman et al. 2007). As both species share a common predator it is beneficial to respond to each other's alarm cues as it will enhance early detection of a predator. Such responses may arise through learning as individuals gain experience with the predator-prey community in their local environment Mirza and Chivers 2003a; Chivers and Smith 1994), or they may be innately fixed through co-habitation with sympatric prey guild members over several generations (Schoeppner and Relyea 2009). Support for this hypothesis is often confounded by the use of wild caught individuals, as it is not possible to control for experience prior to collection. Consequently, it is not possible to make definitive conclusions about how observed responses to heterospecific alarm cues arose. Interestingly, two studies suggest that ecological coexistence may play an important role in modifying responses to phylogenetically conserved responses to heterospecific alarm cues (Dalesman et al. (2007); Dalesman and Rundle (2010)).

For fishes, how responses to heterospecifc cues arise is still open to debate. The ability to acquire learnt recognition of heterospecific cues has been demonstrated across a wide range of fish taxa: minnows (Brown et al. 1995a), sticklebacks (Mathis and Smith 1993), cichlids (Golub et al. 2005), gobies (Smith et al. 1991) and salmonids (Mirza and Chiver 2001c). This suggests that ecological coexistence plays a significant role in acquiring recognition of heterospecifics at the individual level. However, support for the conservation of chemical alarm cues within taxonomic groups varies greatly. Of the taxonomic groups tested to date, alarm cues appear to be highly conserved within the superorder Ostariophysan (where the putative chemical alarm cue hypoxanthine-3-N-oxide has been identified; Brown et al. 2000) and the salmonid family (Mirza and Chivers 2001c). Other studies on wild darters, genus *Etheostoma* (Commens and Mathis 1999; Haney et al. 2001; Commens-Carson and Mathis 2007) and two species of coral reef gobies *Asterropteryx semipunctatus* and *Brachygobius sabanus* (Smith et al. 1991) provide inconclusive support for either hypothesis. Indeed, a more rigorous empirical assessment is still needed to address the phylogenetic relatedness hypothesis among fishes and other vertebrates, and the extent to which phylogeny determines the magnitude of antipredator responses. The answers to these questions are particularly important in understanding antipredator behaviour in species-rich habitats such as coral reefs.

Recent studies have highlighted the importance of chemical alarm cues in predator-prey dynamics for coral reef fishes, particularly for newly settled recruits (Lönnstedt et al. 2012; McCormick and Holmes 2006). Recruits are exposed to a period of extremely high predation following settlement (Almany and Webster 2006) and must rapidly learn to recognise predators to survive. During this period chemical alarm cues play a crucial role in predator recognition (McCormick and Holmes 2006; Chapter 3) and survival (Lönnstedt et al. 2012). Given that many species recruit to reefs around the new moon period and are likely exposed to a similar suit of predators, the ability to access information from heterospecifics will facilitate the rapid acquisition of predator identities and increase an individual's chances of surviving, particularly if they have an innate recognition of alarm cues from heterospecific species that share a common predator. However, to date only a goby, *Asterryoptryx semipunctatus* has been shown to be able to display antipredator responses to heterospecific alarm cues.

The aim of this study was firstly, to see if a common coral reef fish had an innate knowledge of heterospecific alarm cues at the time of settlement and secondly, to assess whether there was a relationship between the intensity of response to heterospecific cues and the extent to which they were related to each other, indicative of a phylogenetically conserved alarm cue. To do this we tested naïve juvenile anemone fish, *Amphiprion percula* (family: Pomacentridae), for an innate antipredator response to a range of chemical alarm cues from four heterospecific species within the pomacentrid family. They were also tested for their response to an alarm cue from a distantly related prey guild member, *Asterropteryx semipunctatus*, and a saltwater control. We then compared the intensity of the response to the heterospecific alarm cues to the time of divergence from the nearest common ancestor shared between *A. percula* and each of the heterospecific species.

2.3 Methods

Study species

Amphiprion percula is a member of the highly diverse and abundant Pomacentridae family that inhabit coral reefs throughout the tropics. While it is found in the same general habitat to the heterospecific species in this study they display distinct micro-habitat difference due to its symbiotic relationship with certain anemone species (Thresher 1985; Elliot et al. 1995; Elliot and Mariscal 2001; Depczynski and Bellwood 2004; Wilson et al. 2008). Consequently the extent to which they are exposed to their alarm cues should be similar for all species. Additionally, all species in this study are targeted by similar range of predators (Mariscal 1970; St John 1995; Graham et al. 2003). The heterospecific species were chosen based on their phylogenetic relationship to *A. percula*; *Amphiprion melanopus* is a closely related congeneric species, *Pomacentrus moluccensis* and *Acanthochromis polyacanthus* are both from different genera with the Pomacentrinae sub-family, *Chromis atripectoralis* is from the Chrominae sub-family, one of the most basal groups with the Pomacentridae [45] and *Asterropteryx semipunctatus* are from the distantly related Gobiidae family. All species are known to possess chemical alarm cues (Smith et al. 1991; Leahy 2011; Chapter 3; Mitchell unpublished data).

Collection and maintenance

A. percula juveniles were captive bred and reared to settlement at the James Cook University aquarium facility, following the methods outlined in Dixon et al. (2010). Juveniles were maintained in three 40-l flow-through aquaria and fed 2/4 NRD marine food pellets (Spectrum Aquaculture) until they reached ~20 mm in length, at which point they were large enough to use in the experiments. Captive breeding ensured that the fish would be completely naïve to the alarm cues of other species.

The five donor species were either taken from captive breed stocks or collected from the wild. *A. melanopus* and *Ac. polyacanthus* were captive bred at the university aquarium facility and reared to the same size as *A. percula*. All other species (*P. moluccensis*, *C. atripectoralis* and *As. semipunctatus*) were collected from coral reefs around Orpheus Island, Great Barrier Reef, Australia. Juveniles of each species were collected using hand nets and anaesthetic clove oil. All fishes were maintained in separate 40-l flow-through aquaria and fed *ad libitum* twice a day with 2/4 NRD marine food pellets (Spectrum Aquaculture).

Stimulus preparation

Alarm cues were prepared fresh directly before being used in each trial. One individual per treatment was sacrificed by a quick blow to the head and placed in a disposable Petri dish. Using a clean scalpel blade, 15 superficial cuts were made along each flank of the fish. Fish were rinsed with 15-ml seawater and the solution was filtered through filter paper to remove any solid material.

Observation tanks

Conditioning and recognition trials were conducted in 11-l flow-through aquaria (30 × 20 × 15 cm). Each tank had a 2-cm layer of gravel, a small terracotta pot (5-cm diameter) for shelter at one end and an air stone at the opposite end. An injection tube was attached to the air stone tube to allow food and odours to be introduced with minimal disturbance to the fish. A 3 x 6 grid (4 × 5 cm) was drawn onto the front of each tank. Each tank was surrounded on three sides with black plastic to visually isolate the fish and a black plastic curtain was hung in front of the tanks to create an observation blind.

Recognition trials

Individual *A. percula* were placed into test aquaria and left to acclimate for two days. On the morning of testing fish were fed 30-ml *Artemia* solution (containing ~200 individuals per ml) and left for at least 1 h before testing began. Trials were conducted between 0800 h and 1600 h each day. Prior to the start of trials the flow-through system was turned off and 10-ml of seawater were withdrawn and discarded from the tube, to remove any stagnant water, and a further 20 ml were withdrawn and retained for flushing. Trials consisted of an initial 2-min feeding period, a 5-min pre-stimulus observation period and a 5 min post-stimulus period. At the start of the 2-min feeding period 30-ml of *Artemia* were injected into the tank followed by 10-ml seawater to flush the tubing to allow feeding rates to stabilise. Once feeding rates had stabilised the 5-min pre-stimulus observation commenced. At the end of the observation period 15-ml of stimulus odour were injected followed by 10-ml of seawater for flushing and the post-stimulus observation period began 1 min later. The stimuli consisted of one of the five skin extracts or a saltwater control. Stimuli were assigned randomly to the tanks. Individuals were tested for their response to one skin extract only. A total of 150 fish were tested (18-20 individuals per treatment).

The behaviour of the focal fish was observed during the pre- and post-stimulus observation periods. We quantified two response variables: foraging rate and distance from shelter. Decreased foraging rate and distance from shelter are well known antipredator responses displayed by a number of prey species, including coral reef fishes (Ferrari et al. 2010a). Foraging rate included all feeding strikes irrespective of whether they were successful at capturing prey. For distance from shelter, the horizontal and vertical locations of the fish in the tank were recorded every 15s, using the grid drawn on the side of the tank. The position of the fish in the tank was then converted into a linear distance from shelter using the dimensions of the grid squares and Pythagoras's theorem.

Identification of phylogenetic relatedness

To assess if the magnitude of an antipredator response to a heterospecific alarm cue is regulated by the phylogenetic relatedness of the focal species to its heterospecific, we used the 'time of divergence' of our focal species (*A. percula*) and the most recent common ancestor (MRCA) to the heterospecific lineage in question. We made use of a recently published chronogram (time-calibrated phylogeny) of the family Pomacentridae (Cowman and Bellwood 2011) to find the divergence time of the MRCA of *A. percula* and the heterospecific alarm cue donors (Table 2.1). The pomacentrid chronogram was reconstructed using Bayesian age estimation methods and fossil calibration techniques (see methods; Cowman and Bellwood 2010). It includes all of the pomacentrid taxa used in this study and all major lineages of the family Pomacentridae. The timing of divergence (T_D) of each pomacentrid heterospecific from *A. percula* was taken as the age of the MRCA of both lineages (T_{MRCA}), minus the age of the node representing the origin of *A. percula* (T_{Ap}; Fig. 2.1a; Table 2.1). This correction for the age of the *A. percula* lineage standardises the MRCA age to a metric that is specific to an ancestor node of *A. percula*.

Table 2.2. Divergence times of heterospecific lineages from *A. percula.* Ages are in millions of years before present and are taken from Cowman and Bellwood (2011).

Species	MRCA age (T_{MRCA}) (MY)	MRCA distance (T_{DP}) (MY)
A. percula	2.7 (T_{AP})	0
A. melanopus	15.3	12.6
P. moluccensis	33.4	30.7
Ac. polyacanthus	34.7	32.0
C. altopectoralis	51.1	48.4

Statistical analysis

The proportional difference between pre- and post-stimulus behavioural observations were calculated and used as the raw data. The effects of test odour (the six fish alarm cues and saltwater) on foraging rate and distance from shelter of *A. percula* were analysed using individual 1-factor ANOVA's. To account for ANOVA's being run on two variables that were potential interrelated a Bonferroni adjustment was employed (adjusted $\alpha = 0.025$). The ANOVAs revealed that only foraging rate was affected by test odour. The subsequent analyses were done on the foraging variable only. Tukey's HSD post-hoc analysis was used to identify significant differences between responses to the test odours.

The relationship between the foraging response of individuals to the different pomacentrid chemical alarm cues and the divergence time between the different pomacentrid species and *A. percula* (T_D) was investigated using a linear regression. Divergence time was used as the predictive variable and mean change in foraging rate was used as the response variable. For both analyses, the data was checked for outliers and residual analyses revealed that all data met the assumptions of homogeneity of variance and normality.

2.4 Results

Test odour had a significant influence on *A. percula* foraging rate ($F_{6,111}$=18.78, p <0.0001). Post-hoc tests showed that individuals displayed a significant reduction in foraging rate when exposed to alarm cues from conspecific *A. percula* and the heterospecifics *A. melanopus*, *P. moluccensis* and *Ac. polyacanthus* compared to the saltwater control and *As. semipunctatus* control (Fig. 2.1b). Individuals also showed a significant reduction foraging rate when exposed to *C. atripectoralis* compared to the saltwater control but not to the *As. semipunctatus* outgroup control. There was no difference in foraging rate between the saltwater control and *As. semipunctatus*, with feeding rate remaining constant throughout the trials. The 1-factor ANOVA on distance from shelter revealed there was no significant effect of test odour on *A. percula* ($F_{6,111}$=1.38, p = 0.23).

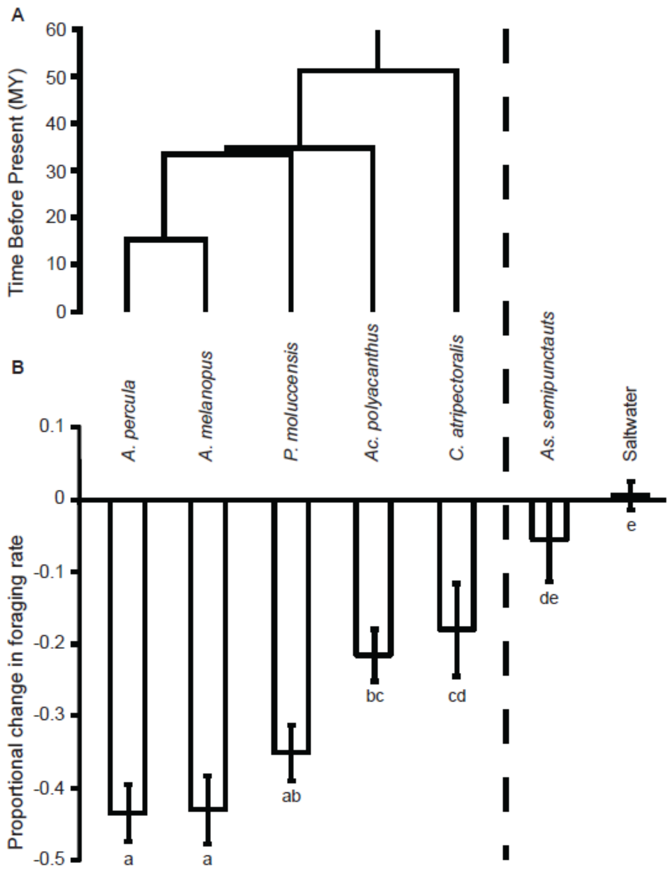

Figure 2.1. The phylogenetic relationship and antipredator response of *Amphiprion percula*, to heterospecific family members (*Amphiprion melanopus*¸ *Pomacentrus moluccensis*, *Acanthochromis polyacanthus* and *Chromis atripectoralis*), a distantly related sympatric prey guild member (*Asterropteryx semipunctatus*) or a saltwater control. **a)** A chronogram (modified from Cowman and Bellwood 2011) displaying the divergence times of the MRCA of the focal species, *A. percula* to each of the heterospecific donor lineages within the family Pomacentridae. Ages are calibrated to millions of years before present. **b)** The mean change in foraging rate (±SE) of juvenile *A. percula* exposed to the chemical alarm cues of five heterospecific species and a saltwater control. Fishes are ordered with respect to their relatedness to *A. percula*. Letters below bars indicate Tukey's groupings.

There was a significant relationship between the response to pomacentrid chemical alarm cues and the timing of divergence of the MRCA of the donor species and *A. percula*, which accounted for 66% of the intensity in antipredator response ($r^2 = 0.66$, $F_{1,88}$=16.72, $p < 0.001$; Fig. 2.2). The greatest reduction in foraging rate was displayed by individuals exposed to alarm cues from conspecifics and *A. melanopus*, the intensity of response then decreased as the donor species became more distantly related (Fig. 2.2).

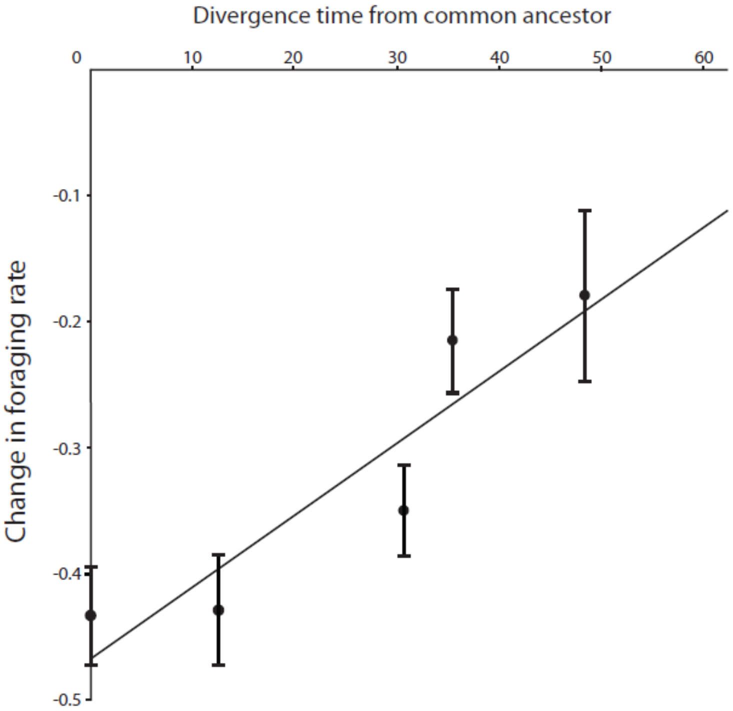

Figure 2.2. The relationship between divergence time and the intensity of antipredator response of juvenile *Amphiprion percula* exposed to chemical alarm cues from various heterospecific species within the family Pomacentridae. Circles represent in the mean change in foraging rate (±SE) of *A. percula* to chemical alarm cues of each heterospecific species.

2.5 Discussion

Our results demonstrate that juvenile reef fish are able to detect and respond to heterospecific chemical alarm cues and that chemical alarm cues are conserved within the Pomacentridae family. Naïve juvenile *A. percula* displayed a significant reduction in foraging rate, when exposed to alarm cues from conspecific and heterospecific family members but not to alarm cues from the distantly related sympatric *As. semipunctatus* or the saltwater control. Additionally, the intensity of antipredator responses to heterospecific alarm cues diminished as the timing of divergence between the heterospecific cue and *A. percula* increased. These results support the findings of similar studies on salmonids (Mirza and Chivers 2001c) and invertebrates (Schoeppner and Relyea 2005, 2009; Dalesman et al. 2007). However, this is the first to demonstrate a strong relationship between phylogenetic relatedness and response intensity to heterospecific chemical alarm cues for a vertebrate species. This strong relationship suggests that the innate recognition of heterospecific cues by *A. percula* resulted from phylogenetic conservation of alarm cues as predicted by the 'phylogenetic relatedness hypothesis'.

The ability to recognise and respond to heterospecific alarm cues will confer a significant survival advantage for reef fish throughout their lives but particularly during critical ontogenetic life history changes. Following an initial pelagic stage, larval reef fish recruit to reefs in pulses around the new moons throughout summer (Milicich and Doherty 1994). During this transition to the reef they enter an environment rich in generalist, opportunistic predators (Goldman and Talbot 1976) and are subject to extremely high mortality (up to 60% in first 2 days; Almany and Webster 2006). Several studies have shown that coral reef fishes lack an innate antipredator responses to predator odours with regards to short term changes in risk perception (Karplus et al. 2006; Chapter 3-5), although Vail and McCormick (2011) and Dixon et al. (2010) suggest there maybe some level of innate recognition of certain predators. In the absence of innate predator recognition, there will be a strong selection pressure to rapidly gain information about potential predators, risky habitats or time periods in respect to predation. Consequently, individuals that are able to detect and respond to heterospecific alarm cues will increase their chances of detecting an active predator in their local vicinity and enhance their chances of surviving any subsequent attack.

The finding that *A. percula* responded to all the heterospecific alarm cues but not to *As. semipunctatus* (a prey guild member) demonstrates that alarm cues are conserved within the pomacentrid family. There was a strong relationship between the intensity of the antipredator response and the time since each heterospecific species diverged from its common ancestor with *A.*

percula. These results support the predictions of the phylogenetic relatedness hypothesis, matching the findings of a number of previous studies on salmonids (Mirza and Chivers 2001c) and invertebrates (Schoeppner and Relyea 2005, 2009; Dalesman et al. 2007). In contrast, other studies investigating antipredator responses to heterospecific alarm cues found that responses to heterospecific cues were highly variable and there appeared to be no support for the phylogenetic relatedness hypothesis and only tentative support for the ecological coexistence hypothesis (Smith et al. 1991; Vadas and Elner 2005; Hazlett and McClay 2005). For example, while *As. semipunctatus* responded to both conspecific cues and heterospecific cues from *Gnatholepis anjerensis*, *G. anjerensis* responded to only conspecific cues (Smith et al. 1991). Similarly, studies on freshwater darters (Commen-Carson and Mathis 2007) and sea urchins (Vadas and Elner 2005) found inconsistent patterns in responses to both conspecific and heterospecific cues. However, the previously mentioned studies were confounded by the fact that they used wild caught individuals rather than naïve individuals. Consequently, any innate responses to phylogenetically conserved alarm cues (if present) may have been modified through experience with coexisting prey guild members, masking any response patterns indicative of phylogenetically conserved cues.

While there is the potential that ecological coexistence could have influenced the innate patterns of response observed here, we would suggest it is unlikely that it caused the responses observed. The heterospecific species in this study were selected based on the consistency of overlap in habitat preference and exposure to common predators between *A. percula* and the heterospecific donor species. Given this, if ecological coexistence was causing the innate response to heterospecific alarm cues we would have expected the responses heterospecific cues to be uniform irrespective of the time of divergence from their common ancestor with *A. percula*. Additionally, we would have expected individuals to respond to *As. semipunctatus* as well. However, as we were unable to include any allopatric Pomacentrid species there is the possibility that ecological coexistence might have influenced the responses observed. Dalesman et al. (2007) and Dalesman and Rundle (2010) demonstrated that ecological coexistence with heterospecific species can modify responses to phylogenetically conserved cues in snails, both at the population level, through coexistence over several generations, and at the individual level, through short term changes in prey guild community structure. Ecological coexistence may therefore play a secondary role in determining responses to phylogenetically conserved cues.

The capacity of any species to use heterospecific cues may depend on a number of intrinsic (e.g. the ability to detect heterospecific alarm cues) and extrinsic factors (e.g. how the individual interprets the relevance of the information once detected). The ability to detect heterospecific cues is dependent on them being sufficiently similar to the focal species' own cues for recognition to occur. As demonstrated here, the intensity of response to heterospecific cues is directly related to the time of divergence from the most recent common ancestor. Species may not recognise heterospecific cues simply because the time since the two species diverged from their common ancestor was sufficient for the cues to become unrecognisable. Similarly, the rate at which such changes to the chemical cues occur will determine recognition patterns. For example, it is thought that chemical alarm cues play a significant role in immune system function for fishes (Chivers et al 2007; Halbergewachs et al. 2009). The composition of the alarm cues may therefore be affected in part by the need to maintain immune system functioning. Consequently, changes in the composition of alarm cues may be driven by changes in the environment (and immune challenges) to which the individual species is exposed. Such drivers may cause a rapid change in the chemical alarm cue of species that have moved into a markedly different ecological niche.

Extrinsic factors, such as the prey species' ecology and life history, or the composition and foraging strategies of the predator community to which they are exposed may also influence how they respond to heterospecific alarm cues. The diversity of predatory species and their preferred foraging mode will likely influence responses of prey to heterospecific cues. Prey exposed to generalist predators (abundant on coral reefs), which target a broad range of species within a preys' guild, will benefit from responding to heterospecific cues. Conversely, prey individuals exposed to specialist predators that target discrete types of species (or ontogenetic stages) within the prey guild may not gain benefits of responding to heterospecific cues, especially if the focal prey is rarely targeted by that predator (Dalesman et al. 2007). Furthermore, life history strategies have the potential to strongly influence responses to heterospecific cues. Hazlett and McLay (2005) suggested that the extent to which various crayfish responded to heterospecific cues did not depend on phylogenetic relatedness, but rather on whether they evolved in specious regions and had the ability to disperse widely. The dispersive pelagic larval phase of reef fish may help to maintain a prey fish's responsiveness to heterospecifics, through the necessity for conservative risk assessment when settling to an environment that is highly patchy and unpredictable.

This study demonstrates that juvenile *A. percula* have an innate ability to recognise and respond to chemical alarm cues from closely related heterospecifics. The patterns of response strongly suggests that responses to heterospecific alarm cues result from a conserved chemical alarm cue within the Pomacentridae family as predicted by the phylogenetic relatedness hypothesis. Given the similarities between early life histories within reef fish, such baseline knowledge will enhance their capacity to detect risky situations and learn about the predators present in their new environment during a critical period in their life history. However, these innate patterns of response may not be permanently fixed. Previous studies have shown that responses to alarm cues can change throughout development, particularly in regards to how individuals perceive heterospecific cues (Golub and Brown 2003). As prey grow not only does perception of risk change with experience (Berejikian et al. 2003) but they will move into new prey guilds composed of different prey species and are exposed to different predators. Consequently, the patterns of responses to heterospecific cues will change throughout their lives to suit their current situation, incorporating new prey guild members and modifying innate responses as the perceived value of the information changes. To further understand the complexities of the predator-prey interactions that affect community composition and diversity on coral reefs, future studies need to look at how perception of risk alters with development and experience.

Chapter 3: Coral reef fish rapidly learn to identify multiple unknown predators upon recruitment to the reef

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Authors: M. D. Mitchell, M. I. McCormick, M. C. O. Ferrari, D. P. Chivers

3.1 Summary

Organisms often undergo shifts in habitats as their requirements change with ontogeny. Upon entering a new environment, it is vitally important to be able to rapidly assess predation risk. Predation pressure should selectively promote mechanisms that enable the rapid identification of novel predators. Here we tested the ability of a juvenile marine fish to simultaneously learn the identity of multiple previously unknown predators. Individuals were conditioned with a ‘cocktail’ of novel odours (from two predators and two non-predators) paired with either a conspecific alarm cue or a saltwater control and then tested for recognition of the four odours individually and two novel odours (one predator and one non-predator) the following day. Individuals conditioned with the ‘cocktail’ and alarm cue responded to the individual ‘cocktail’ odours with an antipredator response compared to controls. These results demonstrate that individuals acquire recognition of novel odours and that the responses were not due to innate recognition of predators or due to a generalised response to novel odours. Upon entering an unfamiliar environment prey species are able to rapidly assess the risk of predation, enhancing their chances of survival, through the assessment of chemical stimuli.

3.2 Introduction

Most organisms live under the constant threat of predation throughout their lives (Lima and Dill 1990). Antipredator behaviours are energetically expensive and reduce time available for other important activities such as foraging, mating and resource defence (Lima and Dill 1990; Lima and Bednekoff 1999). As such, prey must minimise the risk of predation whilst maximising their energetic input to promote growth, reproductive success and ultimately, fitness (Booth and Beretta 2004; Fievet et al. 2008). Prey must therefore be able to reliably identify potential predators, their associated risk and subsequently respond in a way that will optimise the balance between these conflicting demands.

The risks associated with predation vary with both time and space and will change throughout a prey's life (Ferrari and Chivers 2009a). Most organisms undergo ontogenetic shifts during their development, often resulting in individuals switching habitats in order to exploit superior food resources, shelter and establish or enhance mating opportunities (Dahlgren and Eggleston 2000). On entering a new habitat, individuals are exposed to a new community of predators, some of which may be unknown or represent a different level of predation risk (Savino and Stein 1989; Templeton and Shriner 2004). The composition of potential predators within a given habitat will also change as fish grow and switch prey guilds (Brown et al. 2001c; Golub et al. 2005), or as environmental changes introduce new predators (Kristensen and Closs 2004). Predators themselves are also highly variable in space and time, ensuring that the risk of predation is in constant flux. Thus, prey should be dynamic and flexible in their antipredator behaviour.

Prey individuals must be able to develop antipredator strategies that can be adapted to match the current predation risk. The need to adapt antipredator strategies to their current environment explains why fixed innate antipredator strategies are uncommon amongst prey fish (Chivers and Smith 1998; Brown and Chivers 2005). Learning allows individuals to associate novel predators with danger and fine tune their antipredator responses to local environments, reducing the cost of unnecessary antipredator behaviours (Lima and Dill 1990). There is now extensive research showing that prey from a variety of taxa use learning to recognise predators and enhance their antipredator responses, including examples from fish, amphibians, reptiles, molluscs, mammals and birds (Wisenden 2003; Griffin 2004).

In aquatic environments, prey fish are able to access information about local predation risk from environmental cues using their visual, olfactory and mechanical senses (Montgomery and Carton 2008). Predator identity can be learnt through socially transmitted information (Mathis et al. 1996), direct encounters with predators (Mathis and Smith 1993; McCormick and Manassa 2008), or indirectly, by associating a predator’s odour with an alarm cue (chemical released by mechanical damage to the skin during a predation event) (Magurran 1989). Chemosensory information provides reliable information about the identity and potential threat of unknown predators, as alarm cues are only released during direct predation encounters between predators and prey (Wisenden and Chivers 2006). Fish are able to learn the identity of a novel predator by associative learning when the predator odour is presented simultaneously with an alarm cue released by mechanical damage to the skin of a conspecific (Chivers and Smith 1994b; Chivers and Smith 1998). Indeed, the association between a novel odour and an alarm cue is so strong that after a single encounter, prey will respond with an antipredator response to the novel odour alone. This association can last several months (Chivers and Smith 1994b). Furthermore, these associations can even be made using alarm cues from heterospecific fishes in the same prey guild (Chivers and Smith 1998).

The majority of previous studies have investigated the ability of prey to acquire recognition of just one predator at a time under various conditions. However, few environments contain a single predator, with most prey exposed to several predators at any one time (Sih et al. 1998; Griffen and Byers 2006). Prey fish must be able to recognise any new potential risk of predation as fast and efficiently as possible. Learning multiple predators simultaneously would allow fast recognition of predators in a way that maximises time available for fitness promoting activities (Acquistapace et al. 2003). Darwish et al. (2005) conditioned glowlight tetras, *Hemigrammus erythrozonus*, to a ‘cocktail’ of odours containing two predators and one non-predator subsequently demonstrating that tetras learnt to recognise each of the individual odours. They then proceeded to show that odours learnt in this way still confer a survival benefit. Currently it is unknown if these findings can be generalised across all fish or if they are specific to glowlight tetras.

Coral reefs are among the most biologically diverse places on earth and provide a habitat for a rich assortment of fish including a vast array of predators, which can account for up to 50% of biomass in some reef communities (Goldman and Talbot 1976). After a pelagic developmental period, most juvenile reef fish return to coral reefs with little or no experience of the predators they will encounter. Mortality due to predation may reach nearly 60% during the first 2 days post settlement (Almany and Webster 2006). Recruiting juveniles must therefore learn the identity of local predators rapidly to survive. Recent studies have demonstrated that both juvenile and adult coral reef fish utilise chemical alarm cues to assess predation risk and to learn the identity of previously unknown predators through associative learning (Larson and McCormick 2005; Holmes and McCormick 2010). We tested the lemon damselfish (*Pomacentrus moluccensis*) at the end of the pelagic larval phase to see if they could learn the identity of multiple predators during a single conditioning event. Naïve *P. moluccensis* were conditioned with a ‘cocktail’ of odours from two predators and two non-predators paired with a damage-released skin extract (alarm cue) from a conspecific or a seawater control. After conditioning, they were tested for recognition of each odour in the ‘cocktail’, as well as the odour of a novel predator and the odour of a novel non-predator.

3.3 Methods

Study species

Lemon damselfish, *Pomacentrus moluccensis,* are common planktivorous coral reef fish, found throughout the Indo-Pacific region and the Great Barrier Reef. They are particularly abundant on reefs around our study area, Lizard Island, Northern Great Barrier Reef, Australia (14°40’S, 145°28’E). Like many marine organisms, they undergo a planktonic phase, lasting 29 d, after which they settle to the reef (Wellington and Victor 1989). At the time of settlement, they reach ~10mm in length and are preyed upon by multiple predators (Beukers and Jones 1997).

Collection and maintenance

All fish were collected at Lizard Island during November and December 2009. *P. moluccensis* recruits were collected from light traps (see small trap design; Meekan et al. 2001) moored overnight near the reef crest, during the summer larval recruitment pulse. Recruits were captured prior to settling, 50-100 m away from the reef crest. The predators we used are associated with reefs, not open waters (Randall et al. 1997), and hence, the recruits should be naïve to the predators. *P. moluccensis* were maintained in a 60 l aquarium (64.5 x 41.3 x 39.7cm) supplied with aerated seawater and maintained at ambient seawater temperatures (29°C) under a 14:10 light dark photoperiod. Fish were fed ad libitum twice a day with freshly hatched *Artemia sp.* and supplemented with 5/8 NRD marine food pellets (Spectrum Aquaculture). Fish were maintained in the aquaria for at least one day and a maximum of two weeks prior to being placed in experimental tanks.

Three known larval fish predators, brown dottyback, *Pseudochromis fuscus* (Family Pseudochromidae), clearfin lizardfish, *Synodus dermatogenys* (Synodontidae), batu wrasse, *Coris batuensis* (Labridae), and three non-fish predators, picasso triggerfish, *Rhinocanthus aculeatus* (Balistidae), steinitzi goby, *Amblyeleotris steinitzi* (Gobiidae), and bluespot butterflyfish, *Chaetodon plebeius* (Chaetodontidae), were collected from the lagoon at Lizard Island using hand nets, barrier nets and anaesthetic clove oil mixed with alcohol and seawater. The fish were maintained as described above in 32 l aquaria (43.2 x 32.4 x 30.5cm). Fish were fed twice a day with thawed bait squid.

Stimulus preparation

Fresh alarm cues were prepared each day, 10 min prior to the conditioning phase. Six *P. moluccensis* were sacrificed by a quick blow to the head and placed in a plastic disposable Petri dish. Fifteen superficial vertical cuts were made along each side of the body of each fish with a scalpel blade. Each fish was then rinsed in 15 ml of seawater, yielding a total volume of 90 ml of alarm cues from the six fish. This solution was filtered through filter paper to remove any solid material prior to use.

Odours were prepared from pairs of *P. fuscus* (57 and 79 mm standard length (SL)), *S. dermatogenys* (93 and 102 mm SL), *C. batuensis* (124 and 86 mm SL), *R. aculeatus* (109 and 63 mm SL), *A. steinitzi* (65 and 53 mm SL) and *C. plebeius* (68 and 70 mm SL). Pairs of each species was placed in individual 32 l flow-through aquaria (43.2 x 32.4 x 30.5cm). Fish were fed squid twice a day for two days and then starved for two days to remove any potential alarm cues present in their guts (Brown et al. 1995b). On the fourth day, each pair of fish from the same species was placed in a 32 l stimulus collection tank filled with 10 l of seawater, an airstone, and left undisturbed for 6 h. Following this period, the fish were moved back into the original holding tanks and the water from each stimulus collection tank was bagged in either 360 ml or 30 ml aliquots and frozen for later use.

General experimental approach

Our experiment consisted of two phases: a conditioning phase followed by a testing phase. During the conditioning phase, we conditioned individual *P. moluccensis* to recognize a cocktail of four fish odours by exposing them to 120 ml of 'cocktail' odours (30 ml from each of the four 'cocktail' species; *P. fuscus, S. dermatogenys, R. aculeatus* and *C. plebeius*) paired with 15 ml of either conspecific alarm cues (true conditioning) or a water control (pseudo-conditioning). The next day, the fish were tested for their response to one of the four fish odours present in the cocktail or alternatively, the odour of two novel species (*C. batuensis, A. steinitzi*). We tested 15 fish in each of our 12 treatments (2 conditioning groups x 6 odours tested). Although it would have been more rigorous to test for a response to saltwater as a control for the injection process, time constraints and animal limitations prevent us from doing so. In addition, several studies on Pomacentrid fish have demonstrated that they do not respond to the injection process (Larson and McCormick 2005; Manassa and McCormick 2008; Holmes and McCormick 2010). If our larvae have the ability to learn to recognize individual predators from a cocktail mix, then we predict that they would display an antipredator response to each of the four species originally present in the cocktail, but would not respond to the odour of the two novel species. Recent studies have suggested that larval reef fish have an innate recognition of some predators (Dixson et al. 2010; Vail and McCormick 2011). If that is the case, we predict that our larvae would respond more strongly to the two predatory species (*P. fuscus*, *S. dermatogenys*) than the two non-predatory species (*R. aculeatus*, *C. plebeius*). Additionally, we predict that they would also display an antipredator response when exposed to the 'novel' odour of a predator, *C. batuensis*, but not to the odour of a novel non-predator, *A. steinitzi*.

Observation tanks

Conditioning and recognition trials were conducted in 13-l flow-through aquaria (36 x 21 x 20 cm, mean flow-though = 0.6 litres/ min). Each tank had a 3 cm layer of sand and a small terracotta pot (5 cm diameter) for shelter at one end and an air stone at the opposite end. Two injection tubes (a feeding tube and a stimulus tube) were attached to the airstone tube with their ends placed just above the stone to aid rapid dispersal of the chemical stimuli. The injection tubes allowed the food and stimuli to be introduced with minimal disturbance to the fish. A 4 x 6 grid was drawn onto the front of each tank. Each tank was surrounded on three sides with black plastic to visually isolate the fish and a black plastic curtain was hung in front of the tanks to create an observation blind.

Conditioning phase

Single *P. moluccensis* were placed into each tank to acclimate overnight and then conditioned between 1000 h and 1130 h the following day. Prior to conditioning, odours from each of the four 'cocktail' species were thawed and mixed together to form the 'cocktail' of odours containing an equal amount of odour from each species. Prior to conditioning, the flow-through system was turned off to prevent the stimuli from flushing out. After a few minutes, we injected either 15 ml of alarm cue or 15 ml of seawater paired with 120 ml of cocktail odours. The fish were left undisturbed for 1hr, after which the flow-through system was turned on again. We conditioned a total of 180 individuals, 12 fish per day.

Recognition trials

Trials were conducted between 0730 h and 1430 h, the day after conditioning. Each trial consisted of an initial 5 min feeding period, a 5 min pre-stimulus observation and 5 min post-stimulus observation. Prior to the start of the trials, the flow-through system was turned off. Twenty ml of seawater were removed from both injection tubes and discarded to remove any stagnant water. A further 60 ml was removed from the feeding tube and 20 ml from the stimulus tube and retained for flushing. At the start of the 5 min feeding period, we injected 2.5 ml of food (an Artemia solution containing ~250 individuals per ml), followed by 20 ml of seawater (to completely flush the food into the tank), allowing the fish to reach a stable feeding rate before the pre-stimulus observation. At the start of the pre-stimulus observation, an additional 2.5 ml of food was introduced and flushed with 20 ml of seawater. Following the pre-stimulus observation period, we injected 2.5 ml of food, flushed with 20 ml of water, followed by 30 ml of stimulus odour, flushed with 20 ml of seawater. The stimulus odour consisted of the odour from one of the four species used in the ‘cocktail’ or one of the two novel species.

The behaviour of the fish was observed during the pre- and post-stimulus observation periods. We quantified three response variables: foraging rate, distance from shelter and time in shelter. Decreased foraging rate and distance from shelter and increased shelter use are well known antipredator responses in a number of prey species, including coral reef fishes (Ferrari et al. 2010a; Holmes et al. 2010).The foraging rate included all feeding strikes irrespective of whether they were successful at capturing prey. For distance from shelter, the horizontal and vertical locations of the fish in the tank was recorded every 15s, using the grid drawn on the side of the tank. The position of the fish in the tank was then converted into a linear distance from shelter using the dimensions of the grid squares (57 x 42 mm) and Pythagoras's theorem. Time in shelter (in seconds) was defined as total time that the fish spent within one body length of the terracotta pot.

Statistical analysis

The changes between the pre- and post-stimulus behavioural measures were computed and used as our raw data. The effects of conditioning (alarm cues vs. seawater) and testing odours (the six fish odours) were assessed using a 2-factor MANOVA on all three behavioural responses. Univariate ANOVAs revealed that only one behaviour (foraging) was affected by treatments, so the subsequent analyses were done on the foraging variable only. Because of a significant interaction between the 2 factors, we performed two 2-factor ANOVAs, one testing the effect of conditioning and the cocktail odour only, and one testing the effect of conditioning and the non-cocktail odour, on the responses of the fish. Residual analyses revealed that all data met the assumptions of homogeneity of variance and normality.

3.4 Results

The 2-factor MANOVA revealed a significant interaction between conditioning cues and testing odours on the behavioural response of *P. moluccensis* (conditioning x species, $F_{15, 458.7}= 3.3$, $p < 0.0001$). Univariate exploration revealed that foraging was the only behaviour affected by the treatments (Table 1). The 2-factor ANOVAs performed on the 'cocktail' odours only revealed a significant effect of conditioning ($F_{1,112}= 116.0$, $p<0.0001$), but no effect of species ($F_{3,112}= 0.2$, $p= 0.880$) and no interaction between the two factors ($F_{3,112}= 0.2$, $p= 0.910$) on the foraging behaviour of *P. moluccensis*, indicating that the fish learned to recognize the four cocktail species as threatening, and responded to all four with the same intensity (Fig. 3.1). Conversely, the 2-factor ANOVA performed on the response to two novel odours revealed no effects of conditioning ($F_{1,56}= 0.1$, $p= 0.770$), no effect of species ($F_{1,56}= 1.9$, p= 0.168) and no interaction between the two factors ($F_{1,56}=$

0.8, *p*= 0.368), indicating that the fish did not show an antipredator responses to those 2 odours (Fig. 3.1).

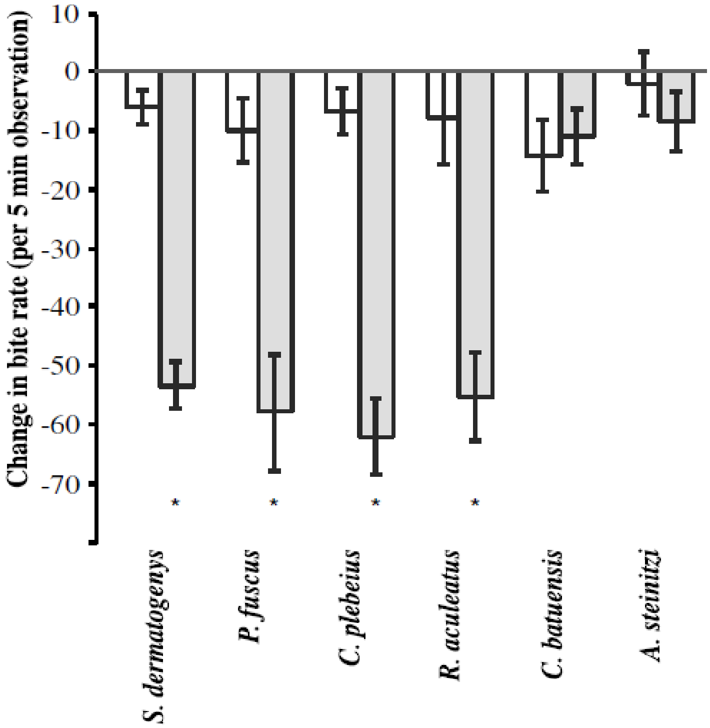

Figure 3.1. Change in foraging rate for *Pomacentrus moluccensis* in response to different odours. *P. moluccensis* were conditioned with the 'cocktail' of *S. dermatogenys*, *P. fuscus*, *R. aculeatus* and *C. plebeius* paired with a) alarm cue (shaded bars) or b) saltwater (open bars) and tested for learned recognition of *S. dermatogenys*, *P. fuscus*, *R. aculeatus* and *C. plebeius* odours alone or the control odours of *C. batuensis* and *A. steinitzi*. * indicate significant differences between conditioning treatments within species.

3.5 Discussion

Our results highlight that juvenile reef fish that are naïve to predators have the ability to rapidly learn multiple unknown predators upon recruitment to the reef. *P. moluccensis* recruits conditioned with a ‘cocktail’ of four odours (predators- *S. dermatogenys* and *P. fuscus* and non-predators- *R. aculeatus* and *C. plebeius*) paired with an alarm cue responded with a clear antipredator response when presented the individual odours from the ‘cocktail’, whereas individuals conditioned with the ‘cocktail’ paired with saltwater did not respond. The learning occurred after a single conditioning event. This is the first study to demonstrate rapid learning of multiple predator cues by marine organisms transitioning to a new environment containing multiple novel predators.

The ability to simultaneously learn the identity of multiple predators is an efficient mechanism that allows prey to rapidly garner information regarding predation risk. Acquired recognition of predator odours enhances an individual’s survival during encounters with predators whether learnt individually or simultaneously as part of a multi-predator ‘cocktail’ (Dawish et al. 2005). Such rapid learning is especially important for reef fishes at the time of settlement. The dispersive nature of planktonic larval reef fishes means that juveniles may settle on non-natal reefs, where the diversity and composition of predators may differ from that of their natal reefs (Holmes and McCormick 2006). During the first two days post settlement, as individuals learn and adapt to their new environment, mortality due to predation is at its most severe (Almany and Webster 2006). Faced with such intense predation pressure, an individual’s ability to rapidly acquire predator recognition will ultimately determine who survives.

Prey displayed a clear antipredator response to the introduction of a ‘cocktail’ odour following a conditioning event where the ‘cocktail’ odour was paired with the alarm cue. The antipredator response was defined by a substantial decline in the foraging rate of individuals. Reductions in foraging rate in response to predator odours has been demonstrated for the closely related *Pomacentrus amboinensis* (Holmes and McCormick 2010) and in other species across several taxa in both marine (Smith and Smith 1989), freshwater (Jachner and Janecki 1999) and terrestrial habitats (Swihart et al. 1991). Such reductions represent a shift in the balance between foraging and antipredator defence in response to an increase in the perceived risk of predation represented by recognition of an odour associated with a potential predator (Chivers and Smith 1998; Kats and Dill 1998).

P. moluccensis did not appear to display an antipredator response suggestive of prior recognition to any predator odours used in this experiment. Individuals conditioned with the ‘cocktail’ and saltwater did not respond to individual ‘cocktail’ odours alone. In addition, fish conditioned under either conditioning regime did not show any significant response to the novel odour of *C. batuensis*, a known predator of juvenile reef fish (Sano et al. 1984). Recent studies on juvenile pomacentrid recruits suggest that individuals have some level of prior knowledge of predators during settlement, as naïve juveniles avoided predator odours in pairwise Y-maze trails (Dixson et al. 2010) and recruits preferentially settled on habitats where the odour of predators was absent (Vail and McCormick 2011). Prior knowledge of predator odours may be used during site selection by *P. mollucensis* but the odours represent a predation risk that is below their behavioural threshold and do not elicit a measurable anti-predator response until presented in combination with an alarm cue. Further work is needed to identify if pomacentrids have prior knowledge of predator odours at settlement, and if so, how this onboard knowledge is used within the decision-making framework to efficiently balance the costs and benefits of antipredator responses.

The antipredator response of *P. moluccensis* to the ‘cocktail’ odours was consistent across all odours (for their respective conditioning regime) irrespective of whether they originated from a predator or a non-predator. The consistency of the antipredator response to individual ‘cocktail’ odours is unsurprising given the apparent absence of prior knowledge of predator odours and the conditioning regime used during the associative learning of the ‘cocktail’ odours. Both *P. moluccensis* and glowlight tetras were simultaneously conditioned to all odours in combination with exactly the same concentration of alarm cue. During associative learning events, the strength of response to the predator odour is directly related to the concentration of the alarm cue during conditioning (Mirza and Chivers 2001d; Mirza and Chivers 2003b). It therefore follows that in the absence of prior knowledge of predators the response to all odours should be consistent for both predators and non-predators.

Simultaneous assessment of the predation risk posed by multiple predators potentially prevents predator specific information from being assessed. Previous studies have highlighted that prey use alarm cues to assess the levels of risk associated with individual predators and are able to respond in a threat sensitive way (Ferrari et al. 2005). European minnows, *Phoxinus phoxinus*, conditioned to recognise predatory pike, *Esox lucius*, or non-predatory tilapia, *Tilapia mariae*, subsequently responded to pike odour with a stronger antipredator response than to tilapia (Magurran 1989). The difference was suggested to be a result of recognition of predators compared to non-predators or an artefact of evolutionary experience. As shown in this study, simultaneously conditioning prey fish to several odours results in all the odours being assigned the same level of risk. This may lead to a disproportionate behavioural response to the relative level of risk posed by the predators during future encounters. Two outcomes are possible from multi-species conditioning which will results in a net loss in fitness or survival. Firstly, predators of low risk may be labelled as a high risk and under these circumstances the prey fish will respond with an excessive anti-predator response, resulting in time and energy being wasted on unnecessary anti-predator behaviour. Secondly, predators of high risk may be labelled as a low risk predator and the prey fish will respond to its odour with an insufficient response, resulting in the prey being captured and consumed by the predator. Immediately after learning the identity of unknown predators, there is a clear need for the prey to start to fine tune its assessment of the relative risk posed by the predator during subsequent encounters. Ferrari and Chivers (2006b) demonstrated that minnows would fine-tune their responses to predators after several encounters but would always place more emphasis on the more recent information.

Studies on associative learning have demonstrated that any unknown stimulus can be recognised as a predation risk through associative learning. In natural environments, fishes are constantly exposed to multiple chemical odours. This study highlights the potential for ecologically irrelevant odours to be learned by association when present during a predation event. Responding to irrelevant cues will negatively impact an individual’s fitness. Association of odours can be prevented with prior exposure to odours through latent inhibition (Acquistapace et al. 2003; Ferrari and Chivers 2006a; Chapter 5) and learned irrelevance (Hazlett 2003) or through experience and constant reassessment of acquired information (Ferrari and Chivers 2006b). However, for reef fish entering a new environment, prior exposure to odours is not possible and some irrelevant odours will be associated with a predation risk. It may pay at first to be overly cautious and learn all odours associated with an alarm cue as a predation risk when entering a new environment and then slowly learn which of those actually do not represent a threat.

Chapter 4: Generalization of learned predator recognition in coral reef ecosystems: how cautious are damselfish?

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Authors: M. D. Mitchell, M. I. McCormick, D. P. Chivers and M. C. O. Ferrari

4.1 Summary

Learnt predator recognition provides animals with an adaptive mechanism to rapidly adjust to current levels of predation risk. Prey may reduce the costs associated with learning if they can use information learned about known predators to respond to cues from closely related predators with which they are unfamiliar. Here, we demonstrated that, in a community where the ability to predict the predatory status novel species is low (i.e. high diversity of closely related predators and non-predators), prey fish generalise predator recognition to novel congeneric species but not confamilial species. Lemon damselfish, *Pomacentrus moluccensis*, conditioned to recognise the odour of a predatory moonwrasse, *Thalassoma lunare*, as a risky stimulus subsequently displayed antipredator responses not only to *T. lunare* odour, but also to the congeneric *T. amblycephalum* and *T. hardwicke* odours. Recognition was not extended to other species within (Labridae; *Coris batuensis* and *Halichoeres melanurus*) or beyond the family (Pseudochromidae; *Pseudochromis fuscus*). Our results demonstrate that reef fishes are able to generalise recognition of a predator to only closely related species and they are unable to distinguish between predators and non-predators when generalising recognition. Recognition patterns may result from uncertainties in predicting the identities of predators in complex communities.

4.2 Introduction

The ability to recognise and respond to predators is essential for prey to survive and reproduce. Individuals that are best able to identify predators and respond appropriately will maximise their fitness and have the greatest chance of surviving to reproduce (Lima and Dill 1990; Kats and Dill 1998). However, predator encounters are often highly variable in time and space and prey must acquire sufficient information about each predator in order to respond in a threat-sensitive way (Helfman 1989; Lima and Bednekoff 1999). Learning, therefore, allows individuals to identify predators and the risk they represent (Griffin 2004; Ferrari et al. 2010a). Learning allows prey to maximise overall fitness through accurate assessment of the risk posed by predators through time. Despite the existence of mechanisms allowing prey to learn to recognize predators from a relatively safe location (via chemical cues or social learning), collecting predator information may frequently come at the cost of prey having to survive a potentially deadly first encounter with a predator (Kelley and Magurran 2003a).

Recent work suggests that prey living in multi-predator systems can mitigate some of the cost associated with learning by generalising recognition of one predator to other closely related novel predators (Griffin et al. 2001; Ferrari et al. 2007; Stankowich and Coss 2007). For instance, Tammar wallabies, *Macropus eugenii*, trained to recognise a model red fox, *Vulpes vulpes*, as a threat, generalised their antipredator response to an unknown predatory feral cat, *Felis catus,* but they did not respond to a non-predatory juvenile goat, *Capra hircus* (Griffin et al. 2001). In a similar study, fathead minnows, *Pimephales promelas*, conditioned to recognise the smell of lake trout, *Salvelinus namaycush,* as a predator subsequently recognised the odour of two other salmonid species (Ferrari et al. 2007). Interestingly, the generalised response to each species diminished as they became more distantly related from the lake trout. This suggests that generalised predator recognition is a specific case of stimulus generalisation, seen in studies from the psychological literature, with odours that are similar to the conditioned odour eliciting a stronger response than odours that are more dissimilar

(Ghirlanda and Enquist 2003; Shettleworth 2010). Such recognition patterns are concordant with the distribution of foraging mode/diet choice within related species, as more closely related species generally have greater overlap in their foraging modes (Bellwood et al. 2006; Cowman et al. 2010). Being able to generalise predator recognition to novel species provides prey with a way to identify potential predators without the cost associated with learning (Griffin et al 2001; Ferrari et al. 2007; Burns et al. 2010).

In accordance with the threat-sensitive predator avoidance hypothesis (Helfman 1989), individuals should be flexible when displaying generalisation patterns. They should match the extent of generalisation to the probability they will respond correctly to more and more distantly related species (Ferrari et al. 2008a). The ‘Predator Recognition Continuum Hypothesis’ (PRCH) suggests that the extent to which individuals generalise recognition depends on the ratio of predators to non-predators within a community (Ferrari et al. 2007; 2008). Where the predator to non-predator ratio is low (i.e. relatively few predators compared to non-predators within a given taxonomic group), individuals should learn each predator as they encounter them, or alternatively have a narrow generalization window, as the costs associated with responding to non-predatory species may be too high. Where the ratio is high (i.e. many predators but few non-predators), individuals should have a wider generalization window, as the benefits from avoiding a potentially deadly encounter with novel predators will override the initial cost of responding to a few non-predatory species.

Interestingly, Griffin et al. (2001) suggested that wallabies might have used characteristics specific to predators (e.g. frontally placed eyes) when generalising recognition to the cat but not the goat. Such cues relate directly to the predators functional morphology and are therefore absent in non-predators. If prey are able to use such functional cues they might be able distinguish between predators and non-predators when generalising recognition. Such abilities would allow prey to reduce the risk of misidentifying non-predators and generalise to a greater range of predators than predicted by the PRCH. However, their results were confounded by the fact that the goat was more distantly related to the fox than the cat was. Thus, a lack of significant overlap between the cues in general rather than the specific cues could have resulted in wallabies not responding to the goat. Furthermore, studies investigating how velvet geckos generalise recognition of predator using olfactory cues showed they could not distinguish between snakes that ate them and those that did not (Webb et al. 2009; 2010).

In aquatic systems, prey often acquire recognition of predators through associative learning, where the predator cue (generally visual or chemical) is encountered simultaneously with a chemical alarm cue (Kelley and Magurran 2003a). Chemical alarm cues (released by mechanical damage to the skin during a predation event) are innately recognised by many prey organisms and elicit dramatic short term increases in antipredator behaviours upon detection (Ferrari et al. 2010a).

If, and to what extent fishes from highly diverse systems such as coral reef, are able to generalise predator recognition is unclear. The predictions of the PRCH suggest that prey species on coral reefs will display a limited use of predator generalisation because of the unreliability of the information. However, if prey are able to utilise cues that are functionally specific to predators, they should distinguish between predators and non-predators and generalise broadly. Therefore, the present study experimentally investigated the ability of a common coral reef fish, the lemon damsel, *Pomacentrus moluccensis* (Bleeker, 1853), to learn to recognise predators, generalise this recognition to other related predators and distinguish between predators and non-predators. After conditioning *P. moluccensis* to recognise the predatory moon wrasse (*Thalassoma lunare*; Linnaeus, 1758) as a threat, individuals were tested for their response to odours from *T. lunare*, a congeneric predator, the six-bar wrasse (*Thalassoma Hardwicke*; Bennett, 1830), a congeneric non-predator, the blueheaded wrasse (*Thalassoma amblycephalum*; Bleeker, 1856), a confamilial predator, the variegated wrasse (*Coris batuensis*; Bleeker, 1856), a confamilial non-predator, the tail-spot wrasse (*Halichoeres melanurus*; Bleeker, 1851), a distantly related predator, the brown dottyback, *Pseudochromis fuscus* (Müller & Troschel, 1849), or a saltwater control. We predicted that if *P. moluccensis* are able to generalise their recognition of *T. lunare*, they should display the strongest response to *T. lunare* odour and show a weaker response to novel wrasse odours as they become more distantly related. Additionally, if *P. moluccensis* are able to distinguish between predatory modes then they should not respond to the odours of the non-predatory *T. amblycephalum* and *H. melanurus*.

4.3 Methods

Study species

Pomacentrus moluccensis are a common and abundant coral reef fish, found throughout the Indo-Pacific Ocean and Great Barrier Reef. They settle at the end of the larval phase to shallow reefs and are preyed upon by a diversity of predators that include *T. lunare* and *Ps. Fuscus* (Beukers and Jones 1997; Feeney et al. 2011). The wrasse *T. hardwicke* has an ecology similar to *T. lunare*, while the congeneric *T. amblycephalum* is a planktivore. The wrasse *C. batuensis* is generalist predators with a diet generally composed of invertebrates, that is known to target early developmental stages of fish (Sano et al. 1984; Steele 1999). *H. melanurus* is a non-piscivorous invertivore. All species co-occur at the shallow reef front at the study location.

Collection and maintenance

This research was conducted with fishes collected from the fringing reef around Lizard Island, Northern Great Barrier Reef, Australia (14°40'S, 145°28'E). *P. moluccensis* recruits were collected prior to settling, using light traps (see small light trap design, Meekan et al. 2001) moored overnight, 50-100 m off the reef crest at Lizard Island, during November 2010. Collecting recruits prior to settlement ensured they would be naïve to the odours of reef-associated predators. The fish were maintained in 60-l flow-through aquaria, at ambient seawater temperature (29oC) under a 14:10 light:dark photoperiod. They were fed freshly hatched *Artemia sp*. twice daily.

Five wrasse species, *T. lunare* (17.1 and 23.5 g, individual weight), *T. hardwicke* (26 and 41.4 g), *T. amblycephalum* (5.3, 6.4 3.7, 4.2 and 6.5 g) *C. batuensis* (21.9 and 14.3 g) and *H. melanurus* (9.3, 8.8, 9.6 and 2.4 g) and a distantly related predator, *Ps. fuscus* (6.4, 5.0, 6.9, 4.8 and 5.0 g) were collected from reefs at Lizard Island using barrier nets and clove oil. All fish were maintained in aerated 60-l aquaria. Fish were fed squid daily. All aquaria were siphoned daily following the feeding period (to remove any uneaten food and faecal matter) and received an 80% water change.

Stimulus preparation

Alarm cues were prepared daily, 10 min before the conditioning phase. One *P. moluccensis* per trial was killed by a quick blow to the head. Using a scalpel blade, fifteen superficial cuts were made along each flank of the fish. Fish were rinsed with 15 ml of seawater and the solution was filtered through filter paper to remove any solid material.

For odour collection, fish were fed squid for two days before the start of the experiment, to remove any potential fish cues from their digestive tract. The aquaria flow-through system was turned off during the experiment, except during the daily water exchange, to allow the odour to concentrate. Eighty percent of the tank water was exchanged at 1600 h daily to ensure odour concentration was constant throughout the experiment. Each aquarium contained at least two individuals of one species. To standardise the concentration of odour between species, the water volume in each aquarium was adjusted so that there was one litre of seawater per 1.1 g of fish. Seawater containing fish odours were collected directly from these maintenance aquaria.

Observation tanks

Experiments were conducted in 13-l flow-through aquaria (36 × 21 × 20 cm, flow at ~ 0.6 litres/min). Tanks were setup as described in Chapter 3 with a layer of sand, shelter (5 cm diameter) at one end, an airstone at the opposite end and 4 × 6 grid (grid size = 5 × 6 cm) drawn onto the tank. One feeding and one stimulus injection tube were attached to the airstone tube.

Conditioning phase

P. moluccensis were acclimated individually in tanks for at least 2 hours before conditioning. The flow-through system was turned off during conditioning to prevent the stimuli from flushing out. We then randomly injected either 15 ml of alarm cue or 15 ml of seawater paired with 30 ml of *T. lunare* odour. The fish were left for 1hr, after which the flow-through system was turned on again. We conditioned at total of 255 fish, 24 fish per day (1.18 cm ± 0.06; mean size ± 1 S.D.), between 1400-1500 h.

Recognition trials

Trials were conducted between 0730 h and 1430 h the day after conditioning. Each trial consisted of an initial 5 min feeding period, a 5 min pre-stimulus observation and 5 min post-stimulus observation. The flow-through system was turned off before the trials started. Fifteen ml of seawater were removed from both injection tubes and discarded to remove stagnant water. A further 65 ml were removed and retained for flushing. At the start of the feeding period, we injected 2.5 ml of food (Artemia solution, ~250 individuals per ml), flushed with 15 ml of seawater, allowing the fish to reach a stable feeding rate before the behavioural observations. At the start of the pre-stimulus observation, an additional 2.5 ml of food was introduced and flushed with 15 ml of seawater. Following the pre-stimulus observation period, we injected 2.5 ml of food, followed by 30 ml of stimulus odour; both were flushed with 15 ml of seawater. The stimulus odours were randomly assigned to each tank and consisted of one of seven odours: *T. lunare, T. hardwicke, T. amblycephalum, C. batuensis, H. melanurus, Ps. fuscus* and a saltwater control. This ensured that all treatments were run simultaneously.

The behaviour of the fish was observed during the pre- and post-observation periods. We quantified two response variables: number of feeding strikes and number of line crosses, as a measure of activity. Decreased foraging rate and activity are well known antipredator responses in a number of prey species (Ferrari et al. 2010a). Foraging rate included all feeding strikes irrespective of whether they were successful at capturing prey. Line crosses were counted every time the entire body of the fish crossed a grid line.

Statistical analysis

Residual analyses revealed that data for feeding strikes met the assumptions of homogeneity of variance and normality but the data for line crosses was arcsine transformed to meet parametric assumptions. The proportional change in behaviour from the pre-stimulus baseline ((post-pre)/pre) were calculated and used in the following analysis. The effects of conditioning (alarm cue vs. saltwater) and test odour (six fish odours and saltwater) were analysed using a two-factor MANOVA's on the number of foraging strikes and line crosses. The MANOVA approach was used due to the correlative nature of the two behaviours (fish feeding more are also likely to be the ones that moved the most).

Due to a significant interaction between the 2 factors, we divided the analysis into 3 parts: (1) we performed a 1-factor MANOVA to test the effect of test cues on the responses of *P. moluccensis* conditioned with *T. lunare* paired with saltwater. This informed us if *P. moluccensis* have an innate response to any of the fish species in the absence of true conditioning. (2) We performed a series of t-tests (or t'-tests) comparing the response of *P. moluccensis* conditioned with water to those conditioned with alarm cues to each of the 7 odours. This told us which odours were generalized by *P. moluccensis*. (3) A 1-factor ANOVA was performed to test if there was a difference in the intensity of antipredator responses displayed to all generalized odours.

4.4 Results

The two-factor MANOVA revealed a significant interaction between conditioning and test odour on the response of *P. moluccensis* ($F_{12, 444} = 2.02$, $p < 0.05$). The univariate tests showed there was a significant interaction between conditioning and test odour for foraging rate but there was no effect of either conditioning or foraging rate on line crosses (Table 1). There was no effect of test odour on foraging rate for fish conditioned with the *T. lunare* odour and saltwater (one-factor ANOVA, $F_{6, 123}$ = 0.581, p = 0.745; Fig. 1). Juvenile *P. molluccensis* did not respond differently to the odour of 6 fishes or the saltwater control, indicating no innate response to any of the fish used in our experiment.

Table 4.1. Comparison of the behaviour of juvenile *Pomacentrus moluccensis* in response to the odours of one of 6 fish species or saltwater ('Test odour') after being conditioned with *Thalassoma lunare* odour paired with either a chemical alarm cue or saltwater ('Conditioning'). Two factor ANOVAs were conducted on: a) foraging rate, and b) line crosses (adjusted α = 0.025).

Source of Variation	*df*	*F*	*p*
(a) *Foraging rate*			
Test odour	6	3.273	< 0.005
Conditioning	1	14.458	< 0.0001
Test odour*Conditioning	6	2.950	< 0.01
Error	239		
(b) *Line crosses*			
Test odour	6	1.128	0.347
Conditioning	1	0.215	0.644
Test odour *Conditioning	6	0.545	0.773
Error	239		

Independent t'-tests revealed that there was a significant effect of conditioning on foraging rate for *P. moluccensis* responding to *T. lunare* ($t_{(23.78)}$ = -3.011, p = 0.004), *T. hardwicke* ($t_{(34)}$ = -3.38, p = 0.002) and *T. amblycephalum* ($t_{(35)}$ = -2.97, p = 0.005) odours. Conditioning had no effect on the response of *P. moluccensis* to *C. batuensis* ($t_{(35)}$ = -0.27, p = 0.79), *H. melanurus* ($t_{(31)}$ = -0.94, p = 0.36), *Ps. fuscus* ($t_{(34)}$ = -0.49, p = 0.62) or saltwater ($t_{(33)}$ = -0.68, p = 0.5). When comparing the intensity of response to the 3 species eliciting antipredator responses, our results indicated that *P. moluccensis* did not respond differently to the 3 congeneric species ($F_{2,51} = 0.38$, $p = 0.69$)

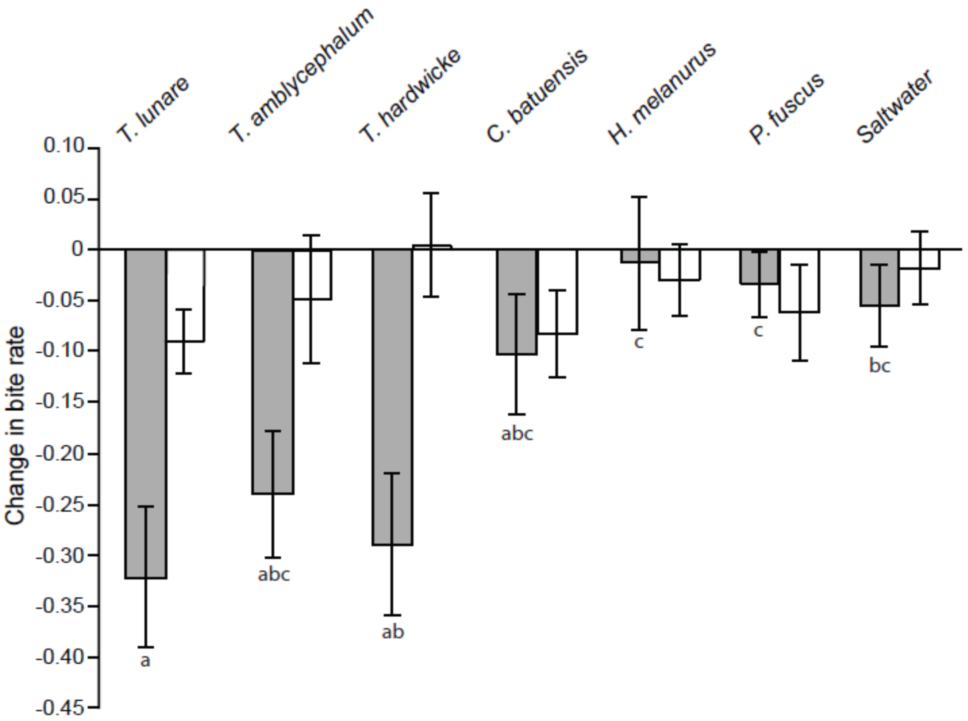

Figure 4.1. Proportional change in bite rate (per 5 min observation; mean ± 1 S.E.) for *Pomacentrus moluccensis* conditioned with *Thalassoma lunare* odour paired with a chemical alarm cue (gray bars) or saltwater (white bars) and tested for their response to the odour of *T. lunare*, *T. hardwicke*, *T. amblycephalum*, *Coris batuensis*, *Halichoeres melanurus*, *Ps. fuscus* or a saltwater control. Wrasse species are presented in order of the degree to which they are related to *T. lunare,* with *T. amblycephalum* being the closest and *H.melanurus* being the most distantly related. Letters below bars indicate Tukey's HSD groupings for the individuals conditioned with chemical alarm cue and *T. lunare* odour only.

4.5 Discussion

Naïve juvenile *P. moluccensis* conditioned *T. lunare* and chemical alarm cues learned to recognise *T. lunnare* odour as a predation risk, displaying a distinct anti-predator response i.e. reduced feeding when subsequently exposed to *T. lunare* odour alone. Juveniles also displayed anti-predator responses to the odours of *T. hardwicke* and *T. amblycephalum* but not to odours of species beyond the *Thalassoma* genus.This result demonstrates that reef fish can generalise the antipredator response associated with a predator odour to the odours of congeneric species that had not previously been associated with risk, similar to findings from fathead minnows (Ferrari et al. 2007), woodfrogs (*Rana sylvatica*, Ferrari et al. 2008a; 2009), tammar wallabies (Griffin et al. 2001) and gecko's (Webb et al. 2009; 2010). Interestingly, our results show that whilst individuals could generalise predator recognition to other related species, they were only able to extend recognition to very closely related species and could not distinguish between predators and non-predators. Differences in the patterns of generalisation may arise from differences in the taxonomy of coral reef fishes compared to other fish groups such as salmonids, with predator odours diverging at a greater rate in confamilial fish from coral reefs. Alternatively, recognition patterns might reflect the difference in predation risk and the reliability of information available to fish on coral reefs.

Generalised predator recognition is clearly advantageous to prey in environments such as coral reefs, even if the chance of accurately identifying a predator is low. Learning allows prey to acquire predator-specific information with which they can then develop anti-predator behaviours to match the risks associated with predators (Helfman et al. 1989; Kelley & Magurran 2003; Griffin 2004). This information is sufficiently valuable that prey are willing to endure potentially deadly encounters with predators to gain such information (Kelley & Magurran 2003). By generalising the recognition of predator cues, they will gain the benefit of identifying novel predators that may be unpredictable in space and time, whilst avoiding the costs associated with learning (Griffin et al. 2001; Ferrari et al. 2007).

As predicted by the PRCH, *P. moluccensis* only generalised recognition to closely related species, within the same genus as the reference predator. By restricting how far they generalised recognition of *T. lunare* they were able to enhance their chances of correctly generalising antipredator responses to closely related predators (Ferrari et al. 2007; 2008). Previous studies have demonstrated that fathead minnows are capable of generalising to species beyond the genus level (Ferrari et al. 2007) and both wallabies (Griffin et al. 2001) and larval woodfrogs (Ferrari et al. 2009) generalised recognition to species from different orders. Whilst these differences between the various studies may arise from methodological idiosyncrasies (such as alarm cue concentration during conditioning) or the rate at which odours differentiate between species within the various taxonomic groups, it is likely that they reflect differences in the composition of the predator community to which prey species were exposed. The high species- and trophic-diversity of genera within tropical systems complicates the prediction of whether a closely related species is a predator or not. This coupled with the inability to distinguish between predatory status of a novel species, would explain why *P. moluccensis* generalised recognition of known predators to only the most closely related species. In contrast, minnows and woodfrogs come from systems where predators are generally closely related phylogenetically to one another, thereby increasing the chances of correctly predicting their identity (Ferrari et al. 2009). By matching the extent to which they generalise with the probability of correctly responding to a novel predator they gain the benefits of generalisation whilst minimising the potential costs of unnecessarily losing time and energy on anti-predator behaviours.

Previous studies found that the intensity of response to novel predators waned as differences between novel predator and reference predator increased (Griffin et al. 2001; Ferrari et al. 2007; Stankowich and Coss 2007). In contrast, we found *P. moluccensis* appeared to display a hypersensitive antipredator response: responding equally to the reference predator, *T. lunare*, and the odours of species recognised through generalisation, matching the findings for juvenile rainbow trout (Brown et al. 2011a). While the consistent responses to odours may reflect the inability of *P. moluccensis* to differentiate between closely related predators (Brown et al. 2011a) the lack of differentiation between species might relate to how individuals perceive risk within their environment.

Response patterns are dependant not only current perception of risk (Kelley & Magurran 2003a,b) but also the predation history to which populations have been exposed (Magurran et al. 1992; Brown et al. 2009). Given the high levels of predation experienced following settlement (Almany & Webster 2006) it may be beneficial to be cautious in how far recognition is extended but response strongly to those species they extend recognition to (Helfman & Winkleman 1997; Brown et al. 2009). However, as predation risk fluctuates through time prey may shift between threat-sensitive and hypersensitive responses to maintain maximal fitness (Brown et al. 2006).

P. moluccensis were unable to distinguish between predators and non-predators when generalising recognition, matching the findings for velvet gecko's using olfactory cues (Webb et al. 2009; 2010) but contrasts with findings for tammar wallabies (Griffin et al. 2001) using visual cues. The disparity between the findings for olfactory and visual cues suggests that prey gain different information from each sensory mode. Visual cues appear to provide predator-specific cues (Griffin et al. 2001). Prey may discriminate between aspects of morphology or behaviour that are functionally specific to predators, allowing them to differentiate between predators and non-predators. Predators that are closely related share specific aspects of their morphology that allow efficient detection, capture and handling of prey, such as eye shape and location, mouth shape and size, or body silhouette (Karplus and Algom 1981; Karplus et al.1982; Blumstein et al. 2000; Griffin et al. 2001; Stankowich and Coss 2007). Our results suggest that such functionally distinct cues are missing from non-diet related predator odours. Future studies should manipulate the availability of predator cues from multiple sensory modes to assess how prey use particular sources of information differentially to assess predation risk within their environment.

While controlled for in the present study, diet cues have the potential to act as olfactory markers of the trophic level of the cue source. As previously mentioned, closely related prey often have a significant overlap in diet composition (Cowman et al. 2010). Prey may therefore be able to recognise differences in diet cues to distinguish between predators and non-predators. Numerous studies have demonstrated the importance of diet cues in predator recognition (Mirza and Chivers 2003c; Ferrari et al. 2010a) and a recent study has even demonstrated that there are predator specific components to urine for carnivorous mammals (Ferrero et al. 2011). 2-phenylethylamine purified from bobcat urine elicited anti-predator responses in rats and mice and was found to be present in high concentration across a range of mammalian carnivores but in low concentrations in herbivores (Ferrero et al. 2011). Such chemical compounds may act to supplement information gained from the predator odour alone, allowing prey to generalise predator recognition across a wide range of predators.

The present study demonstrates that even in highly complex communities, it is still advantageous to generalise predator recognition to closely related species. The extent to which individuals generalise may be a result of their species experience with predators over multiple generations. Interestingly, fish generalised recognition to both predators and non-predators despite the fitness costs (i.e. energy expenditure, lost opportunities to forage or mate etc.) associated with misidentifying non-predators. By being cautious and restricting the extent to which they generalise individuals are able to balance the costs of mislabelling non-predators with the benefits of generalisation. Juvenile fish are subjected to a predation induced bottleneck in the first few days following settlement onto coral reef (60-90% mortality; Almany and Webster 2006). At such critical times during development there is a need to prioritise survival in order to enhance overall fitness. At such times over-estimating risk is likely to be beneficial. Predator recognition can then be refined to enhance fitness with experience (e.g. learnt irrelevance; Hazlett 2003) and latent inhibition (Ferrari and Chivers 2006a, Chapter 5) or by using additional information from other sensory systems (Ferrari et al. 2008b).

Chapter 5: Friend or foe? The role of latent inhibition in predator and non-predator labelling by coral reef fishes

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Authors: M. D. Mitchell, M. I. McCormick, M. C. O. Ferrari, D. P. Chivers

5.1 Summary

In communities of high biodiversity, the ability to distinguish predators from non-predators is crucial for prey success. Learning often plays a vital role in the ability to distinguish species that are threatening from those that are not. Many prey animals learn to recognize predators based on a single conditioning event whereby they are exposed to the unknown predator at the same time as alarm cues released from injured conspecifics. The remarkable efficiency of such learning means that recognition mistakes may occur if prey inadvertently learn that a species is a predator when it is not. Latent inhibition is a means by which prey that are pre-exposed to an unknown species in the absence of negative reinforcement can learn that the unknown animal is likely not a threat. Learning through latent inhibition should be conservative because mistakenly identifying predators as non-predators can have fatal consequences. In this study we demonstrated that a common coral reef fish, lemon damselfish, *Pomacentrus moluccensis* can learn to recognize a predator as non-threatening through latent inhibition. Furthermore, we showed that we could override the latent inhibition effect by conditioning the prey to recognize the predator numerous times. Our results highlight the ability of prey fish to continually update the information regarding the threat posed by other fishes in their vicinity.

5.2 Introduction

Learning allows individuals to make decisions based on prior experience, providing them with a means to respond to changes and fluctuations in the environment in a way that will increase their fitness and survival (Davey 1989). Through learning, prey individuals are able to identify new predators, respond to changes in predator community structure and assess predation risk as it fluctuates in space and time (Kelley and Magurran 2006; Lima and Dill 1990). As a result, prey can fine-tune their anti-predator behaviour to match the current risk and thus balance the costs of predator avoidance with fitness promoting behaviours, such as foraging and mating (Lima and Bednekoff 1999). The ability to assess local predation risk through learning increases an individual’s chances of survival (Mirza and Chivers 2003b).

Chemosensory information provides prey from aquatic environments with a reliable source of information to assess predation risks within their local environment. Chemical alarm cues, released from the skin of an injured conspecific following a predation event, provide a reliable indication of an increase in the current level of predation risk. Detection of alarm cues elicits dramatic short term increases in antipredator behaviour amongst a diverse range of aquatic taxa, enhancing survival (Ferrari et al. 2010a). In the absence of innate predator recognition, alarm cues can be used to learn a novel predator’s identity through associative learning (Chivers and Smith 1998). Learning occurs when individuals detect the predator odour (conditioned stimulus) paired with an alarm cue (unconditioned stimulus) (Chivers and Smith 1998). Subsequent encounters with the predator odour provide individuals with an early warning about presence of a predator in their environment. Given the reliability of chemical alarm cues as indicators of predation risk, pairing an alarm cue with any novel stimulus (e.g. non-predatory goldfish, Chivers and Smith 1994) results in the stimulus being recognised as a predation threat. To date there are no studies that show that the pairing of a chemical alarm cue with a novel stimulus fails to label the stimulus as a predation threat (Ferrari and Chivers 2006a).

The persistence of chemical alarm cues and dispersive nature of chemicals in water (Hazlett 1999) means that conspecifics from a wide area have the potential to detect alarm cues and associate them with any local novel odour. If any novel stimulus can be labelled as a predation threat by pairing it with a chemical alarm cue, the potential exists for individuals to falsely learn to recognize irrelevant cues as a risk. Responding to non-threatening odours will be detrimental to an individual's fitness. Benefits gained from learning predator identities will be negated if individuals do not possess mechanisms to prevent irrelevant cues from being perceived as a threat (Davey 1989). One such mechanism, latent inhibition, is known from the psychological literature (Shettleworth 1998). Latent inhibition occurs when an individual is exposed to a neutral (i.e., without positive or negative reinforcement) odour repeatedly, labelling it as irrelevant or inconsequential, prior to a conditioning event. As a result, the association between the odour and the unconditioned stimulus is retarded (Kaplan and Lubow 2001; Lubow and Moore 1959). Latent inhibition has shown to be effective in preventing non-predators being labelled as a threat for crayfish *Orconectes virilis* and *O. rusticus* (Acquistapace et al. 2003), fish (fathead minnows, *Pimephales promelas,* Ferrari and Chivers 2006a) and amphibians (Ferrari and Chivers 2009b; 2011).

Most aquatic prey species lack innate recognition of their potential predators (Chivers and Smith 1998); hence, latent inhibition has the potential to prevent novel predators from being correctly identified, if their odours were detected in the absence of a risk context. In aquatic environments, ontogenetic shifts in habitats are common (George 1981; Manzur et al. 2010; Mumby et al. 2004), resulting in a number of prey species encountering novel predator communities. There is therefore a high chance that an individual will encounter cues from a potential predator multiple times prior to a conditioning event. For instance, prey may encounter the odour of opportunistic predators several times prior to a predation event; predators that undergo an ontogenetic switch in prey species preference and cryptic ambush predators are likely to be present in an area a long time before a predation event occurs. The threat sensitive learning hypothesis suggests that individuals should continuously learn about predators in order to respond to fluctuations in the threat posed by them (Ferrari and Chivers 2006b). Given the potential consequences associated with a failure to learn the identity of predators, individuals that have experienced predators in previously non-threatening contexts should be able to reverse this initial inhibition and alter the risk-assessment associated with a predator.

Coral reefs support a diverse and abundant assemblage of fishes, comprising many species of predators and non-predators. After an initial planktonic phase, juvenile reef fish return to reefs and must rapidly learn to identify which fish represent a threat. A recent study demonstrated that prey fish on coral reefs are very adept at learning predators through pairing novel odours with chemical alarm cues (Larson and McCormick 2005) and can simultaneously learn multiple predators from a single predation event (Mitchell et al. 2011). The results highlight the potential for non-predators to be wrongly identified as a predation threat if they are present at the time of a predation event. The present study investigated whether latent inhibition was an advantageous mechanism in preventing or modulating learning for a common coral reef fish, the lemon damselfish, *Pomacentrus moluccensis*. Specifically, we address three questions: (1) do juvenile reef fish have an innate antipredator response to predator odours? (2) can latent inhibition prevent the learning of a novel odour in coral reef fishes? (3) if latent inhibition prevents the learning of novel odours, can the effects be reversed as an individual's experience with the odour and its associated threat increases?

5.3 Methods

Study species

Lemon damselfish, *Pomacentrus moluccensis* (family Pomacentridae), is a common planktivorous fish found in association with coral reefs throughout the Indo-Pacific region and the Great Barrier Reef, Australia. After an 18-21 d planktonic phase, they settle to the reef at a size of ~10 mm in length (Wellington and Victor 1989). *P. moluccensis* juveniles are preyed upon by multiple predators, including the brown dottyback, *Pseudochromis fuscus* (Pseudochromidae) (Beukers and Jones 1997). *P. fuscus* are small (maximum size ~ 10 cm total length) crypto-benthic predators, common on reefs

(Messmer et al. 2005). They are found in areas of high coral cover or rubble in association with damselfish.

Collection and maintenance

Fish were collected at Lizard Island, northern Great Barrier Reef (14°40'S, 145°28'E) between November and December 2009. *P. moluccensis* recruits were collected prior to settling using light traps (small trap design, Meekan et al. 2001) deployed overnight, 50-100 m away from the reef edge. As *P. fuscus* is a benthic associated predator (Messmer et al. 2005), collecting *P. moluccensis* recruits from the pelagic environment ensured that they should be naïve to *P. fuscus*. Recruits were maintained in a 60-l aquarium (64 x 41 x 40 cm) supplied with aerated seawater and maintained at ambient seawater temperatures (29^{o}C) under a 14:10 light:dark photoperiod. Fish were fed *ad libitum* twice a day with freshly hatched *Artemia* sp. and supplemented with 5/8 NRD marine food pellets (Spectrum Aquaculture).

P. fuscus were collected on scuba from the lagoon at Lizard Island using hand nets and anaesthetic clove oil mixed with alcohol and seawater. The fish were maintained as described above in 32-l aquaria (43 x 32 x 30 cm). Fish were fed twice a day with thawed bait squid.

Stimulus preparation

Fresh alarm cues were prepared daily prior to the conditioning phase (see below). One *P. moluccensis* per treatment was sacrificed by a quick blow to the head and placed in a plastic disposable dish. Using a clean scalpel blade, 15 superficial cuts were made along each flank of the fish. Fish were rinsed with 15 ml of seawater and the solution was filtered through filter paper to remove any solid material.

P. fuscus odour was prepared from two individuals (57 and 79 mm standard length (SL)) maintained in a 32-l flow-through aquaria (43 x 32 x 30 cm). They were fed twice per day for two days and then starved for two days to remove any potential alarm cues present in their guts. On day 4, the fish were moved into an aerated 32-l stimulus collection tank containing 10 l of seawater and left undisturbed for 6 h. The fish were then returned to their original tanks and the water from the stimulus collection tank was bagged and frozen in 30-ml aliquots.

Observation tanks

Conditioning and pre-exposure were done in 3-l flow-through aquaria (11 x 18 x 12 cm), containing a 2-cm layer of sand and an airstone. Recognition trials were conducted in 13-l flow-through aquaria (36 x 21 x 20 cm; mean flow: 0.6 litres/ min). Tanks contained a 3-cm layer of sand, a small shelter (terracotta pot; 5 cm diameter) at one end and an airstone at the opposite end. A feeding tube and stimulus tube were attached to the airstone tube to aid rapid dispersal of the food and chemical stimuli, whilst minimising disturbance to the fish. The front of each tank was marked with a 4 x 6 grid. Tanks were surrounded on three sides with black plastic to visually isolate the fish and a black plastic curtain was hung in front to create an observation blind.

Experimental overview

Recent studies have suggested that some larval reef fish may have an innate recognition of some predator odours (Dixon et al. 2010; Vale 2009). Hence, the first part of our study investigated if our larvae displayed an innate recognition to the predator species we used in the subsequent experiment (question 1). The second part looked at latent inhibition and its potential reversal (question 2 and 3).

Experiment 1: Innate predator recognition

This experiment consisted of two phases, a conditioning phase followed by a testing phase. After acclimating overnight in individual observation tanks, individual *P. moluccensis* were conditioned for

1 h with either 30 ml *P. fuscus* odour paired with 15 ml alarm cue or with 45ml saltwater. The flow-through system was turned off for the duration of the conditioning phase. The following day, fish were tested for their response to either *P. fuscus* odour or saltwater, using the behavioural assays; feeding rate, distance from shelter, and time spent in shelter. This produced three different treatments (n = 15 fish per treatment): conditioned with *P. fuscus* odour + alarm cue and tested with *P. fuscus* odour; conditioned with saltwater and tested with either *P. fuscus* odour or saltwater. The saltwater conditioning and saltwater recognition trials allowed us to control for the both the conditioning procedure and the injection process. Comparison of the saltwater control with the *P. fuscus* odour conditioning tested for an innate response to *P. fuscus* and assuming a non-response, controlled for the introduction of an unknown odour. Finally, comparison of the alarm cue with *P. fuscus* odour cue conditioning indicated a conditioned antipredator response to *P. fuscus* odour.

Experiment 2: Latent inhibition and reversal

Questions 2 and 3 were investigated using fish that were not used in the innate recognition trials. Both question 2 and 3 were examined using a single experiment, but the questions were answered separately. This allowed comparison among treatments and ensured that there was no temporal or recruitment pulse effect on the trials.

The experiment for latent inhibition consisted of three phases: a pre-exposure phase, a conditioning phase and a testing phase (Appendix Fig. A5.1). Fish were placed in individual 3-l tanks and acclimated over night. During the pre-exposure phase, individuals were exposed to either 30 ml of *P. fuscus* odour (6PO) or 30 ml of saltwater (6SW) twice a day for 1 h over a 3-d period, representing six exposures in total. Following this, individuals were conditioned with either *P. fuscus* odour paired with an alarm cue (PO+AC – true conditioning) or *P. fuscus* odour paired with saltwater (PO+SW – false conditioning). The next day, individuals were tested for their response to PO alone. This 2 x 2 design allowed us to test for the effect of pre-exposure (pre-exposed to saltwater or predator odour) and conditioning cue (with saltwater or alarm cues) on the responses of fish to the predator odour. We predicted that fish that received the false conditioning (PO+SW) would not recognize the predator as threatening, regardless of the pre-exposure cues they received. Fish pre-exposed to saltwater (6SW) and conditioned with alarm cues (PO+AC) should successfully learn to recognize the predator as threatening, while the fish pre-exposed to the predator odour (6PO) should not (i.e., the latent inhibition group).

To assess the potential for reversal, we pre-exposed fish to predator odour (6PO) and conditioned them either twice (2PO+AC), three times (3PO+AC) or four times (4PO+AC). The day following the last conditioning event, individuals were tested for their response to PO alone. As treatments were randomised, pre-exposures and conditioning were staggered to allow testing to be done on the same day. We used the ‘latent inhibition’ group from above (6PO (PO+AC)) and the classical conditioning group from above (6SW (PO+AC)) as negative (response inhibited) and positive (full anti-predator response) controls respectively. Although a complete 2 x 4 design, testing for the effect of pre-exposure (saltwater or alarm cues) and number of conditioning (1 to 4) on the responses of fish to predator odour would have been more rigorous, logistic and animal limitations prevented us from doing so. We predicted that, as the number of conditioning events increased, the fish should respond to the predator odour with an increasing intensity. A total of 124 fish were tested in the 7 treatments.

Conditioning and pre-exposure protocol

The pre-exposure and conditioning treatments followed the same protocol. Individual *P. moluccensis* were allowed to acclimate over night before receiving their first treatment between 1000 h and 1100 h the following day. Fresh *P. fuscus* odour was defrosted each morning and allowed to reach ambient seawater temperature. Prior to stimulus injection, the flow-through system was turned off before the treatment period to prevent the stimuli being flushed out. To remove any stagnant water in the injection tubes 20 ml of seawater were removed and discarded, a further 20 ml were then removed. After a few minutes, we injected the relevant amount of the various stimuli, followed by 20 ml of seawater to flush the tube. The fish were left undisturbed for 1 h before the flow-through system was turned back on, to flush the tanks. We conditioned a total of 191 fish across the three experiments, up to 48 fish per day.

Recognition trials

Trials were conducted between 0730 h and 1430 h, the day after conditioning. Each trial consisted of a 5 min feeding period, a 5 min pre-stimulus observation and 5 min post-stimulus observation. Prior to the start of the trials, saltwater for stimuli injection was removed following the protocol above, a further 60 ml was also removed from the feeding tube. The flow-through system was then turned off. At the start of the 5 min feeding period, we injected 2.5 ml of food (an *Artemia* solution containing ~250 individuals per ml), followed by 20 ml of seawater (to completely flush the food into the tank), allowing the fish to reach a stable feeding rate before the pre-stimulus observation. At the start of the pre-stimulus observation, an additional 2.5 ml of food was introduced and flushed with 20 ml of saltwater. Following the pre-stimulus observation period, we injected 2.5 ml of food, flushed with 20 ml of saltwater, followed by 30 ml of stimulus odour (*P. fuscus* odour or saltwater) flushed with 20 ml of saltwater.

Behavioural bioassay for all experiments

The behaviour of the fish was observed during the pre- and post-stimulus observation periods. We quantified three response variables: foraging rate, time in shelter, and distance from shelter. Decreased foraging rate, average distance from shelter, and increased shelter use are well known antipredator responses in a number of prey species, including coral reef fishes (Ferrari et al. 2010a; Holmes and McCormick 2010). The foraging rate included all feeding strikes irrespective of whether they were successful at capturing prey. Time in shelter (in seconds) was defined as total time that the fish spent within one body length of the terracotta pot. For distance from shelter, the horizontal and vertical locations of the fish in the tank were recorded every 15 s, using the grid drawn on the side of the tank. The position of the fish in the tank was then converted into a linear distance from shelter using the dimensions of the grid squares (57 x 42 mm) and Pythagoras' theorem.

Statistical analysis

The data for the three questions were analysed using separate statistical analyses. The three behaviours were not independent from each other, thus we analysed them together using a MANOVA approach. We used the change in behaviour from the pre-stimulus baseline (post-pre) as our raw data in the analyses.

To test for innate recognition of predators by damselfish larvae (question 1), we performed a one-factor MANOVA, followed by ANOVAs and Tukey's HSD post-hoc comparisons on individual variables to compare the responses of the fish to saltwater, predator alone and to predator odour following conditioning.

To test for latent inhibition, we performed a 2 x 2 MANOVA followed ANOVAs and unequal n HSD post-hoc comparisons on individual variables, looking at the effects of pre-exposure (saltwater vs. predator odour) and conditioning cues (saltwater vs. alarm cues) on the responses of the fish to predator odour.

To test for latent inhibition reversal, we performed a one-factor MANOVA followed ANOVAs and unequal n HSD post-hoc comparisons on individual variables, comparing the responses of 5 experimental groups: the latent inhibition group (6PO (PO+AC) - negative control), the classical conditioning group (6SW (PO+AC) - positive control) and the three groups of fish receiving increasing numbers of conditioning events (6PO followed by 2PO+AC, 3PO+AC or 4PO+AC). Data for the variables foraging rate and distance from shelter were normal and homoscedastic. The data for time spent in shelter were 4th root transformed for the innate predator recognition question while the latent inhibition and latent inhibition reversal data were Log_{10} transformed to meet assumptions of normality and homoscedasticity.

5.4 Results

Experiment 1: Innate predator recognition

There was a significant effect of treatments on the response of *P. moluccensis* to the predator odour (MANOVA, $F_{6,80}$= 13.7, p< 0.001). Univariate exploration revealed that only foraging rate was affected by the treatments (foraging, $F_{2,42}$= 48.12, p< 0.0001, time in shelter, $F_{2,42}$= 3.21, p= 0.055 and distance from shelter, $F_{2,42}$= 0.39, p= 0.679). Post-hoc tests revealed that individuals conditioned with saltwater and tested for their response to saltwater did not vary in their foraging rate from those tested for their response to *P. fuscus* odour (p = 0.201 - Fig. 5.1). However, after being conditioned with *P. fuscus* odour paired with alarm cue, individuals exposed to the *P. fuscus* odour alone displayed a reduction in foraging rate compared to the other two treatments, indicative of an antipredator response (p < 0.001 - Fig. 5.1). The lack of response to *P. fuscus* odour after conditioning with saltwater suggests that individuals do not possess and innate recognition of *P. fuscus* odour.

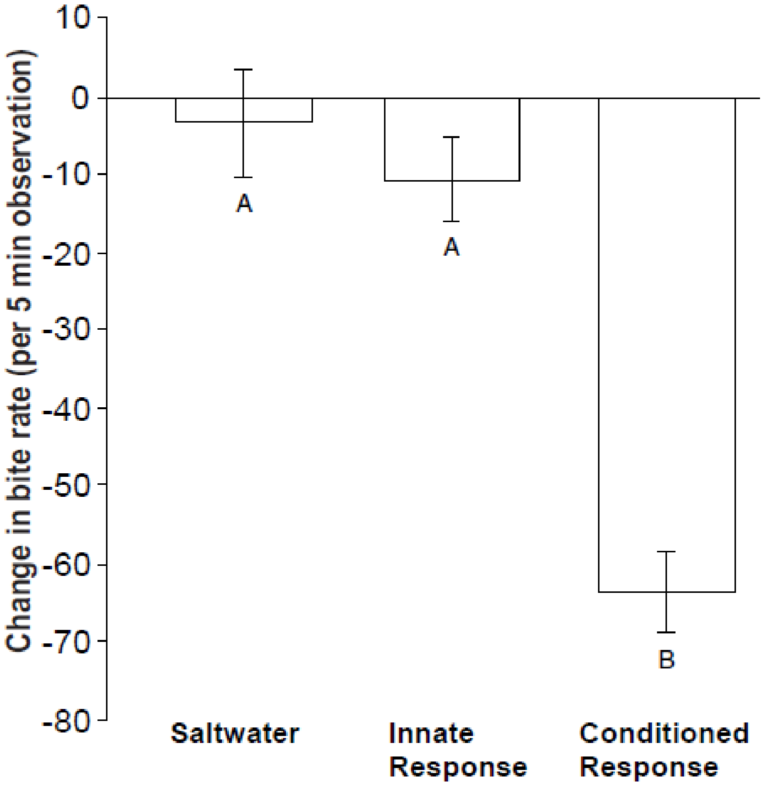

Figure 5.1. Change (+/- SE) in foraging rate for the damselfish *Pomacentrus moluccensis* in response to three treatments: tested for response to saltwater after being conditioned with saltwater (saltwater control), tested for response to *Pseudochromis fuscus* odour after being conditioned with saltwater (innate response) and tested for response to *P. fuscus* odour after being conditioned with *P. fuscus* odour paired with an alarm cue (conditioned response). Letters below bars indicate unequal n HSD groupings ($\alpha = 0.05$).

Experiment 2: Latent inhibition

The two-factorial MANOVA revealed there was no interaction between pre-exposure and conditioning cue ($F_{3,61}= 1.40$, $p = 0.251$) but there was a significant effect of both pre-exposure ($F_{3,61}= 4.29$, $p = 0.008$) and conditioning cue ($F_{3,61}= 5.27$, $p = 0.003$) on the responses of fish to the predator odour, suggesting the importance of latent inhibition. The univariate tests indicated that both foraging rate (pre-exposure, $F_{1,63}= 9.46$, $p = 0.003$ and conditioning cue, $F_{1,63}= 11.51$, $p = 0.001$) and time in shelter (conditioning cue, $F_{1,63}= 6.82$, $p = 0.011$) were affected by the treatments. Unequal n

HSD post hoc analysis revealed that individuals showed a significantly greater reduction in foraging rate after being conditioned with the 6SW (PO+AC) treatment, compared to the 6PO (PO+AC) (p = 0.005) and two control treatments (6SW (PO+SW), p = 0.002 and 6PO (PO+SW), p < 0.001) (Fig. 5.2a). Individuals from the 6PO (PO+AC) treatment did not differ in their response to the PO stimulus compared to the two controls (6SW (PO+SW), p = 0.99 and 6PO (PO+SW), p = 0.81), with individuals from all three treatments showing little difference in their behaviour during the pre- and post-stimulus observations. Unequal n HSD post hoc analysis for time in shelter revealed that there was only a significant difference between individuals from the 6SW (PO+AC) treatment and from the control 6PO (PO+SW) (p = 0.046). There was no difference between individuals from the 6SW (PO+AC) treatment and from the latent inhibition treatment, 6PO (PO+AC) (p = 0.732).

Latent inhibition reversal

There was a significant effect of treatment on the responses of individual *P. moluccensis* to predator odour (MANOVA, $F_{12,\ 190.8}$= 3.45, p < 0.001). The univariate results showed that only foraging rate was affected by the treatments (ANOVA foraging, $F_{4,76}$= 7.08, p < 0.001, time in shelter, $F_{4,76}$= 1.96, p = 0.108 and distance from shelter, $F_{4,76}$= 1.92, p = 0.116). Post-hoc analysis revealed significant differences between treatments (Fig. 5.2b). Individuals that received one or two conditionings with PO+AC failed to override the effects of latent inhibition, as they did not respond to the introduction of PO with an antipredator response. However, after receiving three or four conditionings individuals displayed an antipredator response equal to the control.

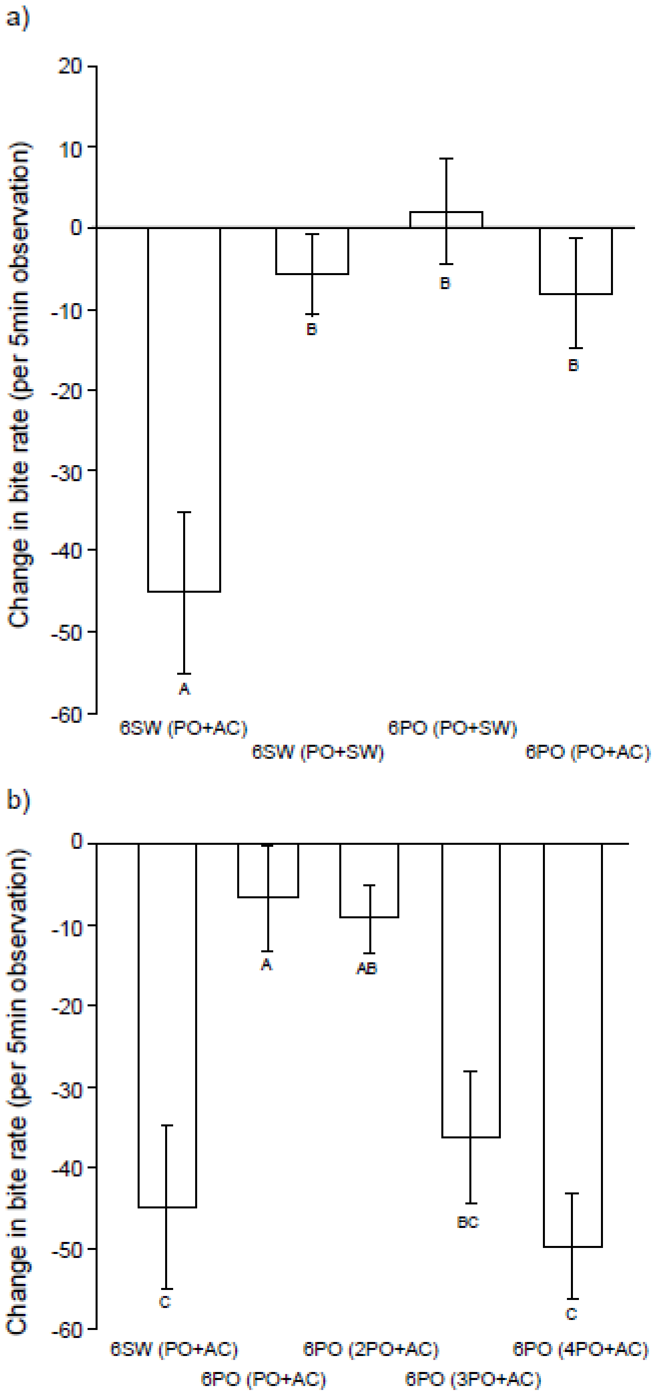

Figure 5.2 Change (+/- SE) in foraging rate for *Pomacentrus moluccensis* in response to *Pseudochromis fuscus* odour, testing for: a) latent inhibition and b) latent inhibition reversal. Individuals were pre-exposed to either *P. fuscus* odour or saltwater six times prior to being conditioned with *P. fuscus* odour paired with an alarm cue or saltwater. Letters below bars indicate unequal n HSD groupings (α = 0.05).

5.5 Discussion

This study clearly demonstrates that prior knowledge of an odour is an effective method to prevent learning irrelevant odours as a predation threat when they are detected in unison with an alarm cue. When *P. moluccensis* experienced six exposures to the odour of *P. fuscus* prior to the coupling of the odour with a conspecific alarm cue they did not respond to *P. fuscus* odour as a predation threat during subsequent encounters. This result confirms that latent inhibition is an advantageous mechanism for preventing irrelevant odours from being learnt as a risk in marine organisms, adding to findings from amphibians (Ferrari and Chivers 2009b; 2011), freshwater fishes (Ferrari and Chivers 2006) and freshwater invertebrates (Aquistapace et al. 2003). Furthermore, by using a predator known to feed on juvenile reef fishes, we were able to demonstrate that, in the absence of an innate antipredator response to predator odours, latent inhibition has the ability to prevent prey from learning the identity of potential predators. However, as an individual's experience with the odour and the associated risk increases, they are able to learn to recognize the odour as a risk, reversing the effects of latent inhibition. To our knowledge, this is the first study to demonstrate the reversal of latent inhibition in a predator/prey context. Our study showed that learning occurs in a graded way reflecting the relative increase in risk associated with the odour, and highlights that individuals learn about predators in a way that is far more complex than a simple one time conditioning with an unknown odour and an alarm cue. The fish are able to learn in a way that can identify and isolate odours that are irrelevant, but they are able to alter their responses when the context of information associated with those odours changes.

For risk assessment in aquatic environments, the benefits of latent inhibition are obvious. Individuals from environments containing a diverse array of species, such as coral reefs, are constantly surrounded by odours from a range of predators and non-predators. Following a predation event, alarm cues are released into the water column. Due to the dispersive nature of chemicals in water and the persistence of alarm cues (Hazlett 1999), there is the potential for conspecifics from a wide area to detect these cues and associate them with ambient odours. Latent inhibition allows individuals to filter out ambient odours and learn only the specific odour associated with the alarm cue as a threat. However, as demonstrated here, in the absence of an innate antipredator response to predators, latent inhibition will prevent an individual learning the identity of a predator if its odour is detected several times prior to a predation event. The consequences of such inhibition could be fatal. Situations where an individual may encounter a predator several times before encountering it in a predation event are likely to be common e.g. *P. fusucus* is a cryptic ambush predator and will be present in an environment for some time prior to its first capture of a prey individual. Other situations may include unsuccessful predation events where no alarm cues is released, opportunistic predators that only feed periodically on the focal species, predators where only part of the population target the focal species and when either the predators or prey undergo an ontogenetic switch in prey species preference or trophic group.

Individuals are able to reverse the effects of latent inhibition as they gain more information regarding risks associated with the predator. Individuals exposed to *P. fuscus* odour prior to conditioning were able to overcome the inhibitory effects and learn to respond to *P. fuscus* odour with an antipredator response after three conditionings with *P. fuscus* odour paired with an alarm cue. This result is unsurprising given the potential consequences of permanently inhibiting an individual's ability to learn about predators and the dynamic nature of the environment. Predation risk in species-diverse environments will be highly variable spatially and temporally. As a result it is adaptive for individuals to be able to respond to changes in the risk associated with a specific odour, even if this means reversing a previously fixed perception.

Individuals appeared to display a threat sensitive anti-predator response to *P. fuscus* odour with increasing numbers of conditionings, following pre-exposure. As the predation risk associated with the *P. fuscus* odour increased with further conditionings so did the antipredator response. Whilst individuals conditioned twice with *P. fuscus* odour paired with alarm cue were still inhibited, individuals conditioned three times displayed an antipredator response that was significantly greater than those conditioned once and the strongest antipredator response was record by individuals that received four conditionings. A previous study showed that, after several conditionings with predator odour paired with an alarm cue of varying strength, fathead minnows displayed an antipredator response to a predator odour that matched the intensity of the most recent conditioning event, rather than integrating the accumulated knowledge into an average response (Ferrari and Chivers 2006b). The disparity between the previous study and our own may reflect the fact that fathead minnows were responding to varying degree of risk, where as *P. moluccensis* had to overcome truly conflicting information. The graded anti-predator response displayed by *P. moluccensis* likely reflects prey gradually learning to pay attention to the *P. fuscus* odour after being conditioned to ignore it or the gradual resolution of the conflicting information represented by the *P. fuscus* odour.

Whilst this study highlights the importance of latent inhibition for individuals learning about local predation risks, it also demonstrates the importance of an individual's experience outside of a conditioning context. To date most studies have focused on how a single conditioning affects an individual's ability to learn. Such conditioning over-simplifies natural conditions. A useful avenue of study will be to investigate how an individual's numerous interactions with other species may, affect its ability to learn and retain the recognition of a specific predator. Indeed, the clear conditioning regimes used in the present experiment were used to test for latent inhibition and any potential overriding effects. Under natural conditions however, it is likely that individuals showing latent inhibition effects toward a specific predator will encounter the predator odour paired with an alarm cue interspersed with encounters with the predator odour alone. In future studies, it is therefore important to know over what time frame these processes occur and how other cues, such as visual and diet cues, aid in resolving such conflicts of information.

Chapter 6: Chemical alarm cues from different developmental stages determine antipredator responses and learnt predator recognition

This chapter was in preparation to be submitted to Behavioral Ecology

Authors: M. D. Mitchell, M. I. McCormick

6.1 Summary

How individuals assess, respond and subsequently learn from alarm cues is crucial to their survival and future fitness. Individuals can receive these cues for risk assessment from a number of sources. In order to maximise fitness they should respond to cues that are most relevant to their current situation. Many organisms exhibit ontogenetic changes in diet and foraging behaviour and they should therefore display threat-sensitive responses to alarm cues and learning about predators from different ontogenetic stages. We tested whether juvenile spiny chromis, *Acanthochromis polyacanthus*, could distinguish between chemical alarm cues originating from donors of different ontogenetic stages and whether cue origin affected its efficacy when learning about predators. Juveniles displayed a significant antipredator response when conditioned with juvenile chemical alarm cues paired with predator odour but failed to respond when conditioned with predator odour paired with either chemical alarm cues from larger conspecifics or saltwater. Subsequently, only individuals conditioned with juvenile alarm cues recognised the predator odour alone as a threat. This demonstrates that prey may be highly selective in how they use information from conspecific alarm cues, responding to and learning from only those cues that are relevant to their developmental stage.

6.2 Introduction

Despite the potential costs associated with encountering predators, learning through experience is critical for many prey species in the development of antipredator strategies. Learned, as opposed to innate, predator recognition provides individuals with the flexibility to adjust responses to match perceived levels of risk as they change through time and space (Lima and Dill 1990; Ferrari et al. 2005). In aquatic environments, chemical alarm cues (released by mechanical damage to the skin during a predation event) serve as a warning about the presence of a predator to local conspecifics and facilitate learnt predator recognition (Chivers and Smith 1998). Using chemical cues, prey may gain recognition of predators through associative learning, when they see or smell a novel predator paired with a chemical alarm cue following a predation event (Chivers and Smith 1998). On the next encounter with the predator, prey are able to recognise the predator as a threat in the absence of an alarm cue. Additionally, the concentration of alarm cues during a conditioning event have been shown to determine the level threat associated with the predator during subsequent encounters (Ferrari et al. 2006), allowing prey to match the intensity of their antipredator response with the level of threat (Helfman 1989). Such threat-sensitive responses are critical to balance the inherent costs of antipredator behaviours with those that promote an individual’s fitness (Helfman 1989; Lima and Bednekoff 1999).

While threat-sensitive learning has been demonstrated with respect to the concentration of alarm cues released during predation events, there is little understanding of how the developmental characteristics of the captured prey affects how conspecifics nearby learn about predators. Threat-sensitive learning should not only depend on the concentration of the alarm cue but also on the reliability or relevance of the indicator of risk (Blumstein and Daniel 2004; Blumstein et al 2004). Prey are exposed to a myriad of cues relating to potential predation risks, yet not all of these cues are relevant or represent an equal level of risk to the individual at any given time. For example, the perceived value of different cues will not only change between conspecific and heterospecific species (Dalesman and Rundle 2007) but will likely change throughout development, coinciding with ontogenetic shifts in habitat use and exposure to different groups of predators (Sih et al. 2000). Under such circumstances individuals should preferentially respond to and learn from individuals that will provide the most relevant information regarding which predators are currently relevant (e.g. similar sized conspecifics). As such, individuals should learn about predators using chemical alarm cues from different developmental stages in a similar way as they do chemical alarm cues of different concentrations; displaying a threat-sensitive response to alarm cues from different developmental stages.

Several studies have demonstrated that responses to chemical alarm cues from both conspecifics and heterospecifics change throughout their development to account for changes in risk perception and dietary requirements (Brown et al. 2002; Golub and Brown 2003; Marcus and Brown 2003; Mirza and Chivers 2003b; Golub et al. 2005). Research suggests that ontogeny strongly influences perception of chemical alarm cues and that individuals should preferentially learn about predators from conspecifics of a similar developmental stage. Indeed, brook charr, *Salvelinus fontinalis*, show a clear threat-sensitive response to alarm cues from different developmental stages, as juvenile brook charr responded to individuals of a similar size and age with a greater intensity than to cues from larger individuals (Mirza and Chivers 2002). However, several studies have shown that individuals respond equally to all developmental stages (Golub and Brown 2003; Brown et al. 2004; Carreau-Green et al. 2008). It seems that the variability in how prey perceive cues from different ontogenetic stages and learn from them may depend on the predator community to which they are exposed.

Little is known about the effects of ontogeny on responses to alarm cues and how individuals learn from different developmental stages in more complex environments such coral reefs. The diversity of predator communities in coral reefs is exceedingly high (Bellwood and Hughes 2001) and prey fish are targeted by an ever changing community of predators throughout development (Holmes and McCormick 2010). Consequently, prey fish are under a strong selective pressure to be highly selective when learning about predators. Recently, Lönnstedt and McCormick (2011) found that the response of juvenile damselfish (*Pomacentrus amboinensis*) to conspecific chemical alarm cues diminished with increasing size of the donor individual and juveniles failed to respond to adult alarm cues. A lack of response may suggest that juveniles do not associate cues from larger individuals with a predation risk and that they should not learn from such cues. However, it has been demonstrated that alarm cues that fail to elicit a behavioural response during a conditioning event may still provide relevant information about a predator, enhancing survival during encounters with the predator (Mirza and Chivers 2003b) and facilitate learnt recognition of a predator (Brown and Smith 1996; Brown et al. 2001b; Ferrari et al. 2005).

The aim of this study was to determine whether coral reef fishes learn about predators from conspecifics of different developmental stages in a threat-sensitive manner. The study was conducted in two stages: firstly, we tested the antipredator responses of juveniles of the damselfish, *Acanthochromis polyacanthus*, exposed to the chemical alarm cues from small (juveniles) and large (sub-adult) individuals. This enabled us to determine whether threat-sensitive responses displayed by *P. amboinensis* (Lönnstedt and McCormick 2011) occurred in other damselfishes. Secondly, we tested the juveniles to see whether they had learnt to recognise the predator after being conditioned with alarm cues from either small or large individuals. Results suggest that *A. polyacanthus* has a sophisticated learning mechanism that is highly selective in its use of information from conspecific alarm cues.

6.3 Methods

Study species

The spiny chromis, *Acanthochromis polyacanthus*, (family: Pomacentridae) is an abundant, widespread damselfish that is site attached and is one of the few reef fishes to lack a pelagic larval stage (Thresher 1983). Juveniles remain with parents until they reach approximately 20mm total length (Connell 2000) and are exposed to range of predators which among others includes *Plectropomus leopardus*, a predator known to target prey that includes all size ranges of *A. polyacanthus* (St John 1999).

Juvenile and adult *A. polyacanthus* were bred and reared from a breeding population at James Cook University MARFU aquarium facility. Both were maintained in 80 l flow-through aquaria and fed daily with NRD marine food pellets (Spectrum Aquaculture). Captive breeding ensured juveniles would be naive to the predator odours. Two dottybacks, *Pseudochromis fuscus* (Pseudochromidae; 6 cm and 7 cm standard length), a known predator of juvenile fishes (Feeney et al. 2012) were purchased commercially, maintained in individual 40 l aquaria and fed with the same NRD food pellets.

Stimulus preparation

Chemical alarm cue were prepared directly before the start of each conditioning trail. One juvenile per trail and one adult per 4 trials were sacrificed by a blow to head (following James Cook University animal ethics permit: A1067). Twelve superficial incisions were made along each flank of the fish. To ensure the amount of cue produced per trial was consistent between juveniles and adults, a piece of plastic with a 3 × 6 mm hole cut in it was placed over the adult body prior to cuts being made. The fish were then rinsed with 10 ml seawater and the solution filtered to remove any particulates.

To make the *P. fuscus* odour, the individuals were placed in separate clean tanks containing 9 l aerated seawater, at 1800 h each night and left overnight. Ten ml of water were then collected from each tank and mixed (20 ml total) prior to the start of each conditioning and testing trial.

Observation tanks

All observations were conducted in 9 l aquaria (30 × 20 × 15 cm) containing a 2 cm layer of gravel, a small terracotta pot (5 cm diameter) at one end and an airstone with an injection tube placed at the opposite end. The injection tube allowed food and alarm cues to be introduced into the aquarium with minimal disturbance. A 6 × 3 grid was drawn onto the front of the tanks and the tanks were surrounded by black plastic curtains, with a small observation hole cut into the front curtain, to visually isolate the fish from conspecifics and the observer.

Behavioural observations

The conditioning and recognition trials were conducted using the following protocol; an initial feeding period, a pre-stimulus observation period and then a post-stimulus observation period. Fish were fed 20 ml of *Artemia* (250 individuals per ml) 1 h prior to the start of each trial. The flow through system was then turned off. Four minutes before the start of the observations 3 ml of *Artemia* were injected into the tank followed by 15 ml seawater (to flush all the *Artemia* through the injection tube), in order to establish a consistent feeding rate. At the start of the pre-observation period a further 2 ml of *Artemia* followed by 15 ml seawater were injected into the tank. The focal fish was then observed for the 4 min pre-stimulus observation. After this 2 ml of *Artemia*, flushed with 15ml of seawater were injected and then the stimulus was injected into the tank followed by 15 ml of seawater. The fish was then observed again for 4 min during the post-stimulus observations. For conditioning trials the stimulus consisted of 10 ml of juvenile chemical alarm cue, adult chemical alarm cue, or saltwater paired with 20 ml of predator odour. For the recognition trials the stimulus consisted of 20 ml of predator odour or 20 ml of saltwater. At the end of each conditioning trial the fish were left to condition to the stimuli for 20 min before the flow-through system was turned back on to flush the tanks. A total of 90 individuals were conditioned and tested across the six treatments.

During the observation periods two behaviours were measured; feeding strikes and line crosses. Feeding strikes were defined as any attempted strike at food irrespective of success. Line crosses (proxy for activity) were defined as every time the entire body of the fish crossed a grid lines. Both feeding strikes and line crosses are well established antipredator behaviours for a range of fishes (Ferrari et al. 2010a).

Statistical analysis

Changes in behavioural counts (feeding strikes and lines crosses) from the pre-stimulus baseline were used as the raw data for the statistical analysis. A one-factor MANOVA was used to test for the behavioural responses to odours (juvenile alarm cue, alarm cues from large individuals and saltwater) during conditioning. Significant effects were investigated using ANOVA's and Tukey's HSD means comparisons. To test for learnt recognition of the predator, the effect of conditioning (juvenile alarm cue vs. adult alarm cue vs. saltwater) and test odour (predator odour vs. water) on behavioural responses was conducted using a two-factor MANOVA. Again, significant effects were investigated using ANOVA's and Tukey's HSD means comparisons. Residual analysis revealed all data was normal and homoscedastic.

6.4 Results

The one-factor MANOVA revealed there was a significant effect of odour type on behavioural response during the conditioning trials ($F_{4, 168} = 28.89$, $p < 0.0001$). Univariate results showed there was a significant effect on feeding strikes ($F_{2, 85} = 66.74$, $p < 0.0001$) and line crosses ($F_{2, 85} = 4.69$, $p < 0.05$). Individuals significantly reduced the number of strikes and line crosses in response to chemical alarm cues from juveniles but to no other cues (Fig. 6.1).

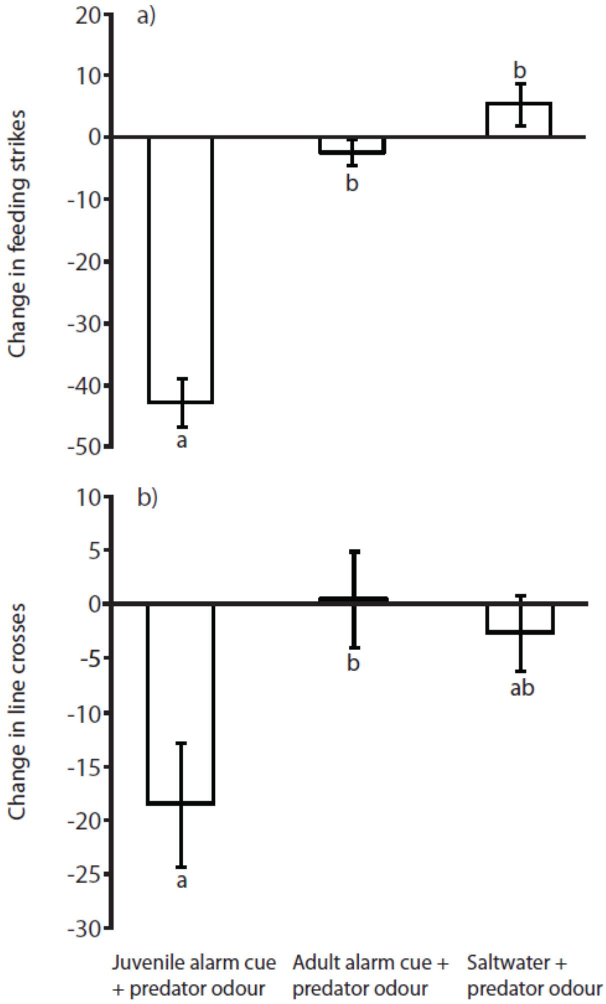

Figure 6.1. Mean change (± S. E.) in a) feeding strikes and b) line crosses of juvenile *Acanthochromis polyacanthus* in response to exposure to predator odour paired with either juvenile chemical alarm cues, adult chemical alarm cues or saltwater. Letters below bars indicate Tukey's HSD comparisons groupings.

The two-factor MANOVA showed there was a significant interaction between conditioning and test odour (table 6.1). This interaction was driven by differences in foraging rate (ANOVA, $F_{2, 82} = 30.75$, $p < 0.0001$). Individuals conditioned with predator odour paired with juvenile alarm cue and tested for their response to predator odour significantly reduced their foraging rate compared to all other treatment combinations, including those conditioned with predator odour and adult alarm cue

(Fig. 6.2). There was no significant difference in response to any conditioning by testing combination for line crosses (table 6.1), although the data showed similar patterns to those seen for feeding strikes.

Table 6.1. Comparison of the effects of conditioning and test odour on behavioural responses of juvenile *Acanthochromis polyacanthus* to different treatments; a) change in feeding strikes, b) change in line crosses.

Source of Variation	***df***	**MS**	***F***	***p***
a) Feeding strikes				
Conditioning	2	2814.6	19.732	< 0.0001
Test odour	1	3218.5	22.564	< 0.0001
Conditioning*Test odour	2	4385.9	30.748	< 0.0001
Error	82	142.6		
b) line crosses				
Conditioning	2	830.4	1.949	0.149
Test odour	1	64.3	0.151	0.699
Conditioning*Test odour	2	328.6	0.771	0.466
Error	82	426.0		

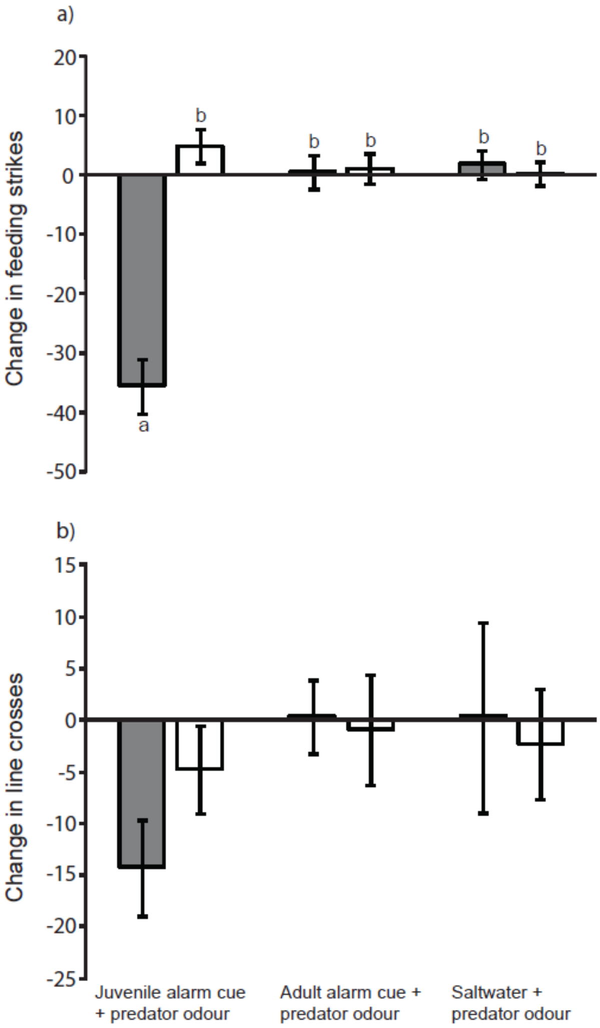

Figure 6.2. Mean change (± S. E.) in a) feeding strikes and b) line crosses of juvenile *Acanthochromis polyacanthus* in response to either predator odour (shaded bars) or saltwater (open bars) after being conditioned with predator odour paired with either juvenile chemical alarm cues, adult chemical alarm cues or saltwater. Letters below bars indicate Tukey's HSD comparisons groupings.

6.5 Discussion

These results demonstrate that juvenile reef fish are able to discriminate between the chemical alarm cues of different ontogenetic stage conspecifics and use these cues to learn about predators in a threat-sensitive manner. During the conditioning phase, juvenile *A. polyacanthus* displayed distinct antipredator responses (reductions in feeding strikes and activity) when exposed to juvenile chemical alarm cues but they did not respond to chemical alarm cues from larger individuals. Subsequently, individuals learned to fear the predator odour alone following conditioning using juvenile alarm cues but failed to recognise the predator when conditioned with alarm cues of larger individuals. These results support previous studies demonstrating that prey have the capacity to distinguish between chemical alarm cues originating from conspecifics of different developmental stages and respond to them in a threat-sensitive manner (Mirza and Chivers 2002; Lönnstedt and McCormick 2011). Additionally, to our knowledge, this is the first study to demonstrate that the use of chemical alarm cues in predator learning is dependent on the ontogenetic developmental stage of the individual from which chemical alarm cues originate.

Ontogeny clearly plays an important role in risk perception as individuals need to alter antipredator responses to account for the variable nature of risk as it changes throughout their life time. Ontogenetic shifts in response to predation cues with development have been demonstrated for a number of fish species (Brown et al. 2001b, 2002; Mirza and Chivers 2003b; Harvey and Brown 2004). For example, largemouth bass, *Micropterus salmonides*, exposed to heterospecific alarm cues show an ontogenetic switch from antipredator responses to a foraging response that reflects their dietary switch to piscivory (Brown et al. 2001b, 2002). Here we found that ontogeny had a significant effect on risk perception in juvenile *A. polyacanthus,* as juveniles displayed antipredator responses when exposed to alarm cues from similar sized individuals but failed to respond to alarm cues originating from larger individuals. Size dependant responses to chemical alarm cues have previously been demonstrated for freshwater brook char, *S. fontinalis*, (Mirza and Chivers 2002) and coral reef fish, *P. amboinensis* (Lönnstedt and McCormick 2011), with juveniles of both species displaying a stronger response to alarm cues of similar sized individuals than to alarm cues of larger individuals. Both *A. polyacanthus* and *P. amboinensis* completely failed to respond to the cues from larger individuals, unlike brook char who still responded to adult cues but with a reduced intensity compared to cues from similar sized individuals (Mirza and Chivers 2002). Additionally, several species of cichlid responded equally to conspecific chemical alarm cues irrespective of size (Golub and Brown 2003; Mirza and Chivers 2003b; Brown et al. 2004; Golub et al. 2005). It appears that, freshwater species appear to use alarm cues from all developmental stages while reef fishes are more selective, responding to only individuals of a similar size. Such result may arise from species specific responses to the predator communities found within their respective environments.

Juveniles that failed to respond to alarm cues from large individuals during the conditioning phase subsequently failed to learn to recognise the predator *P. fuscus* as threat, where as those conditioned with juvenile alarm cues responded to *P. fuscus* as a predator. These findings match similar studies looking at threat sensitive learning in response to different concentrations of chemical alarm cue (Ferrari et al. 2006; Ferrari et al. 2009). In these studies the concentration of alarm cue used during conditioning determined the intensity of the response during recognition trials with the predator odour alone (Ferrari et al. 2006; Ferrari et al. 2009). However, studies have demonstrated that individuals exposed to chemical alarm cue concentrations that fail to elicit a behavioural response may still learn to recognise a predator (Brown and Smith 1996; Brown et al. 2001b; Ferrari et al. 2005). It seems likely that chemical alarm cues from larger individuals act in similar way to very weak concentrations of chemical alarm cue. Although, it is important to note that the underlying processes that result in the threat-sensitive learning using either alarm cue concentration or alarm cues from different developmental stages are likely not the same. Studies where alarm cues of different concentration have been used to teach fish about predators have used cues produced from size matched individuals (Ferrari et al. 2005) or synthetic cues (Brown et al. 2001b). Threat-sensitive responses should therefore be based on perceived changes in the magnitude of risk associated with the concentration of an alarm cue that represents a given level of reliability regarding predation risks. Conversely, threat-sensitive responses based on alarm cues from different developmental stages are based on the preys' perception of the relevance of that specific cue to their current developmental stage.

The difference in responses and ability to learn from chemical alarm cues from different ontogenetic stages may relate to changes in the predator community composition that prey encounter throughout development. Whether or not information regarding predator activity is relevant from other developmental stages probably depends on the diversity and abundance of the predator community to which the prey community is exposed; specifically, the ratio of predators shared by both developmental stages compared to the number of predators specific to each ontogenetic stage. In freshwater systems where predator diversity is low and predators target all sizes of a prey population, responding to alarm cues irrespective of who they originate from will provide relevant information about predation risks. In coral reef systems prey are subjected to intense predation pressure from a highly diverse predator community throughout their development. While predators such as coral trout, *Plectropomus leopardus*, target a broad size range of prey (St John 1999), predators that target both small and large prey are relatively rare compared to predators that target specific size ranges of prey. In this case, prey learning from individuals substantially larger than themselves will result in prey learning and responding to predators that are not yet a relevant threat, reducing time available for activities such as feeding. Prey need to balance the need to learn and respond to current predators with the need to outgrow gape-limited predators and compete with both conspecific and hetrospecific competitors. Coral reef fish are subjected to extremely high levels of predation following settlement due to their small size (Almany and Webster 2006). There is therefore a strong selective pressure to grow and avoid gape-limited predators, to reduce their chances of being preyed upon. Additionally, the species diversity on coral reefs means that there is intense competition for resources and individuals need to grow to maintain their competitive edge (Holmes and McCormick 2006; McCormick 2009). Studies have shown that even a 0.8 mm difference in size results in significantly higher rates of mortality (McCormick 2009). Responding to predation risk is selectively advantageous only when it produces higher fitness than ignoring predation risk (Nonacs and Blumstein 2010). As only a fraction of the information available from large individual is relevant to juveniles, the cost of responding to them is potentially too high compared to just learning from individuals of a similar size.

This study demonstrates that prey display a threat-sensitive selection process in how they utilise information on predation risk from their environment, responding and learning only from cues that are relevant to them at that particular time, matching the predictions of Helfman (1989). In environments where prey are exposed to diverse assemblage of predators, many of whom only target specific size ranges of fish, individuals must be selective with regards to which alarm cues they use to learn about current predation risks. Clearly threat-sensitive responses play an important role in optimising antipredator responses, having been demonstrated for alarm cue concentration (Brown et al. 2001b; Ferrari et al. 2005), heterospecific alarm cues (Dalesman et al. 2007) and through ontogeny. By responding only to those cues that are relevant to their current situation prey enhance their chances of surviving to reproduce.

Chapter 7: General discussion

Predation is a pervasive force that shapes the behaviours and life history of individual prey and influences population patterns and dynamics on an ecosystem and evolutionary time scale (Lima and Dill 1990; Duffy 2003; Lind and Cresswell 2005; Letnic et al. 2012). Understanding the complex dynamics of predator-prey interactions is therefore central to our ability to interpret the processes that regulate communities. By exploring the role chemical alarm cues play in antipredator behaviours of early stage reef fishes, the present study identified the range of information available to prey when assessing predation risk, the mechanisms through which they can rapidly learn about potential predators in a novel environment and the mechanisms that allow them to filter out irrelevant cues ensuring they learn in an efficient way that maximises survival and increases their future fitness.

Informed risk assessment and acquired predator recognition

A central concept to risk assessment is that prey need to make well informed decisions about predation risks in their environment. By matching their antipredator responses to the apparent level of risk prey are able to reduce the costs of antipredator and maximise fitness (Helfman 1989; Lima and Bednekoff 1999; Sih et al. 2001). Prey that can detect a wider range of relevant cues will be able to make better informed decisions (Chivers et al. 2002; Dalesman and Rundle 2010). At the time of settlement when predation is particularly high (Almany and Webster 2006), the ability to innately recognise alarm cues from range of closely related heterospecific species (Chapter 2) with similar early life histories will significantly enhance an individual’s chances of detecting, responding appropriately to and surviving risky situations (Mirza and Chivers 2001a,b,d; Chivers et al. 2002). In the absence of innate predator recognition (Chapters 3-6), the capacity to respond to a range of species at settlement will improve chances of learning to recognise predators. Prey are able to further enhance their ability to assess risk with experience, acquiring recognition of distantly related heterospecific prey guild members through associative learning (Mirza and Chivers 2003a; Pollock and Chivers

2003, 2004). The potential also exists that if associations between non-related prey guild members persist over an evolutionary time scale recognition of the respective alarm cues may become innate, although such hypotheses have yet to be proven (Schoeppner and Relyea 2009).

As seen in a number of species, innate predator recognition allows prey to instantly recognise predators during their first encounter (Berejkian et al. 2003; Vilhunen and Hirvonen 2003; Hawkins et al. 2004). At the time of settlement recognising predators during the first encounter has clear benefits for survival and settlement patterns seen in some studies suggests that prey arrive on reefs with some level knowledge of predators (Dixson et al. 2010; Vail and McCormick 2011). However, prey failed to respond to predator odours (Chapter 3-6) or distinguish between predator and non-predator odours during behavioural observations (Chapter 3, 4), which would suggest prey lack an innate antipredator response to a range of predators. The lack of innate recognition is further highlighted by the finding that learned predator responses can considerably enhance survival rates for newly settled prey (Holmes and McCormick 2006; Lönnstedt et al. 2012). Although, while innate predator recognition may not be apparent from short term behavioural responses, there is the potential that innate predator recognition may result in greater retention of learned responses compared to non-predators (Chivers and Smith 1994c). That reef fishes generally lack innate predator recognition is understandable given the dispersive pelagic stage of reef fishes, potentially high diversity of predators and patchiness of predator community composition at small spatial scales. This combination of characteristics may mean that the identity of predators relevant to juvenile fishes is too variable to result in a consistent selective pressure for innate recognition to develop (Brown and Chivers 2005; Ferrari et al. 2007). Under such variable conditions learned predator recognition clearly enhances survival in reef fishes and allows fish to mould their antipredator responses to the environment in which they settle. That some juveniles appear to have some level of innate recognition of predator odours during settlement but fail to display antipredator response to the same cues during behavioural observations merits further investigation. Furthermore, assuming there is some level of innate recognition, whether this recognition is actually innate (that is genetically fixed) or arises from associative learning during embryonic development, as seen in amphibians (Mathis et al. 2008).

At settlement, coral reef fishes are targeted by multiple predators that opportunistically target recruits (Beukers and Jones 1997; Stewart and Jones 2001). Prey that are able to rapidly learn about the predators in their new environment and respond appropriately will have a greater chance of survival (McCormick and Holmes 2006; Lönnstedt et al. 2012). Recognition of alarm cues from both conspecifcs and heterospecifcs (Chapter 2), simultaneous recognition of multiple predators (Dawish et al. 2005; Chapter 3) and the ability to generalise recognition of risky situations from a learned predator to novel predators (Chapter 4) should significantly reduce the time taken to learn about local predators. Generalisation of predator recognition is particularly useful as it essentially provides prey with innate recognition of novel predators while only having to endure a single predation event (Brown et al. 2011). Through such mechanisms prey can alleviate some of the costs associated with learning and increase time available for foraging and growth. That prey fish have the capacity to rapidly acquire information about predation using a range of different methods demonstrates the potential importance of olfactory cues in risk assessment in coral reef systems.

In order to learn efficiently, prey must be able to distinguish between to odours and predators and those of no-predators that persist in the local environment. By using mechanisms such as latent inhibition, prey can effectively filter out ambient non-predator odours that they frequently encounter allowing them to learn only the novel odours that forewarn them to the presence of a predator (Acquistapace et al. 2003; Ferrari et al. 2006; Chapter 5). Like learnt predator recognition, the ability to label non-predators through latent inhibition can be further enhanced through generalisation of non-predators to other closely related species (Brown et al. 2011). Prey can also reduce the chances of learning irrelevant odours by selectively responding to and learning only from alarm cues that provide a significantly reliable level of risk (Lönnstedt and McCormick 2011; Chapter 6). For example, by learning from size matched individuals within a population prey ensure that they learn about relevant predators that represent a current predation risk (Chapter 6). A potential constraint to mechanisms like latent inhibition is that it requires multiple encounters to inhibit learning and the time period over which such processes occur is currently unknown. In addition to chemical cues, prey are able to gain explicit information regarding risk from a range of other senses (e.g. visual, vibration) and this information is to an extent specific to each sensory system (Karplus and Algom 1981; Karplus et al. 1982; Blumstein et al. 2000; Wisenden et al. 2008; Ferrari et al. 2010). While studies have looked at the synergist effects of multiple cues on risk perception, few have looked how multiple cues interact to resolve potentially conflicting information about novel predators and promote rapid recognition of predators and non-predators. It seems likely that by integrating the unique information available from different senses prey should be able to assess the potential risk of a novel predator with greater accuracy than by relying on cues available from just one sensory system.

After settlement individual survival is dependent on both the physical condition of the recruits at settlement (Holmes and McCormick 2006; Gagliano et al. 2007; Fuiman et al. 2010; Perez-Dominguez and Munch 2010) and variations in specific decisions made regarding life history strategies (Gagliano and McCormick 2007; McCormick and Meekan 2007; Meekan et al. 2010). Theory suggests that prey need to grow rapidly to avoid gape-limited predators (Anderson 1988; Ardent 1997; McCormick and Meekan 2007) and maintain competitive dominance over conspecifics (Holmes and McCormick 2006; McCormick 2009) as dominance is strongly tied to size. The rapid growth of coral reef fish juveniles compared to fishes in other systems (Fonseca & Cabral 2007) and the relationship between growth rates and survival over the first few months following settlement would suggest this is the case (Holmes and McCormick 2006; McCormick and Meekan 2010). However, studies have suggested that individuals that invest heavily in growth during the first 2-3 days post settlement are selectively removed from the population, whereas, individuals that grew slowly immediately after settlement and then invested heavily in growth had greater survival after one month (Gagliano and McCormick 2007). Being cautious initially would allow individuals to sample their new environment and learn about the potential threats. Similarly, bold individuals, a behaviour directly correlated with increased predator inspection (Pellergrini et al. 2010), survived better over a 24 h period following settlement (McCormick and Meekan 2010). Bold recruits tend to be competitive dominants and force subordinates away from safe sites (McCormick 2009). These results would suggest individuals that employ strategies whereby they can sample their environment and learn about local risks, survive better in the long run. Such behavioural strategies are mediated by an individual's ability to accurately assess the predation risks within their environment. Chapters 3-6 demonstrated some of the cognitive processes by which prey can rapidly sample their environment and resolve conflicting information. Studies that address the rate at which individuals can sample their environment and subsequently optimise their life history strategies should help to unravel the complex processes that determine who survives the mortality bottleneck during settlement.

This study has focused on the mechanisms that allow fishes to initially assess risk and learn about predators as they move into new environments or new predators arrive in their local environment, yet there is still a need to investigate how these initial learning events develop into a long term mental map of the local predation risks. The risk of predation is not static through time but varies with season, lunar cycles and diurnal cycles and minute to minute (Lima and Bedneckoff 1999; Sih 2000). Individuals that make behavioural decisions regarding risk based on previously acquired knowledge of high and low risk situations should survive better (Lima and Bedneckoff 1999; Sih 2000; Ferrari et al. 2009). Furthermore, flexible behavioural responses will allow prey to track changes in predation risk and predator community composition as they change through time. So far, coral reef fishes are able to alter their responses to predators as their level of threat changes through time (Chapter 5) and match their intensity of antipredator responses to periods of high and low risk (Bosinger et al. 2012). The extent to which individuals can track changes through time will depend on the cognitive processes that regulate retention of information and the reliability of information in the first place. The capacity for individuals to retain information appears to be flexible (Hirvonan et al. 1999; Ferrari et al. 2010b) and dependent on previous life history (Brown et al. 2011b) and the level of threat during conditioning (Ferrari et al. 2010b,c). For example, growth rate has been shown to directly affect retention of predator recognition with fast growing individuals appearing to forget predators faster than slow growing individuals (Brown et al. 2012). How individuals are able to memorise and retain predator specific recognition given the costs associated with memory in such complex and dynamic communities may provide exciting avenues to explore cognitive processing and retention of information regarding risk assessment.

Concluding remarks

This thesis demonstrates the importance of chemical alarm cues to reef fish for risk assessment and for learning about potential predators within coral reefs, especially during a critical transitional phase in their life history. Following recruitment to coral reefs, fishes arrive relatively naive of the inherent risks in their new environment but by using chemical alarm cues from both conspecifics and heterospecifics they are able to accurately recognise and respond to risky situations. Through mechanisms such as multispecies recognition, generalisation and latent inhibition individuals are able to chemical alarm cues to rapidly learn about relevant predators and avoid making the costly mistakes of misidentifying non-predators. With such sophisticated tools individuals are able to optimise their antipredator behaviours to maximise their ability to forage and grow to reproduce. By integrating our understanding of how individuals use environmental cues to respond and learn about predation threats in their environment into studies of life histories we can start to understand the mechanisms that regulate populations and community composition.

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Appendix List

A5.1 Conditioning diagram for latent inhibition and latent reversal

A8 Publications arising from the thesis

A8.1 Mitchell MD, Cowman PF, McCormick MI. Are chemical alarm cues conserved within the coral reef fish family Pomacentridae? PLoS One. DOI: 10.1371/journal.pone.0047428.

A8.2 Mitchell MD, McCormick MI, Ferrari MCO, Chivers DP (2011) Coral reef fish rapidly learn to identify multiple unknown predators upon recruitment to the reef. PLoS One 6: e15764.

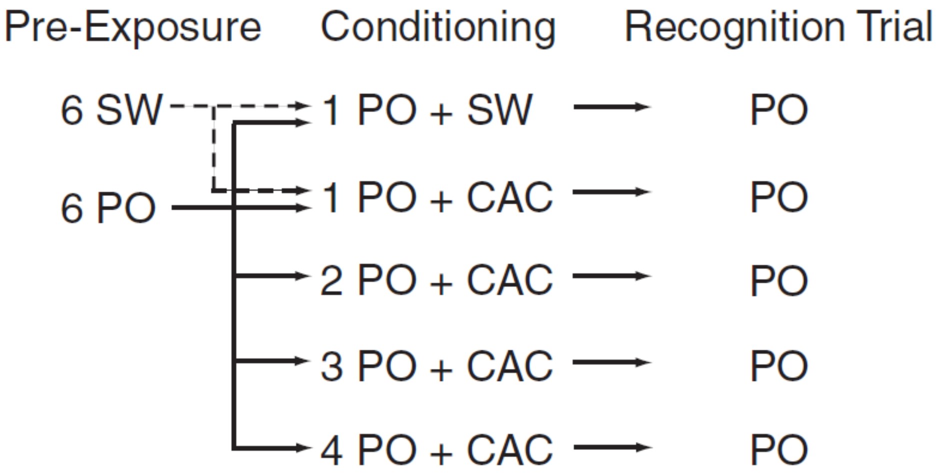
A8.3 Mitchell MD, McCormick MI, Ferrari MCO, Chivers DP. Generalisation of learned predator recognition in coral reef ecosystems: how cautious are damselfish? In final revision at the time of submission, Functional Ecology.

A8.4 Mitchell MD, McCormick MI, Ferrari MCO, Chivers DP (2011) Friend or foe? The role of latent inhibition in predator and non-predator labelling by coral reef fish. Animal Cognition 14: 707-714.

A9 Publications not arising from the thesis

A9.1 Leahy SM, Mitchell MD, McCormick MI, Ferrari MCO (2011) To fear or to feed: the effects of turbidity on perception of risk by a marine fish. Biology Letters 7: 811-813.

A5.1 Conditioning diagram for latent inhibition and latent reversal



Appendix figure A5.1. Diagram showing the combined conditioning regime used to test for both latent inhibition and latent inhibition reversal. Both the latent inhibition and latent inhibition reversal experiments were run simultaneously to allow comparison between the different treatments and control for any temporal or recruitment pulse effects. Numbers indicate the number of times individuals were exposed or conditioned with the relevant odour.

Coral Reef Fish Rapidly Learn to Identify Multiple Unknown Predators upon Recruitment to the Reef

Matthew D. Mitchell[1]*, Mark I. McCormick[1], Maud C. O. Ferrari[2], Douglas P. Chivers[3]

1 Australia Research Council Centre of Excellence for Coral Reef Studies and School of Marine and Tropical Biology, James Cook University, Townsville, Australia, 2 Department of Environmental Science and Policy, University of California Davis, Davis, Davis, California, United States of America, 3 Department of Biology, University of Saskatchewan, Saskatoon, Canada

Abstract

Organisms often undergo shifts in habitats as their requirements change with ontogeny. Upon entering a new environment, it is vitally important to be able to rapidly assess predation risk. Predation pressure should selectively promote mechanisms that enable the rapid identification of novel predators. Here we tested the ability of a juvenile marine fish to simultaneously learn the identity of multiple previously unknown predators. Individuals were conditioned with a 'cocktail' of novel odours (from two predators and two non-predators) paired with either a conspecific alarm cue or a saltwater control and then tested for recognition of the four odours individually and two novel odours (one predator and one non-predator) the following day. Individuals conditioned with the 'cocktail' and alarm cue responded to the individual 'cocktail' odours with an antipredator response compared to controls. These results demonstrate that individuals acquire recognition of novel odours and that the responses were not due to innate recognition of predators or due to a generalised response to novel odours. Upon entering an unfamiliar environment prey species are able to rapidly assess the risk of predation, enhancing their chances of survival, through the assessment of chemical stimuli.

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* E-mail: matthew.mitchell1@jcu.edu.au

Introduction

Most organisms live under the constant threat of predation throughout their lives [1]. Antipredator behaviours are energetically expensive and reduce time available for other important activities such as foraging, mating and resource defence [1,2]. As such, prey must minimise the risk of predation whilst maximising their energetic input to promote growth, reproductive success and ultimately, fitness [3,4]. Prey must therefore be able to reliably identify potential predators, their associated risk and subsequently respond in a way that will optimise the balance between these conflicting demands.

The risks associated with predation vary with both time and space and will change throughout a prey's life [5]. Most organisms undergo ontogenetic shifts during their development, often resulting in individuals switching habitats in order to exploit superior food resources, shelter and establish or enhance mating opportunities [6]. On entering a new habitat, individuals are exposed to a new community of predators, some of which may be unknown or represent a different predation risk [7,8]. The composition of potential predators within a given habitat will also change as fish grow and switch prey guilds [9,10], or as environmental changes introduce new predators [11]. Predators themselves are also highly variable in space and time, ensuring that the risk of predation is in constant flux. Thus, prey should be dynamic and flexible in their antipredator behaviour.

Prey individuals must be able to develop antipredator strategies that can be adapted to match the current predation risk. The need to adapt antipredator strategies to their current environment explains why fixed innate antipredator strategies are uncommon amongst prey fish [12,13]. Learning allows individuals to associate novel predators with danger and fine tune their antipredator responses to local environments, reducing the cost of unnecessary antipredator behaviours [1]. There is now extensive research showing that prey from a variety of taxa use learning to recognise predators and enhance their antipredator responses, including examples from fish, amphibians, reptiles, molluscs, mammals and birds [14,15].

In aquatic environments, prey fish are able to access information about local predation risk from environmental cues using their visual, olfactory and mechanical senses [16]. Predator identity can be learnt through socially transmitted information [17], direct encounters with predators [18,19], or indirectly, by associating a predator's odour with an alarm cue (chemical released by mechanical damage to the skin during a predation event) [20]. Chemosensory information provides reliable information about the identity and potential threat of unknown predators, as alarm cues are only released during direct predation encounters between predators and prey [21]. Fish are able to learn the identity of a novel predator by associative learning when the predator odour is presented simultaneously with an alarm cue released by mechanical damage to the skin of a conspecific [12,22]. Indeed, the association between a novel odour and an alarm cue is so strong that after a single encounter, prey will respond with an antipredator response to the novel odour alone. This association can last several months [23]. Furthermore, these associations can even be made using alarm cues from heterospecific fishes in the same prey guild [12].

The majority of previous studies have investigated the ability of prey to acquire recognition of just one predator at a time under various conditions. However, few environments contain a single predator, with most prey exposed to several predators at any one time [24,25]. Prey fish must be able to recognise any new potential risk of predation as fast and efficiently as possible. Learning multiple predators simultaneously would allow fast recognition of predators in a way that maximises time available for fitness promoting activities [26]. Darwish et al. [27] conditioned glowlight tetras, *Hemigrammus erythrozonus*, to a 'cocktail' of odours containing two predators and one non-predator subsequently demonstrating that tetras learnt to recognise each of the individual odours. They then proceeded to show that odours learnt in this way still confer a survival benefit. Currently it is unknown if these findings can be generalised across all fish or if they are specific to glowlight tetras.

Coral reefs are among the most biodiverse places on earth and provide a habitat for a rich assortment of fish including a vast array of predators, which can account for up to 50% of biomass in some reef communities [28]. After a pelagic developmental period, most juvenile reef fish return to coral reefs with little or no experience of the predators they will encounter. Mortality due to predation may reach nearly 60% during the first 2 days post settlement [29]. Recruiting juveniles must therefore learn the identity of local predators rapidly to survive. Recent studies have demonstrated that both juvenile and adult coral reef fish utilise chemical alarm cues to assess predation risk and to learn the identity of previously unknown predators through associative learning [30,31]. We tested the lemon damselfish (*Pomacentrus moluccensis*) at the end of the pelagic larval phase to see if they could learn the identity of multiple predators during a single conditioning event. Naïve *P. moluccensis* were conditioned with a 'cocktail' of odours from two predators and two non-predators paired with a damage-released skin extract (alarm cue) from a conspecific or a seawater control. After conditioning, they were tested for recognition of each odour in the 'cocktail', as well as the odour of a novel predator and the odour of a novel non-predator.

Methods

Ethics Statement

This research was undertaken with approval of the James Cook University animal ethics committee (permit: A1067) and according to the University's animal ethics guidelines.

Study species

Lemon damselfish, *Pomacentrus moluccensis*, are common planktivorous coral reef fish, found throughout the Indo-Pacific region and the Great Barrier Reef. They are particularly abundant on reefs around our study area, Lizard Island, Northern Great Barrier Reef, Australia (14°40′S, 145°28′E). Like many marine organisms, they undergo a planktonic phase, lasting 29 d, after which they settle to the reef [32]. At the time of settlement, they reach ~10 mm in length and are preyed upon by multiple predators [33].

Collection and maintenance

All fish were collected at Lizard Island during November and December 2009. *Pomacentrus moluccensis* recruits were collected from light traps (see small trap design, [34]) moored overnight near the reef crest, during the summer larval recruitment pulse. Recruits were captured prior to settling, 50–100 m away from the reef crest. The predators we used are associated with reefs, not open waters [35], and hence, the recruits should be naïve to the predators. *Pomacentrus moluccensis* were maintained in a 60 l aquarium (64.5×41.3×39.7 cm) supplied with aerated seawater and maintained at ambient seawater temperatures (29°C) under a 14:10 light dark photoperiod. Fish were fed *ad libitum* twice a day with freshly hatched *Artemia* sp. and supplemented with 5/8 NRD marine food pellets (Spectrum Aquaculture). Fish were maintained in the aquaria for at least one day and a maximum of two weeks prior to being placed in experimental tanks.

Three known larval fish predators, brown dottyback, *Pseudochromis fuscus* (Family Pseudochromidae), clearfin lizardfish, *Synodus dermatogenys* (Synodontidae), batu wrasse, *Coris batuensis* (Labridae), and three non-fish predators, picasso triggerfish, *Rhinocanthus aculeatus* (Balistidae), sand goby, *Amblyeleotris steinitzi* (Gobiidae), and bluespot butterflyfish, *Chaetodon plebeius* (Chaetodontidae), were collected from the lagoon at Lizard Island using hand nets, barrier nets and anaesthetic clove oil mixed with alcohol and seawater. The fish were maintained as described above in 32 l aquaria (43.2×32.4×30.5 cm). Fish were fed twice a day with thawed bait squid.

Stimulus preparation

Fresh alarm cues were prepared each day, 10 min prior to the conditioning phase. Six *P. moluccensis* were sacrificed by a quick blow to the head and placed in a plastic disposable Petri dish. Fifteen superficial vertical cuts were made along each side of the body of each fish with a scalpel blade. Each fish was then rinsed in 15 ml of seawater, yielding a total volume of 90 ml of alarm cues from the six fish. This solution was filtered through filter paper to remove any solid material prior to use.

Odours were prepared from pairs of *P. fuscus* (57 and 79 mm standard length (SL)), *S. dermatogenys* (93 and 102 mm SL), *C. batuensis* (124 and 86 mm SL), *R. aculeatus* (109 and 63 mm SL), *A. steinitzi* (65 and 53 mm SL) and *C. plebeius* (68 and 70 mm SL). Pairs of each species was placed in individual 32 l flow-through aquaria (43.2×32.4×30.5 cm). Fish were fed squid twice a day for two days and then starved for two days to remove any potential alarm cues present in their guts [36]. On the fourth day, each pair of fish from the same species was placed in a 32 l stimulus collection tank filled with 10 l of seawater, an airstone, and left undisturbed for 6 h. Following this period, the fish were moved back into the original holding tanks and the water from each stimulus collection tank was bagged in either 360 ml or 30 ml aliquots and frozen for later use.

General experimental approach

Our experiment consisted of two phases: a conditioning phase followed by a testing phase. During the conditioning phase, we conditioned individual *P. moluccensis* to recognize a cocktail of four fish odours by exposing them to 120 ml of 'cocktail' odours (30 ml from each of the four 'cocktail' species; *P. fuscus*, *S. dermatogenys*, *R. aculeatus* and *C. plebeius*) paired with 15 ml of either conspecific alarm cues (true conditioning) or a water control (pseudo-conditioning). The next day, the fish were tested for their response to one of the four fish odours present in the cocktail or alternatively, the odour of two novel species (*C. batuensis*, *A. steinitzi*). We tested 15 fish in each of our 12 treatments (2 conditioning groups×6 odours tested). Although it would have been more rigorous to test for a response to saltwater as a control for the injection process, time constraints and animal limitations prevent us from doing so. In addition, several studies on Pomacentrid fish have demonstrated that they do not respond to the injection process [19,30,31]. If our larvae have the ability to learn to recognize individual predators from a cocktail mix, then we predict that they would display an antipredator response to each of the four species originally present in the cocktail, but would not respond to the odour of the two novel species. Recent studies have suggested that larval reef fish have an innate recognition of some predators [37,38]. If that is the case, we predict that our larvae would respond more strongly to the two predatory species (*P. fuscus*, *S. dermatogenys*) than the two non-predatory species (*R. aculeatus*, *C. plebeius*). Additionally, we predict that they would also display an antipredator response when exposed to the 'novel' odour of a predator, *C. batuensis*, but not to the odour of a novel non-predator, *A. steinitzi*.

Observation tanks

Conditioning and recognition trials were conducted in 13-l flow-through aquaria (36×21×20 cm, mean flow-though = 0.6 litres/min). Each tank had a 3 cm layer of sand and a small terracotta pot (5 cm diameter) for shelter at one end and an air stone at the opposite end. Two injection tubes (a feeding tube and a stimulus tube) were attached to the airstone tube with their ends placed just above the stone to aid rapid dispersal of the chemical stimuli. The injection tubes allowed the food and stimuli to be introduced with minimal disturbance to the fish. A 4×6 grid was drawn onto the front of each tank. Each tank was surrounded on three sides with black plastic to visually isolate the fish and a black plastic curtain was hung in front of the tanks to create an observation blind.

Conditioning phase

Single *P. moluccensis* were placed into each tank to acclimate overnight and then conditioned between 1000 h and 1130 h the following day. Prior to conditioning, odours from each of the four 'cocktail' species were thawed and mixed together to form the 'cocktail' of odours containing an equal amount of odour from each species. Prior to conditioning, the flow-through system was turned off to prevent the stimuli from flushing out. After a few minutes, we injected either 15 ml of alarm cue or 15 ml of seawater paired with 120 ml of cocktail odours. The fish were left undisturbed for 1 hr, after which the flow-through system was turned on again. We conditioned a total of 180 individuals, 12 fish per day.

Recognition trials

Trials were conducted between 0730 h and 1430 h, the day after conditioning. Each trial consisted of an initial 5 min feeding period, a 5 min pre-stimulus observation and 5 min post-stimulus observation. Prior to the start of the trials, the flow-through system was turned off. Twenty ml of seawater were removed from both injection tubes and discarded to remove any stagnant water. A further 60 ml was removed from the feeding tube and 20 ml from the stimulus tube and retained for flushing. At the start of the 5 min feeding period, we injected 2.5 ml of food (an *Artemia* solution containing ~250 individuals per ml), followed by 20 ml of seawater (to completely flush the food into the tank), allowing the fish to reach a stable feeding rate before the pre-stimulus observation. At the start of the pre-stimulus observation, an additional 2.5 ml of food was introduced and flushed with 20 ml of seawater. Following the pre-stimulus observation period, we injected 2.5 ml of food, flushed with 20 ml of water, followed by 30 ml of stimulus odour, flushed with 20 ml of seawater. The stimulus odour consisted of the odour from one of the four species used in the 'cocktail' or one of the two novel species.

The behaviour of the fish was observed during the pre- and post-observation periods. We quantified three response variables: foraging rate, distance from shelter and time in shelter. Decreased foraging rate and distance from shelter and increased shelter use are well known antipredator responses in a number of prey species, including coral reef fishes [31,39].The foraging rate included all feeding strikes irrespective of whether they were successful at capturing prey. For distance from shelter, the horizontal and vertical locations of the fish in the tank was recorded every 15 s, using the grid drawn on the side of the tank. The position of the fish in the tank was then converted into a linear distance from shelter using the dimensions of the grid squares (57×42 mm) and Pythagoras's theorem. Time in shelter (in seconds) was defined as total time that the fish spent within one body length of the terracotta pot.

Statistical analysis

The changes between the pre- and post-stimulus behavioural measures were computed and used as our raw data. The effects of conditioning (alarm cues vs. seawater) and testing odours (the six fish odours) were assessed using a 2-factor MANOVA on all three behavioural responses. Univariate ANOVAs revealed that only one behaviour (foraging) was affected by treatments, so the subsequent analyses were done on the foraging variable only. Because of a significant interaction between the 2 factors, we performed two 2-factor ANOVAs, one testing the effect of conditioning and the cocktail odour only, and one testing the effect of conditioning and the non-cocktail odour, on the responses of the fish. Residual analyses revealed that all data met the assumptions of homogeneity of variance and normality.

Results

The 2-factor MANOVA revealed a significant interaction between conditioning cues and testing odours on the behavioural response of *P. moluccensis* (conditioning×species, $F_{15,\ 458.7} = 3.3$, $p<0.0001$). Univariate exploration revealed that foraging was the only behaviour affected by the treatments (Table 1). The 2-factor ANOVAs performed on the 'cocktail' odours only revealed a significant effect of conditioning ($F_{1,112} = 116.0$, $p<0.0001$), but no effect of species ($F_{3,112} = 0.2$, $p = 0.880$) and no interaction between the two factors ($F_{3,112} = 0.2$, $p = 0.910$) on the foraging behaviour of *P. moluccensis*, indicating that the fish learned to recognize the four cocktail species as threatening, and responded to all four with the same intensity (fig. 1). Conversely, the 2-factor ANOVA performed on the response to two novel odours revealed no effects of conditioning ($F_{1,56} = 0.1$, $p = 0.770$), no effect of species ($F_{1,56} = 1.9$, $p = 0.168$) and no interaction between the two factors ($F_{1,56} = 0.8$, $p = 0.368$), indicating that the fish did not show an antipredator responses to those 2 odours (fig. 1).

Discussion

Our results highlight that juvenile reef fish that are naïve to predators have the ability to rapidly learn multiple unknown predators upon recruitment to the reef. *Pomacentrus moluccensis* recruits conditioned with a 'cocktail' of four odours (predators- *S. dermatogenys* and *P. fuscus* and non-predators- *R. aculeatus* and *C. plebeius*) paired with an alarm cue responded with a clear antipredator response when presented the individual odours from the 'cocktail', whereas individuals conditioned with the 'cocktail' paired with saltwater did not respond. The learning occurred after a single conditioning event. This is the first study to demonstrate rapid learning of multiple predator cues by marine organisms

Table 1. Univariate results from the 2-factor ANOVA on the effects of Conditioning and Species on behaviour.

Behaviour	Source of Variation	*df*	MS	*F*	*p*
(a) *Foraging rate*	Species	5	4603.1	8.151	<0.0001
	Conditioning	1	50601.8	89.606	<0.0001
	Species*Conditioning	5	4738.0	8.390	<0.0001
	Error	168	564.7		
(b) *Time in shelter*	Species	5	7.36	0.504	0.773
	Conditioning	1	9.34	0.640	0.425
	Species*Conditioning	5	6.99	0.479	0.791
	Error	168	14.59		
(c) *Distance from shelter*	Species	5	10.392	1.069	0.380
	Conditioning	1	9.274	0.954	0.330
	Species*Conditioning	5	5.222	0.537	0.748
	Error	168	9.725		

Comparison of the behaviour of juvenile *Pomacentrus moluccensis* in response to the odours of 6 fish species ('Species') after being conditioned with 'cocktail' odour paired with either a chemical alarm cue or saltwater ('Conditioning').

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transitioning to a new environment containing multiple novel predators.

The ability to simultaneously learn the identity of multiple predators is an efficient mechanism that allows prey to rapidly garner information regarding predation risk. Acquired recognition of predator odours enhances an individual's survival during encounters with predators whether learnt individually or simultaneously as part of a multi-predator 'cocktail' [27]. Such rapid learning is especially important for reef fishes at the time of settlement. The dispersive nature of planktonic larval reef fishes means that juveniles may settle on non-natal reefs, where the diversity and composition of predators may differ from that of their natal reefs [40]. During the first two days post settlement, as individuals learn and adapt to their new environment, mortality due to predation is at its most severe [29]. Faced with such intense predation pressure, an individual's ability to rapidly acquire predator recognition will ultimately determine who survives.

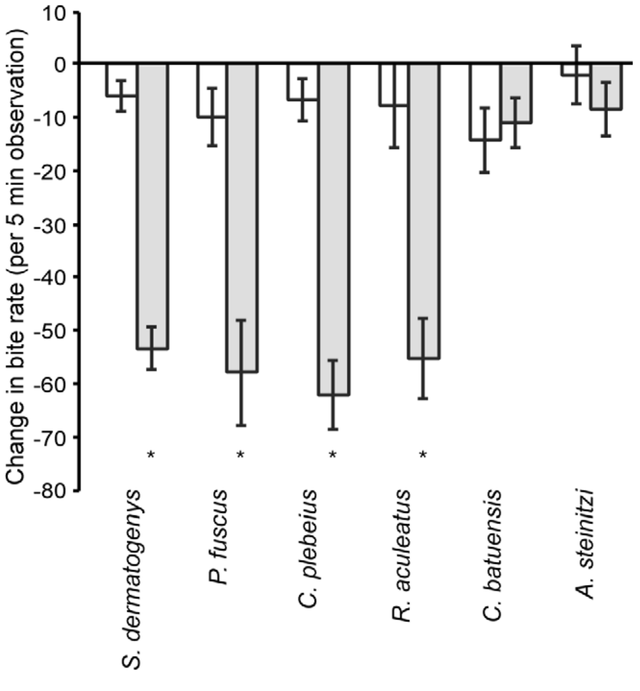

Figure 1. Change in foraging rate for *Pomacentrus moluccensis* in response to different odours. *Pomacentrus moluccensis* were conditioned with the 'cocktail' of *S. dermatogenys*, *P. fuscus*, *R. aculeatus* and *C. plebeius* paired with a) alarm cue (shaded bars) or b) saltwater (open bars) and tested for learned recognition of *S. dermatogenys*, *P. fuscus*, *R. aculeatus* and *C. plebeius* odours alone or the control odours of *C. batuensis* and *A. steinitzi*. * indicate significant differences between conditioning treatments within species.

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Prey displayed a clear antipredator response to the introduction of a 'cocktail' odour following a conditioning event where the 'cocktail' odour was paired with the alarm cue. The antipredator response was defined by a substantial decline in the foraging rate of individuals. Reductions in foraging rate in response to predator odours has been demonstrated for the closely related *Pomacentrus amboinensis* [31] and in other species across several taxa in both marine [41], freshwater [42] and terrestrial habitats [43]. Such reductions represent a shift in the balance between foraging and antipredator defence in response to an increase in the perceived risk of predation represented by recognition of an odour associated with a potential predator [12,44].

Pomacentrus moluccensis did not appear to display an antipredator response suggestive of prior recognition to any predator odours used in this experiment. Individuals conditioned with the 'cocktail' and saltwater did not respond to individual 'cocktail' odours alone. In addition, fish conditioned under either conditioning regime did not show any significant response to the novel odour of *C. batuensis*, a known predator of juvenile reef fish [45]. Recent studies on juvenile pomacentrid recruits suggest that individuals have some level of prior knowledge of predators during settlement, as naïve juveniles avoided predator odours in pairwise Y-maze trails [38] and recruits preferentially settled on habitats where the odour of predators was absent [37]. Prior knowledge of predator odours may be used during site selection by *P. mollucensis* but the odours represent a predation risk that is below their behavioural threshold and do not elicit a measurable anti-predator response until presented in combination with an alarm cue. Further work is needed to identify if pomacentrids have prior knowledge of predator odours at settlement, and if so, how this onboard knowledge is used within the decision-making framework to efficiently balance the costs and benefits of antipredator responses.

The antipredator response of *P. moluccensis* to the 'cocktail' odours was consistent across all odours (for their respective conditioning regime) irrespective of whether they originated from a predator or a non-predator. The consistency of the antipredator response to individual 'cocktail' odours is unsurprising given the apparent absence of prior knowledge of predator odours and the conditioning regime used during the associative learning of the 'cocktail' odours. Both *P. moluccensis* and glowlight tetras were simultaneously conditioned to all odours in combination with exactly the same concentration of alarm cue. During associative learning events, the strength of response to the predator odour is directly related to the concentration of the alarm cue during conditioning [46,47]. It therefore follows that in the absence of prior knowledge of predators the response to all odours should be consistent for both predators and non-predators.

Simultaneous assessment of the predation risk posed by multiple predators potentially prevents predator specific information from being assessed. Previous studies have highlighted that prey use alarm cues to assess the levels of risk associated with individual predators and are able to respond in a threat sensitive way [48]. European minnows, *Phoxinus phoxinus*, conditioned to recognise predatory pike, *Esox lucius*, or non-predatory tilapia, *Tilapia mariae*, subsequently responded to pike odour with a stronger antipredator response than to tilapia [20]. The difference was suggested to be a result of recognition of predators compared to non-predators or an artefact of evolutionary experience. As shown in this study, simultaneously conditioning prey fish to several odours results in all the odours being assigned the same level of risk. This may lead to a disproportionate behavioural response to the relative level of risk posed by the predators during future encounters. Two outcomes are possible from multi-species conditioning which will results in a net loss in fitness or survival. Firstly, predators of low risk may be labelled as a high risk and under these circumstances the prey fish will respond with an excessive anti-predator response, resulting in time and energy being wasted on unnecessary anti-predator behaviour. Secondly, predators of high risk may be labelled as a low risk predator and the prey fish will respond to its odour with an insufficient response, resulting in the prey being captured and consumed by the predator. Immediately after learning the identity of unknown predators, there is a clear need for the prey to start to fine tune its assessment of the relative risk posed by the predator during subsequent encounters. Ferrari & Chivers [49] demonstrated that minnows would fine-tune their responses to predators after several encounters but would always place more emphasis on the more recent information.

Studies on associative learning have demonstrated that any unknown stimulus can be recognised as a predation risk through associative learning. In natural environments, fishes are constantly exposed to multiple chemical odours. This study highlights the potential for ecologically irrelevant odours to be learned by association when present during a predation event. Responding to irrelevant cues will negatively impact an individual's fitness. Association of odours can be prevented with prior exposure to odours through latent inhibition [26,50] and learned irrelevance [51] or through experience and constant reassessment of acquired information [49]. However, for reef fish entering a new environment, prior exposure to odours is not possible and some irrelevant odours will be associated with a predation risk. It may pay at first to be overly cautious and learn all odours associated with an alarm cue as a predation risk when entering a new environment and then slowly learn which of those actually do not represent a threat.

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Author Contributions

Conceived and designed the experiments: MDM MIM MCOF DPC. Performed the experiments: MDM. Analyzed the data: MDM MCOF. Contributed reagents/materials/analysis tools: MIM. Wrote the paper: MDM MIM MCOF DPC.

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