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**SOCIAL LEARNING AND ITS ROLE IN  
ANTI-PREDATOR BEHAVIOUR BY  
CORAL REEF FISHES**

Thesis submitted by

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in August 2013

for the degree of Doctor of Philosophy

School of Marine & Tropical Biology

James Cook University

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## DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7<sup>th</sup> Edition 2004 (the Code)* and in compliance with the *Queensland Animal Care and Protection Act 2001 (Act no. 64 – the Act)*, *James Cook University Policy on Experimentation Ethics – Standard Practices and Guidelines (2001)*, and the *James Cook University Statement and Guidelines on Research Practice (2001)*. The proposed research methodologies received clearance from the James Cook University Experimental Ethics Review Committee (approval numbers A1067, A1593, A1595).

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## **STATEMENT ON THE CONTRIBUTION OF OTHERS**

The chapters of this thesis are also manuscripts that have been published or submitted. This work is a result of collaborations with my supervisors Prof. Mark McCormick, Prof. Douglas Chivers, Dr. Maud Ferrari and Prof. Philip Munday. As part of these collaborations I was responsible for proposal writing, project design, data collection and analysis, along with data interpretation and collation. My co-authors provided technical assistance, intellectual guidance, editorial assistance and financial support. Field assistance was provided by Rohan Brooker and Oona Lönnstedt. Laboratory assistance was provided by Danielle Dixson and Jennifer Donelson.

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They say you don't get to pick your family – but I honestly would not have picked anyone else. I love you three with all my heart and I thank you for being a constant reminder of why life is worth living.

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## GENERAL ABSTRACT

The lifetime fitness of a prey is directly affected by its ability to detect and avoid predators. However, predator avoidance is costly as it reduces the time and energy available for other fitness related activities. Prey must therefore continually modify and update their behaviour towards predators through the process of learning. As a variety of information sources are available to individuals at any one time, knowledge on how animals make decisions is essential for our understanding of animal behaviour. Often an individual's decisions are affected by the presence of others. This thesis investigates the importance of social interactions to the assessment of predation risk, using coral reef fish as model organisms.

Ignoring accurate information on predation risk could lead to death; therefore prey individuals are likely to have evolved the ability to incorporate multiple sources of information, extract important components and respond accordingly. **Chapter 2** explored how juvenile reef fish incorporate multiple sources of information to mediate their risk response and how information sources are prioritised. Naïve anemonefish (*Amphiprion percula*) were exposed to damage-released chemical cues of conspecifics and closely related congeners (*Amphiprion melanopus*), along with additional control cues in the presence and absence of a shoal (conspecifics, congeners or no shoal). *A. percula* responded with anti-predator behaviour to the chemical cues from both conspecifics and congeners, with visual cues dramatically influencing the response elicited. These findings emphasise the ability of coral reef fish to incorporate multiple sources of information into their decision making process, allowing individuals to reduce any uncertainty.

Information can be gained through the process of social learning, where less experienced individuals learn from observing and/or interacting with experienced group members. **Chapter 3** examined the role of social learning in predator recognition in relation to

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the survival of newly settled juvenile reef fish. Naïve damselfish (*Pomacentrus wardi*) were tested for their ability to socially transmit the recognition of a predator odour to conspecifics. Along with this, the study also determined whether there was a difference in the rate of survival between individuals that directly learnt the predator odour and those who acquired the information through social learning. Results showed that *P. wardi* are capable of using social learning to transmit information, with the survival outcome not significantly different from those who directly experienced predator conditioning. As such, this study demonstrates that experience plays a vital role in the outcome of predator-prey interactions, with social learning improving the ability of prey to avoid and/or escape predation.

In a natural setting social learning is likely to occur between more than 2 individuals, as such investigating the effect that group size has on the learning process is vital. **Chapter 4** determined the effect of group size on the ability of the damselfish, *Pomacentrus amboinensis* to socially learn to recognise an unknown predator. Specifically, individuals were tested to see if social learning occurred when the number of less experienced individuals (observers) was increased from 1 to 5, and if the intensity of the anti-predator response differed depending on the size of the group. Regardless of group size, *P. amboinensis* individuals were capable of socially transmitting the recognition of an unknown predator to conspecifics, with the intensity of the response not significantly different between predator-naïve observers who learnt when they were alone compared to when they were one of five observers. Social learning is therefore an important method of acquiring information about predators in aquatic ecosystems, ensuring that the value of the information is transferred in its entirety.

Along with intraspecific social learning (transmission of information between conspecifics), interspecific social learning (transmission of information between species) is likely to be commonplace in biologically complex environments such as coral reefs. Therefore, **Chapter 5** tested if social learning of predator recognition occurs among three species of coral

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reef fishes. Individuals of both *Pomacentrus moluccensis* and *Apogon trimaculatus* were tested for their ability to socially learn from *P. wardi*. Based on a single conditioning event, individuals of both species were able to learn a predator's identity from experienced *P. wardi* individuals. This ability to utilise social information from heterospecifics is likely to confer a significant survival advantage, especially for coral reef fishes as they are faced with constant and unpredictable predation pressures.

Predation pressure is highest during critical life history transitions where the suite of predators one encounters is both diverse and variable. For coral reef fishes, one of the most significant transitions occurs following a planktonic larval stage; settlement into a benthic life. As this stage occurs at night, **Chapter 6** explored whether social learning of predator recognition can occur in total darkness. Results demonstrated that predator-naïve anemonefish, *A. percula*, are capable of socially learning to recognise a novel predator when paired with a predator-experienced conspecific under both light and dark conditions. These results show that visual cues are unlikely to be the sole sensory system responsible, therefore, the study also tested whether when threatened individuals release chemical cues known as disturbance cues into the water. *A. percula* did release disturbance cues following exposure to predator odour; however these cues did not facilitate learnt recognition. It is likely that another sensory modality, possibly mechano-sensory in origin, is responsible for information transfer in the dark, with this study highlighting the diversity of sensory cues available to coral reef fishes.

This thesis demonstrates the use of social learning as an anti-predator mechanism, highlighting the importance of olfactory cues for predator recognition in biologically complex ecosystems. Obtaining accurate information on local predator identities is essential to the decision process of individuals, with the choices made ultimately determining the outcome of predator-prey interactions. Furthermore, this study demonstrates the speed with which

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information can spread through a local prey population without a dilution of importance; highlighting the role of social interactions in the cognitive processes of coral reef fishes.

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## CHAPTER 1 - General introduction

On a day to day basis animals routinely face decisions that are crucial to their fitness including: where to forage, who to mate with, where to breed and who to avoid. Subsequently, the study of how animals make decisions has become an emerging field for behavioural ecologists. One of the most important pressures placed on a prey's life is the ability to detect and avoid predators. Predation is known to significantly impact the dynamics of prey populations and communities, with the mere presence of a predator dramatically influencing a prey's use of space, access to food and allocation of energy into growth, reproduction and maintenance (Lima 1998; Sih *et al.* 1998; Nakaoka 2000). For this reason, individuals that have the ability to rapidly acquire up to date information and modify their behaviour accordingly, are likely to show an increase in life expectancy.

Predator avoidance is costly, as it reduces the time and energy available for other fitness-related activities. Therefore, individuals that are able to minimise the trade-off between predator avoidance behaviours and regular fitness-associated behaviours, and who can best assess risk, are likely to have a higher chance of survival and the highest inclusive fitness. Predation risk can vary in space and time, with predators changing seasonally, daily or from minute to minute (Lima and Bednekoff 1998), resulting in the need for prey to continuously learn new predator identities while adjusting the intensity of their response to match the level of risk posed. In some species, recognition of predators is innate (e.g. Berejikian *et al.* 2003; Wiebe 2004; Dixson *et al.* 2010), in others the identity of predators must be learnt (Brown 2003); however this is an oversimplified view with the response more likely to vary within a continuum. As such, learning plays an important role in the anti-predator responses elicited and in the adjustment of any pre-existing behaviours (Griffin 2004).

A variety of sophisticated mechanisms are used to directly assess risk, including visual, olfactory, tactile and auditory cues. However, regardless of the information source,

each has a different level of uncertainty associated with obtaining the information. For example, unlike olfactory cues, visual cues are spatially and temporally reliable, but the information may be inaccurate (e.g. cues manipulated by predators / cues hard to obtain at night or in topographically complex habitats / visual acuity differs amongst species) and the level of risk associated extreme (e.g. could result in capture and/or death). Conversely, although the risks of using olfactory cues are lower, their propagation is affected by directionality, speed and the temporal patterns of the transporting media (Bradbury and Vehrencamp 1998). As such, to reduce these levels of uncertainty, it is likely that animals incorporate multiple sources of information into their decision making process.

Information on predators can be acquired through either direct experience or through public information (also referred to as social information) made available by other individuals (Valone and Templeton 2002). Obtaining first-hand information on local predators is dangerous, since prey individuals are required to be in close proximity to potential predators, therefore it is not surprising that a significant body of research has demonstrated the ability of prey to use information from other individuals to assess risk. Information gained from others is referred to as public information, which can be acquired through signals such as alarm calls and/or chemical cues (e.g. disturbance cues and/or chemical alarm cues) (Mirza and Chivers 2000; Brown 2003; Ferrari *et al.* 2008). Species of both birds and mammals have been shown to give acoustic signals (alarm calls and/or vocalisation) to warn conspecifics about danger (Caro 2005). In aquatic environments, information on risk can come from either disturbance cues or chemical alarm cues released by nearby individuals (Mirza and Chivers 2002). Disturbance cues are ammonia compounds released by 'disturbed' prey either through the urogenital system or the gills (Kiesecker *et al.* 1999; Vavrek *et al.* 2008). These cues are known to increase vigilance when detected by conspecifics, but to date studies have failed to demonstrate the significance of these cues in learning. In comparison, the use of chemical

alarm cues in predation risk assessment is widespread, with these chemicals involuntarily released by injured prey animals during mechanical skin damage (McCormick and Manassa 2008). Chemical alarm cues are used by a range of species from multiple taxa, including but not limited to marine fishes (e.g. damselfishes (McCormick and Manassa 2008), freshwater fishes (Chivers and Smith 1998; Brown and Chivers 2006), salamanders (Chivers *et al.* 1996), and woodfrogs (Ferrari *et al.* 2007). Along with their use in ‘warning’ nearby individuals of danger, chemical alarm cues have been shown to facilitate learnt recognition of predators through temporal coupling with the chemical and/or visual cues of a predator (Brown 2003; Ferrari and Chivers 2006). A recent study by Mitchell *et al.* (2013) investigated the ability of damselfish to generalise predators based on one odour, with results demonstrating that whilst possible, recognition is limited to congeneric species. This study suggests that at the very least the chemical composition of predator cues may be species specific. However, fish do not necessarily require this coupling to learn a predator’s identity (Manassa and McCormick 2012a).

The social environment in which one lives provides a number of opportunities for learning. Regardless of whether an individual leads a primarily solitary lifestyle, social interactions are important at one point or another. The degree of sociality during an animal’s life changes as it modulates its behaviour to reflect different life stages. For example, social interactions occur for mating, mutual support and parental purposes in even the most solitary species. By being social, individuals are able to acquire public information about their environment rapidly and efficiently without having to engage in potentially hazardous or energetically costly learning trials. Learning that involves the use of public information is referred to as social learning, where less experienced individuals (observers) gain information from experienced group members (demonstrators) (Galaf and Giraldeau 2001; Griffin 2004).

Social learning has been documented in a wide range of human and non-human animals, with its use in predator recognition observed in mammals, birds, amphibians, fish and invertebrates (reviewed in Crane and Ferrari 2013). Just like direct learning there are a number of costs and benefits associated with social learning. Obvious benefits include: low risk (e.g. no need for direct interaction with a predator), the ability of information to be transferred quickly and used immediately, and like non-social learning a rapid increase in both vigilance and survival (reviewed in Crane and Ferrari 2013). Yet as socially acquired information is often based on the behaviour of others, rather than the cues on which these decisions were based, the information may actually be inaccurate, irrelevant and/or erroneous.

Social learning of predator recognition is likely to benefit individuals that live in single or mixed-species aggregations, as the suite of predators that an individual will respond to will quickly equate to the sum of the experienced across the whole group (Mathis *et al.* 1996). Given that social learning allows for the rapid learning of predator identities whilst minimising risk, it is likely that this mechanism would be utilised regularly in systems where predation pressure is constant and predator diversity is variable. Thus, given both the biology of coral reef fishes and the characteristics of coral reefs, it is likely that socially acquired predator recognition may be commonplace in this system. Prior to this thesis, only two species of coral reef fish have been tested and shown to use social learning for predator recognition, *Acanthochromis polyacanthus* (Manassa and McCormick 2012a) and *Pomacentrus amboinensis* (Ferrari *et al.* 2012). Both these studies highlighted the speed with which information on the identity and activities of a predator could be transmitted through a local prey population. The study by Manassa and McCormick (2012a) also demonstrated that the response of the observer to the predator odour was not significantly different to that elicited to the pure chemical alarm cue, suggesting that the importance of the information is retained through the social learning process.

Learned anti-predator behaviour is known to be an important factor driving the outcome of predator-prey interactions (Brown 2003; Kelley and Magurran 2003), with an individual's probability of survival greatly influenced by their ability to recognise and respond to predators (Lönnerstedt *et al.* 2012). Organisms with complex life-cycles, such as coral reef fishes, undergo a series of ecological and life-history transitions, with juveniles seldom learning information about their environment from their parents. Therefore, it is imperative that individuals possess a mechanism which facilitates the rapid identification of predator identities whilst minimising risk, in order to increase survival and overall fitness. McCormick and Holmes (2006) demonstrated for the first time that experience directly leads to higher survival in a study on the damselfish, *Pomacentrus amboinensis*. A further study by Lönnerstedt *et al.* (2012) also demonstrated an increase in survival for the damselfish, *Pomacentrus wardi*. Prior to this thesis the link between social learning of predator identities and survival trajectories was unexplored for any animal species.

Social learning provides individuals with an efficient form of information transfer, adding to the multiple sources of information on which individuals are likely to base decisions, regardless of its accuracy. The majority of experiments following a three-stage process: (1) conditioning of a naïve demonstrator; (2) pairing of the experienced demonstrator with a naïve observer (social learning stage); and (3) testing the observer for learning. Use of this protocol provides researchers with a quantifiable measurement of the capacity for species to socially learn from one another; but in natural systems a 1:1 demonstrator to observer ratio is unlikely. One would assume that the higher the number of knowledgeable individuals (demonstrators) the more effective the information transfer. Prior to this thesis, the only study which tested this theory in fish refuted this. The study conducted by Vilhunen *et al.* (2005) showed that social learning only occurred in Arctic charr (*Salvelinus alpinus*) if there was a lower number of demonstrators. During the study the size of the group did not change with 20 individuals used

during each trial (either a demonstrator to observer ratio of 10:10 or 16:4). In a natural system it is likely that a range of group sizes will exist, with a common assumption being that as you increase group size you decrease the probability of an individual being captured and thus the per capita risk across the whole group (Blumstein *et al.* 1999). Under these conditions it may be possible that the intensity of the learned behaviour is decreased to reflect a reduction in perceived risk. With social interactions playing a pivotal role in the day to day activities of coral reef fish these organisms are an ideal study species to examine this theory.

During periods of high predation pressure, individuals that are capable of detecting and responding to the cues of other species are likely to demonstrate a significant survival advantage. To date, studies have focused almost exclusively on the transmission of information between conspecifics (Ioannou *et al.* 2006); however intraspecific social learning is only one way in which naïve individuals can learn the identities of novel predators. Examples of interspecific social learning (cross-species responses) in aquatic environments whilst rare (e.g. tadpoles (Ferrari and Chivers 2008) and freshwater fishes (Mathis *et al.* 1996)), have been observed in ecologically simple ecosystems, where the number of species that could act as demonstrators was relatively few (Vieth *et al.* 1980; Mathis *et al.* 1996; Ferrari and Chivers 2008). In contrast, the diversity level and overall species richness of coral reefs differ remarkably to freshwater habitats with the Great Barrier Reef known to have over 1500 different fish species (Australian Government – Department of Sustainability, Environment, Water, Population and Communities 2013). Given this diversity and abundance, the opportunities for gaining information are increased, with conspecific or heterospecific individuals almost always in each other's field of view. Consequently, it is suggested that the number of encounters required to gain accurate information may be greater in complex habitats.

Following a larval stage spent in the pelagic, juvenile coral reef fishes are faced with the challenge of locating a suitable benthic settlement site. For most reef fishes this process, from a planktonic to a benthic life, occurs at night (Dufour and Galzin 1993). Once settled, individuals are faced with a myriad of potential predators and non-predators, necessitating the need to identify those which represent a threat and those which do not. Mortality during settlement is exceptionally high, with >60% of individuals preyed upon within the first 48 hours (Almany and Webster 2006). Therefore, if social learning is playing a vital role in the recognition of predator identities it is likely that individuals would utilise this mechanism during this period. Visual cues are unlikely to be the sole sensory system responsible for information transfer in low light conditions; therefore it is possible that individuals rely on other sensory systems during this stage. Fishes may be using either chemical (e.g. disturbance cues) or mechanosensory cues to socially learn, but it is more likely that given the importance of accurate information, individuals are incorporating information from all available cues.

The overall objective of this thesis was to examine the use of social learning as an anti-predator mechanism in coral reef fishes. To address this I conducted five independent but related studies, which each investigate the importance of social interactions to the assessment of predation risk, using coral reef fish as a model organism.

The consequences of ignoring accurate information on local predators could be deadly, so it is likely that individuals have evolved the ability to incorporate multiple sources of information. Visual and chemical cues are known to play a vital role in aquatic systems, therefore **Chapter 2** assesses the use of these senses in the decision making process. This study uses the anemonefish, *Amphiprion percula*, to determine the relative importance of visual and chemical cues from both conspecifics and congeners (*Amphiprion melanopus*).

Prey individuals are capable of acquiring information on predation threats via observation and/or interaction with other non-prey individuals through the process of social

learning. **Chapters 3 to 6** address for the first time the importance of social learning as an anti-predator mechanism in coral reef fish. Specifically, **Chapter 3** examines the role of social learning in predator recognition in relation to the survival benefits for the damselfish, *Pomacentrus wardi*. This study also assesses whether there is a difference in the rate of survival between individuals that directly learnt the predator odour and those who acquired the information through social learning.

Coral reef fishes are highly social; therefore it is unlikely that social learning occurs in the presence of only 2 individuals. **Chapter 4** investigates whether group size affects the ability of individuals to socially learn. This study aimed to determine if social learning occurs in the damselfish, *Pomacentrus amboinensis*, and if so, did an increase in the number of less experienced individuals (observers) influence the acquisition of information.

Not only are coral reefs home to a large density of individuals, but also a large diversity of species. Therefore, it is likely that individuals would benefit from transmitting information between closely related and phylogenetically distant species. **Chapter 5** explores this idea by testing the ability of the damselfish, *P. wardi*, to socially transmit the recognition of an unknown predator to a closely related damselfish species (*Pomacentrus moluccensis*) and a distantly related cardinalfish species (*Apogon trimaculatus*).

Lastly, if social learning is important for predator recognition in coral reef fishes, it is likely to be utilised during settlement, where predation pressure is highest. As settlement usually occurs at night, **Chapter 6** determined whether the anemonefish, *A. percula*, was capable of social learning in total darkness. Further, this study tested whether, when threatened individuals release chemical cues, known as disturbance cues into the water, and if these cues facilitate learnt recognition.

## **CHAPTER 2 – Coral reef fish incorporate multiple sources of visual and chemical information to mediate predation risk**

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### **2.1 Summary**

Behavioural ecology is rife with examples of the way in which prey animals make decisions to alter when, where and how they forage or reproduce in response to variation in predation risk. Given that animals cannot have perfectly accurate information regarding the relative costs and benefits of each decision made, the process of decision making is fraught with uncertainty, particularly given that different sources of information will have different levels of risk associated with them. The consequence of ignoring accurate predator information is potentially death; therefore animals should have evolved the ability to incorporate multiple sources of information, extract important components from each source and respond accordingly. Here, I showed that the anemonefish, *Amphiprion percula* responds with anti-predator behaviour to damage-released chemical cues from conspecifics and congeners. However, the visual cues provided by the presence or absence of conspecifics and congeners dramatically influenced the way in which individuals responded to chemical indicators of risk. The results suggest that anemonefish have a complex decision making process that incorporates multiple sources of information each with different degrees of uncertainty.

## 2.2 Introduction

Predation is a pervasive selective agent that varies over both space and time (Lima and Dill 1990; Ferrari *et al.* 2010a). For this reason, selection should favour those prey individuals that have the ability to rapidly acquire up to date information on predation risk within their local environment. Animals can acquire information through direct experience with predators or through information obtained indirectly by observing nearby individuals (Valone and Templeton 2002). However, regardless of the information source, there is some uncertainty about how information acquired in the past can be used to predict the level of risk in the future. Therefore, to ensure that relevant information or the opportunity for cross-referencing is not overlooked, it would be advantageous for animals to incorporate multiple sources of information into their decision making process. Here, I consider how prey fish use multiple sources of information to make decisions about how to respond to risk.

In aquatic environments, information on predation risk often comes from the presence of damage-released chemical cues produced by injured prey animals (McCormick and Manassa 2008; Ferrari *et al.* 2010a). These cues are released only when the skin is ruptured, and should act as a reliable indicator of the presence of an actively foraging predator (Chivers and Smith 1998). However, small amounts of damage-released chemical cues may be released when pathogens or parasites penetrate the skin (Poulin *et al.* 1999; Chivers *et al.* 2007), reducing the reliability of the chemical cues as indicators of predation risk. Along with this, the propagation of olfactory signals is affected by directionality, speed and temporal patterns, resulting in further uncertainty (Bradbury and Vehrencamp 1998).

As such, the use of chemical alarm cues may be both temporally and spatially inaccurate. A recent study (Chivers *et al.* 2013a) demonstrated that the rate of degradation of chemical alarm cues is highest in the afternoon, implicating solar radiation, temperature, pH and dissolved oxygen levels as possible causes for differences in the rate of degradation.

However, it is currently unknown how the rate of degradation affects the risk level associated with alarm cues. For example, are prey fishes capable of differentiating freshly released chemical cues from those released in the past? An area where chemical cues are detected is likely to be more risky than one where there are no chemical cues; however the level of risk associated with the cues may be unknown without the temporal information component.

Damage-released chemical cues can also be spatially inaccurate. If a prey is close to a predator immediately following an attack, then the concentration of chemical cues should be higher than if the attack occurred at a distance. Nevertheless, the amount of alarm cue that is released during any given predator attack is highly variable, and depends on the amount of skin that is damaged as well as the current speed and direction in which the cues are taken. Studies have shown that prey often respond with a graded response, the higher the concentration the stronger the response (Ferrari *et al.* 2010a). However, we need to be aware that there is no direct relationship between damage-released chemical cue concentration and risk; there is a degree of uncertainty.

When a prey animal is captured by a predator, and damage-released chemical cues are released, the information is available for any other species to utilise (Wisenden and Chivers 2005). Many prey animals which share the same predators often respond to each other's chemical cues (Schoeppner and Relyea 2005; Mitchell *et al.* 2012). In the case of closely related species, innate recognition of each other's chemical(s) is possible, however in more distantly related heterospecifics, prey may need to learn to recognise the damage-released chemical. The role of experience among sympatric prey has also been documented, with several studies demonstrating the response of individuals to heterospecific cues (Mathis *et al.* 1996; Ferrari and Chivers 2008; Manassa *et al.* 2012 – Chapter 5). For example, Pollock *et al.* (2003) showed that fathead minnows (*Pimephales promelas*) were capable of learning the

damage-released chemical cues of brook sticklebacks (*Culaea inconstans*) following their introduction into a previously stickleback free pond.

It is common for prey animals to use the behaviour of nearby individuals as a source of information about predator risk. Both neighbouring conspecifics and heterospecifics can be the source of such visual information. Differences in size, sex, body condition, species, parasitic load and hunger levels could potentially influence differential perception of risk and create uncertainty (Milinski 1985; Mirza *et al.* 2001; Pollock *et al.* 2006a). For example, Mirza *et al.* (2001) demonstrated a difference in response depending on sex (red swordtails – *Xiphophorus helleri*), Milinski (1985) demonstrated that competitively three-spined sticklebacks (*Gasterosteus aculeatus*) were disadvantaged if they were infected with parasites and Pollock *et al.* (2006a) highlighted the importance of species specific chemical cues (fathead minnows - *Pimephales promelas*). Along with this, it is also possible that the lack of visual acuity observed in fishes (e.g. the eyes of larval reef fish rapidly change during early development – Leis *et al.* 2011), likely contributes to the importance of visual information on coral reefs. Manassa *et al.* (2012 - Chapter 5) argued that the sources of uncertainty are the same in both simple and complex ecosystems; however the number of heterospecifics that could provide information is much larger in complex systems, making it more likely that young prey should be overly skeptical when utilising visual information from heterospecifics.

Here, I explored how anemonefish (*Amphiprion percula*) incorporate multiple sources of information to mediate their risk response and how they prioritise different information sources. A common anti-predator response of *A. percula* is a reduction in foraging and activity levels (Chivers and Smith 1998; Holmes and McCormick 2010), thus the response variables measured in this study were the number of feeding strikes and the number of line crosses. The diversity level and overall species richness of marine environments differ remarkably to freshwater habitats, particularly on coral reefs where a greater overall biomass of organisms

are present within a relatively small area. Under these space limiting conditions, predator and prey often occupy the same microhabitat, necessitating the need for accurate risk assessment. As a result, the opportunities for using visual information are increased, with conspecific or heterospecific individuals almost always in each other's field of view. Conspecifics should be an excellent source of information, but the reliability of heterospecific visual information should be minimal unless the prey has considerable experience. In this study, I exposed *A. percula* to damage-released chemical cues of conspecifics and closely related congeners (*Amphiprion melanopus*), along with additional control cues in the presence and absence of a shoal (conspecifics, congeners or no shoal). I tested a series of predictions that specifically address the use of multiple sources of information: 1) I predicted that in the absence of visual cues from conspecifics or congeners, anemonefish will respond to both conspecific and congeneric chemical cues with an anti-predator response, but there will be a stronger response to those from conspecifics; 2) I predicted that visual information about current levels of risk should be able to override chemical information as chemical information is less spatially and temporally reliable; 3) However, given their evolutionary history and limited experience with interspecific visual information, anemonefish should prioritise chemical information about risk over congeneric visual information. If the predictions about uncertainty are correct, I expect that *A. percula* will reduce the intensity of their response to chemical cues when in the presence of unalarmed conspecifics but not in the presence of unalarmed congeners.

## **2.3 Materials and methods**

### **2.3.1 Experimental overview**

In this study, I exposed *Amphiprion percula* to damage-released chemical cues of conspecifics and closely related congeners (*Amphiprion melanopus*), along with additional

control cues (platy cues or saltwater) in the presence and absence of a shoal of conspecifics or congenics.

### 2.3.2 Study species

*A. percula* (Pomacentridae) is a tropical anemonefish that inhabits inshore and lagoon reefs from Northern Queensland to Melanesia (Fautin and Allen 1994). This species is site-attached and forms size-based hierarchal groups, which consist of a monogamous pair and varying numbers of sub-adults or juveniles (Fautin and Allen 1994; Buston 2003). A closely related species, *A. melanopus* (Pomacentridae) was used as a congeneric in this study. *A. melanopus* inhabits similar locations to *A. percula*, including inshore and lagoon reefs in Indonesia, Melanesia, Micronesia, southeastern Polynesia and the Great Barrier Reef (Fautin and Allen 1994). *A. melanopus* form social groups that are much larger than *A. percula* typically consisting of multiple breeding pairs with a greater number of non-breeding sub adults (discussed in Fautin and Allen 1994).

Freshwater platys, *Xiphophorus maculatus*, were used as controls for the addition of damage-released chemical cues as *A. percula* and *A. melanopus* are known not to respond with anti-predator behaviour towards these.

### 2.3.3 Fish collection and maintenance

*A. percula* larvae were reared from adult breeding pairs obtained commercially from A1 Aquarium world (Townsville, Queensland). A total of 14 pairs were purchased and transported individually in oxygenated bags within 20 mins to the Marine and Aquaculture Research Facility (MARFU) at James Cook University. No mortality was recorded during this period. Individuals were slowly acclimated to 70 l aquaria at ambient temperatures (26°C - 29°C).

*A. melanopus* larvae were reared from adult breeding pairs collected from the Great Barrier Reef, Australia under an ethics permit obtained from the Great Barrier Reef Marine Park Authority (G03/3871.1). Individuals were caught using hand nets. Given the behaviour and burst speed of this species, a clove oil-alcohol solution was used to anaesthetise individuals enabling quick and effective capture (Munday and Wilson 1997). A total of 21 pairs were collected which constitutes less than 1% of the local population. Individuals were transported in individual bags away from direct sunlight to the Marine and Aquaculture Research Facility (MARFU) at James Cook University with no mortality recorded during this period. Individuals were slowly acclimated to 70 l aquaria at ambient temperatures (26°C - 29°C).

Adults pairs were maintained in separate 70 l aquaria and fed INVE Aquaculture Nutrition 12/20 pellets three times daily (under Ethics permit #A1595 from James Cook University). A terracotta pot was placed with each breeding pair to allow adequate surface area for egg laying. On the night of hatching (6 - 8 days post-laying, appearance of embryos indicates readiness to hatch) egg clutches (with terracotta pot) were transferred to separate 70 l aquaria. Clutch size varied but averaged 783 eggs ( $\pm$  Standard Deviation (SD): 248) per clutch, resulting in an average of 455 larvae for *A. percula* and 1166 eggs ( $\pm$ SD: 223) per clutch, resulting in an average of 548 larvae for *A. melanopus*.

Following hatching larvae were reared in a semi-closed system, with the only water flow being a slow flush of filtered UV-sterilised seawater each night, until larvae were competent to settle at 11 days. By using a semi-closed system larvae were able to feed ad lib throughout the day, with any unconsumed food removed each night. The larval feeding regime consisted of providing food in proportion to the total volume of the aquaria with rotifers (*Brachionus sp.*) at 5 individuals per ml added each morning for the first 3 days and live brine shrimp nauplii (*Artemia franciscana*) at 1 individual per ml added from day 3 onwards. The

ratio of *A. franciscana* to *Brachionus sp.* was then increased each day until larvae were fed only 5 individuals of *A. franciscana* per ml from day 8. From day 11 onwards, fish were maintained in 70 l tubs (density: approx. 100 per 70 l) at ambient temperatures (26°C - 29°C), under a 12:12 light dark photoperiod, with feeding occurring twice daily (*A. franciscana* and Aquaculture Nutrition NRD 5/8 pellets).

Adult freshwater platys, *X. maculatus*, were obtained commercially from A1 Aquarium world (Townsville, Queensland) and maintained in 20 l tubs (density: approx. 5 individuals per tub) of dechlorinated fresh water at approximately 22.5°C, under a 12:12 light dark photoperiod. Fish were fed ad libitum twice a day with *A. franciscana* and Aquaculture Nutrition NRD 5/8 pellets.

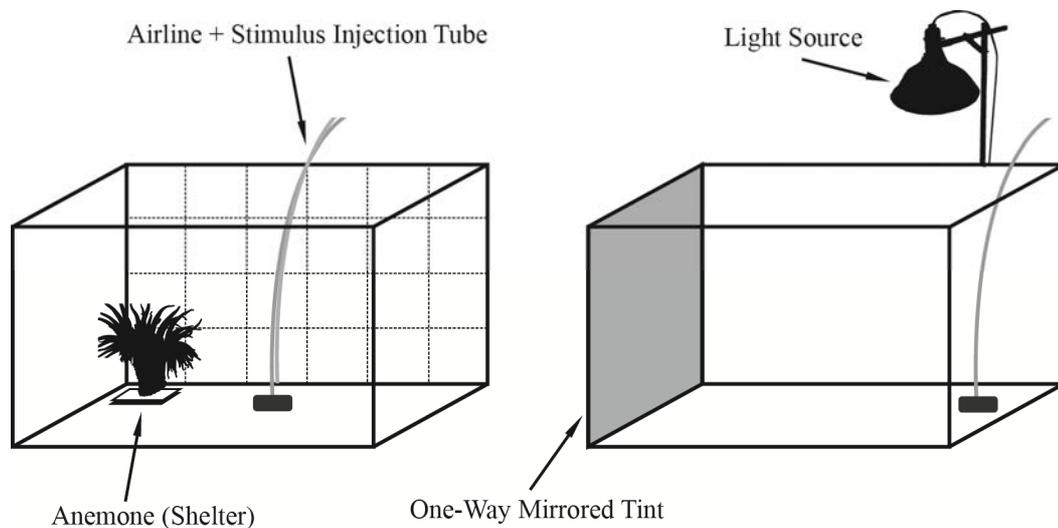
#### **2.3.4 Observation and shoaling tanks**

Experiments and behavioural observations were undertaken in 8 l observation tanks at ambient temperatures (26°C - 29°C) under a 12:12 light dark photoperiod. An air stone was placed in the center of each tank (Figure 2.1). An additional piece of plastic tubing, for cue injection, was attached to the airline with the end fixed approximately 1 cm above the air stone. Trials with dye showed that it took 12 sec for the dye to disperse through the tank. An anemone (*Heteractis magnifica*) was used as a shelter and placed into the observation tank on the left hand side, 24 h prior to experimentation. Each tank was surrounded on three sides with black plastic to avoid the test fish from observing fish in adjacent tanks. The fourth side of the tank was aligned to face an adjacent 8 l tank (shoaling tank) positioned on the right hand side of the observation tank (Figure 2.1). A one-way mirrored film was attached to the left hand side of each shoaling tank, allowing the focal fish in the observation tank to observe fish in the shoaling tank but not vice versa (with the help of a light) (Figure 2.1).

The focal fish species *A. percula* (subsample: mean Standard Length (SL)  $\pm$  Standard Error (SE): 20.61  $\pm$  2.94 mm) was housed individually in the observation tanks, with the occupants of the shoaling tanks either: three similar sized *A. percula*, three *A. melanopus* (subsample: mean SL  $\pm$  SE: 21.21  $\pm$  2.94 mm) or no shoal.

### 2.3.5 Stimulus preparation

Damage-released chemical cues were prepared according to the protocol from McCormick and Manassa (2008), using a total of 60 *A. percula* (mean SL  $\pm$  SE: 20.61  $\pm$  2.94 mm), 60 *A. melanopus* (mean SL  $\pm$  SE: 21.21  $\pm$  2.94 mm) and 30 *X. maculatus* (mean SL  $\pm$  SE: 44.9  $\pm$  5.1 mm) (Ethics permit from James Cook University #A1067). One individual *A. percula* and *A. melanopus* were used to make one chemical cue solution; however one individual *X. maculatus* was used to make two chemical cue solutions, allowing for the



**Figure 2.1** Observation and shoaling tank design.

difference in skin surface ratio between species. The number of individuals sacrificed was necessary to allow for a sufficiently strong olfactory signal to be detected. Rather than using a large number of individuals, experiments took place over several days, allowing the

individuals used the previous day in behavioural trials to be sacrificed for chemical cues and/or used as shoaling individuals. Specimens were euthanised by a quick blow to the head (brain death confirmed following no movement or response after 30 seconds), with 25 superficial (minor flesh damage) cuts made to the skin with a clean razor blade. Specimens were then rinsed in 15 ml of seawater, previously obtained from each test tank. Following this the 15 ml of damage-released chemical cue was filtered prior to use, with the cues used no longer than 20 min after preparation.

### **2.3.6 Experimental protocol**

Individuals were acclimated in the observation and shoaling tanks for 48 h, with each tank receiving live *A. franciscana* daily (~7500 nauplii per tank). Prior to the initial observation period 60 ml of tank water was drawn up the cue injection tube and discarded to remove any stagnant water. A further 135 ml was collected and kept. Immediately prior to the initial observation period 15 ml of live *A. franciscana* (approximately 1 individual per ml of tank water) was injected into the tube followed by 60 ml of previously collected tank water, to flush the tube. The behaviour of the focal *A. percula* (1 individual per observation) was then recorded for 5 min. After initial observations, 15 ml of experimental cue (either *A. percula* damage-released chemical cues, *A. melanopus* damage-released chemical cues, *X. maculatus* damage-released chemical cues or seawater) was injected into the tank, along with a further 15 ml of live *A. franciscana*. Following this 60 ml of previously collected tank water was injected, to ensure all the cue was flushed through. This was followed by a final 5 min observation period, with 20 replicates undertaken for each experimental cue and shoaling condition. The order of testing was randomised among treatments.

### **2.3.7 Quantification of behaviour**

The behavioural responses to all experimental cues were quantified by recording the frequency of two behaviours: the number of feeding strikes and the number of line crosses. The observation tanks were divided into four equal vertical areas and six equal horizontal areas (grid of 4.7 x 4.2 mm rectangles); with every line cross recorded (at least half the body must cross the line). The number of feeding strikes was recorded regardless of success, with the controls in each experiment not expected to show any changes between initial and final observation periods for the variables measured.

### **2.3.8 Statistical analysis**

ANOVA's were used to compare the response of individuals to conspecific and congeneric chemical cues in the absence of visual cues. The difference in the total counts of feeding strikes and line crosses between the initial and final 5 min observation periods were compared among the four experimental cues and three shoaling conditions with a two-factor MANOVA. To further explore the nature of the significant differences found by MANOVA, ANOVAs were undertaken on both variables (feeding strikes and/or line crosses) followed by a series of planned comparisons. Residual analysis found that the assumptions of normality and homogeneity of variance were satisfied.

### **2.3.9 Ethical considerations**

Following each trial, focal individuals were removed from the observation tanks and housed in 70 l aquaria for use as either shoaling individuals or in the preparation of experimental cues. This ensured all individuals that were sacrificed for chemical cues had been used previously in the study. Following the experiment all live individuals (adults and juveniles of *A. percula* and *A. melanopus*) were kept and maintained at the Marine and

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Aquaculture Research Facility (MARFU) at James Cook University for use by other researchers and students. This study was funded through the ARC Centre of Excellence for Coral Reef Studies under the ethics approval guidelines of James Cook University, approval #A1067.

## 2.4 Results

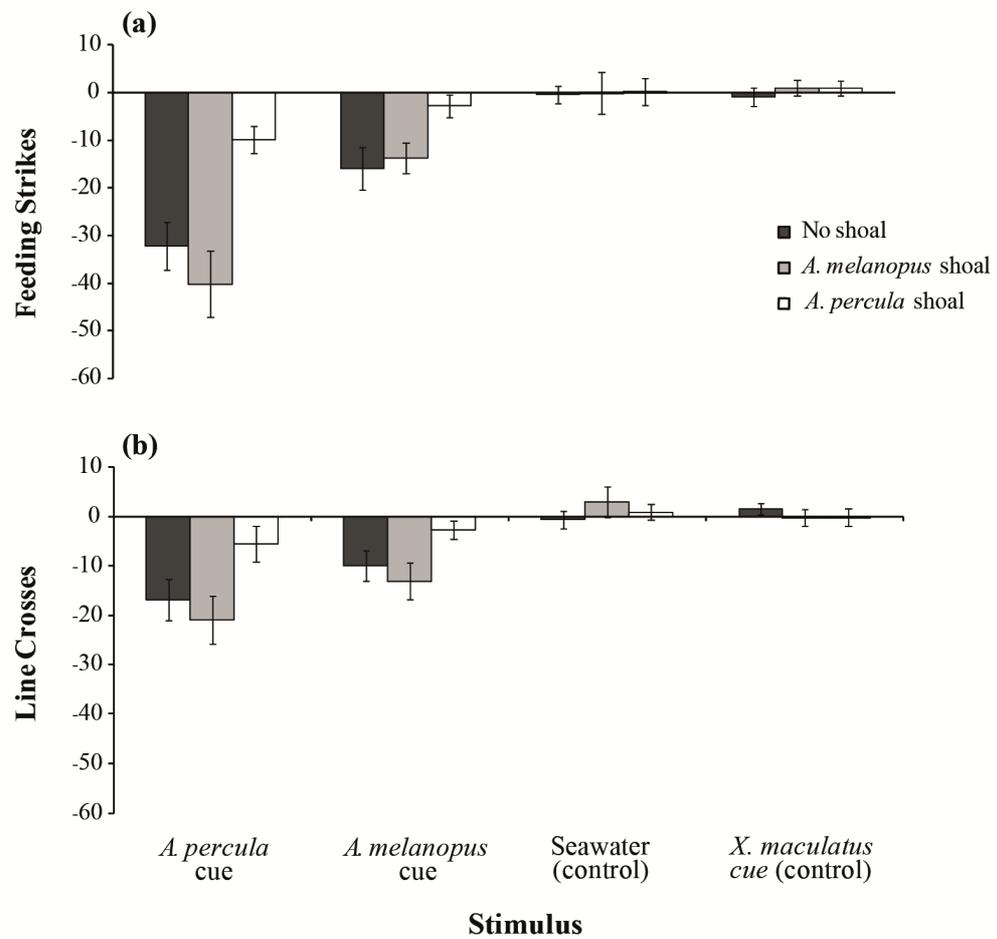
The response of individuals to conspecific and congeneric chemical cues in the absence of visual cues differed significantly in relation to feeding strikes ( $F_{1,38} = 5.93$ ,  $P = 0.02$ ; Figure 2.2), but not line crosses ( $F_{1,38} = 1.73$ ,  $P = 0.2$ ; Figure 2.2). There was a significant interaction in the change in behaviour between initial and final observation periods among the four experimental cues and three shoaling conditions (Pillai's Trace = 0.135,  $F_{12,456} = 2.760$ ,  $P < 0.001$ ; Figure 2.2). Feeding strikes ( $F_{6,228} = 4.144$ ,  $P < 0.001$ ) and line crosses ( $F_{6,228} = 2.151$ ,  $P = 0.049$ ) both caused the significant interaction observed between experimental cues and shoaling condition.

Planned comparisons revealed that *Amphiprion percula* significantly reduced activity between the control cue (*Xiphophorus maculatus* cue and seawater (all shoaling conditions)) and the congeneric (*Amphiprion melanopus*) cue, with either an *A. melanopus* shoal nearby or no shoal (feeding strikes:  $F_{1,228} = 24.979$ ,  $P < 0.001$ ; line crosses:  $F_{1,228} = 25.555$ ,  $P < 0.001$ ). This result suggests that *A. percula* is responding with a reduction in activity to congeneric cues, but the reduction only occurs when alone or in the presence of a congeneric shoal. Planned comparisons also revealed there was no significant change in activity between the control cues (*X. maculatus* cue and seawater (all shoaling conditions)) and the *A. melanopus* cue when a shoal of *A. percula* was nearby (feeding strikes:  $F_{1,228} = 0.549$ ,  $P = 0.460$ ; line crosses:  $F_{1,228} = 1.142$ ,  $P = 0.286$ ). This result shows that the response of *A. percula* to

congeneric cues is negated when a shoal of conspecifics (that are not responding) is visually present.

*A. percula* responded with a similar pattern to conspecific cues to that displayed in response to congeneric cues among shoal treatments (Figure 2.2). *A. percula* reduced activity when exposed to a conspecific cue when alone or when next to a shoal of *A. melanopus* compared to the controls (*X. maculatus* cues and seawater (all shoaling conditions)) (feeding strikes:  $F_{1,228} = 148.837$ ,  $P < 0.001$ ; line crosses:  $F_{1,228} = 65.421$ ,  $P < 0.001$ ). When exposed to a conspecific cue, there was a reduction in feeding strikes when an *A. percula* shoal was present compared to the controls ( $F_{1,228} = 6.433$ ,  $P = 0.012$ ); although the reduction was less than when in the presence of an *A. melanopus* shoal ( $F_{1,228} = 34.627$ ,  $P < 0.001$ ). There was no reduction in line-crosses when exposed to a conspecific cue in the presence of an *A. percula* shoal compared to the controls (*X. maculatus* cues and seawater (all shoaling conditions)) ( $F_{1,228} = 3.771$ ,  $P = 0.053$ ).

Lastly, planned comparisons also revealed that the magnitude of reduction when alone or near an *A. melanopus* shoal was lower when exposed to a conspecific cue than when exposed to a congeneric cue (feeding strikes:  $F_{1,228} = 34.579$ ,  $P < 0.001$ ; line crosses:  $F_{1,228} = 6.133$ ,  $P = 0.014$ ).



**Figure 2.2** Mean change ( $\pm$ SE) in (a) feeding strikes and (b) line crosses by *Amphiprion percula* between initial and final 5 min observation periods, for fish exposed to; four experimental cues (*A. percula* damaged-released chemical cues, *Amphiprion melanopus* damaged-released chemical cues, seawater or *Xiphophorus maculatus* damaged-released chemical cues) and three shoaling conditions (no shoal, three individual *A. melanopus* or three individual *A. percula*).

## 2.5 Discussion

This study highlights the ability of coral reef fish to utilise multiple sources of visual and chemical information to mediate predation risk and how they prioritise these information sources. The results indicate that *Amphiprion percula* respond with anti-predator behaviour to damage-released chemical cues from conspecifics and congeners (*Amphiprion melanopus*). However, the presence or absence of conspecifics and congeners dramatically influenced the

way in which individuals responded to chemical indicators of risk. As hypothesised in the presence of a shoal composed of unalarmed conspecifics, individuals opted to ignore these indicators while maintaining threat-free activity levels. Likewise, despite the ability of individuals to use congeneric chemical cues to assess risk, individuals chose to adopt a conservative approach when near a shoal of congenics.

The reduction in activity levels that *A. percula* displayed towards conspecific and congeneric chemical cues is a common anti-predator response observed among a number of marine fish species (Chivers and Smith 1998; Holmes and McCormick 2010). The ability of individuals to respond to the chemical cues of other species is observed in a variety of aquatic taxa including: amphibians (Schoeppner and Relyea 2005; Schoeppner and Relyea 2009), crustaceans (Hazlett and McLay 2005), gastropods (Dalesman *et al.* 2007) and freshwater fishes (reviewed in Chivers and Smith 1998; Mirza and Chivers 2003). A response to the chemical cues of others may arise via innate (co-habitation with sympatric prey over several generations) or learned (experience from local environment) mechanisms (Schoeppner and Relyea 2009; Mitchell *et al.* 2012). As *A. percula* and *A. melanopus* are closely related and often occupy similar habitats, therefore encountering similar predators, a cross-species response was expected. The response appears to be innate as individuals were laboratory bred with no opportunity for learning. Few studies have examined the extent of innate recognition in coral reef fishes; with a recent study by Mitchell *et al.* (2012) demonstrating a strong relationship between anti-predator behaviour and phylogenetic relatedness.

As chemical cues alone may result in an under or overestimation of risk due to their dependency on water for movement in the environment (lingering with no current or dissipating quickly in areas of high flow) (Bradbury and Vehrencamp 1998), visual cues may provide an additional source of information that, when paired with chemical cues results in a more accurate description of risk. As predicted, a reduction in the intensity of the anti-predator

behaviour displayed by *A. percula* was observed when in the presence of unalarmed conspecifics but not when in the presence of unalarmed congenics. These results suggest that individuals do not react to the visual cues of other species, regarding chemical information about risk of higher importance than congeneric visual information. This may reflect the extensive diversity of species that inhabit complex habitats as opposed to simpler predator-prey systems; as the number of congenics and/or heterospecifics that could provide useful information is significantly increased. Many freshwater lakes/ponds often only contain a few species; therefore it is likely that individuals are aware of one another's role within the community. Previous results of studies conducted on freshwater fishes support this idea, suggesting that visual information is utilised regardless of phylogenetic relatedness (Wisenden et al 2003; Pollock *et al.* 2006a). However, until now it has been unclear as to the extent in which individuals in complex environments utilise visual information.

A greater level of uncertainty surrounds the reliability of visual information in complex ecosystems given the high species diversity and therefore, a greater number of individuals from which useful information can be obtained. Along with a significant increase in species diversity, coral reef fish are often highly site-attached, relying heavily on their habitat for protection from predators (Krause *et al.* 2000). Pitcher and Parrish (1993) suggested that individuals who occupy habitats with high shelter cover benefit more from sheltering rather than shoaling in response to predation. This study suggests that observing the behaviour of conspecifics within visual contact may provide an alternative source of protection from predators. The study species, *A. percula*, is not traditionally a shoaling species, depending on its anemone for defense against predators (Buston 2003). However, although this study did not explicitly examine a choice between shelter and shoaling, the results strongly indicate that individuals rely on the behaviour of others when selecting an appropriate anti-predator response.

As information obtained through the use of chemical cues alone has the potential to be spatially and temporally unreliable and a level of uncertainty also surrounds the use of visual information, incorporating multiple information sources will likely provide prey with the ability to counteract these levels of error. A study by Hartman and Abrahams (2000) found that in the absence of visual cues fishes compensated by responding strongly to chemical cues. Conversely, several studies have shown that fish demonstrate complementary (additive or synergistic) effects of paired visual and chemical cues in response to risk (Brown *et al.* 2004; Ferrari *et al.* 2008; Elvidge *et al.* 2013). In one study, conducted on fathead minnows (*Pimephales promelas*), Wisenden *et al.* (2003) showed that traps labeled with chemical cues caught significantly more individuals if the traps also held a shoal, compared to traps containing only one source of information (alarm cue or shoal). As such, the authors concluded that the benefits of being in a shoal during high risk situations were greater than fleeing the area. Another study, conducted by Pollock *et al.* (2006a) exposed fathead minnows to the damage-released chemical cues of conspecifics while in the presence of one of three shoaling groups (conspecifics, familiar heterospecific or no shoal). Individuals displayed a stronger anti-predator response to chemical cues when no shoal was present than when either a conspecific or heterospecific shoal was observed. It appears that the results of the present study are consistent with the complementary interactions observed in previous studies, suggesting that individuals choose to utilise multiple cues to assess risk.

Given the complexity of coral reef environments, future research on the anti-predator response of coral reef fishes will benefit from observing behaviour under natural conditions where multiple sources of information are available at any one time. The current study emphasises the ability of coral reef fish to incorporate multiple sources of information into their decision making processes. By using multiple cues to assess risk individuals are able to

reduce the levels of uncertainty surrounding each information set, enabling them to minimise erroneous decisions and ultimately increase survival.

## CHAPTER 3 – Social learning improves survivorship at a life history transition

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### 3.1 Summary

During settlement one of the main threats faced by individuals is predation, with the ability to detect and avoid predators vital to survival. Information on predator identities can be gained through either direct experience or from the observation and/or interaction with others, a process known as social learning. In this form of predator recognition, less experienced individuals learn from experienced members, without having to directly interact with a predator. In this study, I examined the role of social learning in predator recognition in relation to the survival benefits for the damselfish, *Pomacentrus wardi* during their settlement transition. Specifically, experiments aimed to determine if *P. wardi* are capable of transmitting the recognition of a predator odour, *Pseudochromis fuscus*, to conspecifics. The experiment also examined whether there was a difference in the rate of survival between individuals that directly learnt the predator odour and those who acquired the information through social learning compared to naïve individuals. Results show that naïve *P. wardi* are able to learn a predator's identity from experienced individuals via social learning. Furthermore, survival between individuals that directly learnt the predator's identity and those that learnt through social learning did not significantly differ, with fish from both treatments surviving at least 5 times better than controls. These results demonstrate that experience may play a vital role in determining the outcome of predator-prey interactions highlighting that social learning improves the ability of prey to avoid and/or escape predation at a life history transition.

## 3.2 Introduction

Animals face a variety of decisions throughout their lives including: where to forage, who to mate with, where to breed and who to avoid. As such, decisions that are based on a large amount of reliable information are most likely to lead to a profitable outcome. Information can either be acquired through direct experience or through public information made available by other individuals (Valone and Templeton 2002). Public information can be acquired through signals (e.g. alarm calls, vocalizations, and chemical alarm cues) or inadvertently through observing the activities of others (Brown 2003; Valone 2007). Learning that involves the use of public information is referred to as social learning, with a wide variety of animals (e.g. birds, eutherian mammals, marsupials and fish) known to benefit from this type of information transmission (Brown and Laland 2001; Griffin 2004; Laland 2004; Manassa and McCormick 2012a). One of the main threats faced by individuals during life history or habitat transitions is predation, with the ability to detect and avoid predators' imperative for survival. As such, any mechanism that allows for the rapid identification of predators and reinforcement of relevant stimuli is likely to be highly advantageous.

Predation risk assessment in aquatic environments often involves the use of chemical information, with a plethora of research demonstrating the use of damage-released chemical cues (released from skin of injured prey) as reliable indicators of predation risk (Chivers and Smith 1998; Ferrari *et al.* 2010a). Likewise studies have shown that upon simultaneous detection of damage-released chemical cues and a novel chemical cue (such as the smell of a predator), prey individuals learn to associate the novel cue with risk (Wisenden 2000). This method of direct experience ensures that prey individuals identify potential predators resulting in anti-predator behaviour in future encounters (Wisenden 2000). However, this method of learning comes at a potential cost, as prey must be in the vicinity of an actively foraging predator before the initial association can be made. Therefore, given the costs associated with

direct learning it is not surprising that social learning is used in a broad range of taxa (including birds, marsupials and freshwater fishes; Brown and Laland 2001; Griffin 2004). No studies to date have demonstrated how social learning affects survival in coral reef fishes, with only two studies documenting the capacity of coral reef fishes to use this mechanism to associate predator odours with danger (Ferrari *et al.* 2012; Manassa and McCormick 2012a).

Coral reef fishes like most marine organisms have a complex lifecycle involving a series of ecological and life-history transitions. Larvae develop in the pelagic and settle into the reef matrix following a period of weeks to months in the open ocean (Leis 2007). During this time the suite of predators they encounter is both diverse and unpredictable (Leis 2007). Mortality during settlement is exceptionally high (>60% in the first 48 hours), highlighting the need for rapid learning and appropriate responses to ensure survival (Gosselin and Qian 1997; Almany and Webster 2006; Holmes and McCormick 2011). Few studies have investigated the importance of prey experience to the survival of coral reef fishes, with the first evidence that experience directly leads to higher survival demonstrated in a study by McCormick and Holmes (2006). A further study by Lönnstedt *et al.* (2012) found that if naïve individuals were conditioned with visual, chemical or a combination of predator cues, survival was eight times greater than those with no experience. However, the link between social learning of predator identities and survival trajectories remains unknown.

This study investigated the role of social learning in predator recognition and the potential survival benefits for a naïve damselfish prey, *Pomacentrus wardi*. Specifically, I investigated: (1) if prey individuals were capable of transmitting the recognition of the odour of a common predator to conspecifics, and (2) whether a difference in the rate of survival occurred between individuals that directly learnt the predator odour and those who acquired the information through social learning compared to naïve individuals.

## 3.3 Materials and methods

### 3.3.1 Fish collection and maintenance

This experiment was conducted at Lizard Island Research Station (14°40'S, 145°28'E) on the Great Barrier Reef, Australia during November and December 2011. Newly settling *Pomacentrus wardi* (mean SL  $\pm$  SE: 15  $\pm$  0.36 mm) were collected from light traps (for design see Meekan *et al.* 2001, small trap) moored overnight near the reef crest, during the summer larval recruitment pulse. Light trap caught individuals were maintained in 32 l aerated flow-through holding tanks (density: approx. 50-100 per 32 l) at ambient temperatures (26°C - 29°C), under a 12:12 light dark photoperiod. Fish were fed ad libitum twice a day with *Artemia franciscana* and Aquaculture Nutrition NRD 5/8 pellets.

*Pseudochromis fuscus*, a common predator of newly settled fish (Feeney *et al.* 2012), were collected on SCUBA using hand nets and a clove oil-ethanol-seawater solution (as an anesthetic). Individuals were maintained in separate compartments within 32 l aerated flow-through holding tanks (density: approx. 6-8 per 32 l). Individuals used to produce predator odours were fed twice daily with INVE Aquaculture Nutrition NRD G12 pellets (commercially manufactured diet); however no feeding occurred 24 h prior to collection of predator odours.

### 3.3.2 Experiment 1 – Social learning

#### 3.3.2.1 Experimental protocol

To determine if social learning of a predator odour occurred in *P. wardi*, experiments were conducted in three separate stages: a) conditioning of naïve demonstrators b) pairing of the demonstrator with an observer (naïve individual); (c) testing for an anti-predator response in the observer (Figure 3.1). If the observer displayed an anti-predator response in Stage 'c' to

the predator odour compared to the controls, it was seen as evidence that the fish had learnt that the predator odour represented a potential threat through social learning.

#### Stage 'a' - conditioning

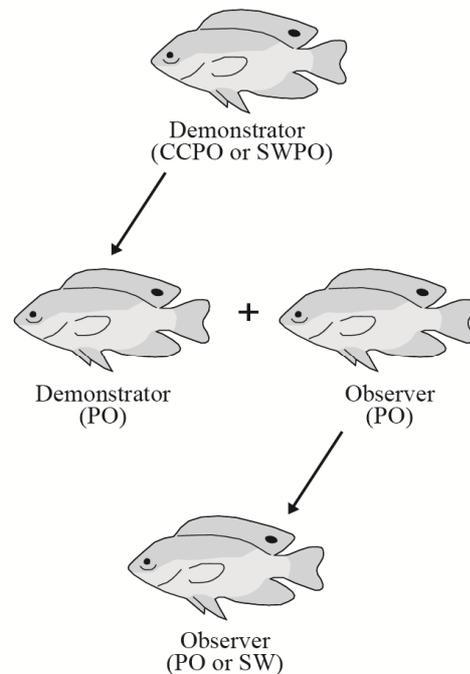
- Initial observation period (3 min)
- Addition of either:
  - 15 ml of damage-released chemical cue and 60 ml of predator odour (CCPO)
  - 15 ml of seawater and 60 ml of predator odour (SWPO)
- Final observation period (3 min)

#### Stage 'b' - pairing

- Addition of 60 ml of predator odour (PO)

#### Stage 'c' - testing

- Initial observation period (3 min)
- Addition of either:
  - 60 ml of predator odour (PO)
  - 60 ml of seawater (SW)
- Final observation period (3 min)



**Figure 3.1** Flow chart illustrating the three-stage experimental design to test the importance of social learning for newly settled *Pomacentrus wardi* (See Table 3.1 for a description of treatment codes).

### 3.3.2.2 Stage 'a' - conditioning of naïve demonstrators

Individuals were acclimated in the observation tanks for a period of 18 h. Prior to the initial observation period the flow-through system was turned off, with 60 ml of tank water drawn up the stimulus injection tube and discarded to remove any stagnant water. A further 135 ml was collected and kept. Immediately prior to the initial observation period 10 ml of live *A. franciscana* (~2500 nauplii per tank) was injected into the tube followed by 60 ml of previously collected tank water, to flush the tube. The behaviour of the focal *P. wardi* was then recorded for 3 min. After initial observations, one of two treatments (a solution of 15 ml of the

damage-released chemical cue and 60 ml of predator odour (*P. fuscus*) (C1) or a solution of 15 ml of the seawater and 60 ml of predator odour (*P. fuscus*) (C2)) (Table 3.1) was injected into the tank, along with a further 10 ml of live *A. franciscana*. Following this 60 ml of previously collected tank water was injected, to ensure all the cue was flushed through. This was followed by a final 3 min observation period, with 30 replicates undertaken for each treatment.

**Table 3.1** Grouping, treatment code, description of cue combinations and cue abbreviations for 7 treatments in the study (see Figure 3.1 for experimental design).

Grouping	Treatment	Cue combination	Cue abbreviation
Conditioning of naïve demonstrators (Stage 'a')	C1	Damage-released chemical cue and predator odour	CCPO
	C2	Seawater and predator odour; conditioning stage control	SWPO
Testing for anti-predator response (Stage 'b')	C3	Predator odour stimulus following conditioning with C1 individual	CCPO+PO+PO
	C4	Predator odour stimulus following conditioning with C2 individual	SWPO+PO+PO
	C5	Seawater stimulus following conditioning with C1 individual	CCPO+PO+SW
	C6	Seawater stimulus following conditioning with C2 individual	SWPO+PO+SW
Control	C7	No conditioning, control	CC

### 3.3.2.3 Stage 'b' – pairing for social learning opportunity

Immediately following the final observation period the individual from Stage 'a' (demonstrator) was dipped in clean seawater then transferred to another observation tank housing a naïve individual (observer) (acclimated for 18 h). To distinguish between the individuals, the observer was tagged with a coloured elastomer injected under the skin behind the dorsal fin. This tagging does not influence the behaviour or survivorship of juvenile damselfishes (Holmes and McCormick 2009). The two individuals were then acclimated in the

tank for 2 h before experiments commenced. After the acclimation period the flow-through system was turned off and 60 ml of tank water was drawn up the stimulus injection tube and discarded, with a further 60 ml collected and kept. A 60 ml aliquot of predator odour along with 10 ml of live *A. franciscana* was injected into the tank followed by 60 ml of previously collected tank water.

#### **3.3.2.4 Stage 'c' - testing for anti-predator response**

Immediately following Stage 'b', the observer was rinsed in clean seawater then transferred to an empty observation tank and acclimated for 2 h before observations commenced. After the acclimation period the flow-through system was turned off and 60 ml of tank water drawn up the stimulus injection tube and discarded with a further 120 ml collected and kept. Immediately prior to the initial observation period 10 ml of *A. franciscana* was injected into the tube followed by 60 ml of previously collected tank water, to flush the tube. The behaviour of the focal *P. wardi* was then recorded for 3 min. After initial observations, one of two treatments (60 ml of predator odour or 60 ml of seawater) (C3 to C6) (Table 3.1) was injected into the tank, along with a further 10 ml of live *A. franciscana*. Following this 60 ml of previously collected tank water was used to flush through the tube. This was followed by a final 3 min observation period, with 15 replicates undertaken for each treatment crossed with each treatment in Stage 'a' (see Figure 3.1). This resulted in a total of four cue combinations (C3 to C6) (refer Table 3.1).

#### **3.3.2.5 Observation tanks**

Experiments were undertaken in observation tanks (height 17 cm x length 27 cm x width 17 cm) with an air stone placed at the back corner of each tank. An additional piece of plastic tubing, for cue injection, was attached to the airline with the end fixed approximately

1 cm above the air stone. Trials with dye showed that it took 12 secs for the dye to disperse through the tank. Aquaria had a 2 cm deep substratum of sand and a shelter consisting of coral rubble located at the opposite corner to the air stone. Each tank was surrounded on three sides by black plastic to avoid test fish observing adjacent tanks. Individual *P. wardi* (mean SL  $\pm$  SE:  $15 \pm 0.36$  mm) were placed in the observation tanks 18 h prior to experimentation.

### **3.3.2.6 Stimulus preparation**

Damage-released chemical cues were prepared as described in Chapter 2 (section 2.3.5) with a total of 30 *P. wardi* individuals sacrificed (mean SL  $\pm$  SE:  $15 \pm 0.36$  mm).

Predator odours were collected in such a way that they were free of possible *P. wardi* damage-released chemical cues. This involved *P. fuscus* (up to 72.4 mm SL) being fed a diet of Aquaculture Nutrition NRD G12 pellets which are manufactured commercially and known to contain no trace elements of chemical cues. The flow-through aquaria system was turned off 2 h prior to experimentation to ensure the predator odours collected just prior to the experiment were concentrated within the holding tanks.

### **3.3.2.7 Quantification of behaviour**

The behavioural responses to all experimental cues were quantified by recording the frequency of two behaviours: the number of feeding strikes and the number of line crosses. The observation tanks were divided into four equal vertical areas and six equal horizontal areas (grid of 4.7 x 4.2 mm rectangles); with every line cross recorded (at least half the body must cross the line). The number of feeding strikes was recorded regardless of success, with the controls in each experiment not expected to show any changes between initial and final observation periods for the variables measured.

### 3.3.2.8 Statistical analysis

The difference in the total counts of feeding strikes and line crosses between the initial and final 3 min observation periods were compared among the two conditioning stage combinations (C1 and C2) (Table 3.1) with one-way ANOVAs. One-way ANOVAs were then used to examine the difference in total counts of feeding strikes and line crosses between the initial and final 3 min observation periods among the four testing stage combinations (C3 to C6) (Table 3.1). Tukey's HSD means comparison tests were used to determine the nature of the significant differences. Residual analysis found that the assumptions of normality and homogeneity of variance were satisfied.

### 3.3.3 Experiment 2 – Survival

#### 3.3.3.1 Experimental protocol

Focal individual *P. wardi* from five cue combinations (C1 to C4 and C7) used in Experiment 1 (refer to Table 3.1) were tested for survival differences over a 48 h period. Individuals exposed to a seawater stimulus (C5 and C6) following conditioning were not tested as it was unclear if social learning had occurred. Individuals were acclimated in a cylindrical opaque standing pipe (height 50 cm x diameter 20 cm) within 300 l observation tanks (height 33 cm, diameter 110 cm) for 1 h along with 1 l of predator odour. Tanks had a 2 cm deep substratum of sand and two shelters consisting of coral rubble located at either sides of the tank. Prior to experimentation the flow through system was turned off and remained so for the duration of the experiment. Following the acclimation period, focal individuals were released from the cylinder and allowed to acclimate for a further 30 min period. An individual *P. fuscus* (predator) was then placed into the centre of the observation tank. The study required a total of 55 individual *P. fuscus*, with a total of 22 replicates per treatment (each individual predator was randomized and used twice).

Focal individuals were released from the cylinder within the observation tanks between 8:30am and 11:00am. Survival of the *P. wardi* individuals was monitored hourly for the first 12 h. Following this, survival was recorded at 24 h, 36 h and a final 48 h. The trial was terminated after a 48 h period with the number of surviving *P. wardi* individuals recorded.

### 3.3.3.2 Statistical analysis

Multi-sample survival analysis using a Cox's proportional hazard model compared the survival of fish in the five cue combinations (C1 to C4 and C7) through the 48 h census period. Survival curves were calculated and plotted using the Kaplan-Meier product-limit method (Kaplan and Meier 1958). The Kaplan-Meier method uses a non-parametric estimator of survival that incorporates incomplete observations, such as those cases in this study where trials were ended before the 48 h period had concluded. Differences in survival between individuals from the conditioning stage treatment (C1) and the testing stage treatment (C3) were compared using a Cox-F statistic.

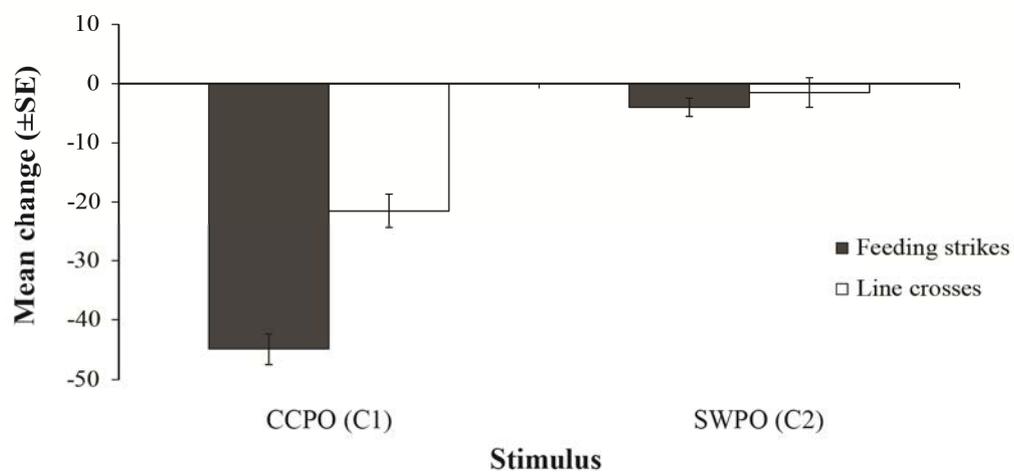
## 3.4 Results

### 3.4.1 Experiment 1 - Social learning

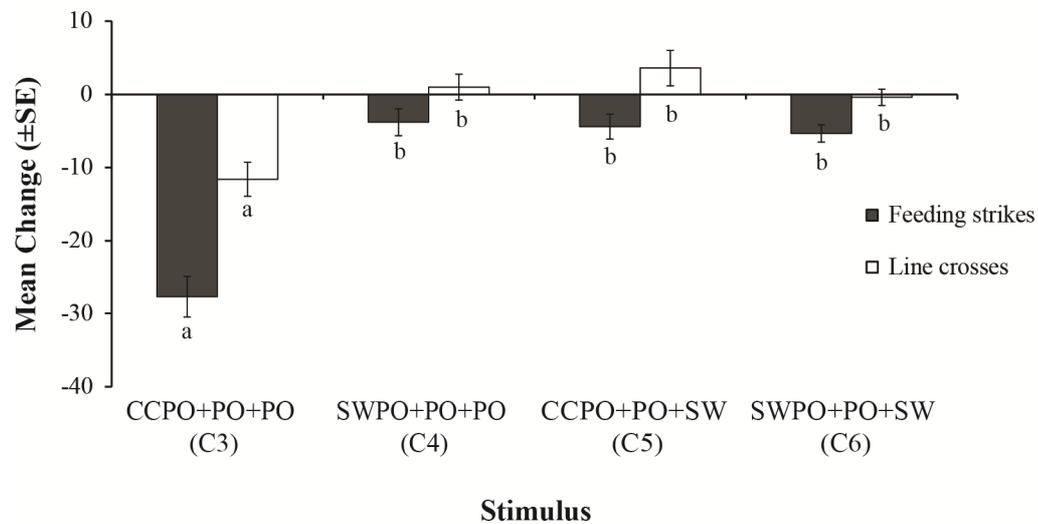
The behavioural response of the demonstrators (*Pomacentrus wardi*) to the conditioning stage treatment (C1) and the conditioning stage control (C2) was examined (refer Table 3.1). There was a significant decrease in both feeding strikes ( $F_{1,58} = 191.607, P < 0.001$ ; Figure 3.2) and line crosses ( $F_{1,58} = 27.495, P < 0.001$ ; Figure 3.2) when the damage-released cue was injected (C1) compared to the control (C2).

The behavioural response of the observers (*P. wardi*) to the four cue combinations was examined (C3 to C6) (refer Table 3.1). There was a significant difference in both feeding

strikes ( $F_{3,56} = 35.315$ ,  $P < 0.001$ ; Figure 3.3) and line crosses ( $F_{3,56} = 11.542$ ,  $P < 0.001$ ; Figure 3.3) between the initial and final observation periods among the four cue combinations. Tukey's HSD means comparison tests highlighted a reduction in both feeding strikes and line crosses in response to the testing stage treatment (C3), compared to the other three cue combinations (C4 to C6; see Table 3.1; Figure 3.3) suggesting that fish had learnt that the predator odour represented a threat through social learning.



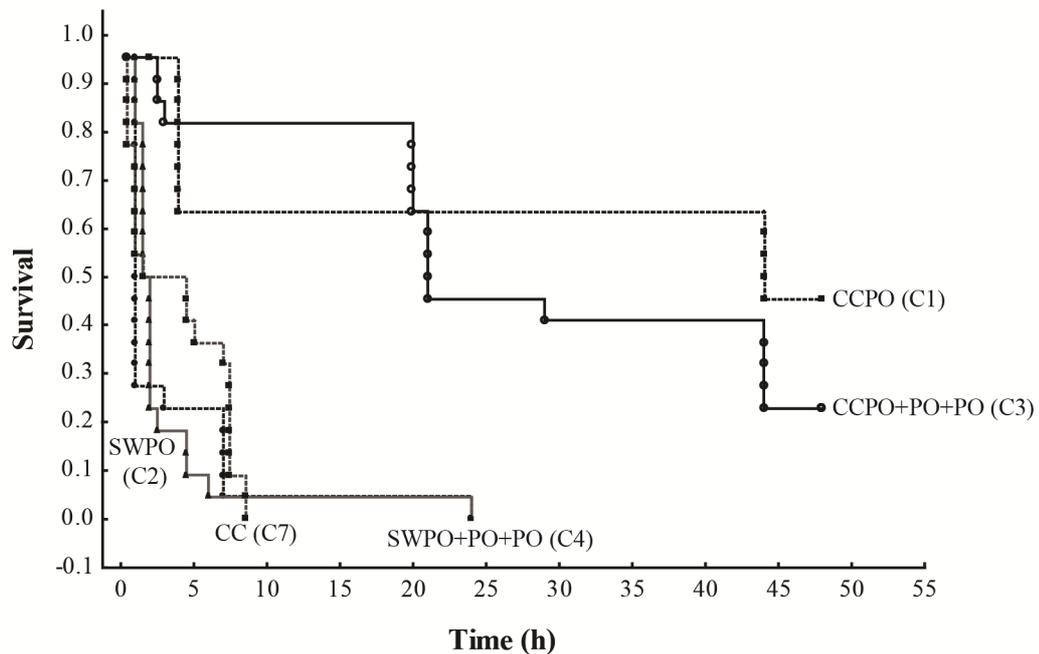
**Figure 3.2** Demonstrator conditioning: change in feeding strikes (foraging level) and line crosses (activity level) by *Pomacentrus wardi* between initial and final observation periods for treated fish (C1) exposed to a solution of damage-released chemical cues and predator odour (CCPO) or control fish (C2) exposed to a solution of seawater and predator odour (SWPO), mean  $\pm$ SE,  $n = 15$  per treatment. Letters below the bars represent Tukey's HSD groupings of means (See Table 3.1 for a description of treatment codes).



**Figure 3.3** Observer testing stage for anti-predator response: change in feeding strikes and line crosses by *Pomacentrus wardi* between initial and final observation periods for fish exposed to predator odour or seawater stimulus following conditioning with either a predator-experienced demonstrator (CCPO, C3 and C5) or a non-experienced demonstrator (SWPO, C4 and C6). Data are means  $\pm$ SE,  $n = 15$  per treatment. Letters below the bars represent Tukey's HSD groupings of means. (See Table 3.1 for a description of treatment codes).

### 3.4.2 Experiment 2 - Survival

There was a significant difference in survival among the five cue combinations (Chi-square = 50.783,  $df = 4$ ,  $P < 0.001$ ; Figure 3.4). Survival was highest for individuals from the conditioning stage treatment (C1) (63 % surviving more than 44 h), with the lowest survival for individuals from the testing stage treatment with conditioning control (C4) (31 % surviving more than 1 h). There was no significant difference between individuals from the conditioning stage treatment (C1) and those from the testing stage treatment (C3) (Cox's F-Test,  $F_{24,34} = 1.682$ ,  $P = 0.081$ ; Figure 3.4).



**Figure 3.4** Observer survival: survival trajectories (Kaplan-Meier plot) of individuals exposed to either: (1) a solution of damage-released chemical cue and predator odour (direct learning; CCPO, C1), (2) a solution of seawater and predator odour (SWPO, C2), (3) predator odour following conditioning with a predator experienced demonstrator (social learning; CCPO+PO+PO, C3), (4) predator odour following conditioning with a non-experienced demonstrator (SWPO+PO+PO, C4), (5) control individuals (no conditioning CC, C7). The time variable represents hours from trials starting between 8:30am and 11:00am,  $n = 22$  per treatment. Note: individuals exposed to a seawater stimulus following conditioning (C5 and C6) were not tested as it was unclear if social learning had occurred.

### 3.5 Discussion

An individual's probability of survival is greatly influenced by their ability to recognise and respond to predation threats (e.g. Lönnstedt *et al.* 2012). Responses to irrelevant information can be costly; therefore reliable information on the local environment and its predators is essential. The results of this study demonstrate that naïve *Pomacentrus wardi* are capable of transmitting the recognition of a predator odour to conspecifics through the process of social learning. Interestingly, results reveal that the survival trajectories between directly-experienced individuals and those who 'learnt' the information through social learning did not

significantly differ. This result suggests that social learning was a rapid and highly effective means of acquiring predator recognition.

This study demonstrates the use of social learning as a mechanism of acquiring predator identities and is similar to the findings by Manassa and McCormick (2012a) and Ferrari *et al.* (2012) for other fish species, highlighting the widespread function of this mechanism amongst coral reef damselfish. As coral reef fishes are exposed to intense predation pressures during early and transitional life-stages (McCormick and Hoey 2004; Almany and Webster 2006; McCormick and Holmes 2006) a mechanism which allows for the rapid spread of information throughout a community, without the costs associated with direct learning, would be beneficial (Kelley *et al.* 2003; Holmes and McCormick 2010). Learning from experienced individuals also allows for fine-tuning of anti-predator behaviours, eliminating responses to non-threatening species (Brown *et al.* 2006). Coral reefs are complex ecosystems containing a high diversity of species within most guilds or trophic groups, with species living in close proximity to one another. This is exactly the sort of system one would expect a large reliance on public information to inform activity patterns of individuals.

This is the first study for any coral reef species to show that survival was strongly influenced by social learning and demonstrated that survival was the same regardless of whether the information on predator identity was learnt directly, or through experience with others. Earlier work on *P. wardi* has shown that regardless of the information type (visual and/or olfactory); direct experience significantly increased the likelihood of survival in the natural environment (Lönnerstedt *et al.* 2012). Studies which have examined the functional importance of social information in freshwater fishes have compared the survival of experienced and non-experienced individuals during staged encounters with predators (Griffin 2004). All studies demonstrated a similar result, highlighting the higher survival rates of fish that had direct experience with predators in both laboratory and field studies (Griffin 2004).

Along with these, a study conducted by Webster and Laland (2008) examined the difference in the use of information, gained either directly or publically, to individuals during varying levels of predation risk. They demonstrated that when information is too costly to acquire or use personally, individual minnows (*Phoxinus phoxinus*) rely more heavily on the information from others (Webster and Laland 2008). Therefore, in conjunction with the results of the present study, one would predict that individuals should increase their use of public information when the costs of obtaining or using direct experience are high (e.g. increased predation levels during critical life history stages).

Demersal marine fishes typically follow a Type III mortality curve following settlement, with the majority of individuals dying within the first 5 days (Hixon 1991; Caley 1998; Planes and Lecaillon 2001; McCormick and Hoey 2004). This curve is likely driven by two factors: selection of individuals with preferred traits by predators and the learning of anti-predator behaviours by prey increasing their ability to avoid consumption (Lönngstedt *et al.* 2012). The relative importance of these factors on the shape of the mortality trajectory is unknown. However, it is very likely that fish who have survived a week on the reef will have experienced predation attempts or will have witnessed strikes at neighbours. Individuals that settle after the first recruitment pulse of the replenishment season will therefore settle into a predator-aware community possibly enhancing their chances of survival through social learning. It is currently unclear whether this temporal survival advantage occurs in natural populations due to the multiple factors that confound such temporal comparisons of per capita mortality (e.g. density dependence, variable size/social structures, temperature etc.).

Prior experience of potential predators significantly influences the survival of juvenile reef fish during the critical life history transition from the pelagic environment to a benthic reef associated lifestyle. The ability of individuals to associate a novel cue with risk and then pass this information onto others, through the process of social learning, allows for the rapid spread

of information throughout a community. Given the survival advantages observed in this study, social learning is likely to benefit a number of marine fish species, especially during the first few days following settlement. However, given the complexity of coral reef ecosystems, field studies are recommended, to ensure the patterns of behaviour and survivorship observed in this study translate into the natural environment. As the type of predators and the level of predation fluctuate with life-stage and environmental conditions, prey are required to continuously learn new predator identities, whilst modifying their response to those that are no longer important. Therefore, social learning is likely to play a vital role throughout an individual's life, as it allows for the continued updating of information on the identity of relevant predators, whilst maximising survival.

## **CHAPTER 4 – Social learning of predators by coral reef fish: does observer number influence acquisition of information?**

This chapter has been submitted to Behavioural Ecology and Sociobiology

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### **4.1 Summary**

The ability of prey animals to survive an encounter with a predator increases for individuals who are able to adjust their responses to match local conditions. This can occur through direct experience or through social learning from experienced individuals. Social learning is common among aquatic taxa; it provides individuals with an effective form of information transfer while reducing the costs associated with direct learning. Under a natural setting social learning is likely to occur between more than 2 individuals. As such, investigating the effect that group size has on the ability of individuals to socially learn is vital to our understanding. Given the characteristics of coral reefs and the biology of coral reef fishes, these systems are ideal for testing the effects of group size on social learning. Using damselfish (*Pomacentrus amboinensis*), I show that: (1) predator recognition is socially transmitted from predator-experienced to predator-naïve individuals regardless of whether the number of observers in the group increases from 1 to 5, and that (2) the intensity of the learned anti-predator response does not differ between predator-naïve observers who learnt when they were alone with the demonstrator compared to when they were one of five observers. This study shows that information on predator identities is able to be passed onto group members quickly without a dilution of information content when the number of observers increases. This study highlights the use of social learning as a method of acquiring information about predators in aquatic ecosystems.

## 4.2 Introduction

Predators have dramatic influence on the behavioural, morphological and life-history traits of prey species and on the dynamics of populations and communities over both ecological and evolutionary timescales (Chivers *et al.* 1999; Sih *et al.* 2000; Hoverman *et al.* 2005). A prey's use of space, access to food and allocation of energy into growth, reproduction and maintenance may all be affected by the presence of predators within a community (Lima 1998). As a result of constant fluctuations in predation risk, prey that are capable of continuously learning new predator identities whilst adjusting the intensity of their response to match the level of risk, are often at a substantive advantage (Brown and Chivers 2006).

In aquatic systems, one effective way for prey to identify predation risk is through the use of damage-released chemical alarm cues. These cues are released from prey following mechanical damage to the skin and are known to induce an intense anti-predator response in nearby individuals (Chivers and Smith 1998; Ferrari *et al.* 2010a). Upon the simultaneous detection of chemical alarm cues and a novel cue (the sight or odour of an unknown predator), individuals learn to associate the novel cue with risk (Chivers and Smith 1994; Wisenden 2000). This method of predator identification requires direct experience, where an individual must be in close proximity to the predator attack in order to be provided with the necessary cues. In addition to direct experience, information regarding predator identification can be acquired through social information made available by more experienced individuals (Heyes 1994; Valone and Templeton 2002). In such cases, prey animals learn the identity of predators by observing nearby conspecifics or heterospecifics responding to the sight, sound or odour of the predator (Manassa *et al.* 2012 - Chapter 5).

Social learning has been documented in a wide range of non-human animals, with its use in predator recognition observed in mammals, birds, amphibians, fish and invertebrates (reviewed in Crane and Ferrari 2013). The majority of the experiments testing for social

learning of predator recognition follow a 3-stage process: 1) conditioning a naïve demonstrator to recognise a predator; 2) pairing of the demonstrator with a naïve observer and exposing the pair to a predator cue; 3) testing the observer alone for an anti-predator response. Use of this protocol provides researchers with a quantifiable measurement of the capacity for species to socially learn from one another; however in aquatic systems a 1:1 demonstrator to observer ratio is unlikely. Intuitively, the higher the number of knowledgeable demonstrators, the more effective information transfer may be. Indeed, Ferrari and Chivers (2008) found that tadpoles (*Pseudacris maculata*) learned to show a stronger anti-predator response to salamanders (*Ambystoma tigrinum*) when the ratio of demonstrators to observers was higher. Vilhunen *et al.* (2005) observed the opposite as Arctic charr (*Salvelinus alpinus*) only learned if there were a lower number of demonstrators. Both of these studies were conducted in ecologically simple systems where the diversity of species and number of individuals is significantly lower than more complex systems such as coral reefs.

Social interactions play a pivotal role in the day to day activities of coral reef fishes. Given the large number of individuals that occupy a relatively small area at any one time, coral reefs are ideal habitats in which to test the effects of group size on social learning. Along with this, the complex lifecycles of coral reef fishes results in exposure to a diverse and unpredictable array of predators and non-predators throughout each life stage; necessitating the need for accurate predator learning (Mitchell *et al.* 2011a,b). A common assumption associated with an increase in group size is the subsequent decrease in the probability of an individual being captured by a predator (Blumstein *et al.* 1999). Per capita risk is thought to decrease as a result of either the dilution effect (increased prey numbers) and/or the detection effect (increased vigilance by the collective prey) (Blumstein *et al.* 1999). If the perception of per capita risk decreases disproportionately to group size so too will the anti-predator response intensity. Several social learning studies have demonstrated a correlation between damage-

released chemical cue concentration and response intensity (Ferrari *et al.* 2005; Zhao and Chivers 2005), with the response of the observers shown to be similar in magnitude to the response given by the demonstrator (Vilhunen *et al.* 2005; Manassa and McCormick 2012a). None of these studies have specifically examined the effect of group size on the intensity of the response displayed.

The present study investigated whether an increase in group size affects the ability of naïve damselfish, *Pomacentrus amboinensis* (Family: Pomacentridae) to socially learn, and if so, is the intensity of the learned response altered. Naïve *P. amboinensis* were used in this study to ensure that individuals had not previously been exposed to the predator odour. This naivety has been demonstrated previously by Ferrari *et al.* (2012); however controls were also undertaken in this study to ensure this. Specifically, the study tested if a predator-naïve observer could socially learn to recognise a novel predator when paired with a predator-experienced conspecific on its own or in the presence of four other observers. I predicted that social learning would occur, however I expected that the intensity of the learned behaviour would decrease to reflect a reduction in perceived risk.

## **4.3 Materials and methods**

### **4.3.1 Fish collection and maintenance**

*Pomacentrus amboinensis* and *Pseudochromis fuscus* individuals were collected, maintained and fed as described in Chapter 3 (section 3.3.1).

### **4.3.2 Stimulus preparation**

Damage-released chemical cues were prepared as described in Chapter 2 (section 2.3.5) with a total of 60 *P. amboinensis* individuals sacrificed (mean SL  $\pm$  SE: 15  $\pm$  0.35 mm). Predator odour (*P. fuscus*) was collected as described in Chapter 3 (section 3.3.2.6).

### 4.3.3 Observation tanks

Experiments were undertaken in observation tanks as described in Chapter 3 (section 3.3.2.5). A single *P. amboinensis* (mean SL  $\pm$  SE: 15  $\pm$  0.35 mm) was placed into each tank and left to acclimate for 18 h prior to experimentation.

### 4.3.4 Experimental protocol

To determine if social learning of a predator odour occurred, a modified version of the well-established 3-stage social learning protocol was used (Manassa and McCormick 2012b – Chapter 3). The modified version uses two ratio conditions during the social learning stage (Stage ‘b’); 1 naïve individual to 1 demonstrator, or 5 naïve individuals to 1 demonstrator.

#### 4.3.4.1 Stage ‘a’ – conditioning of naïve demonstrators

This stage conditions an individual to recognise a novel predator by pairing the predator odour with the innately recognised chemical alarm cues of conspecifics. Along with conditioning, this stage aims to collect baseline data on the behavioural response of an individual to a previously unknown predator odour. The protocol follows that from Chapter 3 (section 3.3.2.2) with one of two treatments (a solution of 15 ml of the damage-released chemical cue and 60 ml of predator odour (*P. fuscus*) or a solution of 15 ml of seawater and 60 ml of predator odour (*P. fuscus*)) injected into the tank. The behaviour of individuals in each of the 2 treatments was recorded, with these individuals later used as non-experienced (control group) and experienced (experimental group) demonstrators in the social learning stage.

#### 4.3.4.2 Stage ‘b’ – pairing for social learning opportunity

Immediately following the final observation period the demonstrator from Stage 'a' was dipped in clean seawater to remove any potential cues, then transferred to another observation tank housing either an acclimated naïve individual, or 5 acclimated naïve individuals (hereafter: the observers). To distinguish between the individuals, the Stage 'a' individual was tagged with a coloured elastomer injected under the skin behind the dorsal fin (prior to Stage 'a'). This tagging does not influence the behaviour or survivorship of juvenile damselfishes (Holmes and McCormick 2009; Ferrari *et al.* 2012). Experiments commenced after a further 2 h acclimation period, ensuring that the Stage 'a' fish was familiar with its surroundings. Following the acclimation period, the flow-through system was turned off and 60 ml of tank water drawn up the stimulus injection tube and discarded, with an additional 60 ml collected and kept for later use. A 60 ml aliquot of predator odour along with 10 ml of live *Artemia franciscana* (~2500 nauplii per tank) was injected into the tank followed by 60 ml of previously collected tank water. No observations were carried out during this stage. I predicted that the presence of predator odour in the tank would elicit an anti-predator response from the predator-experienced demonstrators, but not from the naïve demonstrators. Observers paired with predator-experienced demonstrators should have an opportunity to learn to recognise the predator odour as risky.

#### 4.3.4.3 Stage ‘c’ – testing for anti-predator response

To test whether the observer had acquired recognition of the predator via social learning, the observer was exposed to predator odour on its own, and its anti-predator response measured. Indeed, the anti-predator response displayed in the presence of the demonstrator could indicate learning, but could also result from a mimicking behaviour, in which the observer copies the demonstrator without acquiring new information. Immediately following

Stage 'b' an observer – either the only observer (where Stage 'b' involved 1 naïve individual) or an observer chosen at random (where Stage 'b' involved 5 naïve individuals) were rinsed in clean seawater then transferred to an empty observation tank and acclimated for 2 h. After the acclimation period the flow-through system was turned off and 60 ml of tank water was drawn up the stimulus injection tube and discarded with a further 120 ml collected and kept. Immediately prior to the initial observation period 10 ml of *A. franciscana* was injected into the tube followed by 60 ml of previously collected tank water, to flush the food through the tube. The behaviour of the focal *P. amboinensis* was then recorded for 3 min. After initial observations, one of two stimuli (60 ml of predator odour or 60 ml of seawater) was injected into the tank, along with a further 10 ml of live *A. franciscana*. The remaining 60 ml of previously collected tank water was used to flush the stimulus through the tube. This was followed by a final 3 min observation period.

I predicted that observers that had successfully learned to recognise the predator odour as risky from their demonstrators should display an anti-predator response when exposed to the predator odour. Those that failed to learn should not respond to the predator odour. A total of 15 observers from each of the 8 treatments (exposure to seawater or predator odour following conditioning with non-experienced or experienced demonstrators in the presence of 1 or 5 naïve observers) were tested.

#### **4.3.5 Quantification of behaviour**

Behaviour was quantified as described in Chapter 2 (section 2.3.7).

#### **4.3.6 Statistical analysis**

Change in behaviour between the initial and final observation periods were used as raw data in the analysis. As activity and feeding are correlated, the two responses were

analysed together using a MANOVA approach. The behavioural response of both demonstrators during Stage 'a' (individuals exposed to either seawater and predator odour or damage-released chemical cues and predator odour) were compared using a 2-way MANOVA. To test whether an increase in group size affects the ability of individuals to socially learn, the effect of demonstrator experience (naïve vs. experienced), observer ratio during Stage 'b' (1 observer vs. 5 observers) and Stage 'c' cue (control or experimental stimulus) on the anti-predator response of observers was assessed using a 2x2x2 MANOVA. A series of factorial MANOVAs were then conducted to determine the significance of demonstrator experience (naïve vs. experienced) on observer behaviour. To analyse if the intensity of the learned anti-predator response differs between observers who learnt when they were alone compared to when they were one of five, a 2-way MANOVA was undertaken. Inspection of residuals revealed that the data followed parametric assumptions.

## 4.4 Results

The demonstrators conditioned with chemical alarm cues displayed a significantly stronger anti-predator response than those pseudo-conditioned with seawater ( $F_{2,27} = 34.9$ ,  $P < 0.001$ ). While cue and demonstrator experience significantly interacted to affect the behaviour of the fish during Stage 'b' (social learning stage), observer ratio (and any interaction involving observer ratio) did not significantly explain the variation in the behaviour of observers, indicating that group size did not affect the outcome of learning (Table 4.1).

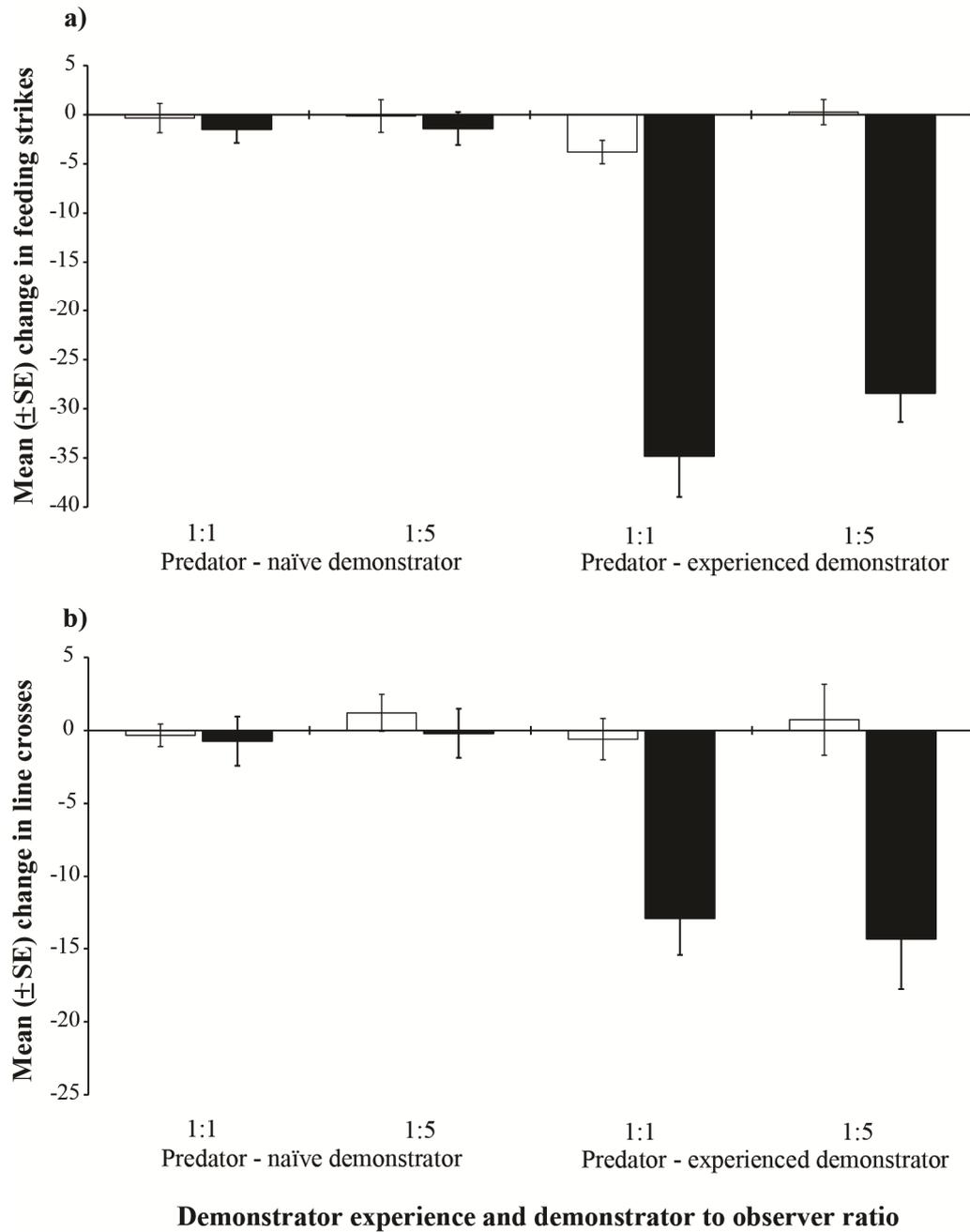
**Table 4.1** Results of the 2x2x2 way MANOVA testing the effect of demonstrator experience (naïve vs. experienced), observer ratio during Stage 'b' (1 observer vs. 5 observers) and Stage 'c' cue (control or experimental stimulus) on the foraging behaviour and activity level of the observers during Stage 'c'.

Source of variance	df	F	Sig.
Demonstrator	2,111	55.9	< 0.001
Observer Ratio	2,111	1.84	0.163
Cue	2,111	52.6	< 0.001
Demonstrator*Ratio	2,111	1.14	0.324
Demonstrator*Cue	2,111	46.2	< 0.001
Ratio*Cue	2,111	0.62	0.537
Demonstrator*Ratio*Cue	2,111	0.03	0.970

When observers were paired with naïve demonstrators, neither observer ratio ( $F_{2,55} = 0.17$ ,  $P = 0.84$ ) nor cue ( $F_{2,55} = 0.7$ ,  $P = 0.5$ ) or any interaction between the two ( $F_{2,55} = 0.33$ ,  $P = 0.72$ ) affected the behaviour of the fish (Figure 4.1). Conversely, observers paired with experienced demonstrators subsequently displayed anti-predator responses when exposed to predator odour but not when exposed to seawater ( $F_{2,55} = 62.1$ ,  $P < 0.001$ ). Again, neither observer ratio ( $F_{2,55} = 2.04$ ,  $P = 0.14$ ) nor the ratio x cue interaction ( $F_{2,55} = 0.34$ ,  $P = 0.72$ ) affected their response.

The behavioural response of the observers who learnt when they were alone compared to when they were one of five observers was examined by comparing the response of individuals to predator odour following conditioning with a predator-experienced demonstrator under each learning ratio (either 1:1 or 1:5). There was no significant change in behaviour between initial and final observation periods regardless of group size ( $F_{2,27} = 1.0$ ,  $P = 0.38$ ),

with no difference in feeding strikes ( $F_{1,28} = 1.6$ ,  $P = 0.22$ ) or line crosses ( $F_{1,28} = 0.1$ ,  $P = 0.73$ ).



**Figure 4.1** Mean change ( $\pm$ SE) in the number of feeding strikes (a) or line crosses (b) between the initial and final observation periods for *Pomacentrus amboinensis* observers exposed to seawater (empty bars) or predator odour (solid bars). The observers were previously paired with either a predator-naïve or predator-experienced demonstrator under two group size conditions (2 individuals or 6 individuals) and exposed to predator odour.

## 4.5 Discussion

This work demonstrates that *Pomacentrus amboinensis* are capable of socially learning regardless of whether group size is increased from 2 to 6. Whilst previous studies have suggested that the demonstrator to observer ratio significantly impacts the ability of aquatic species to socially learn (Vilhunen *et al.* 2005; Ferrari and Chivers 2008), this is the first study to examine ratio effects in coral reef ecosystems. As predicted, by increasing the number of observers from 1 to 5 the ability of individuals to transmit information socially was not altered. With a dilution of predation risk thought to be a common benefit of group membership (Blumstein *et al.* 1999), it was expected that the intensity of an individual's anti-predator response will be decreased; however the response intensity in this study appears to be unchanged.

A large number of studies have examined social learning and the effect of group size on the transmission of information relating to migration/orientation, foraging and mate choice (e.g. Laland and Williams 1997; Reeb 2000; Brown and Laland 2003; Brown *et al.* 2006; Duffy *et al.* 2009), however the impact that group size has on predator recognition was poorly understood until now. Increasing group size leads to a decrease in vigilance and an increase in time available for other biologically important activities (Godin *et al.* 1988). Anti-predator behaviours are thought to drive this 'group size effect', with studies showing that predation pressures are decreased by either the dilution effect or an increase in the opportunity for predator detection (Lima *et al.* 1999). Detection of potential predators is only useful if information is passed onto all members (Godin *et al.* 1988). By using social learning, information on local predators is likely to propagate through the entire group rapidly, resulting in a predator-aware community (Chivers and Smith 1995). The results of the present study demonstrate that social learning occurs regardless of whether the group size increased from 2

to 6, suggesting that by increasing the knowledge of individuals, social learning acts as an important tool for the transfer of predator identities.

Alongside a reduction in per capita risk is the suggestion that the response intensity of individuals is inversely proportional to group size. Previous studies have shown that during social learning trials, the response of the observer matches that of the response given by the demonstrator (Vilhunen *et al.* 2005; Manassa and McCormick 2012a). Unlike the present study, these did not compare the response intensities of observers from different group sizes. Contrary to predictions, the results of the present study found that group size did not significantly affect the intensity of the learned anti-predator response. No significant differences were seen between single observers and when there were a total of 5 observers. These anti-predator responses may reflect the importance that predators have on the lifestyle of coral reef fishes, suggesting that initially individuals will respond with a high level of awareness. It may also be possible that individuals are placing more emphasis on the most recent information, with a study by Ferrari and Chivers (2006) demonstrating that in fathead minnows (*Pimephales promelas*) fine-tuning of anti-predator responses occurred after several encounters. It should also be considered that an effect of group size on the anti-predator response of observers may not have been seen in this study due to either the size of the group, or the demonstrator to observer ratio. Further studies would therefore benefit from combining a larger proportion of demonstrators to observers, or by increasing group size substantially.

During critical life-history transitions coral reef fishes are exposed to an unknown diversity and abundance of predators, thus the ability of individuals to gain continuous information on local predators is essential (Mitchell *et al.* 2011a,b; Lönnstedt *et al.* 2012). Therefore, given the characteristics of coral reefs and the biology of coral reef fishes, it is likely that social learning is commonplace in this system. Social learning allows individuals to gain potentially life-saving information without the need for exposure to potential predators.

Along with this, obtaining information socially reduces individual risk and likely contributes to the increase in vigilance often reported as a benefit of group membership. As demonstrated in the present study, information is able to be passed onto group members without a dilution of information content when the number of observers increases from 1 to 5. This finding highlights the importance of social learning to the behaviour of individuals, demonstrating its use as a method of acquiring information about predators in aquatic ecosystems.

## CHAPTER 5 – Socially acquired predator recognition in complex ecosystems

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### 5.1 Summary

Social animals acquire information on predator identities through social learning, where individuals with no prior experience learn from experienced members of the group. However, a large amount of uncertainty is often associated with socially acquired information especially in cases of cross-species learning. Theory predicts that socially acquired information from heterospecifics should take more repetitions to develop in complex ecosystems where the number of participants is greater. This work focuses on coral reef fish as their social and communal lifestyles, along with their complex life-histories, make them an ideal model to test for socially acquired predator recognition. Specifically, we tested if closely related *Pomacentrus moluccensis* and phylogenetically distant *Apogon trimaculatus* are capable of interpreting the recognition of an unknown predator, *Pseudochromis fuscus*, from experienced *Pomacentrus wardi* individuals. Individuals of both species were able to learn the predator's identity from experienced *Pomacentrus wardi* based on a single conditioning event. It is somewhat surprising how fast social learning occurred particularly for the distantly related cardinalfish. This study demonstrates the widespread nature of social learning as a method of predator recognition in biologically complex ecosystems and highlights that the benefits of responding to uncertain information may override the costs associated with lost foraging opportunities.

## 5.2 Introduction

Whether choosing a mate, deciding where to breed, selecting a foraging area, or knowing who to avoid, an individual's decisions can disclose useful information to others. Therefore, under conditions where individuals can observe one another's actions, it is only logical that animals learn from one another. This interaction, where learning is influenced by the observation of, or interaction with, another animal is referred to as social learning (Brown and Laland 2003; Crane and Ferrari 2013). Studies on social learning in animals date back to at least the 19<sup>th</sup> Century (Romanes 1884), with a wide variety of animals (e.g. insects, birds, eutherian mammals, marsupials and fish) known to benefit from this type of information transmission (Brown and Laland 2001; Griffin 2004; Laland 2004; Manassa and McCormick 2012a).

Social learning allows animals to obtain knowledge on locally adaptive information without the risks associated with individual learning (Brown and Laland 2002). Exploring the environment individually is risky, due to the increased exposure to unknown predators and associated costs in both time and energy. Therefore, given the relatively low risks associated with social learning, it is likely that publically available information will spread rapidly within a population (Swaney *et al.* 2001). The ability to learn quickly may be the difference between life and death, especially for naïve individuals who are required to promptly learn new predators in order to avoid capture and almost certain death.

Predation pressure acts as a strong selective force shaping the behaviour, life history, morphology and distribution of prey animals (Wisenden and Harter 2001; Brown 2003). As such, the ability to assess local predation risk is crucial for survival. A variety of taxa have been shown to acquire information on predator identities through social learning (Griffin 2004; Ferrari and Chivers 2008), with the pattern of acquisition similar across taxa (Griffin 2004). In a typical scenario an avoidance response is evoked in an individual following the simultaneous

detection of a previously neutral stimulus with an alarm stimulus (e.g. alarm call, mobbing call, damage-released chemical cues) (Griffin 2004; Galef and Laland 2005). Following this, the individual who has learnt through association responds to the neutral stimulus in future encounters, thereby highlighting it as a threat to other individuals who learn by this experience.

Studies on social learning about risk have focused almost exclusively on the transmission of information between conspecifics (Ferrari and Chivers 2008); however intraspecific social learning is only one way in which naïve individuals can learn the identities of novel predators. Animals may also learn unfamiliar predators by observing the behaviour of other species (interspecific learning) (Griffin 2004). While research has indicated that this type of learning is possible, such studies are rare and have all been conducted in rather ecologically simple ecosystems where the number of species that could act as demonstrators was relatively few (Vieth *et al.* 1980; Mathis *et al.* 1996; Ferrari and Chivers 2008). Ferrari and Chivers (2008) showed a cross-species response in tadpoles collected from a pond which contained only two species. Likewise, Mathis *et al.* (1996) showed cross-species responses between two freshwater prey fishes, but the lake contained only a handful of other prey fishes. Theory dictates that in biologically complex environments such as coral reefs, where biodiversity is at its highest, interspecific social learning may be commonplace, but will take considerable time to develop. In the case of larval reef fish, for any given individual that recruits to the reef, there are dozens of potential heterospecifics that could provide learning opportunities. Therefore, with such an astonishing array of heterospecifics in the vicinity, which behaviours are relevant and which are not?

Coral reef fish are known to respond to the damage-released chemical cues of heterospecific individuals (Mitchell *et al.* 2012), with studies demonstrating learnt predator recognition following a single-conditioning event (Brown 2003; Ferrari *et al.* 2005; McCormick and Manassa 2008; Holmes and McCormick 2010). However, these direct

learning methods come at a potential cost, as the prey must be in the vicinity of an actively foraging predator. Socially acquired information reduces this risk, however a level of uncertainty surrounds the accuracy of the information. Differences in size, sex, body condition, as well as parasite load and hunger levels could all influence differential perception of risk and create uncertainty (Milinski 1985; Mirza *et al.* 2001; Pollock *et al.* 2006b). The sources of uncertainty are the same in both simple and complex ecosystems; however the number of players is much larger. Consequently the number of encounters required to acquire accurate information should be greater, especially for newly recruited coral reef fishes. As a significant difference in survival has been demonstrated between recruiting fish that forage at high or low levels (food availability), with the latter surviving better due to reduced exposure to predators (Lönstedt *et al.* 2012), selection should cause newly settled reef fish to be less responsive to socially acquired information.

This study explored social learning of predator recognition among three species of coral reef fishes: *Pomacentrus wardi*, *Pomacentrus moluccensis* (Family: Pomacentridae) and *Apogon trimaculatus* (Family: Apogonidae). Specifically, we investigated if individual *P. moluccensis* and *A. trimaculatus* individuals were capable of interpreting the recognition of a common predator odour from *P. wardi*. There should be a greater amount of uncertainty about the quality of socially acquired information in situations where learning occurs from more distantly related species, hence the original goal was to test for an asymmetry in the amount of information required to establish predator recognition from closely and distantly related fishes. However, this proved unnecessary given the high efficiency of the one time learning we observed.

## 5.3 Materials and methods

### 5.3.1 Fish collection and maintenance

*Pomacentrus wardi*, *Pomacentrus moluccensis*, *Apogon trimaculatus* and *Pseudochromis fuscus* individuals were collected, maintained and fed as described in Chapter 3 (section 3.3.1).

### 5.3.2 Experimental protocol

To determine if social learning of a predator odour occurred between *P. wardi* and *P. moluccensis* and/or *A. trimaculatus* a series of experiments using a modified version of the well-established 3-stage social learning protocol was used (Manassa and McCormick 2012b – Chapter 3). Behavioural observations were conducted during Stage ‘a’ to collect baseline data and Stage ‘c’ to determine if social learning occurred. A series of controls were undertaken to ensure that *P. moluccensis* and *A. trimaculatus* did not demonstrate an innate response to the predator odour. If the observer displayed an anti-predator response in Stage ‘c’ to the predator odour compared to the controls, it was seen as evidence that the fish had learnt that the predator odour represented a potential threat through social learning.

#### 5.3.2.1 Stage 'a' - conditioning of naïve demonstrators

The protocol for this stage follows that from Chapter 3 (section 3.3.2.2) with one of two treatments (a solution of 15 ml of the damage-released chemical cue (see below) and 60 ml of predator odour (*P. fuscus*) (see below) (CCPO) or a solution of 15 ml of the seawater and 60 ml of predator odour (*P. fuscus*) (SWPO)) injected into the tank. A total of 60 replicates were undertaken for each treatment. Naïve individuals exposed to the CCPO treatment should have the opportunity to learn that *P. fuscus* is a predator, and hence become experienced

demonstrators. Individuals exposed to the SWPO treatment should not have the opportunity to associate risk with the predator odour and hence act as non-experienced demonstrators.

### **5.3.2.2 Stage 'b' – pairing for social learning opportunity**

Immediately following the final observation period the *P. wardi* individual from Stage 'a' was dipped in clean seawater then transferred to another observation tank housing either a naïve *P. moluccensis* or *A. trimaculatus* (acclimated for 18 h). The two individuals were acclimated in the tank for 2 h before experiments commenced. After the acclimation period the flow-through system was turned off and 60 ml of tank water was drawn up the stimulus injection tube and discarded, with a further 60 ml collected and kept. A 60 ml aliquot of predator odour along with 10 ml of live *Artemia franciscana* was injected into the tank followed by 60 ml of previously collected tank water. Both *P. moluccensis* and *A. trimaculatus* have the opportunity to learn the identity of the predator odour based on the fright response of the *P. wardi* in the tank. Observers who have witnessed the response of experienced *P. wardi* demonstrators should learn that the odour is risky, whereas those who have witnessed the response of non-experienced demonstrators should not.

### **5.3.2.3 Stage 'c' - testing for anti-predator response**

Immediately following Stage 'b', the naïve *P. moluccensis* or *A. trimaculatus* from that stage was rinsed in clean seawater then transferred to an empty observation tank and acclimated for 2 h before observations commenced. After the acclimation period the flow-through system was turned off and 60 ml of tank water was drawn up the stimulus injection tube and discarded, with a further 120 ml collected and kept. Immediately prior to the initial observation period 10 ml of *A. franciscana* was injected into the tube followed by 60 ml of previously collected tank water, to flush the tube. The behaviour of the focal individual was

then recorded for 3 min. After initial observations, one of two treatments (60 ml of predator odour (PO) or 60 ml of seawater (SW)) was injected into the tank, along with a further 10 ml of live *A. franciscana*. Following this 60 ml of previously collected tank water was used to flush through the tube. This was followed by a final 3 min observation period, with 15 replicates undertaken for each species and treatment crossed with each treatment in Stage 'a' (see Figure 5.1). This resulted in a total of four observer testing stage combinations: predator odour stimulus following conditioning with an experienced *P. wardi*, predator odour stimulus following conditioning with a non-experienced *P. wardi*, seawater stimulus following conditioning with an experienced *P. wardi*, seawater stimulus following conditioning with a non-experienced *P. wardi*.

#### Stage 'a' - conditioning

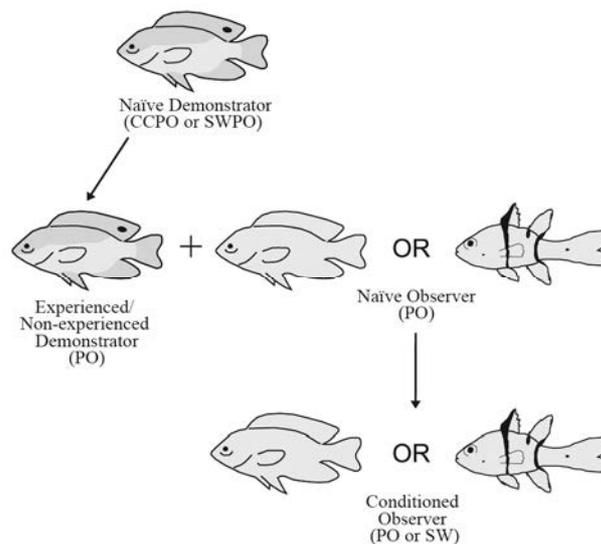
- Initial observation period (3 min)
- Addition of either:
  - 15 ml of damage-released chemical cue and 60 ml of predator odour (CCPO)
  - 15 ml of seawater and 60 ml of predator odour (SWPO)
- Final observation period (3 min)

#### Stage 'b' - pairing

- Addition of 60 ml of predator odour (PO)

#### Stage 'c' - testing

- Initial observation period (3 min)
- Addition of either:
  - 60 ml of predator odour (PO)
  - 60 ml of seawater (SW)
- Final observation period (3 min)



**Figure 5.1** Flow chart illustrating the three-stage experimental design to test the importance of social learning for newly settled fishes.

### 5.3.3 Observation tanks

Experiments were undertaken in observation tanks as described in Chapter 3 (section 3.3.2.5). Individual *P. wardi* (mean SL  $\pm$  SE: 15  $\pm$  0.36 mm), *P. moluccensis* (mean SL  $\pm$  SE: 15  $\pm$  0.71 mm) and *A. trimaculatus* (mean SL  $\pm$  SE: 14  $\pm$  0.28 mm) were placed in the observation tanks 18 h prior to experimentation.

### 5.3.4 Stimulus preparation

Damage-released chemical cues were prepared as described in Chapter 2 (section 2.3.5) with a total of 60 *P. wardi* individuals sacrificed (mean SL  $\pm$  SE: 15  $\pm$  0.36 mm). Predator odour (*P. fuscus*) was collected as described in Chapter 3 (section 3.3.2.6).

### 5.3.5 Quantification of behaviour

Behaviour was quantified as described in Chapter 2 (section 2.3.7).

### 5.3.6 Statistical analysis

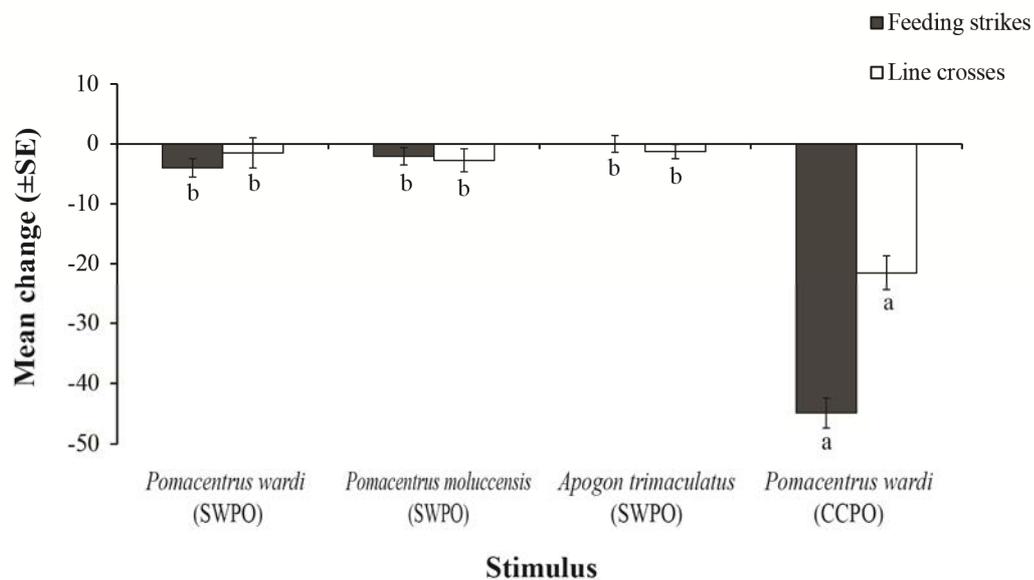
The difference in the total counts of feeding strikes and line crosses between the initial and final observation periods were used for all analyses to control for individual differences. The behavioural responses of *P. wardi* to a solution of damage-released chemical cue and predator odour (CCPO) along with the responses of *P. wardi*, *P. moluccensis* and *A. trimaculatus* to a solution of seawater and predator odour (SWPO), were statistically tested using a one-factor MANOVA. Two one-factor MANOVAs were also used to examine the difference in total counts of feeding strikes and line crosses between the initial and final observation periods for each of the four observer testing stage combinations for each species (*P. moluccensis* and *A. trimaculatus*). To further explore the nature of the significant differences found by MANOVA, univariate ANOVAs were undertaken on both variables

(feeding strikes and/or line crosses) followed by Tukey's HSD means comparison tests. Residual analysis found that the assumptions of normality and homogeneity of variance were satisfied.

## 5.4 Results

### 5.4.1 Controls

The behavioural response of *Pomacentrus wardi*, *Pomacentrus moluccensis* and *Apogon trimaculatus* to a solution of seawater and predator odour (SWPO), along with the response of *P. wardi* to a solution of damage-released chemical cue and predator odour (CCPO) were examined. During the trials (CCPO) *P. wardi* individuals made between 61 and 111 feeding strikes (mean  $\pm$  SE:  $94.3 \pm 4.1$ ) and between 38 and 89 line crosses ( $62.2 \pm 4.5$ ) during the initial observation period and between 25 and 80 feeding strikes ( $49.6 \pm 3.9$ ) and between 13 and 62 line crosses ( $38.3 \pm 3.6$ ) during the final observation period. There was a significant difference in the change in behaviour between treatments (Pillai's Trace = 0.817,  $F_{6,172} = 19.785$ ,  $P < 0.001$ ; Figure 5.2), with a decrease in feeding strikes ( $F_{3,86} = 122.474$ ,  $P < 0.001$ ; Figure 5.2) and line crosses ( $F_{3,86} = 16.439$ ,  $P < 0.001$ ; Figure 5.2) when the damage-released chemical cue was injected compared to the controls.

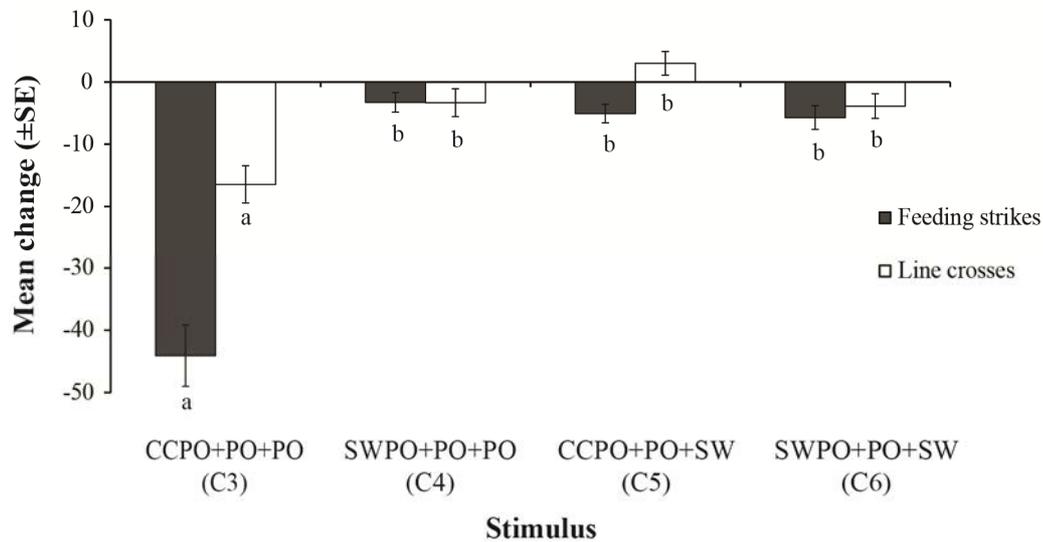


**Figure 5.2** Change in feeding strikes (foraging level) and line crosses (activity level) by *Pomacentrus wardi*, *P. moluccensis* and *Apogon trimaculatus* between initial and final observation periods for fish exposed to a solution of seawater and predator odour (SWPO), along with the response of *P. wardi* to a solution of damage-released chemical cue and predator odour (CCPO), mean  $\pm$ SE,  $n = 15$  per treatment. Letters below the bars represent Tukey's HSD groupings of means.

#### 5.4.2 Stage 'c' response – *P. moluccensis*

The behavioural response of individual *P. moluccensis* to the four observer testing stage combinations was examined. There was a significant change in behaviour between the initial and final observation periods among the four cues (Pillai's Trace = 0.831,  $F_{6,112} = 13.241$ ,  $P < 0.001$ ; Figure 5.3). This difference was caused by significant differences in both feeding strikes ( $F_{3,56} = 47.994$ ,  $P < 0.001$ ; Figure 5.3) and line crosses ( $F_{3,56} = 12.407$ ,  $P < 0.001$ ; Figure 5.3). Tukey's HSD means comparison tests highlighted a significant reduction in both feeding strikes and line crosses in response to the predator odour stimulus following conditioning with an experienced *P. wardi*, compared to the other three observer testing stage combinations. During this treatment stage, *P. moluccensis* individuals made between 24 and 101 feeding strikes (mean  $\pm$  SE:  $73.9 \pm 4.9$ ) and between 29 and 82 line crosses ( $58.2 \pm 3.5$ )

during the initial observation period and between 14 and 61 feeding strikes ( $29.9 \pm 3.1$ ) and between 20 and 69 line crosses ( $41.7 \pm 3.8$ ) during the final observation period.

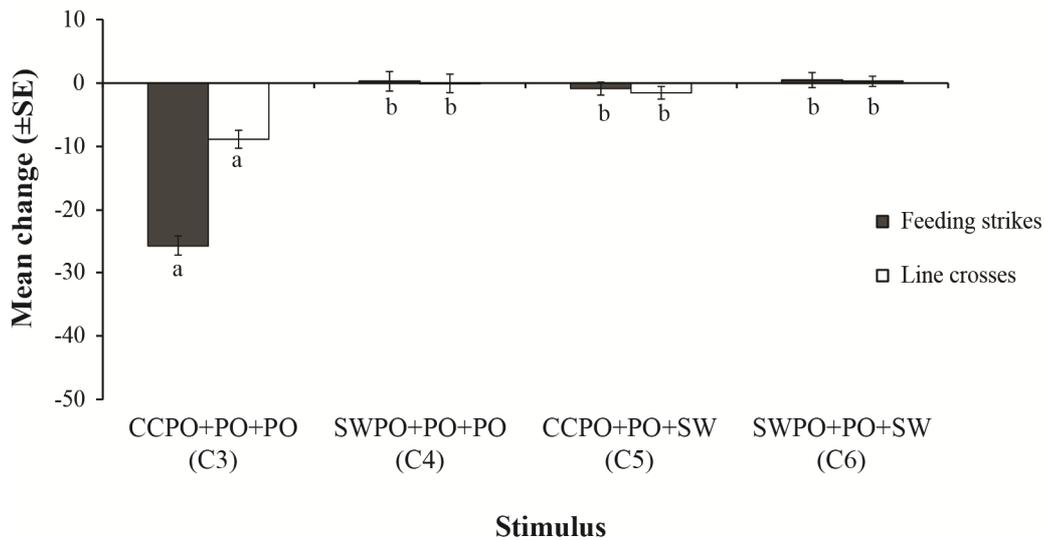


**Figure 5.3** Observer testing stage for anti-predator response: change in feeding strikes and line crosses by *Pomacentrus moluccensis* between initial and final observation periods for fish exposed to predator odour or seawater following conditioning with either a predator-experienced demonstrator (*P. wardi* exposed to a solution of damage-released chemical cue and predator odour (CCPO)) or a non-experienced demonstrator (*P. wardi* exposed to a solution of seawater and predator odour (SWPO)). Data are means  $\pm$ SE,  $n = 15$  per treatment. Letters below the bars represent Tukey's HSD groupings of means.

### 5.4.3 Stage 'c' response – *A. trimaculatus*

The behavioural response of individual *A. trimaculatus* to four observer testing stage combinations was examined. There was a significant change in behaviour between the initial and final observation periods among the four cues (Pillai's Trace = 0.851,  $F_{6,112} = 13.818$ ,  $P < 0.001$ ; Figure 5.4). Significant differences in both feeding strikes ( $F_{3,56} = 89.378$ ,  $P < 0.001$ ; Figure 5.4) and line crosses ( $F_{3,56} = 12.721$ ,  $P < 0.001$ ; Figure 5.4) caused this difference. Tukey's HSD means comparison tests highlighted a significant reduction in both feeding strikes and line crosses in response to the predator odour stimulus following conditioning with an experienced *P. wardi*, compared to the other three observer testing stage combinations.

During this treatment stage *A. trimaculatus* individuals made between 40 and 76 feeding strikes (mean  $\pm$  SE:  $57.6 \pm 2.8$ ) and between 11 and 32 line crosses ( $21.1 \pm 1.8$ ) during the initial observation period and between 17 and 50 feeding strikes ( $32 \pm 2.4$ ) and between 7 and 21 line crosses ( $12.2 \pm 1.1$ ) during the final observation period.



**Figure 5.4** Observer testing stage for anti-predator response: change in feeding strikes and line crosses by *Apogon trimaculatus* between initial and final observation periods for fish exposed to predator odour or seawater following conditioning with either a predator-experienced demonstrator (*Pomacentrus wardi* exposed to a solution of damage-released chemical cue and predator odour (CCPO)) or a non-experienced demonstrator (*P. wardi* exposed to a solution of seawater and predator odour (SWPO)). Data are means  $\pm$ SE, n = 15 per treatment. Letters below the bars represent Tukey's HSD groupings of means.

## 5.5 Discussion

This study highlights the use of social learning as a mechanism of acquiring information on predator identities among three species of coral reef fish. Specifically we demonstrate that a closely related damselfish *Pomacentrus moluccensis* and phylogenetically distant species *Apogon trimaculatus* are capable of learning the recognition of a predator odour through the process of social learning with experienced *Pomacentrus wardi* individuals. While other studies have shown that social learning occurs between conspecifics (Ferrari *et al.* 2012;

Manassa and McCormick 2012a; Manassa and McCormick 2012b – Chapter 3), this is the first study to demonstrate the occurrence of interspecific social learning in species found naturally in highly diverse ecosystems. These results suggest that social learning may act as a useful mechanism for the spread of information between species, ultimately increasing the likelihood of survival (Manassa and McCormick 2012b - Chapter 3).

Previously studies have documented the occurrence of social learning in damselfish species (Ferrari *et al.* 2012; Manassa and McCormick 2012a; Manassa and McCormick 2012b – Chapter 3), with this study extending our knowledge by demonstrating the use of this mechanism in cardinalfishes. As many species of coral reef fish live social lifestyles (Hoare and Krause 2003), it is expected that they could benefit greatly from this method of predator recognition, as it would allow for continuous updates and reinforcement of current predation events within the immediate area. Following a larval phase, coral reef fishes recruit to the reef in large numbers with many individuals settling onto the same habitat patches, resulting in both positive (e.g. schooling) and negative (e.g. competition) interactions (McCormick 2012). Selection of habitat patches is therefore crucial to survival, with those that settle into areas with high food availability, low occurrence of competition and minimal predators likely to have a considerable survival advantage (Feeney *et al.* 2012; McCormick 2012). During the first 48 hours following settlement predation by small piscivores (such as *P. fuscus* used in this study) is high, averaging ~60% (Almany and Webster 2006; Feeney *et al.* 2012). However, these fish settle with little knowledge of the identity of reef based predators (McCormick and Holmes 2006; Lönnstedt *et al.* 2012). Therefore, monitoring the behaviour of individuals that are similar in size, regardless of species, is likely to be beneficial especially within the first few hours following settlement when predator recognition is vital (Feeney *et al.* 2012; Lönnstedt *et al.* 2012). During these first few hours it is likely that individuals are using information from all relevant sources in an attempt to survive. Fine-tuning of anti-predator behaviours is likely

to follow with the assistance of direct learning methods. As such, a reliance on social learning in complex ecosystems with high species diversity, such as coral reefs, is likely immediately following settlement.

In complex ecosystems there is greater uncertainty about the reliability of social information, due to an increase in species diversity and therefore, a greater number of heterospecifics to pay attention to. Thus information will be variable in quality and relevance and may simply overwhelm an individual's ability to decipher the information in an ecologically relevant time-frame. Given this, one may expect that individuals will need multiple learning opportunities to acquire information, particularly from distantly related heterospecifics. However, the results of this study demonstrate that both *P. moluccensis* and *A. trimaculatus* were able to learn the identity of the predator based on a single pairing with an experienced heterospecific. One may expect social transmission of predator avoidance amongst members of the same prey guild irrespective of phylogenetic relatedness, however with the diversity of predators and constant ontogenetic shifts which occur on coral reef this may not occur, therefore additional research is required to further understand this topic. Further studies designed to manipulate the relative uncertainty of information, for example, by using individuals of different size, sex or body condition may also aid our understanding of social information use in complex ecosystems.

An anti-predator response to the alarm cues of other species is common throughout the animal kingdom with studies demonstrating an occurrence in birds, mammals, fishes, amphibians and insects (reviewed in Mitchell *et al.* 2012). However, the costs associated with this type of direct learning are significant, since individuals need to be in close proximity to a potential predator. As such, individuals may adopt a response that minimises risk by using information from all relevant sources. The use of interspecific social cues in predator recognition has been observed in studies conducted on birds (Vieth *et al.* 1980), larval

amphibians (Ferrari and Chivers 2008) and Ostariophysan fishes (Krause 1993; Mathis *et al.* 1996). Since the number of potential information sources are greater in complex ecosystems, individuals who are able to detect and respond to social information provided by other ecologically similar species, are likely to increase their chances of detecting actively foraging predators within their immediate vicinity. However, the mechanisms by which individuals select useful information may differ depending on the type of ecosystem. For example, on coral reefs the intense predation pressures placed on newly settling reef fishes may drive a reliance on social information with the benefits outweighing the costs associated. This may contrast with simpler systems, such as freshwater lakes, where individuals may be more selective when choosing information sources because of the reduced number and type of predators present.

The ability to utilise social information gained from heterospecifics is likely to confer a significant survival advantage for coral reef fish, particularly during critical life history transitions (e.g. settlement) where predation pressure is spatially and temporally unreliable. Likewise, the capacity of individuals to socially learn after a single conditioning event has profound implications for predator-prey interactions. Along with highlighting the widespread nature of social learning as a method of predator recognition this study documents the occurrence of interspecific learning in coral reef fishes, suggesting that the benefits of responding to uncertain information may override the costs associated with reduced foraging.

## **CHAPTER 6 – Social learning of predators in the dark: understanding the role of visual, chemical and mechanical information**

This chapter has been published in Proceedings of the Royal Society B: Biological Sciences.

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doi:10.1098/rspb.2013.0720

### **6.1 Summary**

The ability of prey to observe and learn to recognise potential predators from the behaviour of nearby individuals can dramatically increase survival and not surprisingly, is widespread across animal taxa. A range of sensory modalities are available for this learning, with visual and chemical cues being well established modes of transmission in aquatic systems. The use of other sensory cues in mediating social learning in fishes, including mechano-sensory cues remains unexplored. Here, I examine the role of different sensory cues in social learning of predator recognition, using juvenile damselfish (*Amphiprion percula*). Specifically, I show that a predator-naïve observer can socially learn to recognise a novel predator when paired with a predator-experienced conspecific in total darkness. Further, this study demonstrates that when threatened, individuals release chemical cues, known as disturbance cues into the water. These cues induce an anti-predator response in nearby individuals; however they do not facilitate learnt recognition of the predator. As such, another sensory modality, likely mechano-sensory in origin, is responsible for information transfer in the dark. This study highlights the diversity of sensory cues used by coral reef fishes in a social learning context.

## 6.2 Introduction

To counter the threat of predation, prey individuals have evolved sophisticated mechanisms to assess risk using visual, olfactory, tactile and auditory cues (Kelley and Magurran 2003; Crane and Ferrari 2013). However, obtaining first-hand information on local predators is dangerous since prey are required to be in close proximity of potential predators. In contrast, information obtained indirectly allows prey to gain knowledge about predators without the risk associated with direct experience. Animals that live in close proximity to one another have ample opportunity to acquire information by observing nearby conspecifics and not surprisingly this phenomenon is commonplace among animal taxa (Crane and Ferrari 2013). The process, whereby less experienced prey (observers) learn from experienced individuals (demonstrators) using social cues is known as social learning (Chapman *et al.* 2008). According to reviews by Griffin (2004) and Crane and Ferrari (2013) social cues are defined as any cue emitted (voluntarily or otherwise) by a conspecific, with well-known cues including the mobbing calls of birds and alarm calls in mammals. Changes in the behaviour of the demonstrator, including fleeing or hiding responses, also constitute social cues, just as would chemical cues released by conspecifics that are injured (alarm cues) or disturbed (disturbance cues) by predators (Ferrari *et al.* 2010b).

Social learning of predator recognition has been observed in a range of taxa (Brown and Laland 2001; Griffin 2004; Ferrari and Chivers 2008), however little work has attempted to identify the reliance on or diversity of sensory modes used in the acquisition of information. Studies have shown that social learning can occur through: visual mobbing displays and overt anti-predator responses (mammals, birds, amphibians, fishes and insects), auditory cues such as alarm calls (mammals and birds) and chemical cues such as injury-released conspecific cues (amphibians and fishes) (reviewed in (Crane and Ferrari 2013). It is possible that the range of cues being used reflects environmental constraints on information transfer. In aquatic systems,

the use of both visual and chemical cues is well documented in fishes (Brown 2003; Brown and Chivers 2006). Areas with high visibility and low structural complexity are ideal conditions for reliance on visual cues; however in areas where vision is obstructed, such as low light conditions, high turbidity and topographically complex environments, organisms are likely to demonstrate a well-developed response to chemical cues. Therefore, variation is likely to exist within a habitat, and individuals may acquire information using more than one sensory system or social cue type.

In the majority of studies on social learning in fishes, the demonstrators and observers were placed in the same observation tank (Verheijen 1956; Suboski *et al.* 1990; Mathis *et al.* 1996; Smith 1999; Ferrari *et al.* 2012; Manassa and McCormick 2012a). While these studies have been important in identifying the ability for organisms to socially transmit information about predators, including their level of threat, research now needs to be expanded to isolate the sensory mechanism responsible for the transfer of social information. A study by Ferrari *et al.* (2005) demonstrated learned predator recognition by fishes that observed conspecifics responding to a threat in an adjacent tank. This study was the first to show that the transmission of information could occur in the absence of all but visual cues. For visually oriented animals like humans, this result seems rather intuitive. However, there is huge potential for other sensory systems to be used for recognition as well. Information on sensory cues responsible for social learning are required to better understand the factors that can potentially affect information transfer, especially in light of environmental stressors affecting both visual (turbidity – Chivers *et al.* 2013b) and chemical (ocean acidification and acid rain – Leduc *et al.* 2004; Ferrari *et al.* 2011) properties of aquatic ecosystems.

Further evidence for the importance of non-visual cues is that most reef fishes settle from the plankton to benthic habitats at night (Dufour and Galzin 1993). These site-attached juveniles must quickly learn the identity of local predators as mortality levels are extremely

high within the first 48 hours (Almany and Webster 2006). As such, individuals are faced with a myriad of potential predators and non-predators, necessitating the need to identify those which represent a threat and those which do not. Responding to non-predators wastes valuable time and energy, but failing to respond to predators could cost the prey its life. Under these conditions it is not surprising that coral reef fishes rely heavily on social information to recognise predators. The importance of social learning in mediating survival was highlighted in a study by Manassa and McCormick (2012b - Chapter 3), where it was documented that fish who directly learnt the predator odour and those which acquired the information through social learning survived at least five times better during predator encounters than naïve individuals. As predator detection is an important process where a mistake can equate to death, individuals are likely to utilise all cues available to them.

The present study examines the role of different sensory cues in social learning of predator recognition, using juvenile damselfish, *Amphiprion percula*, as test subjects. The first part of the study investigates whether a predator-naïve observer can socially learn to recognise a novel predator when paired with a predator-experienced conspecific in total darkness. The second part of the study aims to isolate potential sensory cues mediating learning in complete darkness. Specifically, I test whether demonstrators release chemical cues (disturbance cues) upon detecting a threat, and whether or not these cues mediate social learning in the dark. Disturbance cues are ammonia compounds released by 'disturbed' prey either through the urogenital system or gills (Hazlett 1990; Kiesecker *et al.* 1999; Vavrek *et al.* 2008). These cues are known to increase vigilance when detected by conspecifics, but to date studies have failed to demonstrate the role of these cues in learning (reviewed in Ferrari *et al.* 2010b).

## **6.3 Materials and methods**

### **6.3.1 Fish collection and maintenance**

*Amphiprion percula* individuals were reared, maintained and fed as described in Chapter 2 (section 2.3.3).

*Cephalopholis argus* were collected from the Great Barrier Reef, Australia, and maintained at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University in individual 70 l aquaria. Individuals used to produce predator odours were fed twice daily however no feeding occurred 24 h prior to collection of predator odours.

### **6.3.2 Stimulus preparation**

Damage-released chemical cues were prepared as described in Chapter 2 (section 2.3.5) with a total of 75 *A. percula* individuals sacrificed (mean SL  $\pm$  SE: 20.61  $\pm$  2.94 mm).

Predator odour (*C. argus*) was collected as described in Chapter 3 (section 3.3.2.6) with predators fed a diet of Frozen Marine Dinners (46% fish product) which do not contain any damselfish cues.

### **6.3.3 Observation tanks**

Experiments were undertaken in observation tanks as described in Chapter 3 (section 3.3.2.5). A single *A. percula* (mean SL  $\pm$  SE: 20.61  $\pm$  2.94 mm) was placed into each tank and left to acclimate for 48 h prior to experimentation.

### **6.3.4. Quantification of behaviour**

Behaviour was quantified as described in Chapter 2 (section 2.3.7).

### **6.3.5 Experiment 1: Are visual cues necessary for social learning to occur in fish?**

To determine if social learning of a predator odour requires visual cues, a modified version of the well-established 3-stage social learning protocol was used (Manassa and McCormick 2012b – Chapter 3). The modified version uses two light conditions during the social learning stage; normal daylight conditions or total darkness (Figure 6.1).

#### **6.3.5.1 Stage 'a' – conditioning of naïve demonstrators**

Chemical alarm cues are known to elicit a strong anti-predator response and mediate predator learning in a similar way to Pavlovian conditioning. Therefore in this stage, individuals were conditioned to recognise a previously unknown predator by exposing them to either: the novel predator odour paired with chemical alarm cues of conspecifics (true conditioning) or a seawater control (pseudo-conditioning which does not lead to learning). Each demonstrator was conditioned individually in a tank. The protocol for this stage follows that from Chapter 3 (section 3.3.2.2) with one of two treatments (60 ml of predator odour paired with either 15 ml of seawater (pseudo-conditioning) or 15 ml of chemical alarm cue (true conditioning)) injected into the tank. The behaviour of 60 fish in each of the 2 treatments was recorded, with these individuals later used as predator-naïve (control) and predator-experienced (experimental) demonstrators in the social learning stage.

#### **6.3.5.2 Stage 'b' – pairing for social learning opportunity**

Immediately following the final observation period the demonstrator from Stage 'a' was dipped in clean seawater to remove any potential cues, then transferred to another observation tank housing an acclimated naïve individual (hereafter: the observer). To differentiate the two fish, the demonstrator was 5 mm smaller or larger than the observer, with fish randomly matched. The learning during this stage was setup under one of two light

conditions - light (419 - 426 Lux) or total darkness (0.0 - 0.1 Lux). The two individuals were acclimated under either light condition for 2 h before the start of Stage 'b'. Experiments took place in a temperature and light controlled laboratory during daylight hours, with a red-light used by the experimenter to navigate around the room when necessary (during total darkness trials). At the conclusion of the acclimation period, the flow-through system was turned off and 60 ml of tank water was drawn up the stimulus injection tube and discarded, and an additional 60 ml collected and kept for later use. A 60 ml aliquot of predator odour along with 10 ml of live *Artemia franciscana* was injected into the tank and flushed with 60 ml of previously collected tank water. No observations were carried out during this stage, however unquantified observations support that experienced demonstrators displayed the standard anti-predator behaviour. I predict that the presence of predator odour in the tank should elicit an anti-predator response from the predator-experienced demonstrators, but not from the predator-naïve demonstrators. Observers paired with predator-experienced demonstrators should have an opportunity to learn to recognise the predator odour as risky.

#### **6.3.5.3 Stage 'c' - testing for anti-predator response**

This stage tests the ability of the observer to respond to the predator odour on its own. This stage is necessary to ensure the observer can display the response in the absence of a nearby demonstrator (true learning vs. copying behaviour). Immediately following Stage 'b', the observer was rinsed in clean seawater then transferred to an empty observation tank and acclimated for 2 h. After the acclimation period, the experimental procedures from Stage 'a' were repeated, the flow-through system was turned off, 60 ml of tank water was drawn up the stimulus injection tube and discarded with a further 120 ml collected and kept. Immediately prior to the initial observation period, 10 ml of *A. franciscana* was injected into the tube followed by 60 ml of previously collected tank water, to flush the tube. The behaviour of the

observer was then recorded for 3 min. After the initial observation, one of two stimuli - a control (60 ml of seawater) or the experimental stimulus (60 ml of predator odour) was injected into the tank, along with a further 10 ml of live *A. franciscana*. The remaining 60 ml of previously collected tank water was then used to flush the stimulus through the tube. This was followed by a final 3 min observation period.

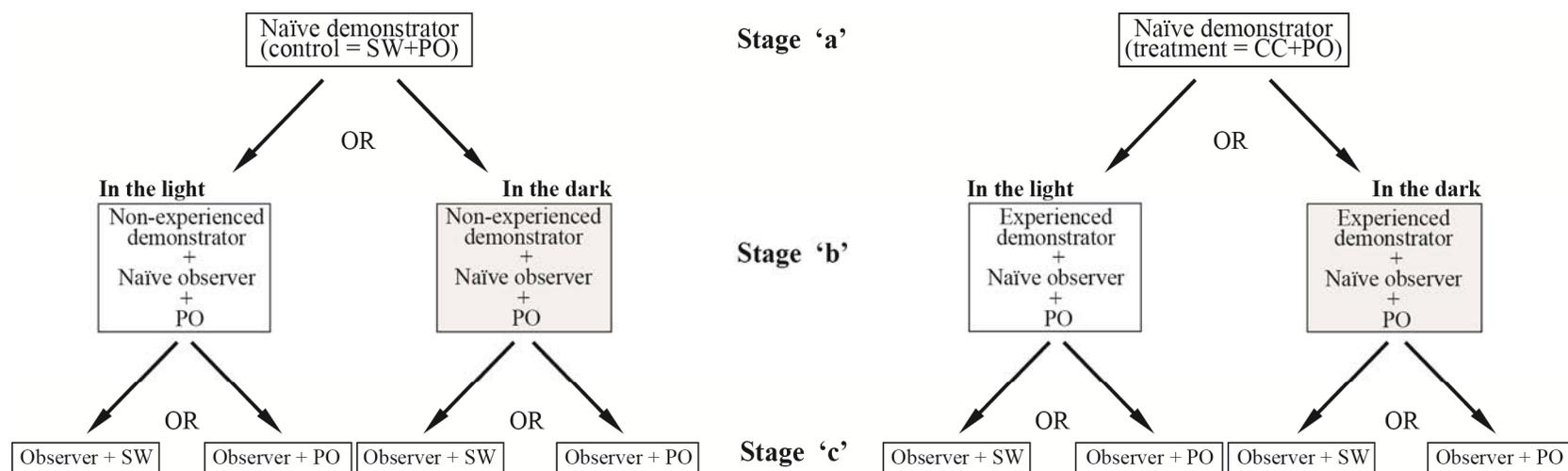
I predicted that observers that had successfully learned to recognise the predator odour as risky from their demonstrators should display an anti-predator response when exposed to the predator odour. Those that failed to learn should not respond to the predator odour. A total of 15 observers from each of the 4 treatments (predator-naïve vs. predator-experienced demonstrators crossed with an observer exposed to seawater or predator odour) were tested.

#### **6.3.5.4 Quantification of behaviour**

Behaviour was quantified as described in Chapter 2 (section 2.3.7).

#### **6.3.5.5 Statistical analysis**

Change in behaviour between the initial and final observation periods were used as raw data in the analysis. As activity and feeding are correlated, the two responses were analysed together using a MANOVA approach. The behavioural response of both demonstrators during Stage 'a' (true conditioning vs. pseudo-conditioning) were compared using a 2-way MANOVA. The effect of demonstrator experience (naïve vs. experienced), light condition during Stage 'b' (light vs. dark) and Stage 'c' cue (control or experimental stimulus) on the anti-predator response of observers was assessed using a 2x2x2 MANOVA. A series of factorial MANOVA's were then conducted to determine the significance of demonstrator experience (naïve vs. experienced) on observer behaviour. Inspection of residuals revealed that the data followed parametric assumptions.



**Figure 6.1** Flow chart illustrating the three-stage experimental design to test if *Amphiprion percula* are able to transmit the recognition of a novel predator odour to conspecifics in the absence of visual cues. Stage 'a' exposes demonstrators to either seawater and predator odour (SW+PO) or conspecific chemical alarm cues and predator odour (CC+PO). Stage 'b' exposes a naïve observer to predator odour (PO) in the presence of either a non-experienced or experienced demonstrator from Stage 'a' under two light conditions, light or dark. Stage 'c' exposes the naïve observer from Stage 'b' to either seawater (SW) or predator odour (PO)

### **6.3.6 Experiment 2 – Do juvenile damselfish release disturbance cues and can they be used as social cues to learn the identity of novel predators?**

The goal of this experiment was to: (1) assess whether damselfish possess disturbance cues, and if they do, (2) whether these cues can mediate learned predator recognition. The experiment was carried out in 4 stages (Figure 6.2).

#### **6.3.6.1 Stage ‘I’ – conditioning of naïve demonstrators**

This stage was identical to Stage ‘a’ in Experiment 1 and follows the same protocol. A total of 60 demonstrators were conditioned - 30 predator-naïve and 30 predator-experienced.

#### **6.3.6.2 Stage ‘II’ – collection of disturbance cues**

This stage exposed the demonstrators from Stage ‘I’ to predator odour. If detection of the predator odour elicited the release of a disturbance cue from the demonstrators these cues would be present in the surrounding water. I predict that predator-naïve demonstrators would not be ‘disturbed’ by the predator odour, hence would not release disturbance cues. Following Stage ‘I’ demonstrators were dipped in clean seawater, to remove any potential cues, and transferred individually to clean observation tanks to acclimate for 2 h. The flow-through system was then turned off, 60 ml of tank water drawn up the stimulus injection tube and discarded, with a further 60 ml collected and kept. A 60 ml aliquot of predator odour was injected into the tank followed by 60 ml of previously collected tank water. After 2 min, 60 ml of tank water containing predator odour and possible disturbance cue was drawn up the stimulus injection tube and retained.

### **6.3.6.3 Stage 'III' – testing disturbance cues on naïve individuals**

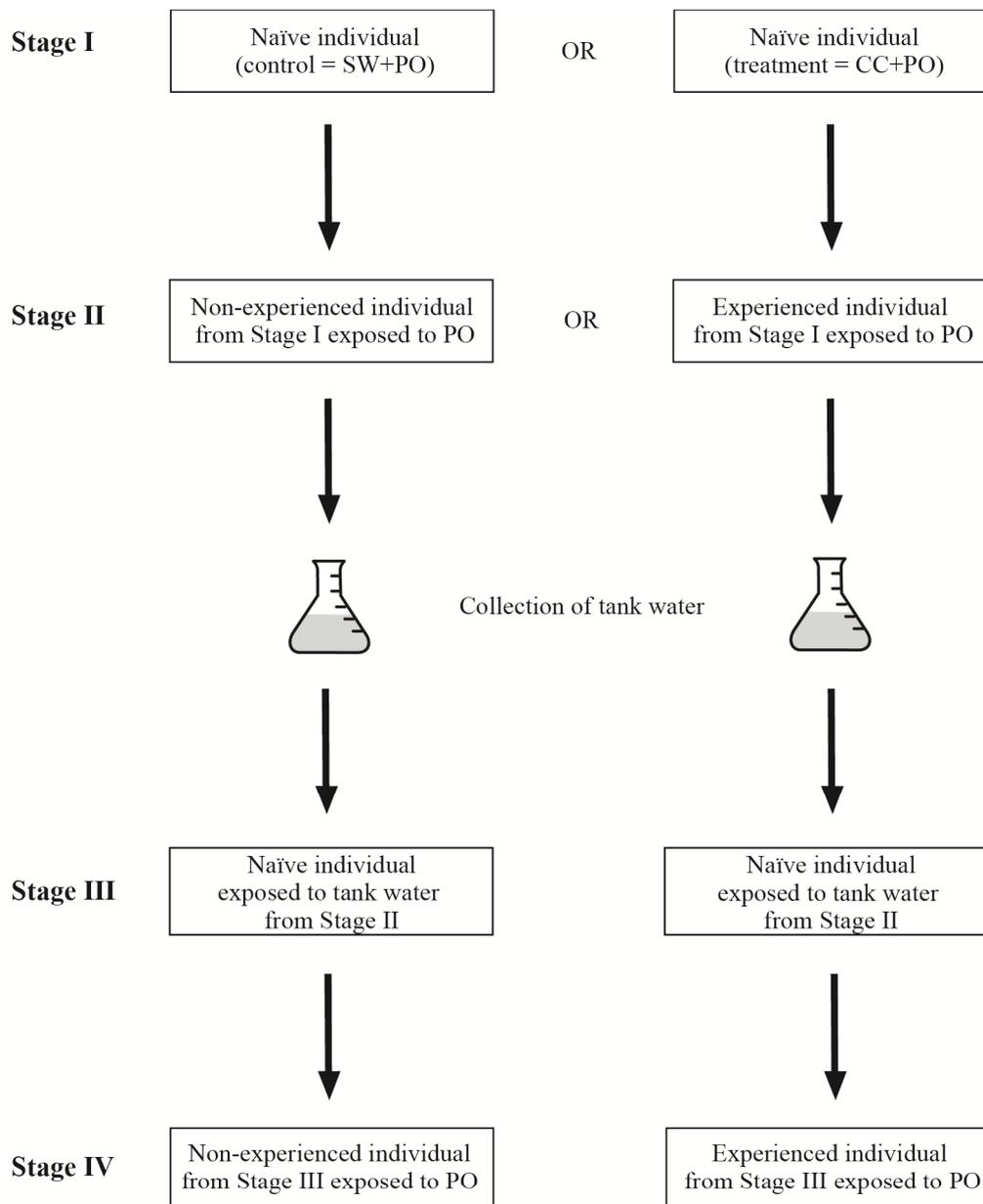
This stage determined whether naïve observers respond to the disturbance cues of conspecifics. Naïve individuals were exposed to the 60 ml of tank water collected during Stage 'II' from predator-naïve and predator-experienced demonstrators (n=15 per treatment). The experimental protocol and behavioural assay for this stage followed that of Stage 'c' in Experiment 1, with the 60 ml of tank water acting as the stimulus.

### **6.3.6.4 Stage 'IV' – testing if learning occurs following exposure to disturbance cues**

The individual from Stage 'III' was transferred to another observation tank to investigate whether exposure to disturbance cues paired with predator odour during Stage 'III' allowed them to acquire recognition of the novel predator. The observers were exposed to predator odour alone and their anti-predator behaviours were recorded. Again the protocol followed was identical to Stage 'c' from Experiment 1, with 60 ml of predator odour acting as the stimulus.

### **6.3.6.5 Statistical analysis**

As for Experiment 1, the behavioural response of the demonstrators during Stage 'I' (true conditioning vs. pseudo-conditioning) was compared using a 2-way MANOVA. A 2-way repeated measures MANOVA to test the effect of demonstrator experience (naïve vs. experienced) and stage (Stage 'III' vs. Stage 'IV') on the behaviour of observers was conducted. The behaviour of the observers was recorded twice, once during Stage 'III' and again during Stage 'IV', thus 'Stage' was the repeated-measure factor. Inspection of residuals revealed that the data followed parametric assumptions.



**Figure 6.2** Flow chart illustrating the experimental design to test firstly if *Amphiprion percula* release a voluntary disturbance cue and secondly if individuals are able to learn to associate a novel predator odour using these cues. Stage 'I' exposes demonstrators to either seawater and predator odour (SW+PO) or conspecific chemical alarm cues and predator odour (CC+PO). Stage 'II' exposes the demonstrators to predator odour (PO), after which tank water is collected for the next stage. Stage 'III' exposes naïve individuals to the tank water collected in Stage 'II' from either non-experienced or experienced demonstrators. The individuals from Stage 'III' are then transferred to new observation tanks, where during Stage 'IV' they are exposed to predator odour (PO)

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## 6.4 Results

### 6.4.1 Experiment 1 – Social learning in the dark

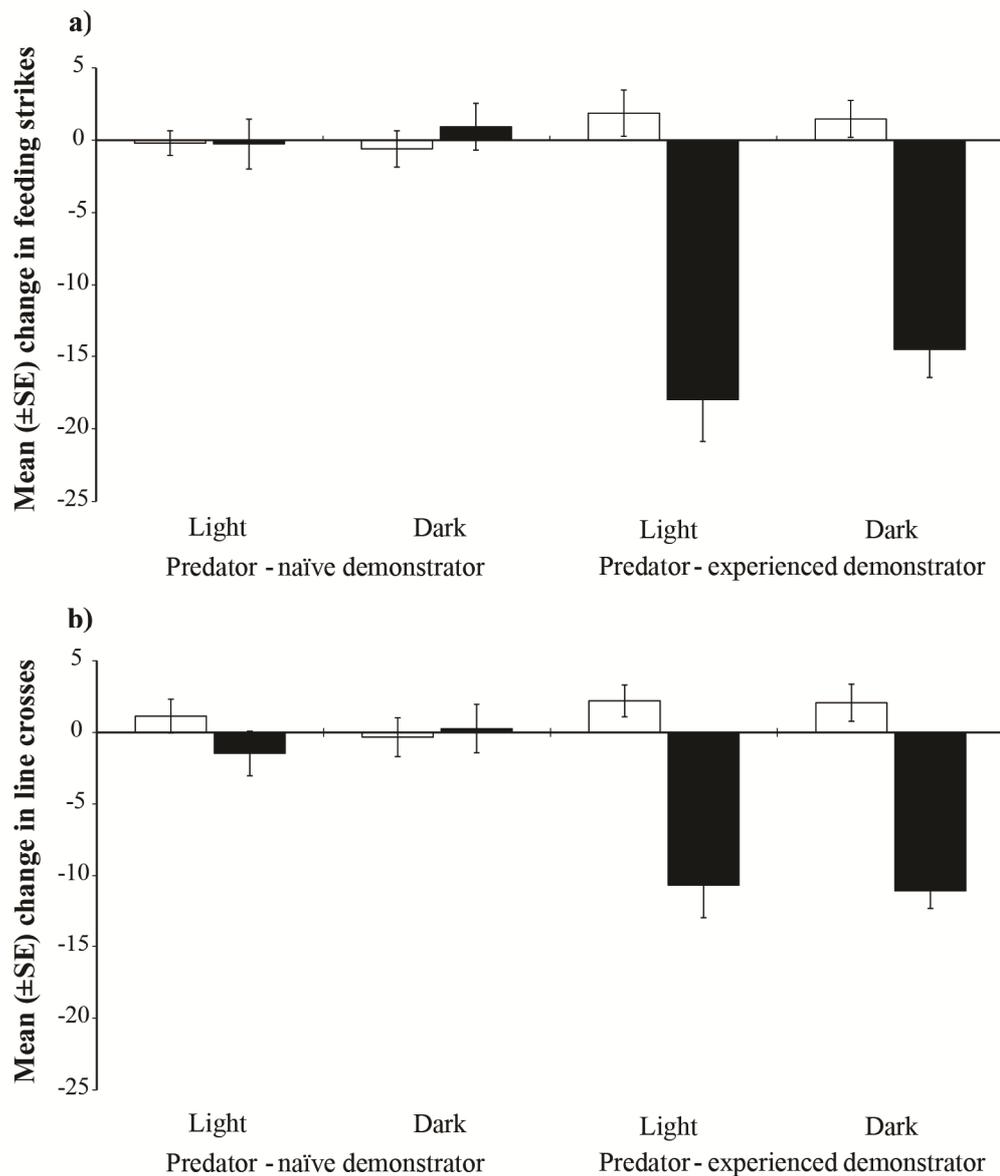
The demonstrators conditioned with chemical alarm cues displayed a significantly stronger anti-predator response than those pseudo-conditioned with seawater (Pillai's Trace = 0.68,  $F_{2,27} = 28.5$ ,  $P < 0.001$ ). While cue and demonstrator experience significantly interacted to affect the behaviour of the fish during Stage 'b' (social learning stage), light conditions (and any interaction involving light conditions) did not significantly explain the variation in the behaviour of observers, indicating that light conditions did not affect the outcome of learning. The results of the 2x2x2 MANOVA are presented in Table 6.1. As expected, when observers were paired with naïve demonstrators, neither cue (Pillai's Trace = 0.02,  $F_{2,55} = 0.7$ ,  $P = 0.5$ ) nor light (Pillai's Trace = 0.001,  $F_{2,55} = 0.04$ ,  $P = 0.96$ ) or any interaction between the two (Pillai's Trace = 0.02,  $F_{2,55} = 0.6$ ,  $P = 0.6$ ) affected the behaviour of the fish (Figure 6.3). The observers responded similarly to water and predator odour, regardless of light conditions during Stage 'b' – in other words, individuals failed to learn the predator odour as risky. Conversely, observers paired with experienced demonstrators subsequently displayed anti-predator responses when exposed to predator odour but not when exposed to seawater (Pillai's Trace = 0.65,  $F_{2,55} = 51.3$ ,  $P < 0.001$ ). Again, neither light (Pillai's Trace = 0.02,  $F_{2,55} = 0.44$ ,  $P = 0.6$ ) nor the light\*cue interaction (Pillai's Trace = 0.02,  $F_{2,55} = 0.6$ ,  $P = 0.5$ ) affected their response.

**Table 6.1** Results of the 2x2x2 way MANOVA testing the effect of demonstrator experience (naïve vs. experienced), light condition during Stage 'b' (light vs. dark) and Stage 'c' cue (control vs. experimental stimulus) on the foraging behaviour and activity level of the observers during Stage 'c' (Experiment 1).

Source of variance	df	F	Sig.
Demonstrator	2,111	18.6	< 0.001
Light	2,111	0.4	0.658
Cue	2,111	31.7	< 0.001
Demonstrator*Light	2,111	0.2	0.814
Demonstrator*Cue	2,111	31.8	< 0.001
Light*Cue	2,111	0.6	0.530
Demonstrator*Light*Cue	2,111	0.8	0.468

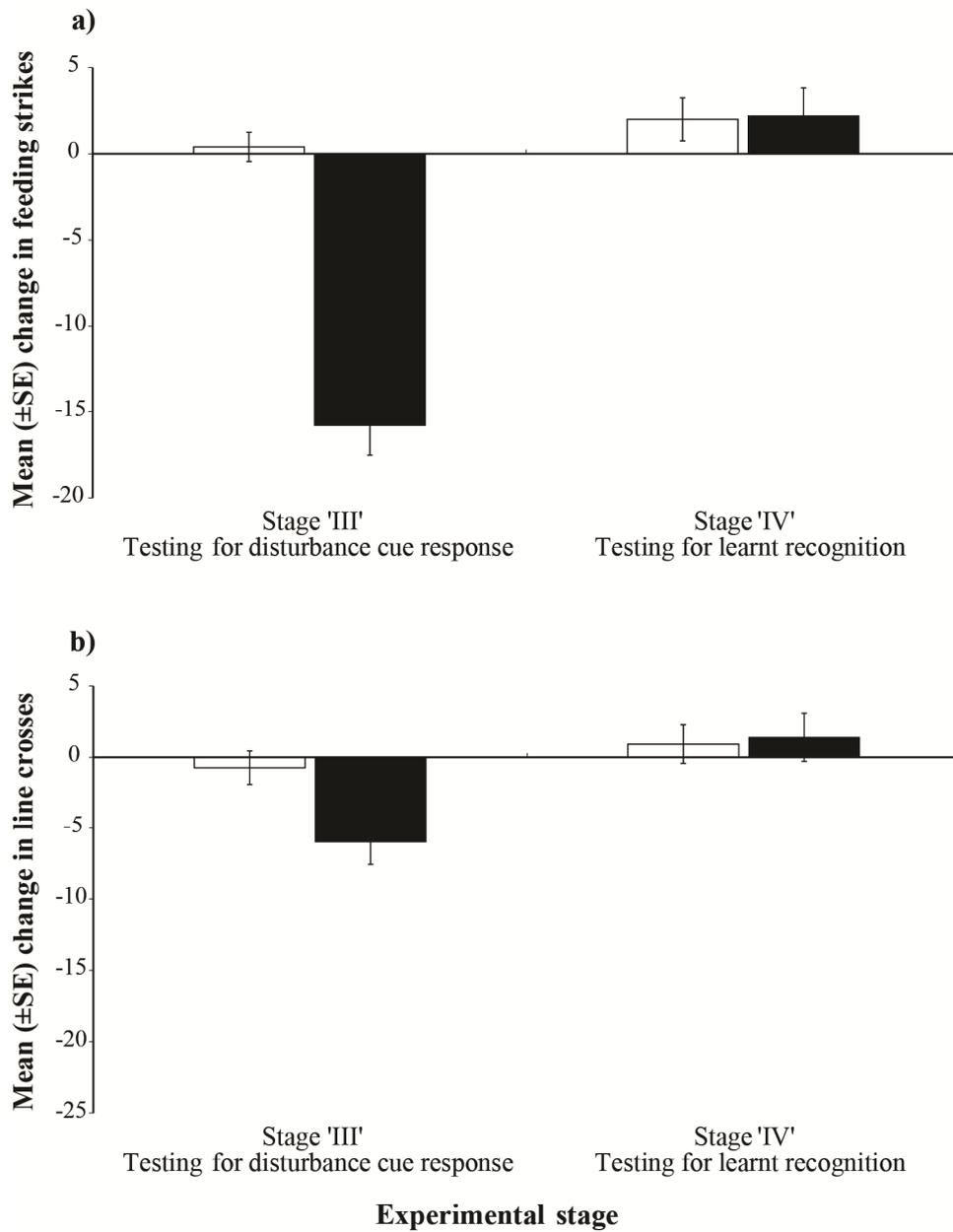
#### 6.4.2 Experiment 2 – Disturbance cues

A stronger anti-predator response was observed from demonstrators conditioned with chemical alarm cues compared to those pseudo-conditioned with water (Pillai's Trace = 0.69,  $F_{2,27} = 29.8$ ,  $P < 0.001$ ). The 2-way repeated-measures MANOVA revealed a significant interaction between demonstrator experience and stage (Pillai's Trace = 0.32,  $F_{2,27} = 18.7$ ,  $P < 0.001$ ; Figure 6.4) on the behavioural response of the observers. During Stage 'III', observers exposed to predator-experienced demonstrators displayed significant anti-predator behaviour while those exposed to cues from predator-naïve demonstrators did not (Pillai's Trace = 0.7,  $F_{2,27} = 34.2$ ,  $P < 0.001$ ). This result indicated that disturbance cues were released by predator-experienced demonstrators exposed to predator odour, with no such cues released by predator-naïve demonstrators. However, during Stage 'IV', demonstrator experience did not explain variation in the behaviour of the observers (Pillai's Trace = 0.003,  $F_{2,27} = 0.04$ ,  $P = 0.9$ ). Neither group responded to the predator odour.



### Demonstrator experience and light condition

**Figure 6.3** Mean change ( $\pm$ SE) in the number of feeding strikes (a) or line crosses (b) between the initial and final observation periods for *Amphiprion percula* observers exposed to seawater (empty bars) or predator odour (solid bars). The observers were previously paired with either a predator-naïve or predator-experienced demonstrator under light or dark conditions and exposed to predator odour.



**Figure 6.4** Mean change ( $\pm$ SE) in the number of feeding strikes (a) or line crosses (b) between the initial and final observation periods for *Amphiprion percula* individuals exposed to potential disturbance cues from predator-naïve (empty bars) and predator-experienced (solid bars) demonstrators. Following exposure to potential disturbance cues, observers were exposed to predator odour to determine if learnt recognition occurred.

## 6.5 Discussion

The results reveal that, in the absence of light, social learning of predator recognition can still occur in damselfish. This indicates that visual information – the sight of a frightened conspecific – is not a necessary cue to mediate learning. While previous studies have shown that visual information is enough to elicit learning (Ferrari *et al.* 2005), this is the first study in fish to demonstrate that visual cues are not the only sensory stimuli relied upon for information transfer; paving the way for future studies into the use of tactile or auditory cues in social learning. It is possible that disturbance cues, released by prey that have detected a predator, could provide the necessary social cues to allow for learning. This study also shows that disturbance cues elicit an anti-predator response in conspecifics; however they do not facilitate learnt recognition of a predator.

If vision is compromised, organisms need to rely on other modes of information transmission. Studies on amphibians and freshwater fishes have shown that when ‘stressed’ individuals release a disturbance cue, resulting in increased vigilance and anti-predator behaviour in nearby conspecifics (e.g. Wisenden *et al.* 1995; Jordão and Volpato 2000; Bryer *et al.* 2001; Mirza and Chivers 2002; Jordão 2004; Vavrek and Brown 2009). Ferrari *et al.* (2008) found that the disturbance cues had an additive effect on the response of fishes to damage-released chemical cues. If individuals had been pre-exposed to disturbance cues, greater response intensity to damage-released chemical cues was observed, suggesting that disturbance cues heighten an individual’s sense of awareness. However, the few studies which have examined the use of disturbance cues in predator learning have failed to provide support for this mechanism (Mirza and Chivers 2000; Ferrari *et al.* 2008). The results of the present study are similar to previous findings from freshwater fishes (Mirza and Chivers 2000; Ferrari *et al.* 2008); damselfish possess disturbance cues which are able to elicit increased anti-predator behaviour when detected; however these cues do not mediate learned predator

recognition. Some have argued that the absence of learning is due to the context in which disturbance cues are released. These cues may be released in response to predators; however they indicate a 'disturbed' individual suggesting that release may also occur as a means of aggression or territorial behaviour (Vavrek *et al.* 2008). Thus, the lack of learned association between disturbance cues and a novel stimulus may be adaptive by decreasing the opportunities for learning of irrelevant stimuli.

If social learning of predator recognition can occur in the absence of visual or chemical cues, other senses (mechanical or electrical) must come into play. Damselfish lack electroreceptors present in other fish species, making this sense an unlikely candidate. This leaves mechanical cues as the remaining sense responsible for the ability of individuals to transmit information about risk. Prey fish can detect mechanical disturbances in the water using their lateral line organs, with studies demonstrating the use of this sensory system during nocturnal predation (e.g. Pohlmann *et al.* 2001). It is possible that damselfish are learning a predator by the burst of activity of a nearby frightened conspecific; however this does not have to be the case. Indeed, it could be a reduction rather than a burst of activity that facilitates learning. Ferrari and Chivers (2008) showed that tadpoles socially transmit recognition of predators among conspecifics and other species with which they co-occur. Predator-experienced demonstrators reduce activity upon detection of known predator odours, and nearby individuals that were naïve to the predator used the reduction in activity as an indication of danger subsequently learning to reduce their activity upon detecting the predator odour. When the ratio of experienced to naïve demonstrators increased, there was a greater reduction in nearby activity and hence greater information transfer to naïve individuals. The same mechanism could be operating in damselfishes, as they often reduce activity upon exposure to predators. An alternative to fish responding to changes in mechanical disturbance is that they may be responding to sounds, possibly alarm calls, emitted by experienced

demonstrators. The ability of coral reef fish to utilise auditory cues has been extensively studied, with the majority conducted on damselfish species (e.g. Radford *et al.* 2001; Leis *et al.* 2002; Simpson *et al.* 2004; Tolimieri *et al.* 2004; Simpson *et al.* 2005; Simpson *et al.* 2008; Parmentier *et al.* 2009; Simpson *et al.* 2010; Simpson *et al.* 2011). As sound has a low attenuation in water, allowing acoustic signals to propagate quickly over large distances (Urick 1983), auditory cues are suggested to be important at night or in poor light conditions when visual cues are limited (Radford *et al.* 2001). With the use of mechanical and auditory cues being common amongst coral reef fishes (discussed in Myrberg and Fuiman 2002), additional experiments which examine the use of these sensory modes are essential to the understanding of social learning.

As predation levels change with life-stage and environmental conditions, prey are required to continuously learn the identity of new predators and modify the risk rating of those that no longer represent a threat (Mitchell *et al.* 2013). Under these conditions it is not surprising that individuals respond to the social cues of others. Given the risks associated with incorrect predator detection (e.g. death) prey are most likely relying on multiple sensory systems for predator recognition. As such, it is likely that cue choice is context dependent, with the spatial and temporal limitations of each sensory modality taken into account prior to use. If however, one or more sensory cue is unavailable, as occurred in this study (i.e. visual cues), prey may be capable of switching to another less reliable sensory cue in order to avoid capture.

Coral reefs are often thought of as clear water environments where visual cues are heavily relied upon. However, the structural complexity of reefs may limit the transmission of visual information. Moreover, for an average of 12 hours a day reefs are blanketed by darkness, a fact that further reduces the use of visual information and hence the utility of visual cues in predator learning. Chemosensory cues are therefore likely to be of benefit under these

conditions. However, chemosensory cues can be limited by water currents, chemicals released by other organisms (e.g. bleaching corals – Lönnstedt *et al.* 2013), and the associated impacts of climate change (e.g. ability of individuals to respond to chemical alarm cues – Luduc *et al.* 2004; Ferrari *et al.* 2010b). This study suggests that mechano-sensory and/or auditory cues can act as social cues enabling individuals to learn the identity of predators in the absence of visual or chemical information. Therefore it is likely that fish simultaneously use information from multiple cues to learn about predators and possess the flexibility to choose the most appropriate and informative sensory modality when one or more are unavailable, highlighting the importance of predation as a pervasive selective force.

## CHAPTER 7 - General discussion

The constant fluctuation of predator diversity and abundance dramatically affects a prey organisms' life both directly and indirectly throughout all life stages. To counteract these levels of predation pressure prey must continuously obtain up to date and relevant information on local threats. With such information being the difference between life and death, studies which examine the influence that one's social environment has on an individual's behaviour are of significant interest to behaviourists. Prior to this thesis, research into the use of social learning as a method of predator recognition in biologically complex ecosystems was limited. The five chapters presented here examined the importance of social interactions to the assessment of predation risk in a number of coral reef fish species.

Until now the ability of coral reef fish to use multiple information sources for risk assessment and prioritise information has not been explored. The results of **Chapter 2** showed that the presence or absence of visual cues dramatically influenced the way in which individuals responded to chemical cues, with a lack of response to the visual cues of other species indicating that individuals regard chemical information of higher importance. Since the reliability of both visual and chemical cues as indicators of predation risk can be limited (visual cues are affected by water clarity and/or topographic complexity whereas chemical cues can be both temporally and spatially inaccurate) it is likely that, as demonstrated in **Chapter 2**, prey incorporate multiple sources of information into their decision making processes.

For social animals, group members can provide information about the local environment directly through communication and/or behavioural responses to external stimuli. The lifestyles of coral reef fish suggest that information gained socially is important to the outcome of predator-prey interactions, as it would allow for continuous updates and reinforcement of current predation events within the immediate area. Social learning of

predation risk has previously been observed in two species of coral reef fishes; *Acanthochromis polyacanthus* (Manassa and McCormick 2012a) and *Pomacentrus amboinensis* (Ferrari *et al.* 2012), with **Chapters 3 – 6** of this thesis expanding our knowledge by demonstrating the use of this mechanism in a further four species (three damselfish species: *Pomacentrus wardi*, *Pomacentrus moluccensis* and *Amphiprion percula*, and one cardinalfish species, *Apogon trimaculatus*). Whilst highlighting the widespread function of social learning amongst coral reef fishes, this thesis demonstrates that the intense predation pressures placed on fish during early and transitional life-stages, and the high diversity and density of species which occupy coral reefs, results in a large reliance on social learning.

The survival of a prey individual depends on its ability to acquire information on local predation threats (e.g. Lönnstedt *et al.* 2012), with experience shown to directly lead to survival in both freshwater (Griffin 2004; Webster and Laland 2008) and coral reef fishes (McCormick and Holmes 2006; Lönnstedt *et al.* 2012). Research presented in **Chapter 3** is the first of its kind for any animal to show that survival was unaffected by the learning mechanism (directly or socially transmitted). In conjunction with previous studies, results predict that individuals should increase their use of socially acquired information when the costs of obtaining information directly are higher. As such, it is likely that social learning is a rapid and highly effective means of acquiring predator identities in coral reef fishes.

The influence that group size has on the behaviour of individual members has been widely studied throughout the animal kingdom (e.g. Brown 1982; Elgar 1989; Roberts 1996). By increasing the size of the group, individual vigilance is thought to decrease, thus allowing more time for other biologically important activities (Godin *et al.* 1988). In terms of social learning, it can be assumed that the higher the number of knowledgeable individuals the more effective information transfer will be. Likewise, a common benefit of group membership is the suggested dilution of predation risk, thus a reduction in the intensity of an individual's anti-

predator response (Blumstein *et al.* 1999). The findings of **Chapter 4** contradicted these hypotheses, by showing that increasing the number of observers (i.e. increasing group size) did not alter the ability of individuals to socially learn, nor did it change the response intensity exhibited. These anti-predator behaviours may reflect the importance that predators have on the lifestyles of coral reef fishes, suggesting that individuals maintain a high level of awareness regardless of group size. While group size used in this experiment may not have been large enough to elicit a significant response from the observer, it is more likely that from an evolutionary perspective there is no benefit in reducing ones response when the threat is extreme. Additional studies are necessary to test this by examining a larger proportion of demonstrators to observers and significantly larger group sizes to further our understanding of group size effects.

Social learning can occur through two pathways: intraspecific learning (learning that occurs between individuals of the same species) and interspecific learning (learning between different species). Intraspecific social learning was demonstrated in **Chapters 3, 4** and **6**, whilst **Chapter 5** focused on interspecific social learning between damselfish and cardinalfish species. Prior to this research interspecific learning had only been shown in birds (Vieth *et al.* 1980), larval amphibians (Ferrari and Chivers 2008) and Ostariophysan fishes (Krause 1993; Mathis *et al.* 1996). In biologically complex environments such as coral reefs, interspecific social learning may be commonplace, especially given that once settlement occurs there are dozens of potential heterospecifics that could provide information. By monitoring the behaviour of similar sized individuals regardless of species, especially at vulnerable life stages such as settlement, coral reef fish are likely to gain a wealth of relevant information. It is then possible that within the days following settlement, pending survival, a fine-tuning of anti-predator behaviours could occur. The levels of uncertainty which surround the accuracy of social learning are likely to be extrapolated in interspecific learning, implying that individuals

may need multiple learning opportunities to acquire the information. Interestingly in **Chapter 5** individuals were able to learn the identity of a predator based on a single pairing with an experienced heterospecific. This discovery indicates that social learning may occur between members of the same prey guild regardless of phylogenetic relatedness. However, additional research which tests individuals of different size, sex or body condition will aid our understanding of social information use in complex ecosystems.

Social learning of predator recognition is likely to occur during periods of high predation pressure. For coral reef fishes this is within the first 48 hours following settlement (Almany and Webster 2006). As settlement occurs at night, it was unclear until now if social learning in low light conditions was possible. **Chapter 6** demonstrated that in the absence of light social learning can still occur, indicating that visual information is not a necessary cue to mediate learning. As vision is compromised in the dark, organisms need to rely on other modes of information transmission. The release of chemical cues (disturbance cues) and their use in predator learning, has been demonstrated in amphibians and freshwater fishes (Bryer *et al.* 2001; Mirza and Chivers 2002; Vavrek and Brown 2009), however their presence in coral reef fishes was previously unknown. **Chapter 6** showed that disturbance cues are released by coral reef fishes, but like those of previous studies on freshwater fishes, these cues do not mediate learned predator recognition. This lack of learning possibly reflects the context under which disturbance cues are released; cues indicate a 'disturbed' individual which could suggest aggression or territorial behaviour rather than a predation threat (Vavrek *et al.* 2008). The flexibility of coral reef fish to switch between sensory modalities in this study highlights the profound influence of predation on coral reefs. However, additional experiments could determine the sensory modality primarily used, with individuals possibly utilising mechanical disturbances in the water or auditory cues emitted by experienced demonstrators.

The research presented in this thesis has focused on the ability of coral reef fish to use social learning as an anti-predator mechanism, with studies indicating its importance during life-history transitions. As predation risk on coral reefs varies on a seasonal, lunar, daily and minute-by-minute basis, prey must gain, assess and accurately utilise information on local predators to ensure survival (Lima and Dill 1990; Smith 1997). Along with this, as individuals grow the predators that pose a threat often change as a result of gape limitation. It is these dynamic predator-prey interactions that likely contribute to the reliance on social learning observed throughout this thesis. By using social learning to acquire information on local predators, coral reef fish are able to increase their lifetime fitness potential by significantly enhancing their survival. This survival benefit may explain the ability of coral reef fish to socially learn from other species; suggesting that individuals are less selective with whom they gain information from, as the number and type of predators is significantly higher compared to most other ecosystems. Interestingly, this thesis also demonstrated that information on predator identities is able to be passed onto group members quickly without a dilution of information content when the number of observers increases. Predation pressure on coral reefs is strongest during dawn and dusk, therefore the ability of coral reef fish to socially transmit the recognition of a predator in low light conditions further highlights the importance of social learning to the anti-predator responses of individuals.

The findings of this thesis raise interesting questions in regards to the role of social learning in the anti-predator behaviour of coral reef fishes. Are individuals able to use social learning as a means of acquiring information on non-threatening cues? If so, is this information able to be attained from heterospecifics? How do individuals reinforce important information whilst forgetting information that is no longer relevant? What is the retention time of information obtained through social learning? Is the value of social information dependent on the accessibility of acquiring direct information? Along with these, understanding how

changing environmental conditions affect the interpretation of information could prove interesting, especially given that results showed an ability to use a wide range of information sources when one or more were unavailable. Further examination into the use of mechanical and auditory cues as information sources is especially important, as coral reef fish are known to use these sensory modalities regularly. Future studies should consider the complexity of coral reefs when interpreting findings, as their chemical composition, species diversity and species abundance have been shown throughout this thesis to significantly alter an individual's behavioural response. However, perhaps more importantly, manipulative field experiments are essential to ensure the patterns of behaviour and survivorship observed throughout translate into the natural environment.

In conclusion, this thesis demonstrates the importance of social interactions to the acquisition of adaptive behavioural patterns in coral reef fishes. Social learning, once thought to be primarily associated with mammals and birds, plays a pivotal role in the life of coral reef fishes, especially during critical life stages such as settlement. The research presented here emphasises the versatility of coral reef fish in regards to choosing an appropriate anti-predator response, with the behaviour of other prey members shown to significantly affect an individual. Given the importance of predation as a pervasive selective force and the ability of coral reef fish to use a variety of sensory modalities to assess risk, social learning is likely to continue to play a vital role in complex ecosystems.

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# Effects of Ocean Acidification on Learning in Coral Reef Fishes

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## Abstract

Ocean acidification has the potential to cause dramatic changes in marine ecosystems. Larval damselfish exposed to concentrations of CO<sub>2</sub> predicted to occur in the mid- to late-century show maladaptive responses to predator cues. However, there is considerable variation both within and between species in CO<sub>2</sub> effects, whereby some individuals are unaffected at particular CO<sub>2</sub> concentrations while others show maladaptive responses to predator odour. Our goal was to test whether learning via chemical or visual information would be impaired by ocean acidification and ultimately, whether learning can mitigate the effects of ocean acidification by restoring the appropriate responses of prey to predators. Using two highly efficient and widespread mechanisms for predator learning, we compared the behaviour of pre-settlement damselfish *Pomacentrus amboinensis* that were exposed to 440 μatm CO<sub>2</sub> (current day levels) or 850 μatm CO<sub>2</sub>, a concentration predicted to occur in the ocean before the end of this century. We found that, regardless of the method of learning, damselfish exposed to elevated CO<sub>2</sub> failed to learn to respond appropriately to a common predator, the dottyback, *Pseudochromis fuscus*. To determine whether the lack of response was due to a failure in learning or rather a short-term shift in trade-offs preventing the fish from displaying overt antipredator responses, we conditioned 440 or 700 μatm-CO<sub>2</sub> fish to learn to recognize a dottyback as a predator using injured conspecific cues, as in Experiment 1. When tested one day post-conditioning, CO<sub>2</sub> exposed fish failed to respond to predator odour. When tested 5 days post-conditioning, CO<sub>2</sub> exposed fish still failed to show an antipredator response to the dottyback odour, despite the fact that both control and CO<sub>2</sub>-treated fish responded to a general risk cue (injured conspecific cues). These results indicate that exposure to CO<sub>2</sub> may alter the cognitive ability of juvenile fish and render learning ineffective.

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## Introduction

Ocean acidification, caused by the uptake of additional carbon dioxide (CO<sub>2</sub>) from the atmosphere, is a significant threat to marine ecosystems [1,2,3,4]. A rapid increase in CO<sub>2</sub> in the atmosphere leads to a lowering of the pH of the oceans, as additional CO<sub>2</sub> reacts with water to release bicarbonate (HCO<sub>3</sub><sup>-</sup>) and hydrogen ions (H<sup>+</sup>). This process has resulted in a drop in oceanic pH by 0.1 pH units since pre-industrial times [5] and a further 0.3–0.4 pH units decrease is predicted by 2100 if current CO<sub>2</sub> emissions trajectories are maintained [4]. Such a decrease is not novel per se, as geologic records indicate similar situations have occurred in the past, such as during the Paleocene-Eocene period some 56 million years ago [6,7]. A key question is how biological life will cope with this rapid change in ocean chemistry [5].

The potential effects of acidification on calcifying organisms, such as corals and invertebrates with calcareous exoskeletons, due

to the reduced saturation of carbonate ions in the ocean at lower pH [3,5,8] is now well-recognised. Much less is known on the consequences of ocean acidification on non-calcifying marine species, such as fish [9,10]. Indeed, a recent meta-analysis [10] shows that only 25% of the 198 tests reporting ocean acidification effects were performed on non-calcifiers, with only 2% of the studies being done on fishes (the other 23% focusing on algae and aquatic plants). Although early research indicated that very high levels of CO<sub>2</sub> (>10,000 ppm) were lethal for a number of fish species [11], some fish species appear to be tolerant of mild increases in pCO<sub>2</sub> [12,13]. However, non-lethal CO<sub>2</sub> levels predicted by the end of the century (up to ~1000 ppm depending on the IPCC scenario chosen) [14] may still lead to negative consequences. For example, Dixon et al. [15] reported that the coral reef clownfish *Amphiprion percula* was affected by CO<sub>2</sub> exposure so that larvae exposed to CO<sub>2</sub> levels of 1000 ppm were not able to respond appropriately to the odour of predatory fishes (the rockcod, *Cephalopholis cyanostigma* and a dottyback, *Pseudochromis*

*fuscus*). Munday et al. [16] provided the first evidence of the fitness costs associated with such effects of CO<sub>2</sub>. Young juveniles of the damselfish, *Pomacentrus wardi*, that were exposed to 850  $\mu\text{atm}$  of CO<sub>2</sub> and released in the wild suffered an 8-fold increase in predation-related mortality in the first 30 h of settlement, compared to control fish exposed to present-day levels of CO<sub>2</sub> (440  $\mu\text{atm}$  CO<sub>2</sub>). These results do not reflect a lack of detection of the cues by the fish, as both Dixson et al [15] and Munday et al. [16] showed that juveniles from controls avoided predator odours, while CO<sub>2</sub>-treated juveniles were *attracted* to predator odours.

Recent studies have shown a surprising amount of intra- and inter-specific variation in the effects of CO<sub>2</sub> on fishes [16,17]. At levels nearing 700  $\mu\text{atm}$ , some individuals consistently display an appropriate response while others consistently show maladaptive responses to predators. Thus, there should exist a time in the future where affected fish (those that do not respond appropriately to predators) will co-occur with unaffected individuals. This could either result in strong directional selection, whereby affected individuals will be removed from the population, or it could delay the effects of CO<sub>2</sub>, by allowing these fish to learn to display the appropriate response by copying the behaviour of non-affected individuals. Thus, the extent to which appropriate responses to predators may be acquired is a key question. If this is possible, it might mitigate the effects of ocean acidification on predator-prey interactions.

Some coral reef fishes do not show innate recognition of predators [18]. Learning is key to acquire new knowledge, skills and behaviours, and interaction and experience with predators are among the most efficient means of learning the identity of predators, due to the immediate costs (i.e., injury or death) associated with a lack of an appropriate response by potential prey. For aquatic species, one way to learn to recognize predators is through the simultaneous detection of novel predators and cues from injured conspecifics (reviewed by [19]). Cues from injured conspecifics (or ‘alarm cues’) are known to elicit immediate and dramatic antipredator responses, due to the highly reliable nature of those cues in a predation context; they are only released through mechanical damage to the skin of the prey, typically during a predator attack. Such learning is highly efficient – one-time learning – and widespread, from flatworms to larval amphibians [19]. The first goal of our study was thus to test if 850  $\mu\text{atm}$  CO<sub>2</sub>-exposed fish would acquire recognition of novel predators through this learning process.

Another form of antipredator learning involves social learning, whereby naive individuals learn by observing more experienced conspecifics respond to a predator ([20,21] for reviews). Social learning may be particularly important for coral reef fishes, as they often colonize corals at high densities and have opportunities to observe the behaviours of resident conspecifics and heterospecifics. If individuals that are affected by CO<sub>2</sub> can learn to recognize predators from unaffected individuals, then the negative effects of CO<sub>2</sub> exposure may be reduced. Thus, the goal of a second experiment was to investigate how exposure to elevated CO<sub>2</sub> affected the ability to acquire recognition of a novel predator from individuals not affected by CO<sub>2</sub>.

A failure to respond to predator cues following a conditioning event may be explained one of two ways: 1) the prey may have failed to learn the predator as a danger, or 2) the prey successfully learned to recognize the predator, but intrinsic factors may prevent them from showing an overt antipredator response to the cues at the time of testing. One such intrinsic factor is hunger level. For instance, Brown et al. [22] showed that hungry fathead minnows, *Pimephales promelas*, still learned to recognize pike, *Esox lucius*, as a predator despite the absence of an alarm response

during conditioning. When subsequently fed, the minnows displayed antipredator responses similar to those of well-fed minnows when exposed to pike odour. These results are explained by a shift in foraging trade-offs whereby the need of prey to forage overrides the behavioural responses to the predator. In our situation, it is possible that CO<sub>2</sub> may alter physiological and foraging needs via another state-dependent factor explaining the lack of response of prey to predator cues. To discriminate between these two options, we performed a third experiment whereby 700- $\mu\text{atm}$  CO<sub>2</sub>-exposed fish that had been conditioned to recognize a predator, via conditioning with injured conspecific cues, were tested for their response to the predator at one day or five days post conditioning. We chose 5 days as previous studies have shown that the CO<sub>2</sub> effects only last up to four days after the fish have been returned to control water [16].

Our study examined these questions in the context of a coral reef ecosystem on the Great Barrier Reef, Australia. Most coral reef fishes have a pelagic larval stage that resides in the plankton for a period of weeks to months [23]. At the end of this phase, juvenile fish must locate suitable benthic habitat and in doing so, face a new and abundant array of predatory reef fishes. Predators may remove up to 60% of newly settling fish in a single night [24], creating population bottlenecks. In the days immediately prior to settlement, juvenile fish can be captured away from the reef in large numbers using light traps [25,26]. Although they have juvenile form and colouration, these individuals are naïve to the suite of predators that await them on the reef. Learning to recognize predators upon settlement is a critical step in the life history of these fish. Our system provides a unique opportunity to examine interactions between learning behaviour, predation and the effects of ocean acidification.

## Methods

### Test subjects and CO<sub>2</sub> treatment

Experiments took place at the Lizard Island Research Station (14°40'S, 145°28'E), on the Great Barrier Reef, Australia, in November and December 2009 (experiments 1 and 2) and 2010 (experiment 3). We used established protocols to capture and treat our fish [16,27]. Pre-settlement juveniles (16–21 days old) of *Pomacentrus amboinensis* were caught overnight in light traps [26] moored >100 m off the reef at Lizard Island. Light traps collect these fish immediately prior to their arrival on the reef at the end of the planktonic larval stage [28]. Every morning, *P. amboinensis* collected in the traps were transferred to 35-L rearing aquariums at 440 (present-day control CO<sub>2</sub> levels), 700 or 850  $\mu\text{atm}$  CO<sub>2</sub>. Pomacentrid larvae exposed to elevated CO<sub>2</sub> over a few days showed identical behavioural impairments as larvae raised under the same CO<sub>2</sub> levels from birth [16], indicating that the alteration in behaviour were not due to a sudden CO<sub>2</sub> exposure. Moreover, given their bipartite life history, juvenile damselfish would naturally be exposed to a change in CO<sub>2</sub> conditions, when they recruit from the open ocean, where CO<sub>2</sub> conditions are relatively stable, to the coral reef where pCO<sub>2</sub> can fluctuate significantly on a daily basis due the net effects of photosynthesis, respiration and calcification [29,30].

CO<sub>2</sub> treatments were maintained by CO<sub>2</sub> dosing to a set pH<sub>NBS</sub> following standard techniques for ocean acidification research, as set out in the Best Practices Guides for Ocean Acidification Research [31]. Seawater was pumped from the ocean into 4×60 L sumps where it was diffused with ambient air (control) or CO<sub>2</sub> to achieve a pH of approximately 8.15 (control), 7.97 or 7.89. The reduced pH values were selected to achieve the approximate CO<sub>2</sub> conditions required, based on preliminary observations of total

alkalinity, salinity and temperature of seawater at Lizard Island. A pH-controller (Tunze Aquarientechnik, Penzberg, Germany) was attached to each of the CO<sub>2</sub> treated sumps to maintain pH at the desired level. A solenoid injected a slow stream of CO<sub>2</sub> into a powerhead at the bottom of the sump whenever the pH of the seawater rose above the set point. The powerhead rapidly dissolved CO<sub>2</sub> into the seawater and also served as a vigorous stirrer. Equilibrated seawater from each sump was supplied at a rate of ~500 ml.min<sup>-1</sup> to four replicate 35-L aquariums, each housing a group of larval fishes. To maintain oxygen levels and the required pCO<sub>2</sub> levels, aquariums were individually aerated with air (control ~440 µatm) or CO<sub>2</sub>-enriched air (~700, or 850 µatm). The concentration of CO<sub>2</sub>-enriched air was controlled by a scientific-grade pressure regulator and precision needle valve and measured continuously with an infrared CO<sub>2</sub> probe (Vaisala GM70, Vaisala, Helsinki, Finland). Temperature and pH<sub>NBS</sub> of each aquarium was measured each morning and afternoon using an HQ40d pH meter (Hach, Loveland, Colorado, USA) calibrated with fresh buffers. Total alkalinity of seawater was estimated by Gran titration from water samples taken twice weekly from each CO<sub>2</sub> treatment. Alkalinity standardizations performed before processing each batch achieved accuracy within 1% of certified reference material from Dr. A. Dickson (Scripps Oceanographic Institute). Average seawater pCO<sub>2</sub> was calculated using these parameters in the program CO<sub>2</sub>SYS and using the constants of Mehrbach et al. [32] refit by Dickson & Millero [33]. Estimated seawater parameters are shown in Table 1.

Young damselfishes were fed freshly hatched *Artemia nauplii* three times a day. The fish were treated for 4 consecutive days and then used in our experiment immediately after the treatment period was over. Due to experimental limitations in the amount of CO<sub>2</sub> water that could be produced daily, it was not possible to test CO<sub>2</sub>-treated fish in CO<sub>2</sub>-enriched water. Thus, the experimental manipulations described thereafter took place in control water. This methodology was successfully used previously [16,17,34]. Juvenile damselfish have also been shown to display the same behavioural alteration in CO<sub>2</sub>-enriched as in control water after a 4-day CO<sub>2</sub> exposure period [16]. Fish treated with 700–850 µatm CO<sub>2</sub> retain their CO<sub>2</sub>-induced impaired behavioural responses for at least 48 h after being transferred back into control water, but no longer than 4 days [16].

### Experiment 1: Acquired predator recognition via pairing with cues from injured conspecifics

Our first experiment investigated the ability of CO<sub>2</sub>-treated fish to respond to predator odour following conditioning with cues from injured conspecifics. The learning procedure is a 2-step process that first involves a conditioning phase where fish are exposed to cues of injured conspecifics paired with those of a novel predator and second, a testing phase, where fish are exposed to the

predator cue alone to measure any learned antipredator response. Our experimental set-up followed a complete 2×2×2 design, consisting of conditioning either control or 850 µatm CO<sub>2</sub>-treated fish with the odour of a predatory dottyback, a common predator of newly-settled damselfishes at Lizard Island [35], paired with either water (pseudo-conditioning) or cues from injured conspecifics (true conditioning). Later, the fish were tested for their response to either the dottyback odour or a water control. The group of individuals that were pseudo-conditioned should not have acquired recognition of the predator, while predator odour should be recognised as a risky stimulus by the group that were exposed to the cues from injured conspecifics.

**Conditioning phase.** At least 6 h prior to conditioning, larvae were removed from their respective CO<sub>2</sub> treatment, and placed individually in 20-L flow-through tanks (32×16×16 cm) equipped with sand, a small piece of dead coral as a shelter, an airstone, and a 1.5 m long injection tube used to introduce stimuli into the tank. Each tank was covered on three sides with black plastic to avoid visual transfer of information from surrounding tanks. In addition, a black plastic curtain was hung in front of the tanks to minimize disturbance to the fish by the movement of the observer. One h after adding fish to the conditioning tanks and again, 1 h prior to conditioning, the fish were fed *ad lib* with *Artemia* larvae. Water flow was turned off 30 min prior to conditioning the fish. In half of the tanks, we introduced 5 mL of injured conspecific cues paired with 20 mL of dottyback odour, while the other half received 5 mL of seawater paired with 20 mL of dottyback odour. The concentrations we used are based on previously published studies [17,18]. After 1 h, we turned the flow-through system back on, transferred the fish into their testing tanks with the water flow on, and fed them *ad lib* 30 min later.

Cues from injured conspecifics were prepared fresh, by gently slicing the side of a sacrificed individual (JCU Animal Ethics Protocol A1067) and rinsing it with fresh seawater. A preliminary experiment showed that cues produced by making 4 cuts on each side of a fish were enough to elicit an overt antipredator response in juvenile damselfish when injected into the tanks. Thus, to minimize the number of fish sacrificed, we made 12 cuts on each side of a fish and rinsed it with 15 mL of seawater in a glass Petri dish to obtain enough cues for 3 conditioning events. We repeated this procedure until we had enough cues to condition all the tanks for that day, and mixed all the cues together prior to injection. All cues were used within 15 min of being made to ensure their potency [18]. Dottyback were collected 3 weeks prior to our experiment while diving in the lagoon at Lizard Island using hand nets and anaesthetic clove oil mixed with alcohol and seawater. Two yellow morph dottybacks (6.5 and 7.1 cm standard length) were maintained in a 70-L tank of aerated water where 60% of the water was changed daily. The dottyback were fed prior to the water change with INVE Aquaculture Nutrition 12/20 pellets. Water taken from the dottyback tank was used as our predator odour and was injected into our experimental tank within 20 min of being collected.

**Testing phase.** Trials began between 4 and 8 hours after transfer of juvenile fish into the testing tank. Test and conditioning tanks were identical, with the exception that a 4×4 cm grid was drawn on the side of the test tank to help the observer record positions of the fish during the experiment. One h prior to testing, the juvenile fish were fed and the flow-through system was tuned off 30 min later. Behavioural observations of the fish were conducted during this phase. The order of treatments was randomized.

**Behavioural bioassay.** To stimulate activity, we injected small quantities of food into the tank, on the opposite side of the

**Table 1.** Mean (± SD) seawater parameters in the experimental system.

pH <sub>NBS</sub>	Temp °C	Salinity ppt	TA (µmol.kg <sup>-1</sup> SW)	pCO <sub>2</sub>
8.15 (0.04)	27.66 (0.98)	35	2269.66 (15.01)	440.53 (44.46)
7.97 (0.06)	27.59 (0.97)	35	2259.87 (11.55)	718.37 (110.82)
7.89 (0.06)	27.74 (0.99)	35	2261.23 (14.92)	879.95 (140.64)

Temperature, pH salinity, and total alkalinity (TA) were measured directly. pCO<sub>2</sub> was estimated from these parameters using CO<sub>2</sub>SYS.

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coral shelter, creating a choice for juveniles to either forage or take refuge within the coral head. During each observation period, we measured 3 behaviours: (1) the total number of feeding strikes displayed by the fish, regardless of whether they were successful at capturing a food item or not; (2) the total number of lines the fish crossed during the observation period, using the 4×4-cm grid drawn on the side of the tank. A line was counted as crossed when the entire body of the fish crossed a line. This behaviour represents a measure of the swimming activity of the fish; (3) the total number of different squares visited during the observation period. This represented the 2-dimensional area of activity of the fish, and is a standard technique used to measure activity [18]. Prey fishes exposed to risk typically decrease or stop feeding, decrease their swimming activity and reduce their area of activity [36,37].

Initially, the juvenile fish were fed 2.5 mL of food (seawater containing ~250 *Artemia* larvae.mL<sup>-1</sup>) to remove the possibility of a “feeding frenzy” effect at the start of the bioassay. Pre-stimulus observation began 5 min later, when another 2.5 mL of food was injected into the tank. At the end of this 5-min pre-stimulus observation period, 20 ml of dottyback odour or 20 mL of water were introduced into the tank followed by 2.5 mL of food. The behaviour of the juvenile was then observed for 5 min. The experimenter was blind to the treatment during the observation. To control for any day effect, we tested the same number of fish from each of the treatment groups each day. We ran 16–17 replicates in each of the 8 treatment groups, testing a total of 129 fish.

## Experiment 2: Acquired predator recognition via visual cues from conspecifics

This experiment was designed to test whether the fish could acquire recognition of predators via visual cues from conspecifics, and in particular, whether this ability was impaired by exposure to 850 µatm CO<sub>2</sub> concentrations. Similar to Experiment 1, the conditioning with odour cues from injured conspecifics and social learning procedures are divided into two phases: the conditioning phase consisting of pairing predator-naïve (hereafter ‘learner’) and predator-experienced (hereafter ‘tutor’) individuals and exposing them to predator cues. At this time, the naïve learner individual has an opportunity to observe the behavioural response displayed by the experienced tutor toward the predator cues and thus acquire recognition of the cues as a potential threat. In the testing phase, the tutor is removed and the naïve fish subjected to the predator cues. Our experimental design followed a 2×2×2 design, consisting of pairing a naïve learner raised under normal or 850 µatm CO<sub>2</sub> (CO<sub>2</sub> effect on learner) to a tutor that was either naïve or experienced with dottybacks (tutor experience), and then exposing the pair to dottyback odour. During the testing phase, the observers were exposed to water or dottyback odour (testing cue) and their antipredator responses were measured. We predicted that learner fish paired with naïve tutors would not learn to recognize the predator as threatening, and that learning to recognize predators from tutors would potentially be reduced if learners were exposed to high CO<sub>2</sub> concentrations.

**Naïve and experienced tutors.** Presettlement juvenile *P. amboinensis* were collected from the light traps in the morning and conditioned to be used as tutors the following day. To distinguish the tutors from learner fish, we marked the tutors with a small colored elastomer tag injected under the skin on their dorsal side behind their dorsal fin. This tagging does not influence the behaviour or survivorship of juvenile damselfishes [38]. Tutors were then randomly placed in conditioning tanks identical to those described in the previous experiment, and underwent a conditioning identical to the one described for Experiment 1.

Half of the tutors were conditioned via pairing of injured conspecific cues and dottyback odour, hence rendering them ‘experienced’ to the dottyback predator, while the other half received dottyback odour paired with water (pseudo-conditioning), which kept them ‘naïve’ with regards to the dottyback.

**Conditioning phase.** In a flow-through conditioning tank, we paired one naïve or one experienced tutor with a learner fish that was raised for 4 days under normal or 850 µatm CO<sub>2</sub> levels. To control for day effects, we conditioned and tested the same number of each of the four pairing combination each day. Thirty min after pairing them, the fish were fed to satiation. The next morning, the fish were fed again. One h after feeding, the flow-through system was turned off and the conditioning phase began 20 min later. To ensure an overt antipredator response from the tutor fish, we injected 5 mL of *Artemia* in the tank 5 min prior to conditioning. We then injected 2.5 mL of *Artemia*, followed by 20 mL of dottyback odour. We left the fish undisturbed for 1 h, then turned the water flow back on and removed the tutor fish.

**Testing phase.** This phase took place between 4 and 8 h following the conditioning phase. The experimental setup, behavioural bioassay and methodology and cues were identical to the ones described for Experiment 1. The fish were tested for a response to 20 mL of seawater or 20 mL of dottyback odour. We ran 16 replicates in each of the 8 treatment groups, testing a total of 128 fish. The order of testing was randomized among treatments.

## Experiment 3: Is CO<sub>2</sub> exposure inducing a lack of learning or simply a lack of response?

This experiment was designed to test whether fish that did not display an antipredator response after being conditioned in elevated-CO<sub>2</sub> water, would subsequently respond to the predator once the CO<sub>2</sub> effects wore off. Juvenile damselfish exposed to control or 700 µatm CO<sub>2</sub>-levels were conditioned via injured conspecific cues to recognize a predatory dottyback following the same methodology as Experiment 1. All fish were exposed to 5 mL of injured conspecific cues paired with 20 mL of dottyback odour. Although the goal of the experiment was to test for residual CO<sub>2</sub> effects post-CO<sub>2</sub> treatment, we needed to ascertain that the results observed in Experiment 1 with 850 µatm CO<sub>2</sub> fish were also observable with 700 µatm CO<sub>2</sub> fish. Thus, as in Experiment 1, a group fish was tested for their behavioural response to the predator odour or a water control one day post-conditioning. The rest of the fish were tested 5 days post-conditioning for their response to the predator odour, a water control or an injured conspecific cue control. The water served as a negative control, while the injured conspecific cues served as a positive control, as they elicit overt antipredator responses independently of experience. Hence, we predicted that if fish are able to display an overt antipredator response to injured conspecific cues, they should also be able to display an antipredator response when exposed to the predator odour, assuming they have successfully learned to recognize the odour as a risky stimulus during the conditioning phase. Conditioning and testing protocols were identical to those described in Experiment 1. We conditioned a total of 75 fish.

## Statistical analysis

For all experiments and all variables, no pre-stimulus difference was found among treatments. Thus, we used the raw data to compute change in activity from the pre-stimulus baseline (post minus pre) for each of the three behaviours. Due to the interdependency of the three behaviours, we analyze them together using a multivariate approach (MANOVA). In cases where the data did not meet parametric assumptions, the data were rank-

transformed and a non-parametric ANOVA approach (extension of the Kruskal-Wallis test) was used on the transformed data [39]. For Experiment 1, we performed a 3-way MANOVA testing the effect of conditioning, CO<sub>2</sub> and testing cue on the behaviour of the fish. Due to a significant 3-way interaction, we performed 2-way MANOVAs on each conditioning type (pseudo-conditioning with water and true conditioning with injured cues) independently, to investigate the effect of CO<sub>2</sub> (control vs 850 μatm) and testing cue (water vs predator odour) on the responses of fish. Similarly, for Experiment 2, we performed a 3-way MANOVA, followed by 2-way MANOVAs on each tutor type (naive and experienced tutors) independently, to investigate the effects of CO<sub>2</sub> (control vs. 850 μatm) and testing cue on the responses of the fish. For experiment 3, we first established that 700 μatm CO<sub>2</sub>-treated fish did not learn to recognize the predator by conducting a 2-way ANOVA, testing the effect of CO<sub>2</sub> treatment (control vs 700 μatm) and testing cue (water vs predator odour) on the antipredator response of the fish one day post-conditioning. We then conducted a 2-way ANOVA, testing the effect of CO<sub>2</sub> (control vs 700 μatm) and testing cue (water vs predator odour vs injured conspecific cues) on the response of the fish 5 days post conditioning.

## Results

### Experiment 1

The antipredator responses displayed by the fish were affected by the cues to which they were exposed, the conditioning they undertook and the CO<sub>2</sub> levels at which they were maintained (3-way non-parametric MANOVA: Pillai's Trace: Cue×CO<sub>2</sub>×Conditioning:  $H_{3,119} = 5.2$ ,  $P = 0.002$ , Figure 1). The responses of fish pseudo-conditioned with water were affected by neither CO<sub>2</sub> nor cue (2-way MANOVA: Pillai's Trace: CO<sub>2</sub>:  $H_{3,59} = 0.8$ ,  $P > 0.4$ , Cue:  $H_{3,59} = 0.4$ ,  $P > 0.7$ ; CO<sub>2</sub>×Cue:  $H_{3,59} = 1.8$ ,  $P = 0.16$ ). However, the responses of fish that were conditioned to recognize the predator with injured conspecific cues (true conditioning) was dependent on both CO<sub>2</sub> and cue (CO<sub>2</sub>×Cue:  $H_{3,58} = 16.3$ ,  $P < 0.001$ ). More specifically, CO<sub>2</sub> did not affect the responses of fish to water ( $F_{3,28} = 1.1$ ,  $P > 0.3$ ), but rather that to predator odour ( $H_{3,28} = 29.5$ ,  $P < 0.001$ ). In addition, fish exposed to 850 μatm CO<sub>2</sub> did not respond differently to water and predator odour ( $F_{3,28} = 0.01$ ,  $P > 0.9$ ).

### Experiment 2

The antipredator responses displayed by the fish were affected by the cues to which they were exposed, the experience of their tutor and the CO<sub>2</sub> levels at which they were maintained (3-way non-parametric MANOVA: Pillai's Trace: Cue×CO<sub>2</sub>×Conditioning:  $H_{3,118} = 9.7$ ,  $P < 0.001$ , Figure 2). The responses of fish conditioned with naive tutors were affected by neither CO<sub>2</sub> nor cue (2-way MANOVA: Pillai's Trace: CO<sub>2</sub>:  $H_{3,58} = 1.0$ ,  $P > 0.4$ , Cue:  $H_{3,58} = 0.2$ ,  $P > 0.9$ ; CO<sub>2</sub>×Cue:  $H_{3,58} = 0.6$ ,  $P > 0.6$ ). However, the responses of fish that were conditioned to recognize the predator with alarm cues (true conditioning) was dependent on both CO<sub>2</sub> and cue (CO<sub>2</sub>×Cue:  $H_{3,58} = 16.7$ ,  $P < 0.001$ ). More specifically, CO<sub>2</sub> did not affect the responses of fish to water ( $F_{3,28} = 1.2$ ,  $P > 0.3$ ), but rather that to predator odour ( $H_{3,28} = 50.6$ ,  $P < 0.001$ ). In addition, fish exposed to 850 μatm CO<sub>2</sub> did not respond differently to water and predator odour ( $F_{3,28} = 0.3$ ,  $P > 0.7$ ).

### Experiment 3

**Test at Day 1.** Changes in antipredator response were influenced both by CO<sub>2</sub> and cue (2×2 non-parametric MANOVA, Pillai's Trace:  $H_{3,78} = 5.7$ ,  $P = 0.001$ , Figure 3). The

responses of fish to water was not affected by CO<sub>2</sub> (non-parametric MANOVA, Pillai's Trace:  $H_{3,37} = 0.1$ ,  $P > 0.9$ ), but their responses to predator odour was ( $H_{3,39} = 6.8$ ,  $P = 0.001$ ).

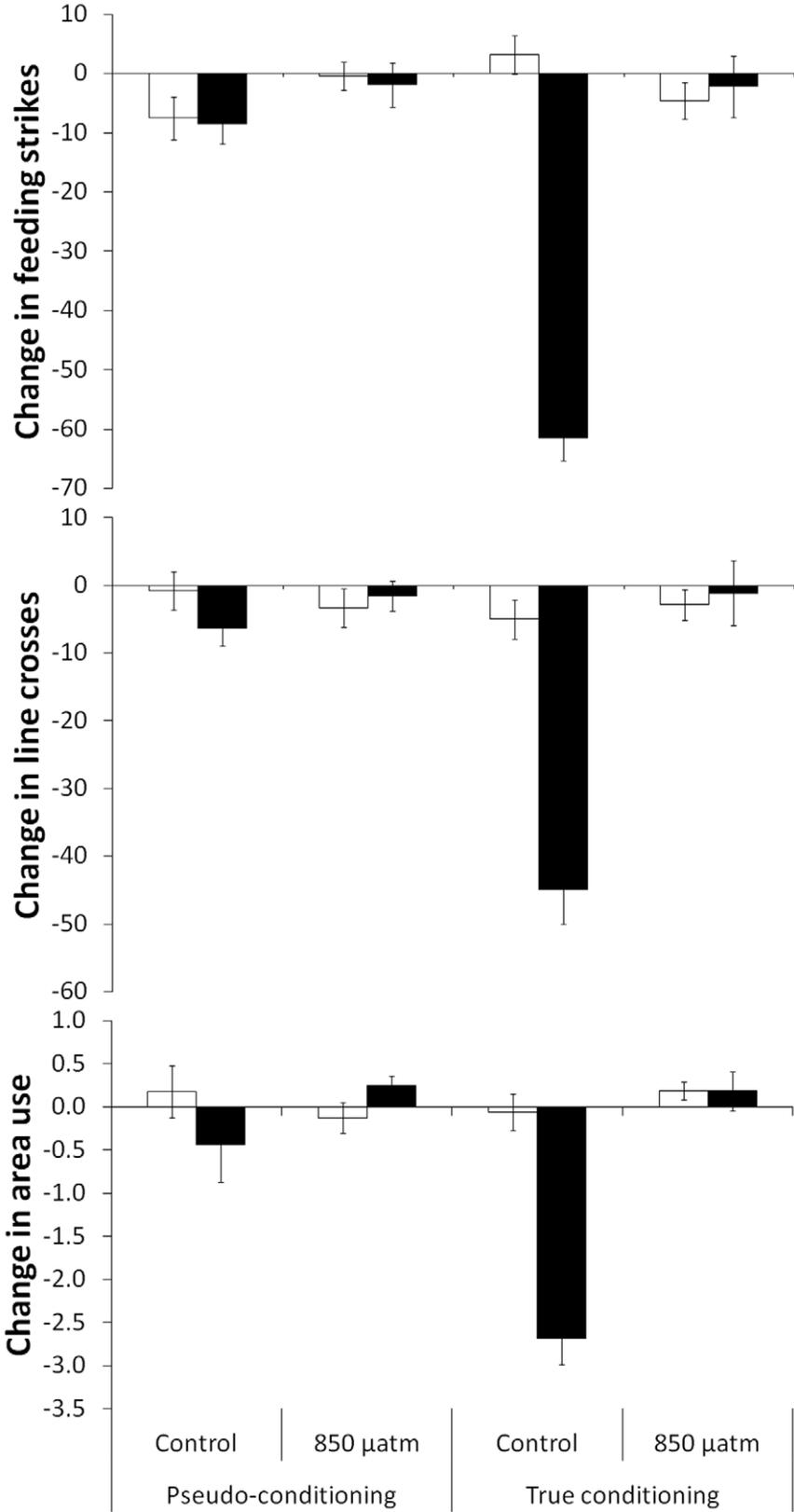
**Test at Day 5.** After the effects of CO<sub>2</sub> wore off, we still found that fish's antipredator responses were influenced by both CO<sub>2</sub> and cue (2×2 MANOVA, Pillai's Trace:  $F_{6,136} = 6.0$ ,  $P < 0.001$ , Figure 4). CO<sub>2</sub> did not affect the responses of fish to water (1-way MANOVA, Pillai's Trace:  $F_{3,20} = 0.2$ ,  $P > 0.8$ ) or a general risk cue like injured conspecific cues ( $F_{3,20} = 1.5$ ,  $P > 0.25$ ), but did affect the responses of fish to predator odour ( $F_{3,23} = 13.7$ ,  $P > 0.001$ ). Post-hoc tests revealed that fish did not respond differently to water and predator odour.

## Discussion

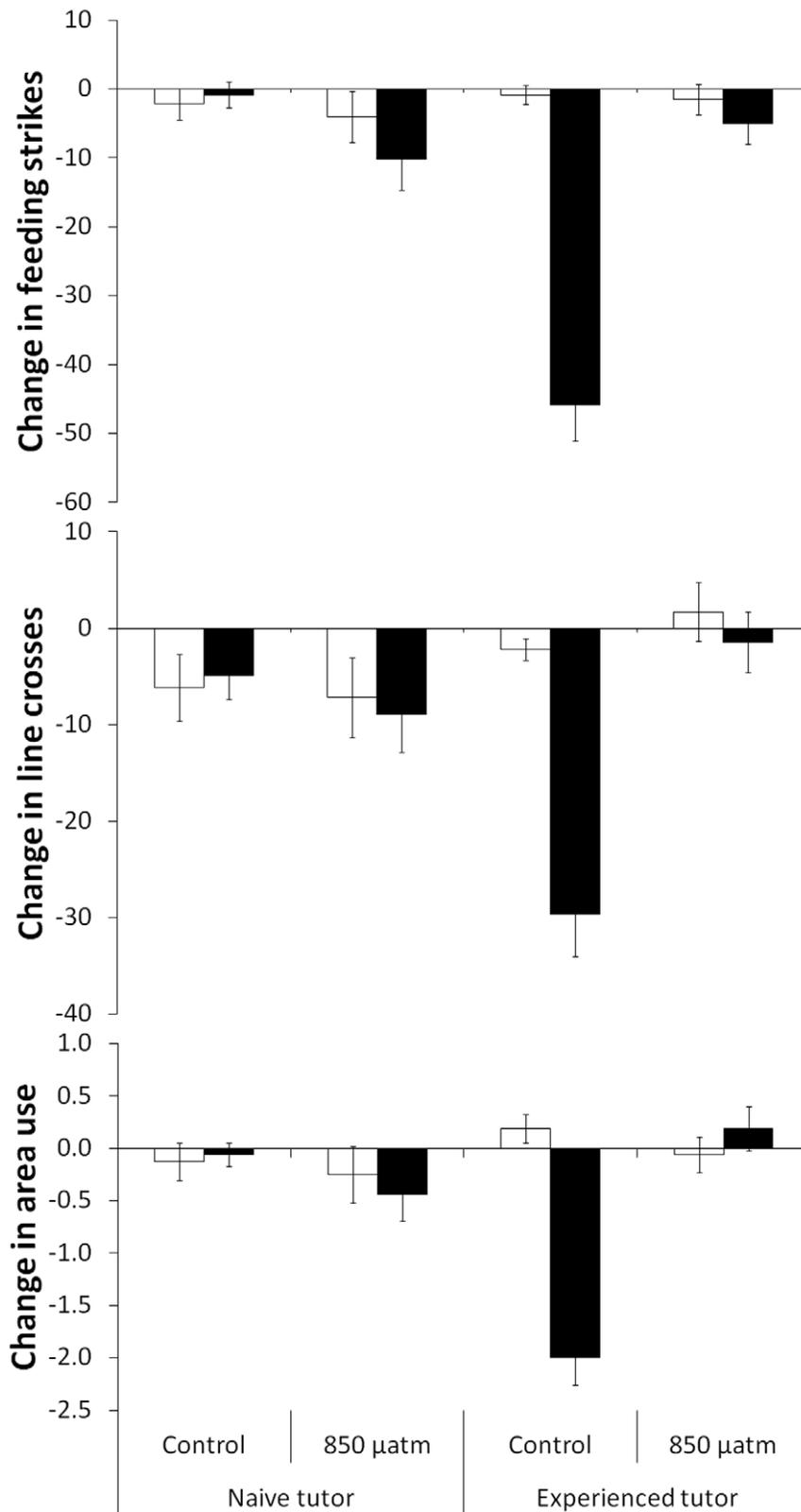
Learning through conditioning with odour cues of injured conspecifics and through observational social learning are very different processes, even though they lead to the same results. In the first, information about risk is provided by a chemical cue, while in the second, the information is provided by a visual source. The results of our study demonstrate that exposure of naive juvenile fish to elevated levels of CO<sub>2</sub> impairs both these processes. If our treatments represent future oceanic conditions on coral reefs, then evidence suggests that new recruit fishes will have a much reduced ability to assess predation risk and will as a consequence have much lower survival.

Our first experiment showed that juvenile damselfish exposed to control levels of CO<sub>2</sub> were able to learn to recognise the odour cue of a predator, but juveniles exposed to 850 μatm CO<sub>2</sub> were not. Our last experiment demonstrated that these effects also held at lower CO<sub>2</sub> concentration (700 μatm CO<sub>2</sub>). In addition, once the CO<sub>2</sub> effect wore off, fish conditioned to recognize the predator in elevated CO<sub>2</sub> conditions still did not respond to the predator odour, but were able to display strong antipredator responses to other risk cues, such as injured conspecific cues. This indicates that elevated CO<sub>2</sub> conditions did prevent learning from occurring.

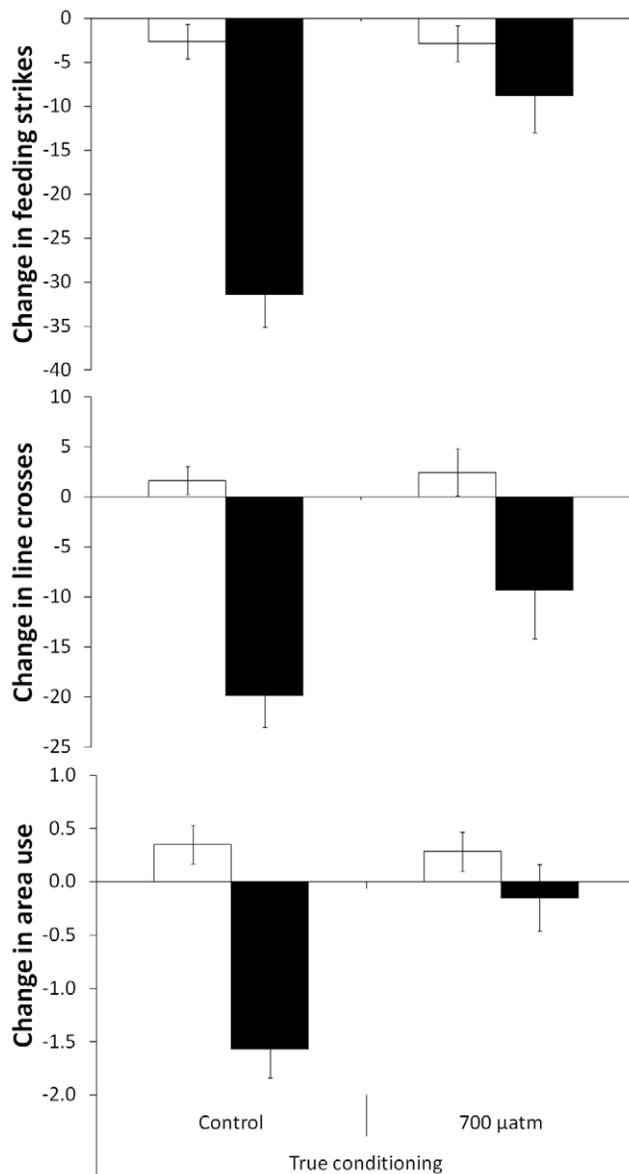
Our work suggests that there is some form of cognitive impairment of the fish exposed to elevated CO<sub>2</sub>. The findings of our second experiment showed that larvae exposed to high levels of CO<sub>2</sub> did not acquire recognition of the predator through cultural learning, whereas the control larvae were able to learn through this mechanism. Recent research showed that exposure to elevated CO<sub>2</sub> affects both olfactory [15,27] auditory [40] and visual [41] senses and a diverse range of behavioural activities in larval [16,17] and adult fishes [42]. Furthermore, Domenici *et al.* [43] provides compelling evidence that elevated CO<sub>2</sub> directly affects brain function in larval fishes, because behavioural lateralization (the propensity for individuals to turn left or right) is impaired by elevated CO<sub>2</sub>. The accumulating experimental evidence indicates that impaired and altered behaviour following exposure to elevated CO<sub>2</sub> is caused by a systemic effect at the neurological level. A new study by Nilsson *et al.* [44] has confirmed this prediction by demonstrating that ionic changes associated with acid-base regulation interfere with brain neurotransmitter function in fish exposed to elevated CO<sub>2</sub>. Therefore, the broad range of behavioural problems identified in larval and juvenile fishes exposed to elevated CO<sub>2</sub>, including the impaired learning ability demonstrated here, appear to be caused by the ionic changes that fish use to prevent acidosis when permanently exposed to high CO<sub>2</sub>. We encourage researchers examining other environmental stressors to consider systemic neurological effects rather than focussing their attention on impaired sensory perception.



**Figure 1. Mean change in number of feeding strikes (top), line crosses (middle) and area use (bottom) from the pre-stimulus period for fish exposed to water (empty bars) or predator odour (solid bars).** Fish were either raised under current-level CO<sub>2</sub> (control) or elevated CO<sub>2</sub> (850 μatm) and conditioned by pairing predator odour paired with either alarm cues (true conditioning) or water (pseudo-conditioning). (N = 16/ treatment).  
doi:10.1371/journal.pone.0031478.g001



**Figure 2. Mean change in number of feeding strikes (top), line crosses (middle) and area use (bottom) from the pre-stimulus period for fish exposed to water (empty bars) or predator odour (solid bars).** Fish were either raised under current-level CO<sub>2</sub> (control) or elevated CO<sub>2</sub> (850 μatm) and conditioned by being paired with either naive or experienced tutors and exposed to predator odour (N = 16–17/treatment). doi:10.1371/journal.pone.0031478.g002



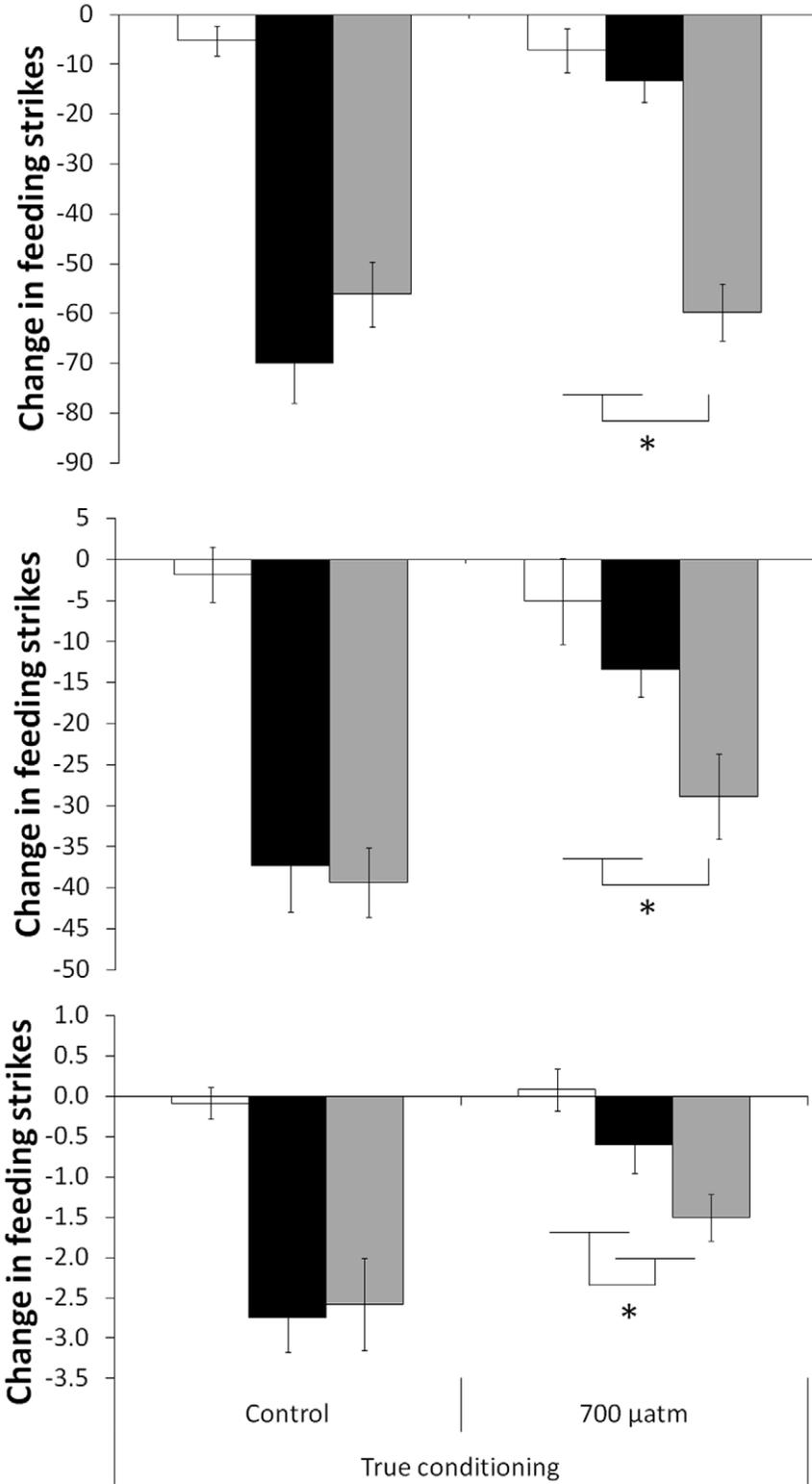
**Figure 3. Mean change in number of feeding strikes (top), line crosses (middle) and area use (bottom) from the pre-stimulus period for fish conditioned to recognize a predator and then exposed to water (empty bars) or predator odour (solid bars) after one day.** Fish were either raised under current-level CO<sub>2</sub> (control) or elevated CO<sub>2</sub> (700 µatm) (N = 20–23/treatment). doi:10.1371/journal.pone.0031478.g003

Like many ocean acidification studies, our CO<sub>2</sub> treatment was short-term; hence we need to consider whether the responses we observed were a result of stress related to our methodology. However, previous studies have shown that raising coral reef fishes in CO<sub>2</sub> from hatching lead to similar alterations in antipredator behaviour as those observed after a 4-day exposure to CO<sub>2</sub> [16]. Hence, if our results were mediated by stress responses related to CO<sub>2</sub> exposure, it seems likely that these effects cannot be dealt with through ontogeny. A possible alternative approach to studying the effects of ocean acidification may be through raising generations of fish in increasing CO<sub>2</sub> conditions. However, beyond the limitation due to the life-history of some species (pelagic larvae), laboratory conditions may relax the selection pressures needed to maintain responses to predators.

Although larval fishes currently experience relatively stable CO<sub>2</sub> conditions that are in equilibrium with the atmosphere during their pelagic stage in the open ocean, they may experience significant diurnal fluctuations in pCO<sub>2</sub> once settled to the reef, temporarily approaching the levels used in our high-CO<sub>2</sub> treatment. An obvious question that arises is: why are the fish able to learn to recognize predators under current conditions with this fluctuating CO<sub>2</sub>? The answer likely lies in the temporal aspect of the exposure regime. Previous research shows that behavioural impairment only occurs after several days of exposure to high CO<sub>2</sub> and that impairment is retained for several days after larvae are returned to low pCO<sub>2</sub> conditions [16]. Therefore, short term fluctuations in CO<sub>2</sub> do not appear to impair learning.

Some conservation research has focused on means of increasing the survival of captive-bred [45] or translocated [46] individuals when released in an environment where these individuals are totally naive to their predators. A number of training programs have been undertaken to mitigate the 'naivete' effects, including social learning and conditioned learning, with some success [47], due to the power and efficacy of learning mechanisms to improve survival. In our situation however, it appears that learning mechanisms may be disrupted by these environmental conditions, which may impact the ability of prey to respond better to predators. The co-existence of affected and non-affected individuals towards mid-century will likely provide a great source of selection towards the elimination of individuals displaying maladaptive behaviour, both in a predation and homing context [16,27]. This lack of response to predators could result in profound effects on coral reef community composition. Damselfish are common prey items for piscivores, especially following larval settlement on the reefs, and learning about predators is a very important way to decrease predation-related mortality [37]. A lack of response by larvae may lead to an increase in consumptive effects, which will change the amount of energy transferred to upper trophic levels. However, more work needs to be done on the effects of CO<sub>2</sub> on foraging behaviours to predict how ocean acidification will affect predator-prey dynamics and trophic interactions.

While organisms typically exhibit a broad range of responses (physiological, morphological, life historical etc) to allow them to cope with current environmental conditions, behaviour is the one type of response that allows individuals flexibility to adjust to a wide range of conditions [48]. In the face of environmental change, behavioural responses typically occur first, as they occur faster, are more plastic and reversible than other forms of adaptations and allow the individual some control over its environment, by simply choosing the type of environment to live in. This crucial behavioural plasticity is often mediated through learning and limited or altered learning abilities may explain interspecific differences in the ability to respond appropriately to human-induced rapid environmental change [49]. Learning, that is, the ability to acquire new knowledge, skills, behaviour through experience, thereby changing the patterns of response to external stimuli, is an ability shared by virtually all animal species [20,50]. Learning is crucial in allowing individuals to identify new suitable habitats or mates [51], food sources [20], new threats [52], and even adjust their behaviour and phenology in the face of environmental change [53,54]. If CO<sub>2</sub> exposure is altering the cognitive ability of species, by either preventing them from learning or by altering the interpretation of environmental cues, the ecological consequences of ocean acidification will be far reaching, and may impinge on any conservation efforts to mitigate the ecological effects of ocean acidification.



**Figure 4. Mean change in number of feeding strikes (top), line crosses (middle) and area use (bottom) from the pre-stimulus baseline for fish conditioned to recognize a predator and then exposed to water (empty bars), predator odour (solid bars) or injured conspecific cues (grey bars) after five days.** Fish were either raised under current-level CO<sub>2</sub> (control) or elevated CO<sub>2</sub> (700 µatm) (N = 12–15/treatment).  
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Conceived and designed the experiments: MCOF DPC PLM MIM MGM. Performed the experiments: MCOF RPM DLD DPC. Analyzed the data: MCOF DPC AS. Contributed reagents/materials/analysis tools: PLM DLD. Wrote the paper: MCOF DPC AS MGM PLM.

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# Ocean acidification erodes crucial auditory behaviour in a marine fish

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Ocean acidification is predicted to affect marine ecosystems in many ways, including modification of fish behaviour. Previous studies have identified effects of CO<sub>2</sub>-enriched conditions on the sensory behaviour of fishes, including the loss of natural responses to odours resulting in ecologically deleterious decisions. Many fishes also rely on hearing for orientation, habitat selection, predator avoidance and communication. We used an auditory choice chamber to study the influence of CO<sub>2</sub>-enriched conditions on directional responses of juvenile clownfish (*Amphiprion percula*) to daytime reef noise. Rearing and test conditions were based on Intergovernmental Panel on Climate Change predictions for the twenty-first century: current-day ambient, 600, 700 and 900 matm pCO<sub>2</sub>. Juveniles from ambient CO<sub>2</sub>-conditions significantly avoided the reef noise, as expected, but this behaviour was absent in juveniles from CO<sub>2</sub>-enriched conditions. This study provides, to our knowledge, the first evidence that ocean acidification affects the auditory response of fishes, with potentially detrimental impacts on early survival.

Keywords: ocean acidification; auditory response; sensory behaviour; clownfish; reef noise

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