



Coral reef fish incorporate multiple sources of visual and chemical information to mediate predation risk



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ARTICLE INFO

Article history:

Received 31 March 2013

Initial acceptance 15 May 2013

Final acceptance 19 June 2013

Available online 14 August 2013

MS. number: 13-00280

Keywords:

Amphiprion percula

anemonefish

coral reef fish

olfactory cue

orange clownfish

predation risk

shoaling

visual information

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. In this study, the anemonefish, *Amphiprion percula*, responded with antipredator 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. Our results suggest that anemonefish have a complex decision-making process that incorporates multiple sources of information each with different degrees of uncertainty.

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Predation is a pervasive selective agent that varies over both space and time (Lima & Dill 1990; Ferrari et al. 2010). For this reason, selection should favour those prey individuals that have the ability to acquire up to date information on predation risk rapidly within their local environment. Animals can acquire information through direct experience with predators or through information obtained indirectly by observing nearby individuals (Valone & 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, it would be advantageous for animals to incorporate multiple sources of information into their decision-making process to reduce these levels of uncertainty. In this study we considered 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 & Manassa 2008; Ferrari et al. 2010). 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 & 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 & Vehrencamp 1998).

As such, the use of chemical alarm cues may be both temporally and spatially inaccurate. A recent study (D.P. Chivers, D. L. Dixson, J. R. White, M. I. McCormick & M. C. O. Ferrari, unpublished data) 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.

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For example, are prey fishes capable of differentiating freshly released chemical cues from those released in the past? An area in which chemical cues are detected is likely to be more risky than one in which 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 sensed. Studies have shown that prey often respond in a graded manner: the higher the concentration the stronger the response (Ferrari et al. 2010). 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 utilize (Wisenden & Chivers 2005). Many prey animals that share the same predators often respond to each other's chemical cues (Schoeppner & 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 recognize 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 & Chivers 2008; Manassa et al. 2013). 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. 2006). Manassa et al. (2013) 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 sceptical when utilizing visual information from heterospecifics.

In this study we explored how anemonefish, *Amphiprion percula*, incorporate multiple sources of information to mediate their risk response and how they prioritize different information sources. 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 is 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, we 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). We tested a series of predictions that specifically address the use of

multiple sources of information. (1) We predicted that, in the absence of visual cues from conspecifics or congeners, anemonefish will respond to both conspecific and congeneric chemical cues with an antipredator response, but there will be a stronger response to those from conspecifics. (2) We 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 prioritize chemical information about risk over congeneric visual information. If our predictions about uncertainty were correct, we expected that *A. percula* would reduce the intensity of their response to chemical cues when in the presence of unalarmed conspecifics but not in the presence of unalarmed congeners.

METHODS

Experimental Overview

We exposed *A. percula* to damage-released chemical cues of conspecifics and closely related congeners (*A. melanopus*), along with additional control cues (platy cues or saltwater) in the presence and absence of a shoal of conspecifics or congeners.

Study Species

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

Freshwater platys, *Xiphophorus maculatus*, were used as controls for the addition of damage-released chemical cues as they are phylogenetically distant from *A. percula* and *A. melanopus* and are therefore unlikely to contain the same chemical alarm cue (Larson & McCormick 2005).

Rearing Procedures and Maintenance

Amphiprion 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 min 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-litre aquaria at ambient temperatures (26–29 °C).

Amphiprion 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 anaesthetize individuals first, enabling quick and effective capture (Munday & 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 MARFU with no mortality recorded during this period. Individuals were slowly acclimated to 70-litre aquaria at ambient temperatures (26–29 °C). Adult pairs were maintained in separate 70-litre aquaria and fed INVE Aquaculture Nutrition 12/20 pellets three times daily (under ethics permit no. 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 postlaying, appearance of embryos indicates readiness to hatch), egg clutches (with terracotta pot) were transferred to separate 70-litre aquaria. Clutch size varied but for *A. percula* averaged 783 eggs (SD 248) per clutch, resulting in an average of 455 larvae, and for *A. melanopus* 1166 eggs (SD 223) per clutch, resulting in an average of 548 larvae.

Following hatching, larvae were reared in a semiclosed system, with the only water flow being a slow flush of filtered UV-sterilized sea water each night, until larvae were competent to settle at 11 days. In the semiclosed system larvae were able to feed ad libitum 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/ml added each morning for the first 3 days and live brine shrimp nauplii, *Artemia franciscana*, at 1 individual/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/ml of *A. franciscana* from day 8. From day 11 onwards, fish were maintained in 70-litre tubs (density: approximately 100 per 70 litres) at ambient temperatures (26–29 °C), under a 12:12 h light:dark photoperiod, with feeding occurring twice daily (*A. franciscana* and Aquaculture Nutrition NRD 5/8 pellets).

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

Observation and Shoaling Tanks

Experiments and behavioural observations were undertaken in 8-litre observation tanks at ambient temperatures (26–29 °C) under a 12:12 h light:dark photoperiod. An air stone was placed in the centre of each tank (Fig. 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 s 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-litre tank (shoaling tank) positioned on the right-hand side of the observation tank (Fig. 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; Fig. 1).

The focal fish species, *A. percula* (subsample: mean standard length, SL ± SE: 20.61 ± 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 ± SE: 21.21 ± 2.94 mm) or no shoal.

Experimental Cue Preparation

Damage-released chemical cues were prepared according to the protocol from McCormick & Manassa (2008), using a total of 60 *A. percula* (mean SL ± SE: 20.61 ± 2.94 mm), 60 *A. melanopus* (mean SL ± SE: 21.21 ± 2.94 mm) and 30 *X. maculatus* (mean SL ± SE: 44.9 ± 5.1 mm), with an ethics permit from James Cook University (no. 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 difference in skin surface ratio between species. The number of individuals killed 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 killed for chemical cues and/or used as shoaling individuals. Specimens were euthanized by a quick blow to the head (brain death confirmed following no movement or response after 30 s), with 25 superficial (minor flesh damage) cuts made to the skin with a clean razor blade. Specimens were then rinsed in 15 ml of sea water, 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.

Experimental Protocol

Individuals were acclimated in the observation and shoaling tanks for 48 h, with each tank receiving live *A. franciscana* daily (about 7500 nauplii per tank). Prior to the initial observation

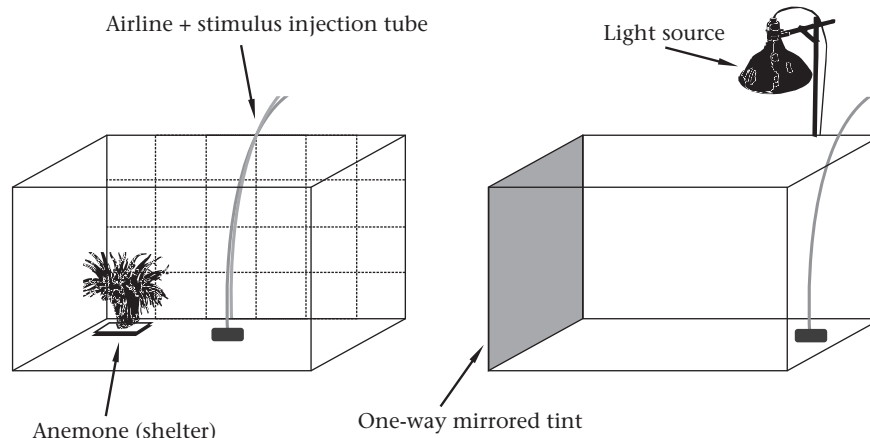


Figure 1. Observation and shoaling tank design.

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/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/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 sea water) 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 randomized among treatments.

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 4.7×4.2 mm rectangles), with every line crossed recorded (at least half the body had to 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.

Statistical Analysis

ANOVAs 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 explore the nature of the significant differences found by MANOVA further, ANOVAs were carried out 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.

Ethical Note

Following each trial, focal individuals were removed from the observation tanks and housed in 70-litre aquaria for use as either shoaling individuals or in the preparation of experimental cues. This ensured that all individuals that were killed 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 MARFU for use by other researchers and students. This study was carried out under the ethics approval guidelines of James Cook University, approval A1067.

RESULTS

The response of individuals to conspecific and congeneric chemical cues in the absence of visual cues differed significantly in relation to feeding strikes ($F_{1,38} = 5.93$, $P = 0.02$; Fig. 2a), but not line crosses ($F_{1,38} = 1.73$, $P = 0.2$; Fig. 2b). There was a significant interaction in the change in behaviour between initial and final observation periods among the four experimental chemical cues

and three shoaling conditions (MANOVA: Pillai's trace = 0.135, $F_{12,456} = 2.760$, $P < 0.001$; Fig. 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 *A. percula* significantly reduced activity between the control cues (*X. maculatus* cue and sea water; all shoaling conditions) and the congeneric (*A. 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 suggests that *A. percula* responded with a reduction in activity to congeneric cues, but the reduction occurred only 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 sea water; 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 shows that the response of *A. percula* to congeneric cues was negated when a shoal of conspecifics (that were not responding) was visually present.

Amphiprion percula responded with a similar pattern to conspecific cues to that displayed in response to congeneric cues among shoal treatments (Fig. 2). The fish 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 sea water; all shoaling conditions; feeding strikes: $F_{1,228} = 148.837$, $P < 0.001$; line crosses: $F_{1,228} = 65.421$, $P < 0.001$). When the fish were 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 the fish were exposed to a conspecific cue in the presence of an *A. percula* shoal compared to the controls (*X. maculatus* cues and sea water; all shoaling conditions; $F_{1,228} = 3.771$, $P = 0.053$).

Lastly, planned comparisons also revealed that the magnitude of reduction when *A. percula* was 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: $F_{1,228} = 6.133$, $P = 0.014$).

DISCUSSION

Our study highlights the ability of coral reef fish to utilize multiple sources of visual and chemical information to mediate predation risk and how they prioritize these information sources. The results indicate that *A. percula* responded with antipredator behaviour to damage-released chemical cues from conspecifics and congeners (*A. melanopus*). However, the presence or absence of conspecifics and congeners dramatically influenced the way in which individuals responded to chemical indicators of risk. As hypothesized, 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 congeners.

The reduction in activity levels that *A. percula* displayed towards conspecific and congeneric chemical cues is a common antipredator response observed among a number of marine fish species (Chivers & Smith 1998; Holmes & McCormick 2010). The ability of individuals to respond to the chemical cues of other species is observed among a variety of aquatic taxa including amphibians (Schoeppner & Relyea 2005, 2009), crustaceans (Hazlett & McLay 2005), gastropods (Dalesman et al. 2007) and freshwater fishes

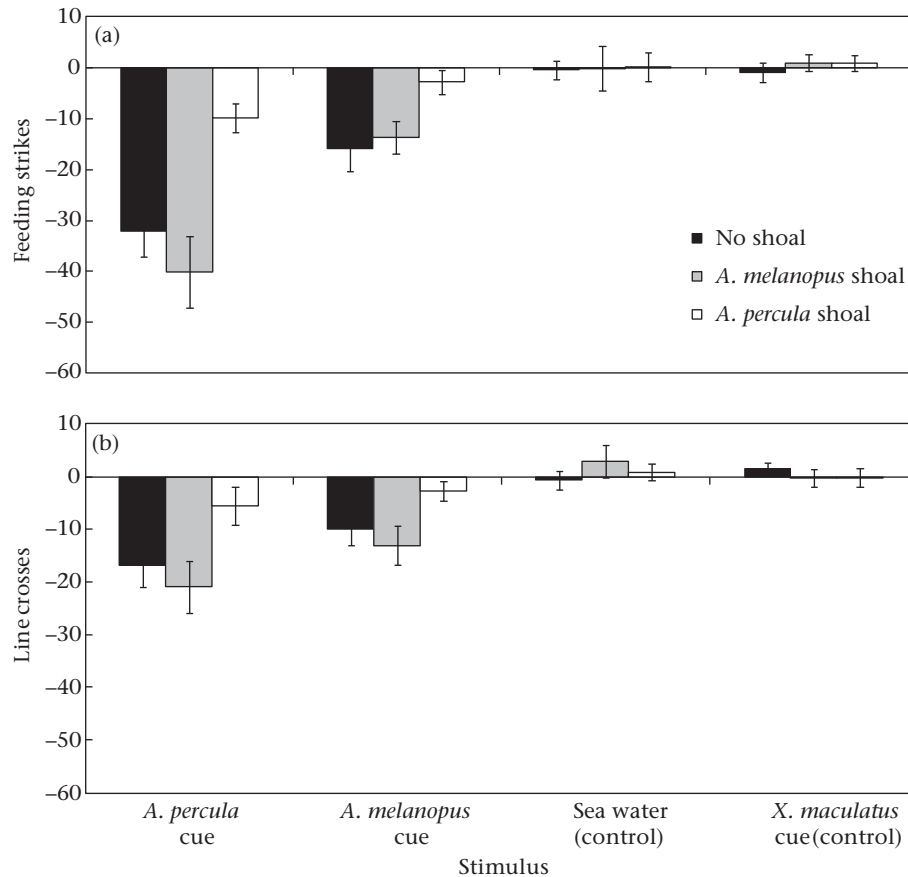


Figure 2. Mean change \pm SE in (a) feeding strikes and (b) line crosses by *Amphiprion percula* between initial and final 5 min observation periods, for fish exposed to four experimental cues (*A. percula* damaged-released chemical cues, *Amphiprion melanopus* damaged-released chemical cues, *Xiphophorus maculatus* damaged-released chemical cues or sea water) and three shoaling conditions (three individual *A. percula*, three individual *A. melanopus* or no shoal).

(reviewed in Chivers & Smith 1998; Mirza & Chivers 2003). A response to the chemical cues of others may arise via innate (cohabitation with sympatric prey over several generations) or learned (experience from local environment) mechanisms (Schoeppner & 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 antipredator behaviour and phylogenetic relatedness.

As chemical cues alone may result in an under- or over-estimation of risk because of their dependency on water for movement in the environment (lingering with no current or dissipating quickly in areas of high flow; Bradbury & 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 antipredator behaviour displayed by *A. percula* was observed when in the presence of unalarmed conspecifics but not when in the presence of unalarmed congeners. 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 congeners and/or heterospecifics that could provide useful information is

significantly increased. Many freshwater lakes/ponds often contain only 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 utilized regardless of phylogenetic relatedness (Wisenden et al. 2003; Pollock et al. 2006). However, until now, the extent to which individuals in complex environments utilize visual information has been unclear.

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 & Parrish (1993) suggested that individuals that occupy habitats with high shelter cover benefit more from sheltering rather than shoaling in response to predation. Our 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 defence 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 antipredator 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 is likely to provide prey

with the ability to counteract these levels of error. A study by Hartman & 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, Wisenden et al. (2003) showed that traps labelled 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 those gained from fleeing the area. Another study, conducted by Pollock et al. (2006), exposed fathead minnows to the damage-released chemical cues of conspecifics while in the presence of one of three shoaling groups (conspecifics, familiar heterospecifics or no shoal). Individuals displayed a stronger antipredator 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 utilize multiple cues to assess risk.

Given the complexity of coral reef environments, future research on the antipredator 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 emphasizes 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 minimize erroneous decisions and ultimately increase survival.

Acknowledgments

We thank J. Donelson for her assistance in the laboratory, O. Lönnstedt for providing useful comments on the manuscript, P. Manassa for his artistic contribution and R. Manassa, L. Manassa and P. Manassa for their continued support. This study was funded through the ARC Centre of Excellence for Coral Reef Studies.

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