



The role of chemical alarm signals in facilitating learned recognition of novel chemical cues in a coral reef fish

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Detection and recognition of chemical cues from predators may lower the risk of predation in prey fish. Responses to particular cues in fish may be inherited or learned. In a laboratory experiment, we tested whether the coral-reef-dwelling goby, *Asterropteryx semipunctatus*, can learn to recognize a novel chemical cue and associate it with danger. Gobies were simultaneously exposed to a neutral chemical cue (the smell of a planktivorous damselfish, *Acanthochromis polyacanthus*) and either conspecific skin extracts (which contains a chemical alarm cue) or skin extracts from the freshwater swordtail, *Xiphophorus helleri*. Swordtails, which are allopatric with *A. semipunctatus*, lack the goby alarm cue and were used to control for a general response to the damaged skin of any fish. Two days after the conditioning, the gobies were exposed to only the neutral chemical cue. Gobies that had been conditioned to the neutral cue paired with conspecific skin extracts on day 1 responded to the neutral cue alone on day 3 with a typical alarm response, including decreased movement and feeding. Gobies that had been conditioned to the neutral cue paired with swordtail skin extracts did not show an alarm response. These results suggest that *A. semipunctatus* can learn to recognize a novel chemical cue and associate it with danger after a single simultaneous exposure to a novel cue and a conspecific alarm signal. This mechanism of rapid learning of novel chemical cues may be an important way in which coral reef fish learn to recognize predators.

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Many prey fish recognize cues emitted from their natural predators and respond by performing appropriate defence behaviour (reviewed by Kats & Dill 1998). However, fish often do not respond with a fright reaction to unfamiliar predators (Smith 1997). Furthermore, individuals from populations that frequently encounter predators typically show stronger antipredator responses than do individuals from populations that rarely experience predators (Gelowitz et al. 1993). In some cases, such behavioural differences between populations may result from genetic differences promoted through differing selection pressures (Gandolfi 1972, cited in Smith 1989). For example, Seghers (1974) found that guppies, *Poecilia reticulata*, collected from different populations in the wild retained differences in schooling behaviour for up to four generations in captivity.

However, not all population differences in antipredator behaviours can be explained by differences in genetic

makeup. Studies of freshwater fish have shown that certain responses to particular predatory fish are not always inherited but need to be learned through experience (Chivers & Smith 1998; Korpi & Wisenden 2001; Gazdewich & Chivers 2002). Many studies have examined the mechanisms by which the recognition of particular predator cues is acquired. Göz (1941) was the first to show that predator-naïve fish can learn to recognize and respond appropriately to cues from a natural predator using conspecific chemical alarm signals. Blinded minnows, *Phoxinus phoxinus*, which initially did not show a fright response to odour from pike, *Esox lucius*, gave a fright reaction to pike odour after the pike had attacked conspecifics in their presence. The authors concluded that alarm substances released during the attack were responsible for the conditioning to the pike odour.

Similar results of learned recognition of a previously neutral stimulus have also been reported for flatworms, *Dugesia dorotocephala* (Wisenden & Millard 2001), damselfly larvae, *Enallagma boreale* (Wisenden et al. 1997), whelks, *Buccinum undatum* (Rochette et al. 1998), amphibians (newts, *Notopthalmus viridescens*: Woody & Mathis 1998; graybelly salamanders, *Eurycea multiplicata*: Whitam

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& Mathis 2000), birds (blackbirds, *Turdus merula*: Curio et al. 1978), squirrel, monkeys, *Saimiri sciureus* (Herzog & Hopf 1984), zebra danios, *Danio rerio*, (Korpi & Wisenden 2001), chinook salmon, *Oncorhynchus tshawytscha* (Berejikian et al. 1999), brook trout, *Salvelinus fontinalis* (Mirza & Chivers 2000), brook sticklebacks, *Culaea inconstans* (Chivers et al. 1995), European minnows, *Phoxinus phoxinus* (Magurran 1989) and fathead minnows (Chivers & Smith 1994b, 1995; Brown & Smith 1996; Brown et al. 1997; Gazdewich & Chivers 2002). This evidence suggests that learned recognition of predators through conditioning with alarm signals may be common.

Fish can also be conditioned to respond to ecologically irrelevant cues that are not naturally related to predators or danger. Magurran (1989) illustrated that, when European minnows were presented with chemical cues from a nonpiscivorous fish, the tilapia, *Tilapia mariae*, in conjunction with conspecific chemical alarm cues, they later reacted with an alarm response to chemical cues from only the tilapia. Fathead minnows have also developed conditioned responses to visual cues, such as the sight of a nonpiscivorous exotic, the goldfish, *Carassius auratus* (Chivers & Smith 1994a), a dark tube or disk (Wisenden & Harter 2001) or a red light (Yunker et al. 1999), using the same learning mechanism. This evidence suggests that this mechanism of learning is flexible and allows prey species to adapt their antipredator behaviour to suit local conditions. This ability may be particularly important for species that undergo long migrations, have a dispersal larval phase or inhabit predator-diverse environments.

On coral reefs, piscivorous fish are high in both abundance and diversity (Hixon 1991), and predators often differ between habitats and between the stages in a prey's life cycle. Most reef fishes have a bipartite life cycle that includes a planktonic larval stage. As a result, larvae may disperse tens to hundreds of kilometres from their parental reef before they metamorphose and settle on a reef as juveniles. The reef that they settle on is likely to be different in the composition and density of predators than at their natal reef. Therefore, there is strong selection pressure for prey to develop ways of recognizing particularly dangerous predators. Prey fish also need to have the flexibility to respond to new predators as they grow or migrate to new habitats (Smith 1997). Many coral reef piscivores are cryptic or ambush predators that hunt by stealth, so potential prey cannot rely entirely on visual stimuli to detect and recognize predators. Consequently, obvious advantages accrue to prey fish that have the ability to detect and recognize chemical cues from predators, which will provide them with an early warning of the presence of a potential predator.

Our aim was to investigate whether the coral-reef-dwelling goby, *Asterropteryx semipunctatus*, could learn to recognize and associate a novel chemical cue with danger. Fish of this species perform a stereotypical alarm behaviour, including decreased feeding and movement, in response to chemicals released from the damaged epidermis of conspecifics as well as to chemicals released from predators (Smith 1989). To simulate an encounter with a novel predator cue, we simultaneously exposed *A. semipunctatus* to a conspecific alarm cue and to

a chemical stimulus that is not likely to be associated with threat under natural conditions (the smell of a planktivorous damselfish). Pairing odours not previously associated with a threat (i.e. a neutral cue) with a conspecific alarm chemical enabled us to determine the capacity of this species to learn novel predator odours.

METHODS

Collection and Maintenance

Gobies were collected in October 2002 in Pioneer Bay, Orpheus Island, Great Barrier Reef, Australia, using anaesthetic clove oil mixed with alcohol (ca. 27 mol/litre) and a hand net, and were brought back to the James Cook University aquarium system. The gobies were maintained in 33-litre tanks in the laboratory in flowing sea water under a 14:10-h light:dark photoperiod at approximately 27°C. The gobies were fed live brine shrimp, *Artemia franciscana* and commercial flake food daily during a minimum of a 2-week acclimation period. Hunger reduces the fright response to conspecific skin extracts in *A. semipunctatus* (J. Larson & M. McCormick, unpublished data), so the gobies received live food ad libitum for at least days before experiments. The research was approved by the James Cook University Ethics Committee and collections were undertaken with a permit from the Great Barrier Reef Marine Park Authority.

Observation Tanks

All observations were conducted in 33-litre tanks with flowing sea water (\bar{X} = 0.4 litres/min). The substratum consisted of a 3-cm-thick layer of aquarium gravel, and an airstone was placed at the back of the tank. Plastic tubing was attached to the airline to enable the injection of extracts into the tank without disturbance. The end of the tubing was attached approximately 1 cm from the airstone, which allowed for rapid dispersal of the extracts in the test tank. Broken terracotta tiles were placed in the middle of the tank, forming a low, structurally complex shelter to mimic the coral rubble shelters that gobies inhabit naturally.

Preparation of Experimental Stimuli

Skin extract was prepared from eight female (\bar{X} ± SE fork length = 34.05 ± 1.57 cm) and seven male *A. semipunctatus* (fork length = 36.8 ± 1.51 cm). Swordtail skin extracts were prepared from 10 swordtail, *Xiphophorus helleri*, females (6.90 ± 2.18 cm) and five males (38.58 ± 1.57 cm). The donor fish were killed by a quick blow to the head. The skin extract was then prepared by putting these fish in a clean disposable plastic petri dish, making 25 superficial vertical cuts on each flank with a razor blade, and rinsing the fish in 15 ml of sea water. The cuts damaged the skin but caused only minor flesh damage. The stimulus water was then filtered through filter paper (qualitative 1) to remove solid

particles such as scales, then drawn up into a disposable syringe for injection into the tank. The skin extracts were used within 20 min of preparation, because extracts may lose potency if frozen or stored for long periods at room temperature (Smith 1989). The neutral stimulus was prepared from a nonpredator damselfish, *Acanthochromis polyacanthus*. The damselfish odour was prepared by placing three juvenile damselfish into a 3-litre aquarium for 3 days. The aquarium was aerated but not filtered. The damselfish were not fed in the stimulus collection aquarium during this period to avoid contaminating the water with food odour. At the end of this period, water was drawn from the aquarium and frozen in 50-ml portions until used. Damselfish were released at their point of capture after the experiment.

Day 1: Initial Response to Skin Extracts

The objective of the first experiment was to determine whether the gobies would react to the paired presentation of a conspecific alarm signal and a neutral chemical cue (i.e. a cue not previously associated with danger). Such paired stimuli could be experienced by a predator-naïve prey exposed simultaneously to a conspecific alarm signal and the smell of a predator, or to chemical cues from a predator that has been eating a conspecific. To control for any stimulus in the conspecific skin extract not related to the alarm cue, we conducted control trials in which the neutral cue was paired with skin extracts from a phylogenetically distantly related and allopatric fish species, the swordtail, which lacks the goby alarm cue.

Test protocol

Groups of three randomly selected *A. semipunctatus* were placed into each observation tank ($N = 41$ males, $\bar{X} \pm \text{SE}$ fork length = 37.25 ± 1.16 mm; $N = 49$ females, 31.53 ± 0.98 mm), and groups were then randomly assigned to be conditioned with one of two pairs of stimuli: either 15 ml of *A. semipunctatus* skin extract (containing an alarm signal) + 15 ml of the neutral cue (damselfish odour) ($N = 15$), or 15 ml of swordtail skin extract (control for alarm signal extract) + 15 ml of the neutral cue ($N = 15$). Pilot tests indicated that individuals in groups of fewer than three gobies per test tank behaved abnormally, spending most of their time under shelter. By putting gobies in groups of three, their activity levels and the group density were similar to those observed in the wild. Fish conditioned in one treatment were not retested in the other treatment.

As a standard test protocol, we quantified the behaviour of three *A. semipunctatus* for 10 min within each tank before injecting the extract into the tank. Immediately before experimental observation, 60 ml of tank water were drawn out through the stimulus injection tube and discarded to remove any stagnant water that may have collected in the tubing. Another 60 ml of tank water were drawn through the tubing and kept for later use. The chemical stimuli were then injected through the tubing followed by 60 ml of tank water to flush the test substance into the tank. The fish were then observed for another

10 min. Trials with vegetable dye showed that it took a mean \pm SE of 39 ± 2.20 s after the injection for the dye to disperse throughout the tank.

Behaviour

The researcher stood behind a blind, observing the tank through small eye slits, and recorded the behaviour with mechanical counters. Two behaviours, moves and feeding strikes, were categorized and recorded. *Asterropteryx semipunctatus* is a cryptic, negatively buoyant, benthic fish that moves episodically, and they remain stationary on the substratum between movements. Each time a goby changed position, it was recorded as a move. All feeding strikes made by a fish were counted regardless of whether the prey item was caught.

Day 3: Conditioned Response to Neutral Stimulus

The objective of this second experiment was to determine whether gobies had learned to recognize and associate the neutral cue (from the damselfish), which had been presented with conspecific alarm cues or swordtail skin extracts, with danger after a single exposure 2 days earlier. We predicted that only gobies that had been conditioned with conspecific skin extracts (containing the alarm cue) and the neutral cue would respond with an alarm reaction to the neutral cue when they were introduced into the tank on their own.

Test protocol

After the conditioning trials, the gobies were maintained and fed ad libitum in the test tanks for 2 days. To determine whether gobies had learned to treat the neutral (damselfish) cue as an alarm signal, we tested gobies on day 3 for a response to the neutral cue alone. The test protocol followed the same procedure as on day 1, except that gobies were exposed only to the neutral cue ($N = 15$ per treatment).

Day 5: Response to Salt Water Control

Mathis & Smith (1993) showed that freshwater fathead minnows can learn to associate an actual experimental protocol with potential danger. Minnows that had been conditioned with conspecific alarm cues later responded to a neutral stimulus (tap water) with increased shelter use when tested under the same conditions. Tap water did not trigger an alarm response when minnows were tested under different experimental conditions (different type of aquarium and group size).

To rule out whether the behavioural responses of gobies in this experiment resulted from a learned recognition of the presentation of the test stimulus, we tested gobies for a response to a saltwater control 4 days after the conditioning trials.

Test protocol

Gobies from both control and treatment trials were tested for a response to salt water on day 5. The test protocol followed the same procedure as on day 3 with the exception that the test stimulus consisted of salt water. We predicted that salt water would not trigger an alarm response in gobies, regardless of whether they had been conditioned with conspecific skin extracts or swordtail skin extracts on day 1.

Statistical Analysis

For all three experiments, we compared the total counts of each behaviour for the 10-min prestimulus period with the total counts for the 10-min poststimulus period using a paired *t* test. We then compared the change in behaviour between pre- and poststimulus periods between gobies that were conditioned with the conspecific skin extract plus the neutral cue and gobies that were conditioned with swordtail skin extracts plus the neutral cue using a Student's *t* test. All data met the assumptions of homogeneity of variances and normality.

RESULTS

Day 1: Initial Response to Skin Extract

There was a significant difference in the responses of gobies exposed to conspecific skin extract (alarm cue) plus neutral cue and gobies exposed to swordtail skin extract (alarm control) plus neutral cue (Student's *t* test: moves: $t_{14} = 3.21$, $P < 0.01$; feeding strikes: $t_{14} = 2.52$, $P < 0.02$; Fig. 1a). Gobies that had been exposed simultaneously to conspecific skin extract and the neutral cue significantly decreased number of moves ($t_{14} = 3.91$, $P < 0.002$) and number of feeding strikes (paired *t* test: $t_{14} = 3.17$, $P < 0.01$). In contrast, gobies that had been exposed simultaneously to swordtail skin extract and the neutral cue significantly increased moves ($t_{14} = 3.39$, $P < 0.005$) but did not significantly change their feeding rates ($t_{14} = 1.66$, $P = 0.12$).

Day 3: Conditioned Responses to Neutral Stimulus

The responses to the neutral cue on day 3 differed significantly between gobies conditioned with conspecific skin extracts and gobies conditioned with swordtail skin extracts (Student's *t* test: moves: $t_{14} = 9.05$, $P < 0.001$; feeding strikes: $t_{14} = 3.39$, $P < 0.003$; Fig. 1b). Gobies that were conditioned with conspecific skin extracts on day 1 significantly decreased their activity levels (paired *t* test: $t_{14} = 9.91$, $P < 0.001$) and the number of feeding strikes ($t_{14} = 2.86$, $P < 0.02$) in response to the neutral cue on day 3. Conversely, gobies that were conditioned with swordtail skin extracts on day 1 responded with a significant increase in number of moves ($t_{14} = -5.05$, $P < 0.001$) but with no significant change in number of

feeding strikes ($t_{14} = -1.86$, $P = 0.085$) in response to the neutral cue on day 3.

Day 5: Response to Water Control

The responses to tank water on day 5 did not differ significantly between gobies conditioned with conspecific skin extracts and gobies conditioned with swordtail skin extracts (Student's *t* test: moves: $t_{14} = -0.56$, $P = 0.58$; feeding strikes: $t_{14} = -0.82$, $P = 0.42$; Fig. 1c). The introduction of salt water significantly increased the number of moves (paired *t* test: $t_{14} = -3.24$, $P < 0.01$) but did not change the number of feeding strikes (paired *t* test: $t_{14} = -1.01$, $P = 0.33$) in gobies that had been conditioned with conspecific skin extracts on day 1. Similarly, gobies that had been conditioned with swordtail skin extracts on day 1 responded with a significant increase in number of moves ($t_{14} = -2.81$, $P < 0.02$) but with no change in number of feeding strikes ($t_{14} = -2.01$, $P = 0.065$) in response to the introduction of salt water on day 5.

DISCUSSION

The results show that *A. semipunctatus* was able to learn to recognize and respond to a previously neutral chemical cue by pairing it with a species-specific chemical alarm signal. Furthermore, the learned recognition of the neutral cue occurred after a single exposure to a mixture of conspecific skin extracts and the neutral cue. Gobies that had been conditioned with conspecific skin extract and the neutral cue on day 1 later reacted to the neutral cue with a stereotypical alarm response. In contrast, gobies that had been conditioned with swordtail skin extract and the neutral cue did not perform an alarm response to the neutral cue on day 3. This mechanism of learned recognition of chemical cues has been reported for freshwater fish in the superorder Ostariophysi (Chivers & Smith 1998; Korpi & Wisenden 2001), as well as in the families Salmonidae (Berejikian et al. 1999; Mirza & Chivers 2000) and Gasterosteidae (Chivers et al. 1995). This is the first study to identify this mechanism of learning in a marine fish, as well as in the family Gobiidae.

The ability to learn to recognize novel cues and associate them with the action of a predator should be advantageous for prey fish that are confronted by a variety of predators, whose identities may change with ontogeny. Furthermore, if the types of predators that the prey will encounter differ throughout its geographical range, then the ability to recognize and remember predators rapidly may be more advantageous than an inherited and inflexible response to a specific predator (Wisenden et al. 1997). *Asterropteryx semipunctatus* is a relatively small (maximum standard length 6.5 cm) benthic fish, and thus has many potential predators on a coral reef. Its geographical distribution ranges from East Africa and the Red Sea to the Hawaiian Islands (Randall et al. 1997), and, like most coral reef fish, it has a bipartite life cycle with a planktonic larval stage (Privitera 2001). Consequently, the types of predators that this species encounters in its life may be highly variable between individuals,

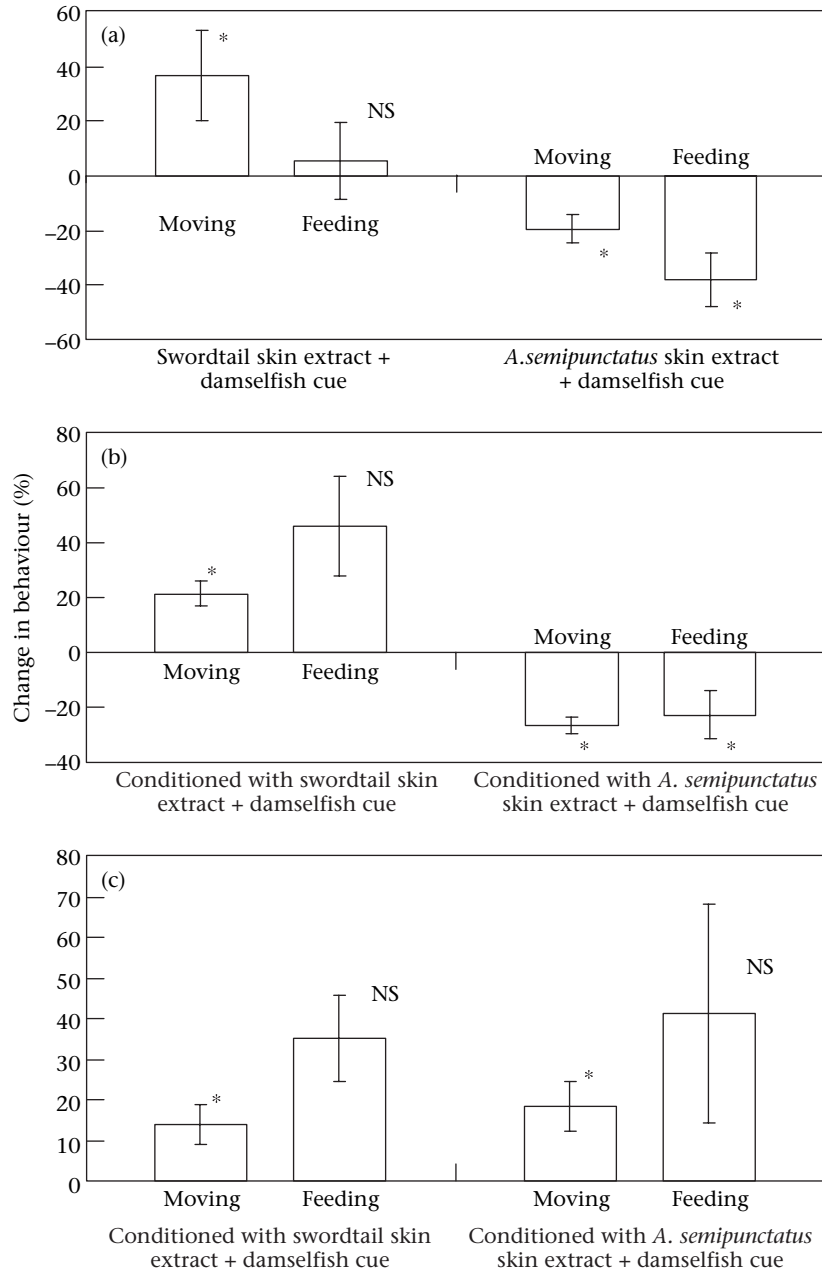


Figure 1. Mean \pm SE percentage of change in moving and feeding behaviours of *Asterropteryx semipunctatus* between 10-min observation periods before and after exposure to (a) conspecific skin extracts + damselfish cue, or swordtail skin extracts + damselfish cue on day 1; (b) damselfish cue on day 3; (c) salt water on day 5. *: Significant changes in behaviour between pre- and poststimulus observation periods ($P < 0.02$).

depending on where they settle at the end of their pelagic larval stage. Our results suggest that *A. semipunctatus* has a strong learning mechanism by which it can acquire information on the identity of relevant predators across its geographical distribution, despite differences in the types of predators present between locations. This has obvious advantages for the gobies in terms of survival, because it allows them to fine-tune the antipredator responses to local conditions.

This learning mechanism is of particular importance because a prey animal does not need to have obtained firsthand knowledge of the predator, either by witnessing

or being the focus of a predator strike. The learned recognition is gained through the coupling of chemical cues from an injured conspecific with other chemical compounds that may be indicative of the action of a predator. Furthermore, in this study, the learned recognition of the neutral stimulus occurred after only a single simultaneous exposure to the neutral cue and conspecific alarm cues. This ability for one-trial learning is important, because it minimizes the costs involved in acquiring this information (Johnston 1982). Repeated naïve encounters with a predator would considerably increase the risk of predation, so a learning mechanism that allows for quick

predator recognition is of obvious survival value to the prey fish (Wisenden et al. 1997).

Several studies have shown that learned recognition of a predator translates into a survival benefit for prey. Mirza & Chivers (2000) found that trout that had been trained to recognize predator odours by simultaneously exposing the trout to conspecific alarm signals and the predator odour showed a greater ability to escape during staged predator encounters and thus had a higher probability of survival than did nontrained fish. Berejikian et al. (1999) trained chinook salmon smolts to recognize predator odours in a similar manner and subsequently released them into a small stream; trained fish had higher survival than did nontrained fish.

Our experiment simulated a natural situation where a goby is exposed simultaneously to a conspecific alarm signal and a neutral smell of a predator. There are at least two situations under which such simultaneous exposure can occur under natural circumstances. A predatory strike on a nearby conspecific could provide a predator-naïve prey fish with a mixture of conspecific alarm cues from the attacked fish and chemical cues from the predator, and thereby facilitate learned recognition of the predator cue (Chivers & Smith 1995). Second, a predator that has recently eaten a conspecific may exude residual chemical alarm cues from the skin of the ingested prey as well as other chemical cues given off the predator regardless of its diet (Gelowitz et al. 1993). This ability to 'label' a predator with chemical alarm cues has been illustrated for fathead minnows (Mathis & Smith 1993), the brook stickleback, *Culaea inconstans* (Gelowitz et al. 1993) and damselfly larvae (Chivers et al. 1996). It is not known whether gobies are able to 'label' their predators by coupling them with chemical alarm cues produced in their epidermis.

In this study, *A. semipunctatus* learned to react with a fright response to a stimulus that is irrelevant to predation risk, that is, the smell of a nonpiscivorous damselfish. This result poses questions about how gobies assign the importance of the many visual and chemical cues that are likely to be present at the time of a predation event when an alarm substance is released. A learned fright response to irrelevant stimuli in nature would bring about costs of lost opportunities for other important activities, such as foraging and mating. We did not evaluate how long gobies retained the learned fright response to the damselfish cue. Similar studies in freshwater systems have shown that fathead minnows retain the recognition of odour from a natural predator, the northern pike, for a year in captivity (Chivers & Smith 1994b) and bluntnose minnows, *Pimephales notatus*, retain recognition of stream stimuli for weeks (Hasler & Wisby 1951). Nevertheless, evidence suggests that learning in fish may be constrained to favour responses that are likely to translate into a survival value. Fathead minnows conditioned to recognize a natural predator, the northern pike, or a nonpiscivorous fish, the goldfish, responded with a similar intensity when tested days after the initial conditioning trial (Chivers & Smith 1994a). However, when tested 2 months after the conditioning, the authors found that minnows responded more strongly to the natural predator than to the goldfish. Similarly, European

minnows conditioned to recognize odours from predatory pike and the nonpiscivorous tilapia reacted more strongly to the pike odour (Magurran 1989). Fish appears to have a predisposition to learn to recognize cues from natural predators, suggesting that both environmental (such as experience with predators) and genetic factors are involved in the antipredator behaviour of fish (Wisenden & Harter 2001).

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