

## Learned recognition of novel predator odour by zebra danios, *Danio rerio*, following time-shifted presentation of alarm cue and predator odour

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### Synopsis

Fishes in the superorder ostariophysi possess specialized epidermal cells that contain an alarm cue. Fish associate novel odours, such as the odour of a predator, with predation risk after a single, simultaneous exposure to the novel odour and alarm cue. Thereafter, the novel cue is recognized as an indicator of risk and its presence induces antipredator behaviour. Two common antipredator behaviours are reduction in activity and movement to the bottom. This phenomenon has been demonstrated many times in the laboratory setting for a variety of aquatic taxa. In nature however, the detection of novel predator odour may be time-shifted with respect to the detection of alarm cues. Is there a critical period immediately upon the detection of alarm cue in which associative learning can occur? We presented zebra danios, *Danio rerio*, with the odour of northern pike, *Esox lucius*, 5 min after presenting them with either alarm cue or water (control). During a predation event, 5 min is a long time. When later retested with pike odour alone, zebra fish conditioned with alarm cue significantly increased antipredator behaviour in terms of decreased activity and movement towards the bottom. Control fish did not recognize pike odour as dangerous when retested. These data show that learned recognition of predation risk is sufficiently robust to accommodate ecologically realistic temporal shifts in stimulus presentation.

### Introduction

Animals assess predation risk in their environment on a moment-to-moment basis. A wide variety of aquatic animals use chemical cues to assess risk (for recent reviews see Dodson et al. 1994, Kats & Dill 1998, Chivers & Smith 1998, Wisenden 2000). There are several classes of chemical cues, released at different times in the predation sequence, that indicate risk (Lima & Dill 1990, Smith 1992, Wisenden 2000). Survival benefits to chemically warned prey have been demonstrated in several studies (Hews 1988, Mathis & Smith 1993a, Wisenden et al. 1999).

In many cases, recognition of odours as dangerous is acquired through experience. Naïve prey from piscivore-free populations do not respond to predator

odour with antipredator behaviour (e.g., Göz 1941, Magurran 1989, Suboski et al. 1990, Mathis et al. 1993, 1996, Mathis & Smith 1993b, Chivers & Smith 1994a,b, Hall & Suboski 1995, Chivers et al. 1995, 1996, Wisenden et al. 1997, Woody & Mathis 1998, Brown & Godin 1999). Prey acquire recognition of predator odour as dangerous when they simultaneously detect the odour of a novel predator and known indicators of risk such as the antipredator behaviour of shoalmates (Suboski et al. 1990, Hall & Suboski 1995, Mathis et al. 1996), or more commonly (in laboratory experiments), injury-released chemical cues. Injury-released chemical cues are generally released only in the context of a predatory attack and serve as a reliable and imminent indicator of risk (Chivers & Smith 1998).

An amplification of this general phenomenon is found in fishes of the superorder Ostariophysi (Frisch 1938, 1941, Pfeiffer 1977, Smith 1992, Wisenden 2000). The ostariophysi represent 6507 species, or approximately 27% of the world's fish species and 64% of all freshwater species, and include the minnows, catfishes, characins, and suckers (Nelson 1994). One of the defining characters of this group is the presence of specialized epidermal cells, called alarm substance cells, that contain an alarm cue (Pfeiffer 1977). These cells lack a duct to the skin surface. The cue contained in alarm substance cells is released only when the skin is damaged, such as by a predatory attack. The cue, thought to be hypoxanthine-3(N)-oxide or related derivative (Pfeiffer et al. 1985, Brown et al. 2000), is potent: 1 cm<sup>2</sup> of skin from fathead minnows, *Pimephales promelas*, is sufficient to create an active volume of approximately 58 000 l, or a sphere 7 m in diameter (Lawrence & Smith 1989). Cell density varies with nutritional status and social context (Wisenden & Smith 1997, 1998).

Zebra danios, *Danio rerio*, are cyprinids and thus, possess alarm substance cells (Waldman 1982). One cm<sup>2</sup> of skin produces approximately 10 000 l of active volume, or a sphere 3.9 m in diameter (Gandolfi et al. 1968). Previous experiments have shown that zebra danios learn to fear novel stimuli when novel stimuli are detected simultaneously with alarm cue (Suboski et al. 1990, Hall & Suboski 1995).

The ability to acquire recognition of predation risk is of obvious fitness benefit. There are a number of plausible scenarios in which simultaneous presentation of novel and alarm cues could occur in nature. However, there are also many plausible scenarios in which the cues may not be encountered simultaneously. For example, if a prey first detects conspecific alarm cue, then 5 min later (an eternity in the context of a predation event) detects a novel odour, can a learned association form between the novel cue and risk? There might be a critical period for the association of novel stimuli with predation risk because there would be a high probability of forming an aversive response to irrelevant stimuli, and the subsequent fitness costs that would ensue (e.g. lost foraging opportunities, etc.). It would be maladaptive if prey associated risk with every novel stimulus they may encounter for an extended period following exposure to alarm cue.

We chose zebra danios as the test species for this study because they are ostariophysian fish, adapt well to the laboratory setting, are readily available, and are fast

becoming a model organism for developmental genetics of vertebrate systems. Moreover, zebra danios are known to associate risk with novel stimuli presented simultaneously with skin extract (Suboski et al. 1990, Hall & Suboski 1995).

In this study, we exposed pairs of zebra danios to either conspecific chemical alarm cues or water (control) then, after a delay of 5 min, to the odour of predatory northern pike, *Esox lucius*. We changed the tank water, then 2 days later, we exposed the same danios to pike odour alone to determine if risk association occurred after time-shifted presentation of alarm cues and predator odour.

## Materials and methods

Adult zebra danios were obtained from a local commercial supplier and housed in a 125-l holding tank held at 26°C on approximately 12 : 12 h light : dark cycle. Fish were fed daily with commercial flake food and live brine shrimp (*Artemia*) nauplii.

Pairs of zebra fish were placed in 38-l tanks containing dechlorinated tap water. The effect of sex, if any, was ignored in this experiment and presumed balanced among treatments. We drew a grid (6.3 cm × 6.3 cm cells) on the broad panel of each test tank. Each tank had a thin layer of naturally coloured gravel and a small air-powered sponge filter. A second airline tube for stimulus injection was wedged into the rigid out-flow tube of the filter. Water currents created by the filter quickly dispersed stimuli injected into the tank.

We recorded 'activity' and 'time near bottom'. Activity was scored as the number of grid lines crossed during each 5-min observation period. 'Time near bottom' was scored as the number of times both fish were in the lowest row of the grid (6.3 cm above bottom). 'Time near bottom' was recorded at 10 second intervals over each 5-min observation period. An antipredator response occurred when there was a significant decrease in 'activity' and a significant increase in 'time near bottom' (Lawrence & Smith 1989).

Experimental protocol was as follows. On day 1, we observed fish for 5 min (prestimulus observation period). Over the sixth minute, we introduced one of two test stimuli: (1) 20 ml dechlorinated tap water (control stimulus), or (2) 20 ml of skin extract (chemical alarm cue). Test stimuli were completely flushed from injection lines with 60 ml of tank water obtained before each trial began. From min 7–11, we observed fish for

5 min (post alarm cue or post water period). During the 12th minute, we introduced 20 ml of pike odour. During min 13–17, we observed fish for a final 5 min observation period (post pike odour observation period). On day 2, we replaced all tank water with fresh dechlorinated water and replaced all injection tubes with fresh tubing. On day 3, we retested all fish using the control protocol from day 1. We recorded the behavioural response of 11 pairs of danios to the control sequence of tests (day 1 and day 3), and another 11 pairs of danios to the experimental sequence of tests (day 1 and day 3).

Conspecific skin extract was prepared by humanely killing one adult zebra danio by cervical dislocation and making 15 cuts with a razor blade on each flank. We then gently dribbled 20 ml of dechlorinated tap water over the zebra fish. Stimulus was used within 10 min of preparation. Alarm cue from one donor fish was used for each trial. Pike odour was collected from a small pike (total length = 18 cm) collected from Boyer Lake, MN, approximately 50 km east of Moorhead, MN. The pike was held in a 38-l aquarium and fed guppies *Poecilia reticulata* for 5 d. Guppies are not in the superorder Ostariophysi and therefore do not possess the ostariophysan alarm cue in their skin (Pfeiffer 1977). This precaution was necessary because injury-released alarm cues remain active after passage through the gut of pike (Mathis & Smith 1993b,c, Brown et al. 1995, Chivers et al. 1996, Mathis & Hoback 1997).

We predicted that fish previously conditioned with alarm cue and pike odour would show an antipredator response to pike odour on day 3 but fish previously conditioned with water and pike odour would show no response to pike odour on day 3. We used Wilcoxon matched-pairs signed ranks tests (Siegel & Castellan 1988) to test for consistent change in activity or time near bottom. All *p* values are based on a 2-tailed probability distribution.

## Results

### Day 1

Zebra fish exposed to chemical alarm cues on day 1 significantly decreased activity in the post-alarm cue period ( $T^- = 62$ ,  $p = 0.007$ ,  $n = 11$ ) and significantly recovered activity again 5 min later after introduction of pike odour ( $T^+ = 60$ ,  $p = 0.014$ ,  $n = 11$ ) (Figure 1). Activity following the introduction of pike

odour was not significantly different from the prestimulus observation period ( $T^- = 50$ ,  $p = 0.147$ ,  $n = 11$ ). Similarly, zebra fish exposed to alarm cue increased time near bottom after introduction of skin extract ( $T^+ = 52$ ,  $p = 0.010$ ,  $n = 10$ ) but did not change significantly after the addition of pike odour ( $T^+ = 15$ ,  $p = 0.061$ ,  $n = 5$ ) (Figure 2). Thus, the complete, overt behavioural reaction to skin extract did not extend at full intensity to the end of the post-pike odour period. There was no behavioural evidence that pike odour elicited additional alarm behaviour in danios.

Zebra fish exposed to water did not change activity in the post water period (signed ranks test:  $T^- = 33$ ,  $p = \sim 1.0$ ,  $n = 11$ ) nor in the post pike odour period ( $T^+ = 42$ ,  $p = 0.464$ ,  $n = 11$ ) (Figure 1). The overall change from the prestimulus period to the post pike odour period was not significant ( $T^+ = 40.5$ ,  $p = 0.548$ ,  $n = 11$ ). Similarly, control fish did not significantly change time near bottom after the introduction of water ( $T^+ = 25.5$ ,  $p = 0.778$ ,  $n = 9$ ) or pike odour ( $T^- = 30$ ,  $p = 0.426$ ,  $n = 9$ ) (Figure 2). The overall change in time near bottom from the prestimulus period to the post pike odour period was not significant ( $T^+ = 29$ ,  $p = 0.922$ ,  $n = 10$ ).

### Day 3

When zebra fish previously conditioned with alarm cue and pike odour were presented with dechlorinated tap water on day 3 there was no significant change in activity ( $T^- = 35$ ,  $p = 0.898$ ,  $n = 11$ , Figure 1) or time near bottom ( $T^- = 33$ ,  $p = 0.250$ ,  $n = 9$ , Figure 2). After presentation of pike odour, conditioned zebra fish significantly decreased activity ( $T^- = 65$ ,  $p = 0.002$ ,  $n = 11$ , Figure 1) and significantly increased time near bottom ( $T^+ = 55$ ,  $p = 0.002$ ,  $n = 10$ , Figure 2). The overall change between prestimulus and post pike odour periods was significant for activity ( $T^- = 59$ ,  $p = 0.019$ ,  $n = 11$ ) but not for time near bottom ( $T^+ = 46.5$ ,  $p = 0.057$ ,  $n = 10$ ).

Conditioned control fish did not change activity after water ( $T^- = 34$ ,  $p = 0.898$ ,  $n = 11$ ) or after pike odour ( $T^- = 38.5$ ,  $p = 0.638$ ,  $n = 11$ ) (Figure 1). Overall difference in activity between prestimulus and post pike periods was not significant ( $T^- = 30$ ,  $p = 0.846$ ,  $n = 10$ ). Similarly, control fish showed no change in time near bottom after presentation of water ( $T^- = 16.5$ ,  $p = 0.266$ ,  $n = 6$ ) or pike odour ( $T^- = 19.5$ ,  $p = 0.894$ ,  $n = 8$ ) (Figure 2).

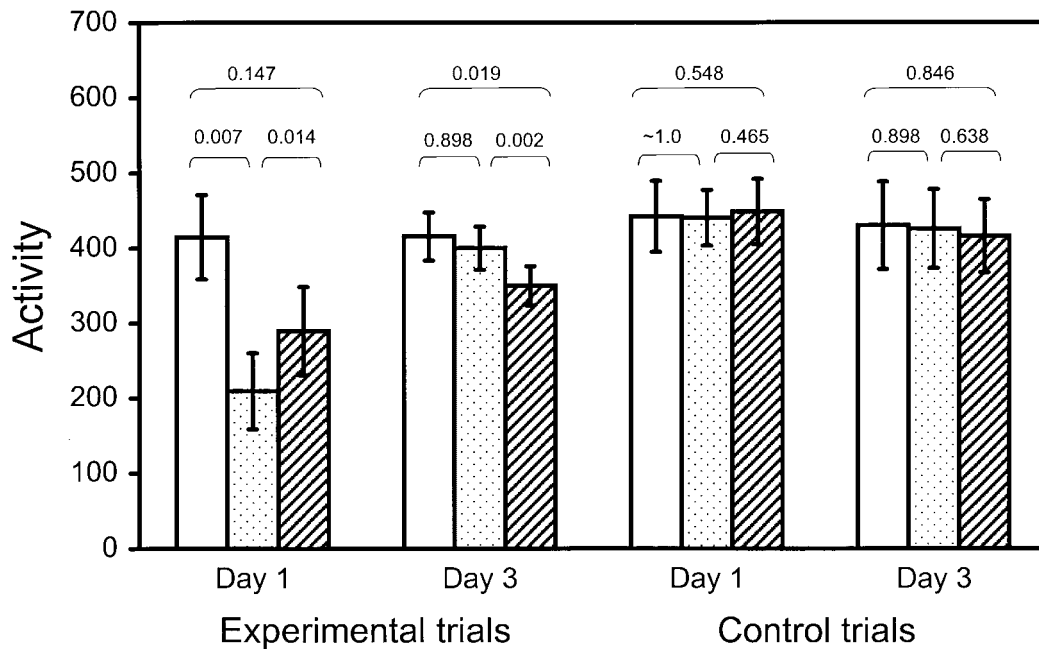


Figure 1. Mean ( $\pm$ SE) activity by zebra danios before (open bars) and after (stippled bars) the introduction of water (control trials) or chemical alarm cues (experimental trials) and after the introduction of pike odour 5 min later (hatched bars). Day 1: fish received water (control) or alarm cues (experimental), followed by pike odour. Day 3: all fish (control and experimental) were retested with water followed by pike odour. Numbers above bars indicate p values from 2-tailed Wilcoxon matched-pairs signed-ranks tests.

Overall change in time near bottom was not significant ( $T+ = 11$ ,  $p = 0.438$ ,  $n = 5$ ).

## Discussion

These data indicate that zebra fish that were previously unresponsive to pike odour learned to recognize and fear it after a single time-shifted presentation of alarm cue and pike odour. The time period during which risk associations can form extends for at least 5 min after alarm cues are first detected. Zebra danios learned to fear pike odour even after the initial fright response to the skin extract had begun to subside. These data corroborate those of Hall & Suboski (1995) and Brown & Smith (1996) in that an overt behavioural response is not required for learning. We cannot say from our data if learned recognition of predation risk would have occurred if the alarm cue had been flushed away completely before the predator odour was introduced because the alarm cues remained in the test aquarium for the entire duration of the data collection on day 1.

Observations of non-ostariophysan *Gambusia* (Poeciliidae) indicate an extended period of predator vigilance following exposure to conspecific injury-released chemical cues (García et al. 1992). Likewise, it appears that an extended period of heightened sensitivity to novel stimuli also occurs in zebra danios, during which they continue to form associations with predation risk.

Given the potency of ostariophysan alarm cues and limited volumes of some ponds and small streams, it is entirely plausible that fish may remain within the active area of the alarm cue for at least 5 min after detection of the alarm cue. Prey may indeed accrue a fitness benefit from remaining close by and maintaining increased vigilance (Brown & Smith 1996). Such behaviour would allow prey to monitor predator activity in the area (Wisenden et al. 1995) and to better learn the identity and nature of the predator (Brown & Godin 1999).

Upon recognition of pike odour, zebra danios reduced activity and increased time spent near the tank bottom. This is the same antipredator behaviour initially exhibited in response to alarm cue, and has been shown to be effective in reducing the probability of

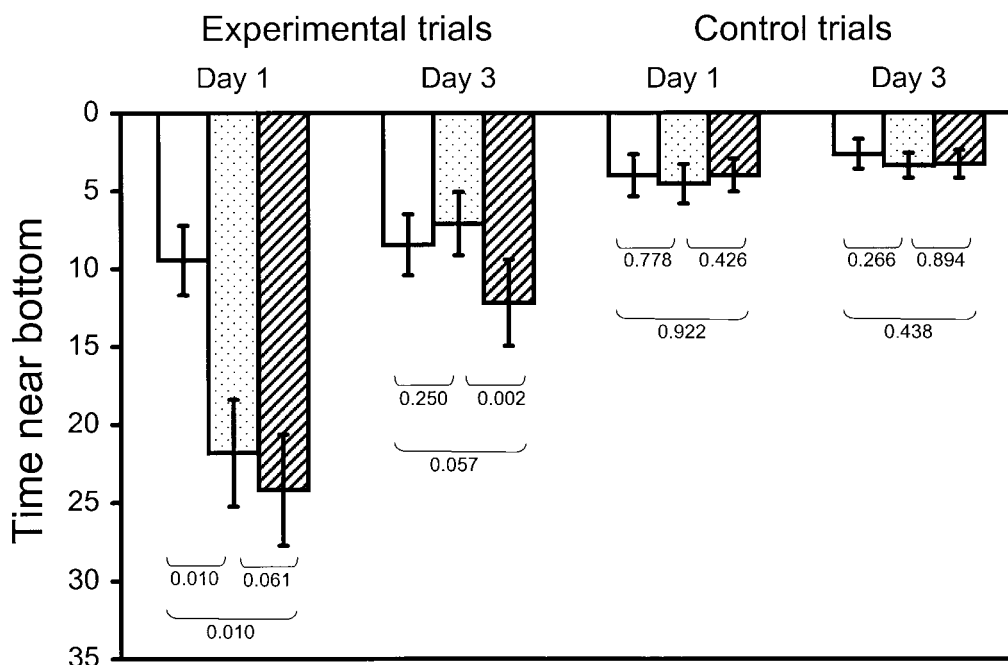


Figure 2. Mean ( $\pm$ SE) time near bottom by zebra danios before (open bars) and after (stippled bars) the introduction of water (control trials) or chemical alarm cues (experimental trials) and after the introduction of pike odour 5 min later (hatched bars). Day 1: fish received water (control) or alarm cues (experimental), followed by pike odour. Day 3: all fish (control and experimental) were retested with water followed by pike odour. Numbers above bars indicate p values from 2-tailed Wilcoxon matched-pairs signed-ranks tests.

predation in other species (Hews 1988, Mathis & Smith 1993a, Wisenden et al. 1999). Danios did not become conditioned to fear the method of stimulus introduction *per se* (Mathis & Smith 1993b) because there was no response to water on day 3 by alarm-conditioned fish.

These data suggest a potentially robust learning mechanism by which zebra danios acquire knowledge of predation risk to accommodate ecologically realistic temporal shifts in stimulus presentation. This form of learning is described as releaser-induced recognition learning (Suboski 1990). An important aspect of this learning is that it occurs after a single presentation. Natural selection has promoted single trial learning because repeated naïve encounters with a predator would greatly increase the risk of predation. While rapid acquisition of risk indicators confers an enormous fitness benefit, acquiring aversive responses to irrelevant stimuli would carry a fitness cost. Using simultaneous (non-time-shifted) presentation of alarm and novel cues as the learning paradigm, zebra danios can be trained to fear non-biological stimuli such as the human-synthesized chemical morpholine, or even a flashing red light (Suboski et al. 1990, Hall &

Suboski 1995). Similarly, fathead minnows have been trained to fear the sight of a common goldfish or a red light (Chivers & Smith 1994a, Yunker et al. 1999). The cognitive hierarchy for assigning risk to novel stimuli is not well studied and is ripe for further experimentation (Brown et al. 1997, Hartman & Abrahams 2000, Wisenden & Harter 2001). The results of the current study seem to indicate that the benefits of rapid recognition of predator identity outweigh costs typically associated with learning to fear irrelevant stimuli. These new data indicate that the potential for predator identity acquisition extends beyond the myriad stimuli that may be present at the precise moment of the first detection of chemical alarm cues.

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