

Responses of American toad tadpoles to predation cues: behavioural response thresholds, threat-sensitivity and acquired predation recognition

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Summary

Predation is one of the most important selective forces acting on prey animals. To respond adaptively to predation threats and increase their chances of survival, prey animals have to be able to recognize their potential predators. Even though a few studies demonstrated innate predator recognition, the vast majority of animals have to rely on learning to acquire this information. Often aquatic prey animals can learn to recognize predators when they detect conspecific alarm cues associated with cues from a novel predator. In this study, we exposed American toad (*Bufo americanus*) tadpoles to varying concentrations of chemical alarm cues (cues from injured conspecifics). We identified a concentration of cues which caused an overt antipredator response (supra-threshold concentration) and a lower concentration for which the prey failed to exhibit a response (sub-threshold concentration). In a second experiment, we attempted to condition the tadpoles to recognize the odour of larval dragonflies (*Anax* sp.) by pairing the dragonfly odour with either the sub-threshold concentration or the supra-threshold concentration of alarm cues. In both cases, the tadpoles learned to recognize the predator based on this single pairing of alarm cues and predator odour. Moreover, the intensity of the learned response was stronger for tadpoles conditioned with the supra-threshold concentration of alarm cues than the sub-threshold concentration. This is the first documented case of this mode of learning in anuran amphibians. Learned recognition of predators has important implications for survival.

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Introduction

Due to the unforgiving nature of predation, prey animals are under intense selection to respond appropriately to predators (Lima & Dill, 1990). The ability of prey to respond appropriately requires that they are able to recognize predators from non-predators. How such an ability arises is of considerable interest to ethologists. In a classic study, Tinbergen (1951) demonstrated that wild turkeys (*Gallopavo melaegris*) exhibited antipredator behaviour when exposed to a model hawk but not a model goose. Likewise, Kiesecker et al. (1996) documented that western toad (*Bufo boreas*) tadpoles responded to cues of backswimmers (*Notonecta* spp.), giant waterbugs (*Lethocerus americanus*) and garter snakes (*Thamnophis sirtalis*), all of which prey on tadpoles. The toads, however, did not respond to cues of rainbow trout (*Oncorhynchus mykiss*) or roughskin newts (*Taricha granulosa*). Trout and newts are potential predators, but rarely feed on toads as they are unpalatable to them.

Throughout their lives, prey are exposed to a myriad of other animals, any of which could be a potential predator. How do prey know which species are potential threats and which are not? Responses of prey to predator cues could result from learning or alternatively could be genetically fixed. Numerous vertebrate taxa have been shown to exhibit innate recognition of predators, e.g. mammals (Barros et al., 2002), birds (Veen et al., 2000; Goth, 2001), amphibians (Kats et al., 1988; Kiesecker & Blaustein, 1997) and fish (Berejikian et al., 2003; Hawkins et al., 2004). For instance, Kiesecker & Blaustein (1997) showed that antipredator responses of red-legged frogs (*Rana aurora*) to cues from introduced bullfrogs, *Rana catesbeiana*, are genetically fixed. In contrast to innate recognition, many studies have demonstrated that recognition of predators is a result of learning. Studies have been done on mammals (McLean et al., 2000; Griffin et al., 2001), birds (McLean et al., 1999; Maloney & McLean, 1995), amphibians (Semlitsch & Ryer, 1992), and fishes (Chivers & Smith, 1994a; Ferrari et al., 2005). In this study, we examine the role of learning in acquired recognition of a predator by larval toads.

Aquatic organisms often rely on chemicals dissolved in water to acquire information regarding predation risk. Chemical cues may be particularly important in environments in which other sensory modalities are obscured, for

example in turbid water and densely vegetated habitats where visual information is limited. These are common conditions in which larval amphibians find themselves. The chemicals to which prey respond may originate from predators, i.e., predator odours, or they may come from injured conspecifics that release chemicals upon encountering or being captured by a predator. Reviews by Kats & Dill (1998) and Chivers & Smith (1998) revealed that responses to predator odours and alarm cues are widespread in amphibians.

Studies with a variety of taxa have demonstrated the importance of alarm cues in mediating the learning of potential predators (review Chivers & Smith, 1998; Brown & Chivers, 2005). For example, Chivers & Smith (1994a,b) demonstrated that fathead minnows could learn to recognize the sight or odour of a predatory pike when they detected the cues of a novel predator associated with minnow alarm cues. Surprisingly, a single conditioning event was enough to elicit the learned response and this response lasted for more than 2 months. Since this finding, numerous other investigators have examined the same phenomenon in fathead minnows (Wisenden & Harter, 2001; Ferrari et al., 2005; Ferrari & Chivers, 2006a) and other fishes, including zebra danios (*Brachydanio rerio*: Suboski et al., 1990), rainbow trout (*Oncorhynchus mykiss*: Brown & Smith, 1998), brook charr (*Salvelinus fontinalis*: Mirza & Chivers, 2000), Chinook salmon (*Oncorhynchus tshawytscha*: Berejikian et al., 1999) and brook stickleback (*Culaea inconstans*: Chivers et al., 1995). Likewise, this mode of learning has been documented in planaria (Wisenden & Millard, 2001), damselflies (Wisenden et al., 1997), snails (Rochette, 1998), crayfish (Hazlett, 2003) and one species of caudate amphibians, the central newts, *Notophthalmus viridescens* (Woody & Mathis, 1998). In all cases, the prey learned to recognize the predator based on a single pairing of conspecific alarm cues and cues of a novel predator.

Some research has investigated the link between the ability of prey to learn to recognize novel predators and the concentration of alarm cues used in the conditioning. In fact, the prey can even learn to recognize the predator when they are exposed to a sub-threshold concentration of alarm cues, i.e., a concentration too low to elicit an overt behavioural response. For example, Ferrari et al. (2005) demonstrated that when minnows were given a sub-threshold concentration of alarm cues paired with the odour of a brook charr, they did not display any overt response. However, when subsequently given the charr odour alone, minnows did exhibit an antipredator response. These

results clearly showed that minnows did acquire predator recognition even in the absence of an alarm response during the conditioning trials. Similar sub-threshold learning effects have also been shown by Brown & Smith (1996) and Brown et al. (2001).

In this study, we conducted the first experiment testing whether an anuran amphibian, the American toad, *Bufo americanus*, could learn the identity of a predator (a dragonfly, *Anax* sp.) based on the pairing of alarm cues and the predator odour. Furthermore, we are the first to investigate the potential for learned predator recognition through sub-threshold concentration of alarm cues in an amphibian.

Methods and results

Collection and maintenance of animals

American toad eggs were collected from seven clutches in a pond in State Gamelands 176, Centre County, PA in June 2003 and transported back to our laboratory at Pennsylvania State University. Eggs were placed into aerated 37 L aquaria at 21°C and were kept on a 12:12 L:D hr photoperiod until they hatched. Once hatched, larval amphibians were removed and placed into plastic containers (31.5 × 17 × 9.5 cm) and maintained on a diet of crushed alfalfa pellets and goldfish flake food. Larvae were allowed to reach Gosner stage 34-36 (Gosner, 1960) before being used in the experiment.

Experiment 1: Responses of toads to varying alarm cue concentrations

The purpose of this experiment was to determine the responses of American toad tadpoles to variation in alarm cue concentrations. Specifically, we attempted to find an alarm cue concentration which evoked an overt antipredator response (supra-threshold concentration) and a sub-threshold concentration which was below the minimum behavioural response threshold, i.e., did not evoke an overt antipredator response. The lack of a response to a low concentration could reflect that the stimulus concentration was too low for the tadpoles to detect. Alternatively, it could reflect a concentration which was detected but was not high enough to evoke an overt response. We exposed tadpoles to one of four different concentrations of conspecific alarm cues or distilled water.

Preparation of stimuli

We created a base solution of toad alarm cue by crushing 10 donor tadpoles (Gosner stage 34-36, mass = 1.725 g) in 50 ml of distilled water with a mortar and pestle, filtering the supernatant through filter floss (75 μm nitex) to remove suspended particles and adding distilled water to make a final volume of 300 ml. We then created dilutions of the base solution (1:1) of 1:10, 1:100 and 1:1000 by diluting the base solution with distilled water. Toad alarm cue and distilled water were frozen in 20 ml aliquots at -20°C until used.

Experimental protocol

Tests were conducted in 5L plastic pails filled with dechlorinated tap water. Tadpoles were allowed to acclimate for 60 minutes. Individual tadpoles (Gosner stage 34-36) were exposed to one of 4 concentrations of conspecific alarm cue (1:1, 1:10, 1:100, 1:1000) or distilled water. Each trial was 12 min in length and consisted of a 6-min pre- and 6-min post-stimulus period. After the pre-stimulus period, 5 ml of stimulus was slowly injected into the pail down the side so as not to disturb the tadpoles. Dye trials indicated that it took approximately 40 sec for the stimulus to distribute to all parts of the pail.

During both the pre- and post-stimulus periods we recorded the time the tadpole spent moving. A reduction in activity is a well-established antipredator response for larval anuran amphibians (Kiesecker et al., 1996; Chivers & Mirza, 2001). Changes between the pre-stimulus and post-stimulus periods were calculated (post-stimulus minus pre-stimulus) and the difference in changes among treatments was analyzed using a one-way analysis of variance (ANOVA) followed by post-hoc Tukey tests. All data met the assumptions for parametric analysis and statistics were calculated using SPSS 13.0.

Results of experiment 1

ANOVA analysis revealed there was a significant difference in time spent moving among the different alarm cue concentration treatments ($F_{4,95} = 82.62$, $p < 0.001$). Based on post-hoc analysis we found that tadpoles exposed to either 1:1, 1:10, or 1:100 concentrations significantly decreased movement compared to tadpoles exposed to an alarm cue concentration of

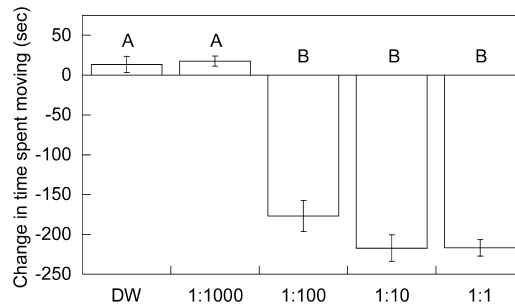


Figure 1. Mean \pm SE change in time spent moving for tadpoles exposed to either distilled water (DW) or various concentrations of chemical alarm cues. Letters over bars indicate significant differences at $p < 0.05$.

1:1000 or distilled water (all $p < 0.001$, Figure 1). There were no significant differences in activity level between tadpoles exposed to concentrations of 1:1, 1:10 or 1:100 (all $p > 0.218$). Similarly, there was no significant difference in activity level in tadpoles exposed to 1:1000 and distilled water ($p = 0.999$).

Experiment 2: Acquired predator recognition

The purpose of this experiment was to determine whether toad tadpoles could learn to recognize chemical cues of larval dragonflies when simultaneously exposed to cues of dragonflies and alarm cues from injured tadpoles. Moreover, we tested whether the toads could learn to recognize the predator when exposed to a sub-threshold concentration of alarm cues, and if so, was there a difference in the intensity of the learned response of tadpoles conditioned with different concentration of alarm cues. We conditioned tadpoles by exposing them to the odour from predatory dragonfly larvae paired with either the 1:100 or 1:1000 alarm cue concentration solution from experiment 1 or a distilled water control. Pairing the dragonfly odour with distilled water served as a control for responses of naïve tadpoles. We subsequently exposed tadpoles to *Anax* odour alone 24 hr later to test for learned recognition.

Stimulus preparation

Alarm cue was used from the previous experiment. The predator odour was created by placing 3 dragonfly larvae individually into plastic containers ($31.5 \times 17 \times 9.5$ cm) and feeding them live bloodworms (*Tubifex* sp.) ad

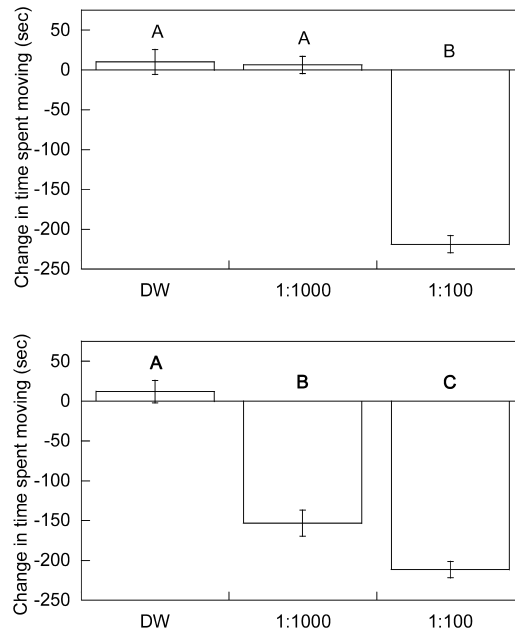


Figure 2. Mean \pm SE change in time spent moving for tadpoles exposed to dragonfly odour paired with either distilled water (DW) or various concentrations of chemical alarm cues during conditioning trials (top panel) and when subsequently exposed to dragonfly odour alone during recognition trials (bottom panel). Letters over bars indicate significant differences at $p < 0.05$.

libitum for 7 days. The dragonflies were then removed from their containers and transferred into clean containers with fresh dechlorinated tap water and left undisturbed for 24 h. We combined the water from each container and the stimulus was frozen in 50 ml aliquots at -20°C until used.

The experimental setup and protocol was identical to that of experiment 1. We exposed toad tadpoles to a paired stimulus of 5 ml of either 1:100 or 1:1000 concentration of alarm cue or distilled water paired with 10 ml of dragonfly larvae odour. We recorded the same response variable as in experiment 1. After the conditioning sessions, we conducted a complete water change and placed tadpoles back in the pails and allowed them to sit for 24 h. On the second day, we exposed tadpoles to 10 ml of dragonfly larvae odour only and recorded their responses. We used the same statistical analysis on both the conditioning and recognition trials as in experiment 1 (ANOVA with post-hoc Tukey comparisons).

Results of experiment 2

Conditioning trials. We found there was a significant difference among groups exposed to the paired stimulus of toad alarm cues or distilled water and dragonfly odour (ANOVA, $F_{2,57} = 109.07$, $p < 0.001$). Tadpoles given *Anax* odour paired with the 1:1000 concentration of alarm cues did not respond differently than those given *Anax* odour paired with distilled water ($p = 0.974$). However, tadpoles given *Anax* odour paired with the 1:100 concentration of alarm cues significantly decrease activity level compared to those exposed to *Anax* odour combined with distilled water ($p < 0.001$) and the 1:1000 concentration ($p < 0.001$; Figure 2).

Recognition trials. Subsequent exposure to dragonfly odour alone resulted in a significant difference in activity among groups (ANOVA, $F_{2,57} = 71.2$, $p < 0.001$). Tadpoles initially exposed to dragonfly odour paired with 1:100 and 1:1000 exhibit a higher intensity of antipredator response than those initially exposed to dragonfly odour paired with distilled water (both $p < 0.001$). Moreover, tadpoles initially exposed to the 1:100 concentration of alarm cues paired with *Anax* odour subsequently exhibited a higher intensity of response (spent less time moving) to *Anax* odour alone than those initially exposed to *Anax* odour paired with the 1:1000 concentration of alarm cues ($p = 0.011$; Figure 2).

Discussion

Our results are the first to document that larval anuran amphibians can learn to recognize unknown predators based on a single pairing of alarm cues and cues of the predator. The ability to learn to recognize unknown predators has important implications for survival (Mirza & Chivers, 2001). In general, predation is highly variable through time and space (Hileman & Brodie, 1994; Sih et al., 2000; Mathis et al., 2003). Consequently, the ability to learn to recognize predators maybe be preferable to innate recognition unless predator communities are constant through long periods of time.

The mode of learning we documented in our study appears to be almost universal among aquatic animals (planaria, snails, damselflies, crayfish, fishes, and amphibians). We know of no studies which have failed to document learning through this means unless the prey were pre-exposed to

the predator cue for several days in a row prior to attempting the conditioning. Failure to learn in such a case results from latent inhibition. Working with crayfish, Acquistapace et al. (2003) was the first to show such an effect. In another study, Ferrari & Chivers (2006b) showed that fathead minnows failed to learn to respond to brook charr when pre-exposed to charr cues for an hour a day during 5 consecutive days prior to attempting the conditioning.

The supposition that prey animals adjust the intensity of their antipredator response to match the degree of threat posed by a predator is known as the threat-sensitive predator avoidance hypothesis (Helfman, 1989; Chivers et al., 2001). Past experiments have documented threat-sensitive responses of prey to changes in concentrations of alarm cues. For example, Zhao & Chivers (2005) showed that goldfish exhibited more intense and longer duration antipredator responses as the concentration of alarm cues increased. Similarly, Dupuch et al. (2004) showed that redbelly dace showed a graded response to increased concentrations of alarm cues in the field. Other authors have failed to find a graded response to alarm cues. For example, Roh et al. (2004) demonstrated that juvenile convict cichlids (*Archocentrus nigrofasciatus*) showed an all-or-nothing response to increased concentration of alarm cues and Marcus & Brown (2003) showed the same effect in pumpkinseed sunfish (*Lepomis gibbosus*). To date, no studies have examined the effect of varying alarm cue concentrations on the intensity of antipredator responses of amphibians. In our first experiment, we found that the tadpoles responded with the same intensity to the 1:1, 1:10 and 1:100 concentrations of alarm cues but showed no overt response to the 1:1000 concentration. More work needs to be done to test whether a graded response exists between the 1:100 and 1:1000 concentrations. It is important to note, however, that the tadpoles could still learn at the 1:1000 concentration, and hence, must have detected this low concentration cue.

Threat-sensitive predator avoidance is widespread in many predator prey systems, including amphibians (Madison et al., 1999; Murray & Jenkins, 1999; Puttlitz et al., 1999; Sullivan et al., 2004). However, how such an ability develops has received little attention. In one study, Ferrari et al. (2005) demonstrated that fathead minnows could learn to recognize brook charr in a threat-sensitive manner, i.e. the intensity of antipredator responses of minnows to varying concentration of alarm cues paired with charr odour during conditioning matched the intensity of antipredator responses of the minnows when they were subsequently exposed to charr odour alone. Moreover, they

demonstrated that the intensity of the learned response when conditioned with the sub-threshold concentration of alarm cues was weaker than the intensity of the learned response when conditioned with a concentration of alarm cues that elicited an overt antipredator response. In our experiment, we also have evidence of threat-sensitive learning. Tadpoles exposed to the 1:100 concentration alarm cue learned to respond with a greater intensity than those exposed to the 1:1000 concentration. It is of particular importance to remember that this threat-sensitive learning develops based on a single pairing of alarm cue and the novel predator odour. Future work should consider the effect of multiple conditionings on the intensity of the learned response to predators.

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