Background risk influences learning but not generalization of predators

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The cognitive abilities of prey animals are closely linked to previous experience with predation risk. For instance, under conditions of high background risk, prey often show an enhanced ability to learn the identity of new predators. When prey learn about predators, they often use the information to make decisions about the predatory nature of unknown, but similar, species. This is referred to as generalization of predator recognition. Often, prey show the strongest response to the species they learned, and a reduced response to other species, with the phylogenetic distance between the learned predator and the other species predicting the degree of generalization. Here, we assessed whether generalization of predator recognition by woodfrog tadpoles was influenced by background level of predation risk in the environment. Given that the likelihood of making a recognition mistake (i.e., failing to respond to an unknown species that is a predator) may be greater in a high-risk environment than in a low-risk environment, we predicted that prey in high risk environments may widen their generalization window and respond with the same intensity to the learned predator and novel predators that are closely related. We found strong evidence that background risk influenced the intensity of antipredator responses that prey exhibit towards learned trout predators. Moreover, tadpoles generalize their recognition to closely-related trout, but the degree of generalization was independent of background risk.

Key words:

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*Lithobates sylvaticus.*
neophobia

risk-assessment
From an evolutionary perspective, there are few events that are as unforgiving as being eaten by a predator (Lima & Dill, 1990). Not surprising, there are an astonishing array of antipredator responses that act to thwart would-be predators (Godin, 1997). Prey animals often develop morphological defences that act to reduce the probability of attack or increase the likelihood of survival in the case of an attack (Bronmark & Miner, 1992; Harvell, 1990; Lönnstedt, McCormick, & Chivers, 2013). Prey may change their life-history traits, including their timing of hatching and metamorphosis to avoid encountering predators (Chivers et al., 2001; Sih & Moore, 1993). Prey also show considerable development of behavioural defences, including the ability to flee from an attack and adjust foraging and reproduction to times and places where predators are absent (Lima, 1998; Stankowich & Blumstein, 2005).

A prerequisite for most behavioural defences to predators is the ability of the prey to recognize potential predators as dangerous (Blumstein, 2006; Ferrari & Chivers, 2011). Consequently, prey have efficient mechanisms to learn the identity of predators (Brown & Laland, 2003; Brown & Chivers, 2005; Kelley & Magurran, 2003). Indeed, when a prey animal learns the identity of a predator, it may generalize this information, effectively making a decision about the predatory status of other similar species. In a pioneering study, Griffin et al. (2001) showed that naïve tammar wallabies (Macropus eugenii) do not show antipredator responses to foxes (Vulpes vulpes), but they can be trained to recognize them as dangerous. Wallabies trained to recognize foxes also display antipredator responses to cats (Felis catus) but not goats (Capra hircus), while wallabies that did not undergo the training fail to respond to either cats or goats. This work shows that wallabies are generalizing their recognition of the fox to the cat based on some characteristics, perhaps frontally-placed eyes, which the animals share in common. Generalization of predator recognition appears to be taxonomically widespread and is not restricted to visual modalities (Brown et al., 2011; Ferrari & Chivers, 2009a; Ferrari, Gonzalo, Messier, & Chivers, 2007; Mitchell, Chivers, McCormick, &
For example, many fishes and amphibians generalize their learned recognition of predator odours to closely-related species. Fathead minnows (*Pimephales promelas*) trained to recognize the odour of a lake trout (*Salvelinus namaycush*) as a threat, generalized their responses to brook trout (*Salvelinus fontinalis*, same genus as lake trout) odour and rainbow trout (*Oncorhynchus mykiss*, same family) odour, but did not generalize to odours of distantly-related predatory pike (*Esox lucius*) or non-predatory suckers (*Catostomus commersoni*). The response was stronger to brook trout than to rainbow trout, reflecting that more closely-related fishes share a greater degree of odour similarity (Ferrari et al., 2007).

The way in which animals learn the identity of predators and maintain responses to those predators appears to be linked to the level of background risk in the environment. For example, newly-settling coral reef damselfish (*Pomacentrus chrysurus*) that were raised under high-risk conditions for 4 days and then taught the identity of a predator, showed stronger learning of the predator than fish raised under low-risk conditions (Chivers, McCormick, Mitchell, Ramasamy, & Ferrari, 2014; Ferrari, McCormick, Meekan, & Chivers, 2015b). Similarly, woodfrog tadpoles (*Lithobates sylvaticus*) raised under high-risk conditions and then taught to recognize a predator, maintained learned responses to the predator for longer than tadpoles raised under low-risk conditions (Mitchell, Chivers, Brown, & Ferrari, 2016). Here, we integrate the concept of differential background risk with predator generalization, specifically testing whether woodfrog tadpoles that are raised under high-risk conditions show stronger learning of trout predators and a change in their degree of generalization. The likelihood of making a recognition mistake (i.e., failing to respond to an unknown animal that is a predator) may be greater in a high-risk environment than in a low-risk environment because there is a greater probability that unknown animals are actually predators. Therefore, we predict that prey from high-risk environments may widen their
generalization window and respond with the same intensity to the learned predator and predators that are closely-related.

METHODS

Test subjects

We collected six, freshly-laid, woodfrog egg masses from a pond in central Alberta, Canada and placed them together into a large water trough to hatch. The trough contained well water that was held for two weeks in a large 1900-L pool with plants taken from the pond. This ensured that the water source contained natural pond odours but was free of predator odours. Upon hatching, the tadpoles were fed ground alfalfa pellets and fish food to supplement the algae present in the pool. The tadpoles were approximately two weeks old when the experiment began.

Experimental design

Our experiment followed a 2 x 2 x 4 design, where we manipulated tadpoles’ background level of risk (low vs. high risk), then their predator training (trained vs. untrained) and tested them for their responses to one of four cues (the predator, two novel closely-related species and a distantly-related control). All manipulations took place outdoors, under natural light and temperature conditions.

Background risk: We placed 30 tadpoles into each of 20, 8-L plastic pails filled with 3 L of well water. Half of the pails were designated high risk and half as low risk. We introduced 20 mL of well water into the low-risk buckets twice a day for eight days. The injections occurred at random times between 0900 and 1800 hours, with a minimum of 1.5 h between injections. For the high-risk treatment, we introduced injured tadpole cues into the pails on the same
schedule. Injured tadpole cues provided tadpoles with a general indication of elevated risk in their environment without providing the specificity of a particular predator which could have biased the antipredator response (Ferrari, Crane, Brown, & Chivers, 2015c). The cues were prepared following a standard protocol: tadpoles from our stock population (i.e. same size and age as the test animals) were euthanized with a blow to the head, crushed with a mortar and pestle and the solution was diluted with well water before being filtered through filter floss. In each high-risk pail, we introduced 20 mL of solution containing the equivalent of three crushed tadpoles.

**Predator training phase:** The day following the completion of the background risk treatment, we removed two tadpoles from each of the 10 high-risk pails (20 tadpoles in total) and placed them in a new high-risk pail. The procedure was repeated 12 times, leading to 12 high-risk pails. Mixing the tadpoles in this manner removed the possibility of any potential pail effects in the risk exposure treatment. We completed the same procedure for the low-risk tadpoles. Unused tadpoles from the high risk and low risk background treatment pails were used for a different experiment. The 12 pails from each risk group were subsequently allocated to the two training treatments, with eight pails receiving the predator training, and the remaining four receiving the no-training (negative control) treatment. The asymmetry reflects the lower sample size required for the control groups. We have conducted several similar learning studies (Chivers & Ferrari, 2013; Ferrari, Brown, Bortolotti, & Chivers, 2010; Ferrari & Chivers, 2009b) and were confident not to expect learned responses in the control groups. Predator training consisted of exposing the tadpoles in each pail to 20 mL of rainbow trout odour paired with 20 mL of injured tadpole cues. Trout odour was prepared from four trout (~15 cm total length) held in 15 L of water for 24 hours. Trout, which were maintained on a diet of trout chow, were starved for 48 hours prior to collecting their odour. The no-training
pails received 20 mL of rainbow trout odour paired with 20 mL of water. One h after training, a 100% water change was performed on all the pails.

**Testing phase:** Tadpoles exposed to high-risk conditions are known to display neophobic tendencies for several days after the end of risk exposure (Mitchell et al., 2016). This phenomenon is problematic for our testing phase, since our high-risk tadpoles would display a fear response to all new odours, regardless of whether or not it was the result of an association with the learned predator. To circumvent this problem, we waited nine days before the tadpoles were tested. This period is likely sufficient to remove any neophobic tendencies (Mitchell et al., 2016). After this waiting period, tadpoles were tested, individually, for their response to one of four fish odours: rainbow trout (the learned predator), brown trout and brook trout (two related, but novel fish) and goldfish (a distantly-related novel fish). The three trout species are in different genera, with rainbow trout (genus *Oncorhynchus*) being more closely related to brown trout (*Salmo*) than to brook trout (*Salvelinus*). The odours for brown trout, brook trout and goldfish were prepared as for rainbow trout. Again all fish were starved for 48 hours to eliminate any diet-related odours.

Our quantification of tadpole anti-predator behaviour followed well established protocols (Chivers & Ferrari, 2014; Ferrari & Chivers, 2009b). Tadpoles, like many prey animals, reduce activity upon exposure to risk cues. Each tadpole was placed in a 0.5-L plastic cup (10 cm diameter, 12 cm high) containing 450 ml of water and left 30 min to acclimate. We then quantified the activity level of the tadpole for 4 min prior to and 4 min following the injection of 5 mL of cue. We recorded activity level by counting the number of times the tadpole crossed a centreline of the cup. We considered the tadpole to have crossed the line when the entire body of the tadpole crossed the line. The 5 mL of cue was injected gently, on the side of the tank using a 10-mL syringe. During testing, the order of treatments was randomized and the observer was blind to the treatments. We tested 11-13 tadpoles in
each of the control no-training treatments and 30-35 tadpoles in each of the predator training treatments. Each tadpole was only tested once.

**Statistical analysis**

After ensuring that no pre-stimulus difference existed among tadpoles from different treatments, we calculated the percent change in line crosses between the pre-stimulus and post-stimulus periods and used this as response variable in our analysis. We performed a 4-way nested ANOVA, testing the effect of risk, training and cue on the activity of tadpoles, and introducing ‘pail’ as a nested factor (type I SS), to account for the non-independence of tadpoles trained in the same pail. To investigate potential interactions, the analysis was rerun for each factor level, using Tukey correction for post-hoc comparisons. All assumptions of homoscedasticity were met.

**Ethical note**

Care was taken to reduce unnecessary stress to the tadpoles during all stages of the experiment. Following hatching, tadpoles were maintained at densities that matched the local conditions and were supplied with an excess of food. Excess food was removed at the end of each day, during water changes a fresh supply food was added. At the end of the experiment all tadpoles were returned to their natal ponds. No collection permit was required for collecting amphibian eggs, but all experimental procedures were carried out in accordance with University Committee on Animal Care and Supply Animal Care Protocol 20060014.

**RESULTS**

**Pre-stimulus activity:** the 4-way nested ANOVA revealed no difference in the activity of tadpoles among treatment (all $P > 0.3$) prior to cue injection. Pail did not have an effect either ($F_{20,322} = 1.4$, $P > 0.1$).
**Percent change in activity:** The 4-way nested ANOVA revealed a significant effect of risk ($F_{1,19.3} = 8.4, P = 0.009$), but no effect of risk $\times$ cue ($F_{1,25.8} = 1.8, P = 0.1$) or risk $\times$ training $\times$ cue ($F_{3,324.9} = 1.2, P = 0.3$), indicating that high-risk tadpoles tended to respond stronger to the cues (figure 1). We also found a significant training $\times$ cue interaction ($F_{3,324.9} = 9.0, P < 0.001$) but no effect of pail ($F_{20,322} = 1.4, P > 0.2$). This interaction stems from the differential response of tadpoles that were trained vs. not trained. Indeed, when we looked at the response pattern of tadpoles in the no-training group, we failed to find an effect of risk ($F_{1,5.8} = 0.1, P > 0.9$), cue ($F_{3,78.6} = 0.7, P > 0.1$) or an interaction between the two ($F_{3,78.6} = 1.1, P > 0.3$), indicating that the tadpoles from all groups did not respond to any of the cues (figure 1). This also confirms that neophobic tendencies were absent. Pail did not have any effect ($F_{14,244} = 1.4, P > 0.1$).

On the other hand, when we looked at the tadpoles that underwent the training, we found that tadpoles from the high-risk groups responded stronger to the cues than those from the low-risk group ($F_{1,13.9} = 9.8, P = 0.007$, figure 1). We also found that tadpoles differed in their responses to different species ($F_{3,245.7} = 42.3, P < 0.001$), with tadpoles responding significantly stronger to rainbow trout (Tukey HSD: $P < 0.04$), but not differing in their response to brook and brown trout ($P = 0.9$, figure 1). There was no interaction between risk and cue ($F_{3,245.7} = 1.8, P = 0.14$).

**DISCUSSION**

The results of our study demonstrate that background risk influences the intensity of antipredator responses that prey exhibit towards learned predators and that prey can use information they acquired about one predator to generalize their recognition to closely related predators. However, the degree of generalization was independent of background risk.
Tadpoles raised in a high-risk environment showed enhanced responses to trout that they were conditioned to recognize. In similar studies, Mitchell et al. (2016) showed that tadpoles raised under high-risk had enhanced memory of predator odours, while Chivers et al. (2014) and Ferrari et al. (2015b) showed enhanced learning of predator odours by damselfishes. Taken together, these results suggest that high-risk environments prime animals to be more responsive to risk. Understanding the mechanism(s) behind such responses deserves consideration. We hypothesize that the differential learning and memory may be related to the level of physiological stress that the prey experience at the time of learning. Stress effects on memory are thought to be driven primarily by the targeted effects of adrenergic and glucocorticoid activity on mineralocorticoid (MR) and glucocorticoid (GR) receptors in hippocampal and basolateral amygdala brain regions (de Kloet, Oitzl, & Joëls, 1999; Dominique, Roozendaal, Nitsch, McGaugh, & Hock, 2000; Roozendaal, 2002).

Differential learning of predators may also be related to the degree of behavioural lateralization. We know that exposure to high-risk conditions for just a few days increases behavioural lateralization (Ferrari et al., 2015a; Ferrari et al., 2015d) and that tadpoles with a greater degree of lateralization show enhanced learning (unpub data).

Here, we showed that tadpoles that were taught to recognize rainbow trout generalized their responses to closely-related brown trout and brook trout but not distantly-related goldfish. This result matches several studies showing that prey generalize their response of the learned predator to other closely related predators but not distantly related predators or non-predators (Brown et al., 2011; Chivers, Al-Batati, Brown, & Ferrari, 2013; Ferrari & Chivers, 2009a; Ferrari et al., 2007). In our current study the intensity of response to the brown trout and brook trout were equal but of lower magnitude than the response to rainbow trout, the predator to which they were trained. Generalization of predators is not fixed but shows considerable flexibility. For example, Ferrari et al. (2008) showed that the
level of threat posed by a predator dictates how much prey will generalize. When fathead minnows were taught brown trout as a high threat predator, they generalized their recognition of brown trout to rainbow trout, but failed to do this when they were taught that the brown trout was a mild threat. In another study, tadpoles showed differential generalization based on how ‘certain’ they were about the identity of the predator (Ferrari, Crane, & Chivers, 2016). Tadpoles were taught that rainbow trout were predators either once (low certainty) or five times (high certainty). High-certainty tadpoles responded more to brown trout (a very close relative) but less to brook trout (a more distant relative), when compared to their low-certainty counterparts. This result highlights the nonlinear nature of stimuli generalization, whereby increasing certainty increases responses to a close relative and decrease responses to more distant relative. Here, we predicted that the width of the generalization window would vary based on background risk. The best strategy should be for tadpoles to respond to all trout equally in the high-risk treatment because the likelihood of a recognition mistake is greater in a high-risk environment than a low-risk environment. Contrary to our prediction, we failed to find evidence that background risk influenced generalization. Tadpoles responded stronger to rainbow trout, the predator they were taught to recognize, than to either brown trout or brook trout. This pattern was the same regardless of background risk. Given our large sample sizes (30-35 per treatment in the predator training groups) we are reasonably confident that the lack of effect in our experiment was not a result of a low sample size.

Examining the role of background risk in generalization of predator recognition deserves more attention because we need to understand whether the negative effect we observed is specific to the experiment we conducted or whether it represents a general pattern. In our experiment, we trained the tadpoles to recognize rainbow trout and tested them for responses to three other species, two of which, the brown trout and brook trout, were closely related, and one of which, the goldfish, was distantly related. Is it possible that we
need to include more phylogenetic diversity into our potential predators for us to observe that background risk influences generalization? Perhaps, systems that have high diversity of predators and non-predators, such as coral reef systems, would provide the opportunity for further testing this hypothesis. Another issue that deserves consideration is the magnitude of the difference in background risk that we employed. Our high risk background consisted of exposing the tadpoles to high risk twice a day and the low risk tadpoles to no risk. Does this difference in background risk constitute enough of a difference to influence generalization? This is the same protocol that we used in past experiments to examine neophobia (e.g. Brown et al. (2013), but perhaps a greater difference is needed to influence generalization.

We think that future researchers examining whether background risk influences generalization should manipulate diet cues of the predators. In our experiment, we fed the trout species commercial trout chow and the goldfish flake food, but starved them prior to collecting the fish odour. This ensured that the results we observed could not be related to diet cues, but rather the odour of the predators themselves (Mitchell et al., 2015). However, if we had collected wild fish as donors, perhaps the differences in diet amongst the trout could have led to greater differences in their odours that could allow the prey more information for generalization.

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Figure 1. Mean (± SE) proportion change in activity (line crosses) for tadpoles exposed to various predator odours. Tadpoles were exposed to high or low background risk for 8 days prior to training to recognize the odour of rainbow trout. Black, dark gray, light gray and white bars represent responses to rainbow trout, brown trout, brook trout and goldfish, respectively. Means with different associated letters are statistically different (P < 0.05) from each other (Tukey HSD tests).