Latent inhibition of predator recognition by embryonic amphibians

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To avoid being captured, prey animals need to be able to distinguish predators from non-predators. Recent studies have shown that amphibians can learn to recognize their future predators while in the egg. Here, we investigated whether amphibians would similarly be able to learn to recognize non-predators while in the egg. We exposed newly laid wood frog eggs to the odour of tiger salamander or a water control daily for 5 days. After hatching, the wood frog larvae were raised for two weeks at which time we tried to condition them to recognize the salamander as a predator. Larvae were exposed to injured conspecific cues paired with salamander odour, a well-established mode of learning for aquatic prey. When subsequently tested for their response to salamander odour, the larvae pre-exposed to water as embryos showed significant anti-predator responses. However, larvae pre-exposed to the salamander odour as embryos showed no learning of the predator, indicating that they had already learned to recognize the salamander as a non-predator. These results indicate that amphibian embryos can (i) learn to recognize stimuli as non-threatening and (ii) remember it for at least two weeks. The widespread ability of prey to learn to recognize non-predators might explain the persistence of injured conspecific cues as a reliable mechanism for learned predator recognition.

Keywords: predator recognition; latent inhibition; embryo; wood frog *Rana sylvatica*

1. INTRODUCTION

Owing to the unforgiving nature of predation, prey have evolved sophisticated behavioural responses to predators (Lima & Dill 1990). A prerequisite for such responses is for prey to distinguish predators from non-predators. While some prey species have an innate ability to recognize predators (Goth 2001; Berejikian *et al.* 2003), some require learning to recognize their potential predators as threatening. Several learning modes allow the acquisition of predator recognition. In aquatic organisms, amphibians (Woody & Mathis 1998; Mirza *et al.* 2006), fishes (Chivers & Smith 1994; Wisenden *et al.* 2008) and a number of invertebrates (Hazlett 2003; Ferrari *et al.* 2008) acquire predator recognition through simultaneous pairing of chemical cues from injured conspecifics with the sight, odour or sound of a novel predator.

Much of the research on embryonic learning has tested whether pre-exposure to a chemical stimulus subsequently results in a preference of the individual for that stimulus as adult (Sneddon *et al.* 1998). Recently, Mathis *et al.* (2008) have been the first to show embryonic learning of predator recognition, reporting that amphibian embryos acquire recognition of future predators through simultaneous pairing of injured tadpole cues with a novel predator odour. Natural selection should favour mechanisms and individuals able to recognize predators upon their first encounter. While this is the first indication that learned predator recognition is possible at such an early age, nothing is known about the ability of embryos to similarly learn to recognize species as non-predators. Failing to recognize and respond to an animal as a predator is very different from recognizing an animal as a non-predator. Recognizing the animal as a non-predator means that the prey may be prevented (in the short term) from subsequently learning that the animal is a threat. In this study, we exposed embryonic wood frogs (*Rana sylvatica*) to the odour of tiger salamanders (*Ambystoma tigrinum*) in the absence of any risk reinforcement. We then attempted to teach the wood frogs, as larvae, to recognize salamander odour as threatening through the simultaneous exposure of salamander odour and conspecific alarm cues. If frog embryos learned that the salamander was non-threatening (i.e. through latent inhibition, see Barad *et al.* 2004), we predicted that the larvae would fail to subsequently learn to recognize the salamander as a threat. If embryos detected salamander odour but did not learn it as a non-predator, then larval wood frogs should associate salamander odour with predation risk through conditioning with chemical cues from injured conspecifics.

2. MATERIAL AND METHODS

(a) Test subjects and stimulus preparation

All eggs and tadpoles used in this experiment were collected from a single pond in Central Alberta, Canada in May 2008. Male and female wood frogs breed only once a season, hence eggs obtained from different clutches are probably unrelated (Halverson *et al.* 2006). Our previous work has shown that wood frogs from this pond do not have 'innate' recognition of their predators (e.g. Ferrari & Chivers 2008).

The salamander odour used for the egg treatment phase was obtained by putting one healthy adult tiger salamander (14.1 cm snout–vent length) into 500 ml of conditioned well water for 24 hours. The water was changed every day.

(b) Experimental set-up

Five freshly laid egg clutches were collected and divided into sub-clutches of approximately 50–60 eggs each. Two sub-clutches from each of the five clutches were then transferred into two 3.5 l buckets filled with 3 l of well water. The sub-clutches consisted of a single mass of eggs with the egg jelly intact. The eggs were at Gosner developmental stage 10–11 (Gosner 1960). At this stage, the neural tube is not yet formed.

(c) Experimental procedure

(i) Embryonic pre-exposure phase

The two sub-clutches from each clutch were randomly assigned to one of the two treatments: 1, water: 20 ml of well water and 2, salamander odour: 20 ml of salamander odour. Hence, we treated 10 buckets, five receiving the water treatment and five receiving the salamander odour treatment. Eggs were treated daily at 15.00 for 5 days; the stimuli were slowly injected on the side of the buckets to minimize disturbance to the eggs. At 17.00, a 100 per cent water change was performed. Eggs were treated until the embryos appeared fully formed (approx. Gosner stage 22–24) but had not hatched. The treatments stopped after day 5. Embryos started hatching the following morning and all the

Figure 1. Mean ($\pm$ s.e.) proportion change in line crosses for tadpoles exposed to salamander odour. Wood frogs were pre-exposed for 5 days to either water (W) or salamander odour (SO) during their embryonic development and later conditioned as tadpoles to learn to recognize salamander as a threat through the pairing of injured conspecific cues and salamander odour (IC + SO) or through a control of water paired with salamander odour (W + SO).

embryos were hatched by the evening. Tadpoles were provided with rabbit food and the water was partially changed every second day. Tadpoles were raised for 12 days prior to being tested.

(ii) Larval conditioning phase

Six tadpoles (Gosner stage 25) from each bucket were arbitrarily chosen and placed individually in 0.5 l plastic cups filled with well water. Three of the tadpoles were subsequently exposed to 5 ml of injured conspecific cues paired with 10 ml of salamander odour (true conditioning), while the other three were exposed to 5 ml of water paired with 10 ml of salamander odour (control). The injured conspecific cues were obtained by crushing one tadpole in 5 ml of water using a mortar and pestle. The salamander odour was obtained by soaking one salamander in 2 l of water for 24 hours. Using a single salamander could have limitations if there is considerable variation in the odour signature of individual salamanders. While variation in the signatures may lead to small variation in the intensity of responses of the tadpoles, it probably will not result in dramatic changes in whether the salamander is recognized or not. Two hours after the conditioning phase, a 100 per cent water change was conducted on all the cups, and food was provided for the tadpoles.

(iii) Larval testing phase

After 24 hours, all the tadpoles were tested for their responses to salamander odour. The salamander odour was obtained using the same methodology as the day before. Tadpoles were tested using a well-established protocol (e.g. Ferrari & Chivers 2008). Accordingly, the trials consisted of a 4 min pre-stimulus followed by a 4 min post-stimulus injection period, during which the activity of the tadpoles was recorded. The two periods were separated by a 30 s injection period, during which 10 ml of salamander odour was slowly introduced on the side of the cup. Tadpoles decrease activity in response to predation cues, hence a line was drawn in the middle of the cup and the number of line crosses was counted during the two observation periods. The trials were performed outdoors ($n=12$ –15 treatment⁻¹), between 14.30 and 18.30. The order of the treatments was randomized throughout the day. The observer was blind to the treatments.

(d) Statistical analysis

We calculated the change in the proportion of line crosses from the pre-stimulus baseline. The data were normally distributed but heteroscedastic. Hence, we analysed the data using a non-parametric ANOVA approach (Scheirer-Ray-Hare extension of the Kruskal–Wallis test (Sokal & Rohlf 2003)). The effects of ‘embryonic pre-exposure’ (water versus salamander odour) and ‘conditioning’ (with water or injured conspecific cues) were treated as fixed factors, whereas ‘clutch’ was included as a random factor, to account for the possibility of maternal effects. Owing to a significant interaction between the two factors, subsequent non-parametric ANOVAs were performed to further investigate the nature of the interaction.

3. RESULTS

The three-way ANOVA revealed an interaction between embryonic pre-exposure and conditioning factors ($F_{1,4.4}=72.6$, $p<0.002$; figure 1). When looking at

the effect of ‘conditioning’ on each ‘pre-exposure’ level, the responses of tadpoles pre-exposed to water as embryos were affected by the conditioning treatment received as tadpoles (conditioning: $F_{1,4}=23.3$, $p=0.008$; clutch: $F_{4,4}=0.9$, $p>0.5$; interaction: $F_{4,19}=0.8$, $p>0.5$). Specifically, tadpoles receiving salamander odour paired with injured cues responded to the salamander odour with stronger anti-predator behaviour than tadpoles receiving the salamander odour paired with water. However, the responses of tadpoles pre-exposed to salamander odour as embryos were not affected by the conditioning treatment received as tadpoles (conditioning: $F_{1,4}=0.9$, $p>0.3$; clutch: $F_{4,4}=1.4$, $p>0.3$; interaction: $F_{4,16}=1.0$, $p>0.4$). When looking at the effect of embryonic pre-exposure on each conditioning level, the treatment received during the embryonic pre-exposure did not affect the responses to salamander odour of tadpoles conditioned with water paired with salamander odour (pre-exposure: $F_{1,4}=0.5$, $p>0.5$; clutch: $F_{4,4}=4.8$, $p>0.05$; interaction: $F_{4,17}=0.6$, $p>0.6$). However, when tadpoles were conditioned with injured cues paired with salamander odour, embryonic pre-exposure had an effect on their responses to salamander (pre-exposure: $F_{1,4}=21.7$, $p=0.009$; clutch: $F_{4,4}=0.5$, $p>0.7$; interaction: $F_{4,18}=0.7$, $p>0.6$). Specifically, tadpoles pre-exposed to water as embryos responded with a stronger intensity to salamander odour than tadpoles pre-exposed to salamander odour as embryos.

4. DISCUSSION

Our results clearly demonstrate that wood frog embryos pre-exposed to salamander odour without risk reinforcement learned that the salamander was a non-predator, as indicated by their failure to subsequently learn to recognize the salamander as threatening through the pairing of salamander odour and injured conspecific cues. This ‘latent inhibition’ phenomenon has been demonstrated in adult crayfish (Acquistapace *et al.* 2003) and adult fathead minnows (Ferrari & Chivers 2006). This is the first time, to our knowledge, that this phenomenon has been demonstrated in embryos. In addition, previous demonstrations of latent inhibition consisted of the

pre-exposure period, immediately followed by the conditioning and testing periods. Here, wood frogs experienced a 15-day lag between the end of the pre-exposure and the conditioning phase. Understanding how much information is needed to override non-predator recognition is a fascinating area for future research.

To optimize their fitness, prey trade-off predator avoidance against other activities. While under-responding to predation threats may have immediate and irreversible consequences (i.e. death), over-responding to non-predators can be costly as a result of missed foraging or mating opportunities. Hence, it is critical for the prey to respond to the appropriate threats and not to respond to non-risky stimuli. The ability of prey to learn to recognize non-predators may explain the success of the secondary evolution of 'alarm cues' as public information. Owing to the high efficiency of learning through pairing with injured conspecific cues, it seems likely that prey could frequently learn to recognize some non-threatening stimuli as dangerous through chance alone. Thus, mechanisms such as latent inhibition may maintain the proportion of erroneous learning events at a low level, explaining the persistence of conditioning with alarm cues as a reliable mechanism for learned predator recognition.

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