Determining sensitive stages for learning to detect predators in larval bronzed frogs: Importance of alarm cues in learning

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Successful survival and reproduction of prey organisms depend on their ability to detect their potential predators accurately and respond effectively with suitable defences. Predator detection can be innate or can be acquired through learning. We studied prey–predator interactions in the larval bronzed frogs (*Sylvirana temporalis*), which have the innate ability to detect certain predators. We conducted a series of experiments to determine if the larval *S. temporalis* rely solely on innate predator detection mechanisms or can also learn to use more specific cues such as conspecific alarm cues for the purpose. The results of our study clearly indicate that larval *S. temporalis* use both innate and learned mechanisms for predator detection. Predator-naïve tadpoles could detect kairomones alone as a potential threat and responded by reducing activity, suggesting an innate predator detection mechanism. Surprisingly, predator-naïve tadpoles failed to detect conspecific alarm cues as a potential threat, but learned to do so through experience. After acquiring the ability to detect conspecific alarm cues, they could associate novel predator cues with conspecific alarm cues. Further, post feeding stages of larval *S. temporalis* are sensitive for learning to detect conspecific alarm cues to label novel predators.

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1. Introduction

Successful survival and reproduction of prey populations depend on their ability to detect predators accurately and respond effectively, because failure to do so increases the probability of the prey's capture/injury, which could lead to death (Lima and Dill 1990). Depending on the species and the ecosystem, prey may use a variety of sensory modes for detection and discrimination of potential predators. In aquatic ecosystems, a most commonly used sensory mode of predator detection and discrimination is chemoreception (Kats and Dill 1998; Ferrer and Zimmer 2007; Ferrari *et al.* 2010). Although other sensory modes (visual, tactile or mechanical disturbance) can be useful in locating predators (Boothby and Roberts 1995; Amo *et al.* 2004; Ferrari *et al.* 2010), chemical cues *per se* are crucial in providing information not only on the spatial and temporal distribution of predators but also on the type of predator and the intensity of predation (Wisenden 2003; Ferrari *et al.* 2010). Moreover, chemical cues can be more reliable signals for prey species that are either nocturnal, inhabiting complex/turbid environments or those encountering diverse predators (Petranka *et al.* 1987; Chivers *et al.* 1996; Amo *et al.* 2004; Cabido *et al.* 2004). Diverse prey species ranging from aquatic invertebrates to mammals are known to use chemical cues for detection and discrimination of potential predators (Kats and Dill 1998; Wisenden 2003; Ferrari *et al.* 2010).

Chemical cues of diverse origin may serve as the signals for predation risk assessment (Wisenden 2003; Ferrari *et al.* 2010). For instance, kairomones (chemicals that are emitted by predators recognized by prey, which will use this cue for their benefit) and alarm cues (chemicals secreted by injured conspecifics), either alone or together, and dietary cues can be important sources of signals in eliciting antipredator responses in many aquatic prey animals (Kats and Dill 1998; Tollrian and Harvell 1999; Wisenden 2003; Ferrari *et al.* 2010). Because predation can be a strong selective force in

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determining spatial and temporal distribution of prey, many aquatic prey including fishes and amphibians use conspecific alarm cues for assessing predation risk (reviewed in Wisenden 2003; Ferrari *et al.* 2010). Alarm cues of heterospecific origin are equally effective in eliciting antipredator responses in some larval amphibians (Ferrari *et al.* 2010).

The ability of prey to detect predators using kairomones or alarm cues may be innate or acquired through experience (Wisenden 2003; Ferrari *et al.* 2010). Innate predator detection mechanisms that confer a high speed of response to a limited spectrum of predators is expected to be favoured in habitats with little variation in predation risk as learning could be costly in such habitats (Wisenden 2003; Brown and Chivers 2005). Numerous aquatic prey including insects, crustaceans, fishes and amphibians have been shown to possess innate mechanisms of predator detection (Wisenden 2003; Ferrari *et al.* 2010). In contrast, learned predator detection is expected to be favoured in complex habitats wherein predator identity varies seasonally and/or spatially across populations (Wisenden 2003; Brown and Chivers 2005). Prey animals that learn to detect predators may adaptively respond to variations in predation risk over time (Chivers and Smith 1998). Thus, learning can greatly enhance the efficiency of prey to detect and evade predation risk.

Whereas some ecosystems may favour the evolution of either innate or learned predator detection mechanisms, others, particularly those harbouring traditional as well as novel/non-native predators, may favour the evolution of both mechanisms in concert for efficient predator detection and avoidance. The prey that detects certain predators innately and also learns to detect novel predators through experience would be at a selective advantage in such complex habitats over those relying on either of the two. Surprisingly, information on such experience-mediated risk-sensitive adjustments to innate avoidance responses by larval amphibians is scarce (Gonzalo *et al.* 2007; Epp and Gabor 2008). Moreover, recent studies have shown that larval amphibians can learn to detect their predators even during embryonic stages (Mathis *et al.* 2008). However, the developmental window (pre-gastrulation, neurulation, post-hatching or post-feeding) during which learning occurs is unclear. Studies focusing on innate responses of naïve prey may inaccurately predict the actual behaviours of prey in natural habitats. Conversely, studies examining behaviours of only experienced prey may fail to detect the extent of innate detection abilities. Therefore, examining avoidance behaviours of both naïve and experienced prey is crucial in understanding both the daily interactions and the selective forces that shape antipredator responses. Such an approach would lend insight into the extent of plasticity in avoidance behaviours of prey animals.

Although prey populations use different chemical cues for detecting their potential predators, ontogenetic changes, if any, in their preference to use different cues for predator detection are not known. Depending on the type of predators, chemical cues used by larval amphibians for assessing predation risk may vary during their ontogeny. For instance, embryonic stages may preferentially use one cue to perceive predation risk while pre-feeding and post-feeding stages may use another cue for the purpose.

In the present study, we used larval bronzed frogs (*Sylvirana temporalis*) with an innate ability to detect certain predators (Mogali *et al.* 2012) to determine if learning also plays a role in predator detection. The streams and rivulets that harbour *S. temporalis* tadpoles are also home to a diverse community of predators. In such diverse, seasonally varying predatory environment, reliance solely on innate predator detection mechanisms could be less efficient in coping with different predator communities. Therefore, learning may be an important component of predator detection (Ferrari *et al.* 2010). Further, we explored the possibility of ontogenetic changes in the preferential use of chemical cues in assessing predation risk. Thus, we designed a series of experiments to determine (1) if predator-naïve (laboratory-reared) and predator-experienced (nature-collected) tadpoles recognize conspecific alarm cues, (2) whether or not larval *S. temporalis* with an innate predator detection mechanism also learn to detect novel predators, (3) if they do, at what developmental stages do they learn, (4) what is the importance of conspecific alarm cues in learning to detect novel predators, and (5) how do predator naïve and experienced tadpoles respond to different chemical cues.

2. Materials and methods

2.1 *Study animal and rearing*

Six egg masses of *S. temporalis* were collected (three on 15 October and three on 5 November 2011) from a gently flowing stream in Koyna forest (17°32′N, 73°45′E), Maharashtra, India, during morning hours (7.00–9.00 a.m.). The eggs were collected in two batches, each with three egg masses. At collection, the first batch was at early neurulation, while the second was at early cleavage. Each egg mass was collected separately in plastic containers and transported quickly to the laboratory, where they were maintained separately in plastic tubs (17 cm diameter) with 8 L of aged tap water. The developmental stages were identified as per Gosner (1960) and used for different experiments depending on the stage of exposure. A total of 1500 tadpoles (750 each from the first and second batches of eggs, mixing 250 eggs from each of the three spawns) were used for experimentation. The rest of the egg masses and all the test tadpoles (after experimentation) were released back into their natural habitat.

Nymphs of the dragonfly (*Bradinopyga geminata*) were used as predators as these are well known predators of anuran tadpoles and are seen along with the *S. temporalis* tadpoles. The study was carried out following the guidelines of the institutional committee for animal ethics (No. 538/CPCSEA).

2.2 *Experiment 1: Behavioural responses of predator-naïve and predator-experienced tadpoles to conspecific alarm cues*

This experiment was designed to assess the responses of predator-naïve (laboratory-reared) and predator-experienced (nature-collected) tadpoles to conspecific alarm cues alone (table 1). Because learning can be mediated through the association of conspecific alarm cues with novel cues, it was essential to test the responses of predator-naïve as well as predator-experienced tadpoles to conspecific alarm cues alone.

The experimental units consisted of large glass aquaria (60 cm × 45 cm × 15 cm) each containing 30 naïve tadpoles reared at a density of 5/L. They were fed on boiled spinach *ad libitum* on alternate days after replenishment of water. Each aquarium was equipped with an empty predator cage of plastic mesh cylinder (10.5 cm diameter). Tadpoles were also collected from nature 10 days before testing and acclimated to the laboratory conditions. The experiment consisted of two groups: predator-naïve and predator-experienced tadpoles. Both the groups were tested (n=30; stage 30–33) for their responses (activity) to conspecific alarm cues using a behavioural assay.

2.3 *Experiment 2: Associative learning in larval S. temporalis*

This experiment was conducted to determine the ability of *S. temporalis* tadpoles to associate novel chemical cues with familiar alarm cues. We used rose water (Dabur Gulabari, Dabur India Limited, at 1%, 2% and 5% concentrations) after confirming its novelty and safety for larval activities and survival. When tested against 1% rose water solution, tadpoles collected from nature did not show any change in activity, suggesting that they do not detect rose water as a potential threat. Therefore, we used rose water as a novel cue to test the associative learning of *S. temporalis*. Tadpoles (stages 30–33) collected from nature, which responded to alarm cues by reducing their post stimulus activity (experiment I), were subjected to Pavlovian conditioning separately by exposing them to either distilled water (control) or rose water (RW) alone or a mixture of rose water and conspecific alarm cues (RW+CC) in equal proportion. The control group received an equal quantity of distilled water to nullify the effect of disturbance, if any, due to cue addition. A group of 30 tadpoles for each treatment was housed in large aquaria (60 cm × 45 cm × 15 cm) with 6 L aged tap water in which 30 mL of cues were added daily. One set of tadpoles was conditioned with this combination of cues for 5 days and the second set for 10 days. Two days after the termination of conditioning, exposed tadpoles were tested for change in activity against rose water only using the behavioural assay (table 1).

2.4 *Experiment 3: Ontogenetic changes in the use of chemical cues for predator detection*

This experiment was conducted to determine (1) if behavioural responses of larval *S. temporalis* to different chemical cues change over time, (2) whether or not larval *S. temporalis* respond similarly to both conspecific and heterospecific alarm cues, (3) the developmental stage (embryonic/post hatching/post feeding) during which larval *S. temporalis* learn to detect conspecific alarm cues, and (4) which chemical cues elicit the maximum antipredator response in *S. temporalis* tadpoles.

The experiment consisted of four sets of predator exposure: cleavage to gastrulation (CG), neurulation to hatching (NH), hatching to feeding (HF), feeding onwards (FO) and two sets of controls (one set for embryonic exposure and one set for larval exposure). Nymphs of the dragonfly, *Bradinopyga geminata* (body length M±SE=25.85±0.05 mm), were reared in plastic tubs (17 cm diameter) with 4 L water until used for experimentation. Each nymph was observed to consume one *S. temporalis* (stage 27–29) tadpole/day and therefore fed with two tadpoles on alternate days. The experimental units consisted of large glass aquaria (60

Table 1. Depicts the details of experiments, different treatment groups and the chemical cues used for testing the antipredator activity of *Sylvirana temporalis* tadpoles in each experiment

Experiment	Treatments	Chemical cues used for testing
Experiment 1	Predator-naïve and predator-experienced	Conspecific alarm cues alone
Experiment 2	Distilled water, RW, RW + CC (5 or 10 days)	Rose water alone
Experiment 3	Predator-naïve and predator-experienced (CG, NH, HF, FO)	Distilled water, P, PCC, PCH, CC, CH, DCC, DCH

cm × 45 cm × 15 cm) containing 50 embryos/hatchlings/tadpoles at a density of 5/L with aged tap water. The tadpoles were fed boiled spinach *ad libitum* on alternate days after replenishment of water. Details of the experimental set up and predator exposure are depicted in table 2. Each of the six sets was replicated five times thus making thirty experimental units in all.

All the aquaria were equipped with a predator cage (10.5 cm diameter) that allowed free diffusion of chemical cues released from the predator during a predation event while preventing direct attack on the prey. Depending on the treatment, each cage was placed either empty or housed a single nymph fed with two *S. temporalis* tadpoles (stage 27–29) on alternate days. Usually, tadpoles were consumed within 15–30 min of addition. Injured or half consumed tadpoles were left in the predator cage for later consumption. Following the exposure, embryos, hatchlings and larvae were reared in a predator-free environment using large aquaria (60 cm × 45 cm × 15 cm) until testing for predator detection ability using the behavioural assay (table 1).

2.4.1 *Behavioural assay:* A specially designed glass aquarium (100 cm × 6 cm × 6 cm) served as a testing chamber. At the bottom, it was marked from the outside with lines at an interval of 5 cm. A dye test was conducted to determine the time required for a chemical to diffuse throughout the test chamber. Five mL of $KMnO_4$ solution was added to the two ends of the testing chamber using a syringe. The time required for the dye to diffuse throughout was recorded. A total of 10 trials were conducted and the mean was taken. $KMnO_4$ solution took ~2 min to spread throughout, suggesting that a minimum time required for the chemical to diffuse throughout the chamber was 2 min. The cues used for testing were as follows:

a) *Distilled water*: 5 mL.
b) *Predator cues (P,* Kairomones*)*: A total of six dragonfly nymphs (body length, M±SE=25.85±0.05 mm) were kept in plastic tubs with 400 mL distilled water and starved for 24 h. This conditioned water was used as a stimulus.
c) *Predator cues + crushed conspecific cues (PCC)*: 2.5 mL each (1:1 proportion) of predator cues and crushed conspecific cues were mixed and used. Predator cues were prepared as described earlier. Crushed conspecific cues were prepared by freezing a conspecific tadpole (stage 27–28) to deep hypothermia at 0°C to −1°C for 10–15 min and then quickly crushing with mortar pestle in 5 mL distilled water. The solution was filtered and used.
d) *Predator cues + crushed heterospecific cues (PCH):* 2.5 mL each (1:1proportion) of predatory cues and crushed heterospecific cues were mixed and used. Predator cues were prepared as described earlier. Crushed heterospecific cues were prepared by quickly crushing a frozen *Clinotarsus curtipes* tadpole (a sympatric close relative), at 0°C to −1°C for 10–15 min, with a mortar pestle in 5 mL distilled water. A heterospecific tadpole of comparable body mass was used.
e) *Crushed conspecific cues (CC,* conspecific alarm cues): Crushed conspecific cues were prepared at a concentration of one tadpole/5 mL as described earlier.
f) *Crushed heterospecific cues (CH,* heterospecific alarm cues): Crushed heterospecific cues were prepared at a concentration of one tadpole/5 mL as described earlier.
g) *Dietary cues of conspecific origin (DCC)*: Six dragonfly nymphs were each fed with two conspecific tadpoles (stages 27–28) and after 2 h, the predators were transferred to a separate plastic tub containing 400 mL distilled water for 12–15 h. This conditioned water was used as the test stimulus.
h) *Dietary cues of heterospecific origin (DCH)*: The predators were fed with *C. curtipes* tadpoles and same protocol was followed as in the case of conspecifics.

Table 2. Depicts various embryonic and post-embryonic (pre-feeding and post-feeding) stages of *Sylvirana temporalis* during which they were exposed to a mixture of predator, alarm and dietary cues and the duration of exposure in each stage

		Exposure to predator	
Sr. no.	Treatment groups	Stage of development	Duration in days
	Embryonic exposure		
1.	Control group (C_1)	–	—
2.	Cleavage to gastrulation (CG)	Up to 12	2
3.	Neurulation to hatching (NH)	13 – 20	3
	Post-embryonic exposure		
1.	Control group (C_2)	–	—
2.	Hatching to feeding (HF)	21 – 24	6
3	Feeding onwards (FO)	25 – 33	~ 90

Tadpoles between stages 30–33 were tested for their antipredator responses to different chemical cues and the trials were conducted between 8.30 a.m. to 5.30 p.m.

2.4.2 *Behavioural assay protocol:* The test chamber was cleaned before each trial and drained to remove traces of water completely, and 1 L of aged tap water was added. A tadpole was introduced at one randomly selected end of the testing chamber and allowed to acclimate for 45 min. Then, as a measure of activity, the number of lines crossed by test tadpole before and after the stimulus addition was recorded. Total testing time was 4 min of pre-stimulus activity, followed by 2.5 min of acclimation period during which the cues were added (5 mL of the solution containing the required stimulus cues was added to the test chamber using a 10 mL syringe within 30 s) and 4 min of post-stimulus activity. 2.5 mL of the stimulus cue was added to both ends of the test tank. A total of 30 trials were carried out for each cue and each treatment. Each tadpole was used separately and only once.

2.5 *Statistical analyses*

Activity data were converted to relative activity using the formula: Relative activity = Number of lines crossed in post-stimulus period − number of lines crossed in pre-stimulus period. The data was confirmed for normality using probability plots and the Anderson-Darling test before subjecting to statistical analyses. A few outliers (11 data points out of 1200, <1%) from some treatments were not included in the analyses.

Differences in the activity of test tadpoles from predator-experienced and predator-naïve groups against conspecific alarms cues alone (Experiment I) were analysed using an independent sample *t*-test. Differences in the activity of tadpoles from three treatment groups against rose water alone (Experiment II) were analysed using a two-way ANOVA with rearing stimulus (distilled water, rose water and rose water + crushed conspecifics cues) and period of conditioning (5 and 10 days) as independent variables and change in activity as a dependent variable followed by Scheffe's test for multiple pair wise comparisons. Differences in the activity levels of predator-naïve tadpoles as well as those of each stage against different cues (Experiment III) were analysed using a two-way ANOVA with stimulus cues (DW, P, CC, CH, PCC, PCH, DCC, DCH) and developmental stages (predator naïve, CG, NH, HF and FO) as independent variables, and relative activity as a dependent variable followed by Scheffe's test. All the tests were two tailed and the significance level was set at 0.05. Analyses were performed using SPSS version 19.0®.

3. Results

3.1 *Experiment 1*

A significant difference was observed in the antipredator response of larval *S. temporalis* from nature and those from laboratory rearing (t_{58}=6.499, p=0.0001). When presented with conspecific alarm cues, tadpoles collected from nature reduced activity significantly in comparison to predator-naïve tadpoles (figure 1). Further, the response of predator-naïve tadpoles to predatory odors was significantly greater than that to crushed heterospecific cues (t_{58}=2.178, p=0.033).

3.2 *Experiment 2*

Conditioning of tadpoles with different stimuli (distilled water, rose water or rose water + crushed conspecific cues) had a significant effect on the activity of tadpoles when tested against rose water alone ($F_{2,177}$=59.18, p=0.0001). Tadpoles conditioned with a combination of rose water + crushed conspecific cues reduced their activity significantly in comparison to those conditioned with either distilled water or rose water alone (p=0.0001; figure 2). Similarly, conditioning period also had a significant impact on the activity of tadpoles when tested against rose water ($F_{1,177}$=5.74, p=0.018). Tadpoles conditioned with rose water + crushed conpsecific cues for 5 and 10 days reduced their activity significantly compared to those conditioned with either

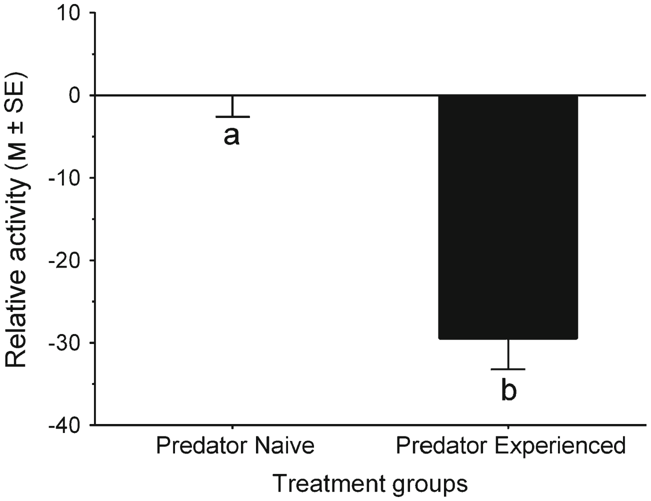

Figure 1. Relative activity (M±SE) of predator-naïve (laboratory-reared) and predator-experienced (nature-collected) tadpoles of *Sylvirana temporalis* when presented with conspecific alarm cues (CC). Dissimilar alphabets over the bars indicate significant difference.

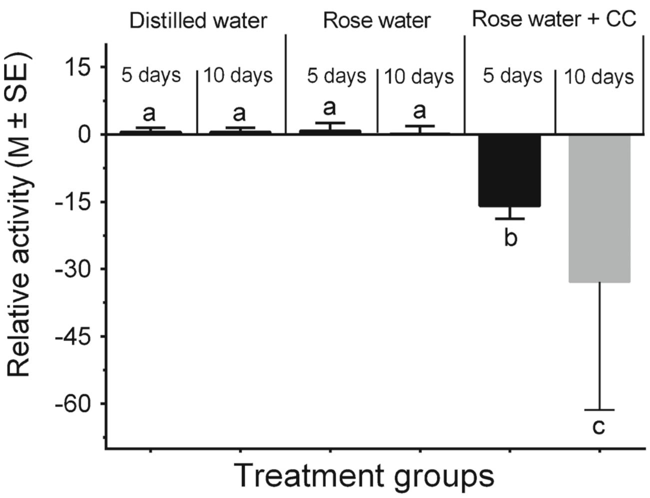

Figure 2. Relative activity (M±SE) of larval *Sylvirana temporalis* to rose water stimulus (RW) following their conditioning with distilled water (control), rose water and rose water + conspecific alarm cues for either 5 or 10 days. Dissimilar alphabets over the bars indicate significant difference.

distilled water or rose water alone (figure 2). However, reduction in the activity of tadpoles conditioned for 10 days was significantly greater than those conditioned for 5 days (*p*=0.0001; figure 2). Further, there was a significant interaction between the conditioning stimulus and the period of conditioning ($F_{2,177}$=4.92, *p*=0.008).

3.3 *Experiment 3*

Both stimulus cues and developmental stages had a significant influence on the activity of tadpoles (table 3). When tested against different chemical cues, both predator-naïve and predator-experienced tadpoles reduced their activity significantly (table 3). Similarly, tadpoles that had been exposed to a mixture of cues (predatory cues + conspecific alarm cues and dietary cues) at specific embryonic, post-hatching and post-feeding stages also exhibited significant reduction in their activity when tested against various cues (table 3). Interestingly, a significant interaction was observed between the cues and the developmental stages (table 3).

When tested against different chemical cues, predator-naïve tadpoles showed a significant reduction in their activity except for distilled water and conspecific alarm cues (table 3; figure 3A). Further, reduction in the activity of test tadpoles was highest against predatory cues (P) followed by PCH, DCH, CH, PCC and DCC (table 3; figure 3A).

Test tadpoles that had been exposed to a mixture of chemical cues from cleavage to gastrulation (CG) showed a significant reduction in their activity when tested against different cues (table 3; figure 3B). Reduction in the activity of tadpoles was highest against DCH followed by PCC, DCC, PCH and P (table 3; figure 3B). Surprisingly, pre-stimulus and post-stimulus activities of tadpoles against DW, CC and CH were comparable (table 3; figure 3B).

Test tadpoles that had been exposed to a mixture of cues from neurulation to hatching (NH) reduced their activity significantly when tested against different chemical cues (table 3; figure 3C). However, reduction in their activity was maximum to predatory cues (P) followed by PCC, DCH, DCC and PCH. As in the CG treatment, these tadpoles too did not reduce their activity to DW, CC and CH. (table 3; figure 3C).

Test tadpoles that had been exposed to a mixture of cues from hatching to feeding (HF), exhibited a significant reduction in their activity when tested against different chemical cues (table 3; figure 3D). Activity reduction was highest against PCC, followed by P, DCC, DCH, PCH and DW.

Table 3. Results of two-way ANOVA followed by Scheffe's test used to analyse the effect of different chemical cues and stage of development on the relative activity of *S. temporalis* tadpoles

Source of variation	*F*	*d. f.*	P
Chemical cues used for testing	63.487	7, 1190	0.0001
Developmental stages	27.303	4,1190	0.0001
Cues x Stages	5.811	28,1190	0.0001

Scheffe's *post hoc* test	Chemical cues used for testing							
Treatment groups	DW	P	PCC	PCH	CC	CH	DCC	DCH
Predator naïve	> 0.05	<0.0001*	0.011*	<0.0001*	1.000	**<0.0001***	0.029*	<0.0001*
Cleavage to Gastrulation (CG)	> 0.05	<0.0001*	<0.0001*	<0.0001*	1.000	1.000	<0.0001*	<0.0001*
Neurulation to Hatching (NH)	> 0.05	<0.0001*	<0.0001*	<0.0001*	1.000	0.999	<0.0001*	<0.0001*
Hatching to Feeding (HF)	> 0.05	<0.0001*	<0.0001*	0.002*	0.999	0.974	<0.0001*	<0.0001*
Feeding Onwards (FO)	> 0.05	<0.0001*	0.002*	<0.0001*	**<0.0001***	0.549	<0.0001*	<0.0001*

Pair-wise comparisons were made between a particular treatment and the control group (predator-naïve tested against distilled water).
* indicates significant difference.

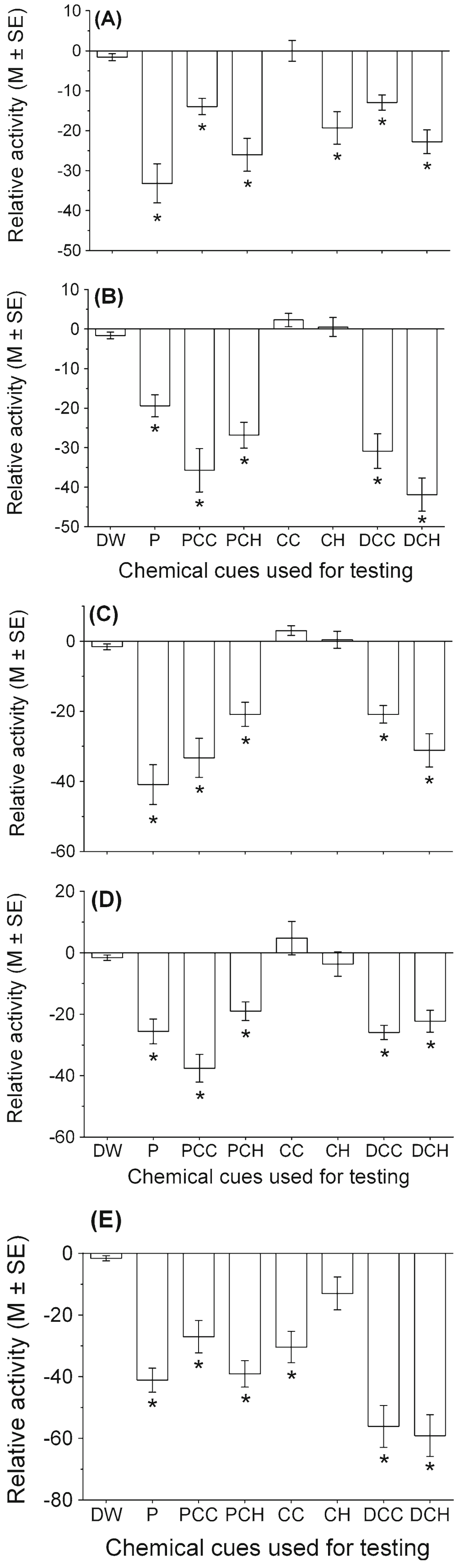

Figure 3. Relative activity (M±SE) of predator-naïve and predator-experienced *Sylvirana temporalis* tadpoles to different chemical cues. (**A**) Predator-naïve tadpoles. (**B**) Tadpoles that had been exposed between cleavage to gastrulation (CG). (**C**) Tadpoles that had been exposed between neurulation to hatching (NH). (**D**) Tadpoles that had been exposed between hatching to feeding (HF). (**E**) Tadpoles that had been exposed from feeding onwards (FO). * indicates significant difference in comparison with distilled water, conspecific cues and heterospecific cues. Note: Embryonic, pre-feeding and feeding stages were exposed to a mixture of cues (predator cues, conspecific alarm cues and dietary cues).

Similar to CG and NH groups, these tadpoles also did not reduce activity against DW, CC and CH (table 3; figure 3D).

Test tadpoles that had been exposed to a mixture of cues during post-feeding stages also reduced their activity significantly when tested against various chemical cues (table 3; figure 3E). However, reduction in their activity was highest towards DCH followed by DCC, P, PCH, CC, PCC and DW. Interestingly, this group did not reduce their activity against DW and CH (table 3; figure 3E).

Overall, predator-naïve tadpoles did not reduce activity when presented with conspecific alarm cues (figure 4A). Similarly, tadpoles that had been exposed to a mixture of cues during CG, NH and HF stages did not reduce their activity to conspecific alarm cues (figure 4A). However, with experience during ontogeny, tadpoles that had been exposed to a mixture of cues during post-feeding stages (FO) reduced their activity to conspecific alarm cues (figure 4A). On the other hand, predator-naïve tadpoles that showed significant reduction in activity against heterospecific cues gradually learned to ignore heterospecific cues with experience (figure 4B).

4. Discussion

In the evolutionary arms race of predator–prey interactions, prey species are under constant selection to evolve novel strategies by which they can successfully detect and evade their potential predators. Recent studies have clearly shown that the goal of predator detection is achieved by two mechanisms: some have the innate ability to detect certain predators while others learn to do so through experience (Ferrari *et al.* 2010).

Reduction in the activity of predator-naïve as well as predator-experienced larval *S. temporalis* to predatory cues (kairomones), predatory odors + crushed conspecific cues (alarm cues) and dietary cues of conspecific and heterospecific origin is consistent with the antipredator response of other amphibian prey (Griffiths *et al.* 1998; Mathis and Vincent 2000; Mathis *et al.* 2003; Mandrillon and Saglio 2005; Epp and Gabor 2008).

Activity reduction by predator-naïve tadpoles to kairomones on the first encounter and their significantly greater response to predatory odors than that for crushed heterospecific cues confirms the existence of an innate

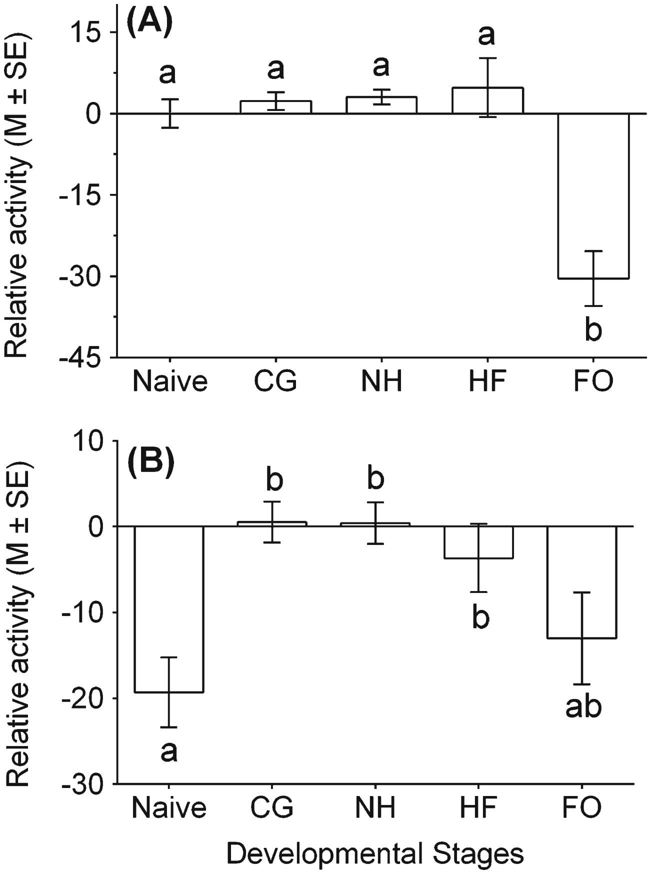

Figure 4. Relative activity (M ± SE) of predator-naïve and predator-experienced (CG, NH, HF and FO) *Sylvirana temporalis* tadpoles to conspecific alarm cues (**A**) and heterospecific cues (**B**). Dissimilar alphabets over the bars indicate significant difference.

predator detection mechanism in *S. temporalis* tadpoles. Similarly, adult salamanders (*Eurycea nana*) and larval anurans (*Rana perezi*) are known to have innate predator detection mechanisms (Gonzalo *et al.* 2007; Epp and Gabor 2008). Although previous studies have shown the existence of innate predator detection mechanisms in amphibians, distinction between detection of kairomones and alarm cues was not made (Ferrari *et al.* 2010). Some studies have considered the antipredator response of predator-naïve tadpoles to alarm cues on the first encounter as the innate predator detection mechanism. However, we consider the antipredator response of predator-naïve prey to kairomones alone on the first encounter as a true measure of an innate predator detection mechanism (Wildy and Blaustein 2001). In this context, *S. temporalis* tadpoles have innate predator detection mechanisms. Further, failure of predator-naïve *S. temporalis* tadpoles to reduce activity to conspecific alarm cues on the first encounter suggests that these tadpoles may not have an innate ability to detect conspecific alarm cues as a threat. In contrast, tadpoles of *R. perezi* and *E. nana* detect conspecific alarm cues on first encounter (Gonzalo *et al.* 2007; Epp and Gabor 2008). Apparently, different species may have innate mechanisms to detect either kairomones or conspecific alarm cues.

4.1 *Ontogenetic changes in chemosensory learning of predator recognition*

Regardless of the existence of innate predator detection mechanisms, prey tadpoles may learn to detect predators using familiar conspecific alarm cues as they are known to be involved in experience-mediated learning of predator detection (Ferrari *et al.* 2010). However, predator-naïve *S. temporalis* tadpoles failed to detect conspecific alarm cues as potential threats. Interestingly, tadpoles that had been conditioned with a mixture of cues at specific embryonic and pre-feeding stages (CG, NH and HF treatments) also failed to detect conspecific alarm cues as threats, suggesting that embryonic and pre-feeding stages of *S. temporalis* are not sensitive to such associative learning. In contrast, reduced activity of tadpoles to conspecific alarm cues that had been exposed to the same cue mixture during feeding stages (FO treatment) suggests the sensitivity of post-feeding stages to associative learning. Failure of embryonic and pre-feeding stages to detect alarm cues as threats might be due to their limited mobility. In the absence of foraging, embryos and hatchlings of *S. temporalis* have a limited opportunity to perceive and learn to detect conspecific alarm cues. However, with the onset of feeding, their increased encounter rates with predators provide them the opportunity to perceive and learn to detect conspecific alarm cues as potential threats. Activity reduction by post-feeding tadpoles indicates that stages from feeding onwards are sensitive to learning to detect alarm cues in larval *S. temporalis*. In contrast, *Ambystoma annulatum* and *Rana sylvatica* are reported to be sensitive to associative learning during egg/embryonic stages (Mathis *et al.* 2008). Possibly, species-specific differences exist in the developmental stages that are sensitive to associative learning.

The present study shows that larval *S. temporalis* have both innate and learned predator detection mechanisms. Certain features of prey species and habitats may promote the evolution of both innate and learned predator detection mechanisms (Gonzalo *et al.* 2007; Epp and Gabor 2008). *Sylvirana temporalis* tadpoles inhabit flowing water bodies, as in *E. nana* (Epp and Gabor 2008), where opportunities for learning to detect predators through conspecifics alarm cues could be limited. This would promote the evolution of innate recognition of certain predators. Further, as in *R. perezi*, larval *S. temporalis* have longer larval periods (3–4 months) and therefore encounter diverse gape dependent predators throughout metamorphosis (Gonzalo *et al.* 2007). In such situations, learning to refine their responses to seasonally varying predator communities through experience may offer a selective advantage to survive with novel predators. Therefore, *S. temporalis* might also have evolved learning-based predator detection. The existence of experience-mediated predator detection with an innate predator detection mechanism has been shown in *Notophthalmus viridescens*, *R. perezi* and *E. nana* (Woody and Mathis 1998; Gonzalo *et al*. 2007; Epp and Gabor 2008).

4.2 *Associative learning in larval S. temporalis*

The ability to learn to detect novel predators could be advantageous over an innate mechanism of predator detection (Gonzalo *et al.* 2007). Reduced activity of *S. temporalis* tadpoles to rose water cues alone following conditioning suggests their ability to associate novel cues with familiar conspecific alarm cues when such novel cues are perceived along with conspecific alarm cues. Similarly, tadpoles of *R. perezi* (Gonzalo *et al.* 2007) and adult *N. viridescens* (Woody and Mathis 1998) detect predatory cues through associative learning. Further, stronger antipredator responses of larval *S. temporalis* to such novel cues following longer conditioning periods suggests time dependency in associative learning. Associative learning can be advantageous in prey tadpoles inhabiting flowing water bodies or in those with extended larval periods such as larval *S. temporalis* since predator densities and communities vary across seasons. By associating novel chemical cues from diverse predators with conspecific alarm cues, they can deal with not only native predators but also non-native novel predators. Thus, our results suggest that associative learning could be a possible mechanism to deal with novel species.

4.3 *Learning to detect heterospecific alarm cues*

An interesting aspect of predator detection is the ability of certain prey to exhibit antipredator responses to heterospecific alarm cues. Two theories have been proposed to explain why and when prey can use heterospecific cues to respond to predation risk. The ‘ecological coexistence theory’ proposes that the response of prey to injury-released alarm cues or dietary cues from coexisting heterospecific species should be stronger than such cues from non-coexisting heterospecifics (Chivers and Mirza 2001). The ‘phylogenetic-relatedness theory’ proposes that response of prey to injury-released alarm cues or dietary cues of closely related heterospecific species should be stronger than such cues from distantly related ones (Parker and Shulman 1986; Mathis and Smith 1993; Sullivan *et al.* 2003; Schoeppner and Relyea 2005). In support of the phylogenetic relatedness theory, larval *R. catesbeiana* show antipredator response to closely related heterospecific cues of *R. septentrionalis* (Raymond and Murray 2008) and fathead minnows (*Pimephales promelas*) respond to heterospecific alarm cues of brook sticklebacks (Mirza and Chivers 2001). However, test prey animals of these studies were conditioned with dietary cues before testing for responses to heterospecific cues alone. Surprisingly, larval *S. temporalis* could not only detect and therefore respond to heterospecific cues, but they could do so in the total absence of any prior experience. Possibly, larval bronzed frogs respond to heterospecific cues of *C. curtipes* because of phylogenetic relatedness. However, this needs to be confirmed with more elaborate experiments.

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