

# Experience window influences development and retention of memory to recognize predators in the larval skipper frogs

Swapnil C. Supekar<sup>1,2</sup> and Narahari P. Gramapurohit<sup>1,\*</sup>

<sup>1</sup>Department of Zoology, Savitribai Phule Pune University, Ganeshkhind, Pune 411 007, India

<sup>2</sup>Modern Education Society's Nowrosjee Wadia College, Pune 411 001, India

**Learning and memory are critical for predator recognition as they allow prey species to develop an adaptive response to a novel situation, thus increasing their chances of survival. In prey species that lack innate predator recognition, alarm cues play a crucial role in learning by their association with novel predator odour. Perception of novel predator odours along with alarm cues allows the prey to learn to detect predatory odours alone in their future encounters. A single event of exposure is considered to be sufficient for the prey to associate predatory cues with alarm cues. However, the minimum time required for learning and memorizing information about predator identity is unknown. Hence we used the tadpoles of *Euphlyctis cyanophlyctis* to determine the association between the experience window and memory development and its retention. We conditioned tadpoles with a mixture of dragonfly nymph odours and alarm cues for 1, 3, 6, 12 and 24 h, and subsequently assessed their antipredator behaviour at different intervals. Our results show that the minimum duration required for associative learning is ~6 h. Interestingly, the intensity of antipredator response was proportional to the duration of conditioning. Moreover, retention of memory increased with an increase in the duration of conditioning. Hence, we show the significance of conditioning duration in learning. We also show an association between the duration of conditioning and retention of memory. In aquatic ecosystems, where the prey encounters a wide array of predatory cues, our findings open a new avenue for understanding the complexities associated with learning and the development of memory.**

**Keywords:** Associative learning, experience window, *Euphlyctis cyanophlyctis*, memory retention, predator and prey.

THE behaviour of an animal is shaped by extrinsic and intrinsic factors that together affect its survival, spatio-temporal distribution and abundance<sup>1,2</sup>. Displaying antipredator responses is a crucial behaviour that directly affects the survival of prey species. Antipredator (defensive) be-

haviour such as dashing, shelter use, freezing and cryptic postures minimize visual detection by predators and hence reduce the chances of prey capture while increasing the chances of prey survival<sup>3,4</sup>. Encounter with predators can be minimized by altering the habitat use and/or time of foraging, type of food and mate choice<sup>4,5</sup>. However, antipredator behaviours are expensive to develop and maintain as they are developed at the cost of certain self-maintenance behaviours, such as foraging, searching for mates and territoriality<sup>6</sup>. In nature, predation acts as a strong selective force, affecting behaviour, morphology, physiology and life-history traits of prey animals. Hence, prey animals inhabiting diverse ecosystems are expected to evolve novel mechanisms of predator detection and deterrence, which can increase their chances of survival and reproduction<sup>7,8</sup>. Prey species as diverse as lower invertebrates to higher vertebrates have evolved various sensory mechanisms for predator recognition using visual, chemical, auditory and vibrational cues released either from predators, conspecific or heterospecific prey animals<sup>9</sup>. These cues serve as a source of information for predator recognition in arboreal, terrestrial and aquatic ecosystems<sup>10</sup>. Prey animals inhabiting aquatic ecosystems often use chemical cues of different origins (kairomones, alarm cues and dietary cues) to detect potential predators, as chemical cues can diffuse rapidly and are highly reliable even at night or in complex environments<sup>2</sup>. Moreover, they play an important role in the recognition of cryptic or sit-and-wait predators<sup>11</sup>.

Developing species-specific responses appropriate for each predator requires precise recognition of a predator, which is achieved by innate and/or acquired recognition mechanisms<sup>8,12-17</sup>. The innate recognition mechanism does not require any prior experience with predators and offers a selective advantage in avoiding a limited spectrum of predators, particularly during early development. On the other hand, acquired (learned) recognition requires prior experience with predators that allows the prey to recognize their predators through associative learning<sup>13,18</sup>. Recent studies have shown that learning plays a critical role in predator recognition in complex habitats as it helps in updating predation-related information during ontogeny<sup>19</sup>. Learning to adjust behaviour

\*For correspondence. (e-mail: naraharipg@unipune.ac.in)

adaptively based on experience affects virtually every aspect of animal behaviour<sup>20</sup>. Chemicals released by injured conspecific prey (alarm cues) can mediate such associative learning in both invertebrate and vertebrate prey, when a naive prey perceives alarm cues along with kairomones of a novel predator simultaneously, such as its image, odour or sound (when alarm cues are paired with predatory cues like its image, odour or sound)<sup>2</sup>. Associative learning can be accomplished even during embryonic development, thus helping the prey to minimize predation risk at a very early stage of development<sup>17,18,21–23</sup>. This mode of learning is highly sophisticated, efficient and can be achieved by a single conditioning event<sup>4,18</sup>. Moreover, it allows the prey to learn to identify the predator, spatio-temporal pattern of predation risk and the level of predation risk<sup>24–27</sup>. Associative learning has been studied in a wide variety of aquatic prey such as flatworms, molluscs, insects, crustaceans, fishes and amphibians<sup>2</sup>, signifying its importance in increasing the survival chances of prey inhabiting diverse habitats.

Another important component of learning is memory. Although learning and memory are linked, they are distinct processes<sup>28</sup>. Learning is simply the acquisition of memory, whereas memory involves retention of learned information and interference in case of distraction during or immediately after learning<sup>28</sup>. Being able to learn and recollect information allows the prey to make adaptive decisions of fleeing or fighting. If the learnt information cannot be memorized, learning becomes non-adaptive. Numerous studies have shown that prey animals can retain the memory of potential predators and subsequently respond to their signals over several days to months<sup>23,29–31</sup>. However, with the passage of time information about predators gathered through learning becomes obsolete in the absence of reinforcement and hence the prey may ignore such information<sup>23,31,32</sup>. Although memories shape how an individual interacts with its environment, the retention of memory about the identity of predators is not fixed but plastic<sup>33</sup>. Ferrari *et al.*<sup>34</sup> have provided a comprehensive framework highlighting intrinsic and extrinsic factors that could affect the memory window of information used by individual prey.

Previous studies have shown that a single conditioning event is sufficient for prey animals to learn to recognize the identity of a predator<sup>2,4,18</sup>. However, the minimum time (an exposure window) required in that single event to learn and memorize information about predator identity is not known. To the best of our knowledge, no study has specifically assessed the minimum time (an experience window) required by a prey to learn to recognize its predators through classical conditioning. This component could be crucial in the development and retention of memory of predator identity. Hence, we used tadpoles of the Indian skipper frog, *Euphlyctis cyanophlyctis* to test the effect of duration of conditioning on associative learning, acquisition and retention of memory.

*Euphlyctis cyanophlyctis* is distributed widely in the Indian subcontinent and inhabits a variety of aquatic ecosystems that include ephemeral and perpetual water bodies, which are either lentic and/or lotic<sup>35</sup>. In such diverse ecosystems, tadpoles of the skipper frog have to coexist with vertebrate and invertebrate predators that vary in their spatio-temporal distribution, abundance and activity pattern. Our previous observations have shown that predator-naïve tadpoles of *E. cyanophlyctis* fail to recognize kairomones of the dragonfly nymphs as a threat, but have the innate ability to recognize conspecific alarm cues. However, they can learn to recognize kairomones of the dragonfly nymphs through associative learning<sup>18</sup>. Moreover, associative learning plays a crucial role in providing them with information on the temporal pattern of predation risk and the intensity of predation risk<sup>26,27</sup>. Hence, we wanted to determine whether the conditioning window has any effect on the learning and memory of larval *E. cyanophlyctis* to kairomones of dragonfly nymphs. We designed an experiment in which tadpoles of *E. cyanophlyctis* were exposed to conspecific alarm cues paired with the kairomones of dragonfly nymphs (henceforth conditioning) for different periods (duration) and assessed their behavioural responses after 24 h and subsequently at a five-day interval.

## Materials and method

### Subjects

During the breeding season of 2016, four pairs of *E. cyanophlyctis* in amplexus were collected from a water body in the Savitribai Phule Pune University campus (18°55'N, 73°82'E), Pune, Maharashtra, India around 23.00 h, quickly transported to the laboratory, where each pair was housed separately in a glass aquarium (60 cm × 40 cm × 45 cm) and left undisturbed for spawning. All the pairs had spawned successfully. The embryos were collected the following morning and placed in another aquarium (45 cm × 30 cm × 10 cm) with aged tap water until hatching, while returning the adults to their habitat. Hatchlings from all the spawns were mixed thoroughly and maintained in aquaria (60 cm × 45 cm × 15 cm) until used for experimentation. The stages of development were identified according to the staging table of Gosner<sup>36</sup>. Water was renewed completely every third day and the tadpoles were fed partially boiled spinach ad libitum. Fourth-instar nymphs of dragonfly (*Bradinopyga geminata*) with a mean body length of 23.95 ± 0.13 mm collected from the same pond were used as predators. Dragonflies are cosmopolitan in distribution and their nymphs feed voraciously on different stages of anuran larvae across the world. They were housed in perforated plastic containers (10.5 cm diameter) and fed small-sized (stage 26–27) tadpoles of *E. cyanophlyctis* ad libitum until used for experimentation.

### Experimental design

The tadpoles (stage 27–28,  $N = 40/\text{group} = 20/\text{tank}$ ) of *E. cyanophlyctis*, which were naïve to the predator, were transferred to a glass aquarium (45 cm × 30 cm × 10 cm) with 6 l aged tap water. A perforated plastic cup (10.5 cm diameter) housing a starved nymph along with two conspecific tadpoles (stage 27–28) was placed at the centre of the aquarium. The starved nymph attacked and injured the tadpoles several times before consuming them. The prey tadpoles were allowed to perceive chemical cues released from the nymph feeding on conspecifics. Kairomones (at a concentration of 5 ml/l) were also added to enhance their simultaneous perception along with conspecific alarm cues. The experimental design was as follows:

Control: Conditioning of tadpoles in aged tap water for 24 h.

Group I: Conditioning of tadpoles with a predator feeding on conspecifics for 1 h.

Group II: Conditioning of tadpoles with a predator feeding on conspecifics for 3 h.

Group III: Conditioning of tadpoles with a predator feeding on conspecifics for 6 h.

Group IV: Conditioning of tadpoles with a predator feeding on conspecifics for 12 h.

Group V: Conditioning of tadpoles with a predator feeding on conspecifics for 24 h.

During the predation event, nymphs consumed the tadpoles in ~15 min following several attacks causing injuries. The nymphs were fed only once during a trial, irrespective of the duration of conditioning. Subsequently, the tadpoles were maintained in cue-free water for 24 h, following which they were tested for antipredator behaviour towards kairomones on days 1, 5, 10, 15 and 20 using an established behavioural assay<sup>8,18</sup>. Kairomones were prepared according to the protocol described earlier. Briefly, six dragonfly nymphs starved for 72 h were housed in a beaker with 400 ml aged tap water without food for 24 h. This conditioned water was filtered and used as a stimulus cue. Thirty tadpoles/group chosen randomly (15 tadpoles/tank) between stages 27 and 28 were used for testing the antipredator response towards kairomones. Each tadpole was tested separately and only once.

### Behavioural assay

A specially designed glass aquarium (50 cm × 6 cm × 6 cm), which served as a testing chamber, was marked with lines at an interval of 5 cm at its bottom from the outside. The test chamber was cleaned thoroughly before each trial and filled with 1 l aged tap water to allow free movement of the tadpoles. A tadpole was introduced randomly at one end of the chamber and allowed to acclimatize for 10 min. Subsequently, its activity was assessed

by counting the number of lines crossed prior to and following the addition of the stimulus cue. The test tadpole was considered to have crossed the line when the whole of its body was on the other side of a line. An equal volume of the stimulus cue (2.5 ml) was added at either end of the testing chamber. The duration of each trial was 10.5 min (4 min pre-stimulus activity recording and 4 min post-stimulus activity recording. Addition of the cues and their diffusion throughout the chamber took 2.5 min).

### Statistical analysis

The data on activity patterns were converted to an index (proportional change in activity) using the formula: (Lines crossed in the post-stimulus period – Lines crossed in the pre-stimulus period)/(Lines crossed in the pre-stimulus period). The data were confirmed for normality using probability plots and the Anderson–Darling test before subjecting them to statistical analyses. Differences in the proportional change in activity on day-one post-conditioning were analysed using a mixed-model analysis of variance (ANOVA) followed by Tukey's post-hoc test, with the duration of conditioning as a fixed factor and rearing tanks as the random factor. Differences in the antipredator responses were analysed using a two-way ANOVA (conditioning period and testing day as independent variables, and antipredator response as a dependent variable), followed by univariate tests and Tukey's post-hoc test. All the tests were two-tailed and the significance level was set at 0.05. All the statistical analyses were performed using SPSS ver. 19.

### Results

Tadpoles of *E. cyanophlyctis* can recognize conspecific alarm cues as a threat without any prior experience (an innate mechanism) and hence reduce their activity. However, they fail to recognize the nymphs of dragonflies as a threat and hence do not alter their activity in response to kairomones. Interestingly, they can learn to recognize the kairomones of nymphs as a threat by associating them with alarm cues. Therefore, a reduction in their activity towards kairomones during the behavioural assay reinforces our earlier finding and confirms associative learning. Two-way ANOVA showed a significant difference in the overall behavioural response of tadpoles (Table 1). Further, both the conditioning period and testing day had a significant impact on the behavioural response of tadpoles. Moreover, there was a significant interaction between the conditioning period and testing day (Table 1). With an increase in the duration of conditioning, the intensity of antipredator response increased.

On day-one post-conditioning, reduction in the activity of tadpoles conditioned for 6, 12 and 24 h was significantly higher than that of the control group and that of the

tadpoles conditioned for 1 h ( $P \leq 0.010$ ; Figure 1). Furthermore, the activity of tadpoles conditioned for 3 h was intermediate and comparable with the control group and that of the tadpoles conditioned for 1 and 6 h ( $P \geq 0.208$ ; Figure 1). Activity reduction of tadpoles conditioned for 24 h was significantly higher than that of the tadpoles conditioned for 6 and 12 h ( $P \leq 0.014$ ; Figure 1). The intensity of activity reduction depended on the duration of conditioning; reduction was highest in tadpoles conditioned for 24 h followed by 12 and 6 h. The behavioural responses of tadpoles conditioned for 1 and 3 h were comparable with that of the control ( $P \geq 0.831$ ; Figure 1).

On day-five post-conditioning, activity reduction of tadpoles conditioned for 6, 12 and 24 h was significantly higher than that of the control group, but weaker than that on day-one post-conditioning (Table 2 and Figure 2). Subsequently, on day-ten post-conditioning, activity reduction of tadpoles conditioned for 12 and 24 h was significantly higher than that of the control group, but weaker than that on day-five post-conditioning. Similarly, on day-15 post-conditioning, activity reduction of tadpoles conditioned for 24 h was significantly higher than that of the control tadpoles, but weaker than that on day-ten post-conditioning. On day-20 post-conditioning, the activity of tadpoles from all groups was comparable (Table 2 and Figure 2).

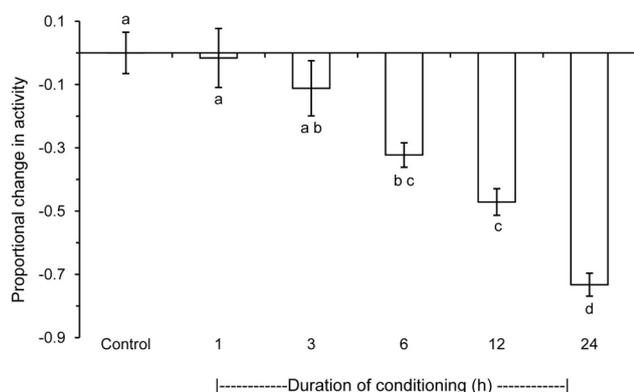
### Discussion

In this study, we have demonstrated that learning to recognize predators and memorize the learnt information in larval skipper frogs is determined by the duration of conditioning. In prey-predator interactions, learning and memorizing are crucial for the prey allowing them to develop an adaptive response to a novel situation, which increases their chance of survival<sup>37,38</sup>. Empirical evidence suggests that in aquatic ecosystems, associative learning allows the prey to update information about the identity of a predator, its size, density, proximity, intensity and temporal pattern of predation risk<sup>2,13,18,26,27,39,40</sup>. Predator recognition is achieved by two mechanisms: innate in which the prey can recognize their potential predators without any experience, and acquired in which the prey require prior experience to recognize their predators. In species with acquired predator recognition, associative learning plays a critical role in associating a novel predator cue (conditional stimulus) with the familiar conspecific alarm cue (unconditional stimulus), when perceived simultaneously. Previous studies have suggested that a single predation event or conditioning is sufficient to learn about a predator or its identity<sup>2,4,18</sup>. However, the minimum time required for a prey to learn to recognize its predators is not known. Results of the present study clearly show that larval skipper frogs learn to recognize dragonfly nymphs as a threat through associative learning. Further, we show

**Table 1.** Results of two-way ANOVA showing the effect of conditioning duration on antipredator response of larval *Euphlyctis cyanophlyctis*

Source of variation	F	Df	P
Duration of conditioning	152.280	4,650	0.000*
Testing day	35.727	4,650	0.002*
Duration of conditioning * testing day	4.968	16,650	0.001*
Tank ID	0.928	1,650	0.514

\*Indicates significant difference.

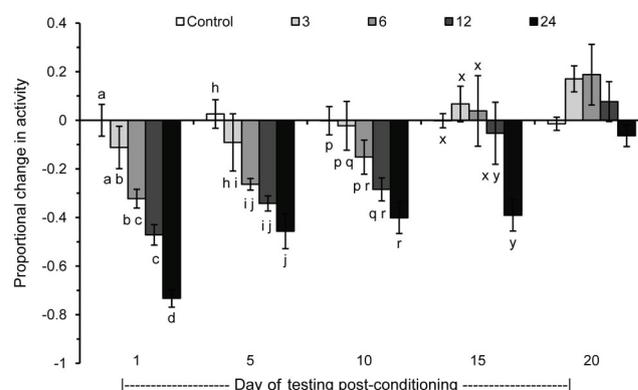


**Figure 1.** Effect of conditioning duration on associative learning and proportional change in activity (mean  $\pm$  SE) of larval *Euphlyctis cyanophlyctis* to kairomones on day 1 post-conditioning. Dissimilar alphabets over the bars indicate significant difference at 0.05 level.

**Table 2.** Effect of conditioning duration on antipredator response of larval *E. cyanophlyctis* among the groups on each testing day

Parameters	F	Df	P
Day 1	46.175	4,130	0.001*
Day 5	16.989	4,130	0.009*
Day 10	7.335	4,130	0.040*
Day 15	140.09	4,130	0.000*
Day 20	4.894	4,130	0.077

\*Indicates significant difference.



**Figure 2.** Effect of conditioning duration on proportional change in activity (mean  $\pm$  SE) of larval *E. cyanophlyctis* to kairomones among the groups on each testing day. Dissimilar alphabets over the bars indicate significant difference at 0.05 level.

that conditioning duration plays a critical role in the development of memory and its retention. For instance, tadpoles conditioned for a short time (1 or 3 h) did not reduce their activity when tested 24 h post-conditioning, indicating that short-term conditioning is not adequate for associative learning. However, an increase in the duration of conditioning for 6 h was effective in reducing the activity of tadpoles, indicating that larval *E. cyanophlyctis* require between 3 and 6 h of conditioning to learn to recognize dragonfly nymphs as their predator. Further, the results of this study indicate that conditioning duration plays an important role in the retention of memory. For instance, when tested 24 h after conditioning, the intensity of antipredator response increased with an increase in the duration of conditioning. With a shorter duration of conditioning, tadpoles had access to only alarm and predatory cues, whereas those conditioned for a longer time (6, 12 and 24 h) had access to dietary cues, in addition to alarm and predatory cues. Dietary cues along with alarm cues could play a key role in reinforcing the predation risk and hence, development and consolidation of memory, thus enhancing the antipredator response of *E. cyanophlyctis* tadpoles. It will be interesting to determine the role of dietary cues in the development of memory and its consolidation in future studies. Threat-sensitive predator avoidance hypothesis assumes that the intensity of antipredator responses should depend on the level of threat posed by the predator<sup>41</sup>. In the present study, conditioning for 24 h enabled *E. cyanophlyctis* tadpoles to perceive predation risk as a high threat causing increased intensity of response when tested 24 h after conditioning. Exposure to predatory odour along with alarm cues (and possibly dietary cues) for a longer period enables larval *E. cyanophlyctis* to perceive it as a high threat situation. Previously, we have shown that tadpoles of the skipper frog learn to recognize dragonfly nymphs as a predator in a single conditioning trial of 12 h (ref. 18). Similarly, wood frog tadpoles learn to recognize predatory tiger salamander, *Ambystoma tigrinum* after a single conditioning event of 1 h (ref. 42). However, species-specific variation exists in the intensity of antipredator responses of prey animals, and is affected by the number of conditioning events (single versus multiple). Species-specific variation can also exist in their capacities to learn and memorize the learnt information to recognize their potential predators. Regardless of the number of conditioning events, the concentration of alarm cues used for conditioning may affect the learning efficiency of prey species. In addition, the presence of dietary cues may enhance the effects of other cues in learning and the consolidation of memory. Empiricists suggest that fish and amphibian tadpoles conditioned with higher concentration of alarm cues perceive the novel predator as a high threat, whereas those conditioned with lower concentration perceive the novel predator as a low threat<sup>2</sup>. Though the concentration of alarm cues used in conditioning trials triggers shifts in

the intensity of antipredator responses<sup>33</sup>, duration of conditioning also plays a crucial role in antipredator responses of the prey. This is evident in the present study; tadpoles exposed to a similar concentration of alarm cues during the conditioning period exhibit increased intensity of antipredator response with an increase in the duration of conditioning, when tested 24 h post-conditioning. Further, the release of dietary cues along with alarm cues may work as additional cues in reinforcing the predation risk, thus aiding in enhancing the antipredator response. Similarly, multiple conditioning events may help prey animals to adjust the intensity of antipredator response to a predator<sup>33</sup>. For instance, fathead minnows (*Pimephales promelas*) subjected to multiple conditioning events use more recent information in their decision making rather than averaging all pieces of information<sup>43</sup>. Similarly, Arctic charr (*Salvelinus alpinus*) subjected to multiple conditioning event had higher survival than those subjected to a single conditioning event<sup>44</sup>. Surprisingly, neither the number of conditioning events nor the size of prey tadpoles affected the intensity of antipredator response of wood frog tadpoles when tested 24 h post-conditioning<sup>42,45</sup>.

The results of the present study show that retention of memory to recognize a predator in larval skipper frogs is affected by conditioning duration. For instance, tadpoles conditioned for 6, 12 or 24 h retained learnt information for 5, 10 or 15 days respectively. Further, the intensity of antipredator response declined with increasing post-conditioning duration. Furthermore, tadpoles conditioned for 6, 12 or 24 h failed to exhibit antipredator response to predator odour on days 10, 15 and 20 post-conditioning respectively. Similarly, tadpoles of *Pelophylax perezi* could memorize learnt information about predator identity for up to nine days<sup>30</sup>. In contrast, *Oncorhynchus mykiss* reared under controlled conditions could memorize information about predator identity for up to 21 days<sup>46</sup>. Different species of crayfish differed in the retention of learned information of predators from one day to four weeks<sup>47</sup>. Similarly, the ability of fish to retain the memory of a predator ranged from a few days to several months<sup>29,48</sup>. Recently, Ferrari *et al.*<sup>23</sup> have shown that predation risk information learned as embryos in wood frogs is memorized for more than five weeks. In contrast, wood frog tadpoles failed to exhibit antipredator behavioural response to the learned predation risk after 10 days<sup>23</sup>. Similarly, in *E. cyanophlyctis* predation risk learnt as embryos could be memorized for up to 41 days (ref. 18). In the present study, learning in *E. cyanophlyctis* was accomplished as tadpoles and hence, the learnt information might have been memorized for up to 15 days in tadpoles that were conditioned for maximum time (24 h). Collectively, these studies indicate that the age of learning could be important in memorizing the learnt information.

Both intrinsic and extrinsic factors are known to affect learning and memory<sup>34</sup>. Intrinsic factors such as body size, growth rate and antipredator response of the prey,

and extrinsic factors such as predator community, diversity and encounter rates are known to influence the memory retrieval window of prey animals<sup>34,42</sup>. For instance, the memory retrieval window of larval *Lithobates sylvaticus* was determined by tadpole size and/or growth rate at the time of learning, and their growth rate post-learning<sup>42</sup>. Similarly, the number of conditioning events also affected the length of the memory window<sup>45</sup>. For instance, *L. sylvaticus* tadpoles conditioned four times memorized the predator identity longer than those conditioned only once<sup>45</sup>. However, the results of the present study indicate that apart from the number of conditioning events, the duration of conditioning might influence the memory retrieval window in larval *E. cyanophlyctis*.

The findings of this study suggest that the waning of antipredator response in larval *E. cyanophlyctis* depends on the duration of conditioning. Tadpoles conditioned for more time responded to the cues longer and with the lapse of time following exposure, the antipredator response of tadpoles gradually waned or they might have ignored the risk as they have outgrown the gape size of their predators<sup>2,34,49</sup>. With the lapse of time following exposure, learnt information about predators becomes old without reinforcement and hence obsolete<sup>23</sup>. Since the development and maintenance of antipredator responses are costly in terms of time and energy spent, it is adaptive to forget the identity of a predator if the prey does not receive warning signals of the predator such as alarm cues and dietary cues. When prey animals are exposed to diverse predators with spatio-temporal variability in predation risk, they have to adopt novel strategies to recognize predation risk, because responding to a non-risky threat is costly resulting in less foraging and losing mating opportunities. Similarly, ignoring a risky threat can be life-threatening. Hence, learning and memorizing information about potential predators may help the prey to survive under predation risk when they are exposed to diverse predators<sup>30</sup>. Studies have shown that even after apparent forgetting, a persisted residual memory can be revealed in a subsequent learning event and prey animals can show antipredator behaviour against predation risk<sup>50–54</sup>. Larval skipper frogs inhabiting almost all kinds of aquatic ecosystems are exposed to a diverse array of invertebrate and vertebrate predators, and hence their ability to learn and memorize cues of potential predators may be especially important for their survivorship<sup>55</sup>. Previous studies have emphasized the significance of conditioning events and the concentration of alarm cues. However, here we demonstrate a novel association between duration of conditioning and development of memory and its retention. In aquatic ecosystems, where prey animals are exposed to a wide array of cues from their predators, our findings open a new avenue in understanding complex prey–predator interaction.

**Conflict of interest:** The authors declare no conflict of interest.

**Ethical approval:** This study was carried out following the guidelines of the Departmental Committee for animal ethics (No. 538/CPCSEA).

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