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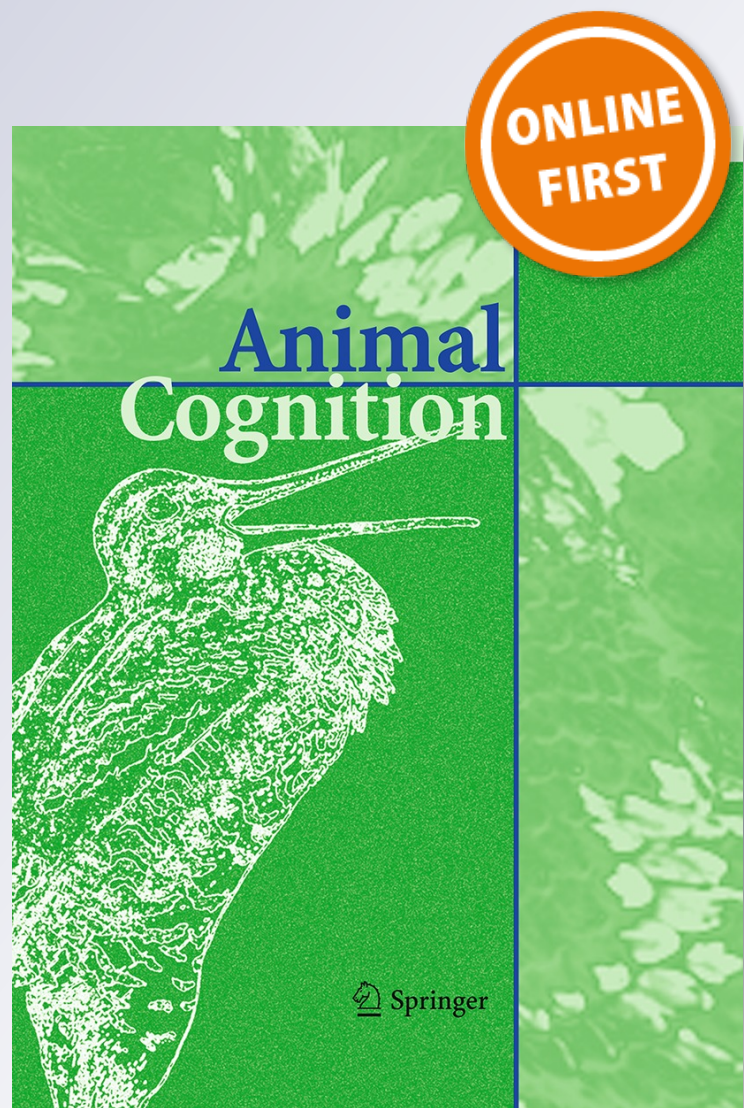
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Anuran tadpoles learn to recognize injury cues from members of the same prey guild

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Abstract Recognition of predation risk from cues released from injured heterospecific could be beneficial when prey belongs to the same prey guild. Here, we performed three experiments. Experiment 1 showed that *P. thaul* tadpoles reduced their activity levels when exposed to conspecific injury cues, but not when exposed to amphipod injury cues. Experiment 2 tested whether *P. thaul* tadpoles can learn to recognize predation risk from chemical cues released from injured heterospecifics from the same prey guild (amphipod, *Hyaletta patagonica*). A group of tadpoles were conditioned by exposing them to a specific concentration of amphipod injury cues paired with conspecific injury cues. Two days later, we evaluated changes in the activity of tadpoles when they were exposed to amphipod cues. As a control of learning, we used an unpaired group. Additionally, we used more control groups to fully investigate the learning mechanism. Our results showed that tadpoles can learn to recognize predation risk from injured amphipods and that the mechanism underlying the observed learned response could be associative. Experiment 3 replicated Experiment 2 and also showed that a low concentration of amphipod cues did not sustain that learning.

Keywords Associative learning · Anuran tadpoles · Amphipods · Predation risk

Introduction

Early detection of predation risk has a key role in the ability of prey to avoid predation. Prey uses a variety of cues and sensory modalities to warn them of predation risk (Ryan et al. 2012; Hermann and Thaler 2014; Jayne et al. 2015). Some animals can recognize risk cues innately, but others need prior experience to recognize some cues as risky or to modify their innate antipredator response (Turner et al. 2006; Gregory 2013; Nelson et al. 2013; Martin et al. 2015). Diverse tactics, mostly behavioral, can be used to avoid risk (Lima and Dill 1990; Ferrari et al. 2010a). In aquatic systems, chemical cues are an essential source of predation risk information used by all taxa (Chivers and Smith 1998). Most aquatic animals appear to have an innate ability to recognize risk from conspecific injury cues (e.g., amphipods: Wisenden et al. 1999; flatworms: Wisenden and Millard 2001; fish: Pollock et al. 2003; and anuran tadpoles: Mirza et al. 2006). While some aquatic species are able to recognize the odor of potential predators without any prior experience, others must learn to recognize predator scents through exposure paired with known predation cues (Ferrari et al. 2010a). Furthermore, several species of animals exhibit antipredator responses to cues from injured heterospecifics, either on their first detection, or as a result of learning (Wisenden et al. 1999; Mirza and Chivers 2001; Crane et al. 2013; Batabyal et al. 2014). Being able to recognize predation risk from injured heterospecific species can be beneficial for prey when heterospecifics are sympatric species and members of the same prey guild because it indicates risk for all members (Avargues-Weber et al. 2013).

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The ability to learn in the context of predation allows prey to respond to novel threats by adjusting their behavior based on specific information acquired from their current environment (Dukas 2013). The learned recognition of native or alien predator odors has been documented in numerous predator–prey systems (Wisenden and Millard 2001; Gonzalo et al. 2010; Nelson et al. 2013; Polo-Cavia and Gomez-Mestre 2014). Typically, experiments to evaluate learned predator recognition teach animals to recognize the scent of previously unknown predators by pairing injury-released chemical cues from conspecifics (innately recognized as risky) with the novel predator cue during a conditioning phase. In some cases, learning is also achieved using innately recognized injury-released chemical cues from heterospecifics instead of from conspecifics (Wisenden et al. 1997).

In addition to the ability to learn the scent of novel predators, some studies have shown the ability of animals to learn to recognize risk from previously unknown chemical cues from injured heterospecifics. For example, some studies have shown evidences that cross-species responses to chemical alarm cues of fish can be learned (Chivers et al. 1995, 2002; Mirza and Chivers 2001, 2003; Pollock et al. 2003; Pollock and Chivers 2004). In these studies, learning was generated by the pre-exposure of fathead minnows to known predators feeding on unknown heterospecific fish, or to unknown predators feeding on unknown heterospecific fish and conspecifics. None of these studies were able to show whether the learned response is due to effects of association between stimuli, or to a mere pre-exposure effect such as sensitization or neophobia (Domjan 2003; Mirza and Chivers 2003).

In anurans, a wide range of evidence has shown the ability of tadpole prey to recognize native or alien predator odors through learning, and has revealed the sophistication of the learned responses (Ferrari et al. 2009; Gonzalo et al. 2010; Polo-Cavia and Gomez-Mestre 2014). For example, in *Rana sylvatica* tadpoles, the generalization of predator recognition is dependent on the concentration of conspecific injury cues (risk level) paired with predator odor during a conditioning phase; this evidences the threat-sensitive learning of predator odor recognition (Ferrari et al. 2009). To our knowledge, no studies have assessed the ability of anuran tadpoles to learn to recognize cues from injured heterospecifics.

Pleurodema thaul (Anura: Leptodactylidae) is one of the most common anuran species in northwestern Patagonia of Argentina. This species breeds primarily in temporary ponds devoid of fish. In these ponds, top predation is exerted by insects, such as odonates, aquatic beetles and water bugs (Jara and Perotti 2010; Jara et al. 2013). These predatory insects feed on the same food resources: zooplankton, herbivorous and omnivorous macroinvertebrates

and anuran tadpoles (Jara et al. 2013; Jara 2014). Predator-naïve *P. thaul* tadpoles appear to exhibit short-term inducible defenses when they undergo predation risk chemical cues. For example, we have previously observed that *P. thaul* tadpoles decrease their activity (any movement) when exposed to cues from injured conspecifics (Pueta et al. 2016).

In the present study, we performed a series of three experiments to test whether *P. thaul* tadpoles recognize injury cues from members of the same prey guild (amphipods). In Experiment 1, we tested the innate response of *P. thaul* tadpoles to cues from injured conspecifics and injured heterospecifics (*Hyaella patagonica*, Amphipoda: Hyalellydidae). This first experiment was intended to help us choose the unconditional stimuli (US) and conditional stimuli (CS) used to assess learning in the next experiment. In Experiment 2, we evaluated whether *P. thaul* tadpoles can learn to recognize predation risk from chemical cues from injured *H. patagonica*. Considering that tadpoles of this species show no aggregation behavior, their ability to learn from heterospecific cues may have a major role. The opportunity to learn in nature results because in Patagonian temporary ponds *P. thaul* is usually associated with amphipods in the benthic zone, and experimental studies have shown that their top predators may prey on both tadpoles and amphipods (Jara 2014). We performed this second experiment using an associative learning protocol. Finally, Experiment 3a was aimed to replicate the results of Experiment 2, whereas Experiment 3b was performed to evaluate whether the concentration of cues from injured amphipods can modulate the learning acquisition. This last experiment was performed because it is known that the salience of the CS is an important determinant in the generation of associative learning (Domjan 2003) and because some studies have demonstrated the modulation of learned responses in anuran tadpoles by the risk level imposed by conspecific cues (Ferrari et al. 2009, 2010b; Gonzalo et al. 2010), but have not assessed the consequences of manipulating the concentration of the novel stimulus during conditioning.

Experiment 1

Materials and methods

Animals and preparation of alarm cues

This section applies to all three experiments. For each experiment, we collected three or four clutches of *P. thaul* (stages 11–13; Gosner 1960) from Laguna Fantasma wetland (41°05'33"S, 71°27'00"W, 794 m above sea level), a temporary pond situated 14 km SW from downtown San

Carlos de Bariloche, Rio Negro Province, Argentina, and reared them until we started the experiments. Two extra clutches of *P. thaul* were collected and maintained outdoors to use them to prepare the alarm cue. We also collected adults of *H. patagonica* (Amphipoda) from the same wetland to prepare chemical cues from injured heterospecifics.

Injury cues were prepared by crushing animals (Wisenden et al. 1999; Ferrari et al. 2006). To minimize suffering, tadpoles were first anesthetized by exposure to low temperature ($-8\text{ }^{\circ}\text{C}$) and immediately killed by decapitation with a single and rapid head cut. Finally, the resulting tadpole tissue mass (1.75 g in total) was crushed using mortar and pestle and then suspended in 25 ml distilled water (conspecific injury cue concentration: 0.07 g/ml). The solution was filtered through filter floss (45 μm nitex) to remove particles. Injury cues from amphipods were prepared similarly to tadpole cues but by crushing *H. patagonica* (6.5 g) and then suspending in 50 ml distilled water (amphipod injury cue concentration: 0.13 g/ml). In all cases, the cue solution was aliquoted into 1-ml doses and frozen at $-20\text{ }^{\circ}\text{C}$ until they were used, no more than 2 days later.

Procedure

To evaluate the effect of cues from injured conspecifics and injured amphipods on the behavioral response of *P. thaul* tadpoles, we performed a one-factor experiment design with cue tested as treatment. Two days before the test, 16 tadpoles (Gosner stages 32–38) were transferred to an indoor experimental room with controlled photoperiod (12:12 L:D) provided by two fluorescent lamps (Philips daylight, TLT 40 W/54RS) at an average room temperature of $17 \pm 0.5\text{ }^{\circ}\text{C}$. Tadpoles were individually placed in plastic cups (10 cm in diameter) with 300 ml of freshwater. On day 1, tadpoles were fed a mix of algae culture (*Scenedesmus* sp. and *Chlamydomonas* sp.) and freshwater fish food (VitaFish[®]). On day 2, the cups were gently cleaned with a bottom cleaner and refilled with freshwater. The day of the behavioral test (day 3), tadpoles were fed during the morning to standardize the nourishing conditions during evaluation. We used eight individual *P. thaul* tadpoles for each of our treatments ($N = 8$).

Experimental trials were conducted in 10-cm-diameter plastic cups (similar to the cups used for indoor maintenance) containing 300 ml of distilled water. The experimental assays took place from 12:00 to 16:00 h. We used a digital video camera (JVC GZ-E300BU: 30 frames s^{-1}) placed 0.70 m above the cups to film tadpoles from 5 min before (pre-stimulus period) to 5 min after (post-stimulus period) the addition of the stimulus. Individual tadpoles were randomly assigned to one of the two stimuli:

conspecific cue [0.07 g/ml] or amphipod cue [0.13 g/ml]. After the pre-stimulus period, 1 ml of one of the stimuli was slowly added on the side opposite to that of the tadpole position to minimize disturbance. The addition was made by moving a 1-ml bulk pipette around for 10 s. To keep tadpoles undisturbed during the test, the observer was present inside the room only when the stimulus was added and to turn on/off the video camera via remote control.

Then, the video recordings were analyzed by an observer blinded to the treatments, who measured the total duration (in seconds) of the overall activity (any movement) for each tadpole during the pre- and the post-stimulus periods. We used a real-time computer-based *ad hoc* program (FoxPro 2.0, Fox Holdings) that allows the behavior to be analyzed using the computer keyboard. Assessing behavior during both the pre- and post-stimulus periods is a method frequently used in behavioral studies where tadpoles are exposed to chemical cues that indicate predation risk (e.g., Mirza et al. 2006; Mathis et al. 2008). A typical antipredator response of larval amphibians, including *P. thaul* tadpoles, is to decrease overall activity after the detection of risk cues.

Statistical analysis

This section applies to all three experiments. We first verified that pre-stimulus activity values did not differ between treatments ($P > 0.05$) and then calculated activity change (*overall activity*) between the post- and the pre-stimulus periods (post-pre). This was used as dependent variable. Although a single observer encoding behavioral activity of all the videos, we verified the observer reliability with a second coder watched a sample of videos ($N = 28$) from different animals and treatments. The inter-observer reliability according to Pearson's correlation coefficient was 0.98; $P < 0.0001$. Data from each experiment met assumptions of normality and homoscedasticity. Hence, we ran parametric one-way ANOVA to investigate the effect of the treatment on the overall activity of *P. thaul* tadpoles. When applicable, analysis was followed by LSD Fisher post hoc test (Fisher's least mean significant differences with a type I error set at 0.05). A rejection criterion of $P < 0.05$ was adopted for all analyses.

Results

Figure 1 shows that tadpoles exposed to the conspecific injury cue reduced their activity and tadpoles exposed to the amphipod injury cue increased their activity during the post-stimulus period. The change in the overall activity of tadpoles during the test as a function of the cue exposure was significantly different between tadpoles exposed to amphipod or conspecific cues [$F(1,14) = 13.06$, $P < 0.0028$] (Fig. 1).

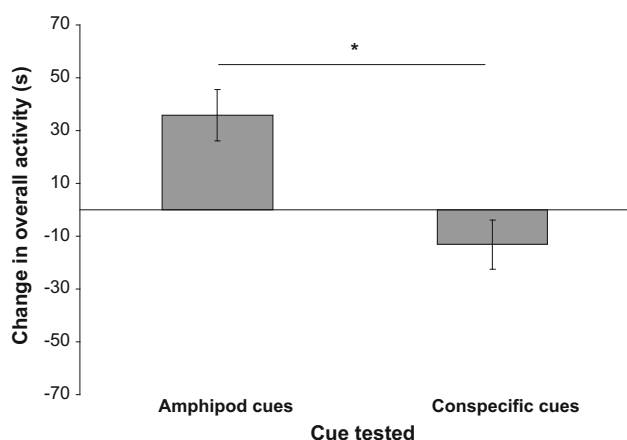


Fig. 1 Mean \pm SE change in overall activity (in seconds) for tadpoles experiencing amphipod or conspecific injury cues. *denotes significant differences

Experiment 2

Materials and methods

Procedure

Based on the results obtained in Experiment 1, we designed Experiment 2 to evaluate whether *P. thaul* tadpoles can learn to recognize the predation risk of chemical cues from injured amphipods. The experiment followed a one-factor design with five experimental groups (paired, unpaired, CS-only, US-only and water). In the morning of the conditioning day (day 1), individual tadpoles (Gosner stages 26–29) were randomly assigned to plastic cups (10 cm in diameter) with 300 ml of well water placed in an indoor experimental room (conditions similar to those of Experiment 1). Then, tadpoles were assigned to one of the five experimental groups. The possibility of learning was assessed using a classical conditioning scheme where a group of tadpoles (paired group) were exposed to chemical cues from injured amphipods (previously unknown cues, CS) paired with chemical cues from injured conspecifics (unlearned biologically relevant cues, US). A learning control group was achieved by presenting tadpoles the same cues as those presented to the paired group but in reverse order and separated in time (unpaired group). Tadpoles in the paired group were exposed to 1 ml of amphipod injury cues immediately followed (10 s) by 1 ml of conspecific injury cues. In the unpaired group, tadpoles were exposed to 1 ml of cues from injured conspecifics followed (4 h) by 1 ml of cues from injured amphipods. We also included three more groups to fully assess the mechanism involved in learning. In CS-only and US-only, tadpoles were exposed to 1 ml of cues from injured amphipods or 1 ml of cues from injured conspecifics, respectively. In the water group, tadpoles were exposed to 1 ml of water (we used previously frozen distilled water). Both the cues

in the paired group and the single cues in the CS-only, US-only and water groups were presented at 13:00 h. In the unpaired group, the cues from injured conspecifics were presented at 12:00 h and the amphipod cue was presented 4 h later; 1 h after the last cue was added, the cups were gently cleaned with a bottom cleaner and refilled with freshwater and tadpoles were fed a mix of algae culture (*Scenedesmus* sp. and *Chlamydomonas* sp.) and freshwater fish food (VitaFish®). On day 2, tadpoles were left undisturbed.

We decided to evaluate learning after 48 h of conditioning to allow the animals to feed ad libitum without disturbances for 24 h, since the results of Experiment 1 showed that after 3 days in the indoor experimental room animals were in good condition. The cups used for the test were similar to those used during conditioning. The day of testing (day 3), tadpoles were fed in the morning and the test took place from 12:00 to 16:00 h in the experimental room. During the test, we first recorded tadpoles for 5 min (pre-stimulus period), and then tadpoles were tested as follows: tadpoles in the paired, unpaired and CS-only groups were exposed to 1 ml of cues from injured amphipods (CS), the tadpoles in the US-only group received 1 ml of cues from injured conspecifics (US), and the tadpoles in the water group received 1 ml of water. Then, we recorded the activity (any movement) for 5 more minutes (post-stimulus period). We used the unpaired group as a learning control because it allowed us to expose the animals to both cues while avoiding any association between them. The additional groups allowed us to test cue pre-exposure effects, manipulation effects and the response magnitude. Specifically, the test responses in the paired and US-only groups informed us about the conditioned and unconditioned responses when tadpoles had one US exposure before (during acquisition). The concentration of the cues from injured conspecifics and injured amphipods was similar to that used in Experiment 1. The final number of tadpoles analyzed per group was 14 ($N = 14$ per group).

To record and analyze behavior, we used conditions and procedures similar to those used in Experiment 1.

Results

Figure 2 shows that tadpoles in the paired and US-only groups reduced their activity from the pre-stimulus period when exposed to the CS and US, respectively (-8.86 ± 9.41 and -55.00 ± 9.70 , respectively), but in the US-only group the magnitude of response was significantly higher. Tadpoles in the unpaired, CS-only and water groups increased their activity during the post-stimulus period and did not differ significantly between them. The change in the overall activity of tadpoles during the test as a function of the treatment (paired, unpaired, CS-only, US-only and water) was significant [$F(4,65) = 34.57$, $P < 0.0000$] (Fig. 2). The post hoc test revealed that the

activity of tadpoles in the paired and US-only groups was significantly different from that of the four remaining groups and that these groups also differed from each other.

Experiment 3

Materials and methods

Procedure

This experiment consisted of two parts. Experiment 3a aimed to replicate the results from Experiment 2 but

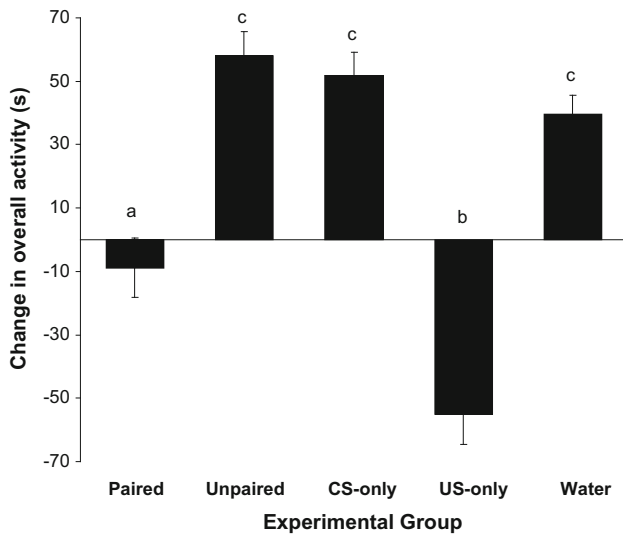
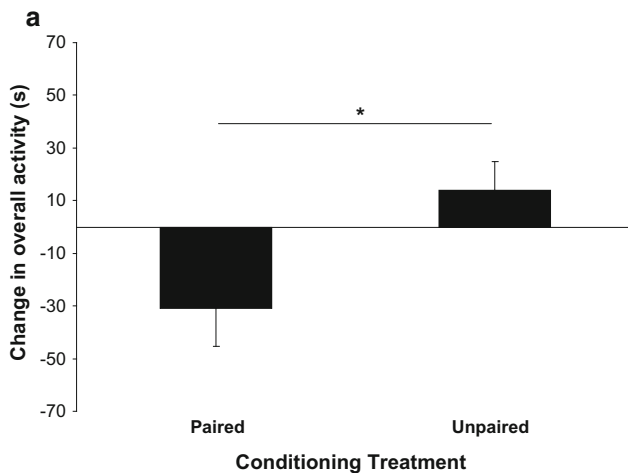


Fig. 2 Mean \pm SE change in overall activity (in seconds) during the test as a function of the experimental treatments. The paired, unpaired and conditioned stimulus-only (CS-only) groups are tested with amphipod injury cues, the unconditioned stimulus-only (US-only) group is tested with conspecific injury cues, and the water group is tested with water. Different letters denote significant differences



using only two experimental groups (paired and unpaired). Experiment 3b was performed to evaluate whether a low concentration (half of that used in the previous experiments) of amphipod cues can support the acquisition of the recognition of injured amphipods as a cue of predation risk. Procedures for Experiments 3a and b were similar to those for Experiment 2, except that in Experiment 3b we used half the concentration of the amphipod cue during conditioning and testing (0.05 g/ml). In Experiment 3a, one tadpole was not considered in the analysis because it remained motionless during the pre-stimulus period. The final number of tadpoles analyzed per group was as follows: Experiment 3a, paired ($N = 10$), unpaired ($N = 9$); Experiment 3b, paired ($N = 10$), unpaired ($N = 10$).

Results

Figure 3a shows that only paired tadpoles displayed a decreased overall activity during the test. The change in the overall activity of tadpoles during the test as a function of the conditioning treatment (paired and unpaired) was significant [$F(1, 17) = 6.04, P < 0.025$] (Exp. 3a). Figure 3b shows that the change in activity of tadpoles in paired and unpaired groups was similar. The change in the overall activity of tadpoles during the test as a function of the conditioning treatment was not significant [$F(1,18) = 0.05, P = 0.822$] (Exp. 3b). It is interesting to note that the values of change in the overall activity of tadpoles in the unpaired group in Experiment 3a and the paired and unpaired groups in Experiment 3b were similar ($14.01 \pm 10.67, 11.15 \pm 14.10$ and 14.97 ± 9.06 s, respectively). This suggests that the concentration of cues from injured amphipods would not affect tadpole basal (unlearned) response.

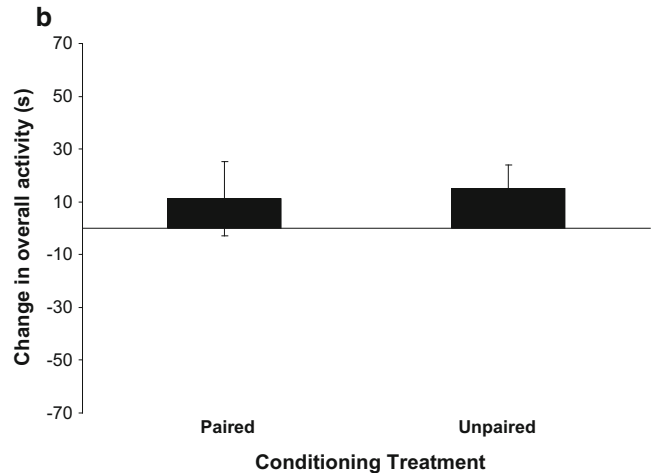


Fig. 3 Mean \pm SE change in overall activity (in seconds) during the test as a function of the conditioning treatment (paired and unpaired) for tadpoles exposed to: **a** a high (Experiment 3a) and **b** a low (Experiment 3b) concentration of amphipod cues. *denotes significant differences

Discussion

Our results show that anuran tadpoles can learn to recognize predation risk from injured amphipods that are members of the same prey guild. In Experiment 1, we observed that the response of tadpoles to cues from injured conspecifics is to reduce their activity, which is consistent with previous results observed in *P. thaul* and other tadpole species as an antipredator response (Mirza et al. 2006; Gonzalo et al. 2010; Pueta et al. 2016). We also noted that cues from injured amphipods did not generate the antipredator response. Our learning experiments allow us to infer that the learned response observed is a consequence of an associative mechanism during acquisition. The unpaired group allowed us to interpret the response to amphipod cues as not being due to potential US pre-exposure effects such as neophobia, which have been previously reported in anuran tadpoles (Brown et al. 2013).

The best way of comparing the magnitude of the conditioned and unconditioned response in our experiments was to compare the test response of the paired group to cues from injured amphipods and that of the US-only to cues from injured conspecifics (Exp. 2). Both groups had previous exposure to the US. We observed that the conditioned response was smaller in magnitude than the unconditioned response (Fig. 2). We do not rule out the possibility that the US-only group might be showing an effect of sensitization; however, if so, this effect seems not to generalize to the scent of amphipods, as shown by the unpaired group.

The acquisition of predator recognition using cues from injured conspecifics has been demonstrated in a wide variety of aquatic species (e.g., anurans: Mirza et al. 2006; fish: Mirza and Chivers 2000; gastropods: Rochette et al. 1998; insects: Wisenden et al. 1997; and flatworms: Wisenden and Millard 2001). This cognitive ability may contribute to reducing the vulnerability of prey to native and alien predators (Mirza and Chivers 2000; Polo-Cavia and Gomez-Mestre 2014). Studies in anurans have shown that tadpoles can learn to identify new predator odors based on a one-time pairing of cues from injured conspecifics and the scent of a novel predator (e.g., Ferrari et al. 2010b; Gonzalo et al. 2010). The present results show a new type of stimulus that tadpoles could learn to use to warn them of the risk of predation, injury-released cues from heterospecifics.

Injury-released chemical cues are released only in the context of a predation event and thus reliably indicate the presence of an actively foraging predator (Wisenden and Chivers 2006). The fact that *P. thaul* tadpoles appear not to acquire an association when the heterospecific cue is in low concentration (Experiment 3b) could be due to the fact that

the salience of the stimulus is not strong enough to make tadpoles *pay attention* to a novel odor in the current context of predation, unlike that indicated by cues from injured conspecifics (Dukas 2002). On the other hand, we cannot reject the possibility that some learning occurred in Experiment 3b, but at a lower magnitude than in Experiments 2 and 3a, and that memory for it lasted less than 48 h (the testing delay we used). The acquisition of an association depending on the concentration of the cue from injured heterospecifics may help tadpoles not to invest energy in acquiring (or retaining) new information concerning heterospecifics for which the predator seems to have little appetite and therefore less relevant. An important fact is that although learning seems to be dependent on the heterospecific cue concentration, tadpoles appear not to have an innate behavioral response to high amphipod cue concentration. This notion arises from the results of the activity change, which was similar when comparing unpaired groups in both experiments.

In summary, this study documents for the first time that anuran tadpoles can learn risk from cues from injured heterospecifics. In fish, learned response to heterospecific fish cues had been previously demonstrated, but the evidence does not allow a conclusion what kind of learning mechanism was involved (Chivers et al. 1995, 2002; Mirza and Chivers 2001, 2003; Pollock et al. 2003; Pollock and Chivers 2004). Our work indicates that the mechanism of learning about heterospecific cues could be associative and that learning can involve associating injury cues from phylogenetically distant animals (like anurans and amphipods). The ability to recognize injury cues from members of the same prey guild could be beneficial because it indicates the presence of an active predator. Learned and innate predation risk recognition mechanisms can coexist and allow animals to use several sources of public information to evaluate risk and act accordingly.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard Experiments were performed under the Guidelines for Use of Live Amphibians and Reptiles in Field Research (ASIH). Experiments followed the ethical norms imposed by Argentina (APN N° 1231). Samplings in Laguna Fantasma were authorized by Secretaria de Medio Ambiente of San Carlos de Bariloche, Río Negro, Argentina (Licence N° 41-AP-2014).

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