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Chemical alarm cues allow prey to adjust their defensive behaviour to cover abundance

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ABSTRACT

Aquatic prey species show sophisticated mechanisms to adjust their antipredator behaviours to the level of risk, which they estimate either by direct experience with predators or from indirect indicators such as chemical alarm cues released by injured conspecifics. For instance, evidence suggests that the alarm cues of tadpoles exposed to high levels of background predation risk elicit a stronger antipredator response compared to alarm cues of tadpoles exposed to low risk. Similarly, the alarm cues of tadpoles from environments with reduced vegetation cover might cause a stronger response than alarm cues of tadpoles from environments with abundant vegetation because tadpoles suffer high predation when vegetation is scarce. I tested this hypothesis in the edible frog, *Pelophylax esculentus*, by comparing the response of focal tadpoles (not exposed to vegetation manipulation) to alarm cues of donor tadpoles raised from eggs in either high- or low-vegetation treatment. I also tested the alarm cues of donor tadpoles switched from high- to low-vegetation treatments and vice versa after hatching because this would enable understanding whether an eventual difference in alarm cues occurred due to the embryonic or larval environments and whether the treatments at the two developmental stages had interactive effects. Alarm cues from the low-vegetation, and thus the high-risk, treatment elicited stronger antipredator response in focal tadpoles in comparison to the alarm cues from the high-vegetation, low-risk treatment. Results from switching donor tadpoles between vegetation treatments after hatching suggested that the observed effect was due to the vegetation treatment experienced by donor tadpoles during the larval stage, with no interactive effects. Chemical alarm cues convey information about cover abundance, an environmental factor that indirectly covaries with predation risk.

1. Introduction

Predation is a major selective force for many species that has triggered the evolution of phenotypically plastic defences (Brown et al., 2013; Domenici et al., 2008). These defences are often costly, for example, in terms of reduced foraging (Sih, 1980). Because predation risk varies across different environments, prey species are expected to tune their responses based on all the available information about risk (Ferrari et al., 2010).

For aquatic species, conspecific alarm cues represent a key source of information about risk (Ferrari et al., 2010). These cues consist of different active substances including blood, chondroitin, and stress hormones that are released through mechanical damage of the tissues and thus indicate a current predation event (Barreto et al., 2013; Dahl et al., 2012; Mathuru et al., 2012). Alarm cues cause typical antipredator behaviours in prey, such as activity reduction (Ferrari et al., 2010). Two recent studies have reported that alarm cues indicate not only an ongoing predation event but also background predation risk: tadpoles exposed to alarm cues from conspecifics that experienced predation show stronger responses than tadpoles exposed to alarm cues from conspecifics that did not experience predation (Bairos-Novak et al., 2017; Lucon-Xiccato et al., 2016).

The amount of vegetation cover is a factor that strongly predicts the level of predation risk for tadpoles: when vegetation is scarce, predation from both vertebrates and invertebrates is high (Baber and Babbitt, 2004; Babbitt and Tanner, 1997, 1998; Figiel and Semlitsch, 1991; Kopp et al., 2006; Tarr and Babbitt, 2002). Because the amount of vegetation indirectly predicts predation risk, I investigated whether edible frog, *Pelophylax esculentus*, tadpoles differ in their response to alarm cues from donors previously exposed to high or low levels of vegetation. I compared the response of focal tadpoles to alarm cues of donor conspecifics entirely raised (i.e., at both the larval and embryonic stages) under low- (LL) and high-vegetation conditions (HH). I also tested the response to alarm cues of donor tadpoles that were switched from the

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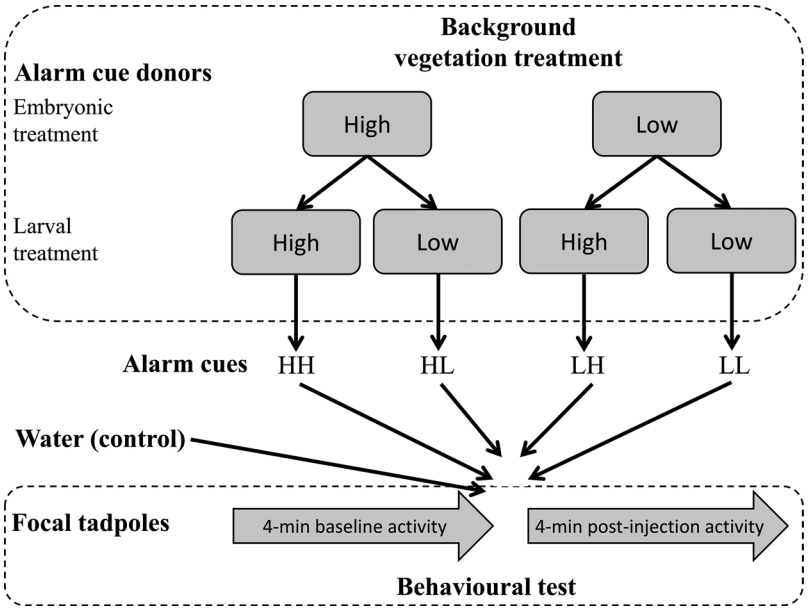

Fig. 1. Experimental design. Focal tadpoles were collected from a pool and were exposed to the alarm cues of donor tadpoles raised in pails under 4 different vegetation treatments across their development (HH: embryonic and larval high-vegetation; HL: embryonic high-vegetation and larval low-vegetation; LH: embryonic low-vegetation and larval high-vegetation; and LL: embryonic and larval low-vegetation). A group of focal tadpoles was also tested with water as control.

high- to low-vegetation treatment after hatching (HL) and vice versa (LH). This design allowed me to test for differences in alarm cue responses due to the environment experienced by the donors at either the embryonic or larval stage and whether any effects of these factors were additive. For example, if differences were due to the embryonic treatment, I expected to observe a significant effect of embryonic donor treatment with focal tadpoles responding more strongly to LL and LH alarm cues (i.e., alarm cues from donor tadpoles exposed to embryonic low-vegetation treatment) compared to HH and HL alarm cues (i.e., alarm cues from donor tadpoles exposed to embryonic high-vegetation treatment). Conversely, if differences were due to the larval stage environment, I expected to observe a significant effect of larval donor treatment, with focal tadpoles responding more strongly to LL and HL alarm cues (i.e., alarm cues from donor tadpoles exposed to larval low-vegetation treatment) compared to HH and LH alarm cues (i.e., alarm cues from donor tadpoles exposed to larval high-vegetation treatment). In case of an interaction, I expected that focal tadpoles responded more strongly to LL alarm cues compared to HL and LH alarm cues.

2. Materials and methods

2.1. Tadpole collection and general maintenance

I collected eggs from 12 egg masses in northeastern Italy and randomly assigned the eggs to two maintenance groups: focal tadpoles maintained in a pool and alarm cue donors maintained in pails with the vegetation treatment (see below). All tadpoles were kept outdoors and thus were exposed to the natural conditions of temperature, illumination, and wind. After hatching, I fed the tadpoles rabbit pellets (alfalfa) every day to complement algae growing in the pails and pool. Overall, I used 164 tadpoles (larval stage) in the experiment. The focal tadpoles used in the experiment and the tadpoles collected but not used in the experiment were released at the sampling site at the end of the experiment. This experiment followed national guidelines (D.L. 4 marzo 2014, n. 26) and was approved by the University of Padova (n. 51/2016).

2.2. Focal tadpole maintenance

I raised focal tadpoles (approx. 80% of the eggs) in an outdoor pool (6 × 4 m, depth: 60 cm), which was made by digging into the ground and covering it with a waterproof plastic sheet. The tadpoles raised in the pool were used as focal tadpoles in the behavioural observations and were not exposed to vegetation treatment. I filled the pool with water 4 weeks before the experiment and added a few submerged aquatic plants (*Hygrophila corymbosa* and *Taxiphyllum barbieri*), which did not cover the water surface. *Leman minor,* which was used for alarm cue donors, was not present in the pool. Focal tadpoles were not exposed to predators because the pool was artificial and filled only for the experiments. Each day, I confirmed that no predators had entered the pool. I used 116 focal tadpoles from the pool.

2.3. Alarm cue donor maintenance

The remaining animals collected at the sampling site (approx. 1000) underwent the background vegetation cover treatment with the aim to use them as alarm cue donors. I raised them in 20 groups of 50, in 20-L pails (50 × 36 cm) filled with 12 cm of pool water (80% daily water change). During the embryonic stage, I kept 10 pails under high-vegetation conditions: the water surface was entirely occluded by L. *minor*. I kept the remaining 10 pails under low-vegetation conditions: L. *minor* covered less than 20% of the water surface. These two treatments reflected natural situations experienced by tadpoles in their environment because similar amounts of vegetation were observed in various parts of the river where frogs reproduce. Soon after hatching, I randomly split each group of pails: I kept half of the pails under high-vegetation conditions and half of the pails under low-vegetation conditions. This resulted in a 2 × 2 experimental treatment for the alarm cue donors (Fig. 1): 5 pails had an embryonic and larval high-vegetation condition (HH); 5 had an embryonic and larval low-vegetation condition (LL); 5 had an embryonic high-vegetation and larval low-vegetation condition (HL); and 5 had an embryonic low-vegetation and larval high-vegetation condition (LH). I used 48 3-week-old tadpoles randomly selected from the pails as alarm cue donors. All the donors were at the larval stage at the time of the experiment.

2.4. Alarm cue preparation

Alarm cues were obtained from tadpoles that underwent the background vegetation treatment. Following the studies on *P. esculentus* and other anurans (Ferrari et al., 2009; Lucon-Xiccato et al., 2018), I randomly selected 2–3 donor tadpoles (total length: approx. 20 mm) from each pail and sacrificed them with a blow to the head. I used this standard physical euthanasia because a study on fish has reported that

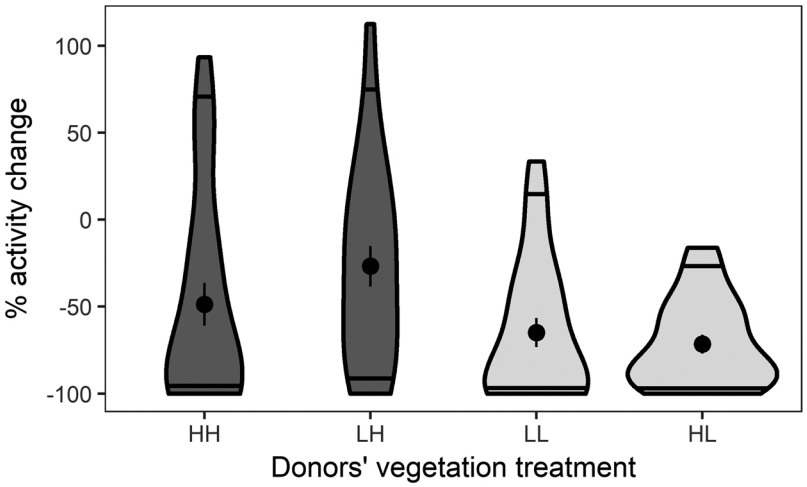

Fig. 2. Violin plot of percentage activity change between baseline and post-cue injection observation. Focal tadpoles were exposed to the alarm cues of conspecifics raised under 4 different vegetation treatments (HH: embryonic and larval high-vegetation; LH: embryonic low-vegetation and larval high-vegetation; LL: embryonic and larval low-vegetation; and HL: embryonic high-vegetation and larval low-vegetation). Focal tadpoles exposed to larval low-vegetation treatment (LL and HL; light grey) showed stronger response than focal tadpoles exposed to larval high-vegetation treatment (HH and LH; dark grey). The plot shows kernel probability density; horizontal lines indicate 5% and 95% percentile of the data; dots and error bars indicate the mean and SE, respectively.

chemical methods can interfere with alarm cue composition and effects (Losey and Hugie, 1994). Immediately after euthanasia, I emulsified the donors with a mortar and pestle, and I suspended the solution in pool water (1 tadpole per 20 mL). The alarm cues from different treatments were kept separate.

2.5. Behavioural response of focal tadpoles

I used a well-established behavioural test to study responses to alarm cues (Ferrari et al., 2009; Lucon-Xiccato et al., 2016). The test consisted of observing the change in activity of focal tadpoles exposed to the alarm cues of interest (i.e., those of donors raised with the 4 different background vegetation treatments) or to water as a control (5 possible testing cues overall). For the test, I moved each focal tadpole into a 0.5-L cup filled with pool water for a 1-h acclimation. I scored the baseline activity of focal tadpoles for 4 min by counting the number of times the tadpole crossed the median of the cup's bottom. Then, I injected in the cup of each focal tadpole 5 mL of one, haphazardly chosen, testing cue: alarm cues from the HH donor tadpoles ($N = 24$), alarm cues from the HL donor tadpoles ($N = 22$), alarm cues from the LL donor tadpoles ($N = 23$), alarm cues from the LH donor tadpoles ($N = 24$; Fig. 1), or pool water as the control ($N = 23$). Sample sizes were unequal because I did not test tadpoles with relatively low levels of baseline activity (< 6 line crossings). I then scored post-injection activity (4 min). Focal tadpoles respond to alarm cues with a reduction in activity compared to focal tadpoles exposed to the water control (Ferrari et al., 2009).

2.6. Statistical analysis

Statistical analysis was performed in R v. 3.4.0. First, I compared the baseline activity of focal tadpoles exposed to the five testing cues (alarm cues from HH, LL, HL, and LH tadpoles, and water control) with ANOVA. Then, I used an index of percentage of activity change of the focal tadpoles as a dependent variable: [(post-injection number of line crossings - baseline number of line crossings)/baseline number of line crossings] × 100. Due to highly skewed distributions, I performed square-root transformation to achieve normality (Kolmogorov-Smirnov test). To verify that the alarm cue exposure resulted in the expected reduction in activity, I compared the percentage of activity change of focal tadpoles exposed to alarm cues (4 alarm cue types pooled) with that of focal tadpoles exposed to control water during the behavioural test (two-sample t test). I subsequently removed the focal subjects exposed to control water and studied the effect of the donor's background vegetation treatment on the percentage activity change of focal tadpoles exposed to alarm cues using an ANOVA fitted with embryonic donor treatment (high or low vegetation) and larval donor treatment (high or low vegetation) as factors.

3. Results

Focal tadpoles assigned to the different testing cues did not differ significantly in terms of baseline activity (number of line crossings, mean ± SD: alarm cues from HH donor tadpoles: 29.54 ± 14.51; alarm cues from LL donor tadpoles: 21.00 ± 12.61; alarm cues from HL donor tadpoles: 31.00 ± 13.28; alarm cues from LH donor tadpoles: 23.75 ± 12.64; water control: 24.74 ± 12.53; $F_{4,111} = 2.293$, $P = 0.064$).

The analysis of the activity change between the baseline and the post-injection observation period revealed that focal tadpoles exposed to alarm cues showed a stronger activity decrease than focal tadpoles exposed to control water (mean ± SD: alarm cues: -52.45 ± 50.70%; water: -13.41 ± 46.07%; $t_{114} = 3.594$, $P = 0.0005$). Analysis of the activity change of the focal tadpoles exposed to alarm cues showed a significant effect of donor larval background treatment ($F_{1,89} = 5.698$, $P = 0.019$): focal tadpoles responded more strongly to the alarm cues of donor tadpoles exposed to the low-vegetation treatment during the larval stage (i.e., LL and HL donor tadpoles: -68.18 ± 33.62% activity decrease) compared to donor tadpoles exposed to the high-vegetation treatment during the larval stage (HH and LH: -37.71 ± 59.28% activity decrease; Fig. 2a). There was no significant effect of donor embryonic background treatment ($F_{1,89} = 1.288$, $P = 0.260$): focal tadpoles responded similarly to alarm cues from donor tadpoles that experienced the low-vegetation treatment during the embryonic stage (i.e., LL and LH donor tadpoles: -45.45 ± 52.92% activity decrease) and alarm cues from donor tadpoles that experienced the high-vegetation treatment during the embryonic stage (HH and HL: -59.61 ± 47.83% activity decrease; Fig. 2b). There was no significant interaction between the embryonic and larval treatment of the donors ($F_{1,89} = 1.466$, $P = 0.229$).

4. Discussion

This study showed that the amount of vegetation experienced by an individual during early ontogeny affects how conspecifics respond to its alarm cues. The alarm cues of donors from a background environment with reduced vegetation elicited a stronger antipredator response. Because tadpoles suffer higher predation in environments with reduced vegetation (e.g., Babbitt and Tanner, 1997), this stronger response is likely adaptive. Tadpoles showing strong responses are more likely to survive an attack, even in the absence of refuge (Lawler, 1989). The key advantage of this response is that it allows tadpoles to cope with high predation risk environments as early as the first days after hatching based on the environment experienced by conspecifics. Tadpoles can

thus indirectly estimate the risk of predation before experiencing predator attacks and exploring the environment.

This finding aligns with previous studies in suggesting that alarm cues provide information about risk beyond the presence of an ongoing predator attack. In tadpoles, alarm cues released by individuals that lived under a high-background risk cause stronger responses (Lucon-Xiccato et al., 2016). In fish, alarm cues elicit a stronger reaction in individuals of the same age as the donor (Mitchell and McCormick, 2013). The novelty of the present study is that the information on background risk provided by alarm cues is not directly related to predators but to an environmental factor (cover abundance) that covaries with predation risk. The effect of vegetation on alarm cue response might also be important for methodological reasons. If different studies on prey response to alarm cues maintain alarm cue donors in different conditions (i.e., with different amounts of vegetation), then the results might not be comparable. Vegetation during maintenance should therefore be taken into account when interpreting previous research and when designing future experiments.

The proximate causes of the effect reported here remain to be addressed. Tissues of tadpoles from habitats with reduced vegetation might have different chemicals compared to those that live in habitats with abundant vegetation. Under this hypothesis, tadpoles might have evolved the capacity to discriminate these differences and use them to infer the abundance of cover in the environment. A similar ability has been observed in fish: three-spined sticklebacks, *Gasterosteus aculeatus*, displayed an association preference for fish that had experienced the same habitat, and were thus characterized by the same odour, compared to fish that experienced a different habitat (Ward et al., 2004, 2005). Furthermore, gravid stickleback females from sympatric species displayed a preference for water scented with males from the same habitat, though it is not clear whether this choice was also affected by other factors (Rafferty and Boughman, 2006). It has been hypothesised that the olfactory cues used by fish in the aforementioned choices derive from water salinity (Ward et al., 2004) or from the type of food consumed by conspecifics in the different environments (Ward et al., 2005).

Alternatively, tadpoles from the two background vegetation treatments might produce different concentrations of those chemicals that act as alarm substances. There is evidence that behavioural responses increase with greater alarm cue concentration (Ferrari et al., 2009). The absence of cover is likely stressful for tadpoles, which might cause an increase in the level of circulating stress hormones (Woodley and Peterson, 2003). Tadpoles have been shown to respond to corticosterone dissolved in water with phenotypic plasticity similar to that caused by predators (Dahl et al., 2012). This suggests the role of stress hormones in the observed effect. However, a recent study on zebrafish, *Danio rerio*, found that social communication about predation risk may be independent from steroid stress hormones (i.e., cortisol; Barcellos et al., 2014); thus, other substances related to the stress response (e.g., catecholamines) should also be considered.

Interestingly, only larval background vegetation treatments elicited a differential response. This suggests that the alarm cues of tadpoles from the two environments diverged after hatching. It is likely that the presence of abundant vegetation also improves survival at the embryos stage, although to the best of my knowledge, there has not been an experimental test of this hypothesis. Accordingly, the fact that I did not detect an effect of embryonic vegetation treatment seems counterintuitive. A possible explanation is that frogs cannot acquire information about the presence of vegetation in the environment when they are embryos. For example, they may have to actively explore their environment, which is only possible after hatching (i.e., at the larval stage).

In conclusion, this experiment suggests that researchers may underappreciate the information available to prey via alarm cues because such cues can convey information about other environmental factors important for survival. Future studies should address whether this chemical communication system can provide information about, for example, the type of predator in the environment (Crane et al., 2018), level of water turbidity (Abrahams and Kattenfeld, 1997), and presence of competitors (Relyea, 2000).

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