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Evidence of the peptide identity of the epidermal alarm cue in tadpoles of the toad *Rhinella arenarum*

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Chemical cues associated with predation attempts allow prey to trigger defensive behaviours. Accordingly, tadpoles of several species of anurans display strong behavioural responses to chemical cues of injured conspecifics. As part of the antipredator response, tadpoles show rapid and sustained inhibition of activity when exposed to chemical cues of predation. Although the ability to respond to cues of conspecifics has been confirmed in a wide variety of anuran species, studies about the tissue source and the chemical aspects of the molecules involved are scarce and contradictory. In the present work, we analysed the chemical characteristics, tissue source and release mechanism of the chemical alarm cue in *Rhinella arenarum* tadpoles. Our results support the hypothesis that a peptide of epidermal origin in mediates amphibian tadpole communication.

Keywords: Amphibia, Anura, tadpoles, anti-predator behaviours, conspecific cues

In anuran tadpoles, chemical cues released during predation can be detected by other tadpoles as a sign of potential predation risk. This information causes changes in the behaviour, morphology, and/or development of the animal receiver (Relyea, 2001; Laurila et al., 2002; Crossland et al., 2019). It has been reported that tadpoles of several species of anurans show a strong reduction in activity in response to chemical cues from injured conspecifics (Marquis et al., 2004; Fraker et al., 2009; Hettyey et al., 2015) or from the predator that had fed on conspecific tadpoles (Hettyey et al., 2015).

Although the ability to respond to cues from conspecifics with anti-predator behaviours has been confirmed in a wide variety of anuran species, little is known about the tissue source and chemical nature of the molecules involved. In *Lithobates sylvaticus* (*Rana sylvatica*), the tadpoles' skin cells produce a peptide alarm pheromone released through an active process of secretion after the predator attack (Fraker et al., 2009). Authors also made a biochemical characterisation of the alarm cue, confirming that it is composed of at least two small peptides (Fraker et al., 2009). On the other hand, *R. aurora* tadpoles increase ammonium secretions during predation attempt, eliciting anti-predator behaviours in conspecifics (Kiesceker et al., 1999). By contrast, in larvae of two frog species, *Lithobates pipiens* and *L. clamitans*, are thought to release a sulfated steroid as a component of the alarm cue (Austin et al., 2018), a result which supports the classic studies of Hrbáček (1950), who postulated that the alarm cue of bufonids is composed of steroids related to the bufotoxins (Hrbáček, 1950). Beyond these studies, there are no other records of the chemical characterisation of the cues that trigger alarm behaviours in tadpoles.

Given the lack of information regarding the tissue source and the chemical nature of conspecific cues in amphibian tadpoles, we sought to analyse these properties in *Rhinella arenarum*. Previously, we confirmed that these tadpoles responded to conspecific homogenates reducing the time they spent swimming (Raices, 2018), which allows us to use them in behavioural assays. In the present work, we provide evidence that these tadpoles use a peptide of epidermal origin similar to that proposed for *L. sylvaticus* (Fraker et al., 2009).

Growth and maintenance of tadpoles: *Rhinella arenarum* embryos were obtained by in vitro fertilisation, according to standard methods (Casco et al., 1992). Tadpoles were staged according to Gosner (1960) and maintained in conditions already standardised in our laboratory: five larvae per litre of dechlorinated tap water are maintained in a 12-hour light/dark cycle, at 22 ° C, and fed ad libitum with boiled chard (Distler et al., 2016). Every other day, food and waste residues were removed and water volume restored. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, Protocol #22/13, and the principles of NIH (publication 8523, revised 1985).

Behaviour essays: *Rhinella arenarum* tadpoles (stage G36-37) were exposed to control and different treatments in a circular glass aquarium (15 cm diameter) filled with 500 ml of dechlorinated tap water at 22 °C. After 15 minutes of acclimatisation, 500 µl of each stimulus (see below for details) was added homogeneously with

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a micropipette at a rate of 100 µl /sec. To avoid having the micropipette produce shadows that could disturb the tadpoles, we determined that the pipet needed to touch the surface of the water at a 45 ° angle. Five seconds after addition of the stimulus, tadpoles were recorded for 6 minutes. Videos were then analysed for the amount of time during which the tadpoles were active (Time spent moving). It was considered as "activity" every time the tadpoles flapped their tails, even if they relocated or not.

Tissue source of the alarm cue: For the preparation of stimulus, *R. arenarum* tadpoles (stage G36-37, n=8) were deeply anaesthetised by immersion in dechlorinated tap water at 0 °C and quickly decapitated to avoid suffering. Tadpoles were dissected into tails (composed by muscle and skin), skin (body skin), and carcass (composed by viscera and muscles). The tissues were then homogenised in 1:1 (weight:volume) of 1 g tissue / 1 ml of dechlorinated tap water. After homogenisation, water was added until a final concentration of 0.1 g/ml. The homogenate was centrifuged at 5000 x g for 5 min and the supernatant was collected (stock solution 1:1). Simultaneously, we prepared aliquots of dechlorinated tap water, without chemical cues, in identical 2 ml vials. We coded all vials to keep the experimenter blind to the treatment. All vials were frozen for no more than 2 weeks at -20 °C prior to use.

Evaluation of potential active release mechanisms: Previous results in *Lithobates clamitans* (*Rana clamitans*) tadpoles suggested that the chemical alarm cue might be released via a voltage coupled stimulus-secretion pathway (Fraker et al., 2009). To test if this paradigm applies to *Rhinella arenarum*, tadpoles were briefly exposed to 5 mM KCl to depolarise cell membranes and thereby activating the release of secretory vesicles via a calcium/potassium-dependent mechanism. Four sets of 5 tadpoles were sequentially immersed in 20 ml 5 mM KCl or 20 ml dechlorinated tap water (as a control group) for 15 min. and then removed (according to Fraker et al., 2009). This generated conditioned media (tadpoles-KCl and tadpoles-water, n=6 and 8, respectively) that were then used as stimuli for behavioural assays. Two additional controls included: 20 ml of 5 mM KCl (n=6) or dechlorinated tap water alone (without tadpoles, n=7) as stimuli for behavioural assays. Behavioural observations were made as described above.

Chemical identity of the alarm cue: To identify the chemical cues in the skin of the tadpole, we used only dissected tails which reduce manipulation time and facilitate the extraction protocol. Homogenates were made as described above and were subjected to different treatments prior to use in the behavioural trials: 1) heat treatment (n=4): the sample was incubated in a water bath at 100 °C for 10 min; 2) Chloroform extraction to remove lipid components (n=4): an equal volume of chloroform was added to the homogenate in a 10 ml conical polypropylene tube, vortexed vigorously, and centrifuged at 2000 x g for 10 min to separate and recover the aqueous phase from the organic phase; 3) Protein digestion (n=3): 400 µl of homogenate sample was incubated with 200 µl Proteinase K solution (Ready to use, Dako Cat. number: S3020) at 50 °C for 15 min. and then boiled for 10 min to inactivate the proteinase activity. A control with Proteinase K solution but without homogenate (n=4) was also included. See Supplementary Figure for protocol details.

Statistical analyses: Statistics were performed using the R software version 3.3.1 (R Development Core Team, 2014) applying a significance level of $\alpha = 0.05$. The results were visualised as median ± quartiles. The mean of "Total activity (min)" was analysed between each group. One-way Analysis Of Variance (ANOVA) was used, with the aov( ) function, to analyse the differences between each group. In every test, Shapiro-Wilk and Levene tests were used to check for normality and homoscedasticity assumptions, respectively. Even when the Levene test result did not show heteroscedasticity, we decided to use the argument "weights" within the function gls( ), and the function varExp( ) to specify an exponential function of the variance. The GLS models were fitted by restricted maximum likelihood (REML). For each experiment, differences in the time spent swimming depending on the stimulus applied were tested with Tukey's Honestly Significant Difference (HSD) tests by glht( ) function. R scripts and datasets are deposited at https://zenodo.org/record/3963025#.Xx9EyvhKhwp.

As proof of the existence of an alarm cue in *R. arenarum*, we found that tadpoles significantly reduced their time spent moving when they were exposed to body skin and tail homogenates compared with water (Fig. 1a: skin=1.77 ± 0.99 min, tail= 0.99 ± 0.52 min, water= 4.66 ± 0.26 min, $p<0.005$ in both cases against water). The responses triggered by cues from the body skin and the tail homogenates did not differ ($p=0.154$). On the other hand, tadpoles exposed to the carcass homogenate did not reduce the time spent moving compared to those exposed to water (carcass=4.67 ± 0.89 min vs. water= 4.66 ± 0.26 min, $p=0.999$). While the tail homogenate was mainly composed of skin and muscles, the latter tissue type can be excluded as a potential source of alarm cues, since the carcass also contained muscles but did not trigger anti-predator behaviours. In *Rana aurora* tadpoles, Kiesecker et al. (1999) proposed ammonia as an alarm cue. However, our results would rule out this possibility since urine levels should be highest in the carcass homogenate. All these results suggest that chemical alarm cues that trigger anti-predator behaviours are stored in the skin of *R. arenarum* tadpoles. Similar results have been observed in other species of *Anura* (Pfeiffer, 1966; Fraker et al., 2009). In tadpoles of the Bufonidae family, including *Rhinella arenarum* (Regueira et al., 2016), a type of epidermal cells called "giant cells" were associated with the production of chemical alarm cues (Pfeiffer, 1966). It is possible that the anti-predator responses observed here, in tadpoles exposed to skin preparations, are related to this epidermal cell type.

We next wanted to address whether the alarm cue from the tadpole is released from the skin by a stimulus-coupled secretion pathway. Therefore, were created conditioned media from tadpoles exposed to 5mM KCl and observed the behaviour of naïve *R.*

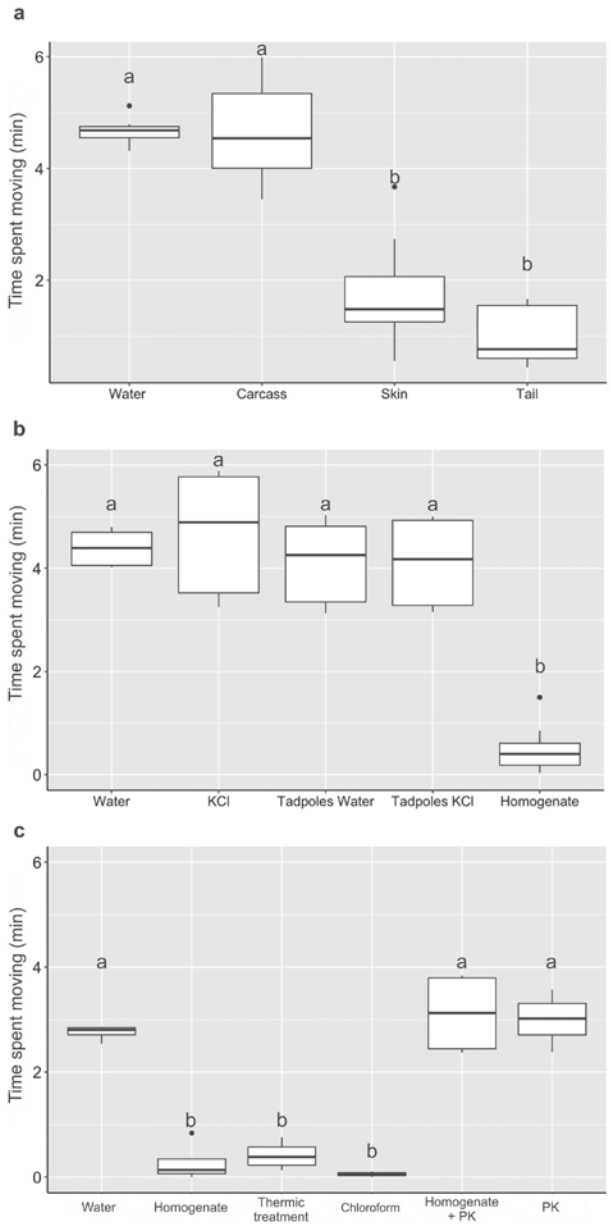

Figure 1. Boxplot representing the time spent moving (min) for larvae of *R. arenarum* exposed to different stimuli. For homogenate preparation and treatment, details see text.
a) The chemical cue of predation is derived from toad tadpole skin. Stimuli: Water (control), Carcass (viscera and tissue), Skin (body skin), Tail (skin and muscle) ($F=56.58$, $p<0.005$, $df=3$).
b) The alarm signal in *R. arenarum* is not released by an active mechanism that involves the opening of ion channels. Stimuli: Water (control), KCl (KCl 5mM), Tadpoles Water (water conditioned by tadpoles that had been immersed in tap water) or Tadpoles KCl (water conditioned by tadpoles that had been immersed in 5mM KCl) and Homogenate (tail homogenate) ($F=37.05$, $p<0.005$, $df=4$).
c) The epidermal chemical cue in *R. arenarum* is a thermostable, water-soluble and peptide-like molecule. Stimuli: Water (control), Homogenate (tail homogenate), Thermic treatment (homogenate preheat at 100 ˚C), Chloroform extraction (homogenate pre-treated with chloroform), Homogenate + PK (homogenate pre-treated with Proteinase K), PK (Proteinase K) ($F=41.09$, $p<0.005$, $df=5$). In a, b and c, thick horizontal lines and boxes represent the medians and interquartile ranges, respectively; whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; circles represent extreme data points. Different letters indicate significant differences with $p<0.05$.

arenarum tadpoles exposed to this conditioned water. Conditioned-water did not result in a reduction of time spent moving compared with water controls (Fig. 1b, tadpoles-KCl=4.11 ±0.9 min vs. water=4.39 ± 0.35 min, $p=0.976$). The conditioned-water obtained from controls (tadpole-water) did not trigger anti-predator behaviours (tadpoles-water= 4.13±0.79 min vs. water=4.39 ± 0.35 min, $p=0.977$). The total time spent moving was not influenced by the addition of 5 mM KCl either (KCl= 4.68 ±1.24 min vs. water=4.39 ± 0.35 min, $p= 0.976$), suggesting that the KCl itself does not affect the normal exploratory activity of the tadpoles. These results indicate that the alarm cue release does not seem to occur via a coupled stimulus-secretion pathway as opposed to what was observed by Fraker (2009) in *L. sylvaticus*, where the cue release occurs by an active mechanism that involves cell membrane depolarisation. Instead, the chemical alarm cue in *R. arenarum* seems to be released by a passive mechanism, surely involving tissue damage.

Since the controversy regarding the chemical nature of alarm cues in amphibian tadpoles, we investigated this feature in *R. arenarum* by treating the removing protein and lipid components of the homogenates (see above). The activity of tadpoles was not affected when they were exposed to homogenates that were heat-treated (100 ˚C) or chloroform extracted (untreated homogenate=0.28 ± 0.38 min vs thermic treatment=0.42 ± 0.28 min $p=0.982$, untreated homogenate vs chloroform extraction=0.06 ± 0.06 min, $p=0.886$). Therefore, we speculate that the alarm cue is a thermostable, non-lipid molecule. In contrast, the anti-predator response was significantly reduced when homogenates were pre-treated with Proteinase K (homogenate + PK = 3.12 ± 0.8 min, untreated homogenate = 0.28 ± 0.38 min, $p <0.001$) supporting the idea of a peptide identity. Since the alarm cue was thermostable, we favour that the cue is a small peptide, because large proteins with complex structures are usually susceptible to heat denaturation.

Information on the chemical nature of alarm cues is scarce and contradictory. Recent work on mass spectrometry has confirmed that an anion characterised by an m/z value of 501 is present in homogenates of larvae of the genus *Lithobates* the exact structure of the molecule is unknown, it was suggested that it is a sulfated steroid of approximately 26 carbon atoms (Austin et al., 2018). Those results coincide with the "steroidal" origin for the tadpoles' alarm cues postulated by Hrbáček (1950). However, other reports describe an anti-predator behaviour in tadpoles exposed to conspecific homogenates prepared in aqueous solutions (Marquis et al., 2004; Fraker et al., 2009; Hettyey et al., 2015; Crane et al., 2017). Additionally, our results in *R. arenarum* also argue against a lipid-based alarm cue because tadpoles exposed to homogenates previously subjected to chloroform extraction showed an anti-predator response as strong as that of tadpoles exposed to untreated homogenates, demonstrating that the alarm cue remained in the aqueous phase. Considering that volatility is a key feature of the molecules involved in chemical communication in the air, some authors have proposed that solubility plays a similar role in water. Supporting this assumption, it is very unlikely that the cues used for chemical communication in larvae of

anurans are large non-polar compounds such as steroids with low solubility in water. Based on this, other authors have postulated peptides as the main candidates in chemical communication in aquatic environments due to their high solubility in water (Wyatt, 2005) in agreement with our results.

In amphibians, there are a variety of bioactive peptides found in adult anuran skin cells, as well as in tadpoles. These peptides are stored in high concentrations and have various functions related to defence against parasites, immunity, and ectohormones (Giuliani et al., 2008). Fraker et al. (2009) characterised an alarm cue coming from the skin of larva*l L. clamitans* that consists of a mix of two components with different chemical properties, the combination of which triggers the anti-predator behavioural response. The components had a molecular weight less than 10 kDa and were not affected by thermal treatments or freezing, but were not extracted with chloroform. The LC-MS/MS analysis identified two small peptides as potential candidates for the alarm cues in *L. clamitans* (Fraker et al., 2009). In our case, preliminary chromatographic studies obtained from skin homogenates of *R. arenarum* tadpoles confirmed that it would be one or more peptides with a molecular weight close to 5 kDa (data not shown). In this regard, Crossland et al. (2019) identified in *Rhinella marina* tadpoles at least four compounds that caused strong avoidance responses and concluded that the alarm cue may involve a mixture of substances, but the identity of that chemical cue (or array of chemical cues) remains unknown.

In summary, the results in our work agree with the assumption that peptides are a solid candidate to participate in tadpole´s chemical communication, partially coinciding with Fraker et al.'s (2009) observations in tadpoles of other anurans. However, unlike what is proposed by Fraker et al. (2009), in *R. arenarum* the release of the alarm cues is not mediated by active secretion mechanisms but would be released by mechanical damage to the skin cells.

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