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Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis

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ABSTRACT

Many species assess predation risk through chemical cues, but the tissue source, chemical nature, and mechanisms of production or action of these cues are often unknown. Amphibian tadpoles show rapid and sustained behavioral inhibition when exposed to chemical cues of predation. Here we show that an alarm pheromone is produced by rapid tadpole skin cells, is released into the medium via an active secretory process upon predator attack, and signals predator presence to conspecifics. The pheromone is composed of two components with distinct biophysical properties that must be combined to elicit the behavioral response. In addition to the behavioral response, exposure to the alarm pheromone caused rapid and strong suppression of the hypothalamo–pituitary–adrenal (HPA) axis, as evidenced by a time and dose-dependent decrease in whole body corticosterone content. Reversing the decline in endogenous corticosterone caused by exposure to the alarm pheromone through addition of corticosterone to the aquarium water (50 nM) partially blocked the anti-predator behavior, suggesting that the suppression of the HPA axis promotes the expression and maintenance of a behaviorally quiescent state. To our knowledge this is the first evidence for aquatic vertebrate prey actively secreting an alarm pheromone in response to predator attack. We also provide a neuroendocrine mechanism by which the behavioral inhibition caused by exposure to the alarm pheromone is maintained until the threat subsides.

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Introduction

Many animals exhibit anti-predator, or alarm behavior when exposed to chemical cues of predation (Mullerschwarze et al., 1984; Lima and Dill, 1990; Dodson et al., 1994; Kats and Dill, 1998; Lima, 1998; Lin et al., 1998; Chivers and Mirza, 2000; Apfelbach et al., 2005; Wyatt, 2005; Thomas et al., 2006). These behaviors are species-specific, and include reducing activity (i.e., freezing, or behavioral inhibition), escape behaviors, shelter seeking, area avoidance, schooling, and colony defense (Wisenden, 2000; Apfelbach et al., 2005; Lamprecht et al., 2008). Informative chemical cues can originate from the predator (e.g., kairomones) or the prey, and prey may use combinations of chemicals in their assessment of predation risk (e.g. Schoeppner and Relyea, 2005; Richardson, 2006). Chemical cues of predation derived from the prey may be released incidentally as a result of tissue damage (and perhaps after digestion of prey by predators), or through active release as part of an anti-predator

mechanism (e.g., disturbance or alarm pheromones; Mullerschwarze et al., 1984; Chivers and Smith, 1998; Madison et al., 2002; Wyatt, 2005; Lamprecht et al., 2008). Here we use the term 'chemical cue of predation' as a general term to describe chemicals derived from prey that may be released actively or passively, and that signal to conspecifics the presence of a predator. We use the term 'alarm pheromone' to distinguish those chemical cues of predation that have been shown to be produced by specialized cells and/or to be released through a regulated secretory process, and that may induce anti-predator behaviors in prey such as escape behavior or freezing.

A common process by which vertebrate prey releases chemical cues of predation is through injury or consumption by a predator (i.e. damage-released cues, reviewed by Chivers and Smith, 1998). These chemical cues are derived from tissues and body fluids of prey, and are often considered to be unintentional information sources for conspecifics; i.e., the emitter does not intentionally signal the presence of a predator (e.g. hemolymph, Smith, 1992; Chivers et al., 1996; Acquistapace et al., 2005; but see Henderson et al., 1997; Cashner, 2004). Disturbed, but uninjured prey also releases chemicals while escaping from a predator or upon capture that act as predation cues for conspecifics; e.g., ammonium waste released by crayfish,

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amphibian tadpoles and fish (Hazlett, 1990; Kiesecker et al., 1999; Manteifel et al., 2005; Wisenden and Barbour, 2005) or skin secretions released by adult salamanders (Graves and Quinn, 2000). Ostariophysan fishes have specialized cells in their epidermis (club cells) that release an alarm pheromone when attacked by a predator (Smith, 1992). It is thought that release requires rupture of the club cells, although the possibility that these cells actively secrete alarm pheromone onto the skin surface cannot be ruled out. Limited biochemical evidence suggests that one component of the alarm pheromone may be the purine derivative hypoxanthine-3-*N*-oxide (Pfeiffer et al., 1985; Brown et al., 2000; Brown et al., 2001; Brown et al., 2003).

Chemical cues of predation cause rapid changes in behavior, but relatively little is known about the neural and physiological processes induced in vertebrate prey that underlie the behavioral responses. In fish, exposure to the putative alarm substance hypoxanthine-3-*N*-oxide leads to enhanced optical alertness, suggesting actions on the central nervous system (CNS) that influence visual acuity (Pfeiffer et al., 1985). In mammals, exposure to predators or predator odor causes behavioral inhibition (freezing behavior), activation of the neuroendocrine stress axis (the hypothalamus–pituitary–adrenal – HPA – axis) and correlated changes in CNS limbic circuitry associated with fear and anxiety (Figueiredo et al., 2003; Apfelbach et al., 2005; Masini et al., 2005; Thomas et al., 2006; Roseboom et al., 2007). The neuroendocrine stress response involves the release of corticotropin-releasing factor (CRF) from the hypothalamus, which stimulates secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, and ACTH acts on adrenal cortical cells to increase biosynthesis and secretion of glucocorticoids (i.e., corticosterone or cortisol), the primary vertebrate stress hormones (reviewed by Denver, in press). Glucocorticoids exert negative feedback at several points along the HPA axis. Glucocorticoids and CRF also have diverse actions on behavior and physiology, including locomotion, food intake, and energy utilization (Sapolsky et al., 2000; Crespi and Denver, 2004; Crespi and Denver, 2005).

Amphibian tadpoles reduce their activity level in response to chemical cues released by caged predators fed tadpoles (e.g., Relyea, 2001; Fraker, 2008a). The level of swimming activity of tadpoles is related to their probability of capture by predators (i.e., the higher the activity, the greater the probability of capture; Skelly, 1994), and reflects a trade-off between predation risk and foraging gain (Werner and Anholt, 1993). However, the tissue source and chemical nature of the chemical cue of predation, and whether it is simply a damage-released, unintentional information source or is actively secreted (i.e., an alarm or disturbance pheromone) is not known. In the current study we used the anti-predator behavioral response of tadpoles as an assay for perceived predation risk following exposure to chemical cues of predation. We investigated responses using tadpoles of two rapid anuran species, the wood frog (*Rana sylvatica*) and the green frog (*Rana clamitans*). These species represent two different life history strategies. In southern Michigan, wood frogs typically breed during late March in ephemeral ponds and their tadpoles metamorphose during June of the same year (Wellborn et al., 1996). Green frogs typically breed from early June through early August in semi-permanent, fishless ponds and overwinter at least once (Wellborn et al., 1996). The differences between the species' life history strategies should favor different anti-predator behavioral strategies (Anholt et al., 2000); this difference seems to be manifested in the duration of the behavioral response (i.e., behavioral inhibition) rather than the speed that it is expressed (M. Fraker, unpublished data). We determined the source of the chemical cue of predation, the means by which it is released, and its biophysical properties. We also analyzed activity of the tadpole HPA axis by changes in whole body corticosterone content following exposure to the chemical cue of predation, and we investigated a role for suppression of corticosterone in the expression of the anti-predator behavioral response.

Materials and methods

Animals

Four to five wood frog (*R. sylvatica*) and green frog (*R. clamitans*) egg masses were collected from several ephemeral and semi-permanent ponds at the University of Michigan's Edwin S. George Reserve (ESGR) near Pinckney, Michigan. The top predators in all of the ponds were invertebrates (i.e., *Anax* dragonflies). The egg masses were cultured in large plastic pools filled with well water that had been inoculated with phytoplankton and zooplankton. The pools were covered with shade cloth to keep predators from colonizing, and tadpoles were fed rabbit chow *ad libitum* (population density approximately 1–2 tadpoles/L). Once the tadpoles reached ~50–100 mg body weight (Gosner stages 26–28; Gosner, 1960), they were transported to the University of Michigan and housed in large holding tanks (172 L, population density 1–2 tadpoles/L) filled with charcoal-purified (dechlorinated), pH-adjusted tap water in a controlled environmental chamber at 21–23 °C on a 12L:12D photoperiod. Tadpoles were then used in experiments two to three days after transporting. Predator-naïve tadpoles were fed boiled spinach and pulverized rabbit chow *ad libitum*.

Predatory larval dragonflies (*Anax junius*) were also collected at the ESGR and housed individually in plastic containers filled with 500 ml dechlorinated tap water. A small piece of fiberglass screen was added to the container as a perch for the dragonfly larvae and they were fed 0.5–1.0 g *R. sylvatica* or *R. clamitans* tadpoles daily. All procedures involving animals were approved by the University of Michigan's University Committee on the Use and Care of Animals.

Behavioral assays

The proportion of time that tadpoles spent swimming vs. resting when exposed to different treatments was analyzed. In this assay, behavioral inhibition is indicated by reduced proportion of time spent swimming. For experiments 1–6, sets of 10 tadpoles were haphazardly selected and distributed among four-to-six 12H×16W×27L cm tanks (containing 3 L dechlorinated tap water) for each treatment (total number of replicates of each treatment is noted in description of the particular experiment). Tadpoles were placed in tanks the day before the behavioral assay was conducted and the tanks were randomly positioned within the environmental chamber to minimize microenvironmental effects. During each behavioral trial, the appropriate treatments were added to all replicate tanks within 5 min. The investigator then exited the chamber, and behavioral data were recorded using two to three CCD cameras mounted on racks over the tanks (each camera recorded a rectangular array of four tanks simultaneously). Recordings were initiated 30 min after the addition of treatments. In experiments with more than three treatments, two or three replicate tanks from each treatment were assayed in multiple blocks due to having only three cameras. In these experiments, treatment additions were delayed so that recording always began 30 min after addition of treatments. The time spent swimming by five haphazardly-selected tadpoles in each tank over a one minute period was measured using the MS-DOS computer program "Tadpole" (Van Buskirk and McCollum, 2000). The five tadpoles were chosen before viewing the recording to avoid bias. The mean proportion of time spent swimming for the tadpoles in each tank served as one replicate. For each experiment, the proportion of time spent swimming by each individual tadpole was log-transformed prior to statistical analysis. The data were analyzed by one-way ANOVA, followed by Scheffe's *post hoc* test, except for Expt. 10 (described below). The $P < 0.05$ criterion was used in the Scheffe's tests. Statistical analyses were conducted using SAS 9.1 software (SAS Institute, 2003).

Analysis of the nature and tissue source of a chemical cue of predation

Experiment 1 was designed to test 1) whether wood frog tadpoles respond behaviorally to water conditioned by a predator fed conspecific tadpoles, and 2) whether the chemical cue of predation is derived from the attacked/consumed tadpole (i.e., homogenizing – ‘macerating’ – the tadpole will release the cue).

The experiment had three treatments: control, predator-conditioned water, and tadpoles homogenized in water. There were four replicate tanks per treatment. For the control treatment we added 500 ml dechlorinated tap water to the tadpole tanks. For predator-conditioned water, *Anax* larvae were housed individually in containers with 500 ml dechlorinated tap water and fed ~0.25 g of live *R. sylvatica* tadpoles. Twenty to twenty four hours after feeding the *Anax* the screens were removed from the container, and the conditioned water strained with a 1 mm mesh net to remove *Anax* waste or tadpole remains. The conditioned water from four *Anax* was pooled, and each tadpole tank (4 replicate tanks per treatment) received 500 ml of predator conditioned water. For the tadpole homogenate treatment, on the day of a behavioral trial tadpoles (~1 g total biomass) were collected from the stock tank, deeply anesthetized by immersion in 0.1% benzocaine for 10 min, and then rinsed in a beaker of fresh water. They were then transferred to a testtube with 4 ml dechlorinated tap water and homogenized for 30 s using a Polytron PT-2000 homogenizer (Kinematica, Littau, Switzerland). The tadpole homogenate was divided into four equal aliquots and each was added to 500 ml dechlorinated tap water before being added to the tanks (~0.25 g tadpole biomass equivalent per tank). This allowed a similar biomass of tadpoles to be used in the predator-conditioned water and tadpole homogenate treatments.

Experiment 2 tested 1) whether the chemical cue of predation is released by living *R. sylvatica* tadpoles (i.e., an *Anax* simply eating and digesting a dead tadpole will not release the cue), and 2) whether the chemical cue is released by living tadpoles in response to a generic predatory event; i.e., its release does not require *Anax per se* or obvious tissue damage.

The experiment had four replicates of four treatments: control, predator-conditioned water (fed live tadpoles), predator-conditioned water (fed dead tadpoles) and water conditioned with poked tadpoles. The predators were fed as described in experiment 1, except that one group was fed tadpoles that were first euthanized by immersion in 0.01% benzocaine for 10 min. Tadpoles do not recover from this dose and duration of anesthesia and are thus euthanized by the treatment. In all cases tadpoles had been consumed by the *Anax* during the 20–24 h period before the experiment. Another treatment included in this experiment involved the addition of water conditioned by tadpoles poked with a hypodermic needle. For this treatment, one large tadpole (~1 g) was selected from the stock tank and submerged in 500 ml dechlorinated tap water within a net. A 26.5 gauge hypodermic needle was used to “poke” the tadpoles’ tail muscle five times for 10 s each poke (i.e. the needle remained in the tail muscle for 10 s). The tadpole remained submerged throughout this process and was subsequently released into the water. This procedure was repeated until a total of five tadpoles per container were held and poked in each of four containers. The tadpoles remained in the water for 20 min to condition the water until they were removed and the water added to the experimental tanks.

Experiment 3 tested whether the chemical cue is released via a classical stimulus-secretion coupled pathway. The focal species used for this experiment was *R. clamitans* owing to its availability and known behavioral response to predator chemical cue (Fraker, 2008a). We exposed tadpoles to 5 mM KCl for a brief period, then tested the conditioned water for a behavioral response by conspecifics. Exposure of cells to KCl is well known to cause plasma membrane depolarization, and the release of secretory vesicles via a calcium-dependent, stimulus-secretion coupled pathway.

Six sets of 10 small (30–50 mg) green frog tadpoles were immersed in 20 ml 5 mM KCl or 20 ml dechlorinated tap water for 15 min, then removed. The tadpole-conditioned KCl solution or dechlorinated tap water was then added to the tanks for behavioral assay. Two control treatments included 20 ml of 5 mM KCl or dechlorinated tap water (without tadpoles having been added) added to the tanks. Behavioral observations were made as described above.

Experiment 4 tested whether the chemical cue of predation is stored in membranous secretory vesicles that can be released upon homogenization in a detergent (Triton X-100) and sonication. The detergent serves to solubilize lipid membranes of secretory vesicles, and sonication uses high-frequency sound waves to promote tissue disruption.

The tadpole homogenate (*R. sylvatica*) was prepared as described in Experiment 1 (above) except that Triton X-100 was added to 4 ml dechlorinated tap water to a final concentration of 1%. The homogenate was then sonicated for 30 s using a Fisher Scientific Sonic Dismembrator, Model 100 (Fisher Scientific, Pittsburgh, PA, USA) and divided into four aliquots, each added to 500 ml dechlorinated tap water (~0.25 g tadpole mass equivalent per tank) which was then added to the assay tanks. The behavioral response resulting from these treatments was compared to a predator-conditioned water treatment as well as to a water only control. There were four replicates of each treatment.

Experiment 5 again tested whether the chemical cue of predation is stored in membranous secretory vesicles, but used both *R. sylvatica* and *R. clamitans* tadpoles as focal species. The experiment was conducted for each species separately.

The procedure used in Experiment 3 was repeated with an additional control group in which Triton X-100 was added to the experimental tanks to the same final concentration as with the Triton X-100 tadpole homogenate (0.0003% in the assay tank) to determine if the detergent alone influenced tadpole behavior. A predator-conditioned water treatment as described in Experiment 1 was also included. There were four replicates of each treatment.

Experiment 6 analyzed the tissue source of the chemical cue in *R. clamitans* tadpoles.

Ten tadpoles (~100 mg BW each) were euthanized in 0.01% benzocaine, then dissected into their dorsal skin, ventral skin, tail, cloaca, nares, and internal organs. Some dorsal or ventral skin was likely included with the cloacae and nares. The tissues were then homogenized, sonicated in water with 1% Triton X-100, and divided into four aliquots, as described above. The behavioral response of tadpoles exposed to each tissue homogenate was recorded as described above. Although there were differences in mass among the tissues, each tissue sample was derived from the same number of animals. Thus, if a specific tissue was the major source of the chemical cue then the activity would be detected in our assay.

Analysis of changes in HPA axis activity following exposure to the chemical cue of predation, and the role of suppression of the HPA axis in the expression of anti-predator behavior

The following experiments tested whether HPA axis activity was altered by exposure to the chemical cue of predation, and a possible role for corticosterone in behavioral inhibition. For experiments 7 and 8, tadpoles were haphazardly selected and distributed three to a tank (15H×9L×22W cm; containing 2 L dechlorinated tap water) with six replicate tanks for each treatment (6 treatments, 36 tanks total). For experiment 9, ten tadpoles were haphazardly selected and distributed among six 12H×16W×27L cm tanks (containing 2 L dechlorinated tap water) for each treatment. The tanks were shielded with opaque plexiglass to minimize stress caused by the investigator entering the room.

Experiment 7 tested whether exposure to the predator chemical cue caused a change in tadpole whole-body corticosterone content. *R. clamitans* tadpoles were exposed to the Triton X-100 tadpole homogenate for 1, 2, and 4 h, then euthanized by immersion in

0.01% benzocaine and snap-frozen in a dry ice/ethanol bath. The control treatment was an equal volume of dechlorinated tap water added to the tank (to control for disturbance effects), and animals were sacrificed at the 4 h time point. Six replicate pools of three tadpoles per pool were collected for each treatment. Tadpoles were extracted and whole body corticosterone content estimated by radioimmunoassay as described previously (Licht, 1983; Denver, 1998). Briefly, tadpoles were homogenized and extracted in ethyl acetate, and extracts were fractionated using thin-layer chromatography (TLC) on silica plates. The region of the TLC lanes containing the corticosterone was collected, and the corticosterone was extracted from the silica using diethyl ether. The extract was dried under nitrogen and resuspended in 0.02 M, pH 7.3 PBS-gelatin for RIA. The extraction efficiency averaged 30%. All samples were analyzed in a single radioimmunoassay, and the intra-assay coefficient of variation was <10%. The corticosterone antiserum was purchased from MP Biomedicals (Solon, OH, USA).

Experiment 8 tested for dose-dependent effects of predator chemical cue on tadpole whole-body corticosterone. *R. clamitans* tadpoles were exposed to different concentrations of the Triton X-100 tadpole homogenate for 4 h before sacrifice. The method used in Experiment 8 was followed, except that tadpoles were exposed to either dechlorinated tap water (control), 0.1 ml, 0.5 ml, 1 ml, or 5 ml of the homogenate (0, 0.1×, 0.5×, 1× or 5×, respectively). Three tadpoles were pooled for each replicate, for a total of six replicates per treatment.

Experiment 9 tested whether the decline in whole-body corticosterone content of tadpoles exposed to predator chemical cue is permissive for the expression of behavioral inhibition. This hypothesis

was tested by reversing the decline in endogenous corticosterone through the addition of corticosterone to the aquarium water with predator chemical cue and monitoring tadpole behavior.

R. clamitans tadpoles were used, and 6 replicates of 10 tadpoles per tank were held in 2 L with 50 nM corticosterone (ICN Biomedicals, Aurora, OH, USA) or dechlorinated tap water (control) for 1 h. The corticosterone was dissolved in 100% ethanol and added to the tanks to a final concentration of 50 nM (final concentration of ethanol 0.001%). Controls were exposed to the ethanol vehicle to the same final concentration. This dose of corticosterone was chosen based on previous studies in tadpoles and frogs that showed that it restores corticosterone to baseline in animals in which corticosterone was reduced by treatment with the corticosteroid synthesis inhibitor metyrapone (Glennemeier and Denver, 2002a,b; Yao et al., 2008). Triton X-100 tadpole homogenate was prepared as described in Experiment 3 and added to the control or corticosterone-treated tanks in aliquots of 20 µl every 15 min (a pilot study showed that this preparation of tadpole homogenate had >10 times the potency of the homogenates used in the previous experiments, thus a lower volume was added to the tanks). Ten minutes after each aliquot of homogenate was added, behavioral observations were made as described above. This process was repeated until 100 µl of homogenate had been added.

Tadpole behavior was analyzed using a repeated-measures general linear mixed-effects model (Proc MIXED), with exogenous corticosterone treatment as a fixed effect, and cue concentration as a repeated factor (Littell et al., 1996). The AIC goodness of fit values were used to determine which covariance structure to use. If a significant corticosterone treatment x cue concentration interaction existed,

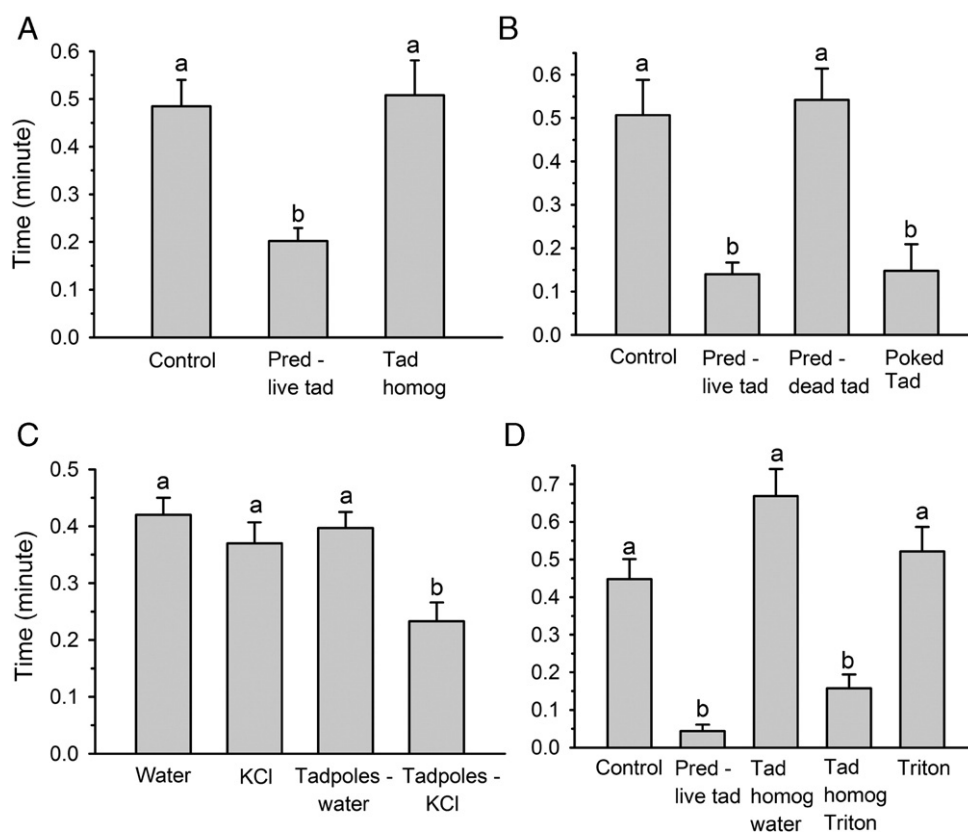


Fig. 1. A chemical cue of predation is produced by living ranid tadpoles and is released via a stimulus-secretion coupled pathway. The graphs show the mean time spent swimming of tadpoles exposed to different water treatments. (A) Tadpoles (*R. sylvatica*) reduced activity when exposed to predator-conditioned water (*Anax* fed tadpoles) but not by euthanized tadpoles homogenized in water. (B) Tadpoles (*R. sylvatica*) reduced activity when exposed to water conditioned by tadpoles poked with a hypodermic needle, but not by predators fed dead tadpoles. (C) Tadpoles (*R. clamitans*) reduced activity when exposed to water conditioned by tadpoles that had been immersed in 5 mM KCl, but not by tadpoles immersed in water alone or the KCl alone. (D) Tadpoles (*R. sylvatica*) reduced activity when exposed to a homogenate made with euthanized tadpoles in 1% Triton X-100, but not tadpoles homogenized in water alone. Similar results were obtained with *R. clamitans* (see Results). Experiments 1, 2, 3, 5 as described in the Materials and methods represented in panels A–D, respectively. Pred – predator; Tad – tadpole; homog – homogenate; Triton – Triton X-100. The bars show the mean \pm SEM; means with the same letter within an experiment are not significantly different ($P < 0.05$).

planned contrasts were performed for each cue concentration between the corticosterone-treated and non-treated tadpoles to identify differences in behavior at each cue concentration (using ESTIMATE and CONTRAST statements; Littell et al., 1996).

Biochemical purification and characterization of a chemical cue of predation

We attempted to purify and to characterize biochemically the chemical cue of predation. We used *R. clamitans* tadpoles as the focal species. Based on the results of Experiment 6 that showed the chemical cue was present in tadpole skin and tail, we harvested tadpole tails for extraction, purification and biochemical characterization of the predator chemical cue. Thirty tadpoles were euthanized by immersion in 0.01% benzocaine, then tails were removed, homogenized and sonicated in 5 ml water plus 1% Triton X-100. We first tested for behavioral responses to samples of the crude homogenate after either boiling for 10 min, freezing and thawing (3 times), or chloroform extraction; to remove lipid components, an equal volume of chloroform was added to the sample, vortexed and centrifuged at 2000 ×g for 10 min to separate and recover the aqueous phase from the organic phase. For each sample, replicated behavioral assays were conducted as described above.

We next size-fractionated the Triton X-100 tail homogenate by first centrifuging at 40,000 ×g for 30 min at 4 °C, then passing the supernatant through Microcon Ultracel YM-10 regenerated cellulose 10 kDa molecular weight cutoff centrifugal filters (Millipore Corp., Bedford, MA) at 18,500 ×g for 30 min at 4 °C to remove high molecular weight components. The flow-through was then run through a Sep-Pak Plus C18 column (Waters Corp., Milford, MA, USA) that was first activated with methanol, then washed with PBS, and eluted with 95% acetonitrile/0.1% trifluoroacetic acid (TFA) before testing in the behavioral assay.

We used reverse phase high performance liquid chromatography (HPLC) to further purify the Sep-Pak purified material from above. We loaded the sample on a 25 cm × 4.6 mm, 5 μm Supelcosil LC-318 HPLC column (Supelco, Bellefonte, PA, USA), ran a 10 to 90% gradient of acetonitrile + 0.1% TFA over 30 min (flow rate 1.5 ml/min) and collected fractions every 0.5 min. We removed the acetonitrile from the fractions through sublimation under a stream of nitrogen before testing in the behavioral assay. Fractions were tested individually or were pooled in different combinations; deionized water with 0.1% TFA and the crude homogenate were included in each behavioral assay as negative and positive controls, respectively.

We further analyzed two of the HPLC fractions by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). HPLC fractions were produced from a large homogenate sample (~400 tails), then lyophilized. Each HPLC fraction was reconstituted in 0.1% formic acid then subjected to trypsinolysis. The LC-MS/MS used a nanospray ion source interface and was conducted using an Ultra Performance LC-Quadrupole-Time of Flight Premier MS (Waters Corp., Milford, MA, USA). The spectra were analyzed using the program PEAKS (Bioinformatics Solutions Inc.) and the search engines Protein Lynx Global Server (Waters Inc.) and Mascot (Matrixscience Inc.) were used to search the SwissProt and NCBI databases.

Results

The source and nature of a chemical cue of predation

Wood frog tadpoles exhibited behavioral inhibition when exposed to water in which predators had consumed live tadpoles (Expt. 1; Fig. 1A; ANOVA: $F_{2,9} = 8.98$, $P = 0.002$). By contrast, tadpoles exposed to tadpole homogenate (euthanized tadpoles homogenized in water) did not change their activity level, which suggested that the chemical cue may be derived from the predator, or a metabolite generated by the

predator upon digestion of the tadpole. However, water conditioned with predators that had been fed dead tadpoles (which they ate) did not cause a change in tadpole activity level (Expt. 2; Fig. 1B; $F_{3,12} = 9.35$, $P = 0.002$), suggesting that generation of the chemical cue depends on the predator having consumed live tadpoles. Notably, the dragonfly predator could be eliminated entirely, as water conditioned by live tadpoles poked with a hypodermic needle (without tissue damage) generated the chemical cue (Fig. 1B).

Our finding that only predators fed live but not dead tadpoles, and tadpoles that were poked with a hypodermic needle released the chemical cue, suggested that the chemical may be released via a coupled stimulus-secretion pathway. To test this hypothesis we briefly exposed green frog tadpoles to 5 mM KCl to depolarize cell membranes and thereby activate cellular secretory processes (Expt. 3). This experiment showed that exposure of tadpoles to 5 mM KCl caused release of the chemical cue (Fig. 1C, ANOVA: $F_{3,12} = 4.70$, $P = 0.02$). Addition of KCl to control assay tanks to the same final concentration (0.033 mM) did not influence tadpole activity level.

Having established that the chemical cue that caused behavioral inhibition was derived from the tadpole, it was puzzling that tadpole homogenate did not elicit the behavioral response. We reasoned that if the chemical cue was packaged into secretory vesicles, then simple

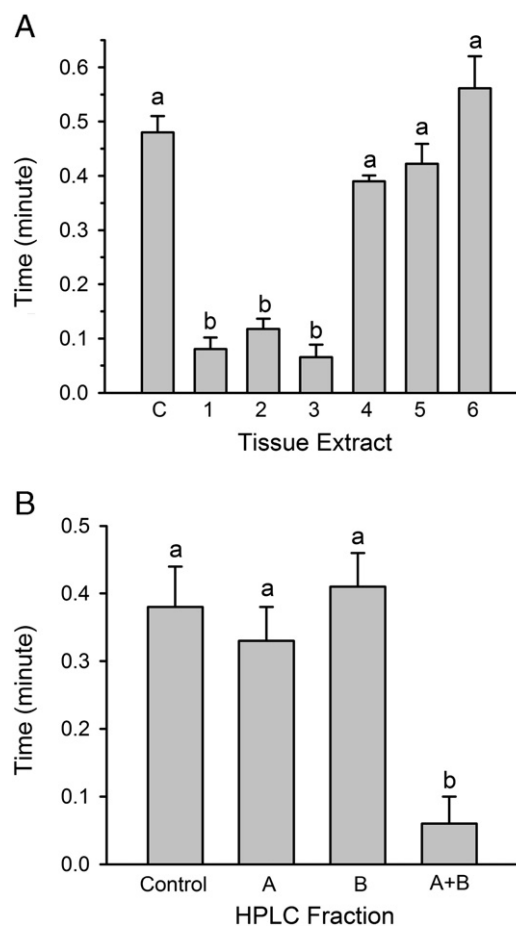


Fig. 2. The chemical cue of predation is derived from ranid tadpole skin and is comprised of two biochemically distinct components. (A) Mean time spent swimming of tadpoles exposed to homogenates of different body tissues. The focal species for this experiment was *R. sylvatica*, and tissues were homogenized in 1% Triton X-100 (see Materials and methods Expt. 6). C – control, 1 – dorsal skin, 2 – ventral skin, 3 – tail, 4 – cloaca, 5 – nares, 6 – internal organs. (B) Fractionation of tadpole tail extract by reversed phase HPLC identified two fractions (fraction A – 14.8–15.2% acetonitrile; fraction B – 52.5–53.0% acetonitrile) that must be combined to elicit a response in the tadpole behavior assay (see Materials and methods). The bars show the mean ± SEM; means with the same letter within an experiment are not significantly different ($P < 0.05$).

Table 1

Peptide sequences identified by LC-MS/MS after tryptic digestion of two HPLC fractions that, when combined, have predator chemical cue activity in the tadpole behavior assay

Fraction A	
TGPTK (100)	
HGAGKANHHGVPPP KPGSGK (58)	
SFMPPSF FGGYALAVMGHMKGPPHTKAAKSLPLLVDLSSDSR (89)	
FADHLVNKGVR (73)	
Fraction B	
VHSPATLGGSR (64)	
VLGVKASGTSK (69)	
TAHSR (67)	
SKYGAKVKLANPR (53)	
AFELLDQDKSGVAADHTHTGTQK (35)	
LVVVLPK (100)	
LASSAETAFLK (61)	
FLRWLPHLK (56)	

The confidence score for each sequence is given in parentheses.

homogenization in water might not release it. Therefore, we included the detergent Triton X-100 (1%) in the homogenization solution to solubilize cell membranes, and this resulted in the release of the predator chemical cue from tadpole tissues causing behavioral inhibition (Expt. 4; time spent swimming in minutes for control: 0.45 ± 0.04 ; predator fed live tadpole: 0.08 ± 0.02 ; tadpole homogenized in Triton X-100: 0.17 ± 0.04 ; $F_{2,9} = 45.74$, $P < 0.001$). The behavioral response was not caused by the Triton X-100 (Expt. 5, *R. sylvatica*; Fig. 1D; $F_{4,15} = 19.14$, $P < 0.001$).

Similar to the wood frogs, green frog tadpoles exhibited behavioral inhibition when exposed to predator-conditioned water (fed live tadpoles) and the tadpole homogenate prepared with Triton X-100, but not to the tadpole homogenate in water alone, or to Triton X-100 alone (Expt. 5, *R. clamitans*; time spent swimming in minutes for control: 0.25 ± 0.08 ; predator fed live tadpole: 0; tadpole homogenized in water: 0.23 ± 0.04 ; tadpole homogenized in 1% Triton X-100: 0.03 ± 0.03 ; Triton X-100: 0.24 ± 0.05 ; ANOVA: $F_{4,15} = 15.44$, $P < 0.001$). Green frog tadpoles were dissected and the tissues homogenized in water with 1% Triton X-100 before assay (Expt. 6). This showed that homogenates of dorsal skin, ventral skin, and tail caused behavioral inhibition, while homogenates of cloaca, nares and internal organs did not (Fig. 2A; $F_{6,27} = 21.42$, $P < 0.001$).

Biochemical purification and characterization of a chemical cue of predation

The activity of the chemical cue was not altered by boiling, freezing or extraction with chloroform, and the activity was present in the flow-through volume from a 10 kDa molecular weight cutoff centrifugal filter (data not shown). We next fractionated tadpole tail extract by reversed phase HPLC. None of the HPLC fractions tested individually had activity in the behavioral assay, but by recombining all fractions we could restore the activity (data not shown). We therefore tested a series of recombinations, and determined that when the fractions that eluted at 14.8–15.2% acetonitrile (fraction 'A') and 52.5–53.0% acetonitrile (fraction 'B') were combined, tadpoles reduced their activity level (Fig. 2B, ANOVA: $F_{3,12} = 16.39$, $P < 0.001$). Further analysis of these HPLC fractions by LC-MS/MS identified a series of small peptide components (Table 1). The best positive identifications were the TGPTK peptide in fraction A, and the LVVVLPK peptide in fraction B (Table 1). The presence of K or R residues at the C terminus suggests that each of these peptides is part of a larger polypeptide (trypsin cleaves at the basic residues K and R.). The presence of internal K residues in some of the peptides was likely due to incomplete trypsinolysis. None of the peptides produced compelling matches to known proteins in genome sequence databases.

Changes in HPA axis activity following exposure to the chemical cue of predation, and the role of suppression of the HPA axis in the expression of anti-predator behavior

We next tested whether the activity of the HPA axis is altered in tadpoles exposed to the chemical cue. We found that addition of the Triton X-100 tadpole homogenate caused a rapid, strong, sustained and dose-dependent reduction in whole body corticosterone content (Expt. 7; Fig. 3). Green frog tadpoles had whole body corticosterone content less than 30% of controls at 2 h, that remained low at 4 h (Fig. 3A; $F_{3,20} = 23.55$, $P < 0.0001$). In a separate experiment we found that wood frog tadpoles exhibited a similar decline in whole body corticosterone content when exposed to the Triton X-100 tadpole homogenate (data not shown). As before, both species became inactive within minutes of addition of the predator chemical cue to the aquaria and remained inactive throughout the exposure period (mean proportions of green frog tadpoles active: control = 0.48 ± 0.07 , 1 h = 0.04 ± 0.02 , 2 h = 0.02 ± 0.02 , 4 h = 0.02 ± 0.02).

Green frog tadpoles exhibited a dose-dependent decrease in whole-body corticosterone content when exposed to increasing concentration of the Triton X-100 tadpole homogenate (Expt. 8; tested at 4 h; Fig. 3B). A statistically significant decrease compared with controls was observed with the 1× dose, and a further decrease was seen with the 5× dose (ANOVA: $F_{4,15} = 7.62$, $P = 0.001$; Fig. 3B). A slight reduction in tadpole activity was observed with the 0.5× dose, and tadpoles became inactive in the 1.0× and 5.0× dose treatments (mean proportions of green frog tadpoles active: control = 0.45 ± 0.05 , 0.1× = 0.49 ± 0.04 , 0.5× = 0.36 ± 0.06 , 1× = 0.01 ± 0.01 , 5× = 0.00 ± 0.00).

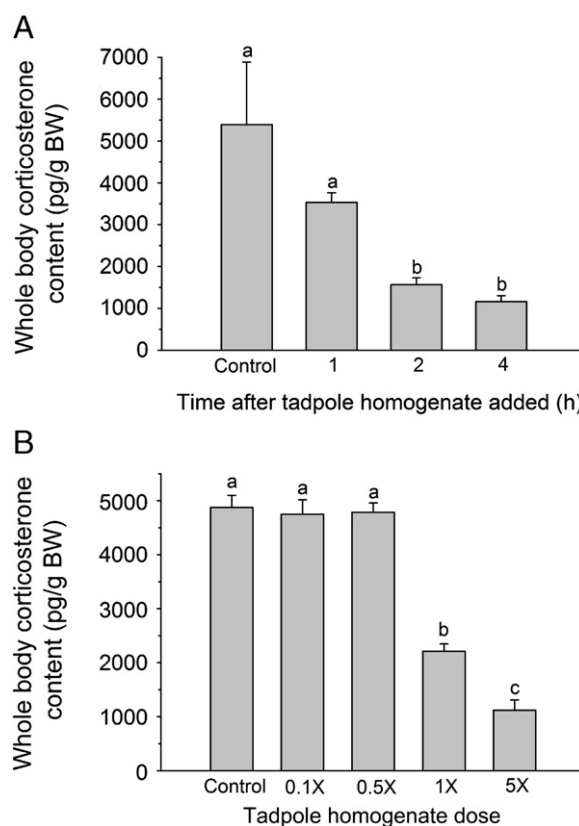


Fig. 3. Exposure of tadpoles to the chemical cue of predation decreases whole body corticosterone content in a time and dose-dependent manner. (A) *R. clamitans* tadpoles were exposed to the tadpole Triton X-100 homogenate and then sacrificed at different times for analysis of whole body corticosterone content (see Materials and methods Expt. 7). (B) The suppression of *R. clamitans* whole body corticosterone content increased with increasing dose of the tadpole Triton X-100 homogenate. Tadpoles were exposed to the homogenate for 4 h before sacrifice and analysis of whole body corticosterone content (see Materials and methods Expt. 9).

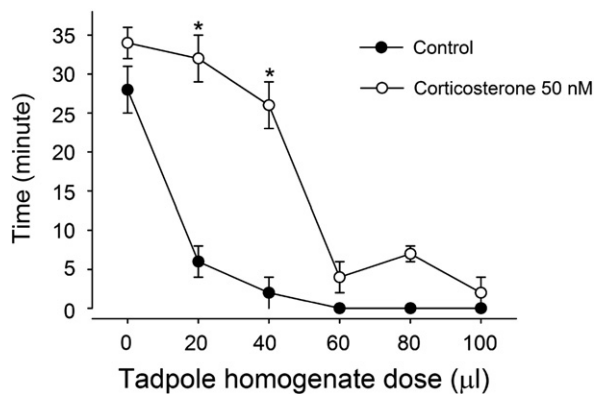


Fig. 4. Reversing the decline in endogenous corticosterone caused by exposure to the chemical cue of predation through treatment with corticosterone partially blocks the anti-predator behavior. Corticosterone was added to the tadpole aquarium to a final concentration of 50 nM. Controls received an equal volume of ethanol vehicle (final concentration 0.001%). Triton X-100 tadpole homogenate was added to the tanks in 20 µl aliquots at 15 min intervals (note that this homogenate preparation was more potent than that used in experiments shown in Figs. 1 and 3). Shown is the mean time *R. clamitans* tadpoles spent swimming \pm SEM. Asterisks indicate significant differences between the corticosterone treated and control groups at the indicated doses ($P < 0.05$).

The correlated, time- and dose-dependent decline in whole body corticosterone content with exposure to the chemical cue suggested that suppression of the HPA axis might be permissive for the expression and/or maintenance of a behaviorally quiescent state. We tested this by exposing tadpoles to increasing doses of Triton X-100 tadpole homogenate with or without a low dose of corticosterone (50 nM; Expt. 9). The addition of corticosterone to the aquarium water was intended to maintain endogenous corticosterone at a constant, basal level (see Yao et al., 2008). Corticosterone-treated tadpoles were more active compared with vehicle treated controls until the Triton X-100 tadpole homogenate dose reached $0.6 \times$ ($F_{5,70} = 5.33$, $P < 0.001$; Fig. 4).

Discussion

Ranid amphibian tadpoles release chemical cues when attacked by a predator that induce rapid, strong and sustained behavioral inhibition in conspecifics. Such anti-predator behavioral responses can provide immediate benefit through reduced predation risk, although the benefits may be traded off with reduced growth rate (Skelly, 1994; Benard, 2004; Relyea, 2007). Here we show that the chemical cue of predation is released upon attack and capture, but does not require extensive tissue damage for its liberation. It is derived from tadpole skin, and is composed of two biochemically distinct components, that may be peptides. In addition to the strong behavioral inhibition, exposure to the chemical cue caused rapid suppression of the neuroendocrine stress axis in conspecifics. Furthermore, the decline in whole body corticosterone content was permissive for the expression and maintenance of a behaviorally quiescent state. Taken together, our findings support the hypothesis that the chemical cue is released from ranid tadpole skin via a stimulus-secretion coupled process, causing rapid behavioral and physiological changes in conspecifics, and thus functions as an alarm pheromone.

Many aquatic animals show alarm reactions when exposed to chemicals released by injured conspecifics (Smith, 1992; Wisenden, 2000). Alarm responses in aquatic species typically involve freezing behavior, in which animals rest on the bottom of the pond or lake, refuge seeking behavior, and tighter schooling in fishes, among others (Smith, 1992; Wisenden, 2000). Alarm reactions in response to chemical cues of predation in amphibian larvae, although less studied than similar responses in invertebrates and fishes (Smith, 1992; Wisenden, 2000), have been known for some time (Pfeiffer, 1974; Hews and Blaustein, 1985; Hews, 1988; Kiesecker et al., 1999; Graves and Quinn, 2000). Like other aquatic prey, tadpoles generally exhibit

freezing behavior when exposed to predator chemical cues (Skelly and Werner, 1990; Relyea, 2001; Fraker, 2008a; but see Hews and Blaustein, (1985) and Hews, (1988), who reported that tadpoles increased activity when exposed to tadpole extracts). However, the tissue origin, chemical nature, and manner in which the chemical cues are released are poorly understood. We found that the chemical cue that predator-naïve wood frog and green frog tadpoles use to assess predation risk was produced when predators fed on living, but not on dead tadpoles (euthanasia achieved by deep anesthesia). This suggested that the cue was not generated by digestion of the prey by the predator, but instead was released by living prey at the time of capture. We discovered that the cue could be released by poking a living tadpole with a hypodermic needle, suggesting that significant tissue disruption is not required for release of the chemical cue. This conclusion is further supported by our finding that immersion in 5 mM KCl caused release of the chemical cue. Potassium chloride is commonly used to cause membrane depolarization in cell culture; it increases calcium influx leading to the fusion of secretory vesicles with the plasma membrane and exocytosis (secretion). Thus, our findings and conclusions contrast with the general assumption that tissue damage is required for the release of chemical cues of predation, and we propose that a simple capture event, with or without tissue damage, may be sufficient to induce release.

Several investigators have generated extracts of ranid and other anuran tadpoles and showed that they contained chemical cues of predation (Hews and Blaustein, 1985; Hews, 1988; Schoeppner and Relyea, 2005). In our initial attempt to make a similar tadpole extract we did not find such activity. We think that the explanation for this failure is that, unlike other investigators, we euthanized tadpoles by deep anesthesia before homogenizing. Since capture and tissue trauma causes release of the chemical cue, the act of sacrificing the animals without anesthesia may have caused release of the chemical and dissolution in the homogenization medium (Hews and Blaustein, 1985; Hews, 1988; Schoeppner and Relyea, 2005). This suggests that release of the chemical cue is dependent upon the animal being conscious (i.e., not anesthetized), which is also supported by our finding that live, but not dead tadpoles fed to predators generated the cue. The deep anesthesia induced by immersion in 0.01% benzocaine in our studies blocked neural activity, implying active release of the chemical mediated by CNS sensory processing.

Further support for active secretion of an alarm pheromone comes from our finding that, while the chemical cue was not liberated by homogenizing euthanized tadpoles in dechlorinated tap water, it was released upon homogenization with the detergent Triton X-100. The detergent, combined with sonication, served to disrupt membranous vesicles and thus cause release of their contents. Taken together with our finding that the cue is released upon membrane depolarization induced by immersion in KCl, we conclude that the alarm pheromone is contained within secretory vesicles and is released via a classical stimulus-secretion coupled process.

In searching for the tissue source of the alarm pheromone, we found that only homogenates derived from tadpole skin (dorsal, ventral, and tail), but not internal organs, cloaca or nares, induced behavioral inhibition. In our initial purification of the pheromone we found that it consisted of at least two components, both of which were < 10 kDa and were neither destroyed by boiling or freezing, nor removed by chloroform extraction. The alarm pheromone of another species, *Rana pipiens*, also appears to consist of at least two components (R.A. Relyea and J. Hempel, unpublished data). Analysis by LC-MS/MS analysis identified several polypeptides within these fractions, but these polypeptides were not found in our search of the available genome databases. Further study is necessary to determine the chemical identities of the two components that comprise the alarm pheromone.

Amphibians produce diverse bioactive peptides in their skins. These peptides are stored at a high concentration and function in

dermal physiological regulation, host defense, immunity and ectohormones (e.g., Bevins and Zasloff, 1990; Erspamer, 1994; Giuliani et al., 2008). While pheromones that are transported through air tend to be small volatile compounds, peptides, which are highly soluble in water, may be a primary form of chemical communication in aquatic systems (Wyatt, 2005). Small peptides, using different combinations of the twenty naturally occurring amino acids, have the potential for extraordinary diversity in their primary sequences, and are used as pheromones in amphibians and mammals (Kikuyama et al., 2005; Wyatt, 2005). Male newts produce decapeptides in their abdominal glands that are released into the environment, and function as sexual attractants, causing pronounced activation of the vomeronasal organ (VNO) epithelium and behavioral changes in the receiver (Kikuyama et al., 2005). The decapeptides of two closely related newt species differ by just two amino acids, which is sufficient to confer species specificity (sodefrin – *Cynops pyrrhogaster*; silefrin – *Cynops ensicauda*; Kikuyama et al., 2005). Schoeppner and Relyea (2005) demonstrated that different tadpole species emit species-specific chemical cues of predation (and also V. Cuddupah, S.A. McCollum and R.J. Denver, unpublished data). Another, recent example of peptides functioning as pheromones are the exocrine-gland-secreting peptides (ESPs) identified in mammals (Kimoto et al., 2007). These molecules are members of a multigene family coding for peptides of different lengths, that increase activity of VNO neurons and are presumed to function as pheromones, although their specific activities have yet to be elucidated (Kimoto et al., 2007).

That the alarm pheromone of ranid tadpoles is comprised of two (or more) components that must be combined for behavioral activity is similar to findings in ostariophysan fishes in which the alarm pheromone system may consist of a suite of compounds, one of which is hypoxanthine-3(N)-oxide (Pfeiffer et al., 1985; Smith, 1992; Brown et al., 2000; Brown et al., 2001). Multicomponent pheromonal systems have been described in different taxa, and the particular combination of compounds may confer species specificity on the response (Brown et al., 2003; Wyatt, 2005). The requirement for two or more components being combined to elicit a behavioral response in ranid tadpoles has implications for the specificity of the chemical signaling system, and the mechanisms by which it occurs. For example, the fact that the signal is comprised of two components supports that this is a specific, alarm pheromone, rather than a nonspecific damage-released cue. The requirement for two or more components may be a means to enhance species specificity, to increase the informational value of the signal and/or to facilitate transport of the pheromone. For example, in terrestrial mammals the activity, and possibly the specificity of small molecule pheromones may be enhanced through interactions with carrier proteins; i.e., small volatile molecules interact with mouse urinary proteins that influence the characteristics of the signal (Wyatt, 2005). For mechanism of action, if the components are comprised of two or more peptides they could form heterologomers, as may occur with anti-microbial peptides derived from frog skin (Giuliani et al., 2008). Such oligomerization could be required for receptor recognition, or for stabilization of the components of the complex forming the alarm pheromone. It is also possible that two or more VNO receptors must be activated by different compounds to generate behavioral and neuroendocrine responses.

While our results show that ranid tadpoles use alarm pheromones secreted by conspecifics to assess predation risk, a number of studies have demonstrated that tadpoles exhibit adaptive, predator-specific behavioral and morphological phenotypes (e.g. Relyea, 2001; Kishida and Nishimura, 2005; Teplitsky et al., 2005; Benard, 2006). These findings suggest that multiple chemical cues may be involved in the induction of a tadpole's overall phenotype (i.e. also predator kairomones). Additionally, more experienced tadpoles may use a wider range of information sources if associative learning has occurred (Suboski, 1992; Mirza et al., 2006).

Rapid suppression of the neuroendocrine stress axis by exposure to ranid tadpole alarm pheromone

Experiencing acutely stressful events such as a predator attack, or visual or auditory cues of predation cause rapid activation of the sympathetic nervous system and the HPA axis (i.e., the classical fight-or-flight response; Sapolsky et al., 2000; Canoine et al., 2002; Figueiredo et al., 2003; Remage-Healey et al., 2006; Bell et al., 2007; Roseboom et al., 2007). Similarly, exposure to predator odor (e.g., 2,5-dihydro-2,4,5-trimethyl thiazoline – TMT – from fox feces) activates the HPA axis in rodents as evidenced by increased plasma [corticosterone] (reviewed by Apfelbach et al., 2005; Thomas et al., 2006).

In contrast to these activational effects, we found a rapid and sustained decrease in whole body corticosterone content, which is reflective of a decline in activity of the tadpole HPA axis, caused by exposure to ranid alarm pheromone. In addition to the time-dependent decline in whole body corticosterone content, we also found that the level of suppression at a single time point (4 h) was dependent on the dose of the alarm pheromone; behavioral inhibition caused by the pheromone was also dose-dependent. This response of the tadpole HPA axis to alarm pheromone contrasts with responses to predator odors in rodents (discussed above), which could be related in part to the nature of the chemical cues and their origin; i.e., in rodents the chemical cue that activates the HPA axis is derived from the predator, while in the ranid tadpole the chemical cue that suppresses the HPA axis is derived from the prey (conspecifics). However, it is interesting that, although the changes in HPA axis activity are in opposite directions, the behavioral responses (i.e., freezing behavior) are very similar in mammals and amphibians. The metabolic actions of glucocorticoids are well known (Sapolsky et al., 2000), and the nature of the HPA response could relate to differences in life history strategy, and metabolic demands, which lead to large differences in behavioral capacities between endotherms and ectotherms (Bennett, 1980).

Given the strong suppression of the HPA axis in tadpoles, and the known roles for corticosteroids in increasing locomotory and feeding behaviors, we hypothesized that the suppression of the stress axis facilitated the expression and maintenance of the anti-predatory behavior. We tested this by replacing corticosterone in animals exposed to the chemical cue through the addition of a low dose of corticosterone to the tadpole's aquarium. Our results with green frog tadpoles show that exogenous corticosterone can block or reduce their anti-predator behavior, depending on the dose of the chemical cue; we obtained similar results with wood frog tadpoles (M. Fraker, unpublished data). Thus, our findings support the hypothesis that the suppression of the HPA axis caused by exposure to an alarm pheromone promotes the expression and maintenance of a behaviorally quiescent state in tadpoles under risk of predator attack. Future studies should examine the recovery of the tadpole HPI axis following release from predation risk, and whether a rebound in corticosterone production plays a role in the recovery of activity (i.e., feeding behavior; see Crespi and Denver, 2004).

The tadpoles used in our experiments were predator-naive and fed to satiation, and we measured their acute response to a single chemical cue exposure. Energetic state and prior experience can influence prey behavioral responses to subsequent chemical cue exposures (Semlitsch and Reyer, 1992; Brown et al. 2006; Fraker, 2008b; Fraker, 2009). Thus, in future studies it will be important to address whether the physiological and behavioral responses of tadpoles and other prey are affected by field conditions, such as chronic predation risk and food limitation.

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