Hormones and Behavior 55 (2009) 520–529

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

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

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ARTICLE INFO

Article history:
Received 17 November 2008
Revised 19 January 2009
Accepted 21 January 2009
Available online 30 January 2009

Keywords:
Amphibian
Tadpole
Anti-predator behavior
Corticosterone
Stress
Predation risk assessment
Skin secretion

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 ranid 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.

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 antipredator 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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doi:10.1016/j.yhbeh.2009.01.007

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 ranid 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 shadecloth 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-naive 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).