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Short communication

Acute changes in whole body corticosterone in response to perceived predation risk: A mechanism for anti-predator behavior in anurans?

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ABSTRACT

Anuran larvae exhibit behavioral and morphological plasticity in response to perceived predation risk, although response type and magnitude varies through ontogeny. Increased baseline corticosterone is related to morphological response to predation risk, whereas the mechanism behind behavioral plasticity remains enigmatic. Since tadpoles alter behavioral responses to risk immediately upon exposure to predator cues, we characterized changes in whole body corticosterone at an acute (<1 h post-exposure) timescale. Tadpoles (*Lithobates sylvaticus*) at Gosner stage (GS) 25 (free-swimming, feeding larvae) increased corticosterone levels to a peak at 10–20 min post-exposure to predator cues, paralleling the acute stress response observed among other taxa. Tadpoles reared for 3 weeks (mean GS29) with predation risk (caged, fed Aeshnid dragonfly nymph) had lower corticosterone levels at 10–20 min post-exposure to dragonfly cues than predator-naïve controls, suggesting habituation, although the magnitude of increase was markedly diminished when compared to younger tadpoles (GS25). These experiments represent the first assessment of tadpole hormonal responses to predation risk at the acute timescale. Further research is required to establish causality between hormonal responses and behavioral changes, and to examine how and why responsiveness changes over ontogeny and with chronic exposure to risk.

1. Introduction

Predators affect prey populations both directly, through injury and mortality, and indirectly, by altering demography through changes in behavior and reproductive output (Mateo, 2010; Zanette et al., 2011). The indirect effects of predation risk have been associated with changes in corticosterone (or cortisol) level typical of the vertebrate glucocorticoid stress response (Clinchy et al., 2013). Anuran larvae detect predation risk via chemical cues that are released when conspecifics are injured and consumed, and they respond to perceived predation risk via plasticity in morphology (Middlemis Maher et al., 2013) and behavior (Fraker et al., 2009). Physiological responses to predator cues are characterized by an initial decline in whole body corticosterone at one to 4 h post-exposure (Fraker et al., 2009), followed by a chronic increase in whole body corticosterone when predation risk is sustained over multiple days or weeks (Middlemis Maher et al., 2013). Exogenous application of corticosterone on a daily basis for 18 days (Glennemeier and Denver, 2002), or bi-daily basis to for 14 days (Middlemis Maher et al., 2013), causes increased tail muscle and tail fin depth in wood frog (*Lithobates sylvaticus*) tadpoles consistent with observed morphological responses to perceived predation risk (Glennemeier and Denver, 2002; Middlemis Maher et al., 2013). Chemically blocking corticosterone synthesis with metyrapone inhibits the morphological response to predation risk cues (Hossie et al., 2010; Middlemis Maher et al., 2013). Whole body corticosterone is also positively correlated with predator density in natural ponds (Middlemis Maher et al., 2013). Taken together, these studies provide strong evidence that morphological plasticity in response to predator cues in tadpoles, an indirect effect of predation risk, is mediated by activation of the glucocorticoid stress response.

In many amphibian species, behavioral and morphological responses to perceived predation risk occur in concert with each other (Relyea, 2003), yet the relationship between behavioral plasticity and the glucocorticoid stress response remains poorly defined (Neuman-Lee et al., 2015). Cessation of activity is a typical tadpole response to perceived predation risk (e.g., Shaffery and Relyea, 2015). In other vertebrates, freezing behavior is associated

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with activation of both the sympathomedullary and hypothalamic–pituitary–adrenocortical (-interrenal (HPI) in amphibians) systems (Hagenaars et al., 2014). Decreased locomotion is associated with increased plasma corticosterone in response to a known stressor in wild Norway rats (*Rattus norvegicus*; Plyusnina and Oskina, 1997), and elevated corticosterone is associated with tree lizard (*Urosaurus ornatus*) flight and hiding behaviors in response to a predator encounter (Thaker et al., 2009). While tadpole activity does not seem to be influenced by corticosterone as measured over several weeks (Glennemeier and Denver, 2002; Hossie et al., 2010), tadpoles typically alter behavior in response to changes in risk level immediately upon detection of risk cues (Fraker, 2008; Orizaola et al., 2012).

We know of no study that has examined the glucocorticoid response to predation risk cues in amphibian larvae at an acute (<1 h post-exposure) time scale. Since behavioral responses to predation risk occur immediately upon cue detection, and morphological responses to predation risk are mediated through the glucocorticoid response, it follows that glucocorticoid responses to predation risk at an acute time scale may be mediating behavioral plasticity. Thus, the objective of this study was to characterize acute corticosteroid response of an amphibian, the wood frog (*L. sylvaticus*), to predation risk cues from Aeshnid dragonfly nymphs, to which wood frogs are known to express pronounced behavioral and morphological anti-predator responses (Relyea, 2001, 2002; Middlemis Maher et al., 2013; Bennett and Murray, 2015). We predicted that perceived predation risk will cause an activation of the HPI system in tadpoles, and that whole body corticosterone will reach a maximum within approximately 30 min post-exposure, followed by a decline to baseline, as is documented in other vertebrate taxa (King and Berlinsky, 2006; Fuzzen et al., 2010). As whole body corticosterone has been shown to increase with chronic exposure to predation risk, we then examined how reactivity of the stress response may change after prolonged exposure, by acutely exposing naïve and predator-conditioned tadpoles to known predator cues.

2. Methods

2.1. Egg collection

Experiments were conducted using wood frog (*L. sylvaticus*) egg masses collected near Peterborough, Ontario, Canada (44°20′N 78°11′W) in the spring of 2012 ($N = 5$) and 2013 ($N = 2$). Predators (late-instar dragonfly nymphs, family: Aeshnidae) were collected from the same or nearby ponds. Egg masses were allowed to hatch in plastic bins filled with 10 L of aged tap water in an outdoor enclosure, and tadpoles used in the experiments represent a homogenous mix of clutches collected for that species in that year. Once tadpoles had reached Gosner stage 25 (free-swimming, feeding larvae; Gosner, 1960), they were brought indoors to environmental chambers in the Trent University Animal Care Facility and maintained at 20 ± 2 °C on a 12:12 light:dark cycle.

2.2. Experiment 1: Acute response to predation risk

Tadpoles were maintained in glass aquaria filled with 30 L of reconstituted reverse-osmosis (RO) water ($N = 30$ per aquarium) and fed *ad libitum* ground algae discs (Omega One Veggie Rounds™) while acute trials were conducted over the course of 1 week. Room temperature was kept at 20 ± 2 °C, on a 12:12 light:dark cycle. Predator cue water was created by housing four late-instar dragonfly larvae (Aeshnidae) in plastic floating breeding cages that were kept in a glass aquarium filled with 30 L of reconstituted RO water. Dragonfly larvae were fed three tadpoles each, twice weekly. Water taken from the predator tank within 24 h of feeding was considered 'predator cue' water (Laurila et al., 2004; Fraker, 2009). At the outset of an acute trial, tadpoles ($N = 3$) were placed in one of ten 1 L plastic containers filled with 750 mL of reconstituted RO water and allowed to acclimate for 1 h. After acclimation, 250 mL of either reconstituted RO water (control) or predator-cue water was added to each container. The first container was sampled immediately after water addition by removing all three tadpoles and euthanizing them with an overdose of tricaine methanesulfonate (MS-222), then tadpoles were flash frozen in centrifuge tubes placed in liquid nitrogen (total time 1–2 min). Containers were sampled every 10 min for a total trial run of 90 min. Trial runs were replicated three times in 5 days ($N = 3$ replicates of control trials, $N = 3$ replicates of predator trials). Samples were stored at −80 °C until extraction and radioimmunoassay (RIA) were performed.

2.3. Experiment 2: Acute response following chronic exposure to predation risk

To create chronically exposed (or control) conditions, tadpoles were reared over 3 weeks in 16 glass aquaria ($N = 30$ per aquarium) filled with 60 L of reconstituted RO water. To increase sample size and maximize available space in our experimental chambers, an additional four aquaria of equivalent density (60 L of reconstituted RO water containing 60 tadpoles each) were added in 2013. Aquaria were cleaned weekly by a half water change and tadpoles were fed ground algae discs (Omega One Veggie Rounds™) *ad libitum*. Half of the aquaria contained a plastic floating breeding cage housing a late-instar dragonfly larvae (Aeshnidae) that was fed three wood frog tadpoles twice weekly. After 3 weeks, acute trials were conducted in the same manner as previously described, with 2–3 tadpoles (mean GS 29.4 ± 0.3 std. err.) per 1 L container and the addition of dragonfly predator water. Samples were flash frozen in liquid nitrogen and then stored at −80 °C, with six replicates per treatment.

2.4. Corticosterone radioimmunoassay

Tadpoles were thawed, weighed, homogenized on ice for 30 seconds in 500 μL of dH_2O (Fisher Scientific Tissuemiser), then kept at −80 °C overnight. Homogenate was then sonicated on ice for 30 seconds at level 3 (Fisher Scientific Sonic Dismembrator, Model 100) and 300 μL of methanol was added to the mixture and vortexed for 30 seconds. The mixture was stored at −80 °C until extractions were performed, at which time homogenate was thawed and centrifuged at 4 °C at 1000 rpm for 15 min. We used dichloromethane for extraction (3 mL × 2); extracts were dried under N_2 at 36 °C, resuspended in 110 μL steroid diluent (MP Biomedicals, Orangeburg, NY, USA), and kept overnight at 4 °C before RIA (adapted from Newman et al., 2008a). Recovery rate was determined by adding 50 pg corticosterone to a subset of the samples ($N = 10$) before extraction.

Corticosterone levels were measured in duplicate with a double antibody RIA (MP Biomedicals, Orangeburg, NY, USA) as per published protocols, using tadpole extractions rather than avian plasma (Washburn et al., 2002; Edwards et al., 2013; Leshyk et al., 2013). The assay was conducted following the manufacturer's direction, with the exception that we diluted the 25 ng mL^{-1} standard by half with the steroid dilutant to produce a 12.5 ng mL^{-1} standard (Washburn et al., 2002), and was validated for wood frogs by serially diluting 110 μL extractions from three individuals and comparing values to a standard curve of known corticosterone concentrations. The serial dilution was parallel to the standard curve, indicating that no further steroid extraction was necessary (Newman et al., 2008b). Whole body