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The role of corticosterone and toxicity in the antipredator behavior of the Rough-skinned Newt (*Taricha granulosa*)

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ABSTRACT

A variety of mechanisms are responsible for enabling an organism to escape a predatory attack, including behavioral changes, alterations in hormone levels, and production and/or secretion of toxins. However, these mechanisms are rarely studied in conjunction with each other. The Rough-skinned Newt (*Taricha granulosa*) is an ideal organism to examine the relationships between these mechanisms because its behavioral displays and toxin secretion during a predator attack are well documented and readily characterized. While we found no direct relationship between antipredator behavior and endogenous levels of corticosterone (CORT), antipredator behavior was inhibited when exogenous CORT and adrenocorticotropic hormone (ACTH) were administered, resulting in high circulating concentrations of CORT, indicating that CORT may play a role in mediating the behavior. There was no correlation between the animal's toxicity and either CORT or behavior. The results of this study provide evidence that CORT plays an important, yet complex, role in the antipredator response of these amphibians.

1. Introduction

Perceiving predators and escaping predator attacks are considered highly stressful events and typically result in the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Cockrem and Silverin, 2002; Figueiredo et al., 2003). During this activation, glucocorticoids (GCs) are released into the blood stream from the adrenal glands. These GCs direct energy away from non-essential life-processes, such as reproduction, and mobilize energy stores in order to promote survival (Sapolsky et al., 2000). Often the intensity and duration of acute stressors can be gauged by the concentration of the glucocorticoid, corticosterone (CORT; Friedman et al., 1967). This release of CORT is considered to both aid in immediate survival as well as prepare for future stressful events, especially predator attacks (Wingfield, 2005).

While predator stimuli, such as visual (Cockrem and Silverin, 2002; Thaker et al., 2009a), olfactory (see review in Apfelbach et al., 2005; Fraker et al., 2009), or a combination of stimuli (Figueiredo et al., 2003) are a well-established source of stress, the interaction between antipredator behavior and the role of CORT has been poorly studied and provides conflicting evidence. In some cases, an acute increase in CORT is associated with a corresponding increase in antipredator behavior. For example, increased acute CORT concentration enhances antipredator behavior in tree lizards (*Urosaurus ornatus*; Thaker et al., 2009a,b) and marine iguanas (*Amblyrhynchus cristatus*; Berger et al., 2007). Conversely, experimentally increased maternal CORT concentration in snakes reduced antipredator behavior in offspring (Robert et al., 2009). Further, when CORT levels were experimentally elevated using dermal patches in both Red-legged salamanders (*Plethodon shermani*; Wack et al., 2013) and Allegheny dusky salamanders (*Desmognathus ochrophaeus*; Ricciardella et al., 2010), there was no change in locomotor behavior, which can be considered antipredator behavior.

When CORT synthesis is inhibited using drugs, such as metyrapone, which blocks the 11β-hydroxylation reaction (Dixon et al., 1985), the relationship between antipredator behavior and the possible mediator corticosterone becomes even more complex. While Thaker et al. (2010) found that application of metyrapone decreased antipredator behavior in lizards, Hossie et al. (2010) discovered no change in behavior associated with metyrapone's

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suppression of CORT in frogs. Therefore, utilizing metyrapone can help determine whether CORT is the primary contributor to controlling the behavior or if upstream/related hormones, such as adrenocorticotropic hormone (ACTH) or corticotropin releasing hormone control the behavior instead (Moore and Miller, 1984).

To further understand the role of CORT in behavior, application of the upstream hormone ACTH can be used to test adrenal sensitivity. There is a complex negative feedback loop and signaling, which regulates the secretion of CORT and resulting behavioral responses (Sapolsky et al., 2000). Therefore, targeting different points in this system will provide a more comprehensive understanding of the relationship between CORT and behavior. ACTH, secreted by the pituitary gland, directly stimulates the adrenal glands to secrete CORT (Wingfield, 2005).

Although amphibians have been the subject of a variety of studies examining the interaction between CORT concentrations and stressors, such as food restriction (Crespi and Denver, 2005), contamination (Hopkins et al., 1997), and captivity (Coddington and Cree, 1995), activation of the HPA axis due to predation as a potential stressor has only been examined in a handful of species, none of which produce potent antipredator toxin (Bliley and Woodley, 2012; Fraker et al., 2009; Hossie et al., 2010; Moore et al., 2005; Moore and Miller, 1984; Ricciardella et al., 2010; Wack et al., 2013). Amphibians provide an excellent model to analyze this interaction given that there are clearly defined and serialized behaviors associated with antipredator behavior (Brodie, 1977; Brodie et al., 1974).

The Rough-skinned Newt (*Taricha granulosa* Skilton; Caudata: Salamandridae) is a common caudate amphibian on the west coast of North America that utilizes two classic mechanisms for avoiding predation (Johnson and Brodie, 1975). They excrete a highly toxic substance, the neurotoxin tetrodotoxin (TTX), and exhibit this toxicity through aposematic coloring on their ventral side (Brodie, 1968). To display this coloring, newts utilize the unken reflex, in which their head and tail curve upwards and often touch each other (Fig. 1). This behavior effectively shows the majority of the ventral surface, and is instigated by predation or the perception of predation (Johnson and Brodie, 1975). Newts have highly variable levels of TTX (Hanifin et al., 1999), but the relationship between individual or population toxicity and the unken reflex has not been examined.

Tetrodotoxin is a highly potent, low molecular weight neurotoxin (Mosher et al., 1964), which inhibits propagation of action potentials by blocking the pore region of voltage-gated sodium channels in nerve and muscle tissue (Narahashi et al., 1967). TTX is found in a wide array of taxa, including both vertebrate and invertebrate groups (reviewed in Chau et al., 2011; Miyazawa and Noguchi, 2001). Though the ecological functions of TTX seem to be wide-ranging (Hwang et al., 2004; Ito et al., 2006; Matsumura, 1995; Zimmer et al., 2006), many species utilize TTX as an antipredator mechanism (Brodie, 1968; Williams et al., 2010). While the evolutionary origins of TTX are currently debated (e.g., Hanifin et al., 2002; Noguchi et al., 1986; Shimizu and Kobayashi, 1983), levels of this toxin are highly variable across populations (Hanifin et al., 2008) and ontogenetic stages (Gall et al., 2011). Theoretical and empirical data have shown positive relationships to aposematic coloration intensity and toxicity (Blount et al., 2009; Maan and Cummings, 2012). Behavioral signal (such as the unken reflex) duration or intensity have been examined, but may demonstrate a similar relationship.

We tested the interaction among different phases in the HPA axis response, the unken reflex duration, and basal TTX levels. We hypothesized that increased unken reflex time would result in a higher concentration of CORT because the newt felt threatened for a longer period of time. We hypothesized newts would display honest signaling, thus the duration of unken reflex would be positively related to the amount of basal levels of TTX. Additionally, we predicted that endogenous CORT levels and adrenal sensitivity (measured by CORT levels after ACTH injection) would be related to unken reflex time. To test these hypotheses, we conducted three experiments: (1) compare CORT levels in animals experiencing a simulated predator attack (stimulated) to animals only experiencing handling in the absence of predator simulation (control), (2) in stimulated animals, measure relationships between endogenous levels of CORT, time spent in unken, and TTX levels, and (3) determine endocrine mechanisms involved in antipredator behavior by injecting exogenous CORT, ACTH, or metyrapone and measuring endogenous CORT levels and time in unken.

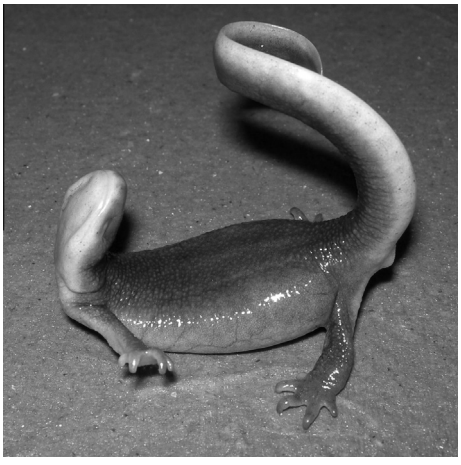

Fig. 1. Antipredator behavior of the Rough-skinned Newt (*Taricha granulosa*). This behavior, known as the unken reflex, allows the newt to display its bright orange ventral surface. Photograph by Edmund D. Brodie, Jr.

2. Materials and methods

2.1. Study animals

Newts were collected by hand, dip net, and minnow trap from Benton and Curry Counties, Oregon, USA. All newts were housed individually in temperature and light-controlled chambers (14 °C; 12 h on:off cycle). Newts were weighed to determine body condition. For all experiments, all individuals were females in non-breeding condition. For experiment 1A and 1B, we randomly assigned 35 newts to two different treatment groups. Ten animals received no predator simulation, while 25 animals were subjected to dorsal stimulation (described below). Animals in experiment 1 were held in captivity for >1 year. For experiment 2, we randomly divided a separate set of 77 individuals into one of four treatment groups (described below). Animals in experiment 2 were held in captivity for 9 months prior to testing. All procedures were approved by Utah State University IACUC (Protocol #1524).

2.2. Experiment 1A: relationship between CORT and stimulation

We removed all of the newts from the temperature-controlled chamber and conducted the experiments in a separate laboratory (approximately 24 °C). To ensure that any change in CORT was associated with predator stimulation and not associated with removing newts from their chamber and handling them, we divided the first set of animals into a control group ($n = 10$) that were removed from their enclosure but not stimulated (described below in *Behavioral analysis*) and an experimental group ($n = 25$). Regardless of whether or not the animal was stimulated, blood samples

were taken 30 min after removal from the chamber and analyzed (collection and analysis described below).

2.2.1. Behavioral analysis

Newts were stimulated (within one minute of removing animal from chamber) to enter the unken position by light tapping of forceps mid-dorsally to simulate a bird predator attack (Johnson and Brodie, 1975). We documented the time from the newt's initiation of the unken posture to the time it left the unken posture (determined by the newt taking one step with a front foot). Regardless of how long the newt remained in unken, we took a blood sample 30 min after initial stimulation via the caudal vein. Heparinized capillary tubes were used to collect approximately 40 μl of blood. All of the blood samples were immediately centrifuged to separate the plasma and red blood cells. The samples were stored at −20 °C until further analysis (see below).

2.2.2. Hormone analysis

For all experiments, we quantified circulating hormone levels using a previously described and established radioimmunoassay (RIA) protocol with a slight modification (French et al., 2010). Extractions were performed using a solution of 30% ethyl acetate:isooctane. Following extraction, we dried the samples, resuspended them in PBS buffer, and assayed in duplicate for CORT (H^3 Fitzgerald 20-CR45, Lot #P0012502; Antibody—MP Biomedicals, LLC, Lot 3R3-PB). Antibody cross-reactivity for corticosterone is 100%, with the next highest cross-reactivity occurring at 2.3% with desoxycorticosterone. All other cross reactivities are <0.05%. For each sample we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and chromatography. These recoveries are used to adjust the final sample concentration values to account for any losses during these procedures. Interassay variation was 11.6% and precision was 93.6%.

2.3. Experiment 1B: TTX toxicity, endogenous CORT concentrations, and time in unken

2.3.1. Tetrodotoxin analysis

For the 25 stimulated and 10 non-stimulated animals, skin biopsies for quantification of TTX were taken following established methods for TTX quantification (Hanifin et al., 2002). Newts were anesthetized using 1% ms222 (Tricaine-S, Western Chemical Inc., Ferndale, WA) to take 3 mm skin-punch biopsies. All biopsies were taken at least one month prior to any experimental trials described here and all wounds were completely healed at time of trials. Tetrodotoxin was extracted from skin tissues using the methods of Hanifin et al. (2002). Quantification of TTX was done using a Competitive Inhibition Enzymatic Immunoassay (Stokes et al., 2012). We used the linear range of the standard curve, which was between 10 and 500 ng/mL, to calculate sample values. Samples were diluted 1:120, 1:300, 1:500, 1:800 or 1:1000 in a 1% Bovine Serum Albumin–PBS solution (BSA) to get them within the linear range of the standard curve. These samples were run against standards also diluted in BSA. Whole newt TTX was calculated using the methods of Hanifin et al. (2002). The average intra-assay variation on each plate was between 4.81% and 7.94%, and the inter-assay variation was 6.68%.

2.4. Experiment 2: effects of exogenous CORT, adrenal sensitivity (ACTH), and metyrapone on antipredator behavior

Seventy-seven animals were randomly assigned to four treatment groups. Each group was randomly divided into two different environmental chambers. Each animal was injected with 100 μl of the following mixed with amphibian ringer's solution (Carolina Biological Supply Company, Burlington, VA): 20 pg CORT (Sigma Aldrich, Inc, St. Louis, MO, USA; $n = 17$), 50 pg ACTH (porcine, Sigma Aldrich, Inc, St. Louis, MO, USA; $n = 20$), 0.4 mg Metyrapone (Sigma Aldrich, Inc, St. Louis, MO, USA) + 20 pg CORT ($n = 20$), or ringer's solution alone ($n = 20$). Injections were given by the same person and animals were then placed back into their enclosures for 60 min.

After 60 min, each animal was retrieved and stimulated to enter unken. The time each newt spent in unken was recorded. Thirty minutes after initial stimulation (90 min after the injection), we collected blood as described above. Blood was collected, processed, stored, and assayed as previously described. No skin biopsies were removed in the second set of animals used in Experiment 2.

2.5. Statistical analyses

We analyzed the mass of the newts for each treatment group (for all experiments) to ensure that there were no statistical differences. For experiment 1A, we log-transformed CORT concentration and TTX concentration to meet the assumptions of normality. We analyzed both of these variables by treatment using a *t*-test. For experiment 1B, we regressed time spent in unken with both log-CORT concentration and logTTX concentration. For experiment 2, we were unable to transform either CORT concentration or time in unken to meet assumptions of normality, so we used nonparametric measures. To compare time in unken and CORT concentrations among the treatment groups, we used a Kruskal–Wallis test. Our *a priori* hypotheses stated that we anticipated differences between treatment groups (not just overall), thus post hoc multiple comparisons were determined using the Wilcoxon Test. Further, we used Spearman rank correlations to determine the relationship between CORT and unken for each group. We were unable to compare newts between experiments 1 and 2 because individuals in experiment 2 were given injections, and time from initial stressor and time in captivity varied. We set our significance level at $\alpha = 0.05$. All statistics were performed using JMP 8.0.1 (SAS Institute Inc., 2009).

3. Results

3.1. Experiment 1A

CORT concentration was significantly higher for newts in the stimulated treatment compared to the non-stimulated treatment group ($t = 2.583$, $df = 11.412$, $p = 0.0124$; Fig. 2).

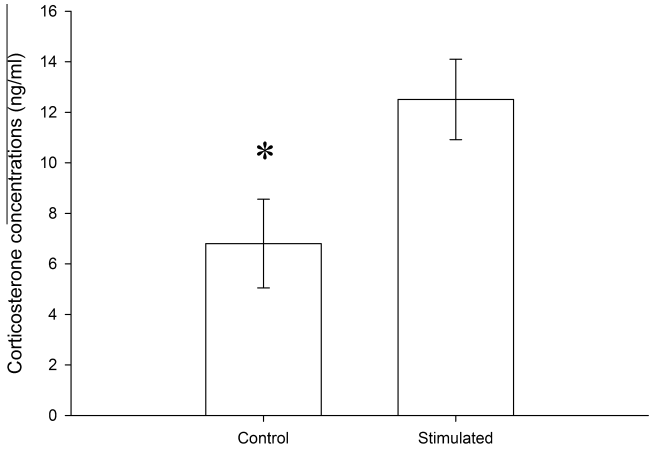

Fig. 2. Experiment 1A—corticosterone levels in the stimulated group compared to the non-stimulated (control) group ($t = 2.583$, $df = 11.412$, $p = 0.0124$).

Table 1
Experiment 1A and 1B—mass, corticosterone concentrations, tetrodotoxin concentrations, and time in unken reflex for *Taricha granulosa* (values are means ± SE).

Treatment	Mass (g)	Corticosterone (ng/ml)	Tetrodotoxin (ng/ml)	Time in unken (s)
Control (n = 10)	13.21 ± 0.58	6.80 ± 1.76	8.72 ± 4.27	–
Stimulated (n = 25)	13.67 ± 0.37	12.51 ± 1.59	8.36 ± 5.43	497 ± 93.76

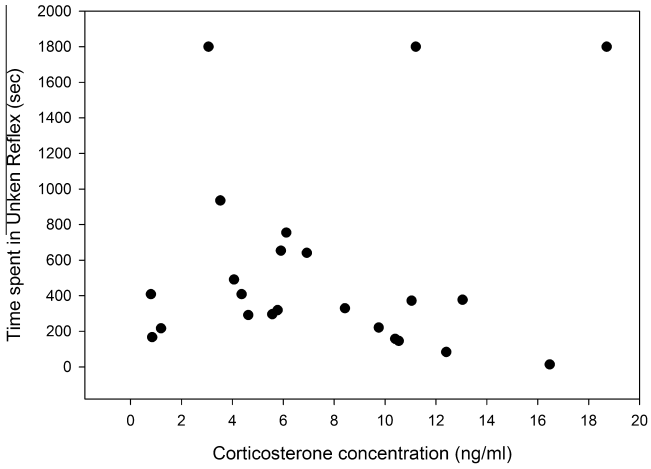

Fig. 3. Experiment 1B—time spent in unken compared to corticosterone concentration 30 min after stimulation in Rough-skinned Newts (R^2 = 0.02, p = 0.48).

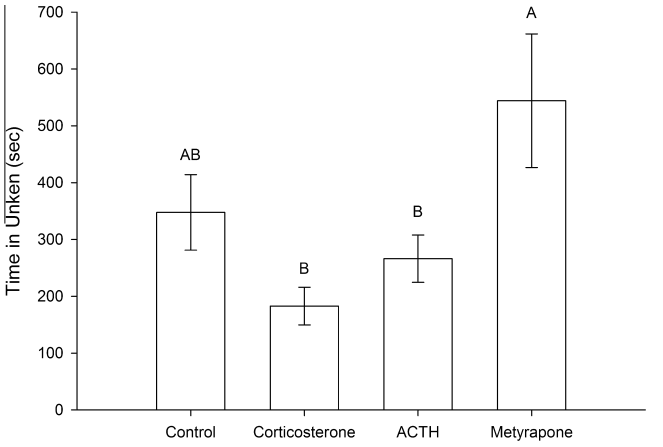

Fig. 5. Experiment 2—time spent in unken in four treatment groups (control, CORT, ACTH, metyrapone) 30 min after stimulation. Different letters indicate significance (p < 0.05).

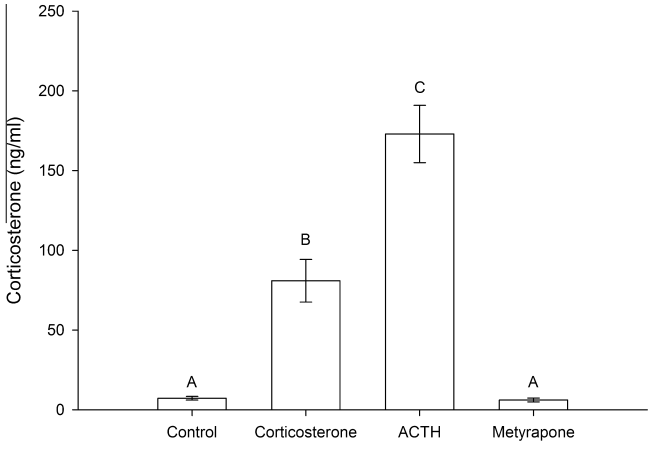

Fig. 4. Experiment 2—corticosterone levels in four treatment groups (control, CORT, ACTH, metyrapone) 30 min after stimulation. Different letters indicate significance (p < 0.05).

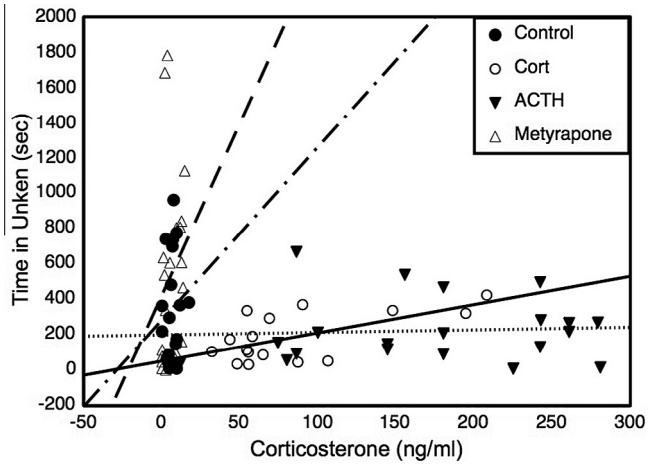

Fig. 6. Experiment 2—corticosterone concentrations compared to time in unken for each of the four treatment groups (control, CORT, ACTH, metyrapone).

3.2. Experiment 1B

All experimental newts displayed the full unken reflex after stimulation (lifting head and tail >90° above ground, Fig. 1) and the mass of newts did not differ between treatment groups (t = 0.660, df = 16.71, p > 0.25). Tetrodotoxin levels did not differ between treatment groups (t = 0.243, df = 14.42, p = 0.81; Table 1), and there was no relationship between time in unken and TTX concentration (R^2 = 0.009, p > 0.5). Further, time in unken reflex was not correlated with CORT concentrations (R^2 = 0.02, p = 0.48, Fig. 3).

3.3. Experiment 2

ACTH and CORT treatment groups had significantly higher levels of CORT than other treatment groups (X = 56.12, df = 3, p < 0.0001, Fig. 4). Thus, metyrapone was effective at blocking CORT response. There was no overall difference in time spent in unken among the treatment groups (X = 5.977, df = 3, p = 0.113), but the CORT and ACTH treatment animals spent significantly less time in unken when compared to the metyrapone treated animals (p < 0.05, Fig. 5). When time in unken was compared to CORT levels within each treatment group, CORT-injected animals demonstrated a positive linear relationship (r = 0.643, p = 0.05), while there was no relationship between the variables in the other three treatment groups (r < 0.20, p > 0.4; Fig. 6).

4. Discussion

The results of our study indicate that exogenous, but not endogenous, CORT may have a suppressive effect on antipredator behavior in newts. However, we found no detectable relationship between the time in unken and levels of the defensive toxin TTX.

Our first study demonstrated the dramatic increase in circulating CORT levels between the control and stimulated animals, indicating that this simulated predator challenge represents an acute stress for these organisms. An acute increase in glucocorticoids (GCs) in response to an unpredictable event, such as a predator attack, can be highly beneficial to an organism (Dhabhar, 2002, 2009; Sapolsky et al., 2000). The increase in GCs can augment the immune system and shift energy resources from non-essential life processes (i.e., reproduction, digestion) to critical ones (i.e., increased cerebral blood flow, increased glucose metabolism; French et al., 2007; Sapolsky et al., 2000), including coping with any consequent wounds from the predator. Acute increases in GC levels also augment memory formation and have a myriad of effects on neuronal processes, which may be critical to avoiding subsequent predator attacks (de Kloet et al., 2008; Sapolsky et al., 2000; Thaker et al., 2010).

Endogenous levels of CORT did not predict time in unken, but in the treatment groups with CORT or ACTH applied there was a suppressed antipredator response and thus with significantly higher levels of circulating corticosterone, unken time was significantly reduced. This may indicate that wild animals already exposed to a stressful situation that have elevated CORT levels may not remain in unken for as long. Given the myriad of other potential dangers for these newts, suppression of this behavior may be beneficial. If, for example, an animal has been repeatedly attacked and has elevated CORT, it may be advantageous to abandon the unken posturing and physically escape a predator. Indeed, Lima and Bednekoff (1999) proposed that organisms may alter their antipredator behavior according to temporal changes in risk. Elevated CORT, in this case, may be a mediator of evaluating the risks between different antipredator strategies (escape or go into unken). It is important to note that levels of measured CORT in experiment 2 were higher than those in experiment 1 and in previously published work (Moore and Zoeller, 1985). These particular levels may not be ecologically relevant in ACTH or CORT animals, however time in captivity, the injection, and increased time from first perception of stressor (injection) may have contributed to the increase and no direct comparisons can be made.

While there is a relationship between these high exogenous CORT levels and time in unken, we cannot make conclusions as to how the two are linked. One puzzling finding is the high positive correlation between CORT levels and unken time in the exogenous CORT treatment group. We see an overall decrease in unken time in the exogenous CORT group when compared with the metyrapone and control groups, but the higher levels of CORT are closely associated with longer unken time. In this case, increased levels of CORT may increase the time that the newt stays in unken or the increased duration of the antipredator behavior may cause an increase in CORT concentrations. This complex relationship may be an example of a positive feedback loop, wherein the increases in CORT levels from the stimulation create a more intense and prolonged response, which, in turn, increases the CORT levels. This positive feedback loop, also known as the "Challenge Hypothesis," has been well-studied and described in testosterone and male/male aggression during reproduction in birds (Wingfield et al., 1990). Interestingly, we did not see this relationship between the circulating CORT levels and unken in the ACTH treatment group. This may be due to the much higher levels of CORT in the ACTH group, which may be sufficient to trigger the negative feedback in the HPA axis (Sapolsky et al., 2000).

There are many factors which evidently alter the relationship between CORT and antipredator behavior, making it highly context-dependent. Individuals may physiologically respond differently depending upon seasons (Ricciardella et al., 2010), reproductive state (Hubbs et al., 2000), previous experience (Sandi et al., 1996), duration of exposure (Øverli et al., 2004), and type of stimulus (Bliley and Woodley, 2012). This context-dependence may be a result of the role of other components of the HPA response, such as changes in corticosteroid binding globulin, a plasma binding steroid (Breuner and Orchinik, 2002; Breuner et al., 2013), expression of membrane receptors (Moore and Orchinik, 1994; Orchinik et al., 1991), and/or the actions of other hormones (e.g., norepinephrine; Morilak et al., 2005).

Tetrodotoxin levels were not predictive of time spent in unken. However, the skin biopsies were taken prior to stimulation and so represent basal TTX concentrations. While there was no relationship between basal TTX levels and either of the other antipredator mechanisms, the amount of TTX released after stimulation is possibly variable and should be examined.

Therefore, there may be a relationship between TTX secretion and time spent in unken that was not detected. However, most work with honest signaling has been conducted with aposematic coloration and toxicity, not behavioral duration, and still no clear pattern has been established (Blount et al., 2009).

Our study presents preliminary evidence that CORT plays an important role in the antipredator behavior of the Rough-skinned Newt, although the direct mechanism linking the two is yet unclear. The relationship between antipredator behavior, toxicity, and glucocorticoids is evidently complex. Given that this behavior is critical to fitness, the relationship between the evolution of this behavior, toxicity, and endocrinological mechanisms should be further examined.

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