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Exposure to coal combustion residues during metamorphosis elevates corticosterone content and adversely affects oral morphology, growth, and development in *Rana sphenocephala*

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ABSTRACT

Coal combustion residues (CCRs) are documented to negatively impact oral morphology, growth, and development in larval amphibians. It is currently unclear what physiological mechanisms may mediate these effects. Corticosterone, a glucocorticoid hormone, is a likely mediator because when administered exogenously it, like CCRs, also negatively influences oral morphology, growth, and development in larval amphibians. In an attempt to identify if corticosterone mediates these effects, we raised larval Southern Leopard Frogs, *Rana sphenocephala*, on either sand or CCR substrate and documented effects of sediment type on whole body corticosterone, oral morphology, and time to and mass at key metamorphic stages. Coal combustion residue treated tadpoles contained significantly more corticosterone than controls throughout metamorphosis. However, significantly more oral abnormalities occurred early in metamorphosis when differences in corticosterone levels between treatments were minimal. Overall, CCR-treated tadpoles took significantly more time to transition between key stages and gained less mass between stages than controls, but these differences between treatments decreased during later stages when corticosterone differences between treatments were greatest. Our results suggest endogenous increase in corticosterone content and its influence on oral morphology, growth and development is more complex than previously thought.

1. Introduction

Anthropogenic pollutants have been suggested to be contributing to global amphibian population declines (Carey and Bryant, 1995). One way pollutants may contribute to declines is via disruption of physiological processes (Carey et al., 1999). Several studies have found that the amphibian stress axis can be influenced by exposure to fungicides, herbicides, and insecticides (Gendron et al., 1997, Cheek et al., 1999; Glennemeier and Denver, 2001; Hayes et al., 2006). Fewer studies have addressed how byproducts of industrial activities influence the amphibian stress axis.

Coal combustion residues (CCRs) have become major global pollutants due to the increased use of energy derived from coal burning power plants worldwide (Hopkins et al., 1999). In 2004, half the electricity in the United States was generated from coal and CCR production tripled that produced in 1970, a total of 111 million tons (United States Geological Survey, 2005; United States Department of Energy, 2007). Approximately, one third of CCRs are disposed of in aquatic settling basins (Electric Power Research Institute, 1997), which are accessible to local wildlife, including amphibians, and contain elevated concentrations of roughly 20 trace elements, several of which (e.g., Se, As, Cd) are high enough to be of toxicological concern (Hopkins et al., 1998; Rowe et al., 2002). Several studies have documented detrimental effects on behavior, morphology, physiology, growth, and development in anurans as a result of exposure to CCRs (Hopkins et al., 1998, 2000; reviewed in Rowe et al., 2002).

Although many studies have observed how CCRs can influence oral morphology, growth, and development during metamorphosis of anurans, to our knowledge, no studies have attempted to document what physiological variables may mediate these changes. Amphibian metamorphosis is primarily regulated by thyroid hormone, but glucocorticoids, such as corticosterone, also induce effects on oral morphology, growth, and development during metamorphosis (Frieden and Naile, 1955; Kobayashi, 1958; Kikuyama et al., 1983; Gray and Janssens, 1990; Hayes et al., 1993; Kikuyama et al., 1993; Wright et al., 1994; Hayes, 1995; Hayes et al., 1997; Denver et al., 2002; Glennemeier and Denver 2002; Belden et al., 2005). Adult male Southern Toads (*Bufo terrestris*) exposed to CCR-contaminated areas at the Savannah River Site, South Carolina exhibited significant increases in plasma corticosterone compared to controls (Hopkins et al., 1997). Because exposure to CCRs alters corticosterone secretion in adult toads and both exogenous corticosterone and CCR exposure induce effects on metamorphosis in tadpoles, CCRs may influence corticosterone secretion which can then mediate changes in oral morphology, growth, and development during metamorphosis.

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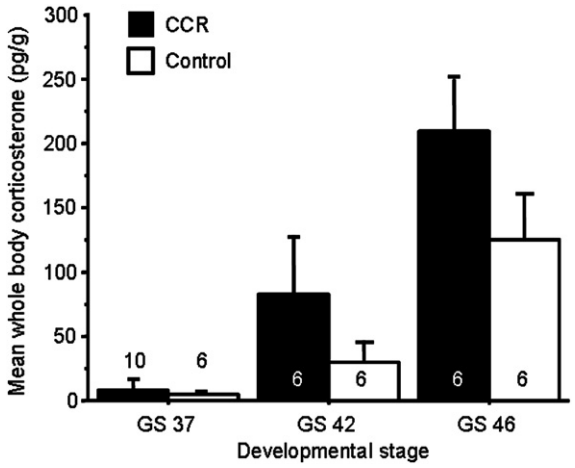

Fig. 1. Mean whole body corticosterone content ± 1 standard error of *Rana sphenocephala* tadpoles exposed to control or coal combustion residue (CCR)-contaminated substrate at early hind limb toe differentiation (GS 37), forelimb emergence (GS 42), and completion of metamorphosis (GS 46). Treatments are significantly different (two way ANOVA; $P=0.04$ $F=4.559$).

Since corticosterone can have differential effects during early and late metamorphosis, we exposed larval Southern Leopard Frogs to CCRs, and documented effects on whole body corticosterone content, oral morphology, growth and development at several time points during metamorphosis. The goals of our experiment were to identify 1) whether significant changes in whole body corticosterone occur during exposure to an anthropogenic stressor and 2) whether these changes may influence oral morphology, growth, and development.

2. Materials and methods

Animal care procedures followed that of Peterson et al. (2008). *Rana sphenocephala* tadpoles hatched from 2 egg masses and were housed communally until they reached Gosner stage 25 (GS 25; Gosner, 1960). Each individual was then placed in their own container with 8 L aerated tap water and either clean sand substrate (control, $n=48$) or substrate collected from a filled basin within the D-area disposal system on the Savannah River Site, South Carolina (CCR, $n=48$). Numerous studies have documented that sediment and organisms from this specific site contain elevated levels of approximately 20 trace elements (e.g., As, Cd, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Se, Sr, V, and Zn; reviewed in Rowe et al., 2002; and more recently assessed in Peterson et al., 2007). Approximately 700 g of dry substrate was added to each bin (approximately 1 cm deep). All animals were weighed at 50 days post exposure (DPE) to substrate. When an individual initiated hind limb toe differentiation (GS 37), completed forelimb emergence (GS 42), and completed tail resorption (GS 46), we determined its mass and the number of days it took each individual to reach each stage from day 1 of exposure to substrate. Ten individuals from each treatment were humanely sacrificed with MS-222 at these stages to determine whole body corticosterone content. All individuals were euthanized and snap frozen in a pentane and dry ice slurry within three minutes of capture. The ten individuals sacrificed at GS 37 were also qualitatively assessed for oral disc abnormalities. Oral disc condition was given a severity score based on how many of the keratinized structures (i.e., tooth rows and jaws) in the oral disc were visible. If an individual had all of the structures, it was given a score of one. Individuals with no apparent anterior tooth rows were given a score of two, individuals with no anterior tooth rows and less than 50% of their anterior jaw were given a score of three, and individuals with no anterior or posterior tooth rows and no anterior jaw were given a score of four.

2.1. Radioimmunoassay

Whole body corticosterone concentrations were obtained via radioimmunoassay protocols reported in Belden et al. (2003) except for the following modifications. Each individual was homogenized with a mass adjusted amount of deionized water (mass×2). Each sample was equilibrated overnight with 3000 cpm of tritiated corticosterone (PerkinElmer). Each sample was extracted twice in 3 ml anhydrous ethyl ether. Treatment groups were separated evenly and run in two assays. Average intraassay variation was 8.3%, interassay variation was 16.3%, and average recoveries were 42%. Standard plasma samples stripped of hormones and spiked with corticosterone were used to calculate variation.

2.2. Statistical analyses

Whole body corticosterone content at each developmental stage was compared between treatments with a two way analysis of variance (ANOVA). Oral disc condition was compared between treatments with a Fisher's exact test. Mass gained or lost between stages and time between stages were compared to determine whether effects due to exposure were more severe in early or late stages and were compared between treatments with repeated measures ANOVAs. We used StatView for Windows (SAS Institute, version 5.0.1) for all statistical analyses. Our low sample sizes in several of the groups reflect either mortality during the study or errors during radioimmunoassay that required removal of the individuals from analysis.

3. Results

3.1. Corticosterone content

Whole body corticosterone content was significantly higher throughout metamorphosis in individuals exposed to CCRs (Two way ANOVA, Treatment: $P=0.04$; $F=4.56$, Developmental stage: $P<0.0001$; $F=18.812$, Treatment×Developmental stage: $P=0.30$, $F=1.236$; Fig. 1). However, treatments were not significantly different at each developmental stage (ANOVA, $P>0.05$). At the completion of metamorphosis, the CCR-treatment group contained, on average, 40% more corticosterone than control individuals. Corticosterone content increased throughout metamorphosis in both treatment groups. Individuals sacrificed for corticosterone content were exposed to

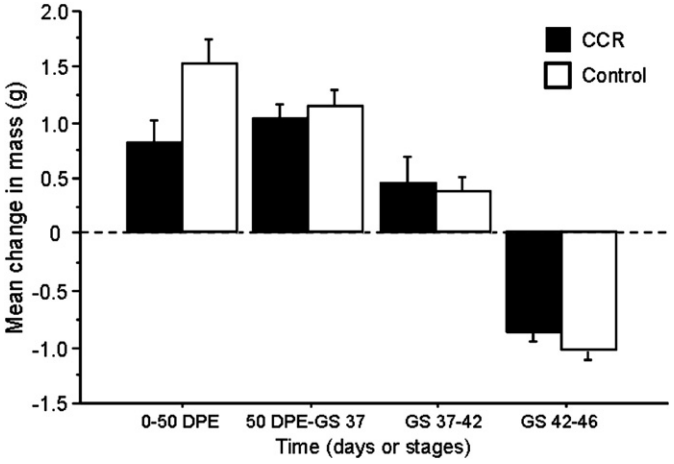

Fig. 2. Mean change in mass ± 1 standard error of *Rana sphenocephala* tadpoles exposed to control ($n=10$) or coal combustion residue (CCR)-contaminated ($n=6$) substrate between zero days post exposure (DPE) and 50 DPE, 50 DPE and early hind limb toe differentiation (GS 37), GS 37 and forelimb emergence (GS 42), and GS 42 and completion of metamorphosis (GS 46). Treatments are significantly different (Repeated measures ANOVA; $P=0.02$; $F=6.872$).

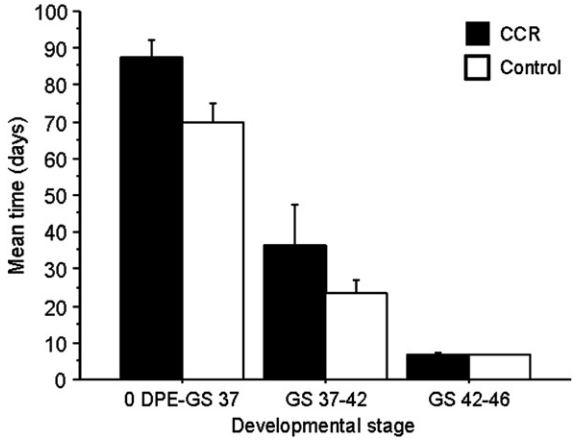

Fig. 3. Mean number of days ± 1 standard error *Rana sphenocephala* tadpoles exposed to control (n = 10) or coal combustion residue (CCR)-contaminated (n = 6) substrate took to transition between zero day post exposure (DPE) and early hind limb toe differentiation (GS 37), GS 37 and forelimb emergence (GS 42), and GS 42 and completion of metamorphosis (GS 46). Treatments are significantly different (Repeated measures ANOVA; P = 0.03; F = 5.79).

control or CCR substrates between 115–143 and 132–158 days for the GS 37 group, 116–158 and 169–211 days for GS 42, and 87–137 and 89–197 for GS 46, respectively.

3.2. Oral disc condition

Significantly more (P<0.001) tadpoles exposed to CCRs displayed abnormal oral morphology than controls (i.e., 9/10 vs. 0/10, respectively). Of the 10 CCR-exposed tadpoles, 1 lacked its anterior tooth rows, 5 lacked anterior tooth rows and at least half of their anterior jaw, and three lacked all anterior and posterior tooth rows and their anterior jaw.

3.3. Mass

Control tadpoles that completed metamorphosis gained significantly more mass between stages throughout metamorphosis than CCR-exposed tadpoles, on average (Repeated measures ANOVA, P = 0.02; F = 6.872; Fig. 2). On average, control tadpoles gained almost twice as much mass as the CCR tadpoles between the start of the experiment and 50 days post exposure (DPE). This difference between treatments decreased between later stages. When we removed the 0–50 DPE time point and analyzed the remaining time points there was no statistical significance between the treatments (Repeated measures ANOVA, P = 0.64, F = 0.226).

3.4. Timing of developmental stages

Tadpoles exposed to CCRs that completed metamorphosis also took significantly more time between stages throughout development (repeated measures ANOVA, P = 0.03; F = 5.79; Fig. 3). On average, the CCR group took 18 more days to transition from GS 25 (0 DPE) to GS 37 than the control group, however, they took a similar amount of time between GS 42 and GS 46.

4. Discussion

To our knowledge, this is the first study to describe an anthropogenic pollutant increasing whole body corticosterone content during amphibian metamorphosis. Tadpoles exposed to CCRs contained significantly higher concentrations of corticosterone throughout metamorphosis, contained significantly more oral abnormalities at GS 37, did not gain as much mass between metamorphic stages, and took significantly longer to transition between metamorphic stages than tadpoles exposed to a clean sand substrate.

It is possible that the elevated levels of body corticosterone could have directly mediated the morphological and developmental effects observed in the CCR-treated group. However, the timing and extent of the increase in corticosterone suggest that instead, CCRs may be the direct cause of these effects and the increase in corticosterone is a secondary physiological response. For example, when the CCR and control treatment were compared, the timing of greatest observed change in corticosterone content did not correspond to that of the greatest change in oral, growth, and developmental effects suggesting that corticosterone may not directly have mediated these effects. In fact, effects on oral morphology, mass, and time between stages were greatest early in metamorphosis (prior to GS 37), while corticosterone content differences between treatments were observed only later, from GS 37 through GS 46 (but see discussion below of increased time to transition between GS 37 and 42 in the CCR-treatment). It is possible, however, that small changes in corticosterone secretion early in development (prior to stage 37) may have influenced the changes observed. Monitoring corticosterone content prior to GS 37 may identify whether corticosterone secretion mediated effects on oral morphology, growth, and development or if corticosterone secretion was a secondary response to these effects.

The uncertainty as to whether the CCRs are directly causing the observed effects or whether the observed effects are being mediated via an increase in corticosterone is further complicated by the fact that it is unclear how or if endogenous levels of corticosterone influence developmental rate during later stages of amphibian metamorphosis (GS 36–42). Denver et al. (2002) predicted that increased glucocorticoid biosynthesis during the transition between GS 36 and 42 might have a unidirectional, acceleratory effect on metamorphosis. This prediction was based on studies in which development was accelerated via exposure to simulated pond drying (which induced a 7 fold increase in corticosterone secretion) or pharmacological concentrations of exogenous glucocorticoids (Frieden and Naile, 1955; Kikuyama et al., 1983; Gray and Janssens, 1990; Hayes et al., 1993; Kikuyama et al., 1993; Hayes, 1995; Denver, 1998). In contrast, our study and another recent study suggest that relatively lower elevations in corticosterone content in response to environmental stressors may actually prolong development between GS 36 and 42. *Rana sphenocephala* in this study experience a 1.7 fold increase in corticosterone content at GS 37 and a slower transition time between GS 37 and 42 while GS 36 *Spea hammondii* (the same species that accelerated development and a 7 fold increase in corticosterone content in response to pond drying) exposed to food deprivation experienced a three fold increase in corticosterone content and a decrease in developmental rate (Denver, 1998; Crespi and Denver, 2005). Collectively, these findings suggest either corticosterone is not the only factor influencing developmental rate or that it is dose dependent. If corticosterone has a dose dependent effect on development, we suggest that the magnitude of the response may be stressor specific. It is important to note that the inhibitory effect we observed on development between GS 37 and 42 may have been influenced by the relatively long amount of time the tadpoles were exposed to the stressor (since GS 25). The studies mentioned above exposed tadpoles to stressors or corticosterone immediately prior to assessing effects on development.

The magnitude of the hypothalamic–pituitary–interrenal-axis response to different stressors may represent the survival threat presented by each stressor (Boorse and Denver, 2003; Crespi and Denver, 2005). Stressors, such as CCRs and decreased food availability, which may not present an immediate threat to survival, may lead to slight increases in corticosterone secretion and prolonged development while a stressor, such as pond desiccation, which presents an immediate threat to survival (Newman, 1992), may lead to greater increases in corticosterone secretion and accelerated development. In

stable water sources, such as aquatic settling basins where CCRs are found, tadpoles may delay metamorphosis, which provides more time to forage and gain weight so they will be larger when they leave the water (Wilbur and Collins, 1973).

The stress response and metamorphosis both involve a complex interaction between the nervous, endocrine, and immune systems and numerous intermediates within each system. Perhaps administration of pharmacological doses of exogenous corticosterone skews the natural role that corticosterone plays in the interaction between these systems. We suggest that more studies investigate the diverse effects of stressors on endogenous levels of corticosterone and amphibian metamorphosis. As anthropogenic pollutants like CCRs become more prevalent in the environment, the need for studies that focus on how these pollutants affect physiology, growth, and development during sensitive life stages in wildlife becomes more urgent.

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