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Increased Circulating Levels of Testosterone and Corticosterone in Southern Toads, *Bufo terrestris*, Exposed to Coal Combustion Waste

William A. Hopkins,*[,1] Mary T. Mendonça,* and Justin D. Congdon†

**Department of Zoology and Wildlife Science, Auburn University, 331 Funchess Hall, Auburn, Alabama 36849; and*
†The Savannah River Ecology Laboratory, P.O. Drawer E, Aiken, South Carolina 29802

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This study describes an interrenal stress response in adult toads, *Bufo terrestris,* after exposure to coal combustion waste (characterized by a variety of trace elements). In the first portion of this study, free-ranging male toads captured at the coal ash polluted site exhibited significantly higher circulating levels of corticosterone (B) in both June/July and August than conspecifics captured at uncontaminated sites. In addition, both calling and noncalling males from the polluted site had higher B levels than conspecifics engaged in the same behaviors at reference sites. Testosterone levels were elevated in toads from the polluted site, regardless of capture month or behavioral state, suggesting altered androgen production, utilization, and/or clearance. In the second portion of this study, male toads from reference sites were transplanted to enclosures at the polluted site or an uncontaminated site. Toads held at the polluted site exhibited significant increases in B after 10 days of exposure compared to toads held at the reference site. B levels remained significantly elevated in toads transplanted to the polluted site after 12 weeks. We hypothesize that high concentrations of various trace elements in the polluted site are responsible for these hormonal responses.

Glucocorticoids help mediate critical processes such as gluconeogenesis, enabling an organism to meet increased energy demands during stressful situations (Kaplan, 1996). Responding to a stressor, however, can interfere with other physiological processes such as digestion, growth, and reproduction (Sapolsky, 1987). Stress-induced elevations in glucocorticoids can potentially interfere with the release of reproductive hormones from the gonadal axis at the hypothalamic, pituitary, and gonadal levels. This inhibitory relationship has been examined in birds (Wingfield *et al.*, 1982), reptiles (Lance and Elsey, 1986), fish (Carragher *et al.*, 1989), mammals (Sapolsky, 1987), and amphibians (Moore and Zoeller, 1985).

Selye (1936) recognized that the stress response is highly conserved across taxa and can be provoked by a wide variety of conditions. Elevations in circulating glucocorticoids can be triggered by diverse stimuli such as competition for resources, captivity, and increased population density (Pickering, 1981; Licht *et al.*, 1983; Sapolsky, 1993). Additionally, it has been demonstrated that many environmental pollutants can act as stressors and provoke a similar adrenal response (Donaldson *et al.*, 1984). The majority of studies have centered on the hormonal response of teleosts to a variety of contaminants (Donaldson *et al.*, 1984; Hontela *et al.*, 1992; McMaster *et al.*, 1994). Sewage, pulp mill effluent, landfill leachate, ammonia, pesticides, resin acids, and a variety of heavy metals have all been

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[1] To whom correspondence should be addressed at present address: The Savannah River Ecology Laboratory, P.O. Drawer E, Aiken, SC 29802. Fax: (803) 952-7413. E-mail: HOPKINS@SREL.EDU.

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shown to increase adrenal activity in teleosts (Donaldson, 1981; for review, see Donaldson *et al.*, 1984).

Coal ash effluent produced by coal burning power plants has become a major source of global pollution. The effluent contains high concentrations of numerous trace elements including various heavy metals. By the end of this century it is estimated that 120 million tons of coal ash waste will be produced annually in the United States by coal burning power plants (USEPA, 1988). There is much concern that leachate from these sites will contaminate ground water since the majority of coal waste management facilities do not have leachate collection systems or protective liners to reduce offsite migration of wastes (USEPA, 1988).

Coal ash effluent has recently been shown to cause developmental abnormalities in bullfrog tadpoles (Rowe *et al.*, 1996). In addition, the waste alters the tadpoles' standard metabolic rates, grazing success, and ability to avoid predators (Rowe *et al.*, 1996; Rowe, Kinney, Nagle, and Congdon, unpublished data; Raimondo, Rowe, and Congdon, unpublished data). Although no studies have evaluated the hormonal response of organisms to coal ash effluent, numerous studies on fish provide evidence that exposure to heavy metals (a significant component of coal ash) results in increased circulating levels of glucocorticoids (Schreck and Lorz, 1978; James and Wigham, 1986; Pratap and Bonga, 1990; Gill *et al.*, 1993). It has also been demonstrated that stress responses to heavy metals can have an inhibitory effect on androgen synthesis (Sangalang and O'Halloran, 1973). Elevated levels of glucocorticoids are energetically costly and can inhibit other physiological processes such as reproduction. Thus, chronic elevations of adrenal hormones could have major systemic effects and potentially affect the health of individuals and populations.

It is possible that other organisms inhabiting contaminated systems exhibit hormonal responses to pollutants similar to those documented for fish. Amphibians seem particularly important to study, since their complex life cycles include water-dependent and terrestrial stages, making them vulnerable to aquatic and edaphic xenobiotics (Dunson *et al.*, 1992). In comparison to other vertebrate groups, little is known about the hormonal cycles in amphibians, let alone their endocrine responses to contaminants. It is likely that amphibians are also affected hormonally when exposed to high concentrations of trace elements in coal ash effluent.

Most studies of physiological responses to pollutants have been lab-based studies of one or a few contaminants. In this study, however, we attempt to quantify the hormonal responses of animals in the field, where they are exposed to the actual combinations and concentrations of xenobiotics that they encounter in polluted habitats. Co-occuring pollutants can have complex synergistic and/or antagonistic relationships (Arnold *et al.*, 1996). Therefore, field studies are critical to understanding the realistic physiological consequences that organisms endure in contaminated ecosystems (see Guillette *et al.*, 1996; Gendron *et al.*, 1997).

This study examines whether exposure to a habitat contaminated by coal combustion wastes triggers an endocrine stress response in the southern toad, *Bufo terrestris.* We also evaluate whether the gonadal axis is affected by pollutant exposure. We report corticosteone and testosterone levels of toads residing naturally in the polluted site as well as in unpolluted sites. An experiment was also conducted in which toads were transplanted to the contaminated site to assess short- and long-term hormonal responses to conditions in the polluted habitat.

METHODS

Study Sites

A coal burning electric power plant with its associated coal ash settling basins is located on the United States Department of Energy Savannah River Site, a National Environmental Research Park near Aiken, South Carolina. The coal ash produced by this plant is mixed with water and then pumped into a primary settling basin (15 ha). The water flows from the primary basin into a smaller secondary basin (6 ha) and then into a 2-ha swamp before finally reaching Beaver Dam Creek, a tributary of the Savannah River. The suspended ash settles as the effluent moves through the basins and the swamp. Previous studies indicate that the water and sediment in the basins and the swamp have elevated levels of a number of trace elements, including cadmium, arsenic, selenium, chromium, copper, and barium (Cherry and Guthrie, 1977;

Alberts *et al.*, 1985; Rowe *et al.*, 1996). In addition, ongoing studies indicate that some vertebrates inhabiting this area have elevated tissue concentrations of these trace elements (Rowe *et al.*, 1996; Hopkins, Mendonça, and Congdon, unpublished data).

The control (reference) habitats were within approximately 13 km of the polluted site. The reference sites are historically uncontaminated by coal ash effluent, and toads sampled from these sites had low tissue concentrations of coal-derived trace elements when compared to toads from the polluted site (Hopkins, Mendonça, and Congdon, unpublished data).

Animal Collection

Hormone levels of both free-ranging toads and transplanted toads were examined in this study. Free-ranging adult male *B. terrestris* were collected at the polluted site and at reference sites between 6 July and 25 August 1996. All individuals were captured by hand between 20:00 and 24:00, and the time of capture was recorded. The behavior of each male (calling or noncalling) as well as the location of capture was also noted. All toads were bled (see below) before being weighed on an Ohaus balance (to nearest 0.1 g) and toe clipped for identification.

The second portion of this study involved transplanting toads from the uncontaminated sites to enclosures located in the polluted site or in another uncontaminated (reference) site (two enclosures / site). Thirty-two males captured between July 6 and 17, 1996, were placed in the enclosures (8 toads / enclosure) within 3 hr of being captured and bled in the field. One enclosure at each site (enclosure A) was sampled after 5 days and the other enclosure (enclosure B) at each site was sampled after 10 days. Individual toads were removed from each enclosure between 21:00 and 23:00 hr on the appropriate sampling day for bleeding. Toads were weighed and then returned to their respective enclosures after all individuals were bled.

Enclosure A and B at each site were subsequently sampled after 12 and 7 weeks, respectively. Following the prolonged exposures, all individuals were returned to the laboratory, where they were weighed and then housed in plastic containers with moist paper towels. Toads were then held for 2 weeks to void gastrointestinal contents before being sacrificed for determination of total body trace element concentrations (Hopkins *et al.*, unpublished data).

Enclosures

Four enclosures (1 × 2 m) constructed of a PVC (polyvinyl chloride) pipe frame and galvanized mesh hardware cloth were stabilized by driving the frames into the polluted and unpolluted sediments. The mesh was anchored to the frames and a small portion was left loosely attached for access during feeding and sampling. Four months prior to the initiation of experiments, two of the enclosures were placed on the western edge of the primary ash settling basin and two were placed on the western edge of Fire Pond (a historically uncontaminated site located approximately 13 km from the ash basins).

By the time experimental manipulations began in June 1996, all enclosures were surrounded by and contained substantial vegetation that provided cover and shade for the toads. For the duration of the experiments, ambient temperatures were recorded within the enclosures every 10 days at 08:00, 12:00, and 21:00 hr. The hardware cloth surrounding the enclosures had 1.5-cm openings which allowed naturally occurring prey items to move in and out of the enclosures freely. Although natural prey items were present, uncontaminated crickets were released in all of the enclosures once a week. Supplemental feeding was done to remove food limitation as a potential stressor.

Blood Collection

Toads were bled within 3 min of capture / removal from enclosure via cardiac puncture. Approximately 100 μl of blood was collected from each individual in heparinized 1-cc syringes, labeled, and placed on ice for transport to the laboratory. Blood collected from toads was centrifuged for 10 min at 3000 rpm. Separated plasma was pipetted off, frozen, and stored at −20° for later analyses.

Plasma Extraction and Radioimmunoassay

Extraction and radioimmunoassay (RIA) procedures follow protocols detailed in Mendonça *et al.* (1996). Thawed plasma samples were incubated with approximately 1000 cpm of the particular tritiated steroid to be assayed. Samples were equilibrated for 1 hr at room temperature before being extracted with 3 ml of anesthesia grade diethyl ether (Aldrich, Milwaukee, WI). Extracted samples were then dried down under nitrogen gas, resuspended in phosphate buffer, and allowed to equilibrate overnight at 4°.

Duplicate aliquots of the resuspended samples were incubated overnight at 4° with the appropriate tritiated hormone (Dupont NEN; testosterone, NET-553; corticosterone, NET-399) and the appropriate antibody (Endocrine Sciences, Calabasas, CA; testosterone, T3-125; corticosterone, B21-42). A third aliquot was used to determine percent extraction efficiency. After being corrected for the plasma volume and percent extraction efficiency, plasma steroid content was expressed as nanograms per milliliter of plasma. Percent extraction efficiency for testosterone and corticosterone averaged 90 and 92%, respectively. Interassay and intraassay variations averaged 11.6 and 6.3% for testosterone and 12.2 and 5.4% for corticosterone. The sensitivity of both assays was 10 pg/ml.

Statistics

Initial plasma samples collected from free-ranging toads inhabiting the polluted and unpolluted sites were tested for normality and homoscedasticity using the Shapiro–Wilk and Hartley's tests, respectively. Data were log transformed. Testosterone and corticosterone levels were compared by one-way and two-way analyses of variance (ANOVA) using the general linear model procedure due to unequal sample sizes. Tukey's pairwise comparisons were used to compare means.

In the transplant experiment, individual toads were treated as replicates for statistical analysis. Corticosterone and testosterone levels in transplanted toads were log transformed and levels taken from the same individuals over time were compared using one-way repeated measures analysis of variance. After using repeated measures, a Student–Newman–Keuls method was used to identify differing groups.

RESULTS

Free-Ranging Toads

Testosterone (T) and corticosterone (B) in toads captured during the last 2 weeks of June and the first 2 weeks of July did not differ significantly ($P = 0.10$ and 0.12, respectively) and were pooled for the analysis (June/July). Male toads captured in June/July and in August at the polluted site had significantly higher circulating levels of B than reference males captured at nearby unpolluted sites ($F(1, 75) = 63.66$; $P < 0.001$; Fig. 1). B levels did not differ between the June/July and August samples in either reference males or males from the polluted site (Fig. 1).

Circulating T levels from males at the polluted site were significantly elevated in comparison to reference males in both June/July and August ($F(1, 75) = 30.53$; $P < 0.001$). In reference males, T decreased significantly as the summer progressed (from $\bar{x} = 29.74 \pm 6.1$ to 4.46 ± 1.1 ng/ml). The opposite pattern was observed in males from the polluted site; T increased significantly from June/July to August (from $\bar{x} = 34.64 \pm 3.7$ to 64.86 ± 11.4 ng/ml) ($F(1, 75) = 6.39$; $P = 0.014$; Fig. 1).

In June/July, males that were captured while calling at the reference sites had significantly higher B than noncalling males from the same sites ($\bar{x} = 27.68 \pm 4.8$ vs 7.67 ± 1.3 ng/ml; $F(1, 47) = 31.39$; $P < 0.001$; Fig. 2). Despite repeated attempts to locate them, no actively calling males were captured at reference sites in August. Noncalling reference males were sampled in August and had B levels similar to those of noncalling reference males in June/July ($F(1, 54) = 0.03$; $P = 0.85$).

In contrast, calling males collected at the polluted site did not differ significantly in levels of B when compared to noncalling males from the same site

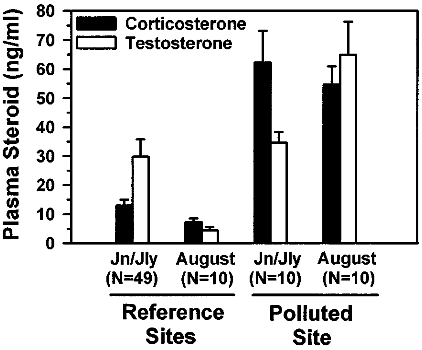

FIG. 1. Circulating corticosterone and testosterone levels in free-ranging male southern toads, *Bufo terrestris*, captured at the polluted site and at nearby reference sites. Toads were captured both in June/July (Jn/Jly) and in August at each site. Values are expressed as means ± 1 SE.

during June/July and August ($F(1, 16) = 2.11$; $P = 0.55$; Fig. 2). There was no significant difference between B levels in calling males from the polluted and reference sites collected in June/July. Males calling at the polluted site in August, however, had higher circulating levels of B than reference males calling in June/July ($\bar{x} = 62.37 \pm 14.0$ ng/ml vs 27.68 ± 4.8; $F(2, 13) = 5.11$; $P = 0.017$). In addition, noncalling males from the polluted site had significantly higher B levels than noncalling reference males in June/July and August ($F(1, 54) = 73.56$; $P < 0.001$; Fig. 2).

T levels in calling males captured in June/July at reference sites were significantly higher than levels in noncalling reference males ($\bar{x} = 83.01 \pm 16.4$ vs 7.81 ± 1.7 ng/ml; $F(1, 47) = 50.79$; $P < 0.001$; Fig. 3). At the polluted site, however, there was no significant difference in circulating T levels between calling and noncalling males during June/July and August ($F(1, 16) = 0.14$; $P = 0.72$).

Noncalling males from the reference sites did not differ in T levels between sample months ($F(1, 54) = 0.02$; $P = 0.88$). Noncalling males from the polluted site had significantly higher T levels than noncalling reference males in June/July and August ($F(1, 54) = 0.02$; $P < 0.001$). T levels in calling reference males were higher than analogous males from the polluted site in June/July ($\bar{x} = 83.16 \pm 14.4$ and 37.40 ± 6.1 ng/ml, respectively); however, these differences were not statistically significant ($F(2, 18) = 1.93$; $P = 0.175$; Fig. 3).

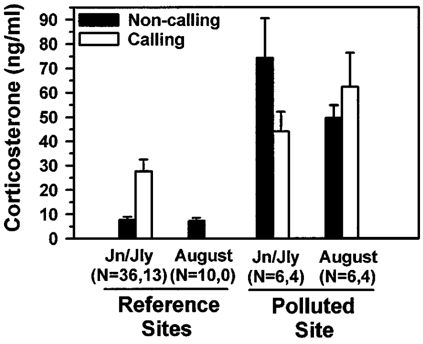

FIG. 2. Circulating corticosterone levels in calling and noncalling male toads, *B. terrestris,* captured at the polluted site and at nearby reference sites. Toads were captured in June/July (Jn/Jly) and August at each site. No calling males were captured at the reference sites in August. Values are expressed as means ± 1 SE.

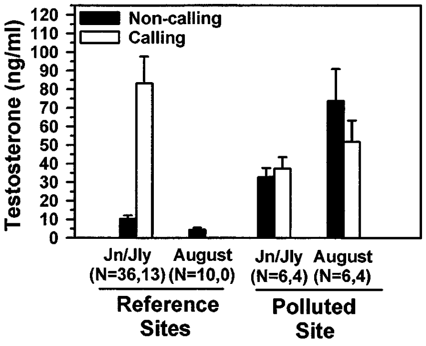

FIG. 3. Circulating testosterone levels in calling and noncalling male toads, *B. terrestris,* captured at the polluted site and nearby reference sites. Toads were captured in June/July (Jn/Jly) and August at each site. No calling males were captured at the reference sites in August. Values are expressed as means ± 1 SE.

Transplanted Toads

In June, July, and August ambient temperatures within enclosures at both sites never differed by more than 1.5°. Toads transplanted from uncontaminated sites to the reference (uncontaminated) site exhibited no significant change in circulating B levels after 5 and 10 days (Figs. 4a and 4b). Toads transplanted from an uncontaminated site to the polluted site, however, exhibited a marked increase in B after 5 days (from $\bar{x} = 7.06 \pm 2.7$ to 14.31 ± 5.5 ng/ml) and a significant increase after 10 days (from $\bar{x} = 3.35 \pm 0.4$ to 19.86 ± 3.6 ng/ml; $F(2, 14) = 16.2$; $P < 0.001$; Figs. 4a and 4b). Following 7 and 12 weeks of enclosure, toads in the reference site exhibited no significant changes in circulating B levels. However, in comparison to their initial B levels toads at the polluted site continued to exhibit elevated B levels in the 7-week sample and significantly elevated levels in the 12-week sample ($F(2, 10) = 4.3$; $P = 0.045$; Figs. 4a and 4b).

T levels decreased in toads transplanted to enclosures at the reference and polluted sites (Figs. 5a and 5b). Five days after being transplanted to each site, circulating T levels decreased in males from the polluted site and significantly decreased in reference males ($F(2, 14) = 7.39$; $P = 0.006$). After 10 days of being transplanted, testosterone levels significantly decreased both in reference toads ($F(2, 14) = 15.3$; $P < 0.001$) and in males transplanted to the polluted site ($F(2, 14) = 14.5$; $P < 0.001$). T levels remained significantly lower in toads at both sites after 7 and 12 weeks (Figs. 5a and 5b).

DISCUSSION

Polluted vs Reference Site

Selye (1976) characterized the stress response as consisting of three phases: alarm reaction, resistance, and exhaustion. Circulating levels of glucocorticoids often increase during the initial alarm reaction. Typically, when an organism is exposed to the stressor for prolonged periods of time, a stage of resistance results, which is characterized by decreased circulating levels of glucocorticoids. Indeed, a number of studies with teleosts indicate that lower levels of circulating cortisol often follow peak levels associated with initial exposure to heavy metals (Donaldson and Dye, 1975; Schreck and Lorz, 1978; James and Wigham, 1986; Pratap and Bonga, 1990). On the other hand, a more recent study indicates that the American eel, *Anguilla rostrata,* exhibits hypercortisolemia even after 16 weeks of exposure to moderate concentrations of cadmium (150 µg Cd/L) (Gill *et al.*, 1993).

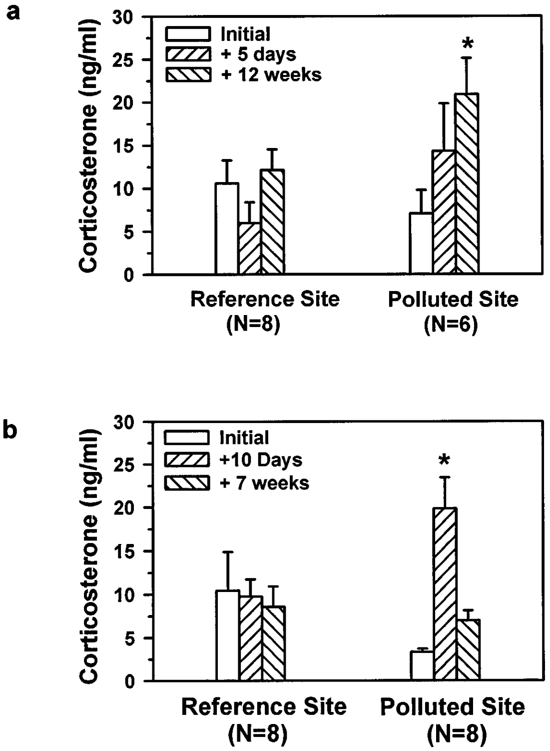

FIG. 4. Circulating corticosterone levels of male toads, *B. terrestris,* transplanted from uncontaminated sites to enclosures located at the polluted and reference site. Toads were bled at (A) 0 days (initial), 5 days, and 12 weeks and at (B) 0 days (initial), 10 days, and 7 weeks. Values are expressed as means ± 1 SE. *Denotes a significant ($P \leq 0.05$) difference.

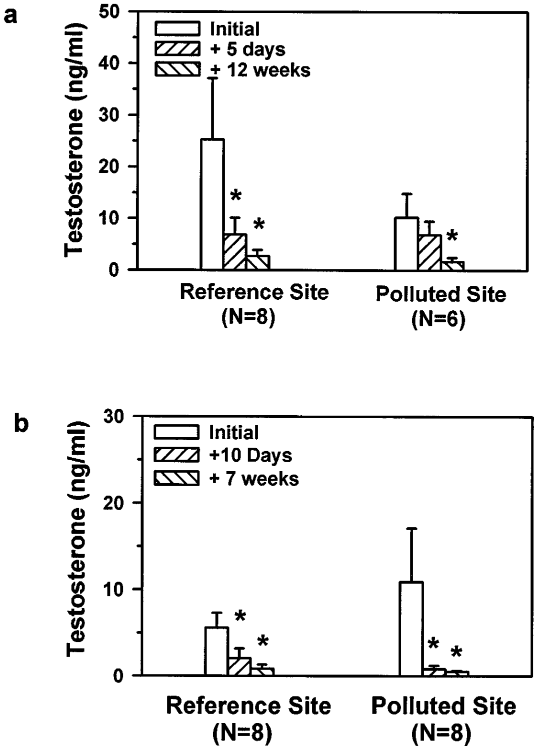

FIG. 5. Circulating testosterone levels of male toads, *B. terrestris,* transplanted from uncontaminated sites to enclosures located at the polluted and reference site. Toads were bled at (A) 0 days (initial), 5 days, and 12 weeks and at (B) 0 days (initial), 10 days, and 7 weeks. Values are expressed as means ± 1 SE. *Denotes a significant ($P \leq 0.05$) difference.

Our study examined populations residing in both the polluted ash basins and nearby uncontaminated reference habitats to determine whether the toads respond hormonally to coal ash effluent. In the reference sites, we found that *B. terrestris* exhibited an apparently normal hormonal profile. That is, corticosterone levels were relatively low and did not change in reference toads as the summer progressed. Testosterone levels in reference toads were higher in June/July (a time of high mating activity) than in late August (a time when breeding is rarely observed) (Fig. 1). The observed correlation between androgens and mating behavior is similar to that found in other spring breeding amphibians (Siboulet, 1981; Houck and Woodley, 1995). In contrast, corticosterone levels in toads from the polluted site were significantly elevated in comparison to reference toads during each of the sampling periods (Fig. 1). Toads captured at the polluted site were also found to have higher levels of several potentially stressful trace elements (including selenium, copper, cadmium, barium, and arsenic) in their tissues than toads from unpolluted sites (Hopkins *et al.*, unpublished data).

It is possible that elevated tissue concentrations of trace elements are provoking the chronic adrenal stress response in toads at the polluted site. Greenburg and Wingfield (1987) recognized that many circumstances can stimulate the release of glucocorticoids. Some more predictable activities, such as migration and reproduction, provoke an adrenal response and often occur while androgen levels are simultaneously elevated. True stressors (e.g., encountering a predator or pollutants), however, also stimulate pronounced adrenal activity but typically result in the inhibition of the hypothalamo–hypophyseal–gonadal axis. Given the pronounced adrenal response of the toads inhabiting the polluted site and that stress affects the gonadal axis, we expected that these animals would exhibit depressed androgen levels. However, T levels were unexpectedly high in toads from the polluted site. Testosterone levels in these toads actually increased in August, a time when levels were declining in the reference toads (Fig. 1). In fact, with the exception of calling toads in June/July at the reference sites, toads from the polluted site had higher circulating testosterone levels than all other reference toads.

It is possible that high concentrations of trace elements at the polluted site alter steroid metabolism, steroid utilization, and/or metabolic clearance rates. Simultaneously elevated levels of both testosterone and corticosterone may be explained by one or any combination of these disruptions. Several studies on cadmium provide insight as to the possible mechanisms that may cause these disruptions to occur. For instance, a study on steroid metabolism in cadmium-treated rats indicated that testosterone synthesis increased in the adrenals but decreased in the testis (Favino *et al.*, 1966). In another study, cadmium levels of only 1 part per billion, an order of magnitude less than the cadmium levels in the polluted ash basin sediment, have been shown to induce elevations in circulating levels of both testosterone and 11-ketotestosterone in the brook trout, *Salvelinus fontinalis* (Sangalang and Freeman, 1974). Additionally, androgen levels remained elevated in these cadmium-treated trout for weeks after testicular regression (Sangalang and Freeman, 1974). A combination of factors, including adrenal androgen synthesis and impaired steroid clearance mechanisms, may have caused these prolonged elevations.

Steroid clearance mechanisms are of particular interest since they are partially dependent on enzymatic processes occurring in the kidneys and liver, two organs that accumulate high concentrations of sequestered metals (Buhler *et al.*, 1977; McCracken, 1987; Gill *et al.*, 1993; Eisler, 1994; Ojeda and Griffin, 1996). Heavy metals such as lead, mercury, and cadmium can bind to thiol or sulfhydryl groups of enzymes and subsequently inactivate the enzyme (Landis and Yu, 1995). In addition, cadmium can replace zinc as a cofactor in some enzymes and, in turn, inactivate enzymatic activity (Landis and Yu, 1995). A plausible explanation could be that by disrupting enzymatic efficiency, trace elements in the tissues of toads from the polluted site may greatly inhibit metabolic clearance of steroid hormones.

Behavior and Steroids

To date, only a few studies have examined the relationship between hormones and reproductive behavior in Bufonids (for review see Houck and Woodley, 1995; Houck *et al.*, 1996). Emerson and Hess (1996) provide androgen and corticosterone levels for a number of calling tropical anurans, including *Bufo asper*, but they did not make intraspecies comparisons of hormone levels between calling and noncalling individuals.

Although Orchnick *et al.* (1988) compared sex steroid levels with amplexus behavior, the present study is, to our knowledge, the first study to examine the hormonal changes associated with calling behavior in a Bufonid. Androgen and corticosterone changes associated with calling have been evaluated in other anurans including the desert spadefoot toad, *Scaphiophus couchi*, and the bullfrog, *Rana catesbiana*, but the relationship of these hormones is quite different in the two species. It appears that *R. catesbiana* exhibits the classic inhibitory relationship between the adrenal and gonadal axis (Licht *et al.*, 1983). Calling *R. catesbiana* have elevated corticosterone but depressed androgen levels in comparison to noncalling individuals in the population (Mendonça *et al.*, 1985). In contrast, circulating testosterone, dihydrotestosterone, and corticosterone levels are elevated in calling *S. couchi* relative to noncalling individuals (Harvey *et al.*, 1997).

The reference populations of *B. terrestris* examined in this study exhibited increases in both testosterone and corticosterone while engaging in calling behavior (Figs. 2 and 3). In this respect, *B. terrestris* exhibits a hormonal pattern similar to that observed in calling spadefoot toads. It is likely that in these two anurans, simultaneous increases in androgens and corticosterone are critical to the initiation and maintenance of energetically costly sexual behaviors (Orchinik *et al.*, 1988; Houck and Woodley, 1995; Harvey *et al.*, 1997). Toads from the polluted site, on the other hand, exhibited no change in B or T levels while actively calling. Instead, both hormones were significantly elevated regardless of month or behavior (Figs. 2 and 3). Chronically elevated hormone levels in noncalling and calling toads from the polluted site may have energetic consequences as well as adverse effects on the timing of reproductive behavior. In fact, calling continued to occur for at least a month longer at the polluted site than at the nearby reference sites.

Transplanted Toads

In addition to sampling free-ranging toads, we also transplanted toads from uncontaminated reference sites to the polluted site to determine if we could mimic the responses exhibited by free-ranging toads. Toads transplanted to the polluted site had increased circulating corticosterone within a short time of exposure and levels remained elevated for the duration of the experiment (Fig. 4). The transplant experiments controlled for as many environmental parameters as possible (e.g., shade, temperature, access to food, toad density, etc.). The primary difference between the enclosures at the polluted and unpolluted sites was the heavily contaminated sediment at the polluted site. It is therefore possible that the incorporation of sediment contaminants in anuran tissues provoked the adrenal stress response observed in the free-ranging and transplanted toads at the polluted site.

The transplant experiments also indicate that the process of bleeding, transport, and subsequent enclosure has a negative impact on the levels of circulating testosterone regardless of the location of the enclosures. Toads transplanted to the polluted and unpolluted sites experienced immediate (≤5 days) suppression of the gonadal axis. These low levels of testosterone persisted for the duration of the experiments (Fig. 5). The results were not surprising, since many studies have shown the direct inhibitory effects of capture and confinement on the gonadal axis (Moore and Zoeller, 1985; Lance and Elsey, 1986; Pickering *et al.*, 1987).

The decrease in T was not the same pattern as observed in free-ranging toads at the polluted site. It could simply be that the additional stress of handling and confinement caused the observed decreases in circulating androgen. It is important to indicate, however, that transplanted toads at each site only remained in the enclosures for 7 and 12 weeks and were fed uncontaminated food. As a result, the toads transplanted to the polluted site did not ingest as much contaminated prey as they would in a more prolonged free-ranging situation. Thus, although whole body metal content was elevated in the toads transplanted to the polluted site, the levels were much lower than in free-ranging toads from the polluted site (Hopkins *et al.*, unpublished data). It is possible that higher levels of the trace elements must be incorporated in order to provoke elevated androgen levels similar to those observed in the free-ranging ash basin toads.

The results observed in the free-ranging toads and in the transplant experiments suggest that coal ash effluent provokes a stress response in *B. terrestris.* The toads exhibit elevated glucocorticoid levels characteristic of the Selyean alarm reaction. The high concentrations of trace elements at the polluted site may be causing the toads to exhibit chronic glucocorticoid elevations due to continual stimulation of the adrenal axis. Typically, organisms chronically exposed to a stressor will undergo a stage of resistance, resulting in lower glucocorticoid levels. The pattern of glucocorticoid secretion in the toads, however, appears to be more similar to the chronic hypercortisolemia exhibited by American eels. In addition, impaired steroid clearance mechanisms may contribute to the observed prolonged elevations of glucocorticoids in toads inhabiting the polluted site. Chronic elevations of glucocorticoids can have a number of adverse consequences including depletion of energy reserves, muscle atrophy, loss of bone matrix, depressed immune responsiveness, and inhibition of reproductive processes (Kaplan, 1996).

The simultaneous chronic elevation of testosterone was a particularly interesting result because it contradicts the accepted paradigm that stress-induced elevations of glucocorticoids (as opposed to reproductively induced) have an inhibitory effect on the gonadal axis. Interference with a variety of mechanisms, including extragonadal androgen secretion and impaired enzymatic clearance capacity, may be responsible for the simultaneous high circulating levels of both steroids in these exposed toads. Sublethal physiological dysfunctions such as these are difficult to detect, but their consequences can be critical both at the individual and population levels. Indeed, by simply lowering the reproductive capacity of organisms or by increasing the energy demands on the organisms, continuous exposure to contaminants might ultimately affect a population as effectively as a single lethal dose (Freeman *et al.*, 1984).

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