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Growth and Developmental Effects of Coal Combustion Residues on Southern Leopard Frog (*Rana sphenocephala*) Tadpoles Exposed throughout Metamorphosis

John D. Peterson[1], Vikki A. Peterson[1], and Mary T. Mendonça[1]

The effects of aquatic deposition of coal combustion residues (CCRs) on amphibian life histories have been the focus of many recent studies. In summer 2005, we raised larval Southern Leopard Frogs, *Rana sphenocephala*, on either sand or CCR substrate (approximately 1 cm deep within plastic bins) and documented effects of sediment type on oral disc condition, as well as time to, mass at, and total body length at key developmental stages, including metamorphosis (Gosner stages [GS] 37, 42, and 46). We found no significant difference in mortality between the two treatments and mortality was relatively low (eight of 48 in the control group and four of 48 in the CCR group). Ninety percent of exposed tadpoles displayed oral disc abnormalities, while no control individuals displayed abnormalities. Tadpoles raised on CCR-contaminated sediment had decreased developmental rates and weighed significantly less at all developmental stages, on average, when compared to controls. The CCR treatment group was also significantly shorter in length than controls at the completion of metamorphosis (GS 46). Collectively, these findings are the most severe sub-lethal effects noted for any amphibian exposed to CCRs to date. More research is needed to understand how these long term effects may contribute to the dynamics of local amphibian populations.

TRACE elements, derived from coal combustion residues (CCRs), have become a major pollutant due to the increased use of energy derived from coal burning power plants worldwide (Hopkins et al., 1999). In fact, in 2004, half of the electricity in the United States was generated from coal (United States Department of Energy, 2007). Total production of CCRs tripled from 35.6 million tons in 1970 to 111 million tons in 2004 (United States Geological Survey, 2005). Roughly one-third of CCRs are disposed of in aquatic settling basins (Electric Power Research Institute, 1997). Several studies have found that CCRs contain elevated concentrations of approximately 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).

Previous research has shown that CCRs pose major threats to wildlife and their natural environments (for a review, see Rowe et al., 2002). While chronic exposure to CCR-contaminated environments is not always lethal, it has been suggested that they have detrimental effects on behavior, development, morphology, and physiology in anurans (Hopkins et al., 1998, 2000). In two previous studies on the hormonal effects of chronic exposure to CCR-contaminated areas, adult male Southern Toads (*Bufo terrestris*) at the Savannah River Site, South Carolina exhibited altered calling behavior and significant increases in blood corticosterone compared to controls (Hopkins et al., 1997, 1999).

Detrimental effects due to CCRs can be manifested early in development, and have impacts later in life. Several studies have documented that American Bullfrog (*Rana catesbeiana*) tadpoles raised in a CCR-contaminated environment display significantly more oral and axial malformations which decreased grazing ability, growth, and swimming speed compared to controls (Rowe et al., 1996, 1998; Hopkins et al., 2000). In a recent study, Snodgrass et al. (2004) reported that chronic exposure to CCRs decreased time to and mass at key developmental stages in Green Frogs (*R. clamitans*) and Wood Frogs (*R. sylvatica*). *Rana clamitans* also experienced decreased survival and metamorphic success. Both *R. clamitans* and *R. sylvatica* exposed to CCR-contaminated substrates accumulated trace elements.

The majority of studies conducted on tadpoles exposed to CCRs suggest that species with relatively long larval periods typically display more pronounced detrimental effects (e.g., increased time to complete metamorphosis, decreased mass, decreased length, and increased trace element accumulation), although high mortality rates have sometimes been observed in rapidly developing species (Rowe et al., 2001). The Southern Leopard Frog, *R. sphenocephala*, has a relatively long developmental period in the laboratory compared to other anurans, and they have been suggested to accumulate

[1] Department of Biological Sciences, Auburn University, 331 Funchess Hall, Auburn, Alabama 36849; E-mail: (JDP) peterj1@auburn.edu. Send reprint requests to JDP.

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more trace elements than *R. catesbeiana* (Burger and Snodgrass, 2001). We exposed Southern Leopard Frog tadpoles to sediment containing CCRs to determine the effect of CCRs on oral disc condition, body size (mass and length), and time to several key developmental stages, including metamorphosis.

MATERIALS AND METHODS

Collection and animal care.—In April 2005 we purchased *R. sphenocephala* egg masses from a commercial supplier. As tadpoles hatched, they were raised communally in four plastic bins with 8 L of dechlorinated and aerated tap water until Gosner stage 25 (GS 25; Gosner, 1960) when they had fully internalized their external gills and their oral discs were fully developed. During this time period, approximately half of the water in the communal bins was replaced with fresh, dechlorinated and aerated tap water each day. All tadpoles reached GS 25 within a two-day period.

When all tadpoles had reached GS 25, individuals were randomly chosen and placed into one of two treatments, control ($n = 48$) or CCRs ($n = 48$). Tadpoles in the control treatment group were housed in plastic bins (one tadpole per bin) with clean sand substrate and 8 L of dechlorinated and aerated tap water. Tadpoles in the CCR treatment group were housed in individual plastic bins with CCR substrate collected from a filled basin within the D-area disposal system on the Savannah River Site, South Carolina. Numerous studies have documented that sediment and organisms collected 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 in Peterson et al., 2007). The CCR-contaminated substrate was added to each bin to a depth of approximately 1 cm (dry mass = 700 g) before water was added. Sediment was given several days to settle before the experiment began.

All tadpoles were fed a diet of ground trout chow (125 g; Aquamax Grower 600, PMI Nutrition International, Brentwood, MO), ground rabbit chow (125 g; Classic Blend Rabbit Food, L/M Animal Farms, Pleasant Plain, OH), agar (20 g; Becton, Dickinson and Company, Sparks, MD), gelatin (14 g; Sigma Chemical Company, St. Louis, MO), and water (1 L). Ingredients were heated to combine, cooled on a cookie sheet, and frozen until use. The first day tadpoles were exposed to their respective substrate, each tadpole was given a small cube of food (approximately 2 × 2 mm). Tadpoles were fed every other day for the length of the experiment. If any individual ate all of the food given within a two-day period, then food amounts were increased for all the tadpoles. Approximately half the water in each bin was replaced with fresh dechlorinated and aerated tap water once a week until algae began to form in the bins, when water changes were increased to twice a week. Great caution was taken during water changes to minimize disruption of the tadpoles and sediment. A small cup was skimmed slowly across the surface of the water during water removal and clean water was ''rained'' into each container from an identical empty container with approximately 100 2 mm diameter holes drilled in the bottom. Very little sediment was agitated when using these methods. A random subsample of bins from each treatment was monitored weekly for water quality. Variables used to assess water quality included alkalinity (120–180 ppm), ammonia (0–0.25 ppm), nitrate (0–20 ppm), nitrite (0–0.5 ppm), pH (6.8–7.2), hardness (50–100 ppm), and temperature. If any variable was outside ''normal'' values provided above then half the water in all bins was changed. Temperature was maintained at a constant 25°C for the first eight weeks of the experiment and at 28°C for the completion of the experiment.

Measurement.—At 50 days post exposure, all tadpoles were weighed to determine initial larval mass. All other measures were recorded as each individual reached a specific developmental stage. When each individual began hind limb toe differentiation (GS 37), forelimb emergence (GS 42), and complete tail resorption (GS 46), we determined its mass and the number of days it took each individual to reach the given stage from day 1 of exposure to substrate. Each individual was weighed using an electronic scale with an accuracy of 0.001 g. No individuals were injured during weighing. Oral disc condition was only assessed at GS 37 because the oral disc begins normal atrophy during the later stages of development. During the experiment, ten individuals from each treatment were sacrificed with MS-222 at each developmental stage for a concurrent experiment. We only assessed oral disc condition and total body length in these individuals immediately following euthanasia, because these methods are potentially harmful to tadpoles. Total body length was determined with calipers with an accuracy of 0.01 mm.

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. All natural fatalities were documented throughout development in order to determine survival and metamorphic success within treatments.

Statistical analyses.—Treatments were compared with repeated measures ANOVAs for time to and mass at key stages of development. Oral disc condition and mortality were compared between treatments with Fisher's exact tests. Total body lengths were compared between treatments via individual ANOVAs for each stage (i.e., GS 37, 42, 46), because different groups of tadpoles were measured at each stage. We used StatView for Windows (SAS Institute, version 5.0.1) for all statistical analyses.

RESULTS

During the course of the experiment, eight of 48 control tadpoles and four of 48 tadpoles exposed to CCRs died. Thus, the percent mortality did not differ statistically between the two treatments ($P = 0.35$). Although there was no difference in mortality between the treatment groups, differences in oral disc condition, time to stages, mass at stages, and total body length at stages were observed.

Oral disc condition.—When control and CCR-exposed tadpoles were sampled at stage 37, significantly more ($P < 0.001$) tadpoles exposed to CCRs displayed abnormal oral morphology than controls (i.e., 9/10 vs. 0/10). One CCR-

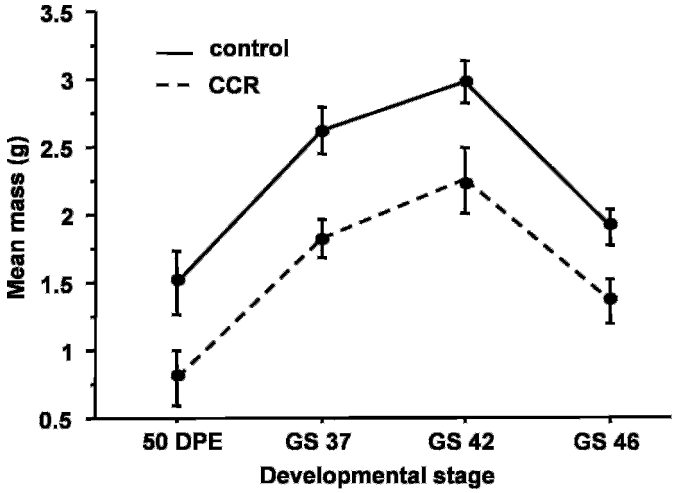

Fig. 1. Mean masses ±1 standard error of *Rana sphenocephala* tadpoles exposed to control (n = 10) or CCR-contaminated (n = 6) substrate at 50 days post exposure (DPE), early hind limb toe differentiation (GS 37), forelimb emergence (GS 42), and completion of metamorphosis (GS 46). Treatments are significantly different (repeated measures ANOVA; P = 0.007, F = 10.063).

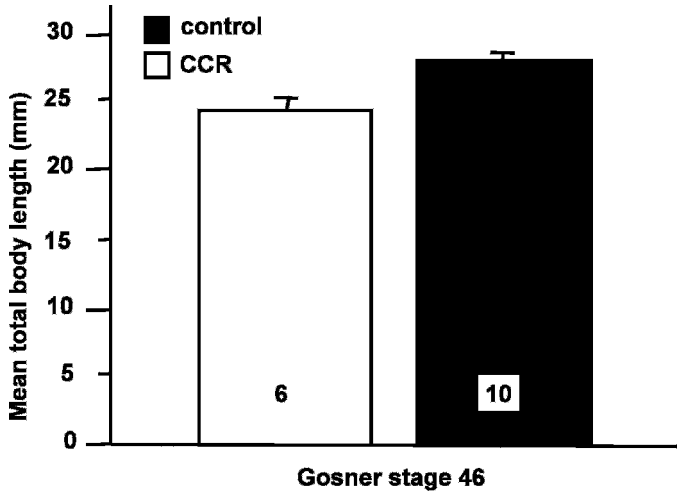

Fig. 2. Mean total body lengths ±1 standard error of *Rana sphenocephala* tadpoles exposed to control (n = 10) or CCR-contaminated (n = 6) substrate at the completion of metamorphosis (GS 46). Treatments are significantly different (ANOVA; P = 0.003, F = 12.864).

treated tadpole was lacking its anterior tooth rows, five were lacking their anterior tooth rows and at least half of their anterior jaw, and three were lacking all anterior and posterior tooth rows and their anterior jaw.

Body size.—On average, CCR-exposed tadpoles weighed significantly less than control tadpoles throughout development (Fig. 1; P = 0.007, F = 10.06). These individuals' average initial larval mass (50 days post exposure to substrate) was 47% less than the controls' average weight. This difference decreased to 29% at the completion of metamorphosis. Tadpoles exposed to CCRs were also 12% shorter in length than control tadpoles at the completion of metamorphosis (Fig. 2; P = 0.003, F = 12.86); however, there was no statistical difference in length between groups at hind limb toe differentiation (GS 37) and forelimb emergence (GS 42).

Time.—Tadpoles exposed to CCR-contaminated substrates took significantly longer to reach hind limb toe differentiation (GS 37), fore limb emergence (GS 42), and complete tail resorption (GS 46; P = 0.024, F = 6.45; Fig. 3). On average, the CCR-treated tadpoles took 31 days longer than the control to complete metamorphosis. We also found a significant time by treatment effect (P = 0.05, F = 3.33).

DISCUSSION

Our findings suggest that *R. sphenocephala* morphology, growth, and development are negatively affected by exposure to CCRs. When compared to previous research, our results indicate that CCRs have more impact on development of this species than all other amphibian species studied to date (Rowe et al., 1996, 1998; Snodgrass et al., 2004). Collectively, these findings suggest aquatic disposal of CCRs may be more problematic for amphibians than previously thought.

Experimental exposure of tadpoles to CCRs resulted in a high frequency of oral abnormalities (90%). The abnormalities we observed were more severe than those noted in American Bullfrog (*R. catesbeiana*) tadpoles from CCR-contaminated wetlands on the Savannah River Site, South Carolina (Rowe et al., 1996, 1998). Exposed *R. catesbeiana* tadpoles were generally missing teeth in all tooth rows and, on average, had 90% fewer teeth in anterior tooth row #1 and 40% fewer teeth in posterior tooth row #2 than reference tadpoles (Rowe et al., 1996). Of the individuals in our study, 80% (8/10) were missing all of their anterior tooth rows and at least 50% of their anterior jaw. The most severe cases in our study were lacking all tooth rows and the entire anterior jaw (30%, 3/10). Thus, our data suggest that *R. sphenocephala* displays more severe oral abnormalities than *R. catesbeiana*, which is the only other species of tadpole in which CCR related oral abnormalities have been described to date. Our findings are also the first laboratory CCR exposure to describe oral abnormalities in tadpoles, suggesting that CCR exposure was the only factor that could have contributed to the oral abnormalities.

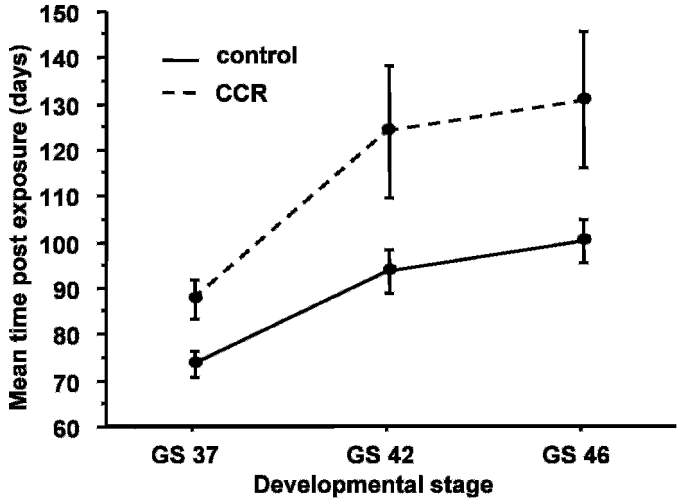

Fig. 3. Mean times ±1 standard error to early hind limb toe differentiation (GS 37), forelimb emergence (GS 42), and completion of metamorphosis (GS 46) post exposure to control (n = 10) or CCR-contaminated (n = 6) substrate for larval *Rana sphenocephala*. Treatments are significantly different (repeated measures ANOVA; P = 0.024, F = 6.451).

On average, *Rana sphenocephala* exposed to CCRs weighed significantly less than control tadpoles at all metamorphic stages and were shorter in length at the completion of metamorphosis. Similar results have been observed in other species exposed to CCRs. Snodgrass et al. (2004) observed that *R. clamitans* and *R. sylvatica* raised on CCRs in the laboratory weighed 10 and 39% less than controls at the completion of metamorphosis, respectively. In our study, newly metamorphosed *R. sphenocephala* (GS 46) raised on CCRs weighed 29% less than controls, on average. However, during initial larval growth (50 days post exposure to substrate), CCR exposed tadpoles weighed 47% less than controls.

Amphibians that are smaller at the completion of metamorphosis may be smaller at first reproduction and, therefore, suffer decreased reproductive success. For example, *R. sylvatica* and *Pseudacris triseriata* that were smaller at completion of metamorphosis were also smaller at first reproduction (Berven and Gill, 1983; Smith, 1987). Breeding success in many amphibians is determined by factors in which a larger body size in males is more favorable than a smaller body size (e.g., call characteristics and male–male competition; Wells, 1979; Arak, 1983; Sullivan, 1983; Olson et al., 1986; Klump and Gerhardt, 1987; Sullivan, 1992). This body size effect is even stronger in females. Several studies have documented that larger females lay larger clutches and larger eggs than smaller females (Salthe, 1969; Howard, 1978; Kaplan and Salthe, 1979; Cummins, 1986; Semlitsch, 1987; Bush et al., 1996). If *R. sphenocephala* exposed to CCRs in their natural environment exhibit smaller body size, as in the current study, they may also experience lowered reproductive success. Future experiments should investigate whether these long term effects do indeed occur and how exposure to CCRs early in life may negatively affect lifetime reproductive success.

In the current study, exposure to CCRs resulted in a significant lengthening of time (days) to reach each key stage of metamorphosis, when compared to control individuals. When compared to other literature, these responses are, again, more pronounced than those documented in other species. Snodgrass et al. (2004) observed the larval period of *R. clamitans* and *R. sylvatica* to be 10 and 11% longer, respectively, for individuals exposed to CCRs than control tadpoles; whereas, we found the larval period of *R. sphenocephala* to be 25% longer than controls. This suggests that, on average, these individuals spend 31 more days in the aquatic environment. This may prolong exposure to CCR-contaminated substrate and aquatic predators, and create a size disadvantage when compared to tadpoles that did not develop on a CCR-contaminated substrate. These effects may be more pronounced in *R. sphenocephala* that hatch during the fall breeding season because of limited time and resources before winter. Previous studies suggest that individuals that take longer to complete metamorphosis may be smaller at time of first reproduction and may experience delayed maturity (Smith, 1987; Semlitsch et al., 1988; Berven, 1990).

Numerous studies to date have documented negative effects of CCRs on amphibians that surround aquatic retention basins. Our data suggest that *R. sphenocephala* appears to be especially susceptible to these environments, and prolonged exposure results in metamorphic characteristics that may be detrimental to reproductive success. In the U.S. one-third of CCRs are disposed of in aquatic settling basins (Electric Power Research Institute, 1997); however, this percentage may be higher in less developed countries. Given the detrimental impacts aquatic disposal of CCRs can have on amphibians, we suggest that this practice be reevaluated and altered to prevent exposure of amphibians to CCR contaminated areas.

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