EXPOSURE TO TRACE ELEMENTS HAS NEGATIVE REPRODUCTIVE CONSEQUENCES FOR SOUTHERN TOADS (*BUFO TERRESTRIS*) EXPOSED TO COAL COMBUSTION WASTE

by

BRIAN SEARS METTS

(Under the Direction of Kurt A. Buhlmann and William A. Hopkins)

ABSTRACT

Bioaccumulation of contaminants and subsequent maternal transfer to offspring could be an important factor that affects amphibian reproduction and embryonic development. My research is among the few to investigate the individual and interactive effects of paternal and maternal exposure to contaminants on amphibian reproductive success, and to document relationships between contaminant concentrations in female amphibians or their eggs and reduced reproductive success. Specifically, southern toads (*Bufo terrestris*) exposed to coal combustion waste (CCW) accumulated several trace elements (e.g. selenium). Exposure to trace elements was associated with adverse effects on clutch size, embryo viability, and overall reproductive success. Compared to reference, reproductive success was reduced by 40% when either the male or the female was from the ash basin and by 58% when both parents were from the ash basin. Reproductive success was negatively correlated with Se concentrations in females and their eggs. In addition, this research demonstrates negative effects of larval exposure to CCW contaminated sediments, latent effects of maternal exposure to CCW derived contaminants, and their interactive effects. Most notably, survival to metamorphosis was reduced dramatically in larvae from females collected near CCW contaminated settling basins; however, the degree of this reduction was dependent on the type of sediment that their larvae developed on. Survival of larvae from contaminated females was lowest when reared on ash basin sediments but highest when reared on ash plume sediments. Negative sublethal effects on larvae (e.g., extended larval period) were attributable to sediment type during the larval period, and not previous maternal exposure. Interestingly, sublethal effects were less pronounced in larvae reared on ash plume sediments compared to ash basin sediments, suggesting the effects may decrease as sediments age. However, larval survival was substantially reduced by exposure to ash plume sediments. My research highlights the need for additional studies investigating the effects of exposure to contaminants on amphibian reproduction. It underscores the importance of further research investigating potential latent effects from contaminant exposure, particularly post-metamorphosis. My study provides evidence that CCW contaminated sites may ultimately serve as ecological traps, and highlights the importance of understanding how individual effects impact local amphibian population viability.

INDEX WORDS: Abnormalities, Amphibian, CCP, CCW, Clutch size, Coal ash, Contaminants, Fecundity, Fly ash, Frog, Hatching success, Malformations, Maternal transfer, Mesocosm, Paternal effects, Reproduction, Viability

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DEDICATION

This dissertation is dedicated to my family:

To my wife, Meridith, I could not have completed this endeavor without her love and support.

To my parents, Olin and Dale Metts, whose unending support and encouragement enabled me to pursue personal and professional goals throughout my life.

To my girls, Sierra and Sydney, whose seemingly limitless energy keeps me young and whose enthusiasm reminds me daily to enjoy the simple things in life.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Amphibian population declines are well documented worldwide (IUCN, 2004, Stuart et al., 2004; Hoffman et al., 2010). Amphibians are important components of aquatic and terrestrial communities because of their high abundance, diverse roles in food webs, and importance for the transfer of energy and nutrients through food webs and across the landscape (Burton and Likens, 1975a,b; Wyman, 1998; Beard et al., 2002; Ranvestel et al., 2004; Regester et al., 2006; Gibbons et al., 2006). Thus, these declines may have important ecological ramifications. Although the causes of many declines have been elusive, habitat loss and alteration, infectious diseases, exotic species, global climate change, and chemical contamination are thought to be the greatest contributors to amphibian declines (Collins and Storfer 2003; Stuart et al., 2004; Hoffmann et al., 2010). Habitat loss and disease are typically considered the primary threats; however, environmental contamination is often an important factor or cofactor in amphibian declines that does not receive the attention it deserves (Alford and Richards, 1999; Blaustein et al., 2003; Lawler et al., 2006).

Exposure to environmental contaminants can have direct sublethal and lethal effects on individuals as well as negative transgenerational effects on their offspring, which are typically attributed to maternal effects (Bernardo, 1996; Mousseau and Fox, 1998). In many vertebrate species maternal exposure to environmental contaminants and subsequent transfer to offspring can reduce reproductive success through malformations and embryo mortality (Di Giulio and Tillitt, 1999). For example, chronic alcoholism in human mothers can lead to congenital malformations of children (Jones et al., 1973; Palmer et al., 1974). In classic case studies in birds, bioaccumulation of dichlorodiphenyltrichloroethane (DDT) caused egg shell thinning and reduced hatching success, resulting in population declines of several North American raptor and piscivorous species (Blus, 1996). However, in amphibians bioaccumulation and maternal transfer of contaminants has received far less attention (Hopkins et al., 2006; Bergeron et al., 2011).

Female amphibians are known to accumulate contaminants and transfer them to their eggs (Kadokami et al., 2004; Wu et al., 2009; Bergeron et al., 2010), which can ultimately lead to reduced hatching success and offspring viability (Kotyzova and Sundeman, 1998; Hopkins et al., 2006; Bergeron et al., 2011a). For instance, African clawed frogs (*Xenopus laevis)* exposed to cadmium (Cd) in laboratory experiments maternally transferred Cd to their eggs, which caused embryological malformations (Kotyzova and Sundeman 1998). Similarly, female eastern narrowmouth toads (*Gastrophryne carolinensis)* exposed to an array of trace elements in the field accumulated and transferred selenium (Se) and strontium (Sr) to their eggs (Hopkins et al., 2006). Embryos from contaminated females exhibited a significant decrease in hatching success and hatchlings exhibited significant increases in developmental and behavioral abnormalities when compared to a reference site (Hopkins et al., 2006). Moreover, when all developmental criteria were considered together, contaminated females produced 19% fewer viable offspring than reference females (Hopkins et al., 2006). Although no correlation was found between any single contaminant transferred to eggs and measures of offspring viability, the importance of maternal transfer as a contaminant route was demonstrated (Hopkins et al., 2006). These studies clearly highlight the need for additional studies on maternal transfer in amphibians and its potential to disrupt early development and impair female reproductive success, which could contribute to localized declines.

Offspring born into the same environment as their parents can be exposed to contaminants of maternal origin and the environment, making it important to determine the relative effects of each exposure route and to identify potential interactions between them (Bergeron et al., 2011b; Todd et al., 2011). For instance, maternal exposure of common snapping turtles (*Chelydra serpentina*) to polychlorinated biphenyls (PCBs) reduced offspring survival, while juvenile dietary exposure to PCBs did not (Eisenreich et al., 2009). In another study, maternal exposure to polycyclic aromatic hydrocarbons (PAHs) in the fish *Fundulus heteroclitus* reduced larval growth and body condition more so than direct larval exposure (Nye et al., 2007). In American toads *(Bufo americanus*), maternal Hg exposure negatively affected offspring growth and size, and increased the number of spinal malformations; however, no effect of offspring dietary exposure was found (Todd et al., 2011). Interestingly, under lower resource abundance conditions, both maternal Hg exposure and offspring dietary Hg exposure negatively influenced offspring growth and development; however, the two routes of exposure acted synergistically, reducing survival by 50% compared to reference larvae (Bergeron et al., 2011b). In addition, the consequences of previous maternal exposure may become apparent later in the development of surviving offspring (i.e., long term and/or latent effects; Bergeron et al., 2011a,b). For example, larvae of female American toads exposed to Hg grew more slowly and were smaller at metamorphosis than larvae from unexposed females (Bergeron et al., 2011a,b). Taken together these studies suggest that traditional research of environmental exposure in amphibians may underestimate the effects of bioaccumulative contaminants and that maternal exposure to contaminants should be considered more frequently.

A limitation of all existing studies on transgenerational effects of contaminants on amphibian offspring is an inherent bias towards maternal effects. Previous studies investigating maternal transfer relied on captive breeding of females and males that had been exposed to contaminants in the field, and then assumed that negative effects were of maternal origin (e.g. Kotyzova and Sundeman, 1998; Hopkins et al., 2006; Bergeron et al., 2010; 2011a,b; Todd et al., 2011). It is possible that observed negative effects were a result of paternal exposure or an interaction between maternal and paternal exposure. Paternal exposure to contaminants may have negative reproductive consequences by influencing sperm count, viability, or mobility resulting in decreased fertilization. For example, male skittering frogs (*Rana cyanophlyctis*) exposed to mercuric chloride had fewer spermatogonia than unexposed males, suggesting the contaminant disrupted sperm production (Kanamadi and Saidapur, 1992), but whether this reduced fertilization was not investigated. In humans, however, exposure of males to contaminants has been linked to decreased reproductive capacity (Irvine 2000) through reduced sperm count and quality (Auger et al., 1995; Swan et al., 2003) and increased incidence of undescended testes (Weidner et al., 1998; Saradha and Mathur, 2006). Paternal exposure to environmental contaminants can increase genetic mutations, impair development of offspring, and create changes in population genetics (Anderson et al., 1994; Belfiore and Anderson, 2001; Shugart and Theodorakis, 1994; Hebert and Luiker, 1996). Furthermore, epigenetic variation (i.e., heritable changes in gene expression not caused by changes in genetic sequence) induced through paternal contaminant exposure can negatively influence offspring (Richards, 2006; Curley et al., 2011). For instance, contaminants can have epigenetic effects that decrease sperm capacity and viability in multiple generations of rats (Anway et al., 2005; Clement et al., 2010). Together these studies suggest that paternal exposure to environmental contaminants could result in reduced reproductive success.

Together, maternal transfer of contaminants, environmental exposure of larvae, and paternal exposure to contaminants may negatively influence the reproductive success of amphibians. I sought to investigate each of these exposure routes to gain a better understanding of how they may influence amphibian populations.

STUDY SYSTEM

Environmental contaminants come from many sources, but my research focuses on waste from coal-fired power plants, one of the largest producers of solid wastes in the U.S. (USDOE, 2005). Coal combustion waste (CCW) contains high concentrations of trace elements [e.g., arsenic (As), selenium (Se), strontium (Sr)] and is often discharged into aquatic settling basins for disposal (Rowe et al., 2002). Amphibians and other wildlife using CCW disposal basins can accumulate elevated concentrations of trace elements, resulting in adverse effects on survival, growth and development, behavior, performance, and recruitment (Hopkins et al., 2000, 2006; Raimondo and Rowe, 1998;

Rowe et al., 1996, 1998a,b, 2001; Roe et al., 2006; Snodgrass et al., 2003, 2004; Snodgrass and Hopkins, 2005). Taken together these studies suggest that CCW disposal basins not only have negative consequences for individual amphibians but may reduce reproductive success, ultimately acting as ecological traps for amphibians (Rowe et al., 2001; Rowe and Hopkins, 2003;). My research focuses on two CCW-contaminated sites and three reference sites on the Savannah River Site near Aiken, South Carolina. The two contaminated sites are associated with the D-area coal-powered steam generation facility (Roe et al., 2005). The reference sites, Ellenton Bay, Rainbow Bay, and Craigs Pond are natural Carolina bay wetlands (Sharitz, 2003; Sharitz and Gibbons, 1982) with no known historical contamination (Davis and Janecek, 1997).

STUDY SPECIES

The study organism used to address the hypotheses in my dissertation is the southern toad (*Bufo terrestris*), a common anuran ideally suited for this research because they are locally abundant, reproduce readily in captivity, have a short embryonic period, and have been the subjects of previous research investigating the effects of CCW (Hopkins et al., 1997, 1998; Rowe et al., 2001; Ward et al., 2006). Like many other amphibians, they have a complex life cycle consisting of aquatic larvae and terrestrial adults. Adult southern toads prefer terrestrial habitats with sandy soils in the non-breeding season. Despite producing toxic skin secretions, southern toads serve as an important food resource for many vertebrate species (Jensen, 2008). Adults migrate up to 1 mile (1.6 km) in the spring and summer to aquatic breeding sites (Bogert, 1947) where females lay 2,500 to 4,000 eggs which typically hatch in 2 to 4 days (Jensen, 2008).

Larvae graze mostly on aquatic vegetation and algae, exhibit rapid growth, and have a relatively short larval period (30-55 days; Jensen, 2008). After metamorphosis juveniles move on to land and disperse across the landscape. In subsequent years, most individuals return to breed in their natal pond, while others disperse to neighboring wetlands (Breden, 1987; Berven, 1990; Semlitsch and Bodie, 2003). Thus, adults may have the opportunity to breed in both contaminated and uncontaminated wetlands within a few kilometers of one another.

OBJECTIVES

The primary goal of my research was to investigate three different pathways that contaminants can take to impact the reproductive success of amphibians. Specifically, I sought to quantify maternal transfer of contaminants and its influence on early development and reproductive success. Second, given that amphibians can be exposed to contaminants of maternal origin and from the environment, I sought to describe the relative influence of and potential interactions between maternal and environmental exposure on larval development. Third, because the effects of paternal exposure to contaminants have been largely overlooked in amphibians, I sought to investigate the consequences of contaminant exposure on the reproductive success of male southern toads. In addition, because trace elements may be less bioavailable in older CCW-contaminated wetlands, I sought to determine if the negative consequences associated with exposure to trace elements would be ameliorated in aged CCW.

In Chapter 2, I investigated the potential role exposure to contaminants has on the reproductive success of female southern toads. My objectives in this chapter were threefold: 1) determine to what extent female southern toads exposed to CCW bioaccumulate and maternally transfer trace elements to their eggs, 2) assess the effects that exposure to CCW has on the reproductive success of southern toads, and 3) determine if the effects of exposure to contaminants are ameliorated in aged CCW soils. In Chapter 3, I investigated the relative influence and interactions of previous maternal exposure and larval environmental exposure to CCW on larval amphibian growth and development. My objectives were to: 1) assess maternal, environmental, and interactive effects on growth, survival, and performance of larval southern toads, and 2) determine whether the effects are ameliorated in aged CCW. In Chapter 4, I investigated the effects of exposure to CCW-contaminated soils on the reproductive success of male southern toads. My objectives were to: 1) determine if exposure of male toads to contaminants would reduce hatching success and increase the frequency of abnormalities in larvae resulting in decreased reproductive success, and 2) determine if paternal and maternal exposure interact to influence reproductive success. Overall, I sought to increase our understanding of how maternal transfer of contaminants to offspring, environmental contaminant exposure of larvae, and paternal impairments influence amphibian populations.

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CHAPTER 2

MATERNAL TRANSFER OF CONTAMINANTS HAS NEGATIVE CONSEQUENCES ON REPRODUCTIVE SUCCESS OF SOUTHERN TOADS (*BUFO TERRESTRIS*) EXPOSED TO COAL COMBUSTION WASTE [1]

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Abstract

Bioaccumulation of contaminants and subsequent maternal transfer to offspring is an important factor that affects the reproductive success of wildlife. However, this process has rarely been investigated in amphibians, even though environmental contamination is thought to be among the greatest contributors to worldwide amphibian declines. We examined maternal transfer of trace elements in southern toads (*Bufo terrestris)* residing in three locations: 1) an active coal ash disposal basin, 2) an adjacent natural wetland contaminated with coal ash over 35 years ago (ash plume), and 3) an uncontaminated reference site. Our study is among the few to document maternal transfer of contaminants and resulting adverse effects in amphibians. We found that females collected from the ash plume and ash basin transferred elevated concentrations of Cd, Cu, Ni, Pb, Se, and Sr to their eggs. After correcting for female body size, clutch size of females collected from the ash basin was 14% smaller than reference females. Overall reproductive success, estimated as a function of clutch size and offspring viability, was reduced 33% and 35% in females collected from the ash plume and ash basin, respectively, compared to reference females. Reproductive success negatively correlated with elevated trace element concentrations, most notably Se, in females and their eggs. In addition, our study highlights the negative effects that maternal transfer of contaminants can have on amphibian reproduction. Our results demonstrate that aging of ash alone does not ameliorate the negative consequences associated with these trace element contaminated sediments.

INTRODUCTION

Bioaccumulation of contaminants in adult amphibians and subsequent maternal transfer to offspring may be an important factor that affects reproductive success. However, the majority of research investigating the effects of contaminants on amphibians has focused on environmental (e.g., aqueous) exposure or trophic uptake and has been heavily biased towards studies during the embryonic and larval stages (Linder and Grillitsch, 2000; Birge et al., 2000) Although environmental exposure to contaminants can have adverse effects on all life stages, maternal transfer of contaminants is an alternative route of exposure that may also negatively affect clutch characteristics and offspring development (Bernardo, 1996; Hopkins et al., 2006). Previous studies have demonstrated that adult female amphibians accumulate contaminants and transfer them to eggs (Kadokami et al., 2004; Wu et al., 2009; Bergeron et al., 2010), which can ultimately lead to reduced hatching success and offspring viability (Hopkins et al., 2006, Kotyzova and Sundeman, 1998; Bergeron et al., 2011a,b) For instance, female African clawed frogs (*Xenopus laevis)* exposed to cadmium (Cd) in laboratory experiments maternally transferred Cd to their eggs, which caused embryological malformations (Kotyzova and Sundeman, 1998). Similarly, female eastern narrowmouth toads (*Gastrophryne carolinensis)* collected from a coal ash settling basin, accumulated and transferred selenium (Se) and strontium (Sr) to their eggs which experienced 19% lower viability than those from a reference site (Hopkins et al., 2006).

Environmental contaminants are thought to be among the greatest contributors to amphibian declines around the world (Collins and Storfer, 2003; Hoffman et al., 2010; Stuart et al., 2004). Although environmental contaminants come from many sources, coal-fired power plants are one of the largest producers of solid waste in the U.S. and this waste has been shown to have a wide array of adverse effects on amphibians. Coal combustion waste (CCW) contains high concentrations of trace elements (e.g., arsenic [As], Se, Sr) and is often discharged into aquatic settling basins for disposal (U.S. DOE, 2005; Rowe et al., 2002). Amphibians and other wildlife using CCW disposal basins can accumulate elevated concentrations of trace elements in their tissues, resulting in adverse effects on survival, growth and development, behavior, performance, and recruitment (Hopkins et al., 2006; Hopkins et al., 2000; Raimondo and Rowe, 1998; Rowe et al., 1996; Rowe et al., 2001; Roe et al., 2006; Snodgrass et al., 2003; Snodgrass et al., 2004; Snodgrass and Hopkins, 2005). Taken together these studies suggest that CCW-contaminated impoundments and wetlands not only influence the health of individual amphibians, but may also serve as ecological traps for amphibian populations (Rowe et al., 2001; Metts et al., 2012; Rowe and Hopkins, 2003).

The objectives of this research were to determine if female southern toads (*Bufo (Anaxyrus) terrestris*; hereafter *Bufo*) exposed to CCW transfer trace elements to their offspring, and to assess whether these contaminants negatively correlate with reproductive success. We also sought to determine whether aging of coal ash would ameliorate the effects on amphibians because trace elements migrate down to lower horizons and are taken up by plants (Sandhu et al., 1993; Carlson and Adrian, 1991; Carlson and Carlson, 1994), thereby potentially reducing bioaccumulation and resultant adverse effects in amphibians (Metts et al., 2012). To achieve our objectives, we examined maternal transfer of contaminants in southern toads residing in three locations: 1) an active CCW disposal basin (ash basin), 2) an adjacent natural wetland previously contaminated with CCW that has undergone natural succession for over 35 years (ash plume; Sandhu et al., 1993; Roe et al., 2005), and 3) a reference site. We tested three hypotheses. First, we hypothesized that female southern toads exposed to CCW would maternally transfer trace elements to their eggs. Second, we hypothesized that, compared to reference animals, females from the ash plume and ash basin would have reduced clutch sizes and hatching success, and their offspring would have increased incidence of abnormalities, ultimately resulting in reduced offspring viability and reproductive success. Finally, because aging of CCW may result in reduced trace element concentrations in surface sediments (Sandhu et al., 1993; Carlson and Adrian, 1991; Sandhu and Mills, 1991), we hypothesized that bioaccumulation and the reproductive effects of contaminants would be ameliorated in female southern toads collected from the 35-yr-old ash plume compared to females from the ash basin currently receiving freshly produced CCW (Metts et al., 2012).

METHODS

Study species. The southern toad is a common anuran in the southeastern U.S. that is well suited for this research because they are locally abundant, reproduce readily in captivity, have a short embryonic period, and have been the subjects of previous research investigating the effects of CCW (Rowe et al., 2001; Metts et al., 2012). Like many other amphibians, southern toads have a complex life cycle consisting of aquatic larvae and terrestrial adults. Adult southern toads prefer terrestrial habitats with sandy soils in the non-breeding season, and migrate up to 1.6 km to aquatic breeding sites in the spring and summer (Bogert, 1947; Jensen, 2008).

Study Sites. The three collection sites in our study are located on the Savannah River Site near Aiken, South Carolina. The two CCW-contaminated sites (ash basin and ash plume) are associated with the D-area steam generation facility, which includes a disposal area where sluiced CCW is discharged into a series of open settling basins, and effluents from these basins flow into Beaver Dam Creek and eventually the Savannah River. Toads were collected from one ash settling basin that is partially filled with CCW and has become re-vegetated. Sediments in this basin are comprised almost entirely of CCW that is enriched with trace elements, the primary contaminants of concern in this waste stream (Hopkins et al., 2006; Rowe et al., 2002). Most organic contaminants are volatilized during the combustion process and remaining organic compounds (e.g., PAHs) tend to not be bioavailable (Norton and Mills, 1987; Shorten et al., 1990; U.S. EPA, 1999). The second contaminated site is the ash plume, a natural wetland located adjacent to the ash basin in the Savannah River floodplain. It was contaminated when CCW was discharged into it from the 1950s to the early 1970s (Roe et al., 2005). Since the cessation of CCW discharge into the ash plume, natural succession has occurred, resulting in elemental uptake by plants and leaching of trace elements to lower horizons (Sandhu et al., 1993; Carlson and Adrian, 1991; Carlson and Carlson, 1994; Sandhu and Mills, 1991), potentially creating an aquatic environment that is less contaminated than the active ash basins. Coal combustion waste in the ash plume sediments extends to a depth of 2.7 m and covers approximately 40 ha, 30% of which becomes occasionally inundated when the Savannah River spills into its surrounding floodplain (Roe et al., 2005). A thin layer (~2.5 cm) of organic material covers the CCW and the ash plume is now vegetated with a mixed floodplain flora community (Roe et al., 2005). The reference site was Rainbow Bay, a ~1 ha Carolina bay wetland (Sharitz, 2003; Sharitz and Gibbons, 1982), with no previously known contamination (Semlitsch and McMillan, 1980; Pechmann et al., 1991), located approximately 12 km from the contaminated sites. All three sites are surrounded by mixed pine-hardwood forest with some open field habitats.

Experimental Design and Data Collection. We collected adult male and female *B. terrestris* from 8 April to 31 May 2010 from the ash basin, ash plume, and the reference site. We transported toads to the lab where we measured (SVL, ± 0.5 mm) and weighed (± 0.01 g) each female. We then injected males and females with human chorionic gonadotropin (males 100 IU, females 250 IU), and bred pairs in 5-10 cm of well water in plastic containers. By allowing females from all sites to oviposit in uncontaminated well water, we were able to isolate maternal transfer as the primary source of trace element contamination to the eggs. After pairs bred, we released males at their location of capture, and kept females in plastic containers in the lab for 48 hours to void their remaining gut contents. We euthanized females by immersion in buffered MS-222 and freeze-dried their carcasses for subsequent elemental analysis.

Forty-four females deposited clutches of eggs: 15 from the reference site, 15 from the ash plume, and 14 from the ash basin. To assess maternal transfer and its effect on the reproductive success of females, we enumerated the total number of eggs in each clutch and allocated a subsample of eggs from each for elemental analysis. We then removed a subsample of 500 fertile eggs from each clutch and held them in a plastic container with 3 L of aged well water at 25 °C until hatching. We counted the number of hatchlings in each subsample and calculated the fraction of the 500 that hatched successfully. To quantify morphological abnormalities, we fixed surviving hatchlings in MEMFA (0.1 M MOPS, pH 7.4, 2.0 mM EGTA, 1.0 mM $MgSO_4$, 3.7% formaldehyde) for 24 hours, and preserved them in 100% ethanol (Hopkins et al., 2006). Using a dissecting microscope, we examined each hatchling for morphological abnormalities (axial, swelling, oral, and blisters) following the methods of Bantle et al., (1991) and ASTM (1998). To estimate offspring viability, we calculated the fraction of the 500 eggs from each clutch that successfully hatched and had no morphological abnormalities (Hopkins et al., 2006). Finally, to estimate overall reproductive success of each female (i.e., predicted number of morphologically normal hatchlings), we multiplied clutch size by offspring viability.

Sample Preparation and Elemental Analysis. For elemental analyses, we freeze dried all tissue samples and then individually digested subsamples of each homogenized female carcass (~250 mg) and eggs in 10 mL of trace metal grade nitric acid (70% HNO_3) using microwave digestion (MarsExpress, CEM Corp., Matthews, NC). After digestion, we brought each sample to a final volume of 15 ml with 18 MΩ deionized water. We used inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer, Norwalk, CT) to determine elemental concentrations of As, Cd, chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni), Se, Sr, vanadium (V), and Zinc (Zn) in each sample. For quality control, we included certified reference material (TORT-2 and LUTS; National Research Council of Canada, Ottawa, Canada) in each analysis. Mean percent recoveries for elements in standard reference material ranged from 81 to 111%. Minimum detection limits in tissue samples were: As, 0.38; Cd, 0.16; Cr, 0.31; Cu, 0.42; Hg, 0.22; Ni, 0.39; Pb, 1.3; Se, 1.59; Sr, 2.1; V, 0.39; and Zn, 3.42 µg/kg. The analytical error expressed as relative percent difference for independent analyses of replicate dilutions ranged from 1.8% for V to 17.0% for Pb. We report elemental concentrations in this manuscript on a dry mass basis.

Statistical Analyses. We performed statistical analyses using SAS 9.1 (SAS Institute, Cary, NC). We examined the assumptions of homogeneity of variance using Levene tests and normality using Shapiro-Wilk tests. Where deviations from these assumptions were found, we performed log or angular (arcsine square-root) transformations to better meet assumptions of the models. For statistical comparisons, we assigned trace element concentrations below the instrument's detection limit (BDL) a value of half the minimum detection limit for that element. In all statistical comparisons, we accepted statistical significance at $\alpha = 0.05$ and present data as mean ± 1 standard error (SE).

We compared trace element concentrations in females and in eggs among sites using multivariate analysis of variance (MANOVA). When we found significant main effects using the multivariate models, we used analysis of variance (ANOVA) followed by Bonferroni corrected pair-wise comparisons to identify differences among treatment means. For elements that were maternally transferred, we used linear regression to determine the strength of the relationship between elemental concentrations in female carcasses and their eggs.

We used ANOVAs to make comparisons among sites for female size, hatching success, frequency of morphological abnormalities, and offspring viability. Because clutch size correlated with female body size, we used ANCOVA to make comparisons among sites for clutch size and reproductive success with female SVL as the covariate. If significant effects were found using ANOVAs we used Bonferroni corrected pair-wise comparisons to identify differences among treatment means. We also used linear regression to determine whether there were relationships between female and egg elemental concentrations and clutch size, hatching success, abnormality frequency, offspring viability, and reproductive success.

RESULTS

Trace Element Concentrations in Females. Overall, elemental concentrations in adult females differed significantly among sites (Pillai's trace = 1.22, $F_{22, 64} = 4.55$, $P \leq 0.001$). Specifically, concentrations of As, Ni, Se, Sr, and V were elevated in females from contaminated sites compared to reference females (in all cases: $F_{2, 41} \geq 7.23$, $P \leq 0.002$; Table 2.1) and Cu concentrations were higher in ash plume females compared to ash basin and reference females ($F_{2, 41} = 3.57$, $P = 0.04$; Table 2.1). Notably, Ni concentrations in females from the ash plume and ash basin were 4.8 and 3 times greater than females from the reference site, respectively (Table 2.1). Similarly, female Se concentrations were nearly twice as high in the ash plume and ash basin compared to reference females (Table 2.1). Strontium concentrations were nearly 3 and 2 times higher in ash plume and ash basin females, respectively, compared to reference females (Table 2.1).

Maternal Transfer of Trace Elements. Elemental concentrations in eggs differed significantly among sites (Pillai's trace = 0.91, $F_{22, 62} = 4.55$, $P = 0.005$). Eggs from females collected from the contaminated sites contained higher concentrations of Cd, Cu, Ni, Pb, Se, and Sr compared to eggs from reference females (Table 2.1). Cadmium concentrations were 3 times higher in ash basin and ash plume eggs than reference eggs ($F_{2, 42}$ = 5.59, P = 0.007). Copper concentrations in ash plume and ash basin eggs were 63% and 39% higher than reference eggs, respectively ($F_{1, 42}$ = 13.78, P < 0.0001). Nickel concentrations were 2 times greater in ash plume and nearly 4 times greater in ash basin eggs than in reference ($F_{1, 42}$ = 10.79, P < 0.001). Concentrations of Pb in eggs were nearly 3 times greater in ash plume and ash basin than reference eggs ($F_{1, 42}$ = 5.30, P = 0.009). Selenium concentrations in eggs were nearly 40% higher in ash plume and ash basin clutches than in reference clutches ($F_{1, 42}$ = 6.46, P < 0.01). Strontium concentrations were over 3 times higher in ash plume and ash basin eggs compared to reference ($F_{1, 42}$ = 8.98, P < 0.001).

Maternal transfer was also supported by correlations between trace element concentrations in females and their eggs. Concentrations of Cu, Pb, and Se were generally higher in eggs than in females. Conversely, concentrations of Cd, Ni, and Sr were lower in eggs than in female tissues. Concentrations of Cu, Se, and Sr in eggs correlated with concentrations in female tissues (Cu: r^2 = 0.14, $F_{1, 41}$ = 6.92, P = 0.01; Se: r^2 = 0.39, $F_{1, 41}$ = 26.73, P = 0.0001, Figure 2.1; Sr: r^2 = 0.25, $F_{1, 41}$ = 13.97, P = 0.001). The correlation between Ni concentrations in females and their eggs was weaker (r^2 = 0.07, $F_{1, 42}$ = 3.01, P = 0.09; Table 2.1). Concentrations of Cd and Pb in females were not correlated with concentrations in their eggs (in all cases: $r^2 \leq 0.03$, $F_{2, 40} \leq 1.29$, $P \geq$ 0.26; Table 2.1).

Effects on Reproduction and Offspring Viability. Female southern toads were similar in size among sites (SVL: $F_{2, 41}$ = 1.01, P = 0.37; mass: $F_{2, 41}$ = 1.57, P = 0.22; Table 2.2). There was a significant positive relationship between female SVL and clutch size (r^2 = 0.39, $F_{1, 40}$ = 25.60, P < 0.0001). After accounting for female SVL, average clutch size of ash basin females was 14% and 20% smaller than reference females and ash plume females, respectively ($F_{3, 40}$ = 3.48, P = 0.04; Table 2.2). Hatching success of larvae from ash basin and ash plume females was reduced by 17% and 28%, respectively, compared to reference females (Table 2.2; $F_{2, 41}$ = 3.25, P = 0.05).

The frequency of developmental abnormalities in hatchlings (~11%) was similar among sites ($F_{2, 39}$ = 0.29, P = 0.75; Table 2.3). Axial malformations were the most abundant abnormality at all sites, comprising approximately 78% of observed abnormalities (Table 2.3). Offspring viability (the percentage of successful hatchlings with no abnormalities) was reduced by 36% in ash plume and 27% in ash basin females compared to reference females ($F_{2, 41}$ = 5.21, P < 0.01; Table 2.2). Overall reproductive success (clutch size X % viability = number of morphologically normal hatchlings) was reduced by 33% in ash plume and 35% in ash basin females, compared to reference females ($F_{3, 40}$ = 3.80, P = 0.03; Table 2.2).

Concentrations of As, Ni, and Se in females and eggs correlated negatively with several reproductive parameters. Maternal concentrations of As negatively correlated with hatching success (r^2 = 0.20, $F_{1, 42}$ = 10.41, P = 0.002), offspring viability (r^2 = 0.28, $F_{1, 42}$ = 16.01, $P \leq$ 0.001), and reproductive success (r^2 = 0.12, $F_{1, 42}$ = 5.73, P = 0.02). Nickel concentrations in eggs negatively correlated with hatching success (r^2 = 0.12, $F_{1, 41}$ = 5.40, P = 0.03) and viability (r^2 = 0.11, $F_{1, 41}$ = 4.99, P = 0.03). Maternal concentrations of Se negatively correlated with hatching success (r^2 = 0.12, $F_{1, 42}$ = 5.59, P = 0.02), viability (r^2 = 0.21, $F_{1, 42}$ = 11.19, P = 0.002), and reproductive success (r^2 = 0.15, $F_{1, 42}$ = 7.38, P = 0.009). Similarly, concentrations of Se in eggs negatively correlated with hatching success ($r^2 = 0.14$, $F_{1, 40} = 6.83$, $P = 0.01$), viability ($r^2 = 0.30$, $F_{1, 40} = 16.95$, $P \leq 0.001$), and reproductive success ($r^2 = 0.24$, $F_{1, 40} = 12.38$, $P = 0.001$, Figure 2.2). Clutch size and frequency of developmental abnormalities did not correlate with any individual elemental concentrations in female carcasses or eggs (in all cases: $r^2 \leq 0.04$, $P \geq 0.22$).

DISCUSSION

Our study is among the first to document relationships between contaminant concentrations in female amphibians or their eggs and reduced reproductive success. Specifically, we found that female southern toads accumulated trace elements from exposure to CCW and subsequent maternal transfer of these elements was associated with adverse effects on clutch size, offspring viability, and overall reproductive success. Reproductive success negatively correlated with Se and As concentrations in females and Se concentrations in eggs. Our findings demonstrate that maternal exposure to CCW can have deleterious effects on amphibian reproduction, underscoring the importance of determining which constituents of CCW contribute to the observed adverse effects.

Like other wildlife exposed to CCW, female southern toads inhabiting the ash plume and ash basin accumulated numerous trace elements and transferred them to their offspring. Female toads collected from contaminated sites transferred Cd, Cu, Ni, Pb, Se, and Sr to their eggs at greater concentrations than females collected from the reference site. Maternal transfer of Cd in laboratory experiments on African clawed frogs increased the frequency of developmental abnormalities (Kotyzova and Sundeman, 1998), but in our study the frequency of gross morphological abnormalities was similar among sites. Copper and Se are essential micronutrients and may have been transferred to eggs as components of egg yolk proteins (Kroll and Doroshov, 1991; Ghosh and Thomas, 1995). Also, Se was likely maternally transferred because it substitutes for sulfur in a variety of egg components (Unrine et al., 2006; Unrine et al., 2007). In contrast, Pb is a non-essential heavy metal that can be toxic to vertebrates including humans even at low doses (Franson and Pain, 2011; ATSDR, 2007). Although Pb concentrations were generally low and concentrations in females were similar among sites, females from the ash plume and ash basin transferred elevated concentrations of Pb to their eggs. Lead and Sr may have been transferred because they both substitute for calcium, and calcium is an essential nutrient that plays an important role in cell division during embryonic development (Hopkins et al., 2006; Goldstein 1993; Fluck et al., 1991). Of the elements we measured, Zn concentrations were the highest. Although Zn concentrations were similar among females and among eggs from different sites, concentrations in eggs were nearly 4 times higher than in female carcasses. This is likely because Zn is an essential trace element for many biological functions.

Comparison of our results to other studies from the same ash basin suggests that southern toads may be more sensitive reproductively to CCW contamination than other vertebrates. Some vertebrate species are unaffected by CCW exposure and others can persist in the ash basin for long periods of time (Rowe et al., 1998a,b; Roe et al., 2004; Staub et al., 2004). However, female eastern narrowmouth toads collected from the ash basin experienced a 19% reduction in offspring viability compared to reference individuals (Hopkins et al., 2006). In our study, southern toads collected from the ash basin experienced a 35% reduction in reproductive success compared to reference toads.

Previous studies demonstrate that southern toads also experience other adverse effects from exposure to CCW, such as abnormal hormone levels, decreased growth and development, and alterations in behavior and performance (Hopkins et al., 1997; Ward et al., 2006) and ultimately reduced survival to metamorphosis and recruitment into the terrestrial environment (Rowe et al., 2001; Metts et al., 2012).

Of the elements maternally transferred, Se is of particular interest because it can disrupt embryonic development in some oviparous vertebrates and has negative effects on reproductive success of fish and birds (Chapman, 2010; Gillespie and Baumann, 1986; Janz et al., 2010; Lemly, 1996; Ohlendorf, 2003; Skorupa, 1998; Young et al., 2012). Selenium reproductive toxicity thresholds identified for fish and birds range from 8-16 µg/g (Lemly et al., 1996; Skorupa, 1998; Heinz, 1996; Skorupa and Ohlendorf, 1991; Fairbrother et al., 1999); however, thresholds for amphibians are not established. In our study, southern toads exposed to CCW accumulated (carcass mean = 4.2 µg/g Se) and maternally transferred relatively low Se concentrations (egg mean = 5.3 µg/g Se) compared to other wildlife species from the same site. In contrast, female eastern narrowmouth toads collected from the ash basin accumulated very high levels of Se (42 µg/g) and transferred similar concentrations to their eggs (Hopkins et al., 2006). Fish, birds, and reptiles from the same ash basin transferred 5.9 - 15.9 µg/g of Se to their offspring (Roe et al., 2004; Staub et al., 2004; Bryan et al., 2003). Despite maternally transferring lower concentrations of Se than all of the above mentioned taxa, southern toads experienced a significant reduction in reproductive success, which also correlated with female Se concentrations. It is possible that this apparent heightened sensitivity to Se is actually due to co-transference of other elements to eggs. For instance, maternal concentrations of As negatively correlated with reproductive success, and Ni concentrations in eggs negatively correlated with hatching success and offspring viability. It is also possible that other effects of CCW could influence egg quality. For example, toads from this site are known to have high plasma corticosterone levels (Hopkins et al., 1997). In birds, high maternal corticosterone levels can impair offspring phenotype and viability (Saino et al., 2005). Thus, disentangling the direct effects of Se in the system from the co-transference of other elements and other physiological effects was not possible in a field study of this nature.

Our hypothesis that bioaccumulation and the reproductive effects of contaminants would be ameliorated in southern toads collected from the 35-yr-old ash plume compared to females from the active ash basin was not supported. Elemental concentrations of female toads residing in the ash plume were similar to those from the ash basin, suggesting contaminants are still bioavailable and may pose risks to wildlife residing in the ash plume. Furthermore, the negative effects on reproductive success were similar between ash plume and ash basin females. Thus, our results demonstrate that aging of CCW-contaminated soils does not ameliorate the negative consequences on reproductive success of adult toads. Interestingly, Metts et al., (2012) suggest that the bioaccumulation and effects of contaminants on larval amphibians may be reduced by aging of CCW sediments. Taken together, these studies suggest that the biogeochemical processes that contribute to aging of CCW have different consequences for aquatic and terrestrial lifestages of amphibians. However, given that adult toads can move around on the landscape and the close proximity of the ash plume wetland to the ash basin, we cannot rule out the possibility that some animals collected from the ash plume actually originated from the ash basin.

Maternal exposure to contaminants negatively affected southern toad reproductive success and could ultimately lead to population declines. Although population-level effects remain unknown, pronounced reductions in reproductive success, such as those documented in our study, could impact local populations. This is particularly true if other adverse effects of maternal transfer emerge during larval and/or juvenile development. For example, in American toads (*Bufo americanus)* maternal Hg exposure and dietary exposure during larval development both had negative effects on offspring health. However, the two routes of exposure acted synergistically, decreasing survival by 50% compared to reference larvae (Bergeron et al., 2011b). Similarly, Metts et al., (2012) found that previous maternal exposure to CCW interacted with larval exposure to reduce survival by 85% compared to reference larvae reared on reference sediments. Together, a 35% reduction in reproductive success and an 85% reduction in survival of remaining larvae to metamorphosis would result in very poor per capita production of recruits to the terrestrial environment. For example, a typical female producing 4,000 eggs in the reference site would be expected to produce 2,243 successful recruits, while a female from the ash basin would be expected to produce only 232 recruits, a 90% reduction in recruitment to the terrestrial environment. Such pronounced effects on recruitment could result in population declines, but reduced larval density due to mortality could decrease the strength of density-dependent interactions among larvae in the ash basins, improving the survival of remaining larvae at the site with no net effect on local population dynamics (Vonesh and De la Cruz, 2002). Future modeling studies such as those by

Willson et al., (In press) are needed to project our observed individual-level responses to potential changes in population dynamics.

In conclusion, bioaccumulation and maternal transfer of trace elements from exposure to CCW is an important factor that negatively affects amphibian reproductive success and may ultimately contribute to localized amphibian declines. Our results yield further evidence that CCW-contaminated basins and wetlands may be ecological traps for amphibians and other wildlife, because animals attracted to these sites accumulate contaminants and experience deleterious effects, including reduced reproductive success, which is not ameliorated by aging of ash. Our study also highlights the need for continued research on maternal transfer of contaminants in amphibians. Because of the correlations between trace element concentrations and reproductive success we attribute the observed reductions to females; however, males were also collected from the same contaminated sites. Thus, the observed effects could have also been attributable to males being exposed to CCW. Future studies examining paternal effects should be a high priority. Finally, investigating the effects of maternal transfer on post-metamorphic terrestrial juveniles is necessary to determine if amphibian populations can persist in CCW-contaminated basins or whether they require immigration from nearby source populations.

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Table 2.1. Elemental composition of postovipositional female southern toads and their eggs collected from a reference site (reference), a natural wetland contaminated with coal combustion waste over 35 years ago (ash plume), and an active coal ash settling basin (ash basin). Data presented are means ± 1 SE. Different letters indicate significant differences.

	Females (µg/g)			Eggs (µg/g)		
Element	Reference	Ash plume	Ash basin	Reference	Ash plume	Ash basin
	n = 15	n = 15	n = 14	n = 15	n = 15	n = 13
As	^{A}0.41 ± 0.01	^{B}0.60 ± 0.02	^{B}0.63 ± 0.01	^{A}0.42 ± 0.01	^{A}0.47 ± 0.01	^{A}0.41 ± 0.02
Cd	^{A}0.24 ± 0.01	^{A}0.15 ± 0.01	^{A}0.15 ± 0.01	^{A}0.01 ± 0.01	^{B}0.03 ± 0.01	^{B}0.03 ± 0.01
Cr	^{A}2.32 ± 0.05	^{A}1.98 ± 0.03	^{A}2.33 ± 0.07	^{A}2.00 ± 0.02	^{A}2.23 ± 0.02	^{A}2.31 ± 0.03
Cu	^{A}11.61 ± 0.37	^{B}18.97 ± 0.77	^{A}11.05 ± 0.24	^{A}12.87 ± 0.18	^{B}21.02 ± 0.31	^{B}17.91 ± 0.40
Hg	^{A}0.63 ± 0.01	^{A}0.53 ± 0.02	^{A}0.69 ± 0.02	^{A}0.32 ± 0.01	^{A}0.31 ± 0.01	^{B}0.22 ± 0.02
Ni	^{A}0.33 ± 0.02	^{B}1.61 ± 0.07	^{B}1.30 ± 0.08	^{A}0.19 ± 0.02	^{B}0.44 ± 0.02	^{B}0.72 ± 0.04
Pb	^{A}1.07 ± 0.03	^{A}0.39 ± 0.02	^{A}0.94 ± 0.05	^{A}0.41 ± 0.02	^{B}1.20 ± 0.08	^{B}1.14 ± 0.07
Se	^{A}2.22 ± 0.03	^{B}4.60 ± 0.10	^{B}3.83 ± 0.06	^{A}3.80 ± 0.06	^{B}5.32 ± 0.10	^{B}5.27 ± 0.13
Sr	^{A}64.79 ± 1.39	^{B}180.86 ± 3.46	^{C}114.01 ± 3.09	^{A}0.48 ± 0.04	^{B}1.78 ± 0.11	^{B}1.51 ± 0.10
V	^{A}0.42 ± 0.02	^{B}0.74 ± 0.02	^{B}0.78 ± 0.02	^{A}0.38 ± 0.02	^{A}0.67 ± 0.03	^{A}0.74 ± 0.04
Zn	^{A}138.57 ± 2.36	^{A}112.6 ± 2.1	^{A}123.98 ± 1.9	^{A}411.85 ± 3.83	^{A}404.8 ± 4.39	^{A}428.14 ± 6.81

Table 2.2 Snout vent length (SVL), mass, clutch size, percent hatching success, abnormality frequency, viability, and reproductive success of female southern toads collected from a reference site (reference), a natural wetland contaminated with coal combustion waste over 35 years ago (ash plume), and an active coal ash settling basin (ash basin). SVL, mass, hatching success, morphological abnormality frequency, and viability data are presented as means ± 1 SE. Clutch size and reproductive success are presented as LS means adjusted for female SVL ± 1 SE. Different letters indicate significant differences.

	Reference n = 15	Ash plume n = 15	Ash basin n = 14
SVL (mm)	^{A}65.13 ± 0.48	^{A}66.8 ± 0.33	^{A}63.71 ± 0.41
Mass (g)	^{A}30.23 ± 0.68	^{A}31.7 ± 0.41	^{A}27.04 ± 0.53
Clutch size (# eggs)	^{A}4,070 ± 98	^{A}4,353 ± 66	^{B}3,484 ± 85
Hatching success (%)	^{A}91.1 ± 0.6	^{B}66.0 ± 2.4	^{B}75.5 ± 1.8
Abnormality frequency (%)	^{A}11.0 ± 0.3	^{A}11.8 ± 0.6	^{A}10.3 ± 0.6
Viability (%)	^{A}71.9 ± 0.6	^{B}46.2 ± 1.8	^{B}52.9 ± 1.6
Reproductive success (predicted number of viable offspring)	^{A}2,938 ± 78	^{B}1,973 ± 93	^{B}1,899 ± 72

Table 2.3. Percentage of southern toad hatchlings from females collected from a reference site (reference), a natural wetland contaminated with coal combustion waste over 35 years ago (ash plume), and an active coal ash settling basin (ash basin) displaying morphologic abnormalities hatchlings. Data presented are means ± 1 SE.

	Reference n = 15	Ash plume n = 14	Ash basin n = 13
Axial	9.4 ± 0.3	9.5 ± 0.5	8.6 ± 0.6
Swelling	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
Oral	0.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
Blister	0.1 ± 0.01	0.4 ± 0.01	0.5 ± 0.01
Total	11.1 ± 0.15	11.7 ± 0.25	10.4 ± 0.26

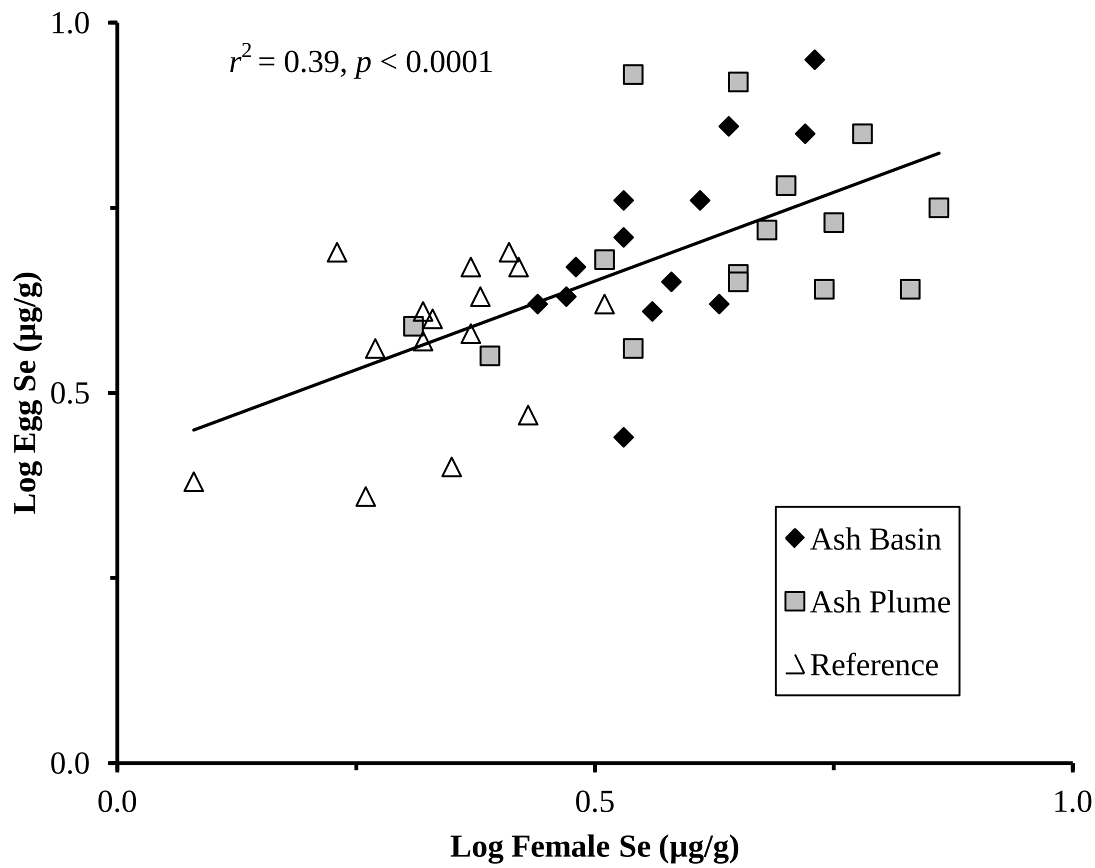

Figure 2.1. Relationship between log selenium concentrations (µg/g dry mass) in carcasses of female southern toads and their eggs collected from an active coal ash settling basin (ash basin), a natural wetland contaminated with coal combustion waste over 35 years ago (ash plume), and an uncontaminated reference site (reference).

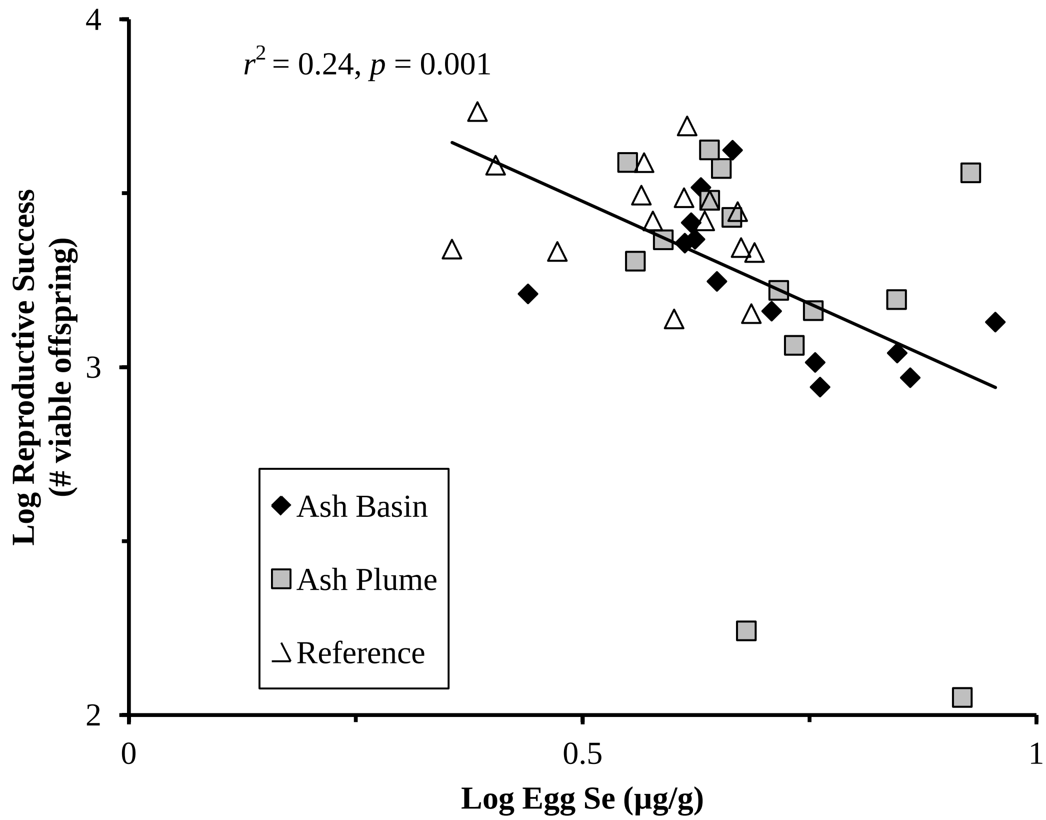

Figure 2.2 Relationship between log selenium concentrations (µg/g dry mass) in eggs and reproductive success of females collected from an active coal ash settling basin (ash basin), a natural wetland contaminated with coal combustion waste over 35 years ago (ash plume), and an uncontaminated reference site (reference).

CHAPTER 3

INTERACTIVE EFFECTS OF MATERNAL AND ENVIRONMENTAL EXPOSURE TO COAL COMBUSTION WASTES DECREASE SURVIVAL OF LARVAL SOUTHERN TOADS (*BUFO TERRESTRIS*)[2]

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[2] Metts, B. S., K. A. Buhlmann, D. E. Scott, T. D. Tuberville, W. A. Hopkins. 2012. *Environmental Pollution* 164: 211-218. Reprinted here with permission of the publisher.

ABSTRACT

We conducted a mesocosm study to assess the individual and interactive effects of previous maternal exposure and larval exposure to trace element-laden sediments on southern toads (*Bufo terrestris*). Previous maternal exposure to coal combustion wastes (CCW) reduced larval survival to metamorphosis up to 57% compared to larvae of unexposed females. Larvae reared on CCW accumulated significant concentrations of trace elements resulting in extended larval periods, reduced growth rates, and reduced mass at metamorphosis. However, the effects were dependent on age of sediments, suggesting the effects of contaminants from CCW may be partially ameliorated over time through the reduced bioavailability of trace elements in aged CCW. Most importantly, maternal exposure to contaminants coupled with larval exposure to fresh CCW interacted to reduce survival to metamorphosis by 85% compared to reference conditions. Our study yields further evidence that disposal of CCW in aquatic basins potentially creates ecological traps for some amphibian populations.

Keywords: Amphibian, Coal combustion wastes, Contaminants, Trace element, Selenium

Introduction

Environmental contamination is thought to be one of the greatest contributors to worldwide amphibian population declines (Collins and Storfer, 2003; Hoffmann et al., 2010; Stuart et al., 2004). Environmental contaminants come from many sources, but coal fired power plants are one of the largest producers of contaminated solid wastes in the U.S. (USDOE, 2005). Coal combustion wastes (CCW) contain high concentrations of trace elements (e.g., arsenic (As), mercury (Hg), selenium (Se)) and are often disposed of in open aquatic settling basins (Rowe et al., 2002). Amphibians and other wildlife using these basins can accumulate elevated concentrations of trace elements, resulting in adverse effects on survival, growth, development, behavior, performance, and recruitment (Hopkins et al., 2000, 2006; Raimondo and Rowe, 1998; Rowe et al., 1996). Taken together these studies suggest that CCW contaminated wetlands may serve as ecological traps to amphibian populations (Roe et al., 2006; Rowe and Hopkins, 2003; Snodgrass et al., 2003, 2004; Snodgrass and Hopkins, 2005).

The majority of research investigating the effects of contaminants on amphibians has focused on environmental exposure (i.e., sediment, soil, and water) or trophic uptake (Linder and Grillitsch, 2000), but other routes of exposure have gained recent attention. Although environmental exposure to contaminants often elicits adverse effects, parental factors such as maternal transfer of contaminants may also negatively affect reproduction and development (Hopkins et al., 2006). Previous studies have demonstrated that adult amphibians can accumulate contaminants and transfer them to their eggs (Kadokami et al., 2004; Bergeron et al., 2010), ultimately leading to reduced hatching success and offspring viability (Kotyzova and Sundeman, 1998; Hopkins et al., 2006; Bergeron et al., 2011a). In addition, the consequences of previous maternal exposure may become apparent later in development of surviving offspring (i.e., long term and/or latent effects; Bergeron et al., 2011b). For example, larvae of female American toads *(Bufo (Anaxyrus) americanus*; hereafter *Bufo*) exposed to Hg grew more slowly as embryos and were smaller at metamorphosis than larvae from unexposed females (Bergeron et al., 2011b). Further, amphibians born into the same environment as their parents can be exposed to contaminants of maternal origin and from the environment, making it important to identify interactions between maternal and environmental or dietary exposure (Bergeron et al., 2011b). For instance, maternal Hg exposure in *B. americanus* had a greater influence on offspring health than larval dietary exposure. However, the two routes of exposure acted synergistically, reducing survival by 50% compared to reference larvae (Bergeron et al., 2011b).

The objectives of our study were to assess the individual and interactive effects of previous maternal exposure and subsequent environmental (i.e., sediment) exposure to trace elements on survival, growth, and performance of larval southern toads (*Bufo terrestris*). We also sought to determine whether reduced availability of trace elements in aged surface sediments would mitigate the effects on amphibians. We conducted a factorial mesocosm experiment designed to simulate conditions in 1) a CCW settling basin, 2) a natural wetland contaminated with CCW that has undergone natural succession for over 35 years, and 3) an uncontaminated reference wetland. We hypothesized that maternal and environmental exposure to trace elements would each independently prolong the larval period and decrease survival, growth rate, size, and performance of metamorphs. Furthermore, we predicted that the interaction of maternal and environmental exposure would exacerbate the individual effects of contaminants on larvae. Because the availability of trace elements in contaminated wetlands may decrease over time, due to downward leaching from surface sediments and other biogeochemical processes (Sandhu et al., 1993), we hypothesized that the effects of contaminants would be less pronounced in larvae reared on wetland sediments contaminated with ash deposited more than 35 years ago compared to sediments from an active ash settling basin.

METHODS

Study species. *Bufo terrestris* is a common anuran in the southeastern U.S. that like many other amphibians, has a complex life cycle consisting of aquatic larvae and terrestrial adults.   Their home range extends up to 1.6 km from wetlands during the non-breeding season (Bogert, 1947) and adults migrate in the spring and summer to aquatic breeding sites (Jensen, 2008). Females lay 2,500 to 4,000 eggs which hatch in 2 to 4 days. Larvae graze mostly on aquatic vegetation and algae, exhibit rapid growth, and have a relatively short larval period (30-55 days; Jensen, 2008). After metamorphosis juveniles move on to land and migrate across the landscape. Once they mature, most individuals return to breed in their natal wetland, while others disperse to neighboring wetlands (Breden, 1987; Berven, 1990). Thus, adults may inhabit and breed in both contaminated and uncontaminated wetlands occurring within a few square kilometers of one another.

Study sites. We collected adult *B. terrestris* from three locations on the Savannah River Site near Aiken, SC, USA: the D-area ash basin, the ash plume wetland, and a reference site (Ellenton Bay, a nearby uncontaminated Carolina bay wetland). The two contaminated sites (ash basin and ash plume wetland) are associated with a coal powered steam generation facility, which includes a disposal area where sluiced CCWs are discharged into a series of open settling basins. Sediments in this basin are comprised entirely of CCWs that are enriched with trace elements (Rowe et al., 2002; Hopkins et al., 2006) and extremely low in organic matter (Hopkins et al., 2004). The second contaminated site is the ash plume wetland, a natural wetland in the Savannah River floodplain that was contaminated when CCWs were discharged into it during the 1950's to early 1970's (Roe et al., 2005). Since the cessation of CCW discharge into the floodplain, vegetational succession has occurred in the ash plume wetland, and possible attenuation of trace elements in the sediments (Sandhu et al., 1993) could create an environment that is less contaminated than the active ash basins. Coal combustion wastes in the ash plume sediments extend to a depth of 2.7 m and cover approximately 40 ha, 30% of which is occasionally inundated when the Savannah River floods into its surrounding floodplain. A thin layer (~2.5 cm) of organic material covers the CCW and the ash plume wetland is vegetated with a mixed floodplain flora community (Roe et al., 2005). The reference site, Ellenton Bay, is a 10-ha natural Carolina bay wetland with no known historical contamination, located approximately 3 km from the D-area facility (Sharitz, 2003; Sharitz and Gibbons, 1982). All three sites are surrounded by mixed pine-hardwoods and open field habitats.

Experimental design and data collection. In February 2009, we created outdoor mesocosms using polyethylene cattle tanks (1.85 m diameter, 1480 L volume). We randomly assigned each mesocosm two treatment components, sediment type and female origin, yielding a 3x3 completely randomized design replicated four times (n=36 mesocosms). We filled mesocosms to a depth of 5 cm with 93 L of sediment from either the ash basin, ash plume wetland, or commercially available river sand. To simulate the low organic content of CCW, we used river sand as reference sediments instead of sediments from a natural wetland, which are typically high in organic content (Sharitz and Gibbons, 1982). The initial conductivity of water containing CCWs can be quite high, thus to help desalinate the water, we filled each mesocosm with 500 L of well water, drained each mesocosm, and refilled them with 1000 L of well water. To provide nutrients to the system, we added 1.5 kg of dry leaf litter from an uncontaminated source to each mesocosm. We inoculated each mesocosm with 10 L of pond water collected from a nearby unpolluted wetland to establish phytoplankton populations.

At the beginning of the study we collected sediment and water samples from each mesocosm for elemental analysis. We collected sediments using a modified 60 mL syringe inserted with backpressure into the sediment at six locations, one at each of the cardinal directions near the mesocosm periphery and two near the center. We homogenized the six subsamples (~15 mL/sample) from each mesocosm into a single sample (90 mL) and froze them for subsequent elemental analyses. We collected water samples from 15-20 cm beneath the surface near the center of the mesocosm using a clean 250 mL plastic bottle. We then acidified water samples to 2% acidity with ultra high pure nitric acid until elemental analyses were performed. We also monitored environmental conditions in each mesocosm weekly by measuring water temperature, specific conductance, dissolved oxygen, pH, and oxidation-reduction potential (ORP) using a YSI 556 MPS handheld probe (YSI Environmental Inc., Yellow Springs, OH).

To quantify periphyton abundance, we suspended a 12.7 x 17.8 cm plastic plate in each mesocosm on 15 April 2009. On 19 June 2009, we removed the plates, scraped both sides with a razor blade, and rinsed the material with deionized water. We dried each periphyton sample at 60° C and then combusted them at 500° C for 24 hours. We measured periphyton dry mass to the nearest 0.1 mg before and after combustion and used the difference between the two as our estimate of organic content in periphyton.

We collected adult *B. terrestris* on 28 - 29 March 2009 from breeding congregations at the ash basin, ash plume, and reference site wetlands. We transported toads to the lab where we measured snout-vent length (SVL, mm) and weighed (mg) each female. We injected males and females with human chorionic gonadotropin (males 100, females 250 IU), and placed the breeding pairs in plastic containers with well water. Thirty-six females (12 from each site) deposited eggs. After pairs bred, we released males at their location of capture. We kept females in plastic containers in the lab for an additional 48 hours to allow them to void their gut contents. We then euthanized females by immersion in buffered tricaine methanesulfonate (MS-222) and freeze-dried the carcasses for subsequent elemental analysis.

We transferred a subsample of 120-150 eggs from each female to a 19 L floating plastic container with screen sides and placed it in the mesocosm to which that female had been assigned. Clutches were not mixed; this approach allowed us to track offspring of individual females and subsequently evaluate female trace element profiles relative to the success of their young. After hatching in the flow-through container, 100 free-swimming larvae were transferred from the embryonic container into the mesocosm for the remainder of the larval period. We monitored larvae in mesocosms for evidence of initiation of metamorphosis (Gosner stage 42), at which time we set two minnow traps in each mesocosm to capture metamorphosing individuals. We collected metamorphs and transferred them to the laboratory where they were kept at 23° C on moist paper towels in plastic containers. After complete tail resorption (typically 2-4 days) we weighed and measured each metamorph. In addition, we assessed performance on a subset of 30 metamorphs from each mesocosm by measuring hopping speed (distance covered in the first 30 seconds of a trial; Walton, 1988) and endurance (total distance covered before no longer responding to ten consecutive proddings) on a 3 m linear track (Beck and Congdon, 2000). In mesocosms that produced fewer than 30 metamorphs we measured performance of all metamorphs from that mesocosm. After performance tests, we euthanized metamorphs, pooled them by mesocosm into samples large enough for elemental analysis (~800 mg wet weight), and freeze-dried them for elemental analyses.

Sample preparation and element analysis. We individually digested a subsample (~250 mg) of each homogenized female carcass, each sample of pooled metamorphs, and mesocosm sediment samples in 10 mL of trace metal grade nitric acid (70% HNO_3) using microwave digestion (MarsExpress, CEM Corp., Matthews, NC). After HNO_3 digestion, we brought tissue samples to a final volume of 15 mL and sediment samples to a final volume of 50 mL with 18 MΩ deionized water. Acidified water samples from mesocosms were not digested or diluted prior to analysis. We determined elemental concentrations of As, Hg, Se, strontium (Sr), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), vanadium (V), and zinc (Zn) in tissue, sediment, and water samples using inductively coupled plasma mass spectrometry (Perkin Elmer, Norwalk, CT). For quality control, we included certified reference material in each analysis

(TORT-2 and LUTS; National Research Council of Canada, Ottawa, Canada). Mean percent recoveries for elements in certified reference material ranged from 84 to 95%. Minimum detection limits in water samples were: As, 0.08; Cd, 0.12; Cr, 0.02; Cu, 0.08; Ni, 0.10; Pb, 0.07; Se, 0.88; Sr, 3.92; V, 0.15; Zn, 0.56 μg/L. Detection limits in sediment and tissue samples were: As, 0.40; Cd, 0.27; Cr, 0.36; Cu, 0.39; Hg, 0.23; Ni, 0.39; Pb, 1.31; Se, 1.70; Sr, 2.86; V, 0.17; Zn, 2.35 μg/kg.

Statistical analyses. We performed statistical analyses using SAS 9.1 (SAS Institute, Cary, NC). We examined the assumptions of homogeneity of variance and normality, and where deviations from these assumptions were found, we performed log or angular transformations to better meet the assumptions. For each mesocosm, we calculated percent survival to metamorphosis, average days to metamorphosis, mass, SVL, growth rate, hopping speed, endurance, and trace element composition of metamorphs. We used mesocosm-specific mean values as the unit of replication for all dependent variables in the models. In all statistical comparisons, we accepted statistical significance at $\alpha = 0.05$ and we present data as mean ±1 standard error (SE). When multivariate models were significant, we used individual ANOVAs followed by Bonferroni corrected pair-wise comparisons to identify differences among treatment groups.

To determine differences in the weekly mesocosm water chemistry parameters (e.g., temperature, dissolved oxygen), we used repeated measures MANOVA with sediment type as the independent variable and time as the repeated measure. We compared dry mass and organic content of periphyton among mesocosms using ANOVA with sediment type as the independent variable.

We compared trace element concentrations in metamorphs, sediments, and water among treatments (sediment type and female origin), using individual MANOVAs. To ensure that the trace element profiles of females from each study site were comparable across the three sediment treatments to which they were assigned, we first used individual MANOVAs for comparisons within each site. After confirming that trace element profiles of females within each site were equivalent, we then compared trace element concentrations in females among sites using MANOVA. For statistical comparisons, we assigned trace element concentrations below the instrument's detection limit (BDL) a value of half the minimum detection limit. However, if more than 10% of the samples from a treatment were BDL for a given element, we excluded that treatment from analysis of that element.

We compared percent survival to metamorphosis among mesocosm treatments using ANOVA. To better quantify the latent effect of previous maternal exposure on survival, we compared survival of larvae from the three female exposure groups that were raised only on reference sediments using contrast statements. To quantify the effects of environmental exposure on larval survival, we compared survival of larvae from each female group reared on their source sediment to survival when reared on the other two sediment types. Because survival within a mesocosm can influence density, which affects amphibian growth rate, size, and larval period (Travis, 1983; Wilbur, 1997) we initially compared SVL, mass, growth rate, and days to metamorphosis among treatments using MANCOVA with survival as the covariate (e.g., Parris and Semlitsch, 1998). However, because survival was influenced by the treatments, making it an inappropriate covariate (Cochran, 1957), and because survival was not significant in the multivariate or individual models, we removed survival from the model in the final analysis. Because size at metamorphosis can influence performance, we compared hopping speed and endurance of metamorphs among treatments using MANCOVA with SVL as the covariate.

RESULTS

Water quality. Water quality changed over time and was dependent on sediment type (time: Pillai's trace = 0.99, $F_{4, 318}$ = 131,635, p < 0.001; sediment: $F_{2, 321}$ = 11.15, p < 0.001; time x sediment: Pillai's trace = 0.92, $F_{8, 638}$ =68.06, p < 0.001). Briefly, water temperature, pH, and specific conductance increased in all mesocosms as summer progressed. We observed differences among mesocosm treatments for specific conductance ($F_{2, 321}$ = 840.98, p < 0.001) and dissolved oxygen ($F_{2, 321}$ = 17.0, p < 0.001), but not temperature, pH, or ORP (temp: $F_{2, 321}$ = 0.08, p = 0.92; pH: $F_{2, 321}$ = 0.99, p = 0.37; ORP: $F_{2, 321}$ = 1.29, p = 0.28). Overall mean specific conductance in reference (48.3 ± 0.6 uS/cm) mesocosms was significantly lower than ash plume (57.1 ± 0.4 uS/cm), which was significantly lower than ash basin mesocosms (93.2 ± 0.8 uS/cm). Similarly, overall mean dissolved oxygen was significantly lower in reference compared to ash plume and ash basin mesocosms (reference: 62.2 ± 0.18%, ash plume: 68.8 ± 0.13%, ash basin: 75.3 ± 0.12%).

Periphyton dry mass was similar between ash plume (62.1 ± 3.9 mg) and reference (52.5 ± 2.9 mg) mesocosms, but was significantly reduced in the ash basin (25.9 ± 3.3 mg) mesocosms (dry mass: $F_{2, 33}$ = 5.33, p = 0.01). In contrast, percent organic material in periphyton was similar between ash plume (30.1 ± 0.7%) and ash basin (23.2 ± 0.4%) mesocosms, but was significantly lower in reference mesocosms (11.1 ± 0.3%; percent organic: $F_{2, 33} = 35.20$, $p < 0.001$).

Elemental concentrations in females. The females within each site that were allocated among the three sediment treatments had similar whole body trace element concentrations (in all three cases: $p > 0.77$). However, as expected, we detected significant differences in elemental concentrations in adult females among sites (Pillai's trace = 1.33, $F_{18, 52} = 5.74$, $p < 0.001$). Specifically, female concentrations of Cu, Pb, Se, and Sr were significantly different among sites (in all cases: $F_{2, 33} > 5.45$, $p < 0.01$; Table 3.1). Female Se and Sr concentrations were 2 to 4 times higher in ash basin and ash plume wetland females compared with reference females (Table 3.1). Copper concentrations in ash plume females were two times that of reference and ash basin females (Table 3.1). Although Pb concentrations were generally low in all females, concentrations in reference females were 46% higher than ash basin females, and also elevated compared to ash plume females, which had Pb levels below detection limits (Table 3.1).

Elemental concentrations in mesocosm sediment. Elemental concentrations differed significantly among the sediments placed in mesocosms (Pillai's trace = 1.33, $F_{22,48} = 35.39$, $p < 0.001$). Concentrations of As, Cr, Cu, Ni, and Zn in sediments were similar between the ash basin and ash plume wetland mesocosms, but were significantly higher compared to reference sediments (in all cases: $F_{2, 33} > 45.96$, $p < 0.001$; Table 3.2). Sediment concentrations of Cd, Hg, Pb, Se, Sr, and V were significantly different among all three sediment types (in all cases: $F_{2, 33} > 109.38$, $p < 0.001$; Table 3.2), with levels in ash basin sediments being generally higher than ash plume sediments, which in turn exceeded reference sediment concentrations. Sediment Hg concentrations were 2 to 4 times higher in both contaminated treatments compared with the reference mesocosms. Selenium was 47 times higher in the ash basin sediments and 12 times higher in the ash plume compared with the reference sediments (Table 3.2). Concentrations of As, Cr, Pb, V, and Zn were 20 to 40 times higher, and Ni, Sr, and Cu concentrations were more than 136 times higher in ash basin and ash plume sediments compared with reference sediments (Table 3.2).

Elemental concentrations in mesocosm water. Elemental concentrations in mesocosm water differed significantly among the sediment treatments (Pillai's trace = 1.92, $F_{18,52}$ = 67.81, p < 0.001). Concentrations that were BDL were excluded from statistical models (Table 3.2). Chromium concentrations in water samples were similar among sediment treatments. However, water concentrations of As, Sr, and V were 13-24 times higher in ash basin mesocosms and 2-3 times higher in ash plume mesocosms compared to reference mesocosms (Table 3.2). Water concentrations of Zn were nearly two times higher in ash basin mesocosms compared with ash plume and reference mesocosms (Table 3.2). Copper concentrations in mesocosm water were 40–50% higher in reference mesocosms compared with ash plume and ash basin (Table 3.2).

Elemental concentrations in metamorphs. Elemental concentrations in metamorphs were dependent on sediment type (Pillai's trace = 1.77, $F_{22, 34}$ = 12.06, p < 0.001), but not female origin (Pillai's trace = 0.87, $F_{22, 34}$ =1.19, p = 0.32) or their interaction (sediment * female interaction; Pillai's trace = 1.68, $F_{44, 76}$ = 1.26, p = 0.19). Specifically, concentrations of As, Se, Sr, V, and Zn in recent metamorphs differed significantly among sediment types (in all cases: $F_{2, 26}$ >7.12, p < 0.003; Table 3.1).

Concentrations of As, Se, and Sr in metamorphs were 15-25 times higher in ash basin and 3-5 times higher in ash plume than reference (Table 3.1). Vanadium concentrations in ash basin metamorphs were 5 times higher than reference, and Zn concentrations were 57% higher in ash basin compared to reference metamorphs (Table 3.1). However, V and Zn concentrations were similar between ash plume and reference metamorphs. Although Cu concentrations in metamorphs reared in ash basin mesocosms were twice as high as those reared in ash plume and reference mesocosms, the differences were not statistically different ($F_{2, 26} = 2.52$, $p = 0.10$; Table 3.1)

Effects on larvae. Bufo terrestris larvae began to metamorphose on 4 May 2009 and continued until 22 June (36-77 day larval period). We observed a significant interaction between sediment type and female origin on survival to metamorphosis (sediment * female: $F_{4, 27} = 2.90$, $p = 0.04$; sediment: $F_{2, 27} = 4.78$, $p = 0.02$, female origin: $F_{2, 27} = 1.18$, $p = 0.32$). Survival to metamorphosis was dramatically reduced in larvae of ash basin and ash plume females compared to those of reference females, but the effect was dependent on sediment type during the larval period (Figure 3.1). Survival to metamorphosis was highest in reference larvae reared on reference sediments and lowest in ash basin larvae reared on ash basin sediments. Survival of larvae from ash basin females reared on reference sediments was reduced by 34%, compared to reference larvae reared on reference sediments ($F_{1, 27} = 2.29$, $p = 0.14$; Figure 3.1). Similarly, survival of larvae from ash plume females reared on reference sediments was reduced by 57% compared to reference larvae reared on reference sediments ($F_{1, 27} = 6.23$, $p = 0.02$; Figure 3.1). When reference larvae were reared on ash basin and ash plume sediments, survival was reduced by 42-60% compared to reference larvae reared on reference sediments (ash basin: $F_{1, 27} = 3.50$, $p = 0.07$, ash plume: $F_{1, 27} = 6.43$, $p = 0.02$; Figure 3.1). Additionally, survival of larvae with mothers originating from the ash plume reared on ash plume sediments was similar when reared on reference sediments ($F_{1, 27} = 1.73$, $p = 0.20$; Figure 3.1), but was reduced by 65% when reared on ash basin sediments ($F_{1, 27} = 6.44$, $p = 0.02$; Figure 3.1). Survival of larvae from ash basin females increased by 427% when reared on ash plume sediments ($F_{1, 27} = 6.47$, $p = 0.02$; Figure 3.1), and by 373% when reared on reference sediments ($F_{1, 27} = 4.39$, $p = 0.05$; Figure 3.1).

Larval exposure to contaminated sediments had sublethal effects on metamorphs (sediment: Pillai's trace = 0.78, $F_{8, 46} = 3.86$, $p = 0.001$; female origin: Pillai's trace = 0.35, $F_{8, 46} = 1.25$, $p = 0.29$; sediment * female: Pillai's trace = 0.41, $F_{16, 104} = 0.74$, $p = 0.75$). Larval period length differed significantly among mesocosm sediment types ($F_{2, 26} = 20.13$, $p < 0.001$, Figure 3.2). After accounting for female origin, larval period duration on ash basin sediments was 27-40% longer than on the other two sediment types (Figure 3.2). Mass of metamorphs reared on reference and ash plume sediments was significantly greater (30%) than metamorphs reared on ash basin sediments (female: $F_{2, 26} = 0.05$, $p = 0.96$, sediment: $F_{2, 26} = 5.84$, $p = 0.01$, sediment * female: $F_{4, 26} = 0.73$, $p = 0.58$; Figure 3.3). However, metamorph body length was statistically similar among treatments (female: $F_{2, 26} = 0.14$, $p = 0.87$, sediment: $F_{2, 26} = 3.0$, $p = 0.07$, sediment * female: $F_{4, 26} = 0.54$, $p = 0.71$). After accounting for female origin, growth rate of larvae reared on ash basin sediments was significantly reduced by an average of 0.4 mg/day compared to those reared on reference and ash plume sediments (female: $F_{2, 26} = 0.83$, $p = 0.45$; sediment: $F_{2, 26} = 13.96$, $p < 0.001$; sediment * female: $F_{4, 26} = 0.84$, $p = 0.51$; Figure 3.4).

After accounting for body length, performance of metamorphs was similar among treatments (sediment: Pillai's trace = 0.33, $F_{4, 50}$ = 2.45, p = 0.06; female: Pillai's trace = 0.02, $F_{4, 50}$ = 0.13, p = 0.97; sediment * female: Pillai's trace = 0.36, $F_{8, 50}$ = 1.36, p = 0.24; covariate (SVL): Pillai's trace = 0.71, $F_{2, 24}$ = 29.68, p < 0.001). Although not statistically significant, overall average hopping speed of metamorphs exposed to ash plume sediments was 15-17% greater than those exposed to ash basin and reference sediments (sediment: $F_{2,25}$ = 3.48, p = 0.05; female: $F_{2,25}$ = 0.05, p = 0.95; sediment * female: $F_{4,25}$ = 1.90, p = 0.14; covariate (SVL): $F_{1,25}$ = 52.67, p < 0.001; Figure 3.5). Similarly, endurance of metamorphs exposed to ash plume sediments was 18-22% greater than those exposed to the other sediments (sediment: $F_{2,25}$ = 4.03, p = 0.05; female: $F_{2,25}$ = 0.095, p = 0.91; sediment * female: $F_{4,25}$ = 1.62, p = 0.20; covariate (SVL): $F_{1,25}$ = 57.77, p < 0.001; Figure 3.6).

DISCUSSION

Our study is the first to document a latent effect of previous maternal exposure to CCW in amphibians. Larval exposure to CCW contaminated sediments reduced survival to metamorphosis, but also had sublethal effects on survivors. Moreover, previous maternal exposure and larval exposure to CCWs interacted to substantially reduce survival to metamorphosis. Taken together, our results suggest that CCW disposal basins may be ecological traps that contribute to amphibian population declines.

Comparison of concentrations of As, Se, Sr, V, and Zn in metamorphs from our factorial experiment revealed that bioaccumulation during early development was primarily attributable to larvae grazing contaminated sediments and not from maternal exposure. Tissue concentrations in our study were consistent with previous mesocosm experiments and other research conducted at our field sites. For instance, mole salamanders (*Ambystoma talpoideum*) reared to metamorphosis in mesocosms containing sediments from the ash basin had mean Se and Sr concentrations of 35 and 250 µg/g (Roe et al., 2006), while male *B. terrestris* had mean concentrations of 17 and 387 µg/g, respectively (Hopkins et al., 1998). Adult *B. terrestris* and southern leopard frogs (*Rana (Lithobates) sphenocephala*) collected from the ash plume wetland had Se and Sr concentrations of 7 and 325 µg/g (Roe et al., 2005), and *B. terrestris* metamorphs had mean concentrations of 46 and 225 µg/g, respectively (Roe et al., 2005). Collectively these studies demonstrate the propensity of amphibians to accumulate trace elements as a result of exposure to CCW, making subsequent transfer of contaminants to terrestrial food webs likely (Snodgrass et al., 2003; Unrine et al., 2007).

We documented a latent effect of previous maternal exposure to CCW on larval survival to metamorphosis. Specifically, when *B. terrestris* larvae of ash basin and ash plume females were reared on reference sediments, maternal exposure to contaminants reduced survival to metamorphosis by 34-57% compared to larvae from reference females raised on reference sediments. In other vertebrate groups, maternal exposure to contaminants can also reduce post-hatching survival of offspring. For example, maternal exposure of snapping turtles (*Chelydra serpentina*) to polychlorinated biphenyls (PCBs) reduced survival of their offspring during the first 14 months of life (Eisenreich et al., 2009). Our findings highlight the importance of tracking the effects of maternal exposure to contaminants beyond embryonic development because important responses may manifest later in ontogeny.

Larval exposure to contaminated sediments also reduced survival to metamorphosis. In our study, survival was highest in reference larvae reared on reference sediments, but exposure of reference larvae to ash basin and ash plume sediments reduced their survival by 42 and 60%, respectively. Larvae of ash plume females reared on ash basin sediments experienced a 65% reduction in survival to metamorphosis compared to those reared on ash plume sediments. In contrast, larvae of ash basin females reared on reference or ash plume sediments had improved survival compared to those reared on ash basin sediments, demonstrating that the combined effect of maternal and environmental exposure to trace elements was much greater than maternal exposure alone. Together, our results suggest that female toads from uncontaminated sites that are attracted to CCW contaminated wetlands to breed can experience a reduction in reproductive success. In contrast, females from ash basins that migrate to less contaminated wetlands to breed may experience improved reproductive success relative to their success when breeding in ash basins.

Our study demonstrated an interaction between previous maternal exposure and subsequent larval environmental exposure to CCW contaminated sediments, resulting in very low recruitment. The combination of maternal exposure and larval exposure to fresh CCW reduced survival to metamorphosis by 85% relative to reference conditions. In a laboratory study, Bergeron et al., (2011b) found that in *B. americanus* previous maternal Hg exposure and larval dietary Hg exposure acted synergistically to reduce survival by 50% compared to reference larvae. In combination, these two studies suggest that the interaction of these two common exposure routes warrant further attention because such dramatic reductions in recruitment could ultimately contribute to amphibian population declines. In addition, because of their high abundance (Burton and Likens, 1975a), diverse roles in food webs (consumers, predators, and prey), and importance for the transfer of energy and nutrients through food webs and across the landscape (Beard et al., 2002; Burton and Likens, 1975b; Gibbons et al., 2006; Ranvestel et al., 2004; Regester et al., 2006; Wyman, 1998), reductions in amphibian recruitment in populations associated with contaminated wetlands may have important ecological ramifications (Rowe et al., 2001).

We found sublethal effects (e.g. size and time to metamorphosis) on larvae exposed to sediments with the highest levels of contaminants. We found that ash basin sediments prolonged the larval period by 11 to 15 days compared to reference and ash plume sediments, respectively. Considering the hot and dry conditions that cause annual pond drying in the southeastern U.S., a two week extension of larval development could limit the number of individuals reaching metamorphosis and reduce the frequency of years with successful recruitment (Pechmann et al., 1989). For example, previous work has shown that exposure to CCW resulted in large numbers of *A. talpoideum* extending their larval period and failing to metamorphose before ponds completely dried (Roe et al., 2006). In our study, larvae reared in mesocosms containing ash basin sediments also grew more slowly and were 30% smaller at metamorphosis than those reared in ash plume or reference mesocosms. Such a considerable reduction in size may negatively influence survival and future reproduction of individuals metamorphosing from ash basins (Berven and Gill, 1983). For instance, Semlitsch et al., (1988) found that size at metamorphosis can influence reproductive success of *A. talpoideum*, although this pattern has not been confirmed for *B. terrestris* (Beck and Congdon, 1999, 2000). Taken together, the sublethal effects on growth and development may further reduce amphibian recruitment from sites contaminated with CCWs under unpredictable environmental conditions (Rowe and Hopkins, 2003).

The reduced size and extended larval periods we observed in larvae reared on ash basin sediments may partially be attributable to lower resource abundance (periphyton biomass) in ash basin mesocosms compared to reference and ash plume mesocosms. The decrease in food resources (i.e., periphyton biomass) we observed in the ash basin mesocosms over the duration of the experiment is consistent with our observations of slower growth, reduced mass at metamorphosis, and prolonged development of larval toads. Similarly, higher resource abundance in ash plume mesocosms may explain the increased growth rate and size of larvae reared on ash plume sediments. Two recent studies suggest that resource abundance may influence the effects of exposure to contaminants on anuran larvae. For instance, under low resource abundance conditions, previous maternal Hg exposure and larval dietary Hg exposure each negatively affected offspring health, but together acted synergistically to cause high mortality (Bergeron et al., 2011b). However, under higher resource abundance conditions, maternal Hg exposure negatively affected offspring, but no effect of dietary exposure was found (Todd et al., 2011).

Our use of river sand low in organic content as reference sediments may have resulted in conservative estimates of the negative effects exposure to contaminants can have on larval growth and size. In our study, periphyton from ash basin and ash plume mesocosms contained greater proportions of organic content than reference mesocosms. Thus, the growth rate and size of larvae in reference mesocosms were likely lower than larvae from natural uncontaminated wetlands where resources are considerably more abundant due to increased nutrient availability. Thus, differences in growth and size between reference and ash plume larvae would likely be greater (Figs. 2-4) if natural wetland sediments were used.

We predicted reduced bioavailability of contaminants in aged ash would ameliorate the effects of CCW exposure on larval amphibians. After 35 years of natural succession, the ash plume area has become revegetated. Elemental concentrations in the sediments were presumably reduced by plant uptake and sequestration, flood events that transported dissolved elements offsite, and other biogeochemical processes that lead to attenuation and/or downward migration of elements in the sediments (Sandhu et al., 1993). Indeed, most trace element concentrations in sediments were lower in ash plume mesocosms compared to ash basin mesocosms. As a result, many of the trace element concentrations bioaccumulated by metamorphs reared on ash plume sediments were lower than those of metamorphs raised on ash basin sediments. In addition, larval period length, mass, and growth rate were negatively influenced by ash basin sediments but not by ash plume sediments. Moreover, survival of larvae from ash basin and ash plume females was similar when reared on reference and ash plume sediments, but was significantly reduced when reared on ash basin sediments. Still, survival to metamorphosis was significantly reduced in reference larvae reared in the ash plume mesocosms compared to reference conditions, suggesting that sediment aging alone does not entirely alleviate the health risks associated with CCW.

Conclusions. Our study demonstrated that larval exposure to CCW contaminated sediments had sublethal and lethal effects, and previous maternal exposure to CCWs significantly reduced survival to metamorphosis. The maternal exposure effects on offspring health we observed are particularly important because they occurred during larval development, long after the embryonic period which has been the predominant focus of most studies on maternal transfer. Our findings suggest that longitudinal studies are needed to fully appreciate the influence of maternal transfer on reproductive success and offspring viability. Importantly, we also demonstrated that the interaction of previous maternal exposure and subsequent larval exposure reduced survival to metamorphosis up to 85% relative to reference conditions. Such a dramatic reduction in recruitment may ultimately result in local amphibian population declines, and suggests that CCW contaminated basins and wetlands may be ecological traps for amphibians.

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Table 3.1. Elemental composition of post-ovipositional female *Bufo terrestris* collected 28-29 March 2009 from reference and contaminated sites, and their recently metamorphosed offspring reared in mesocosms containing sediments from reference and contaminated sites.
Data presented are means[a] ± 1 SE. BDL = below detectable limit.

	Females (µg/g)			Metamorphs (µg/g)		
Element	Reference	Ash plume	Ash basin	Reference	Ash plume	Ash basin
As	BDL	BDL	BDL	A 0.47 ± 0.07	B 3.37 ± 0.02	C 7.44 ± 0.12
Cd	BDL	BDL	BDL	BDL	BDL	BDL
Cr	A 3.95 ± 0.32	A 2.65 ± 0.11	A 4.24 ± 0.23	A 1.65 ± 0.02	A 1.40 ± 0.02	A 2.16 ± 0.08
Cu	A 12.79 ± 0.58	B 26.23 ± 1.29	A 12.97 ± 0.53	A 5.54 ± 0.01	A 6.18 ± 0.01	A 13.13 ± 0.03
Hg	A 0.24 ± 0.01	A 0.22 ± 0.01	A 0.3 ± 0.01	BDL	BDL	BDL
Ni	A 2.42 ± 0.16	A 2.16 ± 0.10	A 3.48 ± 0.17	A 0.43 ± 0.04	A 0.34 ± 0.02	A 0.46 ± 0.02
Pb	A 0.94 ± 0.04	BDL	B 0.51 ± 0.04	BDL	BDL	0.17 ± 0.08
Se	A 1.62 ± 0.03	B 4.1 ± 0.19	C 9.41 ± 1.27	A 2.44 ± 0.13	B 8.07 ± 0.06	C 60.75 ± 0.07
Sr	A 72.04 ± 1.42	B 200.07 ± 7.26	B 162.33 ± 12.71	A 18.62 ± 0.04	B 94.63 ± 0.02	C 274.86 ± 0.05
V	A 0.17 ± 0.01	A 0.27 ± 0.03	C 0.96 ± 0.11	A 0.41 ± 0.14	A 0.25 ± 0.04	B 2.15 ± 0.13
Zn	A 90.73 ± 1.35	A 92.04 ± 1.64	A 98.56 ± 2.24	A 240.54 ± 0.23	A 113.72 ± 0.09	B 377.47 ± 0.42

[a] Significant differences indicated by varying letters

Table 3.2. Elemental concentrations of water and sediment from mesocosms. Data presented are means[a] ± 1SE. BDL = below detectable limit.

	Water (µg/L)			Sediment (µg/g)		
Element	Reference	Ash plume	Ash basin	Reference [b]	Ash plume	Ash basin
As	A0.52 ± 0.04	B1.76 ± 0.06	C6.98 ± 0.22	A1.58 ± 0.06	B50.05 ± 1.34	B44.15 ± 1.08
Cd	BDL	BDL	BDL	A0.93 ± 0.01	B1.21 ± 0.01	C1.11 ± 0.01
Cr	A0.27 ± 0.01	A0.37 ± 0.01	A0.49 ± 0.04	A1.13 ± 0.32	B23.09 ± 0.37	B30.58 ± 0.36
Cu	A1.65 ± 0.09	B0.99 ± 0.03	B0.83 ± 0.04	A0.12 ± 0.02	B42.51 ± 0.49	B69.63 ± 0.66
Hg	BDL	BDL	BDL	A0.14 ± 0.01	B0.32 ± 0.01	C0.66 ± 0.01
Ni	BDL	A0.65 ± 0.03	A0.58 ± 0.01	A0.18 ± 0.02	B24.75 ± 0.38	B39.94 ± 0.39
Pb	A0.15 ± 0.01	A0.1 ± 0.01	BDL	A0.76 ± 0.03	B15.87 ± 0.22	C29.69 ± 0.20
Se	BDL	BDL	7.22 ± 0.27	A0.45 ± 0.05	B5.37 ± 0.15	C21.18 ± 0.14
Sr	A6.97 ± 0.13	B22.01 ± 0.98	C 170.64 ± 3.29	A1.24 ± 0.04	B176.88 ± 3.46	C285.32 ± 5.67
V	A0.29 ± 0.01	B0.54 ± 0.02	C5.24 ± 0.12	A3.41 ± 0.70	B65.56 ± 0.80	C118.69 ± 0.93
Zn	A8.15 ± 0.46	A8.55 ± 0.28	B14.69 ± 0.62	A1.56 ± 0.20	B31.16 ± 0.47	B39.64 ± 0.27

[a] Significant differences indicated by varying letters

[b] Reference sediments were uncontaminated river sand

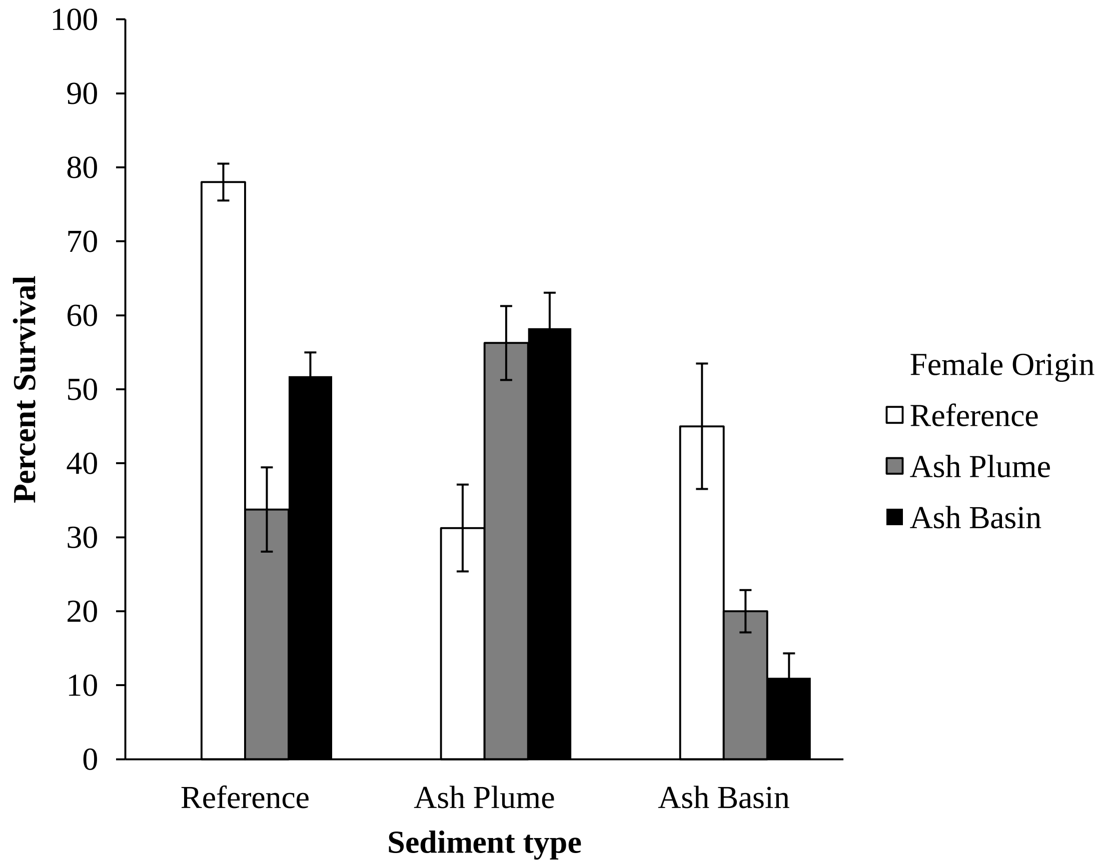

Figure 3.1. Percentage of *Bufo terrestris* larvae that survived to metamorphosis after exposure to sediments from either a reference site (reference), a natural wetland contaminated with coal combustions wastes (ash plume), or a coal ash settling basin (ash basin) in experimental mesocosms. Data are presented as means ± 1 SE.

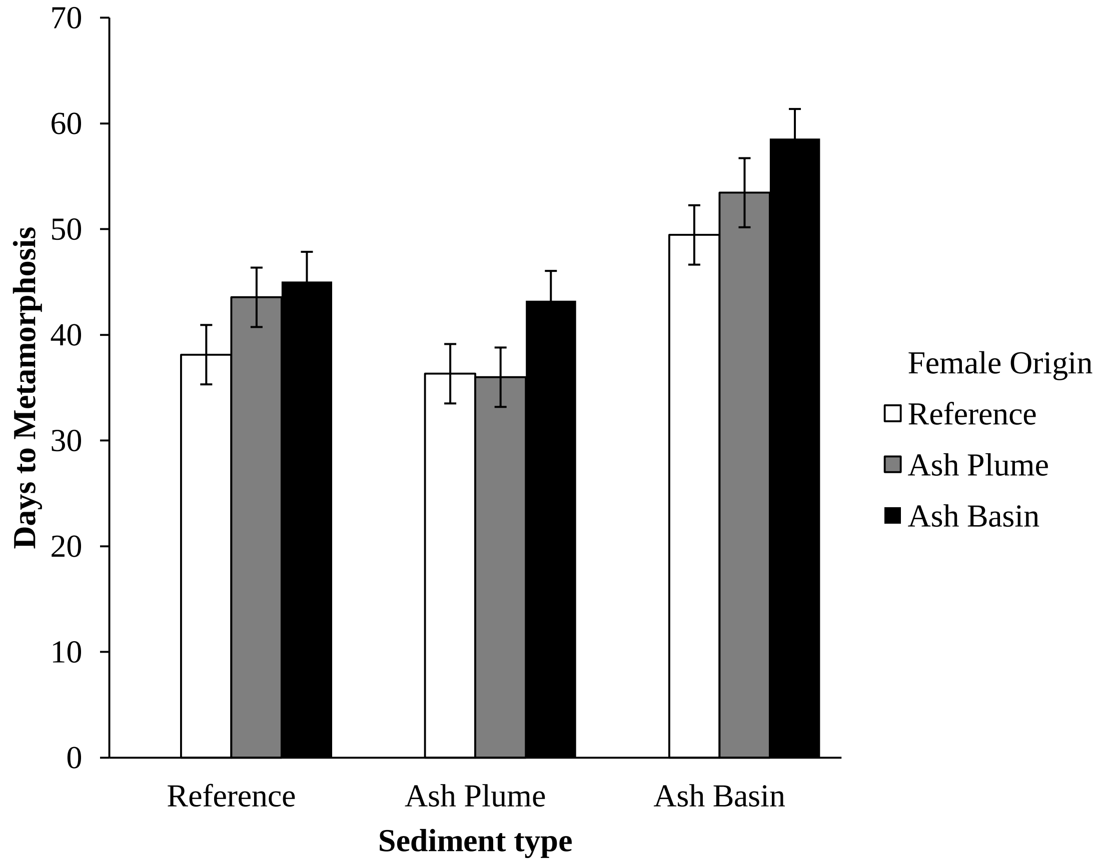

Figure 3.2. Number of days to metamorphosis of *Bufo terrestris* larvae exposed in experimental mesocosms to sediments from either a reference site (reference), a natural wetland contaminated with coal combustions wastes (ash plume), or a coal ash settling basin (ash basin). Data are presented as means ± 1 SE.

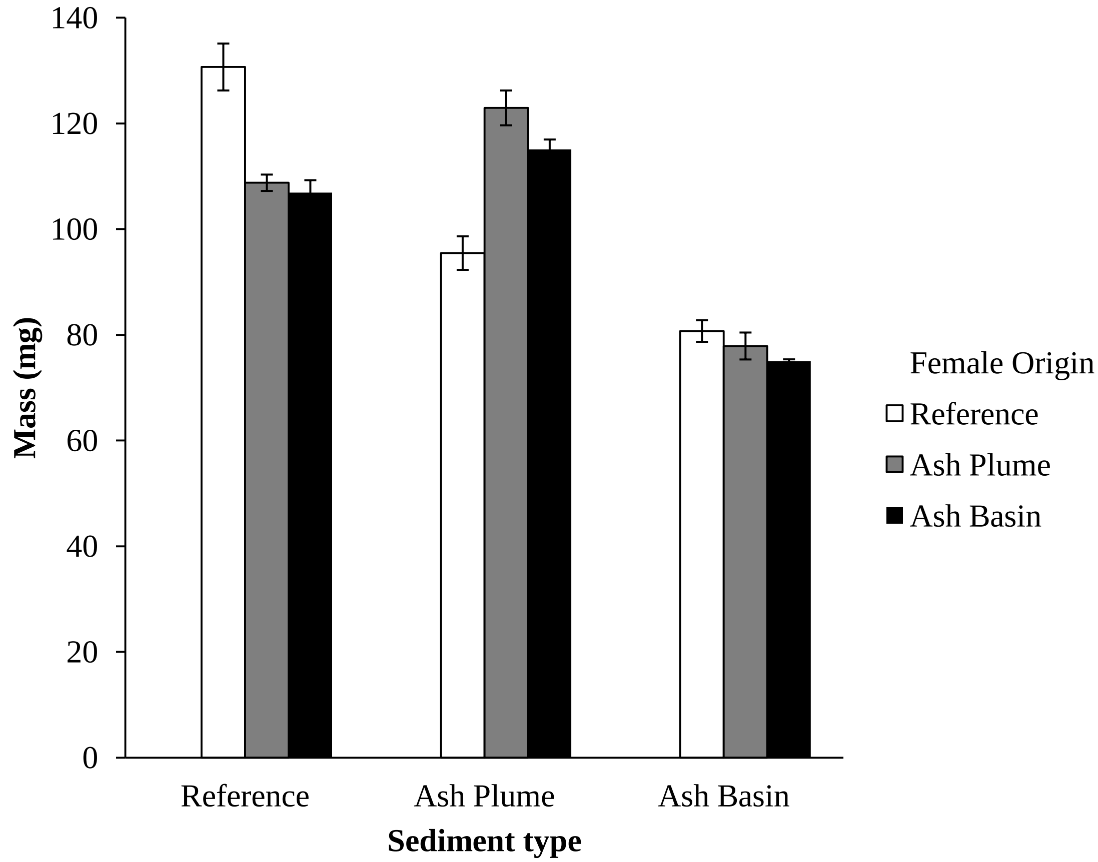

Figure 3.3 Mass (mg) of recently metamorphosed *Bufo terrestris* exposed in experimental mesocosms to sediments from either a reference site (reference), a natural wetland contaminated with coal combustions wastes (ash plume), or a coal ash settling basin (ash basin). Data are presented as means ± 1 SE.

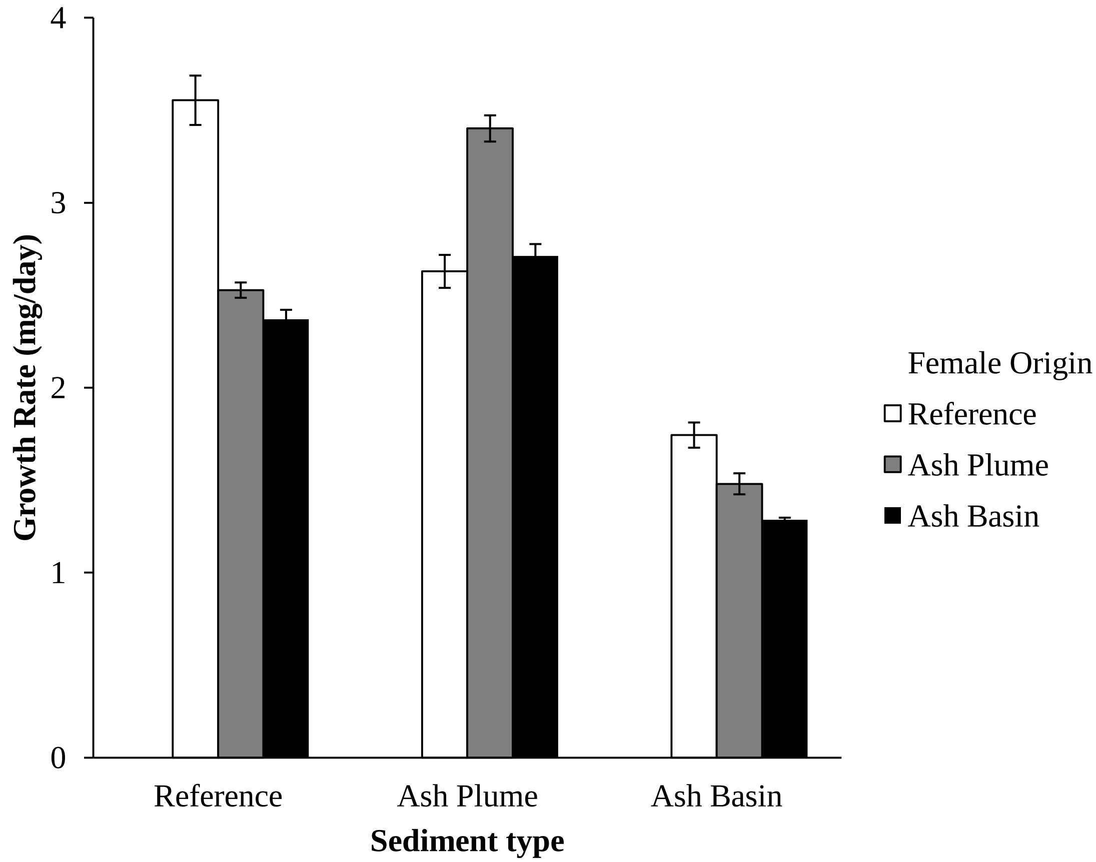

Figure 3.4. Growth rate (mg/day) of *Bufo terrestris* larvae exposed in experimental mesocosms to sediments from either a reference site (reference), a natural wetland contaminated with coal combustions wastes (ash plume), or a coal ash settling basin (ash basin). Data are presented as means ± 1 SE.

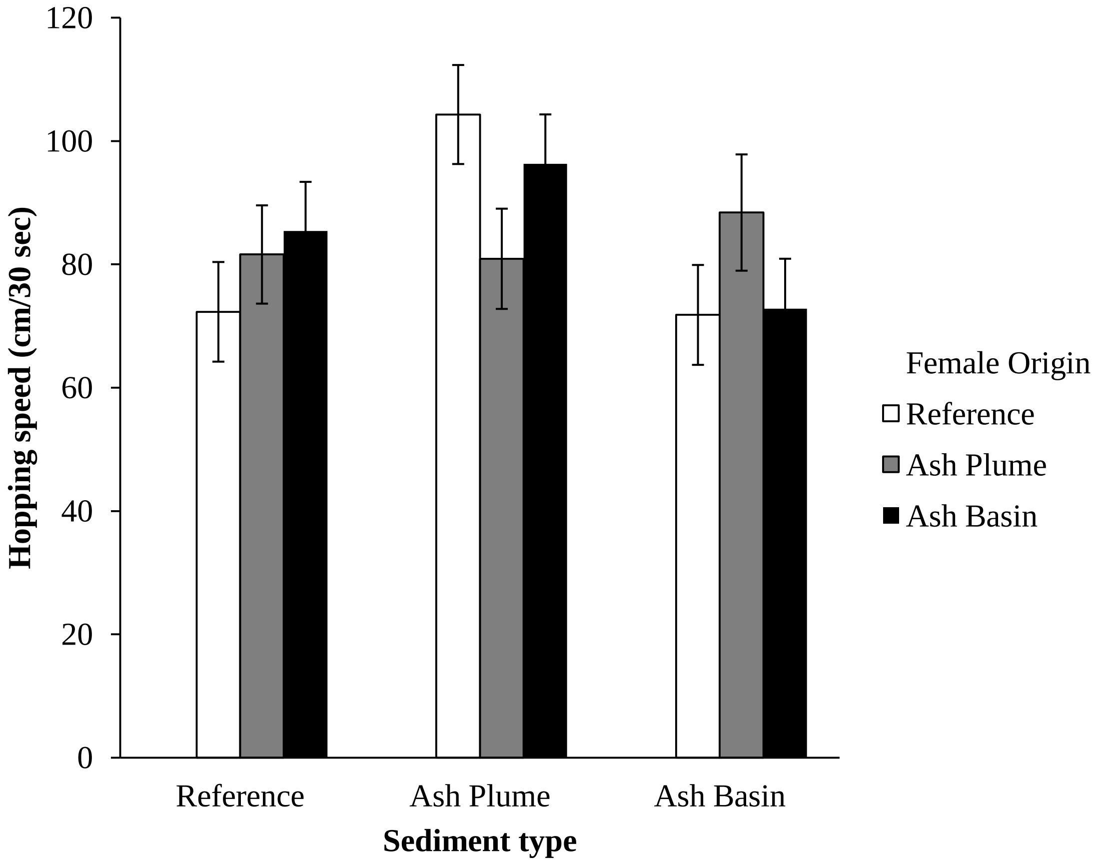

Figure 3.5. Hopping speed (cm/30seconds) of recently metamorphosed *Bufo terrestris* after larval exposure to sediments from either a reference site (reference), a natural wetland contaminated with coal combustions wastes (ash plume), or a coal ash settling basin (ash basin) in experimental mesocosms. Data are presented as LS means (corrected for SVL) ± 1 SE.

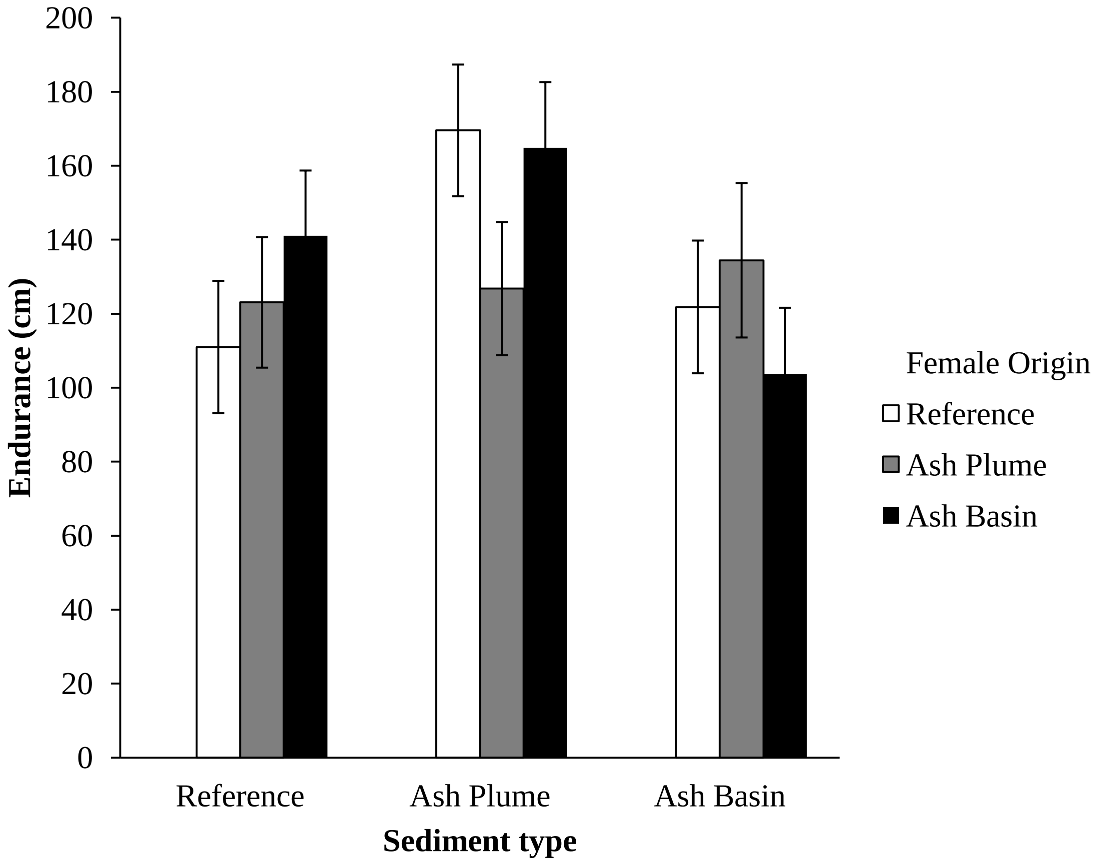

Figure 3.6. Endurance (cm) of recently metamorphosed *Bufo terrestris* after larval exposure to sediments from either a reference site (reference), a natural wetland contaminated with coal combustions wastes (ash plume), or a coal ash settling basin (ash basin) in experimental mesocosms. Data are presented as LS means (corrected for SVL) ± 1 SE.

CHAPTER 4

PATERNAL EXPOSURE TO TRACE ELEMENTS HAS NEGATIVE CONSEQUENCES ON REPRODUCTIVE SUCCESS OF SOUTHERN TOADS (*BUFO TERRESTRIS*)[3]

[3] Metts, B. S., K. A. Buhlmann, T. D. Tuberville, D. E. Scott, W. A. Hopkins. To be submitted to *Environmental Science and Technology*.

ABSTRACT

Exposure of adult amphibians to environmental contaminants has recently been shown to reduce hatching success and increase the frequency of developmental abnormalities in their offspring, ultimately resulting in reduced reproductive success. Available evidence suggests that maternal transfer of contaminants to eggs is responsible for these adverse reproductive effects. However, the effect of prior paternal exposure to contaminants on offspring has been overlooked. We investigated the effects of paternal and maternal exposure to trace elements on the reproductive success of southern toads (*Bufo terrestris*) exposed to coal combustion waste (CCW). We collected adult toads from a CCW-contaminated site and a reference site and crossed male and female pairs using a fully factorial design. Concentrations of several elements including arsenic, selenium (Se), strontium (Sr), and vanadium were higher in males and females collected from the contaminated site than the reference site. Eggs from these females contained elevated concentrations of Se and Sr compared to eggs from reference females. Compared to reference pairs, reproductive success was reduced by 40% when either the male or female was from the contaminated site and by 58% when both adults had been exposed to CCW. Reductions in reproductive success were primarily attributed to reduced hatching success not effects on clutch size or abnormalities. Our study provides evidence that paternal exposure to contaminants can influence reproductive success in amphibians, demonstrating that parental effects are not limited to mothers.

INTRODUCTION

Exposure of animals to environmental contaminants can have negative effects on the offspring of exposed individuals, and these consequences are typically attributed to maternal effects (Bernardo, 1996; Mousseau and Fox, 1998). In many vertebrate species, maternal exposure to environmental contaminants and subsequent transfer of contaminants to offspring can reduce reproductive success through malformations and embryo mortality (Di Giulio and Tillitt, 1999). For example, chronic alcoholism in human mothers can lead to congenital malformations of children (Jones et al., 1973; Palmer et al., 1974). In female birds, bioaccumulation of dichlorodiphenyltrichloroethane (DDT) alters calcium metabolism, causing egg shell thinning and reduced hatching success, resulting in population declines of several North American raptors and piscivorous birds (Blus, 1996). Similarly, in amphibians, maternal transfer of contaminants to offspring can reduce hatching success and increase the frequency of developmental abnormalities, ultimately resulting in reduced offspring viability and reproductive success (Hopkins et al., 2006; Bergeron et al., 2011; Metts et al., 2012). However, in most wildlife the effect of paternal exposure to contaminants on reproductive success has not been evaluated.

Paternal exposure to contaminants may negatively affect reproduction by influencing sperm count, viability, or mobility resulting in decreased fertilization. For instance, in humans exposure of males to contaminants has been linked to decreased reproductive success (Irvine 2000) through reduced sperm count and quality (Auger et al., 1995; Swan et al., 2003) and increased incidence of undescended testes (Weidner et al., 1998; Saradha and Mathur, 2006). In another study, male skittering frogs (*Rana* *cyanophlyctis*) exposed to mercuric chloride had fewer spermatogonia than unexposed males, suggesting mercury disrupted sperm production (Kanamadi and Saidapur, 1992). Whether this reduced fertilization was not investigated. Paternal exposure to environmental contaminants can also increase genetic mutations, impair development of offspring, and increase genetic instability (Anderson et al., 1994; Belfiore and Anderson, 2001; Shugart and Theodorakis, 1994; Hebert and Luiker, 1996). Furthermore, epigenetic variation (i.e., heritable changes in gene expression not caused by changes in genetic sequence) induced through paternal contaminant exposure can negatively influence offspring (Richards, 2006; Curley et al., 2011). For instance, contaminants can have epigenetic effects that decrease sperm capacity and viability in multiple generations of rats (Anway et al., 2005; Clement et al., 2010). Together these studies suggest that paternal exposure to environmental contaminants could result in reduced reproductive success and should be studied in declining groups of wildlife such as amphibians.

Recent research on amphibians has demonstrated that exposure of adults to contaminants can reduce reproductive success (Hopkins et al., 2006; Bergeron et al., 2011; Metts et al., 2012). For example, adult American toads (*Bufo americanus*) collected from mercury (Hg) contaminated sites along the South River in Virginia experienced reduced hatching success of offspring (Bergeron et al., 2011). Eastern narrowmouth toads (*Gastrophryne carolinensis)* exposed to coal combustion waste (CCW) enriched with trace elements exhibited a 19% reduction in offspring viability compared to those from a reference site (Hopkins et al., 2006). Similarly, adult southern toads (*Bufo terrestris)* exposed to CCW experienced a 35% reduction in reproductive success compared to reference adults (Metts et al., *in review*). However, all of these studies relied on captive breeding of females and males that had both been exposed to contaminants in the field. In all cases, the authors concluded that adverse effects were attributable to maternal transfer of contaminants, but it remains possible that the observed effects resulted from paternal exposure or an interaction between maternal and paternal exposure. Therefore, the objectives of this study were to determine if paternal exposure to trace elements has an influence on the reproductive success of southern toads and determine if an interaction between paternal and maternal exposure exists. We hypothesized that male southern toads exposed to CCW would accumulate trace elements, and this paternal exposure to contaminants would reduce hatching success and increase the frequency of abnormalities in larvae, ultimately resulting in decreased reproductive success. We also hypothesized that paternal and maternal exposure to CCW would interact to further reduce reproductive success.

METHODS

Field collection. We collected 54 male and 54 female southern toads 1-31 March 2011 from a contaminated and a reference site on the Savannah River Site near Aiken, South Carolina. The contaminated site is an ash settling basin, associated with a steam generation facility, which is filled with CCW and has become partially re-vegetated. Sediments in this basin are comprised almost entirely of CCW that is enriched with trace elements, the primary contaminants of concern in this waste stream (Hopkins et al., 2006; Rowe et al., 2002). Most organic contaminants are volatilized during the combustion process and remaining organic compounds (e.g., PAHs) tend to not be bioavailable (Norton and Mills, 1987; Shorten et al., 1990; U.S. EPA, 1999). The reference site is

Craigs Pond, a natural Carolina bay wetland (Sharitz and Gibbons, 1982; Sharitz, 2003), 78 ha in size with no known historical contamination, located approximately 26 km from the contaminated site (Davis and Janecek, 1997). Both sites are surrounded by mixed pine-hardwood forest and open field habitats.

Experimental design and data collection. We transported toads to the lab where we randomly allocated them into four groups of breeding pairs using a 2x2 factorial design. The four groups were: 1) reference pairs, 2) ash basin pairs, 3) ash basin females paired with reference males, and 4) reference females paired with ash basin males. We measured (SVL, ± 0.5 mm) and weighed (± 0.01 g) each toad, injected them with human chorionic gonadotropin (males 100, females 250 IU), and placed pairs in 5-10 cm of well water in plastic containers to breed. By allowing females to oviposit in uncontaminated well water, we were able to isolate maternal exposure as the only appreciable source of contamination to the eggs. After breeding, toads were kept in plastic containers in the lab for 48 hours to void their remaining gut contents. Male and female toads were then euthanized by immersion in buffered MS-222 and freeze-dried for subsequent elemental analysis.

Twelve pairs from each of the four groups produced fertilized clutches of eggs (n=48 clutches). To estimate reproductive output of females, we enumerated the total number of eggs in each clutch. From each clutch we removed a subsample of 500 eggs that were polarized and appeared to be fertile (Gosner 1960) and held them in a plastic container with 3 L of aged well water at 25 °C until hatching. The remaining eggs from each clutch were freeze-dried for elemental analysis. We counted the number of hatchlings in each subsample and calculated the fraction of the 500 that hatched successfully. To quantify morphological abnormalities, we preserved the surviving hatchlings in formalin. Using a dissecting microscope, we later examined each hatchling for morphological abnormalities (axial, oral, swelling, and blisters) following the methods of Bantle et al., (1991) and ASTM (1998). To estimate offspring viability, we calculated the fraction of 500 eggs that successfully hatched and had no morphological abnormalities (Hopkins et al., 2006). Finally, to estimate overall reproductive success of each pair, we multiplied clutch size by viability.

Sample preparation and elemental analysis. For elemental analyses, we individually digested subsamples of each homogenized adult toad carcass (~250 mg) and eggs in 10 mL of trace metal grade nitric acid (70% HNO_3) using microwave digestion (MarsExpress, CEM Corp., Matthews, NC). After digestion, we brought each sample to a final volume of 15 ml with 18 MΏ deionized water. We used inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer, Norwalk, CT) to determine elemental concentrations of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), Hg, nickel (Ni), selenium (Se), strontium (Sr), vanadium (V), and zinc (Zn) in each sample. For quality control, we included certified reference material (TORT-2 and LUTS; National Research Council of Canada, Ottawa, Canada) in each analysis. Mean percent recoveries for elements in standard reference material ranged from 95 to 137%. Minimum detection limits in tissue samples were: As, 0.38; Cd, 0.19; Cr, 0.21; Cu, 0.42; Hg, 0.38; Ni, 0.22; Pb, 0.29; Se, 1.18; Sr, 0.21; V, 0.20; Zn, 3.23 µg/kg. We report elemental concentrations as µg/g dry mass.

Statistical analyses. We performed statistical analyses using SAS 9.2 (SAS Institute, Cary, NC). We examined the assumptions of homogeneity of variance using

Levene tests and normality using Shapiro-Wilk tests. We performed log or angular (arcsine square-root) transformations to better meet the model assumptions. For statistical comparisons, we assigned trace element concentrations below the instrument's detection limit (BDL) a value of half the minimum detection limit for that element.

Initially, we used individual analysis of variance (ANOVA) to confirm equivalent distribution of elemental concentrations in males and females from the same study site but allocated among the four groups. We then pooled the data and compared elemental concentrations in males and females between collection sites and sexes using MANOVA. We compared elemental concentrations in eggs between females from the two sites using MANOVA. We used individual ANOVAs to make comparisons between sites and among groups for male and female size (SVL and mass). Because clutch size was influenced by female SVL ($r^2 = 0.62$, $F_{1,46} = 8.65$, $p < 0.001$), we used ANCOVA with female SVL as the covariate to make comparisons among groups for clutch size. We used ANOVA to make comparisons among groups for the frequency of morphological abnormalities. When we found significant main effects using the multivariate models and individual ANOVAs, we used Bonferroni adjusted pair-wise comparisons to identify differences among means.

Hatching success, viability, and reproductive success were not normally distributed after transformations, so we used nonparametric Mann-Whitney tests to make individual comparisons between groups. We used linear regression to determine whether relationships existed between elemental concentrations in males and clutch size, hatching success, abnormality frequency, viability, and reproductive success.

RESULTS

Elemental concentrations. Overall, elemental concentrations in adults varied by site (Pillai's trace = 0.57, $F_{11,77} = 9.47$, $p < 0.001$), and sex (Pillai's trace = 0.35, $F_{11,77} = 3.77$, $p < 0.001$), but not their interaction (Pillai's trace = 0.18, $F_{11,77} = 1.58$, $p = 0.12$). Specifically, As concentrations were 3 times higher in female toads and nearly 2 times higher in male toads collected from the ash basin than reference toads (site: $F_{3,87} = 13.53$, $p < 0.001$; sex: $F_{3,87} = 0.51$, $p = 0.48$; interaction: $F_{3,87} = 0.87$, $p = 0.35$; Table 1). Selenium concentrations were two times higher in male and female toads collected from the ash basin compared to those from the reference site (site: $F_{3,92} = 18.27$, $p < 0.001$; sex: $F_{3,92} = 0.11$, $p = 0.74$; interaction: $F_{3,92} = 0.76$, $p = 0.39$; Table 1). Concentrations of Sr and V were significantly higher in male and female toads from the ash basin than from the reference site (in both cases: site: $F_{3,92} \geq 4.90$, $p \leq 0.03$; sex: $F_{3,92} \geq 0.30$, $p \leq 0.59$; interaction: $F_{3,92} \geq 0.02$, $p \leq 0.89$; Table 1). In contrast, Pb concentrations were higher in male and female toads collected from the reference site than those from the ash basin (site: $F_{3,92} = 30.30$, $p < 0.001$; sex: $F_{3,92} = 1.13$, $p = 0.29$; interaction: $F_{3,92} = 1.43$, $p = 0.23$; Table 1). Mercury concentrations were higher in male and female toads from the reference site than toads from the ash basin, and were also higher in females than in males (site: $F_{3,92} = 15.37$, $p < 0.001$; sex: $F_{3,92} = 14.45$; $p < 0.001$, interaction: $F_{3,92} = 0.13$, $p = 0.72$; Table 1). Chromium and Zn concentrations were higher in females than in males (in both cases: site: $F_{3,92} \geq 0.11$, $p \leq 0.74$; sex: $F_{3,92} \geq 10.10$, $p \leq 0.002$; interaction: $F_{3,92} \geq 0.10$, $p \leq 0.75$; Table 1). Concentrations of Ni were higher in females than in males but this was dependent on site (site: $F_{3,92} = 0.04$, $p = 0.01$; sex: $F_{3,92} = 6.38$, $p = 0.84$; interaction: $F_{3,92} = 4.30$, $p = 0.04$; Table 1).

Overall, elemental concentrations in eggs were dependent on maternal capture site (Pillai's trace = 0.52, $F_{10,37} = 4.06$, $p < 0.001$). Specifically, Se concentrations were 74% higher in eggs from ash basin females compared to eggs from reference females ($F_{1,46} = 20.33$, $p < 0.001$; Table 2). Strontium concentrations were 22% higher in eggs from ash basin females than in eggs from reference females ($F_{1,46} = 5.14$, $p = 0.03$; Table 2). All other elemental concentrations in eggs were similar between collection sites (in all cases: $F_{1,46} \leq 3.57$, $p \geq 0.07$; Table 2)

Effects on reproduction and offspring viability. Both male and female southern toads were similar in size between collection sites (in both cases: SVL: $F_{1,46} \leq 1.96$, $p \geq 0.17$; mass: $F_{1,46} \leq 2.20$, $p \geq 0.15$; Table 3) and among the four experimental groups (in both cases: SVL: $F_{3,44} \leq 2.25$, $p \geq 0.10$; mass: $F_{3,44} \leq 0.59$, $p \geq 0.62$; Table 3). Clutch size of reference females ranged from 1,691 to 7,888 eggs and for ash basin females from 1,302 to 5,596 eggs. After accounting for female SVL, clutch size was similar among the reference group and two out-crossed groups, although clutch size of reference females that mated with ash basin males was 27% higher than ash basin females that mated with ash basin males (group: $F_{3,44} = 3.13$, $p = 0.04$; SVL: $F_{3,44} = 43.66$, $p < 0.001$; Table 3). Compared to reference pairs, hatching success was reduced by at least 36% when one or both mates were from the ash basin, (in all cases: $Z \leq -1.76$, $p \leq 0.04$; Table 3). The frequency of developmental abnormalities was 2.8 – 4.3 times more abundant in hatchlings from ash basin females compared to reference females ($F_{3,35} = 6.04$, $p = 0.002$; Table 3), but the frequency of larval abnormalities was similar between reference males and ash basin males ($F_{3,35} = 1.60$, $p = 0.21$; Table 3). Axial malformations were the most common abnormalities. Compared to reference pairs, offspring viability was reduced by 32% when reference females mated with ash basin males (Z = -1.16, p = 0.12; Table 3) and by 36% when ash basin females mated with reference males (Z = -1.71, p = 0.04; Table 3). Furthermore, when both mates were from the ash basin, offspring viability was reduced by 40% compared to the reference group (Z = -1.94, p = 0.03; Table 3).

Overall reproductive success (defined as clutch size x viability) was reduced by paternal and maternal exposure to CCW (Figure 1). Specifically, when either the male or the female was from the ash basin, reproductive success was reduced by 40% compared to reference pairs (males: Z = -1.53, p = 0.06; females: Z = -1.82, p = 0.03; Figure 1). When both mates were from the ash basin, reproductive success was reduced by nearly 30% compared to ash basin females that mated with reference males (Z = -0.50, p = 0.31; Figure 1). Furthermore, reproductive success was reduced by 58% in ash basin pairs compared to reference pairs (Z = -2.52, p = 0.006; Figure 1). We found no correlation between elemental concentrations in males and any reproductive parameter that we measured (in all cases: $r^2 \leq 0.10$, $F_{1, 46} \leq 2.51$, $p \geq 0.12$).

DISCUSSION

Our study is unique in that we demonstrated that paternal exposure to contaminants can reduce the estimated reproductive success of adult male amphibians. We found that both male and female southern toads from the contaminated site accumulated higher As, Se, Sr, and V concentrations than those from the reference site, and experienced reductions in hatching success, offspring viability, and reproductive success. Our results support previous work demonstrating that maternal exposure to contaminants reduces offspring viability and reproductive success in amphibians (Hopkins et al., 2006; Bergeron et al., 2011; Metts et al., *in review*), but our study is the first to identify a paternal effect of contaminant exposure in amphibians. In addition, exposure of both parents to contaminants reduced reproductive success more than when only one mate was exposed to contaminants, suggesting some degree of interaction between maternal and paternal effects.

In our study, paternal exposure to CCW reduced reproductive success by 40% compared to unexposed males, and this reduction was primarily a function of decreased hatching success. Hatching success was reduced by 36% when male southern toads were exposed to CCW compared to reference males, although the frequency of abnormalities in successful hatchlings was similar between reference and CCW-exposed males. Environmental contaminants can negatively influence sperm count and quality in humans and other vertebrates (Auger et al., 1995; Swan et al., 2003; Facemire et al., 1995; Bayley, et al., 2002; Toft and Guillete, 2005; Fort et al., 2004). It is possible that decreased sperm quality or viability of CCW-exposed males impaired embryo development after fertilization and polarization occurred, thereby resulting in lower hatching success. For example, in humans impaired sperm quality can reduce embryo development and blastocyst formation soon after fertilization occurs (Parinaud et al., 1993; Janny and Menezo, 1994; Tesarik et al., 2002, 2004). Although not completely understood, these paternal effects may be both of genetic and epigenetic origin (Tesarik et al., 2002). In our study, because we estimated reproductive success from a subset of 500 fertilized eggs we may have actually underestimated the paternal effect by not counting the total number of fertilized eggs in each clutch.

In our study, we crossed males and females from reference and contaminated sites, thus, we were able to determine the individual and interactive effects exposure to each parent had on reproductive success. In our study, estimated reproductive success of southern toads was reduced by 40% if either mate was from the ash basin. This is ecologically important because southern toads and other amphibians move around the landscape (e.g. Marsh and Trenham, 2001; Semlitsch, 2008). Thus, contaminated individuals could migrate to non-contaminated breeding sites, resulting in decreased reproductive success of resident animals they mate with. Moreover, if both mates were from the contaminated site reproductive success was reduced by 58% compared to reference pairs. Interestingly, in our study the effects of paternal and maternal exposure on reproductive success were less than additive. Although previous amphibian studies documented similar reductions in reproductive success when both parents were collected from the same contaminated site, the potential effects of contaminants on male reproduction has not previously been considered (Hopkins et al., 2006; Bergeron et al., 2011; Metts et al., *in review*). For instance, eastern narrowmouth toads exposed to CCW at the ash basin as in our study exhibited a 19% reduction in offspring viability compared to those from a reference site (Hopkins et al., 2006), and reproductive success of southern toads exposed to CCW was reduced by 35% compared to reference females (Metts et al., *in review*). Similarly, American toads collected from Hg contaminated sites experienced reduced hatching success of offspring (Bergeron et al., 2011).

For unknown reasons trace element concentrations observed in our study were lower than those previously reported in southern toads collected from the same ash basin (Hopkins et al., 1998; Ward et al., 2009; Metts et al., 2012). For instance, in our study we observed 3.1 µg/g of Se and 113 µg/g of Sr in male southern toads, while Ward et al., (2009) observed 8.5 µg/g of Se and 420 µg/g of Sr. Similarly, Metts et al., (2012) observed Se and Sr concentrations of 9.4 µg/g and 162 µg/g, respectively, in female southern toads collected from the same ash basin. One possible explanation for our findings is that during drought years when many wetlands do not fill, animals from nearby wetlands migrate to the ash basin, a permanent source of water, for breeding. Thus, some toads in our sample may have originated from outside the contaminated area and had not been exposed to CCW. Regardless of the underlying reason for these differences in bioaccumulation patterns, we still observed negative effects on reproductive success of southern toads collected from the ash basin despite lower trace element concentrations than reported in previous studies.

In conclusion, we found that males collected from the ash basin accumulated higher concentrations of As, Se, and Sr than reference males, and paternal exposure to trace elements influenced reproductive success through reduced hatching success. Our study is the first to demonstrate the effects of paternal exposure to contaminants on amphibian reproduction. Moreover, paternal effects combined with maternal effects interact to cause remarkable reduction in reproductive success by breeding pairs at contaminated sites.

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Table 4.1. Elemental composition of postovipositional female and male southern toads collected in March 2011 from a reference site and a CCW contaminated site. Data presented are means ± 1 SE. BDL: Below detection limit.

	Males (μg/g)		Females (μg/g)	
	Reference	Ash basin	Reference	Ash basin
Element	n = 24	n = 24	n = 24	n = 24
As[2]	0.12 ± 0.003	0.22 ± 0.01	0.14 ± 0.01	0.49 ± 0.03
Cd	0.09 ± 0.002	0.08 ± 0.002	0.11 ± 0.003	0.10 ± 0.004
Cr[1,3]	0.71 ± 0.02	1.60 ± 0.08	3.03 ± 0.16	2.19 ± 0.16
Cu	20.02 ± 0.42	16.75 ± 0.20	20.58 ± 0.39	16.47 ± 0.35
Hg[1,2]	0.45 ± 0.01	0.30 ± 0.01	0.67 ± 0.01	0.51 ± 0.02
Ni[1,3]	0.6 ± 0.03	0.88 ± 0.04	1.58 ± 0.08	1.15 ± 0.07
Pb[2]	2.1 ± 0.15	0.53 ± 0.01	1.28 ± 0.02	0.88 ± 0.03
Se[2]	1.51 ± 0.03	3.12 ± 0.08	1.58 ± 0.03	3.27 ± 0.11
Sr[2]	81.60 ± 1.11	113.87 ± 1.56	88.56 ± 1.14	136.31 ± 2.85
V[2]	0.10 ± 0.003	0.20 ± 0.01	0.16 ± 0.01	0.26 ± 0.01
Zn[1]	77.97 ± 0.49	80.14 ± 0.53	95.82 ± 1.68	99.9 ± 1.5

[1] significant differences between sexes
[2] significant differences between sites
[3] significant interaction effect

Table 4.2. Elemental composition of southern toad eggs collected March 2011 from a reference and a CCW contaminated site. Data presented are means ± 1 SE. BDL: Below detection limit.

	Eggs Concentrations (µg/g)	
Element	Reference n = 24	Ash Basin n = 24
As	BDL	BDL
Cd	0.08 ± 0.001	0.09 ± 0.003
Cr	BDL	BDL
Cu	13.89 ± 0.14	12.9 ± 0.11
Hg	BDL	BDL
Ni	0.21 ± 0.001	0.2 ± 0.001
Pb	0.33 ± 0.01	0.43 ± 0.02
Se*	2.39 ± 0.03	4.15 ± 0.08
Sr*	1.47 ± 0.01	1.79 ± 0.02
V	0.12 ± 0.001	0.12 ± 0.002
Zn	266.47 ± 2.16	268.42 ± 2.29

*significantly different

Table 4.3. Mass, snout vent length (SVL), clutch size, percent hatching success, malformation frequency, viability, and successful reproductive output of male and female southern toads collected from a reference site and a CCW contaminated site. SVL, mass, hatching success, morphological abnormality frequency, and viability data are presented as means ± 1 SE. Clutch size is presented as LS means adjusted for female SVL ± 1 SE. Different letters indicate significant differences.

	Group			
Female	Reference	Reference	Ash basin	Ash basin
Male	Reference	Ash basin	Reference	Ash basin
	n = 12	n = 12	n = 12	n = 12
Male mass (g)	^{A}15.9 ± 0.21	^{A}15.35 ± 0.15	^{A}13.89 ± 0.17	^{A}16.16 ± 0.15
Male SVL (mm)	^{A}55.25 ± 0.32	^{A}54.83 ± 0.19	^{A}52.75 ± 0.22	^{A}55.42 ± 0.19
Female mass (g)	^{A}28.51 ± 0.91	^{A}27.53 ± 0.66	^{A}25.78 ± 0.95	^{A}22.71 ± 0.56
Female SVL (mm)	^{A}68 ± 0.59	^{A}67.33 ± 0.56	^{A}64.67 ± 0.71	^{A}65.5 ± 0.62
Clutch size (# eggs)	^{AB}3103 ± 142	^{A}3744 ± 131	^{AB}3580 ± 109	^{B}2725 ± 77
Hatching success (%)	^{A}77.5 ± 2.1	^{B}49.9 ± 3.5	^{B}48.2 ± 3.2	^{B}48.3 ± 3.4
Abnormality Frequency (%)	^{A}1.8 ± 0.1	^{A}1.7 ± 0.1	^{AB}4.9 ± 0.6	^{B}7.6 ± 0.4
Viability (%)	^{A}71.4 ± 1.9	^{AB}48.6 ± 3.4	^{B}45.8 ± 3.1	^{B}42.7 ± 3.1

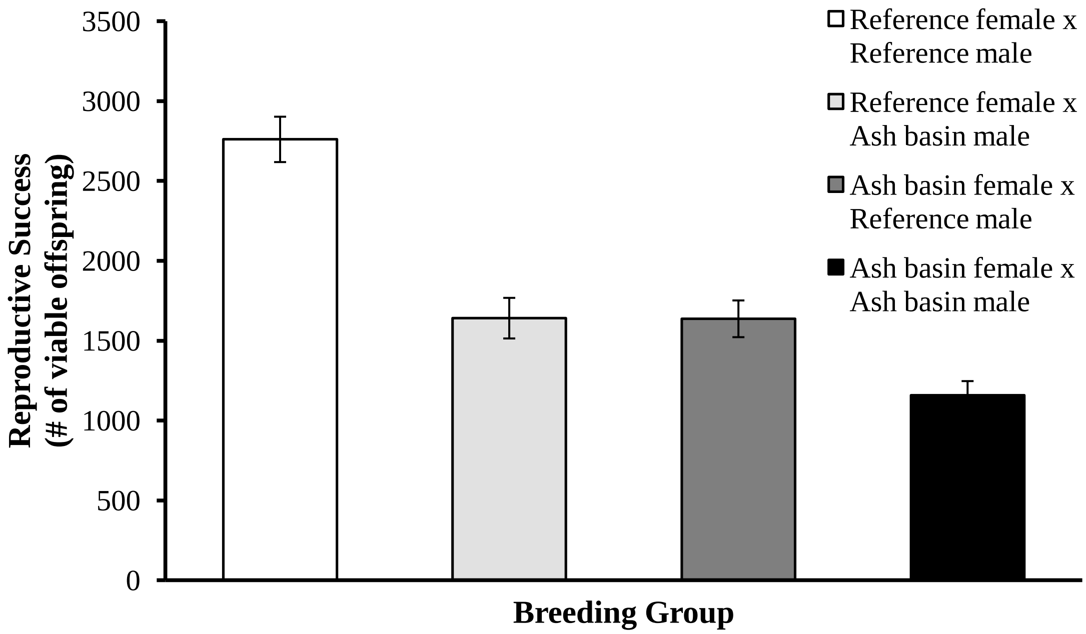

Figure 4.1. Estimated reproductive success of southern toads (*Bufo terrestris*) collected from a reference site and a coal ash settling basin and assigned a mate in a fully crossed factorial design. Data presented are LS means adjusted for female SVL ± 1 SE.

CHAPTER 5

CONCLUSIONS

I had several major objectives with my dissertation research. First, I wanted to quantify maternal transfer of contaminants and its influence on early development and reproductive success of southern toads. My second objective was to describe the relative influence of and potential interactions between maternal and environmental exposure on larval development. My third objective was to investigate the consequences of contaminant exposure on the reproductive success of male southern toads. Finally, I sought to determine if the negative consequences associated with exposure to trace elements would be ameliorated in aged coal combustion waste (CCW). Together, my studies provide insight into how exposure to CCW influences amphibian populations.

Like other wildlife exposed to CCW, female southern toads inhabiting CCW contaminated sites accumulated trace elements and transferred several elements to their offspring. Females collected from the contaminated sites transferred elevated concentrations of Cd, Cu, Ni, Pb, Se, and Sr to their eggs (Chapter 2). Reproductive success was negatively correlated with Se and As concentrations in females and Se concentrations in eggs (Chapter 2). Of the elements maternally transferred, Se is of particular interest because of its known teratogenicity in oviparous vertebrates. Although Se concentrations in southern toads were below toxicity thresholds established for other wildlife, toads experienced a dramatic reduction in reproductive success, suggesting they may be more sensitive to trace element contaminants than other species.

My research demonstrates the negative consequences associated with maternal transfer and environmental exposure of larvae to CCW contaminated sediments, and their interactive effects. Specifically, exposure of larval southern toads to contaminants during the larval period had sublethal effects on growth and development of survivors but previous maternal exposure did not (Chapter 3). Ash basin sediments prolonged the larval development period by 11 to 15 days compared to reference and ash plume sediments, respectively (Chapter 3). Considering the hot and dry conditions that cause annual pond drying in the southeastern U.S., a two week extension of larval development could limit the number of individuals reaching metamorphosis and reduce the frequency of years with successful recruitment. Larval southern toads exposed to ash basin sediments in mesocosms also grew more slowly and were 30% smaller at metamorphosis than larvae reared on ash plume or reference sediments (Chapter 3). Such a considerable reduction in size may negatively influence survival and future reproductive success of individuals that metamorphose from contaminated sites. Most importantly, survival of larvae from females collected near CCW-contaminated settling basins was reduced dramatically; however, the degree of this reduction was dependent on the type of sediment the larvae developed on (Chapter 3). Survival to metamorphosis was highest in reference larvae reared on reference sediments, but exposure of reference larvae to ash basin and ash plume sediments reduced survival 42 and 60%, respectively. Finally, compared to reference larvae reared on reference sediments, maternal exposure to contaminants in combination with the most toxic sediments (i.e., ash basin) reduced survival by 85%, resulting in very low recruitment (Chapter 3). Taken together, my research demonstrates that maternal exposure and environmental exposure of larvae to CCW both negatively impact survival and interact to substantially reduce recruitment to the terrestrial environment.

Previous studies have also demonstrated that exposure to CCW reduces recruitment in amphibians; however, this is the first to demonstrate a latent effect of previous maternal exposure to CCW on larval survival, although the severity of the effect was dependent on which sediment type the larvae were exposed to. In other vertebrate groups, latent maternal effects from contaminant exposure can outweigh the effects of direct or dietary exposure. In this study, when southern toad larvae from females exposed to contaminants were reared on reference sediments, previous maternal exposure to contaminants reduced survival by 34 to 57% compared to larvae from reference females raised on reference sediments (Chapter 3).

Results from my research suggest that aging of CCW contaminants may partially ameliorate the negative reproductive consequences associated with CCW exposure on amphibians. For instance, survival of larvae from contaminated females was highest when larvae were reared on ash plume sediments where aging of sediments had occurred, suggesting that populations residing in contaminated areas may adapt to the contaminated conditions. Over 35 years since the cessation of CCW release into the ash plume the area has become revegetated and a thin organic layer has developed. Elemental concentrations in the sediments have been reduced by downward migration in sediment layers, plant uptake and sequestration, and flood events that transport dissolved elements offsite, additionally, biogeochemical processes can alter elemental bioavailability. In most instances sediment trace element concentrations in ash plume sediments were intermediate between concentrations in reference and ash basin sediments. As a result, many of the trace element concentrations accumulated by metamorphs reared on ash plume sediments were intermediate between those of metamorphs raised on reference and ash basin sediments. In addition, the negative sublethal effects on larvae were reduced in larvae reared in the ash plume wetland environment compared to the ash basin. However, despite this, larval survival was reduced by contaminants in the ash plume sediments suggesting that aging alone may not entirely reduce health risks associated with exposure to CCW. Taken together, my studies suggest that the biogeochemical processes that contribute to aging of CCW have different consequences for aquatic and terrestrial lifestages of amphibians.

My study provides evidence that paternal exposure to contaminants can influence reproductive success in amphibians, demonstrating that parental effects are not limited to mothers. My research is among the first to investigate the individual and interactive effects of paternal and maternal exposure to contaminants on amphibian reproductive success, and to document relationships between contaminant concentrations in female amphibians or their eggs and reduced reproductive success. Specifically, male and female southern toads (*Bufo terrestris*) exposed to CCW, accumulated As, Se, Sr, and V and experienced adverse effects on reproductive success. Specifically, reproductive success was reduced by 40% when either the male or female was from the ash basin, compared to reference pairs (Chapter 4). In addition, when both mates were from the ash basin, reproductive success was reduced by 58% compared to reference pairs.

Although population-level effects remain unknown, the observed reductions in reproductive success and survival to metamorphosis could result in local amphibian population declines. For instance, reproductive success decreased 35% in females from the ash basin compared to reference females, and previous maternal exposure in combination with larval exposure to ash basin sediments reduced survival by 85% compared to reference larvae reared on reference sediments. All else being equal, a typical female producing 4,000 eggs in the ash basin would be expected to produce 90% fewer successful recruits than a female from the reference population. Such pronounced effects on reproduction could result in population declines; however, reduced larval density due to mortality could decrease the strength of density-dependent interactions among larvae, improving the survival of remaining larvae at the site with no net effect on local population dynamics. The persistence of a breeding population of toads around the ash basins suggests that adults from nearby wetlands continue to migrate into the contaminated wetlands to breed. As such, ash basins may serve as ecological traps to amphibian populations.

My results demonstrate that, although elemental concentrations in sediments may decrease as CCW ages, trace elements in the ash plume are bioavailable and can have negative consequences on amphibian recruitment. For example, reproductive success was reduced by 32% in females from the ash plume compared to reference females. Using the example of 4,000 eggs again, a female from the ash plume would produce 54% fewer recruits than a reference female. While this reduction in recruitment is not as dramatic as that observed in ash basin females, exposure to aged CCW clearly has negative consequences on reproductive success of individuals. However, whether this results in population declines remains to be determined as density-dependent interactions may be important. Theoretical modeling approaches that account for density-dependent effects are needed to project the population-level ramifications of these findings.

In conclusion, maternal and paternal exposure to contaminants is an important factor affecting amphibian reproductive success, which together with larval exposure may ultimately contribute to localized amphibian declines. My research provides evidence that CCW contaminated sites may ultimately serve as ecological traps for amphibian populations. Studies investigating the effects of parental exposure on larval development through metamorphosis and the terrestrial juvenile stage are necessary to determine if parental exposure to contaminants contributes to amphibian population declines. Finally, theoretical models are needed to assess the impact of individual-level effects on population dynamics.