Hepatic and renal trace element concentrations in American alligators (*Alligator mississippiensis*) following chronic dietary exposure to coal fly ash contaminated prey

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Abstract

Little is known about the propensity of crocodilians to bioaccumulate trace elements as a result of chronic dietary exposure. We exposed 36 juvenile alligators (*Alligator mississippiensis*) to one of four dietary treatments that varied in the relative frequency of meals containing prey from coal combustion waste (CCW)-contaminated habitats vs. prey from uncontaminated sites, and evaluated tissue residues and growth rates after 12 mo and 25 mo of exposure. Hepatic and renal concentrations of arsenic (As), cadmium (Cd) and selenium (Se) varied significantly among dietary treatment groups in a dose-dependent manner and were higher in kidneys than in livers. Exposure period did not affect Se or As levels but Cd levels were significantly higher after 25 mo than 12 mo of exposure. Kidney As and Se levels were negatively correlated with body size but neither growth rates nor body condition varied significantly among dietary treatment

groups. Our study is among the first to experimentally examine bioaccumulation of trace element contaminants in crocodilians as a result of chronic dietary exposure. A combination of field surveys and laboratory experiments will be required to understand the effects of different exposure scenarios on tissue residues, and ultimately link these concentrations with effects on individual health.

Key words: Crocodilian; Bioaccumulation; Chronic dietary exposure; Growth; Coal combustion waste

Capsule: Chronic dietary exposure can result in significant bioaccumulation of trace elements in juvenile alligators

Introduction

Despite growing interest in alternative energy sources, coal still accounts for 39% of energy production in the US (US EIA, 2014), 80% of power production in China (Shealy and Dorian, 2010), and 30% worldwide (British Petroleum, 2012). The United States alone consumed an estimated 516 million tons of coal in 2013, resulting in the production of more than 114 million tons of coal combustion wastes (American Coal Ash Association, 2013). Prior to 1980, approximately two-thirds of coal combustion wastes (CCWs) in the US were disposed of as a slurry into aquatic settling basins, where particulates are allowed to settle out and the surface waters are often discharged into other aquatic basins or into natural wetlands (Rowe et al. 2002). Currently, these surface impoundments represent 21% of disposal facilities in the United States (Lemly and Skorupa, 2012). Although not intended as wildlife habitat, CCW disposal basins provide a permanent water source that can attract wildlife and, as a result, potentially expose them to an array of trace element contaminants (Lemly and Ohlendorf, 2002, Lemly and Skorupa, 2012). Coal combustion wastes can vary in composition due to the parent coal material as well as the processing and disposal processes (Rowe et al. 2002), but are commonly enriched in arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), and selenium (Se) at levels that can be toxic to wildlife (Hopkins et al., 2001; Rowe et al., 2002).

Exposure to trace element contaminants in CCW aquatic disposal systems can occur through several mechanisms, including direct contact (Hopkins, 2006), maternal transfer to offspring (Metts et al., 2013; Nagle et al., 2001; Van Dyke et al., 2013) and consumption of contaminated prey (Bryan et al., 2012; Hopkins et al., 2002). Thus, an individual's exposure, assimilation, and resulting trace element tissue residues will be influenced by trace element

concentrations in the CCW aquatic system, exposure route(s), exposure period, diet or trophic position, and the propensity of specific trace elements to bioaccumulate. Fish, aquatic invertebrates, amphibians, and birds occupying CCW habitats are among the wildlife species most frequently monitored (Rowe et al., 2002 and references therein). Many of these species have been shown to accumulate tissue residues of trace elements, particularly Se, above proposed toxicity thresholds (Hopkins et al., 2002) or to levels sufficiently high to reduce egg or offspring viability (Hopkins et al., 2006; Metts et al., 2012), induce developmental malformations (Rowe et al., 1996; 1998), or alter metabolic rates (Nagle et al., 2001; Rowe et al., 1998; 2001) and hormone profiles (Hopkins et al., 1997).

Most studies of trace element accumulation in wildlife inhabiting CCW-contaminated wetlands have focused on relatively short-lived species. However, delayed sexual maturity (resulting in potentially long-term exposure prior to first reproduction), longevity, and high trophic status are traits predicted to increase contaminant exposure risk and level of bioaccumulation (Rowe, 2008). The American alligator (*Alligator mississippiensis*) is a conspicuous component of many aquatic habitats in the southeastern United States, where it is an apex predator (Gabrey, 2010; Rosenblatt et al., 2015), feeding on a variety of vertebrates and invertebrates (Delany and Abercrombie, 1986). They can take 10-15 yr to reach sexual maturity, and they may live for 30 yr or more after becoming adults (Dunham et al., 2014). Collectively, these traits make alligators particularly susceptible to chronic exposure to environmental contaminants and their biological effects (Hopkins, 2000; Milnes and Guillette, 2008; Rowe, 2008). However, because alligators are capable of moving between aquatic habitats, particularly over their extended lifetime (Skupien, 2015; Subalusky et al., 2009), it can

be difficult to document their exposure history (Hopkins, 2006). There has been extensive research on alligators in habitats contaminated with organochlorines (Milnes and Guillette, 2008 and references therein) and, to a lesser extent, mercury (Elsey et al., 1999; Moore, 2004; Yanochko et al., 1997). However, other than documenting maternal transfer of Se to offspring by a single female occurring downstream of a coal-fired power plant and lower clutch viability relative to a nearby reference site (Roe et al., 2004), there are no published studies of alligators in CCW-contaminated systems.

We initiated the current experiment to study bioaccumulation of trace elements in juvenile American alligators as a result of chronic dietary exposure to CCW-contaminated prey. For two years, we exposed juvenile alligators to one of four dietary treatments that varied in the relative frequency of meals containing prey from CCW-contaminated habitats vs. prey from uncontaminated sites, and evaluated tissue residues after 12 mo and 25 mo of exposure. Specifically, we sought to determine the following: (1) whether chronic dietary exposure to CCW-contaminated prey results in bioaccumulation of trace elements in liver and kidney of juvenile alligators, (2) if accumulation varies as a function of exposure frequency (i.e., with dietary treatment), (3) if accumulation varies with exposure duration (i.e., 12 mo vs 25 mo), and (4) if growth rates or body condition of juvenile alligators vary as a function of dietary exposure treatment.

Methods

Pre-exposure husbandry

We obtained 36 American alligator hatchlings from Rockefeller Wildlife Refuge, Louisiana in November 2010, and transported them to the University of Georgia's Savannah

River Ecology Lab (SREL) in Aiken County, SC. Each alligator was individually marked and randomly assigned to one of six 270 x 56 x 57-cm living stream tanks (hereafter, tanks; Frigid Units Inc., Toledo, OH) in SREL's aquatic animal facility. The aquatic animal facility is a concrete block frame building with translucent fiberglass roof and upper walls that expose resident animals to natural light (Finger et al. 2015b). Ambient temperatures in the facility were strongly influenced by outdoor temperatures and ranged from 10-37 °C over the course of the study. Each tank was set up as a flow-through system filled with well water to an approximate depth of 11cm. We provided two 38 x 38-cm concrete basking platforms in each tank, with a 100-W incandescent light bulb suspended approximately 25 cm above one of the platforms. Lights were set to a 12 h light/dark cycle using automatic timers. We attempted to maintain water temperatures at 25 °C with 100-W aquarium heaters but were not always able to do so during colder winter months. We drained and cleaned each tank once per week. We fed each group of alligators 60 mL of Mazuri crocodilian chow (Catalog #0062615; PMI Nutrition International LLC, Brentwood, MO) three times per week until dietary exposure treatments were initiated to allow them to become accustomed to their tanks and to consuming dry food pellets.

Dietary exposure treatments

We initiated dietary exposure treatments on 13 June 2011 by randomly assigning tanks (and thereby, the six alligators in each tank) to one of four treatments that varied in relative frequency of CCW-contaminated food. Animals in all diet treatments were fed three times per week. The control treatment received only uncontaminated food ($n = 1$ tank). The other diet treatments alternated between receiving uncontaminated food and CCW-contaminated food. Animals in the CCW treatments received CCW-laden food at the following frequencies: low

dose (LD), once/week ($n = 1$); medium dose (MD), twice/week ($n = 2$); high dose (HD), three times/week ($n = 2$). Space limitations and the ability to supply contaminated food treatments precluded further replication. We elected to replicate the two higher dietary exposure treatments, which we expected to exhibit greater variation in accumulation among individuals due to variation in individual feeding rates and potential variation in trace element composition in prey collected from CCW-contaminated habitats.

We made standardized food for the experimental diets using Mazuri crocodilian chow, mosquitofish (*Gambusia holbrooki*), and crayfish (*Procambarus* and *Cambarus* sp.). For the CCW-contaminated diet, we collected mosquitofish and crayfish from the D-Area coal ash settling basins on the Department of Energy's Savannah River Site (SRS), Aiken, SC (see Alberts et al., 1985; Evans et al., 1980 for further descriptions). For the control diet, we purchased crayfish from a commercially available source and collected mosquitofish from several uncontaminated wetlands on the SRS (White and Gaines, 2002). We froze and subsequently dried prey to a constant weight in a drying oven then ground crayfish, fish, and crocodilian chow separately using a coffee grinder. We prepared CCW-contaminated and control diets by mixing 100 g dried crayfish, 30 g dried fish, 100 g gator chow, and 200 mL deionized water. We homogenized the mixture, pressed it into a plastic grate with uniform squares of 15 mm x 15 mm x 7.5 mm, and dried it in a drying oven. To prevent cross-contamination, we used separate utensils and grinders to prepare contaminated and uncontaminated diets. At each feeding, we offered 60 mL (measured by volume) of gator chow per tank, except during 1 Oct – 31 Mar each year when food rations were halved due to lower ambient temperatures and reduced alligator appetite. We saved samples of both prey items (crayfish, mosquitofish) from contaminated and

reference sites and the formulated contaminated and uncontaminated food pellets for subsequent trace element analysis.

Monitoring & biological sampling

Prior to initiating dietary exposure treatments (time 0; June 2011), we weighed (± 2 g) and measured snout-vent length (SVL; ± 1 mm) of each individual as described by Isberg et al. (2005) and attempted to sex them as described by Allsteadt and Lang (1995). During the experiment, we weighed and measured alligators monthly until immediately prior to culling.

After 12 mo of dietary exposure (June 2012), we euthanized two alligators from each tank ($n = 12$) and euthanized the remaining four individuals per tank ($n = 24$) at 25 mo (July 2013). First, we injected alligators intramuscularly with 5 mg kg⁻¹ xylazine for its analgesic properties. Next, we implemented a two-stage injection of tricaine methanesulfonate (MS222) following Conroy et al. (2009). Briefly, we injected into the coelomic cavity a 250-500 mg kg⁻¹ dose of MS222 buffered with 1% sodium-bicarbonate to a neutral pH, followed by a 0.5 – 1 mL injection of unbuffered 50% (v/v) MS222 injection after the animal was unresponsive and had lost its righting reflex. We then dissected alligators and collected the liver and kidney samples, which were placed individually in whirlpaks (Nasco, Fort Atkinson, Wisconsin) and stored at -80 °C. We also attempted to confirm sex through visual inspection of gonads during dissection. However, because sex could not be determined in a large proportion of individuals due to their small size ($n=13$) and because sex ratio was highly skewed among individuals whose sex was known (19 F, 4 M), we did not consider sex further in any analyses.

Trace element analysis

We freeze-dried and ground tissue samples, then digested them in 10 mL of trace metal grade nitric acid (70% HNO₃) 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. For smaller samples, we digested them in 1 mL of nitric acid and, after digestion, brought them to a final volume of 4 mL with water. We analyzed samples using three separate runs on an inductively coupled plasma mass spectrometer (ICP-MS; Perkin-Elmer, Norwalk, CT) to determine trace element concentrations. For quality control, we included certified reference material (TORT-2 and LUTS; National Research Council) in each analysis. Mean percent recoveries for elements in standard reference material ranged from 91.3% (As) – 127.4% (vanadium; V). Minimum detection limits in tissue samples for the three runs averaged: As, 0.103; Cd, 0.053; Se, 0.132; strontium (Sr), 0.115; and V, 0.053 $\mu\text{g g}^{-1}$ dry mass. We report trace element concentrations in parts per million (PPM) on a dry-mass basis ($\mu\text{g element g}^{-1}$ dry mass tissue).

For analysis of prey items, we freeze dried and ground whole carcasses, then digested and analyzed trace element concentrations as described above for alligator tissues. We collected subsamples of both contaminated and uncontaminated food pellets and analyzed trace elements on the ICP-MS as described above for alligator tissues and prey.

Statistical analyses

Dietary treatment analyses

To determine which trace elements were significantly elevated in CCW-contaminated compared to control food pellets, we compared trace element concentrations in the five batches of each pellet type with a two-tailed t-test using Excel 2007 (all other analyses

performed in SAS 9.3; SAS Institute, Cary, NC). For these comparisons, we only considered the trace elements that had previously been shown to be elevated in the D-Area ash basins relative to uncontaminated reference sites: As, Se, Cd, Se, Sr, and V. Based on mean concentrations in the CCW-laden pellets and control pellets and the relative frequency with which the dietary treatment groups received the two pellet types, we estimated the average dose of each trace element for each group (Table 1).

Tissue residue analyses

We compared trace element concentrations in alligator tissues exposed to the four diet treatments. We limited our analyses to the three trace elements that 1) had been shown in previous field studies to differ significantly in biological tissues from D-Area ash basins compared to reference sites, 2) differed in our initial analysis of trace element concentrations in the prepared diets or in CCW-contaminated prey, and 3) are known to cause potentially severe sublethal effects, particularly in aquatic habitats; these were: Cd, Se, and As (see Burger, 2008; Hamilton, 2004; and Hopkins et al., 1999; respectively). We compared trace element concentrations in liver and kidney tissue at 12 mo and 25 mo. Only Cd occurred at levels that were below the instrument detection limit in some samples [BDL; 42% (5 of 12) of the liver samples at 12 mo, 4 of which were in the Control and LD groups]. Due to the number of left-censored (i.e., BDL) concentration data for liver Cd we used a nonparametric Wilcoxon Wald's χ^2 test in PROC LIFETEST, in which a modified survival analysis can be used to examine group differences in metal concentrations that include several values for BDL from separate analytical runs (Newman, 2012).

We had complete data for all Se and As tissue concentrations, as well as kidney Cd (i.e., no samples BDL), and we tested for dietary treatment, time, body size (SVL) and interaction effects using a general linear model (PROC GLM); SVL was unaffected by the dietary treatments (see Results), and suitable for use as a covariate. Because alligators within a tank cannot be treated as independent observations (Hurlbert, 1984), we tested for a treatment main effect using the TANK(DIET) term as the error term, which is similar to using tank means as the unit of observation, in that it allows for potential covariance in the response variables (Wilbur, 1987). We used the residual error term to test for differences between time periods and a relationship to SVL. When the interaction terms were non-significant ($P > 0.05$) in the full model, we reran the analysis after removing the interactions and tested for main effects in the reduced model. All SVL*DIET interactions were non-significant (i.e., the relationships between SVL and trace element tissue concentrations were similar among diet treatments), which allowed us to test for treatment differences after adjusting for SVL in an analysis of covariance model. As we expected metal concentrations to vary among groups in a dose-dependent manner, we also used a contrast statement to test for a linear increase in mean tissue concentrations of Se, As, and Cd. When we observed significant DIET main effects we used Bonferroni-adjusted pair-wise comparisons of least squares means to identify differences among diet treatments at an experiment-wise error rate of $\alpha = 0.05$. Finally, we compared elemental apportioning to kidneys versus liver by regressing (PROC REG) the $\log_{10}$ of the elemental kidney concentration on the $\log_{10}$ value of the liver concentration; a log-log slope of 1.0 indicates equivalent apportioning to each organ (isometry), while a significant positive intercept indicates higher elemental levels in

kidneys. We $\log_{10}$ -transformed trace element concentrations for all analyses to meet normality and homogeneity of variance assumptions.

Growth analyses

We used a general linear model in PROC GLM to compare growth and body size (wet mass and SVL) among the four diet groups from the beginning to end of the experiment. Using the TANK(DIET) term as the error term, we tested for dietary treatment group differences in body size at the initiation of the diet treatments (TIME 0), at the time of the first culling (TIME 1; 12 mo), and at the end of the study (TIME 2; 25 mo). We also calculated monthly growth rates (SVL) between the three time periods and compared these among treatment groups, again using the statistical model structure described above. Growth rate was calculated as monthly SVL change over the time interval; $\Delta\text{SVL}/12$ (for growth rate 1; GRATE1), and $\Delta\text{SVL}/25$ (for growth rate 2, GRATE2). We examined effects of diet on body condition by regressing mass on SVL and testing for diet treatment effects on residuals. We natural-log transformed size and growth rate variables for all analyses to meet normality and homogeneity of variance assumptions (Levene's test of homogeneity).

RESULTS

Diet comparisons

The following trace elements were significantly elevated in CCW-laden food pellets compared to control food pellets: As (2.7x levels in control diet), Se (8.2x), and Sr (1.6x; $p < 0.001$ for all comparisons; Table 1). Cadmium levels were higher in the CCW-laden pellets than the control food pellets, in which Cd levels were BDL. Trace element concentrations in CCW-laden food were more similar to corresponding levels found in CCW-contaminated crayfish than in

CCW-contaminated mosquitofish, which constituted only 13.0% (by mass) of the dry ingredients used to make the formulated diet (compared to crayfish, which constituted 43.5%; Figure 1).

Treatment group comparisons of Se, As, and Cd concentrations

We compared elemental concentrations (Se, As, Cd) in alligator liver and kidney tissue among diet treatment groups and between exposure periods (12 mo or 25 mo; Figure 2), with SVL as a covariate. The reduced model (no interactions) explained 92% of the variance in liver Se concentrations, and 87% of the variance in kidney Se levels; Se was elevated in higher dose treatments, but was not affected by exposure period (Liver Se: DIET— $F_{3,2} = 170.8$, $P = 0.006$; Kidney Se: DIET— $F_{3,2} = 104.2$, $P = 0.009$). Selenium concentrations in the liver were negatively related to body size ($F_{1,28} = 7.5$, $P = 0.011$), but kidney Se concentrations were not. Arsenic concentrations showed the same pattern—increasingly elevated As in response to diet treatment, (Liver model $R^2 = 90\%$: DIET— $F_{3,2} = 60.6$, $P = 0.016$; Kidney model $R^2 = 84\%$: DIET— $F_{3,2} = 45.2$, $P = 0.022$), no effect of exposure period, and a negative relationship with SVL only in liver tissue ($F_{1,28} = 10.6$, $P = 0.003$). In general the multiple comparisons tests showed that each diet treatment differed from all other groups in tissue concentrations of Se and As; however, liver As concentration did not differ between the control and LD groups ($P = 0.36$), and kidney Se and As concentrations did not differ between the MD and HD treatments ($P > 0.68$). Both Se and As bioaccumulated in a dose-dependent manner, as evidenced by significant positive linear trends in treatment group metal concentrations (Figures 3 and 4; all $P < 0.0001$).

Cadmium concentrations in the kidney were unrelated to body size, but were higher in high-CCW treatments (DIET— $F_{3,2} = 27.0$, $P = 0.036$), increased linearly with dose ($F_{1,2} = 182.1$, P

< 0.0001; Figure 5), and were higher with longer exposure period (i.e., at TIME 2; $F_{1,28} = 50.9$, $P < 0.0001$). All diet treatment groups differed from each other in kidney Cd concentration except the MD and HD groups. Our non-parametric analysis of liver Cd levels showed that liver Cd differed among dietary treatment groups (PROC LIFETEST Wilcoxon test: $\chi^2 = 30.1$, $df = 3$, $P < 0.0001$) and was higher at 25 mo (PROC LIFETEST Wilcoxon test: $\chi^2 = 5.4$, $df = 1$, $P = 0.021$) than at 12 mo.

Allocation patterns

Using the $\log_{10}$ regression models, kidney concentrations of As, Se, and Cd were significantly related to liver concentrations ($R^2 = 68-71\%$, $P < 0.0001$ for all; Figures 3-5). Kidney concentration of all metals were higher than in the liver ($\log_{10}$ intercepts = 0.25 ± 0.02 , 0.39 ± 0.10 , 0.57 ± 0.07 for As, Se, and Cd, respectively; $P < 0.001$ for all). Slopes did not differ from 1.0 for any element ($b = 0.88 - 0.89 \pm 0.10$), indicating isometric apportionment of As, Se, and Cd between the tissue types.

Treatment group comparisons of body size and growth

Initial body sizes at the onset of the dietary treatments did not differ among the four treatment groups (SVL: $F_{3,2} = 2.11$, $P = 0.34$; Mass: $F_{3,2} = 1.49$, $P = 0.43$). Both measures of body size remained similar among groups at 12 mo and 25 mo (Figure 6; all P -values > 0.09). Growth rates did not differ among diet treatments (GRATE1: $F_{3,2} = 5.54$, $P = 0.16$; GRATE2: $F_{3,2} = 0.51$, $P = 0.71$), nor did body condition ($F_{3,2} = 0.31$, $P = 0.822$).

Discussion

Our study demonstrates that chronic dietary exposure to contaminated prey can lead to significant accumulation of trace elements in alligators. Alligators fed CCW-laden food

accumulated hepatic and renal concentrations of As, Cd, and Se that were 2.5-12x levels in alligators fed the control food, and concentrations varied in a dose-dependent manner. Our findings mirror patterns previously documented in banded water snakes (*Nerodia fasciata*) fed fish collected from the same CCW-contaminated system as in our study (Hopkins et al., 2001; 2002). Watersnakes bioaccumulated significant levels of As, Se, and Cd after one (Hopkins et al., 2001) or two years (Hopkins et al., 2002) of exposure, and their renal, hepatic, and gonadal concentrations varied as a function of dose. Diet has been shown to be an important route of exposure for trace elements in laboratory experiments with several other reptile species (Hopkins et al., 2004; 2005; Jones and Holladay, 2006).

In vertebrates, including reptiles, the kidney and liver tend to accumulate the highest levels of metals (Grillitsch and Schiesari 2010; McClellan-Green et al., 2006), making them important target tissues to monitor for contaminant exposure. In our study, As, Se, and Cd concentrations in alligators were strongly correlated between the two tissues, but levels were consistently higher in the kidney than in the liver. Surprisingly, only Cd continued to accumulate after the first 12 mo, whereas neither liver nor kidney concentrations of Se or As differed significantly between animals exposed for 12 mo compared to those exposed for 25 mo. Only Se occurred at levels that exceeded established toxicity benchmarks, although much less is known about toxicity thresholds and biological effects of As and Cd in wildlife species.

Cadmium is considered one of the most toxic heavy metals (Burger, 2008). Cadmium has a long half-life (7-26 yr in human kidneys; Wirth and Mijal, 2010) and low excretion rates due to binding with metallothionein in tissues (Furness, 1996), thus vertebrates accumulate cadmium and appear to be more vulnerable to chronic exposure than to acute exposure (Burger, 2008),

with accumulation increasing with age (Cooke and Johnson, 1996). In sea turtles, Cd levels average 3-5 times more in kidneys compared to liver (Caurant et al., 1999). Little is known about toxicity levels in reptiles, but the highest Cd level observed in any alligators in this experiment (kidney Cd of $3.33 \mu\text{g g}^{-1}$ dry weight after 25 mo exposure) was well below toxicity levels reported for other vertebrates ($100\text{-}200 \mu\text{g g}^{-1}$ kidney wet weight in a variety of experimental animals, primarily rats, Cooke, 2011; $100\text{-}150 \mu\text{g g}^{-1}$ kidney wet weight in birds and small mammals, Pascoe et al., 1996). Kidney or liver Cd concentrations of $10 \mu\text{g g}^{-1}$ (wet weight) have been associated with sublethal effects such as reproductive impairment or reduced growth in vertebrates, and kidney levels of $200 \mu\text{g g}^{-1}$ (wet weight) are considered life threatening (Burger, 2008). Few comparative data are available for liver or kidney concentrations of Cd from wild crocodilians. Burger et al. (2000) reported liver concentrations of Cd of $0.127 \mu\text{g g}^{-1}$ (wet weight) in alligators collected from Lake Apopka, Florida, and Campbell et al. (2010) reported levels of $0.489 \mu\text{g g}^{-1}$ (wet weight) in alligators from coastal South Carolina. Horai et al (2014) reported mean liver Cd concentrations ranging from $0.0962\text{--}0.202 \mu\text{g g}^{-1}$ (dry weight) among three lakes in Florida. If the bioaccumulation rate of Cd that we observed in the high-dose treatment continues linearly over time, then we might expect that alligators living and feeding in a CCW habitat could suffer impairment due to Cd in later reproductive years (>30 yr old).

Arsenic toxicity is rarely observed in wildlife, as the organic forms are quickly excreted unmetabolized and the inorganic forms are detoxified by methylation to organic forms (Eisler, 1988; Pascoe et al., 1996). As a result, As concentrations tend to be highest in the gut and digestive tract, followed by muscle, then kidney and liver (Liao et al., 2003; Saeki et al., 2000;

Sparling and Lowe, 1996). Chronic As toxicity is characterized by paralysis, weakness, conjunctivitis, and liver damage (Pascoe et al., 1996), but sublethal effects have not been thoroughly investigated, particularly in reptiles. The only reported case of a reptile mortality event attributed to As toxicity was at a wetland site in Texas impacted for more than 50 yr by the industrial production of an arsenic-based cotton defoliant (Clark et al., 1998). Despite the apparent rarity of As toxicity, reptiles can accumulate high concentrations of As. Sea turtles collected from the East China Sea and the north Pacific had As concentrations of up to $153 \mu\text{g g}^{-1}$ dry weight in muscle, $32.8 \mu\text{g g}^{-1}$ in liver, and $36.6 \mu\text{g g}^{-1}$ in their kidneys (Saeki et al., 2000). Banded water snakes (*Nerodia fasciata*) inhabiting wetlands contaminated with coal-combustion waste accumulated an average of more than $130 \mu\text{g g}^{-1}$ (dry weight) in their livers (Hopkins et al., 1999). Arsenic concentrations in alligators from our experiment were very low in comparison ($<3.2 \mu\text{g g}^{-1}$ kidney, $<1.5 \mu\text{g g}^{-1}$ liver dry weight), although they were higher than in wild juvenile alligators captured from three lakes in Florida without a history of CCW-contamination (mean liver As concentration $0.152 - 0.271 \mu\text{g g}^{-1}$ dry weight; Horai et al., 2014).

Of the three trace elements we tested, only Se accumulated in alligator kidney and livers to a level that approached toxicity benchmarks established for other vertebrate taxa. In low concentrations, Se is an essential nutrient, but at high concentrations it can displace sulfur (S) during protein synthesis, disrupting the S-to-S bonds critical in protein structure and function (Lemly, 1998). In this study, hepatic and renal Se levels ranged from $6.04 - 35.58 \mu\text{g g}^{-1}$ in alligators from the CCW treatment groups. Most established toxicity benchmarks for oviparous vertebrates are based on egg concentrations. Reproductive, embryotoxic or teratogenic effects tend to occur when egg concentrations exceed $10 \mu\text{g g}^{-1}$ in fish and $16 \mu\text{g g}^{-1}$ in birds

(Fairbrother et al., 1999; Lemly, 1998), although sensitivity varies among species. Few benchmarks based on liver or kidney concentrations are available. Liver Se levels of 29 $\mu\text{g g}^{-1}$ and 34 $\mu\text{g g}^{-1}$ (dry weight) have been associated with reproductive failure and mortality, respectively, in bluegill (Finley, 1985; Hermanutz et al., 1992; cited in Hamilton, 2003). Hamilton (2004) recommended liver thresholds of $<33 \mu\text{g g}^{-1}$ in young and non-laying adult birds, and Heinz (1996) suggested that liver Se concentrations of 3 $\mu\text{g g}^{-1}$ (wet weight) in laying female birds could be associated with reproductive risks. None of the alligators from our study had liver Se concentrations $>33 \mu\text{g g}^{-1}$, although kidney Se concentrations in three (8.3%) individuals exceeded this threshold. Liver Se levels from our study were higher than reported for wild juvenile alligators captured at three Florida lakes without a history of CCW contamination (5.44 – 6.29 $\mu\text{g g}^{-1}$ dry weight), although one adult wild alligator had liver Se concentrations as high as 31.6 $\mu\text{g g}^{-1}$ dry weight (Horai et al., 2014).

Previous studies have suggested that CCW exposure can increase metabolic rate, consequently inducing trade-offs with growth (Hopkins et al., 1999; Rowe et al., 2001; 2002). Thus, one objective of this study was to determine the effect of chronic ingestion of CCWs on the size, growth and body condition of juvenile alligators. We hypothesized that CCW-exposed alligators would exhibit decreased growth and body size relative to control alligators. However, we observed no effect of diet treatment on any morphometric parameter. In fact, alligators continued to grow throughout the duration of the study, regardless of diet treatment (Figure 6). Our results suggest that ingestion of low doses of CCWs, at least for 25 mo, has no effect on alligator growth.

The absence of a diet treatment effect on growth of alligators in our study may have been due to the relatively low exposure levels. For example, alligators from the high dose treatment that received CCW-laden pellets at each feeding (i.e., 3x/week) were only exposed to 12.26 $\mu\text{g g}^{-1}$ of Se per feeding. Previous research suggests that much higher levels ($>40 \mu\text{g g}^{-1}$) are required to impair growth in birds (Ohlendorf, 2003). Reproductive impairment in birds and fish has occurred at lower concentrations than those associated with reduced growth and survival (Lemly, 1996; Ohlendorf, 2003). Thus, reproductive parameters, such as hatching success and offspring viability, may be more sensitive endpoints for evaluating the effects of CCW in wildlife (Hopkins et al., 2004). In the only other investigation of the effects of CCW on alligators, clutches obtained from wetlands receiving CCW-contaminated effluent had higher Se levels and lower clutch viability than those collected from uncontaminated reference wetlands (Roe et al., 2004).

Another possible factor that may have impacted growth irrespective of metal-dose treatment, and therefore masked effects of CCWs on growth/body size, is related to husbandry and housing conditions. Due to space limitations, we housed animals in small groups. However, crocodilians establish hierarchies quickly after hatching (Brien et al., 2013), with larger individuals monopolizing food. Interactions of smaller individuals with larger, more dominant individuals can cause increased corticosterone (the main reptilian stress hormone), and subsequently, diminished growth (Elsey et al., 1990). Therefore, in captive settings (e.g., “gator” farms), crocodilians are often graded (i.e., similar sized individuals are moved into pens together) continuously to prevent variations in size from occurring and larger individuals from monopolizing resources (Finger et al., 2015a, Morpurgo et al. 1993). However, in this study,

grading was not feasible due to differences in the treatment regimens between tanks and because there was a finite amount of space for housing alligators. The non-independence of animals within a tank is one reason we used the tank, not individual animals, as our unit of observation in testing for diet treatment effects.

Interestingly, even though the CCW diet treatments had no effect on growth, body size was negatively related with liver Se and As concentration after accounting for diet and exposure duration. Mercury is one metal that consistently shows increased concentrations at larger body sizes, but size-concentration trends vary widely for other metals (Canli and Atli, 2003). Many factors influence elemental allometry, and size may often not be related to metal concentrations (Newman and Heagler, 1991). A negative relationship between size and metal concentration is a common pattern in both invertebrates (e.g., Mubiana et al., 2006) and vertebrates (e.g., Canli and Atli, 2003), and may indicate somatic growth dilution whereby faster growing individuals attain lower contaminant concentrations than slower individuals subjected to the same exposure conditions (Ward et al., 2010).

Although the dietary concentrations to which alligators were exposed did not result in growth or size differences among treatment groups, our effective exposure treatments were biologically relevant. Trace element concentrations in the two prey types used to make the food pellets – crayfish and mosquitofish – were higher from the CCW-contaminated site than the reference sites (Table 1). In general, they also reflected patterns previously reported from these taxa and from other potential small prey items collected from our study system (Delany, 1990; Nagle et al., 2001; Rowe et al., 2001; Staub et al., 2004). The resulting CCW-laden food pellets had elevated levels of As, Se, and Cd relative to the control food pellets (Table 1).

However, for some trace elements, concentrations varied dramatically between crayfish and mosquitofish collected from the same site (Table 1). Trace element concentrations in food pellets more closely approximated levels measured in crayfish than in mosquitofish, presumably due to the higher proportion of crayfish dry mass used in formulating the pellets. In addition, contaminated prey were mixed with ground, commercially-available crocodilian food pellets to ensure adequate nutrition and to provide a matrix for administering the ground prey items. As a result, overall trace element concentrations in CCW-laden food and in the corresponding CCW diet treatments were somewhat low. For example, Se levels in our CCW treatments ranged from 5.09 – 12.26 $\mu\text{g g}^{-1}$, whereas many prey from the CCW-contaminated site have whole body concentrations of 10 - 37 $\mu\text{g g}^{-1}$ (Roe et al., 2004). Despite the CCW-laden food pellets having trace element levels at the low end of this range, alligators still accumulated significant body burdens of Se, As and Cd in their livers and kidneys and in a dose-dependent manner.

Although it can be difficult to make comparisons with studies of other species due to differences in life history and physiology, our results closely mirror results from two studies in which squamates were chronically exposed to CCW-laden prey. Banded watersnakes were fed fish collected from either CCW-contaminated or reference sites. The CCW-contaminated fish had elevated concentrations of As, Cd, Se, copper (Cu), strontium (Sr), and vanadium (V) compared to fish collected from reference sites, and the watersnakes bioaccumulated significant levels of As, Se, and Cd in their tissues after one (Hopkins et al., 2001) or two years (Hopkins et al. 2002) of exposure. Liver and kidney concentrations of Se, As, and Cd reported for watersnakes were remarkably similar to levels we observed in juvenile alligators. As in our

study, chronic exposure to CCW-contaminated prey had no adverse effects on growth or any other endpoint measured in banded watersnakes (Hopkins et al., 2001; 2002).

Even with biologically relevant doses, trace element concentrations in experimentally exposed animals may not approach levels observed in wild-caught animals. Hopkins et al. (1999; 2001) noted that banded watersnakes in their experiments only accumulated a fraction of the trace element concentrations measured in wild individuals collected from the CCW-contaminated wetlands from which their prey originated (e.g., 22.6 $\mu\text{g g}^{-1}$ vs 140 $\mu\text{g g}^{-1}$ Se). Differences could be due to deviations in exposure period or in diet composition between wild and experimental animals (Hopkins et al., 2001). In addition, because both the watersnake experiments and our alligator experiment focused on dietary exposure, observed trace element concentrations do not reflect other important routes of exposure such as contact exposure or maternal transfer. Unfortunately, we do not have comparative trace element concentrations from the livers or kidneys of wild-caught alligators from our study system. Ongoing long-term mark-recapture studies precluded the collection of destructive samples.

Housing alligators communally made it difficult to control and document individual food consumption and thus contaminant exposure rates. Future manipulative experiments with crocodilians would benefit from housing animals individually when possible, or administering contaminant doses individually. Despite these challenges, crocodilians are an important taxon for understanding the risks associated with chronic exposure to pollutants. Their longevity, delayed sexual maturity and carnivorous diet place them at risk for bioaccumulating toxic levels of contaminants (Finger and Gogal, 2013; Milnes and Guillette, 2008; Moore et al., 2012; Rowe, 2008). However, it can be difficult to link tissue burdens in individuals with their exposure

history, particularly over their extended lifetime. Thus, a combination of field surveys and laboratory experiments will be required to understand the effects of different exposure scenarios on tissue residues, and ultimately link these concentrations with effects on individual health (Hopkins et al., 2004; Moore et al., 2012; Rainwater et al., 2007; Rauschenberger et al., 2007). Experimental manipulations of long-term contaminant exposure in reptiles is rare (Grillitsch and Schiesari, 2010, Hopkins, 2006). Rauschenberger et al. (2007) demonstrated reduced clutch viability in adult alligators dosed with organochlorine pesticides over three years, and Moore et al. (2012) documented that maternal exposure to contaminants has long-term carryover effects on juvenile growth and ovarian gene expression. Except for a single study in which *Crocodylus porosus* were exposed to lead shot-contaminated meat (Hammerton et al., 2003), ours is the only other study to experimentally evaluate the effects of chronic dietary exposure of a trace metal on tissue concentrations in a crocodilian. Although reptiles as a whole appear to be relatively resilient to the direct lethal effects of metals (Grillitsch and Schiesari, 2010; Rainwater et al., 2007), crocodilians remain understudied with respect to the long-term individual- and population-level biological consequences of sublethal effects of trace element contaminants.

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Figure captions

Figure 1: Trace element concentrations ($\mu\text{g g}^{-1}$ dry mass) in crayfish and mosquitofish collected from CCW-contaminated and reference wetlands comprising the formulated food pellets fed to American alligators.

Prey collected from CCW- contaminated and reference wetlands are indicated with open bars and closed bars, respectively. Statistical differences in trace element concentrations between control and CCW-laden food pellets are given in Table 1.

Figure 2: Mean trace element concentrations ($\mu\text{g g}^{-1}$ dry mass; ± 1 SE) in livers and kidneys of American alligators (*Alligator mississippiensis*) after 12 mo (left panel; n=12) and 25 mo (right panel; n=24) exposure to diets with different concentrations of trace element contaminants.

Dietary concentrations ($\mu\text{g g}^{-1}$ dry mass) are based on trace element values of food pellets made from prey collected from either uncontaminated reference sites (control pellets) or CCW-contaminated sites (CCW-laden pellets). Mean dietary concentrations for each treatment group are based on the relative frequency with which they received CCW-laden vs. control pellets. Liver Cd levels could not be plotted for lower dose treatment groups because they were below the instrument's detectable limit.

Figure 3: Relationship between liver and kidney tissue concentration of selenium ($\mu\text{g g}^{-1}$ dry mass) in 36 juvenile *A. mississippiensis* fed four different diets differing in trace element concentrations.

Circles represent reference group animals fed a control, uncontaminated food three times per week; triangles depict a low dose diet (CCW-laden food one of every three feedings), squares a medium dose diet (two of three feedings), and stars a high dose diet (all feedings). Lighter symbols are individuals on the diets for 12 mo (n=12), darker symbols for 25 mo (n=24). Larger symbols represent diet treatment means $\pm$ 1 SE; vertical bars for kidney SE, horizontal bars for liver SE.

Figure 4: Relationship between liver and kidney tissue concentration of arsenic ($\mu\text{g g}^{-1}$ dry mass) in 36 juvenile *A. mississippiensis* fed four different diets differing in trace element concentrations.

Circles represent reference group animals fed a control, uncontaminated food three times per week; triangles depict a low dose diet (CCW-laden food one of every three feedings), squares a medium dose diet (two of three feedings), and stars a high dose diet (all feedings). Lighter symbols are individuals on the diets for 12 mo (n=12), darker symbols for 25 mo (n=24). Larger symbols represent diet treatment means $\pm$ 1 SE; vertical bars for kidney SE, horizontal bars for liver SE.

Figure 5. Relationship between liver and kidney tissue concentration of cadmium ($\mu\text{g g}^{-1}$ dry mass) in 31 juvenile *A. mississippiensis* fed four different diets differing in trace element concentrations.

Circles represent reference group animals fed a control, uncontaminated food three times per week; triangles depict a low dose diet (CCW-laden food one of every three feedings), squares a

778 medium dose diet (two of three feedings), and stars a high dose diet (all feedings). Lighter
779 symbols are individuals on the diets for 12 mo (n=7), darker symbols for 25 mo (n=24). Larger
780 symbols represent diet treatment means $\pm$ 1 SE; vertical bars for kidney SE, horizontal bars for
781 liver SE. At 12-mo liver Cd concentrations in five individuals were below the method detection
782 limit (BDL); 12-mo control and low dose averages are for kidneys only.

783

784 **Figure 6. The effect of dietary treatment (Control, Low, Medium, and High Dose) on alligator**
785 **snout-vent length (SVL; top) and body mass (bottom).**

786 Values shown are means $\pm$ 1 SE at 0 mo (prior to treatment initiation) and after 12 and 25 mo
787 exposure.

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Tables

Table 1: Trace element concentrations ($\mu\text{g g}^{-1}$ dry weight) in crayfish and mosquitofish collected from uncontaminated reference wetlands and a CCW-contaminated wetland and incorporated into control and CCW-laden food pellets, respectively.

Trace element	Reference crayfish	CCW crayfish	Reference mosquitofish	CCW mosquitofish	Control food pellets	CCW-laden food pellets	LD Treatment	MD Treatment	HD Treatment
As*	BDL	4.58	2.98	13.56	1.85 ± 0.04	5.05 ± 0.06	2.92	3.98	5.05
Cd	BDL	1.05	0.22	0.41	BDL	1.95 ± 0.03	0.81	1.38	1.95
Se*	BDL	14.19	1.00	52.18	1.5 ± 0.001	12.26 ± 0.20	5.09	8.67	12.26
Sr*	463.82	688.64	54.24	192.59	203.49 ± 2.68	317.75 ± 1.94	241.58	279.66	317.75
V	161.99	80.4	23.88	66.81	56.58 ± 1.60	40.49 ± 0.50	51.22	45.85	40.49

Concentrations are presented as means $\pm$ 1 SE based on analysis of five separate batches of each food type. Trace elements significantly elevated in CCW-laden food pellets relative to control food pellets (all $P < 0.001$) are indicated with an asterisk (*). Mean dietary concentrations for each experimental diet treatment are estimated based on the relative frequency with which treatments received CCW-laden food pellets (LD [low dose] treatment = 1x/week, MD [medium dose] treatment = 2x/week, HD [high dose] treatment = 3x/week; control group received only control food pellets). BDL = concentration below the detectible limit.

Figure 2. Chronic dietary exposure to coal combustion waste (CCW)-laden food for 12 and 25 mo resulted in bioaccumulation of As, Cd, and Se in kidney and livers of juvenile American alligators.

Figure 1_1 column

Figure 2_2 columns

Figure 3_1 column

Figure 4_1 column

Figure 5_1 column

Figure 6_1 column