Environmental Pollution: Full research paper

Accumulation of coal combustion residues and their immunological effects in the yellow-bellied slider (*Trachemys scripta scripta*)

David L. Haskins^{1,2}, Matthew. T. Hamilton^{1,2}, Amanda L. Jones¹, John W. Finger Jr.^{1,3}, Robert B. Bringolf² and Tracey D. Tuberville^{1,2}

Abstract

Anthropogenic activities such as industrial processes often produce copious amounts of contaminants that have the potential to negatively impact growth, survival, and reproduction of exposed wildlife. Coal combustion residues (CCRs) represent a major source of pollutants globally, resulting in the release of potentially harmful trace elements such as arsenic (As), cadmium (Cd), and selenium (Se) into the environment. In the United States, CCRs are typically stored in aquatic settling basins that may become attractive nuisances to wildlife. Trace element contaminants, such as CCRs, may pose a threat to biota yet little is known about their sublethal effects on reptiles. To assess the effects of CCR exposure in turtles, we sampled 81 yellow-bellied sliders (*Trachemys scripta scripta*) in 2014-2015 from CCR-contaminated and uncontaminated reference wetlands located on the Savannah River Site (Aiken, SC, USA). Specific aims were to (1) compare the accumulation of trace elements in *T. s. scripta* claw and blood samples between reference and CCR-contaminated site types, (2) evaluate potential immunological effects of CCRs via bacterial killing assays and phytohaemagglutinin (PHA) assays, and (3) quantify differences in hemogregarine parasite loads between site types. Claw As, Cd, copper (Cu), and Se (all $p \leq 0.001$) and blood As, Cu, Se, and strontium (Sr; $p \leq 0.015$) were significantly elevated in turtles from CCR-contaminated wetlands compared to turtles from reference wetlands. Turtles from reference wetlands exhibited lower bacterial killing ($p = 0.015$) abilities than individuals from contaminated sites but neither PHA responses ($p = 0.566$) nor parasite loads ($p = 0.980$) differed by site type. Despite relatively high CCR body burdens, sliders did not exhibit apparent impairment of immunological response or parasite load. In addition, the high correlation between claw and blood concentrations within individuals suggests that nonlethal tissue sampling may be useful for monitoring CCR exposure in turtles.

Keywords: reptile, bioaccumulation, coal combustion residues, ecoimmunology, nondestructive sampling

Capsule: Yellow-bellied sliders exposed to coal combustion residues can accumulate high concentrations of trace elements but their immune response does not appear to be negatively impacted.

Introduction

Coal combustion residues (CCRs) are common by-products of energy production globally, are produced in large quantities, and contain high levels of potentially toxic trace elements such as arsenic (As), cadmium (Cd), and selenium (Se; Rowe et al. 2002). The United States produces over 130 million tons of CCRs per year and prior to 1980 approximately 66% of CCRs were placed into aquatic settling basins (Rowe et al. 2002). As of 2010, 40% of these wastes were still being disposed into aquatic settling

basins (ACAA 2010). Trace element contaminants from CCR wastes held in these settling basins can enter the environment in a variety of ways. Coal combustion residues can leach into groundwater, be released from runoff or discharges, or escape as a result of the collapse of retaining walls (Rowe et al. 2002; Ruhl et al. 2012; Van Dyke et al. 2013). Unintentional releases do not have to occur for CCRs to negatively affect local biota. Aquatic settling basins can become attractive nuisances by attracting many species of wildlife for activities such as foraging and reproduction, thereby placing them at risk of exposure to potentially toxic levels of trace elements (Bryan et al. 2012; Lemly and Skorupa 2012).

Previous work has found that reptiles inhabiting CCR-contaminated environments accumulate significant amounts of CCR trace elements (Hopkins et al. 1999; Nagle et al. 2001; Roe et al. 2004). High CCR trace element concentrations are known to cause deleterious effects on reproduction, metabolic processes, and survival in aquatic species including fish, amphibians, and invertebrates (Hopkins et al. 1999; Rowe et al. 2002; Hopkins et al. 2013). Furthermore, CCR trace elements such as As and Se can cause immunotoxic effects in fish and birds in controlled exposure studies (Ghosh et al. 2006; Fairbrother et al. 1994). However, field studies that focus specifically on CCR exposure and its impact on the immune response are sparse. Because mounting an immune response is energetically costly, disruption of metabolic processes and energy allocation due to CCR exposure could lead to compromised immune responses or impair ability to regulate parasite loads (Martin et al. 2010). Immune function can also be negatively impacted by parasite loads in vertebrates (Graham 2002; Martin et al. 2010). Furthermore, little is known overall regarding how trace element contaminants could negatively affect the reptilian immune system, and studies assessing the toxic risk of these contaminants in reptiles have only started in the last two decades (Keller et al. 2006).

In spite of recent advances in the field of reptilian ecotoxicology, sublethal effects associated with chronic exposure to trace element contaminants are relatively unknown. Thus, this study explored the sublethal effects of CCR trace elements on freshwater turtles. Because recent research has suggested that red-eared sliders (*Trachemys scripta elegans*) rely more heavily on innate immune responses rather than adaptive responses (Zimmerman 2013), we investigated the potential effects of exposure to CCR trace

elements on the innate immune responses in wild caught *T. s. scripta*, focusing on two commonly used immunological assays – bacteria killing assays and phytohaemagglutinin skin assays (described further below). Our specific objectives were to: (1) quantify the accumulation of CCR-associated trace elements (As, Cd, Cr, Cu, Se, Sr) in tissues corresponding to different exposure time scales (blood and claw); (2) compare innate immunological responses of sliders inhabiting CCR-contaminated wetlands and reference wetlands; and (3) determine if parasite burdens differ based on exposure to CCR trace elements.

Methods

Study species

The yellow-bellied slider (*T. s. scripta*) is a freshwater turtle common throughout southeastern United States and is a habitat generalist (Gibbons 1990a). Yellow-bellied sliders, like all sliders, tend to have small annual home ranges (although they may move long distances over their lifetime; Morreale et al. 1984), exhibit high site fidelity, are long-lived (20-30 years in the wild), and feed in middle to high trophic levels (Gibbons 1990a; Tucker 2001; Parmenter and Avery 1990). These life history characteristics, along with their wide geographic distribution (North America to South America; Gibbons 1990a), make slider turtles, including *T. s. scripta*, an excellent species for contaminant studies. Slider turtles also experience an ontogenetic diet shift – juveniles tend to be carnivorous but become more omnivorous as adults (Parmenter and Avery 1990), potentially altering patterns of dietary exposure and accumulation of contaminants over their lifetime. On the Savannah River Site (SRS) where our study was conducted (see below), *T. s. scripta* is the most abundant freshwater turtle species (an estimated 61 individuals/ha of aquatic habitat) and accounts for almost 50% of all freshwater turtle biomass (Congdon et al. 1986).

101

102 *Study site*

103 Our study was conducted on the Savannah River Site (SRS), an 800-km² Department of Energy
104 facility located in the southeastern Coastal Plain in west-central, South Carolina, USA. The SRS
105 supports a variety of wetland types, including isolated wetlands (Carolina bays), farm ponds,
106 streams, and bottomland hardwood swamps. A small proportion of these wetlands have been
107 impacted by site operations (*e.g.*, the production of nuclear materials, power production),
108 resulting in wetlands with varying contaminant histories. One of the major sources of industrial
109 contaminants on the SRS was former coal-burning power plants, which produced CCRs that
110 were disposed in surface impoundments (aquatic settling basins). Our study was conducted in the
111 D-Area ash basin system, which has been well-characterized in previous studies (Hopkins et al.
112 1999; Nagle et al. 2001). Coal fly ash was discharged from the power plant into receiving basins,
113 then into large primary and secondary basins (Figure 1), with particulates separating and settling
114 out as water moved through the system. Several natural wetlands in close proximity (< 0.1 km)
115 to the D-Area settling basins receive run-off from the basins. In addition, turtles are known to
116 move between the settling basins and associated wetlands. Thus, for our study D-area turtles
117 were collected from the primary ash settling basin and from wetlands ‘A’ and ‘B’ adjacent to D-
118 area (see Figure 1). Reference animals were collected from 10 natural wetlands on the SRS that
119 have not historically received CCR-effluents and that were 2.4 - 24 km away from D-area and
120 other CCR basins.

121

122 *Field sampling and sample collection*

During May-September 2014 and May-July 2015, *T. s. scripta* were captured with hoop nets baited with sardines and/or creamed corn. Traps were checked once daily, and all turtles were transported to the Savannah River Ecology Laboratory (SREL) for processing. Morphological data including plastron length (PL; to nearest 1 mm), weight (to nearest 2 g), and sex were recorded for each individual. Each turtle was permanently marked by notching or drilling a unique combination of marginal scutes (Cagle 1939; Gibbons 1990b). Whole blood and claw samples were collected to provide a short and long-term measurement of contaminant exposure, respectively (Aresco 2005; Van Dyke et al. 2013). Tips of claws (2-3 mm) from the left forelimb were collected from each turtle and stored at -60 °C until analysis. Blood samples totaling no more than 1% of the animal's body weight, generally <1-1.5 mL, were collected via the subcarapacial sinus with a 27 or 25-gauge needle (Hernandez-Divers et al. 2002). After setting aside half of each whole blood sample for storage at -60 °C for subsequent trace element analysis, a drop of whole blood was also used to make blood smears, which were fixed with 100% methanol (VWR International, Radnor, PA) for *Haemogregarina* parasite quantification. Blood smears were collected opportunistically in 2014 and for all turtles in 2015.

The remaining blood sample was then aliquoted into lithium heparin tubes (Becton Dickson, San Antonio, TX, USA) or SealRite[®] 1.5 mL microcentrifuge tubes (USA Scientific Inc., Ocala, FL, USA) and centrifuged (Heathrow Scientific LLC, Vernon Hills, IL, USA) at 6,000 RPM to collect plasma for the bactericidal assay. Plasma samples were stored at -60°C until further analysis. Finally, phytohaemagglutinin (PHA) skin assays were performed on individuals having at least 100 mm PL. Biological samples were only collected and PHA assays performed for turtles at their first capture during the study period. Assays are described further below.

146

147 *Trace Element Analysis: Blood*

148 All blood samples were analyzed for As, Cd, chromium (Cr), copper (Cu), strontium (Sr),
149 and Se. Blood samples were digested in 2.5 mL of trace metal grade nitric acid (70% HNO₃)
150 using microwave digestion (MarsExpress, CEM Corp., Matthews, NC). Once digestion was
151 complete, each sample was brought to a final volume of 10 mL with 18-MΩ deionized water.
152 Blood samples were analyzed in three separate runs using an inductively-coupled plasma mass
153 spectrometer (ICP-MS, Perkin-Elmer, Norwalk, CT) to quantify trace element concentrations.
154 Certified reference material (TORT-3, National Research Council of Canada, Ottawa, ON) was
155 used as a standard for verifying trace metal recoveries for the blood digests. Mean percent
156 recoveries for trace elements in TORT-3 ranged from 85.7% (Strontium) – 92.3% (Se).
157 Minimum detection limits in blood samples averaged: As, 0.005; Cadmium, 0.002; Chromium,
158 0.002; Copper, 0.005; Se, 0.103; and Sr, 0.003 mg/kg. Blood trace element concentrations are
159 reported as mg/kg on a wet-mass basis.

160

161 *Trace Element Analysis: Claw*

162 Claw tissues were analyzed for As, Cd, Cr, Cu, Sr, and Se. Following acid digestion,
163 claw trace element concentrations were determined by ICP-MS at the Trace Element Analysis
164 Core of Dartmouth College. The methods have been described elsewhere (Van Dyke et al. 2013;
165 Steen et al. 2015) but, briefly, claws were washed with acetone and surfactant, rinsed with
166 deionized water (DI) and dried before acid digestion using 0.5 mL 9:1 HNO₃:HCl at 95°C for 45
167 min. Digested samples were diluted to 10 mL with DI and analyzed by collision/reaction cell

ICP-MS. Procedures used in this analysis followed the protocols from EPA 6020A. Calibration checks and blanks were run every 10 samples, and analysis replicates and spikes were run at a ratio of 1 duplicate and spike for every 20 samples. Mean percent recoveries for trace elements in standard reference materials ranged from 77% (Cu) – 99% (Cd). Minimum detection limits (MDLs) in samples averaged: As, 0.016; Cd, 0.031; Cr, 0.155; Cu, 0.466; Se, 0.031; and Sr, 0.031 mg/kg dry mass. All claw concentrations are reported as mg/kg on a dry mass basis.

Bacteria-killing assays (BKAs)

A bactericidal assay was used to assess the relationship between CCR body burden and innate immunity of wild *T. s. scripta*. Bacteria killing assays are commonly used to quantify an animal's innate immune response to a particular species of bacteria (Millet et al. 2007; French et al. 2010; Zimmerman et al. 2010; Brown and Shine 2014). Our study used *Escherichia coli*, which is commonly used to test bactericidal capacity across vertebrate classes. For each turtle, 7 µL of plasma was mixed with 137 µL of CO₂ independent media containing 5% fetal bovine serum and 4 mM L-glutamine and subsequently added to a 10 µL aliquot of approximately 400 colony-forming units (CFUs) of *E. coli* (ATCC #8739; VWR, Radnor, PA) in phosphate buffered saline (PBS; Finger et al. 2015). Once mixed, 50 µL of the sample mixture was immediately spread with a fish-tail spreader onto agar plates (Time 0 plates; Teknova Tryptic Soy plates, Fisher Scientific Company LLC, Suwanee, GA). The remaining sample mixture was incubated at room temperature for 60 minutes, after which another 50 µL aliquot was placed onto a separate agar plate (Time 60 plates). Plates were incubated overnight at 37°C (Zimmerman et al. 2012). The following morning, the bacterial colonies on each agar plate were counted. The

percentage of bacteria killed by an individual's plasma was calculated using the following formula: $(1 - [\text{Time 60 colony count} / \text{Time 0 colony count}]) * 100$ (Millet et al. 2007).

Phytohaemagglutinin (PHA) skin assays

To further assess the relationship between CCR body burden and immune function, turtles were subjected to an immunological challenge, the PHA skin assay, to measure an individual's ability to mount an immune response to a novel antigen (Finger et al. 2013; 2015). This method requires an injection of a non-pathogenic, antigenic lectin isolated from the red kidney bean (*Phaseolus vulgaris*) and pre- and post-injection measurements of the injection site to measure swelling response (Finger et al. 2013). In this study, we sought only to measure the primary, innate (non-specific) immune response to PHA injection. It has been shown that primary injections with PHA causes a proliferation of cells associated with an innate immune response (i.e., granulocytes, lymphocytes, and macrophages; Martin et al. 2006). Due to the seasonal fluctuations in reptilian immunity (Zapata et al. 1992; Zimmerman et al. 2009), we standardized this assay by only performing the tests during summer months (May-July). PHA (PHA-P #L8754; Sigma-Aldrich, St. Louis, MO, USA) was dissolved into sterile phosphate buffered saline (PBS #10010-023; Gibco by Life Technologies, Carlsbad, CA, USA) to make a 2 mg/mL solution. For each turtle, 20 μ L was injected subcutaneously between the webbing of the third and fourth digit of the right rear foot. As a control, the left foot was injected in the same area with 20 μ L of PBS. Peak swelling response in *Trachemys spp.* occurs at 24 hours (Jones and Finger, unpublished data). Thickness measurements (to the nearest 0.01 mm) at the injection site were taken just prior to injection (time 0) and at 24 hours post-injection using a dial thickness pressure gauge (Peacock G-1A, Ozaki Manufacturing Ltd, Japan). For each foot at each

measurement time, three successive measurements were obtained and averaged for analysis. To minimize sampling errors, measurements were taken quickly and by the same individual (AJ) across both years of data collection.

Parasites: Hemogregarine quantification

Fixed blood smears were stained with Giemsa and viewed in a zig-zag fashion at 1000X magnification using oil immersion and a standard light microscope. Prevalence of hemogregarines (proportion of sampled turtles infected with hemoparasites) was calculated. To quantify total hemogregarine parasite load for each individual, we read an average of approximately 121 fields of view which contained an average of 73 ± 7 (mean $\pm$ 1 SE) erythrocytes. Therefore, we counted a total of 8000 erythrocytes per individual. Total hemogregarine parasite loads were later transformed into a percentage of infection (i.e., parasitemia; Davis and Sterrett 2011). Parasitemia (the percentage of blood cells that are infected with hemogregarines) was calculated using the following equation: $(\# \text{ of parasites} / 8000 * 100)$. Developmental stages of hemogregarines were not characterized.

Statistics

Due to the limited number of turtles collected at individual reference wetlands, all individuals captured at reference sites were grouped into a single site type (Reference). Likewise, turtles captured in D-area's primary basin and 'A' and 'B' wetlands were all grouped as D-area turtles (see Figure 1). If, for a given trace element, at least 50% of blood samples had detectable amounts of a trace element, concentrations below the instrument's minimum detection limits

($<$ MDL) were replaced with half of the original MDL (based on each sample weight) for analysis. If less than 50% of the blood samples had detectable amounts of a trace element, all data for that trace element were excluded from analysis. All claw trace element concentrations were $\geq$ MDL.

Data were analyzed using program R (R Core Team 2016). Data were tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene statistics, respectively. In cases where data did not satisfy the normality assumption, data were log-transformed or rank-transformed (ANCOVA) prior to analysis. If data were not normally distributed but were homoscedastic, nonparametric statistics were used. No data exhibited heteroscedasticity. Blood trace element concentrations and bacterial killing assays were analyzed with nonparametric statistics (Mann-Whitney U tests). Trace element concentrations in claws were compared using an ANCOVA with site type (D-Area vs. Reference) as a main effect and PL as a covariate. Because blood and claw trace element concentrations reflect different exposure time scales, we examined the correlational relationship between these values via Spearman's correlations. We also report the linear relationship between the two sample types. We used ANCOVA to test for possible differences in hemogregarine parasitemia of turtles between site types, using PL as a covariate.

PHA responses were analyzed using a linear mixed model (LMM) repeated measures analysis with a serial correlation structure. Toe web swelling was the response variable and individual turtle was used as a random effect. A likelihood ratio test was performed to ensure that the LMM benefitted from the addition of turtle ID as a random effect. Other variables in the PHA LMM included plastron length (covariate), site type, and the interaction of time (0 vs. 24 hours) and treatment (PBS vs. PHA).

258

259 **Results**

260 A total of 81 *T. s. scripta* (26 females, 27 males, 28 immature) were collected—39 from
261 D-area and 42 from reference locations. At D-Area, mean PL of females ($n = 8$) was $174.5 \pm$
262 17.7 mm (mean ± 1 SE; range of 102-227 mm), of males ($n = 11$) was 154.4 ± 10.9 mm (106-
263 205 mm), and immature individuals ($n = 20$) was 66.6 ± 2.9 mm (42-88 mm). Mean PL of
264 reference females ($n = 18$) was 202.4 ± 9.7 mm (129-261 mm), for males ($n = 16$) was $158.2 \pm$
265 8.5 mm (65-195 mm), and for immature individuals ($n = 8$) was 72.1 ± 3.8 mm (59-91 mm).

266

267 *Trace element burdens (Blood)*

268 Chromium, Cu, and Sr were quantifiable in 100% of blood samples, but As and Se were
269 $<MDL$ in 43% (27/63) and 37% (23/63) of blood samples, respectively. Blood Cd could not be
270 used in our analyses because 86% (54/63) of samples were $<MDL$. Arsenic ($U = 657, p = 0.015$),
271 Cu ($U = 779, p < 0.001$), Se ($U = 836, p < 0.001$), and Sr ($U = 858, p < 0.001$) concentrations
272 were all significantly higher in D-area turtles than in reference turtles. In contrast, Cr ($U = 291.5,$
273 $p = 0.006$) concentrations were significantly higher in animals captured in reference areas than in
274 D-area turtles (Table 1). Two individuals from reference areas had high amounts of Cr in their
275 blood; however, even with these two observations excluded, the difference remained significant
276 ($U = 291.5, p = 0.017$).

277

278 *Trace element burdens (Claw)*

Arsenic, Cd, Cr, Cu, Se, and Sr were detected in 100% (n = 56) of claw samples. ANCOVA (Table 2) revealed that As ($F_{1,52} = 29.37, p < 0.001$), Cd ($F_{1,52} = 15.20, p < 0.001$), Cu ($F_{1,52} = 11.60, p = 0.001$), and Se ($F_{1,52} = 70.99, p < 0.001$), were significantly higher in D-area turtles compared to reference turtles. Neither Cr ($F_{1,52} = 2.30, p = 0.135$) nor Sr claw concentrations ($F_{1,52} = 1.91, p = 0.169$) were significantly different between the site types. Plastron length was a significant covariate in the As ($F_{1,52} = 4.90, R^2 = 0.37, p = 0.030$), Cd ($F_{1,52} = 18.21, R^2 = -0.45, p < 0.001$), and Cu ($F_{1,52} = 21.69, R^2 = -0.37, p < 0.001$) ANCOVA models. Claw Cd and Cu were negatively related to PL (Figures 2 - 3). Although claw As was found to be positively related to PL in turtles, turtles in the reference areas did not contain high amounts of As. Therefore, the significant positive relationship ($p = 0.006, r_s = 0.49$) between As and PL in D-area turtles was further examined via Spearman correlations (Figure 4).

Relationship between blood and claw trace element concentrations

Comparisons of blood and claw within individuals yielded significant relationships for As, Cr, Se, and Sr (Figure 5, n = 50). Selenium had the strongest relationship between blood and claw ($p < 0.001$), with 80% of variation in blood Se explained by claw Se. Arsenic values had a moderately positive relationship ($p < 0.001$), with 37% of variation in blood As explained by claw As. Strontium blood and claw concentrations ($p = 0.033$) had a weak positive relationship, but little variation in blood Sr could be explained by claw Sr. Chromium blood and claw concentrations ($p = 0.029$), exhibited a weak negative relationship; only 16% of blood Cr variation could be explained by claw Cr. It is important to note that upon further inspection the highest Cr claw value was an outlier. Once it was removed, the relationship was no longer

significant ($p = 0.066$), but results are still reported with the original data for readers to interpret both cases. Cd blood and claw could not be examined due to detectability issues.

Bacterial killing assays (BKAs)

Mean percent bacteria killed by D-area turtles (mean $\pm$ 1 SE; 0.91 ± 0.03) was significantly higher than by reference turtles (0.88 ± 0.03), based on Mann-Whitney U test ($U = 1077$, $p = 0.015$). Among D-area turtles, BKA response did not differ between adults and immature individuals ($U = 205$, $p = 0.683$); differences between males ($n = 10$) and females ($n = 8$) could not be investigated due to small sample sizes. Among reference turtles, BKA response did not differ by sex ($U = 157$, $p = 0.666$). Relationships between BKA response and individual trace element concentrations were not statistically evaluated due to the extreme nonnormality of the BKA data.

Phytohaemagglutinin (PHA) skin assays

PHA-treated toe webs had significantly higher swelling responses compared to PBS-treated toe webs ($t_{1,125} = 5.06$, $p = 0.019$), and this difference varied over time (interaction term: treatment group * time; $t_{1,125} = 3.06$, $p = 0.003$; see supplemental material), indicating that the assay was successful in producing a measurable swelling response. PHA responses (i.e., differences in swelling response between PHA- and PBS-injected sites 24 hours post injection) did not differ between D-area and reference turtles when compared using a linear mixed model with turtle ID as a random effect ($t_{1,41} = 0.578$, $p = 0.566$; Table 3). The same model suggested that PHA response did not vary with turtle size (i.e., PL; $t_{1,125} = 1.58$, $p = 0.116$). A second

model to investigate sex-related effects on adult *T. s. scripta* revealed no significant difference in swelling response between males and females ($t_{1,34} = -0.583, p = 0.564$). PHA response also did not vary as a function of claw trace element burdens for any trace element examined (all $p > 0.10$).

Hemogregarine parasitemia

Mean prevalence of hemogregarine infection was 51.85% for turtles at reference sites and 77.78% for D-area (Table 4). When combined across all sites, female *T. s. scripta* had the highest rate of prevalence (15/16) at 94%, followed by males (9/14) at 64%, and immature individuals at 20% (3/15). Overall mean prevalence of hemoparasites among all individuals at all sites sampled was 62.22%. Parasitemia levels were not significantly different between site types (ANCOVA; D-area = 0.030 ± 0.01 ; Reference = 0.043 ± 0.02 ; $F_{1,40} = 0.01, p = 0.98$), but PL was a significant covariate ($F_{1,40} = 21.87, p < 0.001$), with parasitemia increasing with increasing body size. Two turtles did not have PL recorded and were thus not included in the analysis.

Discussion

Concentrations of CCR trace elements As, Cu, Se, and Sr in blood of *T. s. scripta* collected from D-area were significantly elevated compared to those captured in reference wetlands. Our findings corroborate previous results from Nagle et al. (2001) documenting elevated levels of As, Cd, Cr, and Se in liver tissue of adult female *T. s. scripta* collected from D-area compared to reference sites, although their sample sizes were small (4 D-area turtles, 3 reference turtles). Few other published data are available for *T.s. scripta* collected from CCR-

contaminated sites, particularly for non-destructive tissues. However, mean blood As, Cu, and Se were slightly lower in our study (As, 0.07 ± 0.02 mg/kg; Cu, 0.46 ± 0.02 mg/kg; Se, 1.56 ± 0.43 mg/kg) relative to previous data for the same species (As, 0.14 ± 0.01 mg/kg; Cu, 0.52 ± 0.03 mg/kg; Se, 2.19 ± 0.05 mg/kg) from D-area (Tuberville et al., unpublished data). Blood Sr concentrations from this study were also similar (Sr, 0.52 ± 0.06 mg/kg) to previous data (Sr, 0.47 ± 0.01 mg/kg; Tuberville et al., unpublished data). The majority of D-area turtles captured in our study came from wetlands adjacent to the settling basins, and mean blood trace element concentrations of As, Cu, Se, and Sr were higher in animals caught in the primary settling basin. The wetlands adjacent to the settling basins historically did not serve as storage areas for CCR wastes, but were likely contaminated (e.g., through runoff or leaching) because of their close proximity to the settling basins. In addition, they are close enough in proximity (<100 m) to the settling basins that animals can move freely among them. Therefore, the slightly lower trace element values in this study are likely due to a large proportion of the D-area turtles being captured in adjacent wetlands and not the CCR settling basins, which were targeted in the previous study (Tuberville et al., unpublished data). Blood As, Cu, Se, and Sr values in D-area are higher than those reported from *T. s. scripta* living in Tennessee waterways that were heavily contaminated by a coal fly ash spill (Van Dyke et al. 2014). Interestingly, blood As levels in D-area turtles from adjacent wetlands were equal or slightly elevated compared to *T. s. scripta* exposed to the 2008 coal fly ash spill in Tennessee, which released an estimated 3.79×10^9 L of CCR-laden slurry when a retaining wall failed.

An interesting observation from our data is that contrary to expectations, blood Cr concentrations were significantly higher in reference turtles. Two reference turtles (an adult female and an immature individual) had the highest amounts of blood Cr (1.31 and 2.50 mg/kg,

respectively) in our dataset. The reference wetlands where these turtles were captured are within the dispersal range for D-area *T. scripta* (2.4 – 4.3 km). Given that trace element concentrations in blood are a short-term measure of contaminant exposure, it is possible that these individuals had recently migrated from one of the D-area wetlands prior to their capture.

We hypothesized that claw trace element concentrations, like blood values, would also be elevated in D-area turtles relative to reference turtles. Although few studies have quantified claw trace element concentrations in freshwater turtles (Van Dyke et al. 2013; Van Dyke et al. 2014; Steen et al. 2015), claw burdens in this study are among the highest reported in freshwater turtles. Mean claw As, Cd, Cu, and Se concentrations were significantly higher in turtles captured in D-area compared to reference turtles. Although not significant, claw Sr and Cr values were also elevated in D-area turtles compared to turtles from reference sites. Similar to patterns observed for blood, claw trace element values from D-area turtles in this study were much higher than those reported from turtles captured in an area affected by the Tennessee coal ash spill of 2008 (Van Dyke et al. 2014). In addition, likely to due to the longer contaminant history at our study site, the disparity in values between D-Area and the Tennessee site was greater for claw than for blood. In fact, mean concentrations of D-area *T. s. scripta* claw Se were five times higher than mean claw Se values reported for *T. s. scripta* collected near the Tennessee spill site (D-area, 6.65 mg/kg; TVA, ~ 1.25 mg/kg; Van Dyke et al. 2013). *Trachemys s. scripta* captured in D-area had mean claw Sr (47.56 ± 10.49 mg/kg) concentrations 200 times higher than *T. s. scripta* captured near the TVA coal ash spill (0.25 ± 0.04 mg/kg; Steen et al. 2015). Overall, our trace element data agreed with previous studies that found elevated trace element values in turtles that are residents of the D-area system (Nagle et al. 2001).

Claw As, Cd, and Cu concentrations were correlated with individual size (PL) in *T. s. scripta*. Arsenic was the only trace metal that had a positive relationship. Inorganic metals such as As are known to bind to highly keratinized tissues such as the claw tissues that we sampled (Faust et al. 2014), thus, it's possible that the larger, mature *T. s. scripta* simply had more time to accumulate As into their keratinized tissues. Smaller *T. s. scripta* in this study tended to accumulate larger amounts of Cd and Cu in claws relative to larger individuals. A similar pattern for blood Cd has been reported in the Amazonian red-necked turtle (*Podocnemis erythrocephala*; Burger et al. 2009), although no keratinized tissues were collected for comparison with our data. Previous studies suggest that younger, immature *T. s. scripta* consume a higher protein diet compared to adults, who tend to consume more plant matter (Hart 1983; Parmenter and Avery 1990). Indeed, Clark and Gibbons (1969) found that *T. s. scripta* with approximately 80mm PL contained up to 80% animal matter in their digestive tract, while all larger individuals (>80mm PL) sampled had 10% or less animal matter in their tract. Unrine et al. (2007) reported that odonate (*Tramea* and *Erythemis* spp.) and bullfrog (*Lithobates catesbeiana*) larvae sampled near D-area accumulated high amounts of As, Cd, Se, and Cu, and odonate and anuran species have been recorded in the diet of *T. s. scripta* (Parmenter and Avery 1990). Perhaps size-related differences in claw trace element burdens may be due to the ontogenetic diet shift that these turtles experience.

Recent studies of trace element correlations in freshwater turtles found that some trace elements (i.e., Hg and Se) were significantly and positively correlated in blood and claw tissues (Hopkins et al. 2013; Van Dyke et al. 2013). To further explore the validity of blood and claw tissues as non-destructive sampling methods, we investigated correlational relationships in trace element concentrations between the two sample types. Claw trace element concentrations have a

long turnover period (~ 12 months; Aresco 2005). In contrast, blood trace element values are often considered indicators of more recent exposure, although it is important to note that they also include mobilized stored contaminants. Overall, claw concentrations also tended to be higher than blood concentrations in the same individual, corroborating previous studies suggesting that blood and claw trace element concentrations reflect different time scales of exposure (Hopkins et al. 2012; Van Dyke et al. 2014). The generally strong, positive correlations between the two tissue types also support the use of both tissue types as non-destructive methods for monitoring specific trace element concentrations in freshwater turtles (Hopkins et al. 2012). However, more data is necessary (*e.g.*, toxicokinetics of trace elements in *T. s. scripta*) to understand the true relationships between these two tissue types in turtles.

As highlighted in previous studies, there is little information regarding thresholds of trace element concentrations in reptiles (Komoroske et al. 2012; Van Dyke et al. 2013). Thus, we sought to compare our Se data to unpublished exposure experiments in slider turtles and published avian thresholds. In our study, the highest blood Se concentrations in D-area *T. s. scripta* (> 9 mg/kg) were comparable to levels found in a Se exposure experiment that noted immunosuppressive effects and mortality due to chronic Se exposure (Haskins 2016). Because no threshold recommendations are available for reptile keratinized tissue, we chose to compare our Se claw data to recommended thresholds in keratinized bird feathers. Feather Se content in wild birds exceeding 5 mg/kg is considered high enough to warrant further study (Ohlendorf and Heinz 2011). However, it is important to consider that turnover rates may vary between bird feathers and turtles claws. Thus, although our data include Se claw levels high enough in some D-area *T. s. scripta* (range 0.49-39.24 mg/kg) to surpass the level of concern in avian feathers, the temporal differences in these tissue types may limit the utility of this comparison.

Contrary to our expectations, CCR exposure did not appear to negatively impact immune status based upon our ecoimmunological assays. We predicted that *T. s. scripta* captured in D-area would display weakened BKA responses due to CCR exposure; however, innate bacterial killing ability was significantly elevated in D-area *T. s. scripta*. In a study near the Tennessee spill, tree swallows (*Tachycineta bicolor*) sampled at reference and coal ash spill sites were found to have positively correlated Se burdens and BKA responses, and birds sampled downstream of the spill had slightly elevated BKA responses compared to other sites (Beck et al. 2014). Thioredoxin reductase, a selenoenzyme, is involved in the expression of genes associated with innate immune responses (Maggini et al 2007). Because *T. bicolor* blood Se values were correlated with BKA response, the authors hypothesized that increased Se may have increased expression of genes associated with the innate immune response. It is also important to consider that some of these trace elements (i.e., Cu and Se) are essential to vertebrates in small concentrations, thus it is possible that the levels found in our turtles enhanced their bacterial killing ability. Although our results illustrate that D-area turtles had a significantly higher bacterial killing ability than reference turtles, the biological consequences and the physiological conditions contributing to this difference require future study.

In regards to PHA assays, we predicted that *T. s. scripta* with higher trace element burdens would exhibit weaker PHA responses relative to individuals with lower trace element burdens. Although *T. s. scripta* from D-area had elevated trace elements in their blood and claws, site type had no effect on PHA swelling response. Furthermore, PHA swelling response in *T. s. scripta* was not correlated with any claw trace element concentrations. Similar to our results, Finger et al. (2016) found that significant accumulation of trace elements present in CCRs had no effect on PHA swelling response in American alligators (*Alligator mississippiensis*). However,

adult common eiders (*Somateria mollissima*) exposed to Se, a trace element found in high concentrations in coal ash, had weaker PHA responses relative to control birds (Franson et al. 2007). Our results also suggest that in *T. s. scripta* PHA response is not affected by plastron size. Similarly, Zimmerman et al. (2010) found that swelling response was not affected by plastron size in the closely related *T. s. elegans*. In contrast, however, PHA experiments with both saltwater crocodiles (*Crocodylus porosus*) and painted turtles (*Chrysemys picta*) found that PHA swelling response was positively associated with an individual's size (*i.e.*, head length and plastron length; Schwanz et al. 2011; Finger et al. 2013). Perhaps *Trachemys* spp. do not exhibit size-related differences to antigenic stimulation, although future studies are required to further elucidate this.

Contrary to our initial hypothesis, hemogregarine parasite loads did not yield significant differences based on site type. Interestingly, there was a significant positive covariation between hemogregarine parasitemia and PL. Similarly, in past studies, larger freshwater turtles (typically females) exhibited higher hemogregarine parasitemia values, likely due to their larger surface area (McCoy et al. 2007; Davis and Sterrett 2011; Schwanz et al. 2011). An interesting observation in the current study is that 3 out of 15 immature *T. s. scripta* (average PL = 67.3 ± 4.1) were infected with hemogregarines. To our knowledge, ours is the first reported instance of hemogregarine infection in immature *T. s. scripta*. Hemogregarines are thought to be most commonly transmitted to freshwater turtles by leeches (Davis and Sterrett 2011), which have been shown to be sensitive to contaminants such as mercury and atrazine (Suchanek et al. 1995; Brites and Rantin 2004), and are classified as moderately sensitive to contamination on the North Carolina Biotic Index (NCBI; Lenat 1993). It may be that leeches inhabiting D-area wetlands can tolerate environments with high levels of CCR contaminants. Previous observations of

hemogregarine prevalence in southeastern (Georgia, Kentucky, Louisiana, and Tennessee) freshwater turtles range from 45-100% and averaged approximately 75% prevalence (Davis and Sterrett 2011). Thus, our prevalence values (62.22%) are within the range of what has been reported in freshwater turtles.

Our results demonstrate that *T. s. scripta* exposed to CCRs can accumulate high amounts of trace elements, but their immune response is not negatively affected by CCR exposure. This study builds on previous work that shows elevated trace element contaminants in *T. s. scripta* inhabiting CCR-contaminated sites. Furthermore, our study includes a more robust sample size that encompasses a greater range of size classes. Our data also suggests that the size of the individual turtle can be correlated with accumulation of CCR-associated trace elements. Thus, future controlled studies should investigate the toxicological implications of acute and chronic trace element exposure in multiple age classes. Our work adds to a growing body of literature that suggests although reptiles can accumulate high levels of trace element contaminants, there is little evidence of sublethal impacts due to exposure. The reptilian immune system is affected by a wide variety of factors (*i.e.*, age, stress, temperature, season, gender; Keller et al. 2006), and as such, more comprehensive studies are necessary to fully explore the potential effects of CCR-associated contaminants on turtle immune function and host-parasite dynamics. Future studies of contaminant exposure in wild freshwater turtles should seek to incorporate long-term monitoring of hematological parameters, nutrient profiles, parasite loads, and comprehensive measurements of immunity, including the adaptive and humoral immune components.

Acknowledgements

We would like to thank Caitlin Kuper, Naya Eady, Jarad Cochran, Sam Dean, Nick Bossenbroek, Chris Murphy, and Kevin Fouts for their assistance in the field. We are indebted to

Bess Harris for her assistance in processing turtles and to Katrina Woods for performing digestions of blood samples. Dr. Richard Chandler and Dr. Michael Yabsley both provided important feedback for statistical analyses and parasite quantification. We would also like to thank Dr. John Seaman and Dr. Brian P. Jackson for their valuable assistance and interpretations regarding trace element analyses. Earlier versions of this manuscript were improved by comments from Dr. Travis Glenn and Dr. Terry Norton. The Dartmouth Trace Element Analysis Core is supported by the National Institute of Environmental Health Sciences under Award Number P42ES007373. This project was funded by the Department of Energy under award number DE-FC09-07SR22506 to the University of Georgia Research Foundation and by the Savannah River Nuclear Solutions – Area Completions Project.

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Table 1: Trace element concentrations in claw (mg/kg dw) and blood (mg/kg ww) from yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area and reference wetlands on the Savannah River Site, South Carolina. Values are reported as means $\pm$ 1 SE (ranges of raw values reported below respective means in parentheses). Significant differences between site types are marked with bold text. Samples were collected during the summers (May-August) of 2014 and 2015. Note that ‘Reference’ blood As and Se means are not reported in the table but analyses were performed using half of the minimum detection limit (MDL) for each sample.

Element	Blood		Claws	
	D-area	Reference	D-area	Reference
As	0.05 $\pm$ 0.02 (0.00 – 0.42)	<MDL ^a (0.00 – 0.02)	3.94 $\pm$ 1.06 (0.02 – 21.74)	0.06 $\pm$ 0.01 (0.00 – 0.30)
Cd	<MDL <MDL	<MDL <MDL	0.13 $\pm$ 0.02 (0.01 – 0.40)	0.04 $\pm$ 0.01 (0.00 – 0.12)
Cr	0.07 $\pm$ 0.02 (0.02 – 0.44)	0.23 $\pm$ 0.09 (0.02 – 2.50)	0.14 $\pm$ 0.02 (0.01 – 0.32)	0.10 $\pm$ 0.02 (0.02 – 0.50)
Cu	0.46 $\pm$ 0.02 (0.06 – 0.66)	0.33 $\pm$ 0.02 (0.01 – 0.59)	3.13 $\pm$ 0.56 (0.48 – 14.54)	1.45 $\pm$ 0.33 (0.41 – 8.37)
Se	1.43 $\pm$ 0.40 (0.08 – 9.57)	<MDL (0.08 – 0.45)	6.65 $\pm$ 1.59 (0.49 – 39.24)	0.30 $\pm$ 0.03 (0.13 – 0.70)
Sr	0.52 $\pm$ 0.06 (0.05 – 1.45)	0.22 $\pm$ 0.02 (0.01 – 0.51)	71.04 $\pm$ 0.89 (0.17 – 271.44)	47.56 $\pm$ 10.49 (0.11 – 245.40)
<i>n</i> (sample size)	35	28	30	25

^a < 50% of samples from this site type and sample type had detectable trace element concentrations and means could not be presented

Table 2: Results of analysis of covariance of the effects of site type (D-area vs. reference; n = 55) and plastron length (PL) on individual trace element concentrations in yellow-bellied slider (*Trachemys scripta scripta*) claws. Individual reference sites were grouped into one site type for analysis.

Element	Source	<i>df</i>	MS	<i>F</i>	<i>p</i>
As	Site	1	18.29	29.37	<0.001
	PL	1	3.052	4.90	0.030
	Error	52	0.623	-	-
Cd	Site	1	15.203	28.48	<0.001
	PL	1	9.717	18.21	<0.001
	Error	52	0.534	-	-
Cr	Site	1	2.149	2.30	0.135
	PL	1	2.478	2.65	0.109
	Error	52	0.934	-	-
Cu	Site	1	7.307	11.60	0.001
	PL	1	13.67	21.69	<0.001
	Error	52	0.630	-	-
Se	Site	1	31.00	70.99	<0.001
	PL	1	0.032	0.074	0.787
	Error	52	0.437	-	-
Sr	Site	1	1.907	1.948	0.333
	PL	1	0.936	0.956	0.169
	Error	52	0.979	-	-

Table 3: Two types of candidate models with varying correlation structure to explain differences in toe web swelling response between PHA- and PBS-injections in *Trachemys scripta scripta* (n = 44). The most parsimonious model for each correlation structure is indicated in bold. Model terms included treatment (PHA vs PBS; Grp), time of sampling (Time; 0 vs 24 hrs), Site (D-area vs reference), and plastron length (PL). Correlation structures (Corr.) types included ARMA 01 (AR 1), ARMA 02 (AR 2), and none designated (None). Model outputs presented are log likelihood (LL), model degrees of freedom (K), Akaike Information Criteria (AIC), the difference in AIC between given model and most parsimonious model (Δ AIC), and Akaike weights, which displays the weight of each model in the candidate set.

Model	Corr.	LL	K	AIC	Δ AIC	AICWt
Grp*Time + Site + PL	AR 1	94.62	9	-171.24	0	0.91
Grp*Time + Site + PL	None	91.28	8	-166.56	4.68	0.09
Sex + Grp*Time + Site + PL	AR 1	66.17	10	-112.34	0	0.63
Sex + Grp*Time + Site + PL	AR 2	66.50	11	-111.00	1.34	0.32
Sex + Grp*Time + Site + PL	None	62.73	9	-107.47	4.87	0.05

Table 4: Prevalence (proportion of individuals infected) and parasitemia levels (± 1 SE) of hemogregarines in yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area and reference areas on the Savannah River Site, South Carolina. Plastron length (PL) and parasitemia ranges are reported in parentheses below means. Parasitemia is equivalent to the percent of cells (of 8000 surveyed) infected by hemogregarine parasites.

Site type	<i>n</i>	No.		Prevalence (%)	Mean (range) parasitemia
		Infected	Mean (range) PL (mm)		
Reference	18	14	155 ^a	77.78	0.043 $\pm$ 0.02
	-	-	(62 – 234)	-	(0.000 – 0.413)
D-area	27	14	125	51.85	0.030 $\pm$ 0.01
	-	-	(42 – 226)	-	(0.000 – 0.163)
Total	45	28	136	62.22	0.035 $\pm$ 0.01

^a PL was not recorded for two turtles

Figure 1: D-area ash basins and their surrounding wetlands on the Savannah River Site in west-central South Carolina. Historically, effluent containing coal ash would flow into retention basins (R1 and R2), then as particulates settled, surface waters would move into primary and secondary ash basins. Letters ‘A’ and ‘B’ denote natural wetlands adjacent to the primary ash basin that were also sampled.

Figure 2: Relationship between plastron length (PL) and claw Cd values (analysis of covariance, $n = 55$; PL: $F_{1,52} = 18.21$, $R^2 = -0.45$, $p < 0.001$; Site: $F_{1,52} = 28.48$, $p < 0.001$) in yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area and reference sites on the Savannah River Site, South Carolina.

Figure 3: Relationship between plastron length (PL) and claw Cu values (analysis of covariance, $n = 55$; PL: $F_{1,52} = 21.69$, $R^2 = -0.37$, $p < 0.001$; Site: $F_{1,52} = 11.60$, $p = 0.001$) in yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area and reference sites on the Savannah River Site, South Carolina.

Figure 4: Spearman rank correlation between between log plastron length (PL) and log claw As values in yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area on the Savannah River Site, South Carolina.

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796 **Figure 5:** Spearman rank correlations ($n = 50$) between log blood and log claw trace element
 797 (As, Cr, Se, and Sr) concentrations in yellow-bellied sliders (*Trachemys scripta scripta*) captured
 798 on the Savannah River Site, South Carolina.

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