PHYSIOLOGICAL AND IMMUNOLOGICAL EFFECTS OF COAL COMBUSTION RESIDUES IN THE YELLOW-BELLIED SLIDER

by

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(Under the Direction of Tracey D. Tuberville and Robert B. Bringolf)

ABSTRACT

Freshwater turtles are at increased risk for extinction, and although they have been shown to accumulate large amounts of pollutants, little is known how contaminants affect their immune status and overall health. Coal combustion residues (CCRs) contain high amounts of potentially toxic trace elements (i.e. selenium) and are known to cause metabolic aberrations and histopathological abnormalities in some reptilian species. My research sought to ascertain if trace elements associated with CCRs negatively impact the health of the yellow-bellied slider (Trachemys scripta). We performed a field study to examine bioaccumulation of CCRs in wild T. scripta and quantified immune responses across site types. We also acutely exposed T. scripta to Se in a controlled lab study, and we measured Se accumulation, mortality, bactericidal capacity, hematological profiles, and metabolic rates. In the field study we found that wild T. scripta captured in CCR-affected wetlands did accumulate large amounts of CCRs, but did not exhibit diminished immune responses. In the lab study we found that T. scripta exposed to Se exhibit symptoms common in other vertebrates (mortality and altered hematological profiles). Overall, my results further our understanding of contaminant accumulation in a widespread chelonian species, and my results suggest that symptoms associated with Se toxicosis can occur in reptiles.

INDEX WORDS: turtle, bioaccumulation, coal combustion residues, immune system, selenium
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

In present ecosystems, anthropogenic activities have caused contaminants to be pervasive, especially in aquatic systems. Pollutants can enter aquatic systems through multiple pathways, including agricultural run-off, assorted mining methods, and man-made disasters (Dowd et al. 2008; Mishra et al. 2008; Young et al. 2010). Because of large-scale, industrial disasters recently occurring in the United States, pollution is again becoming an increasing public concern. Organisms that make contaminated environments their home are chronically subjected to pollutants, leading to unfavorable biological outcomes. Although anthropogenic contaminants are ubiquitous, pollution continues to be one of the lesser-studied man-made stressors affecting herpetological health and biodiversity in ecosystems (Grillitsch and Schiesari 2010). In particular, reptiles are a class of vertebrates widely underrepresented in ecotoxicological literature (Hopkins 2000; Grillitsch and Schiesari 2010). To help reduce this sizable knowledge gap in vertebrate ecotoxicology, my thesis work intended to explore the sublethal effects of coal combustion residues on chelonians.

Trace Elements and Coal Combustion Residues. In the past 40 years, global energy usage has increased by more than 100%, and it is dominated by the use of oil, gas, and coal resources (80% of energy consumption; Nejat et al. 2015). Furthermore, of these energy sources, coal combustion accounts for approximately 30% of all global energy production (British Petroleum 2015). Coal combustion comprised 40% of all of the United States’ net electricity
The coal combustion process produces copious amounts of coal combustion residues (CCRs; i.e., coal fly ash and bottom ash) that are either disposed in landfills or surface impoundments or are reused for construction (ACAA 2008; Ruhl et al. 2012). CCRs contain high concentrations of trace elements that are known to be toxic to wildlife as well as humans (Rowe et al. 2002; George et al. 2015). In the United States, over 130 million tons of CCRs are produced annually, and roughly 40% of CCRs are placed into surface impoundments for disposal (ACAA 2010).

A major concern of CCR disposal is the contamination of surrounding environments through the release of trace elements via leaching, improper discharge, or failure of storage systems (Izquierdo and Querol 2012; Ruhl et al. 2010). The trace elements that are commonly found in coal fly ash in high concentrations include: arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), selenium (Se), strontium (Sr), vanadium (V), and zinc (Zn) (Rowe et al. 2002). Although each has a complex geochemistry, groups of these elements tend to follow similar patterns in regards to leachability into the environment. The leachability of trace elements within CCRs largely depend upon the pH of the ash-water system. For example, the leachability of Cd, Cu, and Sr is diminished under alkaline conditions; however, alkaline conditions also amplify the release of oxyanionic species of elements including As, Cr, and Se (Izquierdo and Querol 2012). It is important to note that the pH of ash systems is not static and tends to move toward neutrality over time regardless of the initial state. Understanding how these elements disperse from surface impoundments, into the environment, and finally into biota is an important step in assessing risks of CCR exposure.

**Effects of Coal Combustion Residues on Wildlife.** Trace elements contaminants originating from CCR sources are known to cause deleterious effects on a variety of taxa.
Previous field and lab studies demonstrate that acute CCR exposure can be lethal in amphipods, shrimp, Odonates, fish, and amphibians (Magnuson et al. 1980; Guthrie and Cherry 1976; Rowe et al. 2002). Guthrie and Cherry’s (1976) outdoor mesocosm study placed shrimp, Odonate spp., crayfish, many fish species, and salamanders inside of field-cages at CCR-contaminated areas for 5 days. Shrimp, darters, and salamanders all expressed extreme sensitivities to CCRs, while catfish, crayfish, and largemouth bass had much lower mortality rates (Guthrie and Cherry 1976). Furthermore, subsequent CCR-exposure studies have shown that fish species that survive acute exposure may still have high mortality rates when chronically exposed (Hopkins 2001).

In many cases, acute or chronic CCR exposure may not cause direct mortality to the species of interest, but may result in sublethal effects. One of the most researched CCR disposal sites in the U.S., D-Area on the Savannah River Site (SRS; Aiken, SC), has provided researchers with evidence of these sublethal effects. One common response across taxa exposed to CCRs is the disruption of standard metabolic rates (SMRs), which can alter organismal behavior leading to modifications in energy expenditure, foraging needs, and predation risk (Hopkins et al. 1999). Grass shrimp (*Palaemonetes paludosus*), crayfish (*Procambarus acutus*), bullfrog larvae (*Rana catesbeiana*), and banded water snakes (*Nerodia fasciata*) all exhibited higher SMRs than their conspecifics who were not exposed to CCRs (Rowe 1998; Rowe et al. 2001; Rowe et al. 1998; Hopkins et al. 1999).

Increased SMRs in CCR-exposed wildlife has the potential to impact trade-offs related to the redistribution and reallocation of an animal’s nutrients. Alterations of energy allocation and distribution due to oxidative stress may lead to disruptions of other basic life processes that require large amounts of energy. Because mounting an efficient immune response is costly, diverting resources to deal with sublethal costs of CCR exposure could weaken the immune
system (Martin et al. 2010). Trace elements found in high concentrations within CCRs, specifically As and Se, are well-known for their potential to cause immunotoxic effects in fish and birds (Fairbrother and Fowles 1990; Fairbrother et al. 1994; Hermann and Kim 2005; Ghosh et al. 2006; Ghosh et al. 2007). However, studies that deal specifically with CCR exposure and its potential effects on the immune system are sparse. In Tennessee, researchers observed no differences in the immune responses of nestling tree swallows (Tachycineta bicolor) affected by a remediated CCR spill (Beck et al. 2014). Exploring how CCRs may affect vertebrate immunity could have global implications for wildlife populations residing within or close to CCR-affected areas.

**Selenium: A major CCR contaminant of concern.** Coal combustion residues include many trace elements that potentially can move into the surrounding environment and accumulate in resident wildlife. Of these elements, selenium (Se) has received much attention due to its reproductive toxicity in egg-laying vertebrates and potential to cause mass mortality of fish populations (Young et al. 2010). Selenium is released into the environment through natural and anthropogenic sources. Seleniferous deposits from ancient marine basins are a global source of selenium release (Presser et al. 2004). However, there are many sources of anthropogenic release including mining, the coal combustion process, atmospheric deposits, electronic wastes, and even agricultural runoff (Rowe et al. 2002; Wen and Carignan 2007; Young et al. 2010; Seiler et al. 2003). Agricultural runoff becomes a problem in regions where seleniferous soils are common. Irrigation of these Se-laden sediments can mobilize Se (in the form of selenate) and lead to widespread deleterious effects to an ecosystem (i.e., the Kesterson Reservoir disaster; Ohlendorf 1989).
When inorganic Se is released into the environment it is readily transformed by primary producers (i.e., bacterioplankton and phytoplankton) into an organic form (i.e., selenomethionine). Selenomethionine (SeMet) is more bioavailable and can accumulate within wildlife based upon their diet, and oviparous vertebrates tend to be the most sensitive to its toxicity (Chapman et al. 2009; Young et al. 2010). However, selenium’s effects are not limited to reproductive toxicity, but can also include histopathological abnormalities, physical deformities, reduction in growth, and oxidative stress (Janz et al. 2010; Young et al. 2010).

While previous studies have quantified Se burdens in reptiles (Hopkins et al. 1999; Nagle et al. 2001; Roe et al. 2004), little is known about the sublethal effects of Se or SeMet on reptilian species. Hopkins et al. (2004) exposed brown house snakes (*Lamprophis fuliginosus*) to SeMet to observe the effects of trophic and maternal transfer. Similar studies were conducted on fence lizards (*Sceloporous occidentalis*) to ascertain if trophic transfer of SeMet could cause negative effects (Hopkins et al. 2005). Both studies reported no adverse effects of trophic or maternal transfer of SeMet on survival, growth, or body condition. It is important to note these reptiles may not have exhibited effects of Se outwardly, negative effects may be revealed in the form of tissue abnormalities or oxidative stress (Sorensen et al. 1984; Hopkins et al. 1999; Ganser et al. 2003). Understanding the potential of Se to cause lethal or sublethal effects in reptilian species is of utmost importance to the preservation of this threatened group.

**Pollutants and Parasitism.** Immune suppression in wildlife due to pollutants could also lead to increased susceptibility to certain pathogens and parasites (King et al. 2007; Kim et al. 2008; Martin et al. 2010). Weakened immune responses may facilitate increased parasitism, negatively affecting the host population (Kim et al. 2008).
**Haemogregarina** spp. are intraerythrocytic, protozoan parasites (Order Eucoccidiorida, suborder Adeleorina) and are commonly found in reptilian species around the globe (Telford 2009). Although these blood parasites do not typically cause clinical signs of infections, they may cause abnormalities within the turtles’ blood profiles such as anemia (Knotkova et al. 2005). Although these parasites are common, little is known of how they may affect their host’s immune function (Davis et al. 2011). *Haemogregarina* spp. are transferred to intermediate hosts such as chelonians via leeches (*Placobdella* spp; Davies & Johnston 2001).

Previous research suggests that pollutants present in CCRs can affect the abundance and distribution of invertebrate species like leeches. Suchanek et al. (1995) observed that *Placobdella* spp. experienced a significant decline in abundance with increasing sediment concentrations of Hg. In Brazil, *Placobdella bistriata* was absent in atrazine-contaminated portions of the Uberabinha River (Brites and Rantin 2004). The North Carolina Biotic Index (NCBI) characterizes *Placobdella parasitica*, a common parasite of freshwater turtles, as moderately sensitive to contaminants (Lenat 1993). These values are calculated by sampling the species present in the environment and relating presence to distribution of pollutants. If leech species are sensitive to CCRs, this could mean that reptiles that inhabit CCR-affected may have a decreased chance of becoming an intermediate host for parasites (i.e., hemogregarines, trypanosomes, etc.). Furthermore, if leeches are not affected but the immune system of the intermediate host is weakened, this could lead higher parasite loads and subsequent health issues.

**Study species.** The yellow-bellied slider (*Trachemys scripta scripta*) is a subspecies of pond slider belonging to the family Emydidae. The *T. scripta* complex includes three subspecies (*Trachemys scripta scripta, Trachemys scripta elegans, Trachemys scripta troostii*) that are all native to North America. In addition, the Red-eared slider (*Trachemys scripta elegans*), is
commonly utilized as a study organism (Burger et al. 1998, Zimmerman et al. 2010, Yu et al. 2011, Zimmerman et al. 2012). Sliders are semiaquatic turtles that can regularly be found in wetlands with plentiful aquatic vegetation, basking structures, and nearby cleared areas sufficient for nesting (Gibbons 1990a, Spotila et al. 1990). Sliders are opportunistic omnivores and are known to consume a plethora of prey such as vertebrates, invertebrates, and plants (Parmenter & Avery 1990). Juvenile sliders consume a diet higher in protein (carnivorous diet) compared to adults who transition to a more omnivorous diet. Sliders are common throughout the United States and can constitute the highest amount of biomass in some chelonian communities (Congdon et al. 1986). Sliders exhibit behavior (i.e., fidelity to a small home range, opportunistic carnivorous feeding) that puts them at risk for contaminant exposure, and sliders also commonly inhabit bodies of water that could subject them to chronic contaminant exposure (Gibbons 1990b). On the Savannah River Site, previous research has measured CCR concentrations in _T. scripta_, but the sample sizes were small and sublethal effects were not the focus of the study (Nagle et al. 2001).

**Turtles as Environmental Sentinels.** Freshwater turtles in the Southeast represent a large amount of biomass in their respective ecosystems, and they can be exposed to these contaminants through several routes (e.g., dietary, dermal, maternal transfer) (Congdon et al. 1986, Nagle et al. 2001, Ernst & Lovich 2009, Van Dyke et al. 2014). Furthermore, these turtles occupy the middle to upper-level positions in the food chain, have long lifespans, and many have small home ranges (Gibbons 1990a, Ernst & Lovich 2009). These life-history characteristics make freshwater turtle species excellent environmental sentinels (Rowe et al. 2008; Steen et al. 2015). Contaminants may be playing a significant role in the decreasing turtle populations and
more research is needed to understand lethal and sublethal effects of contaminant exposure (Gibbons et al. 2000; Yu et al. 2013).

**Turtles, Pollutants, and Humans.** The accumulation of pollutants by freshwater turtle species puts them at risk of developing health issues; however, another large concern of exposure is humans that consume them (Green et al. 2010). Many contaminants found within CCRs (i.e., As, Hg, and Se) readily accumulate within turtle species and can be detrimental to human health (Nagle et al. 2001; George et al. 2015). Although trace elements within CCRs may pose a human health risk (George et al. 2015), CCR wastes in the United States are not classified as hazardous waste (US EPA 2015).

As mentioned previously, a large amount of CCRs are produced in the coal combustion process and are typically disposed of in surface impoundments (aquatic basins) or landfills. According to Ruhl et al. (2012), more than 1000 aquatic coal ash depositories exist in the United States. Aquatic settling basins used to store CCRs typically produce habitats that are inviting to various species of wildlife (Rowe et al. 2002; Bryan et al. 2011). For example, on the SRS, freshwater turtle species are common inhabitants of aquatic settling basins contaminated with CCRs (Nagle et al. 2001; Haskins personal observation). Indeed, if these animals spend considerable amounts of time within these basins, they are likely to accumulate large amounts of harmful trace elements.

Turtles are a common source of food in many parts of the world, and historically were a popular food item in the United States. Globally, more than 10 million turtles are purchased annually for consumption and these trade systems are not regulated by any government agency (Van Dijk et al. 2000; Green et al. 2010). If trends of global energy consumption through coal combustion continue, it is increasingly possible that individuals that consume turtle will be at
risk for exposure to CCRs. According to the US EPA (2003), only four states have issued consumption advisories for freshwater turtles: Arizona, Massachusetts, Minnesota, and New York. Hopkins (2012) found consumption advisories for snapping turtle consumption in Pennsylvania and Ohio. Risks of human exposure to contaminants through turtle consumption will undoubtedly be exacerbated in areas where consumers lack access to proper consumption guidelines and public education.

**Evaluation of the Physiological and Immunological Effects of Coal Combustion Residues and Selenium in Chelonians.** Overall, my MS thesis research had two overarching goals. My first chapter aimed to investigate the accumulation and sublethal effects of chronic exposure to CCRs in wild-caught yellow-bellied sliders (*Trachemys scripta scripta*). This was accomplished through non-destructive tissue sampling (blood and claws), ecoimmunological assays, and parasite quantification. I hypothesized that turtles from the CCR-affected site would exhibit higher CCR burdens, impaired immunological responses, and lower parasite loads. My second chapter aimed to evaluate the lethal and sublethal impacts of acute selenium (Se) exposure on *T. s. scripta* via a laboratory experiment. I predicted that turtles experimentally exposed to higher doses of Se would exhibit altered hematological profiles (indicative of immunosuppression), weaker immune responses, and depressed metabolic rates.


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CHAPTER 2

ACCUMULATION AND EFFECTS OF COAL COMBUSTION RESIDUES IN THE
YELLOW-BELLIED SLIDER

Introduction

Turtles are a group of reptiles whose life history traits make them particularly useful as environmental sentinels (Meyers-Schöne and Walton 1994; Aguirre and Lutz 2004; Van Dyke et al. 2013). Many turtle species are of high trophic status, are long-lived, typically have small home ranges, and can represent a considerable portion of biomass within their respective ecosystems (Rowe et al. 2008; Steen et al. 2015; Congdon et al. 1986). Furthermore, because they inhabit a variety of environments, turtle species can serve as indicators of contamination in terrestrial, freshwater, and marine environments. For instance, previous studies have documented box turtles (*Terrapene carolina*), slider turtles (*Trachemys scripta*), and sea turtles (*Chelonia mydas*) with high amounts of pollutants (Beresford et al. 1981; Yu et al. 2011; Faust et al. 2014) compared to what would be expected in more pristine reference environments.

Although many previous studies have sought to determine the usefulness of reptiles as bioindicators (Beresford et al. 1981; Yu et al. 2011; Faust et al. 2014), relatively few have attempted to explore the sublethal effects of contaminants in this threatened group (Grillitsch and Schiesari 2010). Indeed, chronic sublethal exposure can be just as harmful as lethal exposure to an individual’s overall fitness and survival (Newman 2014). This is especially concerning due to the current status of many reptilian species around the globe. For example, the 2013 IUCN Red List states that of 40% of all turtle species (134/335 species) have some form of conservation
status (vulnerable, endangered, critically endangered; Turtle Taxonomy Working Group 2014). Turtles are known to accumulate contaminants through diet and maternal transfer (Rowe et al. 2008; Van Dyke et al. 2014). Even a modest increase in adult mortality can have impacts on freshwater turtle populations; thus, the possible effects of pollutants and other anthropogenic stressors (i.e., habitat alteration, poaching) are troubling for freshwater turtle populations (Harden et al. 2009). Therefore, it is of utmost importance that future research seeks to understand the toxicological effects of pollutants on turtle species.

Previous work has documented sublethal effects of pollutants on turtles. In marine species, it has been suggested that organic pollutants (i.e., organochlorine pesticides, polychlorinated biphenyls) can negatively impact sea turtle kidney and salt glands, metabolic status, and hematological parameters (Camacho et al. 2013). Research on declining populations of the western pond turtle (Emys mamorata) in California revealed that mercury exposure can alter plasma thyroid hormones (Meyer et al. 2014). Nagle et al. (2001) measured coal combustion residues (CCRs) in yellow-bellied sliders (Trachemys scripta) and their eggs originating from a coal disposal facility on the Savannah River Site (D-area on the SRS; Aiken, SC). Turtles from D-area contained elevated CCR burdens and they also maternally transferred selenium (Se; a CCR trace element of concern) to eggs and hatchlings. The hatchling T. scripta of contaminated mothers in this study also exhibited lower standard metabolic rates compared to conspecifics from reference mothers.

Coal combustion residues (CCRs) are common waste products around the globe, are produced in large quantities, and contain high levels of potentially toxic trace elements (Rowe et al. 2002). The United States now produces over 130 million tons of CCRs per year, and prior to the 1980, approximately 66% of CCRs were placed into aquatic settling basins (Rowe et al.
2002). As of the 2010, 40% of these wastes were still placed into aquatic settling basins (ACAA 2010). These aquatic basins typically are attractive to many species of wildlife. Wildlife may use these basins to forage and reproduce, placing them at risk of exposure to potentially toxic levels of trace elements (Bryan et al. 2012; Lemly and Skorupa 2012).

In spite of the recent advances of reptilian ecotoxicology, it is relatively unknown what sublethal effects can be caused by chronic exposure to trace element contaminants. Thus, this study explored the sublethal effects of trace elements on freshwater turtles. The D-area on the SRS, is one of the most studied coal ash disposal facilities in the United States (Rowe et al. 2002). Previous research at D-area demonstrated that exposure to trace element contaminants of CCR origin can cause lethal and sublethal effects in biota (Guthrie and Cherry 1976), including metabolic aberrations in exposed individuals (Rowe 1998; Hopkins et al. 1999; Rowe et al. 2001). Trace elements in CCRs such as arsenic (As) and Se are known to cause immunotoxic effects in other vertebrates (Ghosh et al. 2006; Fairbrother et al. 1994). Because the immune system uses copious amounts of energy, disruption of metabolic processes and energy allocation due to CCR exposure could lead to compromised immune responses or the 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 impact 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). The reptilian immune system exhibits many similarities to the immune system of endotherms. Reptiles have all three branches of immunity that are present in mammals including: innate, cell-mediated, and humoral immune responses (Keller et al. 2006). 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), this current study sought to investigate the potential effects of CCR trace elements on the innate immune responses of *T. scripta*. The overall goal of this study was to examine the immunological costs of exposure to trace elements associated with coal combustion in wild yellow-bellied sliders (*Trachemys scripta*). Our specific objectives were to: (1) quantify the accumulation of trace elements (As, Cd, Cr, Cu, Se, Sr) associated with CCRs in tissues corresponding to different time scales (blood and claw); (2) compare innate immunological responses of sliders inhabiting CCR-affected wetlands and reference wetlands; and (3) determine if there are differences in parasite burdens based on capture location.

**Methods**

*Study site*

Our study was conducted on the Savannah River Site (SRS) in west-central, South Carolina, USA. The SRS is an 800-km² Department of Energy facility located in the southeastern Coastal Plain and supports a variety of wetland types, including isolated wetlands (Carolina bays), farm ponds, streams, and bottomland hardwood swamps. A small proportion of these wetlands have been impacted by site operations (*e.g.*, the production of nuclear materials, power production), resulting in wetlands with varying contaminant histories. One of the major industrial sources of contaminants on the SRS and globally is CCRs (Nagle et al. 2001; Lemly and Skorupa 2012). D-area is one of several coal combustion facilities on the SRS and it is one of the most studied coal disposal sites in the United States (Rowe et al. 2002). When D-area was actively burning coal, CCRs were placed into surface impoundments (aquatic settling basins). Coal fly ash would be discharged from the power plant into receiving basins, then into large primary and secondary
basins (Figure 2.1), with particulates separating and settling out as water moved through the system. Because they offer a permanent water source and become vegetated over time, the basins can become attractive nuisances (Bryan et al. 2012). Impoundments often attract wildlife because the habitat offered (i.e., vegetated edges, ample water sources) provides resources necessary for reproduction and overall survival (Rowe et al. 2002). Turtles and other wildlife have been known to inhabit coal ash disposal areas and surrounding wetlands (Nagle et al. 2001; Bryan et al. 2012). D-area has several natural wetlands that are within close proximity (< 0.1 km) of the settling basins. These natural wetlands may receive run-off from the settling basins and are frequented by animals that may use the settling basins. Thus, for our study D-area animals were collected from the primary settling basin and from wetlands ‘A’ and ‘B’ (see Figure 2.1). Reference animals were collected from 10 natural wetlands (encompassing 6 aquatic systems) on the SRS that have not historically received CCR-effluents and that were 2.4 - 24 km away from D-area.

**Study species**

The yellow-bellied slider (*Trachemys scripta scripta*), is considered the most abundant freshwater turtle species at SRS (an estimated 61 individuals/ha of aquatic habitat) and accounts for almost 50% of all freshwater turtle biomass on the site (Congdon et al. 1986). *Trachemys scripta* is common throughout southeastern United States and is considered to be a habitat generalist (Gibbons 1990). Sliders tend to have small home ranges (although they may move long distances over their lifetime; Morreale et al. 1984), high site fidelity, are long-lived (20-30 years in the wild), and feed in middle to high trophic levels (Gibbons 1990; Tucker 2001; Parmenter and Avery 1990). These life history characteristics and their wide geographic
distribution (North America to South America; Gibbons 1990) make them an excellent receptor species for contaminant studies. Sliders also experience an ontogenetic diet shift – juveniles tend to be carnivorous but become more omnivorous as adults (Parmenter & Avery 1990). These diet shifts may impact dietary exposure and accumulation of contaminants in juvenile sliders relative to adults. Previous research on the SRS documented accumulation of CCR trace elements and maternal transfer of Se in *T. scripta* (Nagle et al. 2001).

**Field sampling and sample collection**

*Trachemys scripta* were captured at D-Area wetlands and reference wetlands during May-September 2014 and May-July 2015 with hoop nets baited with sardines or creamed corn. 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 was collected for each individual. Each turtle was individually marked with a permanent ID by notching or drilling unique combinations of scutes to ensure that individuals were only sampled once per trapping season for analysis (Cagle 1939; Gibbons 1990b). Whole blood and claws 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 venipuncture site with a 27 or 25-gauge needle (Hernandez-Divers et al. 2002). Needles used for blood collection were heparinized if needed. 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 with least 100 mm PL.

**Trace Element Analysis: Blood**

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

Claw trace element concentrations were measured via ICP-MS in one batch at the Trace Element Analysis Core of Dartmouth College. The claws were first washed to remove any foreign matter. For washing, claws were placed into individual 7-mL polyethylene vials, 2 mL of acetone were added, and the mixture was placed into an ultrasonic bath for 20 minutes. Claws were then washed with 2 mL of 1% Triton X-100 in an ultrasonic bath for 20 minutes, washed 5 more times with deionized water, and the vial was then placed into a clean box to dry. Once claw samples were dry, claws were placed into a pre-weighed trace metal clean 15-mL polypropylene centrifuge tube (VWR, Radnor, PA) and 0.5 mL of 9:1 HNO₃:HCl (Optima Grade, Fisher Scientific) was added. Weights of claws were recorded on a 4 place balance to the nearest 0.1 mg. Samples were prepped for acid digestion along with certified reference materials, digestion blanks and fortified blanks for quality control. Tubes were then placed into the digestion rack, lightly capped, and set aside for approximately 3 hours. The digestion rack was then placed into a CEM MARS6 Express (Matthews, NC) microwave digestion system for digestions. To monitor the temperature of the MARS unit, a fiber optic probe was placed into a sample tube and the samples were heated to 95°C (ramp to temperature was 15 minutes) and held at this temperature for 45 minutes. Once samples were cooled, 0.1 mL of H₂O₂ (Optima Grade, Fisher Scientific) was added to samples and the samples were put through a second heating cycle. After cooling, the samples were brought to 10 mL with deionized water (Element QPod, Millipore, Billarica, MA) and all measurements were recorded gravimetrically. Digested samples were analyzed for As, Cd, Cr, Cu, Se, and Sr via collision cell ICPMS (7700x, Agilent, Santa Clara, CA). Selenium was measured in hydrogen mode with a flow rate of 6 mL/min. All other trace elements were analyzed in helium mode with a flow rate of ~ 4.5 mL/min. 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 to each 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 on a dry mass (mg/kg) basis.

**Bacteria-killing assays (BKAs)**

A bactericidal assay was used to assess the innate immunity of wild *T. scripta*. Bacterial killing assays are used commonly (birds, amphibians, and reptiles) in ecoimmunological research to quantify an animal’s innate immune response to a species of bacteria (Millet et al. 2007; French et al. 2010; Zimmerman et al. 2010; Brown et al. 2014). For each turtle, 7μL of plasma was mixed with a 10μL aliquot of approximately 400 colony-forming units (CFUs) of *Escherichia coli* (ATCC #8739; VWR, Radnor, PA) in phosphate buffered saline (PBS), and 137μL of CO₂ independent media containing 5% fetal bovine serum and 4mM L-glutamine. Once mixed, 50μL 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 allowed to incubate at room temperature for 60 minutes, after which we placed 50μL onto another agar plate (Time 60 plates). Plates were incubated overnight at 37°C (Zimmerman et al. 2012). The following morning, the number of bacterial colonies were counted for each agar plate. We calculated the percentage of bacterial killed by an individual’s plasma using the following formula: \((1 – [\text{Time 60 colony count / Time 0 colony count}]) * 100\).
**Phytohaemagglutinin (PHA) skin assays**

To further assess innate immunity, animals were subjected to an immunological challenge known as a PHA skin assay. 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). Unlike most studies, we sought only to measure the initial, innate (non-specific) immune response to the PHA injection. The first injection causes a proliferation of cells associated with the 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, 20μL 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 20μL of PBS. Peak swelling occurs in yellow-bellied sliders (*T. scripta*) at 24 hours (Jones and Finger, unpublished data). Thickness measurements (to the nearest 0.01 mm) were taken prior to injection (time 0) and at 24 hours using a dial thickness pressure gauge (Peacock G-1A, Ozaki Manufacturing Ltd, Japan). Three successive measurements were obtained for each foot and averaged for analysis. Measurements were taken quickly and by the same individual (AJ) across both years of data collection to minimize sampling errors.
**Parasites: Hemogregarine quantification**

Fixed blood smears were stained with Giesma 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 loads, we read an average of approximately 121 fields of view which contained an averaged ~ 73 ± 7 (mean ± 1 SE) erythrocytes for each smear (Haskins and Hamilton, unpublished data). 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 & Sterrett 2011). Parasitemia (the percentage of blood cells that are infected with hemogregarines) was calculated using the following equation: (# of parasites / 8000 * 100). Developmental stages of hemogregarines were not characterized.

**Statistics**

Due to the limited number of turtles collected at individual reference locations, 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 (Figure 2.1). 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. All claw trace element concentrations were ≥MDL.

All 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 statistic, respectively. In cases where data did not fit the normality assumption, data were log-transformed.
or rank-transformed (ANCOVA) prior to analysis. If data still did not fit the proper assumptions, nonparametric statistics were used. If correlations were tested (parametric methods) and outliers were suspected, Grubb’s test were used to locate outliers. 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 used ANCOVA to evaluate possible differences in hemogregarine parasitemia of turtles, and PL was used as a covariate. In regards to the description of hemogregarine prevalence, individuals were considered juveniles if they were < 100mm in PL.

PHA responses were analyzed by using a linear mixed model (LMM) repeated measures analysis with a serial correlation structure. Toe web swelling was the response variable within the LMM analysis 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).

Results

D-area vs. Reference populations

Over a 2 year period (2014-2015), a total of 81 T. scripta were collected from D-area and reference locations (D-area, n = 39; Reference, n = 42). Twenty-six individuals were identified as female, 27 were identified as male, and the remaining 28 were too immature to be sexed.
Average plastron size of females (n = 8) collected in D-area was 174.5 ± 17.7 mm (Mean ± SE; range of 102-227 mm), and average plastron size of males (n = 11) in D-area was 154.4 ± 10.9 mm (106-205 mm). Plastron size of juveniles (n = 20) captured in D-area averaged 66.6 ± 2.9 mm (42-88 mm). Average plastron size of reference females (n = 18) was 202.4 ± 9.7 mm (129-261 mm), males (n = 16) averaged 158.2 ± 8.5 mm (65-195 mm), and juveniles (n = 8) averaged 72.1 ± 3.8 mm (59-91 mm).

Trace element burdens (Blood)

Chromium, Cu, and Sr were detected in 100% of blood samples. Arsenic and Se were <MDL in 43% (27/63) and 37% (23/63) of blood samples, respectively. Cadmium was <MDL in 86% (54/63) of all blood samples. Arsenic (U = 657, p = 0.015), Cu (U = 779, p < 0.001), Se (U = 836, p < 0.001), and Sr (U = 858, p < 0.001) concentrations were all significantly higher in D-area turtles than in reference turtles. In contrast, Cr (U = 291.5, p = 0.006) concentrations were significantly higher in animals captured in reference areas than in D-area turtles (Table 2.1). Two individuals from reference areas had high amounts of Cr in their blood; however, even after these two observations were removed the comparison remained significant (U = 291.5, p = 0.017). Because the two outliers were captured at wetlands (Ellenton Bay and Risher Pond) ranging 2.4 - 4.3 km from D-area, it is possible that these individuals had spent time at D-area (or another contaminated area) prior to moving to their capture location.

Trace element burdens (Claw)

Arsenic, Cd, Cr, Cu, Se, and Sr were detected in 100% (n = 56) of claw samples. ANCOVAs were used to test differences in claw trace element values between site types using
PL as a covariate (Table 2.2). Arsenic ($F_{1,52} = 29.37, p < 0.001$), 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, while 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 sites. 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. Cadmium and Cu were negatively related to PL, whereas As concentrations were positively related to PL (Figures 2.2-2.4).

**Relationship between blood and claw trace element concentrations**

To explore the relationship between turtle blood and claw values we tested these data with Spearman’s correlation statistic. We only included in analyses individuals for which trace element concentrations for a given element >MDL for both blood and claw. Thus, due to detectability issues, Cd blood and claw could not be examined and As and Se sample sizes were reduced. Comparisons of blood and claw As, Cr, Se, and Sr yielded significant relationships (Figure 2.5). Arsenic trace element values had a moderately positive relationship ($n = 24, \rho = 0.52, p = 0.009$), with 27% of variation in blood [As] explained by claw [As]. Similarly, Sr blood and claw concentrations ($n = 51, \rho = 0.30, p = 0.034$) had a moderately positive relationship. However, only 9% of variation in blood [Sr] could be explained by claw [Sr]. Chromium blood and claw concentrations ($n = 51, \rho = -0.29, p = 0.042$), exhibited a negative relationship and only 8% of blood [Cr] variation could be explained by claw [Cr]. Selenium blood and claw values ($n = 24, \rho = 0.87, p < 0.001$) exhibited a strong positive relationship, with 75% of variation in blood [Se] explained by claw [Se].
**Bacterial killing assays (BKAs)**

Mean percent bacteria killed by D-area turtles (0.91 ± 0.03) was significantly higher than reference turtles (0.88 ± 0.03; Figure 2.6), based on Mann-Whitney U test \( (U = 1077, \ p = 0.015) \). Differences within site types were also explored to examine possible trends based on sex and age class of turtles. In D-area turtles, BKA response did not differ by age class (adults versus juveniles; \( U = 205, \ p = 0.683 \)); differences in BKA response by sex could not be investigated due to low sample sizes (10 males, 8 females). In reference turtles, BKA response did not differ by sex \( (U = 157, \ p = 0.666) \). Relationships between BKA response and trace element burdens could not be statistically evaluated due to the extreme nonnormality of the BKA data.

**Phytohaemagglutinin (PHA) skin assays**

Originally we ran three separate models to interpret PHA response between site types, with each model containing a different ARMA (autoregressive moving average) serial correlation structure. Another LMM was created to explore if PHA responses were impacted by sex and the same parameters as the previous model. The same model selection methods were used for the second LMM. Linear mixed models for PHA response are reported in Table 2.3. 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; \( t_{1,125} = 3.06, \ p = 0.003 \); Figure 2.7), 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) did not differ between D-area and reference turtles when compared using a linear mixed model with turtle ID as a random effect \( (t_{1,41} = 0.578, \ p = 0.566) \). The same model suggested that PHA response did not vary with turtle size (i.e., PL; \( t_{1,125} = 1.58, \ p = 0.125 \).
A second model to investigate sex-related effects on adult *T. scripta* revealed no significant difference in swelling response between males and females (*t*_{1.34} = -0.583, *p* = 0.564). PHA response 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% and 77.78% for D-area and reference turtles, respectively (Table 2.4). When combined across all sites, female *T. scripta* had the highest rate of prevalence (15/16) at 94%, followed by males (9/14) at 64%, and immatures at 20% (3/15). Overall prevalence of hemoparasites among all individuals at all sites sampled was 62.22%. Parasitemia levels were not significantly different between sites based on an ANCOVA (D-area = 0.030 ± 0.01; Reference = 0.043 ± 0.02; *F*_{1,40} = 0.01, *p* = 0.98). However, PL was a significant covariate in the model (*F*_{1,40} = 21.87, *p* < 0.001; Figure 2.8), with parasitemia increasing with increasing body size. Two observations were not included in the ANCOVA analysis because we did not have plastron length for these individuals. In addition, parasitemia was positively correlated with log-transformed PHA responses (*r* = 0.51, *p* = 0.003). Grubb’s tests detected four outliers (*G* = 4.73, *p* = 0.04; *G* = 3.55, *p* < 0.001). After removing outliers, parasitemia levels were still positively correlated with PHA responses (*r* = 0.8, *p* < 0.001; Figure 2.9).

**Discussion**

Blood trace element concentrations of As, Cu, Se, and Sr in *T. scripta* collected from D-area were significantly elevated compared to those captured in reference wetlands. An
interesting observation from our data is that contrary to our hypothesis, 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 wetlands of origin for these turtles are within the dispersal range for D-area *T. scripta* (2.4 – 4.3 km), and trace element analysis of blood reflects a short-term measure of contaminant exposure. Therefore, it is possible that these individuals had migrated from contaminated sites such D-area prior to their capture. Other blood trace element values (As, Cd, Cu, Se, and Sr) supported our hypothesis that D-area turtles would exhibit higher blood burdens of CCR-associated trace elements compared to reference turtles. Our blood trace element data is similar to recent historical data at the same site (Tuberville et al., unpublished data). 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 (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) compared 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. Therefore, the slightly lower trace element values in this study are likely due a large amount of turtles being captured in adjacent wetlands and not the settling basins. Blood As, Cu, Se, and Sr values in D-area are higher than those reported from *T. scripta* living in Tennessee waterways that were heavily impacted 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. scripta* exposed to the coal fly ash spill in Tennessee. The alkalinity of
ash basins influences mobility of trace elements, and As is one of the more mobile trace elements in CCRs (Izquierdo and Querol 2012).

We hypothesized that claw trace elements, 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, 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. As one might expect, likely due to length and severity of exposure, 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 Valley Authority’s (TVA) coal ash spill of 2008 (Van Dyke et al. 2014). In fact, mean concentrations of D-area T. scripta claw Se were five times higher than mean claw Se values reported for T. scripta collected near the TVA spill site (D-area, 6.65 mg/kg; TVA, ~ 1.25 mg/kg; Van Dyke et al. 2013). Trachemys scripta captured in D-area had mean claw Sr (47.56 ± 10.49 mg/kg) concentrations 200 times higher than T. scripta captured near the TVA coal ash spill (0.25 ± 0.04 mg/kg; Steen et al. 2015). Overall, these data confirmed our hypothesis that claw trace element values would be elevated in turtles that are residents of the D-area system.

Claw As, Cd, and Cu concentrations were influenced by individual size (PL). Although in some cases larger turtles were captured in reference areas, younger turtles in this study tended to accumulate larger amounts of trace elements in claws relative to adults. Previous studies suggest that younger, immature T. scripta consume a higher protein diet compared to adults, who tend to consume more plant matter (Clark and Gibbons 1969; Hart 1983; Parmenter and Avery 1990).
Unrine et al. (2007) reported that odonate larvae (Tramea and Erythemis spp.) and bullfrog (Lithobates catesbeiana) larvae that were sampled near D-area accumulated high amounts of As, Cd, Se, and Cu. Studies of T. scripta have previously recorded odonate and anuran species within their diets (Parmenter and Avery 1990). Perhaps size-related differences in claw trace element burdens can be attributed to the ontogenetic diet shift that these turtles experience. Little is known about the behavioral habits of juvenile freshwater species, and hatchling T. scripta probably exhibit little movement once they have entered the aquatic environment (Gibbons 1990). In contaminated habitats such as D-area, if juvenile T. scripta exhibit higher site fidelity than adults it could expose them to larger amounts of CCR trace elements.

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), while blood trace element values are great indicators of recent exposure. Of the six trace element relationships we examined, As, Cr, Se, and Sr claw concentrations were significantly and positively correlated with corresponding blood concentrations. Arsenic and Se (blood and claw) comparisons yielded the strongest relationships. Recent studies of trace element correlations in freshwater turtles found that some trace elements (Mercury, Se) were significantly and positively correlated in blood and claw tissues (B.C. Hopkins et al. 2013; Van Dyke et al. 2013). As highlighted in previous studies, there is little information regarding thresholds of trace element concentrations in reptilian species (Komoroske et al. 2012; Van Dyke et al. 2013).

Therefore, while it is difficult to relate our non-destructive data to toxicity benchmarks, our data does reveal exposure histories for wild T. scripta,
Although reptiles are experiencing global declines, and contaminants have been proposed as a factor of this decline (Gibbons et al. 2000), few studies have investigated the potential of contaminants to cause immunosuppressive effects in this taxa (Keller et al. 2006). We predicted that *T. scripta* captured in D-area would display weakened BKA responses; however, our results suggest that BKA responses in *T. scripta* were elevated in D-area. In studies near the TVA coal spill, tree swallows 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). Because swallow blood Se values were correlated with BKA response, authors hypothesized that increased Se may have increased gene expression of genes associated with the innate immune response. In regards to our turtles, one must also consider the microbial community of the ash basins when interpreting these results. Previous data from coal ash basins on the SRS found that ash samples contained higher estimates of bacteria relative to control samples, and that microorganism populations increase as the ash basins age (Klubek et al. 1992). D-area’s coal ash basins are over 60 years old, which suggests that microbial populations at D-area could be more robust than those at reference sites. Overall, BKA responses in our turtles were similar between D-area and reference site types (0.91 ± 0.03 and 0.88 ± 0.03, respectively). Our analysis suggested a significant difference between site types but any biological consequences of such a small difference is difficult to interpret.

Assays that measure cell-mediated immune responses, like PHAs, can quantify components of both the innate and adaptive immune responses in vertebrates (Martin et al. 2006; Demas et al. 2011). In our current study, as expected, the administration of PHA in *T. scripta* caused a significantly greater increase in toe web swelling than PBS, and this difference varied
significantly over time. We predicted that *T. scripta* with high trace element burdens would exhibit weaker PHA responses relative to individuals with lower trace element burdens. Although *T. scripta* from D-area had elevated trace elements in their blood and claws, site type was not a significant factor in PHA swelling response. Furthermore, PHA swelling response was not correlated with any claw trace element concentrations in *T. scripta*. Our results also suggest that in *T. scripta* PHA response is not affected by plastron size. Studies of PHA responses in the western pond turtle (*Emys marmorata*) suggested that *E. marmorata* inhabiting a water pollution control plant had weaker PHA responses than individuals residing in reference habitats (Polo-Cavia et al. 2009). 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). PHA experiments in saltwater crocodiles (*Crocodylus porosus*) and painted turtles (*Chrysemys picta*) found that PHA swelling response significantly increases corresponding with an individual’s size (i.e., snout-vent length and plastron length; Schwanz et al. 2011; Finger et al. 2013). However, Zimmerman et al. (2010) found that swelling response was not affected by plastron size in *T.s. elegans*. Perhaps *Trachemys* spp. do not exhibit size-related differences to antigenic stimulation, although, more studies need to be performed before this is determined.

Contrary to our hypothesis, hemogregarine parasite loads did not yield significant differences based on site type. However, we did note a larger prevalence of infected individuals in reference sites. In 2015, we observed leeches (*Placobdella* spp.) on 9 *T. scripta* captured in D-area and we also found leeches on 5 *T. scripta* collected from reference sites. Our analyses did also find that hemogregarine parasitemia covaried significantly and positively with plastron length. An interesting observation in the current study is that 3/15 juvenile *T. scripta* (average PL
were infected with hemogregarines. To our knowledge, this study is the first reported instance of hemogregarine infection in immature *T. 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 moderately sensitive to contamination on the North Carolina Biotic Index (NCBI; Lenat 1993). Thus, it is intriguing that hemogregarines loads did not vary between reference site types and D-area. It may be that leeches inhabiting D-area wetlands can tolerate environments with high amounts of CCR contaminants. In past studies, larger freshwater turtles, typically females, had larger hemogregarine parasitemia values and this is likely due to their larger surface area (McCoy et al. 2007; Davis and Sterrett 2011; Schwanz et al. 2011). Previous observations of hemogregarine prevalence in southeastern (Georgia, Kentucky, Louisiana, and Tennessee) freshwater turtles range from 45-100% and average to 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.

Reptiles are threatened globally and little is known regarding how these species respond if chronically exposed to contaminants of anthropogenic origin. *Trachemys scripta* captured in D-area exhibited some of the highest claw trace element concentrations reported in freshwater turtle species. Arsenic and Se burdens in our study were positively correlated between blood and claw tissues, suggesting that in spite of the discrepancy between blood and claw tissue turnover, either may serve as an acceptable non-destructive sample for monitoring specific trace element burdens. Smaller *T. scripta* also seemed to accumulate higher amounts of claw Cd and Cu, while larger *T. scripta* tended to have elevated claw As concentrations. The observed relationship between plastron size and trace elements may have interesting implications. Immature *T. scripta*
in sites such as D-area may be exposed to preferred prey that contain high concentrations of toxic trace elements. Freshwater turtle species exhibit high juvenile mortality and are sensitive to anthropogenic-based adult mortality (i.e., vehicle collisions, habitat alterations; Gibbons 1990). Thus, future controlled studies should be used to investigate the toxicological implications of acute and chronic trace element exposure in multiple age classes. Furthermore, future work should also aim to discern the nature of relationships among trace element burdens and multiple tissue types.

The reptilian immune system is complex, is affected by a wide variety of factors (i.e., age, population dynamics, stress, temperature, season, gender; Keller et al. 2006), and more comprehensive studies are necessary to fully explore the potential effects of 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 further measurements of immunity (i.e., adaptive and humoral immunity) to complement innate immunity measures. Field and laboratory studies of CCR-exposure in *T. scripta* may allow future researchers to discern if they are affected by trace element concentrations observed in this study.
Literature Cited


Table 2.1: 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 areas on the Savannah River Site, South Carolina. Values are reported as means ± 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 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.

<table>
<thead>
<tr>
<th>Element</th>
<th>D-Area</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Claws</td>
<td>Blood</td>
</tr>
<tr>
<td>As</td>
<td>3.94 ± 1.06</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(0.02 – 21.74)</td>
<td>(0.00 – 0.42)</td>
</tr>
<tr>
<td>Cd</td>
<td>0.13 ± 0.02</td>
<td>&lt;MDL</td>
</tr>
<tr>
<td></td>
<td>(0.01 – 0.40)</td>
<td>&lt;MDL</td>
</tr>
<tr>
<td>Cr</td>
<td>0.14 ± 0.02</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(0.01 – 0.32)</td>
<td>(0.02 – 0.44)</td>
</tr>
<tr>
<td>Cu</td>
<td>3.13 ± 0.56</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(0.48 – 14.54)</td>
<td>(0.06 – 0.66)</td>
</tr>
<tr>
<td>Se</td>
<td>6.65 ± 1.59</td>
<td>1.56 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>(0.49 – 39.24)</td>
<td>(0.08 – 9.57)</td>
</tr>
<tr>
<td>Sr</td>
<td>71.04 ± 0.89</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>(0.17 – 271.44)</td>
<td>(0.05 – 1.45)</td>
</tr>
</tbody>
</table>

n (sample size) | 30 | 35 | 25 | 28
Table 2.2: Results of analysis of covariance of the effects of site type (D-area vs. reference) and plastron length (PL) on individual trace element concentrations in yellow-bellied slider (*Trachemys scripta scripta*) claws. Reference sites were grouped into one site type for analysis.

<table>
<thead>
<tr>
<th>Element</th>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>Site</td>
<td>1</td>
<td>18.29</td>
<td>29.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>1</td>
<td>3.052</td>
<td>4.90</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>52</td>
<td>0.623</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cd</td>
<td>Site</td>
<td>1</td>
<td>15.203</td>
<td>28.48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>1</td>
<td>9.717</td>
<td>18.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>52</td>
<td>0.534</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cr</td>
<td>Site</td>
<td>1</td>
<td>2.149</td>
<td>2.30</td>
<td>0.135</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>1</td>
<td>2.478</td>
<td>2.65</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>52</td>
<td>0.934</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cu</td>
<td>Site</td>
<td>1</td>
<td>7.307</td>
<td>11.60</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>1</td>
<td>13.67</td>
<td>21.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>52</td>
<td>0.630</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Se</td>
<td>Site</td>
<td>1</td>
<td>31.00</td>
<td>70.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>1</td>
<td>0.032</td>
<td>0.074</td>
<td>0.787</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>52</td>
<td>0.437</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sr</td>
<td>Site</td>
<td>1</td>
<td>1.907</td>
<td>1.948</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>1</td>
<td>0.936</td>
<td>0.956</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>52</td>
<td>0.979</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.3: Two types of candidate models with varying correlation structure to explain differences in toe web swelling response between PHA- and PBS-injections. 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) 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 ($\Delta$AIC), and Akaike weights, which displays the weight of each model in the candidate set.

<table>
<thead>
<tr>
<th>Model</th>
<th>Corr.</th>
<th>LL</th>
<th>K</th>
<th>AIC</th>
<th>$\Delta$AIC</th>
<th>AICWt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grp*Time + Site + PL</td>
<td>AR 1</td>
<td>94.62</td>
<td>9</td>
<td>-171.24</td>
<td>0</td>
<td>0.91</td>
</tr>
<tr>
<td>Grp*Time + Site + PL</td>
<td>None</td>
<td>91.28</td>
<td>8</td>
<td>-166.56</td>
<td>4.68</td>
<td>0.09</td>
</tr>
<tr>
<td>Sex + Grp*Time + Site + PL</td>
<td>AR 1</td>
<td>66.17</td>
<td>10</td>
<td>-112.34</td>
<td>0</td>
<td>0.63</td>
</tr>
<tr>
<td>Sex + Grp*Time + Site + PL</td>
<td>AR 2</td>
<td>66.50</td>
<td>11</td>
<td>-111.00</td>
<td>1.34</td>
<td>0.32</td>
</tr>
<tr>
<td>Sex + Grp*Time + Site + PL</td>
<td>None</td>
<td>62.73</td>
<td>9</td>
<td>-107.47</td>
<td>4.87</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 2.4: 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. For all blood smears, approximately 120 fields were observed to examine 8,000 cells per turtle at 1000x magnification. Plastron length and parasitemia ranges are reported below means in parenthesis. PL = plastron length, and parasitemia is equivalent to the percent of cells (of 8000) infected by hemogregarine parasites.

<table>
<thead>
<tr>
<th>Site type</th>
<th>n</th>
<th>No. Infected</th>
<th>Mean (range) PL (mm)</th>
<th>Prevalence (%)</th>
<th>Mean (range) parasitemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>18</td>
<td>14</td>
<td>155&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.78</td>
<td>0.043 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(62 – 234)</td>
<td></td>
<td>(0.000 – 0.413)</td>
</tr>
<tr>
<td>D-area</td>
<td>27</td>
<td>14</td>
<td>125</td>
<td>51.85</td>
<td>0.030 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(42 – 226)</td>
<td></td>
<td>(0.000 – 0.163)</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>28</td>
<td>136</td>
<td>62.22</td>
<td>0.035 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two of 18 observations did not have PL values due to sampling errors in 2014
Figure 2.1: 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 basins. Letters ‘A’ and ‘B’ denote wetlands adjacent to the primary basin that were also sampled.
**Figure 2.2:** Relationship between plastron length (PL) and rank-transformed Claw Cd values (analysis of covariance; 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 2.3: Relationship between plastron length (PL) and rank-transformed Claw Cu values (analysis of covariance; 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 2.4: Relationship between plastron length (PL) and rank-transformed Claw As values (analysis of covariance; PL: $F_{1,52} = 4.90, R^2 = 0.37, p = 0.030$; Site: $F_{1,52} = 29.37, p < 0.001$) in yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area and reference sites on the Savannah River Site, South Carolina.
Figure 2.5: Spearman rank correlations between log blood and log claw trace element (As, Cr, Se, and Sr) concentrations in yellow-bellied sliders (Trachemys scripta scripta). Sample sizes for As and Se (n = 24) were lower than Cr and Sr comparisons, and this was due to detectability issues.
Figure 2.6: Mean percentage of bacteria (Escherichia coli) killed (± 1 SE) by yellow-bellied slider turtles (Trachemys scripta, scripta) from D-area (n = 39) and reference sites (n = 42) on the Savannah River Site, South Carolina.
Figure 2.7: The effects of PHA injection (dashed black line, solid circles) and PBS injection (solid line, hollow circles) on mean (± SE) web swelling response (mm) in yellow-bellied sliders (Trachemys scripta) over 24 hours (n = 44).
Figure 2.8: Relationship between plastron length (PL) and rank-transformed parasitemia values (analysis of covariance; PL: $F_{1,40} = 21.87$, $R^2 = 0.32$, $p < 0.001$; Site: $F_{1,40} = 0.00$, $p = 0.987$) in yellow-bellied slider turtles (*Trachemys scripta scripta*) from D-area and reference sites on the Savannah River Site, South Carolina.
Figure 2.9: Correlation between log PHA response (stimulation index) and parasitemia values in yellow-bellied slider turtles (*Trachemys scripta scripta*) collected during 2014-2015 from the Savannah River Site, South Carolina (PHA responses; $p < 0.001$, $n = 15$).
CHAPTER 3
EFFECTS OF SELENIUM ON THE HEMATOLOGY, INNATE IMMUNITY, AND METABOLIC RATE OF YELLOW-BELLIED SLIDERS

Introduction

Selenium (Se) is a well-known trace element contaminant that also is an essential micronutrient in many vertebrate species (Young et al. 2010). For almost a century and a half (1817-1957) Se was considered to be only a toxic trace element (Wisniak 2000). Selenium is also intriguing because there is a narrow range between the element’s essentiality and toxicity (Janz et al. 2010). The essentiality of Se is best understood in mammalian species, where it is now known that Se is necessary for certain proteins and enzymes (e.g., glutathione peroxidases, thioredoxin reductases) to function properly (Bellinger et al. 2010). In mammals, specific selenoproteins provide protection from reactive oxygen species, assist in detoxification and Se transport, and play a key role in thyroid hormone metabolism (inactivation/activation of iodothyronine deiodinases) (Chapman et al. 2009; Bellinger et al. 2010; Janz et al. 2010).

Selenium in the environment can originate from both natural and anthropogenic sources. Global seleniferous deposits are associated with ancient marine basins (Presser et al. 2004). While natural weathering of these rock formations does slowly release Se, the movement of Se into the environment has been greatly accelerated by anthropogenic activities. Coal combustion, atmospheric deposition, various types of mining, agricultural runoff, and electronic wastes all contribute to Se contamination within the environment (Young et al. 2010). Selenium contamination of aquatic environments is of particular concern. When Se enters these
ecosystems, inorganic Se species are the most common form present (i.e., selenate, \( \text{SeO}_3^{2-} \) and selenite, \( \text{SeO}_4^{2-} \); Fan et al. 2002). However, the most common species of Se that accumulates within wildlife is the organic form, selenomethionine (SeMet), and its toxicity is well-documented in oviparous vertebrate species (Young et al. 2010). Selenium is readily transformed to SeMet and other organic forms (i.e., selenocysteine) by algae, bacteria, and plants, which are then transferred through the food web. Selenomethionine is the main organic form of Se in the environment and its toxicity to birds and fish is well-documented (Chapman et al. 2009).

Selenium can accumulate within vertebrates through trophic, maternal, and environmental exposure (Fan et al. 2002; Ohlendorf 2003; Young et al. 2010). One of the most sensitive endpoints of Se exposure in wildlife is reproductive toxicity in egg-laying vertebrates. For instance, the Se-contamination of Kesterson Reservoir (CA, USA) and other reservoirs in the USA provide classic examples of selenium’s deleterious effects on reproduction. At the Kesterson Reservoir, Se-enriched agricultural runoff and the subsequent accumulation of Se led to impaired reproduction of avian species (Ohlendorf et al. 1986a; Ohlendorf et al. 1986b). Selenium’s adverse effects are not limited to reproduction. Exposure to SeMet and sodium selenite can lead to impaired immune responses in mallards (Fairbrother and Fowles 1990). Oxidative stress is another sublethal effect that is commonly affiliated with Se exposure in vertebrates (Chapman et al 2009). The exact mechanism of SeMet toxicity in vertebrates is still unknown, however, it is hypothesized that toxicity originates from increased cellular oxidative stress and possibly improper protein substitutions (Janz et al. 2010).

Reptilian species whose life histories tie them to aquatic environments are vulnerable to Se contamination. Many reptiles, such as turtles and crocodilians, are at risk of Se exposure because they are long-lived and feed at upper trophic levels within their respective ecosystems.
(Rowe et al. 2008). The effects of Se on vertebrates have been well documented in birds and fish (Janz et al. 2010); however, field and laboratory studies of the effects of Se on reptiles are underrepresented (Young et al. 2010). Research conducted in aquatic settling basins (coal ash) on the Savannah River Site (SRS; Aiken, SC, USA) previously found that Se is maternally transferred in yellow-bellied sliders (Trachemys scripta; Nagle et al. 2001). Mothers from Se-affected areas accumulated significant amounts of Se and hatchlings of contaminated mothers exhibited lower metabolic rates than reference conspecifics. Another study in the same SRS aquatic settling basins found that water snakes captured with elevated Se burdens exhibited increased standard metabolic rates (Hopkins et al. 1999). Rich and Talent (2009) found that dietary exposure to Se in the leopard gecko (Eublepharis macularius) led to reduced growth and reduced food consumption. All other available laboratory studies suggest a lack of adverse effects for the banded water snake (Nerodia fasciata), brown house snake (Lamprophis fuliginosis), and western fence lizards (Sceloporus occidentalis) exposed to concentrations of dietary SeMet ranging 11-23 mg/kg Se (concentrations based on dry weight of prey provided; Hopkins et al. 2001; 2002; 2004; 2005). All endpoints assessed within these studies fell within normal ranges. However, in the Hopkins et al. (2002) study, further analyses of water snake tissues found histological abnormalities associated with contaminant exposure (Ganser et al. 2003).

The objective of the current study was to examine the effects of SeMet exposure in a freshwater turtle species, the yellow-bellied slider (Trachemys scripta scripta). We exposed juvenile T. scripta to SeMet via oral gavage for 6 weeks (5 total doses). We quantified mortality, Se accumulation in multiple tissue types (blood, kidney, liver, muscle), metabolic rates, and
immunologic status. To assess possible immunological effects of Se exposure, we measured bactericidal capacity and hematological profiles.

Methods

Study species

The yellow-bellied slider (Trachemys scripta scripta) is a subspecies of pond slider belonging to the family Emydidae. Sliders are common throughout the United States and, in habitats where they occur, can represent the largest proportion of turtle biomass relative to other species (Congdon et al. 1986). These turtles can regularly be found in a diversity of wetland types that have plentiful aquatic vegetation, basking structures, and adjacent open terrestrial habitats for nesting (Gibbons 1990a, Spotila et al. 1990). Sliders are opportunistic omnivores and are known to consume a plethora of aquatic prey, including plants, vertebrates, and invertebrates (Parmenter & Avery 1990). Adults tend to become more herbivorous, while juvenile sliders are known to consume a diet higher in protein. Sliders commonly inhabit contaminated bodies of water, and they exhibit behaviors (i.e., fidelity to a small home range, opportunistic carnivorous feeding) that may put them at an increased risk for contaminant exposure (Gibbons 1990b). In addition, both yellow-bellied sliders and their close relative, the red-eared slider (Trachemys scripta elegans), are commonly used in field studies and laboratory experiments (Burger et al. 1998, Zimmerman et al. 2010, Yu et al. 2011, Zimmerman et al. 2012), thus their ecology and physiology are well-characterized (Gibbons 1990b). This information about sliders allows researchers to readily place findings in context with known physiological processes.
**Husbandry and experimental protocol**

Seventy juvenile yellow-bellied slider (*Trachemys scripta scripta*; < 111 mm plastron length, PL) were obtained from Concordia Turtle Farms (Jonesville, LA) in September of 2014 and were weighed (to nearest 0.1 g), measured (to nearest 1.0 mm PL), and given a unique identification number by notching their marginal scutes (Cagle 1939). Each turtle was visually inspected and appeared healthy (i.e., no visible injuries or abnormalities). Prior to the initiation of the study, turtles were kept in the living streams tanks inside of the SREL’s Aquatic Animal Holding Facility (AAF). The AAF consists of a concrete block base building with semitransparent fiberglass upper walls and roof, which allows animals housed inside to be exposed to natural light (Hamilton et al. 2016). Each turtle was randomly assigned to one of six 270 x 56 x 57-cm living streams tanks (Frigid Units Inc., Toledo, OH, USA). Each living stream tank was provided two 38 x 38 cm concrete basking platforms, and had at least two 100-W bulbs that were suspended approximately 30 cm above the cement platforms. Basking lights were set on a light/dark cycle using automatic timers (0600-1800). Living streams tanks were drained and cleaned once per week, and turtles were fed Mazuri aquatic turtle chow (Mazuri, PMI Nutrition International LLC, Arden Hills, MN) 3 times per week and were fed ad libitum. Turtles were held in the living streams tanks under these conditions for 8 months to ensure acclimation to their housing facility and the dry food chow.

In May 2015, each turtle was randomly assigned to one of three selenium (Se) treatment groups: control (n=24), 15 mg/kg Se treatment (n=23), and 30 mg/kg treatment group (n=23). Turtles were individually housed in ~ 68 L totes (Sterilite Corporation, Clinton, SC) to prevent confounding interactions among individuals and to allow each individual to represent a true replicate. Turtles were distributed randomly among three separate stalls in the AAF. Stalls were
considered blocks to attempt to control for variation in environmental conditions among stalls. Turtles were given wood basking platforms that were anchored to the wall of each tote, and were maintained under ambient temperatures of 25-32°C. Each turtle tote was cleaned 3-4 times per week and water was replaced at each cleaning. Turtles were fed aquatic turtle chow 3 times per week and were fed ad libitum.

*Selenium exposure via oral gavage*

Originally, we aimed to expose turtles to Se for eight weeks, but due to mortalities in the highest treatment group, we terminated the study after the fifth week. Juvenile sliders were exposed to seleno-L-methionine (SeMet, Sigma Aldrich, St. Louis, MO) via oral gavage for a total of five weeks (~ 37 days). Seleno-L-methionine was used for this study because it is a naturally occurring form of selenium that has a high bioavailability within food webs (Fan et al. 2002). Selenium concentrations in our study also are within the range of Se concentrations reported for contaminated prey species that slider turtles might be expected to consume (Unrine et al. 2007). The SeMet stock solution was made by mixing deionized water with powdered SeMet to a concentration of 5000 mg/kg SeMet. Our stock solution was made to be highly concentrated to minimize the solution volume administered. A minute amount (<15µL) of green food coloring was also added to the mixture to ensure that regurgitation could be detected during and after the gavage procedure. The amount of SeMet given to each turtle was calculated based on their individual body weight (wet weight) and estimated weekly food consumption of young turtles (Equation 1; 4% of body weight per day, CCPA 2006).
Equation (1):

$$Dose (\mu L) = \frac{Turtle \ mass \ (g) \times 0.28 \times \text{[SeMet]} \times 5000 \ (mg/kg \ of \ stock \ solution)}{5000 \ (mg/kg \ of \ stock \ solution)}$$

* where [SeMet] = 15 or 30 mg/kg

Prior to the initiation of the experiment, turtles were weighed again to ensure that the dose they received was accurate. Turtles were then weighed every two weeks to ensure that the dose of SeMet accurately reflected their weight. Control turtles were gavaged with deionized water, and the total volume of water was equivalent to the dosing volumes that 15 mg/kg turtles received. Juvenile turtles were given a dose of SeMet weekly via oral gavage (Turner et al. 2011) using an 18-gauge crop feeding tube (Southpointe Surgical Supply Inc., Coral Springs, FL, USA).

**Metabolic rate**

A total of 8 slider turtles from each treatment group (n = 24) had their standard metabolic rate (SMR) analyzed using a flow-through respirometry system (Field Metabolic System, FMS; Sable Instruments, Las Vegas, NV). Metabolic data was collected after the last dose (5th application) of SeMet was given. Turtles were held without food for a week prior to sampling to ensure that they were post-absorptive.

To collect data, turtles were placed into Rubbermaid tupperware metabolic chambers (14 x 14 cm, 1.2 L), and were placed into a FMS metabolic cooler to minimize visual disturbances to animals during measurements. The FMS metabolic cooler provides dark conditions and is temperature-controlled by a programmable FMS PELT device. Individual turtles were placed into their own Rubbermaid metabolic chamber, which contained ‘in’ and ‘out’ flow airlines. Each metabolic trial collected data on four turtles and a baseline chamber simultaneously. All
individuals experienced constant airflow and flow rates ranged from 105-175 mL/min (dependent upon animal mass in g). All metabolic data was collected between 2100 – 0900 hours (30 June 30 – 2 July; 6 trials for all data).

Metabolic measurements were recorded sequentially for each individual at 25° and 30° C. Turtles (4 per run) were placed into the FMS metabolic cooler 75 minutes prior to initial measurements to acclimate turtles to their chambers and temperature. After the first measurement at 25°C, the automated FMS PELT system increased the FMS metabolic cooler temperature to 30°C. Turtles were also allowed 75 minutes to acclimate to 30°C after their 25°C measurement. Measurements were taken for each individual in twenty minute intervals (for each temperature) and five-minute baseline measurements were taken every hour. A trial would measure the oxygen consumption and CO₂ production (mL/hr) of each turtle sequentially, and control measurements of the baseline chamber would be taken every hour to allow for corrections due to lag and drift.

Euthanasia and sample collection

After five weekly doses, turtles were euthanized to quantify Se body burdens and to collect additional samples. Turtles were euthanized via decapitation followed by pithing of brain (Learly et al. 2013). To minimize lymph contamination, blood was collected from the turtles’ necks immediately following decapitation. Approximately 3 mL of whole blood was transferred into multiple 1-mL lithium heparin tubes to prevent clotting (Becton Dickson, San Antonio, TX, USA). Whole blood was then used to prepare 2-3 blood smears per individual, which were fixed in high-quality methanol (VMW, Radnor, PA) for subsequent leukocyte differentials. Following blood collection, a small aliquot of whole blood was placed into microhematocrit tubes and
centrifuged at 10,000 rpm for 5 minutes to obtain packed cell volume (% PCV) and total solids (g/dL) by refractometer. Approximately 1 mL of whole blood was also centrifuged to collect plasma samples from each individual for bactericidal assays. Any abnormalities in plasma color were noted. Remaining whole blood (~1 mL) was saved for subsequent trace element analysis. Animals were dissected and whole liver, kidney, and muscle samples were weighed to the nearest ± 0.01g. After dissection, all samples were stored at -60°C and saved for trace element analysis. All sample collection procedures were approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC # A2014 08-016-Y1-A2).

**Quantification of selenium burdens: Tissues and blood**

Turtle tissues (liver, kidneys, muscle) were individually lyophilized and homogenized prior to digestion and subsequent trace element analysis, which was performed in six runs using inductively coupled plasma-mass spectrometry (ICP-MS; NexION, Perkin-Elmer, Inc., Waltham, MA, USA). Tissue samples were digested in 150µL of a 9:1 nitric acid (HNO₃, 70%):hydrochloric acid (HCl, 12.4%) mixture prior to digestion in a Mars Express microwave system. After digestion, samples were brought to a volume of approximately 15 mL with 18-MΩ deionized water, and ICP-MS measured samples for Se. The values obtained from analysis were also compared to certified reference materials (TORT-3, National Research Council of Canada, Ottawa, ON) to ensure quality of the data. Mean percent recovery for Se in tissue samples was 98.1%. The minimum detection limit for Se in kidney, liver, and muscle samples was 0.47 mg/kg. Tissue Se concentrations are reported on a dry weight basis (mg/kg).

Blood samples were digested in 2.5 mL of trace metal grade HNO₃ using microwave digestion as described above. Once samples were digested, each sample was brought to a volume
of 10 mL with 18-MΩ deionized water. Blood samples were analyzed in two runs using ICP-MS. This protocol used the same certified reference material as described for tissue samples. Mean percent recovery in blood samples was 92.25%. Instrument detection limit for Se in blood samples was 0.003 mg/kg. Blood Se concentrations were reported on a wet weight basis (mg/kg). All Se values reported (including literature) were in dry weight unless noted otherwise. All tissue samples and >95% of blood samples had Se concentrations above the instrument’s minimum detection limit (>MDL). Samples with Se concentrations <MDL were replaced with calculated 0.5*MDL values (based on sample weight) for analysis.

**Survival**

Survival of turtles was monitored daily throughout the duration of the study. Proportional survival of turtles at the conclusion of the study was compared among treatment groups. If mortalities occurred, deceased individuals were not included in the analysis of hematological data. Tissue accumulation data included two 30 mg/kg turtles that died after the last dose of Se.

**Hematological and immunological measures**

To determine the possible effects of Se exposure on bactericidal capacity of juvenile sliders we used a bacterial killing assay (BKA). Turtle plasma was mixed with phosphate buffered saline (PBS), a standardized aliquot of *Escherichia coli* (ATCC #8739; VWR, Radnor, PA), and CO₂ independent media. Fifty microliters of the mixture was spread onto agar plates (Time 0 plates; Teknova Tryptic Soy plates, Fisher Scientific Company LLC, Suwanee, GA) and 50 μL of the same mixture was plated again one hour later (Time 60 plates). These plates were
incubated overnight and analyzed to determine bactericidal capacity. See Ch. 2 for further information regarding the bactericidal assay protocol.

Methanol-fixed blood films were stained with Wright-Giemsa and blindly evaluated for white blood cell (WBC) estimate, WBC differential (200 cells), and evaluation of blood cell morphology by the same clinical pathologist (N. Stacy). The WBC estimate was performed by multiplying the average number of WBC in 10 microscope fields × the objective power squared (Weiss 1984). The erythroid regenerative response in anemic turtles (PCV ≤25%) was subjectively evaluated by morphologic evaluation of the erythroid cell line for anisocytosis, polychromasia, mitotic figures, and/or early erythroid stages and classified as absent, mild, moderate, or marked. The morphologic evaluation of WBC included evaluation for heterophil toxicity and left-shifting, among other possible morphologic abnormalities.

Statistical analyses

To compare differences in selenium accumulation among treatments groups, one-way Analysis of Variance (ANOVAs) was used. If differences existed, Tukey’s Honest Significant Difference (HSD) post-hoc tests were used to see which groups significantly differed. Possible differences in bacterial killing assays among treatment groups were also tested using ANOVA. Normality and homogeneity of variances were tested using Shapiro-test statistic and the Levene statistic. When necessary, data were transformed using log, square root, or rank transformations to achieve normality and meet assumptions of parametric tests. Due to the nonnormality and heteroscedasticity of hematological and survival data, these data were compared using nonparametric Kruskal-Wallis H tests. Significant differences found in Kruskal-Wallis analyses were tested for multiple pair-wise differences using Dunn’s post-hoc test with Šidák-corrected
significance values. All hematological data were tested for correlations with selenium concentrations in all tissue types. Blood Se data were tested for correlations with all tissue types. Because these data did not fit the assumptions of Pearson’s correlation test, Spearman rank correlation ($R_s$) tests were used.

We used ExpeData-P Data Analysis Software (Sable Systems, Las Vegas, NV, USA) to calculate oxygen consumption data from the metabolic trials. Oxygen consumption (mL/hr) was analyzed using a linear mixed effects model for repeated measures (R package lme). Individual turtle ID was used as a random effect within the model. Stall was included in preliminary models to account for the possible effect of block group but was not significant. Preliminary models also examined an interaction term of treatment and temperature (treatment * temperature), but this was also found to be nonsignificant. Each model investigated was fit with a different kind of ARMA (autoregressive moving average) serial correlation structure. The final model included Se treatment group, trial temperature, and plastron length as fixed effects, with turtle ID serving as a random effect (Table 3.1). All data were analyzed using R (R Core Team 2016).

**Results**

*Selenium body burdens*

Accumulation of total Se in all tissue types was dependent on SeMet dose (Table 3.1). The 30 mg/kg group exhibited the highest Se burdens, followed by 15 mg/kg and control groups. There was a significant difference in mean Se concentrations among treatment groups (ANOVA, $p < 0.001$) for all tissue types. Tukey’s post hoc test found that turtles from the 30 mg/kg group exhibited the highest Se concentrations in all tissue types ($p < 0.001$ for all tissue comparisons, Table 3.1). Blood Se accumulation also significantly differed among treatment groups (Kruskal-
Wallis, $X^2 = 56.40, p < 0.001$). Dunn’s multiple comparisons revealed that all groups differed significantly ($p < 0.005$), and accumulation followed trends previously described in tissues (30 mg/kg > 15 mg/kg > control). For all treatment groups Se concentration was highest in the kidney, followed by liver, muscle, and blood. Blood Se was strongly and positively correlated with kidney Se ($r_s = 0.922, p < 0.001$), liver, ($r_s = 0.860, p < 0.001$), and muscle ($r_s = 0.849, p < 0.001$; Table 3.2 and Figure 3.1).

Survival

Proportional survival of turtles (Table 3.3) was significantly influenced by treatment ($X^2 = 8.55, p = 0.014$). The 30 mg/kg group was the only group that experienced mortality throughout the duration of our study (82.6% survival). The first mortality occurred after the third weekly dose of SeMet, another after the fourth weekly dose, and two more individuals died between the fifth dose and culling of turtles.

Metabolic rates

The most parsimonious model showed that metabolic rates of turtles varied significantly and positively with temperature ($t_{2,17} = 5.55, p < 0.001$) and plastron length ($t_{2,17} = 2.28, p = 0.036$; Table 3.4). Average metabolic rates of all turtles at 25 and 30°C were $6.47 \pm 0.60$ mL/hr and $9.76 \pm 1.01$ mL/hr, respectively. Se treatment group was not a significant factor in any of our models, although at both temperatures turtles from 15 mg/kg group tended to have lower metabolic rates than the other two Se treatment groups (Figure 3.2; $t_{2,17} = -1.86, p = 0.080$).
Hematological and immunological effects of selenium exposure

WBC estimates varied significantly among groups ($X^2 = 6.96, p = 0.031$) and were higher in the 15 mg/kg group compared to control and 30 mg/kg groups (Table 3.5). Dunn’s post-hoc test revealed that only the 15 mg/kg and control groups differed significantly ($p = 0.013$). Treatment groups also significantly differed in H/L ratios ($X^2 = 7.83, p = 0.019$). The H/L ratios of turtles in the 30 mg/kg group were five times higher when compared to both control and 15 mg/kg groups, due to higher heterophil and lower lymphocyte concentrations in the 30 mg/kg group (Table 3.5). Lymphocytes were significantly ($33.72 \pm 3.12 \times 10^9$ cells/L) lower in the 30 mg/kg group relative to control and 15 mg/kg groups (Table 3.5). PCV significantly differed among treatment groups ($X^2 = 14.08, p = 0.043$), and turtles in the 30 mg/kg group exhibited lower PCV results when compared to the control and 15 mg/kg groups. Fifty-five percent (10/18) of turtles from the 30 mg/kg group were anemic (PCV ≤ 25), with PCVs averaging 18.9 ± 1.18. The 30 mg/kg group also had the highest incidence of regenerative anemia, and one-third of turtles (6/18) had green plasma which could be suggestive of hemolytic anemia, liver disease, or starvation. Approximately 17% (4/23) of turtles in the 15 mg/kg group were anemic (mean PCV = 20.8 ± 2.63). Lastly, 13% (3/23) of individuals from the control group had mild anemia (mean PCV = 24). Anemic individuals from the 15 mg/kg and control groups mostly exhibited nonregenerative anemia. All other hematological analytes (heterophils, monocytes, and total solids) did not significantly differ between groups (all $p > 0.235$), although some did trend toward significance (absolute basophils and eosinophils). Heterophil toxicity or left-shifting (a shift towards immaturity in white blood cells) was not observed. Thrombocytes were adequate in all groups.
Spearman correlations revealed a negative relationship between kidney Se concentrations and PCV ($r_s = -0.36, p = 0.003$; Figure 3.3), and also found a positive relationship between blood Se concentrations and total solids ($r_s = 0.26, p = 0.035$; Figure 3.4). All other correlational comparisons of hematological data did not yield significant differences ($p > 0.05$). Mean percent bacteria killed did not significantly differ among treatment groups ($X^2 = 0.39, p = 0.819$). Relationships between BKA responses and other variables were not explored due to the extreme nonnormality of the BKA data.

**Discussion**

The current study shows that *T. scripta* do accumulate SeMet in a dose-dependent manner. Kidney Se concentrations in the current study are among the highest reported in reptiles, if not the highest. The kidneys (renal system) are the main route of elemental elimination in vertebrates, and turtles in all experimental groups partitioned the largest amounts of Se to their kidneys. Correlational relationships in Se accumulation between blood and other tissue types (kidney, liver, and muscle) were strong and positive (all $p < 0.001$, all $R_s > 0.849$), suggesting Se burdens of sensitive organs may be estimated with non-destructive samples in *T. scripta*. The significant correlation between blood and muscle Se concentrations also may assist in predicting the risks of consuming turtle meat. The accumulation patterns (kidney > liver > muscle > blood) of Se in this study are similar to what little information exists for other reptilian species. In reptilian exposure studies, snake species (*Nerodia* and *Lamprophis* spp.) accumulated the highest amounts of Se in their kidney tissues, although these differences were not as dramatic as the amount found in the turtles’ kidneys reported in this study (Hopkins et al. 2002; 2004). However, some hawksbill sea turtles (*Eretmochelys imbricata*) caught in Japanese waters had higher
amounts of Se in their liver relative to their kidneys (Anan et al. 2001). In contrast to reptiles, birds are known to accumulate equal or larger amounts of Se in their liver tissues (Ohlendorf et al. 1988; Ohlendorf and Heinz 2011). The differences in Se accumulation among turtles, snakes, and birds could be of significance when considering these species in ecological risk assessments.

While our treatment concentrations of 15 and 30 mg/kg may reflect a chronic dose, the weekly dose of SeMet was an acute exposure to a week’s worth of SeMet (based on turtle weight). Although the turtles in our study were acutely exposed to SeMet, their Se concentrations are similar to data from previous field studies. Turtles in both of our treatment groups had mean Se liver concentrations similar to those measured in dead birds at the Kesterson Reservoir, and liver Se concentrations of dead birds ranged from 26-86 mg/kg Se (Ohlendorf and Heinz 2011). The highest liver Se concentrations in the 30 mg/kg group (162.01 mg/kg Se) exceeded those reported in banded water snakes (Nerodia fasciata) in CCR settling basins on the SRS (Hopkins et al. 1999). Turtles in the 15 mg/kg group had blood and liver Se concentrations similar to those reported in wild T. scripta captured in CCR settling basins on the SRS. Mean liver Se concentrations of wild T. scripta were 37.18 mg/kg (Nagle et al. 2001), while liver Se in our 15 mg/kg group averaged 34.89 mg/kg. Blood Se concentrations of wild T. scripta in my field study (see chapter 2) were as high as 9.57 mg/kg (ww) Se, and this value falls within the ranges of both SeMet treatment groups (Haskins et al. unpublished data; Table 3.1).

Turtles in both SeMet-treatment groups accumulated large amounts of Se in their tissues, and the Se liver burdens described in the 30 mg/kg turtles are well above known thresholds for lethal responses in mallards (Anas platyrhynchos; Ohlendorf and Heinz 2011). A review of exposure studies suggests that mallard liver concentrations of > 66 mg/kg SeMet can cause mortality (Ohlendorf and Heinz 2011). Turtles in the 30 mg/kg group had mean liver Se
concentrations of 86.37 mg/kg and a mortality rate of 17.4% (4/23) after 5 treatments of SeMet. Furthermore, turtles from the 30 mg/kg group exhibited signs of selenium toxicosis (selenosis). Ohlendorf and Heinz’s (2011) recommended assessment values for selenosis in avian species is > 20 mg/kg liver Se, and selenosis in mallards was characterized by emaciation, hepatic lesions, and the necrosis of maxillary nail tissues. Histopathological analysis of the 30 mg/kg turtles revealed necrosis of maxillary nail tissues, necrosis of kidney tissues, and hepatic lesions (Haskins et al., unpublished data). To our knowledge, this study is the first to report symptoms suggesting selenosis in a reptilian species.

Stressors such as CCRs, which often contain elevated levels of Se, can cause physiological changes in vertebrates that may result in increased energy expenditure (Hopkins et al. 1999; Holliday et al. 2009). Field studies of water snakes, bullfrogs, shrimp, and crayfish showed that all species had increased metabolic demands in response to CCR accumulation (Rowe 1998; Rowe et al. 1998; Hopkins et al. 1999; Rowe et al. 2001). In contrast, Nagle et al. (2001) found metabolic rates of hatchling T. scripta from CCR-exposed mothers were depressed compared to hatchlings from unexposed females. Because these data come from individuals exposed to a mixture of trace elements present in CCRs, it is difficult to discern if Se was the sole cause of metabolic aberrations in these species. In the present study oxygen consumption did not significantly differ among treatment groups. However, turtles from the 15 mg/kg group did have lower oxygen consumption rates than any group, and this difference trended toward significance (p = 0.080). These trends appear to agree with Nagle et al.’s (2001) findings in T. scripta, but it is strange that the 30 mg/kg group did not experience metabolic aberrations. Although metabolic rates did not differ at the conclusion of the 37-d experiment, it could be that the turtles were not exposed long enough to elucidate the changes in metabolic status.
Diamondback terrapins (*Malaclemys terrapin*) exposed to polychlorinated biphenyls (PCBs) did not show a significant decrease in metabolic status until 90 days after exposure (Holliday et al. 2009). Perhaps if individuals in the 15 mg/kg were to be chronically exposed (8-12 weeks) a larger difference would be observed. It is also possible that our sampling period did not observe the initial stress response in the 30 mg/kg group, and that these turtles were so stressed by Se that they could not regulate their metabolic response. Lastly, it is important to note that sampling errors could have impacted our data such as spontaneous animal activity during sampling, which is common when measuring metabolic rates in ectotherms.

Selenium accumulation had a significant influence on numerous hematological parameters including packed cell volume, total white blood cell counts, lymphocyte counts, and heterophil to lymphocyte ratios. Packed cell volume decreased with increasing Se dose. Previous studies exposed broiler chickens to inorganic Se (selenite) or SeMet, and chickens that were supplemented with SeMet had a significantly lower hematocrit count than birds that were fed selenite (da Silva et al. 2010). Fish that were exposed to Se contamination through coal ash wastewater also had lower hematocrit values than fish from reference sites (Lemly 2002). The mean PCV value (24.33 ± 1.67) for 30 mg/kg turtles is below the threshold of mild anemia (< 25), and over half (55%) of the 30 mg/kg turtles were anemic. However, total solids were actually higher in the 30 mg/kg turtles. It is interesting that the 30 mg/kg turtles were anemic but did not show a significant change in total solids, but this observation could be due to hemolysis. One-third (6/18) of turtles in the 30 mg/kg group presented with green plasma; this observation, combined with the significant decreases in PCV, could suggest Se-induced hemolytic anemia. Studies in mammals have shown that hemolysis can alter hematological parameters including albumin and alpha and beta-globulins, which are among the proteins that are quantified when
measuring total solids (Di Martino et al. 2015). Therefore, it is possible that the total solids measured in this study were impacted by hemolysis.

The higher WBC count in the 15mg/kg group this cannot be attributed to heterophilia, as the 30 mg/kg group is the only group that experienced an increase in heterophils. Although the mean heterophil difference among groups was not statistically significant, turtles from the 30 mg/kg group did have elevated heterophil counts (Figure 3.6). Turtles in the 30 mg/kg group also exhibited lymphopenia. Lymphopenia is known to occur when reptiles are stressed, immunosuppressed, or are malnourished (Campbell 2012). Because most Se-treated turtles continued to consume food throughout the study and discernable weight loss was not readily observed, it is unlikely that malnourishment would have caused lymphopenia in the 30 mg/kg group without affecting the 15 mg/kg group. Studies in birds have found that exposure to excessive amounts of dietary Se can cause lymphopenia and cause atrophy in the thymus (Peng et al. 2011). T-lymphocytes are produced in the thymus and are involved in cell-mediated immunity and cytokine responses (Weiser 2012); thus, it could be that damage to the thymus reduced T-lymphocyte counts in the peripheral blood of our turtles. Unfortunately, we did not investigate Se-related effects on the thymus so we cannot speak to the possibility that Se exposure damaged the turtles’ thymuses, leading to lymphopenia.

Heterophil to lymphocyte ratios were about five times higher in the 30 mg/kg group relative to the other two groups in this study. These findings are expected, as the 30 mg/kg turtles experienced heterophilia and lymphopenia. Stress can alter hematological profiles and subsequent H/L ratios. In the current study all turtles were handled, processed, and sacrificed in an identical manner, which suggests it is unlikely these actions would have altered only one group’s hematological profile. Heterophil to lymphocyte ratios have been suggested to be a
useful biomarker to measure stress in avian species (Gross and Seigel 1983). Hormones produced (glucocorticoids) during adrenal stress responses increase heterophil counts and reduce lymphocyte counts (Gross and Seigel 1983; Davis et al. 2008). Studies in avian species have shown that exposure to trace element contaminants (arsenic, boron, Se) can result in increased H/L ratios (Grassman 2002). Furthermore, Keller et al. (2004) found that H/L ratios were significantly elevated in loggerhead sea turtles, and these ratios were positively correlated with organochlorine contaminant burdens. The H/L ratios of the 30 mg/kg group indicates that these turtles also had high glucocorticoid levels, suggesting that prolonged acute exposure to Se causes considerable stress in *T. scripta*.

Surprisingly, turtles across all treatment groups displayed strong bactericidal capacity (all means ≥ 98%). BKA is a useful tool to measure a turtle’s innate immune capacity in response to microbial invaders. The assay measures the innate immune response that involves phagocytes, opsonizing proteins, and natural antibodies (French et al. 2010). Studies in reptiles and amphibians have shown that anthropogenic stress, activity patterns, and time of year can all impact bactericidal response (French et al. 2010; Brown and Shine 2014; Zimmerman et al. 2010). Although the turtles in this experiment were exposed to potentially lethal amounts of SeMet, their bactericidal capacity did not seem to be affected by the SeMet treatments. Bacteria are tolerant of heavy metal exposure and are known to be extremely tolerant to Se-exposure (Janz et al. 2010). Therefore, it is unlikely that the bacteria used in the BKAs were killed by Se exposure (through the SeMet turtles’ plasma). Our results suggest that the innate bactericidal abilities of our turtles were not compromised by Se accumulation.

This experiment is the first to induce selenosis in a reptile. Turtles exposed to a Se-dose of 30 mg/kg had a mortality rate of 17.4%. Turtles in the 30 mg/kg group also exhibited
lymphopenia, heterophilia, increased H/L ratios, and a significant decrease in packed cell volume. The hematological findings of this study support evidence provided by previous Se exposure experiments in vertebrates (fish and birds). Surprisingly, we did not observe statistically significant changes in metabolic status or bactericidal capacity. The current knowledge of Se toxicity in reptiles is insufficient (Young et al. 2010), and further research is necessary to understand what sublethal impacts are caused by Se exposure. Turtles, like *T. scripta*, are known to inhabit Se-contaminated areas such as coal ash basins on the SRS (Nagle 2001). While the Se burdens in our 30 mg/kg group would likely not reflect wild *T. scripta* Se burdens, these reptiles are year-round residents in these contaminated systems, and little is known how Se and other CCRs impact their overall health. Future controlled studies should aim to investigate chronic, sublethal exposure to Se in chelonians and should also seek to study the potential impacts of Se exposure on chelonian reproduction.
Literature Cited


Table 3.1: Differences in selenium accumulation in target tissues (liver, kidney, muscle, and blood) of *Trachemys scripta* exposed to one of three selenium treatments (Control, 15 mg/kg, and 30 mg/kg). Selenium concentrations are reported as means (dry weight for tissues and wet weight for blood) ± 1 SE (range). Letters in the ‘Tukeys’ column designate significant differences among groups within a tissue type.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>n</th>
<th>Se (mg/kg)</th>
<th>F-value, df, p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tukeys&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Control</td>
<td>23*</td>
<td>2.04 ± 0.12</td>
<td>501.8, 2, &lt; 0.001</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>34.89 ± 3.09</td>
<td>(15.08 – 66.18)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>21</td>
<td>86.37 ± 8.03</td>
<td>(25.85 – 162.01)</td>
<td>C</td>
</tr>
<tr>
<td>Kidney</td>
<td>Control</td>
<td>24</td>
<td>4.38 ± 0.12</td>
<td>2733, 2, &lt; 0.001</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>122.74 ± 5.54</td>
<td>(73.75 – 180.77)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>21</td>
<td>229.30 ± 0.12</td>
<td>(141.60 – 306.33)</td>
<td>C</td>
</tr>
<tr>
<td>Muscle</td>
<td>Control</td>
<td>24</td>
<td>1.90 ± 0.07</td>
<td>1322, 2, &lt; 0.001</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>27.70 ± 1.40</td>
<td>(13.50 – 39.92)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>21</td>
<td>42.54 ± 2.10</td>
<td>(26.56 – 64.75)</td>
<td>C</td>
</tr>
<tr>
<td>Blood&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Control</td>
<td>24</td>
<td>0.29 ± 0.02</td>
<td>56.40, 2, &lt; 0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>6.76 ± 0.48</td>
<td>(2.77 – 10.89)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>21</td>
<td>14.85 ± 0.90</td>
<td>(7.68 – 22.36)</td>
<td>C</td>
</tr>
</tbody>
</table>

<sup>a</sup>One-way analysis of variance (ANOVA)

<sup>b</sup>Results from post-hoc Tukey’s (adjusted) Honest Significant Difference tests.

<sup>c</sup>Statistical analysis of blood used nonparametric methods (Kruskal-Wallis H test) and Dunn’s Multiple Comparisons Tests using Šidák-corrected significance values for post-hoc analysis.

* One less sample was available for ‘control’ liver samples due to processing errors.
Table 3.2: Proportional survival of yellow-bellied sliders (*Trachemys scripta scripta*) exposed to three different treatments (control, 15 mg/kg, and 30 mg/kg) of selenium (Se). Chi-square values reported are from a Kruskal-Wallis analysis to compare proportional survival of treatment groups at the end of the study (37 days). ‘Dunn’ letters designate significant differences between groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Proportional Survival</th>
<th>$X^2$, df, p-value</th>
<th>Dunn$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 days</td>
<td>24 days</td>
<td>37 days</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>100</td>
<td>100</td>
<td>100$^a$</td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>23</td>
<td>100</td>
<td>100</td>
<td>100$^a$</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>23</td>
<td>100</td>
<td>95.7</td>
<td>82.6$^b$</td>
</tr>
</tbody>
</table>

$^a$ Results from post-hoc Dunn’s tests using Šidák-corrected significance values
Table 3.3: Linear mixed model for metabolic rate analysis with varying correlation structure to explain differences in metabolic rate among selenium exposure groups. The most parsimonious model is indicated in bold. Model terms included treatment group (Control vs. 15 mg/kg vs. 30 mg/kg), trial temperature at time of sampling (25°C vs. 30°C), and plastron length (PL in mm). Correlation structure (Corr.) types included ARMA 1 (AR 1), ARMA 2 (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 (ΔAIC), and Akaike weights, which displays the weight of each candidate model in the set.

<table>
<thead>
<tr>
<th>Model</th>
<th>Corr.</th>
<th>LL</th>
<th>K</th>
<th>AIC</th>
<th>ΔAIC</th>
<th>AICWt</th>
<th>CumWt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment + Temp + PL</td>
<td>None</td>
<td>-89.26</td>
<td>7</td>
<td>192.52</td>
<td>0</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>Treatment + Temp + PL</td>
<td>AR 1</td>
<td>-89.26</td>
<td>8</td>
<td>194.52</td>
<td>2</td>
<td>0.24</td>
<td>0.91</td>
</tr>
<tr>
<td>Treatment + Temp + PL</td>
<td>AR 2</td>
<td>-89.26</td>
<td>9</td>
<td>196.52</td>
<td>4</td>
<td>0.09</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3.4: Hematological comparisons of *Trachemys scripta* exposed to one of three selenium treatments (Control, 15 mg/kg, and 30 mg/kg). Indices include white blood cell estimate (WBC), heterophil (HET) to lymphocyte (LYM) ratios (H/L), heterophils (HET), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), packed cell volume (PCV), and total solids (TS). ‘Dunn’ letters designate significant differences between groups.

<table>
<thead>
<tr>
<th>Indices</th>
<th>Treatment</th>
<th>n</th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean % count</th>
<th>(X^2), df, (p)-value</th>
<th>Dunn&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>Control</td>
<td>24</td>
<td>9.24 ± 0.45</td>
<td>-</td>
<td>6.96, 2, 0.031</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>12.07 ± 0.80</td>
<td>-</td>
<td>-</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>10.03 ± 1.23</td>
<td>-</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>H/L</td>
<td>Control</td>
<td>24</td>
<td>0.28 ± 0.07</td>
<td>-</td>
<td>7.83, 2, 0.019</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>0.25 ± 0.05</td>
<td>-</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>1.36 ± 0.45</td>
<td>-</td>
<td>-</td>
<td>B</td>
</tr>
<tr>
<td>HET</td>
<td>Control</td>
<td>24</td>
<td>1.05 ± 0.21</td>
<td>10.88 ± 1.77</td>
<td>2.49, 2, 0.286</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>1.21 ± 0.20</td>
<td>9.87 ± 1.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>2.93 ± 0.85</td>
<td>27.78 ± 5.36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LYM</td>
<td>Control</td>
<td>24</td>
<td>4.30 ± 0.28</td>
<td>46.92 ± 2.63</td>
<td>14.08, 2, &lt;0.001</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>5.26 ± 0.34</td>
<td>45.13 ± 2.45</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>3.10 ± 0.43</td>
<td>33.72 ± 3.12</td>
<td>-</td>
<td>B</td>
</tr>
<tr>
<td>MON</td>
<td>Control</td>
<td>24</td>
<td>0.43 ± 0.06</td>
<td>4.54 ± 0.54</td>
<td>1.17, 2, 0.558</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>0.51 ± 0.06</td>
<td>4.65 ± 0.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>0.52 ± 0.10</td>
<td>5.00 ± 0.83</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EOS</td>
<td>Control</td>
<td>24</td>
<td>2.59 ± 0.25</td>
<td>27.50 ± 2.09</td>
<td>5.60, 2, 0.061</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>3.05 ± 0.34</td>
<td>24.26 ± 1.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>2.04 ± 0.41</td>
<td>19.56 ± 2.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BAS</td>
<td>Control</td>
<td>24</td>
<td>0.92 ± 0.10</td>
<td>10.17 ± 1.14</td>
<td>5.80, 2, 0.054</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>2.12 ± 0.36</td>
<td>16.09 ± 2.17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>1.49 ± 0.28</td>
<td>13.94 ± 1.77</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCV</td>
<td>Control</td>
<td>23*</td>
<td>29.78 ± 0.83</td>
<td>-</td>
<td>6.29, 2, 0.043</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>28.91 ± 1.16</td>
<td>-</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>24.33 ± 1.67</td>
<td>-</td>
<td>-</td>
<td>B</td>
</tr>
<tr>
<td>TS</td>
<td>Control</td>
<td>23*</td>
<td>4.07 ± 0.14</td>
<td>-</td>
<td>2.90, 2, 0.235</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>23</td>
<td>4.46 ± 0.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>18</td>
<td>4.34 ± 0.21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of cells x 10<sup>9</sup>/L whole blood

<sup>b</sup>Results from post-hoc Dunn’s tests using Šidák-corrected significance values.

* One PCV and TS sample from a control individual was unusable
Figure 3.1: Spearman rank correlation between blood selenium (Se) and tissue Se concentrations (A - kidney, B - liver, and C - muscle) in yellow-bellied sliders (Trachemys scripta scripta) exposed to three different concentrations of selenium (Se; n = 64).
Figure 3.2: Oxygen consumption (ml O$_2$/hr) of *Trachemys scripta* exposed to one of three selenium treatments (Control = circles, 15 mg/kg = triangles, 30 mg/kg = squares; N = 5, 6, and 8, respectively). Measurements were taken at two separate temperatures (25°C = black and 30°C = grey).
Figure 3.3: Spearman rank correlation between percent packed cell volume and kidney selenium (Se, dry weight) concentrations in yellow-bellied sliders (Trachemys scripta scripta) exposed to three different concentrations of selenium (Se; n = 64).
Figure 3.4: Spearman rank correlation between total solids and blood selenium (Se, wet weight) concentrations in yellow-bellied sliders (*Trachemys scripta scripta*) exposed to three different concentrations of selenium (Se; n = 64).
Figure 3.5: Mean percent (± 1 SE) bacterial killing ability of yellow-bellied sliders (*Trachemys scripta scripta*) exposed to three different treatments of selenium (Se): control (n = 23), 15 mg/kg (n = 23), and 30 mg/kg (n = 18). No statistical differences were observed ($X^2 = 0.39, p = 0.819$). *Escherichia coli* was the bacteria species used in these assays.
Figure 3.6: Mean heterophil (\# of cells x 10^9) count (± 1 SE) of yellow-bellied sliders (Trachemys scripta scripta) exposed to three different treatments of selenium (Se): control (n = 23), 15 mg/kg (n = 24), and 30 mg/kg (n = 18). Kruskal-Wallis analysis found that there were not significant differences among groups ($X^2 = 2.49, p = 0.286$).
CHAPTER 4
CONCLUSIONS

Freshwater turtles are globally declining due to numerous factors, such as invasive species, destruction of suitable habitat, exploitation, and anthropogenic pollutants (Gibbons 2000; Hopkins 2000a). Turtles’ evolutionary history extends over 300 million years, and they have evolved a unique suite of life history characteristics (e.g., including delayed maturity, ontogenetic diet shifts, long life spans, and low adult mortality; Ernst & Lovich 2009). Because of these life history traits, freshwater turtles are particularly susceptible to anthropogenic alterations of their environment (Steen et al. 2015). Turtle population dynamics typically rely on the high survival rates of adult females rather than being dependent upon egg viability and hatchling survival (Congdon et al. 1994). Studies have suggested that compounding stressors, such as increased road mortality and overharvesting, can increase the importance of survival across age classes (Cunningham and Brooks 1996). Contaminants might have a significant role in the decrease of freshwater turtle populations; further research is needed to establish contaminants as a detrimental factor (Gibbons et al. 2000; Grillitsch and Schiesari 2010). Little toxicological information is available for chelonians, and future studies should aim to better assess the sublethal and lethal impacts of contaminants on multiple age classes.

Future field studies of contaminants in freshwater turtles should seek to evaluate the true differences of contaminant accumulation among age classes. Immature slider turtles in my field study tended to accumulate higher amounts of cadmium (Cd) and copper (Cu), though adults tended to accumulate more arsenic (As). The differences in blood and claw trace element
concentrations may be due to the ontogenetic diet shift that slider turtles experience, and subsequent research should seek to confirm this. The consequences of trace element exposure in immature slider turtles may not mirror effects in adults. Results from my field study suggest exposure to coal combustion residues in sliders caused little to no effects on immunological status — an effect opposite to my once-held hypothesis. Previous studies on reptilian species have noted that reptiles seem to be less affected by contaminants that typically cause deleterious effects in other vertebrates (Hopkins et al. 2005; Chin et al. 2013; Perrault et al. 2014). Perhaps the contaminant loads presented in my field study were not enough to cause observable aberrations in slider immune function; however, I sampled only non-destructive tissue types, so it is difficult to estimate the true body burdens of sensitive organs.

Researchers should continue to investigate the effects of controlled contaminant exposure in freshwater turtles. Although my Se study used ecologically unrealistic techniques (oral gavage) to ensure accurate application of Se, we were able to expose slider turtles to two planned concentrations of Se and discern what impacts Se can have on these reptiles. Hematological parameters were impacted by Se treatment, but metabolic rates and bactericidal capacity were not. Turtles in the low dose group (15 mg/kg Se) did not seem to be significantly impacted by the Se exposure. This is fascinating given that these turtles’ Se burdens were much higher than those known to cause mortality in other species (Ohlendorf and Heinz 2011). Turtles in the high dose group (30 mg/kg) exhibited symptoms indicative of excess Se exposure (anemia and increased heterophil:lymphocyte ratios) and Se toxicosis (mortality and histopathological abnormalities; Haskins unpublished data). My Se study also suggests that non-destructive sampling of blood can be a reliable indicator of other tissue burdens. Overall, this study lays a foundation for the investigation of selenium toxicosis in turtles.
The findings of my chapters also have implications that are relevant to chelonian conservation and human health. Forty percent of the world’s turtle species are considered threatened, and further investigation of the effects of contaminants on chelonian populations is warranted (Turtle Taxonomy Working Group 2014). Overall, the immunological status of turtles we captured in D-area did not seem to be negatively impacted by trace element contaminants. This is interesting given the concentrations of selenium (Se) in some D-area turtles’ blood were comparable to individuals in my Se study. I did not collect samples for leukocyte differentials in the field turtles, and perhaps there would have been differences in hematological parameters. The 15 mg/kg Se group in my Se study accumulated liver Se concentrations that resembled previous liver Se data from slider turtles in D-area. Nagle et al. (2001) found that adult, female slider turtles had a mean liver Se concentration of 37.18 (± 10.92; n = 4; dry weight), comparable to the turtles in my 15 mg/kg group with a mean liver Se concentration of 34.89 (± 3.09; n = 23; dry weight). To relate my similar liver Se values to human consumption (0.79 μg/kg of body weight per day; Zhang 2014), I controlled for moisture content in the liver samples and used a tolerable daily intake limit equation (TDIL; JEFCA 2006; Moses et al. 2009). The output of this equation suggests that an average-sized human adult (70 kg) could only eat approximately 3 grams of 15 mg/kg turtle liver before exceeding their daily Se limit. In regard to human health, this could have exposure implications in the United States where turtle consumption still occurs near aquatic coal ash basins. It most certainly has implications for harmful levels of consumption in regions where turtles are more likely to be consumed (e.g., Asia, Central and South America), and these regions include some countries whose coal use rivals or exceeds the United States’.

Toxicological studies of freshwater turtles have shown that these reptiles do indeed accumulate high amounts of contaminants known to cause deleterious effects in other taxa.
(Nagle et al. 2001; Hopkins et al. 2013). My results corroborated past studies’ findings, as slider claw trace element concentrations from coal ash sites are among the highest reported in freshwater turtles. In fact, mean claw trace element concentrations were up to approximately 66 times (Arsenic) higher in turtles captured in coal ash sites when compared to turtles from reference sites. Slider turtles in my Se exposure study also accumulated some of the highest amounts of Se reported in a reptilian species. To my knowledge the Se experiment is the first study to report Se-induced mortality and Se toxicosis in a reptilian species. Historically, most reptilian toxicological studies sought to quantify body burdens through destructive methods and did not focus on the sublethal impacts of contaminant exposure (Hopkins 2000b). Further investigation is necessary to not only discern how and where freshwater turtles accumulate pollutants, but also to properly interpret the sublethal effects of toxicological exposure in these species.
Literature Cited


