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Effects of Heavy Metal Pollution on the Loggerhead Sea Turtle

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Effects of Heavy Metal Pollution on the Loggerhead Sea Turtle

by

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A Thesis submitted in partial satisfaction of
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Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Biology.

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ABBREVIATIONS

AAMo	Absolute Azurophilic Monocytes
AAS	Atomic Absorption Spectrophotometer
AB	Arsenobetaine
AbBa	Absolute Basophils
AbEo	Absolute Eosinophils
AbLy	Absolute Lympocytes
AbMo	Absolute Monocytes
AbNe	Absolute Neutrophils
AbPo	Absolute Heterophils
Al	Aluminum
Albu	Albumin
ALT	Alanine Aminotransferase
Amy	Amylase
AP	Alkaline Phosphatase
As	Arsenic
AsIII	Arsenite
AST	Aspartate Aminotransferase
AzMo	Azurophilic Monocytes
B	Boron
Ba	Barium
Baso	Basophils
Be	Beryllium

BlPa	Blood parasites
BUN	Blood Urea Nitrogen
Calc	Calcium
Cd	Cadmium
Chlo	Chlorine
CK	Creatine Kinase
Co	Cobalt
CPK	Createnine Phosphokinase
Cr	Chromium
Cs	Cesium
Cu	Copper
Da	Dalton
Eosi	Eosinophils
Fe	Iron
GGT	Gamma Glutamyl Transpeptidase
Glob	Globulin
Gluc	Glucose
HCL	Hydrochloric Acid
Hema	Hematocrit
HePo	Heterophils
Hg	Mercury
HGAAS	Hydride Generation Atomic Absorption Spectrometry
HNO_3	Nitric Acid

HPLC	High Profile Liquid Chromatograph
ICPMS	Inductively Coupled Plasma Mass Spectrophotometer
IIRMES	Institute for Integrated Research on Materials, Environment, and Society.
IUCN	International Union for Conservation of Nature
LDH	Lactate Dehydrogenase
Li	Lithium
Lymp	Lymphocytes
mL	Milli-liters
Mn	Manganese
Mo	Molybdenum
Mono	Monocytes
Morp	Morphology
ng	Nano-grams
Ni	Nickel
OC	Organochlorine Contaminants
Pb	Lead
PCB	Polychlorinated Biphenyls
PCV	Packed Cell Volume
Phos	Phosphorus
Pota	Potassium
Rh	Rhodium
SCL	Straight Carapace Length
Sb	Antimony

Sn	Tin
$SnCl_2$	Tin Chloride
Sodi	Sodium
Sr	Strontium
SRM	Standard Reference Material
THg	Total Mercury
Ti	Titanium
Tl	Thallium
Tm	Thulium
ToPr	Total Protein
T3	Triiodothyronine
T4	Thyroxine
Uric	Uric Acid
UrNi	Urea Nitrogen
V	Vanadium
WBC	White Blood Cells
Y	Yttrium
Zn	Zinc
µL	Micro-liters

ABSTRACT OF THE THESIS

Effects of Heavy Metal Pollution on the Loggerhead Sea Turtle

by

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Historically, heavy metal research on sea turtles has been focused on deceased specimens, limiting the ability to determine if the concentrations of heavy metals affected the health of the individuals. More recently, the collection and analysis of blood samples from live turtles has enabled the researcher to investigate the potential health implications of observed metal concentrations.

In this thesis, I present two original studies on the blood concentrations of essential and non-essential heavy metals and their potential physiological correlates on the endangered loggerhead sea turtle (*Caretta caretta*). This work reflects analysis of archived samples collected in 2008 off the southeastern coast of the United States by the South Carolina Department of Natural Resources (SCDNR). Research was funded in part by the Office of Protected Resources and NOAA Fisheries. Samples were obtained through the generous support of Rusty D. Day, MSc.

The first study examined the relationships between body size, sex, geographic location, water depth, and blood concentration of 17 essential and non-essential heavy metals and metalloids. Statistical analysis of these parameters indicated that measures of body size were correlated with several of the metals, whereas sex had no significant

relationship with any of the metals examined. Several metal concentrations also varied with geographic location and depth of water in which the turtles were captured.

The second study examined the potential health effects of these pollutants in *C. caretta*. Regression analyses were used to compare physiological (blood) parameters to metal concentrations. The significant associations between several physiological parameters and several nonessential toxic metals suggest that heavy metal pollution may influence the physiology and, potentially, the health of sea turtles. However, this study is limited in that it can only identify associations and cannot discern causal relationships. Therefore, further research is needed to clarify the effects heavy metal pollution may have on sea turtle health.

A better understanding of the effects of heavy metal pollution on health in this endangered species will facilitate more effective monitoring and protection in the future, enabling us to more effectively conserve these fascinating creatures.

CHAPTER ONE

LIFE HISTORY AND POLLUTION IN THE LOGGERHEAD SEA TURTLE

The Loggerhead Sea Turtle (*Caretta caretta*)

There are seven species of marine turtles: the green sea turtle (*Chelonia mydas*), the loggerhead (*Caretta caretta*), the olive Ridley (*Lepidochelys olivacea*), the Kemp's Ridley (*Lepidochelys kempii*), the leatherback (*Dermochelys coriacea*), the flatback (*Natator depressus*), and the hawksbill (*Eretmochelys imbricata*; (Lutz et al. 1997). All seven species can be found on the IUCN red list (IUCN 2009), ranging from vulnerable (*L. olivacea*) to endangered (*C. caretta*, *C. mydas*) to critically endangered (*D. coriacea, L. kempii, E. imbricata*). Scientific study of these reptiles began in earnest in the 1950's with Dr. Archie Carr. Since then, interest in this field has grown, and all six continents (excluding Antarctica) now have active sea turtle research programs.

Description

The Loggerhead is distinguishable from other marine turtles by several characteristics. The species was named for its relatively large head, which supports powerful jaws and enables it to feed on hard-shelled prey, such as whelks and conch. Loggerheads possess five lateral scutes on their carapace (top shell), which is slightly heart-shaped and longer than it is wide. The carapace is reddish brown, whereas the plastron (bottom shell) is pale yellow (Dodd 1988). They are considered to be a medium to large turtle, with the mean straight carapace length (SCL) of adults being

approximately 92 cm, and a corresponding weight of about 113 kg. Hatchlings can be brown or gray dorsally, lacking the reddish coloration of adults and juveniles. Their flippers are dark gray to brown with white to white-gray margins. The plastron is usually a yellow-tan. At emergence, hatchlings average 45 mm in length and weigh approximately 20 g.

Habitat and Distribution

Loggerheads have a circumglobal distribution, occurring throughout the temperate and tropical regions of the Atlantic, Pacific, and Indian Oceans (Erhart et al. 2003). They occupy three different ecosystems throughout the course of their lives: the terrestrial zone (where nesting occurs), the neritic zone (coastal seawater), and the oceanic zone (deeper offshore seawater). Once loggerhead nestlings hatch, they head to the ocean and swim until reaching areas of downwelling, which are characterized by high volumes of floating material, like seaweeds (Witherington 2002). These areas may be located just miles offshore from the nesting beach (Lohmann et al. 1994; Lohmann et al. 1996; Lohmann et al. 1999), or may be reached through distant travel on the ocean currents.

Between the ages of 7–12 years, the juvenile loggerheads migrate to near-shore coastal habitats, which are in the neritic zone (Bolten 2003). This habitat represents the foraging ground for this species, and individuals remain in this environment through adulthood.

Reproduction

Nesting occurs from May to August throughout most of the loggerhead's range (Meylan et al. 1995). Individuals are known to nest one to seven times during a season, at intervals of approximately 2 weeks. Loggerheads nest on ocean beaches, generally preferring beaches that are high energy, relatively narrow, steeply sloped, and coarse-grained. Clutch size typically varies from 100–126 eggs, with incubation lasting 45–90 days, depending on temperature. Immediately after hatchlings emerge from the nest, they move from their nest to the surf, swim, are swept through the surf zone, and continue swimming away from land for at least one to several days (U.S. Fish and Wildlife Services 2010). To date, no study has tracked the behaviors of individual neonates in the first few weeks of their lives.

Heavy Metals and the Marine Environment

Heavy metals comprise a group of metallic elements with atomic weights greater than 40 g/mol, such as Nickel (Ni), Copper (Cu), Zinc (Zn), Cadmium (Cd), Mercury (Hg), Chromium (Cr), Iron (Fe), Lead (Pb), and Manganese (Mn; Rand 1995). All of these elements are characterized by similar valence electron distribution. Metalloids are nonmetallic elements that behave like heavy metals. Among these are Selenium (Se) and Arsenic (As; Rand 1995).

Both metals and metalloids demonstrate a tendency to form covalent bonds. This characteristic has two toxicological consequences. First, the ability to bind to organic groups creates lipophilic molecules. This property increases the ability of metals to cross cell membranes, and produces some of the most toxic compounds (i.e., tetraalkyl lead,

methyl mercury, and methylated forms of arsenic). Second, these metals can bind to nonmetallic constituents of cellular molecules, such as the sulphydryl groups of proteins, causing toxic effects (Walker et al. 2006).

Unlike organic pollutants, metals not in organometallic complexes are non-biodegradable, meaning that an organism cannot break them down into less toxic forms. As a result, the only options for dealing with metal accumulation are long-term storage, or, if the organism possess the capacity, excretion (Rand 1995).

Some metals are essential for biological function and only become toxic once they reach a threshold level. These include aluminum (Al), selenium (Se), molybdenum (Mo), cobalt (Co), tin (Sn), strontium (Sr), vanadium (V), iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), chromium (Cr), nickel (Ni) and arsenic (As). Nonessential metals, such as mercury (Hg), cadmium (Cd), barium (Ba), beryllium (Be), lead (Pb), antimony (Sb), titanium (Ti), and thallium (Tl) have increased toxicity due to their ability to compete with essential metals at binding sites in important biological molecules (Walker et al. 2006).

Toxicity varies with water quality and among species (Rand 1995). Aquatic and marine species can be exposed to chemicals through water, sediment, and occasionally air. Developmental stage, dietary factors, physiology, and biochemical functions all influence the degree to which any contaminant is toxic in an organism. Concentration, duration of exposure, and chemical speciation of the element also play important roles in toxicity. Water quality influences toxicity through pH, hardness, and salinity interactions.

Heavy Metals and Sea Turtles

The accumulation of heavy metals in sea turtles is an area of interest for multiple reasons. First, turtles generally occupy a high trophic niche. This position enables them to be used as biological indicators of long-term bioaccumulation in the environment. As a result, data gathered on sea turtles can be used to assess the general contaminant status of the environment in which they forage. Second, all seven species can be found on the IUCN red list (IUCN 2009). It is therefore vital that the impact of pollution on these organisms be understood. Finally, interest in this area of research became prevalent very recently. As a result, the dataset remains sparse and the opportunity for contributing valuable knowledge is high. The studies detailed here were conducted in response to these three factors.

The majority of prior research was conducted on tissue samples including the liver, kidney, and muscle. Occasionally, studies included analysis of the stomach, lung, adipose tissue, pancreas, and spleen. All tissue samples were obtained from deceased turtles. The vast majority of studies used tissue only from turtles where time of death was determined to be less than 24 hrs.

Once collected, tissues are usually stored at -80 C^o until analysis. Some studies stored samples at -20 C^o (Godley et al. 1999; Andreani et al. 2008), but standard methodology indicates -80 C^o as the appropriate temperature for tissue preservation. Next, tissue samples are thawed and weighed. Different studies have utilized differing amounts of tissue; however, most studies use approximately 0.5 grams. Samples are oven dried, then digested with HNO_3 in acid-washed Teflon tubes. Standard reference materials (SRMs) are utilized in all studies, and recovery percentages are reported. There

are a variety of instruments available to analyze metal concentrations. Those utilized in the studies reviewed here include hydride generation atomic absorption spectroscopy (HGAAS; Figure 1-1 (Agusa et al. 2008a), high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS; Figures 1-2,1-3; Agusa et al. 2008a; Agusa et al. 2008b), and flame atomic absorption spectroscopy (Figure 1-1; Storelli et al. 1998; Godley et al. 1999; Gardner et al. 2006; Talavera-Saenz et al. 2007; Andreani et al. 2008). Reduction with $SnCl_2$ and readings via cold vapor atomic absorption spectroscopy (Figure 1-1) is the most common method for mercury analysis (Storelli et al. 1998; Godley et al. 1999; Kampalath et al. 2006). Day et al. utilized cold vapor isotope dilution ICP-MS for quantifying mercury (Figure 1-2; Day et al. 2005; Day et al. 2007). All tissue study findings are reported in μg/g dry weight, excepting Day et al., who reported findings in μg/g wet weight.

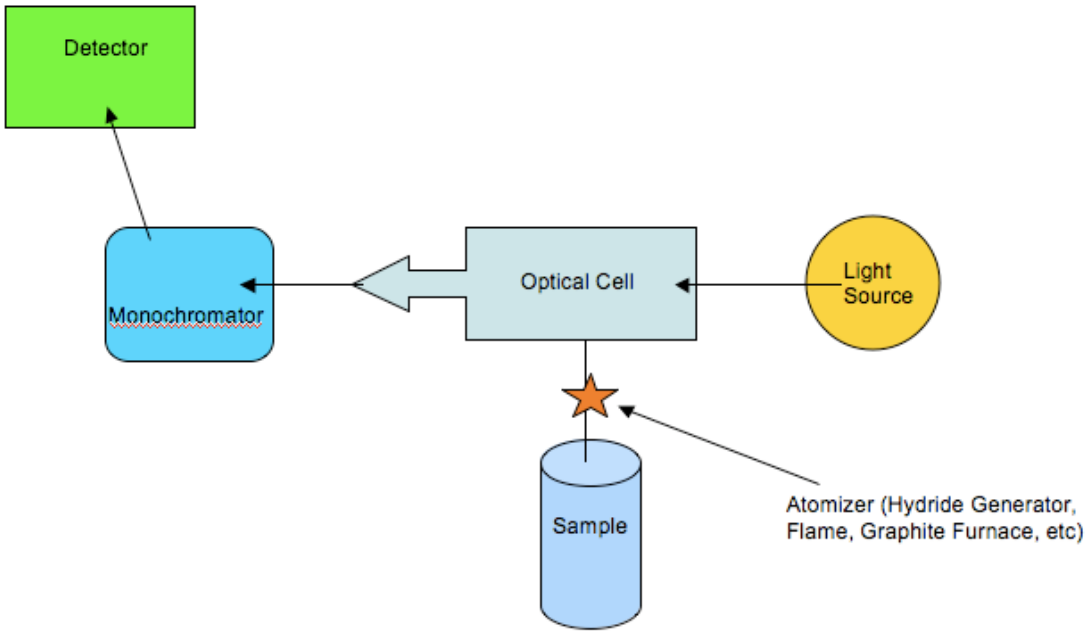

Figure 1-1: Schematic Diagram of an AAS.
The electrons in the atoms are promoted to higher orbitals by the atomizer for a short duration through absorbing a set quantity of energy. The amount of energy absorbed is specific to each element. The amount of energy put into the system by the light source is known, and can be compared with the energy output of the system to determine the concentration of the element.

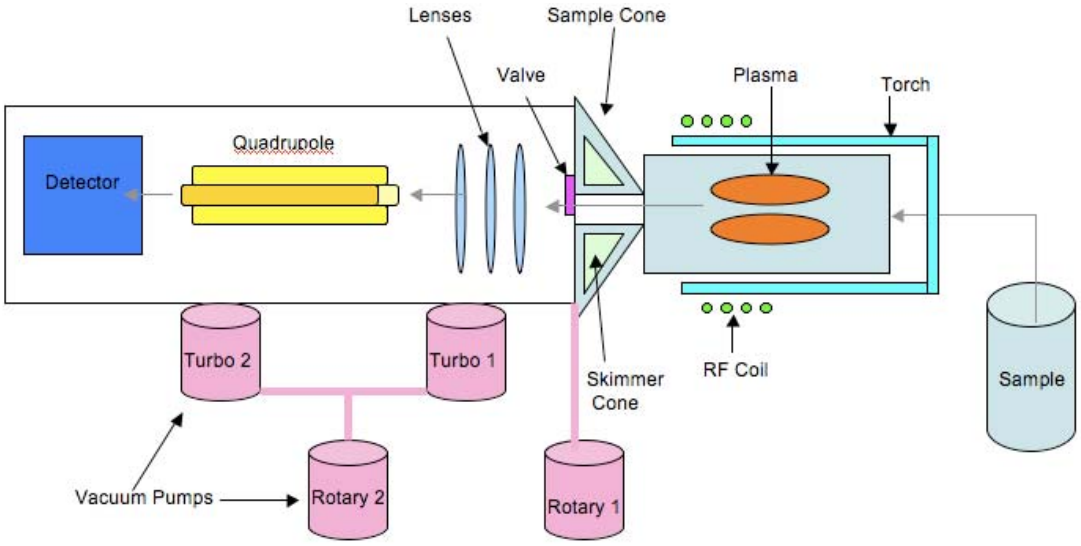

Figure 1-2: Schematic Diagram of an ICPMS. Plasma is a gas containing enough ions and electrons to make it electrically conductive. The torch consists of three tubes that are placed inside a Radio frequency (RF) coil. A flow of gas (typically Argon) is introduced between the two outermost tubes of the torch and an electrical spark is applied to create ions. These ions are rapidly accelerated first in one direction, than the other. They collide with the Argon gas, causing the gas to release an electron, which is in turn rapidly accelerated by the created magnetic field. This process continues until the rate of release of new electrons in collisions is balanced by the rate of recombination of electrons with argon ions. This produces a temperature of around 10,000K. Samples are introduced into this chamber in liquid form through a nebulizer. The liquid evaporates and any solids that were present vaporize and break down into atoms which are then ionized. The ions are extracted into the Mass Spectrophotometer through a series of cones, while the quadrupole separates the ions on the basis of their mass-to-charge ratio. The detector receives a signal proportional to the concentration.

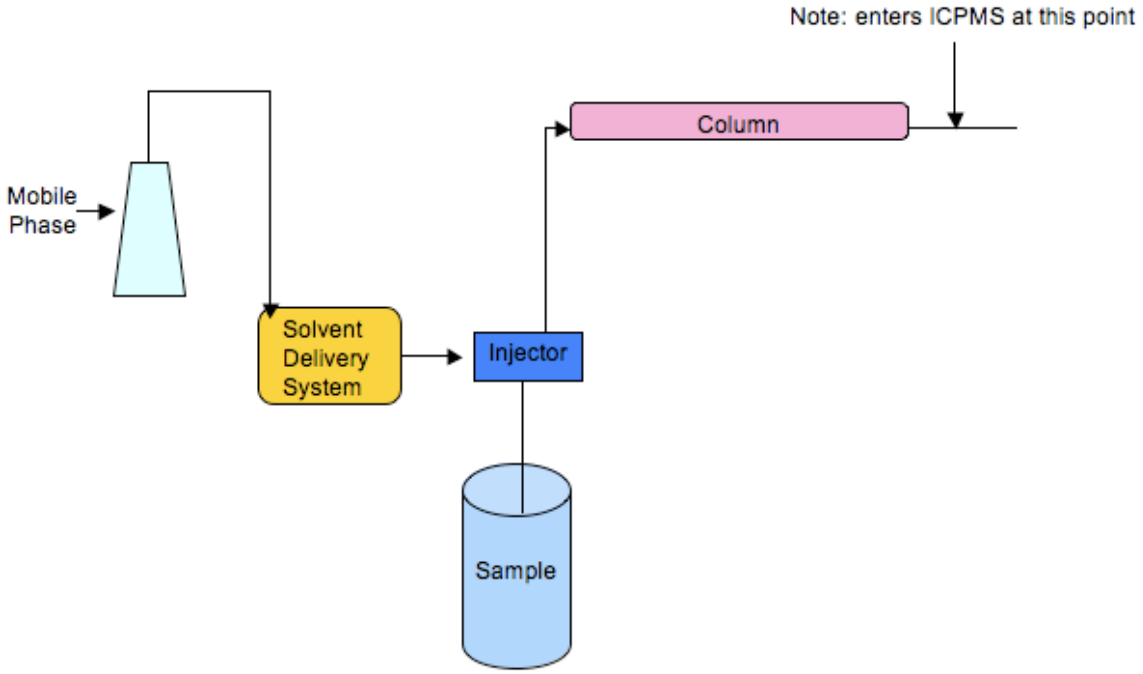

Figure 1-3: Schematic Diagram of a HPLC. The liquid sample is introduced into the system through the injector and the mobile phase. The column is packed with the stationary phase, which slows the progress of the various chemicals in the sample based on chemical and physical interactions between the stationary phase and the sample. This separated the various components of the sample before releasing them to be analyzed. HPLC is not itself an analysis system. It simply separates out chemical compounds to be analyzed.

Arsenic

Arsenic accumulation was studied in *C. mydas*, *C. caretta*, and *E. imbricata* (Storelli et al. 1998; Godley et al. 1999; Andreani et al. 2008; Agusa et al. 2008a; Agusa et al. 2008b). In Agusa et al. (2008a, 2008b), the studies consisted of green turtles gathered by Japanese fishermen for scientific purposes. Arsenic was detected in all tissues analyzed. Liver and kidney concentrations were comparable, and there was a positive correlation noted in As accumulation between these two tissues (Agusa et al. 2008a). Muscle concentrations were significantly higher compared to values found in the liver and kidney (Agusa et al. 2008a; Agusa et al. 2008b). In other studies, similar

relationships were discovered (Storelli et al. 1998; Agusa et al. 2008b). It is notable that, in birds and mammals, there is little As accumulation in the muscle, indicating that the high accumulation of As in the muscle of cheloniids may be family specific (Agusa et al. 2008a; Agusa et al. 2008b).

Chelonia mydas exhibited a significant negative correlation between body size (SCL) and As in the liver (Agusa et al. 2008a; Agusa et al. 2008b), whereas a positive correlation was discovered in *E. imbricata* (Agusa et al. 2008b). The authors attributed the differences to dietary changes between the juvenile and adult in each species. Arsenic (III) was present in the spleen of *E. imbricata* at levels higher than those known to cause endocrine disruption in other organisms (Agusa et al. 2008b); thus, further research should assess the extent of endocrine disruption occurring in this species as a consequence of As accumulation.

Cadmium

Cadmium was studied in *C. caretta*, *C. mydas*, *L. olivacea*, and *E. imbricata* (Storelli et al. 1998; Godley et al. 1999; Gardner et al. 2006; Talavera-Saenz et al. 2007; Andreani et al. 2008). There was only one *E. imbricata* specimen obtained; as a result, the datum were reported but could not be analyzed (Gardner et al. 2006). Andreani et al. (2008) observed higher levels of Cd in *C. mydas* than in *C. caretta*, whereas Godley et al. (1999) documented the opposite. The discrepancy could have resulted from environmental differences, since one study was conducted in Italy (Godley et al. 1999), whereas the other was conducted in the Caribbean (Andreani et al. 2008). Gardner et al. (2006) noted that there were no significant differences in liver metal concentrations

among the three species from Baja California with sufficient data. Cadmium levels were found to be highest in the kidney for all species (Storelli et al. 1998; Godley et al. 1999; Talavera-Saenz et al. 2007). Godley et al. (1999) also studied Cd in nest eggs, where maximum concentrations were recorded in the yolk.

Strong correlations between SCL and metal concentrations were noted in *C. caretta*, but not *C. mydas* or *L. olivacea* (Gardner et al. 2006). However, previous studies noted a strong negative correlation between metal concentrations and SCL in *C. mydas* (Gordon et al. 1998; McKenzie et al. 1999; Saeki et al. 2000; Sakai et al. 2000a). Strong positive correlations among Cd, Pb, and Zn were also noted in liver tissues (Gardner et al. 2006).

Chromium and Selenium

Minimal data are available on both Cr and Se in sea turtles, with levels evaluated in only one study (Storelli et al. 1998), which concluded that Cr was present at high levels in all tissues. Regrettably, this study provides no indication of what the obtained data were compared with to make this statement. No comparable statements were made regarding Se. Both of these elements require further research.

Copper, Iron, and Zinc

Copper, iron, and zinc were studied in *C. caretta*, *C. mydas*, *L. olivacea*, and *E. imbricata* (Gardner et al. 2006; Talavera-Saenz et al. 2007; Andreani et al. 2008). There was no significant difference in liver metal concentrations among the four species studied in Baja (Gardner et al. 2006). A significant positive correlation between SCL and Cu was

noted in the liver of *C. caretta* (Gardner et al. 2006). One study noted substantial levels of Cu in the liver, which the authors postulated to be related to diet (Andreani et al. 2008). The same study also noted that all *C. mydas* specimens analyzed had higher levels of Cu and Fe than *C. caretta*. This observation was attributed to diet, as the algae which *C. mydas* consumes has a higher tendency to bioaccumulate heavy metals than cephalopods, which are the main food source of *C. caretta* in this region (Andreani et al. 2008). One study measured algal metal bioaccumulation in addition to their tissue studies (Talavera-Saenz et al. 2007). No significant difference was noted between Cu or Zn levels in the stomach contents and those obtained from algal samples, while Fe levels were significantly lower in the stomach contents compared to levels observed in algal samples.

Zinc levels found in *C. mydas* were not notably different among kidney, stomach, and liver tissues (Talavera-Saenz et al. 2007). Zinc was most abundant in the adipose tissue of *C. mydas* (Andreani et al. 2008), a finding consistent with other studies. It has also been suggested that the high accumulation of Zn in the adipose tissue of *C. mydas* influences the green pigmentation (Gardner et al. 2006; Andreani et al. 2008), but support of this hypothesis has yet to be obtained.

A potential problem with the Andreani et al. study is that age and gender were not considered in the analysis, as it had been previously determined that these two factors do not significantly influence bioaccumulation (Maffucci et al. 2005). However, there is much evidence to the contrary, as many studies have found statistically significant correlations between SCL, which is associated with age, and metal accumulation in various tissues (Gordon et al. 1998; McKenzie et al. 1999; Sakai et al. 2000a; Sakai et al.

2000b; Gardner et al. 2006; Kampalath et al. 2006; Agusa et al. 2008a; Agusa et al. 2008b). A potential problem with the Talavera-Saenz et al. (2007) study was the time difference between turtle sample and algal sample gathering. Turtle samples were obtained in 2002–2003, while algal samples were collected in 2004–2005. Therefore, the algal samples obtained may or may not be representative of the algae previously consumed by the turtles.

Lead and Manganese

Lead and Mn were studied in *C. caretta*, *C. mydas*, *L. olivacea*, and *E. imbricata* (Storelli et al. 1998; Godley et al. 1999; Gardner et al. 2006; Talavera-Saenz et al. 2007; Andreani et al. 2008). Lead was found to be present at the highest concentrations in the liver (Storelli et al. 1998; Godley et al. 1999). The Talavera-Saenz et al. study found Pb and Mn levels in the stomach to be lower than those found in collected algal samples, but higher than those obtained from the liver. Regrettably, Storelli et al. and Godley et al. did not analyze Pb in the stomach; therefore, no comparisons between their dataset and the Talavera-Saenz et al. findings can be made.

Mercury

Mercury was studied in *C. caretta*, *C. mydas*, and *L. olivacea* (Storelli et al. 1998; Godley et al. 1999; Day et al. 2005; Kampalath et al. 2006; Day et al. 2007; Day et al. 2010). This metal tended to be highest in liver tissue (Storelli et al. 1998; Godley et al. 1999). The Storelli et al. study incorporated age into the comparisons among *C. caretta* specimens, setting adults at 50–100 kg, and youth at 1.8–2.8 kg. Separate correlation

analyses for the two groups noted a stronger correlation between Hg concentration and SCL in juveniles than in adults. The authors postulated that this difference was due to hormones that could potentially change uptake and accumulation mechanisms. This hypothesis has not yet been tested.

Mercury levels found in the Baja, California, population of sea turtles were reportedly lower in comparison with other studies (Kampalath et al. 2006). Differences in accumulation were also noted among species (*L. olivacea* > *C. caretta* > *C. mydas*). Kampalath et al. explained the differences as a consequence of foraging differences. *Caretta caretta* exhibited a positive correlation between total mercury (THg) and SCL, whereas *C. mydas* showed a negative correlation. No correlations were found between body size and THg in *L. olivacea* (Kampalath et al. 2006).

A potential problem with the Kampalath et al. study was the assumption that the turtles being analyzed were healthy. The author states that only turtles caught in fishermen's nets were included in the analysis. By excluding stranded turtles, which are presumed to have died from illnesses, it was assumed that the specimens included in the study did not represent mortally ill individuals. Unfortunately, there was no measurement of animal health to ascertain whether the assumption was sound. Although potential support for Kampalath et al.'s hypothesis can be found in the statement that Hg levels were lower in this study than other studies quantifying Hg (Kampalath et al. 2006), the turtles captured for this study could have occupied an environment containing lower levels of Hg pollution.

Nickel

Nickel was studied in *C. caretta*, *C. mydas*, *L. olivacea*, and *E. imbricata* (Gardner et al. 2006; Talavera-Saenz et al. 2007). Both studies were conducted in Baja, California. No significant results regarding Ni were reported in the Gardner et al. study. The Talavera-Saenz et al. study found that Ni concentrations did not differ between stomach contents and the analyzed algal samples. Nickel concentrations were similar in the liver and kidney, but significantly lower in the stomach (Talavera-Saenz et al. 2007). The Gardner et al. study found a positive correlation between SCL and Ni in the liver of *C. caretta*.

Significance of Metal Accumulation Studies

In light of the previous research within the field of heavy metal contamination in sea turtles, I have conducted a research project utilizing blood as the medium for heavy metal analysis within *C. caretta*. While several of the papers explored in this review utilized *C. caretta* as a study subject, none of the research was performed on live specimens. As a result, there is no information provided in these studies regarding the impact of the observed heavy metal concentrations on the health of the organism. In recent years, the impact of these pollutants on health has become an area of study. At the National Institute for Standards and Technology in Charleston, South Carolina, blood is being utilized to facilitate non-lethal monitoring of mercury with encouraging results (Day et al. 2005; Day et al. 2007).

In the 2005 study, Day et al. tested blood samples and keratinized scutes collected from both live-captured and stranded turtles against liver, kidney, muscle, and spinal cord

tissue collected from the same stranded loggerheads. According to this research, blood levels effectively predicted the total mercury in the muscle and spinal cord, and scute levels corresponded to liver concentrations. The study concluded that the stability of Hg in the scute made it preferable for long-term exposure approximations, whereas the blood was more indicative of recent exposure. The 2007 study focused on blood instead of scutes, and included the monitoring of health parameters. This incorporation enabled comparison of Hg exposure with informative health indicators, such as hematocrit, lysozyme, and lymphocyte proliferation. Statements regarding the physiological effects of metal toxicity to turtles were made previously without supporting evidence (Storelli et al. 2003). As a result, the Day et al. studies marked a turning point in contamination analysis.

In light of these two studies, I sought to utilize blood samples to examine recent exposure levels, and to compare metal and metalloid accumulation with the physiologic state of the animal determined non-lethally. In the next section, I review the studies of health parameters.

Health Parameters in Sea Turtles

Monitoring health indicators in sea turtles is relatively new, with preliminary studies of baseline parameters being initiated fewer than 20 years ago (Jacobson et al. ; Bolten et al. 1992a; Bolten et al. 1994; Aguirre 1996; Day et al. 2005; Day et al. 2007). Health parameters in sea turtles have been monitored in several species, but the ability to control for external influences has been limited. This makes it extremely difficult to determine what factors are affecting the health of these organisms. In fact, little research

has been done relating health parameters to potential causes. The papers detailed here explore health parameters in several species of turtles, and hypothesize about potential causes for the health trends observed.

Investigations regarding baseline health parameters have been conducted in several species of sea turtle, including *C. mydas* (Aguirre et al. 2000), *D. coriacea* (Deem et al. 2006), and *C. caretta* (Casal et al. 2009; Gelli et al. 2009). The earliest of these studies was conducted on *C. mydas* with and without fibropapillomatosis in Hawaii (Aguirre et al. 2000). Two populations of clinically healthy juvenile turtles were studied from Kaneohe Bay (n = 53) and the Kona Coast (n = 37). Turtles with fibropapillomatosis (n = 56) were studied in the Kaneohe Bay area. Turtles were categorized into age/size classes and assigned a fibropapilloma severity score, indicating the presence or absence of the disease, and its severity if present. Blood volumes of 3–10 mL were collected from each individual and stored in lithium heparin vacutainers until processing.

There were 25 different biochemistry analytes examined in this study, including total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), gamma glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine, uric acid, calcium, phosphorus, cholesterol, triglycerides, glucose, iron, sodium, potassium, and chloride.

Several blood enzymatic values were found to differ significantly between both healthy turtle aggregations. The Kona coast group had higher AST and LDH values, whereas the Kaneohe Bay group had higher ALT and AP values. The enzyme ALT also decreased with increasing fibropapilloma severity, whereas AST, AP, and LDH showed

the opposite trend, increasing with fibropapilloma severity. The authors speculated that the difference in enzymatic values between the two healthy groups was the result of a more efficient stress response from the Kona coast group. This argument was based on the fact that fibropapillomatosis had not been diagnosed in the Kona coast population, but had been documented in the Kaneohe Bay population, and fibropapillomatosis was known to be associated with chronic stress and immunosuppression (Aguirre et al. 1995).

Leatherback turtles were studied on the coast of the Republic of Gabon (Deem et al. 2006). To the authors' knowledge, this study represented the first published baseline hematology, plasma biochemistry, and plasma protein values to be published on clinically healthy nesting *D. coriacea*. Blood samples ranging from 5–24 mL were collected from the hind flipper of nesting leatherbacks and stored in lithium heparinized tubes until further analysis. Biochemical parameters analyzed included ALT, amylase, AST, BUN, calcium, cholesterol, carbon dioxide, CK, creatinine, GGT, glucose LDH, lipase, phosphorus, potassium, sodium, total protein, triglyceride, uric acid, and corticosterone. Samples were also tested for organochlorine contaminants (OC's) and polychlorinated biphenyls (PCB's). Additionally, small sample subsets were tested for arsenic (n = 9), lead (n = 9), and mercury (n = 6).

In this study, lower eosinophil counts were obtained than those reported in greens and loggerheads (Arnold 1994; Work et al. 1999). The authors speculated that this might be due to the high level of epibiotic parasites commonly found on greens and loggerheads in comparison to leatherbacks (Deem et al. 2006). Cholesterol and triglyceride levels were found to be higher in nesting leatherbacks than in juvenile wild green turtles (Bolten et al. 1992a) or free ranging loggerheads (Bolten et al. 1992b). These differences could

be due to the physiological changes that accompany nesting. The OC's and PCB's were below detectible limits, possibly due to the dietary preference of leatherbacks for jellyfish, which occupy a low trophic level and subsequently do not bioaccumulate high levels of these chemicals. Arsenic levels were below detectible limits in all but one turtle. Mercury and lead levels were reported to be low and unlikely to cause significant health effects.

Caretta caretta has been studied recently off the coast of Italy (Gelli et al. 2009) and in the eastern Atlantic (Casal et al. 2009). The Italy study examined 65 adult loggerheads that were delivered to the Sea Turtle Rescue Center of Linosa. These turtles were rehabilitated and determined to be clinically healthy before samples were obtained. Samples were taken from the external jugular vein and stored in vacutainer tubes until further analysis. Biochemical parameters included glucose, GGT, ALT, AST, AP, CK, LDH, cholesterol, triglyceride, calcium, phosphorus, total bilirubin, urea, uric acid, creatinine, total protein, and albumin. The AST and LDH values were higher than those previously reported in the literature, whereas triglycerides were found to be lower (Campbell 1996; Wilkinson 2004).

The eastern Atlantic study focused on juvenile loggerheads from the Canary Islands and nesting adults from Cape Verde (Casal et al. 2009). A blood volume of 2 mL was collected from the cervical sinus, then stored in lithium heparin vacutainers until further analysis. Biochemical parameters in this study included total protein, albumin, globulins, calcium, triglycerides, uric acid, glucose, total cholesterol, urea, total bilirubin, creatinine, LDH, AST, ALT, and AP. Regarding enzymes, no significant differences were noted besides LDH activity, which was significantly higher in adult turtles.

Day et al. (2007) conducted a study on *C. caretta* from the eastern coast of the United States to look at the health implications of blood mercury concentrations. Samples were collected according to Antech Diagnostic's specifications, and sent to the laboratory in Memphis, TN for a reptilian blood panel. Results included measures of hematocrit, total protein, albumin, globulin, glucose, urea nitrogen, uric acid, AST, CPK, calcium, phosphorus, sodium, potassium, chlorine, lymphocyte counts, and heterophil counts. This investigation found that total blood mercury concentrations were positively related to measures of hematocrit and CPK, and negatively related to measures of AST, heterophils, and lymphocytes. The study concludes that, relative to what is found in other species, low levels of mercury may be related to altered physiological parameters in *C. caretta*.

Research Objectives

The studies detailed above represent much of the recent work conducted on health parameters in sea turtles. The data set remains sparse, and the studies made little attempt to gather data that would provide insight into the causes of the observed health trends. In light of this, a study correlating heavy metal accumulation with health parameters is clearly needed. Heavy metals are known to cause significant health effects in seabirds, which share the same marine environment (Fujihara et al. 2004; Ikemoto et al. 2005). It is therefore reasonable to hypothesize that heavy metals will have physiologically similar effects on sea turtles.

This thesis developed from collaboration with the National Institute of Standards and Technology (NIST) in Charleston, South Carolina. In the summer of 2008, these researchers collected blood samples from loggerhead sea turtles greater than 5 kg body

weight with a 21 ga needle from the dorsal cervical sinus. Heretofore, this research group had quantified only Hg levels in the blood samples. My contribution to the study was to quantify up to 20 additional metals in samples sent to me under the terms of a collaborative agreement approved by both NIST and Loma Linda University. I obtained measurements of these samples using the inductively coupled plasma mass spectrometer housed at the Institute for Integrated Research on Materials, Environment and Society on the campus of CSU Long Beach. I then subjected the data to statistical analyses to identify potential relationships between heavy metal concentrations and select biological parameters.

The objectives of this thesis are: 1) to provide the first reported blood heavy metal concentrations for metals other than mercury in *C. caretta*; 2) to assess how metal and metalloid accumulations vary with turtle body size and differs between the sexes; 3) to compare heavy metal concentrations with geographic location and depth of water at which turtles were captured; and 3) to compare the metal concentrations with blood physiological parameters, with the intent to identify possible associations. Through these two studies, we hope to gain insight into the accumulation features of various heavy metals and their potential health effects.

Chapter two examines several biological factors that potentially influence metal concentrations, including body size, sex, and the environment from which the turtle was captured. While several previous studies examined the correlations between heavy metal concentrations in storage organs and straight carapace length, no studies have compared blood heavy metal concentrations to measures of body size, excepting the Day et al. (2005, 2007, 2010) studies investigating mercury. This study revealed relationships

between measures of body size and a number of different metals, providing valuable insight into the accumulation features of several heavy metals. It also showed the absence of differences between males and females in accumulation of heavy metals. Moreover, an environmental contribution to heavy metal accumulation was suggested by significant differences between the two primary collecting localities (South Carolina and Georgia/Florida) and by significant associations of several metals with water depth.

Chapter three examines the correlations between heavy metal concentrations and a number of blood-derived physiological parameters. Metal concentrations were subject to principle component analyses, and then compared with individual physiological parameters. Several significant models resulted, with the stronger relationships being with indicators of liver function.

Collectively, these studies are unprecedented in that they represent the first reported blood concentrations for any metal other than mercury in this species, the first reported values for aluminum, antimony, barium, molybdenum, strontium, tin, and titanium in any sea turtle tissues, and the first attempt to determine the health implications of any heavy metal other than mercury in sea turtles. The results of this research enhance our understanding of how heavy metal pollution potentially affects health in sea turtles.

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CHAPTER TWO

RELATIONSHIPS BETWEEN BODY SIZE, SEX, GEOGRAPHIC LOCATION, WATER DEPTH, AND HEAVY METAL CONCENTRATION IN LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*)

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Abstract

To date, little research investigating heavy metal exposure in live sea turtles has been conducted. Since sea turtles are an endangered species, we chose to utilize a non-lethal method of determining heavy metal exposure. We measured concentrations of 17 metals and metalloids in the blood of 81 live loggerhead sea turtles (*Caretta caretta*) captured off the coasts of South Carolina and Georgia/Florida. These concentrations were then examined to identify possible sources of variation, including straight carapace length, weight, sex, geographic location, and water depth of capture location. We found significant positive correlations between turtle body size and arsenic, chromium, titanium, and zinc. This may suggest bioaccumulation or ontogenetic changes in physiology or ecology that alters metal regulation or exposure. We detected no differences for any metal concentrations between the sexes. Several metals varied with geographic location, including barium, cobalt, chromium, and zinc. Two additional metals, molybdenum and strontium, were positively associated with water depth. While there are a variety of potential explanations for the significant relationships identified, the data obtained in this study were unable to provide conclusive answers as to why these associations exist. Further research is needed to elucidate the specific mechanisms of metal biomagnification and potential toxicity in loggerhead sea turtles.

Introduction

Oceanic pollution is an area of growing concern. Much of the waste produced on land eventually ends up in marine ecosystems. Fertilizer, oil, and industrial waste are all examples of pollutants that accumulate in the watershed systems. Run-off from these

watersheds eventually makes it to the ocean. Once in the marine environment, these pollutants can travel for long distances in ocean currents and be transferred through the food chain.

The loggerhead (*Caretta caretta*) is one of seven marine species of turtles (Lutz et al. 1997). Recently, *C. caretta*'s status was upgraded from vulnerable to endangered (IUCN 2010). Sea turtles face a multitude of threats, including habitat loss (Jones 1990; Clarke et al. 2000), by-catch (Lewison et al. 2004; Lewison et al. 2007), and poaching (Dodd 1988; Hutchinson et al. 1991; Laurent et al. 1996). While these threats persist, several recent studies suggest that pollution also poses an increasing danger to sea turtle populations (Hutchinson et al. 1992; Lutcavage et al. 1997).

In response to concerns regarding pollution, studies have been conducted on deceased specimens of *C. caretta* in an attempt to quantify heavy metal pollution accumulating in their tissues (Storelli et al. 1998; Godley et al. 1999; Gardner et al. 2006; Andreani et al. 2008). Samples for these inquiries were collected off the coast of Baja California, Mexico, in the Pacific Ocean; off Italy in the Adriatic Sea; and off northern Cyprus in the Mediterranean. Collectively, these studies investigated the concentrations of 11 metals and metalloids (i.e,. heavy metals, including arsenic [As], cadmium [Cd], chromium [Cr], Copper [Cu], iron [Fe], lead [Pb], manganese [Mn], mercury [Hg], nickel [Ni], selenium [Se], and zinc [Zn]) in various tissues. The findings revealed positive correlations of Hg and Cd with turtle mass (Storelli et al. 1998), and positive correlations of Cd, Cu, Fe, Pb, Mn, Ni, and Zn with turtle length (Gardner et al. 2006).

Studies have recently examined the presence of mercury in *C. caretta* (Day et al. 2005; Day et al. 2007; Day et al. 2010), but there has been no research investigating the

accumulation of other metals in this species. Given the lack of information in the literature regarding the accumulation of metals in this species, there is a definite need for data documenting heavy metal concentrations in loggerheads.

In the present study, we measured the concentrations of 17 different metals and metalloids in the blood of 81 loggerhead sea turtles captured off the Atlantic coast of South Carolina and Georgia/Florida, USA. We then examined several factors that potentially these influence metal concentrations, including body size, sex, and the environment from which the turtle was captured.

Materials and Methods

Sample Collection

Free-ranging sub-adult and adult loggerhead sea turtles (n = 81) were captured as part of a 2008 endocrinology study (Arendt et al. 2009) off the coasts of South Carolina (SC, n = 35) and Georgia/Florida (n = 46; Fig. 2-1). Before release, several biometric and environmental parameters were obtained for each turtle, including straight carapace length (SCL, nearest 0.1 cm) mass (nearest 1 kg), sex (determined by testing blood testosterone levels), mean water depth (nearest 0.1 m), water surface temperature (nearest 0.1 °C), and release location. Blood samples were collected according to the methods detailed in the Arendt et al. (2009) report. Samples were stored at -80^{o}C until further analysis.

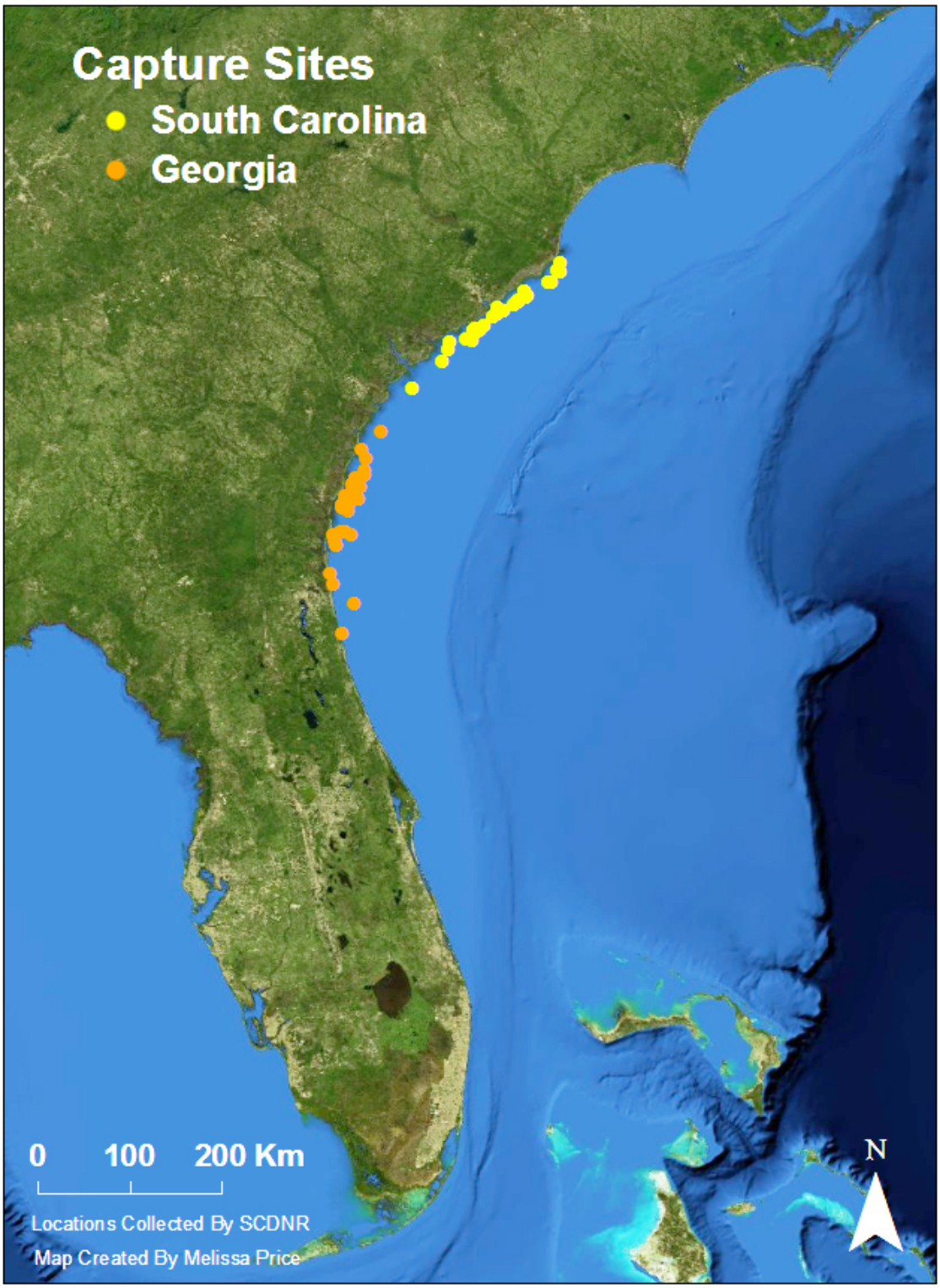

Fig. 2-1. Locations of loggerhead sea turtle (*Caretta caretta*) samples in this study (*n* = 81).

Sample Analyses

Samples were analyzed using inductively coupled plasma–mass spectrometry (ICP-MS; Agilent Hewlett-Packard 4500 Plus Series, Agilent Technologies, Inc., Santa Clara, CA, USA) on an instrument housed at the Institute for Integrated Research in Materials, Environments, and Society (IIRMES), California State University, Long Beach. Sample analysis was conducted following a modified version of EPA 200.8, which had been revised by IIRMES for use with blood. The ICP-MS was tuned before use using a low (lithium, Li), medium (yttrium, Y), and high (thorium, Th) weight element to ascertain instrument sensitivity at these points. An initial demonstration of performance was used to characterize instrument performance and laboratory performance prior to the analysis of samples. This involved establishing linear calibration ranges for each analyte at seven different concentrations. Method detection limits and reporting limits were established for each of the 21 metals being analyzed. The minimum detection limit (MDL) was calculated as follows: MDL = (t) x (S), where t is the student's t value for a 99% confidence level and standard deviation with n-1 degrees of freedom, and S is standard deviation of the replicates analyses. Reporting limits, calculated by IIRMES, were used to determine which values to label as non-detectable (ND) for each metal. A reporting limit was established by taking the mean value of the blanks used and adding three times the standard deviation of this mean to the MDL. Samples were analyzed on three separate days in batches of 27 samples run concurrently with the corresponding blanks as controls, and the calibration curves.

Samples were removed from the -80^{o}C storage freezer and allowed to thaw. Next, 500 µL of the sample was pipetted into a 15 mL plastic vial. We then pipetted 500 µL of

concentrated HNO_3 and 250 µL of concentrated HCl to the sample vials and to separate blank vials. All vials were then placed in a water bath heated to 75°C for a minimum of two hours. After the tissues within samples were digested, 200 µL of an internal standard containing rhodium (Rh) and thulium (Tm) were added. Samples and blanks were then diluted to 10 mL with 2% HNO_3 and stored in styrofoam racks on the counters in the IIRMES facility until analysis.

Three blanks were created with each set of 27 samples processed. One blank contained only the internal standards (Rh and Tm), whereas the other two were spiked with 50 µL of a multi-elemental standard containing 100 mg/L each of aluminum (Al), As, boron (B), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), Cr, cesium (Cs), Cu, Fe, Mn, molybdenum (Mo), Ni, Pb, antimony (Sb), Se, tin (Sn), strontium (Sr), titanium (Ti), thallium (Tl), vanadium (V), and zinc (Zn; SPEX CertiPrep Custom Built Standard, Lot # 4-115CR; SPEX CertiPrep Inc., Metuchen, NJ, USA). Percent blank contributions can be seen in Appendix I. A blank spike and blank spike duplicate were analyzed with each batch of samples. Percent recovery values can be found in Appendix I.

A standard curve was created using dilutions of the multi-elemental stock solution. Sample concentrations run to create the curve included 0, 10, 50, 100, 500, 1000, and 5000 ng/mL. The results of these curves can be seen in Appendix II. A calibration check with a concentration of 500 µL was created using a secondary source multi-elemental standard (SPEX CertiPrep Instrument Calibration Standard 2, Lot # 8-27JB). Percent recoveries for calibration checks can be seen in Appendix I. Quality control for aluminum data did not meet expectations on day 3, so the aluminum data for

the third day were excluded from these analyses. Acid rinses were run between the standard curve, unknown samples, blanks, and known sample checks. A continuing calibration check was run every ten samples using the secondary source calibration check discussed above. These checks were within 15% of the initial calibration curve value. A duplicate analysis of one sample was included in every batch of samples run. Results for these duplicate analyses can be found in Appendix I. Sample preparation was performed by Ashley Register. Operation of the ICPMS and data analysis software was performed by Andrew Hamilton. Adjusted metal concentrations obtained from these analyses can be found in Appendix III. All capture, physiological, and raw metal data are shown in Appendix IV.

Statistical Analyses

All statistical analyses were conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) with $\alpha = 0.05$. All metal concentrations below reporting limits were labeled non-detectable (ND) and discarded from the analyses. After eliminating all values below the reporting limits, the elements Cr, Cu, Sr and Zn were deemed normally distributed. We normalized all other metals using rank transformation (Al, As, Co, Ni, Pb, Sn) or natural log (ln) transformation (Ba, Cd, Mn, Mo, Sb, Se, Ti). Beryllium thallium, and vanadium were excluded from further analyses due to the majority of samples being below reporting limits. Turtle mass and SCL did not require transformation. However, water depth was negatively skewed, and was normalized by ln transformation of reflected data.

Because assumptions were largely met, we relied on parametric tests, including Pearson's correlations (*r*), independent samples *t*-tests, analyses of variance (ANOVA),

and analyses of covariance (ANCOVAs; Zar 1996). We also employed a non-parametric Chi-square test (Zar 1996). We further computed effect sizes, which are independent of sample size (in contrast to statistical significance) and more readily compared across data sets and different studies. For pairwise comparisons (*t*-tests), we calculated Cohen's *d* using pooled standard deviation (Hojat et al. 2004), for which values of ~ 0.2 are considered small, ~ 0.5 moderate, and ≥ 0.8 large effects (Cohen 1988). We expressed bivariate correlations (Pearson's *r*) as coefficients of determination (r^2), with values of ~ 0.1 regarded small, ~ 0.09 moderate, and ≥ 0.25 large (Cohen 1988). For ANOVAs and ANCOVAs, we computed partial eta-squared (η^2), with values of ~ 0.01 deemed small, ~ 0.06 moderate, and ≥ 0.14 large (Cohen 1988). For Chi-square, we used phi (φ), with values of ~0.1 small, ~0.3 moderate and ≥0.5 large. The terms small, moderate, and large are used loosely.

Following Perneger (1998) and Nakagawa (2004), we did not apply Bonferroni adjustments of alpha to the multiple tests. However, considering the high experiment-wise error resulting from multiple tests, we interpreted significant outcomes with appropriate caution.

Results

Summary statistics for heavy metals can be seen in Table 2-1. We could not run omnibus models that included every single variable because of sample size issues resulting in unacceptably low statistical power. Accordingly, we first analyzed heavy metals with respect to intrinsic properties of the sea turtles (body size and sex), and then analyzed potential relationships between heavy metals and environmental variables.

Table 2-1: Summary statistics (ppm) for heavy metals measured in *Caretta caretta*. ND = non-detectable.

Element	*n*	Abbrev	Range (ppm)	Mean ± SD
Aluminum	32	Al	ND–0.16	0.06 ± 0.04
Arsenic	81	As	3.32–46.12	11.17 ± 6.01
Barium	80	Ba	0.004–0.40	0.10± 0.08
Cadmium	81	Cd	0.004–0.41	0.04 ± 0.06
Cobalt	48	Co	ND–0.13	0.007 ± 0.02
Chromium	81	Cr	0.18–0.65	0.4 ± 0.10
Copper	81	Cu	0.29–0.81	0.58 ± 0.09
Manganese	74	Mn	ND–0.11	0.031 ± 0.02
Molybdenum	81	Mo	0.01–0.29	0.05 ± 0.04
Nickel	62	Ni	ND–0.033	0.005 ± 0.005
Lead	76	Pb	ND–0.05	0.01 ± 0.007
Antimony	81	Sb	0.02–0.45	0.08 ± 0.08
Selenium	81	Se	1.18–8.45	3.49 ± 1.59
Tin	81	Sn	0.002–0.24	0.01 ± 0.03
Strontium	81	Sr	0.34–0.85	0.55 ± 0.10
Titanium	81	Ti	0.03–0.72	0.23 ± 0.14
Thallium	6	Tl	ND–0.046	0.01 ± 0.02
Zinc	81	Zn	4.76–15.99	10.50 ± 2.00

Associations with Body Size

We obtained bivariate Pearson's correlations between the two measures of sea turtle body size (SCL, mass) and each of the heavy metal concentrations (Table 2-2). Mass and SCL explained similar variance in metal concentrations, with SCL providing higher r^2 values for 10 metals and mass providing higher values for seven metals (Fig. 2-2). Five heavy metals were significantly associated with body size (Table 2-2). Mass was positively correlated with four metals (As, $r^2 = 0.053$; Cr, $r^2 = 0.067$; Pb, $r^2 = 0.088$; Ti, $r^2 = 0.062$). Length (SCL) was positively correlated with two metals (Pb, $r^2 = 0.120$; Zn, $r^2 = 0.053$). Effect sizes (r^2 values) were in the low to moderate range (0.000–0.120).

Table 2-2: Correlations (*r*) of metal concentrations with *Caretta caretta* body length (SCL) and mass, and results of ANCOVA models for sex and body length.

Metal	*n*	Pearson's *r*		Sex[a]			Body Length[a]		
	♂♂.♀♀	SCL	Mass	*F*	*P*	η^2	*F*	*P*	η^2
Al	26, 6	0.095	-0.006	0.07	0.798	0.002	0.25	0.620	0.009
As	56, 18	0.197	**0.230***	3.00	0.088	0.040	3.84	0.054	0.051
Ba	56, 17	0.108	0.130	0.54	0.467	0.008	1.42	0.237	0.020
Cd	56, 18	-0.207	-0.172	0.08	0.776	0.001	3.12	0.082	0.042
Co	33, 11	-0.200	-0.086	0.17	0.678	0.004	2.15	0.150	0.050
Cr	56, 18	0.209	**0.258***	0.54	0.464	0.008	4.55	**0.036**	0.060
Cu	56, 18	-0.019	-0.011	1.26	0.265	0.017	0.01	0.919	0.000
Mn	50, 18	0.123	0.113	0.20	0.660	0.003	1.40	0.242	0.021
Mo	56, 18	0.137	0.174	0.33	0.568	0.005	1.31	0.257	0.018
Ni	41, 14	-0.101	-0.148	0.49	0.487	0.000	0.95	0.335	0.018
Pb	52, 17	**0.347****	**0.297****	0.23	0.630	0.004	12.41	**0.001**	0.158
Sb	56, 18	0.202	0.178	0.45	0.505	0.006	3.66	0.060	0.049
Se	56, 18	-0.133	-0.132	0.51	0.476	0.007	1.24	0.269	0.017
Sn	56, 18	0.116	0.183	0.13	0.722	0.002	0.77	0.384	0.011
Sr	56, 18	-0.168	-0.089	0.66	0.420	0.009	2.01	0.161	0.027
Ti	56, 18	0.205	**0.249***	0.06	0.805	0.001	4.24	**0.043**	0.056
Zn	56, 18	**0.230***	0.203	0.10	0.749	0.001	4.31	**0.041**	0.057

< 0.05

** $p < 0.01$

[a] Partial η^2 values are reported

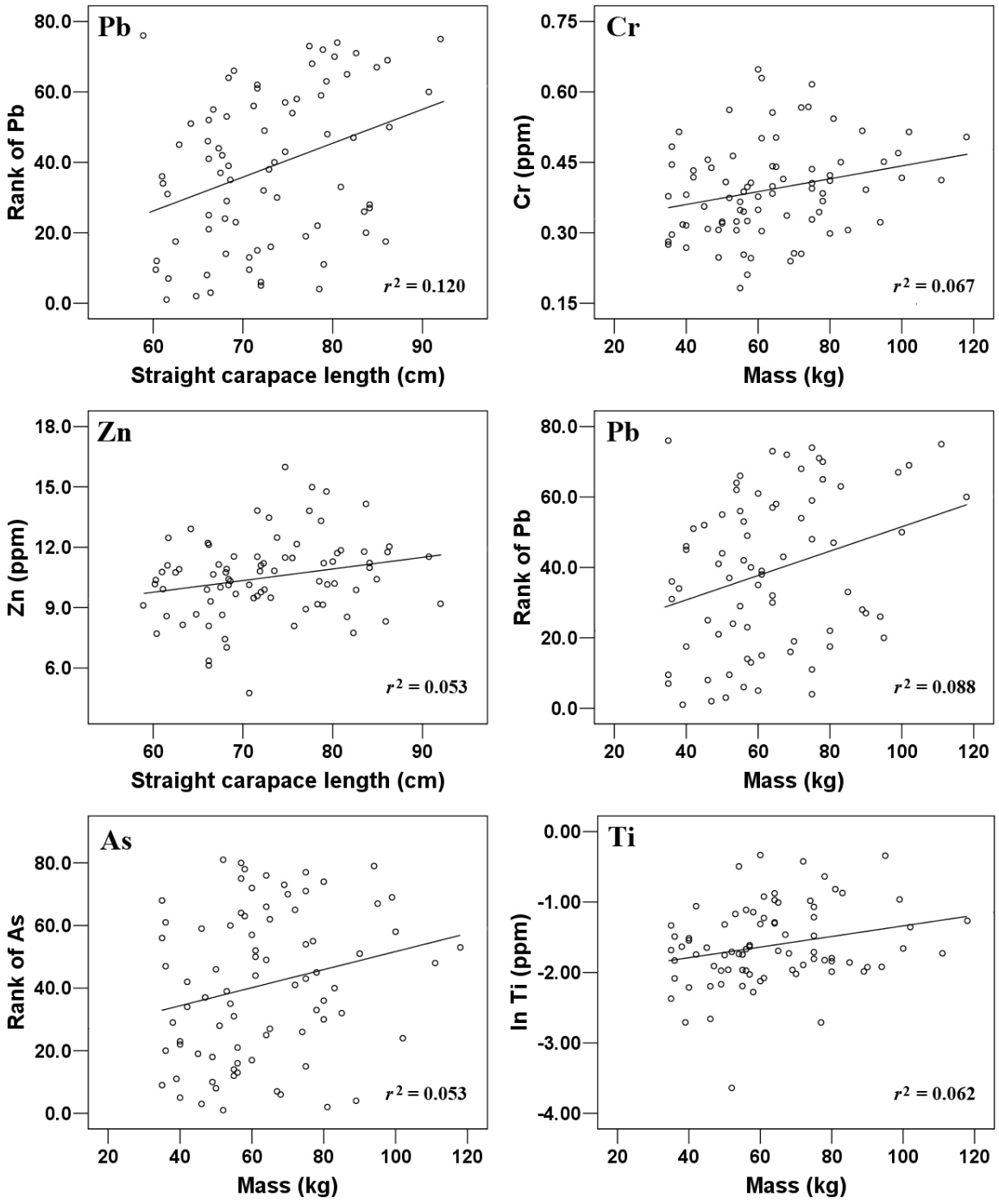

Figure 2-2: Significant relationships between five metals (As, Cr, Pb, Ti, Zn) and measures of body size in *Caretta caretta* (mass: $n = 80$; straight carapace length: $n = 81$).

Differences between Sexes

We used analysis of covariance (ANCOVA) models to compare the relative effects of sex and body size on heavy metal concentrations (Table 2-2). Sex was treated as a between-subjects factor, and body size as a covariate. Mean (± 1 SE) body size did not differ significantly between sexes (mass: male 63.1 ± 2.4 kg, female 61.3 ± 5.8 kg, t = -0.33, df = 71, p = 0.74, Cohen's d = 0.14); SCL: male 72.7 ± 1.1 cm, female 71.5 ± 2.2 cm, t = -0.52, df = 72, p = 0.61, Cohen's d = 0.09). Separate ANCOVAs for each metal revealed no difference between the sexes (Table 2-2). Body size explained significant variation in Cr, Pb, Ti, and Zn, which corresponded well with the bivariate correlations. Effect sizes were consistently small for sex (partial η^2 values = 0.000–0.040), but varied from small to large for body size (0.000–0.158), with Pb having by far the strongest association with body size. Effect sizes for body size exceeded those of sex for 16 (94.1%) of the 17 heavy metals.

Potential Environmental Effects

We examined two potential environmental sources of variation: location (SC and GA/FL) and depth of water at which the turtle was captured. Turtles from the two locations were captured at similar water depths (GA/FL: 11.5 ± 0.4 m, n = 35; SC: 10.9 ± 0.4 m, n = 39), and males and females were captured at similar water depths (males: 11.2 ± 0.5 m, n = 18; females: 11.2 ± 0.4 m, n = 56) at both locations (2 x 2 ANOVA: all P > 0.71 and all partial η^2 < 0.009 for main effects and interactions of location and sex, which were treated as between-subjects factors). Furthermore, body size of both sexes was similar at the two locations (separate 2 x 2 ANOVAs of mass and SCL: all P > 0.31 and

all partial $\eta^2 < 0.013$ for main effects and interactions of location and sex), and the sex ratio was similar at the two locations (80.0% and 71.8% male at SC and GA/FL, respectively; $\chi^2 = 0.41$, df = 1, $P = 0.41$, $\varphi = 0.10$).

We used ANCOVA models to evaluate the effects of location and water depth on heavy metal concentrations. Location was treated as a between-subjects variable and water depth as a cofactor. Because sex differences proved to be non-significant for every metal and showed no bias for water depth and location, we excluded it from these models, which increased our sample size by the addition of turtles for which sex was not determined. We included in the models two additional variables to account for their potential influences: day of sample processing, which was partially confounded with turtle location (day 1: 27 samples from SC; day 2: 8 samples from GA/FL, 19 samples from GA/FL; day 3: 27 samples from GA/FL; treated as a between-subjects factor), and SCL (as a cofactor). Because of missing cells in the location x day of processing matrix, we used type IV sum of squares for computation (Green et al. 1999) and examined the data graphically to verify interpretation of main effects. We computed Pearson's *r* to interpret the direction of significant relationships between water depth and heavy metal concentration.

Results of the ANCOVA models for metals having sufficient samples are shown in Table 2-3. Four (25%) of 16 metals with sufficient data differed significantly between the two capture sites, with effect sizes in the moderate range. Metals Ba, Cr, and Zn showed higher blood levels off the SC coast, whereas Co showed higher levels off the GA/FL coast (Fig. 2-3). Two additional metals, Mo ($r^2 = 0.076$, $n = 81$) and Sr ($r^2 = 0.046$, $n = 81$), were positively associated with water depth (Fig 2-4).

Table 2-3. Results of ANCOVA models for effects of location (South Carolina versus Georgia/Florida) and water depth on heavy metal concentrations in *Caretta caretta*.

Metal	*n*	Location[a]			Water Depth[a]		
		F	*P*	η^2	*F*	*P*	η^2
As	80	1.39	0.243	0.018	0.34	0.561	0.005
Ba	79	9.74	**0.003**	0.118	0.02	0.882	0.000
Cd	80	1.22	0.273	0.016	0.27	0.603	0.004
Co	47	4.65	**0.037**	0.102	0.31	0.581	0.008
Cr	80	5.42	**0.021**	0.069	3.71	0.058	0.048
Cu	80	2.27	0.136	0.030	0.02	0.886	0.000
Mn	73	0.15	0.701	0.002	1.57	0.215	0.023
Mo	80	0.03	0.864	0.000	7.59	**0.007**	0.093
Ni	61	0.36	0.550	0.007	1.56	0.217	0.028
Pb	75	0.04	0.847	0.001	0.13	0.717	0.002
Sb	80	3.44	0.068	0.044	1.13	0.253	0.018
Se	80	3.72	0.058	0.048	0.16	0.689	0.002
Sn	80	0.02	0.897	0.000	3.05	0.085	0.040
Sr	80	1.29	0.260	0.017	4.29	**0.042**	0.055
Ti	80	0.02	0.900	0.000	0.84	0.364	0.011
Zn	80	5.25	**0.025**	0.066	0.34	0.537	0.005

[a] Partial η^2 values are reported

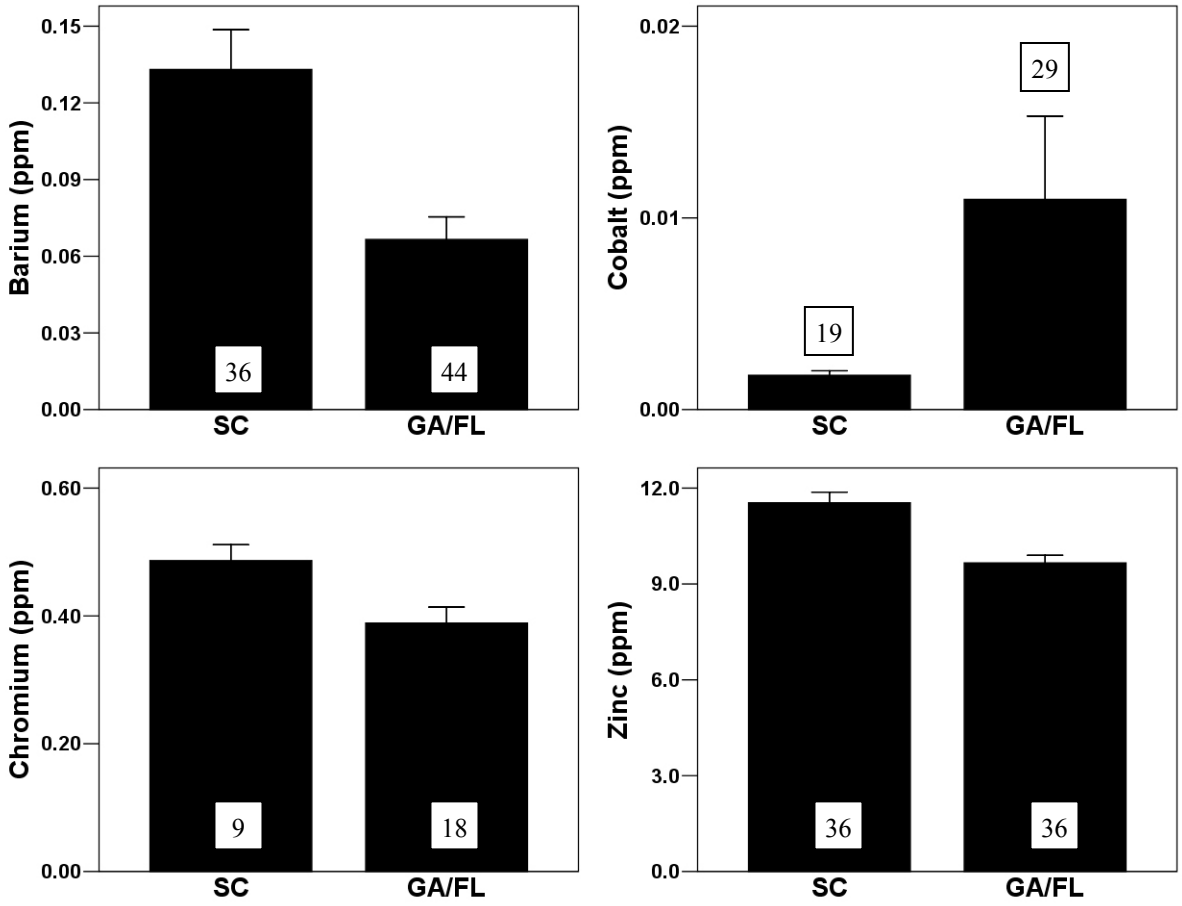

Fig. 2-3. Mean (+ 1 SE) plasma levels of metals that differed significantly between Loggerhead turtles (*Caretta caretta*) captured at South Carolina (SC) and Georgia/Florida (GA/FL) locations. Sample sizes are shown in the boxes. Results for chromium reflect those captured only on day 2 (due to day of processing variation).

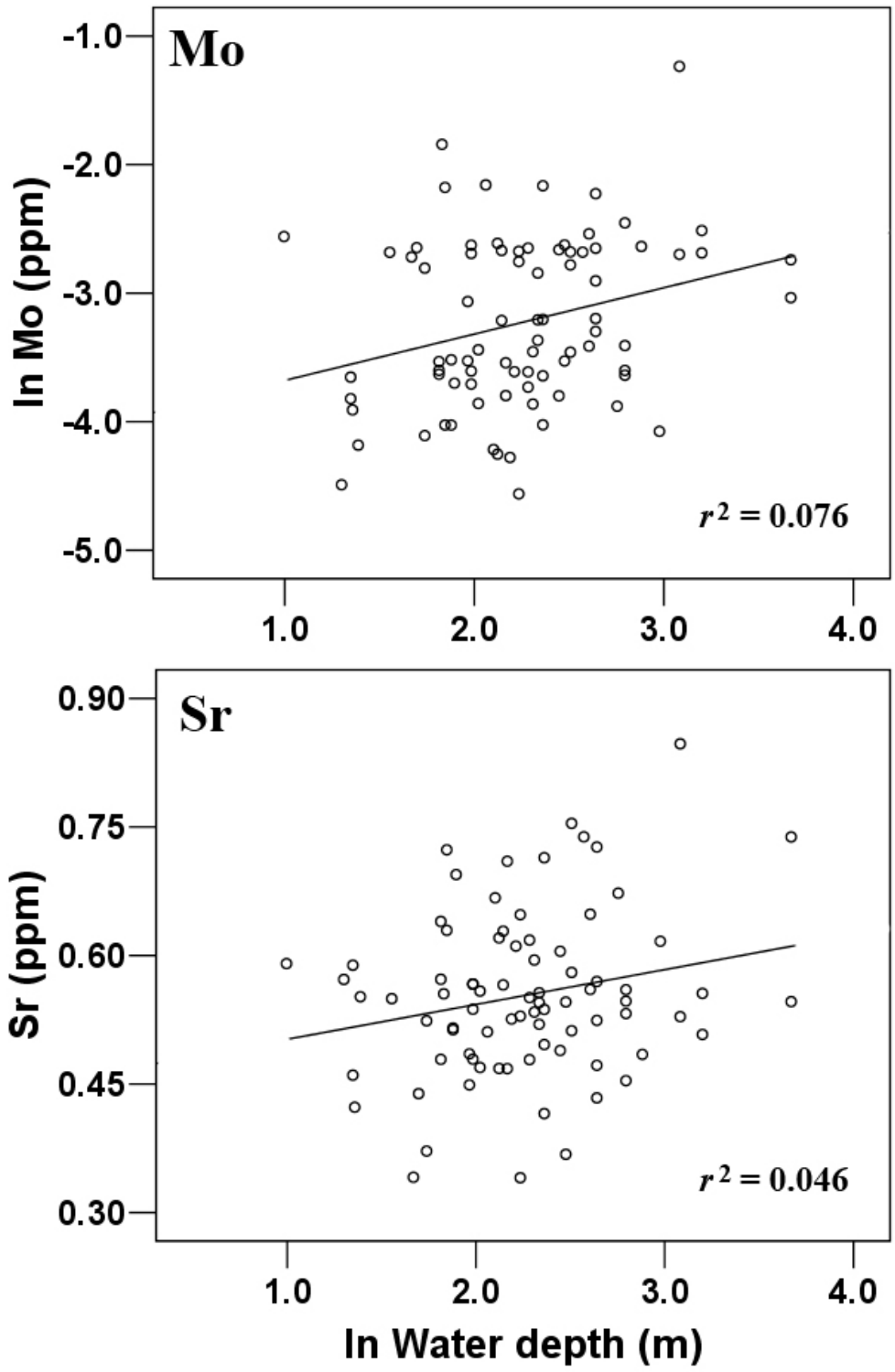

Figure 2-4: Significant relationships between two metals (Mo, Sr) and water depth in *Caretta caretta* (n = 81 for both metals). Water depth was reflected prior to natural log (ln) transformation to correct for negative skew.

Discussion

This study examined the largest number of heavy metals and metalloids to date for any investigation of chelonians. As a consequence, our study resulted in a high experiment-wise error rate. Nevertheless, the number of significant effects we identified exceeded that expected by chance alone (5% of all tests with alpha set at 0.05). Although Bonferroni adjustments of alpha are often recommended to avoid making type I errors (incorrectly identifying a correlation as significant), they often cause more problems than they solve (Perneger 1998; Nakagawa 2004), including failure to identify meaningful relationships (type II errors). Our identification of six metals significantly associated with turtle body size (of 17 examined), and six metals significantly related to environmental variables (of 16 examined), suggests that real relationships exist. In more practical terms, we should emphasize effect sizes, which are more informative than strict statistical significance and are largely independent of sample size.

Effect sizes for heavy metal associations with body size were in the low to moderate range (r^2 = 0.000–0.120), suggesting relatively weak relationships. Previous studies in sea turtles involving diverse species, tissues, and metals showed effect sizes in the moderate to large range (r^2 = 0.173–0.556; see below). We suggest that the weaker relationships found in our study were the result of blood being analyzed rather than a storage tissue.

We found five heavy metals to be significantly associated with turtle body size. Positive correlations existed between body size and accumulations of arsenic, chromium, lead, titanium, and zinc. Previous studies have suggested that variation in diet and metabolism between juvenile and adult turtles could influence patterns of metal

accumulation (Storelli et al. 1998; Gardner et al. 2006; Agusa et al. 2008a), and that regional ecological differences could affect which metals relate to body size within the same species (Storelli et al. 1998). We postulate that these factors may be the primary influences in the relationships between body size and metal accumulation observed in this study.

Saeki et al. (2000) looked at arsenic in deceased *C. mydas* and *E. imbricata* specimens. No relationship between growth and As concentration was noted in *C. mydas*, but a significant negative relationship between As and SCL was seen in *E. imbricata*. A study conducted in freshwater whitefish and trout showed that exposure to higher concentrations of As was related to decreasing mass (Pedlar et al. 2002). It is not yet understood why As was positively correlated with SCL in this study. Previous studies in sea turtles found no relationships between chromium and measures of body size. However, positive relationships between Cr and growth have been observed previously in birds, which share some aspects of physiology with reptiles. Broiler chickens, for example, show increasing Cr levels with body weight gain (Sahin et al. 2003). To our knowledge, no literature provides insight into the positive relationships of either lead or titanium with body size, but the relationships suggest accumulation with age.

Zinc is known to be essential for living organisms. It is an integral part over 200 metalloenzymes and other metabolic compounds, ensuring the stability of many biological molecules and structure (Casey et al. 1980; Leonard et al. 1989). Dietary zinc uptake is highly variable in animals, but absorption generally increases with low body weight (Eisler 1993). We observed the opposite effect in our study. However, the higher

levels of Zn observed in larger animals could be due to dietary and/or metabolic differences.

The associations with body size that we deciphered add to a growing body of knowledge on heavy metal accumulation in sea turtles, which we review in Table 2-4. Previous studies reported species-specific differences in the direction of correlation. Studies in *C. mydas*, for example, showed negative correlations between measures of body size and As in the liver ($r^2 = 0.321$), between body size and Cd in muscle ($r^2 = 0.362$), and between body size and Hg in both the muscle ($r^2 = 0.435$) and kidney ($r^2 = 0.254$; (Saeki et al. 2000; Sakai et al. 2000a; Kampalath et al. 2006; Agusa et al. 2008a; Agusa et al. 2008b). Studies in *E. imbricata*, by contrast, revealed positive correlations between body size and As in the liver ($r^2 = 0.412$; Agusa et al. 2008b). Prior relationships observed between heavy metals and body size in *C. caretta* were consistently positive, including Cd, Cu, and Ni in the liver and kidney (Gardner et al. 2006), and Hg in adipose tissue ($r^2 = 0.556$), muscle ($r^2 = 0.334$), scutes ($r^2 = 0.188$), and blood ($r^2 = 0.173$; (Day et al. 2005; Kampalath et al. 2006).

We found no differences between sexes in the concentrations of heavy metals. By using ANCOVA models, we were able to compare the effect sizes of sex, which were always small (partial $\eta^2 = 0.000–0.040$), with those of SCL, which were generally small (0.000–0.051) except for those metals showing significant associations (0.056-0.158). The absence of differences between the sexes was anticipated, as no study to date has identified such differences in sea turtles (Keller et al. 2004a; Keller et al. 2004b; Maffucci et al. 2005; Burger et al. 2008), and males and females exhibit similar body sizes (Ripple 1996; Seaworld 2011). Nevertheless, behavioral and physiological

differences between the sexes could conceivably lead to different encounter and/or accumulation rates of heavy metals.

Results of the environmental analyses are difficult to assess, as no information regarding water or sediment concentrations of heavy metals was obtained in conjunction with this study. Moreover, we do not know how long the turtles resided in the general vicinity of capture relative to the exposure and accumulation rates of the heavy metals. However, it is interesting that concentrations of barium, chromium, and zinc were higher in the individuals captured in South Carolina waters compared to those captured near Brunswick, GA. Brunswick has four Superfund sights, which were formerly heavily contaminated toxic waste sites (EPA 1995). These include LCP Chemicals Georgia, Inc., Brunswick Wood Preserving, Hercules 009 Landfill, and Terry Creek Dredge Spoil. An investigation into the sediment toxicity of the tributary that LCP Chemicals discharged their waste into revealed high levels of Hg, Cr, Pb, and Zn (Winger et al. 1993). A study conducted on tidal creek and marsh sediments in South Carolina costal estuaries found significantly higher levels of Cu, Cr, Pb, Zn, Cd, and Hg associated with urban and industrial watersheds (Sanger et al. 1999). Sanger et al. also found that the enrichment of these trace metals appears to be mainly related to development activities. Unfortunately, there is not enough information contained in our study to explain why we saw higher concentrations at the South Carolina capture sights in three of the four significantly related metals.

The associations observed between capture trawl depth and blood metal concentrations are even more difficult to understand. While sediment studies have shown that some trace metal concentrations do vary with water depth (Iricanin et al. 1990), why

we would see a relationship with blood metal concentrations remains unclear. Sea turtles do not live at certain depths; rather, they move throughout the water column, and must come to the surface for air. Nevertheless, they do feed on the bottom, where several heavy metals may accumulate in the invertebrate prey they consume. Further investigation is required to clarify the relationships seen between molybdenum, strontium, and trawl depth.

Table 2-4: Summary of heavy metal accumulation studies conducted in Sea Turtles.

Element	Species	Tissue	Range (ppm)	Significant Relationship to Body Size	Source	Sample Size
Al	*C. caretta*	B	ND–0.16		This study	*n* = 32
As	*C. caretta*	B	3.33–46.12	+B(SCL)	This study	*n* = 74
		Li, Lu, K, M	0.83–56.55 (Li); 10.62–44.93 (Lu); 6.09–139.60 (K); 11.21–136.6 (M)		Storelli et al. 1998	*n* = 12
	C. mydas	Li	0.44–5.34	-Li(SCL)	Saeki, et al. 2000	n= 20
		M, K, Li,	11.2–165 (M); 4.6–44.3 (K); 0.9–9.7 (Li)	-Li(SCL)	Agusa et al. 2008a	*n* = 20
		M, K, Li, I, Lu, Sp, St	<0.02–58.4 (M); <0.02–11.7 (K); <0.02–2.1 (Li); <0.02–5.15 (I); <0.02–6.67 (Lu); <0.02–7.04 (Sp); <0.02–2.36 (St)	-Li(SCL); -M(SCL)	Agusa et al. 2008b	*n* = 20
	E. imbricata	Li, K, M, Ey, H, Lu, Sp, St	<0.02–13.2 (Li); <0.02–29.1 (K); <0.02–139 (M); <0.02–12.3 (Ey); <0.02–9.39 (H); <0.02–18.0 (Lu); <0.02–7.71 (Sp); <0.02–8.90 (St)	+Li(SCL); +K(SCL); +M(SCL)	Agusa et al. 2008b	*n* = 11
Ba	*C. caretta*	B	0.00–0.4		This study	*n* = 73
Cd	*C. caretta*	B	ND–0.02		This study	*n* = 74
		Li, Lu, K, M	3.06–20.23 (Li); 0.32–10.50	+Li(m); +Lu(m);	Storelli et al. 1998	*n* = 12

		(Lu); 0.39–64 (K); 0.09–2.21 (M)	+K(m);+M(m)		
	Li, K, M, A	ND–30.62 (Li); 13.72–140 (K); ND–1.45 (M); 0.2–1.37 (A)	+Li(SCL); +K(SCL)	Gardner et al. 2006	*n* = 5
	Li, Lu, K, M, G, H, P	[a] 2.4 (Li) [a] 1.4 (Lu) [a] 5.8 (K) [a] 0.81 (M) [a] 1.3 (G) [a] 2.2 (H) [a] 2.6 (P)		Andreani et al. 2008	*n* = 11(Li) 9(K) 10(M) 3(G) 3(Lu) 3(H) 2(P)
	Ha, Em, Y, Al, Li, K, M	ND–1.45 (Ha); ND–1.09 (Em); 0.23–0.56 (Y&Al); 5.14–12.97 (Li); 18.80–42.20 (K); 0.30–1.43 (M)		Godley et al. 1999	*n* = 7 n^h = 48
C. mydas	Li, K, M, A	ND–102 (Li); 6.09–653 (K); ND–39.24 (M); ND–1.47 (A)		Gardner et al. 2006	*n* = 11
	Li, K, A	[a] 10.6 (Li) [a] 39.2 (K) [a] 0.113 (A)		Andreani et al. 2008	*n* = 33(K) 34(Li) 28(A)
	K, Li	65.08–653 (K); ND–72.57 (Li)		Talavera-Saenz et al. 2007	*n* = 8
	Ha, Em, Y, Al, Li, K, M	ND–0.94 (Ha); ND–0.93 (Em); 0.05–1.22 (Y&Al); 2.53–10.73 (Li); ND (K); 0.12–0.78 (M)		Godley et al. 1999	*n* = 6; n^h = 69
E. imbricata	Li, K, M, A	0.49 (Li); 4.20 (K); 1.02 (M); 0.43 (A)		Gardner et al. 2006	*n* = 1

	L. olivacea	Li, K, M, A	4.98–148 (Li); 0.81–274 (K); ND–8.85 (M); 0.33–2.54 (A)		Gardner et al. 2006	*n* = 6
Co	*C. caretta*	B	ND–0.13		This study	*n* = 44
Cr	*C. caretta*	B	0.18 - 0.65	+B(SCL)	This study	*n* = 74
		Li, Lu, K, M	0.2–2.07 (Li); 0.38–5.41 (Lu); 0.20–6.80 (K); 0.30–2.89 (M)		Storelli et al. 1998	*n* = 12
Cu	*C. caretta*	B	0.29–0.81		This study	*n* = 74
		Li, K, M, A	16.6–58.98 (Li); 1.39–8.23 (K); ND–3.44 (M); 0.53–1.15 (A)	+K(SCL)	Gardner et al. 2006	*n* = 5
		Li, Lu, K, M, G, H, P	[a] 17.5 (Li) [a] 3.76 (Lu) [a] 5.56 (K) [a] 2.4 (M) [a] 5.28 (G) [a] 8.96 (H) [a] 4.28 (P)		Andreani et al. 2008	*n* = 11(Li) 9(K) 10(M) 3(G) 3(Lu) 3(H) 2(P)
	C. mydas	Li, K, M, A	6.79–133 (Li); 1.59–20.36 (K); ND–13.76 (M); ND–9.48 (A)		Gardner et al. 2006	*n* = 11
		Li, K, A	[a] 100 (Li) [a] 8.34 (K) [a] 0.446 (A)		Andreani et al. 2008	*n* = 33(K) 34(Li) 28(A)
		K, Li	1.98–11.6 (K); 6.79–128 (Li)		Talavera-Saenz et al. 2007	*n* = 8
	E. imbricata	Li, K, M, A	2.47 (Li); 3.89 (K); 3.68 (M); 0.72 (A)		Gardner et al. 2006	*n* = 1
	L. olivacea	Li, K, M, A	16.99–100 (Li); 0.81–53.40 (K); 0.7–4.37 (M); 0.47–2.54 (A)		Gardner et al. 2006	*n* = 6

Hg						
	C. caretta	B, Sc, Spc, Li, K, M	0.005–0.188 (B); 0.062–2.837 (Sc); 0.037–0.229 (Spc); 0.346–1.336 (Li); 0.132–0.436 (K); 0.049–0.499 (M)	+B(m); +Sc(m)	Day, et al. 2005	*n* = 34
		B	0.006–0.077		Day, et al. 2007	*n* = 66
		B, Sc			Day, et al. 2010	*n* = 16(B) 44(Sc)
		Li, Lu, K, M	0.35–3.72 (Li); 0.12–0.97 (Lu); 0.30–1.53 (K); 0.17–1.81 (M)	+Li(m); +Lu(m); +K(m);+M(m)	Storelli et al. 1998	*n* = 12
		Li, K, M, A	0.116–0.179 (Li); 0.075–0.108 (K); 0.018–0.041 (M); 0.0002–0.028 (A)	+A(SCL); +M(SCL)	Kampalath et al. 2006	*n* = 23
		Ha, Em, Y, Al, Li, K, M	ND–0.75 (Ha); ND–0.22 (Em); 0.16–0.57 (Y&Al) 0.82–7.50 (Li); 0.13–0.80 (K); ND–1.78 (M)		Godley et al. 1999	*n* = 7; n^h = 48
	C. mydas	Li, K, M, A	0.026–0.153 (Li); 0.003–0.31 (K); 0.003–0.059 (M); ND–0.011 (A)	-M(SCL); -K(SCL)	Kampalath et al. 2006	*n* = 42
		Ha, Em, Y, Al, Li, K, M	ND–0.24 (Ha); ND–0.12 (Em); ND–0.19 (Y&Al) 0.27–1.37 (Li); ND (K); ND–0.37 (M)		Godley et al. 1999	*n* = 6; n^h = 69
	L. olivacea	Li, K, M, A	ND–0.795 (Li) ND–0.372 (K)		Kampalath et al. 2006	*n* = 23

			ND–0.144 (M) ND–0.156 (A)			
Mn	*C. caretta*	B	ND–0.11		This study	*n* = 68
		Li, K, M, A	0.11–8.60 (Li); 2.37–9.97 (K); ND–5.4 (M); 0.8–3.2 (A)		Gardner et al. 2006	*n* = 5
		Li, Lu, K, M, G, H, P	[a] 7.48 (Li) [a] 1.22 (Lu) [a] 7.01 (K) [a] 1.35 (M) [a] 2.46 (G) [a] 1.95 (H) [a] 1.34 (P)		Andreani et al. 2008	*n* = 11(Li); 9(K); 10(M); 3(G); 3(Lu); 3(H); 2(P)
	C. mydas	Li, K, M, A	ND–6.74 (Li); ND–8.12 (K); ND–7.75 (M); ND–0.79 (A)		Gardner et al. 2006	*n* = 11
		Li, K, A	[a] 8.92 (Li) [a] 5.75 (K) [a] 0.826 (A)		Andreani et al. 2008	*n* = 33(K); 34(Li); 28(A)
		K, Li	ND–7.73 (K); ND–5.31 (Li)		Talavera-Saenz et al. 2007	*n* = 8
	E. imbricata	Li, K, M, A	0.74 (Li) 7.62 (K) 1.78 (M) 2.53 (A)		Gardner et al. 2006	*n* = 1
	L. olivacea	Li, K, M, A	ND–9.2 (Li) 3.93–7.52 (K) ND–4.34 (M) 0.88–3.65 (A)		Gardner et al. 2006	*n* = 6
Mo	*C. caretta*	B	0.01–0.29		This study	*n* = 74
Ni	*C. caretta*	B	ND–0.033		This study	*n* = 55
		Li, K, M, A	ND–3.26 (Li) ND–3.38 (K) ND–0.65 (M) ND–0.163 (A)	+K(SCL)	Gardner et al. 2006	*n* = 5
	C. mydas	Li, K,	ND–7.74 (Li);		Gardner et	*n* = 11

		M, A	ND–26.43 (K) ND–4.0 (M) ND–13.42 (A)		al. 2006	
		K, Li	1.19–25.13 (K); ND–30.88 (Li)		Talavera-Saenz et al. 2007	*n* = 8
	E. imbricata	Li, K, M, A	2.48 (Li) 1.61 (K) ND (M) ND (A)		Gardner et al. 2006	*n* = 1
	L. olivacea	Li, K, M, A	ND–3.88 (Li) ND–2.46 (K) ND–0.41 (M) ND–0.51 (A)		Gardner et al. 2006	*n* = 6
Pb	*C. caretta*	B	ND – 0.05	+B(m, SCL)	This study	*n* = 69
		Li, Lu, K, M	ND–3.38 (Li); ND–1.10 (Lu); ND–1.35 (K); ND–0.74 (M)		Storelli et al. 1998	*n* = 12
		Li, K, M, A	ND (Li) ND–69.89 (K) ND–157 (M) ND		Gardner et al. 2006	*n* = 5
		Li, Lu, K, M, G, H, P	[a] 0.1 (Li) [a] ND (Lu) [a] 0.1 (K) [a] ND (M) [a] 0.05 (G) [a] ND (H) [a] ND (P)		Andreani et al. 2008	*n* = 11(Li); 9(K); 10(M); 3(G); 3(Lu); 3(H); 2(P)
		Ha, Em, Y, Al, Li, K, M	ND–10.56 (Ha); ND–6.48 (Em); ND–3.93 (Y&Al); ND–4.90 (Li); ND–4.90 (K); ND–5.53 (M)		Godley et al. 1999	*n* = 7; n^h = 48
	C. mydas	Li, K, M, A	ND (Li) ND–0.36 (K) ND–1.23 (M) ND–1.11 (A)		Gardner et al. 2006	*n* = 11
		Li, K, A	[a] 0.07 (Li)		Andreani et al. 2008	*n* = 33(K);

			[a] 0.044 (K) [a] 0.063 (A)			34(Li); 28(A)
		K, Li	ND–1.74 (K); ND–0.07 (Li)		Talavera-Saenz et al. 2007	*n* = 8
		Ha, Em, Y, Al, Li, K, M	ND–3.86 (Ha); ND–3.41 (Em); ND–1.61 (Y&Al); ND–1.85 (Li); ND (K); ND–2.45 (M)		Godley et al. 1999	*n* = 6; n^h = 69
	E. imbricata	Li, K, M, A	ND (Li) ND (K) 0.38 (M) ND (A)		Gardner et al. 2006	*n* = 1
	L. olivacea	Li, K, M, A	ND (Li) ND–2.63 (K) ND (M) ND (A)		Gardner et al. 2006	*n* = 6
Sb	*C. caretta*	B	0.02 – 0.45		This study	*n* = 74
Se	*C. caretta*	B	1.18 – 8.45		This study	*n* = 74
		Li, Lu, K, M	2.12–27.44 (Li); 4.12–30.52 (Lu); 5.73–15.57 (K); 6.51–15.45 (M)		Storelli et al. 1998	*n* = 12
Sn	*C. caretta*	B	0.00 – 0.24		This study	*n* = 74
Sr	*C. caretta*	B	0.34 – 0.85		This study	*n* = 74
Ti	*C. caretta*	B	0.03 – 0.72	+B (m)	This study	*n* = 74
Zn	*C. caretta*	B	4.76 – 15.99	+B(SCL)	This study	*n* = 74
		Li, K, M, A	42.45–91.87 (Li); 2.68–130 (K); 0.63–100 (M); 0.53–44.76 (A)		Gardner et al. 2006	*n* = 5
		Li, Lu, K, M, G, H,	[a] 103 (Li) [a] 75 (Lu)		Andreani et al. 2008	*n* = 11(Li); 9(K);

	P	[a] 119 (K) [a] 105 (M) [a] 100 (G) [a] 186 (H) [a] 141 (P)		10(M); 3(G); 3(Lu); 3(H); 2(P)
C. mydas	Li, K, M, A	1.32–166 (Li) 1.59–330 (K); 10.44–134 (M); 19.51–163 (A)	Gardner et al. 2006	*n* = 11
	Li, K, A	[a] 82.5 (Li) [a] 77.4 (K) [a] 62.1 (A)	Andreani et al. 2008	*n* = 33(K); 34(Li); 28(A)
	K, Li	102–281 (K); 41.81–109 (Li)	Talavera-Saenz et al. 2007	*n* = 8
E. imbricata	Li, K, M, A	25.89 (Li) 82.45 (K) 102 (M) 42.39 (A)	Gardner et al. 2006	*n* = 1
L. olivacea	Li, K, M, A	18.66–85.75 (Li); 0.43–114 (K); 49.89–107 (M); 0.41–16.65 (A)	Gardner et al. 2006	*n* = 6

Abbreviations: Intestine (I); Eyeball (Ey); Lung (Lu); Heart (H); Spleen (Sp); Liver (Li); Stomach (St); Kidney (K); Adipose (A); Blood (B); Gonads (G); Hatchling (Ha); Pancreas (P); Embryo (Em); Scute (Sc); Yolk (Y); Spinal Chord (Spc); Albumen (Al);
m = mass
SCL = Straight Carapace Length
ND = Non-detectable
Range: [a] indicates a mean value, as no ranges were reported in the study
Relationships: + indicates a positive relationship
- indicates a negative relationship
Sample Size: [h] indicates hatchling; all others are sub-adult and adult

Future Considerations

To the best of our knowledge, this study is the first to find significant correlations between measures of body size and arsenic, chromium, lead, titanium and zinc in *C. caretta*. The number of significant correlations in this study and others suggest that blood metal concentrations have an important relationship to body size in sea turtles (Saeki et al. 2000; Sakai et al. 2000a; Gardner et al. 2006; Kampalath et al. 2006; Agusa et al. 2008a; Agusa et al. 2008b). Several other studies have also reported correlations between tissue concentrations of various metals and body size in *C. mydas*, *L. olivacea*, *D. coriacea*, *E. imbricata*, and *C. caretta* (Storelli et al. 1998; Godley et al. 1999; Gardner et al. 2006; Andreani et al. 2008). Several interesting associations were also observed between blood metal concentrations and environmental parameters. Four metals appeared to have significant variance between capture location, and two showed associations with capture depth. Unfortunately, there are no data regarding the sediment or water concentrations of heavy metals during these captures. As a result, the meaning of these findings remains unclear.

The only studies done investigating the health effects of metal pollution on *C. caretta* considered mercury exclusively (Day et al. 2007; Day et al. 2010). Both Godley et al. (1999) and Storelli et al. (2003) concluded that metal levels observed in their specimens were not high enough to affect the health of the sea turtles. However, without direct data to inform one about the health status of the organism and a poor understanding of the relative sensitivity of different taxa to various contaminants, which may have cumulative effects, it seems inadvisable to make such a claim. Gardner et al. (2006) stated that information regarding the toxicological impact of cadmium in reptiles was

lacking, and suggested that further research to understand the influence of cadmium on reptile health was necessary. We contend that further research regarding the health impacts of all heavy metal pollution in reptiles is necessary. This will enable us to better understand how the turtles are handling the pollution burden they are experiencing, which is a vital aspect of properly managing these endangered creatures.

Conclusions

1. First study to find significant correlations between measures of body size and arsenic, chromium, lead, titanium, and zinc in *C. caretta.*
2. The number of significant correlations observed in this study and others suggest that metal concentrations have an important relationship to body size in sea turtles.
3. No differences were detected between the sexes for any of the metals analyzed.
4. Several metals showed different levels of accumulation at two collection localities.
5. Several metals were found at higher levels in turtles that were captured in deeper water.
6. The number of significant relationships between heavy metals and environmental variables suggests that the environment can influence metal levels in the blood of turtles.
7. Next step: a study investigating the potential health impacts of observed metal concentrations.

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CHAPTER THREE

ASSOCIATIONS OF ESSENTIAL AND NON-ESSENTIAL HEAVY METALS WITH PHYSIOLOGICAL PARAMETERS IN THE LOGGERHEAD SEA TURTLE (*CARETTA CARETTA*)

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Abstract

The presence of heavy metals in the marine environment has attracted heightened attention in recent years. Related to this is a growing concern regarding the occurrence of these metals in marine organisms and their potential role in the deteriorating health of the world's oceans. Given the globally endangered conservation status of the loggerhead sea turtle (*Caretta caretta*), understanding the role of contaminants in sea turtle health is of primary importance. Blood obtained from adult and sub-adult loggerhead sea turtles captured along the southeastern coast of the United States was analyzed for 20 different heavy metals and 30 physiological parameters. Regression analyses of the principle components extracted from the essential metals and the non-essential metals revealed significant associations between these metals and several physiological parameters. We found that albumin, creatinine phosphokinase (CPK), and thyroxine (T4) were positively related to a principle component containing arsenic and mercury. The enzyme CPK was also positively associated with a factor comprised of barium, lead, antimony, and titanium. Chloride concentrations were positively related to a factor containing cadmium and strontium, while thyroxine was negatively related to this factor. Absolute monocytes showed the only relationship observed with essential metals in this study, having a negative association with a principle component containing cobalt, chromium, and nickel. Further research is needed to determine if these associations represent a toxicological threat to the health of *C. caretta*.

Introduction

The loggerhead (*Caretta caretta*) is one of seven species of sea turtles (Lutz et al. 1997). All seven species can be found on the IUCN red list (IUCN 2009), ranging from vulnerable (*L. olivacea*) to critically endangered (*D. coriacea, L. kempii, E. imbricata*). Recently, the IUCN upgraded *C. caretta*'s status from vulnerable to endangered (IUCN 2010). Sea turtles face many threats, including habitat loss (Jones 1990; Clarke et al. 2000) and poaching (Dodd 1988; Hutchinson et al. 1991; Laurent et al. 1996). While these hazards continue to be grave, several recent studies have indicated that pollution also poses an increasing threat to sea turtle populations (Hutchinson et al. 1992; Lutcavage et al. 1997).

On the eastern coast of the United States, studies have recently been conducted investigating the impacts of organic contaminants (Keller et al. 2004; Keller et al. 2004a; Keller et al. 2004b; Keller et al. 2005; Keller et al. 2006) and mercury (Day et al. 2005; Day et al. 2007; Day et al. 2010) on sea turtles, but there has been no regional research investigating the effects of other metals on these organisms. Given the global conservation status of *C. caretta* as threatened or endangered and the known potential for heavy metals to have damaging effects on marine vertebrates (Bull et al. 1983; Nicholson et al. 1983; Rawson et al. 1993; Work et al. 1996; Fujihara et al. 2004; Ikemoto et al. 2005), understanding the role of contaminants in sea turtle health is paramount.

In the present study, we sought to evaluate the relationships between heavy metal contamination, physiological parameters, body size, and sex in loggerhead sea turtles from the Atlantic coast of the southeastern United States. We measured the concentrations of 20 different metals and metalloids (i.e., heavy metals) in the blood of

wild-caught loggerhead sea turtles (n = 81), and obtained clinical measures for 30 physiological parameters. Although we cannot demonstrate cause-effect relationships with our approach, we can identify potentially meaningful associations, which constitutes a reasonable first step toward exploring cause-effect relationships.

Materials and Methods

Sample Collection

As part of an endocrine study being conducted in the summer of 2008, free-ranging sub-adult and adult loggerhead sea turtles (n = 81) were captured off the coasts of South Carolina (n = 35) and Georgia/Florida (n = 46; Arendt et al. 2009; Fig. 2-1). Several biometric and environmental parameters were obtained for each turtle before release, including straight carapace length (SCL, nearest 0.1 cm), mass (nearest 1 kg), sex (determined by testing blood testosterone levels), mean water depth (nearest 0.1 m), water surface temperature (nearest 0.1 °C), and release location. Blood samples were collected according to the methods detailed in the Arendt et al. (2009) report, then stored at -80^{o}C until further analysis.

Heavy Metal Analyses

We quantified the blood concentrations of 20 heavy metals listed with their abbreviations in Table 3-1. Sample analysis was conducted following a modified version of EPA 200.8, which had been revised by IIRMES for use with blood. We measured blood concentrations on an inductively coupled plasma–mass spectrometer (ICP-MS; Agilent Hewlett-Packard 4500 Plus Series, Agilent Technologies, Inc., Santa Clara, CA, USA) housed at the Institute for Integrated Research in Materials, Environments, and

Society (IIRMES), California State University, Long Beach. The ICP-MS was tuned before use using low (lithium; Li), medium (yttrium, Y), and high (thorium, Tl) weight elements to ascertain instrument sensitivity at these points. An initial demonstration of performance was used to characterize instrument performance and laboratory performance prior to the analysis of samples. This involved establishing linear calibration ranges for each analyte at seven different concentrations. Method detection limits and reporting limits were established for each of the 20 metals being analyzed. The minimum detection limit (MDL) was calculated as follows: MDL = (t) x (S), where t is the student's t value for a 99% confidence level and standard deviation with n-1 degrees of freedom, and S is standard deviation of the replicates analyses. Reporting limits, calculated by IIRMES, were used to determine which values to label as non-detectable (ND) for each metal. A reporting limit is established by taking the mean value of the blanks used and adding three times the standard deviation of this mean to the MDL. Samples were analyzed on three separate days because of time and instrument sensitivity constraints. Thus, 27 samples, the corresponding blanks as controls, and the calibration curve were run on each of the three days.

Samples were removed from the -80^{o}C storage freezer. After thawing, 500 µL of the sample was pipetted into a 15mL plastic vial. Next, 500 µL of concentrated HNO_3 and 250 µL of concentrated HCl were added to the sample vials and to separate blank vials. All vials were then placed in a water bath heated to 75^{o}C for a minimum of two hours. After the tissues within samples were digested, 200 µL of an internal standard containing rhodium (Rh) and thulium (Tm) was added. Samples and blanks were then

diluted to 10 mL with 2% HNO_3 and stored in styrofoam racks on the counters in the IIRMES facility until analysis.

Three blanks were created with each set of 27 samples processed. One blank contained only the internal standards (Rh and Tm), whereas the other two were spiked with 50 µL of a multi-elemental standard containing 100 mg/L each of aluminum (Al), As, boron (B), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), Cr, cesium (Cs), Cu, Fe, Mn, molybdenum (Mo), Ni, Pb, antimony (Sb), Se, tin (Sn), strontium (Sr), titanium (Ti), thallium (Tl), vanadium (V), and zinc (Zn; SPEX CertiPrep Custom Built Standard, Lot # 4-115CR; SPEX CertiPrep Inc., Metuchen, NJ, USA). Percent blank contributions can be seen in Appendix I. A blank spike and blank spike duplicate were analyzed with each batch of samples. Percent recovery values can be found in Appendix I.

A standard curve was created using dilutions of the multi-elemental stock solution. Sample concentrations run to create the curve included 0, 10, 50, 100, 500, 1000, and 5000 ng/mL. The results of these curves can be seen in Appendix II. A calibration check with a concentration of 500 µL was created using a secondary source multi-elemental standard (SPEX CertiPrep Instrument Calibration Standard 2, Lot # 8-27JB). Percent recoveries for calibration checks can be seen in Appendix I. Quality control for aluminum data did not meet expectations on day 3, so the aluminum data for the third day were excluded from these analyses. Acid rinses were run between the standard curve, unknown samples, blanks, and known sample checks. A continuing calibration check was run every ten samples using the secondary source calibration check discussed above. These checks were within 15% of the initial calibration curve value. A

duplicate analysis of one sample was included in every batch of samples run. Results for these duplicate analyses can be found in Appendix I. Sample preparation was performed by Ashley Register. Operation of the ICPMS and data analysis software was performed by Andrew Hamilton. Adjusted metal concentrations obtained from these analyses can be found in Appendix III. All capture, physiological, and raw metal data are shown in Appendix IV.

Physiological Parameters

We obtained clinical physiological parameters through Antech Diagnostics (Memphis, TN, USA). This lab ran a complete reptilian profile according to their "test express" option, so that the same laboratory and technician presumably analyzed all samples. We procured measurements for the 30 parameters listed with abbreviations in Table 3-1.

Statistical Analyses

All heavy metal values below reporting limits were labeled non-detectable (ND) and discarded from the analyses. After this, we found that elements Cr, Cu, Sr, and Zn were normally distributed. We normalized all other metals using rank transformation (Al, As, Be, Co, Ni, Pb, Sn) or natural log (ln) transformation (Ba, Cd, Mn, Mo, Sb, Se, Ti, V). Beryllium and vanadium were excluded from further analyses as the results were deemed unreliable. Thallium was excluded due to low sample size. Mercury concentrations for use in the principle component analysis were provided by Rusty Day at the National Institute of Standards and Technology in South Carolina.

Physiological parameters were also examined for normality. Parameters AbNe, AST, Glob, Gluc, HePo, Lymp, Phos, Pota, Sodi, ToPr, T4, Uric, and UrNi were deemed normal in their original form. All other physiological data were normalized using either rank transformation (AAMo, AbEo, AbLy, AbMo, Baso, Calc, Chlo, CPK, Eosi, Hema, PCV, T3, WBC) or ln transformation (AbBa, Mono; with a constant added prior to transformation). Both absolute and relative measures of leukocytes were obtained, but only the absolute values were used in analyses. Turtle mass and SCL did not require transformation.

We conducted analyses of covariance (ANCOVA; Mertler and Vannatta, 2002) to examine the effects of body size (SCL only, as mass covaried strongly) and sex on each physiological parameter (see Chapter 2 for similar analyses on heavy metal concentrations). For effect sizes, we computed partial eta-squared (η^2), with values of ~ 0.01 deemed small, ~ 0.06 moderate, and ≥ 0.14 large (Cohen 1988). We then used principle component analysis (PCA) in conjunction with multiple regression analysis (Mertler et al. 2002) to identify potential predictors of the health parameters. As a rule of thumb, regression analyses should use $n \geq 10$ samples for each independent variable. By subjecting the correlation matrix from a large number of independent variables to PCA, the end result is reduced attribute space and a smaller number of variables (i.e., principle components, PCs).

Metals were divided into two separate groupings for separate PCA analyses. The first included only essential metals (Co, Cr, Cu, Mn, Mo, Ni, Se, Sn, and Zn), and the second consisted of non-essential, toxic metals (As, Ba, Cd, Hg, Pb, Sb, Sr, Ti). We employed the Kaiser criterion to determine which PCs to keep (those with eigenvalues

≥1), and Varimax rotation to more clearly differentiate the factor loading of each heavy metal on a given principle component. We then used multiple regression analysis (Mertler et al. 2002) to identify potential predictors of each physiological parameter from among the heavy metal PCs and, if necessary, body size (SCL) and sex. We used full regression models, as these are generally preferred to stepwise procedures, even though we did not strictly meet the rule of thumb for sample sizes. Regression models were run for each physiological parameter twice: once for the essential metal PC's, and once for the non-essential metal PCs.

All statistical analyses were conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) with $\alpha = 0.05$. Following (Perneger 1998; Nakagawa 2004), we did not apply Bonferroni adjustments of alpha to the multiple tests. However, considering the high experiment-wise error resulting from multiple tests, we interpreted significant outcomes with appropriate caution.

Results

We first examined associations of physiological parameters with turtle body size and sex to learn whether body size and sex would be necessary to include in analyses of associations of physiological parameters with heavy metals. Summary statistics for physiological parameters and heavy metal accumulation in *C. caretta* can be seen in Tables 3-1 and 3-2. Separate analyses for metals revealed a number of significant correlations between body size and heavy metal accumulation, but no differences between the sexes (Chapter 2).

Table 3-1: Summary statistics for physiological parameters measured in *Caretta caretta*.

Parameter	*n*	Abbrev	Range	Mean ± SE
Absolute Azurophilic Monocytes[d]	73	AAMo	0–540	71.0 ± 13.7
Absolute Basophils[d]	73	AbBa	0–5500	134.0 ± 71.8
Absolute Eosinophils[d]	73	AbEo	0–5880	1168.4 ± 143.0
Absolute Lympocytes[d]	73	AbLy	2640–12400	5096.7 ± 206.8
Absolute Monocytes[d]	73	AbMo	0–1750	142.1 ± 29.3
Absolute Neutrophils[d]	73	AbNe	1190–14520	5028.6 ± 319.5
Albumin[a]	81	Albu	0.5–1.7	1.1 ± 0.02
Aspartate Aminotransferase[c]	81	AST	114–458	222.4 ± 7.7
Azurophilic Monocytes[d]	73	AzMo	0–5	0.7 ± 0.1
Basophils[d]	73	Baso	0–22	0.8 ± 0.3
Calcium[b]	81	Calc	1–10.2	7.8 ± 0.2
Chlorine[e]	81	Chlo	78–136	118.2 ± 0.7
Creatine Phosphokinase[c]	81	CPK	253–2676	852.2 ± 54.6
Eosinophils[d]	73	Eosi	0–42	9.5 ± 0.9
Globulin[a]	81	Glob	2–6.5	4.1 ± 0.1
Glucose[b]	81	Gluc	57–202	107.1 ± 2.8
Hematocrit (%)	48	Hema	7–53	33.9 ± 0.8
Heterophils[d]	73	HePo	17–69	42.2 ± 1.5
Lymphocytes[d]	73	Lymp	14–74	45.9 ± 1.5
Monocytes[d]	73	Mono	0–7	1.0 ± 0.2
Packed Cell Volume (%)	81	PCV	18–49	34.8 ± 0.5
Phosphorus[b]	81	Phos	3–9.8	7.5 ± 0.1
Potassium[e]	81	Pota	2.7–7.3	4.8 ± 0.1
Sodium[e]	81	Sodi	102–170	155.4 ± 0.8
Total Protein[a]	81	ToPr	2.6–7.6	5.2 ± 0.1
Triiodothyronine	81	T3	0.1–1.5	0.46 ± 0.03
Thyroxine	79	T4	0.7–9.2	4.4 ± 0.2
Uric Acid[b]	81	Uric	0.1–2.2	0.9 ± 0.05
Urea Nitrogen[b]	81	UrNi	31–174	95.2 ± 2.9
White Blood Cells[d]	73	WBC	6–25	11.7 ± 0.5

[a] = g/dL
[b] = mg/dL
[c] = U/L
[d] = ($x10^3$ /μL)
[e] = mEq/L

Table 3-2: Summary statistics for heavy metals measured in *Caretta caretta*.

Element	*n*	Abbrev	Range (ppm)	Mean ± SD
Aluminum	32	Al	ND–0.16	0.06 ± 0.04
Arsenic	81	As	3.32–46.12	11.17 ± 6.01
Barium	80	Ba	0.004–0.40	0.10± 0.08
Cadmium	81	Cd	0.004–0.41	0.04 ± 0.06
Cobalt	48	Co	ND–0.13	0.007 ± 0.02
Chromium	81	Cr	0.18–0.65	0.4 ± 0.10
Copper	81	Cu	0.29–0.81	0.58 ± 0.09
Manganese	74	Mn	ND–0.11	0.031 ± 0.02
Molybdenum	81	Mo	0.01–0.29	0.05 ± 0.04
Nickel	62	Ni	ND–0.033	0.005 ± 0.005
Lead	76	Pb	ND–0.05	0.01 ± 0.007
Antimony	81	Sb	0.02–0.45	0.08 ± 0.08
Selenium	81	Se	1.18–8.45	3.49 ± 1.59
Tin	81	Sn	0.002–0.24	0.01 ± 0.03
Strontium	81	Sr	0.34–0.85	0.55 ± 0.10
Titanium	81	Ti	0.03–0.72	0.23 ± 0.14
Zinc	81	Zn	4.76–15.99	10.50 ± 2.00

Effects of Turtle Body Size and Sex

The ANCOVA results for physiological parameters yielded a small number of significant effects for both body size and sex (Table 3-3). Absolute neutrophils (AbNe; r^2 = 0.124) and monocytes (Mono; r^2 = 0.102) were negatively associated with SCL, whereas globulins (Glob; r^2 = 0.071) were positively associated with SCL. Thyroxine (T4) was negatively associated with SCL (r^2 = 0.069), and females exhibited higher levels than males (5.1 ± 0.6 and 4.0 ± 0.2, respectively; there was no interaction between SCL and sex). Absolute neutrophil counts (Abne) were greater in females than males (mean ± 1 SE: 6917 ± 797 and 4624 ± 386, respectively), as were white blood cell (WBC) counts (14.8 ± 1.3 and 11.2 ± 0.5, respectively).

Table 3-3: Results from analysis of covariance (ANCOVA) models showing effects of body size (straight carapace length) and sex on physiological parameters in *Caretta caretta*.

Physiological Parameter	*n* (♂♂.♀♀)	Body Size			Sex		
		F	*P*	η^2	*F*	*P*	η^2
AAMo	52.14	1.70	0.197	0.026	0.631	0.430	0.010
AbBa	52.14	0.10	0.756	0.002	0.88	0.351	0.014
AbEo	52.14	1.60	0.211	0.025	0.14	0.710	0.002
AbLy	52.14	0.02	0.877	0.000	0.89	0.348	0.014
AbMo	52.14	**8.96**	**0.004**	**0.125**	0.84	0.364	0.013
AbNe	52.14	1.42	0.237	0.022	**7.26**	**0.009**	**0.103**
Albu	56.18	2.00	0.162	0.027	2.96	0.090	0.040
AST	56.18	1.50	0.225	0.021	1.27	0.263	0.018
AzMo	52.14	1.47	0.230	0.023	0.983	0.325	0.015
Baso	52.14	0.10	0.748	0.002	0.437	0.511	0.007
Calc	56.18	1.20	0.276	0.017	0.39	0.536	0.005
Chlo	56.18	0.08	0.782	0.001	3.55	0.064	0.048
CPK	56.18	2.89	0.094	0.039	1.66	0.202	0.023
Eosi	52.14	2.36	0.130	0.036	0.03	0.871	0.000
Glob	56.18	**5.25**	**0.025**	**0.060**	2.59	0.112	0.035
Gluc	56.18	1.44	0.235	0.020	1.54	0.220	0.021
Hema	33.9	1.83	0.184	0.045	0.21	0.652	0.005
HePo	52.14	1.64	0.206	0.025	2.45	0.122	0.037
Lymp	52.14	0.14	0.714	0.002	3.12	0.082	0.047
Mono	52.14	**7.16**	**0.009**	**0.102**	0.33	0.566	0.005
PCV	56.18	1.92	0.170	0.026	0.15	0.701	0.002
Phos	56.18	1.57	0.215	0.022	0.37	0.545	0.005
Pota	56.18	0.63	0.432	0.009	0.83	0.366	0.012
Sodi	56.18	1.77	0.188	0.024	1.03	0.314	0.014
ToPr	56.18	3.68	0.059	0.049	3.48	0.066	0.047
T3	56.18	0.80	0.373	0.011	1.87	0.176	0.026
T4	55.17	**4.27**	**0.042**	**0.058**	**4.50**	**0.038**	**0.061**
Uric	56.18	0.40	0.527	0.006	1.00	0.320	0.014
UrNi	56.18	0.41	0.522	0.006	0.00	0.969	0.000
WBC	52.14	0.30	0.588	0.005	**6.87**	**0.011**	**0.098**

Significant effects are shown in **bold**.

Table 3-4: Factor loadings for each principle component (PC) extracted from separate principle component analyses of the correlation matrices for essential metals (EPCs; *n* = 39) and for non-essential toxic heavy metals (TPCs; *n* = 75) in *Caretta caretta*.

Metal	EPC1	EPC2	EPC3	TPC1	TPC2	TPC3
Co	0.061	**-0.752**	-0.105	—	—	—
Cr	-0.051	**0.689**	0.163	—	—	—
Cu	-0.059	0.161	**0.869**	—	—	—
Mn	**0.763**	0.184	-0.046	—	—	—
Mo	**0.609**	-0.251	0.558	—	—	—
Ni	-0.382	**-0.605**	0.432	—	—	—
Se	**0.532**	0.385	0.301	—	—	—
Sn	**0.861**	-0.240	0.000	—	—	—
Zn	0.320	0.476	**0.631**	—	—	—
As	—	—	—	0.030	**0.852**	-0.168
Ba	—	—	—	**0.731**	-0.145	-0.002
Cd	—	—	—	0.045	-0.130	**0.826**
Hg	—	—	—	-0.024	**0.777**	0.306
Pb	—	—	—	**0.751**	-0.226	0.213
Sb	—	—	—	**0.854**	0.141	0.017
Sr	—	—	—	0.076	0.189	**0.669**
Ti	—	—	—	**0.744**	0.283	0.016
Variance explained	24.8%	21.8%	19.8%	29.9%	19.4%	16.2%

PC selection based on Kaiser criterion (eigenvalues ≥1); factor loadings computed with Varimax rotation; metals with the highest factor loadings (≥ 0.5) for each PC are shown in **bold**; 66.4% of total variance was extracted from the essential metals, and 65.5% of total variance was extracted from the non-essential metals.

Regression of Metal Principle Components on Physiological Parameters

The PCAs of the heavy metal correlation matrices yielded three principle components for the essential metals and three for the non-essential metals, with each PC comprised of up to four heavy metals. Factor loadings for each PC are shown in Table 3-4. The PCs were then regressed on each physiological parameter to identify significant predictors.

For the essential metals, one of the 23 physiological parameters (4.3%) provided a significant model (Table 3-5), and this could have arisen by chance. Absolute monocytes showed an inverse relationship to EPC2 (Co, Cr, Ni), with bivariate correlations showing absolute monocytes positively associated with Co and Ni and negatively associated with Cr (Table 3-6).

For the non-essential toxic metals, four physiological parameters (17.4%) yielded significant models (Table 3-5), which exceeded the proportion expected from chance alone (5%). Adjusted R^2 values for significant models ranged from 0.091–0.212, indicating robust effect sizes. Concentrations of the serum protein albumin were predicted best by TPC2 (positive associations with As, Hg). The enzyme creatine phosphokinase (CPK) corresponded to TPC1 (positive associations with Ba, Pb, Sb, Ti) and TPC 2 (As, Hg). The thyroid hormone thyroxine (T4) was positively associated with TPC2 (As, Hg) and negatively associated with TPC3 (Cd, Sr). Chlorine (Cl) was best predicted by TPC3 (Cd, Sr). Bivariate correlations between each physiological parameter fitted to a significant model and the individual metals of significant TPCs are shown in Table 3-6.

The larger sample sizes for the regression models of non-essential metals compared to the essential models contributed to the larger number of significant models for the non-essential metals (as evidenced by comparison of model adjusted R^2 values in Table 3-5). For physiological variables influenced by SCL or sex, supplemental analyses confirmed that their exclusion from the regression results in Table 3-5 was inconsequential.

Table 3-5: Results (adjusted R^2 and beta values) from two regression models of principle components from essential (EPCs) and non-essential toxic (TPC) heavy metals on each physiological parameter in *Caretta caretta*.

Health Parameter	*n*	Model adj R^2	EPC1 β	EPC2 β	EPC3 β	*N*	Model adj R^2	TPC1 β	TPC2 B	TPC3 β
AbBa	33	0.007	0.085	-0.206	-0.220	68	0.032	-0.109	-0.090	0.232
AbEo	33	-0.088	0.087	-0.069	-0.060	68	-0.030	-0.031	0.039	0.116
AbLy	33	0.124	-0.159	0.224	0.358*	68	0.070	0.312**	0.081	0.071
AbMo[a]	33	**0.163***	-0.148	**-0.421***	0.146	68	0.011	-0.187	-0.110	0.069
AbNe[b]	33	0.062	-0.015	-0.333	-0.165	68	0.005	-0.164	0.020	-0.158
Albu	39	-0.005	0.037	0.147	0.226	75	**0.212*****	-0.180	**0.460*****	-0.004
AST	39	-0.012	-0.194	-0.170	-0.037	75	0.020	-0.144	-0.035	-0.195
Calc	39	-0.085	-0.102	0.088	0.023	75	0.008	-0.035	0.137	0.169
Chlo	39	0.098	-0.026	-0.402*	-0.081	75	**0.138****	-0.005	0.155	**0.387*****
CPK	39	-0.011	0.031	0.235	0.111	75	**0.163*****	**0.313****	**0.311****	0.050
Glob[a]	39	0.065	-0.117	0.142	0.324*	75	0.054	-0.107	0.124	-0.256*
Gluc	39	-0.018	0.034	-0.077	0.239	75	-0.041	0.001	-0.020	-0.020
Hema	24	0.069	-0.057	0.083	0.422*	47	-0.013	-0.075	0.198	0.075
PCV	39	-0.032	0.152	0.057	0.153	75	0.039	-0.020	0.247*	-0.050
Phos	39	-0.006	-0.232	0.104	-0.092	75	0.012	-0.174	-0.017	-0.146
Pota	39	-0.067	-0.017	-0.117	-0.060	75	-0.023	0.002	-0.099	0.095
Sodi	39	0.037	0.052	-0.333*	0.006	75	0.057	-0.093	0.246*	0.162
ToPr	39	0.074	-0.107	0.150	0.337*	75	0.065	-0.115	0.191	-0.231*
T3	39	-0.024	0.141	-0.134	-0.139	75	0.023	-0.128	0.214	0.008
T4[a]	37	-0.030	0.081	-0.154	-0.169	73	**0.091***	-0.076	**0.260***	**-0.235***
Uric	39	-0.010	-0.157	-0.174	0.122	75	-0.014	-0.013	0.062	-0.151
UrNi	39	-0.078	0.057	-0.073	0.011	75	0.035	-0.054	-0.042	-0.264*
WBC[b]	33	-0.014	-0.043	-0.262	-0.053	63	-0.031	0.086	0.084	-0.053

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; significant predictors from significant models are shown in **bold**.

[a] Supplemental models (results not shown) including straight carapace length yielded identical interpretation

[b] Supplemental models (results not shown) including sex yielded identical interpretations

EPC1: Mn, Mo, Se, Sn
EPC2: Co (inversely), Cr, Ni (nversely)
EPC3: Cu, Zn

TPC1: Ba, Pb, Sb, Ti
TPC2: As, Hg
TPC3: Cd, Sr

Table 3-6: Bivariate Pearson's correlations (*r*) between significant physiological parameters and individual metals of significant principle components (PCs) in Table 3-5.

Physiological Parameter	*n*	Significant PC	Individual Metals	*r*	*P*
AbMo	37	EPC2	Co	0.261	0.059
			Cr	-0.411	**0.006**
			Ni	0.308	**0.032**
Albu	81	TPC2	As	0.396	**<0.001**
			Hg	0.409	**<0.001**
Chlo	81	TPC3	Cd	0.282	**0.005**
			Sr	0.285	**0.005**
CPK	75	TPC1	Ba	0.107	0.180
			Pb	0.198	**0.043**
			Sb	0340	**0.001**
			Ti	0.346	**0.001**
		TPC2	As	0.251	**0.015**
			Hg	0.268	**0.010**
T4	79	TPC2	As	0.253	**0.012**
			Hg	0.085	0.229
		TPC3	Cd	-0.258	**0.011**
			Sr	-0.113	0.180

Significant relationships in **bold**.

Discussion

It is important to understand that this study is a survey of metal concentrations in *C. caretta* blood and their statistical correlation to individual animal blood chemistries and blood cell analyses at the time of blood sampling. As such, it is representative of the physiological state of the animal at a single point in time. Therefore, results need to be interpreted with caution. However, the significant associations between several non-essential toxic metals and physiological parameters suggest that heavy metal pollution may have an impact on the physiological profile and, potentially, the health of sea turtles. We concede that our analyses are exploratory in nature, and at best can show associations between variables, from which we can only predict that a causal relationship exists.

One of the most notable relationships observed was between albumin and TPC2 (As, Hg). Albumin is one of three proteins that are indicative of liver function (albumin, globulin, and total protein). This relationship suggests that liver function may be affected by the metal concentrations of As and Hg observed in this investigation. One of the first indicators of a toxicological effect is a change in liver function. Measurements of albumin, globulin, and total protein levels are the most common tests performed to ascertain liver performance. These measures are used in a wide variety of organisms, and are universally accepted as indicators of liver response to a toxicological interaction.

Creatinine phosphokinase (CPK) is an enzyme that is released into the blood when cellular damage occurs in certain tissues (Evans 2006). A positive relationship between CPK and Hg has been previously reported in *C. caretta* from the southeastern coast of the United States (Day et al. 2007). In this study, we observed a significant positive relationship between CPK and both TPC1 (Ba, Pb, Sb, Ti) and TPC2 (As, Hg).

Moreover, the lack of relationship between CPK and the essential metal PCs indicates that the associations between CPK and these non-essential metal PCs merits further investigation.

The relationship observed between chlorine and TPC3 (positive; Cd, Sr) and the relationships between thyroxine (T4) and both TPC2 (positive; As, Hg) and TPC3 (negative; Cd, Sr) require further study. In reptiles, ovo-exposure to arsenic has been shown to affect several parameters in the hatchling, including thyroid function (Hopkins et al. 1999; Marco et al. 2004).

Significant relationships related to the white blood cells need to be interpreted with caution. White blood cells are represented in this study by absolute measures of monocytes (AbMo), basophils (AbBa), eosinophils (AbEo), lymphocytes (AbLy), neophils (AbNe), and the general grouping WBC. Leukocytes are generally recognized as being extremely difficult to differentiate using machinery, and may become elevated in rapid response to handling of the animal. As a result, the relationship between AbMo and EPC 2 (Co, Cr, Ni) may or may not be meaningful.

Future Considerations

To the best of our knowledge, this is the first study to examine the physiological relationships of heavy metals other than mercury in sea turtles. Some studies looking at heavy metals in deceased individuals suggested that heavy metal pollution did not significantly effect the physiology of sea turtles (Godley et al. 1999), while other studies suggested that more research was necessary to determine if the levels of heavy metal contamination observed were affecting fitness (Gardner et al. 2006). The first study to

look at the potential health effects of metal pollution on sea turtles was published in 2007 on blood samples collected from *C. caretta* along the eastern coast of the United States (Day et al. 2007). This study found significant correlations between several physiological parameters and blood mercury concentrations, with supporting in vitro data for immune function, suggesting mercury may play a role in sea turtle physiology at environmentally relevant levels. Our study similarly found significant associations between several heavy metals and several physiological parameters, suggesting that metal pollution may influence the physiology and, potentially, the health of sea turtles.

Further inquiry needs to be made into the correlations found in this study that were unable to be explained. Since this study is unprecedented, it is difficult to sufficiently explain the relationships observed. Rarely was current literature able to shed adequate light onto the associations observed. Therefore, studies investigating the relationships observed in this study are necessary to correctly interpret future data.

The current study is limited in that it was able to only explore correlations between physiological parameters and metal concentrations. One potential next step in determining the impact of heavy metal pollution in sea turtles is an *in vitro* cell proliferation study similar to the one conducted by Day et. al. (2007). A study such as this would enable the researcher to expose cells to varying concentrations of heavy metals. It would also allow for the testing of various chemical forms of the metal, facilitating determination of which chemical species of each metal is most toxic. Furthermore, a cell proliferation study would be a fundamental first step in determining if causal relationships exist between the metals and the physiological parameters with which they were correlated.

Conclusions

1. Heavy metal pollution appears to have significant associations with the physiological parameters of sea turtle.
2. Albumin was strongly related to TPC2, which contained As and Hg. Bivariate correlation analyses supported the associations.
3. Absolute monocytes were the only parameter to show associations with essential metals (EPC2: Co, Cr, Ni).
4. Chlorine was related to TPC3, which included Cd and Sr. Bivariate correlation analyses reinforced this finding.
5. CPK was related to TPC1 (Ba, Pb, Sb, Ti) and TPC2 (As, Hg). Bivariate correlation analyses supported all of these relationships with the exception of Ba.
6. Thyroxine (T4) showed relationships to TPC2 (As, Hg) and TPC3 (Cd, Sr), but bivariate correlations displayed relationships with only As and Cd.
7. Extensive future research is needed to adequately understand the effects of heavy metal pollution on sea turtle health.

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CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSIONS

The research presented in this thesis is both unprecedented and foundational the study of heavy metal contamination and health in sea turtles. In the first study (Chapter 2), bivariate Pearson's correlation analyses revealed significant relationships between As, Cr, Pb, Ti, Zn and measures of body size (mass and SCL). To the best of our knowledge, this study is the first to report correlations with body size for chromium, lead, titanium and zinc. The implications of these correlations are not yet fully understood, but the observation that sea turtle size, and by extrapolation, age, is related to heavy metal accumulation, could have important implications for the health and survival of this species.

The study in Chapter 2 is further significant to the scientific community as it represents the first published data for relationships between body size and any metal besides mercury in these populations of *C. caretta*. It also contains information relating blood metal concentrations to environmentally associated factors, including capture depth and location. The sea turtles captured along the southeastern coast of the United States face many unique challenges. They reside in waters that are historically subject to high levels of urban run-off, resulting in elevated levels of pollution (Windom et al. 1989). One study investigating the distribution of trace metals in South Carolina found that there was significant enrichment of trace metals in areas that industrial or urban development activities compared to forested reference creek areas (Sanger et al. 1999). Brunswick,

Georgia, is home to four EPA Superfund sights, which have historically introduced high levels of heavy metal contaminants into the environment. Since the vast majority of previous studies investigating metal pollution in sea turtles have been performed on deceased individuals, it has been impossible to make any verifiable statements about the health impacts of this pollutant on sea turtles. Since these organisms are endangered, it is vital for the success of future conservation efforts to adequately understand the threats and challenges these creatures face, and this includes the health effects of human-created pollution.

The second study (Chapter 3) represents the first attempt to examine physiological associations with any heavy metal other than mercury in sea turtles. Health-related data in sea turtles have historically been lacking (Gardner et al. 2006), but recently this gap has been recognized and several studies have been conducted to address the void (Aguirre et al. 2000; Deem et al. 2006; Casal et al. 2009; Gelli et al. 2009). This new focus on collecting health-related data will enable the establishment of baseline physiological parameters for sea turtles. This in turn will facilitate more successful conservation efforts in the future, especially when coupled with an understanding of how environmental threats, such as metal pollution, impact health.

To the best of our knowledge, this study also comprises the first reported values for aluminum, antimony, barium, cobalt, molybdenum, strontium, tin, and titanium in any sea turtle tissues. Multiple regression analyses of heavy metal principle components revealed significant associations between heavy metal concentrations and several physiological parameters, suggesting that heavy metal accumulation may have significant health impacts in *C. caretta*. The nature of this study, however, limits the results to

uncovering associations and cannot reveal causal relationships. Furthermore, no physiologic state regard as "normal" has been established for this population in regards to background metal levels or biochemical parameters. As a result, this study is purely exploratory in nature. However, the results represented in this study are foundational to future efforts understanding the health implications of heavy metal pollution, as this study provides baseline information and insight into which metals and physiological parameters should be investigated in the future.

Future Considerations

Since the studies we conducted were foundational, there are several important questions that need to be addressed in future work. One of these questions is how long the observed metal concentrations remain in the blood. It is postulated that levels of heavy metal pollution observed in the blood reflect recent exposure (Day et al. 2005), but it is unknown how long it takes for these many of metals to be transported from the blood to other organs. Research investigating the half-life of heavy metals in the blood of reptiles is one important future study. Blood is the pathway by which dietary metals are distributed to other tissues, as the blood compartment is intricately linked to internal tissues. Day et al. (2005) found that blood total mercury was significantly related to total mercury in the muscle, spinal cord, kidney and liver, with the strongest relationship between blood and the muscle or spinal cord. Unfortunately, there is a lack of data in this area relating to other metals. The NOAA recovery plans for both *C. caretta* and *L. kempii* sea turtles mention the lack of existing data (USFWS et al. 1992) and the necessity of examining the impact of heavy metals in sea turtle populations (NMFS et al. 2008).

Hence, the relationships between metal pollution in the blood and concentrations found in the internal organs requires further investigated.

Another important question arises from the relationships observed between the blood proteins (albumin and globulin) and the associated metals. While one would be prompted to think that the observed relationships is a result of the metals causing increased protein production, it could be the other way around. Perhaps we see higher levels of these metals in the blood when there are higher levels of protein because the metals are bound to these proteins. Therefore, as the amount of proteins in the blood increases, the concentrations of the metals bound to these proteins also inherently increases. For example, it is known that copper and zinc both have a high binding affinity for albumin (Masuoka et al. 1993), and metallothionein has been shown to have a high affinity for copper and cobalt in sea turtles (Andreani et al. 2008) and for copper, zinc, and mercury in *Spongia officinalis* (Berthet et al. 2005). A study examining the proteins transporting metals in the blood would be a valuable first step in further understanding half-life and ultimate destinations of blood metal concentrations.

Another important question to address is the chemical speciation of the total metal concentrations observed in the blood. It is well documented that heavy metals tend to form bonds with organic groups, forming organometallic compounds (Rand 1995). Agusa et al. (2008a,b) found five different forms of organic arsenic in tissue samples from *C. mydas*. Research in sea birds also found several different forms of organic arsenic in the tissues analyzed (Fujihara et al. 2004; Ikemoto et al. 2005). Studies on mercury suggest that much of the observed total mercury is in the form of methyl mercury (Day et al. 2005). Furthermore, it was discovered in the sea bird studies that different tissues tended

to have different chemical species as the most prevalent form of arsenic. Research investigating the organic forms of the metals found in the blood would be both important foundational information, and potentially provide insight into the final destination of the metals being found in the blood.

Habitat utilization is also an important area for future research in heavy metal pollution to consider. How each sea turtle interacts with its habitat will affect the type and quantity of metal pollution they encounter. Prey items will have tendencies to accumulate different metals, sediments will intrinsically have differing levels of contamination, distance from a watershed and the urbanization around that watershed will all impact the heavy metal exposure each individual experiences. Previous studies have verified that sea turtles use their habitat in different ways (van Dam et al. 1998; Troeng et al. 2005; Hatase et al. 2006); even differences between sub-adult and adult individuals (Meylan 1999a; Houghton et al. 2003) and between males and females (van Dam et al. 2007) have been noted. With all these different variables, more study needs to be conducted on habitat utilization to understand the relationships between habitat use and exposure to heavy metal contaminants.

Finally, further studies examining health in these populations of sea turtles need to be conducted. This will enable baseline biochemical values to be established for the population, which in turn will allow researchers to distinguish between healthy and unhealthy individuals. While broad parameters for reptile health, or even sea turtle health in different species or different locations, will shed light on the results for this population, they cannot necessarily considered equivalent. As mentioned above, habitat utilization plays a significant role in establishing the normal physiological state for each individual.

Therefore, each population needs background health data relating specifically to their location. Understanding baseline levels of biochemical markers is essential for placing observed metal concentrations in their proper context.

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APPENDIX A

QUALITY CONTROL VERIFICATION CALCULATIONS

The first table presented below contains the percent recovery values for the blank spikes, blank spike duplicates, and secondary source calibration check samples for all three days of processing. The second table contains comparisons between the blank spike and blank spike duplicates, as well as the three unknown samples that were analyzed in duplicate.

Table A3-1: Percent recovery for blank spike, blank spike duplicate, and secondary source calibration checks.

Processing Day	Sample ID	Element ID	Expected Value	Reported Value	% Recovery
1	Blank Spike	Al	500	504	100.8
		Sb	500	444	88.8
		As	500	435.1	87.02
		Ba	500	505.4	101.08
		Be	500	455.9	91.18
		Cd	500	452.3	90.46
		Cr	500	502.8	100.56
		Co	500	A	NA
		Cu	500	487.2	97.44
		Pb	500	480.9	96.18
		Mn	500	495.3	99.06
		Mo	500	549.9	109.98
		Ni	500	503.5	100.7
		Se	500	374.1	74.82
		Sr	500	A	NA
		Tl	500	A	NA
		Sn	500	568.2	113.64
		Ti	500	486.5	97.3
		V	500	A	NA
		Zn	500	403.7	80.74
1	Blank Spike Dup.	Al	500	500.5	100.1
		Sb	500	439.9	87.98
		As	500	424.5	84.9
		Ba	500	503.6	100.72
		Be	500	460.3	92.06
		Cd	500	447.5	89.5
		Cr	500	495	99
		Co	500	481.3	96.26
		Cu	500	478.9	95.78
		Pb	500	489.4	97.88
		Mn	500	485	97
		Mo	500	544.8	108.96
		Ni	500	489.5	97.9
		Se	500	363	72.6
		Sr	500	491.6	98.32
		Tl	500	A	NA
		Sn	500	564.5	112.9
		Ti	500	483.2	96.64
		V	500	474.7	94.94
		Zn	500	393.9	78.78

1	Calibration Check	Al	500	541.2	108.24
		Sb	500	430.8	86.16
		As	500	510.5	102.1
		Ba	500	500.8	100.16
		Be	500	541.5	108.3
		Cd	500	506.4	101.28
		Cr	500	515.4	103.08
		Co	500	512	102.4
		Cu	500	507.7	101.54
		Pb	500	494.6	98.92
		Mn	500	499.2	99.84
		Mo	500	514.3	102.86
		Ni	500	512.3	102.46
		Se	500	510.2	102.04
		Sr	500	471.4	94.28
		Tl	500	508.3	101.66
		Sn	500	524.8	104.96
		Ti	500	522.2	104.44
		V	500	514.1	102.82
		Zn	500	500.7	100.14
2	Blank Spike	Al	500	389.7	77.94
		Sb	500	463.9	92.78
		As	500	417.9	83.58
		Ba	500	488.7	97.74
		Be	500	420.7	84.14
		Cd	500	423.4	84.68
		Cr	500	477.2	95.44
		Co	500	455.9	91.18
		Cu	500	466.7	93.34
		Pb	500	434.8	86.96
		Mn	500	474.6	94.92
		Mo	500	534.2	106.84
		Ni	500	476.4	95.28
		Se	500	363.4	72.68
		Sr	500	472.4	94.48
		Tl	500	421	84.2
		Sn	500	553.2	110.64
		Ti	500	465	93
		V	500	442.8	88.56
		Zn	500	403.5	80.7
2	Blank Spike Dup.	Al	500	506.4	101.28
		Sb	500	481.9	96.38
		As	500	432.8	86.56

		Ba	500	521.9	104.38
		Be	500	527.3	105.46
		Cd	500	454.6	90.92
		Cr	500	528.6	105.72
		Co	500	480.1	96.02
		Cu	500	474.3	94.86
		Pb	500	448.1	89.62
		Mn	500	521.8	104.36
		Mo	500	572	114.4
		Ni	500	483.2	96.64
		Se	500	368.6	73.72
		Sr	500	490.1	98.02
		Tl	500	432.6	86.52
		Sn	500	607.2	121.44
		Ti	500	528.2	105.64
		V	500	506.7	101.34
		Zn	500	414.7	82.94
2	Calibration Check	Al	500	445.1	89.02
		Sb	500	459.2	91.84
		As	500	517.9	103.58
		Ba	500	505.4	101.08
		Be	500	499.9	99.98
		Cd	500	505.2	101.04
		Cr	500	482.1	96.42
		Co	500	484.9	96.98
		Cu	500	484.9	96.98
		Pb	500	512.2	102.44
		Mn	500	497.6	99.52
		Mo	500	576.8	115.36
		Ni	500	482.3	96.46
		Se	500	547.3	109.46
		Sr	500	504.2	100.84
		Tl	500	514.1	102.82
		Sn	500	521.3	104.26
		Ti	500	488.3	97.66
		V	500	470.9	94.18
		Zn	500	523	104.6
3	Calibration Check	Al	500	A	NA
		Sb	500	433	86.6
		As	500	521.2	104.24
		Ba	500	507	101.4
		Be	500	499.9	99.98
		Cd	500	472.2	94.44
		Cr	500	515.2	103.04

	Co	500	515.4	103.08
	Cu	500	532.6	106.52
	Pb	500	512.7	102.54
	Mn	500	517.1	103.42
	Mo	500	545.9	109.18
	Ni	500	483.2	96.64
	Se	500	516.6	103.32
	Sr	500	517.1	103.42
	Tl	500	533	106.6
	Sn	500	503.9	100.78
	Ti	500	513.6	102.72
	V	500	481.4	96.28
	Zn	500	529.4	105.88