Toxic elements and associations with hematology, plasma biochemistry, and protein electrophoresis in nesting loggerhead sea turtles (*Caretta caretta*) from Casey Key, Florida

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ABSTRACT Toxic elements (arsenic, cadmium, lead, mercury, selenium, thallium) are a group of contaminants that are known to have developmental, reproductive, general health, and immune system effects in reptiles, even at low concentrations. Reptiles, including marine turtles, are susceptible to accumulation of toxic elements due to their long life span, low metabolic rate, and highly efficient conversion of prey into biomass. The objectives of this study were to (1) document concentrations of arsenic, cadmium, lead, mercury, selenium, and thallium in whole blood and keratin from nesting loggerhead sea turtles (*Caretta caretta*) from Casey Key, Florida and document correlations thereof and (2) correlate whole blood toxic element concentrations to various hematological and plasma biochemistry analytes. Baselines for various hematological and plasma analytes and toxic elements in whole blood and keratin (*i.e.*, scute) in nesting loggerheads are documented. Various correlations between the toxic elements and hematological and plasma biochemistry analytes were identified; however, the most intriguing were negative correlations between arsenic, cadmium, lead, and selenium with α - and γ -globulins. Although various extrinsic and intrinsic variables such as dietary and feeding changes in nesting loggerheads need to be considered, this finding may suggest a link to altered humoral immunity. This study documents a suite of health variables of nesting loggerheads in correlation to contaminants and identifies the potential of toxic elements to impact the overall health of nesting turtles, thus presenting important implications for the conservation and management of this species.

Keywords: electrophoresis; heavy metal; keratin; inorganic contaminant; marine turtle; toxicology

Abbreviations: α , alpha; A:G, albumin:globulin ratio; ALAD, aminolevulinic acid dehydratase; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AU, absorbance unit; β , beta; BDL, below detection limits; BUN, blood urea nitrogen; CBC, complete blood count; CPK, creatine phosphokinase; CV, coefficient of variation; CV-AAS, cold vapor atomic absorption spectrometry; DBS, dried blood spots; DCF, dichlorodihydrofluorescein; dw, dry weight; γ , gamma;

GGT, gamma-glutamyl transferase; HEL, hen egg white lysozyme; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; ICP-MS, inductively coupled plasma mass spectrometry; IgG, immunoglobulin G; LDH, lactate dehydrogenase; LoD, limit(s) of detection; LPO, lipid peroxidation; NIST, National Institute of Standards and Technology; PC: principal component; PCA: principal component analysis; PCV, packed cell volume; PIT, passive integrated transponder; QC, quality control; ROS, reactive oxygen species; RNS, reactive nitrogen species; rpm, revolutions per minute; SCL_{min}: minimum straight carapace length; SOD, superoxide dismutase; SRM, standard reference material; TEPC, toxic element principal component; UMDP, University of Miami's Department of Pathology; URTD, upper respiratory tract disease; WB, whole blood; WBC, white blood cells; ww, wet weight

Capsule: Toxic elements in nesting loggerhead sea turtles were negatively correlated with γ -globulins, potentially suggesting immunosuppression as a result of exposure to these contaminants.

Introduction

Toxic elements (*e.g.*, arsenic, cadmium, lead, mercury, selenium, thallium) in the environment are extremely persistent and despite coping mechanisms such as selenium accumulation, they have the capability of negatively affecting wildlife health (Storelli et al., 2005). These elements can bioaccumulate in organisms and biomagnify within the food web (Caurant et al., 1999; Anan et al., 2001; Camacho et al., 2013). Numerous studies have documented the diverse effects of toxic elements on wildlife species, which include abnormal development, decreased reproductive output, and altered endocrine and immune function in fish, birds, and mammals (Beyer et al., 1996; Hoffman et al., 2003). The effects of these chemicals on reptiles have been documented less frequently in comparison to other vertebrates (Gardner, 2006); even fewer are studies on the effects of toxic elements on marine turtles (Finlayson et al., 2016 for review). Reptilian species have physiological characteristics that might affect contaminant accumulation such as long life spans (Day et al., 2007), highly efficient conversion of prey into biomass, and less developed detoxification mechanisms (Hopkins, 2006). Although sea turtles reportedly have lower contaminant concentrations in their tissues in comparison to other reptiles, birds, and mammals (Keller, 2013), negative effects may still occur from these stressors (Milton and Lutz, 2003; Keller et al., 2004; Day et al., 2007).

In particular, loggerhead sea turtles (*Caretta caretta*) are an internationally threatened species and are protected under the U.S Endangered Species Act. Factors contributing to their decline include habitat loss, nest predation, strikes by watercrafts, disorientation of hatchlings, as well as marine pollution and debris (NMFS and USFSW, 2008; Casale and Tucker, 2015). Understanding the effects of pollutants on marine turtles has been elevated to a global research priority (Hamann et al., 2010; Rees et al., 2016). A recent publication reviewed toxicity studies in marine turtles and found 49 toxicology studies relating to marine turtles, and of those, 33 identified toxic effects of various contaminants (*e.g.*, inorganic and organic; Finlayson et al., 2016). Furthermore, just four of these studies examined correlations with toxic elements and health parameters (loggerheads: Day et al., 2007; Camacho et al., 2013; green turtles, *Chelonia mydas*: Komoroske et al., 2011; Kemp's ridley, *Lepidochelys kempii*: Innis et al., 2008). Since the publication of Finlayson et al. (2016), three additional studies examined correlations between hematological and plasma biochemistry analytes and toxic elements in marine turtles (green turtles: Villa et al., 2016; Perrault et al., 2017; loggerheads: Ley-Quinónez et al., 2017; Kemp's ridleys: Perrault et al., 2017). These seven studies documented correlations between toxic elements and indicators of disease (*e.g.*, fibropapillomatosis), body condition, organ function, immune function, ion regulation, oxidative stress, and overall health; however, these results are often not discussed due to confounding factors (*e.g.*, size, season, sex) or due to lack of understanding of reptilian toxicology (Innis et al., 2008; Komoroske et al., 2011; Labrada-Martagón et al., 2011; Camacho et al., 2013;). Additionally, four publications have reported risk assessments of toxic elements in freshwater and marine turtles. Those studies found that arsenic, cadmium, mercury, and selenium may impose risks to marine turtle embryos and hatchlings (Lam et al., 2006; van de Merwe et al., 2009; Perrault et al., 2013; Dyc et al., 2016). Interestingly, due to these

risk assessment studies, it is thought that metals may present a higher risk to marine turtle eggs/embryos than organic contaminants (Finlayson et al., 2016).

Very few studies report toxic elements in the keratinized scutes of marine turtles (Sakai et al., 2000; Day et al., 2005; van de Merwe, 2008; Komoroske et al., 2011). It is known that toxic elements bind to keratin proteins, making this tissue a suitable indicator of contamination and bodily toxic element load (Sakai et al., 2000; Day et al., 2007). Additionally, it is likely that keratin in scutes represents growth over the course of multiple years, suggesting this tissue as a good indicator for long-term contaminant status within the body (Day et al., 2007). Lastly, it has been shown that toxic elements in keratin correlate with various health indicators, which enhances the value of this tissue analysis (Komoroske et al., 2011).

Further understanding of toxicological effects includes establishing the impacts of heavy metals on overall health, oxidative stress, and immune function of nesting loggerheads and is an imperative aspect of sea turtle conservation. The objectives of this study were to (1) document concentrations of arsenic, cadmium, lead, mercury, selenium, and thallium in whole blood and keratin from nesting loggerhead sea turtles (*Caretta caretta*) from Casey Key, Florida and document correlations thereof and (2) determine relationships between whole blood toxic element concentrations and various hematological and plasma biochemistry analytes using linear regressions. Blood and keratin were selected, since blood provides information on recent or active exposure to toxic elements, while keratin provides an analysis of long-term contaminant accumulation.

Materials and methods

Sample collection

Nesting loggerhead sea turtles were encountered during nightly surveys of 6 km of sea turtle nesting habitat from May 21 to July 31, 2015 on Nokomis Beach of Casey Key, Florida USA (28.7°N, 82.3°W). This time frame spanned over 9 weeks and represents the main nesting activity in this area. There are usually very few turtles before and after that time frame. Individual females were identified based on their flipper and/or subcutaneous passive integrated transponder (PIT) tags (Biomark® Inc., Boise, Idaho USA). For documentation of unregistered turtles in the tagging database, both types of tags were applied if neither type of tag was present. After the turtles entered their nesting fixed action pattern, approximately 8–10 mL of whole blood were from the subcarapacial sinus using a 20 mL syringe and 3” BD heparin-coated needle (Becton, Dickinson and Company, Franklin Lakes, New Jersey USA). The blood was then placed into two 10 mL lithium-heparin coated BD Vacutainers®. Before blood collection, the entire area was thoroughly swabbed with sterile 70% isopropyl alcohol pads (Tyco Healthcare Group LP, Mansfield, Massachusetts USA). The blood sample was carefully inverted to ensure good mixing of blood and anticoagulant and subsequently placed on wet ice in the field for up to 8 h until processing. Direct contact of blood tubes and ice was prevented to avoid hemolysis. The venipuncture site was then disinfected with a new alcohol swab and pressure was applied to promote hemostasis. After sample collection, minimum straight carapace length was recorded (SCL_{min}).

Keratin samples from the posterior marginal scutes were collected during oviposition. The sampling area was prepared by scrubbing with three large 70% alcohol swabs to remove epibionts. Approximately 0.5–1.0 g of keratin (~1 mm thick) was collected from the radial edge of the carapace using a stainless steel biopsy tool. After sample collection, the entire area was swabbed with isopropanol and pressure was applied to promote hemostasis. Keratin samples were further soaked in 70% isopropanol in the lab to remove further epibionts. The samples were considered free of epibionts when the isopropanol was free of color and completely transparent after addition of the keratin. Keratin samples were stored at –80°C until analyses were conducted.

Toxic element analyses

Whole blood and keratin samples were dried at 60°C for 24 h in a Quincy Lab GC 20 drying oven (Quincy Lab Corporation, Chicago, Illinois USA). Samples were weighed before and after drying to determine moisture content. All samples were shipped to Michigan State University's Diagnostic Center for Population and Animal Health. Toxic elements (arsenic, cadmium, lead, mercury, selenium, thallium) were analyzed by inductively-coupled plasma mass spectrometry (ICP-MS; Agilent 7500ce ICP-MS, Agilent Technologies, Santa Clara, California USA). Dried blood samples were treated by a procedure essentially identical to that of dried blood spots (DBS; Lehner et al., 2013). In brief, 100 mg of dried blood were weighed and digested with 500 µL concentrated nitric acid (Ultrex II, Mallinckrodt-J.T. Baker) in 15 mL PFA digestion vessels (Savillex, Eden Prairie, Minnesota USA) in a 95°C oven overnight. Lypocheck 2 (medium range) and Lypocheck 3 (high range) Whole Blood Metals Controls (Bio-Rad, Hercules, California USA) standard reference materials (SRMs) were used as quality control (QC) reference materials for this process. Average QC results for these materials (percent recovery = average result/expected result) were as follows: Lypo 2: arsenic (100.8%); cadmium (97.0%); lead (104.2%); mercury (96.3%); thallium (101.2%); Lypo 3: arsenic (99.4%); cadmium (103.0%); lead (104.3%); mercury (92.8%); thallium (102.5%). Selenium was assessed by reference to Alfa Aesar (Haverhill, Massachusetts USA) Multi-Element QC-21 (Ward Hill, Massachusetts USA) standard, where average result/expected result values were 101.8%. Average coefficient of variation (CV) for DBS elements were as follows: arsenic, 4.1%; cadmium, 5.1%; lead, 3.7%; mercury, 5.0%; selenium, 3.5%; thallium, 5.2%. Dried blood limits of detection (LoD) were typically 10 ppb for arsenic, cadmium, lead, selenium, and thallium and 20 ppb for mercury.

Dried keratin was treated as a tissue sample for analysis by ICP-MS. Keratin mercury results that fell below detection limits (BDL) on ICP-MS were reanalyzed using cold vapor atomic absorption spectrometry (CV-AAS; Cetac M-6000A, Cetac Technologies Inc., Omaha, Nebraska USA) to provide a lower LoD. Up to 1 g of sample (in most cases 0.1–0.5 g) was used in keratin analyses. The samples were digested overnight at 95 °C after addition of a 10-fold excess on a milliliter per gram (ml/g) basis of concentrated nitric acid (Avantor Performance Materials, Center Valley, Pennsylvania USA). The National Institute of Standards and Technology (NIST, Gaithersburg, Maryland USA) Standard Reference Materials (SRM) 1577c Bovine Liver and 2976 Mussel Tissue and Alfa Aesar (Haverhill, Massachusetts USA) Multi-Element QC-21 standard solutions were used as the QC materials for keratin ICP-MS analyses. Daily QC determination for each of the elements in QC materials typically shows them to be within one standard deviation of the expected mean concentration, with average CVs as follows: (i) NIST Mussel Tissue: arsenic: 2.6%; cadmium: 7.0%; lead: 6.3%; mercury: 35% (owing to negligible amounts of mercury); selenium: 3.6%; (ii) Alfa Aesar Multi-Element-21: arsenic, 3.2%; cadmium: 4.5%, lead, 4.3%; selenium, 3.5%; and (iii) NIST Bovine Liver: selenium 3.3%. LoDs for keratin were as follows: arsenic, cadmium, lead, selenium, and thallium: 0.1 µg/g; mercury: 0.005 µg/g. NIST Mussel SRM contained the following concentrations: 13 µg/g arsenic; 0.8 µg/g cadmium; 1.0 µg/g lead; 0.061 µg/g mercury; 1.8 µg/g selenium. Multi-Element 21 contained 100 µg/mL of each of the following: arsenic, cadmium, lead, selenium, and thallium. NIST Bovine Liver was certified at 2.44 mg/kg selenium. Low level mercury analyses on CV-AAS used the following QC materials with the included CV values on mercury: NIST Mussel, 10.2%; GFS Chemical (Powell, Ohio USA) 5 pg/g standard solution, 3.4%. The low level mercury instrumental LoD is 1.8 pg/g.

Plasma biochemistry, PCV, and hematology

The packed cell volume (PCV) was determined from whole blood collected into two microcapillary tubes (Fisher Health-Care, Houston, Texas USA) with Critoseal® (Sherwood Medical Co., Deland, Florida USA) as the sealant. The capillary tubes were spun for 5 min at 14,800 g (12,000 rpm) using a microhematocrit centrifuge (LW Scientific, Inc., LWS-M24, Lawrenceville, Georgia USA). A hematocrit microcapillary tube reader was used to read the PCV (%). Two blood films were prepared from well-mixed whole blood, dried, and stained with Wright-Giemsa. Blood film evaluation included

white blood cell (WBC) estimate, WBC differential, and morphologic evaluation of red blood cells (RBC), WBC, and thrombocytes.

The remainder of whole blood was centrifuged at 1318 g (3400 rpm) for 10 minutes to harvest the plasma. Whole blood and plasma aliquots were stored at -80°C for ~2 months until further analyses were conducted. Plasma samples were used for biochemical analyses, which were carried out at the University of Miami Avian and Wildlife Laboratory (UMAW). Biochemical analysis included alanine aminotransferase activity (ALT), albumin, alkaline phosphatase activity (ALKP), amylase, aspartate aminotransferase activity (AST), blood urea nitrogen (BUN), calcium, calculated osmolality, chloride, cholesterol, creatine phosphokinase activity (CPK), creatinine, gamma-glutamyl transferase (GGT), globulin, glucose, iron, lactate dehydrogenase activity (LDH), magnesium, phosphorus, potassium, sodium, total protein, triglycerides, and uric acid using a dry chemistry analyzer (Ortho Clinical Diagnostics, Rochester, New York, USA).

Immune function, oxidative stress, and electrophoresis

Lysozyme activity of plasma samples was measured using modifications of standard turbidity assays performed by Walsh et al. (2010). A 1 mg/ml stock solution of hen egg white lysozyme (HEL; Sigma-Aldrich, St. Louis, Missouri USA) was prepared fresh in 0.1 M phosphate buffer (pH 5.9). *Micrococcus lysodeikticus* (Sigma-Aldrich) cell suspension was prepared by dissolving 50 mg of lyophilized cells in 100 mL of 0.1 M phosphate buffer. Hen egg white lysozyme was serially diluted by successive 1:1 dilutions in phosphate buffer to produce a standard curve of 0, 0.31, 0.63, 1.25, 2.50, 5.00, 10.0, 20.0 and 40.0 $\mu\text{g/ml}$. Aliquots (25 μL) of each concentration and 25 μL of test plasma were added to a 96-well plate in quadruplicate. *M. lysodeikticus* solution (175 μL) was quickly added to the first three rows of the sample wells and to each of the standard wells. The same amount of phosphate buffer was added to the fourth sample well to serve as a blank. Absorbance was measured at 450 nm using a BioTek[®] ELx800 microplate reader (BioTek US, Winooski, Vermont USA). Readings were conducted immediately (T_0) and after 5 min. Absorbance unit (AU) values at 5 min were subtracted from AU values at T_0 to determine the change in absorbance. The AU value for the blank sample well was subtracted from the average of the triplicate sample wells to compensate for sample hemolysis. The resulting AU values were converted to HEL concentration ($\mu\text{g/ml}$) by linear regression of the standard curve.

Presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were evaluated using an OxiSelect[™] *In Vitro* ROS/RNS Assay Kit (Green Fluorescence, Cell Biolabs, Inc., San Diego, California USA). In this assay, a reduced fluorophore is oxidized to a fluorescent molecule (2',7'-dichlorodihydrofluorescein [DCF]) in the presence of ROS and RNS. This microplate-based assay provides a measurement that indicates total free radical population within a sample. Fluorescence was measured with 480 nm excitation and 530 nm emission on a BioTek[®] FLx800 microplate reader. Free radical content of samples was determined using a DCF standard curve.

Total plasma superoxide dismutase (SOD) activity was measured using a commercially available SOD Assay Kit (Cayman Chemical Co., Ann Arbor, Michigan USA). A tetrazolium salt solution was used to detect superoxide radicals generated by xanthine oxidase in a 96-well plate. Absorbance was read at 440 nm using a BioTek[®] ELx800 microplate reader. Units of SOD activity per mL of plasma (U/mL) were determined using a standard curve. 1 U/mL of SOD is equal to the amount of enzyme needed to cause 50% dismutation of the superoxide radical.

Total solids in plasma were estimated using a Reichert VET 360 handheld refractometer (Reichert Technologies Analytical Instruments, Depew, New York USA). Total protein was quantified using the Biuret method at the UMAW. Plasma protein electrophoresis (albumin, alpha- [α_1 - and α_2 -] beta- [β], and gamma- [δ] globulins) was carried out at UMAW using an SPIFE 3000 system (Helena Laboratories Inc., Beaumont, Texas USA). The albumin:globulin (A:G) ratio was calculated.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 24 software (SPSS, Inc., Chicago, Illinois USA). Mean, standard deviation, median and range are reported for all data. In turtles sampled more than once for blood and/or scute, the average value of all sampling events was calculated before measures of central tendency, standard deviation, standard error, and range were determined. A number of toxic elements values fell BDL; this is indicated where appropriate. Blood toxic element concentrations that fell BDL were assigned to half of the detection limit for statistical analyses. This is standard for toxicology studies and was only used for five blood samples (twice for cadmium, three times for mercury) (Paustenback and Madl, 2008).

To test for relationships between blood toxic element concentrations and clinical parameters (plasma biochemistry, hematology, protein electrophoresis, etc.), least-squares linear regressions were carried out for each element and clinical parameter, separately. A linear regression was also performed between the sums of toxic elements in whole blood (arsenic, cadmium, lead, mercury, and selenium; excluding thallium) and clinical parameters to determine potential synergistic effects. For regressions where the residuals were non-normal, data were transformed to meet the assumptions of the linear regression analyses. To test for relationships between blood and keratin toxic element concentrations, least-squares linear regressions were performed for each element separately. Lastly, to determine if toxic elements in blood significantly decreased across the nesting season, linear regressions were carried out between toxic elements in whole blood and date of the nesting season. Repeated-measures ANOVAs were also carried out to determine if whole blood toxic element concentrations significantly differed between our first nesting encounter with the turtle and our subsequent nesting encounter with the turtle (this does not necessarily mean that our second encounter was her second clutch). The number of days after the first sampling event was treated as a covariate.

Results

Nesting females

A total of 40 blood samples (from 26 turtles) and 31 keratin samples (from 24 turtles) were collected from female loggerhead sea turtles during the 2015 nesting season on Casey Key, Florida. Seven turtles were sampled twice for blood, two turtles three times, and one individual four times. Seven turtles were sampled twice for keratin. The minimum straight carapace length (SCL_{min}) of the turtles ranged from 78–103 cm ($mean \pm SD = 88 \pm 7$ cm).

Toxic elements in blood and keratin

Concentrations of toxic elements in whole blood and keratin and the moisture content of whole blood and keratin are presented in **Table 1**. Both dry weight (dw) and wet weight (ww) values are reported for accurate comparisons to the literature (**Table 2; Supplemental Fig. 1**). Positive relationships were detected between the following toxic elements in the blood for $N = 26$ (r^2 and P values are reported in **Table 3**): arsenic and mercury, arsenic and selenium, and cadmium and selenium.

There was no significant relationship between date of the nesting season and whole blood toxic element concentrations ($P > 0.05$). Repeated-measures ANOVAs revealed no significant differences in whole blood toxic element concentrations between our first and subsequent nesting encounter (**Fig. 1**).

Positive relationships between whole blood and keratin toxic element concentrations were observed (**Table 3**): arsenic in blood and arsenic in keratin ($N = 22$), arsenic in blood and mercury in keratin ($N = 23$), arsenic in blood and selenium in keratin ($N = 22$), cadmium in blood and selenium in keratin ($N = 22$), lead in blood and cadmium in keratin ($N = 23$), mercury in blood and mercury in keratin ($N = 23$), selenium in blood and arsenic in keratin ($N = 22$), and selenium in blood and selenium in keratin ($N = 22$). Additionally, keratin selenium was positively correlated with keratin arsenic ($N = 22$) concentrations ($N = 22$). **Fig. 2** demonstrates the significant relationships between whole blood and keratin arsenic, mercury, and selenium concentrations.

Linear regressions between whole blood toxic elements and hematological and plasma analytes

The results of health, immune, and oxidative stress parameters are reported in **Table 4**. Results of linear regressions between whole blood toxic elements and measured hematological and plasma analytes are reported in **Table 5**. A marginally significant ($P = 0.06$) negative relationship between whole blood arsenic and γ -globulins was observed ($N = 19$).

Negative relationships between whole blood cadmium and γ -globulins ($N = 19$; marginally significant; $P = 0.07$) and total globulins ($N = 19$) were observed. A positive relationship between whole blood cadmium and the A:G ratio was observed ($N = 19$).

Negative relationships between whole blood lead and albumin ($N = 19$), α_2 -globulins ($N = 19$), total solids ($N = 28$), and iron ($N = 19$) were observed. Positive relationships between whole blood lead and magnesium ($N = 19$) and sodium ($N = 19$) were observed.

A positive relationship was observed between whole blood mercury and BUN ($N = 19$); however, this correlation is the result of a single, very high BUN outlier ($= 42$ mg/dl). Removing this data point results in a correlation that is no longer significant.

Negative relationships between whole blood selenium and total protein ($N = 19$), γ -globulins ($N = 19$), and total globulins ($N = 19$) were observed. Positive relationships between whole blood selenium and lymphocyte counts ($N = 31$) and monocyte ($N = 31$) were observed.

Negative relationships between the sum of whole blood toxic elements and γ -globulins ($N = 19$; **Fig. 3a**) and total globulins ($N = 19$) were observed.

Discussion

This is the first report of a suite of health variables and the toxic elements arsenic, cadmium, lead, mercury, selenium, and thallium in whole blood and keratin of nesting loggerhead sea turtles in western Florida. Our results indicate that toxic elements remain constant throughout the nesting season (**Fig. 1**), suggesting that any correlations observed with toxic elements are a result of past exposure and not due to changes in food intake or from tissue mobilization during the nesting season. These results add to the existing literature regarding accumulation and the potential effects of toxic elements on marine turtle health (Day et al., 2007; Innis et al., 2008; Komoroske et al., 2011; Register, 2011; Camacho et al., 2013; Villa et al., 2017; Ley-Quinónez et al., 2017; Perrault et al., 2017).

Blood and keratin toxic element concentrations

Whole blood arsenic concentrations (**Table 2**) in nesting loggerhead sea turtles from this study were similar to blood arsenic concentrations of foraging and nesting loggerheads from Georgia, USA (Deem et al., 2009), free-ranging loggerheads from Baja California Sur, Mexico (Ley-Quinónez et al., 2011), and green turtles in rehabilitation in Queensland, Australia (van de Merwe et al., 2010). However, blood arsenic concentrations in turtles from this study were one order of magnitude higher than blood arsenic concentrations in nesting loggerheads from Cape Verde, West Africa (Camacho et al., 2013), foraging green turtles from San Diego Bay, USA (Komoroske et al., 2011), four green turtle subpopulations in Australia (Villa et al., 2016), and one to two orders of magnitude higher than blood arsenic concentrations in leatherbacks (*Dermochelys coriacea*) from Gabon, Africa (Deem et al., 2006). Arsenic may be higher in loggerheads compared to green turtles, since their diet (*e.g.*, crustaceans) has been shown to be high in this toxic element (Ley-Quinónez et al., 2011). Additionally, arsenic may be high in tissues of turtles from the Gulf of Mexico due to effluent from natural phosphate mineral deposits that exist in the area and contain high levels of arsenic (Neff, 1997; Perry et al., 2015). Furthermore, the 2010 Deepwater Horizon oil spill could have affected arsenic levels in this area, since oil decreases the ocean's natural ability to filter arsenic when it combines with sediment on the ocean floor. Oils combine with sediment where arsenic is stored and redistribute it within the water column, causing a concentration

increase in the water column (Wainippee et al., 2010). Because arsenic can biomagnify, marine turtles, may accumulate this toxic element in their tissues. All of these are plausible explanations as to why arsenic concentrations are higher in turtles from this population compared to other geographic regions. However, arsenic in keratin of green turtles from California were one order of magnitude higher than turtles from this study, suggesting that turtles foraging in San Diego bay may also be at increased risk to arsenic exposure due to developmental, commercial, and residential activities in the area (Komoroske et al., 2011).

Whole blood cadmium concentrations (**Table 2**) from this study were similar (on the same order of magnitude) to nesting leatherbacks from French Guiana and St. Croix, USVI (Guirlet et al., 2008; Harris et al., 2011) and green turtles from Queensland, Australia and California, USA (van de Merwe et al., 2010; Komoroske et al., 2011). Cadmium concentrations in loggerheads from this study are one to two orders of magnitude lower than foraging loggerheads from Baja California Sur, Mexico and nesting loggerheads from western Australia (Ley-Quinónez et al., 2011; Trocini, 2013). Like arsenic, cadmium concentrations differ by life stage class, species, and location. Cadmium in whole blood has been shown to correlate with storage organs (*e.g.*, liver, kidney), suggesting this tissue as an accurate indicator of total body burden in marine turtles (van de Merwe et al., 2010). Keratin cadmium concentrations in loggerheads from this study fell BDL for 25 of 31 samples. These values are ~one order of magnitude lower than keratin cadmium concentrations in green turtles from California, USA (Komoroske et al., 2011) and loggerheads from Japan (Sakai et al., 2000), suggesting that cadmium exposure is low in the nesting loggerhead population of Casey Key; however, the carapace represents only a small proportion of the storage of this element in marine turtles and may not be representative of total body burden (Sakai et al., 2000).

The whole blood lead concentrations (**Table 2**) of nesting females from the present study were similar to whole blood lead in leatherbacks from Gabon, Africa (Deem et al., 2006), loggerheads from Cape Verde, West Africa (Camacho et al., 2013), and wild-caught Kemp's ridleys from the Gulf of Mexico (Kenyon et al., 2001). Kemp's ridleys and loggerheads exhibit dietary overlap (Jones and Seminoff, 2013), which suggests that diet and location are the primary influences of lead in the blood of marine turtles. It is likely that species, geographic area, life stage class, and diet all influence lead concentrations in marine turtles, as blood lead concentrations from our study are one order of magnitude lower than nesting leatherbacks from French Guiana (Guirlet et al., 2008) and wild-caught green turtles from California, USA (Komoroske et al., 2011). It is possible that blood lead concentrations might be higher in nesting females compared to foraging marine turtles due to calcium mobilization during egg production, and lead following calcium after release from bone tissue (Guirlet et al. 2008); however, Deem et al. (2009) and Harris et al. (2011) found no significant differences between lead levels in nesting females compared to foraging turtles. Lead concentrations in keratin in loggerheads from this study were low in comparison to green turtles and loggerheads from Japan and green turtles from California, USA (Sakai et al., 2000; Komoroske et al., 2011).

Whole blood mercury concentrations (**Table 2**) in turtles from this study are similar to foraging Kemp's ridleys from the Gulf of Mexico (Kenyon et al., 2001), foraging loggerheads from the southeastern United States (Day et al., 2005, 2007), foraging leatherbacks from the northwest Atlantic Ocean and California (Innis et al., 2010; Harris et al., 2011), and nesting leatherbacks from French Guiana, St. Croix, and Florida (Guirlet et al., 2008; Harris et al., 2011; Perrault et al., 2011, 2013). However, blood mercury concentrations in loggerheads from this study were one order of magnitude lower than blood mercury concentrations in nesting leatherbacks from Gabon (Deem et al., 2006) and foraging and nesting loggerheads from Georgia (Deem et al., 2009). Loggerheads from this study had keratin mercury concentrations that were one order of magnitude higher than green turtles from Japan, Australia, and California (Sakai et al., 2000; van de Merwe, 2008; Komoroske et al., 2011), but were similar to loggerheads from the southeastern United States, suggesting a higher level of mercury contamination for turtles that are residents in waters off of the eastern United States (Day et al., 2005, 2007). Mercury concentrations in keratin tend to be higher than mercury concentrations in the blood, which is opposite of the trend observed with other toxic elements (arsenic, cadmium, lead, selenium; Day

et al., 2005; van de Merwe, 2008; this study). Mercury has been shown to bind heavily with keratin (Crewther et al., 1965), which explains this trend. It is likely that differences in mercury concentrations exist between species and populations due to differences in diet and/or feeding locale. For example, some estuaries in Georgia experience extreme mercury contamination, due to the proximity to a superfund site and a coal power plant (Day et al., 2005). The elevated concentrations of mercury in loggerheads from Georgia (Day et al., 2005; Deem et al., 2009) are likely due to residence of turtles in this area during their foraging periods. In our study, five loggerheads had mercury concentrations one order of magnitude higher than the rest of the loggerheads from this study, which may be attributed to mercury contamination, likely on foraging grounds (Day et al., 2005). The authors of the present study are examining toxic element concentrations in eggs and tissues of satellite-tracked loggerheads from Casey Key (Tucker et al., 2014) to determine if certain foraging grounds have turtles with higher contaminant loads than others.

Whole blood selenium concentrations (**Table 2**) in marine turtles tend to be similar across all species (*i.e.*, of the same order of magnitude; Guirlet et al., 2008; Innis et al., 2010; Jerez et al., 2010; van de Merwe et al., 2010; Komoroske et al., 2011; Ley-Quinónez et al., 2011; Perrault et al., 2011, 2013; Register, 2011; Trocini, 2013; Villa et al., 2016); however, green turtles have the lowest concentrations, perhaps due to their low trophic position (van de Merwe et al., 2010; Komoroske et al., 2011; Jones and Seminoff, 2013). Leatherbacks have been shown to have the highest selenium concentrations in blood for reasons that remain unknown, but this has been attributed to their diet (Innis et al., 2010) and high daily food intake (Heaslip et al., 2012; Perrault, 2014). It is likely that blood selenium concentrations are accurate predictors of diet, as turtle toxicokinetic studies have observed increasing blood selenium concentrations with an increase in selenium dosage (Dyc et al., 2016). This suggests that loggerheads are less exposed to dietary selenium than leatherbacks, but have a greater intake than green turtles due to diet and volume of food intake. The results from blood are confounded by keratin selenium concentrations, as turtles from this study had lower keratin selenium concentrations than green turtles from San Diego Bay, again suggesting that this location may have an increased input of toxic elements into the system (Komoroske et al., 2011).

Comparisons to thallium concentrations in other marine turtle species are difficult, as all blood thallium samples from this study except three were BDL ($\sim <0.0002 \mu\text{g/g ww}$); all keratin samples also fell BDL (**Table 2**). Prioste et al. (2015) reported median blood thallium concentration in green turtles from Brazil to be $0.00008 \mu\text{g/g ww}$, while nesting flatbacks (*Natator depressus*) from Australia had thallium concentrations $<0.0001 \mu\text{g/g ww}$ (Ikonomopoulou et al., 2011). Free-ranging subadult and adult loggerheads from South Carolina, Georgia, and Florida USA had thallium concentrations that ranged from BDL to $0.046 \mu\text{g/g ww}$ (Register, 2011).

Blood and keratin toxic element correlations

A number of other marine turtle studies have observed correlations among various toxic elements in whole blood (Innis et al., 2008; Jerez et al., 2010; Komoroske et al., 2011; Ley-Quinónez et al., 2011; Camacho et al., 2013; Trocini, 2013; Prioste et al., 2015). These correlations likely exist for several reasons: (1) loggerheads have simultaneous exposure to these elements through prey items, (2) these elements have similar transport mechanisms and physiological pathways in the body (heavy metals and metalloids are bound to red blood cells), and/or (3) there are antagonistic effects of the elements (Innis et al., 2008; Komoroske et al., 2011; Prioste et al., 2015).

The keratinized scutes surround and protect the carapace in chelonids. It has been suggested that keratin is more indicative of long-term toxic element exposure and represents a non-lethal sampling method for determining total body toxic element burden (Sakai et al., 2000; Day et al., 2005; van de Merwe, 2008; Komoroske et al., 2011; Dyc et al., 2016). In turtles from this study, we found that arsenic and selenium were higher in blood compared to keratin, suggesting a more recent exposure to these elements, while mercury was lower in the blood compared to keratin, indicative of a more historical exposure to this element.

We observed a number of significant correlations between whole blood and keratin toxic element concentrations (**Fig. 2**). These findings are similar to those reported in Day et al. (2005) and van de Merwe (2008) and it is likely that these elements are binding with the keratin in the carapace of loggerheads (Crewther et al., 1965). A number of factors are known to influence toxic element concentrations in keratin including layering (*i.e.*, upper layers might represent exposure further in the past than lower layers), migration, diet, and time (Day et al., 2005; van de Merwe, 2008). Because scutes likely represent years of growth (Day et al., 2005), concentrations in this tissue more accurately represent total body burden/long-term exposure in comparison to blood, which is a more recent indicator of toxic element exposure. Additionally, it has been suggested that juvenile/subadult turtles may have stronger correlations between blood and keratin (van de Merwe, 2008) in comparison to nesting females, as larger juveniles/subadults recruit back to neritic zones (*i.e.*, foraging grounds) where they remain until maturity, making their exposure constant (Mansfield and Putman, 2013). Nesting females undertake extensive migrations every two to three years from foraging to nesting grounds, on average (Mansfield and Putman, 2013), making their exposure to different toxic elements less constant. This may explain the lack of correlations between some elements (lead and cadmium). It is also likely that lack of correlations with lead are due to differing toxicokinetics, as lead has been shown to associate more with bone than other soft tissues (Sakai et al., 2000).

Arsenic and hematological and plasma biochemistry analytes

Arsenic is toxic to numerous organ systems (Grillitsch and Schiesari, 2010; Jomova et al., 2011) and is released into the environment through smelting, chemical and glass manufacturing, and pesticide and herbicide applications (Fowler et al., 2007). In this study, we observed a marginally significant negative relationship between whole blood arsenic and γ -globulins (**Table 5**), suggesting altered humoral immunity. Several studies on vertebrates have established links between arsenic and plasma proteins. Elevated arsenic concentrations in mice have been associated with suppression of the primary antibody response (Sikorski et al., 1991). Arsenic has also been linked to decreased albumin and WBC counts in loggerheads (Register, 2011; Camacho et al., 2013), decreased total globulins in green turtles (Komoroske et al., 2011), and upper respiratory tract disease (URTD) in desert tortoises (*Gopherus agassizii*) (Berry et al., 2001). While URTD is caused by a bacterial infection of *Mycoplasma agassizii* (Brown et al., 1999), an altered immune response may increase the risk of other diseases (Hultman, 2007). Thus, arsenic contamination may prevent the immune system from fighting off the bacteria, promoting infection (Brown et al., 1999). Future studies should establish the concentrations of the different arsenic congeners in blood, as toxicity differs among the compounds. Inorganic arsenic compounds tend to be the most toxic (Storelli and Marcotrigiano, 2000; Jomova et al., 2011), while the less toxic organic compounds (*e.g.*, arsenobetaine, arsenochlorine, tetramethylarsonium) have been shown to be the most dominant form of arsenic in loggerhead prey items as well as in loggerhead tissues (*e.g.*, muscle, liver, kidney). Inorganic arsenic has been detected in trace amounts in liver and muscle (Storelli and Marcotrigiano, 2000). Saeki et al. (2000) suggest that turtles are likely not affected by arsenic, as the organic forms are the most predominant in marine turtles. Arsenic alone may not be enough to elicit negative effects in marine turtles, but this study suggests a possible cumulative negative effect of toxic elements on the humoral immune response in loggerhead turtles (**Fig. 3**).

Cadmium and hematological and plasma biochemistry analytes

All congeners of cadmium are extremely toxic (Remelli et al., 2016). This heavy metal has no known biological function and is increasing in the atmosphere due to mining, smelting, refining of ores, burning of fossil fuels, and waste incineration (Guirlet and Das, 2012). Cadmium exposure has shown to result in oxidative stress, damage to DNA and RNA, disruption in bone formation, and toxicity to the kidney and liver, the target organs (Jin et al., 2016); yet, information on the effects of cadmium on the health and immune function of marine turtles are lacking (Komoroske et al., 2011; Camacho et al., 2013).

We observed a marginal decrease in γ -globulins with increasing whole blood cadmium concentrations (**Table 5**). Due to this trend, we also observed a decrease in total globulins and an increase in the A:G ratio with increasing whole blood cadmium concentrations. In green turtles from San Diego Bay, blood cadmium concentrations negatively correlated with total globulins; however, protein electrophoretic profiles were not reported in that study. Given differences in methodologies, it cannot be determined which of the globulin proteins was related to a decrease in the total globulins (Komoroske et al., 2011), but a suppression of immune responses could have occurred. In mice exposed to cadmium, there was a significant decrease in immunoglobulin G (IgG), an antibody forming cell (Koller et al., 1975). While reptiles are not known to possess this class of immunoglobulins (Keller et al., 2006), these results suggest a potential similar immunosuppressive effect on the reptilian immune system due to exposure. In addition to a suppression of antibody production, numerous studies have documented a suppression of B-lymphocyte (*i.e.*, the antibody producing lymphocytes) production in response to cadmium exposure in vertebrates (goldfish, *Carassius auratus*: Murad and Houston, 1988; mice: Daum et al., 1993; Jin et al., 2016; bottlenose dolphins, *Tursiops truncatus*: Pellissó et al., 2008) perhaps through diminished lymphocyte proliferative capacity. Within loggerheads, we would expect to observe a simultaneous decrease in lymphocytes with a decrease in γ -globulins since B-lymphocytes produce antibodies, but no correlations between cadmium and lymphocytes were observed, although separate B- and T-lymphocyte populations were not defined. Perhaps cadmium may result in suppression of humoral immunity (B-lymphocytes), but not cell-mediated immunity (T-lymphocytes). Further research on both B- and T-lymphocytes should be undertaken to determine if this is the case.

Lead and hematological and plasma biochemistry analytes

Lead is released into the atmosphere through exhaust from motor vehicles, sewage sludge, ammunition, paint, and piping (Skerfving and Bergdahl, 2007). It causes reproductive, developmental, and endocrine disruption, has negative effects on the immune, renal, hepatic, and nervous systems, and is a carcinogen (Grillitsch and Schiesari, 2010). In marine turtles, whole blood lead has negatively correlated with the A:G ratio, glucose, lymphocytes, and sodium, and positively correlated with globulin, phosphorus, total protein, and uric acid (Komoroske et al., 2011; Camacho et al., 2013).

We observed negative relationships between whole blood lead and albumin and α_2 -globulins (and total solids likely as a result of these relationships). Albumin is a negative acute phase protein that decreases in response to inflammation (Osborne et al., 2011; Evans, 2011). The α_2 -globulins are positive acute phase proteins that increase with inflammation (Evans, 2011). In Swiss albino mice (*Mus musculus albinus*) dietary lead exposure resulted in immunosuppression, as evidenced by decreases in α -, β -, and γ -globulins. This suggested that fewer acute phase proteins and antibodies are being produced (*e.g.*, IgA, IgM, IgG, IgE) as a result of lead exposure (Rekha, 2014). Similarly, lead exposure may interfere with the humoral immune response in nesting loggerheads.

We also observed a negative relationship between whole blood lead and iron. Lead is also known to inhibit heme biosynthesis and iron utilization (Lilis et al., 1978; Pattee and Pain, 2003). Lead can hinder heme-synthetase (an enzyme responsible for assimilating iron into protoporphyrin 9), whereby zinc is substituted into protoporphyrin 9 in the place of iron and therefore may interfere with iron metabolism and transport in plasma, which would explain the negative correlation (**Table 5**) (Pattee and Pain, 2003).

Whole blood lead concentrations of $\sim 1.1 \mu\text{g/g ww}$ ($7.3 \mu\text{g/g dw}$) have been suggested as the toxic threshold for marine turtles (da Silva et al., 2016). Additionally, Buekers et al. (2009) proposed a toxic threshold concentration of $0.71 \mu\text{g/g ww}$ ($\sim 4.6 \mu\text{g/g dw}$) in blood for birds. Both toxic thresholds are one order of magnitude above the concentrations found in our study. Even the highest lead concentrations in loggerheads from this study did not approach these values, suggesting lower toxic lead thresholds for loggerhead sea turtles in Florida, given the above described correlations.

Positive correlations between whole blood lead and magnesium and sodium (**Table 5**) were difficult to explain. It is possible that this heavy metal could impact salt gland function, similar to other

contaminants (Lutcavage et al., 1995). Lead was low in the salt gland of dead stranded leatherbacks (Perrault, unpublished data), but was found at increased concentrations in the salt gland in comparison to other tissues (e.g., heart, kidney, liver, muscle) in birds (Burger et al., 2000). This explanation is purely speculative and needs further investigation.

Mercury and hematological and plasma biochemistry analytes

Mercury is a potent neurotoxicant with known adverse effects on reproduction, development, and endocrine, immune, renal, and hepatic functions and is released into the atmosphere through pesticides, paper mills, fossil fuel burning, waste disposal, and the chlorine industry (Berlin et al., 2007). All species of mercury are toxic; however, mercury in the methylated form is ~100 times more toxic than the inorganic forms. While we did not analyze for the presence of methyl-mercury in this study, previous studies have shown that methyl-mercury is the dominant mercury congener in whole blood (Henny et al., 2002). The toxicological consequences of mercury to vertebrate wildlife have been well-documented, but the effects of mercury on reptiles remains poorly understood (Wolfe et al., 1998; Wiener et al., 2003; Gardner, 2006; Grillitsch and Schiesari 2010). In this study, whole blood mercury concentrations were positively related to blood urea nitrogen (**Table 5**); however, this correlation was due to one outlier with BUN (= 42 mg/dL, 2.6 times higher than the next highest value) in a turtle with the highest whole blood mercury concentration in this study. In contrast to other species, lower BUN in sea turtles is often associated with reduced food intake as can be observed in nesting loggerhead turtles during the nesting season (Casal et al., 2009). Consequently, higher BUN is interpreted with active feeding and thus suggests dietary exposure of mercury in an actively feeding female sea turtle.

Interestingly, Day et al. (2007) found that the no observed effect level (NOEL) for suppression of B-lymphocytes in loggerhead turtles was 0.05 µg/g methyl-mercury, while the effective dose to produce a 50% response (ED50) was 0.08 µg/g methyl-mercury. In turtles from this study, 32% had blood mercury concentrations above the NOEL, while 24% fell above the ED50; yet, no significant relationships between whole blood mercury concentrations and indicators of immune function were observed. This suggests that there is individual and/or population variability with physiological responses to toxic elements in loggerhead turtles (Ganser et al., 2003).

Lastly, loggerhead turtles have the lowest Se:Hg ratios in comparison to green turtles and leatherbacks (**Fig. 2f**), suggesting that selenium may be used to detoxify methyl-mercury, depleting the body's selenium load. Because loggerheads forage higher on the food chain than green and leatherback turtles (Jones and Seminoff, 2013), they may have a greater exposure to mercury and may be at greater risk of mercury toxicity than other species.

Selenium and hematological and plasma biochemistry analytes

Selenium is an essential nutrient to animals in low quantities, but high body burdens can result in toxicity (Ohlendorf, 2003). This element is released through anthropogenic practices including smelting, phosphate mining, oil refinement, coal-fired power plants, and agricultural drainwater (Hoffman, 2002; Högberg and Alexander, 2007). Negative relationships between whole blood selenium and total γ-globulins were observed in this study (and total protein and total globulins as a result; **Table 5**). Selenium is known as an essential element for proper immune function and has been shown to counteract immunosuppressive effects due to toxic element exposure (El-Boshy et al., 2015). Therefore, it was unexpected that selenium toxicity could be related to a suppression of the immune response (McKenzie et al., 1998). However, both selenium deficiency and selenium toxicity have been shown to result in impairments of the immune system (Kiremidjian-Schumacher and Stotzky, 1987). Komoroske et al. (2011) found that blood selenium concentrations were negatively correlated with total globulins. This indicates a potential suppression of the immune response. Mallards (*Anas platyrhynchos*) exposed to selenomethionine treated water for 78 days exhibited a delayed hypersensitivity reaction to tuberculin, an indicator of suppressed immune response (Fairbrother and Fowles, 1990). In common eiders (*Somateria*

mollissima) fed selenium supplemented diets (as L-selenomethionine), γ -globulins were lower (although non-significant) in the high exposure group compared to controls and the low exposure group (Franson et al., 2007). The liver selenium concentrations of the birds in high exposure group ranged from 370–910 ppm dw (Franson et al., 2007). If we use the blood/liver relationship equation in van de Merwe et al. (2010) and an estimated liver moisture content of ~70% (Ohlendorf and Heinz, 2011), we can estimate that the liver selenium concentrations of the loggerheads from this study range from ~5–227 $\mu\text{g/g dw}$; these values are much lower than the eiders. Perhaps marine turtles are more sensitive to selenium exposure than birds as several hazard quotient studies show that selenium may be at or above harmful levels in marine turtle tissues (Lam et al., 2006; van de Merwe et al., 2009; Perrault et al., 2013).

There was also a positive relationship between whole blood selenium and absolute lymphocytes and monocytes (**Table 5**). These results conflict with results from Innis et al. (2008) and Camacho et al. (2013), where selenium was negatively correlated with lymphocytes and total WBC counts, respectively. T-lymphocytes are involved in the regulation of antibody production, but do not themselves produce antibodies like B-lymphocytes (Zimmerman et al., 2010). The negative correlation of selenium with γ -globulins and the positive correlation with T-lymphocytes imply that the humoral immune system (B-lymphocytes; *i.e.*, antibodies or γ -globulins) may be suppressed/more sensitive to selenium exposure, while the cell-mediated immune system (T-lymphocytes) may be modulated. It is worth mentioning that γ -globulins are on the low end for marine turtles, yet still within normal limits, while WBC counts are within normal ranges. Other factors (*e.g.*, diet, anorexia during the nesting season, etc.) may have altered the immune analytes in this dataset. Further research is needed regarding the effects of selenium on marine turtles, as bird studies have shown that effects on reproduction, health, and fitness cannot be predicted by a small range of blood selenium concentrations. It is possible that the associations between selenium and health are related to the antagonist role selenium plays in detoxification and the correlations of selenium with plasma proteins could be the result of selenium associating with toxic elements through detoxification and binding with the toxic elements (arsenic, cadmium, mercury; Högberg and Alexander, 2007).

Thallium and hematological and plasma biochemistry analytes

Thallium is present naturally in the environment, but is increasing due to anthropogenic activities (*e.g.*, smelting, coal-burning; Karbowska, 2016). It is an extremely toxic element with effects on liver and kidney function (Peter and Viraraghavan, 2005). Inferences into the effects of thallium cannot be made as most samples fell BDL. In humans, thallium concentrations of more than 0.1 $\mu\text{g/g}$ in the blood are known to cause toxicity (Lansdown, 2013), which is much higher than the blood thallium concentrations observed here. Anan et al. (2001) found that thallium accumulated highest in the salt gland of green and hawksbill sea turtles (*Eretmochelys imbricata*), likely due to the interference of thallium with potassium due to their similar chemistry. Because thallium concentrations are low in marine turtles, it is likely that thallium is not negatively impacting their health at these concentrations (Anan et al. 2001).

Conclusions

All nesting females from this study had detectable levels of toxic elements in their blood, which documents exposure to contaminants of loggerheads at the west coast of Florida including five marine turtles with exceptionally high concentrations of toxicants (one to two orders of magnitude higher than the mean). Our results provide lines of inquiry for future studies that focus on toxic elements and various health variables in marine turtles. Future studies should focus on documenting contaminant concentrations by foraging ground (Stoneburner et al., 1980; Alava et al., 2006) to determine exposure risks. Additionally, it has been suggested that there may be individual variability in physiological responses to toxicant exposure and individuals from certain populations may have different physiological consequences than others (Ganser et al., 2003). The results presented here highlight the need for more toxicological studies in marine turtles, as this study was correlative, and therefore had its limitations as

the identification of the exact effects of toxicants could not be differentiated from potential effects of other extrinsic and intrinsic factors (e.g., maternal health, seasonal changes, size, synergistic effects: **Fig. 3**, etc.). Other marine turtle studies have observed correlations with toxic elements that are suggestive of liver damage and suppression of the inflammatory response and immune system (Day et al., 2007; Innis et al., 2008; Komoroske et al., 2011; Register, 2011; Camacho et al., 2013; Perrault et al., 2017). Therefore, it can be presumed that toxic elements can result in negative effects in these animals and that some life stages such as nesting marine turtles may be more prone to negative health effects from tissue release during fasting; also, marine turtles may be more sensitive to contaminants compared with other vertebrates (Keller et al., 2004; Day et al., 2007). Although a number of interesting relationships between toxic elements and health variables were identified in this study, health variables were within normal limits for nesting loggerheads. However, data at the lower or higher end of normal ranges may indicate subtle effects from toxic elements, giving this dataset importance for population monitoring as well as for spatial and temporal comparative studies in the future.

Our results highlight the need for more *in vitro* exposure studies by life stage similar to Day et al. (2007), Webb et al. (2014), Wise et al. (2014), and Young et al. (2015). Future contaminant research that report concentrations on a dw basis should report moisture content and conversions from dw to ww, so that comparisons can be made across all studies. Lastly, conversion factors between toxic element concentrations in RBCs, whole blood, and plasma should be established for cross study comparisons.

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Figure Legends

Fig. 1. There were no significant differences in whole blood toxic element concentrations between the first nesting encounter and the second nesting encounter for (A) arsenic and selenium and (B) cadmium, lead, and mercury. Points represent the mean concentration with standard error bars.

Fig. 2. Least-squares linear regressions between whole blood and keratin (A) arsenic, (B) mercury, and (C) selenium.

Fig. 3. Least-squares linear regression between the sum of toxic elements in blood and γ -globulins.

Table 1. Concentrations of toxic elements in whole blood (WB) and keratin ($\mu\text{g/g}$ dw and ww) of nesting loggerhead sea turtles from Casey Key, Florida. Samples are reported as dw and ww for more accurate comparisons to the literature. Additionally, the ww sample size is less than dw sample size because moisture content was not collected for all samples. For turtles sampled more than once for blood or keratin, values were averaged for all samples collected.

	Mean	SD	SE	Median	Min	Max	N
WB % Moisture	84.63	1.88	0.38	85.06	80.31	89.68	25
Keratin % Moisture	29.10	5.70	1.22	30.31	18.96	39.69	22
WB arsenic dw	21.94	22.93	4.50	13.76	1.31	78.92	26
WB arsenic ww	3.42	3.69	0.74	2.35	0.24	13.45	25
Keratin arsenic dw	0.39	0.20	0.04	0.30	0.16	0.85	23
Keratin arsenic ww	0.25	0.12	0.03	0.20	0.12	0.55	21
WB cadmium dw	0.12	0.16	0.03	0.06	0.005	0.64	26
WB cadmium ww	0.02	0.02	0.005	0.01	0.001	0.094	25
Keratin cadmium dw	NA	NA	NA	<0.10	<0.10	0.21	24
Keratin cadmium ww	NA	NA	NA	<0.03	<0.03	0.15	22
WB lead dw	0.24	0.12	0.02	0.21	0.07	0.52	26
WB lead ww	0.03	0.02	0.003	0.04	0.01	0.07	25
Keratin lead dw	NA	NA	NA	0.10	<0.10	0.38	24
Keratin lead ww	NA	NA	NA	<0.10	<0.03	0.30	22
WB mercury dw	0.28	0.37	0.07	0.10	0.02	1.62	26
WB mercury ww	0.04	0.06	0.01	0.02	0.003	0.25	25
Keratin mercury dw	0.59	0.71	0.15	0.25	0.01	3.01	24
Keratin mercury ww	0.43	0.54	0.11	0.17	0.03	2.30	22
WB selenium dw	18.38	19.95	3.91	12.11	2.42	80.15	26
WB selenium ww	2.78	3.02	0.60	1.78	0.39	11.79	25
Keratin selenium dw	1.42	1.45	0.0	0.91	0.35	6.98	23
Keratin selenium ww	1.04	1.05	0.23	0.65	0.34	4.84	21
WB thallium dw	NA	NA	NA	<0.001	<0.001	0.001	26
WB thallium ww	NA	NA	NA	<0.0002	<0.0002	0.0002	25
Keratin thallium dw	NA	NA	NA	<0.10	<0.10	0.10	24
Keratin thallium ww	NA	NA	NA	<0.03	<0.03	0.04	22

Table 2. Concentrations of toxic elements in whole blood (WB) and keratin from this study and the literature. Concentrations are given as mean±SD or median in µg/g ww. Range (R) is given when mean or median was not reported.

Species	Tissue	Location	Year	SCL/CCL (cm)	n	Arsenic	Cadmium	Lead	Mercury	Selenium	Thallium	Ref.
Flatback	WB	Australia	06	<90->96	12	–	<0.0001	<0.0001	<0.0001	–	<0.0001	1
Green	WB	Australia	06–07	33–82	16	4.4±1.4	0.04±0.01	0.02±0.01	0.003±0.001	2.5±0.6	–	2
Green	WB	California	07–09	52–117	19–30	0.2±0.03	0.01±0.004	1.3±0.2	0.001±0.0002	0.8±0.3	–	3
Green	WB	Australia	14	61–95	131	R: 0.02–2.8	R: 0.001–0.03	R: 0.004–0.2	–	R: 0.03–1.4	R: 0.00003–0.0007	4
Green	WB	Brazil	13	Median = 72	31	0.2 ^a	0.01 ^a	0.02 ^a	–	0.3 ^a	0.00008 ^a	5
Hawksbill	WB	Iran	11	63.54 ± 10.23	12	–	0.05±0.01 ^b	0.09±0.04 ^b	0.03±0.01 ^b	–	–	6
Kemp's ridley	WB	Gulf of Mexico	94–95	22–66	106	–	–	0.01	0.02	–	–	7
Leatherback	WB	Africa	01–02	139–169	6–9	<0.1	–	0.09±0.03	0.2±0.2	–	–	8
Leatherback	WB	French Guiana	06	143–170	78	–	0.08±0.03	0.2±0.05	0.01±0.003	10.0±0.1	–	9
Leatherback	WB	W. Atlantic	07–08	136–162	16	–	0.07±0.02	R: 0.07–0.20	0.01±0.00	7.6±2.8	–	10
Leatherback	WB	St. Croix	05–07	NA	23	–	R: 0.01–0.18	R: 0.08–0.31	R: <0.005–0.05	–	–	11
Leatherback	WB	Florida USA	07–08	125–174	52–71	–	–	–	0.03±0.02	8.6±5.2	–	12
Leatherback	WB	St. Croix	09	138–174	206–207	–	–	–	0.01	9.5	–	13
Loggerhead	WB	W. Atlantic	01	50–95	34	–	–	–	0.03±0.008	–	–	14
Loggerhead	WB	W. Atlantic	03	51–95	66	–	–	–	0.03±0.002	–	–	15
Loggerhead	WB	Georgia	00–04	87–108	12	1.8±1.3	–	0.1±0.04	0.3	–	–	16
Loggerhead	WB	Spain	03–06	29–65	5	1.1±1.4 ^b	0.02±0.03 ^b	0.05±0.05 ^b	0.003±0.002 ^b	0.4±0.2 ^b	–	17
Loggerhead	WB	W. Atlantic	08	73±1; 72±2	6–81	11.2±6.0	0.04±0.06	0.01±0.007	–	3.5±1.6	0.01±0.02	18
Loggerhead	WB	Mexico	–	49–84	22	4.1±2.6	1.8±0.6	–	–	6.1±3.6	–	19
Loggerhead	WB	W. Africa	10–11	82±3, 96±4	201	0.6±1.0	0.3±0.3	0.06±0.02	0.04±0.04	2.5±2.2	–	20
Loggerhead	WB	Australia	06–08	87.5±4.6	98	2.8±3.5	0.3±0.3	0.02±0.01	0.02±0.01	2.8±5.0	–	21
Loggerhead	WB	Florida	15	78–103	37	3.1±3.3	0.02±0.02	0.03±0.02	0.04±0.05	2.4±2.7	<0.0002	This study
Green	Keratin	Japan	90	93–97	2	–	0.04±0.02	2.7±0.6	0.002±0.001	–	–	22
Green	Keratin	Australia	06–07	33–82	16	0.4±0.08	0.2±0.03	0.2±0.06	0.06±0.02	0.6±0.1	–	23
Green	Keratin	California	07–09	52–117	31	1.1±0.2 ^c	0.3±0.04 ^c	5.2±1.7 ^c	0.03±0.01 ^c	1.2±0.2 ^c	–	3
Loggerhead	Keratin	Japan	90	83±6	6	–	0.1±0.03	2.4±0.5	0.04±0.03	–	–	22
Loggerhead	Keratin	W. Atlantic	01	50–95	34	–	–	–	0.5±0.09	–	–	14
Loggerhead	Keratin	Gulf of Mexico	15	78–103	27–28	0.3±0.1	<0.1	<0.1	0.4±0.5	1.0±1.0	<0.1	This study

1: Ikononopoulou et al. (2011); 2: van de Merwe et al. (2010); 3: Komoroske et al. (2011); 4: Villa et al. (2016); 5: Prioste et al. (2015); 6: Ehsanpour et al. (2013); 7: Kenyon et al. (2001); 8: Deem et al. (2006); 9: Guirlet et al. (2008); 10: Innis et al. (2010); 11: Harris et al. (2011); 12: Perrault et al. (2011); 13: Perrault et al. (2013); 14: Day et al. (2005); 15: Day et al. (2007); 16: Deem et al. (2009); 17: Jerez et al. (2010); 18: Register (2011); 19: Ley-Quinónez et al. (2011); 20: Camacho et al. (2013); 21: Trocini (2013); 22: Sakai et al. (2000); 23: van de Merwe (2008)

^a First and third quartiles reported, not total range

^b Data originally presented as dw, values were converted for comparison purposes to wet weight using a moisture percentage of 84.62%

^c Data originally presented as dw, values were converted for comparison purposes to wet weight using a moisture percentage of 28.02%

Table 3. Results of least-squares linear regressions between toxic element concentrations in whole blood and keratin. The coefficient of variation is given (r^2) with the P value in parentheses. Keratin cadmium and lead were not included in the table as the majority of values were BDL and/or no correlations were observed. All observed relationships are positive. NS represents “non-significant” regressions.

	WB arsenic	WB cadmium	WB lead	WB mercury	WB selenium	Keratin arsenic	Keratin cadmium	Keratin lead	Keratin mercury	Keratin selenium
WB arsenic	–	NS	NS	$0.16 (0.04)^a$	$0.40 (0.001)^b$	$0.43 (0.001)^a$	NS	NS	$0.33 (0.004)$	$0.46 (0.001)^b$
WB cadmium		–	NS	NS	$0.23 (0.01)^b$	NS	NS	NS	NS	$0.35 (0.004)$
WB lead			–	NS	NS	NS	$0.29 (0.01)$	NS	NS	NS
WB mercury				–	NS	NS	NS	NS	$0.96 (<0.001)$	NS
WB selenium					–	$0.32 (0.01)^b$	NS	NS	NS	$0.70 (<0.001)^b$
Keratin arsenic						–	NS	NS	NS	$0.24 (0.02)^b$
Keratin cadmium							–	NS	NS	NS
Keratin lead								–	NS	NS
Keratin mercury									–	NS

^a Data was square-root transformed

^b Data was log-transformed

Table 4. Plasma protein electrophoresis, hematology, plasma chemistry, immune function, and oxidative stress data in nesting loggerhead sea turtles. Cells assigned “NA” indicate that some sample concentrations were BDL.

Health index	Mean	SD	SE	Median	Min	Max	N
<i>Plasma proteins</i>							
Total protein by Biuret (g/dl)	3.8	0.5	0.1	3.8	2.9	4.5	19
Prealbumin (g/dl)	0.1	0.1	0.01	0.1	0.04	0.2	19
Albumin (g/dl)	0.7	0.1	0.03	0.7	0.3	0.9	19
α_1 -globulin (g/dl)	0.2	0.1	0.01	0.2	0.1	0.3	19
α_2 -globulin (g/dl)	0.7	0.1	0.03	0.7	0.5	1.0	19
β -globulin (g/dl)	1.1	0.2	0.04	1.2	0.8	1.4	19
γ -globulin (g/dl)	1.0	0.2	0.04	1.0	0.7	1.3	19
Total globulin (g/dl)	3.0	0.4	0.1	3.0	2.2	3.5	19
Albumin:Globulin ratio	0.26	0.05	0.01	0.28	0.15	0.33	19
Total solids (g/dl)	4.6	0.8	0.2	4.4	3.4	6.4	28
<i>Hematology</i>							
PCV (%)	27	4	1	28	16	33	36
WBCs x 10 ³ / μ l	13.4	2.1	0.4	13.3	8.9	18.0	31
Heterophils x 10 ³ / μ l	7.9	2.1	0.4	7.9	4.5	12.0	31
Lymphocytes x 10 ³ / μ l	4.1	1.1	0.2	3.9	2.2	5.9	31
Monocytes x 10 ³ / μ l	0.7	0.3	0.06	0.7	0.1	1.3	31
Eosinophils x 10 ³ / μ l	0.6	0.5	0.08	0.6	0	1.9	31
Basophils x 10 ³ / μ l	0.05	0.1	0.02	0	0	0.4	31
<i>Immune/oxidative stress</i>							
Lysozyme (μ g HEL/ml)	5.0	2.5	0.5	4.3	1.7	14.1	24
SOD (U/ml)	92.5	58.6	9.8	80.3	23.7	310.5	36
ROS/RNS (nM)	2898	2169	362	2198	177	7820	36
<i>Plasma biochemistry/PCV</i>							
ALT (U/L)	20	6	1	21	7	28	16
ALKP (U/L)	19	8	2	19	<10	34	19
Amylase (U/L)	529	151	35	570	257	770	19
AST (U/L)	180	39	9	180	114	263	19
BUN (mg/dl)	13	8	2	11	5	42	19
Calcium (mg/dl)	5.3	3.3	0.8	4.3	1.0	11.9	19
Calculated osmolality (mmol/l)	271	18	4	271	238	309	19
Chloride (mmol/l)	112	5	1	112	102	122	19
Cholesterol (mg/dl)	184	42	10	190	112	269	19
CPK (U/L)	381	188	43	288	208	846	19
GGT (U/L)	<5	NA	NA	<5	<5	<5	19
Glucose (mg/dl)	92	16	4	88	68	126	19
Iron (μ g/dl)	40	18	4	39	9	69	19
Magnesium (mg/dl)	5.5	0.8	0.2	5.3	4.2	7.7	19
Phosphorus (mg/dl)	8.5	1.7	0.4	8.1	6.5	13.3	19
Potassium (mmol/l)	4.0	0.5	0.1	4.0	3.1	5.1	19
Sodium (mmol/l)	135	9	2	135	119	155	19
Triglycerides (mg/dl)	419	217	50	442	46	1039	19
Uric Acid (mg/dl)	0.5	0.2	0.04	0.5	<0.2	0.7	19

Table 5. Significant linear regressions between the whole blood toxic element concentrations (and the sum of all toxic elements) and measured blood analytes. The coefficient of variation (r^2) is given with the P value in parentheses. Blank cells indicate no significant correlation. Negative relationships are **bolded**. Positive relationships are *italicized*.

Blood analyte	Arsenic	Cadmium	Lead	Mercury	Selenium	Sum
Total protein					0.27 (0.02)	
Albumin			0.21 (0.046)			
α_2 -globulins			0.43 (0.002)^a			
γ -globulins	0.19 (0.06)	0.18 (0.07)			0.38 (0.01)	0.40 (0.004)
Total globulin		0.23 (0.04)^b			0.34 (0.01)	0.27 (0.02)
Albumin:globulin ratio		<i>0.32 (0.01)</i>				
Total solids			0.25 (0.01)			
Lymphocytes					<i>0.19 (0.02)</i>	
Monocytes					<i>0.15 (0.03)</i>	
BUN				<i>0.67 (0.0001)^c</i>		
Calculated osmolality						
Iron			0.22 (0.045)			
Magnesium			<i>0.21 (0.049)</i>			
Sodium			<i>0.26 (0.03)</i>			
Triglycerides						

^a Data were log-transformed

^b Data were cube-root transformed

^c Regression was significant due to one outlier of BUN

Humoral Immune Response

Toxic Element Concentration