Maternal transfer and sublethal immune system effects of brevetoxin exposure in nesting loggerhead sea turtles (*Caretta caretta*) from western Florida

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Abbreviations: ACN, acetonitrile; ALT, alanine aminotransferase; ALKP, alkaline phosphatase; AST, aspartate aminotransferase; BDL, below detection limits; BUN, blood urea nitrogen; CK, creatine kinase; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography-mass spectrometry; LOD, limit of detection; LDH, lactate dehydrogenase; MeOH, methanol; OC, organic contaminants; PCV, packed cell volume; PbTx-3, brevetoxin-3; PIT, passive integrated transponder; ROS, reactive oxygen species; RNS, reactive nitrogen species; SCL_{min}, minimum straight carapace length; SOD, superoxide dismutase

ABSTRACT

Blooms of *Karenia brevis* (also called red tides) occur almost annually in the Gulf of Mexico. The health effects of the neurotoxins (*i.e.*, brevetoxins) produced by this toxic dinoflagellate on marine turtles are poorly understood. Florida's Gulf Coast represents an important foraging and nesting area for a number of marine turtle species. Most studies investigating brevetoxin exposure in marine turtles thus far focus on dead and/or stranded individuals and rarely examine the effects in apparently "healthy" free-ranging individuals. From May–July 2014, one year after the last red tide bloom, we collected blood from nesting loggerhead sea turtles (*Caretta caretta*) on Casey Key, Florida USA. These organisms show both strong nesting and foraging site fidelity. The plasma was analyzed for brevetoxin concentrations in addition to a number of health and immune-related parameters in an effort to establish sublethal effects of this toxin. Lastly, from July–September 2014, we collected unhatched eggs and liver and yolk sacs from dead-in-nest hatchlings from nests laid by the sampled females and tested these samples for brevetoxin concentrations to determine maternal transfer and effects on reproductive success. Using a competitive ELISA, all plasma samples from nesting females tested positive for brevetoxin (reported as ng brevetoxin-3[PbTx-3] equivalents [eq]/mL) exposure (2.1–26.7 ng PbTx-3 eq/mL). Additionally, 100% of livers (1.4–13.3 ng PbTx-3 eq/mL) and yolk sacs (1.7–6.6 ng PbTx-3 eq/mL) from dead-in-nest hatchlings and 70% of eggs (<1.0–24.4 ng PbTx-3 eq/mL) tested positive for brevetoxin exposure with the ELISA. We found that plasma brevetoxin concentrations determined by ELISA in nesting females positively correlated with gamma-globulins, indicating a potential for immunomodulation as a result of brevetoxin exposure. While the sample sizes were small, we also found that plasma brevetoxin concentrations determined by ELISA in nesting females significantly correlated with liver brevetoxin concentrations of dead-in-nest hatchlings and that brevetoxins could be related to a decreased reproductive success in this species. This study suggests that brevetoxins can still elicit negative effects on marine life long after a bloom has dissipated. These results improve our understanding of maternal transfer and sublethal effects of brevetoxin exposure in marine turtles.

Keywords: brevetoxin, eggs, hatchling, Gulf of Mexico, immune system, liver, marine turtle

1. Introduction

Marine turtles face a number of natural and anthropogenic threats including bycatch from fisheries, coastal development and erosion, artificial lighting, and low survival rate of hatchlings (Wallace et al., 2011). Marine turtles in the eastern Gulf of Mexico must also contend with harmful blooms of the dinoflagellate, *Karenia brevis*. Blooms of *K. brevis* occur almost annually in the Gulf of Mexico and result in the release of neurotoxins (*i.e.*, brevetoxins) that are known to cause massive fish kills, increased mortalities of marine mammals and turtles, and adverse effects on human health (Fauquier et al., 2013). Both nesting and foraging loggerhead sea turtles (*Caretta caretta*) on Florida's west coast can be affected by brevetoxins through two possible routes of exposure: inhalation of aerosolized toxins and/or ingestion of red-tide exposed prey (Flewelling et al., 2005). Loggerheads may be especially vulnerable to the effects of brevetoxin bioaccumulation as they prey on filter-feeding invertebrates (Bjorndal, 1997) that may serve as brevetoxin vectors (Fauquier et al., 2013).

The presence of brevetoxins in marine turtles is an issue of concern as little is known about the potential sublethal effects of these organic toxins on these organisms. Previous studies of laboratory animals, freshwater fishes and marine turtles suggest that the immune system, the reproductive system, and overall survival are impacted by exposure to brevetoxins (Kimm-Brinson and Ramsdell, 2001; Benson et al., 2004, 2005; Walsh et al., 2010, 2015; Perrault et al., 2014a). These impacts include reduced survival and hatching success, a higher incidence of stranding, suppressed immune function (*e.g.*, decreased lymphocyte proliferation), oxidative stress and inflammation (Walsh et al., 2010; Flewelling et al., 2013; Perrault et al., 2014a); however, limited research exists regarding brevetoxin exposure in marine turtles aside from reported concentrations in tissues (Capper et al., 2013; Flewelling et al. 2013).

Additionally, only one study has been conducted that documents long-term storage of this toxin in marine turtle tissues (*e.g.*, loggerheads had detectable brevetoxin concentrations in their plasma ≤ 80 days post-exposure to a red tide bloom: Fauquier et al. 2013).

Marine turtles are capital breeders, whereby they accumulate lipid reserves on foraging grounds and forage little, if any, during the nesting season (Hamann et al., 2003; Goldberg et al., 2013; Plot et al., 2013; Perrault et al., 2014b, 2016). Brevetoxins are lipid soluble (Poli et al., 1986) and it is likely that female loggerheads store and accumulate these toxins in numerous tissues (*e.g.*, fat, liver), as well as pass on these toxins to their offspring through the egg yolk (Kennedy et al., 1992; Cattet and Geraci, 1993; Flewelling et al., 2010). Thus, even if no major red tide events occur during the nesting season, toxins stored in the females' fat from previous exposure events could be released as their lipid stores are metabolized (Kwan, 1994; Keller et al., 2014). This route of exposure has the potential to continually affect marine turtle health and reproductive success long after a bloom has dissipated (Naar et al., 2007). Brevetoxins persist both in the environment and in loggerhead prey items for extended periods, in some cases over a year, which could also result in prolonged brevetoxin exposure (Naar et al., 2007; Flewelling, 2008).

Brevetoxins include at least 14 closely related toxic congeners (*e.g.*, PbTx-1, PbTx-2, PbTx-3, PbTx-4, Cysteine PbTx-A, etc.) all with two distinct backbone structures: PbTx-1, or type A, and PbTx-2, or type B (Fauquier et al., 2013). Establishing which of these brevetoxins are present in marine turtle tissues is critical to understanding the sublethal immune system effects, as parent brevetoxins are more toxic than brevetoxin metabolites (Shimizu et al., 1986). Our objectives were to document (1) brevetoxin concentrations in nesting loggerhead sea turtles over a year after the last major red tide event, (2) the effects of red tide exposure on immune function and overall health in nesting loggerheads, (3) brevetoxin concentrations in egg contents and hatchling tissues (liver, yolk sac) and (4) the concentrations of specific parent brevetoxin congeners in tissues of nesting and hatchling loggerhead turtles.

2. Materials and Methods

2.1. 2013 and 2014 red tides

The last major red tide event prior to the 2014 nesting season occurred from January–March, 2013, where medium to high levels ($>100,000$ – $>1,000,000$ cells/L) of *K. brevis* were detected immediately offshore in southwest Florida waters. The bloom spanned ~160 km, over four counties (Collier, Lee, Charlotte, Sarasota; FFWCC and FWRI, 2015). It is known from satellite-tagging studies that Casey Key (our study site discussed below) nesting loggerheads forage at or near the areas where the red tide was present (Tucker et al., 2014). Additionally, towards the end of the 2014 nesting season a red tide was present at in an area ~150–200 km north of the nesting beach (FFWCC and FWRI, 2015). The potential effects of both of these blooms are subsequently discussed.

2.2. Sample collection from nesting females and nest inventory

Nest monitoring and exposure assessment of Gulf of Mexico loggerhead sea turtles were accomplished simultaneously through on-going field sampling efforts along the southwest Florida coastline. Routine nightly surveys of 6 km of loggerhead nesting habitat were conducted from June 1–August 1, 2014 on Nokomis Beach of Casey Key, Florida USA (28.7° N, 82.3°W). Approximately 125 nesting loggerheads were previously satellite-tagged on Casey Key for a separate study in an effort to determine their foraging grounds (Tucker et al., 2014). These turtles were targeted for our study as they show strong site fidelity to both foraging and nesting grounds. Non-satellite tagged animals were also sampled. Individual female loggerhead turtles were identified based on their flipper and/or internal passive integrated transponder (PIT) tags. These tags were applied if neither type of tag was present. After the turtles entered their nesting fixed action pattern (Dutton and Dutton, 1994), approximately 8–10 mL of blood were collected from the subcarapacial sinus using a 20 mL syringe and 3" BD heparin-coated

spinal needle (Becton, Dickinson and Company, Franklin Lakes, New Jersey USA). Before insertion of the needle, the entire area was swabbed with a sterile 70% isopropyl alcohol swab. The blood was collected into a 10 mL lithium-heparin coated Vacutainer® and subsequently placed on ice in the field for up to 8h. The venipuncture site was then disinfected with a new alcohol swab and pressure was applied to promote hemostasis. After blood collection, straight carapace length was recorded (SCL_{min}). Plasma was collected from the whole blood by centrifugation, transferred to cryovials, and stored at -80°C until analyses were conducted.

After sample collection from nesting females, nests were marked and monitored for signs of hatchling emergence. Nests were excavated 3 days after the mass hatchling emergence. When hatches were not observed, nests were inventoried at 70 d from the date laid; average incubation time in loggerhead is ~50 days. Several measures of reproductive success were calculated, including: total clutch size (total hatched, pipped and unhatched eggs), hatching success (hatched eggs/total clutch size), and emergence success (number of hatchlings that emerged independently from the nest prior to nest excavation/total clutch size). Up to 3 unhatched eggs with no evidence of embryonic development from each clutch were collected for analysis of brevetoxin concentrations. Eggs from each nest were pooled by clutch. When available, non-autolyzed dead-in-nest hatchlings were also collected during nest inventories. Liver and yolk sac were removed from each hatchling. Collected samples were placed into cold storage containers in the field for up to 8 h and subsequently frozen at -80°C until analyses.

2.3. *Extraction of plasma, unhatched eggs and hatchling tissues for liquid chromatography-mass spectrometry (LC-MS/MS)*

Brevetoxins consist of a suite of ~14 related congeners (Fauquier et al. 2013). We analyzed for two parent brevetoxin compounds (PbTx-1 and PbTx-2: the brevetoxins produced within the dinoflagellate), two derivatives of the parent compound PbTx-2 (PbTx-3 and PbTx-2CA: produced as the cells lyse) and the brevetoxin antagonist, brevenal (Pierce et al., 2011). Brevetoxins were extracted from loggerhead plasma using a modification of the turtle bile extraction method by Fauquier et al. (2013). Briefly, 0.5–1.0 ml of plasma were added to a Sep-Pak C-18 solid phase extraction column (Varian 1000mg/6mL C18-E cartridges; Phenomenex®, Torrance, California USA) and eluted with 25% MeOH in water. Brevetoxins were recovered in 100% MeOH. Quantitative and qualitative analyses were conducted with a high performance liquid chromatograph interfaced with tandem mass spectrometer detection (LC-MS/MS, described below).

Whole egg contents (*i.e.*, albumen and yolk) of up to 3 unhatched eggs per nest were pooled by clutch. Liver and yolk sac samples from dead-in-nest hatchlings were also collected and extracted according to the method of Abraham et al. (2012). Briefly, tissues were extracted in acetone, defatted with hexane, cleaned by Sep-Pak SPE, and analyzed with ELISA (see below) and/or LC-MS/MS.

Brevetoxin congeners (*e.g.*, PbTx-1, PbTx-2, PbTx-3, PbTx-CA, brevenal) in the plasma, liver and yolk sac extracts were structurally confirmed and quantitated by using a Thermo Electron Quantum Access LC-MS/MS system. The LC consists of an Accela Ultra High Performance Liquid Chromatography pumping system, coupled with an Accela autosampler and degasser. Mass spectral detection was performed using a Quantum Access triple quadrupole MS/MS. The analytical column was a Thermo Fisher Hypersil Gold (100 x 2.1 mm) with 5 µm particles. The solvent gradient was composed of acetonitrile (ACN, 0.1 % Formic Acid) and H₂O with initial conditions of 30:70 ACN:H₂O to 95:5 ACN:H₂O over 30 min returning to 30:70 ACN:H₂O over 5 min by a hold of 30:70 ACN:H₂O for 5 min for a total of 40 min at a flow rate of 200 µl/min.

2.4. *Brevetoxin ELISA*

Total brevetoxin concentrations in plasma, liver and yolk sac were analyzed using modifications of a competitive enzyme-linked immunosorbent assay (ELISA; MARBIONC, Wilmington, North Carolina USA) described by Naar et al. (2002). The ELISA developed by Naar et al. (2002) detects and

measures brevetoxin congeners with the dominant (80%) B-type backbone (PbTx-2, 3, 5, 6, 8, 9); however, those with the A-type backbone are recognized, but at reduced affinities (Fauquier et al., 2013). The resulting concentration from the ELISA is essentially a sum of the detected congeners. Briefly, 96-well plates were coated with bovine serum albumin (BSA)-linked brevetoxin-3 [PbTx-3] (Reagent A). Both samples and the standard were added to the BSA-coated wells and were serially diluted seven times in PGT (phosphate buffered saline [PBS], 0.5% gelatin and 0.1% Tween-20) to create a standard curve of 0, 0.15625, 0.125, 0.625, 1.25, 2.5, 5 and 10 ng PbTx-3 equivalents/ml (for the standard wells; ng PbTx-3 eq/ml). Goat anti-PbTx-3 (Reagent C) was added to each well (100µL) and the plate was incubated at room temperature for 1 h using an orbital shaker. The wells were washed three times in PBS-Tween (PBS-T) followed by the addition of horseradish peroxidase-linked rabbit anti-goat IgG (Reagent D). The plate was then incubated for 1 h at room temperature. The wells were washed six additional times, three with PBS-T followed by three with PBS. Finally, 3,3',5,5'-tetramethylbenzidine substrate (Thermo Fisher Scientific, Inc., Tampa, Florida USA) was added for 2 min in the absence of light until a blue color change was observed. The reactions were stopped by adding 100 µL of 0.5 M H₂SO₄. Absorbance was read at 450 nm using a BioTek® ELx800 microplate reader (BioTek® Instruments, Inc., Winooski, Vermont USA). Concentrations were calculated using a standard curve of PbTx-3. The limit of detection (LOD) for this assay was 1 ng PbTx-3 eq/mL or g.

2.5. Immune function, inflammation and oxidative stress

Lysozyme is an enzyme used to measure innate immune function. It is used as a marker for pro-inflammatory responses and has been shown to correlate with toxins and toxicants (Walsh et al. 2010, 2015). 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) solution 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 in phosphate buffer to produce a standard curve of 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg/ml. Aliquots (25 µl) of each concentration were added to a 96-well plate in quadruplicate and 25 µl of test plasma were added in quadruplicate to the plate. *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. Readings were conducted immediately (T₀) and after 5 min. Absorbance unit (AU) values at 5 min were subtracted from AU values at T₀ 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 (µg/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. One U/mL of SOD is equal to the amount of enzyme needed to cause 50% dismutation of the superoxide radical.

Total protein in plasma was measured using a handheld refractometer. Plasma protein fractions including albumin, alpha- (α_1 - and α_2 -), beta- (β), and gamma (γ)-globulins were determined using a QuickGel™ Serum Protein Electrophoresis (SPE) Chamber and agarose gels (QuickGel® Split Beta SPE; Helena Laboratories, Beaumont, Texas USA). Gels were stained with an acid blue stain and destained with citric acid. Relative densities of bands were determined using a gel imager (Bio-Rad ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc., Hercules, California USA). The albumin:globulin (A:G) ratio was also calculated. Quality control was carried out using plasma protein electrophoresis (PPE) normal and PPE abnormal controls (Helena) on each gel run.

2.6. *PCV and plasma biochemistry*

Packed cell volume (PCV) was determined from a subsample of whole blood collected into microcapillary tubes (Fisher HealthCare, Houston, Texas USA) with Critoseal® (Sherwood Medical Co., Deland, Florida USA) as the sealant. The samples were spun for 5 minutes 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 measure PCV.

Plasma samples were used for biochemical analyses, which were carried out at Sarasota Memorial Health Care Center (Sarasota, Florida USA). Single aliquots of plasma were analyzed for alanine aminotransferase activity (ALT), alkaline phosphatase activity (ALKP), aspartate aminotransferase activity (AST), blood urea nitrogen (BUN), calcium, chloride, creatine kinase activity (CK), creatinine, glucose, iron, lactate dehydrogenase activity (LDH), phosphorus, potassium, sodium, total bilirubin, and uric acid.

2.7. *Statistical analyses*

All statistical analyses were performed using All statistics were performed using IBM SPSS Statistics 22 (SPSS, Inc, Chicago, IL, USA). The Shapiro-Wilk statistic was used to determine if the data were normally distributed. Mean, standard deviation, median and range are reported for data that did not fall below detection limits (BDL). Mean and standard deviation were not calculated for parameters with values that fell BDL.

In an effort to determine if plasma brevetoxin concentrations in nesting females or eggs changed across the nesting season, we subtracted plasma brevetoxin concentrations from the first sampling event from concentrations during the second sampling event. To establish if days in between sampling events impacted the change in brevetoxin concentration, we performed a Pearson correlation between the change in concentration and the number of days in between sampling events. Because no statistical correlation was found between days in between sampling events and change in brevetoxin concentration, we eliminated the sampling interval from the statistical analyses and analyzed the change in concentration in plasma and eggs using a repeated measures ANOVA.

Statistical analyses were also conducted to determine relationships between brevetoxin concentrations and the measured health and immune parameters using correlation analyses (Pearson or Spearman, depending on normality). A Kruskal-Wallis test with a post hoc Dunn's test was used to determine differences in brevetoxin concentrations in plasma or egg contents by foraging ground (west Florida shelf, Florida Keys, offshore Yucatan, Caribbean; Tucker et al., 2014). Spearman rank-order correlations were used to assess the relationship between maternal plasma brevetoxin concentrations and hatchling liver and yolk sac brevetoxin concentrations. Spearman correlations were also used to determine if correlations existed between hatchling tissue brevetoxin concentrations and hatching and emergence success (Zar, 1999).

3. **Results**

3.1. *Plasma brevetoxins in nesting females using a competitive ELISA*

Thirty-four nesting loggerheads were sampled during the 2014 nesting season. Average SCL_{min} was 86.1 ± 6.4 cm, with a range of 70.9–97.2 cm. Twelve nesting females were sampled twice and one individual was sampled three times for brevetoxin concentrations yielding a total of 48 samples. All of the sampled females tested positive for brevetoxin exposure (**Table 1**).

Clutch size ranged from 23–148 eggs ($mean \pm SD = 95.4 \pm 23.9$ eggs). Excluding depredated nests, hatching success ranged from 12.4%–99.0% (median = 89.7%), while emergence success ranged from 12.4%–99.0% (median = 87.6%). Using Pearson or Spearman correlations (depending on normality), minimum SCL, clutch size, hatching success, and emergence success did not significantly correlate with maternal plasma brevetoxin concentrations ($P > 0.05$). Plasma brevetoxin concentrations did not change across the season (*i.e.*, in nesting females sampled more than once; $N = 13$; $P > 0.05$) when analyzed using a repeated measures ANOVA. Lastly, results of the Kruskal-Wallis test with a post hoc Dunn's test revealed that plasma brevetoxins did not significantly differ among foraging grounds ($P > 0.05$; **Fig. 1**).

3.2. Plasma brevetoxins in nesting females using LC-MS/MS

All plasma samples ($N = 19$) from nesting females fell BDL for PbTx-1 (LOD: 0.76 ng/ml), PbTx-2 (LOD: 0.13 ng/ml), PbTx-2CA (LOD: 0.36 ng/ml) and brevenal (LOD: 6.69 ng/ml). One sample came back positive for the PbTx-3 congener (2.04 ng/ml); the LOD for PbTx-3 was 0.08 ng/ml. PbTx-3 in the plasma accounted for <0.3%–19.3% of the measured ELISA activity when comparing the results of LC-MS/MS to the values given by the ELISA. To determine the percentages, the amount of PbTx-3 in the samples determined by LC-MS/MS (*e.g.*, 2.04 ng/ml) was divided by the ELISA result for the same sample (*e.g.*, 10.6 ng PbTx-3 eq/ml; $2.04/10.6 = 19.3\%$). For values that were BDL with LC-MS/MS, the detection limit of PbTx-3 (0.08 ng/ml) was divided by the ELISA result to give an estimate of the percentage (*e.g.*, $0.08 \text{ ng} \cdot \text{ml}^{-1} / 26.7 \text{ ng PbTx-3 eq} \cdot \text{ml}^{-1} = <0.3\%$).

3.3. Brevetoxins measured by a competitive ELISA and maternal health

Lysozyme activity, PCV, plasma biochemistry, ROS/RNS, SOD activity, total protein and protein electrophoresis results are presented in **Table 2**. Of all measured blood parameters, only albumin ($N = 37$; $r = 0.39$, $P = 0.02$; **Fig. 2**), γ -globulins ($N = 37$; $r = 0.57$, $P = 0.0002$; **Fig. 3**) and total globulins ($N = 37$; $r = 0.42$, $P = 0.01$; **Fig. 3**) correlated with plasma brevetoxin concentrations using Pearson or Spearman correlations.

3.4. Brevetoxins in egg contents and hatchling tissues using a competitive ELISA

Forty-seven eggs were analyzed for brevetoxin concentrations, 14 of which fell BDL. Results of brevetoxin analyses from egg contents and hatchling tissues are reported in **Table 1**. Results of repeated measures ANOVA show that brevetoxin concentrations in the egg contents did not change from the first clutch to the second clutch ($N = 6$, $P > 0.05$). Additionally, brevetoxin concentrations in egg contents did not significantly differ among foraging grounds ($P > 0.05$; **Fig. 1**) using a Kruskal-Wallis test with a post hoc Dunn's test.

Using Spearman correlations, we found that nesting female plasma brevetoxin concentrations did not significantly correlate with brevetoxin concentrations in the egg contents ($N = 30$; $P > 0.05$) or yolk sacs ($N = 4$; $P > 0.05$), but did correlate with brevetoxin concentrations in the livers of dead-in-nest hatchlings ($N = 5$; $r_s > 0.99$; $P < 0.0001$; **Fig. 3**). Hatchling liver brevetoxin concentrations did not significantly correlate with yolk sac brevetoxin concentrations ($N = 6$; $P > 0.05$) or hatching and emergence success ($N = 7$; $P > 0.05$) using Spearman correlations. Lastly, dead-in-nest hatchling yolk sac brevetoxin concentrations negatively correlated with hatching ($N = 6$; $r_s = -0.89$; $P = 0.02$; **Fig. 4**) and emergence success ($N = 6$; $r_s = -0.94$, $P = 0.005$; **Fig. 4**) using Spearman correlations.

3.5. *Brevetoxins in egg contents and hatchling tissues using LC-MS/MS*

All egg samples ($N = 12$), hatchling liver samples ($N = 7$), and hatchling yolk sac samples ($N = 4$) fell BDL for PbTx-1, PbTx-2, PbTx-2CA, and brevenal using LC-MS/MS. Two egg samples tested positive for the PbTx-3 congener (3.02 ng/ml and 4.76 ng/ml). PbTx-3 in the egg contents accounted for <0.3%–44.5% of the measured ELISA activity when comparing the results of LC-MS/MS to the values measured by the ELISA. One liver sampled tested positive for the PbTx-3 congener (0.15 ng/ml); PbTx-3 accounted for <0.6–10.7% of the measured ELISA activity in liver samples. All yolk sac samples fell BDL for PbTx-3, with the PbTx-3 congener accounting for <1.2%–<4.7% of the measured ELISA activity.

4. Discussion

4.1. *Brevetoxins in nesting females*

In this study, we set out to document brevetoxin concentrations (using a competitive ELISA) in nesting loggerhead sea turtles during the 2014 nesting season, one year after the last major red tide event, and compare those concentrations to measures of immune function and overall health. We also sought to establish, for the first time, brevetoxin concentrations in egg contents and tissues (liver and yolk sac) of loggerhead hatchlings. The ELISA assay provides a measure of total brevetoxins in plasma, but does not provide information on specific congeners, derivatives, or metabolites present. In order to obtain information about specific congeners present, we analyzed a subset of our samples using LC-MS/MS to assess the presence of specific parent and derivative brevetoxin congeners (PbTx-1, PbTx-2, PbTx-3, PbTx-2CA, Brevenal) in tissues of nesting and hatchling loggerhead turtles.

Using a competitive ELISA, we found that every nesting female tested positive for brevetoxin exposure during the 2014 nesting season. This finding was unexpected, as the last red tide bloom (>100,000–>1,000,000 cells/L) occurred from January–March 2013 in waters surrounding the nesting beach (FFWCC and FWRI, 2015). Brevetoxin concentrations in nesting females from this study averaged 9.1 ± 6.1 ng PbTx-3 eq/ml, which is lower than plasma brevetoxin concentrations of loggerhead (Walsh et al., 2010: 68.0 ± 30.7 ng PbTx-3 eq/ml; Fauquier et al., 2013: 32 ng PbTx-3 eq/ml) and Kemp's ridley turtles (*Lepidochelys kempii*; Fauquier et al., 2013: 63 ng PbTx-3 eq/ml; Perrault et al., 2014a: 22.6 ± 6.5 ng PbTx-3 eq/ml) that were sampled during a red tide event. The low plasma brevetoxin concentrations observed in this study were likely due to the absence of a *K. brevis* bloom and reduced food intake during the nesting season, while turtles from the other studies were consuming red-tide exposed prey and inhaling aerosolized toxin. Kemp's ridleys present during red tide blooms of 2012–2013 had significantly higher plasma brevetoxin concentrations in comparison to turtles sampled while a bloom was not present, further substantiating our findings (Perrault et al., 2014a).

Marine turtles are capital breeders that fast during the nesting season (Hamann et al., 2003; Goldberg et al., 2013; Plot et al., 2013; Perrault et al., 2014b, 2016). Because brevetoxins are lipid-soluble (Poli et al., 1986), these toxins likely accumulate in adipose tissue when these organisms consume red-tide exposed prey on foraging grounds. Brevetoxins have been shown to accumulate in fat after oral and intratracheal exposure in rats (Cattet and Geraci, 1993; Benson et al., 1999) and red-eared sliders (*Trachemys scripta*; Cocilova et al., 2014). Loggerheads forage on shellfish and crustaceans (Bjorndal, 1997), organisms in which brevetoxins can persist for months after a bloom has dissipated (Flewelling, 2008). During the nesting season, fat reserves are utilized as an energy source (Stephens et al., 2009) and lipid-soluble contaminants stored in fats are metabolized into the bloodstream (similar to organic contaminants [OCs]; Keller et al., 2004; Guirlet et al., 2010). Therefore, it is plausible that the nesting females from this study tested positive for brevetoxin exposure as a result of fat metabolism or mobilization from the liver during vitellogenesis (Guirlet et al., 2010). This is likely as brevetoxins in the livers of fishes are present for more than a year after the cessation of a bloom (Naar et al., 2007).

A red tide was present at the end of the nesting season (July 25, 2014) in an area ~150–200 km north of the nesting beach. The bloom was ~10,000 km² and was present 60–145 km offshore (FFWCC and FWRI, 2015). It is possible that nesting females traveled to this area during their internesting intervals (T. Tucker, pers. comm.); however, Casey Key loggerheads tend to stay within 100 km of the nesting beach during the nesting season (Tucker et al., 2010). Therefore, the bloom of July 2014 most likely had little impact on plasma brevetoxin concentrations in Casey Key loggerheads during the nesting season. If the turtles were exposed to the bloom, it would be expected that plasma brevetoxin concentrations would have increased towards the end of the nesting season; such a trend was not observed. Additionally, it appears that Kemp's ridleys exhibit an avoidance behavior to red tides; however, the same may not apply to nesting and post-nesting loggerheads, as some satellite-tracked individuals appear in the epicenter of red tide blooms (J. Schmid, pers. comm.). Lastly, LC-MS/MS did not reveal any parent compounds (PbTx-1, PbTx-2, PbTx-3, PbTx-2CA) in the plasma, suggesting that this bloom did not affect turtles from our study, as parent compounds would have likely been more prevalent in the plasma from the more recent bloom. The percentage of PbTx-3 in the ELISA was <1.1% for 18 of the 19 samples, further suggesting the presence of metabolites in the plasma of nesting females.

Loggerheads that were previously satellite-tagged for a separate study were targeted for this project, although non-satellite tagged turtles were also sampled. Upon completion of the nesting season, Casey Key loggerheads return to one of five potential foraging grounds and exhibit a strong fidelity to these foraging sites (Wider Caribbean, Florida Keys, northern Gulf of Mexico, West Florida shelf, and the Yucatan Peninsula; Tucker et al., 2014). During 2014, we sampled turtles from every foraging ground except the northern Gulf of Mexico. Differences in plasma brevetoxin concentrations (by ELISA) among foraging grounds were not significant likely due to small sample sizes (Caribbean: $N = 2$; Florida Keys: $N = 4$; West Florida Shelf: $N = 4$; Yucatan: $N = 2$; **Fig. 1**). Further sampling during subsequent seasons will determine if West Florida loggerheads (where red tides are frequent) have significantly higher brevetoxin concentrations than turtles that forage in the Caribbean and the Florida Keys (where red tides are rare; Flewelling et al., 2010).

The patrol area for loggerheads on Casey Key is ~6 km and it was rare to encounter the same nesting female multiple times during the season. We were able to sample 13 turtles twice and found that there was little to no change in plasma brevetoxin concentrations across the nesting season. Concentrations of OCs also tend to remain constant during the nesting season when turtles expend high amounts of energy due to the demands of nesting (*e.g.*, migration to the nesting beach, production of numerous egg clutches, internesting migrations; Guirlet et al., 2010). Brevetoxins, like OCs, are likely mobilized from fat stores during the nesting season and will remain constant during periods of high activity and low food intake (Keller et al., 2004; Guirlet et al., 2010). Constant plasma brevetoxin concentrations across the season suggest that the adipose reserves have already been mobilized during migration to the nesting beach (Guirlet et al., 2010).

4.2. *Brevetoxins and maternal health*

We found that albumins positively correlated with brevetoxin concentrations (**Fig. 2**). Previous studies have found no correlations between hematologic and biochemical indices (*e.g.*, liver and kidney enzymes) and brevetoxin concentrations (bottlenose dolphin, *Tursiops truncatus*: Twiner et al., 2011; sea birds: Barron et al., 2013); however, Twiner et al. (2011) compared health parameters to brevetoxin concentrations in urine and feces instead of plasma. The positive correlation between plasma brevetoxins and albumin is puzzling as brevetoxins in plasma are rarely bound to this plasma protein (Woofter et al., 2005), although more recent evidence suggests brevetoxin can form adducts with serum albumin (Wang and Ramsdell, 2011). Because albumin is related to nutritional status (Zaias and Cray, 2002), individuals that feed more while on foraging grounds could have higher plasma albumin concentrations as a result. Tissue brevetoxin concentrations are influenced by a number of factors, including food intake and frequency and extent of exposure (Flewelling et al., 2010). Loggerheads that forage at higher rates could both increase plasma albumin concentrations and tissue brevetoxin concentrations, as brevetoxins can

bioaccumulate with increased food intake even in the absence of a bloom (Flewelling et al., 2010). Additionally, proteins including albumins could be mobilized during the nesting season (Deem et al., 2009) as plasma albumins are a large component of egg albumen (Woodward, 1990). Simultaneous mobilization of brevetoxins and albumin could explain the positive correlation.

We also observed an increase in γ -globulins (and total globulins as a result of the γ fraction) with increasing brevetoxin concentrations (**Fig. 2**). In the body, γ -globulins (*i.e.*, antibodies) will increase in response to certain pathogens (Zaias and Cray, 2002); however, the highest γ -globulin concentration from our study (2.26 g/dL) fell within the “normal” reference range for loggerhead turtles (0.48–2.38 g/dL; $N = 437$ turtles; Osborne et al., 2010). Other studies have reported γ -globulin concentrations outside of this reference range for loggerhead turtles (Gicking et al., 2004: max of 2.98 g/dL for adult females; Deem et al., 2009: 2.88 g/dL for foraging turtles). Therefore, while immunomodulation may be occurring as a result of brevetoxin exposure, it is not causing the measured health parameters to increase outside of the normal biological range (Twiner et al., 2011). In the plasma, only one sample fell above detection limits for one parent compound (PbTx-3) with LC-MS/MS analyses. This indicates increased presence of less toxic metabolites (*e.g.*, Cysteine PbTx-A, Cysteine PbTx-A sulfoxide, Cysteine PbTx-B, Cysteine PbTx-B sulfoxide) over the more toxic parent compounds (Shimizu et al., 1986), explaining why we did not find correlations with other immune and health parameters. However, metabolites can still exert toxic effects (Rein et al., 1994; Fauquier et al., 2013). Additionally, brevetoxin concentrations may be too low to elicit observable effects or the effects may have occurred upon initial exposure. Cocilova et al. (2014) observed decreased lysozyme activity, phagocytosis and lymphocyte proliferation in red-eared sliders exposed (orally and intratracheally) to PbTx-3 for 2–4 weeks. These results indicate that upon initial exposure, immune-related effects may occur in turtles. Our results suggest that nesting turtles may still be susceptible to immunomodulation by brevetoxin over a year after a red-tide event due to the metabolism of fat stores and mobilization of brevetoxins from the liver during vitellogenesis.

4.3. *Maternal transfer of brevetoxins and correlations with reproductive success*

Brevetoxins were detected in egg contents of 33 of 47 eggs analyzed during the 2014 nesting season, verifying maternal transfer in loggerhead sea turtles (**Table 1**). Brevetoxins were also present in the yolk sacs of dead-in-nest hatchlings (**Table 1**). Yolk sac brevetoxin concentrations were nearly double the brevetoxin concentrations of the egg contents within the same nest. This is likely due to water absorption and concentration of lipids of the yolk sac during embryonic development (Keller, 2013a). Brevetoxin concentrations in loggerhead turtle eggs (median = 4.2 ng PbTx-3 eq/ml) were similar to concentrations in green turtle (*Chelonia mydas*) eggs also collected during the 2014 nesting season on Casey Key (median = 4.3 ng PbTx-3 eq/ml; range = <1.0–8.5 ng PbTx-3 eq/ml; $N = 9$; Perrault, unpublished data). Adult loggerheads are omnivorous while green turtles are herbivorous (Bjorndal, 1997); therefore, it would be expected that brevetoxins would be higher in loggerheads due to their dietary preference of filter-feeding organisms. Similarities between egg brevetoxin concentrations of the two species suggest similar rates of exposure and/or similar foraging areas.

We also found that brevetoxin concentrations in 14 of 47 eggs were BDL. It is possible that brevetoxin concentrations in these eggs were diluted due to the analysis of the yolk with the albumen (*i.e.*, egg white). Egg albumen is protein-rich and brevetoxins may not bind as readily to this compartment in comparison to the lipid-rich yolk. Additionally, albumen is formed during the nesting season (Owens, 1980); therefore, this compartment may adequately reflect brevetoxin exposure on nesting grounds. Future studies should analyze for the presence of brevetoxins in egg yolk and albumen separately, as brevetoxins present in albumen of the egg will be also be consumed and utilized by the embryo (Romanoff, 1967) and could influence hatchling tissue concentrations. Lastly, because brevetoxins are lipid soluble, lipid normalized values of brevetoxins in eggs and/or tissues may be beneficial in future studies (similar to OCs; Keller, 2013a).

Red tide was absent during the majority of the 2014 nesting season. Vitellogenesis begins 8–10 months prior to the breeding season (Wibbels et al., 1990); therefore, red tide conditions during vitellogenesis while marine turtles are on foraging grounds are likely more important than red tide conditions during the nesting season (Flewelling et al., 2010), as it has been hypothesized that the diet on foraging grounds is an important factor influencing contaminant loads in egg follicles (Wolfe et al., 1998). While not significant, eggs of loggerheads that forage in the West Florida Shelf and the Yucatan Peninsula had higher, on average, brevetoxin concentrations in comparison to eggs of loggerheads that forage in the Caribbean and the Florida Keys (**Fig. 1**). As previously mentioned, red tides are rare on the Atlantic coast and in the Florida Keys (Flewelling et al., 2010), while the Yucatan Peninsula has experienced blooms of *K. brevis* in the past (Magaña et al., 2003) and blooms are common in West Florida waters; therefore, not all loggerheads nesting on Casey Key are exposed equally to *K. brevis*. It has been suggested that concentrations of OCs in marine turtle tissues and eggs are affected by their foraging area (van de Merwe et al., 2010) and it is not uncommon for contaminants in loggerhead eggs to vary by foraging ground (Stoneburger et al., 1980; Alava et al., 2011). It is likely that variations in egg brevetoxin concentrations are related to maternal exposure on foraging grounds, during their migration to nesting grounds and/or during vitellogenesis. Continued sampling of marine turtle eggs for brevetoxins may allow us to reveal significant trends by foraging ground.

Just two egg samples and one liver sample yielded a result above detection limits for parent compounds (PbTx-3) with the LC-MS/MS analyses of brevetoxins. The percentage of PbTx-3 in the ELISA was <1.0% for ten of the 12 egg samples, <2.6% for six of the seven liver samples, and <4.7% for the four yolk sac samples. These results suggest that parent compounds are low/absent in eggs and hatchling tissues and that metabolites are predominantly passed on to the offspring. It is difficult to make far-reaching conclusions with our LC-MS/MS results as the majority of samples fell BDL.

Hatchling liver brevetoxin concentrations ranged from 1.4–13.3 ng PbTx-3 eq/ml (median = 6.7 ng PbTx-3 eq/ml; **Table 1**). These values are lower than liver concentrations of dead stranded loggerheads (Fauquier et al., 2013: median = 71 ng PbTx-3 eq/ml; range = <5–683 ng PbTx-3 eq/ml), green turtles (Capper et al., 2013: median = 18.6 ng PbTx-3 eq/ml; range = 5.6–40.9 ng PbTx-3 eq/ml; Fauquier et al., 2013: median = 239 ng PbTx-3 eq/ml; range = <5–345 ng PbTx-3 eq/ml) and Kemp's ridleys (Fauquier et al., 2013: median = 195 ng PbTx-3 eq/ml; range = <5–1006 ng PbTx-3 eq/ml). Hatchling liver brevetoxin concentrations were lower than, but overlap with live stranded loggerhead turtles that later died in captivity (Fauquier et al., 2013: median = 21 ng PbTx-3 eq/ml; range = <5–470 ng PbTx-3 eq/ml). Because early life stages of organisms are more sensitive to contaminants than adults (Walker et al., 1996; Thompson et al., 1998), this overlap in tissue concentration indicates that brevetoxins may elicit negative effects on developing marine turtles (see discussion below on hatching and emergence success).

It is possible that developing eggs/embryos and hatchlings are exposed to brevetoxins while in the nest, as brevetoxins have been detected in sand samples during red tide blooms (Castle et al., 2013). However, we assume that this is unlikely for three reasons. First, we found that brevetoxins in maternal plasma positively correlated with hatchling liver brevetoxin concentrations. This indicates that the majority, if not all, of the brevetoxins found in the hatchlings is present due to maternal transfer. Second, 14 of 47 eggs fell below the limits of detection. If brevetoxins were present in the sand, it is likely that all samples would have yielded positive results. Lastly, the sand samples that were collected in Castle et al. (2013) were collected during or immediately after a bloom and it is likely that the sand in our study had no brevetoxins, as no bloom had occurred in this area for well over one year.

While our sample size was small, we observed a significant positive correlation between maternal plasma brevetoxin concentrations and hatchling liver brevetoxin concentrations (**Fig. 3**). Maternal transfer of lipid-soluble contaminants is well documented in the marine turtle literature (Guirlet et al., 2010; van de Merwe et al., 2010; Stewart et al., 2011). Additionally, maternal transfer of brevetoxins into embryonic tissues has been documented in mice (Benson et al., 2006), bonnethead sharks (*Sphyrna tiburo*; Flewelling et al., 2010) and Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*; Flewelling et al., 2010). Brevetoxins were absent in tissues of Atlantic guitarfish (*Rhinobatos lentiginosus*) embryos from

southwest Florida, despite high concentrations in maternal tissues (Flewelling et al., 2010). Atlantic guitarfish are ovoviviparous and the embryos have no connection to the maternal blood supply and rely on nutrients strictly from the yolk sac. The absence of brevetoxins in embryonic guitarfish tissues was likely due to the absence of a red tide during egg development; however, the authors suggested that brevetoxins in the yolk are likely transferred to developing embryos (Flewelling et al., 2010). Here, we confirm that hypothesis and show that brevetoxins accumulate in liver tissue of developing marine turtles as a result of exposure through the yolk sac. This was not surprising as the liver accumulates and metabolizes brevetoxins (Kennedy et al., 1992; Cattet and Geraci, 1993). In subsequent years, we will continue sampling maternal and hatchling tissues to determine if this trend remains and to determine if dead-in-nest hatchling concentrations can be used to predict brevetoxin concentrations in nesting females.

We found that both hatching and emergence success decreased with increasing yolk sac brevetoxin concentrations (**Fig. 4**). While far-reaching conclusions cannot be made due to our low sample size, it is not uncommon for measures of hatchling health and reproductive success in marine turtles to be correlated with contaminants. Observed correlations include lower hatchling body condition index with increased egg Σ POP (persistent organic pollutant) concentrations (van de Merwe et al., 2010), decreased hatching and emergence success with decreased hatchling liver selenium:mercury ratios (Perrault et al., 2011), and decreased egg mass with increased egg polychlorinated biphenyl (PCB) 138, cis-nonachlor and total polybrominated diphenyl ether (PBDE) concentrations (Keller, 2013b). Additionally, other marine biotoxins (*e.g.*, domoic acid) are thought to be major factors in reproductive failure of marine mammals (Bossart, 2011), while brevetoxins have been suggested as a potential reproductive threat to bonnethead sharks (Flewelling et al., 2010). During development, embryonic marine turtles mobilize lipids from the egg yolk, which are then used by hatchlings as an energy source (Miller, 1985). It is during this time that lipid-soluble contaminants such as brevetoxins are distributed to hatchling tissues. Brevetoxins exert their effects on voltage-gated sodium channels (Poli et al., 1986; Wang and Wang, 2003), thereby negatively affecting development, growth, and hatchling survival through alterations of differentiation of nervous tissue (Colman and Ramsdell, 2003), although neurons from turtles may be more resistant to the effects of PbTx-3 in comparison to other vertebrates (Cocilova et al., 2016). Hatchlings exposed to brevetoxin through the yolk may be forced to utilize these nutrients to combat toxic stress instead of to grow and develop (van de Merwe et al., 2010). Medaka (*Oryzias latipes*) embryos exposed to PbTx-3 resulted in tachycardia, sustained convulsions, and an observed LD₅₀ of 4.0 ng/egg (~4.0 µg/g in tissue; Colman and Ramsdell, 2003), while medaka exposed to PbTx-1 exhibited spinal defects, abnormal hatching, herniation of the brain and meninges, abnormal positioning of the eyes and failure to hatch (Kimm-Brinson and Ramsdell, 2001). While the LD₅₀ observed for the medaka (~4 µg/g) was much higher than the highest concentration (13.3 ng PbTx-3 eq/g) observed in dead-in-nest hatchling tissues from this study, developmental effects of brevetoxins could still occur in embryonic marine turtles due to the sensitivity of early life stages to toxins and toxicants (Walker et al., 1996; Thompson et al., 1998). Abnormalities during development (*i.e.*, muscle weakness and lethargy) that occur as a result of brevetoxin exposure may result in a decrease in reproductive success of these organisms. We will increase our sample size in future seasons to determine if the trend of decreased hatching and emergence success with increased hatchling yolk sac brevetoxin concentrations remains.

5. Conclusions

Here, we provide the first evidence that maternal transfer of brevetoxins occurs in marine turtles. This represents an important discovery, as these natural biotoxins could negatively affect reproductive success in these internationally vulnerable animals. Additionally, we confirm that these organisms are potentially susceptible to the effects of brevetoxins long after a bloom has dissipated. Metabolism of these toxins from bodily stores can lead to immunomodulation; therefore, nesting marine turtles may be subject to the effects of these toxins twice: upon initial exposure and during metabolism of fat stores during the nesting season. Future studies on dead stranded animals and dead-in-nest hatchlings should measure brevetoxins in fat in an effort to determine if this is a potential storage site for this toxin.

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Figure Legends

Fig. 1. Brevetoxins measured by a competitive ELISA in maternal plasma (white columns) and egg (gray columns) by foraging ground. Each bar represents the mean $\pm$ SE of the samples from each foraging ground. No error bars are present for the Florida Keys' egg samples as only one sample was collected. There were no significant differences ($P > 0.05$) in brevetoxin concentrations by foraging ground in maternal plasma or egg samples using a Kruskal-Wallis test with a post hoc Dunn's test.

Fig. 2. Plasma brevetoxin concentrations (ng PbTx-3 eq/ml; square-root transformed) measured by a competitive ELISA positively correlated with albumin (black circles; square-root transformed), γ -globulins (grey squares) and total globulins (white triangles) using Pearson correlations.

Fig. 3. Maternal brevetoxin concentrations measured by competitive a ELISA positively correlated with dead-in-nest hatchling liver concentrations using Spearman correlations.

Fig. 4. Hatchling yolk sac brevetoxin concentrations measured by a competitive ELISA negatively correlated with hatching success (open black circles, black line) and emergence success (open gray squares, gray line line) using Spearman correlations.

Table 1. Brevetoxin concentrations (ng PbTx-3 eq/ml or g) in tissues of nesting loggerhead sea turtles and their eggs and hatchlings as determined by competitive ELISA. Cells assigned “NA” indicate samples with concentrations BDL.

Tissue	Mean	SD	Median	Min	Max	<i>N</i>
Maternal plasma	9.1	6.1	8.2	2.1	26.7	48
Egg contents	NA	NA	4.2	<1.0	24.4	47
Liver	7.6	4.5	6.7	1.4	13.3	7
Yolk sac	4.7	1.7	5.0	1.7	6.6	6

Table 2

Table 2. Plasma protein electrophoresis, immune parameters, plasma biochemistry values, and PCV in nesting female loggerhead sea turtles. Cells assigned “NA” indicate that some sample concentrations were BDL. Results of Pearson or Spearman correlations comparing health parameters to brevetoxin concentrations are also presented. Significant correlations are bolded.

Health index	Mean	SD	Median	Min	Max	N	r or r_s	P
<i>Plasma proteins</i>								
Total protein (g/dl)	4.3	1.1	4.2	1.8	7.2	54	0.26	0.07
Albumin (g/dl)	0.98	0.38	0.88	0.40	2.28	43	0.41	0.01
α_1 -globulin (g/dl)	0.14	0.07	0.14	0.02	0.39	43	-0.20	0.24
α_2 -globulin (g/dl)	0.78	0.43	0.78	0.09	1.84	43	0.11	0.50
Total α -globulin (g/dl)	0.91	0.45	0.88	0.19	2.10	43	0.08	0.63
β -globulin (g/dl)	0.99	0.44	0.98	0.13	1.98	43	0.27	0.10
γ -globulin (g/dl)	1.52	0.38	1.49	0.55	2.26	43	0.57	<0.001
Total globulin (g/dl)	3.42	0.89	3.43	1.01	4.94	43	0.42	0.01
Albumin:Globulin ratio (g/dl)	0.30	0.13	0.25	0.17	0.78	43	0.07	0.69
<i>Immune/oxidative stress</i>								
Lysozyme (μ g HEL/ml)	5.2	2.0	4.8	1.8	10.2	47	-0.01	0.94
SOD (U/ml)	169	83	140	51	354	47	0.05	0.72
ROS/RNS (nM)	4046	3072	2823	193	9302	33	0.06	0.73
<i>Plasma biochemistry/PCV</i>								
ALKP (U/L)	NA	NA	14	<4	40	30	0.29	0.13
ALT (U/L)	NA	NA	<6	<6	8	30	0.18	0.35
AST (U/L)	215	53	204	109	328	30	0.01	0.94
BUN (mg/dl)	9	6	8	4	35	30	-0.16	0.39
Calcium (mg/dl)	NA	NA	10.5	<5.0	15.8	30	0.11	0.57
Ca:P ratio	NA	NA	1.5	<0.6	2.4	30	-0.04	0.84
Chloride (mmol/l)	112	5	112	102	121	30	0.10	0.59
CK (U/L)	407	206	364	62	836	30	0.01	0.96
CRN (mg/dl)	NA	NA	0.2	<0.1	0.4	30	-0.26	0.16
Iron (μ g/dl)	61	28	56	24	134	30	0.03	0.86
Glucose (mg/dl)	85	19	85	43	136	30	0.22	0.24
LDH(U/L)	277	264	158	25	998	30	-0.14	0.45
PCV (%)	26	5	27	16	39	45	0.26	0.09
Phosphorus (mg/dl)	7.5	1.4	7.8	3.8	9.3	30	0.16	0.40
Potassium (mmol/l)	NA	NA	3.8	2.9	>10.0	30	0.01	0.98
Sodium (mmol/l)	148	5	148	139	159	30	-0.09	0.62
Uric Acid (mg/dl)	1.3	0.9	0.9	0.3	3.9	30	0.12	0.54

Figure 1

Figure 2

Figure 3

Figure 4