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SHORT NOTES

Note on the unique physiologic state of loggerhead sea turtles (*Caretta caretta*) during nesting season as evidenced by a suite of health variables

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Abstract
Baseline hematologic and plasma biochemical analytes provide insight into wildlife health. In recent years, blood analytes have been used to infer foraging strategies and nutritional status of female sea turtles of different species during nesting season in an effort to determine if turtles at this life stage are capital breeders that forage little to none during nesting season. These changes in foraging during nesting have not been documented in loggerhead sea turtles (*Caretta caretta*). The objective of this study was to evaluate correlations between hematologic, plasma chemistry, immune function, and antioxidative analytes of female loggerhead turtles during nesting season to determine evidence of reduced foraging. We found that chloride tended to increase, while total protein, various plasma protein fractions (pre-albumin, alpha-1-globulins, beta-globulins, total globulins), total white blood cells, superoxide dismutase, reactive oxygen species, iron, and triglycerides decreased over the course of nesting season. These results suggest that loggerhead turtles rely on fat stores accumulated on foraging grounds to fuel their energetic costs during nesting. Our results also indicate alterations in hemodynamics, metabolism, and antioxidative capacity due to reduced foraging and high energy efforts of nesting, which lend further insight into the physiologic dynamics and catabolic state of sea turtles during nesting season.

Introduction

Capital breeding is a strategy whereby little to no food is ingested during the reproductive season, allowing organisms to spatially and temporally separate suitable foraging and breeding areas (Bonnet et al. 1998). Sea turtles are known to employ the capital breeding strategy by accumulating substantial fat stores on foraging grounds so that they may spend months in nesting grounds laying multiple clutches of hundreds of eggs. This reproductive strategy has been verified in a number of sea turtle species through analysis of morphometrics (green turtles, *Chelonia mydas*: Hays et al. 2002; hawksbill turtles, *Eretmochelys imbricata*: Goldberg et al. 2013; leatherback turtles, *Dermochelys coriacea*: Plot et al. 2013), transmitters and stomach temperature pills (leatherback turtles: Casey et al. 2010), stomach contents (green turtles: Tucker and Read 2001), and physiologic measures of health (green turtles: Hamann et al. 2002; leatherback turtles: Honarvar et al. 2011; Perrault et al. 2012, 2014, 2016b; Plot et al. 2013; hawksbill turtles: Goldberg et al. 2013).

Specific alterations in blood health analytes of nesting sea turtles include variable changes in cholesterol and triglycerides, and declines in electrolytes and minerals (e.g., calcium, phosphorus, potassium, sodium), glucose, packed cell volume, proteins (e.g., albumin, leptin, globulins), nitrogenous compounds (e.g., blood urea nitrogen, uric acid), and white blood cells (Hamann et al. 2002; Honarvar et al. 2011; Perrault et al. 2012, 2014, 2016b; Goldberg et al. 2013; Plot et al. 2013). These blood analyte changes are presumptively associated with periods of reduced foraging and concurrent mobilization and depletion of energy stores, physiologic stress (including bone marrow effects such as reduced blood cell production), environmental variability, and a return to

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"normal" physiologic state after alterations associated with vitellogenesis/folliculogenesis (e.g., calcium, proteins; Stacy and Innis 2017). Other analytes indicative of antioxidant defenses (e.g., superoxide dismutase) and oxidative stress (e.g., reactive oxygen species) have been evaluated in nesting sea turtles (Perrault et al. 2016a), but changes across nesting season have not been investigated.

The capital breeding strategy in loggerhead sea turtles (*Caretta caretta*) has not been thoroughly investigated. It is likely that loggerheads employ this breeding strategy, as they have similar migratory and reproductive behaviors as other sea turtle species, including long distance migrations (up to 1200 km) from foraging grounds to nesting grounds and the deposition of multiple clutches (5–6 on average) of eggs during nesting season (Tucker 2010; Tucker et al. 2014). Given the observations in other sea turtle species, we anticipated that reduced food intake combined with high energy efforts of nesting likely impact physiologic measures of health and that antioxidative capacity and indicators of oxidative stress would decrease (Butler et al. 2016) during nesting season in loggerhead turtles. The objective of this study was to evaluate correlations between hematologic, plasma chemistry, immune function, and antioxidative analytes of female loggerhead turtles during nesting season to determine evidence of reduced foraging or other physiologic alterations during a time of high energy demand.

Materials and methods

Sample collection and processing follows the procedures outlined in Perrault et al. (2017b). Briefly, blood was collected from nesting loggerhead turtles during the 2015 nesting season on Casey Key, Florida USA. Whole blood was used to determine packed cell volume and to prepare two blood films for white blood cell (WBC) estimates, WBC differentials, and complete morphologic evaluation. The remaining whole blood was centrifuged at 1318*g* (3400 rpm) for 10 min to separate plasma. Plasma was analyzed for lysozyme activity (an aspect of immune function), reactive oxygen and nitrogen species (ROS/RNS; indicators of oxidative stress), superoxide dismutase (SOD; an indicator of antioxidant defense), total protein, protein electrophoresis (e.g., pre-albumin, albumin, alpha-1-, alpha-2-, beta-, gamma-, and total globulins), and plasma biochemistry (e.g., alanine aminotransferase activity, alkaline phosphatase activity, amylase activity, aspartate aminotransferase activity, blood urea nitrogen, calcium, calculated osmolality, chloride, cholesterol, creatine kinase activity, creatinine, gamma-glutamyl transferase activity, globulin, glucose, iron, lactate dehydrogenase activity, magnesium, phosphorus, potassium, sodium, total protein, triglycerides, and uric acid).

Statistical analyses were performed using IBM SPSS Statistics 24 (SPSS, Inc, Chicago IL, USA). Regression analyses were appropriately fitted to the trends (e.g., exponential or polynomial) by comparing date of nesting season with the measured hematologic and plasma analytes. The residuals of the regressions were tested for normality using the Shapiro-Wilk statistic and data were square-root transformed if they did not meet the assumptions of normality.

Results

We sampled 25 individual nesting loggerheads for this study, yielding up to 27–36 samples (some turtles were sampled more than once). The sample sizes differ between analytes due to insufficient sample quantity or quality in some instances. Two samples from 2 turtles were collected in May, 28 samples from 21 turtles in June, and 6 samples from 6 turtles in July. The sample size of loggerheads sampled more than once during nesting season was too low to detect statistical trends in individual turtles; therefore, we used changes in plasma analytes compared to date of nesting season to detect trends over time.

All measured hematologic and plasma analytes were previously reported in Perrault et al. (2017b). We found that a number of these analytes changed significantly across nesting season (Fig. 1) using regression analyses. There was a significant increase across nesting season with chloride ($r^2=0.37$; $P=0.004$; $N=27$). Significant decreases occurred with total protein ($r^2=0.28$; $P=0.019$; $N=27$), pre-albumin ($r^2=0.25$; $P=0.034$; $N=27$), alpha-1-globulins ($r^2=0.23$; $P=0.011$; $N=27$), beta-globulins ($r^2=0.22$; $P=0.048$; $N=27$), total globulins ($r^2=0.24$; $P=0.036$; $N=27$), total white blood cells ($r^2=0.28$; $P=0.011$; $N=31$), SOD ($r^2=0.24$; $P=0.003$; $N=35$), ROS ($r^2=0.11$; $P=0.045$; $N=36$), iron ($r^2=0.33$; $P=0.002$; $N=27$), and triglycerides ($r^2=0.24$; $P=0.038$; $N=27$). There was a marginally significant decrease across nesting season with heterophils ($r^2=0.19$; $P=0.057$; $N=31$). All remaining analytes showed no significant trends with date of nesting season.

Discussion

We report a number of changes in hematologic, plasma biochemical, immune function, and antioxidative analytes in loggerhead turtles indicative of reduced foraging during nesting season. Although a number of analytes decreased across nesting season, the values still remained within normal reference ranges for this species during this life stage (Deem et al. 2009; Stacy and Innis 2017). Multiple studies have documented the capital breeding strategy in sea turtles (green turtles: Hamann et al. 2002; leatherback turtles:

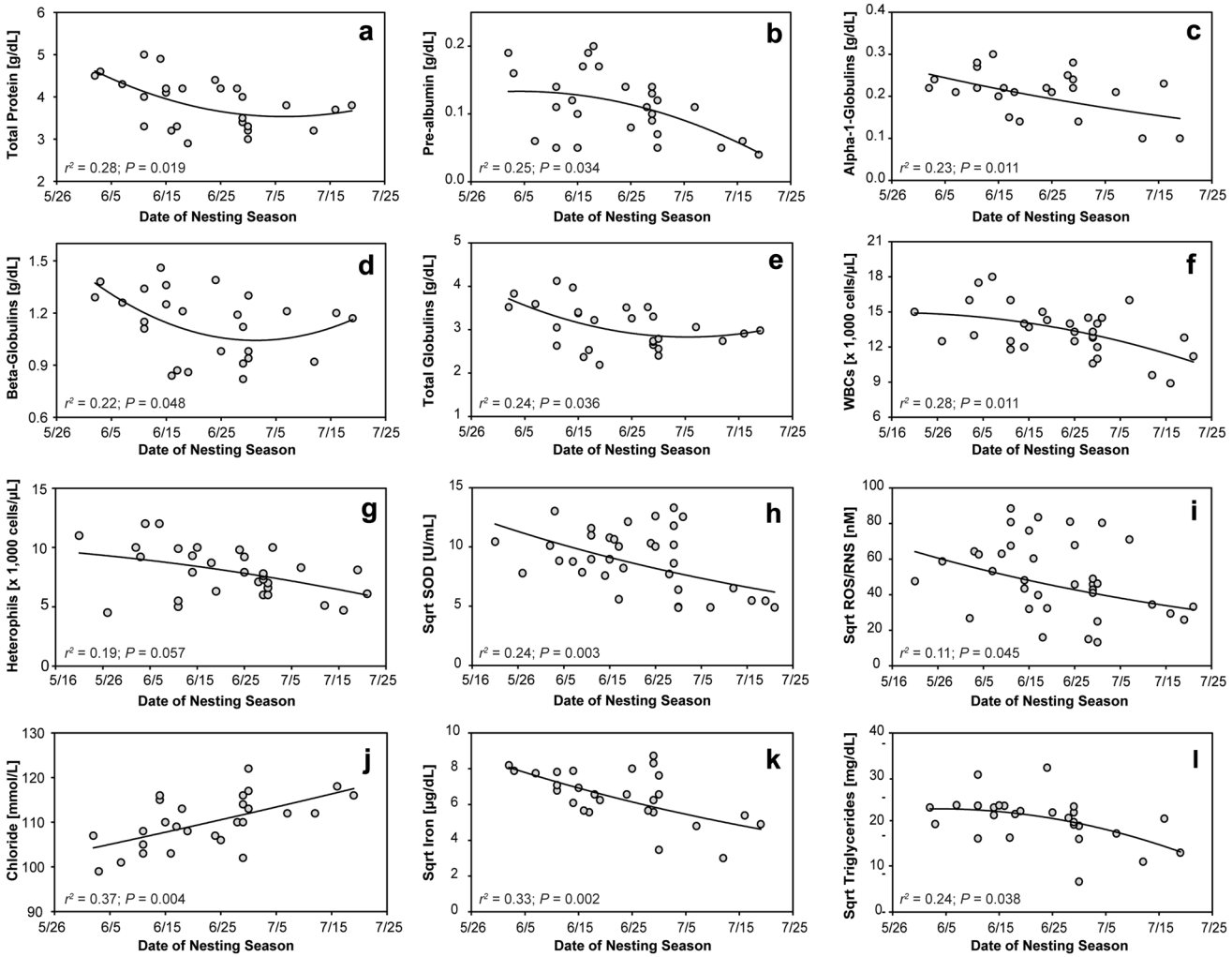

Fig. 1 Changes in **a** total protein, **b** pre-albumin, **c** alpha-1-globulins, **d** beta-globulins, **e** total globulins, **f** white blood cells (WBC), **g** heterophils, **h** superoxide dismutase (SOD), **i** reactive oxygen and nitrogen species (ROS/RNS), **j** chloride, **k** iron, and **l** triglycerides of nesting loggerhead turtles across nesting season. "Sqrt" indicates data that were square-root transformed, so that the residuals of the regressions met the assumptions of normality

Honarvar et al. 2011; Perrault et al. 2012, 2014, 2016b; Plot et al. 2013; hawksbill turtles: Goldberg et al. 2013); however, to the authors' knowledge, evidence of capital breeding has not been documented in loggerhead turtles using physiologic measures of health.

Regressing analyte concentrations with date of nesting season was chosen for the investigation of capital breeding in this group of loggerhead turtles in Casey Key, FL USA. Although few turtles were observed more than twice during nesting season, it was difficult to track changes within individuals across the season as is commonly done in other studies where observations of the same individuals were more feasible (Honarvar et al. 2011; Goldberg et al. 2013; Plot et al. 2013; Perrault et al. 2014, 2016b). It is likely that more convincing trends would have been uncovered if increased serial sampling of individuals had taken place, as not all turtles within a nesting season lay their first clutch of eggs at the same time (Tucker 2010). For example, one loggerhead on Casey Key could lay her first clutch in May, while another may lay her first clutch in June. Therefore, declines in blood analytes are not as obvious using date of nesting season instead of individual variation.

Various observed trends in proteins indicated metabolic and hormonal changes during nesting season. Decreases in total protein were associated with concurrently lower trending protein fractions of pre-albumin, alpha-1-, beta-, and total globulins. The observed decrease in pre-albumin may be associated with changes of thyroid hormone-binding and/or other proteins during nesting season, similar to observations in birds and mammals (Chang et al. 1999). Declines in alpha-1-globulins and beta-globulins suggest changes in reproductive proteins such as vitellogenin and

other proteins involved with active reproduction, such as apolipoproteins or yolk proteins, as reported in other reptile species (Gerstle and Callard 1972; Yaron and Widzer 1978). In addition to hormone-related changes, lower trending proteins can result from reduced foraging and/or increased protein catabolism, as described in nesting Floridian and Caribbean leatherback turtles (Perrault et al. 2012, 2014). The decline in plasma iron likely mirrors the lower trending protein as plasma iron reflects the protein-bound iron in plasma (Stacy and Innis 2017). This is documented in debilitated turtles in which plasma iron does not reflect total iron body stores (Manire et al. 2017).

The lack of significant seasonal trends of BUN was unexpected. In nesting leatherback turtles, declines in BUN across the first 80% of nesting season have been documented, after which BUN started to significantly increase. The authors attributed this to a shift from catabolism of lipids to proteins as the body's energy stores become depleted (Plot et al. 2013). Conversely, in nesting hawksbills, BUN significantly increased throughout the entire nesting season with a concomitant decrease in plasma protein, suggesting that muscle catabolism in hard-shelled sea turtles occurs throughout the entire season (Goldberg et al. 2013). Therefore, differences in nesting physiology between hard-shelled cheloniids and soft-shelled dermochelyids likely occur. While BUN in loggerheads from this study slightly increased across nesting season, the trend was not significant, likely due to the lack of serial sampling of individual animals from this study.

Lipid metabolism appears to play an important role in capital breeding as observed in loggerhead turtles from this study and in other species. Nesting sea turtles reportedly have elevated concentrations of triglycerides in comparison to non-vitellogenic females as a result of lipid mobilization and follicle development (Hamann et al. 2002; Deem et al. 2009). Declines in triglycerides across nesting season have been observed in nesting hawksbill turtles from Brazil due to reductions in food intake (Goldberg et al. 2013; Price et al. 2013). In nesting green turtles with 0–19% of reproductive investment remaining during nesting season, triglycerides were significantly lower in comparison to triglyceride concentrations in turtles from early- to mid-season (Hamann et al. 2002). This is similar to triglyceride concentrations in the current study between the first and last sample collected on Casey Key, which is likely associated with reduced foraging and lipid mobilization from adipose tissues, resulting in a catabolic state during nesting season. As nesting season progresses, triglycerides from adipose tissue are catabolized and the byproducts are utilized for energy and as a substitute for glucose (Goldberg et al. 2013). Nutrients from the breakdown of amino acids in muscle tissue provide the necessary carbon to maintain blood glucose levels during times of fasting, which is likely why declines in glucose were not observed (Finn and Dice 2006; Goldberg et al. 2013).

The observed increase in chloride across nesting season is interesting, as this electrolyte has been shown to decline in nesting leatherbacks and hawksbills (Honarvar et al. 2011; Goldberg et al. 2013). This decline was hypothesized to be related to decreased food intake and/or mineral depletion across nesting season. Although not extensively evaluated, it has been suggested that marine turtles might have altered hemodynamics towards the end of nesting season due to water loss, catabolic state, and production of egg albumen (Plot et al. 2013; Perrault et al. 2016b). Perhaps loggerhead turtles experience hemodynamic changes from protein catabolism or energy-related alterations in salt gland metabolism. Species-level differences in electrolytes during nesting season likely exist due to differences in reproductive output and migratory distances covered to nesting grounds.

Trends in immune parameters across nesting season have not been well-documented in sea turtles (Perrault et al. 2012). Our study reports a significant decrease in WBC (with a marginally significant decrease in heterophils). In nesting leatherback turtles from Florida, total WBC counts decreased across nesting season, attributed to the physiologic stress on hematopoietic tissues caused by reduced feeding during migration and reproduction (Perrault et al. 2012; Stacy and Innis 2017). Since WBC did not decrease below normal ranges in loggerhead turtles, this trend may indicate individual variability in stress responses or stress exposure during nesting, or catabolic effects on hematopoietic tissues.

The observed declines with ROS and SOD across nesting season are intriguing, and warrant future study. SOD, an antioxidant defense enzyme, counteracts ROS (an indicator of oxidative stress) production (Afonso et al. 2007), and in sea turtles these two analytes have been shown to be positively correlated (Perrault et al. 2017a). In capital breeding mammals, oxidative stress is associated with extended periods of fasting, resulting in increases in ROS, inflammation, and oxidative damage (Sharick et al. 2015) and decreases in antioxidant capacity (Mårtensson 1986). Conversely, in corn snakes (*Pantherophis guttatus*), oxidative damage (measured by reactive oxygen metabolites in plasma) was significantly lower in snakes in a "baseline" (i.e., non-absorptive) state compared to those that were digesting a meal (i.e., absorptive state). We observed a significant, albeit slight decrease in ROS across nesting season, which is contradictory to what is seen in capital breeding marine mammals (Sharick et al. 2015). In loggerheads from this study, ROS and SOD production decreased across the season, possibly as a result of the need to combat fewer oxidative stressors after initial phases of increased lipid metabolism and peroxidation and/or changes in protective mechanisms from circulating vitellogenin, and SOD consumption, respectively (Olsson et al. 2009; Sun and Zhang 2015; Butler et al. 2016). It is likely

that physiologic differences in oxidative stressors and antioxidant defenses exist between marine mammals and reptiles, which may explain these opposing trends.

Conclusions

We present evidence for temporal changes during nesting season of loggerhead turtles supporting evidence for the capital breeding hypothesis in this species. The observed trends demonstrate the dynamic physiologic changes of various health variables during the high energy demanding phase of active reproduction in nesting loggerhead turtles and highlight the importance of evaluating blood analytes in the context of physiologic state (i.e., intrinsic factors). The results from this study indicate changes in foraging strategies during nesting and a unique physiologic dynamic resulting from nutrient depletion during nesting. Other populations of loggerhead turtles and additional analyses (e.g., hormonal, metabolomics: Schock et al. 2013; Bailey et al. 2017) should be examined for this foraging strategy to better understand physiologic and catabolic effects during nesting season.

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Compliance with ethical standards

Our study was carried out in accordance with a Florida Fish and Wildlife Conservation Commission permit #15-205 and MML IACUC approval #15-01-JP2. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This work was supported by a MML Postdoctoral Research Fellowship.

Conflict of interest JRP and NIS declare no conflicts of interest. All authors approved the final version of this manuscript.

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