Effects of *In Situ* Incubation Temperatures on Hatchling Loggerhead Sea Turtle (*Caretta caretta*) Morphology, Health Indices, and Locomotor Performance

by

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ABSTRACT

Incubation temperatures, in addition to an embryo's genetic makeup, play crucial roles in development and alter a variety of characteristics in sea turtle embryos. Atmospheric temperatures are currently predicted to rise at least 1.5°C above pre-industrial levels by 2052, potentially impacting embryonic development. Most sea turtle temperature studies document the effects of "high" and "low" incubation temperatures in laboratory-reared nests. This study's objective was to examine impacts of varying *in situ* incubation temperatures on loggerhead sea turtle (*Caretta caretta*) hatchling morphology, health, and locomotor performance. Temperature data loggers were deployed in 15 individual loggerhead nests on Juno Beach, Florida between June and August 2018. In total, 144 hatchlings were measured and sampled. Carapacial scute abnormalities, mass, and morphological measurements (straight carapace length (SCL), straight carapace width (SCW), body depth (BD), front flipper length (FFL), umbilical scar length, and umbilical scar width) were taken. Heart rate was measured using a portable ultrasound and blood was collected to analyze for glucose, packed cell volume, hemoglobin, total solids, and white blood cell estimates with differentials. Lastly, righting response in water was tested. After mass emergence, 14 nests were excavated to identify hatching success and developmental stage of unhatched eggs. Unsurprisingly, incubation temperatures were significantly lower in May compared to June and July. The results of the Kruskal-Wallis and Pearson's Chi-squared tests indicated that warmer months resulted in higher

values in umbilical scar size and abnormal scutes present, slower righting time, increased levels of several blood health analytes, and a higher number of unhatched embryos at full-term. The results of the linear regression and Kruskal-Wallis tests corroborate previous studies on hatchlings in laboratory-reared nests, with increasing temperatures resulting in smaller hatchling size (SCL, SCW, BD, FFL) and slower righting time. I suggest possible lower survival rates in hatchlings from warmer nests due to increased risk of predation from smaller body sizes, decreased physical responses, altered hemodynamic balance (e.g., dehydration) and potential inflammation due to increased temperatures. Furthermore, the higher number of unhatched embryos at full-term in warmer nests may indicate nest temperatures that are exceeding their lethal limit resulting in egg mortality. This study adds novel health reference intervals for loggerhead hatchlings and demonstrates that even sublethal increases in sand temperatures will likely affect sea turtle hatchling health and have the potential to negatively impact hatchling survival.

CHAPTER 1: INTRODUCTION

Incubation temperatures, in addition to an embryo's genetic makeup, have a number of consequences to developing reptile embryos. Increased incubation temperatures have the potential to negatively affect embryonic metabolism (Ligon and Lovern 2012), immune function (Dang et al. 2015), reproductive success, hatchling body condition, and locomotor performance (Elphick and Shine 1998; Du and Ji 2003; Booth et al. 2004; Tang et al. 2012). Additional effects of increased incubation temperatures include alterations in growth rates (Rhen and Lang 1995), yolk conversion rates (Booth 2000; Booth and Astill 2001; Booth et al. 2004), and behavior (Vervust et al. 2011; Sivitier et al. 2017). As of 2015, Earth's atmospheric temperatures have risen 1°C above pre-industrial levels and are expected to increase anywhere from 0.3°C to 4.7°C, with a likely increase of at least 0.5°C by 2052 at the current rate (IPCC 2018). It is probable that this increase in temperature will impact embryonic development in sea turtles, altering their morphology, health, and locomotor performance and therefore impacting their ability to survive.

Several studies have examined the effects of "high" and "low" incubation temperatures on hatchling size, quality, and performance. Overall, these studies demonstrated that hatchlings from warmer nests were smaller in size with reduced locomotor performance abilities compared to those from cooler nests. However, the majority of these studies were conducted on laboratory-reared nests exposed to constant

incubation temperatures throughout the incubation period (Booth et al. 2004; Fisher et al. 2014). Oftentimes, only a fraction of the eggs from these nests were selected for temperature-exposure experiments. This does not mimic the natural nest environment due to differences in water uptake, gas exchange, metabolic heating, and fluctuating temperatures (Horne et al. 2014; Booth 2017). The few studies that have evaluated the effects of natural nest temperatures on hatchling locomotor performance and quality showed similar results (Glen et al. 2003; Michelson and Downie 2010; Read et al. 2012; Sim et al. 2015). To my knowledge, no studies have evaluated baseline blood health indices in sea turtle hatchlings, especially in regards to natural temperature regimes.

Temperature effects on morphology: hatchling size

Hatchling size is one of the most commonly measured traits known to be affected by incubation temperatures. Elevated incubation temperatures have been shown to result in significantly reduced carapace lengths (green turtles, *Chelonia mydas*: Glen et al. 2003; leatherbacks, *Dermochelys coriacea*: Michelson and Downie 2010; loggerheads, *Caretta caretta*: Read et al. 2012, Sim et al. 2015), fore and hind limb area (green turtles: Glen et al. 2003; leatherbacks: Michelson and Downie 2010; loggerheads: Read et al. 2012), and mass (green turtles: Glen et al. 2003). Larger body sizes produced from cooler nests might be advantageous to avoid gape-limited predators, meaning what they consume is limited by how far they can open their mouth (Salmon and Scholl 2014; Salmon et al. 2015). Hatchling survival has been shown to increase with body size in red-eared sliders (*Trachemys scripta elegans*) and green turtles (Gyuris 2000; Janzen et al. 2000).

Temperature effects on morphology: abnormal scutes

The evolution of the turtle shell dates back about 220 million years in the fossil record. Even with the variety of different shells, the scute arrangement in the diversity of extant shells has remained relatively unchanged (Li et al. 2008; Zimm et al. 2017). Nevertheless, individual variations in scute patterns are observed for all six scute-possessing sea turtle species, with researchers suggesting both a genetic and an environmental component driving scute abnormalities (Mast and Carr 1989). Examining the genetic factor, Velo-Antón et al. (2011) found a higher prevalence of abnormal scutes in European pond turtles (*Emys orbicularis*) with lower genetic diversity. However, different environmental factors can also influence the development of scute patterns including the handling of eggs, which is a common phenomenon when nests are relocated (Mast and Carr 1989). Eggs with minimal handling had fewer scute abnormalities than those with intermediate and extreme levels of handling, while dead hatchlings had more extreme scute abnormalities than the live hatchlings (Mast and Carr 1989).

In regard to incubation temperatures, painted turtle (*Chrysemys picta*) hatchlings had more scute abnormalities when they were exposed to extremely hot temperatures (>34° C) for at least 60 hours at some point during incubation (Telemeco et al. 2013). In addition, scute abnormalities in nesting females were rare, suggesting that these irregularities may be related to decreased survival and fitness (Telemeco et al. 2013). In sea turtles, hotter and dryer conditions in incubating nests often lead to a higher prevalence of abnormal scute patterns in comparison to cooler, wetter nests (Zimm et al. 2017). These results indicate that temperatures during the incubation period can play a

crucial role in the development of carapacial traits, especially with regard to scute abnormalities.

Temperature effects on morphology: umbilical scar as a proxy for yolk mass

The umbilicus is the site of attachment of the yolk sac in developing reptilian embryos (Keller 2017). As the yolk sac becomes internalized and nutrients are transferred from the yolk to the developing embryo, the embryo increases in size while the yolk decreases, resulting in the closing of the umbilicus (Keller 2017). Thus, the umbilical scar is used as a proxy for yolk mass, with a smaller umbilical scar serving as a proxy for a smaller yolk mass. Incubation temperatures affect the amount of energy (*i.e.,* yolk) utilized during embryonic development (i.e., higher temperatures lead to higher embryonic metabolism), which then impacts the amount of yolk reserves left at hatching. Eggs laid in cooler nests incubate longer and convert more yolk material to tissue, thus resulting in hatchlings with a larger yolk-free mass (Booth 2000; Glen et al. 2003; Booth and Evans 2011); however, smaller hatchlings from warmer nests have larger yolk reserves and are more likely to be able to survive longer without eating (Booth 2006). Green turtle hatchlings incubated at 26°C were greater in mass, had a larger yolk-free mass, and a smaller residual yolk compared with hatchlings incubated at 30°C (Booth and Astill 2001). Furthermore, yolk quantity was found to be significantly less in flatback turtle hatchlings (*Natator depressus*) when incubated at 26°C and 29°C, compared to 32°C (Hewavisenthi and Parmenter 2001).

Temperature effects on health indices

Baseline health indices (*e.g.*, hematology, biochemistry) can also be influenced by environmental temperatures. These analytes can indicate various health conditions such as inflammation, dehydration, stress, or anemia and serve as important diagnostic indicators in sea turtles (Stacy and Boylan 2014; Stacy and Innis 2017). In juvenile loggerheads captured in Core Sound, North Carolina, total estimated white blood cell counts and heterophils were negatively correlated with water temperature, while PCV and glucose were positively correlated with temperature (Stamper et al. 2005; Campbell 2006; Kelly et al. 2015). Water temperature also potentially impacted PCV in nesting leatherbacks in St. Croix, U.S. Virgin Islands (Perrault et al. 2016).

Additionally, incubation temperatures significantly altered immune function through a higher expression of genes involved in acquired immunity in soft-shelled turtle (*Pelodiscus sinensis*) hatchlings incubated at lower temperatures when compared to hatchlings incubated at higher temperatures (Dang et al. 2015). Hatchlings incubated at lower temperatures also had increased survival rates, potentially resulting from a more well-developed immune system (Dang et al. 2015). A higher immune response, specifically a higher hemolytic complement activity (plasma proteins that help fight infection by causing inflammatory responses and target pathogens for destruction) was also found in hatchling map turtles (*Graptemys ouachitensis*) incubated at lower temperatures (Freedberg et al. 2008). Although changes in baseline health can be related to temperature, how this relates to hatchlings and *in situ* incubation temperatures is unknown.

Incubation temperatures not only influence various health indices but can also impact heart rate. Embryonic heart rates have been observed to increase with ambient

temperature in various oviparous reptiles, including turtles, and can be negatively associated with shorter incubation periods (Du et al. 2010, 2011). In addition, increasing heart rates have been found to rise as body temperature increases in red-eared slider turtles (*T. scripta elegans*) and ornate box turtles (*Terrapene ornata ornate*, Gatten 1974). Yet, there are no published studies analyzing the influence of incubation temperatures on heart rates of hatchlings immediately following emergence. Therefore, studies are needed to determine if incubation temperature profiles influence heart rate and the cardiovascular system.

Temperature effects on locomotor performance

Understanding the effects of temperature on sea turtle hatchling locomotor performance is critical as it can impact the hatchlings' ability to evade beach and near shore predators and survive in the open ocean. This ability to survive is directly related to their locomotor performance and since the probability of being predated upon is directly proportional to the time spent in coastal waters (Gyuris 1994), the ability of a hatchling to quickly upright itself is important. The self-righting response is a common test used to examine locomotor performance in hatchlings. A faster righting time can be assumed to be related to increased hatchling survival (*i.e.*, better predator avoidance), which can have lasting effects on long-term survival and performance (Janzen 1993; Freedberg et al. 2004). The self-righting test refers to the hatchling's ability to turn itself over from its back. This is beneficial when sea turtles nest on rocky shores or when hatchlings crawl over beach debris. In these situations, overturning is a common phenomenon as

hatchlings make their trek to the sea (Booth 2017). Additionally, hatchlings can be overturned in coastal waters in strong waves.

Hatchlings right themselves to avoid predation and thermal stress, so a better response time is likely indicative of better abilities in other performance tests (Burger 1976; Freedberg et al. 2004). Sim et al. (2015) found that relocated loggerhead hatchlings performed better in crawling and swimming tests when the mean three day maximum incubation temperature was below 34°C. Additionally, Fisher et al. (2014) found when loggerhead sea turtle (*C. caretta*) eggs were incubated at constant temperatures, ranging from 27–33°C , the optimal incubation range for peak performance on righting, crawling, and swimming tests was 28.5–31°C. Given that incubation temperatures are frequently higher than 31°C in southeast Florida, with annual high nest temperatures ranging from 34.2–36.5°C (Hanson et al. 1998; Wyneken and Lolavar 2015), it is possible hatchlings in Florida from later-season nests will have sub-optimal performances.

Temperature effects on stage of development

At present, there are few studies analyzing the influence of *in situ* incubation temperatures on the stage of development of unhatched sea turtle embryos. Embryology of sea turtles has been described by Miller (1985) and divided into 31 developmental stages. Development starts within the female (stages 1–5) and extends through oviposition (stage 6), pipping (stage 30), and hatch out (stage 31). Visual staging begins at stage 19 (Miller 1985). Using this information and the field guide for staging (Miller et al. 2017), Bladow (2017) examined stage of embryonic death in loggerheads and green

turtles in relation to nest temperatures. The majority of embryos for both species died in later stages of development with a significant influence of time when incubation temperatures were above 34–35°C (Bladow 2017). Contrary results were observed in a study conducted by Blanck and Sawyer (1981) that found that most of the unhatched loggerhead eggs in their study contained embryos that were in the pre-carapace stage, suggesting an occurrence of mortality during the early embryonic stages. However, they did not specify differences between their naturally incubated nests and the ones reared at 28°C.

Using a different classification system, development stage of leatherback and green turtle embryos were analyzed (Whitmore and Dutton 1985). They were classified into three categories: early embryonic development (eggs with blood vessel formation or a small embryo without pigmentation usually < 10 mm long), mid embryonic development (eggs containing an embryo usually 10–30 mm with an unpigmented body but pigmented eye), and late embryonic development (eggs containing a pigmented embryo generally > 30 mm long; Whitmore and Dutton 1985). For leatherbacks and green turtles in Suriname, embryonic mortality occured mainly during early development, followed by late development, with a very low percentage of mortality happening during mid embryonic development (Whitmore and Dutton 1985). Similar findings were observed in freshwater species, Northern snake-necked turtle (*Chelodina oblongea*) and Murrary River turtle (*Emydura macquarii*), with peaks of embryonic death occurring at stage 0 when incubated at 16°C and at stage 25 when incubated at 22°C (Rafferty and Reina 2014).

More research is needed to evaluate the effects of incubation temperatures on stage of development at death for sea turtle embryos, specifically how long embryos can withstand lethal temperatures and within what periods of development that they are able to do so. If hatchlings are developing to full-term and then dying due to extremely hot incubation temperatures, gaining a better understanding of why and when death occurs could assist in creating more effective solutions by lowering incubation temperatures by providing shade protection or by splitting nests into multiple cluthes to lower metabolic heating.

Loggerhead sea turtles

Of the sea turtle species, the loggerhead (*Caretta caretta*) is the most commonly found in Florida (Florida FWC 2017). The species is listed as vulnerable internationally by the International Union for Conservation of Nature (IUCN) with several subpopulations listed as endangered or critically endangered (Casale and Tucker 2017); however, the Northwest Atlantic subpopulation is listed as a species of least concern (Ceriani and Meylan 2017). This Northwest Atlantic subpopulation is one of the two most abundant subpopulations (Casale and Tucker 2017), with approximately 87% of its nesting effort occurring on the peninsular region of Florida, making it the largest nesting population in the western hemisphere (Ehrhart et al. 2003; Ceriani et al. 2012). Between the years 2011–2015, an average of 84,000 nests were laid annually (Brost et al. 2015; Florida FWC 2017). Due to the high concentration of nesting turtles, and the fact that these Florida beaches will likely be impacted by global climate change (Wyneken and Lolavar 2015), this area is of high conservation importance.

Objectives and hypotheses

The main objective of this study was to examine the impacts of varying *in situ* incubation temperatures on loggerhead sea turtle hatchling morphology, health, and locomotor performance. The hypotheses were as follows:

(1) Early, cooler season nests would produce larger hatchlings (in terms of mass, straight carapace length (SCL), straight carapace width (SCW), body depth (BD), front flipper length (FFL)), with fewer abnormal scutes and smaller umbilical scars when compared to the hatchlings from later, warmer season nests.

(2) Early, cooler season nests would produce offspring with health indices (*e.g.*, glucose concentrations, PCV, hemoglobin concentration, total solids, WBC estimates with differentials) that differ from offspring from late, warmer season nests. Therefore, I predicted that hatchlings from May would have overall different health indices when compared to hatchlings in July. In addition, early season nests would produce hatchlings with lower heart rates when compared to those from later season nests.

(3) Early, cooler season nests would produce hatchlings with faster righting times when compared to hatchlings from late, warmer season nests. Thus, hatchlings from the May nests would have better locomotor performances when compared to the locomotor performance by hatchlings from the nests in July.

(4) Early, cooler season nests would have unhatched embryos that died at different stages of development when compared to unhatched embryos in later, warmer season nests. I therefore predicted nests from May would produce fewer unhatched full-term embryos compared to those in July.

CHAPTER 2: MATERIALS AND METHODS

Study site

My sampling site was located along Florida's east coast (Juno Beach, Florida USA), primarily in the 9.63 km area of peak loggerhead sea turtle nesting activity (26°55'13.71" N, 80°3'56.76" W – 26°50'11.37"N, 80°2'28.85"W; Fig. 1). Juno Beach is located in Palm Beach County and was selected as the study site due to the high nesting activity experienced there every year. The majority of loggerhead nesting in Florida occurs in five counties: Brevard, Indian River, St. Lucie, Martin, and Palm Beach (Florida FWC 2017). Over the past five years, Palm Beach County experienced the highest number of loggerhead nests in all Florida counties (Florida FWC2019).

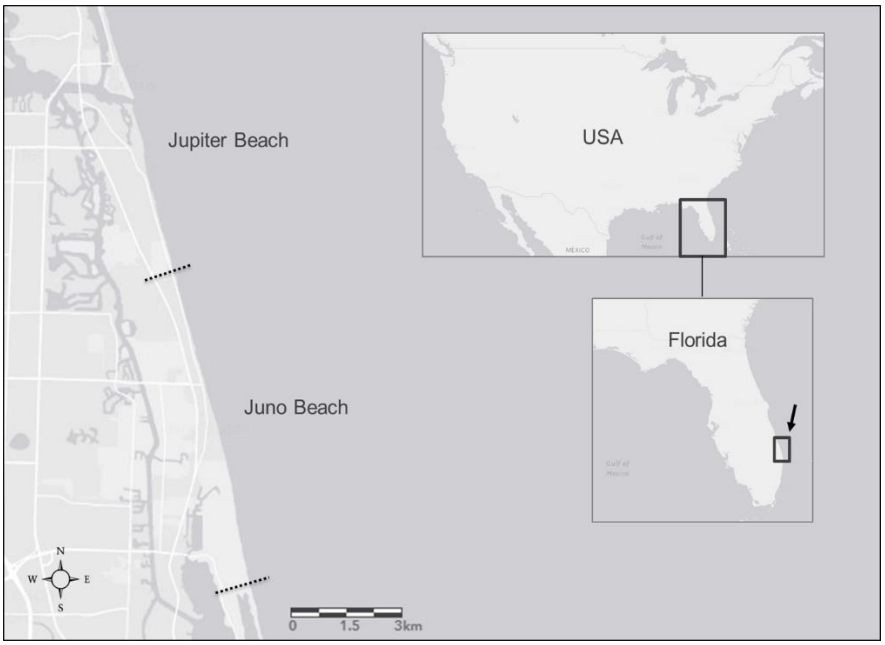

Figure 1. The study site for this project was Florida's east coast on Juno Beach located in Palm Beach County, Florida, primarily in the 9.63 km area of peak nesting. The north and south end of the study site are represented by dotted lines.

Data loggers

Onset® Hobo® Water Temperature Pro v2 data loggers (Onset Computer Corporation Bourne, Massachusetts, USA) were utilized to determine incubation temperatures. These devices have an accuracy of ±0.21°C and are operational between -40° to 70°C. A temperature-accuracy check of the data loggers was conducted prior to use in the field to ensure accurate readings.

Nest selection/monitoring and hatchling collection

Nesting season of loggerhead sea turtles occurs from April through September and peaks from June to July. The data loggers were placed in the center of a total of 15 clutches on Juno Beach, Florida during May (N = 5 nests), June (N = 5 nests), and July (N

= 5 nests) of the 2018 nesting season. The data loggers were placed on May 8th and 9th, June 13th and 14th, and July 11th and 12th into the middle of each clutch after approximately 50 eggs had been laid. The data loggers were programmed to record temperature to 0.01°C every thirty minutes for the duration of incubation. All encountered nesting females were tagged using a combination of passive integrated transponder (PIT) and Inconel flipper tags. This study was carried out with the approval of the Institutional Animal Care and Use Committee (University of South Florida protocol #WIS00004563) and permitted by Florida Fish and Wildlife Conservation Commission, permit #MTP-205.

Nests were monitored for hatchling emergence starting 40–45 days after the clutch was deposited. The hatchlings were collected as they naturally emerged. An attempt to collect 10 hatchlings from each of the nests was made; however; in June, only nine were collected from one nest and five from another due to missing the mass emergence event (N = 144 hatchlings total; May = 50 hatchlings, June = 44 hatchlings, July = 50 hatchlings). Upon collection, hatchlings were placed in labeled coolers with damp sand for transportation to Loggerhead Marinelife Center's (LMC) Research Laboratory for testing. All sampling was conducted at constant temperatures and with all sources of artificial white light eliminated. Red LED headlamps were used during the sampling process.

Morphological measurements

Mass was recorded to the nearest 0.01g using an electronic balance scale. SCL, SCW, BD, FFL, and umbilical scar length and width were measured using stainless steel

digital calipers (±0.01 mm). The umbilical scar was measured in lieu of the yolk sac to prevent having to sacrifice any hatchlings. SCL was measured from the mid-point of the nuchal scute to the posterior notch where the two most posterior marginal scutes meet and SCW was measured at the widest part of the carapace (Wyneken 2001). BD was measured at the deepest part of the carapace and FFL was measured on the front right flipper from the elbow to the tip of the flipper (Wyneken 2001). A carapace size index (CSI) was calculated by multiplying SCL by SCW (Sim et al. 2015). Body condition index (BCI) was also calculated using the following method:

$$BCI = \frac{mass}{SCL^3} \times 10{,}000 \text{ (after Bjorndal et al. 2000)}$$

The number and location of carapacial scute abnormalities were also noted for each hatchling. Scute abnormalities include either the presence of an additional scute or the absence of one typically found on loggerheads. The total number of hatchlings with abnormal scutes per month was added, and then divided by, the total number of hatchlings per month to achieve a percentage.

Health indices

Heart rate was taken manually using a portable ultrasound (EI Medical Imaging®, Ibex® EVO®; Loveland, Colorado, USA) with a 6.4 MHz CL3E transducer placed on the plastron. Beats were counted for fifteen seconds and then multiplied by four to determine beats/min.

Whole blood was then collected from the external jugular vein using 1 ml 26-gauge BD allergy syringes (Becton-Dickinson and Co. Franklin Lakes, New Jersey, USA) using safe blood collection practices for reptiles outlined by Strik et al. (2007).

Baseline health indices measured included glucose, PCV, hemoglobin, total solids, and WBC counts with differentials.

The whole blood samples were well-mixed and analyzed for glucose using an EasyTouch® glucose monitoring system (MHC® Medical Products, Fairfield, Ohio, USA) based on glucose oxidase and potentiometry with test strips for use in capillary whole blood. The test strips were adequately filled with whole blood per the manufacturer's recommendations. This system has been shown to be an accurate method for determining blood glucose concentrations in sea turtles (Perrault et al. 2018).

Next, we determined PCV from whole blood collected into a microcapillary tube (Fisher Health-Care, Houston, Texas USA) with Critoseal® (Sherwood Medical Co., Deland, Florida, USA) as the sealant. The capillary tubes were spun for 5 minutes at 1,300 *g* (5,000 rpm) using a ZipCombo microhematocrit centrifuge (LW Scientific, Inc., Lawrenceville, Georgia, USA). A hematocrit microcapillary tube reader was used to read the PCV as a percentage.

Hemoglobin concentrations in whole blood were analyzed using a portable hemoglobinometer (HemoCue 201+ analyzer, HemoCue Inc, Lake Forest, California, USA). The blood sample was drawn into a cuvette by capillary action, where the hemoglobin was released by disintegrating the erythrocyte membranes by sodium deoxycholate. The hemoglobin iron was then oxidized from the ferrous to the ferric state by sodium nitrite to form methemoglobin and then combined with azide to form azidemethemoglobin (HemoCue Inc., 2016).

Plasma from the hematocrit tubes was used to obtain total solids and was estimated using a Reichert VET 360 handheld refractometer (Reichert Technologies Analytical Instruments, Depew, New York, USA).

Total WBC estimates with differentials were determined using one blood film prepared from well-mixed whole blood, followed by drying and staining with Wright-Giemsa. Blood film evaluation included WBC estimates (Weiss 1984), WBC differentials (including heterophils, lymphocytes, monocytes, eosinophils, basophils), and morphologic evaluation of red blood cells (RBC), WBCs, and thrombocytes.

Locomotor performance

Following blood collection, hatchlings were subjected to the self-righting performance test. The time it took the hatchlings to complete the test was determined using a stopwatch to the nearest 0.01 second. A five-gallon bucket was filled halfway with seawater. Hatchlings were placed upside down on their carapace one at a time at the surface of the water. The time it took the hatchlings to right themselves was recorded and then repeated three times for an average. Once the experiment was completed, the hatchlings were released the same night on Juno Beach.

Hatching success and stage of development

Three days after the mass emergence event, nests were excavated. Data loggers were removed and hatching success (*i.e.* # hatched eggs divided by the total amount of eggs) was recorded. Nest depth was calculated along with the classification of the eggs in the nest. Classification of the eggs followed guidelines provided in Florida Fish and

Wildlife Conservation Commission Marine Turtle Conservation Handbook (Florida FWC 2016) and included hatched eggs (empty eggshells), unhatched eggs, live-in-nest hatchlings, dead-in-nest hatchlings, live-pipped hatchlings, dead-pipped hatchlings, and spacers/shelled albumen globs (i.e., packets of albumin with an outer shell). Live- and dead-pipped hatchlings are defined as turtles that had broken through the egg but were not completely free of the eggshell. The unhatched eggs were further evaluated to determine stage of development at death. All unhatched eggs were opened and assigned a classification (no discernable embryo: no development; partially developed embryo: early pigmented eye, late pigmented eye, early pigmentated body, late pigmentated body; fully developed embryo: full-term; or unknown, Florida FWC 2016).

Statistical analyses

Data were pooled by month and tested for normality using the Shapiro-Wilk test. Given the violation of normality, nonparametric Kruskal-Wallis tests were performed to test the effects of average incubation temperature on hatching morphology, health indices, and locomotor performance. If the results of the Kruskal-Wallis were significant, Bonferroni post hoc tests were also performed to examine which monthly differences were significant. Any values with associated hemolysis scores of 2+ were removed from PCV and total solids analysis for both Kruskal-Wallis tests and linear regressions.

Previous researchers examined the effects of mean three day maximum temperature (T_{3dm}) and found nests with a T_{3dm} above 34°C had lower emergence success and hatchlings performed worse on locomotor performance tests, despite having a mean temperature within normal limits (Maulany et al., 2012; Sim et al., 2015). Thus, a linear

regression was performed to test the association between hatching success and T_{3dm}. We also calculated overall mean incubation temperature (T_{inc}), along with the mean temperature in the middle third of incubation (T_{mid}), and the mean temperature during the last two weeks of incubation (T_{2w}).

Additionally, linear regressions were performed to test the association between continuous temperature data (T_{inc}) and morphology, health indices, and locomotor performance. Linear regressions were also performed to test the association between righting time and hemoglobin and heart rate. Assumptions of the linear regressions were checked with diagnostic plots. Variables were log 10 transformed to comply with the assumptions of parametric tests.

Reference intervals for glucose concentrations, PCV, hemoglobin concentrations, total solids, and white blood cell estimates with differentials were calculated after Friedrichs et al. (2012) using nonparametric methods for sample sizes ≥120. Hemolysis scores of ≥2+ were eliminated from calculated reference intervals for PCV, total solids, glucose, and hemoglobin.

I performed Pearson's Chi-squared to test the effects of incubation temperature on (1) the percentage of abnormal scutes by month and (2) the percentage of unhatched eggs at each developmental stage.

Hatchling morphology, health, and performance variables were corrected for size by dividing by the hatchling's SCL to ensure differences seen were not due to size. Statistical significance was assumed if $p \leq 0.05$. All statistical tests were performed using R 3.4.1 (R Core Team 2017).

CHAPTER 3: RESULTS

Incubation temperature

The overall mean incubation temperature (T_{inc}; $\chi^2(2) = 9.98$, $p = 0.007$) and mean incubation temperature during the middle third of incubation (T_{mid}; $\chi^2(2) = 10.52$, $p = 0.005$) were significantly lower in May than in June and July. No significant differences were found in the mean temperature during the last 2 weeks of incubation (T_{2w}; $\chi^2(2) = 4.37$, $p = 0.11$) or the maximum temperature for three consecutive days (T_{3dm}; $\chi^2(2) = 4.34$, $p = 0.11$, Table 1) between May, June, and July. All nests except one experienced a T_{3dm} of >34°C, with eight out of 15 (53.33%) nests experiencing a T_{3dm} of over 35°C. Temperature data for all 15 nests obtained from the data loggers are shown in Fig. 2 and Fig. 3. Temperature data plotted against incubation date representing the entire incubation period for all nests is shown in Fig. 2, while Fig. 3 displays the significant lower temperatures experienced in May, with the start of the x-axis representing day one of incubation throughout hatch-out (Fig. 3).

Table 1. Summary of *in situ* incubation temperatures for loggerhead sea turtle nests in May, June, and July 2018 (N=5 for each month). Data are represented in terms of mean ± SE (min–max). Superscript letters indicate significant differences between months ($p < 0.05$).

	May	June	July	*P* Value
T_{inc} (°C)	$29.84^{a} \pm 0.04$ (29.74–29.97)	$32.50^{b} \pm 0.37$ (31.06–33.21)	$32.99^{b} \pm 0.07$ (32.75–33.19)	0.007
T_{mid} (°C)	$28.776^{a} \pm 0.02$ (28.72–28.86)	$32.44^{b} \pm 0.4$ (30.92–33.18)	$32.29^{b} \pm 0.06$ (32.13–32.46)	0.005
T_{2w} (°C)	34.08 ± 0.15 (33.61–34.45)	33.99 ± 0.65 (31.49–35.31)	34.52 ± 0.11 (34.13–34.77)	0.112
T_{3dm} (°C)	34.55 ± 0.19 (34.01–35.04)	34.76 ± 0.6 (32.46–35.92)	35.27 ± 0.15 (34.73–35.58)	0.114

Abbreviations: T_{inc}, mean temperature during the entire incubation period (May v June: $p = 0.024$; May v July: $p = 0.007$; June v July: $p = 0.437$); T_{mid}, mean temperature in the middle third of incubation (May v June: $p = 0.004$; May v July: $p = 0.034$; June v July: $p = 0.865$); T_{2w}, mean temperature during the last 2 weeks of incubation; T_{3dm}, maximum temperature experienced by a nest for three consecutive days.

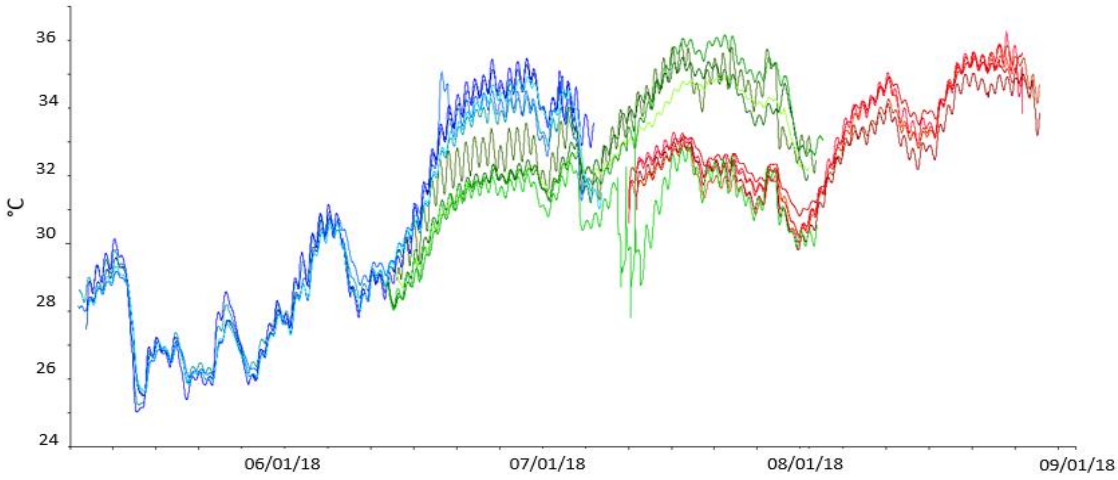

Figure 2. *In situ* incubation temperature profiles for all 15 loggerhead nests obtained from the data loggers. Temperature (°C) throughout the entire incubation period is portrayed on the y-axis with days of incubation on the x-axis. May nests are represented by the blue lines, June nests by the green lines, and July nests by the red lines.

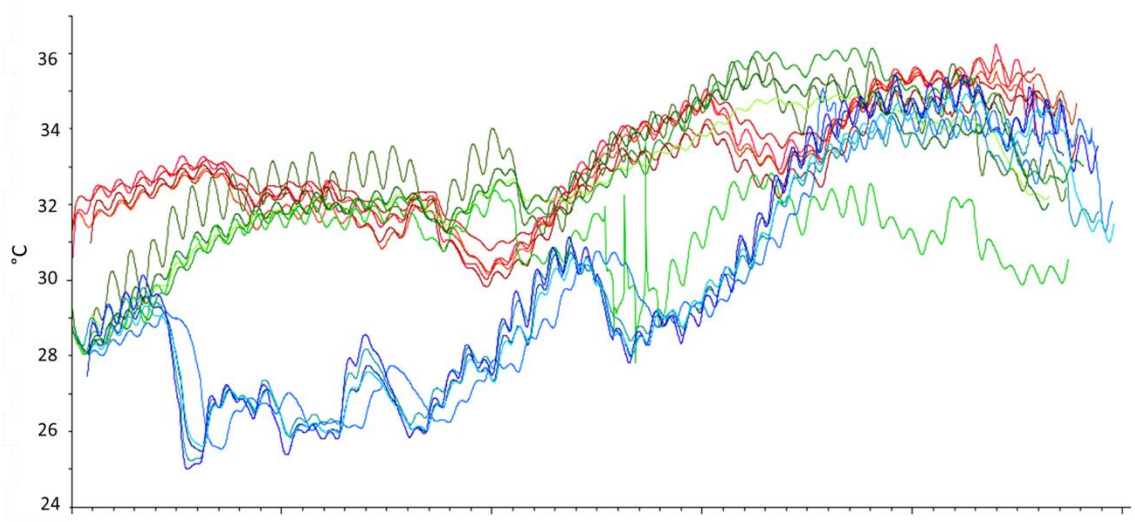

Figure 3. Transposed incubation temperatures for all nests to demonstrate differences between months. Incubation temperatures (°C) throughout the entire incubation period are portrayed on the y-axis with the start of the x-axis representing day one of incubation for all nests and continuing throughout hatch out. May nests are represented by the blue lines, June nests by the green lines, and July nests by the red lines.

Hatching success

The average hatching success (# hatched eggs divided by the total # of eggs) of nests laid in May was 83.75%, ranging from 72.92–93.98%. June's average rate ranged from 52.48–82.71% with an average success rate of 68.24%. July's success rate was similar to June's with an average rate of 66.55% but had a larger range of 48.53–91.36%. Average hatching success rates were found to be negatively associated with T_{3dm} (R^2 = 0.23, p = 0.047). Despite the fact that a significant difference was not found between the three months, the average success of nests with a T_{3dm} of under 35°C was 84.75%, while the average success of nests above 35°C was 65.92%. Previous research has shown nests with a T_{3dm} above 34°C had lower hatching success (Maulany et al. 2012; Sim et al. 2015); however, all of the nests for which I had hatching success data recorded

experienced a T_{3dm} of over 34°C, so the temperature of 35°C was chosen as the temperature for T_{3dm} analysis.

Temperature effects on morphology: hatchling size

Results from all morphological analyses are presented in Table 2. A significant difference was found for SCL ($\chi^2(2) = 39.12$, $p < 0.001$), with May producing the longest hatchlings (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.514$). SCW was significantly different between months ($\chi^2(2) = 17.21$, $p < 0.001$), with May also producing the widest hatchlings (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.972$). BD varied significantly ($\chi^2(2) = 46.98$, $p < 0.001$), with May having a significant smaller depth (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.363$). FFL decreased significantly ($\chi^2(2) = 43.31$, $p < 0.001$) between months (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.084$). Hatchling mass ranged from 14.4g to 21.4g and was highest in May and lowest in June; however, differences were not significant. Results from the CSI and BCI calculations indicate that hatchlings from May had the largest CSI ($\chi^2(2) = 28.79$, $p < 0.001$), but smallest BCI ($\chi^2(2) = 32.76$, $p < 0.001$). CSI was significantly higher in May compared to June and July (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.731$). However, BCI was significantly higher in June and July than in May (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.954$).

Results of the linear regressions showed incubation temperature to be negatively associated with SCL ($R^2 = 0.34$, $p < 0.001$) and CSI ($R^2 = 0.23$, $p < 0.001$) and positively associated with BD ($R^2 = 0.49$, $p < 0.001$) and BCI ($R^2 = 0.25$, $p < 0.001$).

Table 2. The effects of incubation temperatures on loggerhead (*Caretta caretta*) hatchling morphology. Data are represented in terms of mean ± SE (min–max). Superscript letters indicate significant differences between months.

	May	June	July	*P* Value
SCL (mm)	43.72^{a} ± 0.13 (41.67–45.64)	42.01^{b} ± 0.31 (36.93–46.09)	42.03^{b} ± 0.18 (39.11–44.55)	$p < 0.001$
SCW (mm)	32.53^{a} ± 0.13 (30.21–33.91)	31.31^{b} ± 0.29 (26.22–34.26)	31.66^{b} ± 0.16 (28.96–34.05)	$p < 0.001$
BD (mm)	17.79^{a} ± 0.09 (16.33–19.17)	18.98^{b} ± 0.11 (17.34–20.58)	18.83b ± 0.13 (16.88–21.11)	$p < 0.001$
FFL (mm)	36.60^{a} ± 0.21 (32.76–39.83)	34.15^{b} ± 0.29 (30.55–37.61)	35.12b ± 0.18 (32.20–38.10)	$p < 0.001$
Mass (g)	18.36^{a} ± 0.12 (16.30–20.10)	17.73^{a} ± 0.25 (14.50–21.00)	17.90^{a} ± 0.24 (14.40–21.40)	$p = 0.142$
CSI (mm2)	1415.94^{a} ± 8.90 (1232.84–1510.35)	1311.23^{b} ± 22.39 (915.16–1561.99)	1331.55^{b} ± 10.93 (1155.79–1499.55)	$p < 0.001$
BCI	2.21^{a} ± 0.02 (1.91–2.48)	2.39^{b} ± 0.03 (2.10–3.07)	2.41^{b} ± 0.03 (2.09–2.99)	$p < 0.001$

Abbreviations: SCL, straight carapace length (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.514$); SCW, straight carapace width (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.972$); BD, body depth (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.363$); FFL, front (right) flipper length (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.084$); CSI, the carapace size index (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.731$); BCI, body condition index (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.954$).

Temperature effects on morphology: abnormal scutes

There was a significantly larger percentage of turtles with abnormal scutes in July compared to May and June (May v June: $p = 0.404$; May v July: $p < 0.001$; June v July: $p = 0.002$ Fig. 4). Overall, out of the 144 hatchlings examined during this study, 42 had abnormal scute patterns (29.17%) with the most common abnormality occurring with the vertebral scutes (Table 3).

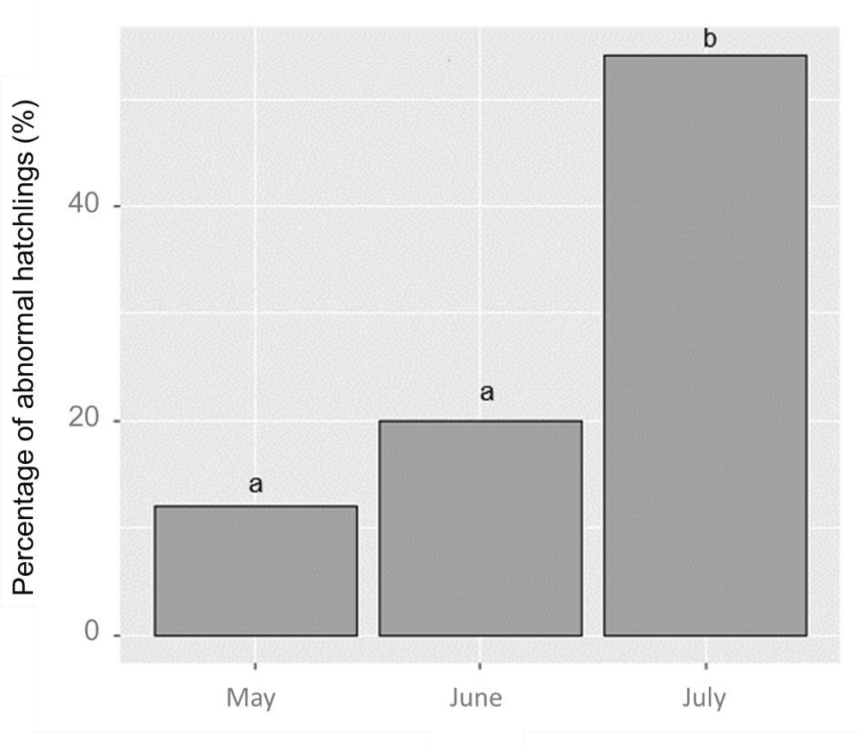

Figure 4. Percentage of hatchlings with at least one abnormal scute. The total number of hatchlings with abnormal scutes per month was added and then divided by the total number of hatchlings per month to achieve a percentage. Different lowercase letters represent statistically significant differences between months.

Table 3. Anatomical location, number, and percentage of abnormal scutes by month.

	May	June	July	Total
Individuals[a]	6/50 (12%)	9/44 (20.5%)	27/50 (54.0%)	42/144 (29.2%)
Total extra scutes	12	14	31	57
Type	Vertebral: 2 Costal: 5 Marginal: 1 Notch: 4	Vertebral: 7 Costal: 4 Marginal: 0 Notch: 3	Vertebral: 28 Costal: 0 Marginal: 2 Notch: 1	Vertebral: 37 Costal: 9 Marginal: 3 Notch: 8

[a]Individuals refers to the number of hatchlings that had at least one abnormal scute present.

Temperature effects on morphology: umbilical scar length and width

There was a significant difference between months for both umbilical scar length ($\chi^2(2) = 28.92$, $p < 0.001$), and width ($\chi^2(2) = 28.34$, $p < 0.001$; Fig. 5). Loggerhead hatchling umbilical scar length was largest in hatchlings from nests laid in July (7.45mm ± 0.20 mm), intermediate in hatchlings from nests laid in June (6.85mm ± 0.19mm), and smallest in hatchlings from nests laid in May (6.06mm ± 0.10 mm). Umbilical scar length was significantly larger in July (May v June: $p = 0.015$; May v July: $p < 0.001$, June v July: $p = 0.054$). A similar trend was observed for umbilical scar width with hatchlings from July having the largest width (5.64mm ± 0.18mm), followed by June (4.62mm ± 0.12mm), then May (4.49mm ± 0.09mm). Umbilical scar width was significantly larger in July (May v June: $p = 0.889$; May v July: $p < 0.001$, June v July: $p < 0.001$)

Linear regression analysis revealed a positive association between umbilical scar length ($R^2 = 0.34$, $p < 0.001$) and incubation temperature, along with umbilical scar width ($R^2 = 0.20$, $p < 0.001$) and incubation temperature.

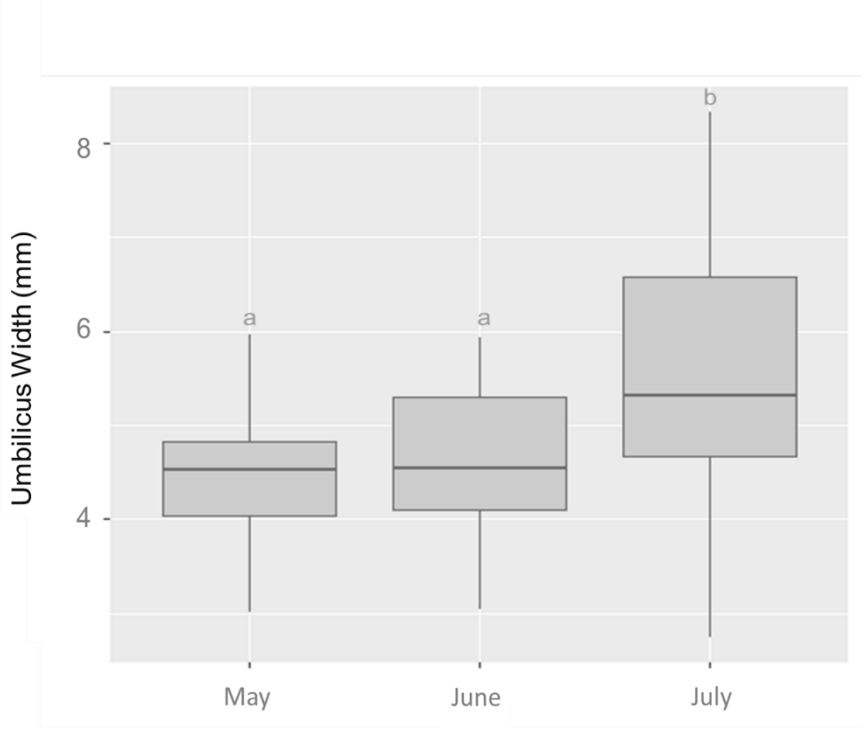

Figure 5. Boxplot of effects of incubation temperature on umbilical scar width. Boxplots show the median (solid line within box), 1st and 3rd quartiles (lower and upper box limits), and range (whiskers) for May, June, and July. Different lowercase letters represent statistically significant differences between months.

Temperature effects on health indices

Measures of central tendency and reference intervals for all health indices are shown in Table 4. Ten blood samples were not included in statistical analyses regarding glucose, PCV, hemoglobin, and total solids due to a hemolysis score of 2+ (Stacy and Innis 2017). Additionally, one hatchling in July had blood that clotted and twenty hatchlings in May were removed for PCV due to inaccurate readings. Significant differences for PCV ($\chi^2(2) = 51.44$, $p < 0.001$), hemoglobin ($\chi^2(2) = 42.79$, $p < 0.001$), and total solids ($\chi^2(2) = 48.75$, $p < 0.001$) were found between the three months. All three values were found to be the highest in June: PCV (May v June: $p < 0.001$; May v

July: p = 0.671; June v July: p < 0.001, Fig. 6), hemoglobin (May v June: p < 0.001; May v July: p = 0.623; June v July: p < 0.001, Fig. 7), and total solids (May v June: p < 0.001; May v July: p = 0.089; June v July: p < 0.001, Fig. 8). Glucose and total white blood cell estimates did not significantly differ between the three months.

The results of the linear regressions indicated similar influences of temperature. Incubation temperature was significantly and positively associated with PCV (R^2 = 0.10, p = 0.012), hemoglobin (R^2 = 0.17, p < 0.001), and total solids (R^2 = 0.29, p < 0.001).

Several white blood cell types varied significantly between the three months including heterophils ($\chi^2(2) = 11.6$, p=0.003), immature heterophils ($\chi^2(2) = 18.78$, p < 0.001), monocytes ($\chi^2(2) = 6.79$, p = 0.034), and basophils ($\chi^2(2) = 8.19$, p = 0.017). Eosinophils, hemoparasites, and heterophil toxicity were absent for all hatchlings. Anisocytosis was mild for all hatchlings and polychromasia ranged from minimal to mild.

Hatchling heart rate varied significantly between the three months ($\chi^2(2) = 21.59$, p < 0.001), with May having the highest rate and July having the lowest rate (May v June: p = 0.665; May v July: p < 0.001; June v July: p = 0.005). Heart rate was highest in May (79.29 ± 1.2), intermediate in June (77.91 ± 0.97), and lowest in July (73.14 ± 0.93).

Table 4. Measures of central tendency and reference intervals for packed cell volume, total solids, glucose, hemoglobin, heart rate, and white blood cell counts with differentials for hatchling loggerhead (*Caretta caretta)* sea turtles from Florida. Nonparametric methods for sample sizes ≥120 were used to calculate reference intervals after Friedrichs et al. 2012. For packed cell volume, total solids, and glucose, and hemoglobin, samples with a hemolysis scores ≥2+ were eliminated during calculation of reference intervals.

Parameter	Mean±SD	Median	Range	*N*	Lower limit (90% CI)	Upper limit (90% CI)
Packed cell volume [%]	34±6	34	23–46	128	24 (23–26)	45 (44–46)
Total solids [g/dl]	3.2±0.5	3.2	2.0–5.9[a]	132	2.3 (2.0–2.5)	4.2 (4.0–4.4)
Glucose [mg/dl]	92±21	91	39–152	130	55 (39–60)	134 (127–152)
Hemoglobin (g/dl)	8.6±1.1	8.7	6.0–10.9	130	6.5 (6.0–7.0)	10.6 (10.3–10.9)
Heart rate (beats/min)	77±8	76	60–98	142	62 (60–68)	94 (90–98)
Total wbc counts [cells/µl]	8570±2412	8100	4700–14100	134	4938 (4700–5200)	13725 (12800–14100)
Heterophils [cells/µl]	4726±1600	4300	2200–9300	134	2400 (2200–2600)	8550 (7800–9300)
Immature heterophils [cells/µl]	138±165	95	0–830	134	0 (0)	729 (390–830)
Lymphocytes [cells/µl]	2679±888	2500	800–5000	134	1175 (800–1500)	4800 (4400–5000)
Monocytes [cells/µl]	835±523	710	150–2800	134	198 (150–270)	2263 (1800–2800)

Eosinophils [cells/µl]	0	0	0	134	0 (0)	0 (0)
Basophils [cells/µl]	315±478	200	0–3600	134	60 (0–60)	1950 (700–3600)

[A] 5.9 g/dl is an outlier. The next highest value was 4.4 g/dl. Reference intervals were calculated with this value removed.

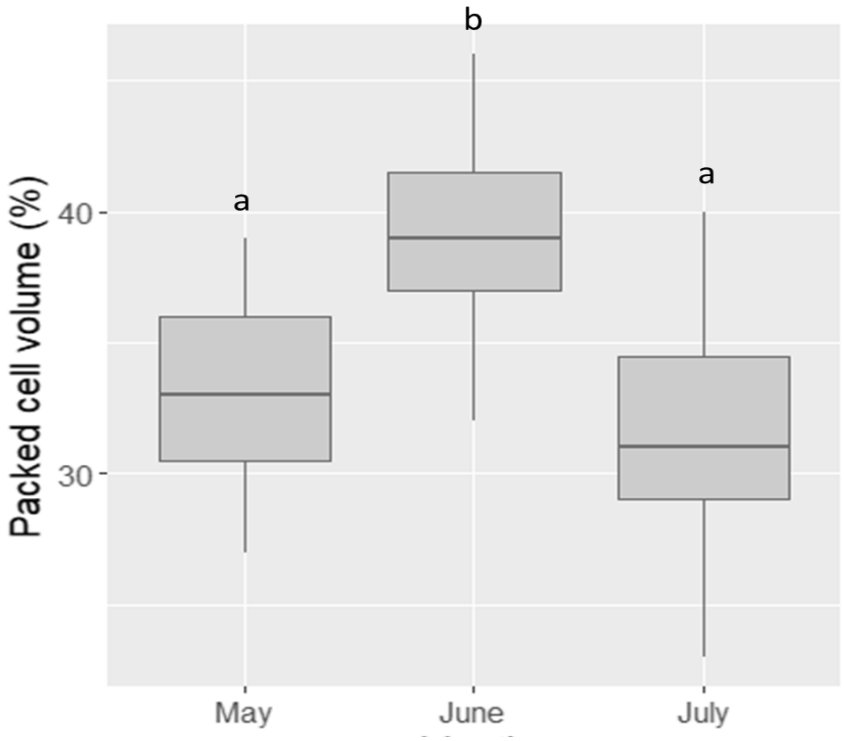

Figure 6. Effects of incubation temperatures on packed cell volume. Boxplots show the median (solid line within box), 1st and 3rd quartiles (lower and upper box limits), and range (whiskers) for May, June, and July. Different lowercase letters represent statistically significant differences between months.

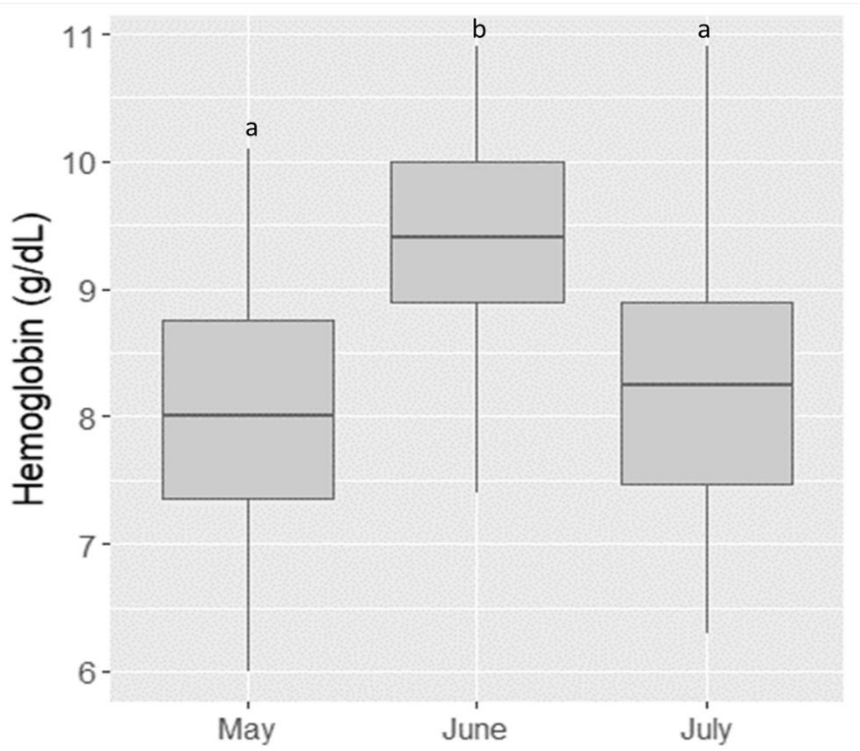

Figure 7. Effects of incubation temperature on hemoglobin. Boxplots show the median (solid line within box), 1st and 3rd quartiles (lower and upper box limits), and range (whiskers) for May, June, and July. Different lowercase letters represent statistically significant differences between months.

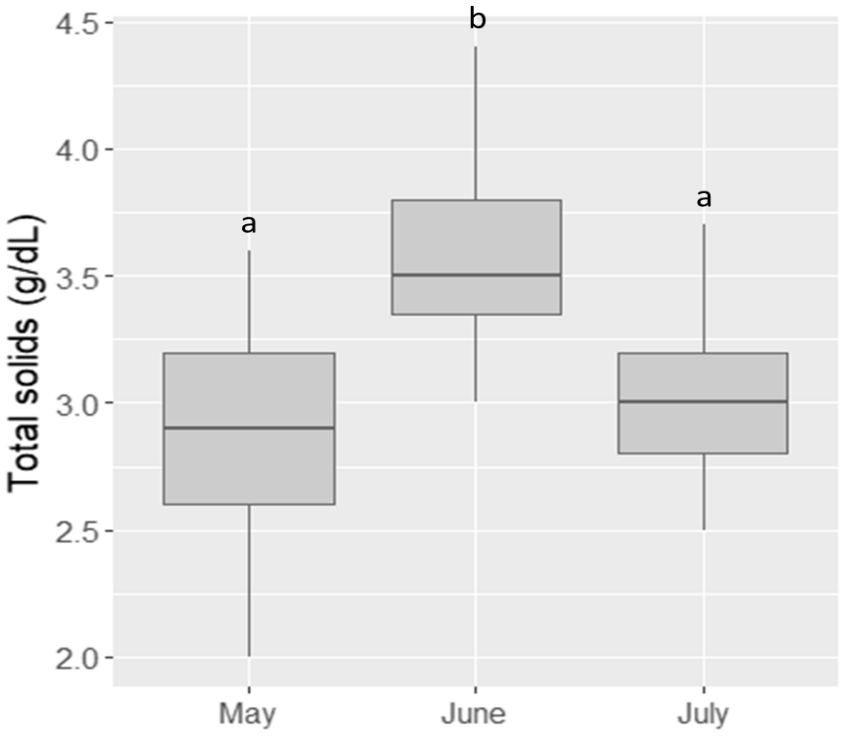

Figure 8. Effects of incubation temperature on total solids. Boxplots show the median (solid line within box), 1st and 3rd quartiles (lower and upper box limits), and range (whiskers) for May, June, and July. Different lowercase letters represent statistically significant differences between months.

Temperature effects on locomotor performance

There was a significant difference in self-righting response time ($\chi^2(2) = 26.74$, $p < 0.001$, Fig. 9) with righting time being significantly faster in May (May v June: $p < 0.001$; May v July: $p < 0.001$; June v July: $p = 0.957$). Overall, righting time ranged from 0.72 to 2.22 seconds. Mean times averaged to 1.23 ± 0.04 seconds in May, 1.50 ± 0.05 seconds in June, and 1.52 ± 0.04 seconds in July.

In addition, incubation temperature was also found to be negatively associated with self-righting time ($R^2 = 0.29$, $p < 0.001$). In contrast, hemoglobin was positively associated with self-righting time ($R^2 = 0.16$, $p < 0.001$). Self-righting response time was not significantly related with heart rate.

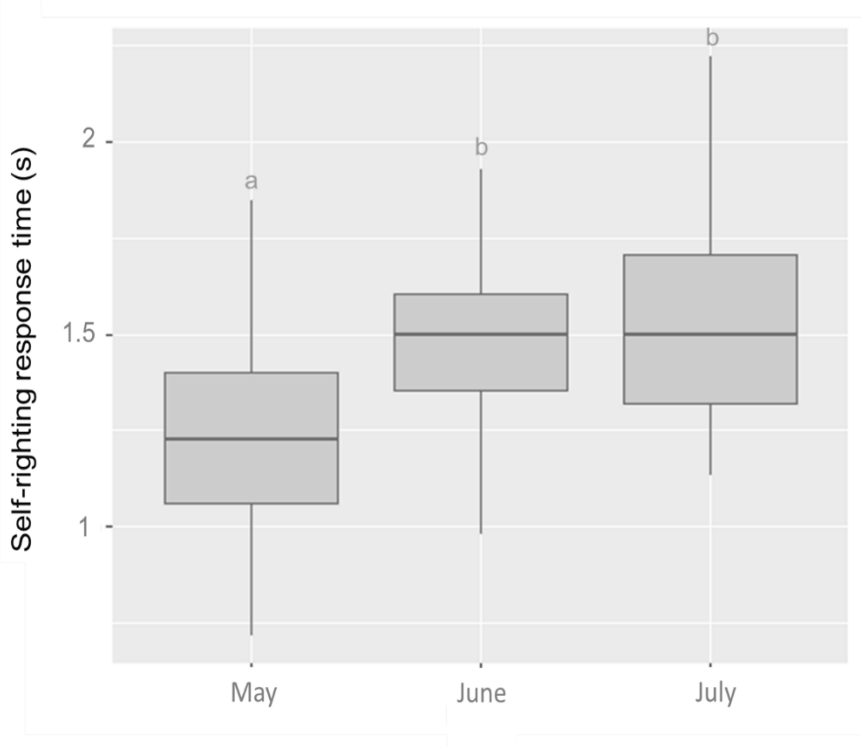

Figure 9. Differences in self-righting response (seconds) by month. Boxplots showing median (solid line within box) and 1st and 3rd quartiles (lower and upper box limits), and range (whiskers). Different lowercase letters represent statistically significant differences between months.

Temperature effects on stage of development at death

Overall, the number of embryos at full-term represented 41% of unhatched eggs, followed by 27% with no development, with the middle four stages (early pigmented eye, late pigmented eye, early pigmented body, late pigmented body) representing a combined 16%. The stage of development for the remaining eggs could not be determined and represented 16% of unhatched eggs (Table 5). May had 28 eggs with no development out of the 51 unhatched eggs, resulting in a higher percentage of eggs with no development (n = 28/51: 54.9%), compared to June (n = 27/129: 20.9%) and July (n = 11/62: 17.7%).

May had no unhatched eggs in the late pigmented eye, early pigmentated body, or late pigmentated body stages. May also had a lower percentage of embryos that died at full-term (n = 4/51: 7.8%), compared to June (n = 73/129: 56.6%, $\chi^2(6) = 55.94$, $p < 0.001$) and July (n = 22/62: 35.5%**,** $\chi^2(6) = 36.698$, $p < 0.001$). There was also a significant difference for percentage of dead, full-term embryos between June and July ($\chi^2(6) = 18.58$, $p = 0.005$) with June having a significantly higher amount.

Table 5. Percentages of stage of development at death by month for loggerhead (*Caretta caretta*) embryos.

	May (n = 51)	June (n = 129)	July (n = 62)
No development	54.90% (28)	20.93% (27)	18.03% (11)
Early pigmented eye	5.88% (3)	2.33% (3)	1.64% (1)
Late pigmented eye	0% (0)	3.88% (5)	3.28% (2)
Early pigmented body	0% (0)	4.65% (6)	4.92% (3)
Late pigmented body	0% (0)	3.88% (5)	16.39% (10)
Full term	7.84% (4)	56.59% (73)	36.07% (22)
Unknown	31.37% (16)	7.75% (10)	21.31% (13)

Total number of hatchlings in each stage of development from each nest were added by month and divided by the total number of unhatched eggs. (n = number of unhatched eggs). Data represented in percentages and (number of individuals).

CHAPTER 4: DISCUSSION

Understanding and planning for how species will respond to the threat of climate change is imperative when developing species management and recovery plans. Rapid climate warming is expected to severely threaten sea turtles due to potential alterations in sex ratios (feminization of hatchlings, Cavallo et al. 2015), high nesting site fidelity (shifting and decreasing suitable nesting habitat, Butt et al. 2016; Patrício et al. 2018), long generation times (decreasing growth rates, Bjorndal et al. 2016; Bjorndal et al. 2017), decreased foraging grounds and food supply (Chaloupka et al. 2008), and increased nest temperatures. This study aimed to examine the latter and provide critical information about how *in situ* incubation temperatures will affect a variety of loggerhead hatchling traits. Comprehensive studies have not been conducted regarding the effects of incubation temperatures on both the numerous physical and physiological characteristics documented here, especially with natural incubation temperatures. Generally, I found hatchlings that incubated in May during cooler temperatures were larger, had smaller umbilical scars, less abnormal scutes, took a shorter time to right themselves, had a higher heart rate and lower values for health indices, and had more unhatched eggs with no development and fewer that were full-term when compared to June and July's warmer nest temperatures.

Incubation temperatures

Nest temperatures recorded during this study provide unique information regarding *in situ* incubation temperatures. Obtaining data from *in situ* nests is important since laboratory experiments do not mimic the natural nest environment due to differences in water uptake, gas exchange, and metabolic heating (Horne et al. 2014; Booth 2017). Additionally, almost 90% of the nesting effort of this loggerhead subpopulation (Northwest Atlantic) occurs on the peninsular region of Florida, making it the largest nesting population in the western hemisphere (Ehrhart et al. 2003; Ceriani et al. 2012). This makes the data obtained from *in situ* studies critical, especially for this important nesting population.

Since the current estimated lethal threshold for constant incubation temperatures is between 33°C and 35°C (Howard et al. 2014), it is alarming that the majority of the nests are experiencing temperatures within this range, specifically, 93% of nests experienced a T_{3dm} of over 34°C, and 53% experienced a T_{3dm} of over 35°C. More so, a T_{3dm} of over 35°C was experienced in a nest in May, meaning that even nests laid in the cooler part of the nesting season may already be experiencing temperatures above lethal limits for some time during incubation. One nest in June and another in July experienced a T_{3dm} of over 35.5°C, with the highest T_{3dm} recorded in this study reaching 35.92°C. The one nest that did not experience a T_{3dm} of over 34°C was an overwashed nest in June. The mean hatching success of nests with a T_{3dm} below 35°C (81%) in this study was higher than the mean success of nests above 35°C (69%), which is similar to findings in other studies that looked at a T_{3dm} of above and below 34°C (Maulany et al., 2012; Sim et al., 2015). Knowing when, and for how long, embryos are exposed to temperatures above

these limits would be useful for developing management strategies. Loggerhead sea turtle hatching success was significantly influenced by time spent at incubation temperatures between 34–35.5°C in Florida (Bladow 2017).

The incubation temperatures from this study were not significantly different between June and July. More studies are needed to evaluate these traits in order to achieve a wider representation of incubation temperatures. Future studies should analyze nests in April to see if incubation temperatures are significantly cooler than May, and if this correlates to significant differences in morphology, health, performance, or embryology. Since loggerhead nests usually incubate between a mean nest temperature of 24–33°C (Howard et al. 2014), knowing the effects of *in situ* incubation temperatures throughout the incubation period is important to achieve a more comprehensive understanding of the impact incubation temperature has on hatchling traits. Future studies should also include the position of the nest on the beach to determine if distance from the high tide line or vegetation line influences the results. Additionally, studies should evaluate the effects of shade and moisture on incubation temperatures, as both have been observed to impact hatchling fitness and performance in turtles (Janzen et al. 1990; McGehee 1990; Wood et al. 2014).

Temperature effects on morphology: hatchling size

It is now well known that incubation temperatures affect sea turtle hatchling morphological characteristics. Hatchlings from May nests had significantly different SCL, SCW, BD, FFL, CSI, and BCI compared to hatchlings from nests laid in June and July. This supports previous studies that found increased incubation temperatures resulted

in smaller hatchling sizes (Glen et al. 2003; Michelson and Downie 2010; Read et al. 2012; Sim et al. 2015). BCI was the lowest in May, likely resulting from a longer carapace length and smaller yolk sac, whereas hatchlings from June and July had larger yolk sacs and shorter carapace lengths. For juveniles and adults, a larger BCI score is thought to correspond to a healthier individual; however, few studies analyze BCI in hatchlings. Future studies should include BCI in hatchling measurements to achieve a baseline of a typical BCI score for hatchlings. The larger sizes produced in May might be advantageous in avoiding predation, yet the hatchlings produced in June and July have larger yolk reserves and are more likely to be able to survive longer without eating (Booth 2006).

Development and the timing of developmental stages depend on incubation temperature (Miller et al. 2017). When the incubation temperature is at 26°C, the duration of incubation is approximately 23 days longer than at 29°C and 8 days longer than at 32°C compared to 29°C (Miller et al. 2017). The differences in time taken to reach different developmental stages may be a result of higher metabolism in warmer nests and thus a faster growth rate; whereas, incubating longer gives hatchlings more time to convert yolk material to tissue (Booth 2000, Booth and Evans 2011).

Incubating longer and converting more yolk contents to tissue is beneficial in order to achieve a larger size and thus better locomotor abilities for making the trek from nest to open ocean (Booth 2000; Sime et al. 2015). With ever-increasing atmospheric temperatures comes an increase in sea turtle nest incubation temperatures. This results in shorter incubation times, smaller hatchlings, and larger yolk reserves (Glen et al. 2003; Booth and Evans 2011). However, with the amount of suitable nesting space likely to

diminish with sea level rise (Butt et al. 2016; Patrício et al. 2018), shorter incubation times might be beneficial for nests in unfavorable locations. However, a decrease in hatchling recruitment to the population might occur if the smaller hatchlings experience a higher rate of predation after emergence due to poor locomotor performance abilities. If the hatchlings make it past the predator-rich coastal waters, the larger yolk reserves present might be able to compensate for the longer time it takes them to reach their open ocean foraging grounds due to decreased locomotor abilities.

Temperature effects on morphology: abnormal scutes

More hatchlings with abnormal scutes were documented in nests from July, in comparison to May and June, supporting previous research (Zimm et al. 2017). The higher percentage of abnormal scutes present in warmer nests could indicate a disadvantage for fitness and survival, as abnormal scute patterns have been shown to be selected against since they occur more often in dead hatchlings and are less prevalent in adults (Mast and Carr 1989; Türkozan et al. 2001; Telemeco et al., 2013). However, the underlying cause of abnormal scute formation needs to be better understood before the implications of such abnormalities can be realized. Currently, the source of these variations is under discussion and include environmental stress (Telemeco et al. 2013, Zimm et al. 2017), DNA methylation (Caracappa et al. 2016), and genetic diversity (Velo-Antón et al. 2011). The fact that hatchlings from July had significantly more abnormal scutes than June, even though the mean incubation temperature between them was not significant, could suggest the influence of other unmeasured environmental factors, such as rainfall.

Future studies should evaluate the internal anatomy in hatchlings with normal and abnormal scute patterns, with the degree of abnormality taken into account, to determine if abnormal scute patterns correspond to abnormal internal anatomy. Additionally, Türkozan et al. (2001) found that nesting loggerhead adults did not have any abnormal vertebral, costal, nuchal, or supracaudal scutes. Thus, future studies could investigate the number of abnormal scutes present during different life stages to see when selection against abnormal scute patterns occur.

Temperature effects on morphology: umbilical scar length and width

Umbilical scar length was significantly smaller in May compared to June and July, and umbilical scar width was significantly smaller in May and June compared to July. If umbilical scar size is representative of the amount of yolk reserve, then this study supports previous studies, whereby cooler nests produce hatchlings that utilize their yolk reserves faster by converting more yolk material to tissue, thus having smaller residual yolk remaining after hatching (Booth 2000; Booth and Astill 2001; Hewavisenthi and Parmenter 2001; Glen et al. 2003; Booth and Evans 2011).

The amount of yolk reserves left in hatchlings is directly proportional to the amount of energy they have to expend (Kraemer and Bennett 1981). Yolk reserves were found to decrease considerably from the time of hatching to 96 hours after emergence, without evidence of fat growth or storage (Kraemer and Bennett 1981). It is then inferred that hatchlings must rely on yolk reserves for the metabolic demands of hatching, emergence, and the trek from nest to open ocean (Kraemer and Bennett 1981). Therefore, hatchlings from warmer nests that have larger umbilical scars (yolk reserves) are more

likely to be able to swim farther and survive longer without eating. However, this produces a phenotypic trade-off as hatchlings from May (cooler nests) had larger body sizes and might have an advantage in avoiding predation (Booth 2006; Gyuris 2000; Janzen et al. 2000; Salmon and Scholl 2014; Salmon et al. 2015).

There are currently no published studies that have looked at umbilical scar size in relation to nest temperatures or in relation to the amount of residual yolk reserve present. Future research should measure umbilical scar size and dissect the yolk reserve for measurement to verify that umbilical scar size correlates to yolk reserve size. This was attempted during the study but there were too few dead-in-nest hatchlings to accurately carry out this part of the experiment.

Temperature effects on health: heart rate

Heart rate varied significantly, by month, but was surprisingly lower in July than in May and June and was not significantly associated with temperature, contradicting previous studies. Embryonic heart rates have been found to increase with incubation temperature in various oviparous reptiles, including turtles (*Chelydra serpentine*, Trionychidae and Emydidae), and can be negatively associated with shorter incubation periods (Du et al. 2010, 2011). However, these studies evaluated heart rate in a laboratory with a wide range of incubation temperatures (20.0–33.5°C), so it is possible we did not achieve similar results due to the smaller range of mean incubation temperatures (29.7–33.2°C) experienced in our fluctuating *in situ* nest temperatures (Du et al. 2010, 2011). Additionally, heart rate measurements have typically been established in embryos, which might not correspond to post-emergence. It is possible that other factors are impacting

heart rate, such as dehydration, oxygen levels, and moisture; however, Du and Shine (2008) found that moisture levels did not significantly impact mean embryonic heart rates in common garden skinks (*Lampropholis guichenoti*).

Temperature effects on health: baseline indices

Several health indices were significantly associated with incubation temperatures; the highest variations occuring in June. PCV, a useful indicator of dehydration or anemia (Stacy and Innis 2017), represents the proportion of RBC in comparison to the total volume of blood and has been found to vary with water temperature (Stamper et al. 2005; Kelly et al. 2015; Perrault et al. 2016). The mean PCV reported from this study (34%) is higher than that reported for green turtle post-hatchlings in Suriname (29%; Frair 1977) and green turtle hatchlings from Heron Island, Australia (28%; Wells and Baldwin 1993). It is also higher than the mean (22%) reported for juvenile loggerheads in Azorean waters (Stacy et al. 2018). The results of this study indicate that incubation temperatures can have a significant influence on hatchling PCV. Dehydration is a common cause of high PCV (Selleri and Hernandez-Divers 2006) and has been shown to significantly increase PCV in leatherback hatchlings that were withheld from water for 12 hours following emergence (Reina et al. 2002). Therefore, it is possible that increasing environmental temperatures will lead to dehydration in sea turtle hatchlings.

Like PCV, hemoglobin, the oxygen-transporting protein in red blood cells (Wells and Baldwin 1994), can also indicate dehydration or anemia as it is an indirect measurement of the amount of red blood cells in the blood. Few studies have included hemoglobin in their analysis of blood analytes in sea turtles. The mean concentration

obtained from this study (8.6 g/dl) was higher than the range (4.0–6.5 g/dl) obtained from captive-reared 4-month-old loggerhead post-hatchlings in South Carolina (Bradley et al. 1998) but were more similar to hemoglobin concentrations of green turtle hatchlings (7.765 g/dl) from Heron Island, Australia (Wells and Baldwin 1994). Increased incubation temperatures could potentially decrease the affinity of hemoglobin to oxygen, enhancing the unloading of oxygen in the tissues (Malte and Lykkeboe 2018; Patel and Cooper 2018). PCV and hemoglobin were highest in June and were significantly different from readings in May and July. Mean incubation temperatures did not vary significantly between June and July; therefore, it would be expected that readings in June and July would have similar health indices. Nonetheless, linear regressions showed that both values were significantly correlated with incubation temperatures.

Measurement of total solids provides information regarding the amount of proteins, lipids, and electrolytes in the plasma. The mean total solids concentration for this study (3.2 g/dL) was similar to that recorded for juvenile green turtles from North Carolina (median = 3.0 g/dL; Anderson et al. 2011). Total solids measurements can indicate dehydration, inflammation when elevated, or malabsorption/maldigestion when low (Stacy and Boylan 2014). Total solids concentrations in loggerhead hatchlings from this study were significantly associated with incubation temperature; whereby total solids were June was significantly higher than May and July. Total solids, PCV, and hemoglobin concentrations were all higher in warmer months (June or July) when compared to cooler months (May); therefore, these three concomitant increases provide convincing evidence that high nest incubation temperatures could be related to dehydration in loggerhead hatchlings. Dehydration during incubation has been found to

reduce embryonic mass, alter morphological phenotype, and result in bradycardic (abnormally slow heart rate) embryos of the American alligator (*Alligator mississipiensis*; Tate et al. 2012). This slow heart rate could potentially lead to exhaustion and decreased locomotor performance potentially impacting survival.

Glucose is an important diagnostic analyte in sea turtles and can indicate stress, disease, or starvation, depending on the concentrations (Stacy and Boylan 2014). It has also been observed to vary with environmental temperatures (Coulson and Hernandez 1953; Callard et al. 1975; Stacy and Boylan 2014); however, glucose was not found to vary significantly between the three months of this study or as a result of temperature. The median glucose value reported for this study, (91 mg/dL), is the most similar to that reported for neritic juvenile and adult loggerheads from Cape Canaveral, Florida, using an Olympus AU5061 autoanalyzer (95 mg/dL; Stacy et al. 2018). The median value for this study is lower than that of juvenile loggerheads in Core Sound, North Carolina (105 mg/dL; Kelly et al. 2015), lower than juvenile loggerheads in Azorean waters (114 mg/dL; Stacy et al. 2018), and lower than juvenile green turtles near Harkers Island, North Carolina (124 mg/dL; Anderson et al. 2011). The differences in values reported might be due to the differing methods used to analyze glucose; however, similar results in whole blood glucose, while using a glucometer compared to a traditional plasma chemistry analyzer, have been observed (Perrault et al. 2018).

How incubation temperatures influence health indices is important to understand because reduced health and immunity due to high incubation temperatures might increase their vulnerability to disease with an increasingly warmer climate. The varying health indices in relation to nest temperatures found in this study could suggest altered

hemodynamic balance, especially in regards to dehydration with warmer incubation temperatures. Dehydration may influence hatchling metabolism, locomotor performance, and critical thermal maximum (the temperature above which most individuals do not survive; Plummer et al. 2003). Hatchlings face a multitude of threats upon emergence and already have decreased survival rates (Crouse et al. 1987). The reduced health due to higher incubation temperatures will further disadvantage hatchlings before emergence even occurs, potentially decreasing their chance at survival.

Temperature effects on health: WBC counts

Total WBC counts are good diagnostic indicators of immune function and maturity status (Stacy and Innis 2017) and have been observed to be influenced by water temperature (Kelly et al. 2015). Total WBC estimates were not significantly different between the three months of this study and were not correlated with incubation temperature; however, higher means for June (9150 cells/µl) and July (8770 cells/µl) were recorded than for May (7890/µl). The median WBC estimate in loggerhead hatchlings from this study (8100 cells/µL) was lower than that reported for juvenile loggerheads in Core Sound, North Carolina (9000 cells/µL; Kelly et al. 2018), but higher than WBC estimates for juvenile green turtles from Core Sound (7000 cells/µL; Anderson et al. 2011). The median for heterophils in hatchling loggerheads from this study (4300 cells/µL) were lower than the reported values for the loggerheads in Core Sound (4700 cells/µL; Kelly et al. 2018). The median for lymphocytes in this study (2500 cells/µL) were also lower (3400 cells/µL: Kelly et al. 2018). However, this study had higher monocytes (710 cells/µL) and basophils (200 cells/µL) compared to the juvenile

loggerheads in Core Sound (monocytes: 140 cells/µL, basophils: 0 cells/µL; Kelly et al. 2018). Basophil variations in reptiles are poorly understood, but are generally assumed to be associated with immune stimulation (Strik et al. 2007). This is the first study to report WBC counts in hatchling sea turtles. This is important because if hatchlings from warmer nests have increased WBC counts or variations in WBC proportions, this could suggest potential systemic inflammation and/or immune stimulation.

This is the first study to examine baseline health analyses of sea turtle hatchlings in relation to nest temperature. Unfortunately, limited data are available with which to compare the reference intervals calculated from this study to other blood indices in hatchling sea turtles. Additional studies are needed to better establish baselines for loggerhead hatchlings. Baselines should also be evaluated for other sea turtle species across geographic locations. Having baselines for hatchlings will be useful in understanding how sea turtle health changes across life stages (*e.g.*, juvenile, subadult, adult). Baselines will also be useful in determining the health status of washback hatchlings (post-hatchling turtles that get washed back ashore during high winds, large storms, or large seaweed events).

Temperature effects on locomotor performance

Righting response time was found to be significantly and negatively associated with incubation temperatures, with the hatchlings from May's cooler nests having a faster righting response time. Similar findings have been observed in studies looking at righting response on land and in other locomotor performance tests, such as crawling and swimming (Burgess et al. 2006; Ischer et al. 2009; Booth and Evans 2011; Booth et al.

2013; Fisher et al. 2014; Sim et al. 2015). Mean righting time on land was found to increase significantly when incubation temperatures were above 30°C in loggerhead hatchlings (Fisher et al. 2014), which is similar to the results in this study since hatchlings in June (T_{inc} 32.5°C) and July (T_{inc} 32.99°C) had significantly longer righting times compared to hatchlings in May (T_{inc} 29.84°C). *G. ouachitensis* and *T. scripta elegans* hatchlings that incubated at 30°C, compared to 25°C, righted significantly faster at hatching and at one year of age, suggesting that incubation temperature might have a long-lasting influence on physiological performance (Freedberg et al. 2004). The self-righting response test took place after blood collection, and although safe blood collection practices were followed, it cannot be ruled out that blood collection did not impact performance.

Since nesting season for loggerheads in Florida does not typically peak until June, it is probable that most hatchlings produced on these beaches have slower righting response times, consequently hindering their survival. The few nests that would have a mean incubation temperature under 30°C are likely produced in April and May, and with the threat of increasing nest temperatures due to climate change, these nest temperatures will likely rise. This could result in all nests producing hatchlings with decreased locomotor abilities.

This is the first study to examine a righting response in water, as other studies typically measure this response on land. The implications of this test in water are important as hatchlings can be overturned in the near-shore waves. Understanding the effects of temperature on sea turtle hatchling locomotor performance in general is critical since it can impact the hatchlings' ability to evade beach and near shore predators and

survive in the open ocean. Hatchlings in May might be more likely to survive to adulthood since an improved locomotor ability has been found to increase predator avoidance and have lasting impacts on long-term survival and performance (Janzen 1993; Freedberg et al. 2004).

Several studies have examined the influence of incubation temperature on hatchling performance, but none have analyzed the underlying causes of these differences. Following the recommendations of Booth (2017), I attempted to analyze heart rate, heart size, and hemoglobin levels as possible underlying causes for reductions in locomotor performance. Heart rate was observed not to be significantly associated with righting response time, indicating that it might not contribute to differences in locomotor performance. However, since the righting time experienced in the water was a rapid activity, heart rate might still influence other prolonged activities that require aerobic metabolism and thus effective delivery of oxygen by the cardiovascular system (Booth 2017). These could include activities such as digging out of the nest or during the swimming frenzy en route to the open ocean (Booth 2017). Future studies should evaluate heart rate to establish baselines in hatchlings and to explore the potential influences it may have on different performance tests. There were too few dead-in-nest hatchlings from this study to determine heart size. Analyzing heart size would be beneficial in determining what differences in the cardiovascular system, if any, influence variations in locomotor performance (Booth 2017).

Hemoglobin was determined to be significantly and positively associated with locomotor performance, contradicting what was expected. It was anticipated that higher hemoglobin levels would result in faster righting times due to a higher oxygen carrying

capacity in the blood. This might indicate that other variables have a greater influence on righting time than the oxygen-transporting abilities of the hatchling's blood, although no published studies have evaluated this interaction. Future studies should investigate hemoglobin values in relation to longer performance tests conducted on hatchlings from differing nest temperatures. This will assist in understanding if differences seen in locomotor performance are due to hemoglobin values and if the increasing incubation temperatures due to climate change will impact these results.

Temperature effects on stage of development at death

Eggs with little to no development were found more frequently in May, while more full-term embryos were found in June and July. There was a low percentage of unhatched eggs with embryos in the middle stages of development across all three months. It should be noted, however, that our study contained a low percentage of unhatched eggs (n = 242 unhatched eggs / 1620 total eggs = 14.94%). The low percentage of eggs containing embryos in the middle stages of development found in this study has also been documented in other sea turtle species and populations (Rafferty and Reina 2014; Whitmore and Dutton 1985). Leatherback eggs incubating at similar temperatures (29–31°C) as the recorded mean for our May loggerhead nests (29.84°C), were found to be primarily in the early stages, similar to the findings from this study (Bell et al. 2004). Bladow (2017) discovered that the majority of loggerhead and green turtle embryos in Boca Raton, Florida, died in later stages of development. This could support our findings for the months of June and July; however, Bladow (2017) did not provide mean incubation temperatures, nor the months in which the clutch was deposited,

making comparisons to this study difficult. However, incubation temperature was observed to play a significant role in embryonic death, with the percentage of time spent above a set temperature (34°C, 34.5°C, and 35°C) being the primary cause of embryonic death (Bladow 2017).

The higher percentage of unhatched eggs at full-term in June and July could suggest incubation temperatures that are within normal limits during the majority of incubation; however, embryonic death might occur due to increased air temperatures, resulting from prolonged exposure above lethal thermal tolerance limits or a spike above lethal thermal tolerance limits due to metabolic heating. This could explain the number of full-term embryos that had not pipped. Further studies are needed to determine the relationship between embryonic death patterns and nest temperatures. This information would be useful in determining if the number of dead embryos at full-term increase with increasing metabolic heat and whether this correlates to the amount of eggs in the nest. If so, possible management plans could include splitting up nests to reduce the potential of full-term embryonic death.

Conclusions

Climate is changing worldwide, and sea turtles are particularly vulnerable. In the face of climate change, the fundamental information provided in this study could be vital in helping to formulate effective management plans for loggerhead sea turtles in Florida. Sand temperatures during nesting season are projected to increase 0.57–5.68°C by 2030 and 1.05–6.69°C by 2070 for rookeries in northern Australia (Fuentes et al. 2009). An additional study predicting sand temperatures in Australia found that average yearly sand

temperatures are predicted to rise between 2.17 and 3.34°C over the next 60 years (Cavallo et al. 2015). The anticipated increase of 2.17–3.34°C is forecasted to decrease yolk-free hatchling mass by 6–12%, power-stroke rate by 15–40%, and swimming speed by 15–38%, under conservative emissions (Cavallo et al. 2015). The impacts of this decline in quality will have substantial impacts on sea turtle hatchlings.

In addition, feminization of green turtles is already occurring in Australia (Jensen et al. 2018). The cooler nesting beaches have a 65–69% female bias and warmer beaches are almost completely feminized (Jensen et al. 2018). If these predictions for Australia hold true, many nests might also reach the lethal incubation limit resulting in egg mortality (Cavallo et al. 2015). A similar study modeling future climate change scenarios, found that with an increase of 2°C, complete feminization would occur in the loggerhead population at Cape Canaveral, Florida, with an increase of 3°C resulting in nests with an incubation temperature above the lethal limit (Hawkes et al. 2007).

Climate change is likely to progress at a faster pace than sea turtles can evolve (Janzen 1994). Since adaptation will be slow and insufficient in mitigating the increasing temperatures, it will be up to us to develop and implement management strategies to ensure sea turtle species survive. If global temperatures rise by the predicted 0.5°C, and nest temperatures follow suit, climate change management plans need to include a strategy to decrease incubation temperatures of loggerhead hatchling nests, such as shade protection. The combined use of shade cloths and trees as shade protection have been found to lower mean nest incubation temperature by 1.9°C (Wood et al. 2014).

Increasing incubation temperature is just one aspect of climate change that will influence nesting beach habitats and the survivability of sea turtles. Additional threats

include sea level rise, an increase in hurricanes, and changes in precipitation, none of which will help facilitate the recovery of this already vulnerable species. As these impacts become greater, management strategies will need to use an integrative approach of mitigation measures in order to address these concerns and to protect not only sea turtles and their nests, but also the beaches their vulnerable embryos rely upon.

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