DIFFERENCES IN RESOURCE ALLOCATION BETWEEN POST-MATING MALE AND FEMALE OLIVE RIDLEY SEA TURTLES (*LEPIDOCHELYS OLIVACEA*)

An Undergraduate Research Scholars Thesis

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ABSTRACT

Differences in Resource Allocation Between Post-Mating Male and Female Olive Ridley Sea Turtles (*Lepidochelys olivacea*)

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Olive ridley sea turtles (*Lepidochelys olivacea*) are a vulnerable species. During their reproductive season these turtles migrate great distances to mate and lay eggs. Poor nutrition before this migration can result in fitness costs including skipped breeding years, longer inter-nesting intervals, and fewer eggs, thus hindering species recovery. Recovery could be facilitated by better information on nutrition in wild populations. Subcutaneous fat ultrasonography and blood β-hydroxybutyrate measurement are considerably less invasive than traditional techniques to evaluate nutritional state, and have provided useful results in other sea turtle species, but have not been applied to olive ridleys. This study aimed to evaluate if these methods are effective for determining possible changes in fat storage and mobilization in reproducing olive ridleys. Both subcutaneous fat and blood β-hydroxybutyrate were measurable in samples collected from a nesting turtle population in Costa Rica, thus showing promise of these techniques in nutritional assessment of wild olive ridleys.

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NOMENCLATURE

BHB	β-hydroxybutyrate
SubQ	Subcutaneous
FCF	Fulton's Condition Factor
CCL	Curved Carapace Length
CCW	Curved Carapace Width
CV	Coefficient of Variation
F	Follicles
FAF	Follicles and Atretic Follicles
EF	Eggs and Follicles
EFAF	Eggs, Follicles, and Atretic Follicles
EAF	Eggs and Atretic Follicles

CHAPTER I

INTRODUCTION

Olive ridley sea turtles are a vulnerable species according to the IUCN Red List (Abreu-Grobois and Plotkin, 2012). Their population decline is partially due to habitat loss, which can lead to a decrease in food availability. Poor nutrition in sea turtles has resulted in reproductive consequences such as skipped breeding years, longer nesting intervals, and fewer laid eggs (Bjorndal, 1982; Broderick et al., 2001; Hatase and Tsukamoto, 2008; Solow et al., 2002). If undernourished turtles are producing fewer eggs, hatching success will decline, making species recovery more difficult. It would therefore be helpful to be able to assess nutrient storage and utilization to better understand how turtles fuel reproduction, an energetically expensive physiological process.

The general explanation for how sea turtles nutritionally support reproductive costs is that they are capital breeders (Miller, 1997). Capital breeders build up energy reserves at foraging grounds and then cease feeding before migration from feeding grounds to nesting beaches where reproductive effort is fueled by fat stores (Plot et al., 2013). This breeding model is controversial, however, as some studies have shown that there is feeding behavior during the reproductive season (Casey et al., 2010; Hochscheid et al., 1999; Schoefield et al., 2006). The question of the nutritional condition of nesting turtles has historically been difficult to address since sea turtles are migratory and recapturing them is difficult. Additionally, traditional methods for assessing nutrient intake in wild turtles have serious limitations.

Stomach flushing is the oldest and most widely used non-lethal technique to study nutrient consumption. Forbes and Limpus (1993) developed this methodology and it has been

effective in five sea turtle species with no observed ill effects or deaths. The advantages of this technique are that it takes less than ten minutes, is rarely unsuccessful, is relatively inexpensive, and can be used in field conditions (Forbes and Limpus, 1993). Although a great alternative to lethal techniques, it is still highly invasive and is not without risks. It consists of prying a turtle's mouth open, followed by inserting a lavage and retrieval tube down the esophagus (Forbes and Limpus, 1993). Salt water is then pumped through the lavage tube to force the stomach contents through the retrieval tube (Forbes and Limpus, 1993). This is a highly invasive process that can be damaging to the turtle. Forbes and Limpus (1993) also point out that the interpretation of the significance of the results should be done with care since the results are dependent on the size of the retrieval tube, the size of diet components in the anterior digestive tract, and the experience of the person performing the procedure. Further limitations of this technique are it only provides information on what was eaten immediately before capture (Forbes and Limpus, 1993). It does not give information about long-term access to nutrition, i.e. if a turtle has consistently been feeding. There is also a high bias of false negatives (showing that the turtle has not recently fed when it actually has) when food might be farther down the gut (Forbes and Limpus, 1993). In an effort to develop less potentially damaging means to assess sea turtle nutritional state I have evaluated the feasibility of two less invasive techniques in wild olive ridleys sea turtles: ultrasonography and ketone body analysis.

Ultrasonography has been used in leatherback sea turtles (*Dermochelys coriacea*) to measure the subcutaneous (SubQ) fat (Harris et al., 2016). This is the tissue layer below the epidermis and dermis where fat is stored. If a turtle is not eating, this layer will thin as fat is mobilized from this depot into the blood and distributed to other tissues for energy. Turtles that are well nourished (i.e. eating consistently) should have thicker SubQ fat than turtles that are not

(Harris et al., 2016). Therefore, SubQ fat measurement may provide information about a turtle's long-term access to food. Since this technique has been validated with necropsy in leatherbacks, I propose that SubQ fat ultrasonography will also provide information about nutrient storage in olive ridleys. Ultrasonography may also have application for evaluation of body condition scores based on animal mass and length, such as the Fulton's Condition Factor (FCF). These scores have been widely used in fisheries and sea turtle studies to estimate nutritional health of organisms (Froese, 2006; Labrada-Martagon et al., 2010); however, they have limitations. These morphometrics do not differentiate between fat, muscle, and reproductive tissue, and thus do not accurately reflect nutrient storage in depots such as SubQ fat. Furthermore, whereas these indices have been used successfully for a variety of fish, sea turtles present a unique challenge because they are encased in shells that do not flex with fat gain or loss. Therefore, I will also compare SubQ fat layer depth to these body condition scores to evaluate how representative these scores are when estimating nutritional status.

Blood β-hydroxybutyrate (BHB) measurement is another less invasive technique that has been used to study nutrient mobilization in green sea turtles (*Chelonia mydas*). BHB is a ketone body that is produced by the liver during fasting as an alternate energy source to glucose (Price et al., 2013). Price et al. (2013) conducted a feeding study in captive juvenile green sea turtles and concluded that BHB was an effective indicator of fasting status since it increases rapidly during fasting. They also concluded that serum triglycerides might be a good indicator of fasting; however, plasma triglycerides are elevated in reproductively active sea turtles (Hamann et al., 2002b), so this may limit the use of this metabolite as a feeding marker. Therefore, blood BHB may be a more reliable index of fasting behavior in reproductively active sea turtles, and I propose that it could serve as a fasting indicator in other sea turtle species as well. I am applying

this method in a novel way by evaluating its use as a potential indicator of nutritional status in olive ridley turtles in field conditions - a non-controlled environment.

I propose to apply these techniques to olive ridleys because they are the most abundant sea turtle species with an estimated global population of 800,000 individuals (Abreu-Grobois and Plotkin, 2012). This species also reproduces year-round and returns annually to nest, unlike many other sea turtle species. Furthermore, this species mass nests in monthly nesting events called *arribadas*, thus making it possible to catch post-mating couples and recapture nesting females throughout the season. Extensive sampling, including repeat sampling of individual females, can therefore be achieved at locations such as Ostional, Costa Rica, a mass breeding and nesting beach. The objectives of this project are therefore to determine whether SubQ fat and blood BHB measurement are potentially effective methods for determining whether there are changes in fat storage and mobilization in female olive ridleys associated with reproductive activity. Currently there are no sea turtle prey monitoring or management conservation policies in Costa Rica, which hosts two mass nesting beaches and several solitary nesting beaches. If successful, this research will contribute to the better understanding of the nutritional state of these animals in the wild, thus potentially resulting in new conservation policies that can help sustain these sea turtle populations.

These techniques could also be used to elucidate physiological differences in feeding versus fasting between males and females. Understanding differences in fat storage and utilization between sexes is needed to increase understanding of the differences of energetic and nutritional needs to maximize reproductive output of this species. Female sea turtles are expected to be capital breeders who fast during reproduction (Miller, 1997). During migration to mating and nesting beaches, females produce unfertilized oocytes surrounded by follicle cells called

follicles (Owens, 1980). During ovulation, these unfertilized oocytes leave the follicle for the oviduct where they begin the first few stages of development (Owens, 1980). The oocytes are then fertilized by sperm during mating in the anterior portion of the oviduct and are then coated by albumen and a calcium carbonate shell resulting in shelled eggs (Owens, 1980). These shelled eggs travel to the distal end of the oviduct waiting to be laid (Owens, 1980).

Mating typically occurs in a distinct period prior to a female's first nest of the season (Erhart, 1982; Owens and Morris, 1985; Limpus, 1993). One mating event is often sufficient to fertilize all of a female's clutches for a nesting season (Pearse and Avise, 2001; Hamann et al., 2003). However, sea turtles are promiscuous (Miller, 1997; Hamann et al., 2003) and olive ridleys nesting at Ostional have the highest observed multiple paternities of any sea turtle species (Jensen et al., 2006). Therefore, during this mating period, a female may mate with several different males. At nesting beaches, females lay multiple clutches over a four-month period either during monthly mass-nesting events or solitarily. These clutches are fertilized with sperm retained in the oviduct from mating earlier in the reproductive season (Owens, 1980; Plotkin, 2007). After a female has laid her last nest for the season, the remaining unfertilized oocytes are absorbed by the female's body and are called atretic follicles (Owens, 1980).

Males are not as commonly studied as females, but they are not expected to be fasting during migration. Therefore, females are expected to have higher BHB levels than males at arrival to nesting beaches and post-mating (i.e. immediately after mating). Fasting turtles should also have a lower FCF since they weigh less than turtles that have been consistently eating; therefore, females are also expected to have lower FCFs than males. Since egg and sperm production occur during migration and eggs are more energetically expensive to produce (Miller,

1997), females are expected to mobilize more fat and have thinner SubQ fat than males at post-mating.

CHAPTER II

METHODS

Field studies were conducted at Ostional National Wildlife Refuge in Ostional, Costa Rica from June to September in 2016 through 2018. Ostional Beach is one of two mass mating and nesting sites for olive ridley sea turtles in Costa Rica where hundreds to thousands of olive ridleys migrate to mate and nest. Females nest two to three times a year and nesting occurs year-round. Once a month, nesting occurs synchronously in mass-nesting events called *arribadas*.

Sample Collection

All sampling was covered under a 3-year CONAGEBIO permit, number R-029-2016-OT-CONAGEBIO as well as a 3-year TAMU IACUC approval number 2016-0169. Samples were imported to the United States under CITES permit number 18US78340C/9. Adult olive ridleys were initially captured in the water immediately after mating, designated post-mating. The curved carapace length (CCL) and width (CCW), and total circumference were measured. Mass was measured with a handmade harness and a CAMRY 100 kg hanging scale. Ultrasound images of the SubQ fat and blood samples were taken within 5 minutes for males and 25 minutes for females (for additional details see "Ultrasonography" and "Blood Sampling and β-hydroxybutyrate Measurement" below). Gonadal ultrasounds were also taken of females (for additional details see "Ultrasonography" below). The third scale of the front left and right flipper was disinfected with ethanol and then a unique Inconel metal tag was applied to later identify and re-sample females during the *arribadas.*

During the *arribadas*, any nesting females with our tags were recaptured and resampled. A second blood sample was taken and a second SubQ fat and gonadal ultrasound was collected.

Ultrasonography

Ultrasound images of the SubQ fat were taken in the dorsal shoulder region using a Sonosite 180+ with a 2.0 MHz transducer in 2016 and 2017, and a Wristscan V9 version SW201001E01 with a 5.0 MHz transducer in 2018 as described in Harris et al. (2016) (Figure 1). Three images of this region were taken per turtle using the shoulder joint as a landmark to keep the measurements consistent. Image J software was used to measure the SubQ fat in the sonograms (Figure 2). Three SubQ fat measurements were averaged to give the most representative measurement.

Figure 1. Ultrasonography of the SubQ fat taken in the dorsal shoulder region using a Wristscan V9 version SW201001E01 with a 5.0 MHz transducer.

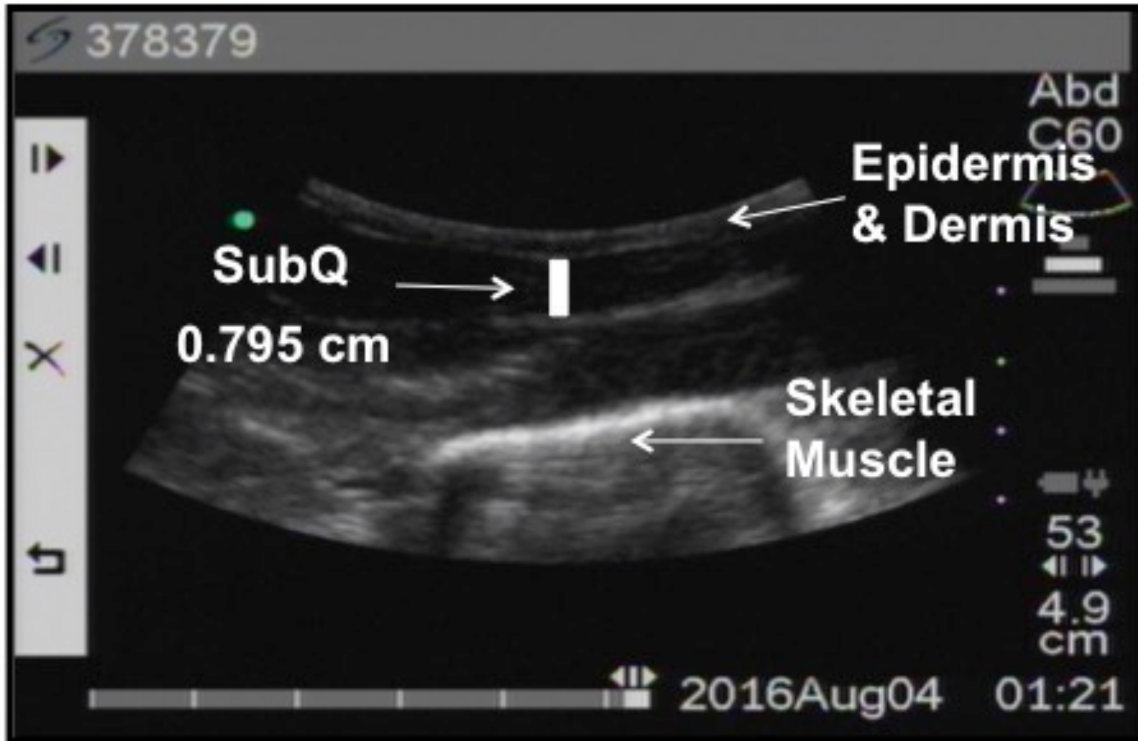


Figure 2. A representative sonogram of the dorsal shoulder region used for SubQ fat measurement. The SubQ fat measurement is indicated by the white line and is the anechoic (black) region between the hyperechoic (white) regions of the epidermis/dermis and the skeletal muscle.

To assess reproductive state, females were placed in dorsal recumbency in a car tire and gonadal ultrasounds were taken in the inguinal region as described in Rostal et al. (1990) (Figure 3). This technique was used to determine presence or absence of preovulatory or atretic ovarian follicles and oviductal shelled eggs, but was not used to determine exact counts of these structures because the turtle's plastron and carapace prevents penetration of the ultrasound signal to the entire reproductive (Harris et al., 2016). Males were not ultrasounded.

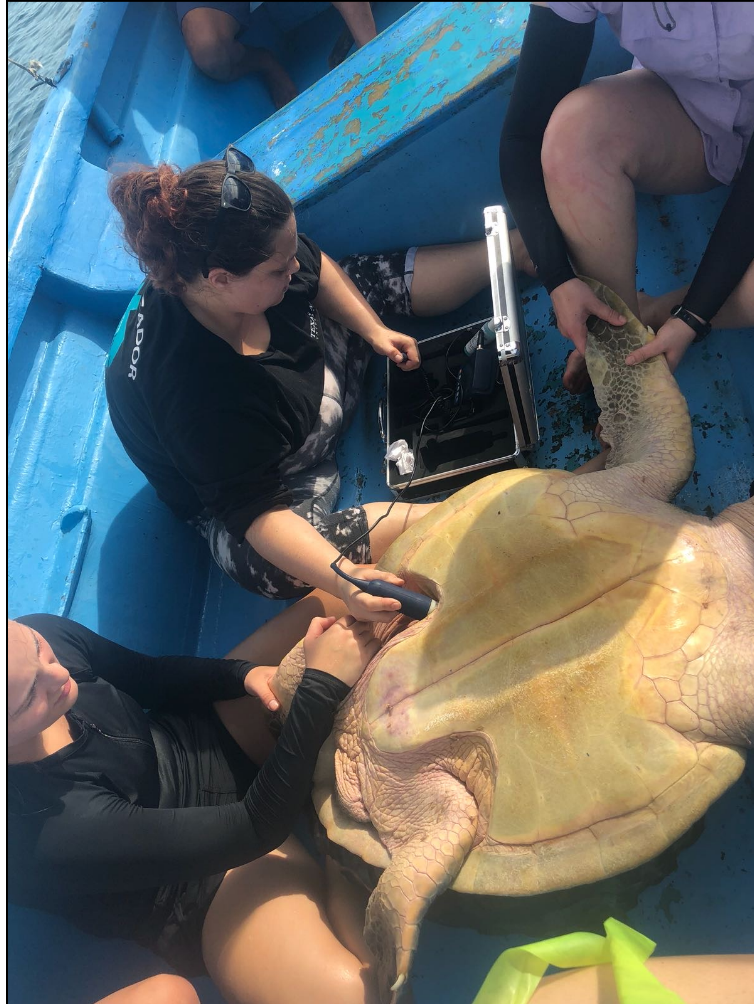


Figure 3. Ultrasonography of the gonads in the inguinal region of a female in dorsal recumbency. The female's head is to the right and her tail is to the left of the image.

Females were assigned categories based on reproductive structures visible in the gonadal sonograms (Figure 4). Females with mature ovarian follicles but no shelled eggs in the oviduct were assigned as "follicles only" (F); females with mature ovarian follicles and shelled eggs in the oviduct as "eggs and follicles" (EF); females with mature follicles, shelled eggs in the oviduct, and follicles in atresia as "eggs, follicles, and atretic follicles" (EFAF); and females with ovarian follicles and atretic follicles as "follicles and atretic follicles" (FAF) (Myre et al., 2016). Females with shelled eggs in the oviduct and atretic follicles were assigned "eggs and atretic

follicles" (EAF). Atresia of follicles resulting in atretic follicles occurs later in the nesting season (Owens 1980). Females with atretic follicles (the categories EFAF, FAF, EAF) were therefore further classified as late season females. Females without atretic follicles (F, EF) were further classified as early season females. EAF females were considered reproductively depleted, with all other reproductive categories considered reproductively gravid.

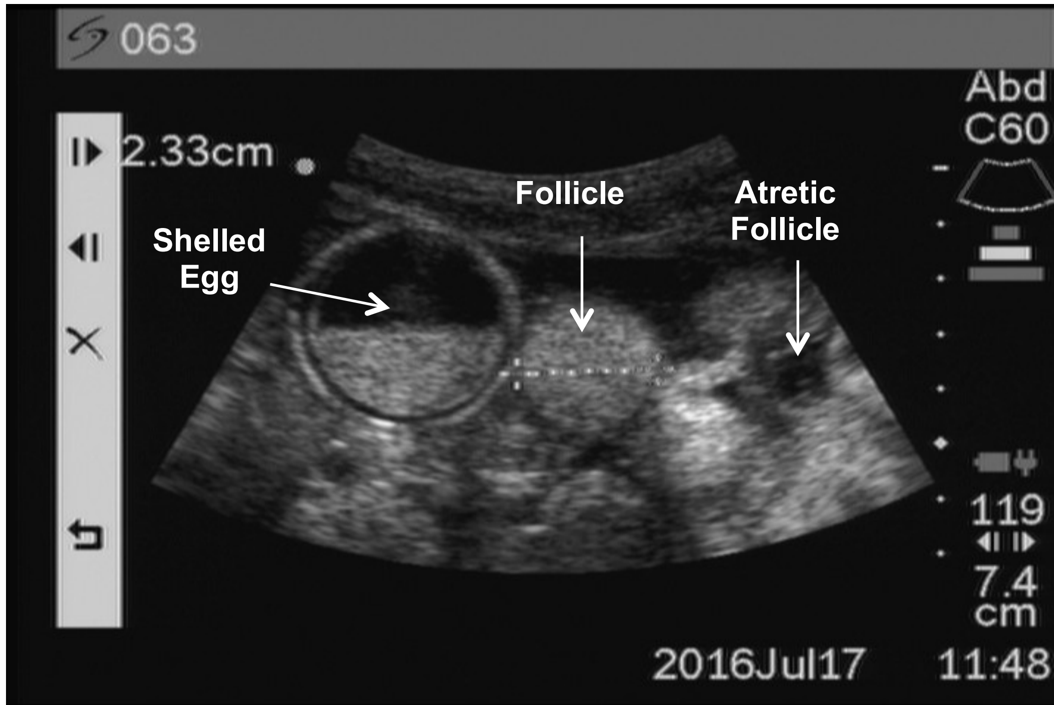
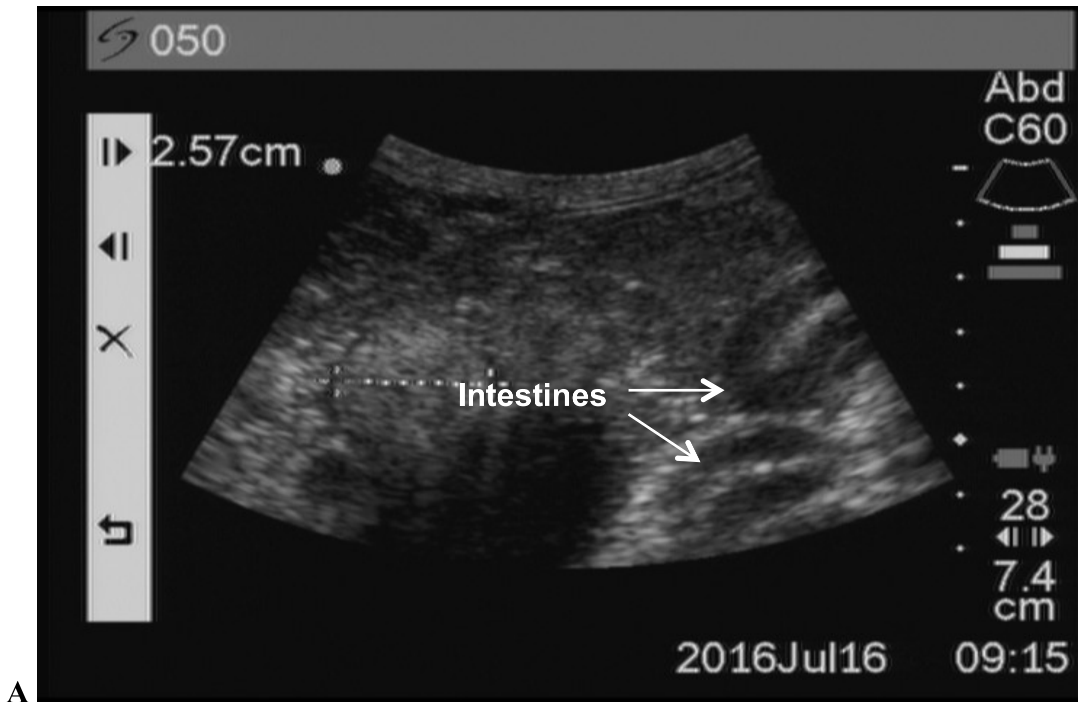


Figure 4. A gonadal ultrasound image depicting examples of shelled eggs in the oviduct, mature follicles, and atretic follicles. This turtle would be classified as EFAF and as a late season female.

Gonadal ultrasounds were also used to detect if intestinal contents were present. Intestines were only discernible in the sonograms if they were full (Valente et al., 2007) (Figure 5). Only the distal end of the intestine is visible due to conformation of the shell.



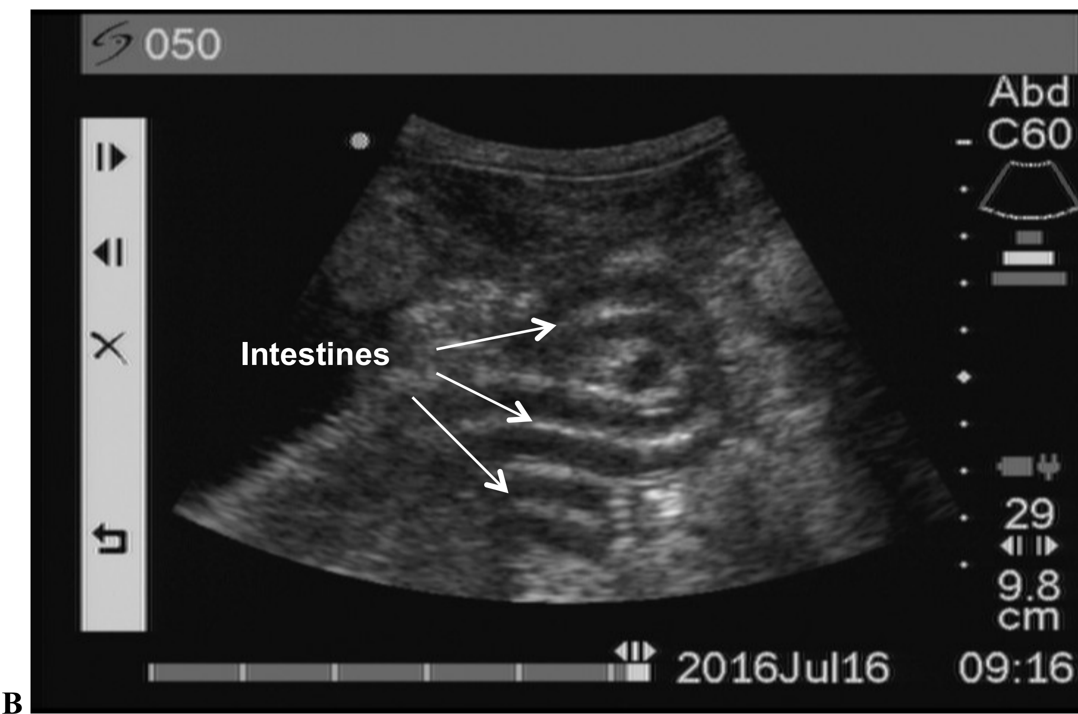


Figure 5. A gonadal sonogram with full intestines. The white arrows indicate the intestines. (A) Coronal view of the intestines. (B) Longitudinal view of the intestines.

Blood Sampling and β-hydroxybutyrate Measurement

Blood samples (10mL) were drawn from the dorsal cervical sinus from each animal after disinfecting with ethanol, using a 21-gauge 1.5-inch heparinized needle as described in Owens and Ruiz (1980) (Figure 6). All blood samples were placed immediately on ice and centrifuged at the conclusion of the sampling to separate out the plasma, which was stored in liquid nitrogen. Samples were transported in liquid nitrogen to *la Universidad de Costa Rica* and stored at -80ºC. Samples were later imported to the United States on dry ice, and then re-stored at -80ºC until analysis.

Figure 6. Blood sampling from the dorsal cervical sinus. The turtle's head is covered with a wet black cloth to keep the turtle calm. The head is also held extended to prevent injury.

Circulating blood BHB was measured with a commercial BHB kit (Sigma Aldrich) and read using a microplate spectrophotometer (Bio-TEK Synergy HT) at 450 nm. Undiluted serum

samples were out of the functional range of the standard curve, so a serial dilution of 1:1, 1:2, 1:4, 1:8, and 1:16 was performed. The 1:16 dilution was the only dilution within the functional range of the assay; therefore, serum samples were diluted to 1:16 with BHB buffer from the kit before being run, and true concentration was determined by multiplying the resulting values by the dilution factor, 16 in this case.

Statistical Analysis

Normality and homogeneity of the variance in the SubQ fat, BHB and FCF were tested using Microsoft Excel with males versus females and early versus late females used as the parameters. For each variable, the residuals for each parameter were graphed to test for homogeneity and a histogram was made for each parameter to determine a normal distribution. All parameters met the assumptions of normality and homogeneity, so data was analyzed with a series of two-way ANOVAs. Two-way ANOVAs included comparing means of males and females for BHB, then SubQ fat and FCF. Next, the same series of ANOVAs was run contrasting early versus late season females. Additionally, an R^2 value was calculated using linear regression to determine the correlation of SubQ fat and FCF. Lastly, a two-sample t-test was run assuming unequal variance for visible intestines and BHB.

CHAPTER III

RESULTS

Tables 1, 2, and 3, summarize mean BHB, SubQ fat, and FCF for each turtle classification, as well as the number of turtles in each classification with visible intestines. Females were first classified based on present reproductive structures to distinguish differences in SubQ fat, FCF, and BHB (Table 1). However, it was not possible to run ANOVAs across female reproductive category due to insufficient sample size in some ultrasound categories. Therefore, females were alternatively classified as early and late season females for analysis (Table 2). Next, an ANOVA comparing females and males was conducted to determine differences in SubQ fat, FCF, and BHB based on sex (Table 3). In Table 1, F females were the largest category with EAF females as the smallest category, indicating that depleted females are less likely to be mating than gravid females.

Table 1.

Turtle Category	**BHB N**	**Mean BHB**	**SubQ & FCF N**	**Mean SubQ Fat**	**Mean FCF**	**Visible Intestines**
F	9	92.25 ± 43.14	26	1.00 ± 0.47	1.36 ± 0.08	15
FAF	9	86.96 ± 27.18	12	0.79 ± 0.40	1.32 ± 0.10	12
EF	3	121.25 ± 46.54	7	1.09 ± 0.50	1.37 ± 0.09	1
EFAF	3	79.62 ± 28.73	3	0.82 ± 0.46	1.35 ± 0.11	1
EAF	2	63.21 ± 51.69	2	1.17 ± 0.64	1.28 ± 0.20	1

Females classified based on present ovarian reproductive structures. Follicles denoted as (F), atretic follicles (AF), and shelled eggs (E). Mean (± standard error) values for β-hydroxybutyrate (BHB), subcutaneous (SubQ) fat, and Fulton's Condition Factor (FCF) analyses.

Table 2.

Turtle Category	BHB N	Mean BHB	SubQ & FCF N	Mean SubQ Fat	Mean FCF	Visible Intestines
Early Season Females	12	99.50 ± 43.81	33	1.02 ± 0.47	1.36 ± 0.08	16
Late Season Females	14	82.00 ± 29.32	17	0.84 ± 0.42	1.32 ± 0.10	14

Females alternatively classified based on whether they are early or late in their reproductive season based on present ovarian structures. Abbreviations are the same as in Table 1. These turtles were used for the BHB versus visible intestines ttest.

Table 3.

Turtle Category	BHB N	Mean BHB	SubQ & FCF N	Mean SubQ Fat	Mean FCF	Visible Intestines
Total Females	26	90.08 ± 37.02	50	0.96 ± 0.46	1.35 ± 0.09	30
Males	16	99.61 ± 30.84	32	0.84 ± 0.41	1.17 ± 0.11	Not Determined

Total females and males mean (± standard error) values for β-hydroxybutyrate (BHB), subcutaneous (SubQ) fat, and Fulton's Condition Factor (FCF) analyses to determine differences between sexes.

Tables 4, 5, and 6 follow the same classifications as Tables 1,2, and 3, but summarize the morphometric means for each category. As can be seen in the tables, none of the morphometric data differed largely between classifications.

Table 4.

Turtle Category	N	Mean CCL	Mean CCW	Mean Circ	Mean Mass
F	26	67.21 ± 2.83	71.58 ± 2.85	129.57 ± 4.10	41.46 ± 4.40
FAF	12	67.38 ± 3.60	70.78 ± 3.25	128.27 ± 6.16	40.38 ± 4.86
EF	7	67.31 ± 2.81	72.59 ± 3.09	131.31 ± 4.40	42.01 ± 6.98
EFAF	3	68.57 ± 2.48	71.87 ± 2.75	129.73 ± 4.90	43.33 ± 2.84
EAF	2	66.65 ± 0.07	71.20 ± 1.13	126.15 ± 1.34	38.00 ± 5.66

Mean (± standard error) values for morphometric data of females classified based on present ovarian structures. Turtle category abbreviations same as in Table 1. Curved carapace length (CCL), curved carapace width (CCW), and total turtle circumference (Circ).

Table 5.

Turtle Category	N	Mean CCL	Mean CCW	Mean Circ	Mean Mass
Early Season Females	33	67.23 ± 2.78	71.79 ± 2.89	129.94 ± 4.16	41.58 ± 4.93
Late Season Females	17	67.50 ± 3.16	71.02 ± 2.91	128.28 ± 5.49	40.62 ± 4.64

Mean (± standard error) values for morphometric data of females alternatively classified based on whether they are early or late in their reproductive season according to present ovarian structures. Abbreviations are the same as in Table 4.

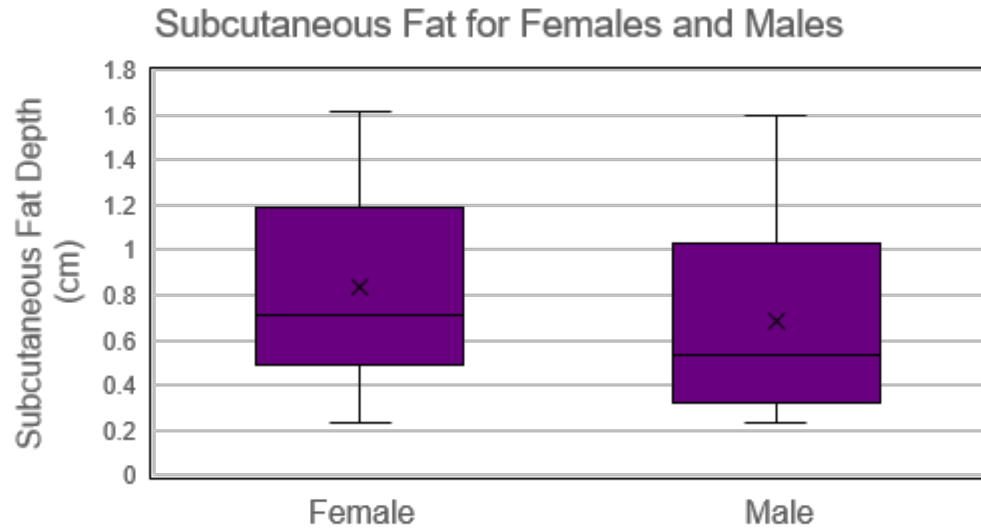
Table 6.

Turtle Category	N	Mean CCL	Mean CCW	Mean Circ	Mean Mass
Total Females	50	67.32 ± 2.89	71.53 ± 2.89	129.37 ± 4.67	41.25 ± 4.81
Males	32	64.93 ± 2.01	68.41 ± 2.23	124.74 ± 4.24	32.11 ± 3.92

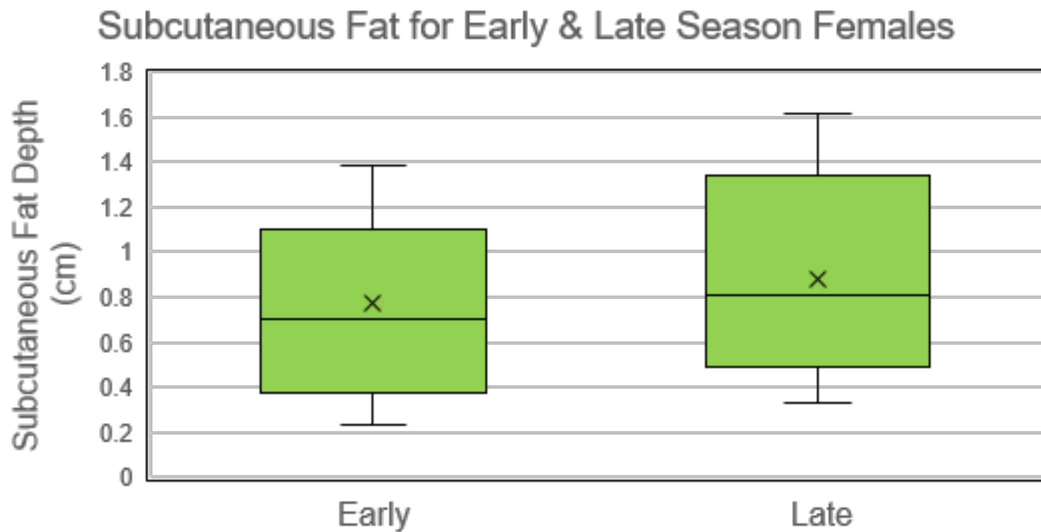
Mean (± standard error) values for morphometric data of total females and males. Abbreviations are the same as in Table 4.

Ultrasonography and Body Condition

Every gonadal ultrasound category was observed except for females with no follicles, atretic follicles, or shelled eggs. Ultrasonography showed easily discernable and measurable SubQ fat in all categories (Tables 1, 2, and 3). SubQ fat did not differ significantly between sexes or in early versus late season females (Tables 2 and 3, Figure7).



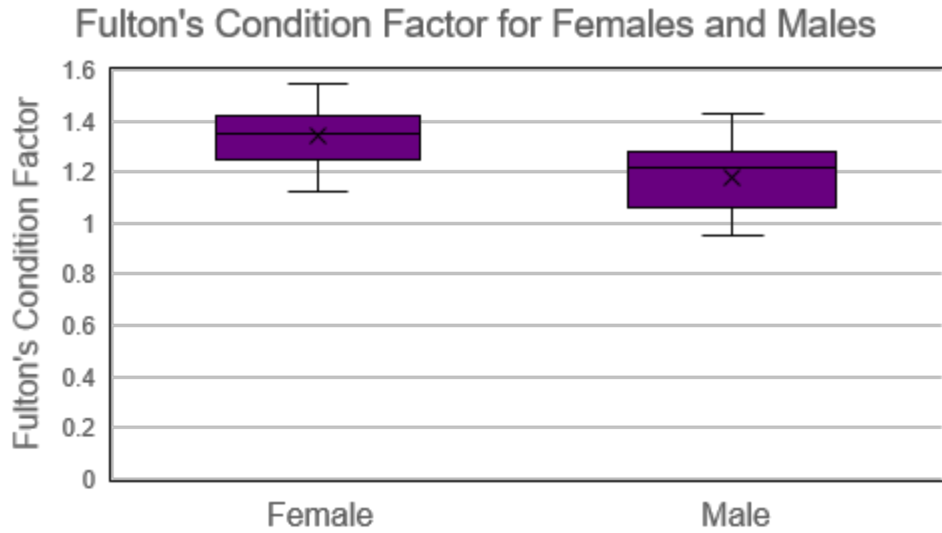
A



B

Figure 7. No significant difference in SubQ fat was found between (A) sexes, or (B) early and late season females at post-mating. Error bars represent ± standard error.

Differences in FCF were also tested between sexes and early versus late season females (Tables 2, 3). FCF fat did not differ significantly between early versus late season females or in sexes (Figure 8). Furthermore, no significant correlation between SubQ fat and FCF was found (R^2=0.005, P=0.6).



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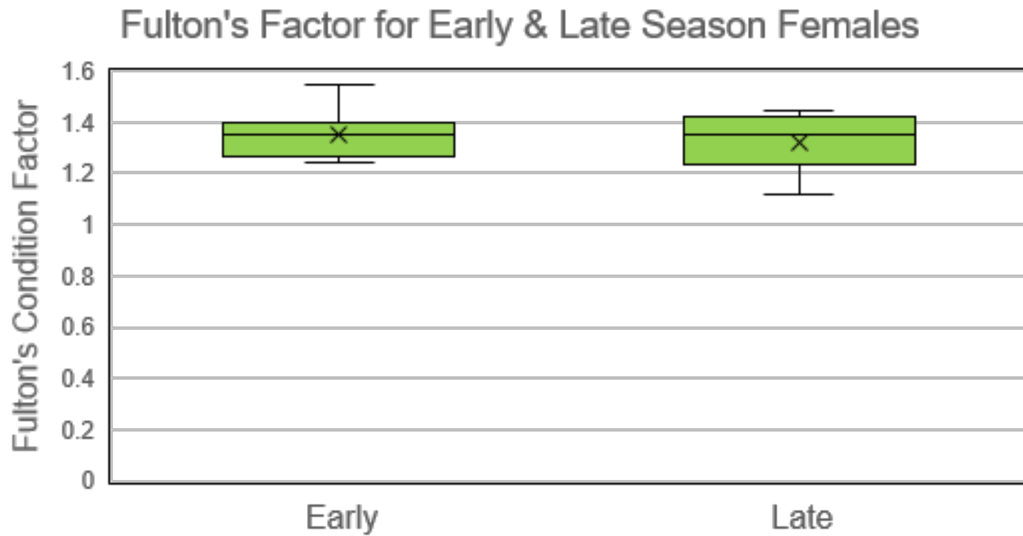


B

Figure 8. No significant difference in FCF was found between (A) sexes, or (B) early and late season females at post-mating. Error bars represent ± standard error.

β-hydroxybutyrate Measurement

A standard curve was first generated to determine the functional range of the BHB assay. The curve was measured twice, an hour apart, to evaluate the stability of the colorimetric change (Figure 9). The functional range was determined to be 0-10 mM, and the assay was stable over this time period (Figure 9).

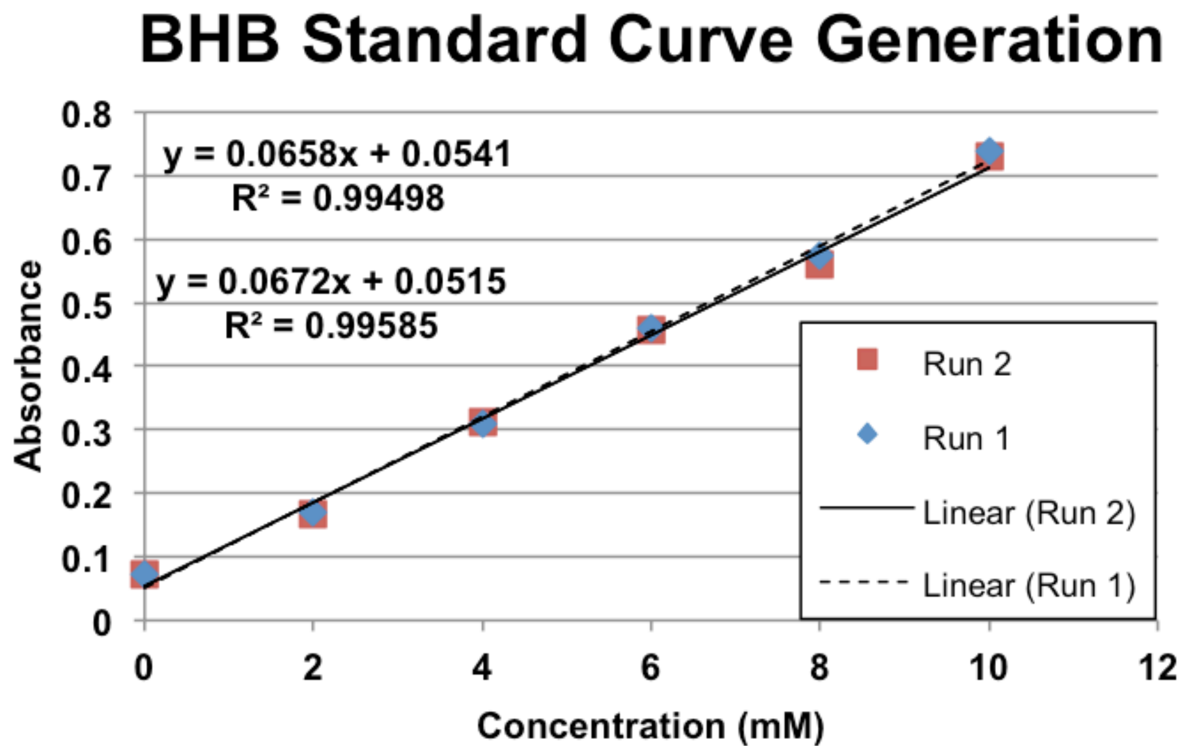


Figure 9. Standard curves for first BHB assay showing that the colorimetric change of the assay was stable over an hour time period.

A range finding assay was then conducted to determine if blood samples needed to be diluted before assay. Undiluted blood BHB concentrations were outside the standard curve, but a 1:16 dilution gave concentrations within the functional range of the assay (Figures 10 and 11). Another BHB assay was then run with blood samples diluted 1:16. BHB was then measurable in all samples (Figure 12).

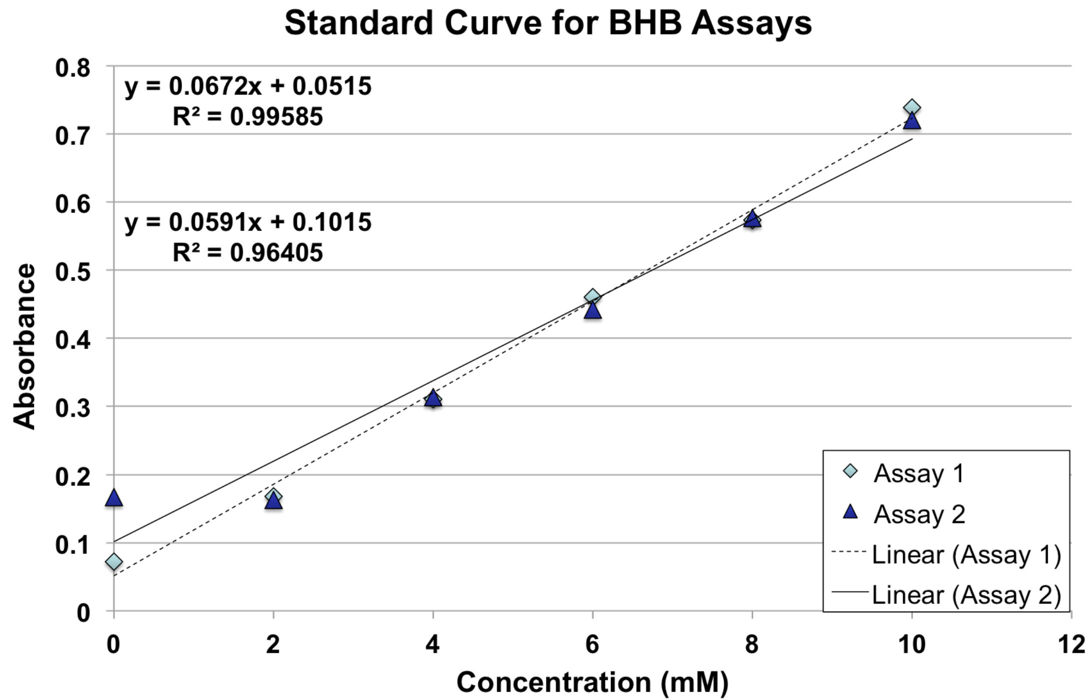


Figure 10. Standard Curves for the first and second BHB assays. The functional range of the standard curve is 0-10 mM.

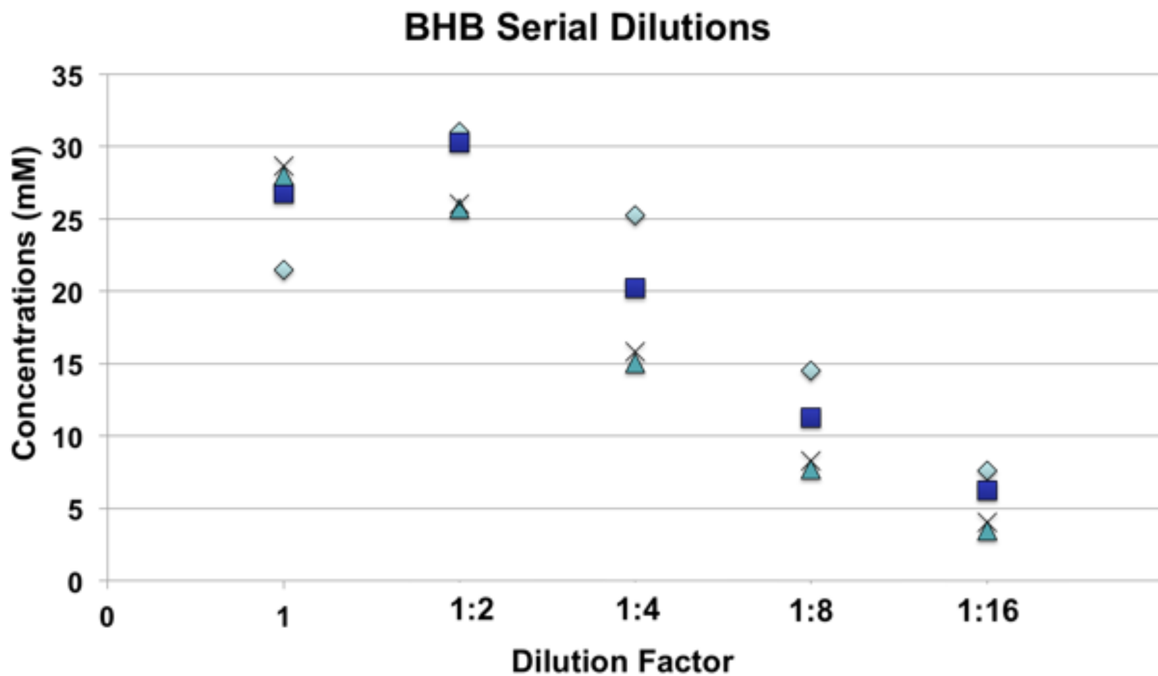


Figure 11. Serial dilutions of four plasma samples show that a 1:16 dilution is needed to bring samples into the functional range of the standard curve.

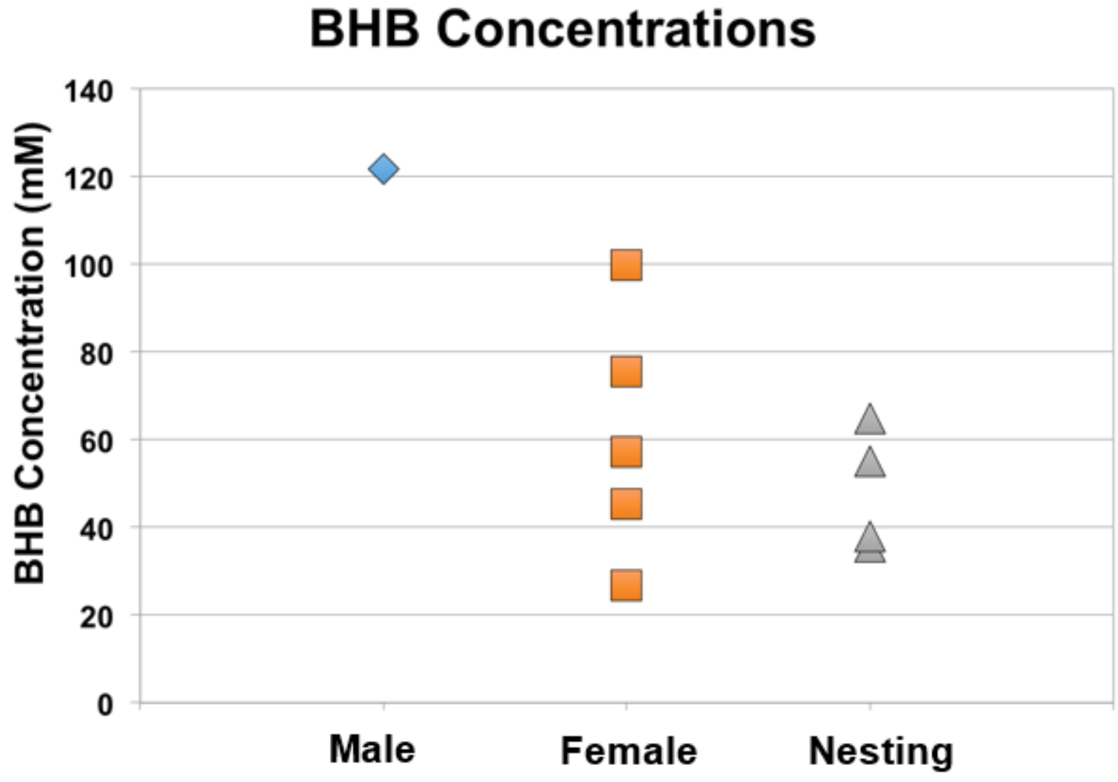
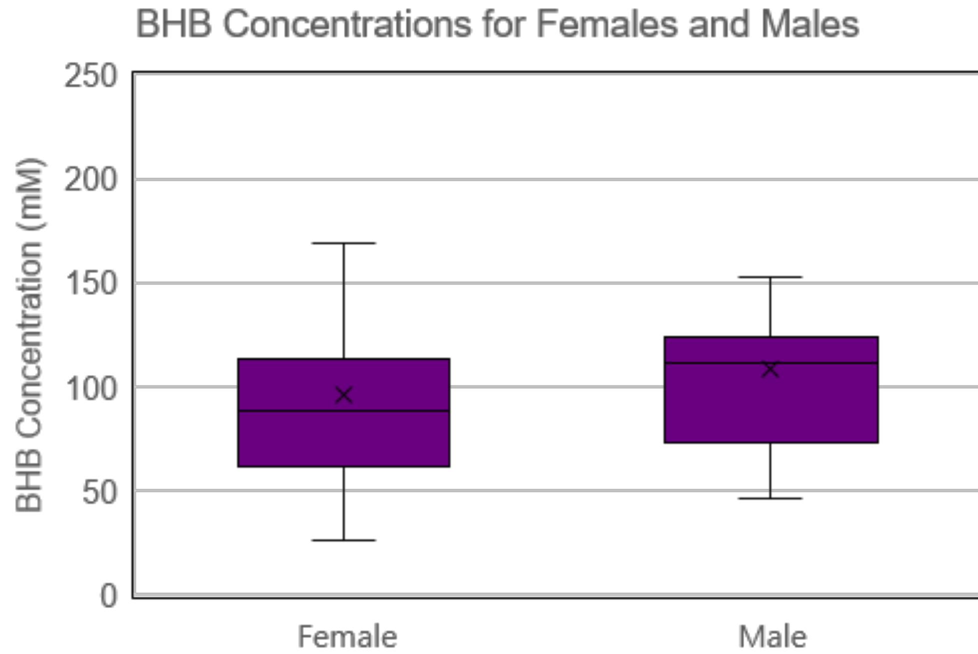


Figure 12. BHB concentrations for ten turtles diluted to 1:16 in the second BHB assay. All had BHB concentrations within the detectable range of the assay.

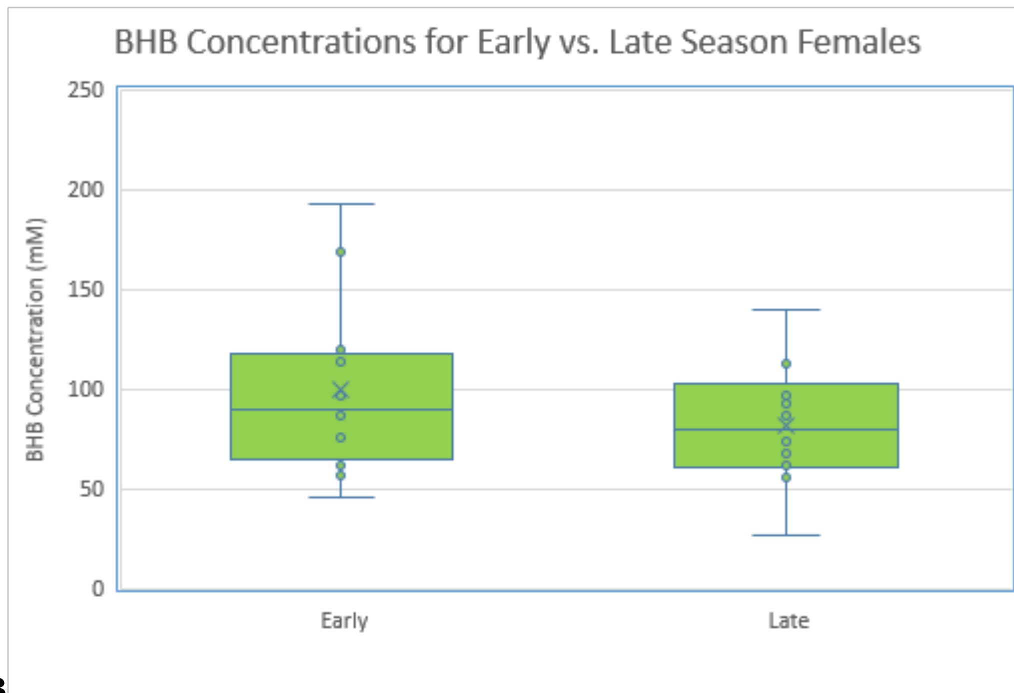
A third BHB assay was run to compare BHB between sexes and female reproductive categories, as well as to determine if BHB concentrations between assay plates could be used in the same comparison. One male and one female blood sample that had been run in the previous assay were used to calculate the inter-assay coefficient of variation (CV). These controls were plated at the beginning and end of the third assay plate to test for intra-assay variation. The male intra-assay CV was 4% and the female was 2.5%. The CV for the inter-assay male control was 8% and was 33% for the female control. Therefore, BHB concentrations could be compared across plates.

There was one F female and one male sample in addition to the samples in Tables 1, 2, and 3 that were not included in the BHB analyses because they were above the functional range of the standard curve. It was not possible to run ANOVAs across female reproductive category

due to insufficient sample size across ultrasound category; therefore, females were classified as early and late season females (Table 2). There was no significant difference in BHB either by sex or in early versus late season categories (Tables 2 and 3, Figure 13). Furthermore, BHB and visible intestines were compared to determine if BHB varies by intestinal state. There was a significant difference ($P<0.001$) with no visible intestines having a BHB average of 98.3 and visible intestines having a BHB average of 85.7.



A



B

Figure 13. No significant difference in BHB was found between (A) sexes, or (B) early and late season females. Error bars represent ± standard error.

CHAPTER IV

CONCLUSION

The results of this study indicate that SubQ fat and blood BHB are easily detectable in wild olive ridleys. Therefore, ultrasonography and blood BHB ketone body analysis should be practical techniques for further evaluation. These techniques have been used successfully in other sea turtle species (Harris et al., 2016; Price et al., 2013) and this study expanded their potential use in wild olive ridleys.

BHB concentrations had only previously been measured in captive green sea turtles (Price et al., 2013). We tested the applicability of this technique to wild sea turtles for the first time and found that BHB was measurable in all categories. Not only was it measurable, but also BHB was sufficiently elevated that blood samples had to be diluted for analysis. Furthermore, our BHB levels were approximately 30-fold higher than those in Price et al. (2013). A primary reason for this difference could be that their study was done with captive juvenile turtles and ours was with wild adults, or because of species differences. Nutritional status of wild animals can differ substantially from that of captive animals fed a defined diet at regular intervals.

Ultrasonography of subcutaneous fat is another technique that has only been previously applied in a single sea turtle study, using leatherbacks (Harris et al., 2016). Our study was the first to apply this technique to wild olive ridleys and found that SubQ fat was also easily measurable in this species. However, we were unable to confirm by necropsy, as was done in that previous study, that the anechoic layer we observed was indeed SubQ fat. In future studies, SubQ fat ultrasonography measurements will need to be validated by comparing fat layers in

dead olive ridleys at necropsy to ultrasonographic measurement of SubQ fat in the dorsal shoulder region (Harris et al., 2016) to confirm the validity of this measurement.

The finding that FCF and SubQ fat were not correlated brings into light the shortcomings of current body condition estimates. FCF does not discriminate between reproductive tissues, muscle, and fat storage. A further limitation of FCF is that it relies on length of the carapace or shell. Shell morphology does not change with fat gain and loss, so ultrasonography should be better able to measure fat fluctuations. Ultrasonography of the SubQ fat is also more informative because it allows for measurement of nutrient storage (Harris et al., 2016). Furthermore, SubQ fat may be used in conjunction with carapace length to create a new, more informative body condition score. Having a body condition score that encompasses fat mobilization would be useful for these organisms because they are thought to be mobilizing large amounts of fat to energetically support reproductive costs. My results suggest that once validated, ultrasonography would be an effective technique to measure this fat mobilization.

In addition to being the first study to assess the practical applicability of ultrasonography and blood BHB measurement in wild conditions, this is the first study to simultaneously apply ultrasonography of SubQ fat, FCF, and BHB in in sea turtles. These techniques appear to provide practical alternatives to stomach flushing to assess feeding status. I found that they can be used for a large number of animals under wild conditions, on the beach or in the water, just like the current method of stomach flushing, but are significantly less invasive and less harmful to sea turtles.

This study also found that females of all reproductive categories, except for females with no reproductive structures, were identified in our post-mating sample. Females with shelled eggs and atretic follicles were not expected to be mating since females primarily mate before their

first clutch is ovulated, before shelled eggs and atretic follicles are formed (Owens, 1980). These females may have been mating because they were fatigued from nesting and could not dissuade aggressive males (Owens, 1980). F females were the most numerous category of females sampled, which matched expectations since they are in the recognized mating period (Owens, 1980). EAF females were the smallest category sampled indicating that depleted females are mating less then gravid females. Fortunately for this study, these late season females allowed for a comparison in SubQ fat, FCF, and BHB over the reproductive season.

Ultrasonography was also used to detect intestine visibility in sonograms of females. BHB was higher when intestines were not visible or empty and was lower when intestines were visible or full in the sonograms. This result matched expectations since BHB is expected to increase during fasting, a period when the intestines should be empty. This result also suggests that BHB may be functioning in sea turtles in the same capacity as in mammals.

In comparing SubQ fat, FCF, and BHB between sexes, females were expected to have thinner SubQ fat layers, lower FCFs, and higher BHB concentrations. However, there was no difference between sexes in any of these categories. If females are capital breeders and are fasting there should be a difference in these measurements between the sexes at post-mating because females should be fueling yolking of eggs through lipid mobilization while males are fueling reproductive effort with food intake before arrival at nesting beaches (Miller, 1997). Therefore, our results suggest that females may be feeding at the mating grounds. To further test this possibility, these same parameters were compared in early and late season females at post-mating. If females are capital breeders and are fasting during the reproductive season, late season females should have thinner SubQ fat layers, lower FCFs, and higher BHB concentrations than early season females. However, there was no difference between early and late season females in

any of these categories, thus providing more support for the possibility of females fueling reproductive costs with food intake at mating and nesting grounds.

Further studies should determine how SubQ fat and blood BHB change during the four-month nesting period in nesting females. Females are expected to be fasting during this period so blood BHB is expected to increase as SubQ fat decreases, as fat and ketone bodies are mobilized for energy (Harris et al., 2016). Additional blood samples and ultrasounds can be taken from females previously sampled at post-mating during the monthly mass-nesting events to track changes in nutrient storage and utilization. Other parameters that can be analyzed include the hunger hormones leptin and ghrelin. Leptin functions as an anorexigenic signal (Denver et al., 2011; Niewiarski et al., 2000), and ghrelin has been shown to function as a feeding signal in hawksbill sea turtles (*Eretmochelys imbricata*) (Goldberg et al., 2013). Therefore if females are capital breeders, leptin should decrease during the reproductive season as ghrelin increases. Testosterone can also be analyzed to further classify females as it increases at the start of the reproductive season (Owens, 1997), decreases after ovulation and continues to decrease to basal levels at the end of the nesting period (Licht et al., 1982; Wibbels et al., 1990).

Expanding the applicability of ultrasonography of SubQ fat and blood BHB measurement to other sea turtle species and for use in field conditions should provide less invasive and more informative methods than stomach flushing to increase understanding about how food availability and nutrition impacts sea turtle reproduction. These methods should also lead to an increase in understanding about physiological differences between males and females during reproduction to determine if both sexes have access to the nutrients they need to maximize egg production and population recovery. If females are feeding during the reproductive season as our results suggest, Ostional National Wildlife Refuge should be reclassified to administer sea turtle

prey species monitoring and management policies to increase food availability for this population. This will in turn improve the nutritional health of these organisms and will increase reproductive output to help sustain these populations.

REFERENCES

Abreu-Grobois, A., Plotkin, P., 2012. *Lepidochelys olivacea*. IUCN Red List of Threatened Species.

Bjorndal, K., 1982. The consequences of herbivory for the life history pattern of the Caribbean green turtle, *Chelonia mydas*. Biology and conservation of sea turtles, 111-116.

Broderick, A.C., Godley, B.J., Hays, G.C., 2001. Trophic status drives interannual variability in nesting numbers of marine turtles. Proceedings of the Royal Society of London Series B Biological Sciences. 268, 1481-1487.

Casey, J., Garner, J., Garner, S., Williard, A.S., 2010. Diel foraging behavior of gravid leatherback sea turtles in deep waters of the Caribbean Sea. Journal of Experimental Biology. 213, 3961-3971.

Denver, R.J., Bonett, R.M., Boorse, G.C., 2011. Evolution of leptin structure and function. Neuroendocrinology. 94, 21-38.

Ehrhart, L.M., 1982. A review of sea turtle reproduction, Bjorndal, K.A. (Ed.), Biology and Conservation of Sea Turtles. Smithsonian Institution Press, Washington, DC, pp.29-38.

Forbes, G.A., Limpus, C.J., 1993. A Non-lethal Method for Retrieving Stomach Contents from Sea Turtles. Wildlife Research. 20, 339-343.

Froese, R., 2006. Cube law, condition factor and weight-length relationships: history, meta-analysis and recommendations. Journal of Applied Ichthyology. 22, 241-253.

Goldberg, D.W., Leitao, S.A.T., Godfrey, M.H., Lopez, G.G., Santos, A.J.B., Neves, F.A., de Souza, E.P.G., Moura, A.S., Bastos, J.d.C., Bastos, V.L.F.d.C., 2013. Ghrelin and leptin modulate the feeding behaviour of the hawksbill turtle *Eretmochelys imbricata* during nesting season. Conservation Physiology. 1.

Hamann, M., Limpus, C.J., Owens, D.W., 2003. Reproductive Cycles of Males and Females. The Biology of Sea Turtles. 2, 135-162.

Hamann, M., Limpus, C.J., Whittier, J.M., 2002. Patterns of lipid storage and mobilisation in the female green sea turtle (*Chelonia mydas*). Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology. 172, 485-493.

Harris, H.S., Benson, S.R., James, M.C., Martin, K.J., Stacy, B.A., Daoust, P.Y., Rist, P.M., Work, T.M., Balazs, G.H., Seminoff, J.A., 2016. Validation of Ultrasound as a Noninvasive Tool to Measure Subcutaneous Fat Depth in Leatherback Sea Turtles (*Dermochelys coriacea*). Journal of Zoo and Wildlife Medicine. 47, 275-279.

Hatase, H., Tsukamoto, K., 2008. Smaller longer, larger shorter: energy budget calculations explain intrapopulation variation in remigration intervals for loggerhead sea turtles (*Caretta caretta*). Canadian Journal of Zoology. 86, 595-600.

Hochscheid, S., Godley, B.J., Broderick, A.C., Wilson, R.P., 1999. Reptilian diving: highly variable dive patterns in the green turtle *Chelonia mydas*. Marine Ecology Progress Series. 185, 101-112.

Jensen, M.P., Abreu-Grobois, F.A., Frydenberg, J., Loeschcke, V., 2006. Microsatellites provide insight into contrasting mating patterns in arribada vs. non-arribada olive ridley sea turtle rookeries. Molecular Ecology. 15, 2567-2575.

Labrada-Martagon, V. Mendez-Rozriguez, L. C., Gardner, S.C., Cruz-Escalona, V. H., Zenteno-Savin, T., 2010. Health Indices of the Green Turtle (*Chelonia mydas*) Along the Pacific Coast of Baja California Sur, Mexico. Chelonia Conservation and Biology. 9, 173-183.

Licht, P., 1982. Endocrine patterns in the reproductive cycle of turtles. Herpetologica. 38, 51-61.

Limpus, C.J., 1993. The green turtle, *Chelonia mydas*, in Queensland: breeding males in the southern Great Barrier Reef. Wildlife Research. 20, 513-523.

Miller, J.D., 1997. Reproduction in Sea Turtles, Lutz, P.L., Musick, J.A., Wyneken, J. (Eds.), The Biology of Sea Turtles. CRC Press, Boca Raton, FL, pp. 51-83.

Myre, B.L.M., Guertin, J., Selcer, K., Valverde, R.A., 2016. Ovarian Dynamics in Free-Ranging Loggerhead Sea Turtles (*Caretta caretta*). *Copecia*. 104, 921-929.

Niewiarski, P.H., Balk, M.L., Londraville, R.L., 2000. Phenotypic effects of leptin in an ectotherm: a new tool to study the evolution of life histories and endothermy? The Journal of Experimental Biology. 203, 295-300.

Owens, D.W.M., 1980. Behavioral and Reproductive Biology of Sea Turtles. American Zoologist. 20, 549-563.

Owens, D.W., 1997. Hormones in the life history of sea turtles, Lutz, P.L., Musick, J.A., and Wyneken, J. (Eds.), The Biology of Sea Turtles. CRC Press, Boca Raton, FL, pp. 549-563.

Owens, D.W., Morris, Y.A., 1985. The comparative endocrinology of sea turtles. *Copeia*. 3, 723-735.

Owens, D.W., Ruiz, G.J., 1980. New Methods of Obtaining Blood and Cerebrospinal Fluid from Marine Turtles. Herpetologica. 36, 17-20.

Pearse, D.E., Avise, J.C., 2001. Turtle mating systems: behavior, sperm storage and genetic paternity. Journal of Heredity. 92, 206-211.

Plotkin, P.T., 2007. Biology and Conservation of Ridley Sea Turtles. Johns Hopkins University Press, Baltimore, ML.

Plot, V., Jenkins, T., Robin, J.P., Fossette, S., Georges, J.Y., 2013. Leatherback turtles are capital breeders: morphometric and physiological evidence from longitudinal monitoring. Physiological and Biochemical Zoology. 86, 385-397.

Price, E.R., Jones, T.T., Wallace, B.P., Guglielmo, C.G., 2013. Serum triglycerides and ß-hydroxybutyrate predict feeding status in green turtles (*Chelonia mydas*): Evaluating a single blood sample method for assessing feeding/fasting in reptiles. Journal of Experimental Marine Biology and Ecology. 439, 176-180.

Rostal, D.C., Robeck, T.R., Owens, D.W., Kraemer, D.C., 1990. Ultrasound imaging of ovaries and eggs in Kemp’s ridley sea turtles (*Lepidochelys kempii*), Journal of Zoo and Wildlife Medicine. 21, 27-35.

Schoefield, G., Katselidis, K.A., Dimopoulos, D., Pantis, J.D., Hays, G.C., 2006. Behaviour analysis of the loggerhead sea turtle *Caretta caretta* from direct in-water observation. Endangered Species Research. 2, 71-79.

Solow, A.R., Bjorndal, K.a., Bolten, a.B., 2002. Annual variation in nesting numbers of marine turtles: the effect of sea surface temperature on re-migration intervals. Ecology Letters, 742-746.

Valente, A.L., Parga, M.L., Espada, Y., Lavin, S., Alegre, F., Marco, I., Cuenca, R., 2007. Ultrasonographic imaging of loggerhead sea turtles (*Caretta caretta*). Veterinary Record.

Wibbels, T., Owens, D.W., Limpus, C.J., Reed, P.C., Amoss, M.S. Jr., 1990. Seasonal changes in serum gonadal steroids associated with migration, mating, and nesting in the loggerhead sea turtle (*Caretta caretta*). General and Comparative Endocrinology. 79, 154-164.