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# Variation in Egg Components: A Study of Maternal Investment and Resource Partitioning in the Nesting Loggerhead Sea Turtle; Estimating Genic Variation and Mapping Genetic Lineage of a Rare Captive Helodermatid Population: *Heloderma Horridum Charlesbogerti*

Ketan V. Patel

VARIATION IN EGG COMPONENTS: A STUDY OF MATERNAL INVESTMENT AND RESOURCE PARTITIONING IN THE NESTING LOGGERHEAD SEA TURTLE

ESTIMATING GENIC VARIATION AND MAPPING GENETIC LINEAGE OF A RARE CAPTIVE HELODERMATID POPULATION: *HELODERMA HORRIDUM CHARLESBOGERTI*

by

KETAN V. PATEL

(Under the Direction of David C. Rostal)

ABSTRACT

Loggerhead sea turtles have the highest egg yields of any oviparous non-avian reptiles. Blood urea nitrogen levels increased as the season progressed while total blood albumen and total protein levels decreased. Wet egg mass (H = 1.7719 *df* = 2 p = 0.0375*) and albumen mass (H = 6.0507 *df* = 2 p = 0.0485*) significantly decreased across the nesting season however, wet yolk did not. Analysis of dried egg components showed that dry yolk and albumen did not differ across the nesting season. In addition on no seasonal change in dried egg components, hatchling size was conserved across the nesting season. This study shows that maternal condition declined across the nesting season and this decline correlated with the decrease in egg size, specifically the amount of water allocated to eggs. late season clutches were incubating at the same time that seasonal rainfall increased (r = -0.7251 *df* = 2, p = 0.0271*) and were compensated with water from increased rainfall. This study suggests that in loggerhead sea turtles, natural selection favors an optimal hatchling size rather than an optimal egg size.

*Heloderma horridum charlesbogerti* is the rarest recognized subspecies of *H. horridum* with approximately 200-250 individuals in the wild. Using five microsatellite markers, we found that the nine wild caught individuals, show very little genetic variation across the microsatellites used. In terms of effective population size, three wild caught females and three males are reproductively active. So far, three of the hatchings were produced by male A06107 and female A06110 in 2003, two were produced by male A06106 and female A06100 in 2006 at the San Diego Zoo. The final hatchling was produced by A06104 and A06105 in 2012 at Zoo Atlanta. Assessments of genetic markers used in this study show a high $P_{ID}$ value 0.0792, suggesting a low specificity in terms of identifying individuals by genetic profiles therefore, more genetic markers must be developed to better identify these animals.

INDEX WORDS: Reptilia; Cheloniidae; Maternal investment; Maternal condition; Loggerhead sea turtle; *Caretta caretta*; Egg component analysis; Ash free dry mass; Bomb calorimetry. Guatemalan Beaded Lizard, microsatellites, captive breeding program

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# CHAPTER 1

# INTRODUCTION

## Purpose of this study

This study comprised of two different experiments: the first study focused on the seasonal variation in maternal investment due to tradeoffs in nesting loggerhead sea turtles while the second study focused on the effects of low genetic diversity on captive breeding programs. The purpose of the first study was to monitor the relationship between maternal condition and resource partitioning in the nesting loggerhead sea turtle and account for the effect of rainfall to clutch survivorship. The second purpose was to determine how abiotic factors influence nest survivorship. The purpose of the second study was to determine the genetic diversity in a captive population of beaded lizards and construct a pedigree with genetic data and zoo records. In addition to the first two goals of this study, measuring the accuracy of currently available markers was also achieved.

# CHAPTER 2

# VARIATION IN EGG COMPONENTS: A STUDY OF MATERNAL INVESTMENT AND RESOURCE PARTITIONING IN THE NESTING LOGGERHEAD SEA TURTLE

## INTRODUCTION

Maternal investment is defined as any expenditure that benefits the offspring at a cost to the mother's ability to invest in other aspects of her fitness (Trivers 1972). Maternal investment is shaped by trade-offs between competing functions, such as energy allocated to mating behaviors and resource allocated to number, size, and post-birth/ hatching care of offspring (Congdon 1989; Roff 1992; Wallace et al. 2007). However, resources are finite, and an increase in the amount of maternal investment toward individual offspring typically results in decreased allocation to other offspring and other aspects to a female's fitness.

Post-ovulatory parental investment occurs in crocodilians (Hunt 1975), but rarely in snakes, lizards, and turtles (Hughes and Brooks 2006). For turtles, the allocation of yolk and nutrients to eggs for follicle production, embryogenesis, and post-hatching survival is a direct reflection of the maternal investment in reproduction (Congdon 1983a; Congdon et al. 1989). Since there is rarely post-hatching parental care in turtles (Miller 1997), the appropriate rationing of the number and size of eggs and clutches per reproductive period is equal to the total amount of investment allocated to offspring. Therefore, quantifying how much energy a female allocates into reproductive output could account for maternal investment in offspring survivorship (Wallace et al. 2006a).

Yolk supplies the hatchling with energy and nutrients for growth and the yolk hatchling hypothesis suggests that yolk is most closely tied to hatchling size (Wallace et.al. 2006). With respect to sea turtles, eggs consist of approximately equal proportions of yolk and albumen (Miller, 1985; Hewavisenthi and Paramenter 2002), while eggs of other turtle species contain higher proportions of yolk than albumen (Finkler and Claussen 1997). However, sea turtle yolk contains a higher proportion of lipids than the yolk of other turtle species (Kramer and Bennett 1981; Congdon et al. 1983b; Hewavisenthi and Paramenter 2002). Therefore, a female sea turtle can allocate less yolk supply in eggs compared to other oviparous reptiles. The albumen is a mixture of proteins that sequesters water to hydrate the embryo in a jelly-like coat. The amount of water in albumen can vary across taxa. Albumen in chicken eggs contains about 83% water while alligator eggs contain albumen with 96% water (Nelson et. al. 2010). In turtle eggs, albumen is 80.1% water in eggs of E. singata and ranged from 81.2-71.2% based in egg size in C. expansa (Booth 2002).

Sea turtles opt for more offspring rather than larger offspring. This trend is most likely due to proportion of eggs surviving to adulthood lying between 0.0009 and 0.0018 (Frazier 1986). Due to the high mortality rates of sea turtles at early life stages (Dodd 1988, Miller et al. (2003), loggerhead females lay many small eggs rather than a few large eggs (Wallace et al. 2007).

Loggerhead females will nest every 2-3 years and will produce between 50-130 eggs per clutch. In addition to a large clutch size female loggerheads will lay, on average, more than five clutches per year. Because of the large number of eggs produced, studies suggest that an optimal egg size is maintained to manage tradeoffs in energy allocation due to female size and available resources (Erhart 1979; Congdon & Gibbons 1987; Hays & Speakman 1991; Congdon et. al. 1999; Broderick et. al. 2003; Bowden et.al. 2004). In order to survive though the nesting season the female must acquire energy stores before arriving to the nesting beaches close in proximity to their natal beach (Carr et. al. 1978; Miller 1997; Miller et al. 2003). These energy stores are from food found at the foraging grounds such as crustaceans, jellies, etc. (Reich et al. 2009). During the entire nesting period, females must ration energy costs of nesting over her total number of clutches in a nesting period, as well as maintain enough reserve for migration back to foraging grounds (Hamann et al. 2002). Groups of male loggerheads will intercept and mate females on their way to reaching the nesting beach (Hawkes et.al.2007). In sea turtles ovarian follicular development occurs prior to arrival at the nesting beach (Rostal et al. 1990, 1996; Miller et al. 2003), therefore the females will arrive with hundreds of eggs in her celomic cavity as well as energy stores that she will utilize across the nesting season. Typically, nesting females will not forage, but will live off fat reserves stored in their body (Limpus and Limpus 2003), this is possibly due to the celomic cavity being filled with ova and eggs, as well as energy constraints for the female and availability of food at the nesting grounds. In hawksbills, nesting females lost 5.46 kg of body mass across the season suggesting that they were not taking in food; however the net loss of energy due to laying eggs was not

great enough to take in extra energy while nesting (Santos et.al. 2010). Blood profiles, blood urea nitrogen, blood albumen, and total protein show that nesting female health declines across the nesting season (Deem et al. 2009; Honovar et. al. 2011)due to the female fasting or eating less across the nesting season. The albumen portion of the egg is laid down in the oviduct prior to each nesting interval (Aitken and Solomon 1976; Miller 1985), requiring energy sources continually throughout the nesting period. Because albumen is needed throughout the season, maternal condition will affect the amount of albumen allocated to eggs across the nesting season.

Off the coast of Georgia female Caretta caretta nest on Wassaw Island from mid-May until early August, and hatchlings emerge from late June until early October. Frazer and Richardson (1985) and Le Blanc (2004) reported that clutch size, in Georgia, decreases over the nesting season. LeBlanc (2004) has also reported individual females produce significantly smaller eggs later in the nesting season. This is also observed in green turtles (Bjorndal & Carr 1989). Ultrasonography data (Rostal et al. 1990, 1996; LeBlanc 2004) showed that there was little variation in egg yolk size yet the major variation in egg size in loggerheads was the result of albumen laid down in the oviduct. Therefore, it is assumed that the first clutches laid in the nesting season contain more albumen due to the hydration state of the female earlier in the nesting season as compared to later on in the season.

Maternal resources are not the only factor influencing egg components. Environmental factors also play a significant role in embryo development therefore nest site selection is important (Wood & Bjorndal 2000). Females use a variety of cues such as elevation, temperature, and moisture to select sites that would ensure successful incubation (Wood & Bjorndal 2000).

Nest environment factors such as temperature, moisture, pH, salinity, influence the development of embryos (Packard & Packard 2001). Like painted turtle eggs, sea turtle eggs have the ability to absorb water from the nest environment allowing for them to become turgid soon after deposition (Miller 1997). Environmental moisture while incubation affects egg size during development (Packard & Packard 2001). In addition eggs that incubate in wetter environments produce larger hatchlings compared to eggs in dryer environments (Packard 1999; Roosenburg & Dennis 2005). On coastal Georgia, increases in seasonal rainfall occur simultaneously with the nesting season. The increased rainfall is thought to compensate late clutches with necessary water for proper development. Therefore, seasonal rainfall patterns have a great effect on nest survivorship across the nesting season. Because environmental factors are often interrelated (Ackerman et al. 1985; Ackerman 1997), investigating rainfall patterns may provide evidence for the effects of abiotic factors on hatchling morphology and physiology.

Examining the patterns of seasonal variation in maternal condition can quantitatively describe the cause for lower allocation of water later in the nesting season. Blood profiles are highly influenced by externals factors, such as nutritional and environmental conditions (Lutz and Dunbar-Cooper 1987; Bolton and Bjorndal 1992; Bolton et. al. 1992; Deem et al. 2009; Honovar et. al. 2011). Monitoring blood biochemistry detail the health and condition of nesting females and provide a better understanding for how females utilizes and allocate her stored energy over the nesting period.

Gravimetrically measuring egg components with and without water, ash content, and calorimetry will reflect how the nesting female allocated resources to multiple clutches and show any variation in maternal investment to clutches across the nesting season due to the decline in her condition as the season progresses. Blood profiles coupled with measurements of egg size across the nesting season will quantitatively show how a nesting female can manage tradeoffs with the limited amount of available nutrients and allocate nutrients to clutches across the nesting season. This data then can separate biotic factors from the abiotic factors contributing to offspring survivorship.

The focus of this study is to quantify nesting female health parameters and relate them to the maternal investment contributions in her eggs. The hypotheses tested were:(1) If an adult female is fasting during the nesting period, then body condition will decline over the course of the nesting period (May - August). This will result in decreased levels of total albumen levels, and an increase in blood urea nitrogen levels. (2) If the adult female is fasting during the nesting period, egg mass should decrease with female condition over the course of the nesting season (May - August). (3) If hatchling size remains constant across the nesting period, then rainfall must compensate dehydrated eggs with water.

# METHODOLOGY

Study site. – The study site was Wassaw National Wildlife Refuge (WASI). Wassaw Island is approximately 23 km south of Savannah, Georgia and has 11 km of beach. Female C. caretta nest from mid-May until early August, and hatching occurs from late June until early October. The nesting period was divided into three portions: May 15- June 10 were designated as early nests (first and second nests), June 11- July 8 as middle nests (third and fourth nests), and July 9- August 4 as late nests (fifth and sixth nests).

Adult female condition. – Adult females were located during the nightly patrols. Each female was tagged and measured according to U.S. Fish and Wildlife protocols. Curved carapace length (from the nuchal notch to the tip of the posterior marginal; CCL, N-T) and curved carapace width at the widest width (CCW); were recorded on each female sampled. Blood samples were taken from the cervical sinus and retained in 6 ml sodium heparin vacutainers. No individual was sampled more than once.

Blood samples were stored on ice for up to 4 hours before being centrifuged for 15 minutes. Plasma and red blood cells were stored at -20°C in a cooler for several days before being taken to Georgia Southern University for analysis. Indications of the adult female's hydration and energy levels were quantified by creating a blood profile for each female. Blood profiles consisted of three variables: Blood urea nitrogen level (BUN), total protein, and albumin level. Blood urea nitrogen and blood albumin were quantified from plasma. Plasma biochemisty profiles were processed by using the dry slide IDEXX VetTest autoanalyzer at Ogeechee Technical College. A Schuco clinical hand held refractometer was used to indicate total plasma protein levels.

Egg Components

Wet egg components. – All eggs from field nests were removed and counted within 6 h of oviposition. Six eggs per nest were taken nest for composition analysis, for a total of 540 eggs. Each egg was brushed free of sand and weighed using a digital scale to the nearest 0.1 g before being stored in -20 °C freezer and taken to Georgia Southern University for composition analysis. Frozen eggs were thawed and re-weighed. Once the total mass was obtained, a small incision was made into the shell to separate the albumen for weighing. The shell was then opened to transfer the yolk into another weigh boat to be weighed and the shell was subsequently was weighed. The yolk, albumen and shell were transferred into a whirl pack bag and frozen at -20 °C for analysis of dried components.

Dry components. – Dry mass was used to assess the amount of usable nutrients allocated into each egg (i.e. pure yolk and pure albumen) as well as determine any changes in component mass (minus water) over the nesting season. Thawed component samples were transferred into aluminum pans and their initial weights were recorded before being placed into the drying oven. To separate water mass from component mass, samples were placed in a drying oven at 60$^{o}$C for a period no less than 48 hours, adapted form Booth (2002). Immediately after drying, samples were taken out of the drying oven, re-weighed and their weights were recorded. Subsequently, the samples were placed in plastic containers stored in a cool dry room.

Ash free dry mass. – Stored dry yolk and albumen samples from three eggs taken from two sample nests, from each of the nine nesting periods in the study (n = 54 eggs) were placed into a muffle furnace at 450oC adapted from Booth (2002). Immediately after being taken out of the muffle furnace, samples were re-weighed. Inorganic matter was weighed as ash free dry mass (AFDM) weight and the difference between dry component weight and AFDM weight was recorded as organic weight.

Bomb Calorimetry. –Two yolk samples (0.5 grams) from two nests in each of the nine periods of the study were placed into a bomb calorimeter and ignited (n = 36 samples). To ensure a complete burn, the experiment was set to run for 900 seconds taking a temperature measurement every 12 seconds. The resulting temperature change in the water around the bomb was used to ascertain the caloric content (J) in the sample. Data from all the samples were extrapolated to account for the total dry mass of the yolk.

Hatchling measurements. – Determined by the nest temperature, hatchlings began to emerge after 45-60 days of incubation. Field nests were closely monitored for hatchling emergence as hatching dates approached. Corrals were placed over the nests for aid in hatchling capture, and each nest was monitored at least twice daily. Upon emergence, each hatchling was brushed free of sand, straight carapace length (SCL), width (SCW), height (or depth, SCH), and mass (g) of approximately 20 live hatchlings was measured. Hatchlings were weighed (g) during all years. Hatchling SCL was used as the measurement of the size of hatchlings.

Abiotic Factors.

Historically, seasonal weather patterns are marked by an increase in rainfall as the nesting season progresses (Davis 2007). To understand how abiotic factors affected incubating eggs, average rainfall patterns were obtained from the Georgia Automated Environmental Monitoring Network to correlate with seasonal variation in egg mass. Clutches from early, middle, and late nesting periods were matched with corresponding monthly rainfall averages. Clutches were matched with an offset of one month to ensure that the clutches in the study were incubating on the beach as rainfall increased. This means that clutch mass for the month of May was matched with monthly rainfall patterns in June, mass for June clutches were matched with average rainfall patterns in July and, mass for clutches deposited in July were matched with August average rainfall patterns.

Data analysis. – All analyses were performed using JMP statistical software package version 4.04 (©2001, SAS). Research nests were chosen randomly throughout the nesting period. No individual female was sampled more than once for blood profiles or egg composition. Data from all study years were combined for analyses unless otherwise noted. Averages are reported as mean ± 1 standard error. The assumptions of normal distribution and equal variances were tested prior to performing analyses of variance (ANOVA) and linear regressions. If variables showed unequal variance and/or were not normally distributed,, then a Kruskal-Wallis test was used.

Adult female condition –An ANOVA, was used to test seasonal variability in total protein. A Kruskal-Wallis test was used to test seasonal variability in blood urea nitrogen and blood albumen.

Egg composition. – Yolk and albumen mass from six eggs in each clutch were averaged into a mean yolk and albumen mass. This average clutch mass was used for analysis for all nesting seasons. Clutch averages for all three years of the study were taken and were sorted into data from the early, middle, or late periods.

Wet mass and dry mass. – Kruskal Wallis tests was used to test for seasonal variation in mean egg mass, mean yolk mass, and mean albumen mass for nesting period between 2008-2010. A Kruskal Wallis test was used to test for seasonal variation in dry yolk and dry albumen across the nesting season.

Ash free dry mass. – Kruskal-Wallis tests were used to test for seasonal variation in ash free dry mass for both yolk and albumen. Comparisons between dry weights and AFDM weights for both yolk and albumen were also made.

Bomb Calorimetry. – A Kruskal Wallis test was used to test the seasonal variation in average caloric content of dried yolks. A regression graph was created to correlate caloric content respective to dry yolk mass.

Hatchling measurements. – An ANOVA was used to test for differences in mean hatchling SCL. All hatchling measurements were averaged and the mean measurement per clutch was used for analysis for all nesting seasons.

Abiotic factors. - Monthly rainfall averages for 2008-2010 were ascertained and averages among years were plotted. A Pearson's correlation was used to determine if there was any association between the trend in decreasing egg mass and the trend increasing rainfall across the nesting season.

## RESULTS

Adult female condition. –Blood urea nitrogen (BUN) levels for nesting females ranged between 3.0 - 24.0 mg/dL (mean = 11.39 ± 0.7 mg/dL), blood albumin (ALB) ranged between 0.8 - 2.1 g/dL (mean = 1.3 ± 0.09 g/dL), and Total Protein (TP) levels ranged between 3.8 - 7.4 g/dL (mean = 5.36 ± 0.1 g/dL). Blood albumin levels (H =12.10 df = 2; P = 0.0023*) and blood urea nitrogen levels (H = 12.56 *df* = 2; P = 0.019*) showed a significant change over the course of the nesting period. Total protein levels increased significantly over the course of the nesting period ($F_{(2, 81)}$ = 10.09; P = 0.0002*). Blood urea nitrogen and blood albumen did not show a significant correlation(r = - 0.205; P = 0.0613), however, total protein had significant relationships with blood albumen and blood urea nitrogen (r = 0.7331: P = 0.0001*) and (r = - 0.4305; P = 0.0001*) respectively. When compared to total egg mass, albumen levels had a significant positive correlation (r = 0.2281, P = 0.0406*).

Egg composition. – Wet component data showed a significant difference of total egg mass across the nesting season (H= 6.6638 *df* = 2; P = 0.0375*). Wet yolk mass does not significantly differ throughout the season (Early, Middle, Late) (H= 0.7915 *df* = 2; P = 0.6732) however, wet albumen mass does (H= 6.0507 *df* = 2; P = 0.0485Neither dry yolk mass (H = 3.3753 *df* = 2; P = 0.1849) nor dry albumen mass (H = 4.3663 *df* = 2; P = 0.1227) differed significantly across periods. There was no significant difference in AFDM yolk (H= 1.8667, df = 2; P = 0.3932) or albumen (H = 0.3556, *df* = 2; P = 0.8371) across the periods of the nesting season. The Kruskal Wallis test showed no difference in the caloric content of eggs across the nesting season (H = 2.00 *df* = 2; P = 0.3679).

Hatchling measurements. – Hatchling SCL (mm) was measured to determine if it displayed any seasonal variation. Mean hatchling SCL did not display any seasonal variation over the course of the nesting period ($F_{(2,8)}$ = 0.2248; P = 0.8051).

Abiotic factors. –There is no difference in mean rainfall across the nesting period (H= 8.5468 *df* = 5; P = 0.1286) between 2008-2010. A significant negative correlation exists between the decreases in egg mass and seasonal rainfall pattern, egg mass was offset by one month (r = - 0.0271 *df* = 2; P = 0.0271*).

## DISCUSSION

Adult female condition. – Since nesting females fast throughout the nesting season (Limpus and Limpus 2003), initial hydration, fat reserves, and protein in muscle tissue are the only sources of nutrients available to the female. Females arrive to the nesting beach in optimal health and start off metabolizing fat stores. As the nesting season progresses, fat reserves are depleted and females switch to metabolizing muscle tissue (Stockham & Scott 2008; Honarvar 2011). Total protein levels in the blood significantly decreased across the nesting season suggesting that the female is metabolizing stores of energy and subsequently skeletal muscle tissue from limbs. With the increase of nitrogenous waste from the increase in protein metabolism, the kidneys cannot effectively filter nitrogenous waste products. This leads to a higher content of urea and nitrogen in the bloodstream (Stockham & Scott 2008; Honarvar 2011). A significant correlation between total protein and blood urea nitrogen levels support that when fasting, the nesting female is living off stores and then muscle tissue. This trend shows that tradeoffs between available energy and physiological demands a compromise in which the females health declines so that her offspring have enough to survive yet no so much that the female is risking death.

Fasting also correlates to the hydration state of the female; a correlation between total protein and blood albumen levels suggests that nesting sea turtles do not maintain their hydration state across the nesting season. Blood albumen levels are indicators of hydration; if the nesting female is dehydrated, the total blood albumen content will consequently decrease. A correlation between blood urea nitrogen and blood albumen further support the females are simultaneously fasting and becoming dehydrated (Figure 1). Blood urea nitrogen levels in turtles as a poor indicator of renal disease; however, it probably depicts nutritional status and protein metabolism (Campbell 1996). Deem et al. (2009) found high levels of blood albumen and low blood urea nitrogen levels in nesting female loggerheads when compared to foraging loggerheads and migrating loggerheads. Blood albumen and blood urea nitrogen results for this study are within the range of nesting loggerheads found by Deem et. al. (2009). Nevertheless, the significant decline in blood albumen and increase in blood urea nitrogen levels in nesting females suggest that female condition is declining significantly across the nesting season (Table 1).

Egg composition/ Hatchling measurements. – Since turtles display lecithotrophy, a consistent supply of yolk would ensure optimal efficiency in clutch survivorship and hatching size. Egg mass decreases over the course of the nesting period (H= 6.6638 df = 2; P = 0.0375*). LeBlanc (2004) suggested that there are less resources allocated to clutches laid later in the season compared to earlier in the season. Since resources allocated to eggs are limited by the health of the female, eggs laid later in the season should have fewer resources. Miller et. al., (2003) found that the female will allocate the yolk to all of the follicles prior to the arrival to the nesting ground. Ultrasonography data which followed multiple females across a nesting period suggests there is little variation in egg yolk size in Lepidochelys kempi (Rostal et al. 1990, 1996), C. caretta (LeBlanc 2004). This study supported the idea that follicle size, (yolk) in loggerheads was consistent during the nesting period among females (Figure 3). In this study, yolk mass accounted for half of egg mass; similar findings were reported by Finkler and Claussen with Chelydria serpentinia (Finkler and Claussen 1997). The amount of wet, dry, ash mass, or energy content of yolk showed no variation in amount across the season (Figure 2) supporting the notion that allocation of yolk happens prior to fasting and that fluctuation of egg mass is not related to the amount of yolk allocation (Figure 3 & 4). The female will allocate equal resources to as many eggs as possible rather than preferentially allocate resources to certain clutches; this will ensure a consistent hatchling size. This trend is consistent with previous studies and suggests that the female manages tradeoffs to optimize the number and size of hatchlings. In leatherback sea turtles, yolk was consistent across all clutches and was most closely tied to hatchling size (Wallace et al. 2006). Because yolk is utilized by the hatchling as a source of growth

and development during embryogenesis (Hewavisenthi and Parmenter 2002), equal allocation of yolk may explain the little variation in hatchling size found in this study and therefore accepting the yolk – hatchling hypothesis. Consistent with Booth (2002), smaller eggs had higher dry mass to wet mass ratio than larger eggs (Table 2). This suggests that the amount of nutrients is allocated equally to all eggs, however the amount of water allocated to eggs is contingent on the condition of the mother at the time of oviposition. The pattern found in this study was different than seen in eggs of freshwater turtle and other sea turtle species, where the variation in yolk mass was attributed to most of the variation in egg mass (Finkler and Claussen 1997; Hewavisenthi and Parmenter 2002).

Increases of egg size are correlated to increases in the solid content of the egg, i.e. yolk (Congdon et al. 1983b; Sinervo 1993). Yet other studies found the amount of albumen available to the developing embryo may influence hatchling size and quality (Finkler and Claussen 1997). Rostal et al. (1990, 1996) and LeBlanc (2004) also suggest that the major variation in egg size of loggerheads is the result of albumen laid down in the oviduct. Albumin is used to sequester water and therefore if the proteins in albumen are equally allocated, the eggs can still attain an adequate hydration state provide the nest environment has water available for uptake. Wet albumen decreased significantly as the nesting period progressed (Figure 2). Similar to other turtle species C. serpentinia (Finkler & Claussen 1997) and Dermochelys coriacea (Wallace et al. 2006b), albumen (proteins and fresh water) accounted for half of egg mass. Since fresh albumen must be laid down before each nesting event, allocation equal amounts of albumen to all clutches is problematic if the nesting female is not adequately hydrated throughout the nesting season.

Females are becoming dehydrated and therefore cannot allocate the equal amounts of albumen to all clutches laid over the nesting season. Our study suggests that blood albumen levels significantly correlate with decreased egg size ($r = 0.2281$; $P = 0.0406$*). Early clutches contain more water due to the hydration state of the female early in the nesting period versus later in the nesting period (Table 2). Simultaneously with the decline in the females' hydration state, the amount of wet albumen allocated to eggs significantly decreases over the nesting season accounting for the fluctuation in egg mass across the nesting season.

Figure 2 shows that the amount of wet albumen decreases as the nesting season progresses. Dry component and ash free dry mass analysis showed that neither the mass of dry albumen nor AFDM albumen allocated into eggs changed (Figure 3, Figure 4) therefore, water accounts for the difference in egg mass across the nesting season. Because females are dehydrated, they allocate less water in the albumen as the season progresses. (Figure 3, Figure 4).

Abiotic factors. – The amount of water available to an egg can be related back to water reserves in the female before leaving the foraging grounds (Wallace et al. 2006a) where vitellogenesis occurred (Rostal et al. 1996) and rainfall during incubation. Environmental factors, such as rainfall, are often interrelated to the incubation duration and temperature of a nest. Any fluctuations of the nesting environment can influence sex, size/mass of hatchlings, the amount of residual yolk used post hatching and ultimately hatchling fitness (Miller et al. 1987). Between 2008-2010 on Wassaw Island NWF, monthly rainfall trends show no significant change in the amount of rainfall; however this result can be attributed to small number of data points. Since statistics were run on monthly averages rather than all rainfall data points, within group variation was much exceeded the amount needed to show significant different in monthly averages (Figure 5). The a correlation between the decrease in egg mass and the increase in rainfall ($r = -0.7251$ $df = 2$; $P = 0.0271$*) suggests that the eggs from the later nesting periods (June, July) are incubating coincidently with the increase in rainfall (July, August). This means that eggs which do not have adequate amounts of water are oviposited at the same time that seasonal rainfall levels increase. This shared timing allows less hydrated eggs the opportunity to uptake enough water for proper embryogenesis and grow to the same size as hatchlings from the early eggs that were adequately hydrated by the nesting female (Figure 5). From these results, it is suggested that seasonal rainfall plays a crucial part in hydrating clutches that are laid in the middle and late nesting periods. Since females nest two to three years, they undergo an ultimate cue and ration an average amount of nutrients and water for optimal clutch survivorship across all clutches.

The loggerhead sea turtle is threatened throughout its entire range, thus reproductive research on this reptile is critical to its survival. Future research should emphasize the interactions of the environment associated with genotypic variation within this population of loggerheads. Further studies with component analysis could also shed light on how important rainfall patterns are to late nests. With increasing global temperatures during nesting months, rainfall patterns will surely change. This will impact the survivorship of clutches laid. Further awareness of this factor and other related factors are necessary to protect this northern subpopulation from the effects of changing weather patterns. In addition, such studies would improve knowledge of how abiotic factors influence the reproduction strategies of nesting reptiles as well as provide baseline data of any potential differences in the conditions of nesting beaches used by different C. caretta population.

Table 1. Variations in blood parameters of loggerhead females across the nesting season and years in the study. Blood urea nitrogen levels show increase while total protein and blood albumen levels decrease.

| Blood Parameters | Year | Nesting Period | | | |
|---|---|---|---|---|---|
| | | Early | Middle | | Late |
| Blood Albumen (g/dL) | 2008 | 1.38 | 1.2333 | | 1.11 |
| | 2009 | 1.4333 | 1.21 | | 1.16 |
| | 2010 | 1.71 | 1.516 | | 1.15 |
| | | | | | |
| Blod Urea Nitrogen (mg/dL) | 2008 | 6.6 | 10.5 | | 12.2 |
| | 2009 | 13.47 | 13.3 | | 14.61 |
| | 2010 | 8 | 11.16 | | 12.7 |
| | | | | | |
| Total Protien (g/dL) | 2008 | 5.7 | 5.3556 | | 5 |
| | 2009 | 6.1333 | 5.16 | | 4.84 |
| | 2010 | 6.122 | 5.667 | | 4.3 |

- Table 2. Variation in egg components, hatchling size and hatchling mass.

| | | | Early | Middle | Late |
|---|---|---|---|---|---|
| Yolk | 2008 | Mass | 18.339 | 18.622 | 20.27 |
| | | Percent Water | | | |
| | 2009 | Mass | 20.81 | 21.86 | 21.07 |
| | | Percent Water | | | |
| | 2010 | Mass | 18.25 | 18.52 | 19.24 |
| | | Percent Water | | | |
| Albumen | 2008 | Mass | 17.453 | 14.95 | 11.99 |
| | | Percent Water | | | |
| | 2009 | Mass | 11.74 | 18.54 | 16.26 |
| | | Percent Water | | | |
| | 2010 | Mass | 16.27 | 18.547 | 16.26 |
| | | Percent Water | | | |
| | | | | | |
| | | | Early | Middle | Late |
| Dry Yolk | 2008 | Dry mass | 6.79 | 6.94 | 6.6 |
| | | AFDM | 1.29 | 1.41 | 1.52 |
| | 2009 | Dry mass | 5.1 | 5.28 | 4.32 |
| | | AFDM | 1.25 | 1.36 | 1.37 |
| | 2010 | Dry mass | 5.14 | 5.41 | 5.72 |
| | | AFDM | 1.53 | 2.1 | 3.13 |
| Dry Albumen | 2008 | Dry mass | 0.47 | 0.36 | 0.34 |
| | | AFDM | 0.25 | 0.16 | 0.14 |
| | 2009 | Dry mass | 0.32 | 0.45 | 0.48 |
| | | AFDM | 0.15 | 0.24 | 0.22 |
| | 2010 | Dry mass | 0.39 | 0.34 | 0.27 |
| | | AFDM | 0.17 | 0.23 | 0.24 |
| | | | | | |
| Caloric Content (J) | 2008 | | 11697.51 | 11883.86 | 13597.58 |
| | 2009 | | 15112.48 | 14243.78 | 15151.48 |
| | 2010 | | 12832.16 | 10252.45 | 13350.31 |
| | | | | | |
| Hatchling SCL (cm) | 2008 | | 43.23 | 44.39 | 44.50 |
| | 2009 | | 44.35 | 44.86 | 44.82 |
| | 2010 | | 44.36 | 43.85 | 43.02 |
| | | | | | |
| Hatchling mass (g) | 2008 | | 18.16 | 18.88 | 18.21 |
| | 2009 | | 18.80 | 19.72 | 19.80 |
| | 2010 | | 19.05 | 17.99 | 18.88 |

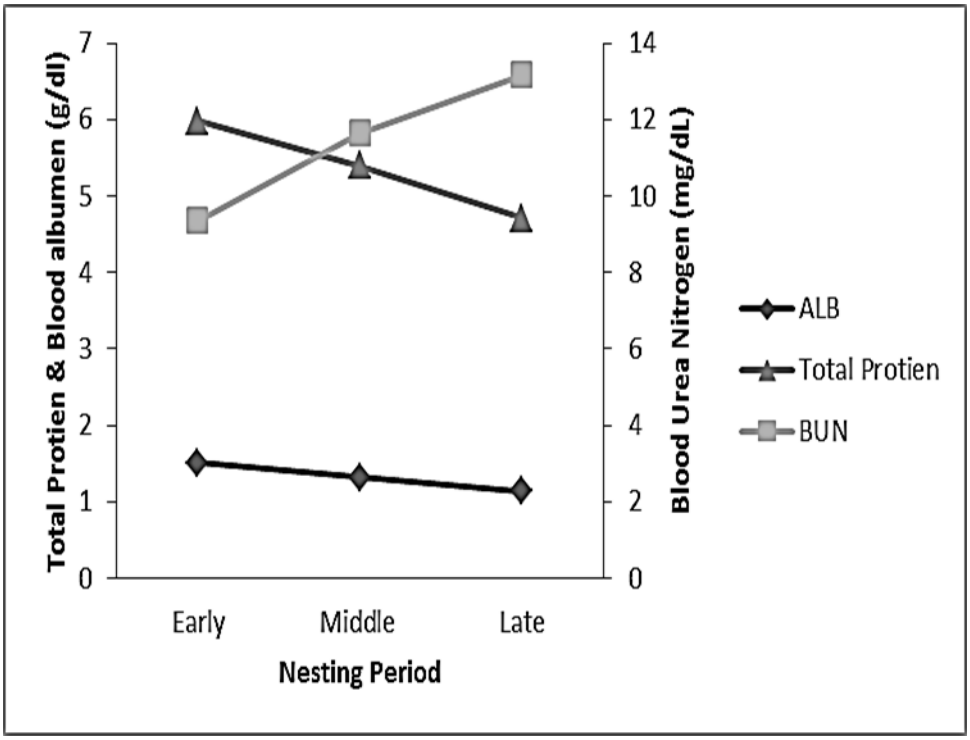


Figure 1. Trends in maternal condition across the nesting season show that both blood albumen levels (H = 12.10 *df* = 2; P = 0.0023*), Blood Urea Nitrogen levels (H = 12.56 *df* = 2; P = 0.0406*) and Total Protein ($F_{(2,81)}$ = 10.09; P = 0.0002*) significantly change across the nesting season. Total protein correlated significantly with Blood urea nitrogen and blood albumen (r = 0.7331: P = 0.0001*) and (r = - 0.4305; P = 0.0001*) respectively. Blood urea nitrogen levels do not significantly correlate negatively with blood albumen levels but not significantly (r = - 0.2051; P = 0.0613).

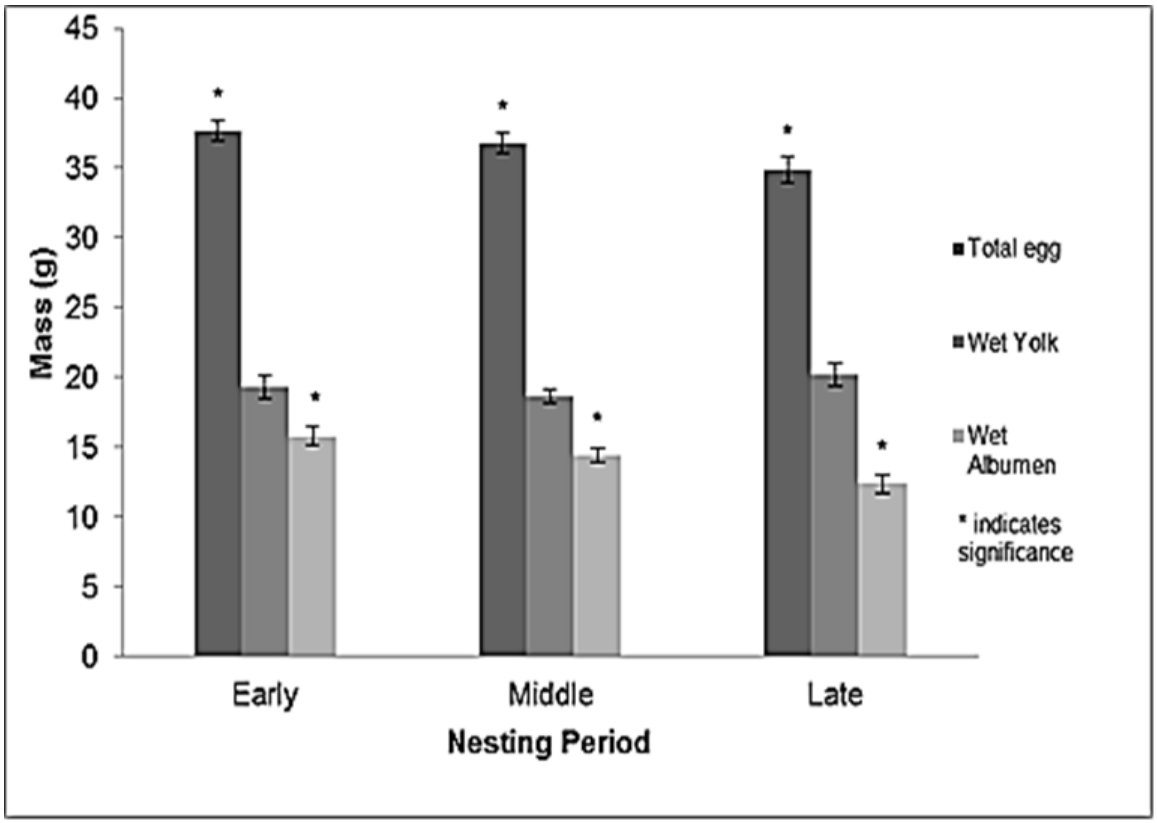


Figure 2. Comparisons of average total egg mass, wet yolk mass and wet albumen mass across the nesting season from 2008-2009-2010. Total egg mass significantly shows significant seasonal variability across the nesting season (H = 6.6638 *df* = 5; P = 0.0375*). Wet yolk mass data shows no significant seasonal variation (H = 0.7915 *df* = 2; P = 0.6732). Wet albumen mass shows significant decrease as the nesting season progresses (H = 6.0507 *df* = 2; P = 0.0485*).

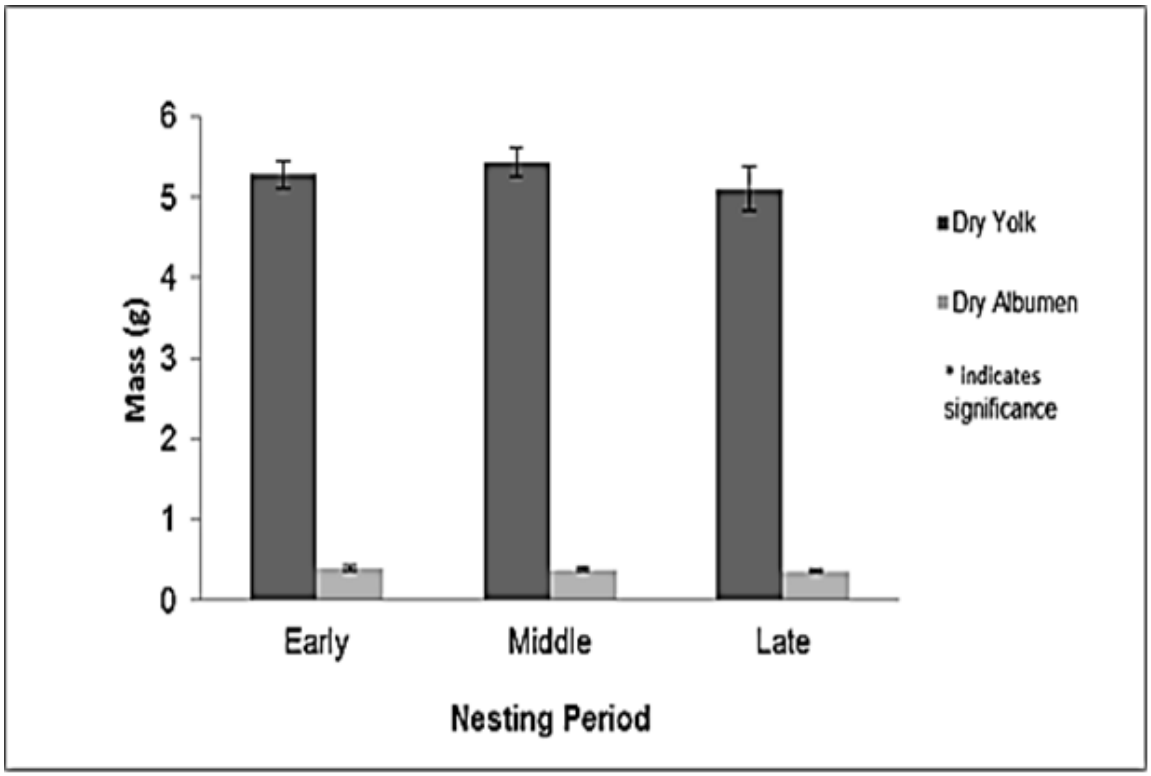


Figure 3. Comparisons of average dry yolk and dry albumen across the nesting season taken from 2008, 2009, 2010. Dry yolk data shows no seasonal variation (H = 3.3753 *df* = 2; P = 0.1849). Dry albumen also shows no significant difference across the nesting season (H = 4.3663 *df* = 2, P = 0.1227).

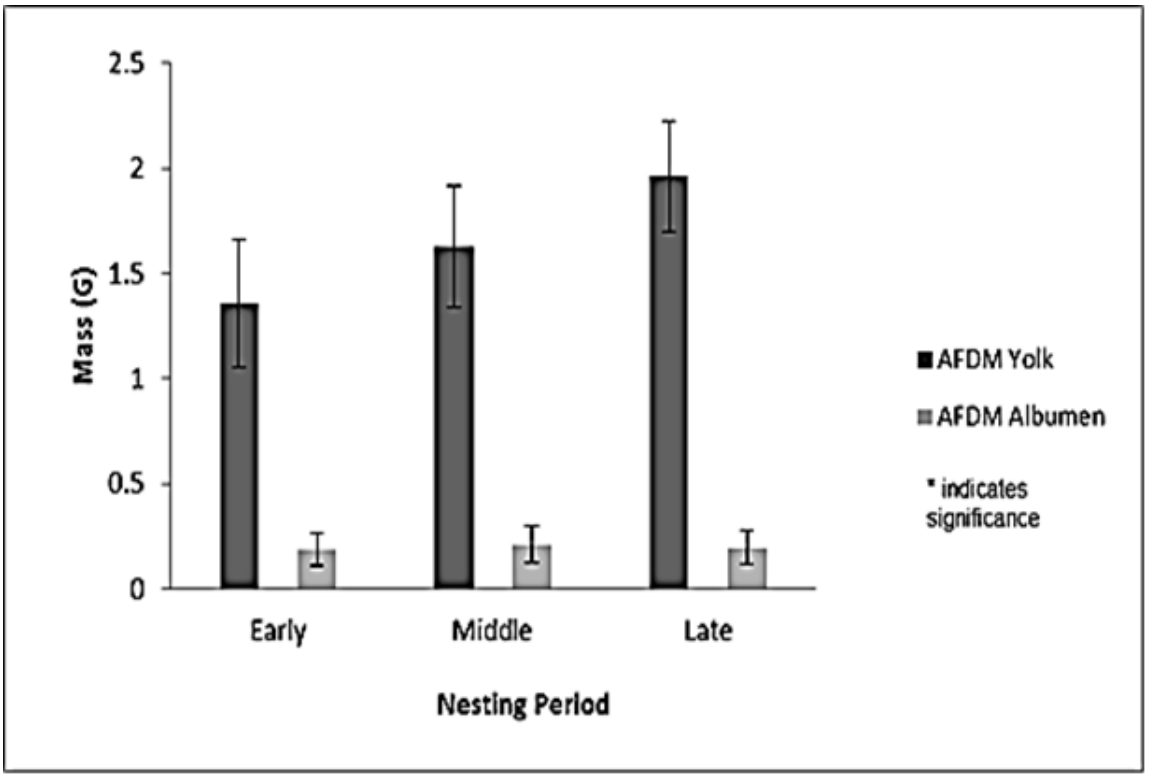


Figure 4. Comparisons of average ash free dry mass yolk and ash free dry mass albumen across the nesting season taken from 2008 - 2010. AFDM yolk shows no significant change across the nesting season (H= 1.8667 *df* = 2; P = 0.3932). AFDM albumen also shows no significant difference in mass across the nesting season (H = 0.3556 *df* = 2; P = 0.8371).

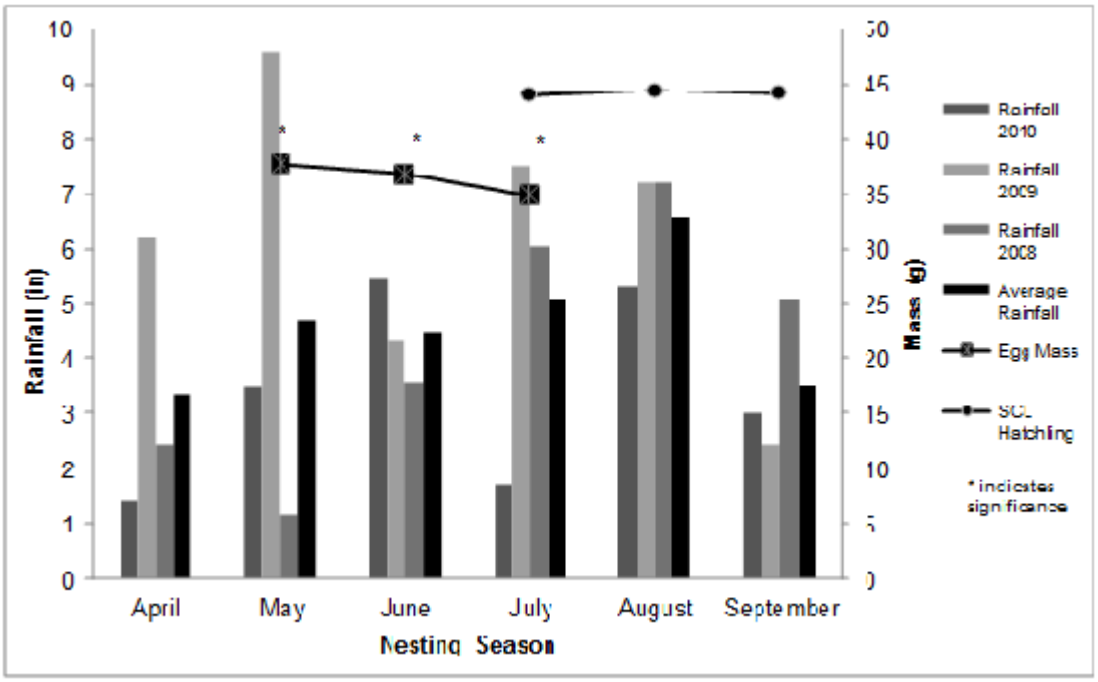


Figure 5. Comparisons of rainfall, egg size, and hatchling size across the nesting season. Rainfall showed no significant variation across the nesting season (H = 8.5468 *df* = 5; P = 0.1286). Total egg size shows seasonal variation, specifically a decrease in mass as the season progresses (H = 6.6638 *df* = 2, P = 0.0375*). Average hatchling SCL does not show significant change across the nesting season ($F_{(2,8)}$ = 02248; P = 0.8051). A Pearson's correlation (r = -0.7251; P = 0.0271*) shows a significant correlation between decrease in egg mass (offset by 1 month) and rainfall patterns when eggs are incubating.

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# CHAPTER 2
# ESTIMATING GENIC VARIATION AND MAPPING GENETIC LINEAGE OF A RARE CAPTIVE HELODERMATID POPULATION: *HELODERMA HORRIDUM CHARLESBOGERTI*

## INTRODUCTION

Over the last century, species near extinction have been successfully re-introduced from captive assurance populations in zoos and aquariums (Gippoliti & Amori, 2007). To preserve genetic diversity in a captive population, managers utilize pedigree information on reproductive pairings, hatching events, and genetic relationships in species-specific studbooks (Ballou & Foose, 1995). It has been suggested that captive breeding programs should strive to maintain at least 90% of the total genetic diversity of a species in their captive assurance population (Frankham et al., 2003). However, this objective is contingent on the number of founder individuals and is magnified when dealing with taxa that have historically small population sizes or that have experienced severe genetic bottlenecks in the wild (Jones et al., 2002).

Loss of diversity in captive populations is mostly due to genetic drift (Fernandez et. al., 2001). Genotypes of captive individuals are similar as a result from small effective populations in the wild, and a high number of alleles that are common in all members due to co-ancestry (Fernandez et. al., 2001). This low variability lowers the specificity of genetic markers in identifying individuals in a population.

Therefore, when species that have low genetic diversity in the wild are the focus of captive breeding programs, maintenance of accurate studbooks is critical to avoid further inbreeding and reductions in genetic diversity (Jones et. al., 2002).

Microsatellite markers are preferred for use in diversity and parentage analysis because thee variation among individuals is mostly due to genetic drift (Guerier et. al., 2012). Microsatellites bind to tandem repeats of nucleotide bases found on certain non-coding regions of genomic DNA. These repeats are found in every individual in the population and inferences of relatedness can be determined by the similarity of the number and size of repeats among individuals in a population (Beebee & Rowe, 2008). Since genotypic identities are by descent, it is assumed that parent and offspring should have similar genetic profiles (Beebee & Rowe, 2008). By determining the genetic profile of all animals and aligning offspring genotypes with parental genotypes, researchers can, by the principle of exclusion, exclude all genetically dissimilar individuals and determine true parentage.

In wild populations where the genotypes of all individuals cannot be determined, conservationists determine the variation in a natural population by the Hardy- Wienberg equilibrium and probability of identity (Beebee & Rowe, 2008). The Hardy Weinberg Equilibrium is used to determine whether genetic diversity in natural populations are under selective forces by comparing the proportion of homozygotes to heterozygotes, observed in a population to the expected proportion of homozygotes to heterozygotes for examined loci.

If there is no significant deviation from the expected ratio, then the allelic variation in the natural population is assumed to be neutral and is influenced by genetic drift (Beebee & Rowe, 2008). The probability of identity determines the likelihood that two individuals share the same genotype across the loci examined. Since the genotype of individuals is comprised of the genotypes of its parents, probability of identity ($P_{ID}$) values can be a useful tool to determine the diversity of the population as well as determine likely parentage of offspring using allele frequencies. This principle is contingent on the number and specificity of markers used (Guerier et. al., 2012). With markers that target the most diverse regions of DNA, differences among individuals are greatened and thus will enhance the confidence in the ability to identify one individual from every other in the population. Simulations from computational programs allow for confident assignments of parentage when all individual are unknown. This method is also useful in determining the specificity of markers used for genetic analysis (Guerier et. al., 2012). Combining existing pedigree and studbook information with molecular techniques is a useful strategy to maximizing genetic diversity by ensuring that individuals that have the least amount of co-ancestry breed. This will assure that the captive breeding program produce the most genetically variable offspring for re-introduction. In this study we will address the conservation efforts associated with managing a captive breeding population of *Heloderma horridum charlesbogerti*.

The Guatemalan beaded lizard, *Heloderma horridum charlesbogerti*, with 200 to 250 wild individuals endemic to the Rio Motagua and Rio Lagarto valleys of Guatemala, is

critically endangered (Campbell & Vannini, 1988) (Anzueto & Campbell, 2010). Geography of the native range lead speculations that this sub-species arose from individuals of Mexican beaded lizards that migrated into the Motagua and Lagarto Valleys via a Pacific path (Rodrico & Campbell, 2010). With the expanse of human population, leading to native range and habitat fragmentation, this subspecies is at risk of extinction (Beck, 2005). In 2005, Zoo Atlanta began maintaining a captive population of nine wild caught adults and six offspring produced in captivity (Janice, 2011). Breeding information for captive born offspring was logged into the North American Regional Studbook on the Mexican Beaded Lizard (Janice, 2011). Captive bred offspring were the result of parings in 2002 (three offspring), and in 2005 (two offspring) at the San Diego Zoo (Janice, 2011). The sixth offspring was born at Zoo Atlanta in March of 2012 as a result of pairings in 2011.

The objectives of this study are to: 1. Use microsatellite loci to estimate genetic diversity of wild caught individuals in the captive Zoo Atlanta population. 2. To identify the sires for offspring born in 2006 and 2012 and use genetic data to revise the existing. 3. Assess the accuracy microsatellite markers for their application to paternity.

## METHODS

### Blood collection:

Blood was collected from the caudal vein of the tail from all 15 individuals at Zoo Atlanta and centrifuged for 10 minutes to separate red blood cells (RBC) and plasma. Plasma and RBC's were stored frozen at -20$^{o}$C until analysis.

### DNA extraction:

DNA was extracted by adding 0.5µl of RBC to 70 µl cell-lysis/proteinase-K buffer (10 mM of Tris, 50 mM KCl, , 0.2 mg/ml proteinase-K, pH. 8.0) followed by incubation for 1 hour at 65 ˚C and 15 min at 99 ˚C (Lee & Frost, 2002). Supernatant was separated from cellular debris by centrifuging at 1400 RPM for 5min.

### Genotyping:

Individuals were genotyped at five microsatellite loci (Helo 1, Helo2, Helo3, Helo 5, and Helo6) designed by Feltoon et. al., (2007). Polymerase chain reactions were performed in 10 µL volumes: 7µL of 1.5mM $MgCl_2$Taq master mix and ultrapure $H_2O$, 1µL of 1µM labeled forward and reverse primer, and 1µL of DNA. Amplifications consisted of 30 cycles of 95 $^{o}$C for 30 s, 60 $^{o}$C for 15 s, and 72 $^{o}$C for 10 s, followed by a final extension at 72 $^{o}$C for 5 min.

PCR products were run on an ABI 3500 Genetic Analyzer with an internal size standard. In one well, 1µL of PCR product labeled with of Helo1 primer, and 0.94µL of PCR product labeled with Helo2 and 1 µL of PCR product labeled with Helo3 was mixed. A second well was used, to mix 1.7 µL of PCR product labeled with Helo5 and 1 µL of

PCR product labeled with Helo6. Genotypes for each individual were determined manually using the program Gene Mapper v 4.1® (Park, S. D. E.,2001).

Statistical Analysis:

Genetic Diversity

Allelic frequencies, observed and expected heterozygosity, and deviations from the expected heterozygosities found in populations under Hardy-Weinberg Equilibrium (HWE) for wild caught individuals were calculated using Genpop 4.0.10® (Raymond M. & Rousset F., 1995), (Rousset, F., 2008).

Pedigree Analysis

To create an accurate pedigree we consulted the 2011 North American Regional Mexican Beaded Lizard Studbook 7th Edition (Janice, 2011). Records show that pairings of male A06107 and female A06110 led to the offspring female A96101, male 11R059, and male 11R060 in 2004. In 2006, a trio comprised of female, male A06107 and male A06106, produced in two offspring: male 11R061, and female 11R062. Herpetology department staff stated that in 2011 a trio comprised of female A06105, male A060104 and male A06109 produced individual 12R009. Genetic profiles of both males in the 2006 and 2011 trios were aligned with the genotypes of respective offspring and females.

Males were excluded is if their genotype profile, mixed with the known dam, mismatched with the resulting offspring's genotype. This information was used to produce a pedigree for all individuals at Zoo Atlanta (Figure 1b).

Marker Accuracy

We also wanted to assess specificity microsatellite marker specificity in terms of captive breeding planning. Using computational methods, the same mother/offspring pairs described above but included all males as potential sires in the analysis. Using CERVUS Version 3.0.3® simulations of parentage of all offspring, were generated. Confidence was calculated using Likelihood of Difference (LOD) scores, with a 95% (fixed) and 80% (relax) levels. Scores were calculated by allelic frequency of polymorphic markers (Helo1, Helo2, and Helo3) (Kalinowski, ST, Taper, ML & Marshall, TC, 2007). We allowed the program to select the most likely sire and the second most likely sire; higher scores were associated with greater confidence in parentage assignment. Paternity assignments based computational methods were compared to results (most likely sire) formulated by exclusion analysis (Figure1a, b). For the purpose of determining how specific individual markers were at identifying individuals in this population, Probability of Identity ($P_{ID}$) was also calculated. Specifically to this group, $P_{ID}$ shows the likely number of individuals who share the same genotype across loci tested and whether or not markers alone can identify one individual from every other individual in this population.

# RESULTS

## Genetic Diversity

Three of the five loci tested were polymorphic. In addition, observed heterozygosity was not significantly different from expected heterozygosity under Hardy-Weinberg Equilibrium: H = 3.4692, *df* = 6 P = 0.7481 (Table 1). The number of alleles per locus average 1.8, ranging from 1-3. The observed number of heterozygotes ranged from 0.0 - 0.7 while the expected number of heterozygotes ranged from 0.27 – 0.45 (Table 1). Loci Helo5 and Helo6 were found to be monomorphic for this population.

## Pedigree Analysis

The current effective population at Zoo Atlanta is comprised of six individuals in which all wild caught females and three of the six males sired offspring (Figure 1 b). In 2003, male A06107 sired female A96101, male 11R059, and male 11R060. For offspring born in 2006, both A06107's genotype and A06106's genotype had the potential to match correctly with the genotype of offspring female 11R062 however, with information from herpetology staff at Zoo Atlanta; A06106 was deemed the sire of female 11R062. The genotypic profile of 12R009, born in 2012, was homozygous at 214 for Helo3, while A06109 was homozygous for Helo3 at 210, therefore we excluded A06109 as a potential father due to genotype mismatch at Helo3 and assigned A06104 as the sire of 12R009 (Figure1 b).

Marker Accuracy

To test the specificity of microsatellites used in this study, true pedigree information was compared to parentage assignments generated by the program CERVUS© (Figure 1a). We found that available markers are not accurate enough to identify all individuals. Using CERVUS©, parentage assignments were correct for 11R059 (LOD = 0.662), 11R060 (LOD = 0.662), and 11R062 (LOD = 0.208). For the trio in 2011, candidate father A06108 was assigned is the most likely sire however, A06108 was never in the enclosure with female A06105. The correct sire (A06104) was assigned as the second most likely sire, with a LOD score of 0.378. In addition to 12R009 being incorrectly matched with A06108; neither offspring A96101 nor 11R061 were correctly assigned their true sires, A06107 and A06106 respectively. Thus using only genetic markers, we only determined the true sire only 50 percent of the time (Figure 1 a, b). This is consistent with, the $P_{ID}$ values for the three polymorphic loci: 0.5254 for Helo1, 0.3883 for Helo2, and 0.3883 for Helo3 (Table 1). We found that male A06108 and male A06104 share the exact genotype for across the loci tested.

# DISCUSSION

## Genetic Diversity

Characteristics of a long lived species (Beck 2005) combined with most likely small founder and effective populations (Ariano-Sánchez 2006), accurate estimates of genetic diversity using microsatellite markers among individuals of *H. h. charlesbogerti* prove difficult to make. Markers used in this study were designed for the entire Heloderma horridum group which comprises of four subspecies ranging from Sonoran Mexico to central Guatemala (Feltoon et. al. 2007). Therefore they display low specificity when utilized on small number of individuals isolated in a 24,000 acre range. In this study, wild caught individuals in the captive breeding *H.h.charlesbogerti* population show little allelic variability across the loci examined. Feltoon et.al. (2007) which also showed three allele polymorphisms for the loci marked by Helo 1 and, unlike our results, showed that Helo5 and Helo6 mark polymorphic loci suggesting that the genetic diversity is highly influenced by sample size. Our genetic diversity sample only includes the original nine wild caught founder individuals, compared to 18 individuals by Feltoon et.al. (2007), suggesting that this discrepancy may be attributed to sampling different individuals and wild caught individuals dying in between the time of the two studies. With this in mind, our group of wild caught individuals in the breeding population may have lost the genetic diversity in which Helo5 and Helo6 may have marked polymorphic loci. As a result of small sample size, observed heterozygosities showed no deviation from heterozygosities calculated under HWE conditions, however, there is little statistical confidence associated

with the results because of the limited number of tested individuals. Therefore, across the loci tested, we assume that genetic variation found in these wild caught individuals was attributed to variation in the sampled individuals who were breeding not outside selective or migratory forces.

For our population, we conclude allelic similarity across the loci tested is a result of genetic drift driving the loci to uniformity (Beebee & Rowe, 2008). Since the accuracy of serviceable markers used in this study was poor, we cannot accurately assess the overall genetic diversity of Guatemalan beaded lizards with available markers. In addition, we cannot conclude whether or not the genetic diversity in the assurance population at Zoo Atlanta accurately reflects the genetic diversity in the wild population. However, with a sample size equaling three-four percent of the wild consensus population (Ariano-Sánchez, 2006); we assume that the diversity in wild caught individuals accurately reflects diversity in the wild population. However, without sampling wild individuals and comparing genotypes, we cannot conclude how accurately the genetic diversity in the captive assurance population reflects genetic diversity in the wild population.

Pedigree Analysis

With the production of six offspring from three different breeding events, breeding Guatemalan beaded lizards in the United States has had success (Janice 2011). Studbook and pairing information was critical to determining paternity, without this information to support genetic information; we could not be able to confidently assign correct sires to all

offspring (Figure 1b). The pedigree shows that half of the wild caught breeding males are reproductively successful and all of the wild caught females are able to produce viable offspring (Figure 1). Pedigree results show that all offspring born in 2004 were sired by A06107. Offspring in 2006 were sired by A06106 and their genotypes reflect 50% of alleles from A06106 and 50% of alleles from A06100 (dam). Microsatellite markers were useful in determining the paternity of 12R009. The offspring born in 2012 (12R009) was sired by A06104. There are three males (A06108, A06103, and A06109) that have not sired offspring and potentially could sire offspring in the future (Figure1b).

Marker Accuracy

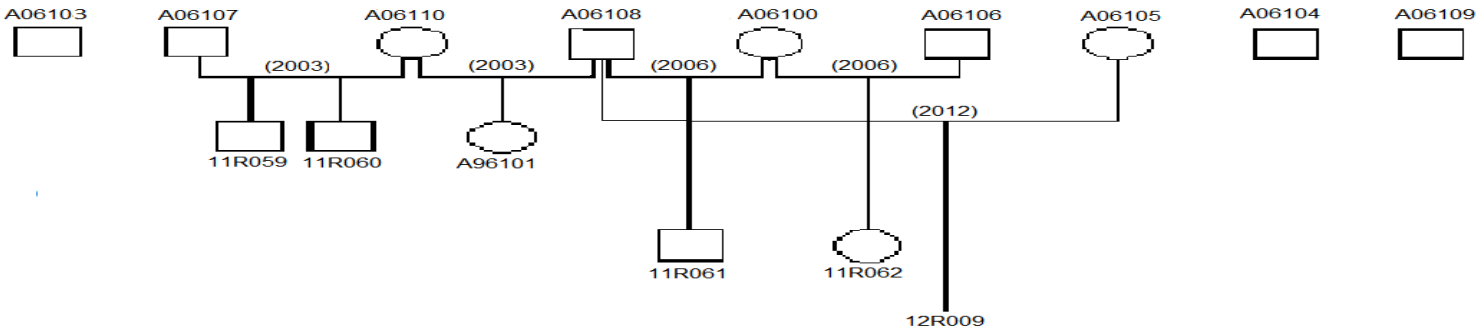
Maintaining diversity is critical to the success of assurance populations. However, captive breeding inherently creates a founder population effect because of the limited number of individuals that can be kept in a program compared to the wild. This is compounded by long- lived animals that stem from a geographically small area. Genetic drift is the main reason for loss of alleles in captive populations (Falconer & Makay, 1996) and the minimization of genetic drift is essential to the management of assurance populations. For captive breeding planning, pairing of the most genetically diverse adults in the captive population will ensure the production of most genetically diverse offspring. In our population, high genetic similarity impeded markers to identify individuals based on genetic differences. Using current markers, we can only accurately assign paternity to an offspring in this population 50% of the time (Figure 1a). With a combined $P_{ID}$ value of

0.0792 and a breeding population of nine individuals we can say that 1.2 out of 15 individuals share the same genotype across the loci analyzed. This low variability is due to combination of high co-ancestry among individuals and a long regeneration time. Thus, markers used in this study provide little use in captive breeding planning without supporting information from zoo records and studbooks. This low specificity illustrates the difficulties in captive breeding planning when the individuals of focus suffer from high genetic similarity. In addition, this study calls for new, more specific markers, to be developed for populations with high co-ancestry.

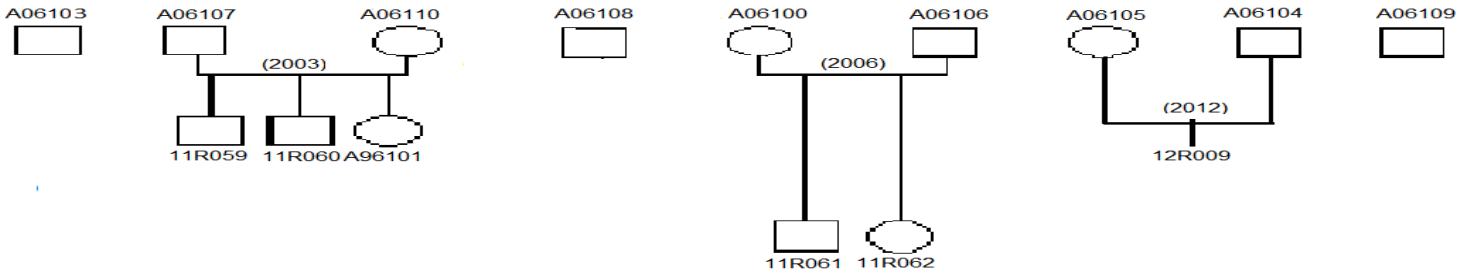
This study shows the value of combining genetic data with zoo records to maintain genetic diversity in captive populations. The combination of genetic analysis and zoo records allowed us to determine true paternities of all offspring in the captive population. This data allows Herpetology staff at Zoo Atlanta to pair individuals (or create breeding groups) that would yield the most genetically variable offspring and avoid inbreeding depression for their assurance population. In the future new individuals can contribute to the gene pool of the captive population and Zoo Atlanta can have a genetically diverse reservoir population that can continue to survive in captivity. In addition, Zoo Atlanta can produce genetically variable individuals that could be released into their native habitat so that a wild population can be re-established and lessen the threat of extinction.

Table 1. Genetic Diversity of the Population. The table shows the variation in the microsatellites used in the study; number of alleles (A), allelic frequency (AF), observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), significance value between observed and expected heterozygosity (P). The probability of Identity ($P_{ID}$) for each marker was also calculated using allelic frequency data. The $P_{ID}$ value for all markers combined is 0.0791.The Hardy- Weinberg equilibrium shows the Chi-Square value (H), degrees of freedom (*df*), significance (P) for all wild caught individuals in the captive population.

| Variation Across Five Microsattelites Used in the Study | | | | | | | |
|---|---|---|---|---|---|---|---|
| Primer | A | AF | $H_o$ | $H_e$ | P | $P_{ID}$ | |
| HELO1 | 1 | 0.056 | 0.3000 | 0.2760 | 1.0000 | 0.5254 | |
| | 2 | 0.0833 | | | | | |
| | 3 | 0.011 | | | | | |
| Helo2 | 1 | 0.389 | 0.5000 | 0.4520 | 1.0000 | 0.3883 | |
| | 2 | 0.611 | | | | | |
| Helo3 | 1 | 0.611 | 0.7000 | 0.4520 | 0.9174 | 0.3883 | |
| | 2 | 0.389 | | | | | |
| Helo5 | 1 | 1 | 0.0000 | 0.0000 | 1.0000 | 1.0000 | |
| Helo6 | 1 | 1 | 0.0000 | 0.0000 | 1.0000 | 1.0000 | |
| | | | | | | | |
| Hardy Weinberg Equilebrium | | | | | | | |
| Population | N | H | *df* | P | | | |
| A | 9 | 3.46 | 6 | 0.748 | | | |




a)




b)

Figure1. Revised pedigree of parent offspring relationships in Zoo Aatlanta's breeding colony. Top pedigree (a) was generated by soley genetic data. A96101, 11R061, and 12R009 were all incorrectly assigned when tested by the principle of identity by descent alone. The bottom pedigree (b) is the true pedigree derived from a combined approach. Pedigree (b) was a result of the exclusion principle: comprised of genetic data, pairing records and studbook information. Identification numbers are assigned to all individuals, birth years are assigned to captive-bred offspring resutling from the three breeding events.

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