REPRODUCTIVE ENDOCRINOLOGY OF NESTING LEATHERBACK SEA TURTLES IN ST. CROIX, U.S. VIRGIN ISLANDS

A Dissertation

by

JEANNE ALEXANDER GARNER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Wildlife and Fisheries Sciences

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Approved by:

Co-Chairs of Committee,	Duncan MacKenzie
	Delbert Gatlin
Committee Members,	Andre Landry
	Rosemary Walzem
Head of Department,	John Carey

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ABSTRACT

Reproductive Endocrinology of Nesting Leatherback Sea Turtles in St. Croix, U.S. Virgin Islands.

(May 2012)

Jeanne Alexander Garner, B.S., Cornell University; M.S., Florida Atlantic University

Co-Chairs of Advisory Committee: Dr. Duncan Mackenzie
Dr. Delbert Gatlin III

The global population of leatherback sea turtles is decreasing worldwide, with extinction predicted for some populations within 15 years. The population of leatherbacks nesting at Sandy Point National Wildlife Refuge (SPNWR), St. Croix, USVI, displayed a significant population increase from 1982 - 2001 but has experienced a slowed recovery since then. To better understand the causes of this decline, a historical database of SPNWR nesting female data was utilized to investigate trends in reproductive indices. Since 2001, average remigration interval (RI) has increased significantly, while average number of clutches laid, hatch success, hatchling production, and the percentage of neophytes recruited annually have decreased. Annual remigrant numbers have been stable to increasing, suggesting that adult survivorship remains high.

To assess whether maternally-derived factors may be influencing clutch production and low hatch success, blood samples were collected by saturation sampling during nesting. Circulating estradiol, testosterone, and progesterone were evaluated in

conjunction with reproductive data. All hormones were highest at deposition of the first clutch and declined progressively with each consecutive clutch, as previously observed in other sea turtle species. Increased clutch production in remigrants was associated with higher estradiol levels compared to neophytes, presumably due to ovarian size and maturity. Contrary to observations in Pacific leatherbacks, progesterone decreased significantly with successive nests and total levels of estrogen were significantly lower, suggesting Atlantic leatherbacks may undergo a longer migration or spend more time in the feeding grounds prior to migrating.

Linear Mixed Effect (LME) modeling was employed to determine whether hormone levels at nesting might serve as indicators of reproductive variables. Because models for all hormones were individual specific, a population model could not be developed that effectively utilized hormone levels at nesting to predict clutch size, hatch success, age or RI. However, number of clutches laid may potentially be predicted based on individually tailored estrogen models. Decreased recruitment (due to increased mortality of early life stages, altered sex ratios, or delayed age to sexual maturity), decreased productivity, and increased RI (possibly due to diminished foraging ground productivity) appear primarily responsible for current population trends which threaten the population's future.

DEDICATION

To AAM 898 and NNE 239.

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CHAPTER I

GENERAL INTRODUCTION : LEATHERBACK REPRODUCTIVE BIOLOGY AND CONSERVATION

Seven sea turtle species exist worldwide, with six of the seven listed as either threatened or endangered on the International Union for Conservation of Nature (IUCN) Redlist (Sarti Martinez, 2000). Of these seven species, the leatherback (*Dermochelys coriacea*) (Fig. 1.1) is morphologically and physiologically unique when compared to all other species of sea turtles. The leatherback is a critically endangered species that inhabits all of the world's oceans (except the Arctic and Antarctic). It is the oldest living, largest, and most divergent species of sea turtle with a unique life history and reproductive characteristics. Leatherback sea turtles are pelagic from hatching to adulthood (Bolten, 2003), and feed on jellyfish, salps, and other soft-bodied prey throughout their lives (Leary, 1957; Bleakney, 1965; Lazell, 1980; Collard, 1990; Bjorndal, 1997). The foraging grounds and diving patterns of leatherbacks are determined by the presence of their jellyfish prey. Daily dives tend to follow the migration of jellyfish in the deep scattering layer, with deeper dives occurring during the day, and shallower dives occurring at night (Eckert, et al. 1989).

This dissertation follows the style of *General and Comparative Endocrinology*.

Unlike other species of marine turtles, the leatherback grows to a very large size in a very short period of time (Zug and Parham,1996). The average adult leatherback is approximately 270-360 Kg (600-800 pounds) and will reach this size in a period of 9-14 years, the estimated age of maturity for this species (Zug and Parham, 1996; Dutton personal communication). Rapid growth to such a large size, on what has been determined to be a very nutrient poor diet, still puzzles biologists (Lutcavage and Lutz, 1986). It is estimated that an adult leatherback must consume the equivalent of 91 Kg (200 lb) of jellyfish each day to maintain itself (Bjorndal, 1997).

FIG. 1.1. Adult and hatchling leatherback sea turtle (*Dermochelys coriacea*).

The large size of the leatherback is an important factor in its ability to migrate to colder latitudes, allowing movement into waters colder than 26 °C when a sufficient size (curved carapace length (CCL) >100 cm) is reached (Eckert, 2002). After attaining this size, turtles over 100 cm CCL may be found in waters as cold as 8 °C, indicating an onset of partial endothermy (commonly known as gigantothermy) at this size (Eckert, 2002). In addition to large size, large amounts of adipose tissue and high oil content in the shell, the leatherback has another unique adaptation for thermoregulation. This adaptation is a counter-current exchange system of blood vessels in the extremities that assists in regulation of body temperature (Paladino et al., 1990).

The ability to thermoregulate is unique when compared to other sea turtles and reptiles in general. In addition, the leatherback also possesses exceptional diving capabilities. The leatherback is one of the deepest divers in the ocean, diving to depths greater than 1200 meters (Eckert, 1992). In order to dive to these depths, the leatherback has evolved to store oxygen primarily in the blood and tissues, rather than in the lungs (Lutcavage and Lutz, 2003). The leatherback also has significantly higher levels of hemoglobin, myoglobin and hematocrit when compared to those of other sea turtles (Lutcavage and Lutz, 2003).

Leatherback sea turtles will migrate thousands of miles from foraging grounds in the North to tropical and sub tropical nesting beaches (Figure 1.2). In the Atlantic, leatherbacks nest during the spring and summer (from March through August), while in the Pacific they nest during the winter. Leatherback turtles prefer dynamic beaches (Hendrickson and Balasingman, 1966; Schulz, 1975), with open sand, such as that at

Sandy Point National Wildlife Refuge, St. Croix (in the Atlantic) (Figure 1.3) and Las Baulas, Costa Rica (in the Pacific).

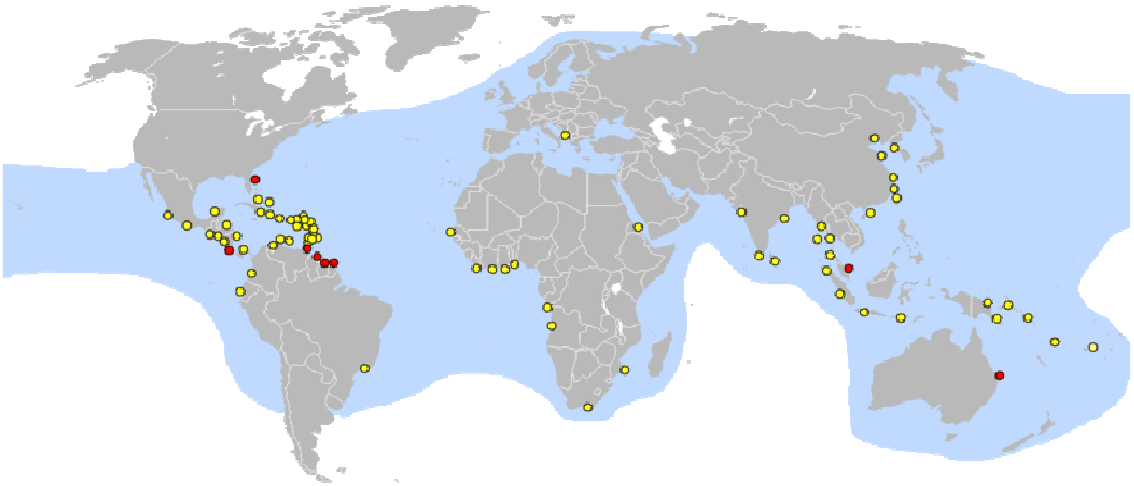

FIG. 1.2. Global distribution of leatherback nesting beaches. (From Wikipedia).

FIG 1.3. Location of St. Croix. This Caribbean island (17° 45′ 0″ N, 64° 45′ 0″ W) is the home to a key nesting population of Atlantic leatherback sea turtles.

The global population of leatherbacks is decreasing significantly worldwide, with the Pacific population declining by over 80 % in the last 15 years, and extinction predicted within the next 15 years (Spotila et al., 1996; Sarti Martinez, 2000; Spotila et al., 2000). Although the decline in the Atlantic population has not been as severe, nesting numbers in the Atlantic have been significantly lower than those observed in the Pacific in the previous decade (Sarti Martinez, 2000; Spotila et al., 1996; Spotila et al., 2000).

Leatherback population demise has been attributed to the harvest of eggs and adults, fisheries interactions and incidental capture, and loss of nesting habitat (Ross,

1979; Spotila et al., 1996; Sarti Martinez, 2000; Spotila et al., 2000). Although leatherbacks are hypothesized to reach sexual maturity at an earlier age than most sea turtle species (9-14 years versus 20-40 years for most species) (Zug and Parham, 1996; Dutton personal communication), population recovery has been difficult and slow. Leatherbacks nest up to 11 times in one season (with an average of 6 clutches per season), have an average inter-nesting interval of 9-10 days, and an average remigration interval (RI, or number of years since previous observed nesting) of 2-3 years (Boulon et al., 1996). This is unique when compared to other turtle species that lay significantly fewer clutches per season (only 2-4) and have a significantly greater inter-nesting interval (Miller, 1997). The unique reproductive biology of this species suggests that with protection and conservation efforts, recovery could be swift and significant when compared to that of other species. However, this is not necessarily the case. Although leatherbacks lay more clutches and have a decreased remigration interval, additional reproductive characteristics of this species may be impeding reproductive success and thus, ultimately, population recovery.

Several reproductive factors have contributed to the population decline observed in leatherbacks. Leatherbacks lay fewer eggs per clutch when compared to other sea turtle species. Average clutch size for leatherbacks is 80 yolked eggs with 20-40 yolkless eggs, or shelled albumin globs (SAGs) (Bell et al., 2003) also deposited. Leatherbacks are the only sea turtle species to consistently lay SAGs. The purpose of laying SAGs has not yet been determined, but hypotheses include: Addition of moisture

to the nest, assistance with pore spacing, predator avoidance, and excretion of left-over material (Caut et al., 2006; Blanvillain et al., 2011).

Leatherbacks also have the lowest hatch success (50-55 %) of any sea turtle species (Whitmore and Dutton, 1985; Boulon, 1992; Chan and Liew, 1996). This is extremely low when compared to the greater than 80% average observed in other sea turtles (Miller, 1997). Low hatch success in leatherback nests has been determined to be due to high embryonic mortality rather than to infertility (Bell et al., 2003), with mortality occurring during latter developmental stages in at least one population (Garner et al., 2006; Garner and Garner, 2007). Although extrinsic factors such as temperature, moisture, partial pressure of oxygen and carbon dioxide have been investigated, conventional studies of the nest environment have not yielded definitive explanations for low hatch success (Wallace et al., 2004; Garrett et al., 2010). Lack of conclusive extrinsic data suggests intrinsic, maternally-derived factors contribute to the low hatch success observed in leatherbacks. To date, few attempts have been made to identify and correlate intrinsic factors with hatch success. This is due mainly to a lack of available, well studied populations in which both hatch success and intrinsic factors can be examined in individual females with known nesting histories.

Compounding the nest mortality problem is a progressive decline over time in the average number of nests laid per female (Garner and Garner, 2009), which significantly impacts hatchling production. In one well studied population the average number of nests laid per female has decreased over the past 30 years from almost seven per individual to less than four (Garner and Garner, 2009). This may be due in part to

the increased number of neophytes in the nesting population. Neophyte turtles (no nesting experience) lay fewer clutches in a given season when compared to remigrant nesters (with previous nesting experience) (Garner, unpublished results). This observation suggests an ontogeny of fecundity, with the number of clutches laid increasing with increased reproductive experience. However, managers are currently unable to determine an individual's reproductive capacity (number of potential clutches), or to differentiate remigrant turtles from neophyte turtles without a significant investment of resources, impeding efforts to effectively predict reproductive output on specific beaches. Such information could also provide a foundation for studies that may identify potential environmental and anthropogenic factors that impact the number of nests laid.

Traditionally, studies of sea turtle reproductive output have focused on saturation tagging and basic data collection (species identification, morphometrics, behavior, egg counts) to monitor population trends and understand the basic biology of this unique animal. However, there are limitations to this approach. Saturation tagging programs require multiple years of intense, comprehensive beach coverage to ensure that all females are observed, tagged, and recaptured. This requires substantial resources (money, time, staff, and equipment) over multiple years. A minimum of 2-3 years is required before initial tag returns will be observed, and several more years are required before a project can ensure that untagged individuals are indeed new recruits (neophytes) rather than remigrants from a previous year who nested prior to the project inception. In addition to the extensive resource and funding requirements, the data collected are

limited to direct extrinsic observations. This approach, therefore, will not quantify maternally-derived factors that might serve as indicators of hatch success, clutch number, or reproductive age in this species. Future research is needed to test whether techniques to quantify intrinsic, maternal characteristics can be effectively applied to wild leatherback populations and provide predictive information on reproductive capacity of individual females. Intrinsic characteristics that may be applicable and bear further investigation are circulating sex steroid hormone levels.

Steroid hormones and reproduction in sea turtles

Sampling and analysis of plasma steroid hormones throughout a nesting season may prove a valuable resource for sea turtle biologists, allowing them to further understand and potentially predict specific reproductive parameters within a nesting population (Owens, 1997). The three primary steroid hormones studied in the regulation of reproduction are estradiol, testosterone, and progesterone. These hormones are produced via enzymatic modification of the steroid precursor cholesterol in gonadal tissues (as well as fat, adrenal glands and the central nervous system) (Hadley, 2000). Changes in levels of these steroid hormones in marine turtles initiate physiological and behavioral processes such as vitellogenesis, follicular development, ovulation, courtship behavior, and receptivity (Owens and Morris, 1985). They have been investigated in numerous vertebrate species, including sea turtles, in order to better understand the

endocrine regulation of reproduction, in the hopes that endocrine data may be used to evaluate the reproductive status of wild populations.

Analysis of blood samples from adult Kemp's ridley sea turtles (*Lepidochelys kempii*) revealed distinct, seasonal cycles in steroid hormones, particularly testosterone. Testosterone, the most commonly measured steroid hormone in sea turtles, has important roles in the reproductive cycles of both male and female sea turtles. Knowledge of circulating testosterone levels has been applied to predict the sex of immature sea turtles in multiple species, as well as determine reproductive condition (Owens, 1997). Testosterone, in association with gonadotropin, triggers the development of secondary sexual characteristics in male sea turtles (i.e., elongated tail and penis, curved front claws, and softened plastron) as well as stimulating courtship and mating behavior in adult individuals (Owens, 1997). Male Kemp's ridley turtles exhibit a dramatic rise in testosterone approximately 4-5 months before mating (Rostal et al., 1997). This increase is associated with spermatogenesis, while a subsequent decrease in testosterone occurs during the actual mating period (Rostal et al., 1997). This pattern of declining testosterone has also been observed in captive, as well as wild male green sea turtles (*Chelonia mydas*) (Licht 1982; Wibbels et al., 1990).

Female Kemp's ridley and green sea turtles display a similar spike in testosterone levels prior to mating (Rostal et al., 1997; Haman et al., 2002). A rise in testosterone in females is associated with follicular development and possibly stimulation of female receptivity and courtship behavior (Hamann et al., 2002). As in the male, levels drop off during the courtship and mating period. Additionally, the sequential loss of follicles

due to consecutive ovulations results in a progressive decline in circulating testosterone levels to basal levels at the end of the nesting season (Owens, 1997; Rostal et al., 1998). Measurement of testosterone levels in sea turtle species may thus provide a useful indicator of receptivity, fertility, and reproductive condition.

Estradiol is another frequently-measured reproductive steroid hormone. Estradiol triggers vitellogenesis (yolk deposition) in reptiles (Ho, 1987), and increased circulating levels are directly correlated with mobilization of vitellogenin, increased oviducal weight, and increased circulating serum protein and calcium levels (Owens and Morris, 1985; Owens, 1997; Heck et al., 1997). Increased estrogen is also linked to appetite suppression in sea turtles (Owens and Morris, 1985). Maturation of the ovary and a concomitant increase in estradiol are observed 4-6 months prior to mating in multiple sea turtle species (Rostal et al., 1997; Owens, 1997; Rostal et al., 2001).

Declining estrogen levels imply that vitellogenesis in sea turtles may be complete or near completion prior to mating and arrival at nesting beaches (Wibbels et al., 1990; Rostal et al., 1997; Rostal et al., 1998; Rostal et al., 2001). However, since sea turtles may not arrive at the nesting beach with a full complement of mature follicles, but instead contain multiple size classes of follicles that will mature consecutively for successive clutches, it is also possible that a minimal level of estradiol may be required for continued vitellogenesis and follicle maturation throughout the nesting season in some species (Owens and Morris, 1985; Blanvillain et al., 2011).

A significant and consistent decline in estrogen (together with a dramatic surge in circulating testosterone levels to their apex) may serve as the stimulus for migration to

the nesting grounds (Owens, 1997). Estradiol levels continue to decrease with each consecutive nesting event to basal levels at the end of the nesting season (Owens, 1997; Rostal et al., 1998). Estradiol, therefore, has both physiological and behavioral roles in reproductively-active female sea turtles and has also been applied, in association with ultrasonography, to determine the reproductive condition of individual female turtles (i.e., inactive versus reproductively active, which is evidenced by the presence of oviducal eggs and vitellogenic follicles) (Owens, 1997; Rostal et al., 1998). Analysis of estrogen levels in reproductive females may therefore provide a useful indicator of reproductive condition, as well as provide an indicator of the reproductive potential (i.e., number of clutches to be laid) for an individual female.

Serum progesterone has also been monitored in reproductively-active sea turtles turtles (Owens and Morris, 1985; Miller, 1997). Progesterone is released from the corpus luteum post ovulation and stimulates production of albumin proteins in the oviduct as well as the subsequent secretion of albumin from the anterior portion of the oviduct (albumin gland) in sea turtles (Miller, 1997; Owens and Morris, 1985). Progesterone increases sharply 24-48 hours post-nesting in the olive ridley, green, and loggerhead sea turtles (Licht et al. 1979; Licht et al., 1982; Wibbels et al., 1992). A high level of serum progesterone is also observed early in the season and is directly correlated with nesting and ovulation of the first clutch of eggs (Rostal et al., 1997). Additionally, levels in the Kemp's ridley have been shown to decrease with each clutch laid throughout the nesting season (Rostal et al., 1997). Progesterone is vital to albumin deposition and has also been suggested to play a role in egg retention in sea turtles

exhibiting arribadas (Owens and Morris, 1985). Progesterone measurements, in addition to testosterone and estrogen, may thus serve as an additional, useful indicator of reproductive potential in female sea turtles.

Basic functions and seasonal trends for reproductive steroid hormones have been investigated in multiple sea turtle species, with similar trends being observed across species. Rostal et al. (2001) conducted a study on Pacific leatherbacks and confirmed that, in spite of their unique physiology when compared to that of other sea turtle species, leatherbacks exhibit similar hormone trends as other sea turtles with regard to estrogen and testosterone. The leatherback exhibits the step-wise reduction in testosterone and estradiol throughout the nesting season (as evidenced by other species of sea turtles), although circulating levels of these hormones are much higher when compared to those of other species (Rostal et al., 2001).

Testosterone cycles have been reported for the Pacific leatherback turtle, with initial plasma testosterone levels ranging from 3.5 to 22.0 ng/ml at the beginning of the season, and declining to low levels (1.73 ± 0.34 ng/ml) at the close of the nesting season (Rostal et al., 2001). In the beginning of the nesting season plasma estradiol levels in female leatherback sea turtles ranged from 57.7 to 480.5 pg/ml, with an average of approximately 191 pg/ml estradiol (Rostal et al., 2001). Levels subsequently declined significantly to an average of 76.5 pg/ml at the end of the nesting cycle (Rostal et al., 2001). The phenomenon of a step-wise decrease in progesterone, however, has not been observed in the leatherback sea turtle. In this unique species, levels of progesterone appear to remain constant over the course of the nesting season (Rostal et al., 2001).

This may be related to the production of shelled albumin globules, an additional unique reproductive attribute of this species, although further investigation is required to determine if this is the case. The difference in magnitude of the hormone levels observed between leatherbacks and other sea turtles may be due to a difference in ovary size, receptor affinity, or a unique hormone threshold and bears further investigation. However, since the leatherback exhibits similar estradiol and testosterone trends as other species, it is believed that circulating hormone levels may also be applied in leatherbacks to determine individual sex, reproductive potential and reproductive condition, as well as address other potential research and conservation applications previously employed with other species.

Application of steroid hormones in sea turtle conservation

Maternal steroid hormone levels have recently been utilized to address fundamental reproductive questions in several species of oviparous vertebrates. Work conducted with Kemp's ridley and green sea turtles (Owens and Morris, 1985; Rostal et al., 1998; Hamann et al., 2002) has supported the use of steroid hormones as indicators of clutch number (Rostal et al., 2001). Levels of testosterone, estrogen, and progesterone were observed to decrease as follicles were ovulated, with levels decreasing proportionally in accordance with each nesting event. This decline was ascribed to a progressive depletion of mature follicles in the ovary with progressing clutch number (Rostal et al., 2001). This endocrine approach was successfully utilized to predict clutch

number of the Kemp's ridley (Rostal et al., 1998), thus suggesting that a simple, single blood sample could serve as an effective metric for identifying future nest production in this species.

Maternal testosterone, estrogen, and progesterone levels have also been proposed to be useful indicators of hatchling development and hatch success in oviparous species (McCormick, 1999; Eising et al., 2001; Lovern and Wade, 2001). Maternal steroids are deposited in the yolk of eggs during vitellogenesis (Lovern and Wade, 2001). In many bird species rate of embryonic development, hatch success, hatchling mass, and survivability are directly correlated with yolk testosterone levels (McCormick, 1999; Eising et al., 2001; Lovern and Wade, 2001). Increased maternal androgen levels in the yolk generally result in increased hatch success and juvenile survivorship. Testosterone levels are also correlated with incubation length and may influence sex ratios in freshwater turtles (Janzen et al., 1998). Although exact mechanisms are unknown, experimental evidence supports the hypothesis that steroid levels in the egg yolks of turtles reflect the circulating concentrations at the time of follicular development (Janzen et al., 2002). This is contrary to the hypothesis in birds, where evidence supports selective allocation of hormones to individual eggs within a clutch. If maternal levels in leatherbacks similarly determine yolk hormone levels, circulating maternal steroid hormones may potentially play a significant role in determining mortality and hatch success in leatherback clutches as well.

Maternal hormones are also related to physiological parameters such as the age and size of an individual animal (Milnes et al., 2002). Estrogen and testosterone have

been shown to vary with age and body size in American alligator (*Alligator mississippiensis*) populations. In alligators, a direct correlation is observed between hormone levels and the age of individual animals (Milnes et al., 2002). Greater reproductive maturity and experience may impact the size of ovaries that serve as the main source of estradiol and testosterone in the alligator, as well as in sea turtles (Owens, 1997). Thus older, more mature leatherback females with larger ovaries may exhibit higher testosterone levels when compared to those of younger, smaller individuals within the same population. In addition to ovary size, maturity may also impact the total number of follicles, thus affecting reproductive output (clutch size and number). Correlating hormone levels with animal size and age may prove a valid method of distinguishing neophyte from remigrant turtles, as well as help identify the physiological basis for the observed difference in nest production between neophyte and remigrant leatherback turtles.

Steroid hormones play a central role in regulating critical reproductive processes however, their application as a management tool remains unstudied in leatherback turtles. Presumably, since steroid hormone levels decrease as follicles are ovulated, levels will decrease proportionally in accordance with each nesting event, as has been observed in other sea turtle species such as the Kemp's ridley (Owens and Morris, 1985; Rostal et al., 1998; Rostal, 2001; Hamann et al., 2002). In the only study of circulating reproductive steroid hormones in leatherback turtles, Rostal et al. (2001) reported declining levels of estrogen and testosterone for the Pacific leatherback turtle. Highest estrogen levels were observed in females at the beginning of their nesting cycle, with

lowest levels observed at the end of their cycle. Likewise, testosterone levels were highest at initial nesting events and declined to the lowest levels in females at the end of their nesting cycle (Rostal et al., 2001). Although these trends are similar to those observed in the hard shelled turtles (Kemp's ridley, green, and loggerhead) with regard to estrogen and testosterone, overall hormone levels in leatherbacks are higher, and the decline is more gradual. Smaller turtles lay fewer clutches and thus may ovulate up to half of their available follicles at a single nesting event. As a result, steroid hormone levels also dramatically decrease by as much as 50% between consecutive nesting events (Rostal et al., 2001). As with other species, Rostal et al. (2001) concluded that the gradual decline in these hormones was associated with a decline in the number of ovarian follicles and a decrease in size of the ovaries. However, since leatherbacks lay up to 11 clutches their observed decline is more gradual. The phenomenon of a step-wise decrease in the hormone progesterone, however, was not observed in leatherbacks. In this study, levels of progesterone were highly variable and did not correlate with individual nesting events over the course of the nesting season (Rostal et al., 2001). This may be due to the time of sampling or may imply that progesterone levels are somehow impacted by the production of shelled albumin globs (SAGs), a unique reproductive characteristic of this species.

Rostal et al. (2001) confirmed that leatherbacks exhibit similar hormone trends with regard to estrogen and testosterone, thus suggesting that maternal reproductive steroid measurements may provide a powerful tool for answering reproductive questions and may serve as a useful predictor of clutch number in leatherbacks. Rostal et al. (2001)

did not, however, investigate the predictive capabilities of these hormones, and they were unable to associate hormone data with individual reproductive histories or apply endocrine data to answer key conservation and management questions. This was not possible due to low nesting numbers, limited access to neophyte turtles, and insufficient data with regard to reproductive history. Additionally, their data are limited to a single Pacific population. Although similar with regards to basic biology and behavior, Atlantic leatherbacks do exhibit some unique reproductive characteristics when compared to those of the Pacific leatherbacks. Atlantic leatherbacks consistently lay more clutches, produce more eggs per clutch, and have a decreased remigration interval when compared to their Pacific conspecifics (Reina et al., 2002; Dutton et al., 2005; Wallace et al., 2006). The average size (carapace width, length, and weight) of reproductive Atlantic leatherbacks is also greater than that of nesting Pacific leatherbacks, and age to sexual maturity is presumably less. Analysis of hormones in an additional population of Atlantic turtles will serve to expand our knowledge of leatherbacks as a whole, and will provide insight into their unique reproductive physiology.

Objectives

This study will describe the basic reproductive biology for a population of Atlantic leatherback sea turtles. Steroid hormone values and trends will be documented for nesting female turtles. The results will be evaluated in association with analyses of

the long-term reproductive histories documented in the 30 year database. Information will be assimilated and utilized to develop a predictive model for reproductive Atlantic leatherback sea turtles.

This study will serve specifically to:

- Collect consecutive blood samples from known individual female Atlantic leatherbacks throughout the nesting season and measure estrogen, testosterone and progesterone levels.

- Analyze estrogen, testosterone and progesterone data in association with historical data to determine if hormone levels correlate with individual characteristics such as age, size, nesting frequency, and hatch success.

- Utilize observed changes in hormone levels to develop a predictive reproductive model.

- Test the model using additional hormone data.

The results provided in these studies will determine whether reproductive steroid hormone measurement will help answer important questions that plague many sea turtle conservation and management programs, such as: 1) How do I identify which nesters

may have laid early-season nests? 2) Have nesters that arrive late in the season previously laid elsewhere, or are they just late migrators? 3) How can I determine definitively if neophytes are indeed new recruits, or if they are remigrants from another population or beach which have evaded previous observation and tagging? Individual blood samples may ultimately be utilized by beach managers to resolve these issues, assuming useful indicators of clutch production and individual age can be identified. Currently, no specific indicators for these important reproductive parameters have been identified in this species. This study will test whether estrogen, progesterone, and testosterone levels, evaluated in the context of historical reproductive data can serve as the much needed indicators for answering the aforementioned questions. This approach could benefit startup programs as well as large or diffuse nesting assemblages where the ability to successfully tag all turtles is limited. The ability to accurately designate turtles as either remigrants or neophytes, especially when tagging data are limited or lacking, is an extremely valuable conservation and management tool. Evaluation of these indicators may also provide additional insight with regard to hormonal influences on other reproductive characteristics of a population, such as low hatch success, remigration interval, advanced age, and size. Hormone levels may also be characterized and compared among leatherback populations and among sea turtle species to enhance our basic knowledge of hormone trends and function in these unique reptiles.

In order to effectively evaluate the utility of endocrine data for the identification of the reproductive status of individual turtles, hormone levels must be obtained from females of known reproductive history in a well documented nesting leatherback population. The nesting Atlantic leatherback population at Sandy Point National Wildlife Refuge (SPNWR), St. Croix, (Fig. 1.4) has been comprehensively monitored and studied for 30 years. With nesting numbers that range between 92 and 202 female turtles annually (Garner and Garner, 2010) (Fig. 1.5), it provides a large sample size where saturation blood sampling of a well known, established nesting population may be implemented in association with the saturation tagging and nest excavation programs. An established 30 year database contains all tagging and nesting data collected for every individual female in the population, and provides a unique opportunity to evaluate endocrine studies in the context of historical data on nesting frequency, hatch success, and female age to determine if a significant relationship exists between nesting steroid hormones and these parameters in Atlantic leatherbacks.

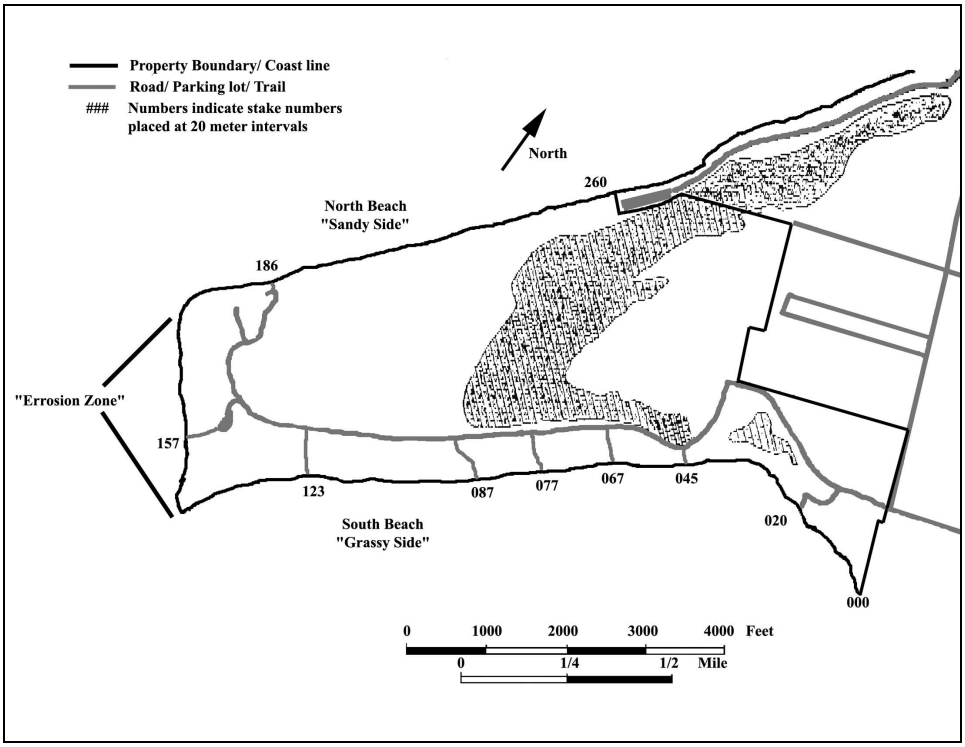

FIG. 1.4. Schematic of Sandy Point National Wildlife Refuge. (From Garner and Garner 2010). The drawing shows boundaries, coastline, roads, trails and stake numbers at the study site. The stippled areas represent salt ponds, which may be seasonally filled with water.

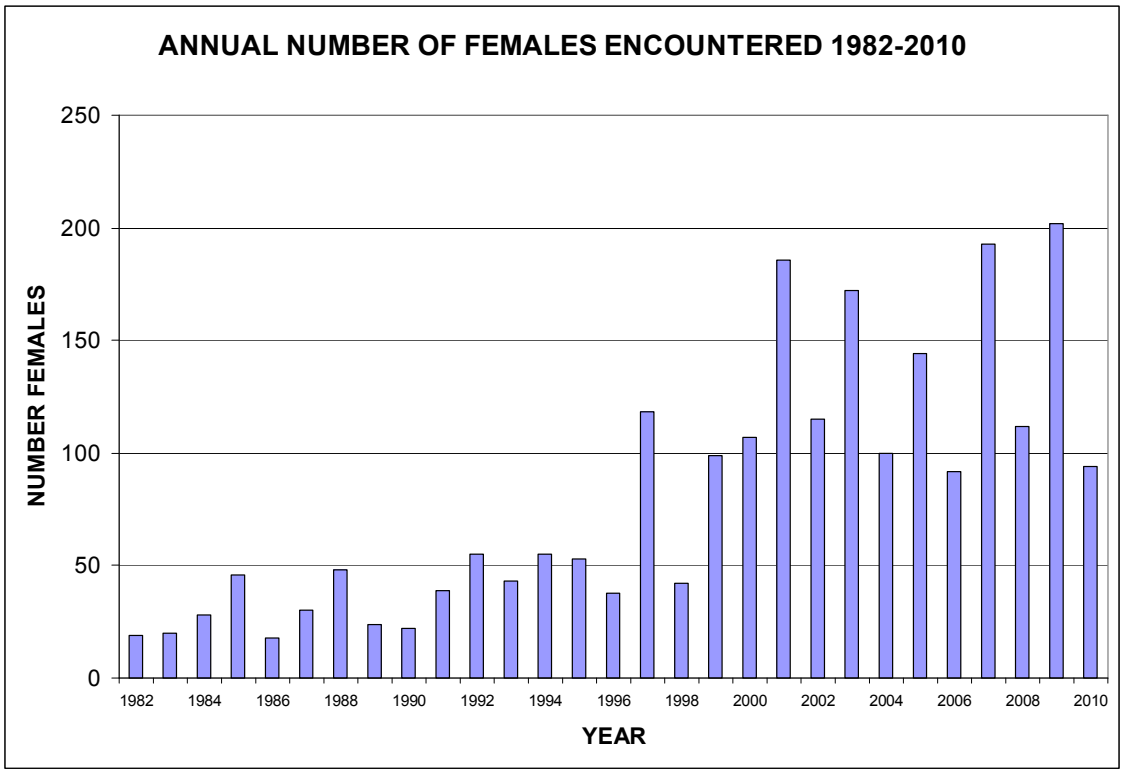

FIG. 1.5. Annual Number of Nesting Leatherback Turtles Encountered at Sandy Point, St. Croix, USVI from 1982 through 2010.

CHAPTER II

REPRODUCTIVE BIOLOGY OF ATLANTIC LEATHERBACK SEA TURTLES AT SANDY POINT, ST. CROIX

Introduction

The leatherback sea turtle (*Dermochelys coriacea*) is morphologically and physiologically unique when compared to all other species of sea turtles. It is a critically endangered species that inhabits all of the worlds' oceans (except the Arctic and Antarctic). It is the oldest living, largest, and most divergent species of sea turtle with a unique life history and reproductive characteristics. Leatherbacks nest up to 11 times in one season (with an average of 6 clutches per season), have an average inter-nesting interval of 9-10 days, and an average remigration interval of 2-3 years (Boulon et al., 1996). This is unique when compared to other turtle species that lay significantly fewer clutches per season (only 2-4), and have a significantly greater inter-nesting interval (Miller, 1997). Leatherbacks are also hypothesized to reach sexual maturity at an earlier age than most sea turtle species (9-14 years versus 20-40 years for most species) (Zug and Parham, 1996; Dutton personal communication). The unique reproductive biology of this species suggests that with protection and conservation efforts, recovery could be swift and significant when compared to that of other species. However, this is not necessarily the case. Other reproductive characteristics of this species, such as a diminished number of eggs per clutch (80-85 on average) and low

hatch success (only 50-55%), may impede reproductive success and thus, ultimately, population recovery. Additionally, the potential for recovery of a population may be influenced by environmental factors such as changing climate and increased sea temperatures, which may impact migration, food availability, hatch success, or sex ratios (Wallace et al., 2006; Witherington, 2009). Identifying factors that negatively impact the successful recovery of a population is essential for promoting conservation of sea turtle species. Because sea turtles exhibit inter-annual variation in nesting numbers and productivity (Broderick et al., 2001; Heppell et al., 2003), long-term evaluation of a defined population is required. However, few opportunities exist for long-term monitoring of reproductive parameters in established wild sea turtle populations.

The Atlantic population of leatherback sea turtles nesting at Sandy Point National Wildlife Refuge (SPNWR), St. Croix, has been studied comprehensively for 30 years. Since 1981, the annual leatherback sea turtle research and conservation project has provided long-term protection of this population, in conjunction with saturation tagging and nest management programs. Historical data show a population that initially comprised twenty or fewer individual nesting females annually (Fig. 2.1) (Boulon et al., 1996).

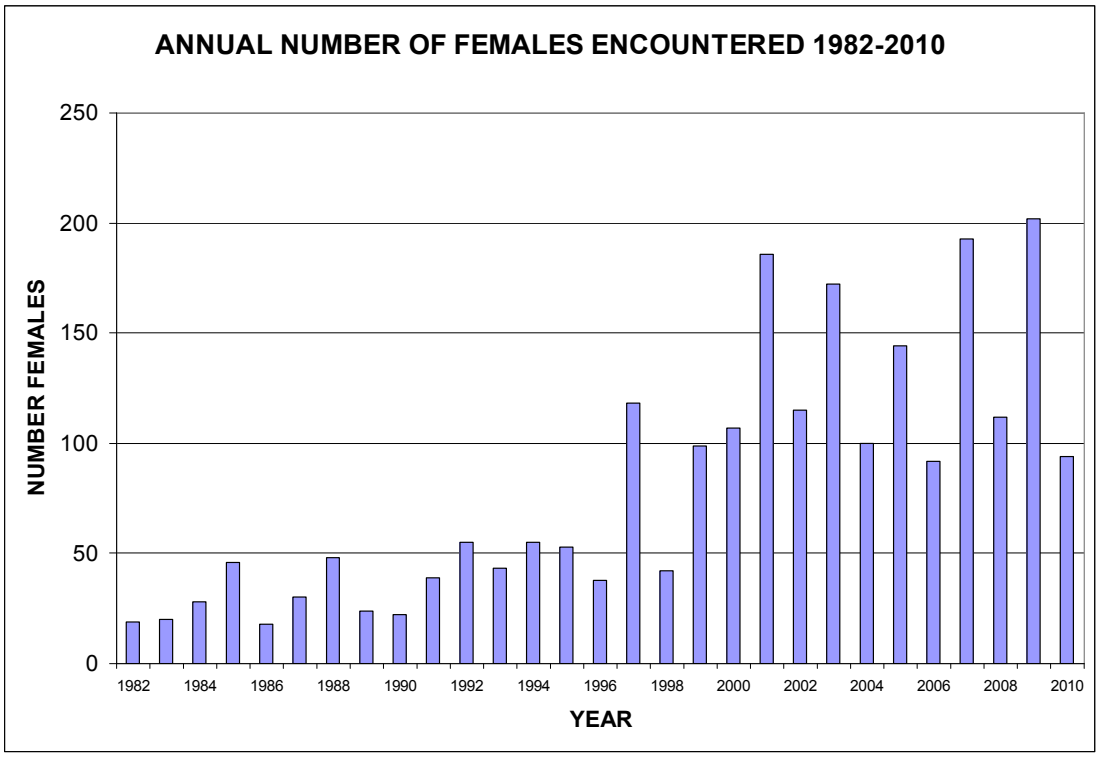

FIG. 2.1. Annual number of leatherback turtles nesting at Sandy Point National Wildlife Refuge from 1982 through 2010.

This low number was due to lack of protection for nesting females, nesting habitat, and a high incidence of egg poaching (Boulon et al., 1996). With protection of nesting adults and hatchlings, and relocation of nests in danger of erosion or inundation, population numbers increased to a record of 202 individual turtles in 2009 (Garner and Garner, 2010). Although the population has increased since the project's inception, the most dramatic increase occurred between 1997 and 2001 and is attributed to the increased hatchling production in the eighties (Dutton et al., 2005; Garner and Garner, 2010). This increase includes a significant number of neophytes recruited into the population each year and coincides with the proposed age of sexual maturity (9-14

years) for the hatchlings produced in the 80's (Dutton et al., 2005). The population numbers in the last 10 years have generally been stable, however the dramatic increase observed in the years prior to 2002 has halted and recovery has slowed. Multiple factors including: increased mortality, decreased recruitment rate, increased remigration interval, increased age to reach sexual maturity, remigrants entering senescence, or decreased productivity (decreased number of nests laid per turtle, and/or decreased hatch success) (Chaloupka and Musick, 1997; Hays et al., 2000; Heppell et al., 2003; Dutton et al., 2005; Witherington et al., 2009) have been attributed to similar declines in other sea turtle species (Hays, 2000; Witherington et al., 2009). These fundamental factors are believed to drive both short-term variability and long-term population trends and have not been evaluated for most sea turtle populations due to the lack of long-term comprehensive data collection (Heppell et al., 2003). Comprehensive mark-recapture programs that tag every female and confirm repeated observations of nesting activity and productivity minimize the error caused by unobserved females, and provide the most reliable estimates for population trends and analysis (Witherington et al., 2009). The historical, comprehensive data available for the St. Croix population provides an opportunity for the analysis of specific reproductive parameters to determine which variables may be responsible for impeding continued population recovery at Sandy Point. Changes in productivity (number of clutches laid), recruitment rate, and remigration interval are hypothesized to be the primary factors contributing to the slowed growth in this population. These parameters have been attributed to declining nest counts and population numbers in other sea turtle populations (Hays, 2000;

Witherington et. al., 2009). Analysis of population structure and trends over the last 20 years is necessary to test these hypotheses, and confirm which parameters are contributing to the slowed recovery observed recently at SPNWR. This should provide significant insight into the biological basis for recent trends in nesting numbers as well as the future of the population. This evaluation is imperative to our understating of the reproductive biology of this species, and for the modification of recovery and management plans to ensure species survival. This study utilizes the unique, long-term database available at Sandy Point to evaluate which parameters might be most influential in impeding the continued recovery of this population.

Methods

Basic reproductive data collection

The nesting leatherback population at SPNWR served as the study group. Patrols were conducted by the West Indies Research and Conservation Service (WIMARCS) personnel and local volunteers as part of the annual Leatherback Research and Conservation project funded by the Virgin Islands Department of Planning and Natural Resources (VIDPNR) and WIMARCS. For the past 30 seasons a basic beach protocol has been conserved. Nightly beach patrols at SPNWR are initiated annually April 1st and continue until approximately 10 days after the last female leatherback has nested. The beach is patrolled nightly on foot during this timeframe, starting around 2000 hours until either 0500 hours or until the last female has finished nesting.

The 3 km beach was divided into multiple sections and each respective study area was patrolled at approximately 45 minute intervals to ensure that all nesting turtles were observed, tagged, and recorded. Every time a turtle was encountered on the beach a nesting data sheet was completed and all data regarding nesting, identification, morphology, activity and nest location, nest parameters, and behavior were recorded. All nests in danger of erosion, inundation, or with standing water in the nest were relocated per standard protocol (Garner et al., 2005). Nests were constructed in suitable habitat to specific shape and dimensions (Dutton et al., 1992). Yolked egg and shelled albumin globules (SAG) counts were obtained for each relocated nest. The time and date of every encounter were also recorded. Date of emergence and excavation were also recorded once hatchlings emerged and the nests were successfully excavated. Upon excavation, all nest contents (i.e., whole eggs, pipped eggs, hatched shells, live and dead hatchlings) were categorized to determine nest success and any unhatched eggs were opened to determine stage of development at the time of mortality. This information was recorded on a separate hatchling data sheet. All methods for tagging, basic data collection, and data analyses followed the standard protocol employed at SPNWR (see Garner et al., 2005).

Statistical analyses

Basic reproductive statistics were compiled at the end of each season. Mean ± one standard deviation are provided annually for: turtle length, turtle width, number of

yolked and SAGs laid, hatch success (hatched shells/total yolked eggs), and emergence success [hatched shells - (dead hatchlings + live in nest)/total yolked eggs]. Annual hatchling production was calculated as (average number of eggs per clutch x number of *in situ* nests x *in situ* emergence success) + (average number of eggs per clutch x number of relocated nests x relocated emergence success). Number of individual neophytes (untagged females who are first time nesters) and remigrants (tagged females with a reproductive history) were counted and reported. Average remigration interval for the population (sum of the RI's for each individual/total number of turtles) was calculated for each year, as well as average number of nests laid (number of nests recorded per turtle/number of turtles). RI = number of years since previous recorded nesting. Non linear regression analysis was conducted to establish trends in number of neophytes, remigrants, and total turtles observed annually, as well as average number of nests laid and average hatch success over various project durations. Analysis of variance was conducted to determine if significant differences ($p < 0.05$) were observed among even and odd years for the total number of turtles observed, number of neophytes recruited into the population, and number of remigrants observed.

Results

The number of annual nesting turtles ranged from a record low of 92 individuals in 2006 to a record high of 202 in 2009 (Fig. 2.1). The 2001 and 2007 seasons also exhibited high numbers with 186 and 193 individuals, respectively. The 2010 season

recorded the second lowest number of nesting individuals (94) since 2006. The number of nesting individuals was lower in even years when compared to that in odd years, with record highs recorded during odd years and record lows recorded during even years (Fig. 2.1). The average number of turtles observed in odd years versus that in even years was significantly higher from 1997 to 2010 ($p < 0.05$). The remigration interval for all years ranged from 1 to 11 years, with the most common intervals being 2 then 3 years (Table 2.1). The average remigration interval decreased from 2000 through 2003, then increased steadily to a record high (3.41 years) in 2008 (Fig. 2.2). The number of observed one-year remigrants has increased over the course of the project, with 5 one-year remigrants observed in 2010 (Table 2.1).

Table 2.1. Leatherback Remigration Intervals to SPNWR from 1977 to 2010 (From Garner and Garner 2010).

Year	Total Turtles Encountered	Remigration Interval						Un-known[1]	Total Remigrants
		1	2	3	4	5	>5		
1977	10[2]	0	0	0	0	0	0	0	0
1979	6[2]	0	0	0	0	0	0	0	0
1981	20[2]	0	3	0	0	0	0	0	3 (15.0%)[3]
1982	19	0	0	0	0	0	0	1	1 (5.3%)[3]
1983	20	0	7	0	0	0	0	2	9 (45.0%)
1984	28	0	4	0	0	0	0	0	4 (14.3%)
1985	46	1	10	3	0	0	0	2	16 (34.8%)

[1] Represents turtles that either through tags or tag scars are deemed as "new remigrants". Origin of tagging is unknown and therefore remigration history is unknown.
[2] May or may not represent the total number of turtles nesting.
[3] Not accurate due to incomplete tagging in previous years.

Table 2.1. Continued.

Year	Total Turtles Encountered	Remigration Interval						Un-known	Total Remigrants
		1	2	3	4	5	>5		
1986	18	0	1	2	0	0	0	0	3 (16.7%)
1987	30	0	9	5	0	0	0	0	14 (48.3%)
1988	48	0	5	7	1	0	0	4	17 (35.4%)
1989	24	0	7	0	0	0	0	0	7 (29.2%)
1990	22	0	2	3	1	0	0	0	6 (27.3%)
1991	39	0	8	8	0	0	0	1	17 (43.6%)
1992	55	0	6	4	7	0	0	4	21 (38.2%)
1993	43	0	13	4	0	0	0	7	24 (55.8%)
1994	55	0	14	8	1	1	0	14	38 (69.1%)
1995	53	0	16	7	5	0	0	0	28 (52.8%)
1996	38	0	13	5	4	2	0	0	24 (63.2%)
1997	118	0	27	22	5	3	0	0	57 (48.3%)
1998	42	0	15	6	3	1	0	0	25 (59.5%)
1999	99	1	32	9	4	2	2	0	50 (50.5%)
2000	107	0	10	28	2	3	2	0	45 (42.1%)
2001	186	1	45	12	26	1	2	9	96 (51.6%)
2002	115	1	35	23	5	3	1	2	70 (60.9%)
2003	172	0	84	12	6	3	3	6	114 (66.3%)
2004	100	0	37	13	8	4	0	0	62 (62%)
2005	144[11]	0	70	15	6	5	3	0	99 (68.8%)
2006	92[12]	0	19	29	5	7	1	0	61 (66.3%)
2007	193	0	56	31	26	8	14	0	135 (69.95%)
2008	112	1	27	25	9	6	10	0	80 (71.4%)
2009	202	0	90	16	11	5	5	6	127 (62.87%)
2010	94	5	32	20	7	1	4	0	69 (73.40%)
Totals		**10**	**697**	**317**	**142**	**55**	**47**	**58**	**1326**

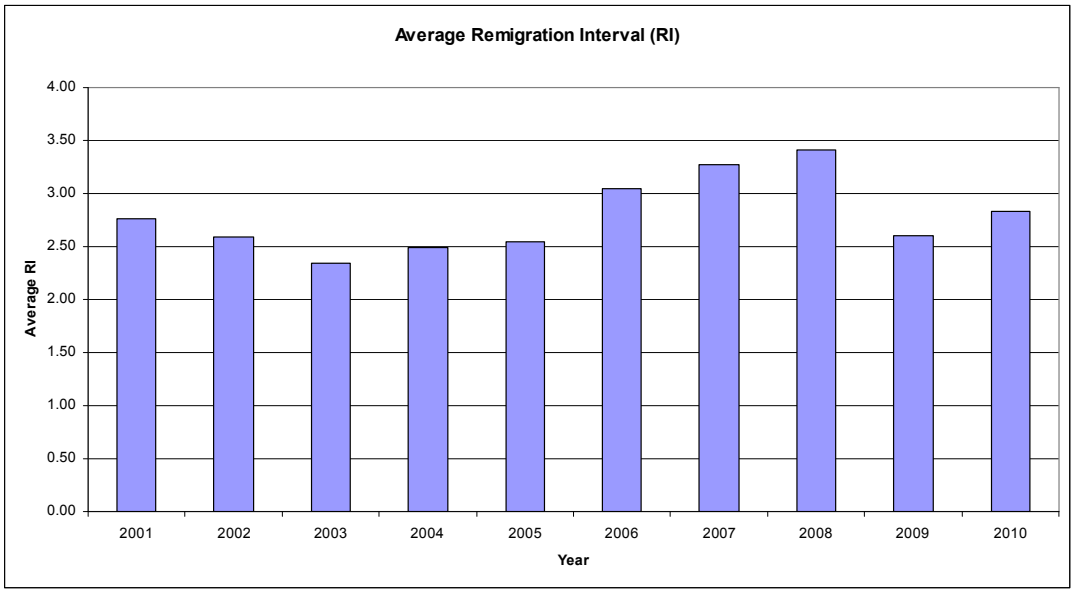

FIG. 2.2. Average remigration interval of SPNWR remigrants from 2001 to 2010.

The number of remigrants observed annually in the Sandy Point population has remained stable (Fig. 2.3) over the last 10 years. Odd year remigrant numbers ranged from 96 to 133 individuals, with an average of 115.6 individuals. A slight increase in remigrants was observed in 2007 and 2009 (with 136 and 133 turtles, respectively). Even year remigrant numbers remained steady (ranging between 61-80 individuals, with an average of 68.4 remigrants per even year). The average number of remigrants observed during odd years was significantly higher when compared to that in even years beginning in 1997 (to current) ($p < 0.05$). While the average number of remigrants has remained steady, the percentage of the annual nesting population represented by remigrants has increased over the last 20 years ($R^2 = 0.69$) (Fig. 2.4.).

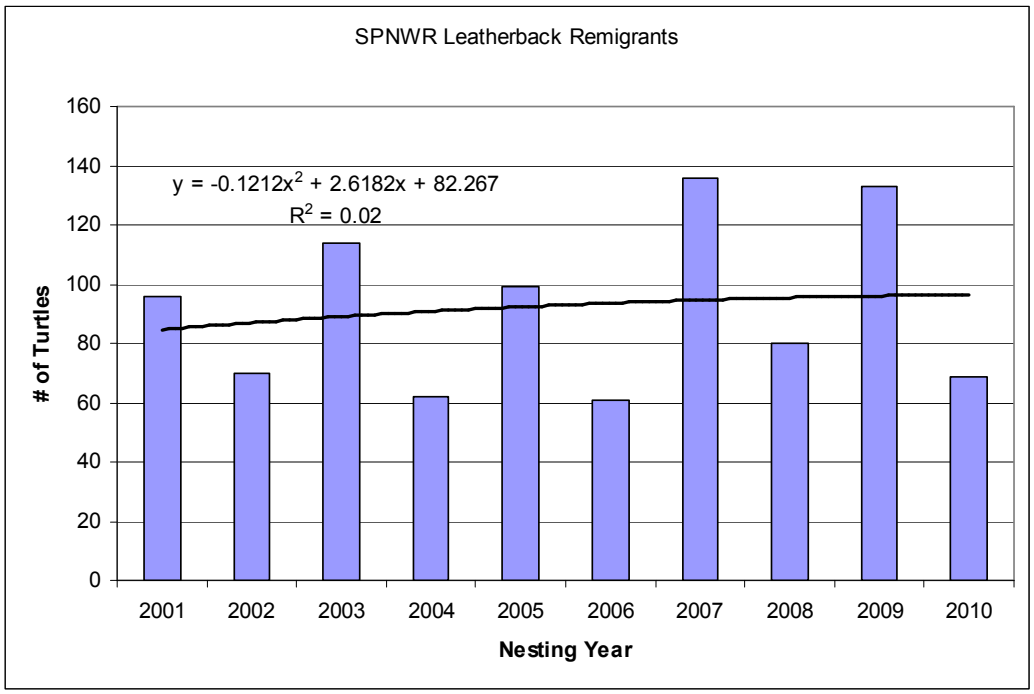

FIG. 2.3. Number of individual remigrants observed at SPNWR from 2001 to 2010.

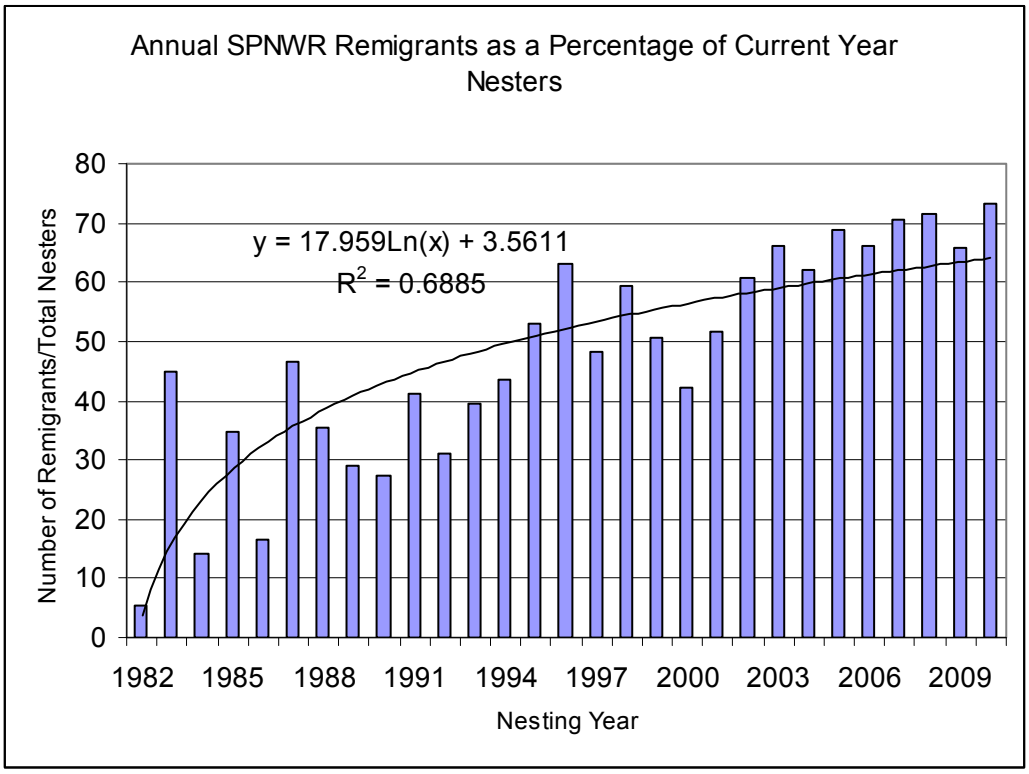

FIG. 2.4. Number of remigrants nesting annually at SPNWR as a percentage of each year's nesting population 1982 – 2010.

The number of neophytes arriving to nest at Sandy Point varied annually, but number of neophytes decreased through 2008 ($R^2 = 0.58$). A small increase in recruits occurred in 2009, but was followed by a further decrease in 2010 (Fig. 2.5). The 2001 season boasted a project record, with 90 individuals tagged (Fig. 2.5). After 2001, recruitment (identified as the number of neophytes recruited into the nesting population for a given year) generally decreased through 2006 ($R^2 = 0.77$). While all even years remained low, even year numbers decreased from 2002 through 2006 ($R^2 = 0.77$). Odd years (2007 and 2009) showed a slight increase in numbers with 57 and 69 individual neophytes tagged, respectively. The 2010 season recorded the lowest number of

neophytes in the last 10 years (Fig.2.5). In general, the average number of neophytes observed in odd years was also significantly greater than that in even years beginning in 1997 (to current) ($p < 0.05$).

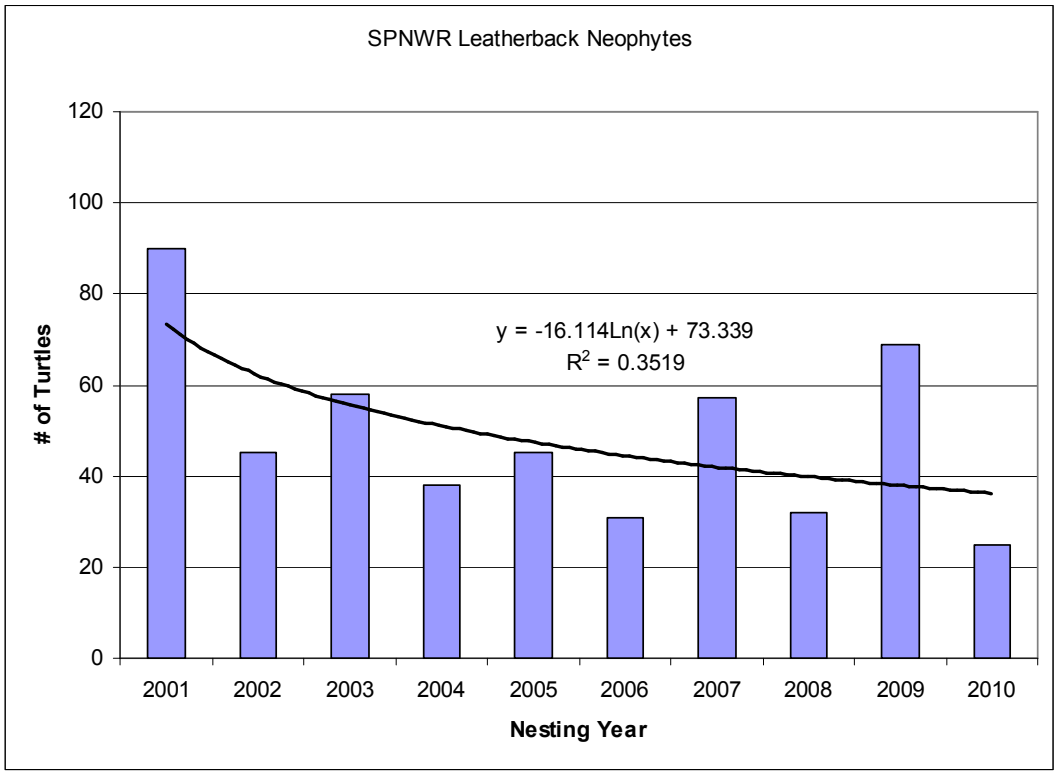

FIG. 2.5. Neophyte nesting population at SPNWR from 2001 to 2010.

Since the number of total nesting females varies annually, as well as significantly between even and odd years, the percentage of neophytes observed (number of neophytes/total number of nesting turtles) was also calculated for the last 10 years. The overall percentage of neophytes recorded decreased over the last 10 years ($R^2 = 0.77$) (See Fig. 2.6). Odd years decreased 14.23% from 48.39% in 2001 to 34.16% in 2010.

Even years decreased 10.56% from 39.13% in 2002 to 28.57% in 2010. The 2007 nesting season recorded the third lowest percentage of neophytes in project history, with 29.53%, while 2008 and 2010 recorded the lowest percentages of neophytes in project history with 28.57 and 26.59%, respectively. Overall, the percentage of neophytes observed has decreased since the inception of the project ($R^2 = 0.80$) (Fig. 2.7).

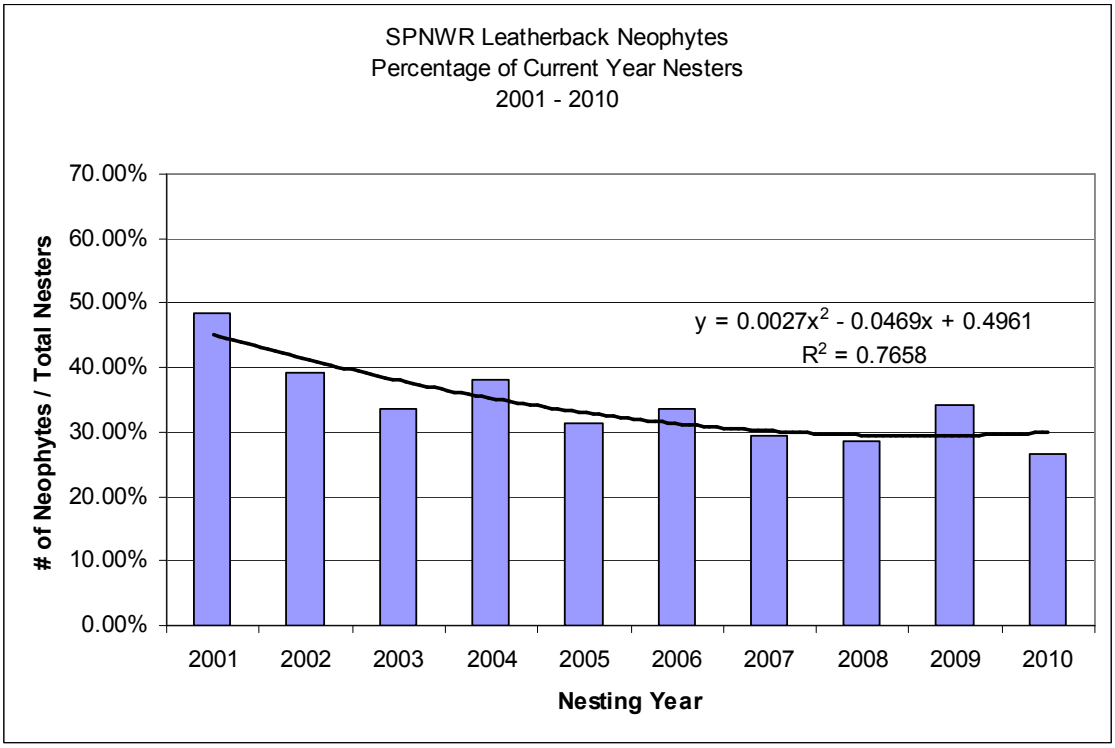

FIG. 2.6. SPNWR neophytes divided by the number of nesting turtles from 2001 – 2010.

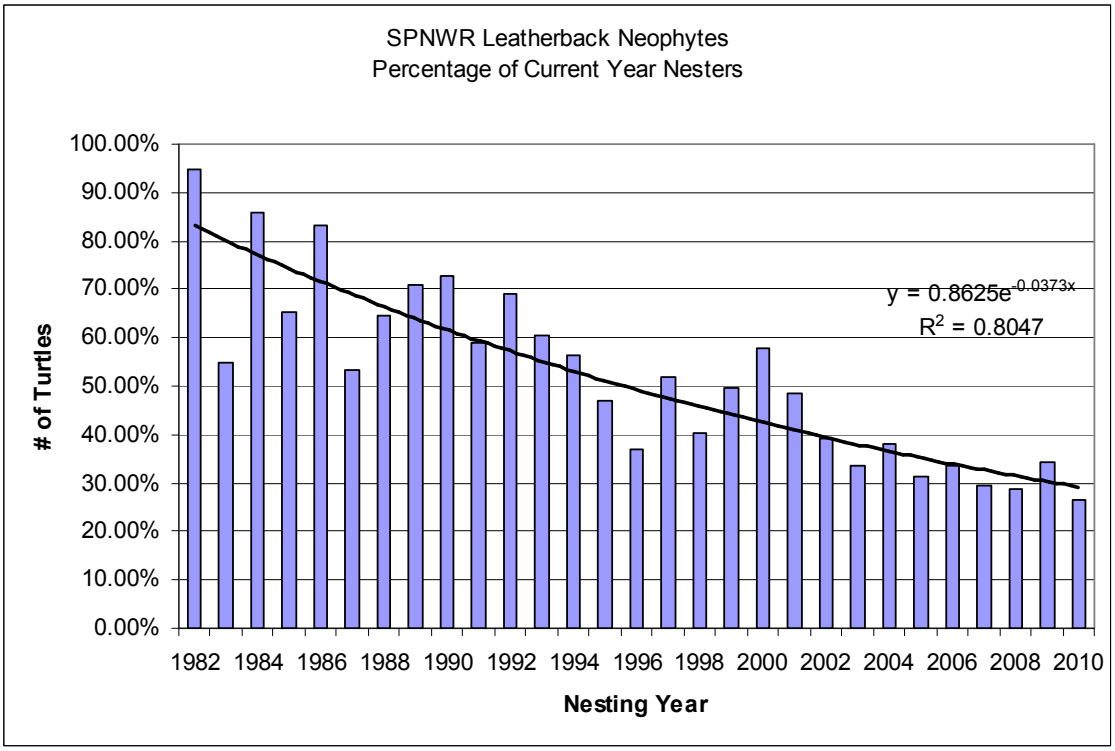

FIG. 2.7. SPNWR neophytes divided by the number of nesting turtles from 1982 – 2010.

The average number of nests produced per turtle has also decreased over the last 10 years (Fig. 2.8). The highest average was observed in 2003 with an average 6.17 nests per turtle. Even years steadily decreased to a project low of 3.60 nests per turtle. Odd years were stable from 2005 through 2009 with 4.40, 4.54, and 4.66 average nests per turtle. The statistic of decreased nest production extends back 20 years to 1991 (Fig. 2.9).

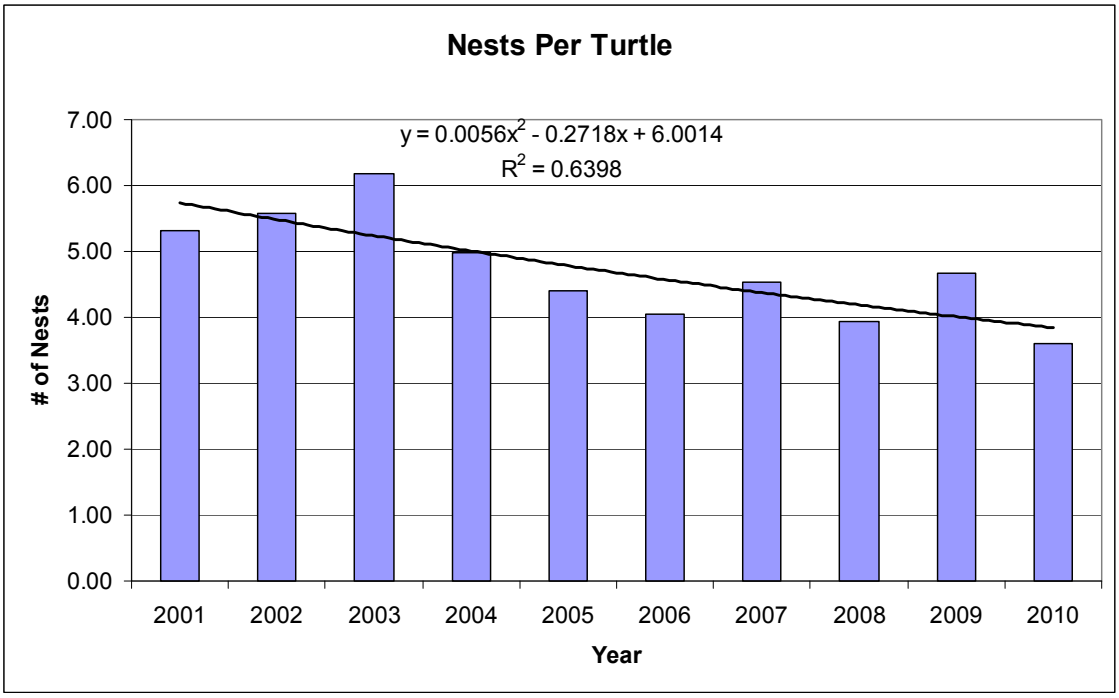

FIG. 2.8. Average nest production per turtle at SPNWR from 2001 through 2010.

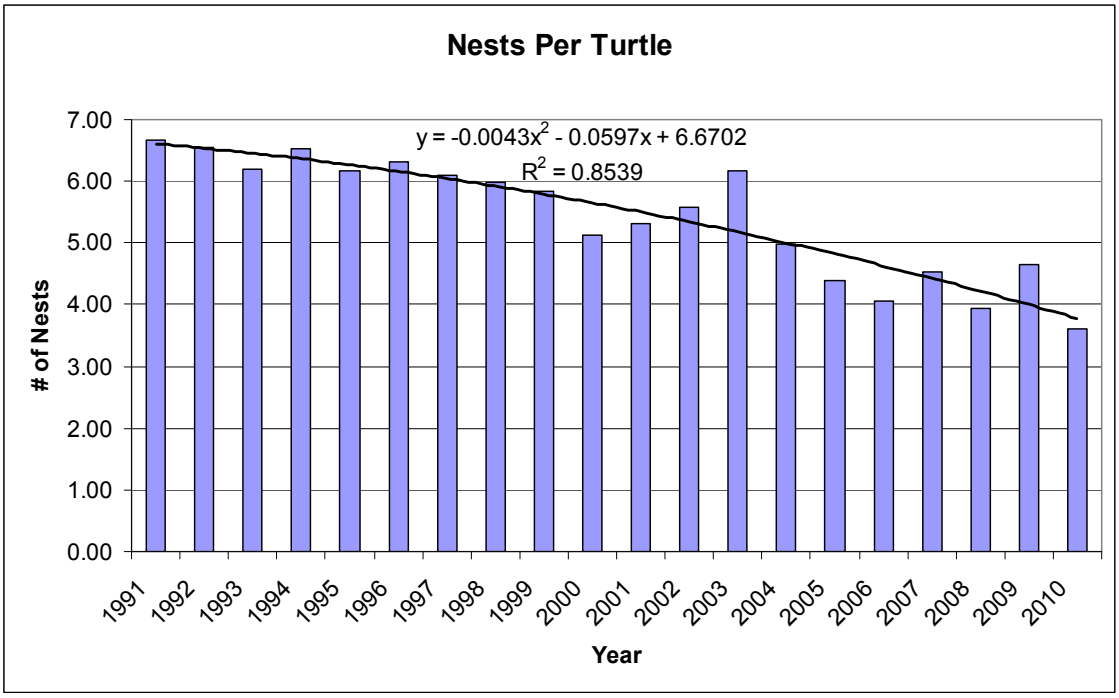

FIG. 2.9. Nest production per turtle at Sandy Point from 1991 through 2010.

Hatch success has also varied over the last 10 years (Fig. 2.10). The 2003 season recorded the highest average hatch success in the last 10 years at 59.50%, while 2005 exhibited the lowest average hatch success in the last 10 years (and the lowest average in project history) at 37.88%. The 2007 season exhibited the second lowest success in project history at 39.65%. Hatch success generally decreased between 2001 and 2007 ($R^2 = 0.65$), then increased between 2008 and 2010 (Fig. 2.10). Over the last 20 years, average overall hatch success has ranged from 40.28% to 67.80%, with a mean hatch success of 56.56%, and has declined overall (Fig. 2.11). Hatch success in 2010 was 12.77% lower than that in 1991 (Fig. 2.11).

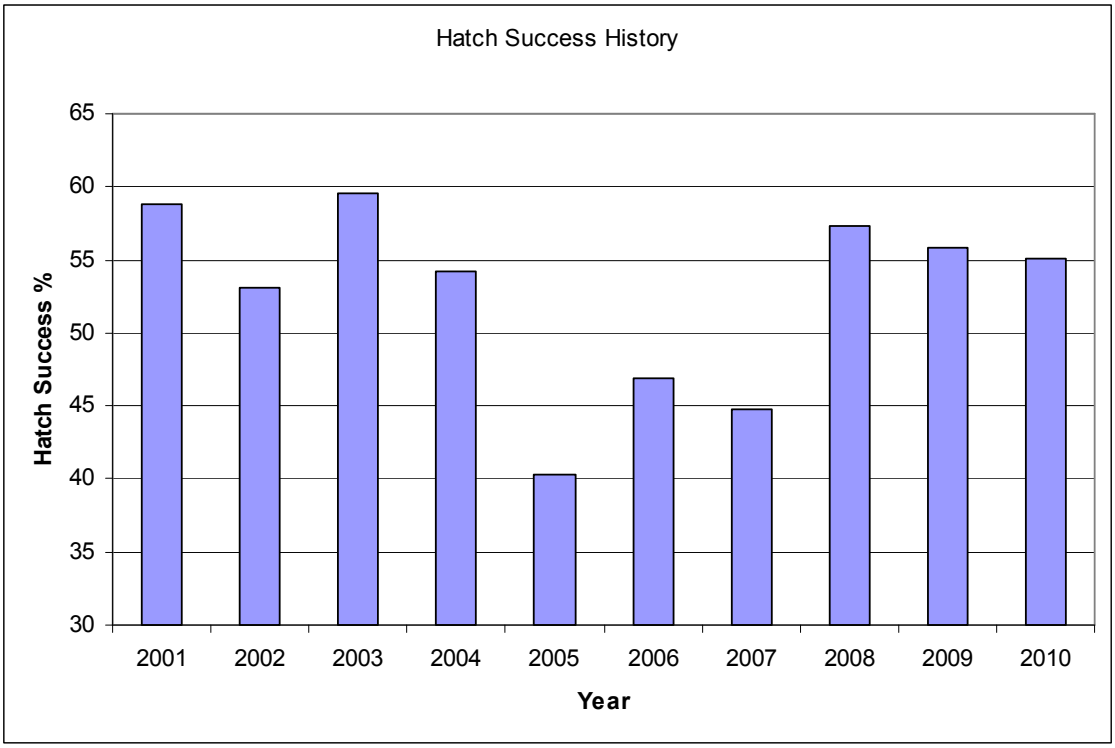

FIG. 2.10. Average hatch success for nests at SPNWR from 2001 – 2010.

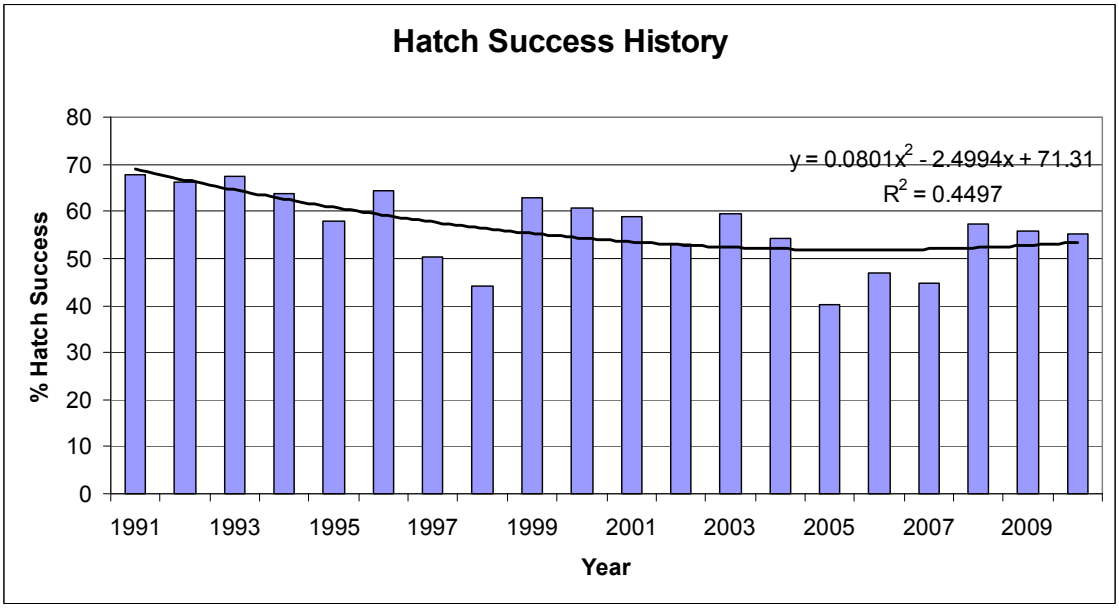

FIG. 2.11. Average hatch success for nests laid at SPNWR from 1991 – 2010.

Hatchling production has varied over the last 10 years, with a general decline in production. The greatest number of hatchlings produced in the last 10 years, as well as project history (44,325 hatchlings) occurred in 2001 (Fig. 2.12). The 2003 and 2009 seasons produced slightly lower, but more similar numbers with 43,282 and 37,669 hatchlings, respectively. The 2006 (11,567) and 2010 (15,866) seasons produced the lowest number of hatchlings in the last 10 years. Although hatchling production has decreased since 2001 (Fig. 2.12), overall production has increased since the project's inception (Fig. 2.13).

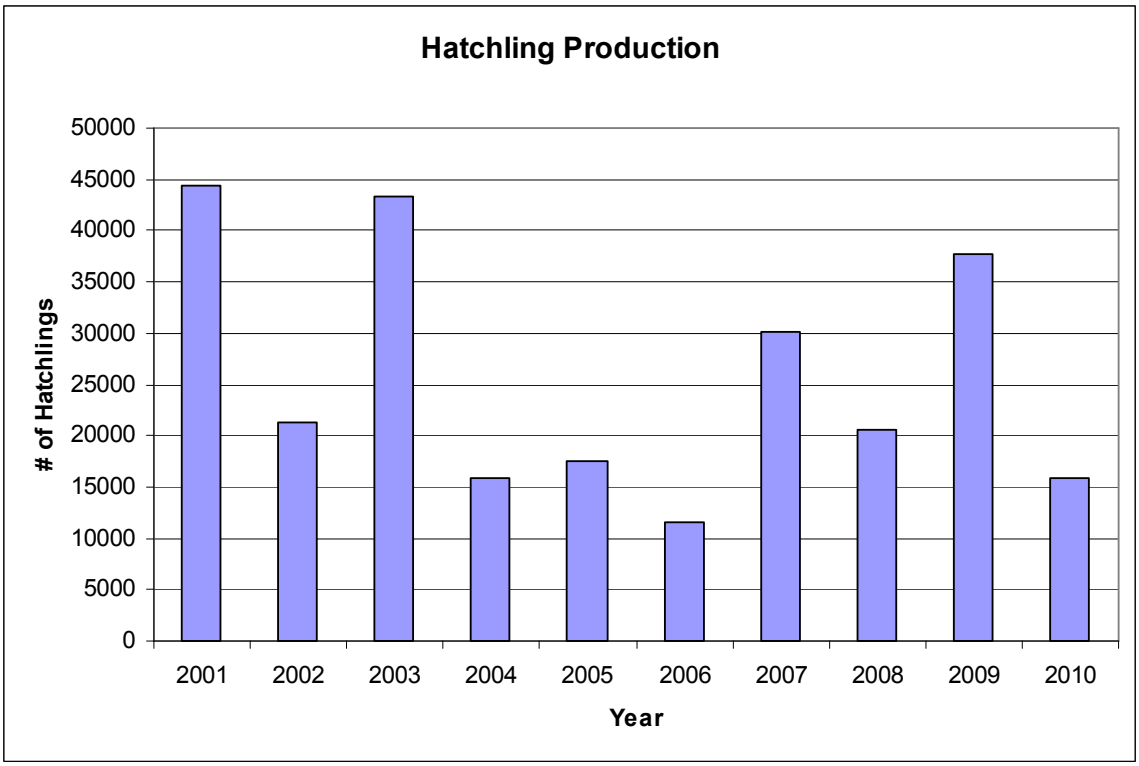

FIG. 2.12. Leatherback hatchling production at SPNWR from 2001 - 2010.

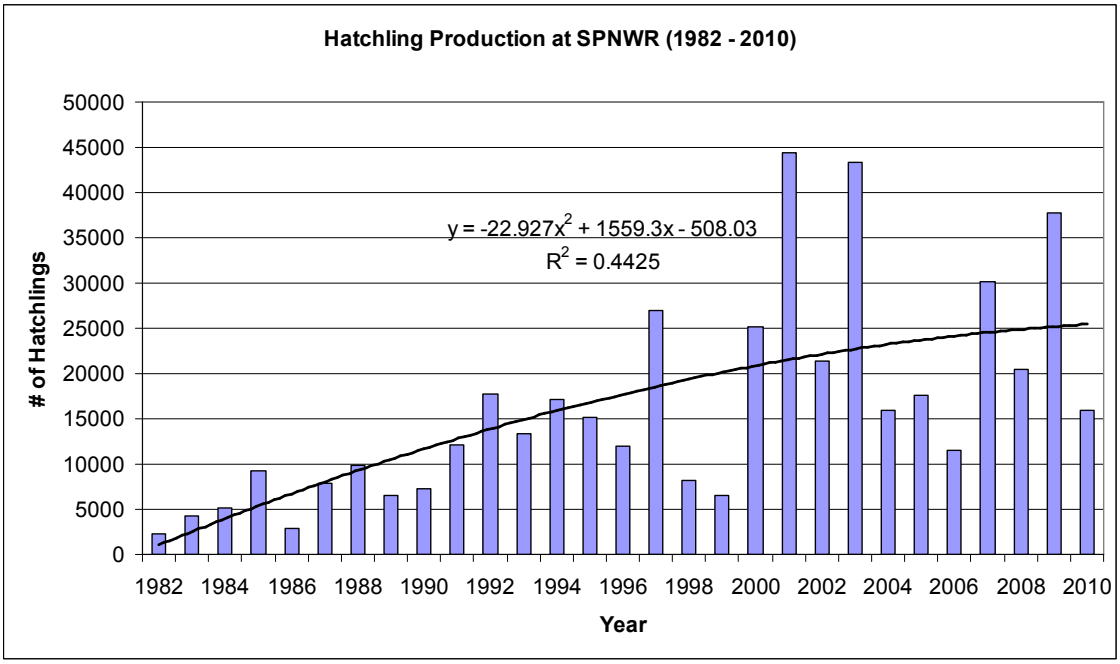

FIG. 2.13. Leatherback hatchling production at SPNWR from 1982 - 2010.

Discussion

Inter-annual variation in nesting numbers is common in sea turtle populations, therefore long-term data are necessary to truly evaluate population trends. The population of endangered leatherbacks at Sandy Point, St. Croix showed a significant increase in the first 20 years of the project, increasing from less than 20 turtles in 1981 to an initial record of 186 turtles in 2001 (Dutton et al., 2005; Garner et al., 2005). A concomitant increase in hatchling production was also observed during this period, with the number of hatchlings produced increasing from 2,000 to over 49,000 hatchlings (Dutton et al., 2005; Garner et al., 2005). The annual population growth rate (approximately 13% per year) observed since the early 1990s was not ascribed to

increased survival probability of adults, because survivorship was determined to remain high and constant during this period (at approximately 0.893 minimal survival rate for nesters annually) (Dutton et al., 2005). The population increase was instead associated with increased hatchling production. Nest protection and relocation programs began in the early 1980s and relocated 30 – 40% of the clutches laid annually at Sandy Point (Dutton et al., 2005, Garner and Garner 2010). This program resulted in significantly increased hatchling production and a subsequent population boost due to neophyte recruitment approximately 12-14 years later, within the approximate duration to reach sexual maturity (Dutton et al., 2005; Zug and Parham, 1996). In spite of inter-annual variability in the population, the Sandy Point nesters showed a steady increase through 2001. Trends for the last 10 years have not previously been reported, but suggest a population that may be in decline. Odd years traditionally boast greater nesting numbers than do even years. Odd years in the last decade did not continue the exponential increase observed from 1991 to 2001, and the 200 nester threshold was not breached until 2009, later than predicted (Dutton et al., 2005; Garner and Garner 2010). The last decade has also yielded record low nesting numbers during even years. The inter-annual variability in nesting numbers within the last 10 years is generally associated with varying remigration intervals (Broderick et al., 2001; Hays, 2000). Remigration intervals vary for each individual nester and are based on reaching a nutritional threshold for reproduction and migration (Broderick et al., 2001; Hays, 2000). The ability to reproduce in a given year is directly linked to foraging ground productivity (and effective exploitation of these areas by individuals) and ultimately affects population

numbers annually at nesting beaches (Hays, 2000). Environmental stochasticity, such as el nino southern oscillation (ENSO) events, climate change, and sea surface temperatures can directly influence the foraging capacity of turtles and ultimately the remigration interval (RI) (Wallace et al., 2006). Remigration intervals, when increasing consistently on an annual basis over multiple years (Fig. 2.2) may negatively impact population productivity and result in a low number of annual nesters (Witherington et al., 2009), such as that observed at SPNWR during multiple seasons since 2001.

The number of nesting remigrants appears to be constant to slightly increasing (Fig. 2.4), thus supporting the observed trend of high adult survivorship described by Dutton et al. (2005). Therefore, adult survivorship does not appear to be directly related to any observed increase, or decrease in population numbers. Survivorship of hatchlings and juveniles has not been evaluated, however, and changes in survivorship of earlier life stages may significantly impact recruitment (Dutton et al., 2005; Witherington et al., 2009). In addition to impacting RI, nutritional status will also directly impact the time it takes for a juvenile to reach sexual maturity thus, the decreasing number of annual nesters. In addition, the lows observed over the last 10 years may be due to delayed recruitment and an increasing RI (Witherington et al., 2009). This hypothesis is supported by the decreased number and percentage of neophytes observed in the last 10 years (Fig. 2.5 and 2.6).

Factors directly affecting reproductive productivity, such as number of nests laid per turtle and average hatch success, will impact hatchling production. Hatchling production has been linked to the drastic population increase within the first 20 years of

the project (Dutton et al., 2005) and decreases may result in a continued decline in future recruitment and delayed population recovery. Hatch success at SPNWR decreased from 2001 through 2007, and reached a historical low in 2005. Abnormal erosion patterns may account for some of these results (Garner and Garner, 2010), as well as variations in sand properties and bacterial load. Previous studies conducted at SPNWR could not pin-point the specific biotic or abiotic factors in the nest environment responsible for the low average hatch success observed (only 50-55%) in this population (Garrett et al., 2010). Additionally, low hatch success in other populations, such as that at Las Baulas, Costa Rica, has been attributed to high embryonic mortality rather than to infertility (Bell et al., 2003), suggesting that intrinsic maternal influences may be involved.

The number of nests laid per turtle also decreased from 2001 through 2010 (Fig. 2.8) and has been decreasing consistently for the last 20 years (Fig. 2.9). The number of nests produced by an individual female is likely linked to nutritional status and maternal health. A decreased number of nests produced per female, in conjunction with decreased hatch success rates has resulted in the decreased hatchling productivity numbers observed between 2001 and 2010 (Fig. 2.12), in spite of some years exhibiting increased nesting numbers. Long-term decreases in hatchling productivity will be detrimental to population recovery, with effects delayed for 12-14 years, the interval required for sexual maturation.

Decreased productivity (the number of nests laid, hatch success), in association with the increased remigration intervals and the decreased recruitment rate observed over the last 10 years appears to be impacting the continued recovery of the Sandy Point

population. Although the population of remigrants and adult survivorship are generally stable, these same individuals are taking longer to return to reproduce, and producing fewer nests and hatchlings over time. New population models need to be developed that take into account these changing reproductive factors over the last 10 years. This will help managers visualize future population trends and amend management plans accordingly. Reproductive status and RI in these animals are linked to foraging ground productivity, environmental changes, and nutritional status. These factors may ultimately correlate to additional reproductive parameters, such as the number of nests laid and hatch success but they are difficult to measure.

Satellite telemetry, satellite relayed data loggers (SRDL) and time depth recorders (TDR) have previously been utilized to assess the habitat use and movement patterns of leatherback sea turtles (Hays et al., 2004; Eckert et al., 1989; Eckert, 2006; Meyers et al., 2006; Sherrill-Mix et al., 2008; Fossette et al., 2010). Spatio-temporal behavior has been linked to differential foraging success in sea turtles (Fossette et al., 2010), a maternal factor that ultimately impacts reproductive success. The diving behavior and location of leatherbacks from Grenada, French Guiana, and Suriname were tracked during their migration to North Atlantic foraging grounds and data showed that the animals exhibited three main migration strategies (including round-trip, Northern, and equatorial patterns) (Fossette et al., 2010). Turtles also traveled slower and had shallower dives when in areas of high foraging success (located at high latitudes (> 30° N) and in the sub-equatorial zone) (Fossette et al., 2010). Areas of low foraging success fell between these two zones and turtles exhibited deeper dives and traveled at higher

speeds (Fossette et al., 2010). Overall, leatherback movements were correlated to the distribution of meso-zooplankton in the Atlantic (Fossette et al., 2010).

Sherril-Mix et al. (2008) tracked the southward migration of leatherback turtles from the foraging grounds in the N. Atlantic and evaluated the timing of migration with location (latitude and longitude), sea surface temperature and chlorophyll concentration. Higher temperatures and increased chlorophyll levels 1week prior to the animals departure correlated with an increased probability of migration (Sherrill-Mix et al., 2008). This was attributed to increased prey productivity and feeding efficiency in areas of increased temperature and chlorophyll values (Sherrill-Mix et al., 2008).

Based on these studies, analysis of SPNWR turtle migratory behavior would help elucidate if these animals are utilizing different, less efficient foraging strategies when compared to other Atlantic populations; if decreased prey availability requires SPNWR turtles to travel greater distances from the nesting grounds to obtain sufficient nutrition, or if they remain at the foraging grounds longer prior to remigration back to Sandy Point. These hypotheses could account for the recent observed trends in annual nesting numbers and reproductive parameters observed at SPNWR.

Analysis of sea surface temperatures and chlorophyll levels have been linked to turtle migration routes and foraging areas (Sherrill-Mix et al., 2008) and should be analyzed in conjunction with future telemetry data in this population to answer these questions. The analysis of carbon and nitrogen isotope ratios is an alternative method that has also been utilized to reflect the foraging strategies and locations of different leatherback populations (Wallace et al., 2006). Isotope signatures prove valuable in

evaluating primary productivity and habitat preferences among and within sea turtle populations (Wallace et al., 2006). Ultimately, a better understanding of foraging ecology will help us determine the impact of climate change and increased sea surface temperatures on the behavior of migrating sea turtles, and ultimately how variations in the pelagic environment may translate to altered reproductive output at the nesting beach. Additional migration data is also vital to establishing the primary migratory corridors utilized by Atlantic turtles. This provides regions on which to focus conservation and protection efforts for turtles outside of the nesting habitat.

Additionally, the physiological mechanisms through which environmental influences (and nutritional status) act to influence reproductive output have not been evaluated. Evaluation of the maternal impacts and possible physiological factors that may direct these changes in reproductive parameters are recommended to further elucidate physiological processes influencing individual and ultimately population fecundity. Recently, steroid hormones have been utilized to evaluate reproductive status in sea turtles, with hormones correlating to clutch number in hard shelled sea turtles. Work conducted with Kemp's ridley and green sea turtles (Owens and Morris, 1985; Rostal et al., 1998; Hamann et al., 2002) provided the first endocrine model for sea turtles and supported the use of steroid hormones as indicators of clutch number (Rostal et al., 1998). Concentrations of testosterone, estrogen, and progesterone were observed to decrease as follicles were ovulated, with levels decreasing proportionally in accordance with each nesting event. Evaluation of steroid hormones in the Sandy Point population may provide a useful indicator of reproductive status in leatherbacks.

Additional analysis of hormones in association with reproductive data (clutch number, clutch size, and hatch success), such as that provided for Sandy point, St. Croix, will serve to expand our knowledge of leatherbacks as a whole, and will provide insight into the physiological mechanisms that underlie their unique reproductive biology.

CHAPTER III

REPRODUCTIVE STEROID HORMONES IN NESTING ATLANTIC LEATHERBACK SEA TURTLES

Introduction

Traditional studies of sea turtle population trends and reproductive biology rely on saturation tagging and extrinsic data collection (identification, morphometrics, behavior, egg counts). These approaches provide valuable information regarding nesting behavior, population structure, annual reproductive output, recruitment, and growth rates for reproductive females (Boulon et al., 1996; Dutton et al., 2005; Garner and Garner, 2010) thus allowing managers to track population trends over time and identify changes in characteristics of particular interest for species conservation, such as the number of nesters observed annually, recruitment rate, and changes in egg and hatchling production (Boulon et al., 1996; Dutton et al., 2005; Garner and Garner, 2010). However, these approaches generally limit data collection to external observations and basic number counts, and do not identify environmental or maternally-derived factors that might help explain the observed changes in egg production, hatch success, clutch number, or reproductive maturity in sea turtle species. Additionally, extrinsic data do not account for physiological mechanisms impacting reproductive output and maturity. Evaluation of maternally-derived, intrinsic factors may thus contribute an additional perspective to

our understanding of clutch production in leatherbacks (Garner and Garner, 2010; Perrault, 2012).

This approach was previously utilized to evaluate clutch production in the hard-shelled sea turtle species, and correlated clutch number to maternal reproductive steroid hormone measurements. Work conducted with Kemp's ridley (*Lepidochelys kempii*) and green (*Chelonia mydas*) sea turtles (Owens and Morris, 1985; Rostal et al., 1998; Hamann et al., 2002) provided the first endocrine model for sea turtles and supported the use of steroid hormones as indicators of clutch number (Rostal et al., 1998). Levels of testosterone, estrogen, and progesterone were observed to decrease as follicles were ovulated, with levels decreasing proportionally in accordance with each nesting event. This decline was ascribed to a progressive depletion of mature follicles in the ovary with progressing clutch number (Rostal et al., 1998). The observed step-wise decrease in testosterone with each subsequent clutch was utilized with the endangered Kemp's ridley sea turtle to predict remaining numbers of clutches for nesting females (Rostal et al., 1998). Hormone measurement may therefore provide significant insight into clutch production in the leatherback (*Dermochelys coriacea*) as well.

Rostal et al. (2001) have provided the only description of reproductive endocrinology in leatherbacks, evaluating steroid hormone levels over time in nesting females at La Baulas, Costa Rica. Rostal et al. (2001) determined that, as in the hard-shelled species, there was a simultaneous decline in testosterone and estradiol over the course of the nesting season in Pacific leatherbacks. This is logical as estradiol and testosterone generally decrease in association with decreased ovarian size and numbers

of follicles as progressive ovulations occur in nesting sea turtles (Owens, 1997). However, leatherbacks exhibited higher overall hormone levels and a more gradual decline with each nesting event when compared to these trends in other sea turtle species (Rostal et al., 2001). This may be attributed to the increased number of clutches produced by leatherbacks when compared to that in other species (Rostal et al., 2001). This study provided the first major confirmation that leatherback turtles exhibit similar steroid hormones trends during nesting as other sea turtle species. It also suggests that clutch number is correlated with hormone concentrations in this species as well. The applicability of this descriptive model to Atlantic leatherbacks, however, has not been evaluated. Although similar with regards to basic biology and behavior, Atlantic leatherbacks exhibit some unique reproductive characteristics when compared to Pacific leatherbacks. Atlantic leatherbacks consistently lay more clutches, produce more eggs per clutch, and have a decreased RI when compared to the Pacific leatherback (Reina et al., 2002; Dutton, 2005; Wallace et al., 2006). The average size (carapace width, length, and weight) of nesting Atlantic leatherbacks is also greater when compared to those of Pacific turtles, and age to sexual maturity is presumably less. Rostal et al.'s (2001) hormone data pertains solely to Pacific turtles, which exhibit multiple unique reproductive characteristics when compared to Atlantic turtles. Therefore, evaluation of hormones in an Atlantic population is necessary to confirm whether Atlantic turtles behave similarly to Pacific turtles with regard to hormones. The leatherback population at Sandy Point also provides a greater sample size, including a large percentage of neophytes and remigrant turtles (tagged up to 30 years prior), and a comprehensive,

historical reproductive history for every turtle observed. The Pacific study population did not possess these attributes. Additionally, the Pacific data did not delineate between neophytes and remigrants who may differentially contribute to clutch production (Tucker and Frazer, 1991) and thus the observed hormone averages. Further analysis of hormone levels in association with the comprehensive reproductive data available for the Sandy Point leatherbacks may determine if this is the case, identify any significant hormone differences between neophytes and remigrants, and provide insight into potential intrinsic influences on clutch production in this population. This evaluation will confirm whether independent populations of leatherbacks exhibit similar hormone values and trends, and will provide critical baseline data which, in the future, may help identify endocrine anomalies associated with declining reproductive output in this species.

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Methods

Basic reproductive data collection

The nesting leatherback population at Sandy Point National Wildlife Refuge (SPNWR), St. Croix, U.S. Virgin Islands, served as the study group. Patrols were conducted by the West Indies Research and Conservation Service (WIMARCS) personnel and local volunteers as part of the annual Leatherback Research and Conservation project funded by the Virgin Islands Department of Planning and Natural Resources (VIDPNR) and WIMARCS. For the past 30 seasons a basic beach protocol

has been conserved. Annual nightly beach patrols at SPNWR were initiated April 1st and continue until approximately 10 days after the last female leatherback has nested (generally July 15th – August 1st). During this timeframe the beach was patrolled nightly on foot, starting around 2000 hours until either 0500 hours or until the last female has finished nesting.

The 3 km beach was divided into multiple sections and each respective study area was patrolled at approximately 45-minute intervals to ensure that all nesting turtles were observed, tagged, and recorded. Every time a turtle was encountered on the beach a nesting data sheet was completed and all data regarding nesting, identification, morphology, activity and nest location, nest parameters, and behavior were recorded. All nests in danger of erosion, inundation, or with standing water in the nest were relocated per standard protocol (Garner et al., 2005). Nests were constructed in suitable habitat to specific shape and dimensions (Dutton et al., 1992). Yolked egg and SAG counts were obtained for each relocated nest. The time and date of every encounter were also recorded. Date of emergence and excavation were also recorded once hatchlings emerged and the nests were successfully excavated. Upon excavation, all nest contents were categorized to determine nest success and any unhatched eggs were opened to determine stage of development at the time of mortality. This information was recorded on a separate hatchling data sheet. All methods for tagging, basic data collection, and data analyses followed the standard protocol employed at SPNWR (see Garner et al., 2005).

Blood sampling

In association with the ongoing conservation project blood sampling was conducted during the nesting season for two consecutive years to ensure repeatability of results, as well as account for the inter-annual nesting variability observed in sea turtle species. Sampling was funded by WIMARCS. To ensure that enough blood samples were obtained from a representative sample of turtles (various ages, sizes, remigration intervals) and clutches (1 through 10), an attempt was made to sample each individual turtle every time she successfully nested throughout the season (April-August). Remigration interval (RI) = number of years since previous observed nesting. Blood samples were obtained from turtles only after successful initiation of egg deposition to minimize disruption of the nesting process, once the turtle had entered the nesting "trance" and deposited approximately 4 eggs. Blood samples were taken from the femoral rete system in the rear, covering flipper (approximately 10 cm posterior to the genual joint) (Dutton, 1996). Before insertion of the needle, the entire area was swabbed with Betadine solution. Blood was collected aseptically using a 20G 1½ venous collection needle fitted in a Vacutainer® tube holder, and drawn with 5-mL or 10-mL BD Vacutainer™ tubes (Becton, Dickinson and Co., Franklin Lakes, NJ). Two blood samples were collected for each turtle. One sample was collected in a BD Serum Vacutainer™ containing no anticoagulant and maintained in the upright position. The second sample was collected in a BD Vacutainer™ containing lithium heparin and inverted gently five times after collection to assure mixing of the anticoagulant solution with the blood. Both samples were obtained in one draw, subsequently labeled with the

date and turtle identification number, and placed upright in a cooler containing ice packs (FreezPak© and CryoPak©) to ensure refrigeration while in the field (up to 9 hours).

Upon return to the WIMARCS laboratory, each sample was refrigerated (up to 8 hours) until centrifugation. Each sample was centrifuged for 10 minutes at 3,000 rpm (to provide relative centrifugal force) using a Junior Angle Centrifuge (Model no. 1600). After separation, serum and plasma samples were removed using Fisherbrand sterilized disposable polyethylene transfer pipettes. One pipette was used for each sample. Individual serum and plasma samples were transferred to a sterile 2-mL self-standing Cryogenic vial (Non-pyrogenic, polypropylene, Corning®), labeled with the female's original tag number and date, then frozen and stored at -20°C for future analysis. After transport to the United States, analysis of blood parameters was conducted at the Endocrine Diagnostic Laboratory of the Texas Veterinary Medical Diagnostic Laboratory (TVMDL), College Station, TX.

Hormone analyses

Prior to conducting the assays all blood samples were allowed to thaw to room temperature (approximately 15 – 28 °C) and were subsequently vortexed gently for approximately 3 seconds. Approximately 0.75 ml of each sample was aliquoted into labeled 1.5-ml tubes (RNAase, DNAase, DNA, and pyrogen free natural micro-centrifuge tubes, USA Scientific Inc) to avoid repeated thawing and freezing. All blood samples were subsequently assayed in duplicate at a volume of 50 µl. A Genesys 5000 gamma counter was used for counting and concentration calculations.

Testosterone

The Coat-A-Count® total testosterone (# TKTT2 and PITKTT-4, Siemens, Los Angeles, CA) solid-phase kit was utilized. ^{125}I labeled testosterone competed for 3 hours with the study sample for binding to an immobilized polyclonal rabbit anti-testosterone antibody. After incubation, separation of the bound from free hormone was achieved by decanting the supernatant. Bound hormone was quantified (ng/ml) using a Genesys 5000 gamma counter and potencies were calculated using Genesys software (Logit (B/B_0) vs Log (Dose)). Sensitivity (defined as 2 standard deviations above B_0) of the assay was 0.25 ng/ml. Intra-assay and inter-assay coefficient of variation (% CV) for testosterone were 5.2 and 9.8%, respectively. The cross-reactivity values (manufacturer's data) were: 0.6% for androstenedione, 2% for 5α-dhydrotestosterone, 0.7% for Methyl testosterone, and 0.1% for progesterone, with 17 other compounds (steroids or metabolites) undetectable (manufacturer's data). To determine whether leatherback samples diluted in a parallel fashion to the standard curve, 10 samples were assayed in duplicate at doses of 50 µl and 100 µl. These samples yielded identical potencies when corrected for dilution, indicating parallelism to the standard over this dose range. Recovery of known amounts of cold testosterone spiked into leatherback plasma (n = 10) was 89.4% .

Estradiol

The Coat-A-Count® (#KE2D1 and PITKE2-7, Siemens, Los Angeles, CA) no

extraction, solid-phase double antibody estradiol test was utilized. The sample was pre-incubated with anti-estradiol antiserum raised in rabbits. ^{125}I labeled estradiol then competed for 3 hours with the study sample for binding to immobilized polyclonal rabbit anti-estradiol antibody. After incubation, separation of the bound from free hormone was achieved by decanting the supernatant. Bound hormone was quantified (ng/ml) using a Genesys 5000 gamma counter and potencies were calculated using Genesys software (Logit (B/B_0) vs Log (Dose)). Results were reported in pg/ml. Cross-reactivity value for 17β-Estradiol-3β-D-Glucuronide was 6.0%, with 21 other estrogens, and metabolites less than 0.5% (manufacturer's data). Sensitivity of the assay was 0.22 ul. Intra-assay and inter-assay coefficient of variation (% CV) for estradiol were 9.6 and 12.4% respectively. To determine whether leatherback samples diluted in a parallel fashion to the standard curve, 10 samples were assayed in duplicate at doses of 50 µl and 100 µl. These samples yielded identical potencies when corrected for dilution, indicating parallelism to the standard over this dose range. Recovery of known amounts of cold estradiol spiked into leatherback plasma (n = 10) was 82.6%.

Progesterone

The Coat-A-Count® (#TKPG5, PITKPG-5, Siemens, Los Angeles, CA) solid-phase progesterone immunoassay was used. Results were reported in ng/ml. ^{125}I labeled progesterone competed for 3 hours with the study sample for binding to immobilized polyclonal rabbit anti-progesterone antibody. After incubation, separation of the bound

from free hormone was achieved by decanting the supernatant. Bound hormone was quantified (ng/ml) using a Genesys 5000 gamma counter and potencies were calculated using Genesys software (Logit (B/B_0) vs Log (Dose)). The cross-reactivity values were: corticosterone 0.9%, 11-deoxy-corticosterone 2.2% , 17α- hydroxy-progesterone 3.4%, pregnenolone 0.1% and testosterone 0.1%, with 13 other compounds low or undetectable (manufacturer's data). Sensitivity of the assay was 0.01 ng/ml. Intra-assay and inter-assay coefficient of variation (% CV) for progesterone were 5.6 and 9.8%, respectively. To determine whether leatherback samples diluted in a parallel fashion to the standard curve, 10 samples were assayed in duplicate at doses of 50 µl and 100 µl. These samples yielded identical potencies when corrected for dilution, indicating parallelism to the standard over this dose range. Recovery of known amounts of progesterone spiked into leatherback plasma (n = 10) was 87.93%.

Statistical analyses

The number of individuals (neophytes and remigrants) nesting varies on an annual basis in sea turtles and is based on individual RI, the number of years between successive nesting seasons, and recruitment rate (Chapter II, Heppell et al., 2003). At Sandy Point, this has resulted in high nesting leatherback numbers in odd years and significantly lower nesting numbers in even years. Due to this inter-annual nesting variability, statistics were evaluated in association with hormone values for two consecutive years, including both an odd (2005) and even year (2006) to ensure that all

age/size classes were sufficiently represented and to address the odd-even year dichotomy in nesters (Chapter II). Basic reproductive statistics were compiled at the end of each season to evaluate trends in population structure (remigration interval, number of neophytes and remigrants) and productivity (including average clutch size, number of nests laid, average hatch and emergence success). Mean ± one standard deviation is provided for the number of yolked and "yolkless" eggs (SAGs) laid and hatch success (hatched shells / total yolked eggs). The Mann-Whitney test was used to determine if significant differences were observed in reproductive indices (eggs laid, average number of clutches, hatch success) between neophytes and remigrants. A Kruskal-Wallace one-way ANOVA, followed by a Dunn multiple pairwise comparison test were utilized to identify significant ($p \leq 0.05$) differences in egg production between each nesting event. Mean plasma values (testosterone, estradiol, and progesterone) ± standard deviation (SD) were established for the individuals sampled during clutch deposition in 2005 and 2006. Significant changes in steroid hormones from initial (i.e., clutch 1) to final nesting (i.e., clutch 10), as well as between consecutive clutches were determined using a Kruskal Wallace ANOVA, followed by a multiple pairwise comparison test. ($p \leq 0.05$). A trend analysis was also conducted to evaluate the correlation between clutch number and hormone levels. Significant difference in average hormone levels between nesting years for a given clutch was determined using a Mann-Whitney test.

Results

Nesting Statistics

2005 Remigrants

There were 144 individual turtles documented during the 2005 nesting season, including 45 neophytes. A total of 580 nests were laid. Number of nests laid per individual ranged from 1- 10, with a mean of 4.03 ± 2.2 nests per turtle (Table 3.1). No significant difference in average clutches laid was observed among experienced nesters (turtles with RIs of 2 – 6 years). The number of yolked eggs per clutch ranged from 10-131 eggs, with production of yolked eggs decreasing significantly over the course of the nesting season ($p = 0.021$) (Figure 3.1). Remigrants laid the greatest number of eggs in the third clutch (95.44 ± 13.58) and this varied significantly from all other clutches ($p < 0.05$ for clutches 1, 2, and 5, and $p < 0.01$ for clutches 6, 7, and 8) except clutch 4 ($p = 0.09$). Overall hatch success for all clutches laid in 2005 ranged from 0 – 92.59% (Table 3.1) and did not differ significantly over the nesting season (Fig. 3.1).

The number of yolkless eggs or SAGs laid per clutch ranged from 0 – 88 SAGs, with a mean of 31.00 ± 14.72 SAGs per clutch (Table 3.1). Production of SAGs increased significantly over the course of the nesting season ($p < 0.001$) (Fig. 3.1) for remigrants. Remigrants laid the least number of SAGs in the first clutch and this varied significantly with clutches 2 through 8 ($p < 0.01$ for clutches 2 – 6; $P = 0.043$ and 0.018 for 7 and 8, respectively).

Table 3.1. Summary of nesting data for the 2005 and 2006 seasons.

Year	Condition	N	Nests	Nest per Turtle	Average Yolked Eggs per Clutch	Average SAGs	Hatch Success
2005	Total	144	580	4.03 ± 2.20	84.46 ± 17.60	31 ± 14.72	40.36[f] ± 23.21
	Neophyte	45	162	3.14 ± 1.41	78.95 ± 13.36	27.97[e] ±15.10	41.93 ± 25.00
	Remigrant	99	471	5.29[a] ± 1.91	85.21[b,c] ± 17.25	31.69[d] ±14.43	38.50 ± 22.90
2006	Total	92	373	3.7 ± 2.08	77.68 ± 19.72	37.7 ± 20.31	47.61[f] ± 25.31
	Neophyte	31	112	2.86 ± 1.53	77.68 ± 21.61	40.03[e] ±16.47	47.95 ± 28.14
	Remigrant	61	261	4.62[a] ± 1.95	77.69[c] ± 18.35	36.93[d] ±20.57	47.39 ± 24.39

[a] Significant difference observed between neophytes and remigrants within year ($p < 0.001$)
[b] Significant difference observed between neophytes and remigrants within year ($p = 0.019$)
[c] Significant difference observed between years for remigrants ($p = 0.001$)
[d] Significant difference observed between years for remigrants ($p = 0.041$)
[e] Significant difference observed between years for neophytes ($p < 0.001$)
f Significant difference between years ($p < 0.01$)

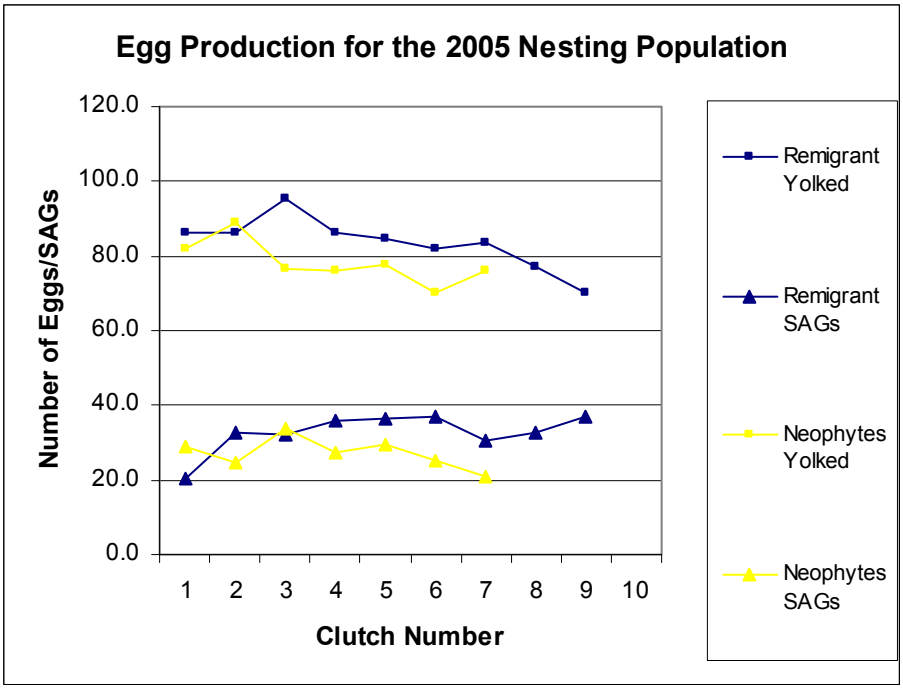

FIG. 3.1. Average yolked egg and SAG counts versus clutch number for the 2005 nesting season.

2006 Remigrants

There were 92 individual turtles observed during the 2006 nesting season, including 31 neophytes. A total of 373 nests were laid. Number of nests laid per individual ranged from 0- 10, with a mean of 3.70 ± 2.08 nests per turtle (Table 3.1). No significant difference in average clutches laid was observed among experienced nesters (turtles with RI's of 2 – 6 years). The number of yolked eggs per clutch ranged from 7-128 eggs. Production of yolked eggs did not vary significantly among clutches over the course of the nesting season. Clutch size did not decrease with increased clutch number as in 2005 (Fig.3.2). Overall hatch success for all clutches laid in 2006 ranged from 0 - 100% and, as in 2005, average hatch success did not differ significantly over the nesting season.

The number of yolkless eggs, or SAGs laid per clutch ranged from 0 – 109 SAGs, with a mean of 37.70 ± 20.31 SAGs per clutch (Table 3.1). As in 2005, production of SAGs varied significantly over the course of the nesting season with remigrants showing a significant increase in SAGs with clutch number ($p < 0.035$). Remigrants laid the least number of SAGs in the first clutch (21.43 ± 9.41) and this varied significantly with clutch 2 (39.86 ± 15.60, $p = 0.017$), clutch 3 (47.44 ± 15.08, $p < 0.0001$), and clutch 4 (38.87 ± 19.82, $p = 0.015$) (Fig. 3.2).

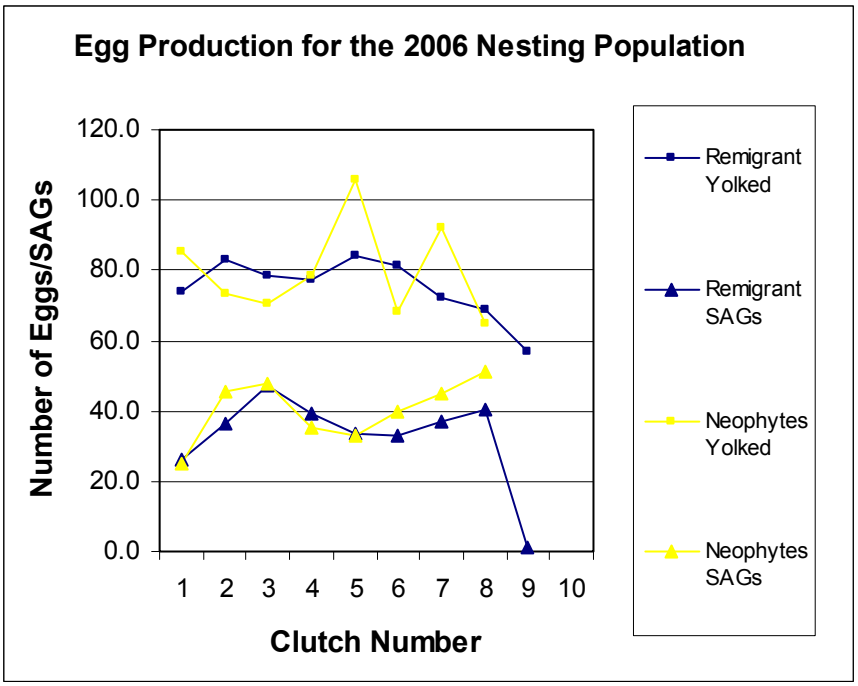

FIG. 3.2. Average yolked egg and SAG counts versus clutch number for the 2006 nesting population.

2005 Neophytes

Neophytes produced fewer nests on average than did the experienced remigrants in 2005 (3.14 ± 1.41 versus 5.29 ± 1.91, respectively, $p < 0.001$ After comparing neophytes (RI = 0, n = 28) with remigrants of differing remigration intervals: RI = 2 (n = 59), RI = 3 (n = 15), RI = 4 (n = 3), RI = 5 (n = 5), RI = 6 (n = 3) a significant difference in average nests laid was observed among neophytes (3.14 ± 1.41, RI = 0) and remigrants with an RI of 2 (5.42 ± 2.06, $p < 0.0001$) and 3 (4.93 ± 1.75, $p = 0.021$) years.

In addition to producing fewer nests, neophytes also laid significantly fewer eggs per clutch when compared to that by remigrants (78.95 ± 13.36 versus 85.21 ± 17.25 respectively, $p = 0.019$) and egg production was consistent throughout the season.

Unlike that for the 2005 remigrants, no significant decrease in yolked egg production was observed over the course of the nesting season for neophytes.

SAG production also remained consistent throughout the season. Unlike the 2005 remigrants, no significant increase in SAG production was observed over the course of the nesting season for neophytes. Additionally, no significant difference in hatch success was found between neophytes and remigrants and hatch success did not differ over the course of the nesting season for either neophytes or remigrants.

2006 Neophytes

As in 2005, neophytes produced significantly fewer nests than did remigrants (4.62 ± 1.95 versus 2.86 ± 1.53 respectively, $p < 0.0001$) (Table 3.1). After comparing neophytes (RI = 0, n = 28) with experienced nesters of differing remigration intervals: RI = 2 (n = 17), RI = 3 (n = 27), RI = 4 (n = 5), RI = 5 (n = 7), a significant difference in average nests laid was observed among neophytes (2.86 ± 1.53, RI = 0) and remigrants with an RI of 2 (4.12 ± 1.93, $p = 0.028$) and 3 (5.00 ± 1.75, $p < 0.0001$) years. Unlike that in 2005, the average number of yolked eggs laid per clutch did not vary significantly between neophytes (77.68 ± 21.61) and remigrants (77.69 ± 18.35) and clutch size did not decrease with increased clutch number for either neophytes or remigrants (Fig. 3.2).

As in 2005, no significant change in SAG production was observed over the course of the season for neophytes. Additionally, as in 2005, no significant difference in

hatch success was found between neophytes and remigrants and hatch success did not differ over the course of the nesting season for either neophytes or remigrants.

2005 Versus 2006

Among remigrants, there was a significant difference between the average number of yolked eggs and SAGs laid between 2005 and 2006, with significantly more yolked ($p = 0.001$) eggs and significantly fewer SAGs laid in 2005 ($p = 0.041$). There was no significant difference observed between 2005 and 2006 among neophytes for average yolked eggs laid. However, neophytes laid significantly more SAGs per clutch in 2006. Hatch success was significantly greater in 2006 ($p < 0.001$) than in 2005.

Steroid hormones

A total of 423 blood samples were collected from 113 individual turtles in 2005. A total of 250 blood samples were collected from 88 individual turtles in 2006. The 2005 and 2006 data combined represents a minimum of 3 to a maximum of 101 samples per given clutch number. Overall sample size per clutch generally decreased as number of clutches increased since most females average only 5-6 nests per season. More samples were collected for first ($n = 100$) and second ($n = 101$) than for third ($n = 88$), fourth ($n = 94$), fifth ($n = 64$), sixth ($n = 61$), seventh ($n = 44$), eighth ($n = 19$), ninth ($n = 8$), or tenth ($n = 3$) clutches deposited.

Plasma testosterone

Initial plasma testosterone levels in 2005 averaged 10.27 ± 6.69 ng/ml, and declined significantly over the course of the nesting season to a low of 5.00 ± 4.55 ng/ml (df = 9, $p < 0.001$) (Fig. 3.3). During the 2006 season initial plasma testosterone levels averaged 8.31 ± 5.18 ng/ml, and declined significantly (df = 9, $p < 0.001$) with increased clutch number over the course of the nesting season to a low of 1.05 ± 0.64 ng/ml (Fig. 3.3). Multiple pairwise comparisons yielded a significant difference in testosterone levels between all clutches ($p < 0.01$ for all), except 8 and 9, 8 and10, 9 and 10 for both the 2005 and 2006 nesting seasons. Similar trends in declining testosterone levels with increased clutch number were observed over both seasons ($R^2 = 0.949$, and $R^2 = 0.922$ for 2005 and 2006, respectively) (Fig. 3.3). Mean testosterone levels for a given clutch did not differ significantly between seasons, except for clutches 2, 3, and 7 (P = 0.021, 0.030, and 0.039, respectively) (Table 3.2). The average testosterone values were significantly higher for these clutches in 2005. There was no significant difference between neophytes and remigrants with regard to initial testosterone levels. There was no significant correlation between hormone values and hatch success. A summary of testosterone data for each clutch is presented for 2005 (Table 3.2) and 2006 (Table 3.3).

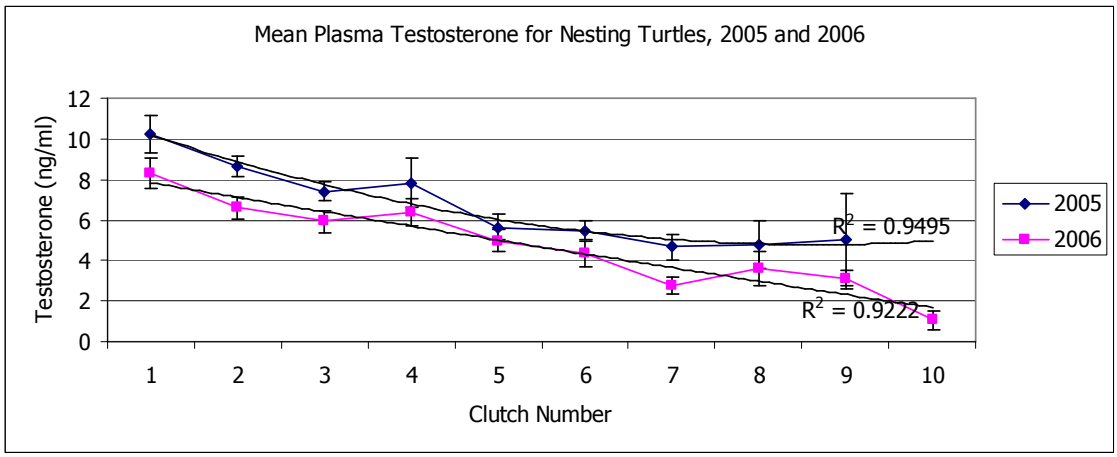

FIG. 3.3. Mean plasma testosterone levels plotted relative to clutch number for leatherback sea turtles (*Dermochelys coriacea*) at Sandy Point, St. Croix during the 2005 and 2006 nesting seasons. Values are means ± SE. A trendline with associated R^2 value is displayed.

Table 3. 2. Testosterone values for 2005 nesting population.

Clutch	N	Minimum	Maximum	Mean	Std. deviation
1	53	1.610	42.700	10.271	6.690
2	59	1.105	22.850	8.643*	4.113
3	51	0.000	14.850	7.405*	3.248
4	60	0.000	75.200	7.837	9.465
5	42	1.210	15.800	5.653	4.030
6	40	0.910	12.120	5.489	2.966
7	26	0.410	12.560	4.696*	3.218
8	11	0.680	10.950	4.779	3.832
9	4	1.440	11.210	5.003	4.550
10	1	11.410	11.410	11.410	

*Significantly greater than 2006 values for the same clutch ($p < 0.05$)

Table 3.3. Testosterone values for 2006 nesting population.

Clutch	N	Minimum	Maximum	Mean	Std. deviation
1	47	0.662	21.500	8.309	5.186
2	42	1.100	13.560	6.596*	3.415
3	37	1.100	14.210	5.925*	3.324
4	34	0.350	13.230	6.374	3.706
5	22	1.400	9.600	4.982	2.449
6	21	0.620	13.310	4.326	3.021
7	18	0.360	6.460	2.802*	1.750
8	8	0.750	7.890	3.623	2.369
9	4	1.670	3.850	3.068	0.969
10	2	0.600	1.500	1.050	0.636

*Significantly lower than 2006 values for the same clutch ($p < 0.05$)

Plasma estradiol

Circulating levels of estradiol also varied significantly over the course of the nesting season in 2005 (Fig.3.4). Initial plasma estradiol levels averaged 12.87 ± 14.50 pg/ml, and declined significantly over the course of the nesting season to a mean low of 2.0 ± 2.82 pg/ml (df = 9, $p < 0.001$) (Table 3.4). During the 2006 season initial plasma levels averaged 9.15 ± 6.59 pg/ml, and declined significantly (df = 9, $p < 0.001$) over the course of the nesting season to a low of 0.1 ± 0 pg/ml (Figure 3.4). Multiple pairwise comparison yielded a significant difference in estradiol levels between all clutches ($p < 0.01$ for all), except 8 and 9, 8 and 10, 9 and 10 for both the 2005 and 2006 nesting seasons. Similar trends in declining estradiol levels with increased clutch number were observed over both seasons ($R^2 = 0.949$, and $R^2 = 0.922$ for 2005 and 2006 respectively) (Fig. 3.4) and mean estrogen levels for a given clutch did not differ significantly between seasons (Tables 3.4 and 3.5). Therefore, data for 2005 and 2006 were pooled

exhibiting a significant decline ($p < 0.001$, $R^2 = 0.958$) in circulating estrogen levels from an average 11.14 ± 1.16 pg/ml (ranging from 0 – 61 pg/ml) at initial clutch deposition to an average low of 1.0 ± 1.0 pg/ml (ranging from 0 – 4.0 pg/ml). A significant difference in estradiol levels was observed between neophytes and remigrants with regards to initial estradiol levels. There was no significant correlation between hatch success and hormone values. A summary of estradiol data for each clutch is presented for 2005 (Table 3.4) and 2006 (Table 3.5).

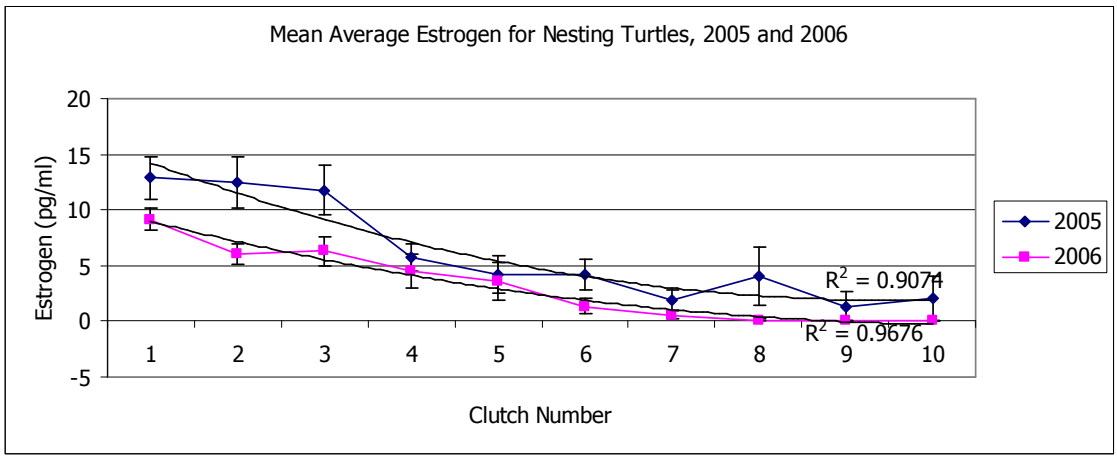

FIG. 3.4. Mean plasma estradiol levels plotted relative to clutch number for leatherback sea turtles at Sandy Point, St. Croix during the 2005 and 2006 nesting seasons. Values are means ± SE. A trendline with associated R^2 value is displayed.

Table 3.4. Summary of estradiol data for the 2005 nesting population.

Clutch	N	Minimum	Maximum	Mean	Std. deviation
1	54	0.100	61.000	12.870	14.508
2	52	0.100	85.000	12.462	16.235
3	48	0.100	62.000	11.750	15.461
4	54	0.100	38.000	5.722	9.374
5	36	0.100	56.000	4.194	10.150
6	35	0.100	40.000	4.200	8.460
7	20	0.100	13.000	2.050	4.273
8	10	0.100	26.000	4.000	8.206
9	3	0.100	4.000	1.333	2.309
10	2	0.100	4.000	2.000	2.828

Table 3.5. Summary of estradiol data for the 2006 nesting population.

Clutch	N	Minimum	Maximum	Mean	Std. deviation
1	47	0.100	22.000	9.149	6.590
2	42	0.100	19.000	6.048	5.996
3	37	0.100	40.000	6.297	8.171
4	35	0.100	47.000	4.514	9.214
5	21	0.100	37.000	3.762	8.142
6	20	0.100	9.000	1.350	2.815
7	17	0.100	5.000	0.588	1.372
8	8	0.100	1.000	0.125	0.354
9	4	0.100	0.100	0.100	0.000
10	2	0.100	0.100	0.100	0.000

Plasma progesterone

Circulating levels of progesterone also varied significantly over the course of the nesting season in 2005 (Fig. 3.5). Initial plasma progesterone levels averaged 1.73 ± 0.59 ng/ml and declined significantly over the course of the nesting season to a low of 0.35 ± 0.35 ng/ml (df = 9, $p < 0.001$) (Table 3.6). During the 2006 season initial plasma progesterone levels averaged 1.99 ± 1.60 ng/ml, and declined significantly (df = 9, p <

0.001) over the course of the nesting season to a low of 0.18 ± 0.09 ng/ml (Fig. 3.5, Table 3.7). Multiple pairwise comparisons yielded a significant difference in progesterone levels between all nesting events ($p < 0.01$ for all), except 8 and 9, 8 and 10, 9 and 10 for both the 2005 and 2006 nesting seasons. Similar trends in decreasing progesterone levels with increased clutch number were observed over both seasons ($R^2 = 0.800$, and $R^2 = 0.861$ for 2005 and 2006, respectively) (Fig. 3.5) and mean progesterone levels for a given clutch did not differ significantly between seasons, except for clutch 5. The average progesterone level for clutch 5 was significantly higher in 2005 ($p = 0.047$) (Table 3.6). There was no significant difference between neophytes and remigrants with regards to initial progesterone levels. There was no significant correlation between hatch success and hormone values. A summary of progesterone data for each clutch is presented for 2005 (Table 3.6) and 2006 (Table 3.7).

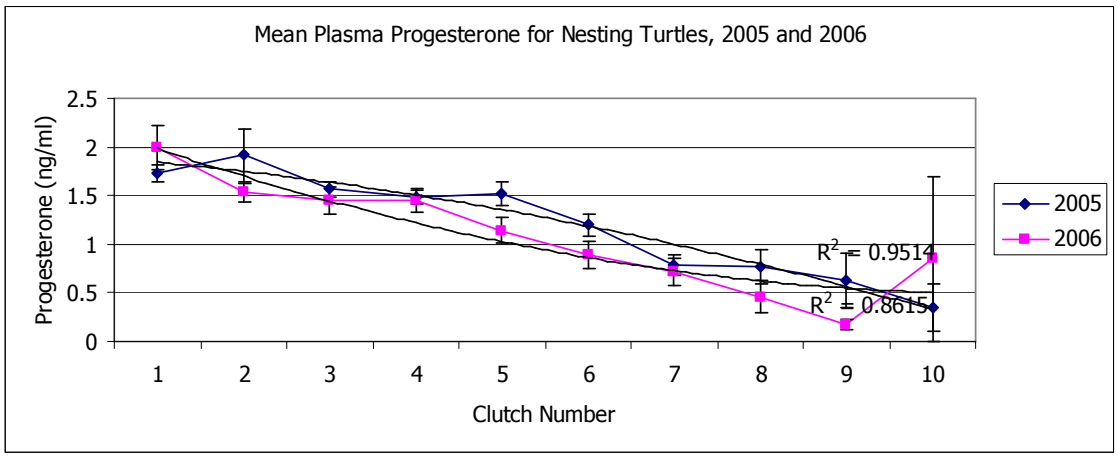

FIG. 3.5. Mean plasma progesterone levels plotted relative to clutch number for leatherback sea turtles (*Dermochelys coriacea*) at Sandy Point, St. Croix during the 2005 and 2006 nesting seasons. Values are means ± SE. A trendline with associated R^2 value is displayed.

Table. 3.6 Summary of progesterone data for the 2005 nesting population.

Clutch	N	Minimum	Maximum	Mean	Std. deviation
1	55	0.500	4.100	1.733	0.596
2	57	0.200	16.800	1.919	2.076
3	53	0.100	2.900	1.568	0.587
4	60	0.000	2.500	1.492	0.579
5	43	0.000	3.400	1.519*	0.765
6	44	0.000	3.300	1.198	0.735
7	29	0.100	2.300	0.797	0.596
8	12	0.100	1.700	0.767	0.580
9	4	0.100	1.200	0.625	0.556
10	2	0.100	0.600	0.350	0.354

*Significantly greater than 2006 values for the same clutch (p = 0.047)

Table. 3.7 Summary of progesterone data for the 2006 nesting population.

Clutch	N	Minimum	Maximum	Mean	Std. deviation
1	47	0.600	12.000	1.991	1.606
2	42	0.200	2.500	1.531	0.598
3	37	0.400	4.100	1.454	0.870
4	35	0.200	3.700	1.449	0.793
5	22	0.000	2.100	1.141*	0.599
6	21	0.100	2.900	0.895	0.645
7	18	0.000	1.900	0.711	0.581
8	8	0.000	1.300	0.463	0.472
9	4	0.100	0.300	0.175	0.096
10	2	0.000	1.700	0.850	1.202

*Significantly lower than 2006 values for the same clutch (p = 0.047))

Neophytes versus remigrants

There was no significant difference between neophytes and remigrants with regards to initial testosterone and progesterone levels. However, there was a significant difference observed between neophytes and remigrants with regards to estradiol (p = 0.008). Remigrants (with RI = 2 and 3 years) exhibited higher levels of estrogen when compared to those of neophytes (RI = 0). Within the remigrant class, initial estradiol

levels were significantly different between the 2005 and 2006 nesting populations ($p = 0.013$), with higher levels documented in 2005 (17.27 ± 16.11 pg/ml) than 2006 (9.82 ± 5.98 pg/ml).

For all steroid hormones (testosterone, estrogen, and progesterone), there was no significant correlation between hatch success and hormone values.

Discussion

This study provides the first description of steroid hormones during nesting in Atlantic leatherback sea turtles. It is also the first to evaluate hormone profiles in conjunction with specific reproductive indices of Atlantic turtles, including RI (neophytes versus remigrants), clutch production, and egg and SAG production. Population composition during the study years (percentage represented by neophytes versus remigrants, and animals representing different RIs) were similar to those of other seasons documented at SPNWR and are typical of the species (Garner et al., 2006). Overall seasonal patterns of circulating testosterone, estradiol, and progesterone in the Atlantic *D. coriacea* population were similar to those observed in the hard-shelled sea turtle species, with all hormones decreasing significantly with increased clutch number. When compared to hard-shelled turtles however, Atlantic leatherbacks exhibited a more gradual decline in these steroids across subsequent clutches, a trend also observed by Rostal et al. (2001) in Pacific leatherbacks. This is attributed to the greater number of clutches produced in this species (Rostal et al., 2001), on average 5 - 6 clutches per

season (Boulon et al., 1996; Garner and Garner, 2010) versus 2 – 4 observed in other species (Miller, 1997). Declines in both testosterone and estradiol are associated with a decrease in the number of follicles following the ovulation of consecutive clutches (Owens and Morris, 1985). Since leatherbacks ovulate a smaller portion of their total follicles to deposit each clutch, the decline in these hormones should be more gradual (Rostal et al., 2001).

Overall testosterone and progesterone levels were similar in both magnitude and profile between Atlantic and Pacific leatherbacks. Plasma estradiol levels, however, were observed to be distinctly different among the two populations, with Pacific turtles exhibiting higher levels (57.7 pg/ml - 480.5 pg/ml; Rostal 2001) when compared to those of Atlantic turtles (0 - 61 pg/ml). Despite lower levels of circulating estradiol, Atlantic leatherbacks historically lay more clutches, produce more eggs per clutch, and have a shorter remigration interval when compared to Pacific leatherbacks (Reina et al., 2002; Dutton et al., 2005; Wallace et al., 2006).

Estradiol is associated with growth and maturation of female reproductive organs and deposition of reproductive resources, such as fat (Hadley, 2000) necessary for migration to the nesting beach. An increase in estradiol has been observed in Kemp's ridley and green sea turtles 4-6 months prior to mating (Owens, 1997; Rostal et al., 1997). This is correlated with an increase in the serum proteins, calcium levels, and phosphoproteins associated with vitellogenesis in reptiles (Ho, 1987; Heck et al., 1997). Declining levels imply that vitellogenesis in sea turtles may be complete or near completion prior to mating and arrival at nesting beaches (Wibbels et al., 1990; Rostal et

al., 1996; Rostal et al., 2001). Leatherbacks exhibit high intra- and inter- population variability with regard to habitat use and migratory patterns (Fossette et al., 2010) therefore, the lower estradiol levels observed in Atlantic leatherbacks suggest that they may undergo a longer migration or spend more time in the feeding grounds prior to migrating, while vitellogenesis is occurring. Thus, there is more time for depletion of estradiol levels in Atlantic leatherbacks prior to arrival at the nesting beach.

Overall average estradiol levels within the Atlantic population did not vary significantly between years. This was expected since turtles in both years are subsets of the same population and exhibit the same general reproductive characteristics. However, observed differences in clutch productivity between years among neophytes and remigrants elicited further analysis of hormone profiles. This analysis is novel and has not previously been conducted for any other sea turtle population.

Clutch size varied significantly between neophytes and remigrants in 2005, with remigrants laying significantly more eggs per clutch. Clutch size did not vary significantly between these groups in the 2006 nesting season, however, 2- and 3-year remigrants still produced significantly more clutches than did neophytes. Based on these reproductive data, the population was divided based on RI and a significant difference was observed with regards to estradiol levels. Remigrants (with RI = 2 and 3 years) exhibited significantly higher levels of estradiol when compared to those of neophytes (RI = 0). Higher levels of estradiol within remigrants are attributed to increased reproductive maturity and an increased number of follicles. Maternal hormones have previously been correlated to physiological parameters such as the age and size of an

individual animal. Estrogen and testosterone have been shown to vary with age and body size in populations of the American alligator (*Alligator mississippiensis*). In alligators, a direct correlation is observed between hormone levels and the age of individual animals (Milnes et al., 2002). Greater reproductive maturity and experience impacts the size of ovaries which serve as the main source of estradiol and testosterone in the alligator, as well as in sea turtles (Owens, 1997). Thus, older, more mature leatherback females with larger ovaries have more follicles and will exhibit higher estradiol levels when compared to those of younger, smaller, less mature individuals within the same population. Correlating hormone levels with animal size and age may thus prove a valid method of distinguishing neophyte from remigrant turtles, as well as age of an individual animal, and potential reproductive output. Trends in clutch number and size were therefore further evaluated in conjunction with hormone values for remigrant turtles over both years since remigrants laid more clutches on average and significantly more eggs per clutch in 2005 versus 2006.

Evaluation of only "mature", remigrant animals showed initial estradiol levels were significantly different between the 2005 and 2006 nesting populations ($p = 0.013$), with higher levels documented in 2005 (17.27 ± 16.11 pg/ml) than in 2006 (9.82 ± 5.98 pg/ml). This correlates to the different reproductive output observed between the two years for remigrant turtles. Greater reproductive output was documented in the year initial estradiol levels were higher. No significant difference was observed in estradiol levels between 2005 and 2006 for neophytes only, and neophytes produced a similar number of clutches and eggs per clutch over both years. This further supports the

hypothesis that estradiol levels reflect the potential reproductive output of individual animals in different age classes. Similar analyses of testosterone and progesterone values revealed no correlation between these hormones and potential reproductive output.

Trends in SAG production varied annually between those of neophytes and remigrants within the nesting population. This suggests a species that exhibits an ontogeny of reproductive output as well as reproductive plasticity in yolked egg, SAG and clutch production depending on nesting year. The 2005 remigrants showed a decrease in the number of yolked eggs per clutch, and a concomitant increase in SAGs with nesting chronology. This was not observed in neophytes for either the 2005 or 2006 nesting season. The average number of eggs and SAGs produced per clutch remained constant throughout the season for neophytes. In 2006, the remigrant turtles did not exhibit a decline in average clutch size but exhibited an increase in SAG production over time. Rostal et al. (2001), observed a trend similar to that for the 2005 Sandy Point remigrants during the 1996 and 1997 nesting seasons at Las Baulas, Costa Rica. Pacific leatherbacks showed the greatest clutch size and fewest SAGs at the initial nesting event with the fewest eggs, and greatest number of SAGs observed at the final nesting event (Rostal et al., 2001). The purpose of laying SAGs has not yet been determined, but hypotheses include addition of moisture to the nest, assistance with pore spacing, predator avoidance, and excretion of left-over albumin. The physiological mechanism for SAG production has yet to be determined, but is believed to be linked to progesterone due to the hormone's role in regulating albumin production in other species

(Blanvillain et al., 2011). In this species, levels of progesterone appeared to remain constant as documented over the course of the nesting season for Pacific leatherbacks (Rostal et al., 2001). High, constant levels of progesterone were hypothesized to be directly related to deposition of a significant number of shelled albumin globules or SAGs in each clutch (Rostal et al., 2001). SAG's are not commonly produced by other sea turtle species and these species also show a significant decline in progesterone over the nesting season (Owens and Morris, 1985; Rostal et al., 1998; Hamann et al. 2002). In addition, the observation of constant, elevated progesterone levels in the Pacific leatherback supported this hypothesis.

The seasonal trend in progesterone for Atlantic leatherbacks was similar to that for the hard-shelled species (but more gradual), and differed from that observed in Pacific leatherbacks (Rostal et al., 2001). Atlantic leatherbacks exhibited a significant decrease in progesterone levels with increased clutch number, while no significant decrease was observed in Pacific turtles (Rostal et al., 2001). This was true for both years, in spite of the differing trends in SAG production observed in 2005 versus 2006. Atlantic turtles exhibit decreasing hormone values irrespective of the annual trend in SAG production. This further complicates the theory of how SAGs are made and suggests that progesterone may not be the primary stimulus for SAG production. However, it does not rule out the potential role of progesterone in the production of these unique entities.

While estradiol correlated with trends in clutch production, there was no correlation between hatch success and estradiol. Additionally, there was also no

correlation between hatch success and progesterone values. Testosterone values varied significantly between nesting seasons for several clutches (e.g., 2, 3, and 7), with highest values observed in 2005 when production of clutches and eggs was higher. Hatch success was significantly higher in 2006, however, and there was no correlation between hatch success and testosterone values for either year. Despite slight variations in absolute values between years, similar trends were observed for all three hormones, thus confirming that a decrease in estradiol, testosterone, and progesterone levels over consecutive nesting events (clutches) is a characteristic of this population. These data serve as a biological validation of the assays conducted and support the hypothesis that leatherbacks are also similar to other sea turtle species with regard to their basic reproductive steroid endocrinology at nesting.

Although hormones were not correlated with hatch success, and no specific correlation was observed between hormones and SAG production, this study determined that there is a correlation between estradiol levels and reproductive output. Additionally, reproductive experience plays an important role in reproductive output in leatherbacks and correlated to increased estradiol levels. The increased size and reproductive maturity of the ovaries in remigrants is associated with increased estradiol production. The increased estradiol production correlates to greater clutch production and greater reproductive plasticity in remigrants when compared to that of neophyte turtles. The Atlantic population at St. Croix provides an opportunity to further evaluate the relationship between hormones and age due to the availability of a significant number of neophytes annually, as well as females tagged between 2 and 25 years prior. It also

affords the opportunity to further investigate the use of hormones as a predictor of reproductive output and trends in clutch production. Egg output is directly related to hormone levels in many species (Jones et al., 1976; Sinervo and Licht, 1991) and this is supported in leatherbacks as well. Estradiol levels may therefore serve as a useful predictor of clutch number and clutch size, and ultimately predict years of high versus low reproductive output within a population. Baseline data are also needed to provide the foundation for determining any impact of endocrine disruptors on reproduction in this species.

The potential role of hormones in SAG production is still inconclusive and also requires further evaluation. This study illustrated that leatherbacks exhibit a reproductive ontogeny with age and a reproductive plasticity between years which correlate to specific hormone values. To further understand these processes it is necessary for individual animals to be sampled over multiple remigration intervals. This is feasible within the Atlantic population at Sandy Point, a population which provides great opportunity for enhancing our knowledge of reproductive endocrinology in this species.

CHAPTER IV

EVALUATING THE POTENTIAL FOR PREDICTIVE REPRODUCTIVE MODELING IN ENDANGERED LEATHERBACK SEA TURTLES

Introduction

Recent analysis of maternal reproductive steroid hormones in a population of leatherback sea turtles (*Dermochelys coriacea*) in St. Croix, provided the first description of steroid hormones during nesting in Atlantic leatherback sea turtles (see Chapter III). This was also the first study to evaluate hormone profiles in conjunction with specific reproductive indices of Atlantic leatherbacks, including remigration interval (RI), (neophytes or first time nesters, versus remigrants or returning nesters with RIs of 2, 3, or more years), clutch production, and yolked egg and shelled albumin globule (SAG) production. For the Atlantic population, overall seasonal patterns of circulating testosterone, estradiol, and progesterone were similar to those observed in the hard-shelled sea turtle species, with all hormones decreasing significantly with increased clutch number (Chapter III). In comparison to that for hard-shelled turtles, however, Atlantic leatherbacks exhibited a more gradual decline in these steroids across subsequent clutches, a trend also observed by Rostal et al. (2001) in Pacific leatherbacks (see Chapter III). This is attributed to the greater number of clutches produced in this species (Rostal et al., 2001), on average 5 - 6 clutches per season (Boulon et al., 1996; Garner and Garner, 2010) versus 2 – 4 observed in other species (Miller, 1997). Decline

in both testosterone and estradiol are associated with a decrease in the number of follicles following the ovulation of consecutive clutches (Owens and Morris, 1985). Because leatherbacks ovulate a smaller proportion of their total follicles to deposit each clutch, the decline in these hormones is more gradual.

Previous work conducted with Kemp's ridley (*Lepidochelys kempii*) and green (*Chelonia mydas*) sea turtles (Owens and Morris, 1985; Rostal et al., 1998; Hamann et al., 2002) has supported the use of these steroid hormones as predictors of clutch number (Rostal et al., 2001). Since leatherbacks exhibit similar hormone trends when compared to those of the aforementioned species, it is therefore feasible that a blood sample may serve as a predictor of reproductive output (clutch number) in this species as well. Based on analyses of reproductive data in conjunction with hormone values in this population, it is also hypothesized that steroid hormones may serve as valid predictors of additional reproductive parameters.

Hormone levels may serve as a predictor of reproductive age and maturity in this species, and serve as a useful tool for distinguishing neophyte from remigrant turtles. A significant difference was observed in reproductive output between neophytes and remigrants within the Sandy Point population, with remigrants producing significantly more clutches per season than did neophytes (Chapter III). Remigrants, with RIs of 2 and 3 years, also exhibited significantly higher levels of estradiol when compared to neophytes (RI = 0) (Chapter III). Higher levels of estradiol within the remigrant class are attributed to increased reproductive maturity, greater ovarian size, and an increased number of follicles (Owens, 1997; Milnes et al., 2002). Maternal hormones have

previously been correlated to physiological parameters such as the age and size of an individual animal in other reptile species (Milnes et al., 2002). Thus, hormone levels may serve as a valid predictor of reproductive age and maturity in this species. Older, more mature leatherback females with larger ovaries and more follicles should exhibit higher estradiol levels when compared to those of younger, smaller, less mature individuals within the same population.

Evaluation of hormone levels in only "mature" remigrant animals in this population also showed initial estradiol levels were significantly different between the 2005 and 2006 nesting populations (Chapter III). Higher initial estradiol levels were documented in 2005 when reproductive output (average number of clutches laid and average number of eggs laid per clutch) was higher. No significant difference was observed in estradiol levels between 2005 and 2006 for neophytes only, and neophytes produced a similar number of clutches and a similar number of eggs per clutch over both years (Chapter III). Egg output (number of eggs laid) has been shown to be directly related to hormone levels in many species (Jones et al., 1976; Sinervo and Licht, 1991) and this is supported in leatherbacks as well. Thus, it is hypothesized that estradiol levels reflect the productivity (both clutch number and clutch size) of individual animals in different age classes for this population. However, the ability to utilize hormone levels to predict the number of clutches laid has not been tested in this species. Additionally, the feasibility and validity of utilizing a blood sample to predict overall reproductive output (clutches laid, clutch size), age, and maturity of an individual animal has not been evaluated either.

The Atlantic population at St. Croix provides an opportunity to develop and test a predictive reproductive model utilizing these parameters in association with hormone values. This population boasts a significant number of neophytes annually, as well as females tagged between 2 and 25 years prior, thus allowing evaluation of hormone parameters with increased reproductive age. It also boasts a comprehensive reproductive database for each individual within a given year, and historically over the past 30 years. If a predictive model can be developed for this population, it may allow managers to predict years of high versus low reproductive output within a population, as well as identify neophyte from remigrant animals from a blood sample. This would be useful on start-up projects and projects that lack saturation tagging. Additionally, a valid reproductive model may help identify animals that have nested prior to being documented on SPNWR. It is also possible that similar models could be developed for other wild populations which exhibit unique reproductive characteristics.

Methods

Basic reproductive data collection

The nesting leatherback population at Sandy Point National Wildlife Refuge (SPNWR), St. Croix, U.S. Virgin Islands, served as the study group. Patrols were conducted by the West Indies Research and Conservation Service (WIMARCS) personnel and local volunteers as part of the annual Leatherback Research and Conservation project funded by the Virgin Islands Department of Planning and Natural Resources (VIDPNR) and WIMARCS. For the past 30 seasons a basic beach protocol

has been conserved. Annual nightly beach patrols at SPNWR are initiated April 1st and continue until approximately 10 days after the last female leatherback has nested (generally July 15th – August 1st). During this timeframe the beach is patrolled nightly on foot, starting around 2000 hours until either 0500 hours or until the last female has finished nesting. Specific protocols for data collection are described in Chapter II. Number of nesting turtles, number of neophytes versus remigrants, average hatch success, curved carapace length (CCL), number of yolked and yolkless eggs, remigration interval (RI, number of years since previous nesting), and reproductive age for the 2005 and 2006 population (Chapter II) were utilized for modeling purposes.

Blood sampling

In association with the ongoing conservation project blood sampling was conducted during the nesting season for two consecutive years to ensure repeatability of results, as well as account for the inter-annual nesting variability observed in sea turtle species. To ensure that enough blood samples were obtained from a representative sample of turtles (various ages, sizes, remigration intervals) and clutches (1 through 10), an attempt was made to sample each individual turtle every time she successfully nested throughout the season (April-August). Blood samples were obtained from turtles only after successful initiation of egg deposition to minimize disruption of the nesting process, once the turtle had entered the nesting "trance" and deposited approximately 4 eggs. Specific blood sampling and handling protocol is described in Chapter II.

Analysis of hormones

All analyses were conducted at the endocrine diagnostic laboratory of Texas Veterinary Medical Diagnostic Laboratory (TVMDL). Plasma steroid hormone levels were measured using commercially available radioimmunoassay (RIA) kits including:

- Testosterone: Coat-A-Count® Total Testosterone solid-phase ^{125}I radioimmunoassay kit, # TKTT2 Diagnostics Products Corporation (DPC), Los Angeles, CA
- Estradiol: DPC Coat-A-Count® Estradiol no-extraction, solid-phase ^{125}I radioimmunoassay kit #KE2D1
- Progesterone: DPC Coat-A-Count® Progesterone, solid-phase ^{125}I radioimmunoassay kit #TKPG5

All blood samples were assayed in duplicate at a volume of 50 µl. A Genesys 5000 gamma counter was used for counting and concentration calculations. Validation results are provided in Chapter III.

Statistical analyses

The number of individuals (neophytes and remigrants) nesting varies on an annual basis in sea turtles and is based on individual RI, or the number of years between successive nesting seasons (usually 2 - 3 years) and recruitment rate. For leatherbacks at Sandy Point this results in higher numbers of nesting females in odd numbered years and smaller numbers of nesting females in even numbered years (Chapter II). Due to this

inter-annual nesting variability, statistics were evaluated in association with hormone values for two consecutive years, including both odd (2005) and even year (2006) data to ensure that all age/size classes were sufficiently represented and to address the odd-even year dichotomy in nesters. Basic reproductive statistics were compiled at the end of each season to evaluate trends in population structure (remigration interval (RI), number of neophytes and remigrants) and productivity (including average clutch size, number of nests laid, average hatch and emergence success). Turtle ID, year tagged, hormone (estradiol, testosterone, progesterone) and reproductive data (clutch number, clutch size, turtle size/age, RI) were factored into a mixed effects model utilizing R Version 2.12.1 (© 2010, The R Foundation for Statistical Computing, Platform i386-pc-mingw32/i386). Linear mixed effects models were used to determine the effects of designated variables on hormone levels. Individual turtles were treated as a random effect and repeated measures within each individual were accounted for as statistically nested. Effects of independent variables were fit by maximum likelihood and assessed by using AIC (Akaike Information Criteria; Akaike, 1974) and BIC (Bayesian Information Criteria; Schwarz ,1978). This approach accounts for all possible factors in one analysis and looks at the overall fit simultaneously. Turtle size (as curved carapace length (CCL)), reproductive age (number of years since first tagged), RI and clutch size (including yolked and yolkless eggs) were the factors analyzed to determine if they contributed significantly to the hormone model. These parameters were incorporated into a "kitchen sink" model e.g.: Testo ~ 1 + nDays * nYrs * RI * CCL. The non-significant interaction terms and fixed effects were sequentially removed. Additionally, number of days since

first nesting within each year replaced clutch number as a variable to account for the possibility that the turtle may have laid prior to initial sampling at SPNWR. Average slopes ± standard error are provided for each model.

Results

All three hormones were analyzed in association with number of days since first nesting, turtle size (as CCL), reproductive age, RI, clutch size (including yolked and yolkless eggs (or SAGs)) and hatch success. The most parsimonious model for testosterone included the number of days (nDays) since first sampling within each year. The relationship was linear and the slope and intercepts were individual specific. No significant relationship was found for any other factors, including reproductive age, CCL, remigration interval, clutch size and hatch success, thus these factors did not contribute to the model. The average slope for testosterone utilizing nDays was -0.084 ± 0.009 (CI: - 0.069, -0.098). The intercept (y: testosterone (ng/ml)) varied significantly between neophytes and remigrants (Table 4.1) ($p<0.0001$), with remigrants exhibiting a larger mean intercept value. The slope (nDays) also differed significantly between neophytes and remigrants ($p < 0.0001$) with remigrants exhibiting a greater decrease in testosterone over time

Table 4.1.
Parameter estimates for Testosterone Model.

		Mean	S.E.	DF	t-value	p-value
All Turtles	Intercept	8.845	0.403	172	21.925	0
	nDays	-0.084	0.009	172	-9.631	0
Neophytes	Intercept	7.699*	0.668	43	11.527	0
	nDays	-0.058*	0.024	43	-2.446	0.0186
Remigrants	Intercept	9.505*	0.503	128	18.895	0
	nDays	-0.094*	0.009	128	-9.549	0

* Significantly different between neophytes and remigrants

The most parsimonious model for progesterone included the number of days since first nesting within each year. The relationship was linear and the intercepts were individual specific. A common slope was the best model for progesterone. The average slope for the progesterone model utilizing nDays was -0.016 ± 0.0018 (CI: -0.013, -0.019). No significant relationship was found for any other factors, including reproductive age, CCL, remigration interval, clutch size and hatch success, thus these factors did not contribute to the model. Slope (nDays) and intercept (y, progesterone ng/ml) did not differ significantly between neophytes and remigrants (Table 4.2)

Table 4.2.
Parameter estimates for Progesterone Model.

		Mean	S.E.	DF	t-value	p-value
All Turtles	Intercept	1.764	0.068	171	26.069	0
	nDays	-0.016	0.002	171	-8.873	0
Neophytes	Intercept	1.869	0.154	41	12.177	0
	nDays	-0.031	0.005	41	-6.432	0
Remigrants	Intercept	1.763	0.076	129	23.253	0
	nDays	-0.015	0.002	129	-7.796	0

The most parsimonious model for estrogen was log transformed (i.e. ln(y) = mx + b) and included the number of days since first nesting within each year (nDays), the number of years since first nesting (reproductive age: nYrs), and the interaction between these two factors (nDays*nYrs). Both factors were linear with respect to hormone level and intercepts were individual specific. A common slope was the best model for estrogen. Estrogen decreased linearly in association with increased number of days within a season, but increased with the number of years since first nesting. The average slope for nDays was -0.011 ± 0.002 (approximate 95% CI: -0.008, -0.014). The average slope for nYrs was 0.038 ± 0.01 (approximate 95% CI: 0.056, 0.207). The slope did not differ significantly between neophytes and remigrants; however the intercept (y, estrogen (pg/ml)) differed significantly between the two groups (p = 0.0001) (Table 4.3).

Table 4.3.
Parameter Estimates for Estrogen Model.

		Mean	S.E.	DF	t-value	p-value
All Turtles	Intercept	0.631	0.067	161	9.48	0
	nDays	-0.011	0.002	161	-6.45	0
	nYrs	0.038	0.01	94	3.715	0.0003
	nDays:nYrs	-0.0005	0.0002	161	-2.409	0.017
Neophytes	Intercept	0.549*	0.079	39	6.879	0
	nDays	-0.01	0.002	39	-4.129	0
	nYrs	N/A	N/A	N/A	N/A	N/A
	nDays:nYrs	N/A	N/A	N/A	N/A	N/A
Remigrants	Intercept	0.804*	0.117	121	6.869	0
	nDays	-0.012	0.002	121	-5.291	0
	nYrs	0.019	0.014	59	1.374	0.175
	nDays:nYrs	-0.0004	0.0003	121	-1.453	0.149

*Differed significantly between neophytes and remigrants

Discussion

This study utilized linear mixed effects modeling (LME) to evaluate numerous reproductive variables and determine if they contributed significantly to a reproductive hormone model for nesting Atlantic leatherback sea turtles. Linear mixed effects modeling evaluates the effect of both fixed and random factors on a response variable, and estimates the range of response variable values that would be produced in the population based on these factors. In this study, LME was employed to assess the contribution of clutch number (identified by number of days since first sampling), turtle size (as CCL), age, remigration interval, clutch size (yolked and yolkless eggs) and hatch success to the documented hormone values and trends observed in the St. Croix population. Variables deemed significant were factored into the reproductive model for a given hormone, non-significant factors were removed.

The relationship for all three hormones (testosterone, progesterone and estrogen) was linear and decreased with increased number of days from initial sampling. Although trends in average clutch production, clutch size and SAG production varied annually between neophytes and remigrants within the SPNWR nesting population (Chapter III), no relationship was found between hormone values, clutch size and SAG production; therefore these factors were not included in the model. Nesting year (odd versus even) was considered a random effect based upon the 2-year data set. It is recommended, however, that future studies further evaluate the impact of nesting year

on reproductive indices. To determine the impact of odd versus even years replicates are needed for both odd and even years, thus a minimum four year dataset is required.

Testosterone

Both male and female sea turtles exhibit distinct seasonal cycles for testosterone (Owens and Morris 1985; Licht et al., 1985; Rostal et al., 1998; Rostal et al., 2001; Hamann et al., 2003, Blanvillain et al., 2011). Testosterone triggers the development of secondary sexual characteristics and stimulates courtship and mating behavior in individuals (Owens, 1997). Testosterone levels in males rise approximately 4-5 months prior to mating in association with spermatogenesis and testicular recrudescence (Rostal et al., 1997). Levels subsequently decrease during the actual mating period (Licht et al., 1985; Wibbels et al., 1990; Owens, 1997; Rostal et al., 1997; Hamann et al., 2003).

Female Kemp's ridley and green sea turtles show a similar spike in testosterone levels prior to mating (Rostal et al., 1997; Hamann et al., 2002). A rise in testosterone in females is associated with follicular development and possibly stimulation of migration, female receptivity and courtship behavior (Hamann et al., 2002). As in the male, levels drop off during the courtship and mating period. Additionally, circulating testosterone levels progressively decline to basal concentrations in conjunction with follicle loss to consecutive ovulations with each nesting event until the nesting season ends and the ovary regresses (Owens, 1997; Rostal et al., 1998).

Testosterone in nesting female leatherback sea turtles at SPNWR was the most variable hormone evaluated, with both slope and intercept being individually specific.

The magnitude of testosterone values observed at initial nesting is therefore largely dependent on the individual animal. The rate of decrease in testosterone observed over the course of the nesting season is also individually dependent.

Although number of years since tagging (reproductive age) did not contribute significantly to the model, once the individually based testosterone model was applied to neophyte versus remigrant turtles it was determined that remigrants have a significantly greater Y intercept and slope when compared to that of neophytes. Thus, remigrants have higher testosterone levels at initial nesting and show a greater decrement in testosterone between consecutive nesting events over the course of the nesting season. Ovaries are the main source of testosterone in sea turtles; therefore this is likely due to increased reproductive maturity, greater ovarian size, and an increased number of follicles in mature, remigrant turtles when compared to that of neophytes (Milnes et al., 2002; Owens, 1997). The decrease in testosterone is associated with ovulation of follicles at each nesting event and the subsequent cessation of testosterone production by the granulosa cells (Owens and Morris, 1985; Hamann et al., 2003). Although slope and intercepts for testosterone do vary significantly between neophytes and remigrants based upon the model, testosterone levels are highly individual and do not correlate significantly with increased age once a turtle has reproductive experience.

Testosterone levels are also linked to initiation and maintenance of mating behavior (Licht et al., 1985; Hamann et al., 2002) and therefore increased testosterone levels at initial nesting (intercepts) may reflect individuals that are more sexually active (Licht et al., 1985). Testosterone in male green turtles was determined to cycle

seasonally on a population, as well as on an individual level, and levels were significantly correlated with active mating activity (identified by the number of mates and the time spent copulating) (Licht et al., 1985). If this is true for females as well, initial plasma testosterone levels may therefore reflect increased sexual activity. This is likely the case for remigrants who generally arrive at the nesting beach earlier than neophytes (when males are likely more abundant in offshore waters) (personal observation; Tucker and Frazer, 1992; Limpus et al., 2001; Hamann *et al*., 2002; Hamman et al., 2003) and also produce more clutches.

Since the model for testosterone deemed both the slope and intercept terms are highly dependent on the individual, it is not possible to present a common equation (i.e., $y = mx + b$) for the population. The equations are individual specific and utilize different values for slope and intercept that are dependent upon each animal.

Progesterone

Intercepts were also individually specific for progesterone, however, the most parsimonious model also utilized a common slope. As with testosterone, the magnitude of progesterone concentration observed at initial nesting is highly dependent on the individual animal. Although no significant difference was observed between intercepts for neophytes versus remigrants, the best fitting model for progesterone did not utilize a common Y intercept. The best fitting regression lines did, however, utilize a common slope for all turtles, thus suggesting all turtles (including neophytes and remigrants) exhibit a similar decrement in progesterone between nesting events.

Progesterone is released from the corpus luteum post ovulation and stimulates production of albumin proteins in the oviduct as well as the subsequent secretion of albumin from the albumin glands in sea turtles (Owens and Morris, 1985). Progesterone increases sharply 24-48 hours post-nesting in the olive ridley, green, and loggerhead sea turtles (Licht et al., 1979; Licht et al., 1982; Wibbels et al., 1992). A high level of serum progesterone is also observed early in the season and is directly correlated with nesting and ovulation of the first clutch of eggs (Rostal et al., 1997). Additionally, levels in the Kemp's ridley have been shown to decrease with each clutch laid throughout the nesting season (Rostal et al., 1997). Progesterone is vital to albumin deposition and has been suggested to play a role in egg retention in sea turtles, as well as production of SAGs in leatherbacks (Licht et al., 1979; Owens and Morris, 1985). Similar levels of progesterone observed in both neophyte and remigrant individuals are consistent with progesterone cycles and the timing of successful oviposition. Unlike those for testosterone and estrogen, progesterone levels have been shown to vary depending on timing of sampling (e.g., during nesting, post nesting on the beach, 24-48 hours post nesting in water) within the nesting season. Progesterone does not increase until a turtle successfully deposits eggs, and is associated with a surge in luteinizing hormone and the formation of new corpora lutea (Al-Habisi et al., 2006). All turtles in this study were sampled during oviposition (which lasts approximately 10 – 20 minutes (personal observation), thus accounting for similar hormone values. The Y intercept also reflects the progesterone level at initial nesting, therefore accounting for the fact no corpora lutea have been formed in any of the turtles, irrespective of age. Progesterone also decreased

significantly over time, with both neophytes and remigrants exhibiting similar slopes (decrements in hormone levels over time). Decrease in progesterone is due to regression of corpora lutea (Licht et al., 1979, Hamann et al., 2003, Al-Habisi et al., 2006); therefore, this is likely due to copora lutea regressing at the same rate.

Estrogen

Intercepts were also individually specific for estrogen with the most parsimonious model utilizing a common slope. Therefore, all turtles (including neophytes and remigrants) exhibited similar decrements in estradiol over the nesting season, although remigrants exhibited significantly greater initial hormone levels (Y intercept). Increased initial estradiol levels in remigrants, in association with increased clutch and egg production (Chapter III), suggests that remigrants in this population are still undergoing vitellogenesis. Vitellogenesis has been hypothesized to continue after initial nestings in multiple species of sea turtles (although possibly at a decreased rate), especially in animals that exhibit higher initial estradiol levels (Wibbells et al., 1990; Dobbs et al., 2007).

Estradiol triggers vitellogenesis (yolk deposition) in reptiles (Ho, 1987), and is directly correlated with hepatic synthesis and secretion of vitellogenin, increased oviducal weight, and increased circulating serum reproductive protein and calcium levels (Owens and Morris, 1985; Owens, 1997). Maturation of the ovary and a concomitant increase in estradiol are observed 4-6 months prior to mating in multiple sea turtle species (Owens, 1997; Rostal et al., 1997; Rostal et al., 2001). Declining estrogen levels

imply that vitellogenesis in sea turtles may be complete or near completion prior to mating and arrival at nesting beaches (Wibbels et al., 1990, Rostal et al., 1996). However, since sea turtles do not arrive at the nesting beach with a full complement of mature follicles, but instead contain multiple size classes of follicles that will mature consecutively for successive clutches, it is believed that a minimal level of estradiol is required for continued vitellogenesis and follicle maturation throughout the nesting season (Owens and Morris, 1985).

Remigrants generally lay more clutches and more eggs per clutch than do neophytes (Chapter III), therefore increased estradiol levels indicate they are further from the completion of vitellogenesis when compared to that of neophytes. Further evaluation of serum vitellogenin or Zinc in neophyte versus remigrant turtles would serve to further validate this theory.

Estradiol levels decrease with each consecutive nesting event to basal levels at the end of the nesting season (Owens, 1997; Rostal et al., 1998). The slope did not vary significantly between neophytes and remigrants, thus suggesting a similar rate of decrease in estradiol during the nesting season in spite of reproductive experience.

Additional factors

Previous work (see Chapter III) suggested a relationship between hormone profiles and RI (neophytes versus remigrants with RIs of 2, 3, or more years), (Chapter III). Remigrants laid significantly more nests than did neophytes in both 2005 and 2006 and exhibited higher initial estradiol levels when compared to those of neophytes

(Chapter III). Based upon the dichotomy in reproductive output and initial estradiol levels previously observed in this population, RI and age since first reproduction were also evaluated as potential factors. Although RI did not contribute to the model, age since first reproduction was significant. A direct correlation has previously been observed between hormone levels and the age of individual animals (Milnes et al., 2002) and this was observed for leatherbacks as well.

The most parsimonious model for estradiol also included both the number of years since first nesting (reproductive age as identified by year initially tagged), and the interaction between age and days since first nesting. Estradiol levels thus increased with the number of years since first tagging and older remigrant animals exhibited a greater decrement in estradiol levels between successive nesting events. Greater reproductive maturity and experience impacts the size of ovaries which serve as the main source of estradiol in sea turtles (Owens, 1997). Thus, older, more mature leatherback females with larger ovaries produce more follicles and subsequently exhibit higher estradiol levels when compared to those of younger, less mature individuals within the same population.

Increased estradiol, in association with consistently higher reproductive output in turtles tagged 2 to 25 years prior also supports the theory that sea turtles, like other species of reptiles, do not experience senescence (Vom Saal et al., 1994; Girondot and Garcia, 1999; Congdon et al., 2003). Additionally, the transition from neophyte to remigrant appears to have the greatest impact on reproductive output and behavior as observed in long-lived freshwater turtles (Congdon et al., 2003).

In addition to age, maternal hormones have also been correlated to physiological parameters such as the size of an individual animal, since size generally increases with age (Milnes et al., 2002). Estrogen and testosterone have been shown to vary with age and body size in populations of the American alligator (*Alligator mississippiensis*) (Milnes et al., 2002). The length- mass relationship of (kg) = 0.000214*SCL (cm) 2.86 has previously been reported for leatherbacks, therefore turtle size (expressed as CCL) was evaluated as a factor (Jones et al., 2011). Although estradiol levels increased linearly with reproductive age, no correlation was found between hormone levels and animal size, thus size did not contribute to the model.

Predictability of models

After evaluation of multiple models and independent variables utilizing LME in R, the model for all three hormones was based upon nDays with the addition of reproductive age and nDays*Age in the estrogen model. While population values showed smaller variation, individual variation was large. There was smaller variation among remigrants than in neophytes, which suggests among remigrants it may be more feasible to predict age since first nesting and number of days since first nesting. Number of days since first nesting within each year should replace clutch number as the predictive variable to account for the possibility that the turtle may have laid elsewhere prior to nesting and sampling at SPNWR. Taking into account individual turtle nesting history and total number of days nesting, nDays may then be transformed into potential total number of clutches laid utilizing the standard 9-10 day inter-nesting interval

(Garner and Garner, 2010). Although this type of modeling may be possible with additional data collection, it is not reliable at this point.

The sample size for neophytes with an effective number of blood samples was limited due to the decreased number of nests laid by neophytes and the smaller number of neophytes nesting in a given season. Future models will therefore need to be individual based. This means that it is necessary to continue to track individuals over time and over multiple remigration intervals. Due to the importance of age in the model, additional data collection over multiple remigration intervals and in turtles transitioning from neophyte to remigrant status is necessary. A minimum of 4 years is recommended to account for potential odd/even year effects. At this time it is not feasible to complete an effective, predictive reproductive model based on short-term data collection of only two years.

This was the first study to determine the implications of reproductive experience and age on steroid hormone levels and profiles for nesting endangered leatherback sea turtles. It was also the first to determine if additional reproductive indices contributed to these profiles, and ultimately reproductive hormone models. It does not appear, based on current data that multiple variables contribute effectively to the hormone models. In conjunction with high individual variability for model parameters, this means the models can not effectively predict clutch size, age, RI, or hatch success. It is feasible that number of days since first nesting (and ultimately number of clutches laid) may be predicted based on individually tailored models. Reproductive modeling shows promise for the future, with the potential to determine if individual animals have nested prior to

being sampled. This would help solve the mystery of late-season arrivals. Additionally, sampling of odd and even year populations would help determine if they are unique sub-populations that exhibit reproductive parameters specific to the year of nesting.

CHAPTER V

SUMMARY AND CONCLUSIONS

Sandy Point, St. Croix boasts the largest nesting population of critically endangered leatherback sea turtles (*Dermochelys coriacea*) in the U.S. or any of its territories. Beach patrols and conservation strategies were implemented in the early 1980's and the population exhibited exponential recovery within the first 20 years due to adult and nest protection and the implementation of nest management strategies (Dutton et al., 2005). In spite of inter-annual variability in the population, the Sandy Point nesters showed a steady increase through 2001 (Dutton et al., 2005). At this point, the sea turtle community touted the SPNWR population as a success story. Data from the population continued to contribute to numerous studies, even as concern over the status of the population waned. Although data have been recorded since 2001, in depth analysis of population trends in productivity have not been published. This study, therefore, evaluated data obtained over the last decade at SPNWR to elucidate more recent population trends. This analysis exposed areas of great concern for population conservation and recovery.

Since 2001, odd years continued to show significantly greater nesting numbers than did even years, but did not continue the exponential increase observed from 1991 to 2001 (Chapter II), and the 200 nester threshold was breached later than predicted based upon population projections (Dutton et al., 2005; Garner and Garner, 2010). Even years continued to exhibit significantly lower nesting numbers when compared to those of odd

years, with record, historical lows observed in recent years (Chapter II). Based upon these data (and taking into account inter-annual variability in nesting seas turtle populations), the SPNWR population recovery appears to have slowed. The historical reproductive data were further analyzed in an attempt to identify the reproductive parameters primarily responsible for this decline.

The number of adult females nesting annually is primarily dependent upon the number of return nesters (based upon RI) and the recruitment rate of neophytes into the population. I therefore investigated these parameters to determine if they are impacting the annual nesting numbers observed at SPNWR (Chapter II). Analysis of RI showed that the average number of years it takes for a turtle to return to nest at SNPWR has increased over the last decade. The remigration interval for all years ranged from 1 to 11 years, with the most common intervals being 2 then 3 years (Chapter II). The average remigration interval decreased from 2000 through 2003, then increased steadily to a record high (3.41 years) in 2008 (Chapter II), thus illustrating that it is indeed taking remigrant turtles longer to return to the nesting beach. The RI, when increasing consistently on an annual basis over multiple years, may negatively impact population productivity and result in a low number of annual nesters (Witherington et al., 2009), as observed in the last decade in this study.

Remigration intervals vary for each individual nester and are based on reaching a nutritional threshold for reproduction and migration (Hays, 2000). An individual's ability to reproduce in a given year is directly linked to foraging ground productivity and effective exploitation of these areas. Increased RI in this population suggests that there

may be decreased productivity in the North Atlantic foraging grounds or that turtles are having difficulty locating and exploiting available resources.

In addition to impacting RI, nutritional status will also directly impact the time it takes for a juvenile to reach sexual maturity, thus impacting annual recruitment rate. This hypothesis is supported by the decreased number and percentage of neophytes observed in the last 10 years at Sandy Point (Chapter II). Delayed age to sexual maturity also suggests decreased productivity in the North Atlantic foraging grounds or that individual animals are experiencing difficulty finding and utilizing available resources. Decreased recruitment may also be due to increased mortality rates. Sea turtles generally exhibit differential survivorship, with greater mortality at earlier life stages (Dutton et al., 2005; Witherington et al., 2009). The average number of remigrants observed annually has remained steady at SPNWR; however, the percentage of the annual nesting population represented by remigrants has increased over the last 20 years (Chapter II), thus supporting the observed trend of high adult survivorship described by Dutton et al. (2005) in this species. Therefore, based upon data evaluated in this study, adult survivorship does not appear to be directly related to any observed change in population numbers at SPNWR.

Increased mortality of juveniles is another potential cause for decreased recruitment. Survivorship of hatchlings and juveniles has not been evaluated, however, and changes in survivorship of earlier life stages may significantly impact recruitment (Dutton et al., 2005; Witherington et al., 2009). Additionally, if hatchling productivity has decreased significantly, then there will be fewer animals to potentially contribute to

the population in future years. Hatch success at SPNWR decreased from 2001 through 2007 and reached a historical low in 2005 (Chapter II). While this decline is too recent to contribute to the current adult numbers observed at SPNWR, this decreased productivity may result in a continued decline in future recruitment (approx 10-14 years later (i.e., beginning 2011-2015)) and further delay population recovery in the future.

This study also determined that the average number of nests laid annually per turtle decreased from 2001 through 2010 and has been decreasing consistently for the last 20 years. In conjunction with low (50-55% average hatch success for this species) and decreased hatch success rates observed for this population (Chapter II), this resulted in the decreased hatchling productivity numbers observed between 2001 and 2010 (Chapter II). This study also determined that neophytes lay significantly fewer clutches than remigrants (Chapter III). However, neophytes do not account for the decreased average nests laid annually in the population, as the percentage of neophytes contributing nests has decreased (Chapter II). Low hatchling productivity was also observed in spite of some years exhibiting increased and record nesting numbers. The number of nests produced by an individual female is likely linked to nutritional status, again pointing to issues in the foraging ground. Increased follicular atresia may also account for decreased productivity. Follicular atresia has been shown to increase in animals that have difficulty nesting or experience increased stress, and may occur in animals that encounter long difficult migrations (Hamann et al., 2003).

Decreased production may also be related to alterations in endocrine function, since clutch production has been correlated with hormone values in multiple sea turtle

species (Rostal et al., 1998; Rostal et al., 2001). Steroid hormones have been utilized to evaluate clutch production in the hard shelled sea turtle species, with clutch number correlated to maternal reproductive steroid hormone measurements. Work conducted with Kemp's ridley (*Lepidochelys kempii*) and green (*Chelonia mydas*) sea turtles (Owens and Morris, 1985; Rostal et al., 1998; Hamann et al., 2002) provided the first endocrine model for sea turtles and supported the use of steroid hormones as indicators of clutch number (Rostal et al., 1998). Based on these studies I determined blood concentrations of the principal reproductive steroids, in conjunction with historical reproductive data, to potentially provide novel insight into the reproductive decline observed in leatherbacks at SPNWR.

In Chapter III, I analyzed hormone values over the nesting season for consecutive years and established steroid hormone profiles for an Atlantic leatherback population. Overall seasonal patterns of circulating testosterone, estradiol, and progesterone in the Atlantic *D. coriacea* population were similar to those observed in the hard-shelled sea turtle species, with all hormones decreasing progressively with increased clutch number. In comparison to that of hard-shelled turtles however, I found that Atlantic leatherbacks exhibited a more gradual decline in circulating steroids across subsequent clutches (Chapter III), a trend also observed by Rostal et al. (2001) in Pacific leatherbacks. I also found that Atlantic leatherbacks exhibit a significant decline in progesterone, a trend that was not observed in Pacific leatherbacks (Rostal et al., 2001), thus suggesting that the two populations have diverged in their reproductive physiology. Additionally, I found that Atlantic turtles exhibit decreasing progesterone values irrespective of the annual

trend in SAG production. This observation does not support the currently popular theories of how SAGs are made and suggests that progesterone may not be the primary stimulus for SAG production. However, it does not rule out any potential role for progesterone in the production of these unique reproductive products.

Estradiol levels were significantly lower in Atlantic leatherbacks when compared to those of Pacific turtles suggesting that they may be spending more time in the foraging ground, or have longer (more difficult migrations) when compared to those of Pacific turtles, thus allowing more time for estradiol levels to decrease prior to arrival at the nesting beach. This theory compliments the similar theories presented in this chapter regarding the possible impacts of foraging ground productivity (and or individual ability to exploit available resources) on reproductive parameters (as evidenced by indicators such as RI and age to sexual maturity).

The impact of reproductive experience and age on steroid hormone levels and profiles for nesting endangered leatherback sea turtles was also evaluated (Chapters III and IV). Estradiol and testosterone levels were increased for remigrants and represent increased clutch productivity (and possibly sexual activity) in remigrants. Increased estradiol levels in remigrants, who also produce increased clutches (Chapter III) suggests that remigrants may still be undergoing vitellogenesis, perhaps at a decreased rate, upon arrival to the nesting beach (Blanvillain et al., 2011). This is unique when compared to that of Pacific leatherbacks which are presumed to have completed vitellogenesis prior to arrival at the nesting beach (Rostal et al., 2001). This is likely due to the fact that Atlantic turtles lay more clutches on average than do Pacific turtles.

Increased estradiol values observed in this study, in association with consistently higher reproductive output in turtles tagged 2 to 25 years prior, also support the theory that sea turtles, like other species of reptiles, do not experience senescence (Vom Saal et al., 1994; Girondot and Garcia, 1999; Congdon et al., 2003). Additionally, the transition from neophyte to remigrant appears to have the greatest impact on reproductive output and behavior as observed in long-lived freshwater turtles (Congdon et al., 2003).

Although informative, these results have limitations. Samples were obtained over 2 years, but given the striking biannual pattern for nesting in this species I recommend a minimum of 4 years to account for potential odd/even year effects. Additional hormone values for non-nesting and pre-nesting turtles are also necessary to complete the documentation of seasonal hormone cycles for endangered leatherbacks. Vitellogenin and zinc assays would also be beneficial in determining whether or not remigrant females are still undergoing vitellogenesis upon arrival to SPNWR.

In Chapter IV, I used LME to assess the contribution of clutch number (identified by number of days since first sampling), turtle size (as CCL), age, RI, clutch size (yolked and yolkless eggs) and hatch success to the documented hormone values and trends observed in the St. Croix population. Variables deemed significant were factored into a reproductive model for the given hormone; non-significant factors were removed. For all three models the number of days since first nesting was a significant factor. Reproductive age and the interaction between age and the slope (nDays) also contributed to the estrogen model. The predictability of these models was evaluated (Chapter IV) and it appears, given the high individual variability of the current data, that the models

can not effectively predict clutch size, age, RI, or hatch success. It is feasible that number of days since first nesting (and ultimately number of clutches laid) may be predicted based on individually tailored models. Additional samples over multiple remigration intervals for individual turtles would be beneficial. Reproductive modeling shows promise for the future, with the potential to determine if individual animals have nested prior to being sampled. This would help solve the mystery of late-season arrivals and may help explain nesting activities on satellite beaches. Identifying animals who have laid pre-season nests (prior to April 1st) is also a benefit to beach managers when funding for early season patrols is lacking. Additionally, continued sampling of odd and even year populations would help determine if they are unique sub-populations that exhibit reproductive parameters specific to the year of nesting.

Most population models are based upon data for the first 20 years of the leatherback project. It is recommended that future models factor in more current data as many productivity parameters have significantly changed. This will provide more accurate estimates of survivorship and projections of population numbers. The contribution of neophytes versus remigrants to the population should also be considered differential. The number of nests laid is commonly used as a way to estimate number of female nesters on a particular beach (dividing the total nests by average number of nests laid for the species). It is important to evaluate this parameter accurately and ensure that the appropriate number of average nests is used. Utilizing the wrong number will result in either under- or over-estimating the number of females in a population. Conservation

programs must establish accurate population estimates to ensure appropriate protection for their population and funding for their programs.

Studies at SPNWR have failed to successfully identify biotic or abiotic factors impacting hatch success, and nesting beach studies are not capable of determining the physiological mechanisms responsible for altered reproductive output. This study confirmed that reproductive output and age are correlated with blood hormone concentrations in this species, and it is therefore recommended that future studies focus more on maternally derived factors impacting reproductive success. This study also provides necessary baseline hormone data that will serve as the foundation for determining any impact of endocrine disruptors on reproduction in this species in the future. Climate change and contaminants may seriously impact an individual's health, the timing of reproduction and reproductive capacity. In-water sampling and additional work are necessary. This includes the evaluation of foraging ground productivity and sea surface temperatures which may impact rate of maturation, remigration interval and migration timing and routes for sea turtles. To ensure the conservation of this widely distributed, long-lived species, it is necessary to better understand physiological and behavioral mechanisms outside the nesting beach.

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