BIOLOGICAL, ECOLOGICAL AND CONSERVATIONAL IMPLICATIONS OF TEMPERATURE-DEPENDENT SEX DETERMINATION IN SEA TURTLE POPULATIONS

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ABSTRACT

Aspects of TSD were examined in two different sea turtle populations, representing two sea turtle species. Sex ratio was examined in the juvenile portion of the loggerhead sea turtle population in the southeastern U.S. Blood samples were analyzed in a testosterone radioimmunoassay. The results suggest a significant female-biased sex ratio (2.5F:1.0M). The presence of a female-biased sex ratio has significant implications for the conservation of this population.

Another study examined nesting beach temperatures throughout the range of loggerhead nesting in the southeastern U.S. from 2004-2009. Beach temperatures were simultaneously recorded at nest depth on nesting beaches used by this population. The results indicate that temperatures vary significantly between nesting beaches, with potential sex ratios ranging from highly female biased to male biased depending on the beach. The results provide an initial data set for evaluating long-term changes in beach temperatures associated with global climate change.

A third study investigated temperature-dependent sex determination in the Hawaiian green turtle. Beach and nest temperatures from 2003-2009 were evaluated. Collectively, the data indicate that temperatures at FFS were low compared to those reported for other sea turtle nesting beaches. Such cool temperatures are not conducive to the production of female-biases which have been reported for other green turtle populations. An experiment was conducted to evaluate the effects of temperatures similar to those from the nesting beach on sex determination in the Hawaiian green. The hatchlings were subsequently reared in captivity and were sexed by laparoscopy prior to their release. The results indicate that the pivotal temperature for the Hawaiian green is not lower than those reported for other green populations. This finding plus the relatively cool temperatures recorded at FFS, indicate the overall hatchling sex ratio of Hawaiian greens is predicted to be unbiased or even male-biased.

The purpose of the final chapter was to develop an educational module for high school and college students, which demonstrates how modern molecular genetics can be utilized for conservation of endangered species. This chapter includes implementation of the module into local high schools, UAB CORD Summer Science Institute, and the college curriculum at Dauphin Island Sea Lab.

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°C	degrees Celsius
CM	Centimeter
CPM	Counts per minute
FFS	French Frigate Shoals
ML	Milliliter
PG	Picograms
PIFSC	Pacific Islands Fisheries Science Center
SCL	Straight carapace length
SLP	Sea Life Park
TRT	Transitional Range of Temperatures
TSD	Temperature-dependent sex determination

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INTRODUCTION

Sex Determining Systems in Vertebrates:

Sex determining systems employed by vertebrates generally fall into two distinct types: genotypic sex determination (GSD) and environmental sex determination (ESD). In genotypic sex determining systems, the inherited genotype determines sex. The genotype is based on the presence or absence of particular sex chromosomes at conception. Environmental sex determining systems are characterized by the establishment of sex at some point in the life cycle after conception. In ESD systems, sex is based on the influence of environmental factors, such as temperature or social environment. In addition to vertebrates, ESD has been documented in a variety of organisms such as plants, nematodes, echiurid worms, and amphipods (Korpelainen, 1990, 1998; Janzen and Philips, 2006). TSD is a common form of ESD found to occur in fishes, amphibians, and in all extant orders of reptiles (Bull, 1980, 1983; Deeming and Ferguson, 1988).

Vertebrates employ many different types of sex determining systems (SDS). Mammals possess a system of male heterogamety in which an individual inheriting two different sex chromosomes (XY) will become male and individuals inheriting homogenous sex chromosomes (XX) will be female. Birds possess a similar system, but with female heterogamety (ZW in females versus ZZ in males) (Bull et al., 1985). Reptiles utilize a variety of sex determining systems including male heterogamety, female heterogamety, some unisexual systems, and even environment-dependant sex determining systems (ESD), specifically temperature-dependent sex determination. Amphibians employ a variety of these including male heterogamety, female heterogamety, unisexual systems, ESD systems, as well as hermaphrodism. Finally fishes are also found to have many types of sex determination including male heterogamety, female heterogamety, hermaphrodism, and ESD.

Characteristics of TSD in Reptiles:

Since its first discovery in the rainbow lizard (*Agama agama*) by Charnier in 1966, TSD has been characterized in a variety of reptiles (Charnier, 1966; Cree et al., 1995; Janzen and Paukstis, 1991; Ferguson and Joanen, 1983). The incubation temperature of the egg determines the sex of the hatchling in TSD systems (Pieau, 1971, 1974). Although the patterns and specific parameters of TSD may vary between species, there are general characteristics that appear common to all species. The typical characterization of TSD in a species includes a range of temperatures called the transitional range of temperatures or TRT, in which the sex ratio changes from 100% male to 100% female. The TRT encompasses a pivotal temperature at which a 1:1 sex ratio is produced. Temperatures above or below this threshold temperature will produce most or all of one sex (Vogt and Bull, 1982). Three patterns of TSD have been reported; only two have been well verified and appear commonly distributed. The female/male/female (FMF) pattern occurs when females are produced at high and low temperatures and males are produced at intermediate temperatures (Figure 1). Several species of crocodilians, one lizard species, and most turtle species posses this FMF pattern of TSD (Bull, 1980, 1983; Ewert, et al., 1994; Pieau, 1996). The male/female (MF) pattern is a system in which males are produced at low temperatures and females are produced at high temperatures (Figure 2). The MF pattern of TSD is representative of many turtle species. The female/male pattern occurs when females are produced at low temperatures and male produced at high incubation temperatures; this pattern has rarely been reported. (Ewert, et al., 1994).

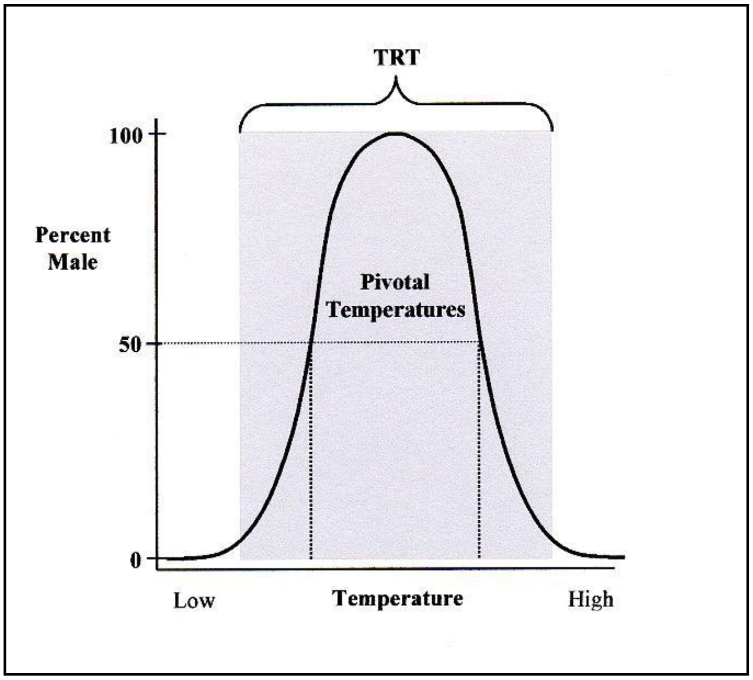

Figure 1: The FMF pattern of TSD occurs when females are produced at high and low temperatures and males are produced at intermediate temperatures. A 1:1 sex ratio is produced at the pivotal temperature, which occurs at two points in the FMF pattern.

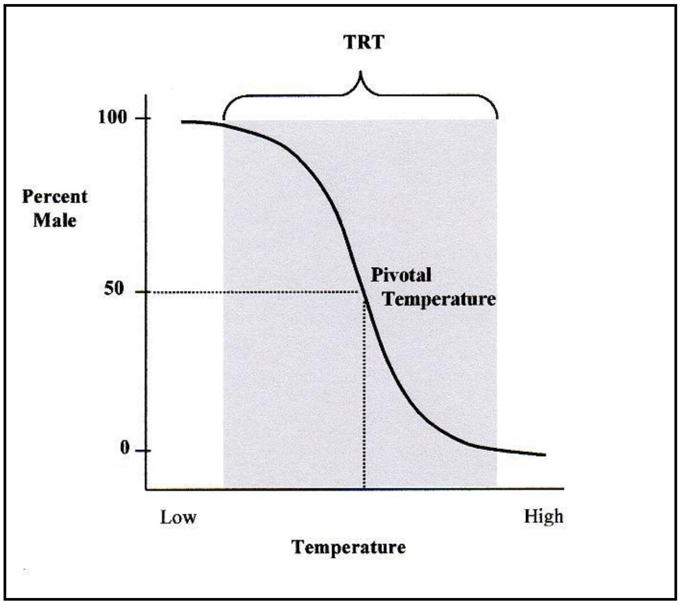

Figure 2: The MF pattern of TSD occurs when females are produced at high temperatures and males are produced at low temperatures. A 1:1 sex ratio is produced at the pivotal temperature.

Effects of Temperature during TSD:

Previous studies have indicated that in many reptiles with TSD, temperature affects sex determination during the approximate middle third portion of the incubation period (Mrosovsky, 1994; Wibbels et al., 1994; Wibbels, 2003). This critical period or thermo sensitive period overlaps with the stages of development when the gonad begins to differentiate (Lance, 1997; Wibbels et al., 1991). After the thermo-sensitive period, sex remains constant throughout life (Crews et al., 1988).

TSD physiology and Estrogen hypothesis:

Studies indicate that the thermo-sensitive period is also the period that the differentiation of the gonad occurs (Wibbels et al., 1991). The mechanism by which temperature affects the gonad during the thermo-sensitive period is not well understood. It appears that temperature activates genes in the embryo that begin a cascade of molecular events, which lead to the production of hormones particularly estrogen. The production of estrogen is hypothesized to stimulate ovarian differentiation. Molecular studies have identified several genes in reptiles with TSD that appear common in the sex differentiation pathways of other amniotic vertebrates, including birds and mammals. However, the specific roles of these genes in sex determination and differentiation in reptiles are not well understood (Lance, 1997). These common genes include SOX-9, DMRT-1, AMH, SF-1, DAX1, and WT1, FOX-L2 (Crews et al., 1988; Wibbels et al., 1998; Smith et al., 1999).

Evolution of TSD in Amniotic Vertebrates:

TSD was first described in the rainbow lizard (*Agama agama*) by Charnier in 1966 (Charnier, 1966). TSD has been suggested to be the ancestral form of sex determination in reptiles, and thus in amniotic vertebrates (Marshall-Graves and Shetty, 2001). TSD has been reported to occur in the most primitive extant reptilian groups including most turtles, all crocodilians (Ferguson and Joanen, 1983), and the tuatara (Cree et al., 1995). These reptilian groups can date back 200 million years or more, to the early Triassic Period. Many amniotic groups with GSD appear more recently in the fossil record and may share a common ancestry with these reptile groups. For example, birds appear to be closely related to Therapod dinosaurs like Velociraptor, but appear in the fossil record millions of years after the first appearance of turtles, crocodiles, and the tuatara. Thus, it has been hypothesized that the GSD systems of birds and mammals have been derived from the TSD systems of ancestral reptilian groups (Marshall-Graves and Shetty, 2001).

The TSD system in turtles may represent the most primitive SDS of the extant amniotic vertebrates. In general, turtles represent an ancient amniotic vertebrate that appears to have changed very little in the last 200 million years. The first turtles evolved more than 200 million years ago (mya). Sea turtles first appear approximately 100 mya in the mid-Cretaceous Period and appear conservative in their evolution. By the late Cretaceous, approximately 65 mya, there were four distinct families of sea turtles Protostrgidae, Toxochelidae, Dermochelyidae, and Cheloniidae (Gulko and Eckert, 2004). Dermochelyidae and Cheloniidae include the seven species of sea turtles that exist today. Dermochelyidae includes only one living species. Extinct members of Dermochelyidae are reported to have existed during the Tertiary period (Ernst et al., 1994). Cheloniidae includes the remaining six extant sea turtle species. This family dates to the late Cretaceous (Ernst et al., 1994; Nicholls et al., 1990). Thus, in examining TSD in sea turtles, we are potentially addressing a very primitive sex determining system.

Adaptive Significance of TSD:

Even though TSD is widespread in reptiles, its evolutionary significance remains hypothetical. According to Janzen and Philips, 2006, the adaptive significance of ESD has been clarified in the case of many non reptilian organisms, but is not so clear in amniotic vertebrates. This is interesting because the amniotic vertebrates are the largest group of organisms to possess TSD (Janzen and Philips, 2006). In his 1999 article, Shine reviewed the evolutionary origin of TSD in reptiles (Shine, 1999). He discussed several basic hypotheses for the adaptive significance of TSD in reptiles. These hypotheses attempt to explain the widespread occurrence of TSD in reptiles, with the most accepted hypothesis being one of differential fitness, or the Charnov-Bull model (Charnov and Bull, 1977). Shine's review of the adaptive advantages of TSD remains one of the most comprehensive reviews on this topic.

One potential explanation for the persistence of TSD in some reptilian groups is the idea of phylogenetic inertia (Shine, 1999). This hypothesis is based on the idea that there may no longer be any adaptive significance of TSD, but it continues to exist because the shift from TSD to GSD could be difficult due to the required genetic and physiological changes. Thus the probability of such a change occurring may be low. There are lines of reasoning that would not support phylogenetic inertia as a plausible hypothesis for the occurrence of TSD. First, there are several lines of amniotic vertebrates that have shifted to GSD. For example, there are closely related species of lizards in which some species posses GSD and others have TSD. Additionally, TSD may have survived because not because of Phylogenetic inertia, but because of its adaptive significance.

A second hypothesis is that of group adaptation; that TSD evolved because it facilitates group fitness through sex ratio skewing (Shine, 1999). This hypothesis relies on assumptions of group selection and is inconsistent with accepted evolutionary theory that suggests that selection acts at the level of the individual and not the group. For example, it may be advantageous for a population to have a female biased sex ratio in order to enhance reproductive output, but for an individual within that group it would be advantageous to be male because that would ensure a better probability of the individual's reproductive success.

A third hypothesis is one of inbreeding avoidance (Shine, 1999); since TSD can result in single-sex clutches from natural nests, TSD could reduce the effects of inbreeding among siblings. This hypothesis is inconsistent with the prevalence of TSD occurring in long-lived species that produce multiple clutches annually. These clutches could have the same father, thus the same genetics, and they could likely breed with each other. Therefore, this does not seem to be a reasonable explanation for the adaptive significance of TSD in reptiles.

Shine, 1999, indicates that the most plausible theoretical models for the evolution of TSD are based on differential fitness of male and female offspring incubated under different thermal regimes, the Charnov-Bull model (Charnov and Bull, 1977). If incubation temperature affects the fitness of male and female offspring differently, then TSD can enhance fitness by enabling embryos to develop as the sex best suited for survival at a given time. This hypothesis is the most consistent with the available data on reptiles with TSD (reviewed by Crews et al., 2008), and has been supported by some empirical data (Warner and Shine, 2008). However, the mechanism by which differential fitness was achieved was not defined in that study, but has been suggested in a more recent study (Therrien, 2008).

There are several possible models for the differential fitness hypothesis. One model for the differential fitness hypothesis suggests that TSD enables the mother to adjust sex ratio relative to offspring size (Shine, 1999). As a hypothetical example, in reptiles that lay multiple clutches throughout a season the first clutch may have larger egg size due to the availability of stored nutrients (LeBlanc, in review; Rosenburg, 1996). Larger eggs could provide more nutrients to initiate a faster growth rate early in life. A faster growth rate might be more beneficial to female than males if larger sizes correlate with higher fecundity. This could allow females to reach reproductive maturity faster and to have greater energy stores to produce more eggs. Whereas, male hatchlings might not need the same increased energy stores or body size because they do not have to undergo egg production. It is possible that the early season eggs would be exposed to environmental temperatures conducive to the production of females, while the late season eggs would be exposed to mostly male producing temperatures. An alternative scenario would be one in which it benefits the male to have a large body size, such as in territorial species. These species could benefit from an environment that produces males from the first clutch of the season. These scenarios describe how TSD could optimize the fitness of the hatchlings relative to sex.

A similar model relates hatchling sex to seasonal time of hatching in species that produce multiple clutches with similar egg size (Shine, 1999). Individuals hatched from early season nests could benefit by having a longer time to feed and grow before the first winter. Growing larger during the first year the hatchlings could potentially establish a faster growth rate throughout life. For example, in a species in which one sex is larger than the other, it may be advantageous for the larger sex to be produced early in the nesting season.

Another model suggests that sex of the hatchling is linked to nest site philopatry (Reinhold, 1998; Shine, 1999). For example, female sea turtles may have site fidelity for the natal nest site and return to this site to lay their own eggs (Bowen et al., 1993). Although males have been less studied, they are believed to have site fidelity for courtship areas for opportunistic mating (Fitzsimmons, 1997; Limpus, 1985). This model suggests that there are differences in nesting beach quality and in the degree of nest site fidelity among females. Consequently, it is advantageous for a female to have high nest site fidelity for a good nesting beach because the quality of the nesting beach is paramount to the survival of their hatchlings. For example, females nesting on a good nesting beach would produce greater numbers of offspring, and potentially greater numbers of female offspring, that would return to the same nesting beach over time. This model also suggests that nest site fidelity in females together with nesting beach quality could determine the location of optimal nesting beaches in a given population.

The first empirical evidence to support the differential fitness hypothesis for the adaptive advantage of TSD was published by Warner and Shine in 2008. The lab experiment with the Jacky dragon (*Amphibolurus muricatus*) indicated that incubation temperatures which naturally produced a specific sex, best improved the fitness of animals in terms of lifetime reproductive output. For example, male lizards produced in the lab at optimal, natural, male-producing temperatures had greater reproductive success in terms of reproductive output than did males produced at higher or lower temperatures; and vice versa with females (Warner and Shine, 2008). Thus, the reproductive success of each sex was optimized in the lab by the incubation temperature that produces that sex in nature. The results of this study provide evidence that the incubation temperature affects male and female fitness differentially in a way that favors the selection for TSD.

Significance of Sex Ratios Resulting from TSD:

There are many ramifications in regard to the ecology, evolution, and conservation of sex ratios resulting from TSD because this form of sex determination has the potential to produce a wide variety of sex ratios, including highly skewed sex ratios in certain environmental conditions. TSD is of evolutionary interest because of its ability to produce a wide range of sex ratios, including some that not conform to a 1:1 sex ratio, predicted by some evolutionary theory (Fisher, 1930). Specifically, it is not clear if TSD has survived because it provides an adaptive advantage (see discussion above regarding differential fitness). TSD also has implications for ecology and conservation, since the resulting sex ratios can affect the reproductive ecology of a population. For example, TSD can produce sex ratios that can enhance or hinder the recovery of endangered populations. Furthermore, both natural factors and human influence on environmental conditions could affect the sex ratios produced in species employing TSD. Although sex ratios resulting from TSD have been reported in some studies, the implications regarding conservation, ecology and evolution are not well understood.

There are a wide variety of questions that need to be addressed in order to provide insight into the conservation, ecological, and evolutionary significance of TSD. The ultimate questions include: What is the adaptive significance of TSD? Does TSD produce a 1:1 sex ratio in nature, similar to GSD? If not, why does TSD produces biased sex ratios? In addition to these ultimate questions, there are a multitude of proximate questions which need to be addressed. These include: What is the range of population sex ratios that occur in reptiles with TSD? What are the proximate factors that affect sex ratios on a given nesting beach? Does pivotal temperature evolve to match nesting beach temperatures in order to produce a specific sex ratio for a population? How does the quality of a nesting beach relate to beach temperature, sex ratio, and long-term productivity of a given nesting beach? Are sex ratios from a given nesting beach stable over multi-year intervals? How does sex ratio affect the recovery of a population? How might climate change affect sex ratios within a population? Does sex ratio vary within a population relative to age class? Within a population, do hatchling sex ratios vary significantly between nesting beaches? What effects do anthropogenic factors, such as beach development, nourishment, etc., have on nesting beach quality and hatchling sex ratio? What are the impacts of weather events such as rain, tropical storms, hurricanes, etc., on hatchling sex ratios? A logical first step for addressing such questions would be to evaluate naturally occurring sex ratios. To effectively answer such questions, a comprehensive knowledge of naturally occurring sex ratios is required relative to the environmental conditions. Further, an understanding of the effect of sex ratios on the reproductive ecology of a population is required.

The Question of TSD and Climate Change:

Finally, a current topic of prime interest is how climate change may affect reptiles with TSD. Possessing TSD allows reptiles to maintain a mixed sex ratio as long as their pivotal temperatures remain near the environmental temperatures. A variety of studies have indicated that global temperatures have significantly increased by 0.74°C during the last century (air temperature near the Earth's surface; IPCC, 2007). If this warming trend continues, climate change could potentially alter the ecology and survival of TSD species (Janzen, 1994; Janzen and Philips, 2006). It is plausible that reptiles with TSD could alter their ecology and physiology to adapt to climate change. However, the genetic changes necessary to alter the physiology of TSD (e.g. change of pivotal temperature) may require relatively long time periods in comparison to the current rate of climate change. As such, the physiology may not be able to change quickly enough to adapt to the current warming trend. Alternatively, reptiles may be more capable of altering their ecology in order to adapt to rapid climate change. For example, sea turtles might utilize different nesting beaches or different locations on a nesting beach. They could also alter the seasonal timing of their reproduction. However, such changes may have constraints, such as lack of alternate nesting sites, which would limit the extent of possible adaptation.

Considering the Earth appears to be in a period of rapid climate change, there is a distinct need for long-term evaluation of the nesting beach temperatures of reptiles with TSD. It is plausible that temperature change over the last century could already be impacting such species. In the case of sea turtles, previous studies suggest that female biases often predominate in many populations (Wibbels, 2003). It is not clear if such biases would be naturally produced by TSD or if they are a reflection of increased environmental temperatures. This exemplifies the need for long-term temperature and sex ratio studies on nesting beaches.

Sea Turtles as a Research Model:

There are a wide variety of reptiles that could serve as model systems for TSD studies. Each species possesses potential advantages and disadvantages as models in the study of TSD. Sea turtles provide a useful model for studying TSD for a number of reasons. All species of sea turtles have been documented to possess TSD. Sea turtles have the widest distribution of any reptile on Earth. They inhabit tropical and temperate waters world-wide and thus are dispersed throughout many different environments, which give these experiments a global approach. In this way, sea turtles provide an optimal model for studying the effects of TSD in multiple and varied environments. Further, the various species and populations exhibit a wide range in the timing and behavior of nesting. As such, sea turtles represent an advantageous model for evolutionary and ecological studies examining how TSD facilitates adaptation to a wide range of nesting habitats. These relatively large populations of long-lived species also provide a good model for studying sex ratio variability within the population structure. Additionally, sea turtles represent an advantageous system for examining the impact of TSD on the recovery of depleted populations. Conservation programs monitoring nesting activity exist world-wide and provide a viable means for monitoring the recovery of species. Therefore, these conservation programs provide an efficient platform for implementing studies examining the effects of TSD on species recovery.

The current dissertation addresses the ecology and evolution of TSD in sea turtles by addressing a variety of questions, including the following:

1) What sex ratios are produced in a sea turtle population as a result of TSD?
2) How variable are the sex ratios produced from TSD?
    (a) Do sex ratios produced from TSD vary significantly within a population (nesting beaches, between age groups, etc.)?
    (b) Do hatchling sex ratios vary over a single nesting season?
    (c) Do hatchling sex ratios vary over multiple nesting seasons?
    (d) Do sex ratios vary between populations?
3) Do naturally occurring sex ratios approximate 1:1 in sea turtle populations?
4) Does TSD evolve to adapt a particular species or population to a particular habitat (i.e. nesting beach)?

5) How does the quality of a nesting beach relate to beach temperature, sex ratio, and long-term productivity of a given nesting beach? Why are certain nesting beaches the primary nesting location for a population? What effects do anthropogenic factors, such as beach development, nourishment, etc., have on nesting beach quality and hatchling sex ratio? What are the impacts of weather events such as rain, tropical storms, hurricanes, etc., on hatchling sex ratios?
6) How might climate change affect sex ratios within a population?

In order to address the proposed questions, various aspects of TSD were examined in two different sea turtle populations, representing two sea turtle species. The research evaluated TSD in sea turtle populations that are widely distributed geographically. These studies provided a unique opportunity evaluate similarities as well as variability in the TSD of sea turtles.

Chapter 1 of this dissertation directly examines sex ratios of juvenile loggerheads from the southeastern U.S. This study examines the sex ratio of juvenile turtles captured at the St. Lucie Nuclear Power Plant, on the Atlantic coast of Florida. This sampling location is located in a region that includes developmental habitats, foraging grounds, and is where the majority of loggerhead nesting occurs in the southeastern U.S.

Chapter 2 of this dissertation describes a study that examines nesting beach temperatures throughout the range of loggerheads nesting in the southeastern U.S. This includes a long-term database that provides insight on the potential hatchling sex ratios being produced from the various nesting beaches. The study includes simultaneous recording of temperatures from nesting beaches throughout the nesting range of loggerheads in the southeastern U.S. Additionally, the general effects of beach renourishment were examined relative to nesting beach temperature. Finally, it also includes the examination of loggerhead sex ratios based on the histological evaluation of hatchlings that were found dead in nests at a subset of nesting beaches in the southeastern United States. In particular, the results suggest which beaches may be of conservation significance regarding the management of this population. Further, these data provide a foundation for a continuing long-term evaluation of the effects of global climate change on hatchling sex ratio production in this population.

Chapter 3 focuses on temperature-dependent sex determination in the Hawaiian green turtle. This includes a long-term analysis of temperatures on the primary nesting beach at French Frigate Shoals, Northwestern Hawaiian Islands National Monument. The results suggest relatively cool nesting beaches relative to green turtle beaches in other areas of the world. Previous studies of green turtles in other areas of the world suggested that these temperatures would produce strong male biases; however, necropsy studies of stranded Hawaiian green (Koga and Balazs, 1996), as well as testosterone-based study of juveniles (Wibbels and Balazs, 1993) suggested a nonbiased sex ratio. For that reason, a study was initiated to characterize TSD in the Hawaiian green turtle, that is, to determine which temperatures produce which sex in this population of green turtle. This study included the incubation of eggs in laboratory incubators, followed by the captive-rearing of approximately 100 turtles for 1 year. The sex of the yearlings was verified by laparoscopy, and the sex of any turtle that died during incubation or captive rearing was verified by histology of the gonad.

Chapter 4 focuses on the genetic variation in the loggerhead population in the southeastern U.S. The purpose of this chapter was to develop an educational module for high school and college students, which exemplifies how state-of-the-art molecular genetics can be utilized for the conservation of endangered species. This chapter includes the implementation of this teaching module into local area high schools, UAB CORD Summer Science Institute, and into the summer curriculum for marine biology college students at Dauphin Island Sea Lab.

CHAPTER 1

JUVENILE SEX RATIO OF LOGGERHEAD SEA TURTLES INHABITING THE ATLANTIC COASTAL WATERS OF FLORIDA

By

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ABSTRACT

The loggerhead sea turtle population inhabiting the southeastern U.S. represents one of the largest loggerhead populations in the world. The coastal waters acts as both a developmental habitat and foraging grounds for the loggerhead, and the majority of nesting in this population occurs along the Atlantic coast of Florida. In the current study, juvenile loggerheads were captured in the intake channel of the St. Lucie Nuclear Power plant on the central Atlantic coast of Florida. Evaluation of the juvenile portion of the population represents an accurate means of evaluating the total population’s sex ratio because juveniles represents a condensation of many years of hatchling production and the sampling of juveniles should not be confounded by the sex-specific migratory behavior that occurs with adults. Blood samples were collected from a total of 111 juvenile turtles from May 2005 through April 2007. The samples were analyzed in a testosterone radioimmunoassay that was validated for use as a sexing technique for sea turtles. The results suggest a significant female-biased sex ratio (2.5F:1.0M). No significant differences were detected between the sex ratios of different size classes of turtles. These findings are consistent with several previous studies suggesting the female-biased sex ratio in the loggerhead population inhabiting the southeastern U.S. Further, comparison of these data to those of a study from several decades earlier, indicate that a significant female bias may be a relative stable characteristic of the juvenile portion of this sea turtle population. The presence of a female-biased sex ratio has significant implications for the ecology and conservation of the loggerhead turtle in the southeastern U.S.

INTRODUCTION

The loggerhead sea turtle, *Caretta caretta*, is one of the largest members of the family Cheloniidae (the hard-shelled sea turtles) and is the most common sea turtle encountered in the coastal waters of the continental United States (U.S.). Loggerheads inhabit temperate and subtropical waters throughout the world and are known to have substantial nesting aggregations in the U.S., the Mediterranean, Africa, the Middle East, Asia, and Australia. One of the world's largest populations of loggerhead sea turtles nests in the southeastern U.S. and is currently listed as Threatened under U.S. Federal Endangered Species Act (ESA). The number of nesting loggerheads in this population has steadily and significantly declined since 1998 (NMFS/USFWS Loggerhead Sea Turtle 5 year status review, August 2007). For example, during 1989-2007 the overall nesting of loggerheads in Florida declined by 37% (TEWG, 2000; FWCC, 2007). Therefore, the conservation of this population is currently a major concern.

Like all sea turtles, the loggerhead possesses temperature-dependent sex determination. This form of sex determination has the potential to produce biased sex ratios and, therefore has significant implications regarding the ecology, evolution, and conservation of the loggerhead. As such, it is of interest to evaluate naturally occurring sex ratios in loggerhead populations. This type of information can prove valuable when attempting to understand the reproductive ecology of sea turtles and when developing conservation strategies for enhancing the recovery of threatened and endangered populations.

The current study evaluated the sex ratio of juvenile loggerheads inhabiting the Atlantic coastal waters of Florida. Evaluation of the juvenile portion of the population represents an accurate means of evaluating the total population's sex ratio because juveniles represent a condensation of many years of hatchling production and the sampling of juveniles should not be confounded by sex-specific migratory behavior that occurs with adults. However, studies of juvenile loggerhead sex ratios are rare (Limpus, 1985; Wibbels, 1988; Wibbels et al., 1991; Casale, 1998) because it is not possible to sex juvenile sea turtles based on external morphology. The current study overcomes this logistical hurdle by utilizing a radioimmunoassay to evaluate blood testosterone levels as an indicator of sex (Wibbels et al., 2000). The juvenile loggerheads examined in current study inhabited the Atlantic coastal waters of Florida and were captured at the St. Lucie Nuclear Power Plant located on the south central portion of Florida's Atlantic coast.

The knowledge of the juvenile sex ratio will provide basic information regarding the reproductive ecology of this loggerhead population. The current study addresses the temporal stability of population sex ratios, since this same population was examined over two decades ago (Wibbels et al., 1981). The results also address whether population sex ratios are static or dynamic in sea turtle species with TSD.

METHODS

Study Location, Capture Methods, and Blood Sampling

The turtles used in the current study were captured in the cooling water intake channel of the St. Lucie Nuclear Power Plant on the Atlantic coast of Florida. Turtles were captured over a three year period ranging from May 2005 through April 2007. This power plant uses sea water for cooling and has three intake heads located 365 m offshore. Water travels from the intake heads through three concrete conduits ranging from 3.7 to 4.9 m in diameter. The water then enters the intake cooling channel which is approximately 40 m wide by 1.6 km long. The turtles used in the current study were taken into the intake channel along with the cooling water. After traveling through the concrete conduits, the turtles surfaced in the initial portion of the intake channel and were prevented from moving farther than approximately 100 m up the channel by nets that transected the entire channel. Turtles were captured using wide mesh tangle nets.

Blood samples were drawn within 10 minutes of capture using standard methodology (Owens and Ruiz, 1978; Wibbels et al., 1990). The samples were immediately placed on ice. Samples were spun in a centrifuge in order to separate the plasma and cellular fractions of the blood. The plasma was then frozen and stored in a -80 C freezer until assayed. Turtles were released approximately 1 hr after capture back into the Atlantic Ocean from the beach adjacent to the power plant.

RIA Based Evaluation of Juvenile Sex Ratio:

The sex of the juvenile turtles was identified based on circulating levels of testosterone. Circulating levels of testosterone have been used in a variety of other studies to evaluate the sex of juveniles of a variety of sea turtle species (Owens et al., 1978; Morris, 1982; Wibbels et al., 1987; Wibbels et al., 1991; Bolton et al., 1992, Coyne et al., 1994; Gregory, 1996; Casale et al., 1998; Coyne, 2000; Geis et al., 2005; Witzell et al., 2005; review by Wibbels et al., 2000).

The testosterone RIA has previously been described in Geis et al., 2005. Briefly, 250 ul of serum was extracted from each sample using 2 ml of diethyl ether. Samples were reconstituted in 1 ml of assay buffer. Samples were run in duplicate in the RIA with 100 ul of testosterone antisera (Fitzgerald Industries International, Acton, MA), and approximately 10,000 cpm of tritiated testosterone (Perkin Elmer). A standard curve was run with each assay using testosterone standards of 15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000pg/assay tube. The assay proved optimal when using a 1:10 dilution of the testosterone antisera stock solution. A total of five assays were conducted to evaluate all samples in duplicate. Intra-assay and inter-assay control samples were run with each assay. The intrassay coefficient of variation was 8.43 % and interassay coefficient of variation was 18.5%

Previous studies of circulating testosterone levels in sea turtles included laparoscopic verification of sex, and provide validation for using this technique with juvenile loggerheads in this study (Wibbels et al., 1991; Wibbels et al., 2000; Geis et al., 2005; Witzell et al., 2005). Based on those studies, juveniles with levels greater than 500 pg/ml of plasma testosterone in the current study were predicted to males and those with levels less than 400 were predicted to be female. Turtles with levels between 400 and 500 pg/ml were designated as sex undetermined.

Carapace Measurements

Straight carapace measurements were taken for each turtle using calipers and consisted of the straight-line measurement from the front of the nuchal scute to the notch between the two postcentral scutes.

Exclusion of Adults

In order to exclude adults from this study, individuals with carapace lengths greater than 76 cm were not included in the analysis. This value was chosen after consideration of the minimum size of nesting females in this area (Ehrhart, 1980) as well as the size ranges of immature turtles whose reproductive status was verified by laparoscopy (Limpus, 1985; Wibbels, 1988; Wibbels et al., 1991).

RESULTS

A total of 111 juvenile turtles, captured from May 2005 to April 2007, were examined in this study. The turtles' straight line carapace lengths ranged from 28.9 cm to 76.2 cm. Turtles above that size range were excluded from analysis since they are nearing the size of the smaller adult loggerheads. Testosterone levels ranged from less than 100pg/ml to approximately 2800pg/ml (Figure 1). As indicated above, the prediction of sex was based on previous validation studies in our laboratory (Wibbels et al., 2000;

Geis et al., 2005; Witzell et al., 2005. The predicted sexes of the turtles are shown in Table 1 relative to the general size classes of turtles captured in the study. Of the 111 turtles evaluated, 71 fell within the female range and were thus predicted to be females, and 29 fell into the female range and were thus predicted to be female (Figure 1). Eleven turtles were designated as “sex undetermined” because their testosterone levels fell between the validated ranges of the males and female. The results indicate an overall predicted sex ratio of approximately 2.5 F:1.0F. This sex ratio is significantly different that 1:1 sex ratio (Fisher’s exact test, $P<0.05$). Even if the 11 undetermined turtles in this study were all males, the sex ratio (1.8F:1.0M, n =111) the sex ratio is still significantly different from a 1:1 sex ratio (Fisher’s exact test, $P<0.05$). Table 1 shows sex ratios in different size classes of the turtles in this study (size classes were arbitrarily derived using 10 cm intervals). The sex ratios of the different size classes were not significantly different from one another (Replicated Goodness of Fit test, $P>0.05$). The overall sex ratio from the current study was also compared to the sex ratio (2.1F:1.0M, n = 148) from a similar study conducted approximately two decades earlier (Wibbels et al., 1991). No significant difference was detected between the overall sex ratios from the two studies (Fisher’s exact test, $P>0.05$). Additionally, the sex ratios from each size class were compared between the two studies, and no significant differences were detected (Fisher’s exact tests, $P<0.05$).

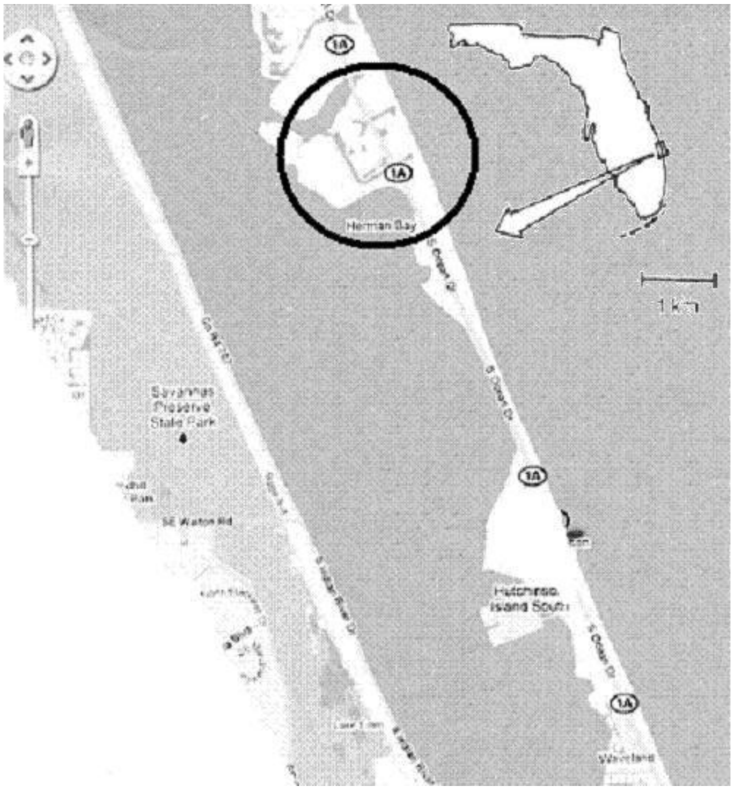

Figure 1. Turtle were captured in the intake channel of the St. Lucie Nuclear Power Plant on the Atlantic coast of Florida

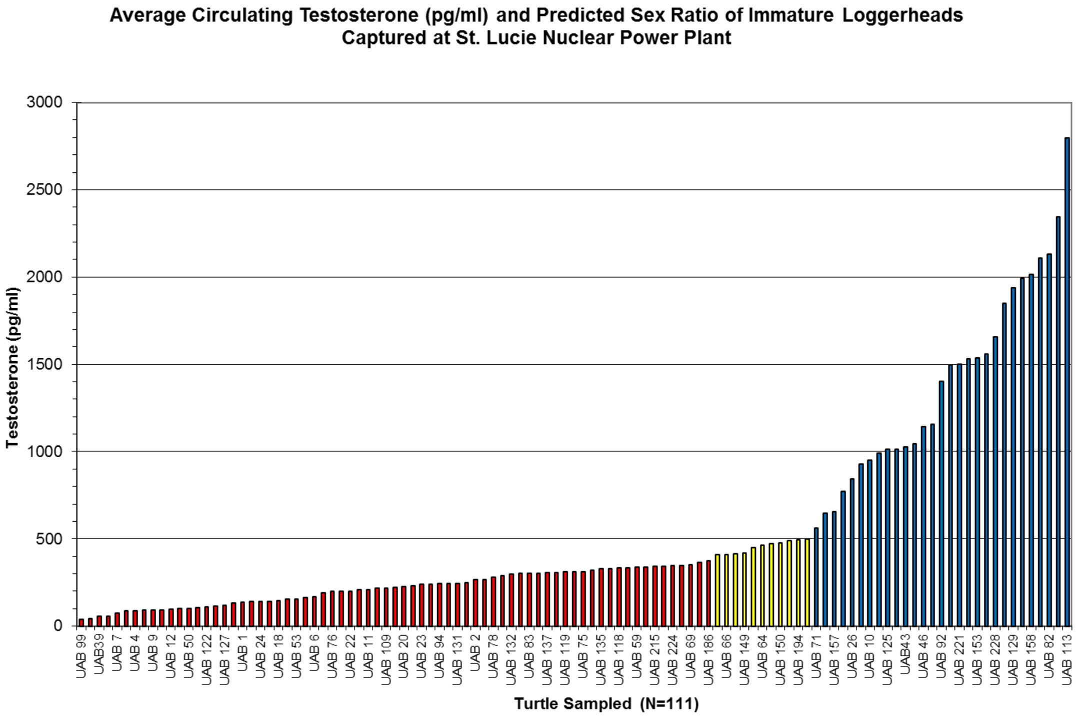

Figure 2. Circulating testosterone levels of juvenile loggerhead sea turtle captured at the St. Lucie Nuclear Power Plant, Florida. Blue lines indicate individuals predicted to be male, red lines are individuals predicted to be females and yellow lines indicate individuals with intermediate levels of testosterone that fall in a range for which sex has not been verified by laparoscopy. All turtles were less than 76.5cm and did not show any external sex characteristics.

Table1: Sex ratios of different size classes of loggerheads captured at the St. Lucie Nuclear Power Plant. Size classes were chosen on 10 cm intervals and are identical to those used in a previous study by Wibbels et al. (1991).

Carapace Length	**No. of females**	**No. of males**	**No. of unknown sex**	**n**	**Predicted Sex Ratio (F:M)**	**p value**
70.0-76.4 cm	12	5	3	20	2.40:1.00	p>0.05
60.0-69.9 cm	47	20	6	73	2.35:1.00	p>0.05
50.0-59.9 cm	11	4	2	17	2.75:1.00	p>0.05
< 50.0 cm	1	0	0	1	--	--
Totals	71	29	11	111	2.50:1.00	p>0.05

DISCUSSION

The life history of the Chelonid sea turtles includes an initial oceanic stage followed by a neritic stage in which the turtles inhabit near shore areas which act as foraging grounds (Bolten, 2003). In the case of loggerheads in the Atlantic this transition normally begins when the turtles reach an approximate size of 45 to 55 cm in curved carapace length (Bolten, 2003). The turtles then inhabit the neritic foraging ground and gradually grow to maturity. Typical loggerheads nesting along the Atlantic coast of the U.S. must attain a size of approximately 80 cm straight carapace length before they reach maturity and begin nesting (Kamezaki, 2003). This life history stage from when they first transition to a neritic habitat until they reach sexual maturity may take one or two decades (Musik and Limpus, 1997). The current study examined the sex ratio of this life history stage in loggerheads inhabiting the Atlantic coast of Florida. There are distinct advantages of using this life history stage when examining population sex ratios. This stage represents a condensation of many years of hatchling production and evaluation of its sex ratio should not be confounded by sex-specific migration patterns of adults (Wibbels, 2003).

The results of the current study suggest that a significant female bias exists in the neritic juvenile portion of this loggerhead population (2.4F:1.0M). There were 11 "sex undetermined" turtles using the RIA-based sexing technique, but even if they were all males, the sex ratio would still be significantly female biased. A similar study of juvenile sea loggerheads (Wibbels, et al., 1991) utilizing the same sampling location and same sexing methodology obtained a similar sex ratio (2.1F:1.0M, n =148). These findings suggest that a female bias exists in the juvenile portion of the loggerhead population inhabiting the Atlantic coast of Florida. These findings are also supported by several previous studies. A study used a testosterone RIA to sex juvenile loggerheads (n = 256) from four different locations along the U.S. Atlantic coast, and reported an approximate 2F:1M sex ratio (Wibbels et al., 1987).   Another study used necropsy to examine the sex ratio of immature loggerheads stranded on the Atlantic coast of Georgia (Shoop et al., 1998), and found an approximate 2F:1M (n = 129). Additionally, an approximate 2F:1M sex ratio has also been reported for loggerheads stranded on the upper Texas coast (Stabenau et al., 1996). Thus, there are a number of previous studies that support the current finding of a significant female bias in this loggerhead population.

The presence of a female bias is also supported by studies of nesting beach temperatures and hatchlings sex ratios. Several previous studies have examined hatchling sex ratios of loggerheads produced on the Atlantic coast (Mrosovsky et al., 1984; Mrosovsky and Provancha, 1989; 1992: Mrosovsky et al., 1992; Hanson et al., 1998; review by Wibbels, 2003). Most of these studies have generally suggested female-biased sex ratios (as high as 90% or more).

The results of the current study also suggest that the female bias may be a consistent characteristic in the population structure of this population. No significant differences were detected between the sex ratios of the different size classes of turtles. Further, the sex ratio from the current study is similar to that reported by study from approximately two decades ago (Wibbels, et al., 1991), thus suggesting that the female bias may be a temporally stable characteristic of this population.

A wide variety of sex ratios have been reported in studies of sea turtle sex ratios (reviewed by Mrosovsky, 1994; Wibbels, 2003). However, the majority of studies have focused on hatchling sex ratios from specific beaches. Studies focusing on the sex ratios in the juvenile and adult portions of a population sex are much more infrequent. Henwood (1987) found seasonal changes in the sex ratio of adult loggerheads in the Cape Canaveral Ship channel ranging from male to female biased. Ross (1968) examined adult sex ratios in green turtles in Oman and found that it varied by location ranging from male to female biases. These results exemplify the problems associated with potential sex-specific migration behavior.

Chalupka and Limpus (2001) conducted a comprehensive survey of that loggerhead and green sea turtles in the southern portion of the Great Barrier Reef. The study included the laparoscopic verification of sex of over one thousand immature turtles. The sex ratio of greens fluctuated over the 8 year study, but was consistently female biased. In contrast, they recorded a significant male bias for the loggerhead population. An unbiased sex ratio has reported in two studies of the Hawaiian green turtle (Koga and Balaz, 1996; Wibbels et al., 1993). Bolten et al. (1992) recorded a 1.4F:1.0M sex ratio of 111 immature green turtles in the Bahamas in which sex was predicted by circulating testosterone levels. However, that sex ratio was not significantly different from a 1:1 sex ratio. A number of studies have addressed sex ratios in the adult and juvenile portion of the Kemp's ridley population in the Atlantic and Gulf of Mexico (Coyne and Landry, 2007; Wibbels, 2007). Those studies included a variety of sexing methodologies (i.e. necropsy, histology, testosterone RIA) and, collectively suggested a strong female bias in the Kemp's ridley population (Wibbels, 2007). The concept of an overall female bias in the Kemp's ridley population is also supported by studies using nest temperatures to estimate hatchling sex ratios at the primary nesting beach at Rancho Nuevo, Mexico (Wibbels, 2007). Thus, the findings from numerous studies suggest that sex ratios in sea turtle populations can vary and do not always conform to a 1:1 ratio.

The findings from the current study suggest a significant female bias (2.5F:1.0) male. Evolutionary theory proposed by Fisher (1930) suggested that population sex ratios should conform to a 1:1 sex ratio if there is equal parental investment in the sex of each offspring. As exemplified by the findings in the current study, it would seem that sea turtles do not consistently adhere to a 1:1 sex ratio. A variety of hypotheses have been suggested to account for skewed sex ratios in reptiles with TSD (Rheinhold, 1998; Shine 1999). The most widely accepted is the "differential fitness" hypothesis, which suggests that TSD may allow the nesting female to alter the sex ratio to enhance the fitness of her offspring. For example, a female may be able to match the sex of the offspring to a particular environment (e.g. time of year, or quality of a particular nesting beach) which enhances the fitness of one sex versus the other (Rheinhold, 1998; Shine, 1999). Alternatively, several other hypotheses have been proposed such as phylogenetic inertia, sibling avoidance, and group selection (Shine, 1999), but the differential fitness hypothesis is currently the most accepted hypothesis and also the only one currently supported by experimental data from a TSD species (Warner and Shine, 2008). Thus, it is plausible that the biased sex ratio reported for loggerheads in the current study represents an evolutionarily stable phenomenon.

The presence of a significant female bias has implications for the conservation of the loggerhead population in the southeastern U.S. Assuming that males do not become a limiting factor, a significant female bias could enhance the recovery of this population by significantly increasing egg production in comparison to a population with a 1:1 sex ratio (Coyne et al., 2007; Wibbels, 2007). For example, data from the Kemp's Ridley Recovery Program suggest that the recovery of this endangered species is being enhanced by a significant female bias in the population (Coyne et al., 2007; Wibbels, 2007). Therefore, from a conservation management viewpoint, the loggerhead population in the southeastern U.S. is currently producing a sex ratio that is advantageous for its survival, so no mitigation activities would be recommended, however, the long-term monitoring of sex ratios is recommended.

The findings from the current study can provide insight on the potential effects of global climate change on population sex ratio. Analysis of sea turtle sex ratios could prove to be a bioassay system for assessing the impact of global climate change. Considering the TSD pattern in sea turtles, one might predict increasing female biases over time. It is of interest that the predicted sex ratio (2.5F:1.0M) in the current study was higher than, but did not differ significantly, from that of a study two decades earlier (2.1.F:1.0M, Wibbels et al., 1988). However, in the case of the current study the magnitude of any change over the past two decades may not have been great enough to be detected when analyzing the current sample sizes. Another factor that should be considered is that the sex ratio analyzed during the current study reflects hatchling production from several decades ago. It is not clear to what extent (if any) temperature changes have occurred on the nesting beaches for loggerheads in southeastern U.S. over the past two decades. This exemplifies the need for long-term temperature studies as well as long-term sex ratio studies to evaluate any shifts associated with potential climate change. However, it is of particular importance, that models project significant increases in temperature over the 21st Century (IPCC, 2007). In the case of the loggerhead's temperature-dependent sex determination, a 1.0 ºC increase temperature could result in a significant shift in sex ratio (Yntema and Mrososvsky, 1982; Mrosovsky, 1988; Mrosovsky et al., 1992; Wibbels, 2003), and current models suggest a temperature increase ranging from 1.8 to 4.0 C during the 21st Century (IPCC, 2007). Considering a female bias is already being produced, it plausible that this bias could become extreme, and that males could become a limiting factor in the reproductive output of this population.

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CHAPTER 2

CONSERVATION, ECOLOGICAL, AND EVOLUTIONARY IMPLICATIONS OF NESTING BEACH TEMPATURES AND HATCHLING SEX RATIOS IN THE LOGGERHEAD SEA TURTLE POPULATION OF THE SOUTHEASTERN UNITED STATES

By

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ABSTRACT

The loggerhead sea turtle (*Caretta caretta*) population inhabiting the southeastern U.S. is one of the largest loggerhead populations in the world. Loggerheads in this population nest on a variety of beaches ranging from the mid-Atlantic states to the northern Gulf of Mexico, with the great majority of nesting occurring along the Atlantic coast of Florida. The current study represents a multi-year project (2004-2009) investigating beach temperatures that were simultaneously recorded at nest depth on a range of nesting beaches used by this population. The study also includes the histological evaluation of the sex of a subset of hatchlings that were found dead in nests on several of the beaches. The results indicate that temperatures vary significantly between nesting beaches. The results also indicate that the spectrum of beaches used by the loggerhead population in the southeastern U.S. include a wide variety of thermal profiles, with potential sex ratios ranging from highly female biases to male biases depending on the specific beach. In general, nesting beaches temperatures along the Atlantic coast of Florida (e.g. Melbourne Beach, Juno Beach, Hutchinson Island), where the majority of nesting occurs, were relatively warm suggesting the production of female-biased sex ratios. The histology-based hatchling sex ratio data support the hypothesis that female biases may often be produced on nesting beaches on the Atlantic coast of Florida. In contrast, a few locations (e.g. Cape San Blas on the Florida panhandle) were consistently cooler suggesting the production of a greater proportion of males. However, these locations represented minor nesting beaches compared to those on the Atlantic coast. These finding are consistent with the hypothesis that sex ratio production is beach-

specific, but the loggerhead population in the southeastern U.S. is producing an overall female-biased sex ratio. The results provide a basis for suggesting which beaches in the southeastern U.S. are important for the production of female and male loggerheads. The results also provide an initial data set for evaluating potential long-term changes in beach temperatures associated with global climate change.

INTRODUCTION

The loggerhead sea turtle (*Caretta caretta*) population inhabiting the southeastern U.S. is considered one the largest loggerhead populations in the world (TEWG, 2000). Although it is the most common sea turtle in the waters of the southeastern U.S., it is listed as “threatened” under U.S. Federal Endangered Species Act (ESA) and the number of nesting loggerheads in this population has steadily and significantly declined since 1998 (NMFS/USFWS Loggerhead Sea Turtle 5 year status review, August 2007). For example, during 1989-2007 the overall nesting of loggerheads in Florida declined by 37% (FWCC, 2007). Therefore, the conservation of this population is currently a major concern.

Like all sea turtles, the loggerhead possesses temperature-dependent sex determination. This form of sex determination can produce a variety of sex ratio, some of which may not conform to 1:1 (Wibbels, 2003). Sex ratios have significant implications for the ecology of sea turtles since they can affect the reproductive output in a population. Sex ratios also have significant implications for conservation since they can affect the recovery rate of a population (Coyne, 2004; Wibbels, 2003; 2007). Therefore, knowledge of naturally occurring sex ratios is a prerequisite for understanding the reproductive ecology of a sea turtle population. Further, such information can be useful when developing management strategy for threatened and endangered populations (Wibbels, 2003).

The loggerhead was the first sea turtle to be examined for the presence of temperature-dependent sex determination (Mrosovsky and Yntema, 1980; Yntema and Mrosovsky, 1980). Subsequently it was shown that that temperature during the approximate middle third of incubation determines sex in this species (Yntema and Mrosovsky, 1982). Studies of the loggerhead have also examined the pivotal temperature (temperature that produced a 1:1 sex ratio) and transitional range of temperatures (range in which sex ratio changes from all males to all females) for the loggerhead. The pivotal temperature for loggerheads in the western north Atlantic have been estimated to be approximately 29.0ºC, with temperatures at or below 27.5ºC producing 100% male hatchlings and temperatures 30.4ºC and higher producing 100% female hatchlings (Mrosovsky, 1988, 1994; Yntema and Mrosovsky, 1982). This availability of this information can facilitate the prediction of sex ratios based on incubation temperatures or beach temperature (Mrososky and Provancha, 1989; Georges et al., 1994; Wibbels, 2003).

Several studies have examined sex ratios within this population, but most of these studies were limited to specific beaches and/or specific nesting seasons. Many of the studies focused on specific beaches on the Atlantic coast of Florida ratios (Mrosovsky and Provancha, 1989; 1992; Hanson et al., 1998; Blair, 2004) and most have indicated female-biased sex ratios. While previous studies have provided insight on sex ratios produced from specific beaches, they have not provided a comprehensive overview of sex ratios produced throughout the population. Further, they represent sex ratios produced at a specific point in time and thus, do not address long term stability or variability of sex ratio production from nesting beaches. Therefore, it would be useful to generate comprehensive and long-term studies on sex ratio production. Unfortunately, large scale sex ratio studies have been avoided in the past because they require the sacrifice of large numbers of hatchlings in a threatened population. Therefore many studies have adopted indirect methods for predicting hatchling sex ratios such as incubation temperatures, beach temperatures, and incubation durations (Mrosovsky and Provancha, 1989; 1992; Godfrey et al., 1996; Marcovaldi et al., 1997; Kaska et al., 1998; Hanson et al., 1998; Hays et al., 1999; Mrosovsky et al., 1999; Broderick et al. 2000; Godley et al., 2001).

The current study examines beach temperatures as a method of gaining insight on hatchling sex ratio production and utilizes a comparative approach in which beach temperatures are simultaneously recorded on a variety of the nesting beaches for loggerheads in the southeastern U.S. This study uses data loggers buried at nest depth to record temperatures in areas of the beach where the majority of loggerheads nest. The results are presented relative to the pivotal temperature and transitional range of temperatures for the loggerhead. In addition to the temperature data, sex ratio data based on hatchlings found dead in nests are also analyzed. The study also includes the simultaneous comparisons of two beaches in the same general area of Florida; one is a beach that had recently been “renourished” with sand and the other is a natural beach that has never been renourished. Additionally, a sand transfer experiment was conducted to examine the effects of two different sand types on beach temperature. Collectively, the results provide insight on spatial and temporal dynamics of beach temperatures and hatchling sex ratio production for loggerheads in the southeastern U.S. For example, the results indicate that some beaches have a propensity for producing female biases, whereas other beaches may produce greater proportions of males. Such information is of interest for evaluating the dynamics of sex ratio production within the loggerhead population and it provides data which are useful for the development of management strategy for the threatened loggerhead population. Finally, these data also provide an initial step in establishing a long-term study of beach temperatures for assessing potential shifts associated with global climate change.

METHODS

Nesting Beach Temperature Monitoring

The comprehensive nature of this study was facilitated by a large network of collaborators from major loggerhead nesting beaches ranging from South Carolina to the northern Gulf of Mexico. The bulk of loggerhead nesting in the southeast U.S. occurs in the state of Florida; therefore, much effort was focused on Florida. However, beaches throughout the range were be monitored. This study was conducted over six nesting season (2004-2009).

Temperature data loggers (Onset Computer Corporation, Pocassette, MA) were used to record beach temperatures. These are relatively small, battery powered units consisting of a microprocessor and a temperature probe, which accurately record temperatures to approximately +/- 0.3 - 0.4° C. HOBOs, Optic stowaways, and HOBO pendants models were used to record temperatures. The data loggers were programmed in the laboratory to record temperature every hour. They were heat-sealed in plastic bags with containers of desiccant. The data loggers were placed on loggerhead nesting beaches in areas that were representative of most loggerhead nesting as indicated by collaborators familiar with each particular beach. In general, the topography of the beaches included an intertidal zone, then a gently sloping portion of the beach that connects with a steeper-sloped region that is continuous with a vegetative zone. Most of the nesting typically occurs well above the high tide line on the gently sloping portion of the beach near the start of the steeply sloped beach. Therefore data logger locations were similar on all beaches.

Approximately 5 data loggers were deployed at each nesting beach over a 0.5 to 1.0 km area where high density nesting normally occurred. The location of each data logger was triangulated to two stakes in the dune and GPS coordinates were taken to assist in recovery of the data loggers. Additionally, we employed the use of radar golf balls to aid in relocating the data loggers (Tucker et al., in press).

Each data logger was placed at a 40 cm depth which approximates the center of a loggerhead nest (Dodd, 1988; DeGroot and Shaw, 1993; Broderick 1997). The data loggers were retrieved at the end of the nesting season and the data were downloaded using HOBOWARE software and then exported to Microsoft Excel for analysis.

The nesting beaches listed below were utilized during this study. These nesting beaches provide a broad range of the beaches used by the loggerhead population in the southeastern U.S. and include the major nesting beaches (central and southern Atlantic beaches in Florida). Due to logistical difficulties related to placing and retrieving data loggers, not all beaches were utilized during every year of the study. However, the major nesting beaches for the loggerhead were recorded every year (e.g. Melbourne Beach, Juno Beach, and Hutchinson Island). Additionally, erosion of beaches during major storms often resulted in the loss of some data loggers. The locations are shown in Figure 1 and listed below:

<u>Nesting Beaches Used in This Study (Location and Collaborating Organization):</u>

1) New Smyrna Beach, FL (Ecological Associates, Jensen Beach, FL)
2) Melbourne Beach, FL (University of Central Florida, Orlando, FL)
3) St. Lucie Nuclear Power Plant, FL (Quantum Resources, St. Lucie, FL)
4) Hutchinson Island, FL (Ecological Associates, Jensen Beach, FL)
5) Hobe Sound, FL (Ecological Associates, Jensen Beach, FL)
6) Juno Beach, FL (Loggerhead Marine Life Center, Juno Beach, FL)
7) Boca Raton, FL (Florida Atlantic University, Boca Raton, FL)
8) Cape Romano, FL (Florida Fish and Wildlife Commission)
9) Sanibel Island, FL (Sanibel/Captiva Conservation Foundation, Sanibel Island, FL)
10) Venice Beach, FL, South (Mote Marine Lab, Sarasota, FL)
11) Venice Beach, FL, North (Mote Marine Lab, Sarasota, FL)
12) Nokomis Beach Park, Casey Key, FL South (Mote Marine Lab, Sarasota,FL)
13) Casey Key, FL, Central (Mote Marine Lab, Sarasota,FL)
14) Casey Key, FL, North (Mote Marine Lab, Sarasota,FL)
15) Turtle Beach, Central Siesta Key, FL, South (Mote Marine Lab, Sarasota,FL)
16) Siesta Key, FL, North (Mote Marine Lab, Sarasota,FL)
17) Lido Key (Mote Marine Lab, Sarasota,FL)
18) Longboat Key, FL, South (Mote Marine Lab, Sarasota,FL)

19) Longboat Key, FL, Central (Mote Marine Lab, Sarasota,FL)

20) Longboat Key, FL, North (Mote Marine Lab, Sarasota,FL)

21) St. George Island, FL (Apalachicola National Estuarine Research Reserve)

22) Cape San Blas, FL (Ray Carthy, University of Florida, Gainesville, Fl)

23) Panama City, FL (St. Andrew Bay Resource Management Association)

24) Bon Secour National Wildlife Refuge, AL (U.S. Fish and Wildlife Service)

25) Dauphin Island, AL (Dauphin Island Sea Lab, Alabama)

26) Cape Romain National Wildlife Refuge, SC (U.S. Fish and Wildlife Service)

Figure 1: Locations of beaches monitored during this study. Beaches range from Alabama to South Carolina, with the majority located in areas of highest nesting activity in Florida.

<u>"Renourished" Beach Study</u>

A comparison was conducted to evaluate the potential effects of beach renourishment (Steinitz et al., 1998) on beach temperatures. Two beaches near Sarasota, FL, were utilized. Temperatures were examined on Longboat Key which was recently renourished with relatively dark sand that is dredged from offshore, and Casey Key which represented a natural non-renourished beach. The comparison was conducted over two nesting seasons from 2005 and 2006. Five data loggers were buried on each beach per nesting season in locations where the majority of nesting occurred

During the summer of 2007, an additional experiment was conducted on Longboat Key. This involved a sand transfer experiment examining the effects of sand type on incubation temperature. The experiment was conducted near an area where a lighter colored beach is located adjacent to a beach renourished with darker sand. The darker sand that was often used during renourishment in this area, was transferred onto lighter colored beach and placed into a hole that was 1 meter in diameter and approximately 0.75 meter deep (Figure 2). A reciprocal experiment was also conducted in which light colored sand was transferred to a hole on the dark sand beach. Five data loggers were used in each experiment, all buried at 40 cm in depth, with two near the center of the plot (place about 5 cm from one another), one at the periphery of the plot (interface between the two sand types), one that was placed one meter out from the periphery of the sand plot, and one that was placed two meters out from the periphery of the sand plot.

Figure 2: Sand transfer experiment showing dark sand tranferred to a light sand beach (Longboat Key, FL).

Temperature Data Analysis

Loggerhead nesting in the southeastern U.S. normally begins in mid to late May, is heavy during June and July, and declines in August. Beach temperatures were compared from the last week of June through the first week of August which represents the main portion of the nesting season and includes the time period when the majority of the nests would be experiencing their middle third of incubation (i.e. the temperature sensitive period of sex determination. Two-way ANOVA were used to examine both intra-beach and inter-beach variation in temperatures. Two-way ANOVA were also used to compare the results of the nourished versus natural beaches and for comparisons in the sand transfer experiment. T-tests were used for specific comparisons of two beaches to one another.

Histological Analysis of Hatchling Sex Ratios

Histology-based hatchling sex ratio data were analyzed from a collaborative study with Florida Atlantic University, the U.S. Fish and Wildlife Service at Bon Secour National Wildlife Refuge, Alabama (U.S. Fish and Wildlife Service) and Florida Fish and Wildlife Commission. The study included several loggerhead beaches on the Atlantic and Gulf coasts of the southeastern U.S. during the 2003 and 2004 nesting seasons. Histological analysis was conducted to determine the sex of hatchlings from certain nesting beaches (dependent on specific projects and available permits). In the case of nesting beach studies, tissues used for histology were from hatchlings that died of natural causes in the nest and were collected after all live hatchlings emerged. The tissues were preserved in neutral buffered formalin and the kidney/adrenal/gonad (AKG) were removed for histological processing. The AKG's were infiltrated with paraffin wax, imbedded in wax blocks, and cut into 8 um cross-sections of tissue using a microtome. The cross-sections were mounted onto slides and stained using standard hematoxylin and eosin staining methodology. The processed tissues were examined on a microscope to determine sex. The sex of an individual is determined by the structure of the gonad (Yntema and Mrosovsky, 1980). In the case of a female, the ovary has a relatively well-developed outer region (the cortex), which stains heavily. The inner portion of the ovary (medulla) does not show any distinct organization. Additionally, the mullerian ducts of the female are well developed. In contrast to the female, the gonad of a male lacks a well-developed cortex, and the inner portion of the gonad (the medulla) shows well-developed cells that become organized into sex cords that will form the seminiferous tubules. During embryonic development, males will develop mullerian ducts, which then regress (Wibbels et al., 1999).

RESULTS

Nesting beach temperatures during the six years study are shown in Figures 3 - 8 relative to the pivotal temperature for the loggerhead. Temperatures varied significantly between data loggers on a given beach (two-way ANOVA, $P<0.05$), and between beaches (two-way ANOVA, $P <0.05$). Table 1 shows the average temperatures recorded on each of the nesting beaches during the main portion of the nesting season (last week of June through first week of August) during each year of the study (shown in geographical order). The warmest beaches were located on the Atlantic coast of Florida, whereas the coolest beaches were located on the Florida panhandle. Melbourne Beach was the beach that topographically was the most consistent throughout the study and was not renourished during the study. It is also the beach with the highest loggerhead nesting activity in Florida. Figure 9 shows a year- by-year comparison of temperatures on Melbourne beach. Figures 10 - 15 show the average beach temperatures for the various beaches during 2004 -2009, relative to the estimated pivotal temperature and transitional range of temperatures for the loggerhead. Figures 16 - 17 show comparisons of the beach temperatures for Longboat Key (a renourished beach) versus Casey Key (a nearby natural beach) for 2005 and 2006. The temperatures were significantly warmer on Longboat Key for both years of the study (two-way ANOVA, $P<0.001$). On average, Longboat was approximately 1.52°C higher in temperature than Casey Key during the 2005 nesting season, and 0.81°C warmer than Casey Key during the 2006 nesting season.

Figures 18 and 19 show the temperatures recorded during the sand transfer experiment. During the sand transfer experiment, the darker-colored sand area was significantly warmer than the lighter-colored sand area (two-way ANOVA, $P<0.001$). The dark sand plot was 0.57°C warmer than the surrounding light sand on the beach, and in the reciprocal experiment, the light sand plot 0.80°C cooler than the surrounding dark sand.

Table 1. Comparison of average temperatures at nest depth of nesting beaches during the main portion of the nesting season when most nests are experiencing their temperatures sensitive period. Key to symbols: $ indicates final 1 of 6 weeks; * indicates final 2 of 6 weeks; ^ indicates final 3 of 6 weeks; # indicates final 4 of 6 weeks; & indicates final 5 of 6 weeks; N/A indicates there are data from that year, but not during this 6-week time frame.

	Location	2004	2005	2006	2007	2008	2009	Multi-Year Avg.
ATLANTIC COAST	Cape Romain NWR, SC				# 30.8±0.4	30.6±0.2	31.1±0.3	30.8 ± 0.3
	Volusia Co., FL					28.7±0.3	28.7±0.1	28.7 ± 0.0
	Melbourne Beach, FL	30.9 ±0.2	30.7± 0.6	30.4±0.2	30.2±0.2		31.0±0.3	30.6 ± 0.6
	Boca Raton, FL	32.4±0.6	32.5±0.2	31.3±0.4	31.4±0.5	31.8±0.5	& 31.9±0.4	31.7 ± 0.3
	Hobe Sound NWR, FL	30.9±0.3	31.9±0.5	30.9±0.5	30.2±0.4	30.2±0.4	31.3±0.4	30.6 ±0.6
	St. Lucie, FL		30.9±0.5	30.7±0.4	30.4±0.4		31.4±0.2	30.9 ± 0.7
	Juno Beach, FL		33.3±0.2	31.8±0.5	31.7±0.5	31.7±0.5	31.6±0.3	31.7 ± 0.1
	Hutchinson Is., FL					30.1±0.4	31.7±0.3	30.9 ± 1.1
WESTERN GULF COAST, FL	Sanibel Is., FL	29.9±0.3	29.9±0.4	* 29.2±0.4	^ 30.6±0.7	& 30.0±0.4		30.3 ± 0.4
	Cape Romano, FL		29.6±0.5	28.6±0.2	& 30.1±0.2	29.4±0.3	29.9±0.3	29.8 ± 0.4
	Manisota Key (Casperson), FL						32.7±0.3	32.7
	Venice Beach (North), FL			30.2±0.3	31.7±0.5	30.7±0.3	32.1±0.3	31.5 ± 0.7
	Venice Beach (South), FL		31.5±0.5	29.9±0.3	31.8±0.4	31.1±0.3	32.4±0.3	31.8 ± 0.7
	Casey Key (King), FL		29.0±0.4	28.4±0.3	30.7±0.4		31.6±0.3	31.2 ±0.6
	Casey Key (Bush's), FL		29.5±0.2	28.3±0.2	30.4±0.5	29.5±0.3	30.8±0.3	30.2 ± 0.7
	Casey Key (Nokomis), FL			28.9±0.2	29.6±0.3	28.7±0.3	30.0±0.2	29.4 ± 0.7
	Siesta Key (White's), FL		29.8±0.3	28.3±0.2	30.8±0.3	31.2±0.2	32.3±0.4	31.4 ± 0.8
	Siesta Key (Turtle Beach), FL		29.9±0.5	28.4±0.2	31.0±0.4	30.0±0.3	31.6±0.3	30.9 ± 0.8
	Lido Key, FL		29.9±0.3	28.8±0.3	30.4±0.5	29.4±0.2		29.9 ± 0.7
	Longboat Key (North), FL			28.4±0.3	29.4±0.4	29.4±0.2	29.4±0.2	29.4 ± 0.0
	Longboat Key (South), FL		28.7±0.3	29.8±0.3	31.3±0.6	30.6±0.2	31.1±0.4	31.0 ± 0.4
NORTHEASTERN GULF COAST	Cape San Blas, FL	28.4±0.1	* 27.8±1.2	# 28.7±0.1	# 29.3±0.3	29.0±0.3	^ 30.1±0.2	29.5 ± 0.6
	St. George Is., FL				^ 29.2±0.6	28.7±0.2	^ 28.9±0.2	28.9 ± 0.3
	Panama City Beach, FL				^ 28.7±0.1	28.1±0.3	$ 28.5±0.0	28.4 ± 0.3
	Dauphin Is., AL			29.3±0.2	29.1±0.2	29.6±0.2	29.5±0.3	29.4 ± 0.3
	Bon Secour NWR, AL				^ 28.1±0.1		N/A	28.1

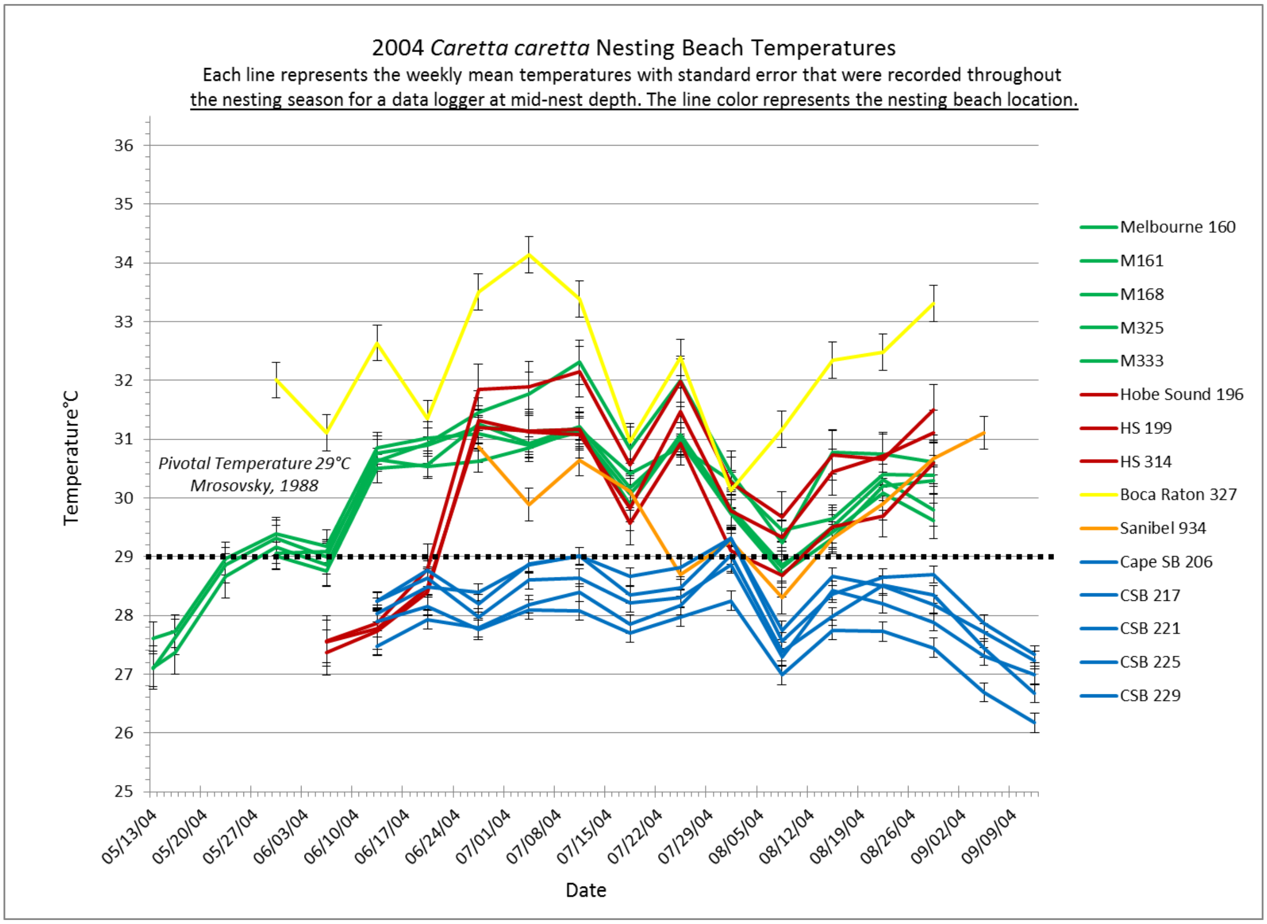


Figure 3. Beach temperatures at nest depth during the 2004 nesting season. Each beach is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs. M = Melbourne Beach, HS = Hobe Sound, CSB = Cape San Blas.

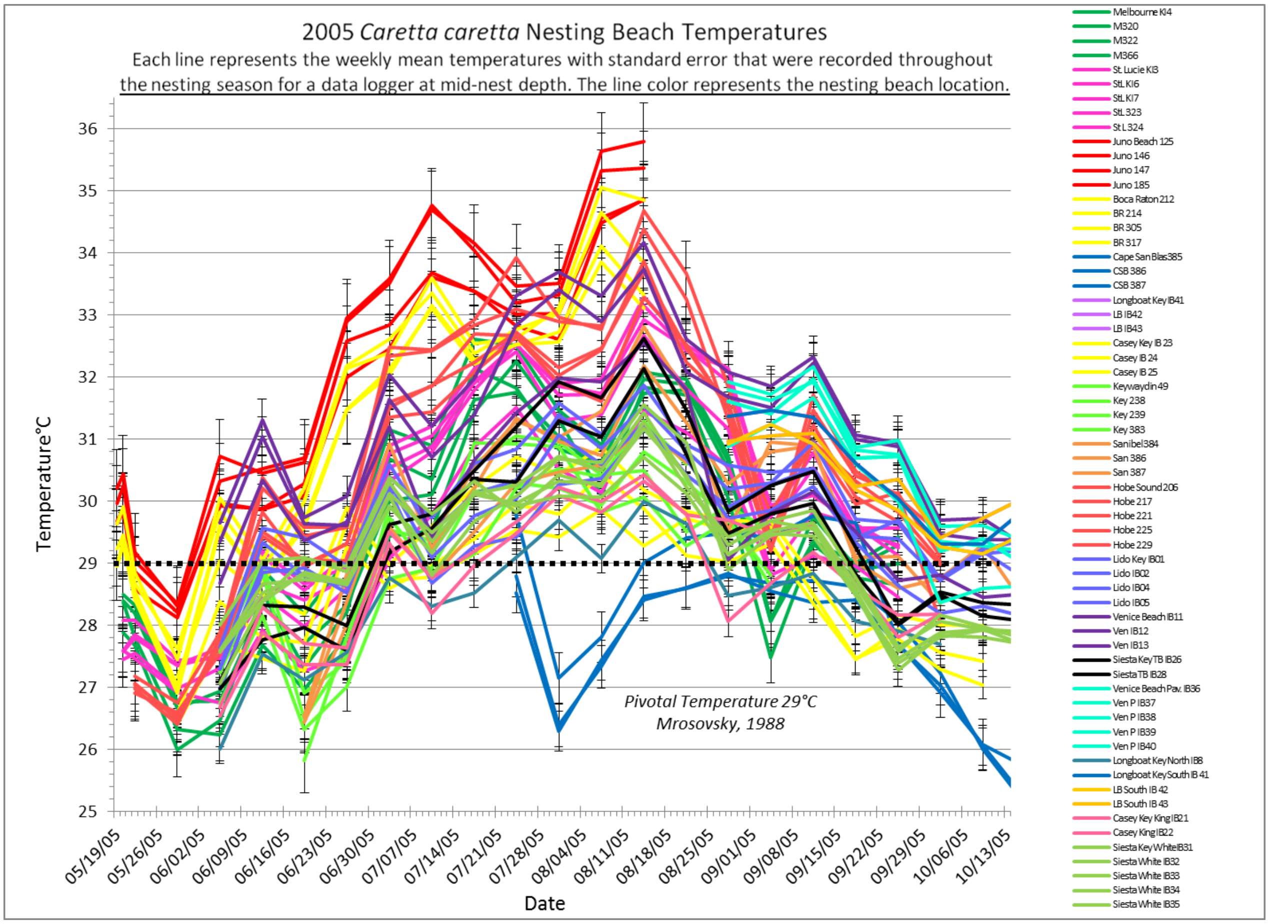


Figure 4. Beach temperatures at nest depth during the 2005 nesting season. Each beach is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs.

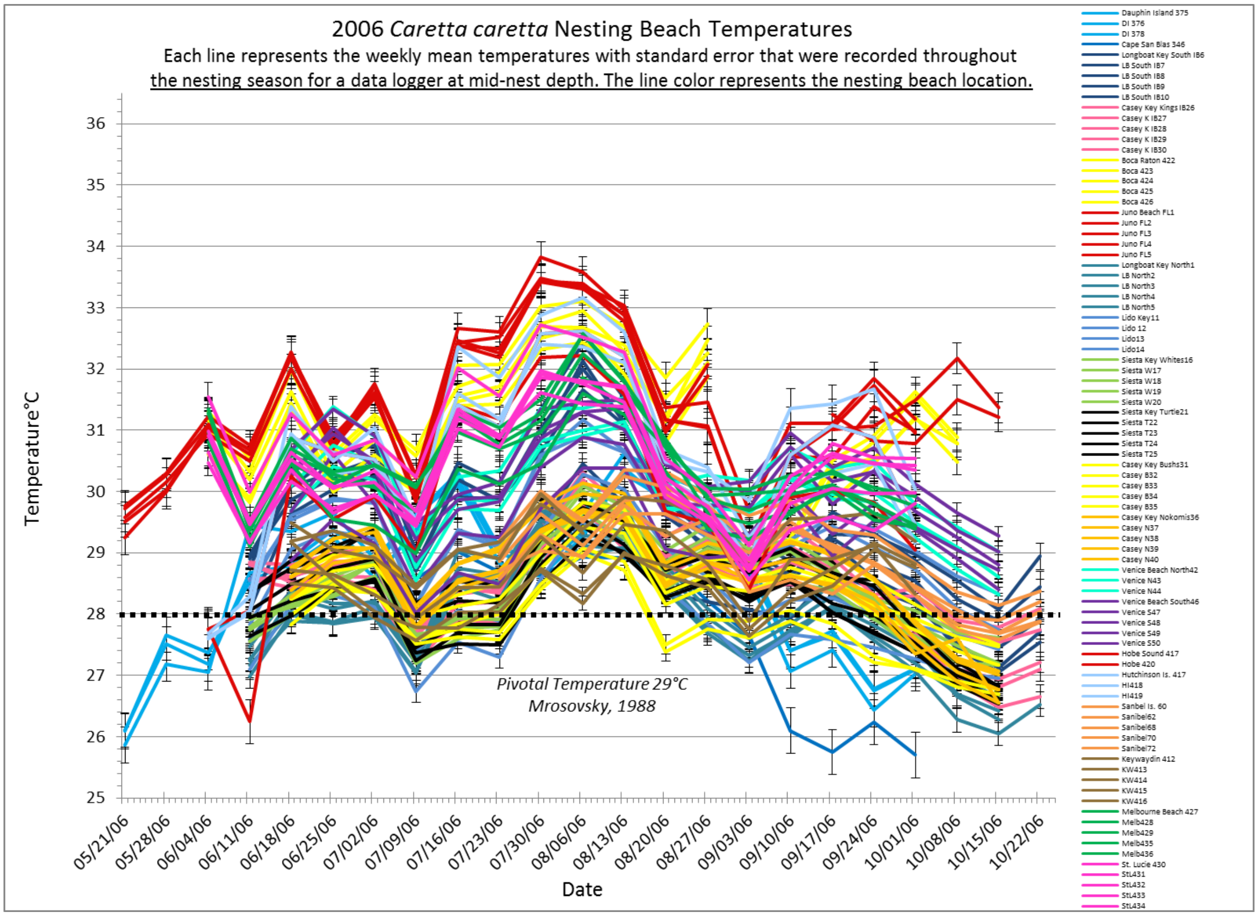


Figure 5. Beach temperatures at nest depth during the 2006 nesting season. Each beach is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs.

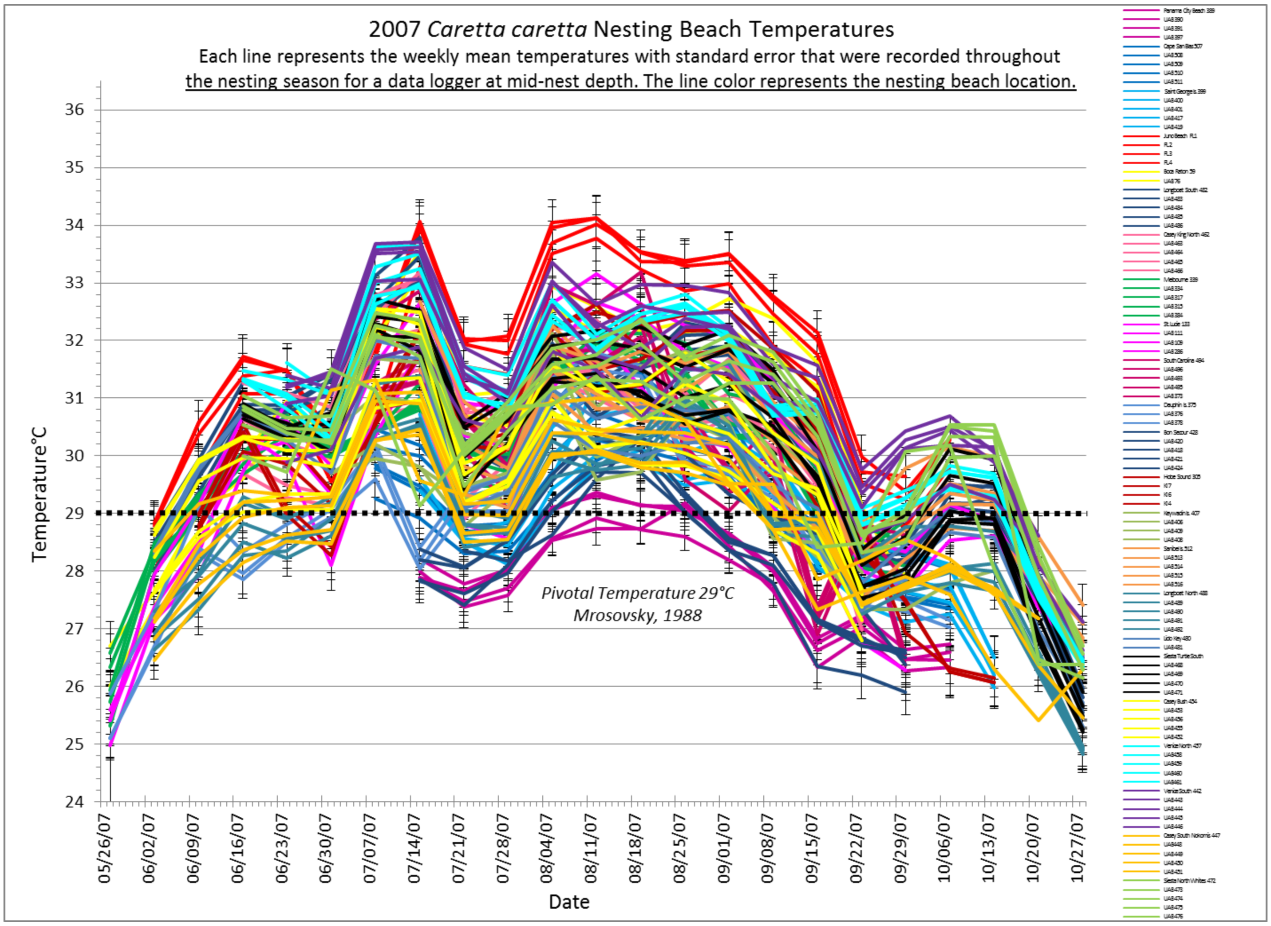


Figure 6. Beach temperatures at nest depth during the 2007 nesting season. Each beach is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs.

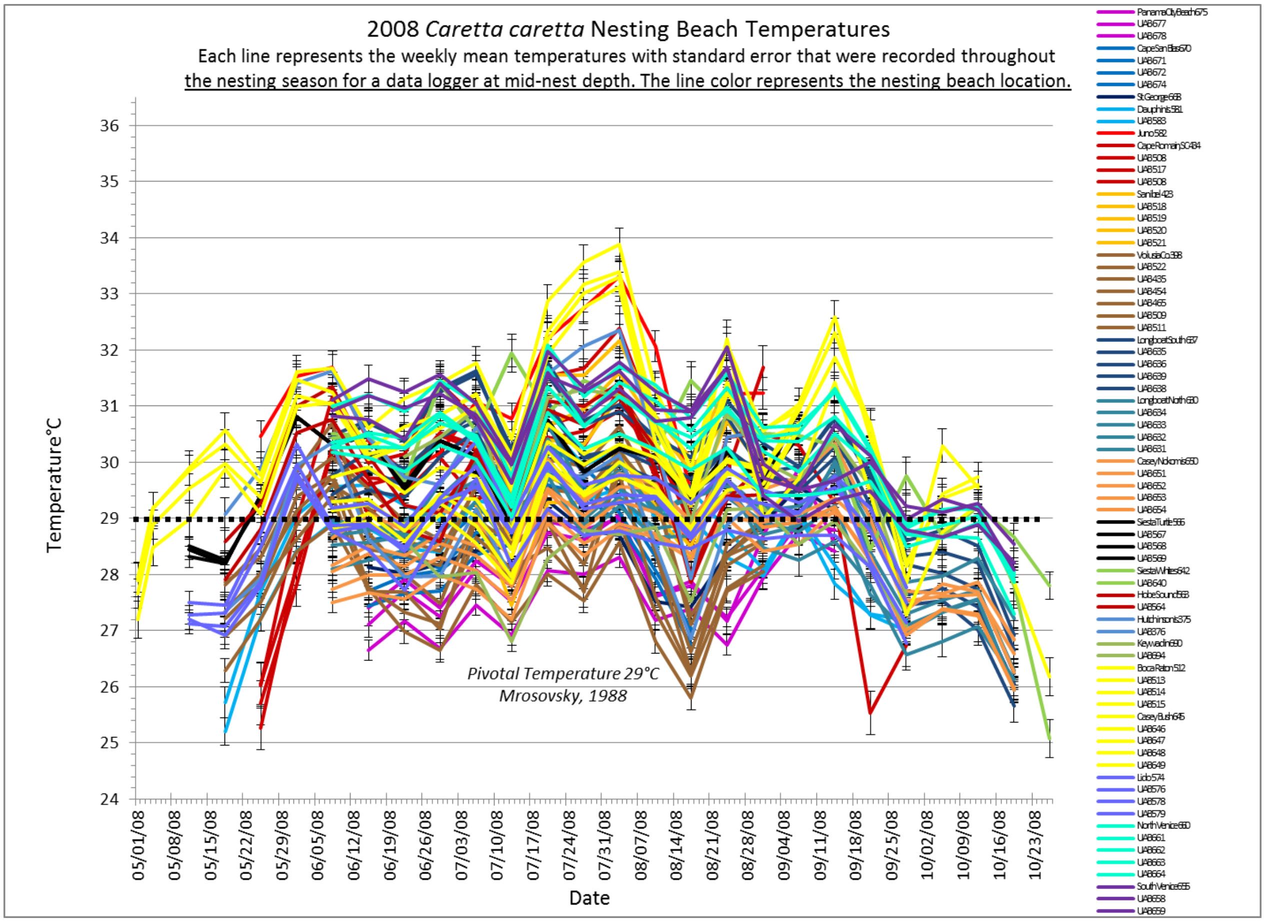


Figure 7. Beach temperatures at nest depth during the 2008 nesting season. Each beach is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs.

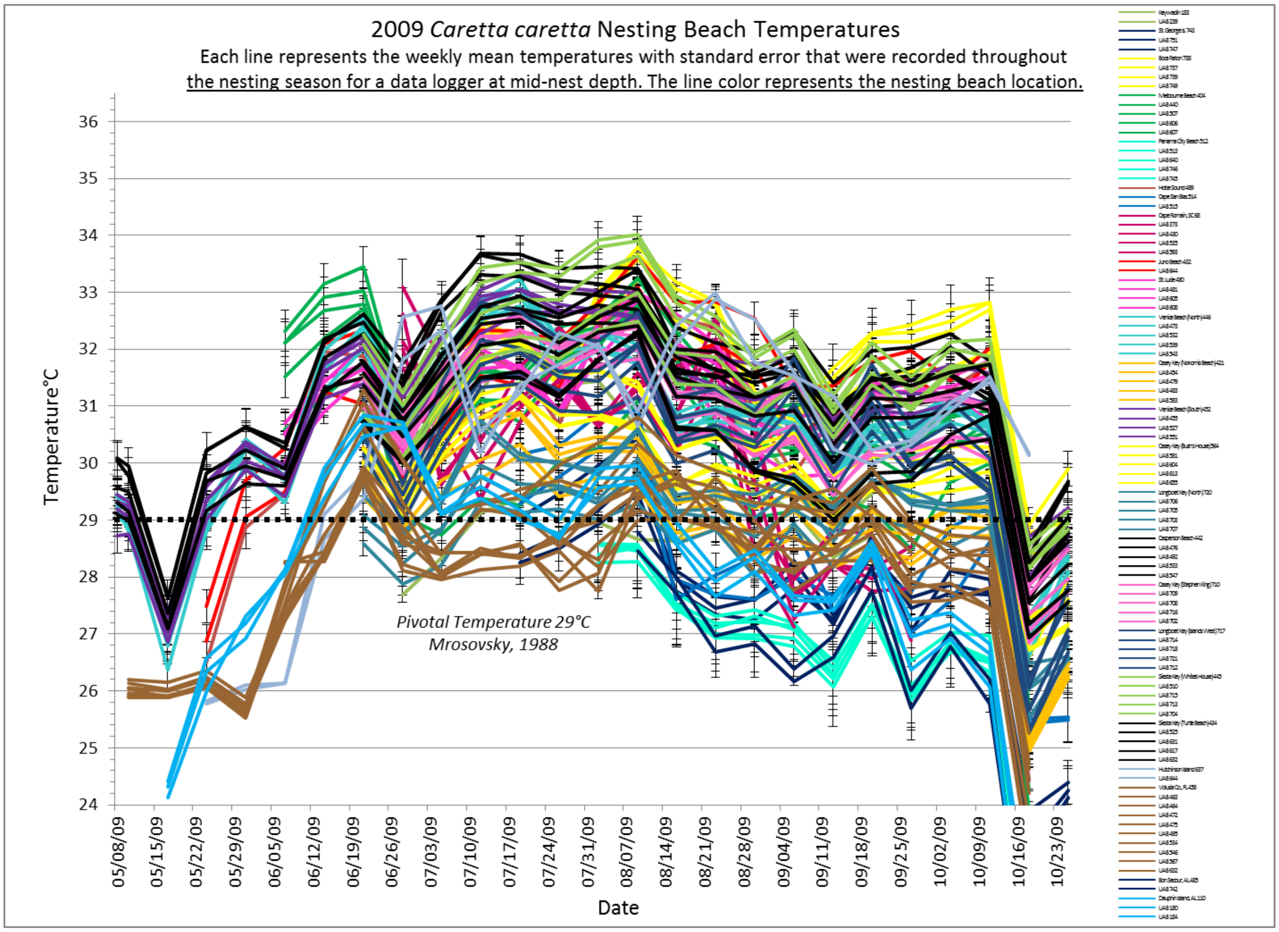


Figure 8. Beach temperatures at nest depth during the 2009 nesting season. Each beach is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs.

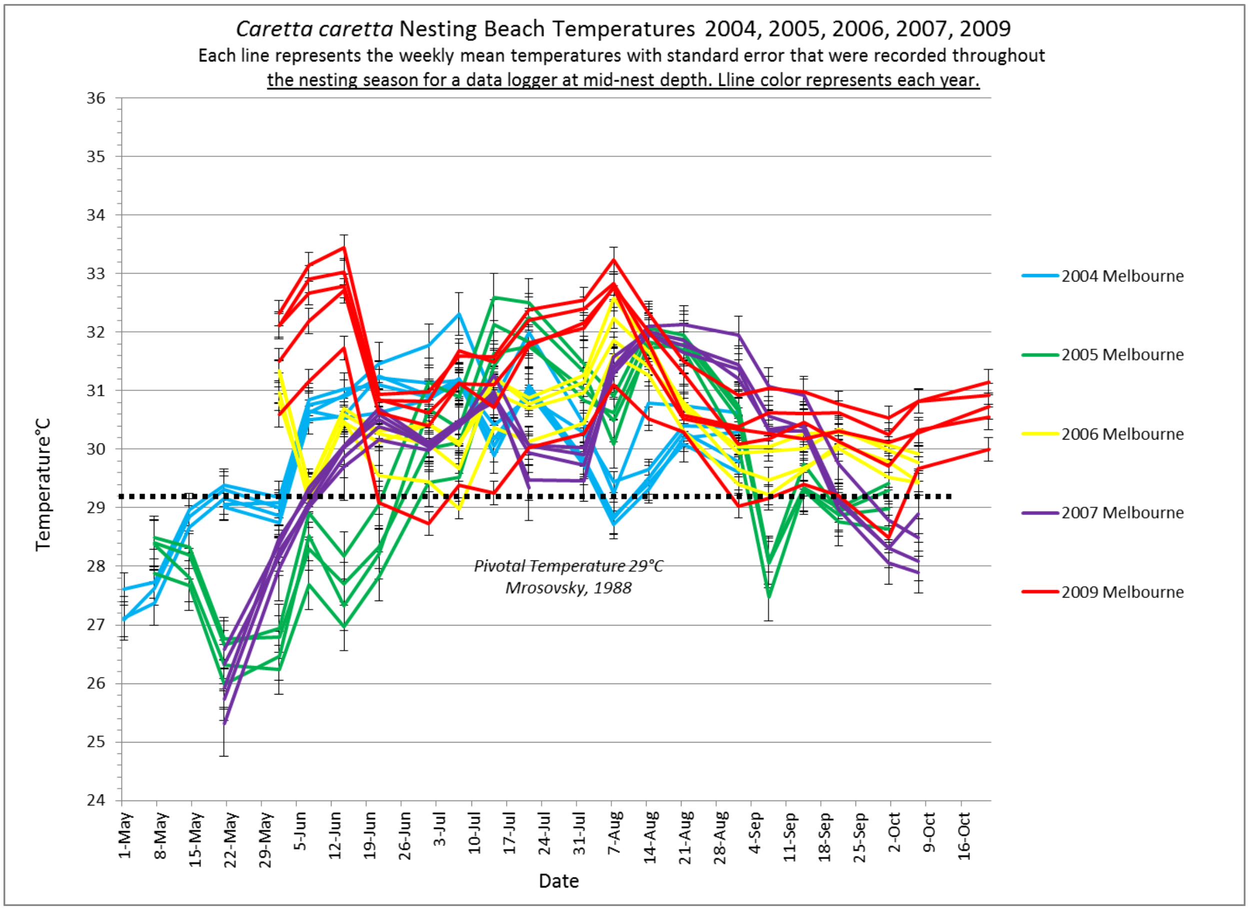
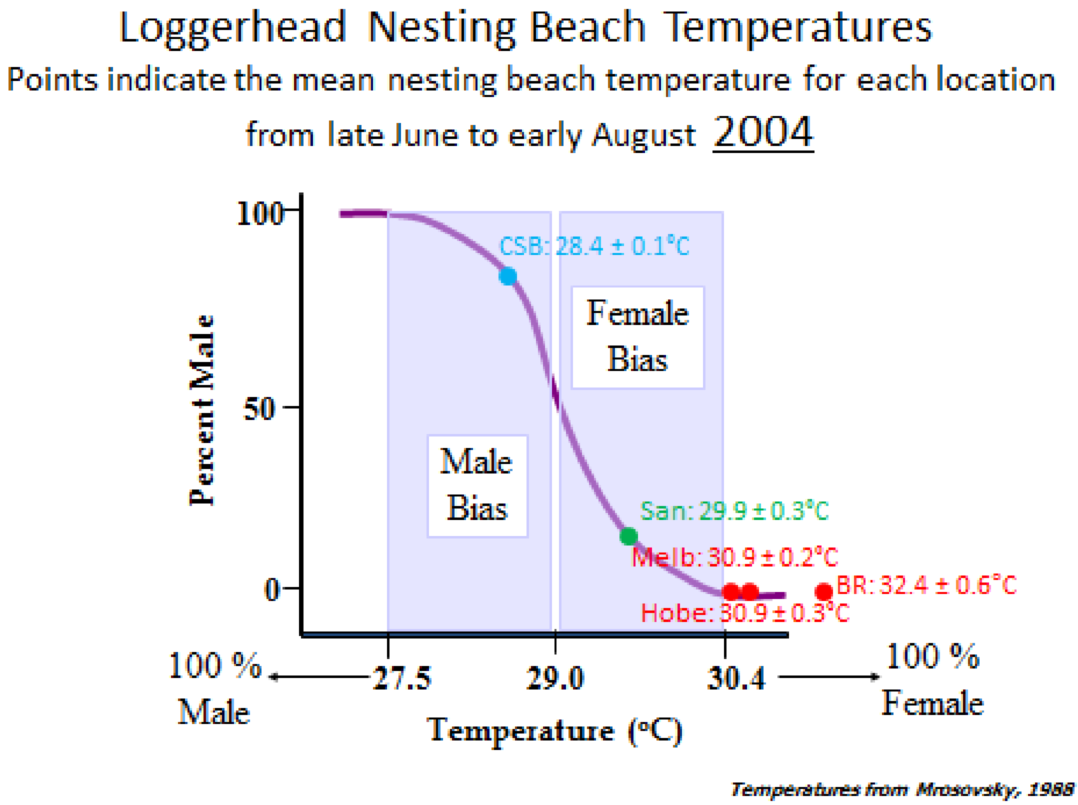


Figure 9. Year-by-year comparison of beach temperatures at nest depth on Melbourne Beach, FL. This beach experiences the highest loggerhead nesting activity in the southeastern U.S. and it was not renourished during this study. Each year is designated by a different color as indicated. Each line represents the weekly averages from a single data logger buried at nest depth in a region of the beach were most nesting occurs. No data is available for 2008 due to the loss of data loggers during a severe storm.



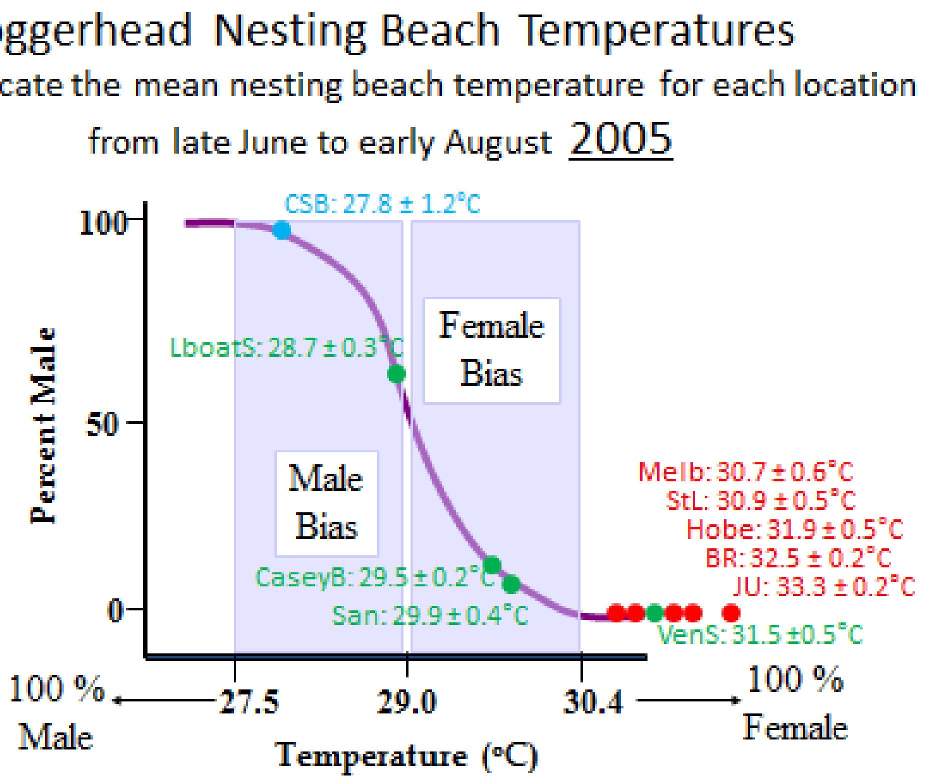
Loggerhead Nesting Beach Temperatures

Points indicate the mean nesting beach temperature for each location from late June to early August 2004

Temperatures from Mrosovsky, 1988

Figure 10. Mean temperatures (± SE) for 2004 from index nesting beaches representing different regions throughout the range of loggerhead nesting beaches (i.e. Florida Atlantic Coast, western Gulf coast of Florida, and northwestern Gulf coast). Temperatures are compared to the pivotal temperature and transitional range estimated for the loggerhead (Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination. The nesting beaches representing the northwestern Gulf coast are: Cape San Blas, FL (CSB), Dauphin Is., AL (DI), and St. George Island, FL (StG) and were plotted using blue points and text. The nesting beaches representing the western Gulf coast of Florida are: Sanibel Island (San), Venice Beach South (VenS), Casey Key Middle (CaseyB), and Longboat South (LboatS) and were plotted using green points and text.

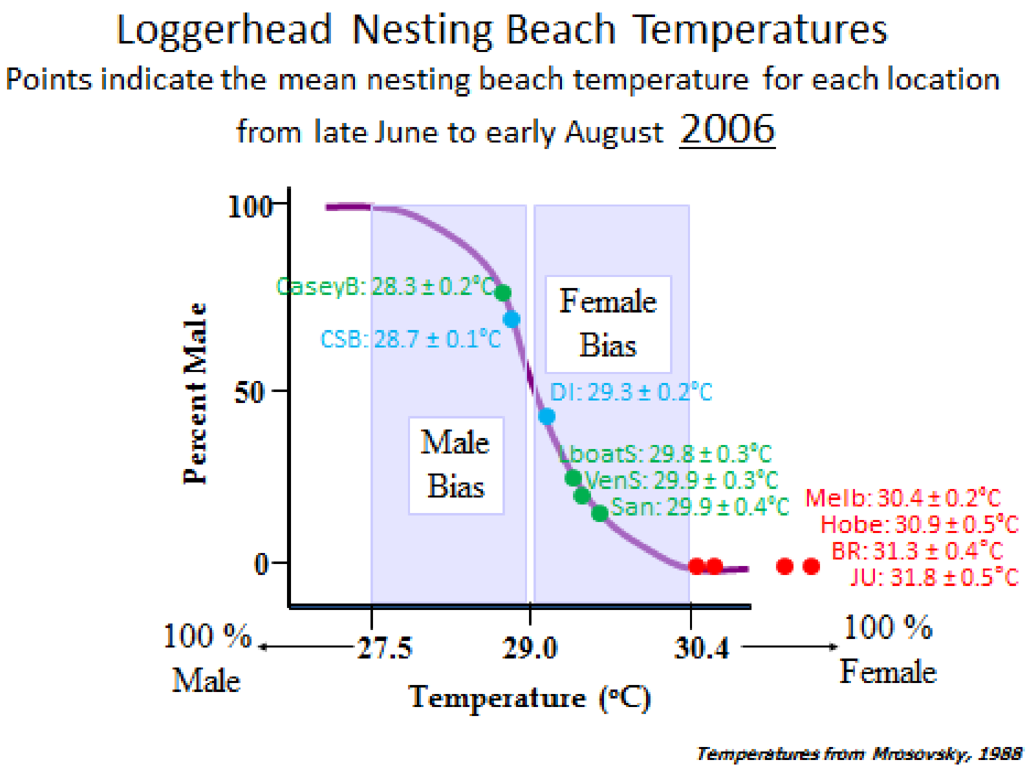
The nesting beaches representing the Atlantic coast of Florida are: Melbourne Beach (Melb), Boca Raton (BR), Hobe Sound NWR (Hobe), and Juno Beach (JU) and were plotted using red points and text.



Temperatures from Mrosovsky, 1988

Figure 11. Mean temperatures (± SE) for 2005 from index nesting beaches representing different regions throughout the range of loggerhead nesting beaches (i.e. Florida Atlantic Coast, western Gulf coast of Florida, and northwestern Gulf coast). Temperatures are compared to the pivotal temperature and transitional range estimated for the loggerhead (Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination. The nesting beaches representing the northwestern Gulf coast are: Cape

San Blas, FL (CSB), Dauphin Is., AL (DI), and St. George Island, FL (StG) and were plotted using blue points and text. The nesting beaches representing the western Gulf coast of Florida are: Sanibel Island (San), Venice Beach South (VenS), Casey Key Middle (CaseyB), and Longboat South (LboatS) and were plotted using green points and text. The nesting beaches representing the Atlantic coast of Florida are: Melbourne Beach (Melb), Boca Raton (BR), Hobe Sound NWR (Hobe), and Juno Beach (JU) and were plotted using red points and text.

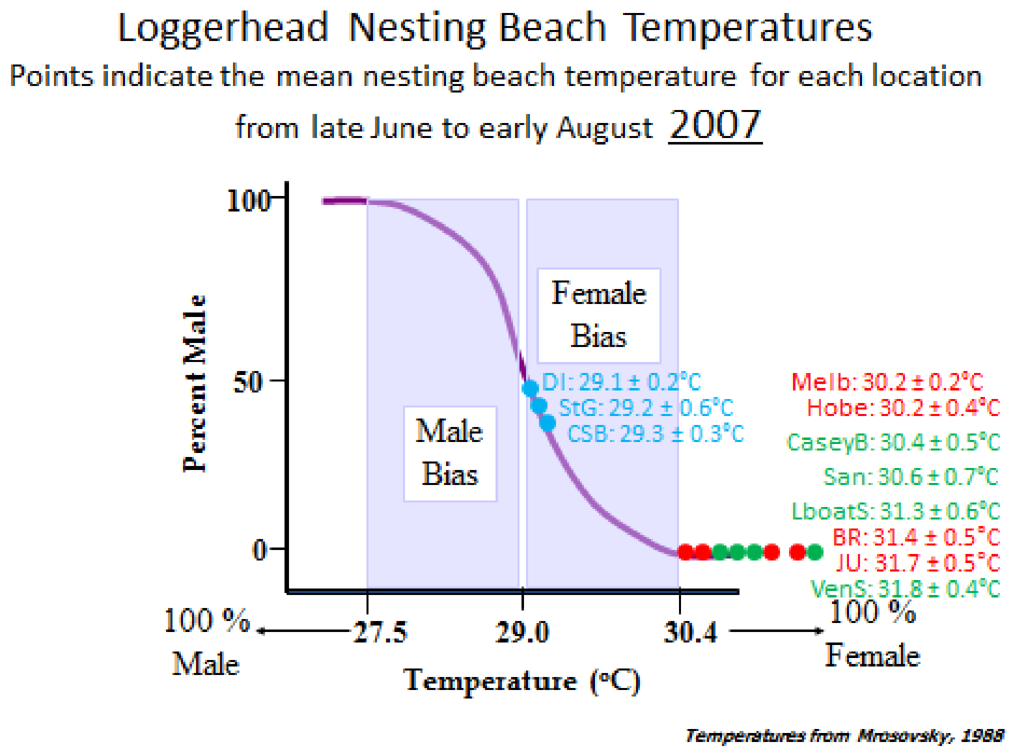

Loggerhead Nesting Beach Temperatures

Points indicate the mean nesting beach temperature for each location from late June to early August 2006

Temperatures from Mrosovsky, 1988

Figure 12. Mean temperatures (+ SE) for 2006 from index nesting beaches representing different regions throughout the range of loggerhead nesting beaches (i.e. Florida Atlantic Coast, western Gulf coast of Florida, and northwestern Gulf coast). Temperatures are

compared to the pivotal temperature and transitional range estimated for the loggerhead (Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination. The nesting beaches representing the northwestern Gulf coast are: Cape San Blas, FL (CSB), Dauphin Is., AL (DI), and St. George Island, FL (StG) and were plotted using blue points and text. The nesting beaches representing the western Gulf coast of Florida are: Sanibel Island (San), Venice Beach South (VenS), Casey Key Middle (CaseyB), and Longboat South (LboatS) and were plotted using green points and text. The nesting beaches representing the Atlantic coast of Florida are: Melbourne Beach (Melb), Boca Raton (BR), Hobe Sound NWR (Hobe), and Juno Beach (JU) and were plotted using red points and text.



Temperatures from Mrosovsky, 1988

Figure 13. Mean temperatures (± SE) for 2007 from index nesting beaches representing different regions throughout the range of loggerhead nesting beaches (i.e. Florida Atlantic Coast, western Gulf coast of Florida, and northwestern Gulf coast). Temperatures are compared to the pivotal temperature and transitional range estimated for the loggerhead (Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination. The nesting beaches representing the northwestern Gulf coast are: Cape San Blas, FL (CSB), Dauphin Is., AL (DI), and St. George Island, FL (StG) and were plotted using blue points and text. The nesting beaches representing the western Gulf coast of Florida are: Sanibel Island (San), Venice Beach South (VenS), Casey Key Middle (CaseyB), and Longboat South (LboatS) and were plotted using green points and text. The nesting beaches representing the Atlantic coast of Florida are: Melbourne Beach

(Melb), Boca Raton (BR), Hobe Sound NWR (Hobe), and Juno Beach (JU) and were plotted using red points and text.

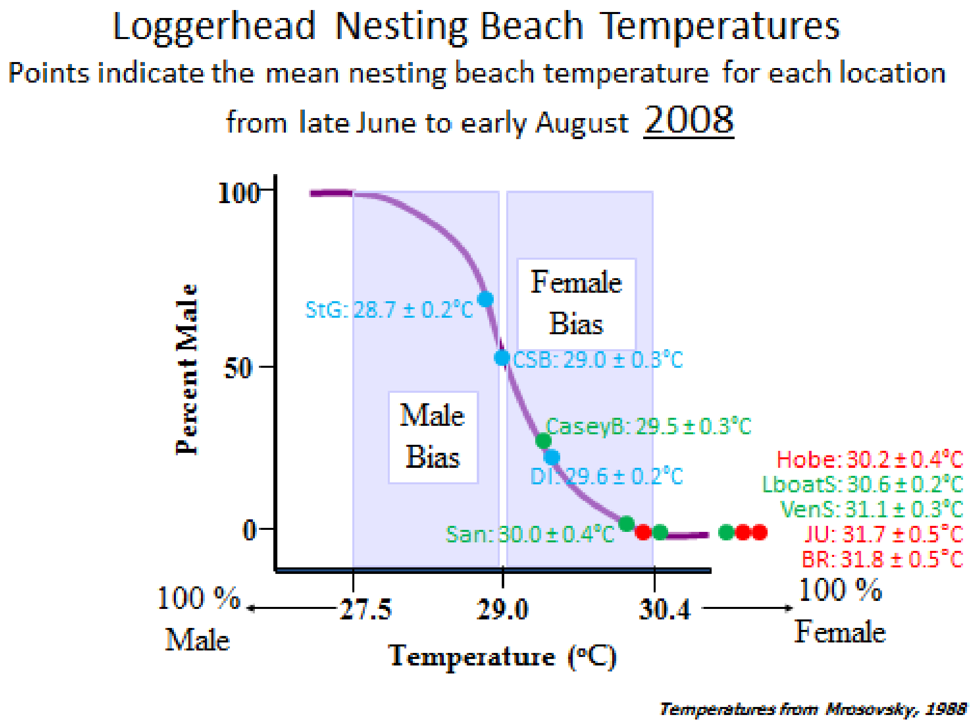
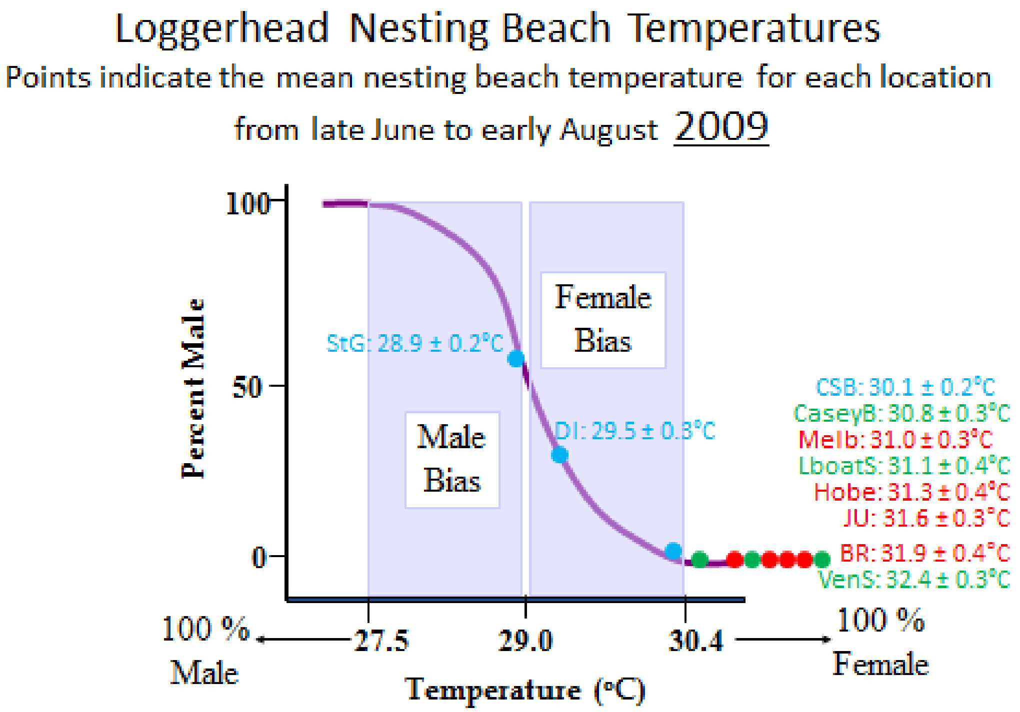


Figure 14. Mean temperatures (± SE) for 2008 from index nesting beaches representing different regions throughout the range of loggerhead nesting beaches (i.e. Florida Atlantic Coast, western Gulf coast of Florida, and northwestern Gulf coast). Temperatures are compared to the pivotal temperature and transitional range estimated for the loggerhead (Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination. The nesting beaches representing the northwestern Gulf coast are: Cape San Blas, FL (CSB), Dauphin Is., AL (DI), and St. George Island, FL (StG) and were

plotted using blue points and text. The nesting beaches representing the western Gulf coast of Florida are: Sanibel Island (San), Venice Beach South (VenS), Casey Key Middle (CaseyB), and Longboat South (LboatS) and were plotted using green points and text. The nesting beaches representing the Atlantic coast of Florida are: Melbourne Beach (Melb), Boca Raton (BR), Hobe Sound NWR (Hobe), and Juno Beach (JU) and were plotted using red points and text.



Temperatures from Mrosovsky, 1988

Figure 15. Mean temperatures (± SE) for 2009 from index nesting beaches representing different regions throughout the range of loggerhead nesting beaches (i.e. Florida Atlantic Coast, western Gulf coast of Florida, and northwestern Gulf coast). Temperatures are compared to the pivotal temperature and transitional range estimated for the loggerhead

(Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination. The nesting beaches representing the northwestern Gulf coast are: Cape San Blas, FL (CSB), Dauphin Is., AL (DI), and St. George Island, FL (StG) and were plotted using blue points and text. The nesting beaches representing the western Gulf coast of Florida are: Sanibel Island (San), Venice Beach South (VenS), Casey Key Middle (CaseyB), and Longboat South (LboatS) and were plotted using green points and text. The nesting beaches representing the Atlantic coast of Florida are: Melbourne Beach (Melb), Boca Raton (BR), Hobe Sound NWR (Hobe), and Juno Beach (JU) and were plotted using red points and text.

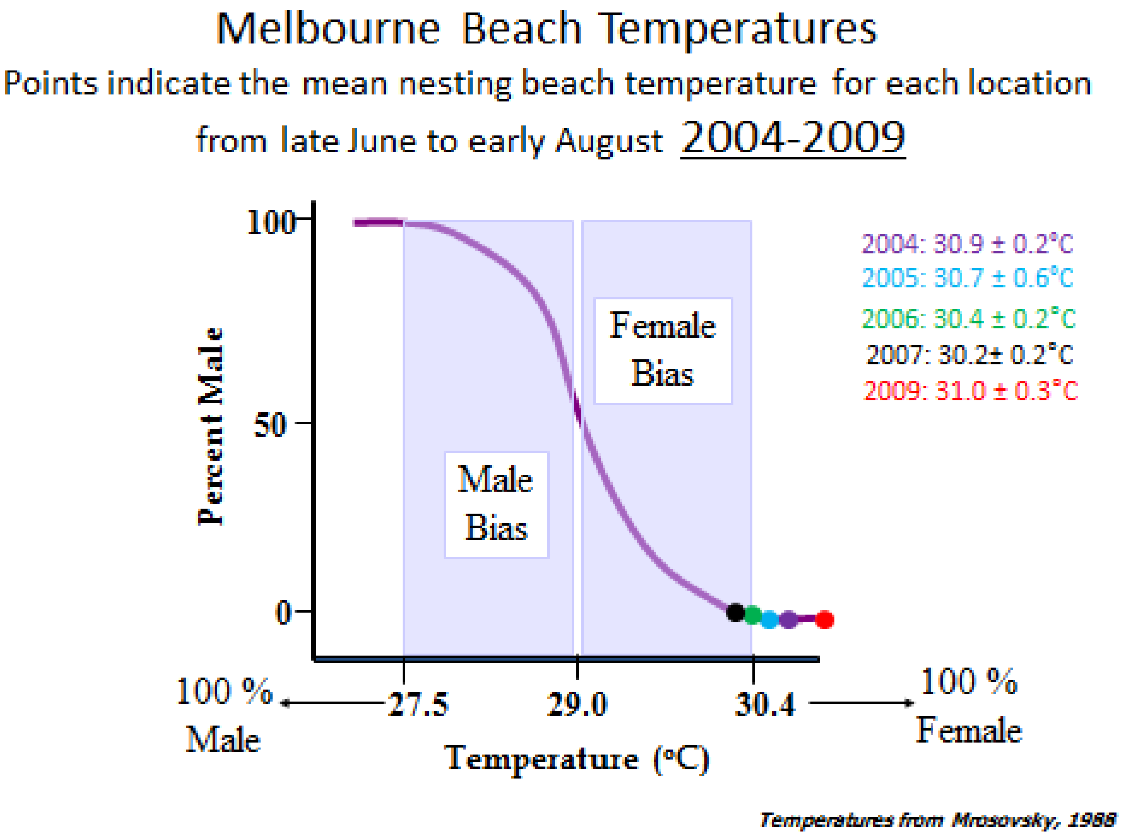


Figure 16. Example of a year-by-year comparison of beach temperatures at nest depth on Melbourne Beach, FL. This beach experiences the highest loggerhead nesting activity in the southeastern U.S. and it was not renourished during this study. Each year is designated by a different color as indicated. No data is available for 2008 due to the loss of data loggers during a severe storm. Temperatures are compared to the pivotal temperature and transitional range estimated for the loggerhead (Mrosovsky, 1988). Temperatures shown are from the portion of the nesting season when the majority of nests are experiencing the temperatures sensitive period of sex determination

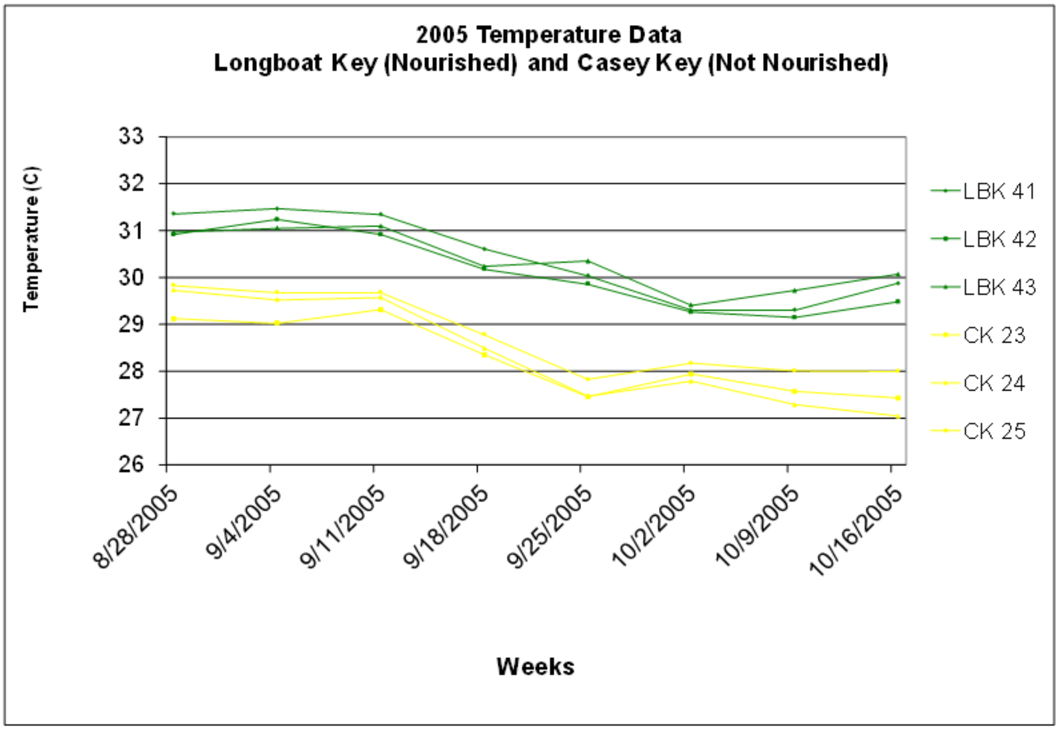


Figure 17. Beach temperatures at nest depth on a nourished versus a natural beach near Sarasota, FL, during 2005. Longboat Key is a nourished beach and is shown in green. Casey Key is a natural beach and is shown in yellow. Each line represents a data logger. Temperatures on the natural beach were significantly cooler than those on the renourished beach (ANOVA, P<0.05).

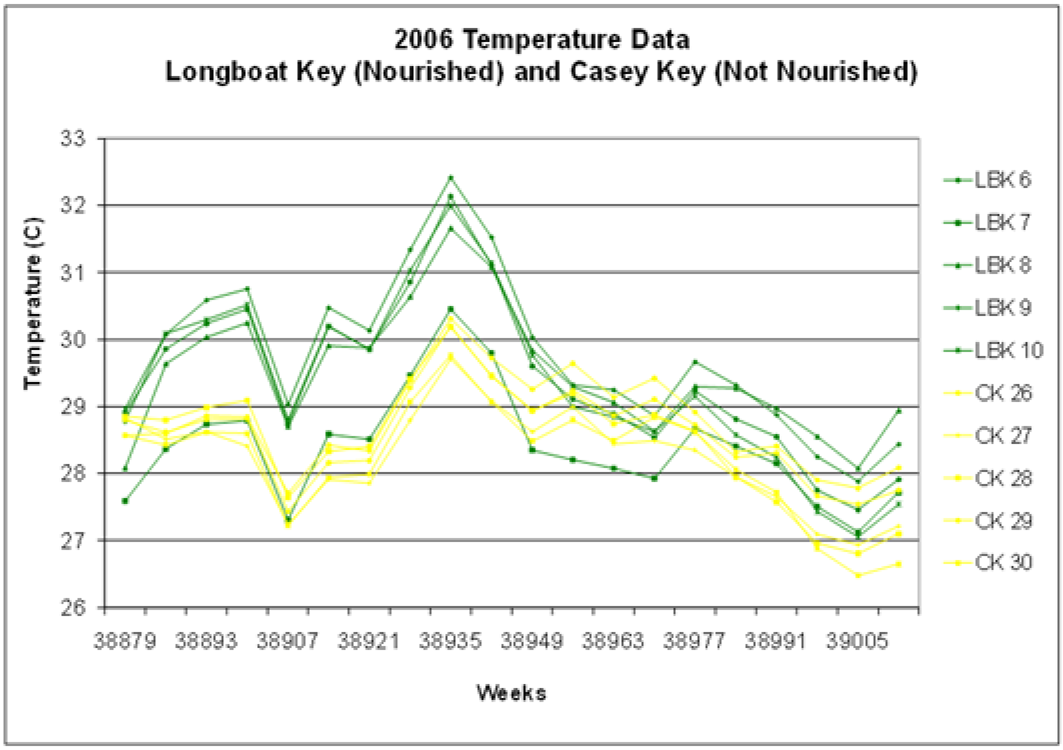


Figure 18. Beach temperatures at nest depth on a nourished versus a natural beach near Sarasota, FL, during 2006. Longboat Key is a nourished beach and is shown in green. Casey Key is a natural beach and is shown in yellow. Each line represents a data logger. Temperatures on the natural beach were significantly cooler than those on the renourished beach (ANOVA, P<0.05).

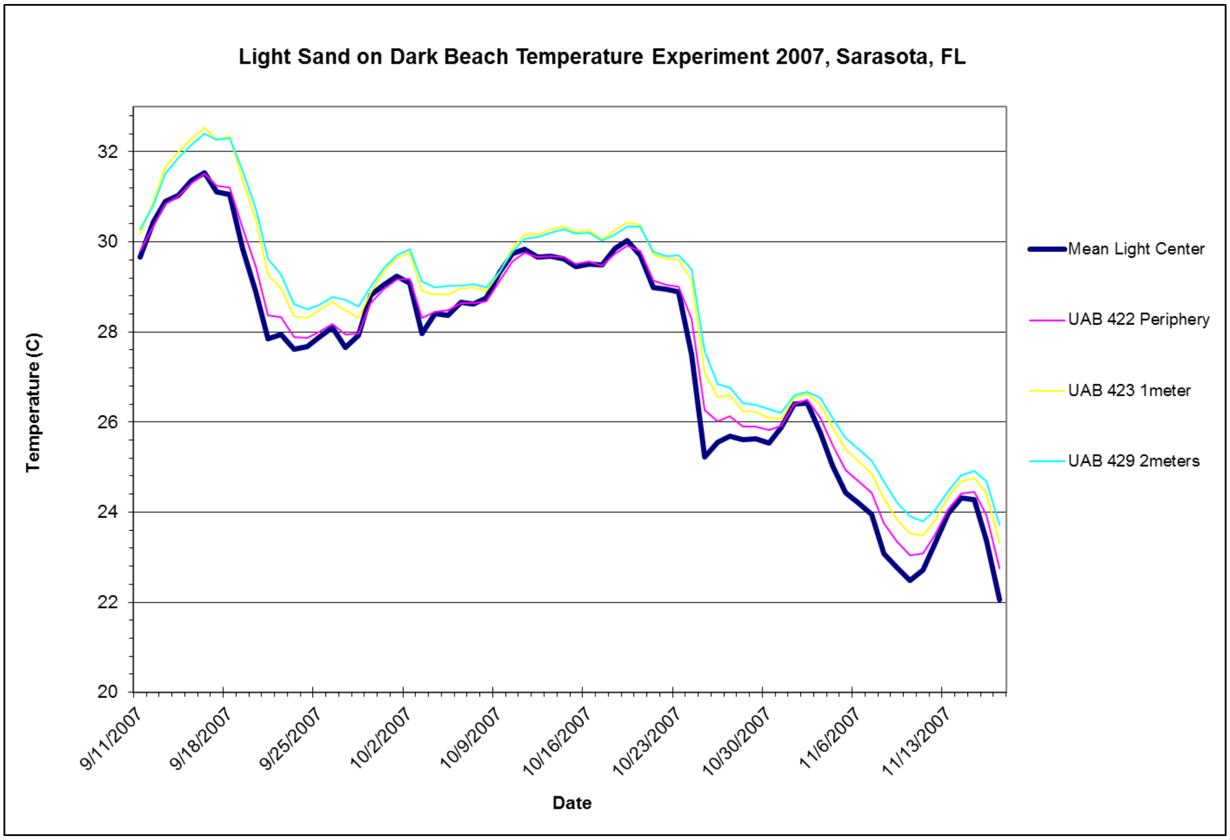


Figure 19. Light sand versus dark sand transfer experiment. A plot of light sand 1.0 m in diameter and 0.75 m deep was placed on a dark sand beach. Temperatures in the middle of the light sand plot were significantly cooler than the surrounding dark sand (ANOVA, $P<0.05$).

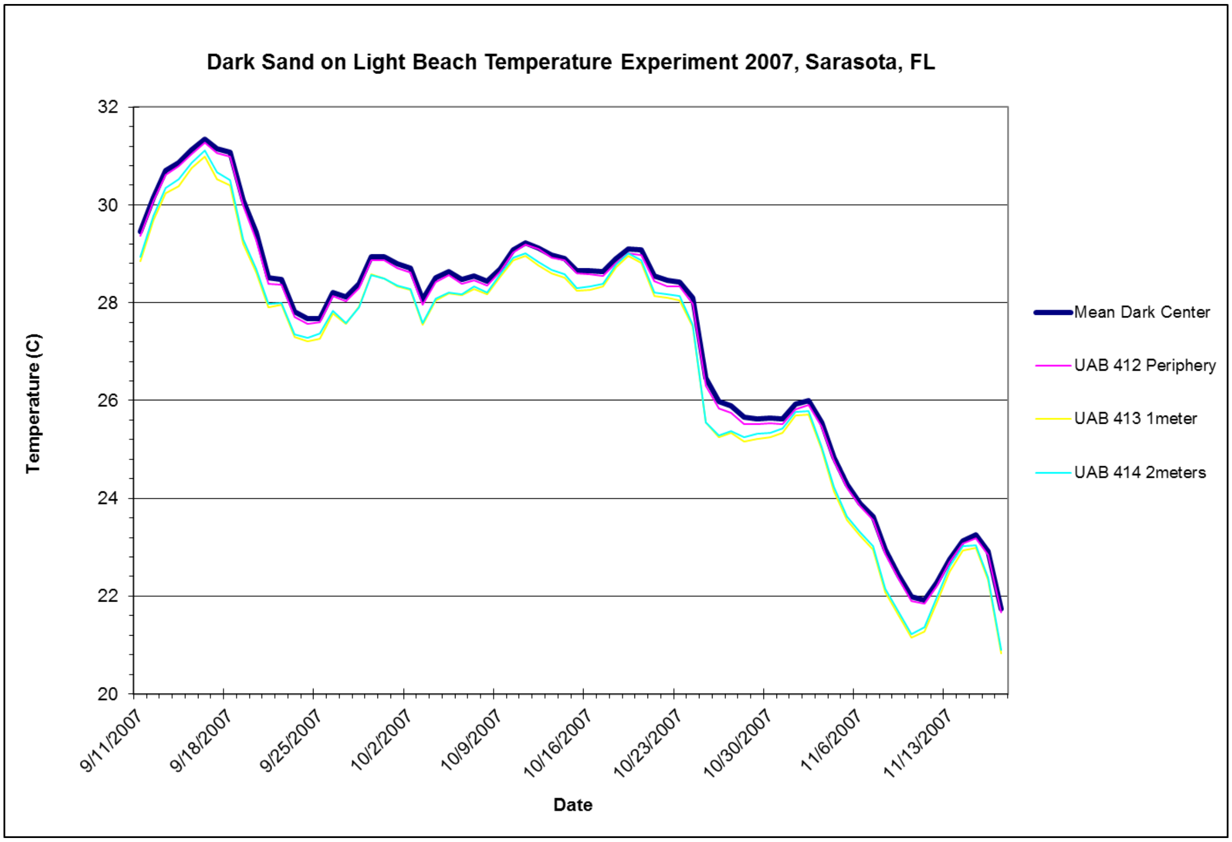


Figure 20. Light sand versus dark sand transfer experiment. A plot of dark sand 1.0 m in diameter and 0.75 m deep was placed on a light sand beach. Temperatures in the middle of the dark sand plot were significantly warmer than the surrounding dark sand (ANOVA, $P<0.05$).

The results of the histological analyses are shown in Table 2 relative to location and year. Hatchlings from a total of seven beaches were examined, and these included beaches on the Atlantic and Gulf coasts. The number of nests examined per beach per nesting season ranged from 3 to 66, with a range of 1 to 22 hatchings examined per nest. The results show that both males and female were produced at most beaches, but females predominated at each location. Combining the data from all beaches resulted in an overall sex ratio of 69.1% female (n =686).

Table 2. Summary of the histological analysis of hatching loggerheads collected from nests on beaches in the southeastern U.S. The hatchlings used in this study were found dead in the nest after all living hatchlings had emerged.

Location	Year	No. Nests	Samples per nest	No. Hatchlings	No. Female	No. Male	Unknown Sex	%Female
Miami, FL	2003	25	1 to 9	66	39	9	18	81%
Boca Raton, FL	2003	19	1 to 5	63	46	15	2	75%
Melbourne Beach, FL	2002	66	1 to 7 (plus 1 nest of 22)	197	138	46	13	75%
	2003	45	1 to 10	169	96	30	43	76%
Hutchinson Island, FL	2003	10	1 to 12	34	18	13	3	58%
Cape Romano, FL	2001	11	1 to 14	43	18	24	1	43%
	2002	4	3 to 10	26	22	0	4	100%
	2005	3	14 to 18	47	14	32	1	30%
Sanibel Island, FL	2002	22	1	22	3	5	14	38%
Bon Secour, AL	2007	8	1 to 4	19	6	5	8	55%
Total:				686	400	179	107	69%

DISCUSSION

Nesting Beach Temperatures and Sex Ratios

Each beach included in this study had its own unique thermal profile (Figures 3-8). Although data loggers on a given beach showed similar thermal profiles, the specific location on a beach could affect the magnitude of the temperature. However, temperature variation between beaches was much greater than the variation within a specific beach (Figures 3-8). The temperatures recorded during the main portion of the nesting season (June and July) significantly varied among the beaches (Figures 10-15). Table 1 provides a summary of beach temperatures relative to geographical location. The results suggest that the diverse range of beaches used by the loggerhead population in the southeastern U.S. spans a relatively wide range of temperatures, with the potential of producing a wide range of sex depending on the specific beach. The results exemplify that the overall hatchling sex ratio produced each year in a sea turtle population represents a condensation of many different sex ratios produced from beaches throughout the nesting range of the population.

Although there were yearly and seasonal variations in temperatures, certain trends are evident. The beaches on the central and southern Atlantic coast of Florida were generally the warmest beaches with temperatures averaging above 30°C. In contrast, the beaches in the northeastern Gulf of Mexico in FL and AL were generally the coolest beaches with temperatures ranging from approximately 28 -29°C. The beaches on the western Gulf coast of Florida were more variable, with some years being cooler than the beaches on the Atlantic coast of FL, while other years appearing similar to those beaches.

The temperatures on the warmest beaches (e.g. beaches on the central and southern Atlantic coast of FL) were generally above the pivotal temperature of sex determination, thus suggesting the production of female-biased sex ratios. Further, these temperatures were consistently warm over the six year study. In fact, the average temperature during the peak portion of the nesting season was near or above the upper range of the transitional range of temperatures on some of the beaches (Figures 10 – 15) suggesting the production of extreme female biases. Although the histology data from the current study were based on small sample sizes, it shows that females were being produced and that females predominated in the samples that were collected from nests from Atlantic coast beaches. Thus, the results support the hypothesis that female-biased hatchling sex ratios may predominate on many of the loggerhead nesting beaches, especially beaches examined on the central and southern Atlantic coast of Florida.

There were also relatively cool beaches (e.g. beaches on the north eastern Gulf coast) which had temperatures that were normally within the transitional range of temperatures and were often near or below pivotal temperature suggesting the production of mixed sex ratios, unbiased sex ratios or even male-biased sex ratios. This finding is consistent with a previous study suggesting that some loggerhead nesting beaches in the southeastern U.S. may produce sex ratios near 1:1 (Mrosovsky et al., 1984). The specific factors affecting temperature on the beaches were not investigated, but factors could relate to beach topography, location in terms of latitude, sand characteristics, and microclimate. The current results exemplify the variety of sex ratios that could potentially be produced in this population (including male-biased sex ratios) and emphasizes the concept that the overall population sex ratio reflects a confluence of many sex ratios from a diverse range of nesting beaches. However, nesting is not uniform across beaches and approximately 80% or more of the nesting in this population occurs along the central and southern Atlantic coast of Florida (TEWG, 2000). The nesting beaches examined from this area (e.g. Melbourne Beach, Hutchinson Island, Juno Beach, Boca Raton) had relatively high nesting beach temperatures indicative of female-biased sex ratios, if not extreme female biases. For example, Melbourne Beach represents the beach with the high nesting activity of loggerheads in the southeastern U.S. and its temperatures were indicative of extreme female biases (Figures 9 and 16). The production of female-biased sex ratios from the Atlantic coast of Florida has been reported in several previous studies (Mrosovsky et al., 1984; Mrosovsky and Provancha, 1989; 1992; Hanson et al., 1998). The results of these studies support the hypothesis that beaches on the central and south Atlantic coast of Florida produce significant female biases, with some predictions as high as approximately 90% or more (Mrosovsky and Provancha, 1988: 1992; Hanson et al., 1998).

Considering the Atlantic beaches in Florida produce the majority of hatchlings in this population, the predicted female bias should be reflected in the population sex ratio. Studies of the juvenile portion of the loggerhead population support the hypothesis of a significant female bias. A study of juvenile sea loggerheads (Wibbels, et al., 1991) captured in the St. Lucie Nuclear Power Plant used a testosterone sexing technique to predict a 2.1F:1.0M sex ratio (n =148). A similar study examined immature loggerheads from four different locations along the Atlantic coast of the U.S. and reported an approximate 2F:1M sex ratio (Wibbels et al., 1987). Shoop et al. (1998) used necropsy to examine the sex ratio of immature loggerheads stranded on the Atlantic coast of Georgia, and found an approximate 2F:1M (n = 129). Additionally, an approximate 2F:1M sex ratio has also been reported for loggerheads stranded on the upper Texas coast (Stabenau et al., 1996). Finally, in a more recent study by Layton et al. (Chapter 1, this dissertation) found a 2.5F:1.0 sex ratio of juvenile loggerheads (n = 111) at the St. Lucie Nuclear Power Plant, FL. Thus, there are numerous studies consistent with the current findings indicating that the nesting beaches on Florida's Atlantic coast may be producing a female bias and that bias is reflected in the loggerhead population sex ratio.

Interestingly, the approximate 2F:1M sex ratio reported in studies of juvenile sex ratios is much less biased than the hatchling sex ratios suggested by previous studies (Wibbels, 2003). Some previous studies have suggested hatchling sex ratios as high as 90% female from some beaches on the Atlantic coast of Florida (Mrosovsky and Provancha, 1988; 1992; Hanson et al., 1998). This discrepancy could be accounted for by factors such as sex-specific differential mortality (Hopkins-Murphy and Owens, 2003). Differential mortality could be related to factors such as the environmental factors present at time of hatching or temperature-specific fitness. However, it may also reflect other factors. The population sex ratio represents a combination of sex ratios from many beaches as well as many years of hatchling production. As indicated in the current study, some of those beaches may produce near 1:1 or even male-biased sex ratios. Although many of these beaches represent only a small percentage of the overall loggerhead nesting, they may collectively have a significant impact on the population sex ratio. This concept emphasizes how some of the lower density nesting beaches may be of significance to the reproductive ecology of the loggerhead if they are producing males, thus enhancing the conservational importance of those beaches. For example the current results indicate that some nesting beaches in the northeastern Gulf of Mexico (e.g. Cape San Blas) have temperatures that are consistently near or below pivotal. These nesting beaches could play a valuable role in the reproductive ecology of the loggerhead by contributing males to the population. It should be noted that the movements of male sea turtles is not well documented due to the logistical difficulties associated with the capture, tagging, and monitoring of adult male sea turtles. However, considering that at least 80% of loggerhead nesting occurs along the Atlantic coast of Florida (TEWG, 2000), that region may also produce a larger proportion of the males in the population, even if they represents a small percentage of the hatchling sex ratio produced on those beaches.

It is also plausible that the discrepancy between juvenile and hatchling sex ratio reflects sampling bias. For example, the current study used a broad geographic approach in comparison to past studies, but it was still limited to a few of the primary nesting sites along the Atlantic coast. It is plausible that other beaches in that area could produce more males (Blair, 2004).

Conservation Implications

The results have several implications for the conservation of the loggerhead population in the southeastern U.S. This population has significantly declined since 1998 (NMFS/USFWS Loggerhead Sea Turtle 5 year status review, August 2007). As an example, the number of nesting loggerheads in Florida declined by 37% from 1998-2007 (FWCC, 2007). It is plausible that possessing TSD could be advantageous to the recovery of the loggerhead population. It has been proposed that biased sex ratios could significantly impact the recovery of sea turtle populations (Coyne, 2007; Wibbels, 2003; 2007). Coyne (2007) provides a model that shows increased recovery rates in populations with female–biased sex ratios, based on the hypothesis that increasing the number of females will increase the number of eggs in future years. As an example, the artificial skewing of sex ratios could be one of the factors that is accelerating the recovery rate of the Kemp's ridley population (Wibbels, 2007). These projections are based upon the assumption that males do not become a limiting factor as the percentage of females increases.

The current study, as well as previous studies, supports the hypothesis that a female-biased sex ratio exists in the loggerhead population in the southeastern U.S. From a conservation viewpoint, this may represent a beneficial component of the population's ecology. Therefore, the production of a female-biased sex ratio should be considered when assessing factors that could affect hatchling sex ratios, such as the moving of nests, and the renourishment of beaches. Incorporating management strategy that would ensure the maintenance of the female bias could help maximize the recovery rate in the loggerhead turtle recovery program. However, one should also be cautious since the effect of sex ratio on the reproductive ecology of sea turtles has not been well studied. It is not clear at what point the decreased proportion of males may become a limiting factor in reproduction. Therefore it is important to ensure that the production of males is maintained (i.e. protection of male-producing beaches) to make certain that they do not become a limiting factor. This is especially relevant due to the potential decrease in male production in response to global climate change (see discussion below). The current study has identified several beaches with relatively cool temperatures which may need to be emphasized in management strategy due to their potential production of males.

Loggerhead Sex Ratios and Beach Renourishment

As suggested above, effective management strategy for the loggerhead should assess the impact of factors which could affect hatchling sex ratio. The renourishment experiments in the current study are clear examples of the effects of sand types on beach temperature. During both the nesting beach comparison (Figures 17 – 18), and the sand transfer experiment (19 -20), the darker sand had significantly increased beach temperatures at nest depth, ranging from about a 0.5 to 0.8°C increase. Such a difference could have a significant effect on sex determination. For example if a loggerhead nest were normally incubating near the pivotal temperature, a 0.5 to 0.8°C increase could shift from 1:1 sex ratio to a strong female bias (Yntema and Mrosovsky, 1982; Mrosovsky, 1988: 1994; Wibbels, 2003). Sand for renourishment is often obtained from offshore sources and can have a variety of different qualities, including color as well as particle size which can affect the thermal characteristics of the sand (Steinitz et al., 1998). These findings indicate that planned renourishment projects should always be assessed in regard to their impact on beach temperature.

Evolutionary Implications of a Female-Biased Sex Ratio

Past studies have reported a range of sex ratios in sea turtle populations (reviewed by Wibbels, 2003). This suggests that temperature-dependent sex determination does not always produce the 1:1 sex ratio suggested by evolutionary sex allocation theory (Fisher, 1930). A variety of hypotheses have been proposed to account for why sex ratios produced from TSD might differ from 1:1 (Rheinhold, 1998; Girondot, 1999; Shine, 1999). At first glance, it might appear evolutionarily advantageous because it could be beneficial for the population, but this would be based on the concept of "group selection" which is not a well-accepted hypothesis in evolutionary science. Shine (1999) suggests that the most probable explanation of skewed sex ratios is the "differential fitness" hypothesis and it has been supported by experimental data from a TSD species (Warner and Shine, 2008). This hypothesis suggests that TSD may allow the nesting female to produce a sex ratio that maximizes the fitness of her offspring. A female may be able to match the sex of the offspring to a particular environment (e.g. time of year, quality of a particular nesting beach, food availability at the time of hatching, etc.) which could enhances the fitness of one sex versus the other (Rheinhold, 1998; Shine, 1999). For example, it may be advantageous to produce females on a good quality nesting beach, because those hatchlings would then return to, and use that nesting beach as adults due to imprinting(Rheinhold, 1988). The loggerhead population in the southeastern U.S. could well be a model for this hypothesis, with the nesting beaches on the Atlantic coast of Florida representing good quality nesting beaches. The population could have evolved a pivotal temperature and TRT which assures the production of a female bias on those beaches, and those female hatchlings would eventually return to those good quality beaches. This is just one hypothetical scenario, and variety of other factors could also be selecting for the biased sex ratio in regards to the differential fitness hypothesis. Regardless, it is plausible that the biased sex ratio reported for loggerheads in the current study represents an evolutionarily stable phenomenon that is selected for by the specific environment inhabited by this population.

Global Climate Change and Loggerhead Sex Ratios

Figures 10 -15 show the average temperature on nesting beaches during the main portion of the loggerhead nesting season. On many of the beaches these temperatures are near or even above the top the TRT for the loggerhead, indicating the production of highly female-biased or all-female sex ratios. This included Melbourne Beach (Figure 16) which has the highest nesting activity of any beach in the southeastern U.S. This could potentially pose a problem if future temperatures increase due to global climate change. Current models project significant increases in temperature over the 21st Century (IPCC, 2007). In the case of the loggerhead's temperature-dependent sex determination, a 1.0 ºC increase temperature could result in a significant shift in sex ratio (Yntema and Mrosovsky, 1982; Mrosovsky, 1988; 1994; Wibbels, 2003), and current models suggest a temperature increase ranging from 1.8 to 4.0 C during the 21st Century (IPCC, 2007). Considering the temperatures recorded in the current study (Figure 10 – 15), the minimal projected increase of 1.8 º C increase would shift temperatures during the main portion of the nesting season to the production of 100% female or highly biased female sex ratios. It is plausible that the reproductive ecology and physiology of loggerheads may try to adapt to rising temperatures. The rapid rate of temperature change would probably preclude the adaptation of the actual sex determination mechanism (i.e. changes in pivotal temperature and TRT). However, seasonal timing of reproduction could potentially change rapidly to help compensate for changes in environmental temperature. Temperature appears to be a main factor controlling the timing seasonal reproduction in many reptiles (Marion, 1982; Ganzhorn and Licht, 1983; Mendonça, 1987), and increases in seasonal temperatures can accelerate the timing of nesting (Schwantz and Janzen, 2008). While that could decrease the temperature for initial nests in a season, loggerheads produce multiple clutches and the later clutches would have a high probability of producing extreme sex ratios. Loggerheads could also adapt through behavioral changes. A variety of environmental factors have been suggested to affect nest site selection in sea turtles including beach temperature (Stoneburner and Richardson, 1981; Hays and Speakman, 1993; Wood and Bjorndal, 2000). Turtles could potentially compensate for increasing beach temperatures by choosing cooler areas on a beach or by selecting a new beach with a cooler temperature profile. As an example, there may be very few hatchlings that survive the extremely hot temperatures on some nesting beaches, or hatchlings produced at extremely high temperatures may be less fit, therefore natural selection would favor those hatchlings from nests placed in cooler areas, perhaps under shade from nearby vegetation, or a slightly cooler beach adjacent to the mother's natal beach. If the latter occurs, there could be a shift in the nesting distribution of loggerheads associated with global climate change. It is unclear, however, if changes in the timing of the nesting season, and changes in nesting locations and beaches could compensate for a 1.8 to 4.0°C during the 21st Century (IPCC, 2007).

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CHAPTER 3

TEMPERATURE-DEPENDENT SEX DETERMINATION AND THE HAWAIIAN GREEN TURTLE: CONSERVATION, ECOLOGICAL, AND EVOLUTIONARY IMPLICATIONS

By

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ABSTRACT

Like all sea turtles, the Hawaiian green turtle (*Chelonia mydas*) possesses temperature-dependent sex determination (TSD). This form of sex determination can produce a wide variety of sex ratios. Hatchling sex ratios resulting from TSD are of interest for a variety of reasons. For example, sex ratios affect the reproductive output of a population and, therefore, have ecological and conservational implications. The Hawaiian green is a genetically isolated population of greens, and it primary nesting habitat is on French Frigate Shoals (FFS) located in the Northwestern Hawaiian Islands. This location accounts for over 90% of green turtle nesting in the Hawaiian Archipelago. The purpose of this study was to evaluate beach temperatures at FFS and predict their effect on sex ratio. This study also included an experiment for characterizing the effects of specific temperature on TSD in the Hawaiian green turtle. Previous studies suggested that beach temperatures at FFS were relatively low, thus indicative of the production of unbiased or male-biased sex ratios (the latter has only rarely been reported for sea turtle populations). As such the Hawaiian green represents an interesting population for sex ratio studies. It has been hypothesized that the Hawaiian green turtle may have evolved a relatively low pivotal temperature to match the thermal profile of the nesting beach at FFS. The current study included an extension of a long-term evaluation of nesting beach temperatures at French Frigate Shoals by our laboratory (started in 1996). Beach temperature data and nest temperature data from a period ranging from 2003 - 2009 were evaluated and compared to previously published data from 1996 - 2002. Collectively, the data indicate that temperatures at FFS were cooler compared to those reported for other sea turtle nesting beaches. Such temperatures would not be conducive to the production

of female-biases which have been reported for several other sea turtle populations. Alternatively, the Hawaiian green could have evolved a lower pivotal temperature which could facilitate the production of female biases at lower temperatures. To test this hypothesis, an experiment was conducted to evaluate the effects of relatively low temperatures (similar to those recorded on the nesting beach) on sex determination in the Hawaiian green turtle (i.e. temperatures spanning the lower portion of the reported transitional range of temperatures for other green turtles). Eggs from captively-bred green turtles were placed into laboratory incubators that were set at approximately 1 °C intervals (actual average temperatures were 26.8, 27.9 and 28.6 °C). The hatchlings from the three temperatures were subsequently reared in captivity for approximately one year and were sexed by laparoscopy prior to their release. Additionally, the sex of any turtle that died during late development or captive-rearing was determined by histological evaluation of the gonad. The results indicate that the pivotal temperature for the Hawaiian green is not distinctly lower and may approximate those previously reported for other green turtle populations (approximately 29°C or slightly higher). This finding together with the relatively cool temperatures recorded at FFS, indicate that the overall hatchling sex ratio of Hawaiian greens is not female-biased, rather it is predicted to be unbiased or even male-biased.

INTRODUCTION

Many previous studies have examined hatchling sex ratios in sea turtle populations (reviewed by Wibbels, 2003), and the great majority have reported female-biased sex ratios. For example, examination of hatchling sex ratios on a wide range of green sea turtle nesting beaches have consistently indicated the production of female-biases: Sarawak (Standora and Spotila, 1985), Suriname (Mrosovsky et. al., 1984; Godfrey et. al., 1996), Costa Rica (Spotila et. al., 1987), Cyprus (Kaska et al., 1998; Broderick et. al., 2000), Australia (Booth and Freeman, 2006), Although female-biases predominate, a few studies have suggested unbiased or male-biased sex ratios can be produced from some sea turtle nesting beaches. Examples include an unbiased sex ratio from a leatherback nesting beach in French Guiana (Rimblot-Baly et al., 1987), and a "not female biased" sex ratio from a hawksbill nesting beach in Antigua (Mrosovsky et al., 1992). An unbiased sex ratio was also reported for immature hawkbills captured near Mona Island, Puerto Rico (Bolten etal., 1992; Deiz and van Dam, 2003). Further, evidence from a comprehensive study that included laparoscopy-verified sex of immature turtles reported a significant male bias in immature loggerhead inhabiting the southern Great Barrier Reef (Chalupka and Limpus, 2001), thus suggesting the production of male-biased hatchling sex ratios in that area. However, no significantly male-biased sex hatchling ratios have been reported for the green sea turtle.

Green sea turtles, *Chelonia mydas,* can be found world-wide in tropical and temperate oceans. Green turtles are The Hawaiian green turtle is an interesting sea turtle for sex determination studies for many reasons. Hawaiian green turtles are currently a protected species under the U.S. Endangered Species Act, and they comprise a genetically distinct population. The majority of the Hawaiian green turtle nesting activity (as much as 90%) occurs on a specific, isolated, nesting ground named French Frigate Shoals (Balazs, 1976, 1980; Dutton et al, 1996; Dutton et al., 2008). French Frigate Shoals is an uninhabited reef with several sandy islets located in the middle of the Hawaiian archipelago, approximately 800 km northwest of Oahu (Balazs, 1980).

Previous temperature data collected on French Frigate Shoals indicate that nesting beach temperatures were relatively cool in comparison to other sea turtle nesting beaches (Hanson, 1997; Estes, 2003; Estes et al., 2005), suggesting the production of unbiased or male-biased sex ratios. Male-biased or unbiased population sex ratios are rare in sea turtles (reviewed by Wibbels, 2003), therefore, studies of the Hawaiian green turtle could shed light on the range of sex ratios that naturally occur in sea turtle populations.

Previous sex ratio predictions for the Hawaiian green were based on pivotal temperatures published for the green turtle in other areas of the world because the pivotal temperature and the transitional range of temperatures (TRT) for the Hawaiian green turtle have not been characterized. Specifically, pivotal temperature and TRT studies have been conducted for green turtles in Suriname and Costa Rica (Mrosovsky et al., 1984a; Standora and Spotila, 1985; Spotila et al, 1987). These studies suggest a pivotal temperature of approximately 28.8°C (Figure 1) and temperatures less than 28°C produce primarily males, while temperatures 30.5°C and higher produce primarily females (Mrosovsky et al., 1984a, Spotila et al., 1987).

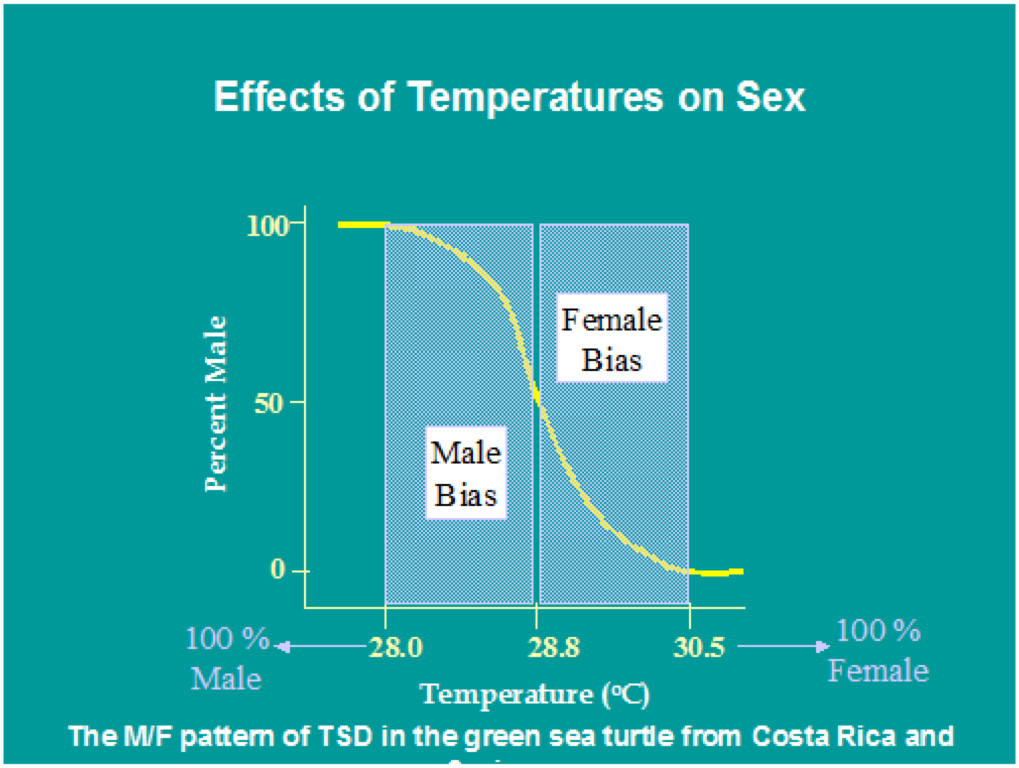

Figure 1. Estimated pivotal temperature and transitional range of temperatures for green turtles in Costa Rica and Suriname (Mrosovsky et al., 1984a, Spotila et al., 1987).

Although nesting beach temperatures appear relatively cool at FFS, they may be more conducive to producing an unbiased sex ratio rather than a male-biased sex ratio since necropsy data on stranded turtles (Koga and Balazs, 1996; Work et al., 2004; Chaloupka et al., 2008) as well as data on immature turtles sexed via hormone analysis (Wibbels et al., 1993) have indicated a 1:1 sex ratio in the juvenile and adult Hawaiian green turtle population. This suggests that the nesting areas at FFS may be producing unbiased hatching sex ratios, although sex ratio dynamics within sea turtle populations are not well understood (Wibbels, 2003). However, previously recorded beach temperatures at FFS appear cool enough for the production of male-biased sex ratios (Hanson, 1997; Estes, 2003). Thus, it is of interest to determine if the beaches at FFS are indeed producing unbiased sex ratios or alternatively, male-biases. In either case, it would represent a rare condition in sea turtle populations.

It is plausible that this isolated sea turtle population could have evolved a lower pivotal temperature (in comparison to other green populations) to match the cooler beach temperatures (Bowen, et al., 1992; Broderick, et al., 2000). Alternatively, the balanced sex ratios observed in the Hawaiian green turtle juvenile and adult studies may have resulted from slightly warmer incubation temperatures at FFS during years previous to the start of our temperature studies at French Frigate Shoals. Fluctuations in hatchling sex ratio production have been observed in other sea turtle species (Wibbels, 2007). In the case of the Hawaiian green sea turtle, which is a slow-to-mature species (25-40 years; Balazs, 1982), the effects of a hatchling sex ratio may not manifest itself in the sub-adult and adult portions of the population for several decades.

The purpose of this study is twofold; A) to evaluate nesting beach temperatures and nest temperatures at FFS to provide insight on the naturally occurring sex ratios, and B) to evaluate if Hawaiian green turtle have a lower pivotal temperature and the pivotal temperature and TRT compared to other green turtle populations. These findings have significant implications for the ecology, conservation, and evolution of the Hawaiian green turtle.

METHODS

Study Site for Naturally Occurring Hatchling Sex Ratios

Data loggers were used to monitor beach and nest temperatures at FFS. As indicated above, FFS (Figure 1) is an uninhabited reef with several sandy islets located in the middle of the Hawaiian archipelago, approximately 800 km northwest of Oahu

(Balazs, 1980). Approximately 90% of Hawaiian green sea turtles nesting activity occurs at FFS (Balazs, 1980). Access to FFS is restricted by the National Marine Fisheries Service due to the presence of the critically endangered monk seal. Therefore, the placement of data loggers on this remote nesting beach was facilitated through collaboration with researchers at NMFS Pacific Islands Fisheries Science Center (PIFSC) in Honolulu. The specific sites for the study at FFS include East Island and Tern Island. These sites are the major nesting grounds for green turtles at FFS. East Island is a natural, undisturbed nesting area. Tern Island was modified and utilized as a landing strip during World War II; however, it still receives large numbers of nests each season (Balazs, 1976, 1980).

Monitoring Temperature as a Means of Predicting Hatchling Sex Ratios:

Direct sexing of hatchlings sea turtles requires the histological evaluation of the gonads (Wibbels, 2003). Although this is the most accurate method of determining sex ratios, it is not practical when working with protected species. Therefore many studies have adopted indirect methods for predicting hatchling sex ratios such as incubation temperatures, beach temperatures, and incubation durations (Mrosovsky and Provancha, 1989; 1992; Godfrey et al., 1996; Marcovaldi et al., 1997; Kaska et al., 1998; Hanson et al., 1998; Hays et al., 1999; Mrosovsky et al., 1999; Broderick et al. 2000; Godley et al., 2001). The use of incubation temperatures and beach temperatures have become popular methods of estimating hatchling sex ratios. Previous studies of sea turtles have shown that the temperature during the approximate middle third of incubation represent the thermo-sensitive period that determines the sex of the hatchlings (Mrosovsky and

Yntema, 1980; Yntema and Mrosovsky, 1982; Merchant-Larios et al., 1997). Furthermore, the average temperature during the thermo-sensitive period can be used to predict of sex ratio (Georges et al., 1994). The use of incubation temperature or even nesting beach temperature, to predict sex ratios is especially useful in threatened and endangered species because it limits potential impacts on the hatchlings. For example, the use of data loggers is a logistically feasible method for monitoring nesting beach temperatures throughout an entire nesting season without requiring the continuing presence of personnel on the beach. This methodology has commonly been used in order to predict hatchling sex ratios (Mrosovsky et al., 1992; Mrosovsky and Provancha, 1992; Godfrey et al., 1996; Hanson et al., 1998, Wibbels et al., 1999).

A knowledge of which temperatures produce each sex is a pre-requisite for the accurate use of this method. The TRT and pivotal temperatures have been reported for many sea turtle species (Wibbels, 2003). These studies employed the use of incubators and histologically verified the sex of hatchlings. Additionally, beach temperatures at nest depth have been shown to be similar to temperatures within adjacent nests (Estes, 2003; Park, 2006). This kind of information provides the groundwork for predicting hatchling sex ratios from natural nesting beaches based on incubation temperature and/or beach temperature.

Methods for Recording Beach and Incubation Temperatures:

Data loggers were used to monitor nest temperatures and sand temperatures at FFS. Data logger placement was accomplished by collaborators working for the National Marine Fisheries Service, Pacific Islands Fisheries Science Center. The data loggers used to record nest temperatures were placed in the approximate center of the egg mass in each nest and the data loggers used to record sand temperatures were buried at mid-nest depth (approximately 45 cm) in locations where the majority of nesting occurred. Temperature data loggers from Onset Computer Corporation were calibrated in the laboratory to verify accuracy. The data loggers are relatively small, battery powered units consisting of a microprocessor and a temperature probe, which can accurately record temperatures to approximately +/- 0.3-0.4°C. The data loggers were programmed to record temperatures once per hour. They were heat-sealed in plastic bags with containers of desiccant. GPS coordinates were recorded for each nest. Additionally, the locations were noted relative to distance from the surf, dune, and vegetation.

During a typical nesting season at FFS, nesting increases during May, remains relatively high through early August, and then declines through late August and early September (Niethammer et. al., 1997). Data loggers were deployed toward the beginning of the nesting season (May) and were retrieved after the majority of nesting (September) had occurred and progressed through the middle third of incubation.

The data loggers were downloaded and exported to Microsoft Excel for analysis. For analyzing nest temperatures, the middle third of incubation was determined for each nest; and the maximum temperature, minimum temperature, and average temperature during the thermo-sensitive period were calculated. The average temperature during the middle third of incubation was used as a predictor of sex ratio in each nest. The pivotal temperatures used for sex ratio predictions were based on previous studies that examined the effects of specific incubation temperatures on the sex determination of green turtle hatchlings (Mrosovsky et al., 1984a; Standora and Spotila, 1985; Spotila et al, 1987). A conservative approach was adopted in which predicted sex ratios were placed into one of three categories based on average incubation temperature. Each nest was assigned either a designation of male bias, unbiased (or 1:1 sex ratio), female bias.

Experimental Evaluation of the Effects of Specific Incubation Temperatures in the Hawaiian Green Sea Turtle

Previous studies indicated that temperatures at FFS were cool relative to those reported for other sea turtle nesting beaches (Estes, 2003). This has prompted the hypothesis that Hawaiian green turtle may have evolved a lower pivotal than other green turtles populations (Estes, 2003). Although the pivotal temperature and TRT have been estimated for green turtle populations in Costa Rica and Suriname, they have not been examined in the Hawaiian green turtle. Therefore, the current study investigated the effects of specific temperatures on sex determination in the Hawaiian green turtle. In particular, the hypothesis of a lower pivotal temperature was addressed by designing an experiment to evaluate the effects of relatively low temperatures (similar to those recorded on the nesting beach) on sex determination in the Hawaiian green turtle (i.e. temperatures spanning the lower portion of the reported transitional range of temperatures reported for other green turtle populations).

The incubation study was a collaborative effort between Sea Life Park (SLP), the National Marine Fisheries Service PIFSC, and the University of Alabama at Birmingham. Eggs were obtained during the 2006 nesting season from captive-bred (pre-Endangered Species Act) Hawaiian green turtles that nest on an artificial nesting beach at Sea Life Park (SLP), Oahu, Hawaii. During the spring of 2006, custom incubators were constructed and installed at Sea Life Park for incubating eggs from the captive breeding colony of Hawaiian greens. Over an approximate two-month period, eggs were obtained from captive (pre-Endangered Species Act) Hawaiian green turtles that nested on the artificial nesting beach at Sea Life Park. Incubator temperatures were monitored using a Hobo Pro data logger that recorded temperatures every hour and the data were downloaded on a weekly basis. This data logger had a probe that was inserted into the incubator into one of the egg baskets. Additionally, three Hobo Pendant data loggers were placed into the incubators and were allowed to record temperatures at various locations within the incubator for the entire incubation period. The incubators allowed for the controlled incubation of eggs at specific temperatures +/- 0.2°C. The experimental design of this project was limited by the number of clutches and the number of experimental incubators (i.e. three incubators). Approximately100 eggs from each incubator were used in this study. Incubators were set at approximately 1°C intervals (actual average temperatures were 26.8, 27.9 and 28.6 °C). Hatchlings were raised in captivity for approximately six to eight months until they attained a size at which they could be examined by laparoscopy to verify their sex (approximately 150 grams or larger). Embryos and hatchlings that died during the study were preserved and examined by histology to verify sex.

Laparoscopy of Post-Hatchling Turtles

Laparoscopy was used to directly examine the gonad of post-hatchling turtles once they attained a size of approximately 150 grams. The laparoscopic analyses were performed under the veterinary oversight of a marine-turtle veterinarian specialist (Dr.

Robert Morris, D.V.M.) at the facilities of the Makai Animal Clinic in Kailua, Hawaii, using standard methodology for sea turtle laparoscopy (Owens, 1999; J. Wyneken, Florida Atlantic University, pers. comm. regarding laparoscopy of post-hatchlings). The morphology of the gonad and the presence or absence of a well-developed oviduct are used as accurate indicators of sex. A support team of personnel from the National Marine Fisheries Service, Pacific Islands Fisheries Science Center (organized and coordinated by George Balazs) prepped the turtles for surgery and maintained the turtles during recovery under the supervision of marine turtle veterinarian specialist. The internal anatomy observed during laparoscopy was recorded on video for all turtles, and the sex was verified by evaluating the gross morphology of the gonadal tissue and the mullerian duct. The hatchlings were released by NMFS, PIFSC personnel a minimum of one week after laparoscopy.

Methods for Histological Analysis of Sex

Histological analysis was conducted in order to verify the sex of embryos or hatchlings that died, but survived through the thermo-sensitive period. The tissues were preserved in neutral buffered formalin and the kidney/adrenal/gonad (AKG) removed for standard paraffin histological processing (Humason, 1979). The AKG's were infiltrated with paraffin wax, imbedded into wax blocks, and cut into 8 µm cross-sections of tissue using a microtome. The cross-sections were mounted onto slides and the slides were stained using standard hematoxylin and eosin staining methodology. The processed tissues were examined using microscopy to determine sex. The sex of an individual was determined by the structure of the gonad (Mrosovsky and Yntema, 1980; Wibbels, 2003).

In the case of a female, the ovary has a relatively well-developed outer region (the cortex), which stains heavily with hematoxylin, and the inner portion of the ovary (medulla) does not show distinct organization. Additionally, the mullerian ducts of the female are well developed. In contrast to the female, the gonad of a male lacks a well-developed cortex, and the inner portion of the gonad (the medulla) shows well-developed cells that become organized into sex cords that will form the seminiferous tubules. During embryonic development, male will develop mullerian ducts, which then regress, so by the time of hatching the oviduct is absent or degenerate.

Evaluation of Circulating Testosterone Levels of Turtles Prior to Their Release

The availability of six month old turtles whose sex is verified via laparoscopy provided an opportunity to evaluate circulating testosterone as a potential sexing technique that could be used in future studies of sex ratio (thus circumventing the need for laparoscopy). Blood samples were taken from the bilateral dorsal sinus of the neck (Owens and Ruiz, 1980) of the captive-reared turtles prior to their release. These samples were analyzed to determine the circulating testosterone levels in the turtles (Wibbels et al., 2000).

The testosterone RIA has previously been described in Geis et al., 2005. Briefly, 250 ul of serum was extracted from each sample using 2 ml of diethyl ether. Samples were reconstituted in 1 ml of assay buffer. Samples were run in duplicate in the RIA with 100 ul of testosterone antisera (Fitzgerald Industries International, Acton, MA), and approximately 10,000 cpm of tritiated testosterone (Perkin Elmer). A standard curve was run with each assay using testosterone standards of 15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000pg/assay tube. The assay proved optimal when using a 1:10 dilution of the testosterone antisera stock solution.

RESULTS

Nest and Sand Temperature Data

Temperature data were collected from FFS from the beach and from nests during five nesting seasons, 2003 – 2004, and 2007 – 2009. Due to logistics data loggers were not deployed during 2005 and 2006.

Nest temperature data from 7 to 22 nests per year were analyzed on East Island (Table 1). The average temperature during the middle third of incubation ranged from 23.2 to 29.8°C, but most nests were below the estimated pivotal temperature published for other green turtle populations (approximately 29.2°C). The average temperature in most nests was between the estimated pivotal temperature and the lower range of the TRT, indicating a male-biased sex ratio (Table 1). Of the total of 68 nests examined, 60 (88%) were predicted to produce males biases and 8 (12%) were predicted to produce female biases.

Table 1. Analysis of incubation temperatures in nests of the Hawaiian green turtle on East Island in the French Frigate Shoals.

	Nests laid on East Island									
SN#	**Lay**	**Est. Hatch**	**Incubation**	**Temp Sensitive Period**	**Mid 1/3**	**Mid 1/3**	**Mid 1/3**	**Mid 1/3**	**Mid 1/3**	**28.8C Pivotal**
Filename	**Date**	**Date**	**Duration (days)**	**Mid 1/3 Dates**	**Max T**	**Min T**	**Mean T**	**Avg SD**	**Avg SE**	***Mrosovsky***
FFS Hawaii 2003										
243570	6/12/2003	8/16/2003	66	7/4/2003-7/25/2003	27.69	27.16	27.25	0.15	0.01	Male Bias
243573	6/3/2003	8/3/2003	62	6/24/2003-7/14/2003	28.57	26.94	27.77	0.40	0.03	Male Bias
266016	6/27/2003	8/23/2003	58	7/16/2003-8/4/2003	29.33	27.69	28.18	0.44	0.04	Male Bias
383500	5/31/2003	8/2/2003	64	6/21/2003-7/12/2003	28.71	26.90	27.91	0.53	0.04	Male Bias
557677	6/9/2003	8/9/2003	62	6/30/2003-7/20/2003	29.06	27.78	28.05	0.27	0.02	Male Bias
557680	5/27/2003	8/7/2003	73	6/20/2003-7/14/2003	27.57	26.16	26.92	0.41	0.03	Male Bias
557681	06/19/03	08/23/03	66	7/11/2003-8/1/2003	27.83	26.58	26.91	0.35	0.03	Male Bias
557682	DL malfunction									
			64		**28.39**	**27.03**	**27.57**	**0.37**	**0.03**	
FFS Hawaii 2004										
744615	06/05/04	08/21/04	78	7/16/2004-8/3/2004	30.77	29.11	29.81	0.57	0.05	Female Bias
744611	06/13/04	08/26/04	75	7/8/2004-8/1/2004	28.52	26.02	27.52	0.47	0.03	Male Bias
744614	06/14/04	09/01/04	80	7/27/2004-8/14-2004	30.24	28.94	29.58	0.43	0.04	Female Bias
744617	06/18/04	08/08/04	52	7/5/2004-7/22/2004	28.99	28.08	28.44	0.25	0.02	Male Bias
744607	06/20/04	08/29/04	71	7/14/2004-8/6/2004	28.32	26.34	27.27	0.55	0.04	Male Bias
744608	06/21/04	08/30/04	71	7/15/2004-8/7/2004	28.11	26.51	27.19	0.44	0.03	Male Bias
744610	06/22/04	08/31/04	71	7/16/2004-8/8/2004	28.31	26.70	27.28	0.49	0.04	Male Bias
744605	06/24/04	09/02/04	71	7/18/2004-8/10/2004	27.48	26.95	27.21	0.16	0.01	Male Bias
742776	06/25/04	09/01/04	69	7/18/2004-8/9/2004	27.67	26.61	27.08	0.36	0.03	Male Bias
744606	DL malfunction									
742730	DL malfunction									
			71		**28.71**	**27.25**	**27.93**	**0.41**	**0.03**	
FFS Hawaii 2007										
641021	06/10/07	08/19/07	71	7/4/2007-7/27/2007	25.66	24.45	25.19	0.32	0.02	Male Bias
641022	06/27/07	09/01/07	67	7/19/2007-8/10/2007	27.66	25.53	26.31	0.61	0.03	Male Bias
641024	06/14/07	08/14/07	62	7/5/2007-7/25/2007	27.51	25.56	26.57	0.48	0.03	Male Bias
641025	06/08/07	08/09/07	63	6/29/2007-7/19/2007	24.68	22.97	23.89	0.58	0.04	Male Bias
742718	07/03/07	09/07/07	67	7/25/2007-8/16/2007	23.94	22.58	23.15	0.32	0.02	Male Bias
742730	06/24/07	08/26/07	64	7/15/2007-8/5/2007	29.27	26.73	27.44	0.62	0.04	Male Bias
744609	06/13/07	08/20/07	69	7/6/2007-7/28/2007	22.91	21.41	22.25	0.30	0.02	Male Bias
1155711	06/16/07	08/16/07	62	7/7/2007-7/26/2007	29.95	27.67	28.75	0.45	0.03	Male Bias
1155713	06/06/07	08/01/07	57	6/25/2007-7/13/2007	29.85	27.76	28.52	0.59	0.04	Male Bias
1155715	06/26/07	08/21/07	57	7/14/2007-8/1/2007	30.15	28.16	28.91	0.50	0.03	Female Bias
1155717	06/18/07	08/27/07	71	7/12/2007-8/1/2007	27.57	26.59	27.01	0.22	0.01	Male Bias
1155718	06/20/07	08/28/07	70	7/13/2007-8/5/2007	28.46	26.88	27.30	0.44	0.03	Male Bias
1155720	07/08/07	09/06/07	60	7/29/2007-8/17/2007	28.95	27.96	28.55	0.23	0.01	Male Bias
1155726	06/07/07	08/09/07	64	6/28/2007-7/19/2007	47.50	24.93	27.60	2.06	0.13	Male Bias
1155727	06/15/07	08/17/07	63	6/16/2007-8/17/2007	28.36	26.78	27.54	0.33	0.02	Male Bias
1155728	06/17/07	08/22/07	67	7/9/2007-7/31/2007	28.66	26.98	27.57	0.43	0.03	Male Bias
1155729	06/13/07	08/07/07	56	7/2/2007-7/20/2007	30.46	28.26	29.12	0.66	0.04	Female Bias
1155730	06/20/07	09/01/07	74	7/15/2007-8/8/2007	28.56	26.78	27.35	0.51	0.03	Male Bias
1155732	07/03/07	09/04/07	64	7/24/2007-8/14/2007	28.56	26.68	27.38	0.53	0.03	Male Bias
744608	06/20/07	08/17/07	59	7/10/2007 to 7/29/2007	23.74	18.64	20.23	1.61	0.10	Male Bias
1155710	07/02/07	08/27/07	57	7/21/2007 to 8/08/2007	27.57	26.88	27.22	0.22	0.01	Male Bias
1155722	07/05/07	08/28/07	55	7/23/2007 to 8/10/2007	31.27	28.46	29.47	0.75	0.05	Female Bias
742777	DL malfunction									
1155714	DL malfunction									
			64		**28.69**	**25.85**	**26.70**	**0.58**	**0.04**	
FFS Hawaii 2008										
1155712	06/05/08	08/10/08	67	6/27/2008-7/19/2008	28.16	26.20	27.13	0.58	0.04	Male Bias
1155715	06/08/08	08/16/08	70	7/1/2008-7/24/2008	29.15	27.47	28.34	0.60	0.04	Male Bias
1155717	06/23/08	09/01/08	71	7/17/2008-8/9/2008	27.27	26.29	26.64	0.28	0.02	Male Bias
1155720	06/20/08	08/26/08	68	7/13/2008-8/4/2008	28.46	27.37	27.55	0.27	0.02	Male Bias
1155725	07/04/08	09/15/08	74	7/29/2008-8/22/2008	27.67	26.20	26.81	0.48	0.03	Male Bias
1155733	06/17/08	08/27/08	72	7/11/2008-8/3/2008	26.88	26.49	26.67	0.11	0.01	Male Bias
1262189	06/17/08	08/28/08	73	7/11/2008-8/4/2008	27.96	26.49	27.08	0.40	0.02	Male Bias
1262190	06/28/08	09/03/08	68	7/21/2008-8/12/2008	27.96	26.39	26.86	0.47	0.03	Male Bias
1262192	06/10/08	08/29/08	81	7/7/2008-8/2/2008	28.66	26.68	27.64	0.39	0.02	Male Bias
1262195	06/24/08	09/24/08	93	7/25/2008-8/24/2008	26.59	26.10	26.30	0.15	0.01	Male Bias
1262198	07/07/08	09/15/08	71	7/31/2008-8/23/2008	28.56	26.88	27.73	0.48	0.03	Male Bias
1262199	06/07/08	08/13/08	68	6/30/2008-7/22/2008	29.15	27.08	28.00	0.64	0.04	Male Bias
1262202	06/19/08	08/17/08	60	7/9/2008-7/28/2008	28.85	28.26	28.73	0.13	0.01	Male Bias
1262203	06/06/08	08/19/08	75	7/1/2008-7/25/2008	27.47	26.49	27.13	0.26	0.01	Male Bias
1262205	06/28/08	09/17/08	82	7/25/2008-8/21/2008	27.96	26.00	26.69	0.61	0.03	Male Bias
1262206	06/07/08	08/13/08	68	6/30/2008-7/22/2008	27.47	26.29	26.87	0.40	0.02	Male Bias
1262207	07/04/08	09/10/08	69	7/27/2008-8/18/2008	28.06	26.49	26.98	0.46	0.03	Male Bias
1266472	07/09/08	09/09/08	63	7/30/2008-8/19/2008	28.95	26.39	27.69	0.68	0.04	Male Bias
1262188	DL malfunction									
1266471	DL malfunction									
			72		**28.07**	**26.64**	**27.27**	**0.41**	**0.02**	
FFS Hawaii 2009										
1155716	5/25/2009	7/29/2009	66	6/16/2009-7/7/2009	29.15	26.68	27.43	0.69	0.04	Male Bias
1155729	5/29/2009	7/27/2009	60	6/18/2009-7/7/2009	29.25	27.37	27.99	0.56	0.04	Male Bias
1262191	5/15/2009	7/20/2009	67	6/6/2009-6/28/2009	28.26	27.37	27.72	0.20	0.01	Male Bias
1262196	5/27/2009	8/7/2009	73	6/20/2009-7/14/2009	27.76	26.00	26.85	0.59	0.03	Male Bias
1262194	5/26/2009	7/28/2009	64	6/16/2009-7/7/2009	29.35	27.67	28.17	0.47	0.03	Male Bias
2311270	5/31/2009	8/1/2009	63	6/21/2009-7/11/2009	29.25	26.98	28.02	0.85	0.05	Male Bias
2311265	6/3/2009	8/8/2009	67	6/25/2009-7/17/2009	27.47	26.10	26.77	0.47	0.03	Male Bias
2311266	6/4/2009	7/30/2009	57	6/23/2009-7/11/2009	30.46	27.47	28.88	1.01	0.07	Female Bias
2311269	6/7/2009	8/3/2009	58	6/26/2009-7/15/2009	30.15	27.76	29.20	0.70	0.05	Female Bias
2311267	6/8/2009	8/3/2009	57	6/27/2009-7/15/2009	30.15	27.67	28.95	0.88	0.06	Female Bias
1262200	6/8/2009	8/15/2009	69	7/1/2009-7/23/2009	28.06	26.49	27.42	0.33	0.02	Male Bias
1262190	6/10/2009	8/19/2009	62	7/4/2009-7/27/2009	28.26	26.29	26.87	0.45	0.03	Male Bias
1266470	DL malfunction									
			64		**28.97**	**26.99**	**27.86**	**0.60**	**0.04**	

Data loggers were placed at mid-nest depth to monitor the nesting beach temperatures at French Frigate Shoals (Figures 2-6). Data loggers during a given year showed similar temperature profiles, but significant variation in the magnitude of the temperatures was detected between data loggers (ANOVA, $P < 0.05$). This indicated that the location of the data logger on a given beach could affect temperature. However, beach temperatures at nest depth were generally consistent each year and were relatively cool (Figure 2 – 6). During all years examined beach temperatures were typically near or below the pivotal temperature published for other green sea turtle populations examined (Mrosovsky et al., 1984a, Spotila et al., 1987).

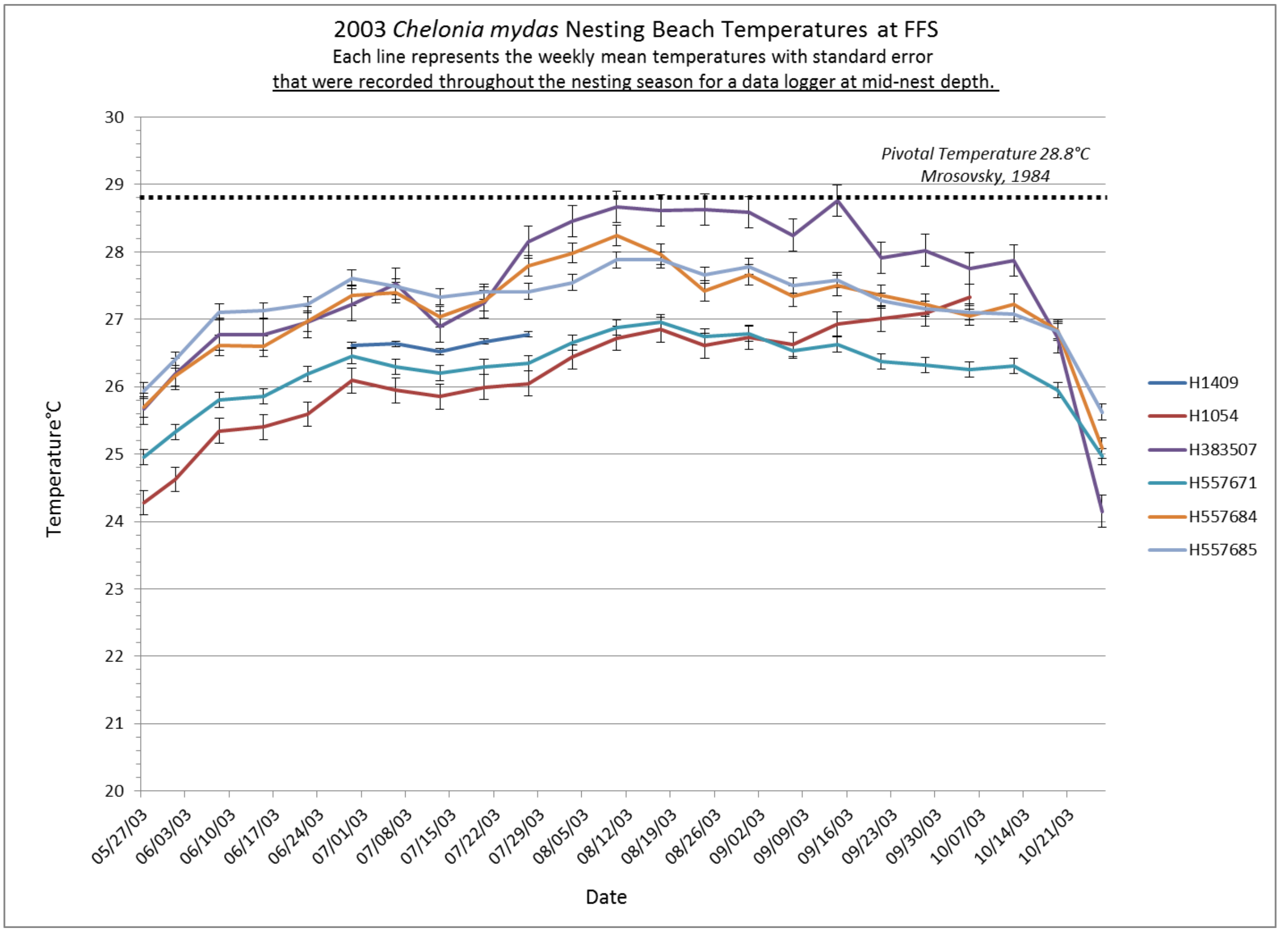

Figure 2. Beach temperatures at nest depth during 2003 on East Island, French Frigate Shoals. The pivotal temperature reported for green turtles in Suriname and Costa Rica is shown (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987).

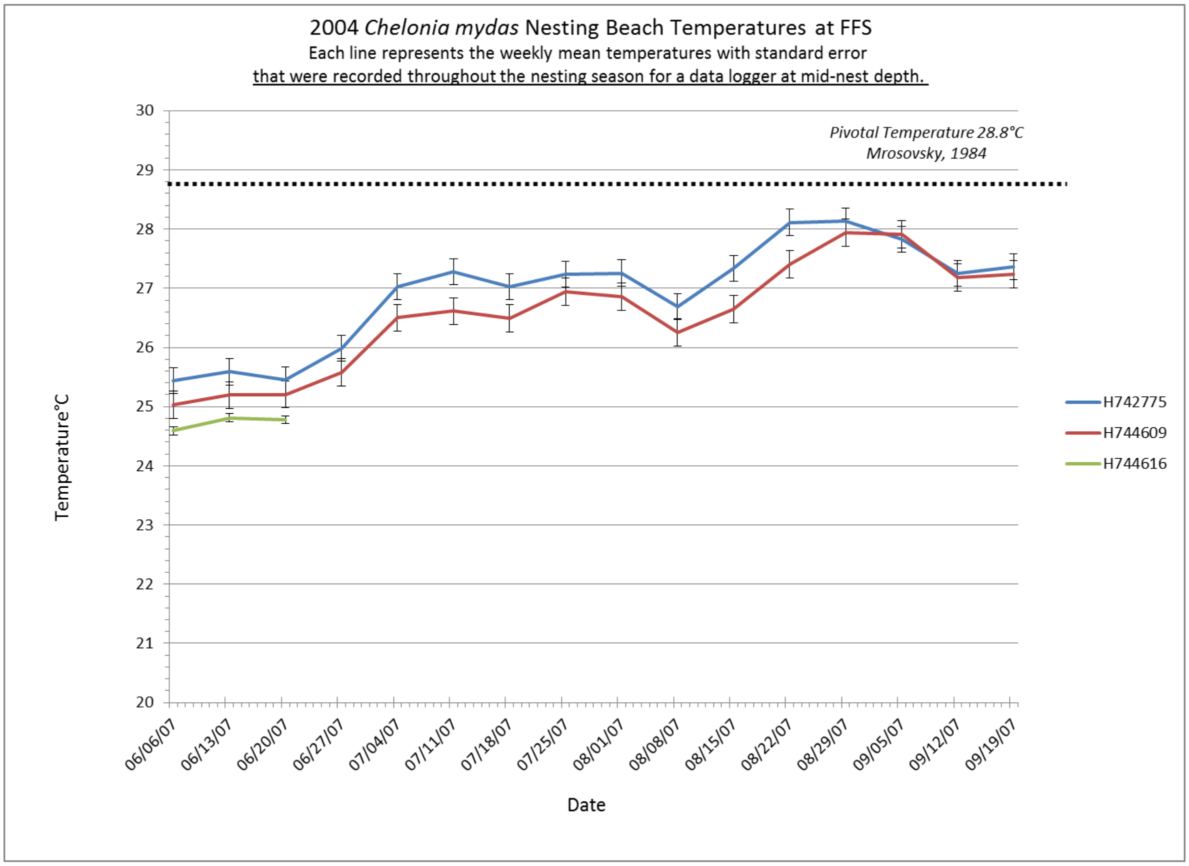

Figure 3. Beach temperatures at nest depth during 2004 on East Island, French Frigate Shoals. The pivotal temperature reported for green turtles in Suriname and Costa Rica is shown (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987).

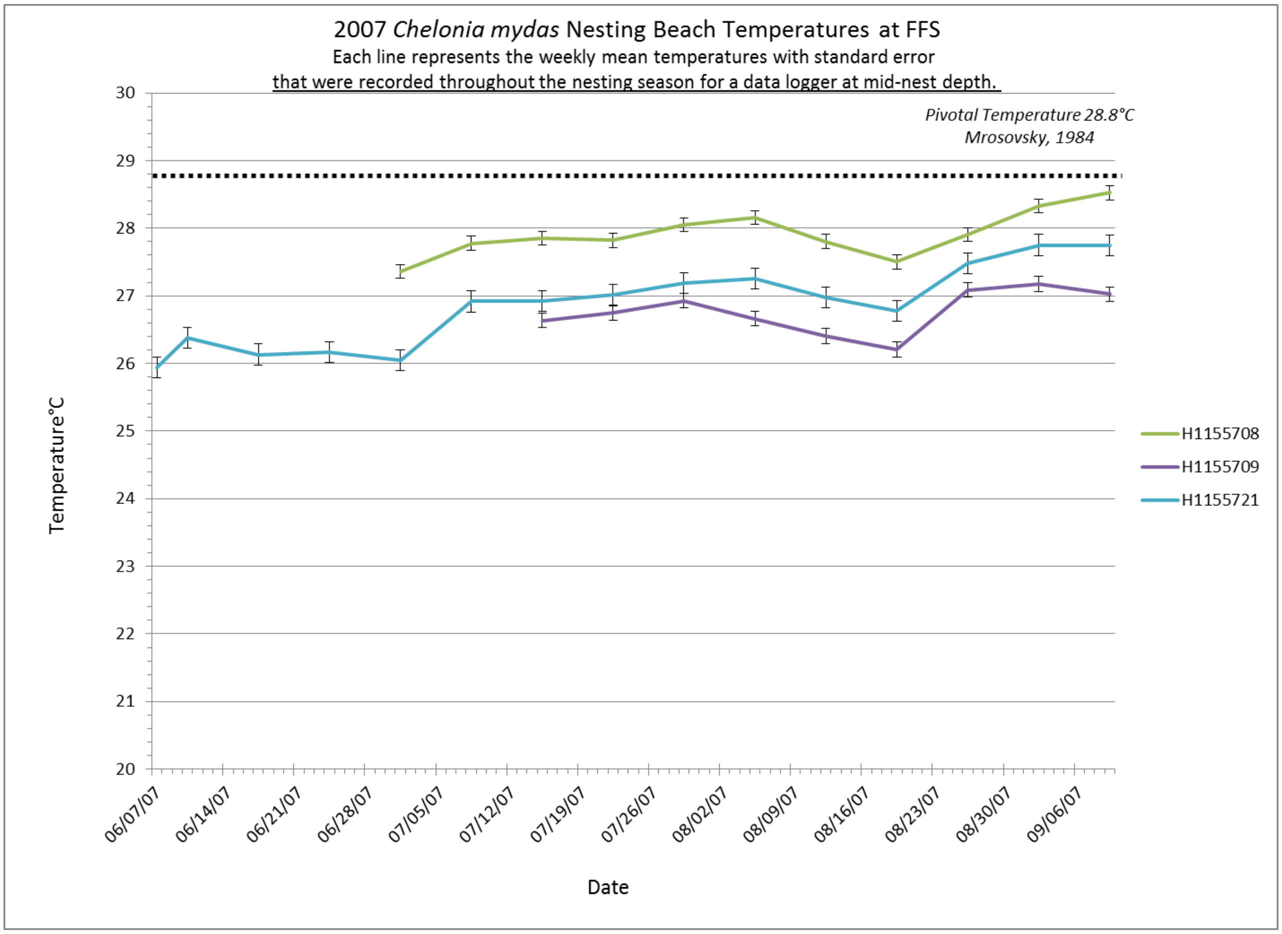

Figure 4. Beach temperatures at nest depth during 2007 on East Island, French Frigate Shoals. The pivotal temperature reported for green turtles in Suriname and Costa Rica is shown (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987).

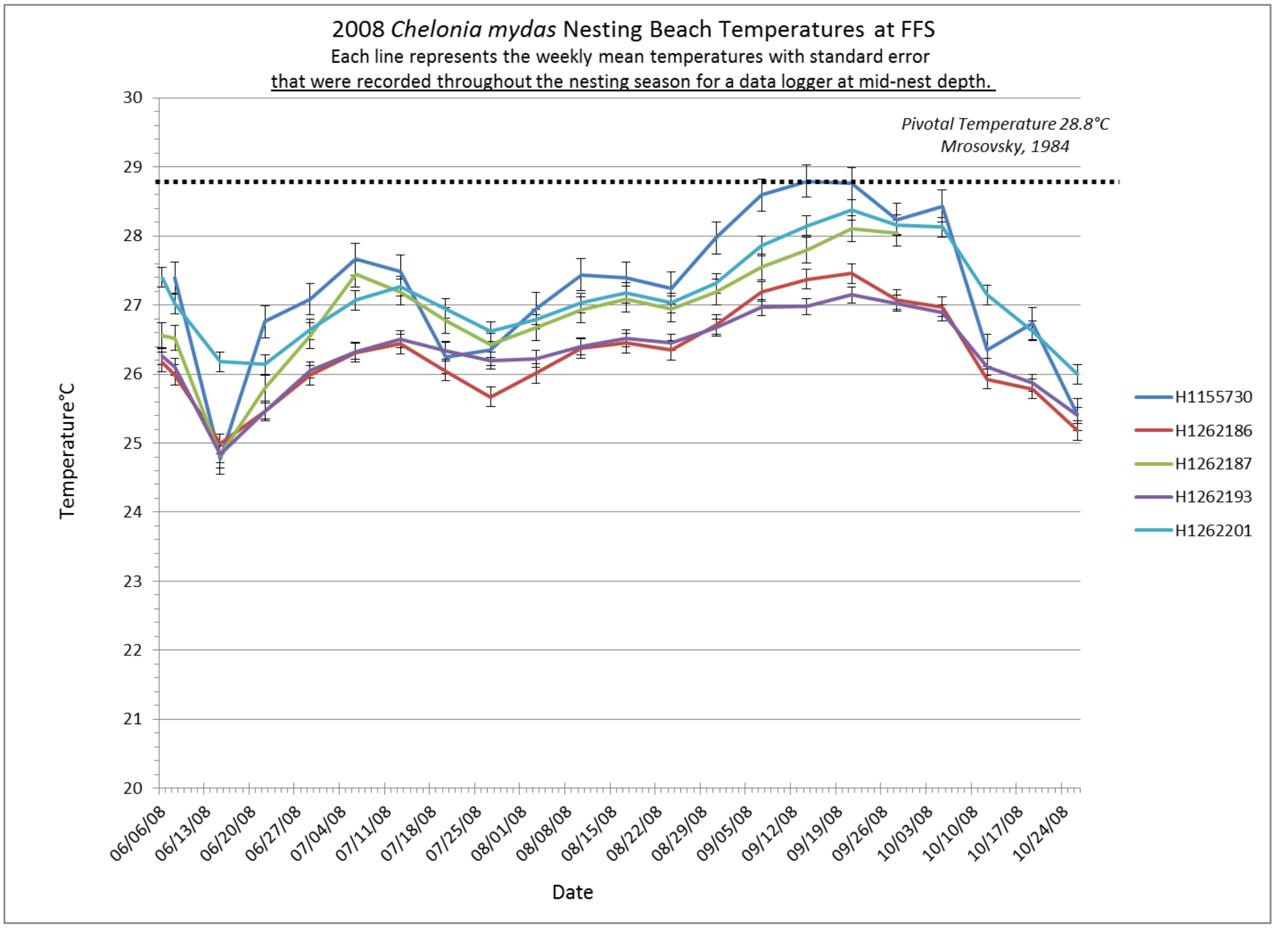

Figure 5. Beach temperatures at nest depth during 2008 on East Island, French Frigate Shoals. The pivotal temperature reported for green turtles in Suriname and Costa Rica is shown (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987).

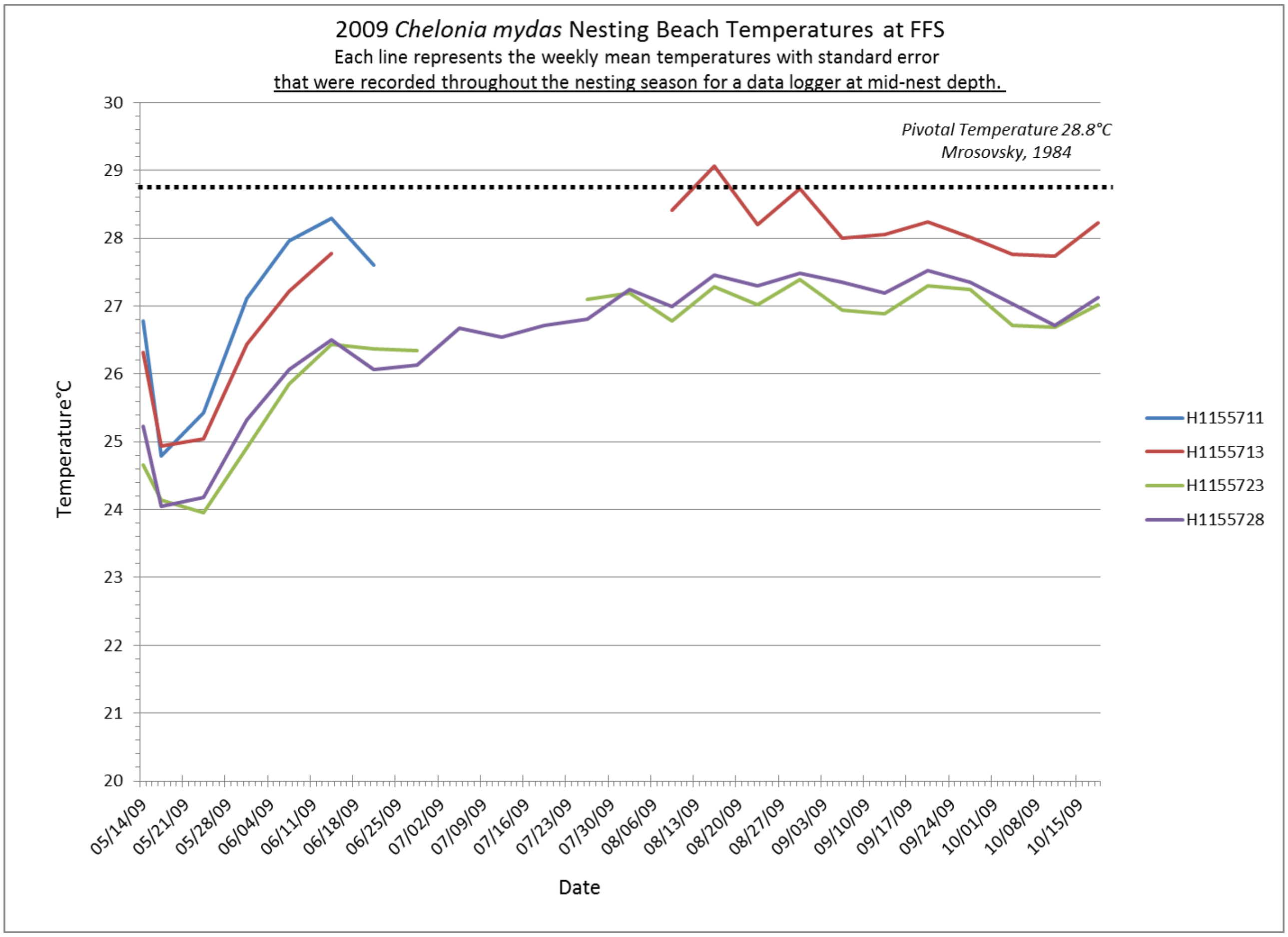

Figure 6. Beach temperatures at nest depth during 2009 on East Island, French Frigate Shoals. The pivotal temperature reported for green turtles in Suriname and Costa Rica is shown (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987).

Incubation Study

Temperatures within the incubators averaged 26.8 ± 0.10, 27.9 ± 0.13 and 28.6± 0.05 (mean + SE) respectively for the three incubators. Eggs were obtained from 15 clutches laid on the artificial beach at SLP. Eggs from each clutch were divided evenly between the three incubators. Approximately 100 eggs per incubator were used in the current study. Eggs were examined for viability on a daily basis and removed if they were not viable. Any embryos that died near the time of hatching were preserved for histological determination of sex. Hatchlings were reared at SLP by SLP personnel for approximately six months. Any hatchlngs or post-hatchlings that died during captive-rearing were preserved in formalin and their gonads were examined by histology to verify sex.

A total of 189 late stage embyros, hatchlings, or post hatchlngs died during development or captive rearing and were analyzed by histology. Of the 181 tissues analyzed, 28 were too decomposed to evaluate, and the sex of the other 161 was verified by examining the structure of the gonad and oviduct as shown in Figure 7 – 11.

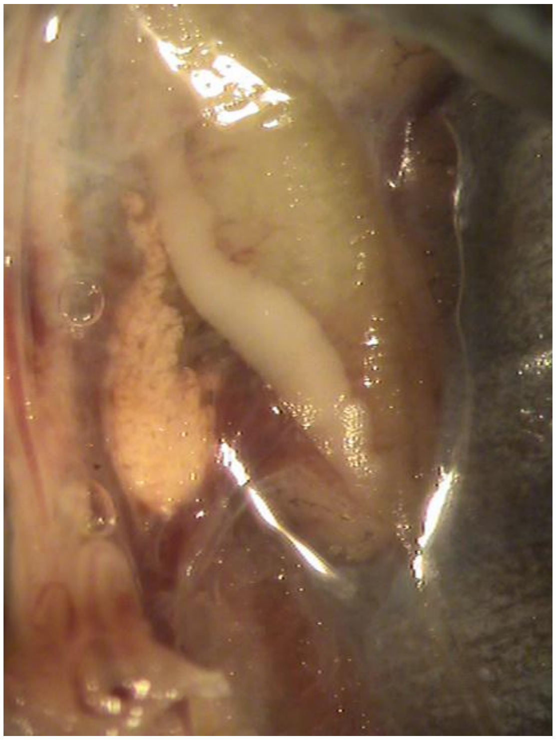

Figure 7. Adrenal, gonad, and kidney tissue from a Hawaiian green turtle. The yellow tissue to the left is the adrenal tissue, the long white structure to the right of the adrenal tissue is the gonad, and the kidney is the large structure underneath the gonad and adrenal.

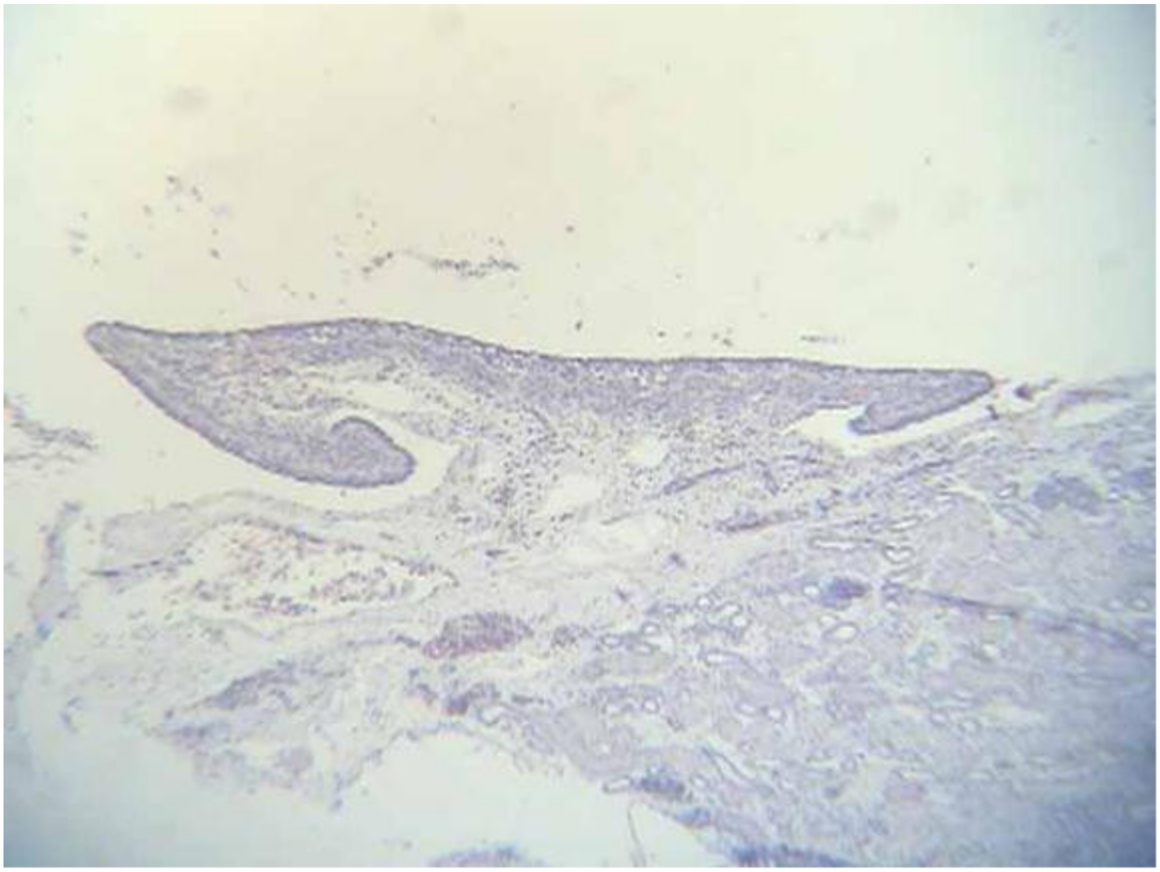

Figure 8. Cross-section of an ovary from a Hawaiian green turtle. Note the dark purple staining on the outer layer of cells (cortex). The inner portion of the gonad (medulla) does not show any distinct organization.

Figure 9. This figure shows an oviduct extending from the kidney of a female. The oviduct is connected to the kidney via a mesentery (i.e. the mesovarium).

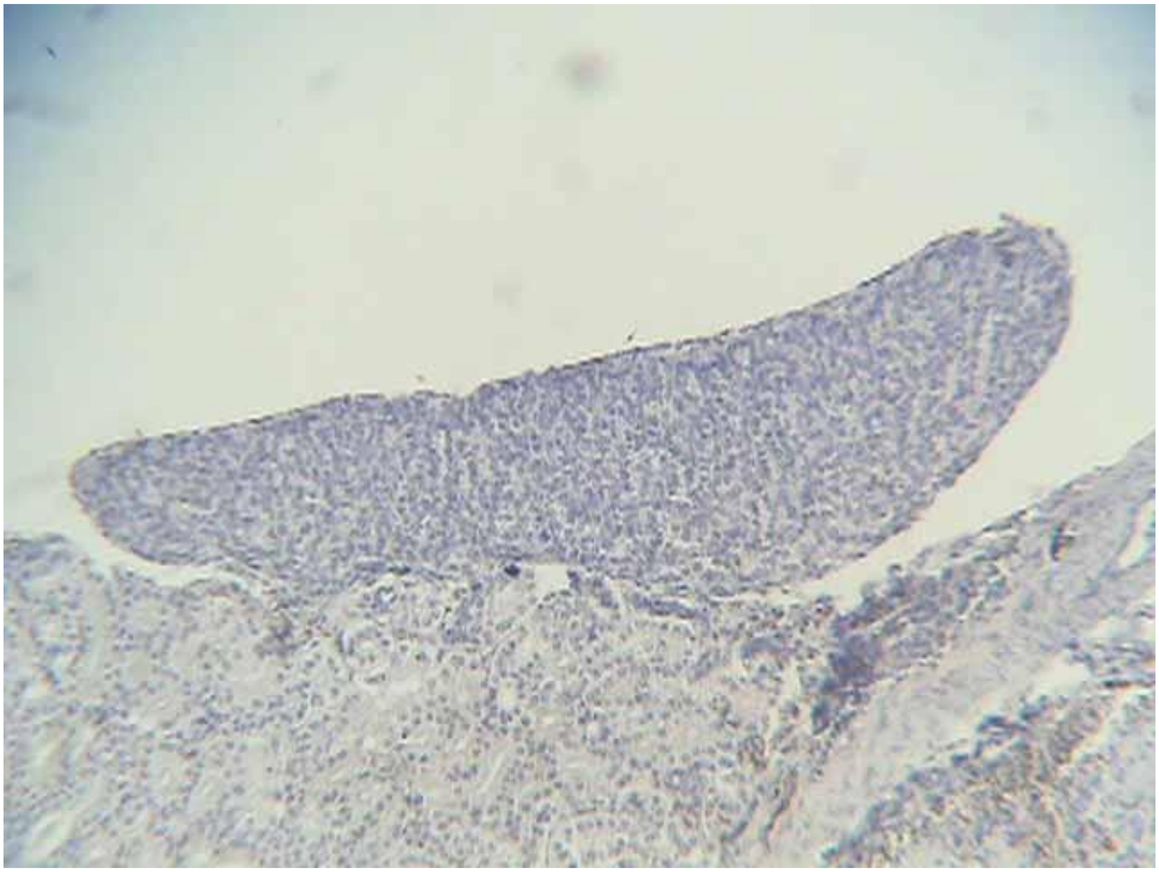

Figure 10. Gonad from a male Hawaiian green turtle. The testis lacks a well-developed cortex (no cortical staining as in the female), and has well developed inner portions of the gonad (medulla) with distinct cells that are grouping together into tubular sex cords.

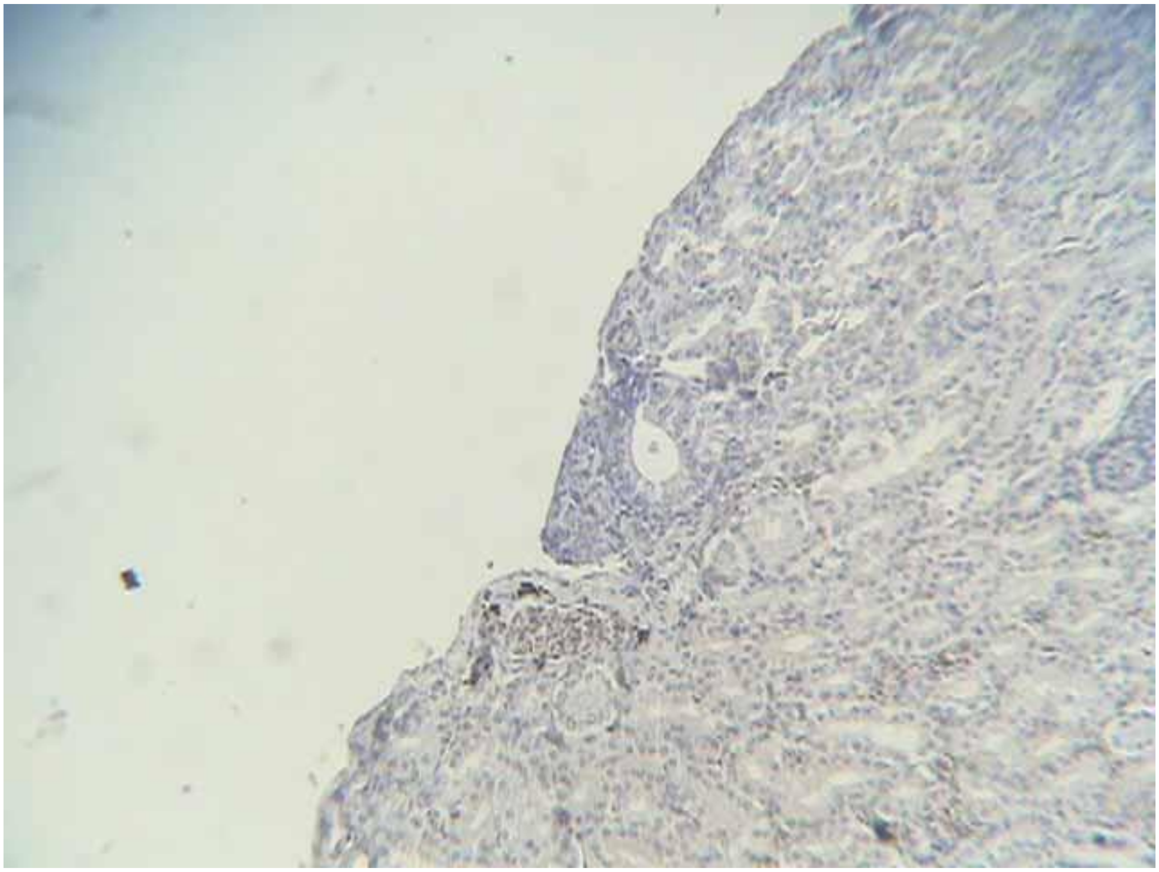

Figure 11. Regressed region of a male Hawaiian green turtle that was originally an oviduct during embryonic development.

A total of 66 turtles were examined by laparoscopy following captive-rearing. In the case of 4 of the 66 turtles, the initial laparoscopic examination on the left side of the turtle was inconclusive, so a second laparoscopy was conducted on the right side of the turtle. The morphology of the gonad and presence or absence of the oviduct proved to be clear indicators of sex (Owens, 1999; J. Wyneken, Florida Atlantic University, pers. comm). The internal anatomy observed during laparoscopy was recorded on video for all turtles. All turtles were responsive and active following laparoscopy and no mortality was associated with the laparoscopic examinations.

The results of the histological analysis are summarized along with the laparoscopy data in Table 2 which shows incubation temperature versus sex ratios produced relative to clutch. Both males and females were produced at each of the three temperatures and none of the three temperatures produced a female bias. The sex ratios produced at each of the three temperatures did not differ from one another (Fisher exact tests, $P > 0.5$).

TABLE 2. Sexes of turtles produced by each clutch relative to incubation temperature. The table includes a total of 227 turtles with sex verified through histology or laparoscopy.

CLUTCH	TEMPERATURE (^oC)	n	SEX RATIO
1	27.9	2	2 Male : 0 Female
2	26.8	1	0 Male : 1 Female
2	27.9	1	0 Male : 1 Female
2	28.6	5	1 Male : 4 Females
3	26.8	2	2 Males : 0 Females
3	28.6	1	1 Male : 0 Females
4	26.8	14	9 Males : 5 Females
4	27.9	21	9 Males : 12 Females
4	28.6	15	7 Males : 8 Females
5	27.9	10	4 Males : 6 Females
5	28.6	2	2 Males : 0 Females
6	26.8	3	3 Males : 0 Females
6	27.9	16	15 Males : 1 Female
6	28.6	9	9 Males : 0 Females
7	26.8	7	6 Males : 1 Female
7	27.9	2	2 Males : 0 Females
7	28.6	10	9 Males :1 Female
8	26.8	14	10 Males : 4 Females
8	27.9	15	13 Males : 2 Females
8	28.6	15	12 Males : 3 Females
9	26.8	16	6 Males : 10 Females
10	26.8	2	1 Male : 1 Female
11	26.8	16	14 Males : 2 Females
11	27.9	11	10 Males : 1 Female
11	28.6	15	15 Males : 0 Females
12	26.8	1	1 Male : 0 Females
12	27.9	1	1 Male : 0 Females
Totals	26.8	76	52 M : 24 F (31.6% Female)
	27.9	79	56 M : 23 F (29.1% Female)
	28.6	72	56 M : 16 F (22.2% Female)
Grand Total		227	164 M: 63 F (27.8% Female)

Circulating Testosterone in Post-hatchling Turtles

Blood samples were successfully collected from 51 of the 70 captive reared turtles prior to their release. The circulating testosterone of these turtles is shown in Figure 12. Many of the turtles had detectable levels of testosterone; however, a bimodal distribution (males versus females) was not evident.

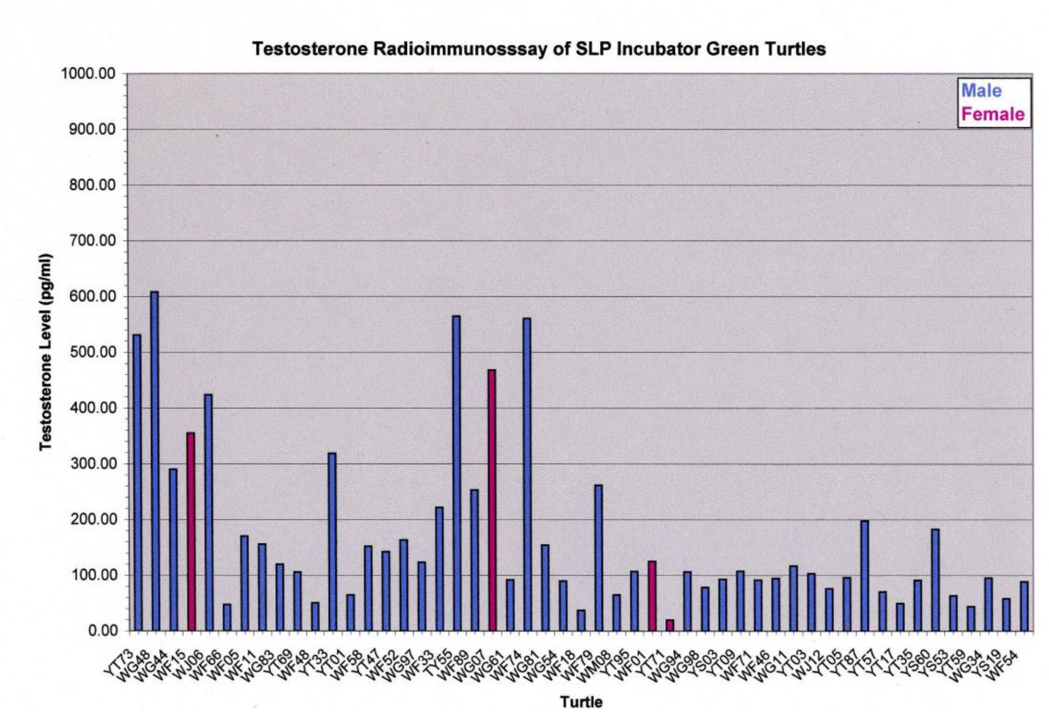

Figure 12. Testosterone levels in the post-hatchling Hawaiian green turtles the examined by laparoscopy (turtles were approximately six month old). Red bars represent females (as indicated by laparoscopy) and blue bars represent males. At this age, a bimodal distribution of testosterone is not evident between sexes.

DISCUSSION

The beach temperature data suggest that temperatures were relatively cool during all of the years analyzed. These results are similar to those reported by Hanson, 1997 and

Estes, 2003. The beach sites were monitored generally from June through October, which encompasses the majority of the nesting season. Overall, the sand temperatures generally remained below the pivotal temperatures of green turtle nesting beaches in Suriname and Costa Rica (Mrosovsky et al., 1984a; Standora and Spotila, 1985; Spotila et al, 1987). Thus, the beach temperatures recorded in the current study suggest the production of male-biased hatchling sex ratios on FFS.

The current study also included the evaluation of incubation temperatures within nests on FFS. The results indicate that both male- and female-biased nests can be produced on FFS, however, male biases predominated in the nests examined. Based on the pivotal temperature data from Suriname and Costa Rica, of the 68 nests that were examined, 60 nests (88%) would be predicted to produce male-biased sex ratio, with a only 8 nests (12%) predicted to produced female-biases. The incubation duration of the nests is consistent with this hypothesis in that incubation durations were relatively long (Table 1), averaging 66.8 + 7.25 days (mean + SD). Similar finding were reported in regards to nest incubation durations at FFS in a previous study (Niethammer et al., 1997). The nesting incubation data and temperature data are consistent with the beach temperature data and support the hypothesis that temperatures on FFS are conducive to overall production of male biases.

The findings of the natural nesting beach study suggest that male-biased hatchling sex ratios should be produced at FFS. However, two previous studies of sex ratios in the juvenile and adult portion of the population suggest an unbiased sex ratio for Hawaiian green turtles (Koga and Balazs, 1996; Wibbels, et al., 1993). This discrepancy could relate to yearly variation in temperatures from previous years or decades at FFS, but temperatures were relatively consistent during this study and previous studies in our lab that span over a decade (Hanson, 1997; Estes, 2003).

An alternative hypothesis would be that this isolated nesting population has evolved a cooler pivotal temperature range relative to the cooler beach temperatures at FFS. If the pivotal temperature of the Hawaiian green is significantly lower than that reported for other green turtle populations in Suriname and Costa Rica (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987), it is plausible that unbiased sex ratios could have been produced at the cooler temperatures recorded during the current study. If the pivotal temperature of the Hawaiian green turtle is relatively low, this could represent a prime example of how nesting beach temperatures may select for a specific pivotal temperature in a population. That was the rationale for conducting the incubation temperature experiments in the current study.

During the incubation experiment, temperatures similar to those recorded at FFS were utilized. Those temperatures represented the lower half of the TRT reported for green turtles in Suriname and Costa Rica. If the Hawaiian green turtle had evolved a lower pivotal temperature it was predicted that the temperatures in the study would encompass the pivotal, and the range of temperatures would produce a full spectrum of sex ratios ranging from male biased to female biased.

As shown in Table 2, the results do not support the hypothesis that the pivotal temperature in the Hawaiian green turtle is distinctly lower than those reported for green turtles in Suriname and Costa Rica. Each of the three temperature used in the current study produced male-biased sex ratios, indicating that they are in the lower portion of the TRT for the Hawaiian green turtle. In fact, even the warmest incubator (28.6 °C) still produced a male-bias indicating it was below the pivotal temperature. The current data suggest that the Hawaiian green turtle has a pivotal temperature near or above 29.0°C which is consistent with previous studies of other green turtle populations (Mrosovsky et al., 1984; Standora and Spotila, 1985; Spotila et al, 1987).

A variety of previous studies have examined pivotal temperatures in sea turtle populations (reviewed by Wibbels, 2003). The majority of these studies suggest pivotal temperatures that are near 29.0°C. Considering the wide range of nesting beaches utilized by sea turtles world-wide, it is interesting that there appears to be a consistency among pivotal temperatures. It is plausible that temperature may be selected by factors other than just sex determination. For example, optimal embryonic development may require a specific range of temperature which could restrict the range of pivotal temperatures. Regardless, the results of the current study support the hypothesis that the Hawaiian green turtle pivotal temperature and TRT may be similar to those reported for other green turtle population. It would be of distinct interest in future studies to examine effects of higher temperatures on sex determination in the Hawaiian green turtle.

If this is the case, how does one explain the discrepancy between the male-biased sex ratios predicted from the beach temperatures and incubation temperature, versus the unbiased sex ratios suggested by necropsy studies of stranded turtles and analysis of juveniles by a testosterone sexing teachnique (Koga and Balazs, 1996; Wibbels, et al., 1993). As suggested previously, this could represent temporal differences in hatchling sex ratio production. The sex ratios predicted in the current study may not be indicated in the juvenile and adult portion of the population for many years considering the slow growth and maturation for Hawaiian green turtles (Balazs, 1982). It could also reflect factors such as sex-specific mortality in the Hawaiian green population. This has been suggested as a possible factor to explain a discrepancy between hatchling and immature sex ratios in loggerhead sea turtles inhabiting waters along the southeastern U.S. (Hopkins-Murphy et al., 2003). It is also possible that sampling bias in regards to data logger location could contribute to this discrepancy. It is possible that certain areas in FFS are conducive to the production of female and that they have yet to be examined relative to beach temperature. Although the current study has focused on two of the main nesting locations in FFS, in light of the current findings it would be of interest to broaden the approach in evaluating beach temperatures. Finally, it is possible that the two studies looking at sex ratios of immatures and adults may have underestimated the number of males (Koga and Balazs, 1996; Wibbels, et al., 1993).

Finally, the results of the current study also provide information on the use of circulating testosterone as a method for sexing post-hatchling green turtles. The turtles used in the current study were approximately six months old and did not exhibit a sex-specific testosterone levels. Previous studies have indicated that green turtles between one and two years of age begin showing a bimodal distribution of testosterone levels with little or no overlap between males and female (Wibbels et al., 2000). The results suggest that turtle may have to reach a certain size/age before testosterone levels become sex-specific.

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CHAPTER 4

SEA TURTLE CSI (Crime Scene Investigation):
A TEACHING MODULE IN SEA TURTLE GENETICS

By

JENNIFER E. LAYTON AND THANE WIBBELS

Formatted for the Journal The Science Teacher

ABSTRACT

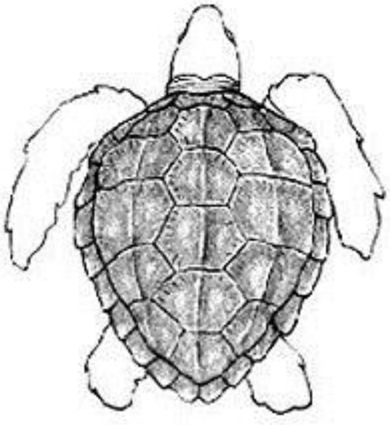
This chapter is formatted for the journal The Science Teacher. The format for this journal requires an abstract written in conversational style that shares activities and ideas tried in the classroom along with a copy of the activity and assessment.

This teaching module was developed for upper level high school and lower level college level students based on sea turtle conservation and genetics. The module is targeted to teach students concepts of wildlife conservation and management through the application of genetic techniques. This module is designed as a portable experiment that can be conducted in the classroom. Students are initially given a series of multimedia presentations to provide a background in sea turtle biology, conservation, and molecular genetics. Students are given a scenario in which they are wildlife conservation agents in charge of identifying sea turtle tissue samples that have been confiscated by customs officials. Students are assigned unknown samples of loggerhead sea turtle DNA. Their goal is to identify the genetic haplotype (and potential origin) of the unknown sample via PCR, gel electrophoresis, and DNA sequencing analysis. The protocol teaches the students basic pipetting skills and the students amplify a fragment of the mtDNA d-loop using PCR. The students make their own gels and separate their amplified mtDNA using gel electrophoresis. During the final portion of the module, the students use a sequence alignment program to analyze their sequence and determine the halpotype of their turtle. Based on previous data that examined the distribution of various loggerhead haplotypes, they are then able to infer the location that the confiscated tissue originated (i.e. the country of origin) in order to protect the turtles and help prosecute the "bad guys". The goal of the module is to give the students a basic understanding of molecular biology and

its applications to conservation. This module has been successfully implemented over the last five years for high school students through the UAB Center for Community Outreach Development (CORD) Summer Science Institute; at the Jefferson County International Baccalaureate High School; at G.W. Carver High School for Health Professions, Engineering, and Technology (a Birmingham City school); and for college students at Dauphin Island Sea Lab as part of the Conservation of Marine Turtles course. The module requires that instructors have equipment available for PCR and gel electrophoresis and provide safety equipment in the form of gloves. The DNA template, primers, sequence files, and audiovisual PowerPoint are available to educators by contacting: twibbels@uab.edu.

SEA TURTLE CSI:
Crime Scene Investigation

An Experiment in
Molecular Biology and Conservation



Jenny Estes Layton and Thane Wibbels
The University of Alabama at Birmingham

HOW TO USE THIS GUIDE:

The text written in **Times New Roman** *is the student lab protocol. The "text boxes" (written in Hevetica italics) contain the information and questions you need to successfully facilitate the lab and teach your students.*
The key to a successful lab is getting the students to actively participate. Generate a sense of excitement and fun!

All of the information below is important. The students have the same information. Make sure that you cover each of these points throughout the lab. Take a moment to look at the key words in **bold***. These are your landmarks, and indicate the key points to cover in the lab. Remember, have fun, and* engage the students*!* ***Use every opportunity to let them do the work and challenge them to think****.*

Before you begin, *orient the students to the bench. Name everything. Ask them if anything is familiar*.

If you haven't done so already, here's a good place to introduce pipetting. Spend 5 or 10 minutes making sure everyone knows how to use pipets, and WHY we use small volumes. Students can practice with pipets using colored water.
-Get the students to figure out the quickest way to get 1.5 ml (1500 μL) into their tube.
(Answer: set the P1000 to 750 μL and pipet twice.)

SEA TURTLE GENETICS using Polymerase Chain Reaction

All species of sea turtles are considered threatened or endangered. The U.S. Fish and Wildlife Service continually seizes illegally imported sea turtle products such as meat, stuffed turtles, shells, and leather goods. In the current experiment you will be working with DNA extracted from meat believed to be from loggerhead sea turtles. The various samples were confiscated at a several different Ports of Entry. These tissues were associated with other sea turtle items such as shell and leather goods that suggested they were from loggerheads. This meat could potentially be from various areas throughout the world. Your goal is to determine the origin of the confiscated goods. In order to prosecute, Biologists must confirm the identity and origin of the illegal products using genetics.

The loggerhead sea turtle (*Caretta caretta*) is distributed in temperate and subtropical waters around the world. Like most sea turtles, loggerheads are known to travel long distances and use a variety of habitats at different stages of their life cycle. When it is time to nest adult females will return to their natal nesting beach to lay their eggs. In the past, when a sea turtle was encountered away from its nesting beach it was impossible to know where that turtle originated. Now with the use of state-of-the-art molecular techniques, we are able to solve this mystery.

Each group of students will **amplify** and **sequence** samples of unknown **mitochondrial DNA (mtDNA)** from sea turtles. **Your task is to help us determine the origin of each "mystery" turtle**. In order to do this, you will use **PCR** to amplify mtDNA that has been isolated from tissue samples from the confiscated sea turtle meat (Figure 1). Then, you will find out the "**genetic identification**" (i.e. **haplotype**) of your turtle. A variety of haplotypes have been identified for loggerhead sea turtles based on their unique sequence of mtDNA (Table 1). You will then use BIO EDIT, a sequence matching computer program, to match the haplotype of your mystery turtle to the haplotypes of known origin. Once your mystery turtle's haplotype has been determined, your group will be able to deduce its possible origin based on previous research that matched specific haplotypes to specific nesting beaches throughout the world.

The source of template DNA is a portion of the D-loop control region of mtDNA that has been isolated from tissue samples of sea turtles. The mitochondria or "powerhouse of the cell" has its own DNA, which it uses to produce organelle specific proteins, and is capable of reproducing on its own. mtDNA has a high mutation rate (10X that of DNA), therefore, sequence changes accumulate quickly. mtDNA is maternally inherited; therefore individuals with the same mtDNA share a common maternal ancestry. In effect, mtDNA markers observed in nesting populations are employed as "genetic tags" to identify the origin of turtles found in distant foraging habitats.

A sample containing mtDNA is combined with a buffered solution of heat-stable ***Taq* polymerase**, two oligonucleotide primers, and the four deoxynucleotide building blocks of DNA. The PCR mixture is placed in a DNA thermal cycler and taken through 20 cycles consisting of:

- 30 seconds at 94°C   DNA is **denatured** into single strands
- 1 minute at 58°C   primers **anneal** to their complementary sequences on

either side of the locus via hydrogen bonds

- 1 minute at 72°C *Taq* polymerase **extends** a complementary DNA strand from each primer

The primers used in the experiment bracket the area of interest and selectively amplify that region of DNA. Following PCR amplification, the DNA is separated according to size using **agarose gel electrophoresis**. After staining with **Ethidium Bromide**, one band will be visible in each student lane, indicating successful amplification. Different sized fragments of DNA appear as distinct bands each composed of several billion copies of the amplified sample. A band's position in the gel indicates the size of the amplified DNA: smaller fragments move a longer distance from their origin, while larger fragments move a shorter distance. After amplification, sequence-matching analysis will be conducted to determine the haplotype and origin of the mystery turtle.

LABORATORY PROCEDURE

Procedure A: *Set Up PCR Reaction*

Each group will determine the origin of one of the sea turtles sampled.

1. Use a permanent marker to label the cap of a 1.5 ml tube with your group # and transfer 99µl of master mix. Now, ask your facilitator to add 0.5 µl Taq polymerase to the master mix. Spin down in centrifuge to mix.
2. Use a permanent marker to label the cap of a PCR tube with your initials. Add the following reagents to the 0.2 ml PCR reaction tube.

	Reagent		*Role in reaction*
☐	22 µl Master Mix		
	containing:	*Water*	*mimics cellular environment*
		dNTPs	*Building blocks of DNA (N = A, G, C, T)*
		10x buffer	*Keeps pH of reaction neutral*
		Taq Polymerase	*enzyme that helps during extension*
☐	1 µl Forward (5') primers		*Oligonucleotide that brackets gene of interest*
☐	1 µl Reverse (3') primers		*Oligonucleotide that brackets gene of interest*
☐	1 µl Sea Turtle DNA		*Substrate for PCR, DNA of interest*

-Taq polymerase comes from Thermophilus aquaticus*. What do those words mean?*
-Where is Taq found in nature?
-Why does Taq have polymerase?
-Why do we use Taq polymerase instead of, eukaryotic polymerase?

3. Carefully close cap to PCR tube. Mix reagents by gently tapping tube bottom on lab bench.

4. Place your sample in the thermal cycler and wait for the other groups to load their samples. (You could store your sample on ice or in the refrigerator until ready for amplification along with other student samples.)

5. Program and start thermal cycler with a step file: (Your facilitator will help you do this!) Reaction
volume is 25 µL.

Hold	94°C	5 minutes
20 cycles	**94°C**	30 seconds
	58°C	1 minute
	72°C	1 minute
Holds	72°C	3 minutes
	4°C	Hold at end of run

Draw the three steps of each cycle on the board along with their temperatures and name.

94°C = Denaturation

58°C *=* ***Annealing*** *of Primers*

72°C = Extension *of DNA by Taq polymerase*

Make sure the students understand the idea of amplification. We are making approximately 1 billion copies so that we can actually see it on an agarose gel.

It helps to draw one stand of DNA, then 2, then 4 etc. Emphasize that this is exponential growth.

Prepare an agarose gel while your PCR reaction is in progress.

Procedure B: Preparation of 1.5% Agarose Gel

1. Your facilitator will show each group how to prepare their 1.5% agarose gels, in 1x TAE buffer. Weigh 0.600 g agarose and transfer to an erlenmeyer flask. Add 40 ml 1X TAE buffer. Melt agarose until clear in microwave or on a hot plate, swirling frequently. Let the agarose cool to the touch (but not solidify). Then add 5 µl of Ethidium Bromide stock (2 mg/ml) to agarose. Pour the agarose into a prepared gel casting tray.

NOTE – FACILITATORS SHOULD RESTRICT HANDLING OF ETHIDIUM BROMIDE SINCE IT IS A HEALTH HAZARD.

Take a minute to let them work this problem out. Use the following formula:

$$\% = g/mL \times 100$$

Using the board, have them figure out how to make a 1% gel in 100 ml of buffer. Then move to a 1.5% gel in 100 ml. Once they have figured this out, move on to a 1.5% gel in 40 ml.

-What is Agarose? (Sugar from a seaweed.)

-What are some other sugars. Get the students to realize sugars end in –ose

-Ask the students if they can see DNA in a gel?

–Ask them what DNA looks like in a microfuge tube.

-We need to ***stain*** *the DNA, we use* ***Ethidium Bromide*** *as our stain.*

-Ethiduim Bromide (EtBr) actually intercalates inside the double helix, binding to the DNA.

-EtBr also fluoresces under UV Light.

-So, we put EtBr in our agarose gel to stain the DNA and then we analyze the gel using UV light. Where ever we see pink/orange florescence; we know that's where the DNA is.

-It's a good idea to draw EtBr binding or intercalating with DNA and then "glowing" under UV light.

-Have 2 students weigh out agarose.

-Have 2 students measure 40 ml of TAE buffer.
-Have a student mix the agarose with the buffer in an Erlenmeyer flask.
-Have a student boil the mixture in the microwave for 60 seconds in 20 second intervals (this keeps the mixture from boiling over). Have all the students watch the flask as the mixture boils and cools.
-Have a student hold the flask up to the light and swirl it. Ask all the students to look for agarose crystals. Once the solution is completely clear, take the flask back to the bench.
-Add EtBr when the flask is cool enough to hold comfortably with a latex glove.
-Cast the gel in the gel box and allow it to solidify.
-Everyone will load and run their gels after lunch.

Procedure C. Agarose Gel Electrophoresis

Before you begin:

-Draw a gel and include charge, DNA marker, and several wells. (If you want, use the example gel on page 7 as

template for your drawing)

-Explain the principle of electrophoresis (moving molecules with an electric current).
-Ask students what molecule we are analyzing.
-On what basis does DNA separate in an agarose gel?
-I like to explain it this way: gel concentration, current, and time are constants. The only variable is the size of the

1. Add 4 µl of loading dye to the PCR sample. Close tube and mix by tapping tube on bottom of lab bench or by pulsing in a microcentrifuge.

-Loading Dye does 2 things.
-1. It weighs the DNA down so it sits in the wells.
-2. It has a dye front that allows us to see the DNA travel through the gel.

2. Load 15 µl of the PCR/loading dye sample into your assigned well of the 1.5% agarose gel. Expel any air in the tip before loading and be careful not to punch the tip of the pipet through the bottom of the sample well. Record your groups' names and lane assignments, in the table on page 8.

3. In each gel, run one lane with a DNA marker. Load 10 µl DNA marker into the gel as described above. The DNA marker contains a ladder of DNA fragments ranging in size from 100 bp to 1500 bp. Sizes are 100, 200, 300, 400, **500**, 600, 700, 800, 900, **1000**, 1100, etc. The 500 bp,1000bp, and 1500 bp bands are especially bright and easy to locate. The size of your PCR product can be estimated by comparing its position to the DNA marker.

4. Electrophorese agarose gels at 175 volts for approximately 30 minutes. Adequate separation will have occurred when the bromophenol dye front has moved at least 2/3 of the way down the gel.

-Have the students adjust the voltage and timer, and press the start button.

5. Following electrophoresis turn off power supply, disconnect power leads and remove gel. Wear gloves at this step.

6. Wearing protective glasses each group can photograph gels on trans-illuminator. Setting may be adjusted but try a setting of 10 and printer on the "SN" mode.

RESULTS AND DISCUSSION

1. Examine the photograph of the stained gel containing your sample and those from other individuals. Orient the photograph with the sample wells at the top. First, look for a diffuse (fuzzy) band of “primer dimers” that might appear toward the bottom of the gel, at the same position in each lane. Primer dimer is not amplified DNA, but is an artifact of the PCR reaction that results from primers amplifying themselves. Excluding primer dimer, interpret the bands in each lane of the gel:

    a. No bands visible. This usually results from an error during sample preparation.

    b. One band visible. If this band is in the 800-900 bp range, you have successfully amplified the sea turtle mtDNA.

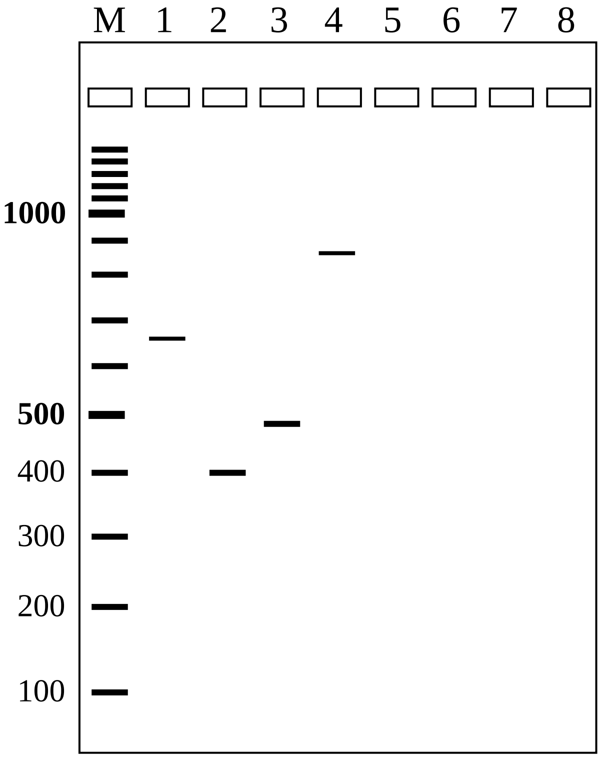
    c. Two or more bands visible. The brightest band is likely the true mtDNA. Additional bands may occur when the primers bind nonspecifically to DNA other than the area of interest and give rise to additional amplification products.

EXAMINING YOUR DATA

Analysis of Sea Turtle PCR products using agarose gel electorphoresis:

The figure below is an example of an agarose gel containing DNA. In the first lane there is molecular weight marker; the other lanes contain PCR products. Use the marker to approximate the size, in base pairs, of the PCR products in each sample lane.

For example, in the diagram below, the PCR products in lane 1 can be approximated at 670 base pairs (bp). In lane 2, we see a single band at 400 bp. How would you interpret lanes 3 and 4?



Determine the size of the PCR products:
Record the name of each member of your group next to the lane number that they loaded their sample. Compare the size of each band to the DNA molecular weight marker and record it in the table. List the number of the unknown sea turtle DNA you were given. After analysis using BIO EDIT, record the haplotype that you identified for your mystery turtle.

lane	name	size of DNA (base pairs)	unknown #	matching Haplotype A-I

Label and tape the picture of your gel in the space below.

Sequence analysis of Sea Turtle PCR products:

Now you have successfully amplified your sea turtle mtDNA! At this point, you will send it out for sequencing.
A variety of haplotypes have been identified for loggerhead sea turtles based on their unique sequence of mtDNA (Table 1) including A, B, C, D, E, F, G, H, J, and I . You will now use BIO EDIT, a sequence matching computer program, to match the haplotype of your mystery turtle to the loggerhead haplotypes of known origin. Once your mystery turtle's haplotype has been determined, your group will be able to deduce its possible origin based on previous research that matched specific loggerhead haplotypes to specific nesting beaches throughout the world.
Your group will receive the specific sequence of your mystery turtle's mtDNA on a disk. You will also receive a disk containing the known sequences for the 10 loggerhead haplotypes. In order to determine your turtle's haplotype, use the program BIO EDIT to match your unknown turtle to one of the sequences of known loggerhead haplotypes:
A, B, C, D, E, F, G, H, J, and I.

Once your group identifies the haplotype of the unknown turtle, you can deduce your turtle's origin based on Figure 2.

I. Using BIO EDIT:

- ☐ Open the BIO EDIT program and insert your disk
- ☐ Click on FILE, select your "unknown" file, and click OPEN
- ☐ Next, go to OPEN, go to IMPORT, SEQUENCE ALIGNMENT FILES, make sure that you have chosen ALL FILES at the bottom of the window and choose "Hap A"
  Do this for all sequences until they are all in the same window (B, C, D, E, F, G, H, I, J).

- ☐ Click on the button for "Normal View Mode" (VIEW, VIEW MODE, NORMAL)
- ☐ Go to ALIGNMENT, click on "PLOT IDENTITIES TO FIRST SEQUENCE WITH A DOT"

  - ➢ Since your unknown sequence is the first one listed, BIO EDIT will automatically compare all of the other sequences to yours.
  - ➢ LETTERS indicate areas that contain DIFFERENT nucleotides between each sequence and your unknown.
  - ➢ DOTS indicate areas where the nucleotide sequences MATCH your unknown.

Answer the following questions:

1. Does your sequence match any of the known haplotypes? Which one(s)?
If so, record this in your data table on page 8.

2. Now, look at the distribution map for loggerhead turtles (Figure 2). What are the possible origin(s) of your mystery turtle?

II. BLAST Search:

If time permits your instructor will help you perform a BLAST search analysis of your 'unknown' haplotype sequence.

A BLAST (Basic Local Alignment Search Tool) search is often the most convenient method for detecting homology (identical match) of a biological sequence to existing characterized sequences. BLAST looks for homology by searching for locally aligned regions of identity and/or similarity between a query sequence and sequences in a database.

Answer the following questions:

1. Were you able to find your sequence using the BLAST search?
2. What are the 3 closest matches to your sequence in the database?
3. Which of those haplotypes is the most similar to the haplotype of your turtle?
4. According to Figure 2, where is the origin of that haplotype (*from question #3*)?

ASSESSMENT

Groups will compete to answer the following questions for points. The group with the top score wins.

1. What does PCR stand for?
2. What percentage of gel did you make today?
3. What kind of gel did you use to separate the DNA?
4. How many milliliters are in 1 liter?
5. From what organism was Taq Polymerase isolated?
6. To make 100 milliliters of a 1.5% agarose gel, how much agarose should you weigh out?
7. Why do we use a DNA marker?
8. What kind of light is shined through the agarose gel to allow us to see DNA?
9. What is the purpose of a buffer?
10. What is the purpose of ethidium bromide?
11. What is the central dogma of molecular biology?
12. What is the abbreviation for microliters?
13. To make 100 milliliters of a 1.5% agarose gel, how much agarose should you weigh out?
14. How many cycles of PCR will be run today?
15. What are the 4 nitrogenous bases that make up DNA? (no abbreviations)
16. What makes DNA negatively charged?
17. What metal ion is required to make Taq polymerase work?
18. What is the optimal temperature at which Taq works?
19. At what temperature does annealing occur?
20. In PCR, what is the name of the step occurring at 94 degrees Celsius?
21. Adenine bonds with thymine, and guanine bonds with cytosine. What is the name of the bonds?

22. Draw out the steps of PCR, including temperature, time, and the name of each step.

23. How many eggs does a sea turtle normally lay? __________

24. How many years does it take a sea turtle to reach adulthood? ______________

25. T or F. Sea turtles are prehistoric and were around at the time of dinosaurs.

26. T or F. Sea turtles need to come to the surface to breath, because they have lungs.

27. T or F. The largest sea turtle in the world is the Kemp's ridley.

28. T or F. Tortoise shell jewelry is made from the shell of the hawksbill sea turtle.

29. T or F. Hatchling sea turtles normally are attracted toward dark areas of the beach.

30. T or F. If you incubate sea turtle eggs at relatively warm temperatures, the eggs produce female hatchlings.

31. T or F. When attached by a shark, a sea turtle will pull it head into its shell.

32. T or F. Turtle soup was historically made from the green sea turtle.

33. T or F. When it is time to nest, a female sea turtle will migrate back to the beach where she originally hatched.

34. T or F. The loggerhead turtle nests on the coast of Alabama.

35. T or F. Genes are composed of RNA.

36. T or F. When a gene is turned on, it produces RNA.

37. T or F. The double strands of DNA are connected by "bases".

38. T or F. Taq polymerase is the enzyme used in PCR.

39. T or F. PCR is used to amplify (make millions of copies) of a specific gene.

40. T or F. An enzyme is an example of a protein.

GENERAL CONCLUSIONS

There are many ramifications in regard to the ecology, evolution, and conservation of sex ratios resulting from temperature-dependent sex determination (TSD) because this form of sex determination has the potential to produce a wide variety of sex ratios, including highly skewed sex ratios in certain environmental conditions. TSD is of evolutionary interest because of its ability to produce a wide range of sex ratios, including some that not conform to a 1:1 sex ratio, predicted by some evolutionary theory (Fisher, 1930). Specifically, it is not clear if TSD has survived because it provides an adaptive advantage (see discussion above regarding differential fitness). TSD also has implications for ecology and conservation, since the resulting sex ratios can affect the reproductive ecology of a population. For example, TSD can produce sex ratios that can enhance or hinder the recovery of endangered populations. Furthermore, both natural factors and human influence on environmental conditions could affect the sex ratios produced in species employing TSD. Although sex ratios resulting from TSD have been reported in some studies, the implications regarding conservation, ecology, and evolution are not well understood.

In order to address the proposed questions, various aspects of TSD were examined in two different sea turtle populations, representing two sea turtle species. The research evaluated TSD in sea turtle populations that are widely distributed geographically. These studies provided a unique opportunity evaluate similarities as well as variability in the TSD of sea turtles.

Chapter 1 of this dissertation directly examined sex ratios of juvenile loggerheads from the southeastern U.S. The loggerhead sea turtle population inhabiting the southeastern U.S. represents one of the largest loggerhead populations in the world. The Atlantic coastal waters acts as both a developmental habitat and foraging grounds for the loggerhead, and the majority of nesting in this population occurs along the Atlantic coast of Florida. In the current study, juvenile loggerheads were captured in the intake channel of the St. Lucie Nuclear Power plant on the central Atlantic coast of Florida. Evaluation of the juvenile portion of the population represents an accurate means of evaluating population sex ratio because juveniles represents a condensation of many years of hatchling production and the sampling of juveniles should not be confounded by sex-specific migratory behavior that occurs with adults. Blood samples were collected from a total of 111 juvenile turtles from May 2005 through April 2007. The samples were analyzed in a testosterone radioimmunoassay that was validated for use as a sexing technique for sea turtles. The results suggest a significant female-biased sex ratio (2.5F:1.0M). No significant differences were detected between the sex ratios of different size classes of turtles.

These findings are consistent with several previous studies suggesting the female-biased sex ratio in the loggerhead population inhabiting the southeastern. Further, comparison of these data to those of a study from several decades earlier (Wibbels et al., 1989), indicate that a significant female bias may be a relative stable characteristic of the juvenile portion of this sea turtle population. The presence of a female-biased sex ratio has significant implications for the ecology and conservation of the loggerhead turtle in the southeastern U.S.

The results of this study also suggest that sea turtle populations can have sex ratios which significantly differ from a 1:1 sex ratio suggested by evolutionary theory (Fisher, 1930). A variety of hypotheses have been suggested to account for biased sex ratios such as the one predicted in Chapter 1 (Shine, 1999).

Chapter 2 of this dissertation describes a study that examined nesting beach temperatures throughout the range of loggerheads nesting in the southeastern U.S. As indicated above, the loggerhead sea turtle (*Caretta caretta*) population inhabiting the southeastern U.S. is one of the largest loggerhead populations in the world. Loggerheads in this population nest on a variety of beaches ranging from the mid-Atlantic states to the northern Gulf of Mexico, with the great majority of nesting occurring along the Atlantic coast of Florida. This study represented a multi-year project (2004-2009) investigating beach temperatures that were simultaneously recorded at nest depth on a range of nesting beaches used by this population. The study also included the histological evaluation of the sex of a subset of hatchlings that were found dead in nests on several of the beaches.

The results indicated that temperatures vary significantly between nesting beaches. The results also indicated that the spectrum of beaches used by the loggerhead population in the southeastern U.S. include a wide variety of thermal profiles, with potential sex ratios ranging from highly female biases to male biases depending on the specific beach. In general, nesting beaches temperatures along the Atlantic coast of Florida (e.g. Melbourne Beach, Juno Beach, Hutchinson Island), where the majority of nesting occurs, were relatively warm suggesting the production of female-biased sex ratios. The histology-based hatchling sex ratio data support the hypothesis that female biases may often be produced on nesting beaches on the Atlantic coast of Florida. In contrast, a few locations (e.g. Cape San Blas on the Florida panhandle) were consistently cooler suggesting the production of a greater proportion of males. However, these locations represented minor nesting beaches compared to those on the Atlantic coast.

These finding are consistent with the hypothesis that sex ratio production is beach-specific, but the loggerhead population in the southeastern U.S. is producing an overall female-biased sex ratio. The results provide a basis for suggesting which beaches in the southeastern U.S. are important for the production of females and male loggerheads. The results also provide an initial data set for evaluating potential long-term changes in beach temperatures associated with global climate change.

Chapter 3 focused on temperature-dependent sex determination in the Hawaiian green turtle. Like all sea turtles, the Hawaiian green turtle (*Chelonia mydas*) possesses temperature-dependent sex determination (TSD), and this form of sex determination can produce a wide variety of sex ratios. Therefore, hatchling sex ratios resulting from TSD are of interest for a variety of reasons. For example, sex ratios affect the reproductive output of a population and, therefore, have ecological and conservational implications.

The Hawaiian green is a genetically isolated population of greens, and it primary nesting habitat is on French Frigate Shoals (FFS) located in the Northwestern Hawaiian Islands. This location accounts for over 90% of green turtle nesting in the Hawaiian Archipelago. The purpose of the study described in Chapter 3 was to evaluate beach temperatures at FFS and predict their effect on sex ratio. The study also included an experiment for characterizing the effects of specific temperatures on TSD in the Hawaiian green turtle.

Previous studies suggested that beach temperatures at FFS were relatively low, thus indicative of the production of unbiased or male-biased sex ratios (the latter has only rarely been reported for sea turtle populations). As such the Hawaiian green represents an interesting population for sex ratio studies. It has been hypothesized that the Hawaiian green turtle may have evolved a relatively low pivotal temperature to match the thermal profile of the nesting beach at FFS. The current study included an extension of a long-term evaluation of nesting beach temperatures at French Frigate Shoals by our laboratory (started in 1996). Beach temperature data and nest temperature data from a period ranging from 2003 - 2009 were evaluated and compared to previously published data from 1996 - 2002. Collectively, the data indicate that temperatures at FFS were relatively cool compared to those reported for other sea turtle nesting beaches. Such temperatures would not be conducive to the production of female-biases which have been reported for several other sea turtle populations. Alternatively, the Hawaiian green could have evolved a lower pivotal temperature which could facilitate the production of female biases at lower temperatures.

To test the “low pivotal” hypothesis, an experiment was conducted to evaluate the effects of relatively low temperatures (similar to those recorded on the nesting beach) on sex determination in the Hawaiian green turtle (i.e. temperatures spanning the lower portion of the reported transitional range of temperatures for other green turtles). Eggs from captive-bred green turtles were placed into laboratory incubators that were set at approximately 1 °C intervals (actual average temperatures were 26.8, 27.9 and 28.6 °C). The hatchlings from the three temperatures were subsequently reared in captivity for approximately one year and were sexed by laparoscopy prior to their release.

Additionally, the sex of any turtle that died during late development or captive-rearing was determined by histological evaluation of the gonad. The results indicate that the pivotal temperature for the Hawaiian green is not distinctly lower and may approximate those previously reported for other green turtle populations (approximately 29°C or higher). This finding together with the relatively cool temperatures recorded at FFS, indicate that the overall hatchling sex ratio of Hawaiian greens is not female-biased, rather it is predicted to be unbiased or even male-biased. This is consistent with previous studies that examined sex ratios in the juvenile and adult portion of the Hawaiian green turtle population (Koga and Balazs, 1996; Wibbels and Balazs, 1993).

Chapter 4 focused on the genetic variation in the loggerhead species. The purpose of this chapter was to develop an educational module for high school and college students, which exemplifies how state-of-the-art molecular genetics can be utilized for the conservation of endangered species. This chapter includes the implementation of this teaching module into local area high schools, UAB CORD Summer Science Institute, and into the summer curriculum for marine biology college students at Dauphin Island Sea Lab.

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