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Conservational Implications Of Temperature-Dependent Sex Determination

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ABSTRACT

A variety of reptiles have temperature-dependent sex determination (TSD), where incubation temperature determines gender. This form of sex determination has significant conservational implications because it has the potential of producing highly biased sex ratios which can affect the reproductive output of a population. TSD is of particular interest to conservation programs that use hatcheries to artificially incubate the eggs of endangered sea turtles because such programs need to select specific incubation temperatures in an effort to produce a desired sex ratio. This type of sex determination also has evolutionary implications as it may be advantageous for an animal to produce a specific gender under certain circumstances. The theory of differential fitness in regard to TSD implies that certain incubation temperatures may produce individuals of a particular sex due to a fitness advantage. However, actual physiological mechanisms behind this have received little or no attention.

To address the evolutionary and conservational implications of TSD, this study evaluated the effect of specific incubation temperatures on the morphology and endocrinology of the gonads and reproductive tracts in a turtle with TSD. Through comparison, many significant differences were found that could be a potential mechanism

for differential fitness in these reptiles and could provide insight into which incubation temperatures are optimal for artificial hatchery programs.

This study also estimated the hatchling, immature, and adult sex ratios of leatherback sea turtles from nesting beaches on the Atlantic coast of Florida (through nest temperature analysis) and from in-water capture off the coast of Nova Scotia (through blood plasma radioimmunoassay). Estimated sex ratios were found to be primarily female biased. This information can provide guidance for conservation programs with this species.

Collectively, the results provide insight into two key topics concerning TSD. First, the natural sex ratio data are a prerequisite for understanding the ecology of endangered sea turtles and for developing conservation strategies for their recovery. Secondly, the incubation results provide a possible link between temperature and reproductive fitness, thus providing a potential mechanism for the evolutionary advantage of TSD. Both findings have significant implications for the conservation, ecology, and evolution of reptiles with TSD.

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LIST OF ABREVIATIONS

AKG	adrenal-kidney-gonad complex
CCL	curved carapace length
CSTN	Canadian Sea Turtle Network
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
ESD	environmental sex determination
FAU	Florida Atlantic University
FM	female-male sex determination pattern
FMF	female-male-female sex determination pattern
GSD	genotypic sex determination
MF	male-female sex determination pattern
RIA	radioimmunoassay
SRY	sex determining region of the Y chromosome
SWFSC	Southwest Fisheries Science Center
TRT	transitional range of temperatures
TSD	temperature-dependent sex determination
TSP	temperature sensitive period or thermosensitive period
UAB	University of Alabama at Birmingham

INTRODUCTION

A variety of reptiles possess temperature-dependent sex determination, TSD (reviewed below). This sort of sex determination has significant implications for the ecology, conservation, and evolution of these reptiles. For example, the sex ratios resulting from TSD can affect the reproductive ecology and survival status of a particular population and TSD has the potential of producing highly biased sex ratios which may not conform to those predicted by evolutionary theory (Fisher, 1930).

This thesis addresses several aspects of TSD. The first chapter of this thesis evaluates sex ratios produced in the Atlantic leatherback population. Specifically, hatching, immature, and adult sex ratios are examined in this unique and endangered sea turtle. This is one of the few reports of sex ratios in the leatherback sea turtle, and is the only study to examine the sex ratio in immatures. The immature sex ratio is of particular importance because it represents a condensation of many years of hatching production, thus providing insight into the history of sex ratios produced from the population's nesting beaches. Information of this sort is a prerequisite for generating effective management strategy for the Atlantic leatherback. For example, knowledge of naturally occurring sex ratios can provide insight into what incubation temperatures should be used in egg hatchery programs. Further, these data can indicate if naturally occurring leatherback sex ratios conform to those predicted by evolutionary theory (Fisher, 1930).

The second chapter of this thesis addresses a subject that is of importance to the evolution and conservation of reptiles with TSD. Since the first report of TSD in 1966 by Charnier, a basic question has confronted biologists: Is there an evolutionary advantage of TSD? It has been hypothesized that different incubation temperatures could result in altered sex and fitness of individuals (Shine, 1999; Warner and Shine, 2008). For example, TSD may provide a flexibility that allows the production of males at a time when the fitness of an individual is enhanced by being a male, and vice versa when it is advantageous to be a female. If this is the case, TSD may provide a distinct advantage over systems such as the XX/XY and ZZ/ZW systems seen in mammals and birds respectively.

To optimally address this hypothesis it would be advantageous to evaluate the reproductive fitness (reproductive success) of adults produced from known incubation temperatures. Unfortunately, such studies are logistically difficult in turtles and impossible in the short term due to the long period to maturity in turtles. It may be possible, however, to infer the fitness of individuals by evaluating specific characteristics or traits. The second chapter of this thesis addresses this subject by evaluating the reproductive tracts of individuals from different incubation temperatures. This includes the evaluation of gross morphology, histology, and steroid endocrinology.

The results of the second chapter also provide insight into developing optimal management strategy for the conservation of endangered reptiles with TSD. Specifically, it addresses the question: Which incubation temperatures are best for egg hatcheries?

Presented below is a general introduction to the subjects addressed in this thesis. Initially an overview of TSD is provided. This includes general characteristics, the

phylogeny, and the physiology of TSD. Additionally, the ecological and evolutionary implications of sex ratios produced from TSD are discussed. Finally, the implications of TSD for the conservation of sea turtles are presented including the various methodologies used to evaluate sea turtle sex ratios.

Temperature-Dependent Sex Determination

Vertebrate species employ various strategies for determining the gender of offspring. In general, sex is determined in one of two ways. The first method is through genotypic sex determination (GSD), where different genotypes yield different sexes. For example, mammals exhibit a male heterogametic system with XX genotype for females and a XY genotype for males, whereas birds display a female heterogametic system where ZZ genotypes are males and ZW genotypes are females (Bull, 1985). An alternative method of sex determination, environmental sex determination (ESD), occurs when certain environmental factors that occur during the embryonic or larval stages of development determine the sex of the animal.

Many reptiles have temperature-dependent sex determination (TSD), a type of ESD in which the incubation temperature of the egg determines sex (reviewed by Bull, 1980; Janzen and Paukstis, 1991) as opposed to sex-specific chromosomes. This type of development was first noted in the African rainbow lizard, *Agama agama*, by Charnier in 1966. It has since been documented in certain lizards (Pieau, 1974), all crocodilians (Ferguson and Joanen, 1982 and 1983; Deeming and Ferguson, 1988), the tuatara (Cree et al., 1995; Bull, 1980), and most turtles (Ewert et al., 2005). In sea turtles, TSD was initially reported in the loggerhead, *Caretta caretta,* (Yntema and Morsovsky, 1980) and

has since been noted in every species of sea turtle (reviewed by Mrosovsky, 1994; Wibbels, 2003).

Patterns and Characteristics of TSD

Within TSD, there are three main patterns of sex determination. All sea turtles and most other turtles have been noted as having a male-female (MF) pattern (also called Pattern Ia, Ewert and Nelson, 1991), where cooler incubation temperatures produce males and warmer incubation temperatures produce females (Bull, 1980; Ewert and Nelson, 1991; Wibbels, 2003) (Figure 1). That is to say that at certain high temperatures, 100% of the eggs will be female and at certain low temperatures, 100% of the eggs will be males. These temperatures have been well documented in all species of sea turtles and can vary between species as well as within a species, depending on factors such as its nesting range.

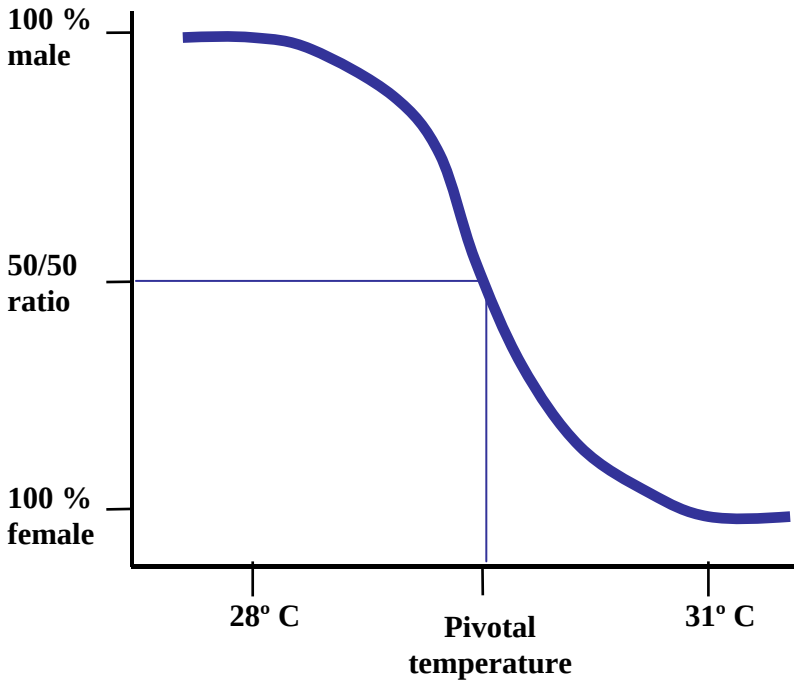

Figure 1. The MF (male-female) pattern of sex determination in reptiles.

Between the two temperatures that produce 100% of one gender or the other, there is a range of temperatures called the TRT, or the transitional range of temperatures, in which the sex of the hatchlings gradually switches from 100% male to 100% female (Wibbels, 2003). Within the TRT is a temperature that will produce a 1:1 ratio of males and females, called the pivotal temperature (Mrosovsky and Pieau, 1991; Wibbels, 2003.) The TRT and pivotal temperatures have also been well documented in many species. Wibbels et al. (1991a) found that the TRT could occur over a several degrees centigrade interval in some species and the pivotal temperature in sea turtles may vary among species by as much as one degree (Mrosovsky, 1994; Wibbels et al., 1998).

In addition to the MF pattern, there are also FMF (female-male-female, or pattern II) and FM (female-male, or pattern Ib) patterns. The FMF pattern, in which males are produced at intermediate temperatures and females are produced at higher and lower temperatures (Mrosovsky, 1983, Crews et al., 1988, Ewert et al., 1994), has been documented in most crocodilians (Lang and Andrews, 1994) as well as some turtles and lizards (Figure 2). The FMF pattern is unique in that it has two pivotal temperatures. The third pattern of sex determination, the FM pattern, was once thought to be fairly prevalent, but in some species it was later shown to be an FMF pattern with females again being produced as temperatures rose above the original lab settings (Ewert et al., 1994).

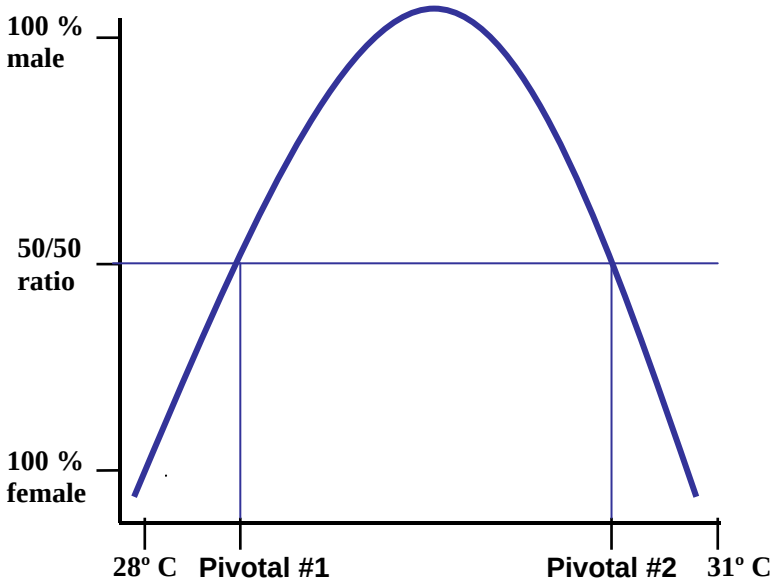

Figure 2. The FMF (female-male-female) pattern of sex determination in reptiles.

Animals exhibiting TSD go through a critical period of incubation during which their sex is determined. This period of time, called the thermosensitive or temperature-sensitive period (TSP), occurs during the approximate middle third of incubation in many species, which often coincides with the time when the bipotential gonad begins to sexually differentiate (Mrosovsky, 1994; Wibbels et al., 1994). Before and after this period of time, the temperature of the nest or incubator does not affect the sexual phenotype of the hatchling (Pieau, 1974; Crews et al., 1988). Further, after the thermosensitive period, its gender remains constant throughout life (Pieau, 1974; Crews et al., 1988).

The length of the thermosensitive period is related to specific incubation temperatures, with the warmer temperatures having a shorter thermosensitive period, and thus shorter incubation time overall, than cooler temperatures (reviewed by Wibbels et al., 1994). The length of the thermosenstitive period can also very among sea turtle groups (Mrosovsky and Pieau, 1991).

The incubation temperature seems to also have a cumulative effect in which embryos must be exposed to a male- or female-producing temperature for a prolonged period of time in order to irreversibly determine sex (Deeming and Ferguson, 1988; Wibbels et al., 1991a; Wibbels et al., 1991b). Hatchlings that were switched from male to female temperatures (or vice versa) within the early portion of the thermosensitive period were determined to be the gender produced by the last temperature encountered (Wibbels et al., 1991b). This particular study also found that female sex became fixed at an earlier time than with embryos at male temperatures.

Evolution of TSD

It is generally believed that TSD is the ancestral form of sex determination in amniotic vertebrates and that GSD arose from it (Marshall-Graves and Shetty, 2001). Because heteromorphic sex chromosomes are observed in some species of lizards and snakes, and in a few species of turtles (Bull, 1980), but not many reptile species, it is suggested that GSD independently arose at different times from TSD in reptiles, four to six times within the turtle families (Ewert and Nelson, 1991). The GSD systems in mammals and birds also appear to be independently derived from TSD systems (Marshall-Graves and Shetty, 2001). (Figure 3).

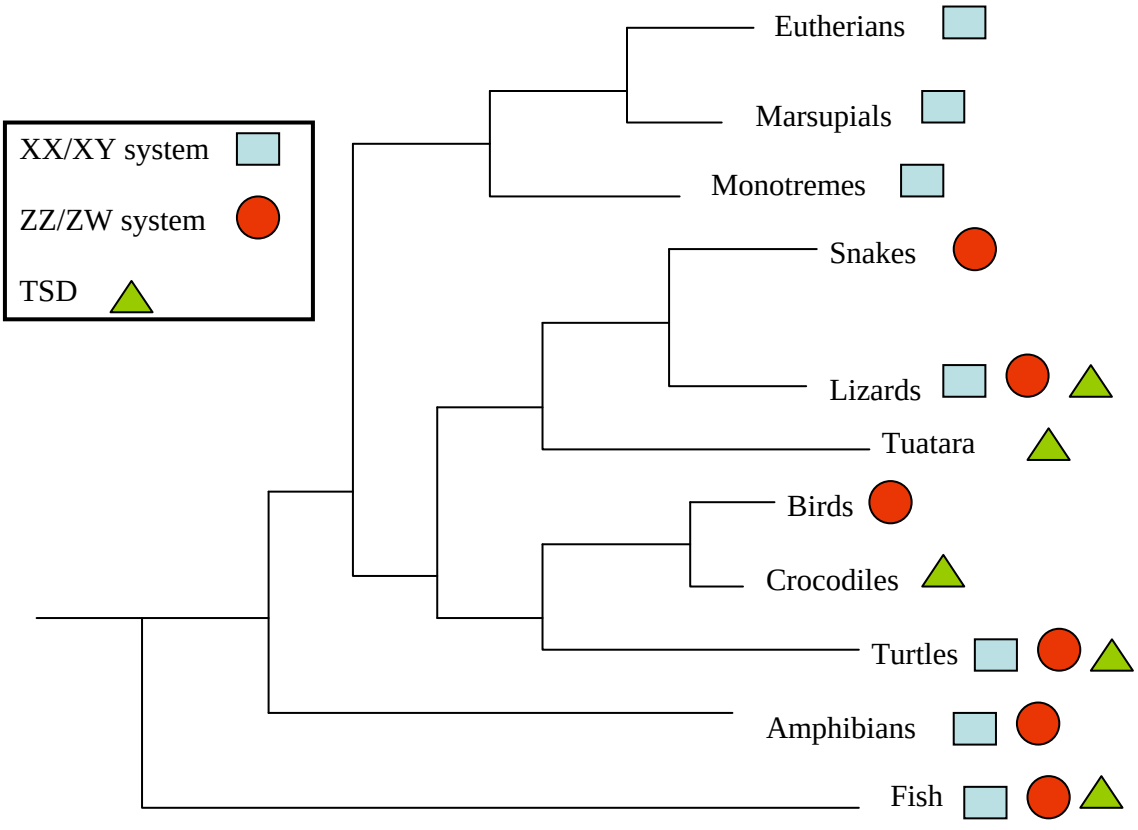

Figure 3. The phylogenetic occurrences of TSD and GSD.

The persistence of TSD in many reptilian groups suggests that it may have an adaptive advantage. Shine (1999) reviews a series of hypotheses regarding the possible advantages of TSD, including the possibility of different fitness levels of offspring related to incubation temperature and sex. The differential fitness hypothesis was originally proposed by Charnov and Bull (1977) and predicts that TSD enhances the fitness of offspring by matching offspring sex to incubation conditions. That is, eggs should produce males when conditions are favorable for males and females when conditions are favorable for females. A recent study by Warner and Shine (2008) provides evidence supporting the differential fitness hypothesis in a lizard with TSD, the jacky dragon (*Amphibolurus muricatus*). That study evaluated the reproductive success of individuals produced at different temperatures, including experimentally sex-reversed individuals. The results indicated that fitness was sex-specific at a given temperature. That is, certain temperatures appeared to optimize the fitness of males whereas others optimized the fitness of females. Similar results were suggested for leopard gecko (*Eublepharis macularius*) in which specific temperatures produced individuals of greater reproductive fitness (Rhen and Crews, 1999).

Several other hypotheses have suggested why TSD has been retained by many reptilian groups (reviewed by Shine, 1999). These include the concepts of phylogenetic inertia and sib-avoidance, both of which were investigated by Ewert and Nelson (1991). Phylogenetic inertia suggests that TSD persists in most groups of reptiles because the organisms lack the genetic variation to allow GSD to evolve (Bull, 1980, 1983; Mrosovsky, 1980; Ewert and Nelson, 1991). Another concept is sib-avoidance, where

producing mostly unisexual clutches reduces the chances of inbreeding between siblings (Ewert and Nelson, 1991), and unisex clutches would be more probable with TSD.

Physiology of TSD

The physiology of sex determination in vertebrates is not fully understood at this time. Sexual differentiation, and the steps that lead to it, have been mostly studied in mammals, so studies on reptiles have been guided by these findings. There are some similarities between mammalian and reptilian sex differentiation and these similarities have lead to an evaluation of specific genes expressed during reptilian sexual differentiation. Additionally, there have been many studies examining the endocrinology associated with sex determination and sex differentiation of reptiles. Two species, the red-eared slider (*Trachemys scripta*) and the European pond turtle (*Emys obicularis*), have been studied in great detail (Lance, 1997) and much of what is know about this subject comes from these two animals.

Sex determination and sex differentiation in placental mammals has received more attention than any other vertebrate and provides a detailed model which can be used to evaluate potential factors that could be involved in the reptilian sex determination and sex differentiation cascade. Mammalian sex determination begins with the expression of the SRY gene, the sex determining region of the Y chromosome. If this gene is present, meaning that the Y chromosome is present, testis formation is triggered. If the Y chromosome, and thus the SRY gene, is not present, a female will develop as the "default" sex (Lance, 1997). The SRY gene has not been found in egg-laying mammals, birds, amphibians, fish, or reptiles, so SRY appears to be a recently evolved triggering

mechanism unique to most mammals. While this sex determining gene appears to be unique to mammals, many other genes involved in sex differentiation appear to be conserved and have been reported in other vertebrates, including reptiles. Several of those genes are mentioned below under "Other Factors and TSD".

The specific physiological mechanisms of sex determination in reptiles with TSD are not well understood, but the effect of temperature on sex determination has been characterized in many species. As indicated previously, the thermosensitive period approximates the middle third of incubation in many reptiles with TSD (reviewed by Wibbels et al., 1994). In the case of the red-eared slider turtle, the TSP begins just prior to and even overlaps the time of sexual differentiation of the gonads, so temperature could be directly affecting the physiological events associated with gonadal differentiation (Wibbels et al., 1991b).

It has been suggested that temperature may be controlling the expression of certain genes and/or the production of specific hormones that may play a role in reptile sex determination and gonadal differentiation (Bull, 1985; Crews, 2003). It is hypothesized that incubation temperature alters steroid hormone production in the embryo, which then leads to sexual differentiation of the gonad (Lance, 1997). Crews (2003) suggested that temperature also activates the genes that encode for the hormones and this, in turn, is what determines the gender of the animal. The hormones that have been most studied have been estrogen and testosterone.

Estrogen hypothesis. Estrogen was first linked to sexual differentiation in reptiles through a study with the Greek tortoise, *Testudo graeca*, since the application of exogenous estrogens to the eggs resulted in female sex determination even when eggs were at male-producing temperatures (Pieau, 1969). Additional studies confirmed that the administration of sex steroids such as estrogen and testosterone to developing embryos during the TSP can alter the gonadal development and cause complete sex reversal when administered at high doses (Pieau, 1974; Crews et al., 1991; Dorizzi et al., 1991; Gahr et al., 1992; Wibbels and Crews, 1992). Histological studies indicate that exogenous estrogens stimulate the regression of the medullary cords and possibly stimulate the cortex (outer region) of the ovary to develop (Wibbels et al., 1993).

In concordance with these studies, Pieau et al. (1995) found a strong connection between aromatase activity (the enzyme that mediates the aromatization of steroid hormones, such as testosterone, to produce estrogens) (Figure 4) and the resulting estrogen production in the gonads of reptiles incubated at female-producing temperatures. Higher aromatase levels were detected in diamondback terrapin embryos (*Malaclemys terrapin*) from female temperatures during the TSP (Jeyasuria and Place, 1998) and Willingham et al (2000) found that the aromatase activity in the AKG of red-eared sliders was significantly higher after the TSP. In other studies, it was found that the addition of aromatase-inhibitors to eggs at female-producing temperatures will block female development and allow the embryos to develop as males (Wibbels and Crews, 1994; Crews and Bergeron, 1994; Dorizzi et al., 1994; Rhen and Lang, 1994; Richard-Mercier et al., 1995). All of these studies support the hypothesis that aromatase plays a vital role in sex determination in these reptiles.

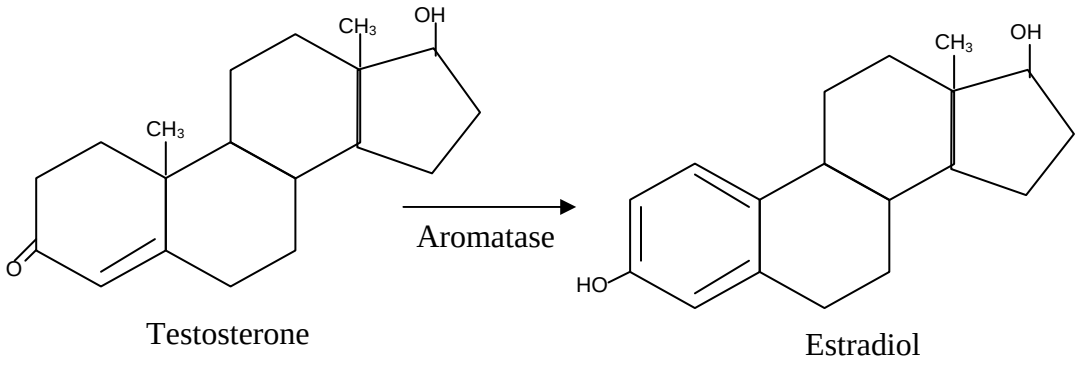

Figure 4. The conversion of testosterone to estradiol by the enzyme aromatase.

Results from these hormone studies prompted researchers to propose that the endogenous production of steroids may be involved in TSD and it was found that the effects of estrogen and temperature act synergistically during TSD (Wibbels et al., 1991a) by producing more females when acting together than would be expected if the two acted separately. This particular study found that the periods of time that the embryos were sensitive to incubation temperatures and to estrogens were similar, meaning that they both act on the embryo during the same time of development. Pieau and Dorizzi (2004) found that estrogens produced by the gonads (specifically estradiol) initiate ovarian differentiation. Embryos exposed to estrogens or warmer temperatures outside of this sensitive time frame were not affected by either of them. The later studies that found that aromatase inhibitors blocked female sex determination (Wibbels and Crews, 1994; Crews and Bergeron, 1994; Dorizzi et al., 1994; Rhen and Lang, 1994; Richard-Mercier et al., 1995), verified the connection. Based on this information, it was proposed that female-producing incubation temperatures during the TSP activate aromatase activity, thus

increasing the levels of estrogen in the gonad and causing ovarian differentiation (Pieau et al., 1994; Pieau, 1996).

Other factors and TSD. Treating eggs with androgens such as testosterone, seems to have an effect similar to estrogen (Pieau, 1974; Wibbels and Crews, 1992). It has been hypothesized that this is due to the aromatization of the testosterone to estradiol (Pieau, 1974). In contrast, DHT (dihydrotestosterone), a non-aromatizable androgen, has been shown to stimulate male sex determination in *Trachemys scripta*, but only when used near the pivotal temperature and only when used at very high doses (Wibbels and Crews, 1992 and 1995).

It is possible that other genes that control steroid hormone production, such as the steroidogenic factor-1 (SF-1), can play an important role in sex determination as well. SF-1 appears to be a global regulator of steroidogenesis and it binds to the promoter region of steroidogenic genes. It can act as a transcription factor for the aromatase gene (Lance, 1997) thus becoming a key determinant of gonadal development. Luo et al. (1994) found that disrupting this gene in mice caused the mice to be born without gonads. Several other genes have been implicated in the sex determination/sex differentiation cascade of reptiles with TSD. For example, Dmrt1, SOX9, the gene for anti-mullerian hormone, DAX1, and FoxL2 are expressed in the developing gonads of reptiles with TSD (Wibbels et al., 1998; Western et al., 1999; Torres Maldonado et al., 2002; Pieau and Dorizzi, 2004; Murdock and Wibbels, 2003; Shoemaker et al., 2007). More than likely, sex determination and sex differentiation in reptiles results from many of these factors working together, not just one alone.

TSD and Sex Ratios

Animals with GSD tend to produce sex ratios that conform to a 1:1 ratio, because each new individual has an equal chance of receiving either sexual genotype from the two parents. It was predicted by Fisher (1930) that this 1:1 sex ratio would be the natural occurrence in species that provide equal parental investment in each gender of young. However, animals with temperature-dependent sex determination exhibit a wide range of sex ratios (Mrosovsky, 1994) despite the fact that the parental involvement is only the investment in the egg and is equal for male and female hatchlings. This range of sex ratios is due to factors such as the incredible variety of beach conditions, environmental temperatures, and species-specific variability in TSD. In order to fully understand the evolutionary and ecological significance of TSD, an understanding of the natural sex ratios of a population is needed (Bull and Charnov, 1989).

Sex ratios in sea turtles can vary not only between species that nest on different beaches around the world but also within the same nesting groups over a single nesting season or over several nesting seasons (Mrosovsky et al., 1984b). Climatic changes can drastically affect the nesting temperatures and thus the sex ratio for any particular nesting season (Vogt and Bull, 1982). For example, nesting seasons that include tropical storms, hurricanes, or high rainfall amounts can experience cooler overall temperatures than seasons without rain (Wibbels et al., 1999) and thus produce a male-biased sex ratio.

Other factors on the beach can affect the sex ratio of a nest, including nest location, presence or lack of shading and vegetation, sand color, nest depth, and time of nesting within the nesting season. Nests laid in open beach without vegetation will

generally experience warmer temperatures than a nest in the closed, shady area of the beach, thus producing more females (Standora and Spotila, 1985). Darker sand will absorb more solar radiation and also possibly produce more females than lighter sand (Hays et al., 2001). It has also been shown that, in some locations, temperatures gradually rise throughout the nesting season, so that nests laid at the end of the season will generally produce more females than nests laid at the beginning of the season (Mrosovsky et al., 1984b). Additionally, Booth and Astill (2001) found that deeper nests are generally cooler and produce more males than nests that are more shallow. Other sand characteristics such as substrate water potentials (moisture content), particle diameter, electrical conductivity and air volume can also affect nest temperature and thus the sex ratio (Mortimer, 1990).

An additional way in which sex ratios are affected is through predation. Sea turtles have many different predators (including raccoons, armadillos, dogs, wild cats and fire ants) and the way those predators function on a particular beach can vary from looking for nests strictly on open beach to combing the vegetation line and predating those nests. Thus predation can alter the overall sex ratio of hatchlings produced from a beach as well (Fowler, 1979).

All of these factors that can affect sex ratios make predicting natural sex ratios very difficult, but an understanding of the natural sex ratios is important for conservational interests because sex ratios can affect the recovery of endangered populations (Mrosovsky, 1983; Hanson et al., 1998).

Sea Turtle Conservation, Sex Ratios, and Egg Hatcheries

For many years, sea turtles were hunted for their shells and meat. Some of their shells were used to make tortoise shell jewelry and their nests were poached by human and animal predators (Fowler, 1979). Increased population growth of humans nearer nesting beaches has not only reduced the available nesting area due to development, but has also increased the number of encounters between humans and sea turtles and resulted in drastically reduced nest numbers and hatch rates (Hart et al., 2006) because of the effects of artificial lighting, the impact of foot traffic over nests, disturbed nesting activity, or ingestion of debris left in the ocean by humans (Lutcavage et al., 1997). Increases in fishing and shrimping industries has also played a role in the decrease of sea turtle populations due to the turtles getting caught in the nets and drowning (Crowder et al., 1994).

The Endangered Species Act of 1973 covers sea turtles and sea turtle activities such as nesting, and gave conservationists guidelines for protecting them. Sea turtle populations that are critically endangered at this time, such as the Kemp's ridley (*Lepidochelys kempi*) and the Pacific leatherback sea turtles (*Dermochelys coriacea)*, as well as other species that are threatened, are currently involved in conservation programs around the world. These programs can involve protection of natural beaches, public education, transplanting eggs to nest corrals, and/or artificial incubation of the eggs. Protection of the nesting beaches, if possible, is the optimal method, as the nest temperature will not be affected and eggs will not be disturbed (Morreale et al., 1982). This, however, is not always possible.

Programs have been in place since 1968 for the Kemp's ridley on its main nesting beach in Rancho Nuevo, Mexico, in which the majority of the nests are transplanted into guarded egg corrals immediately after deposition (Chavez et al, 1968; Marquez 1994). This has steadily increased the size of this dwindling population but has resulted in a strong female-bias, as the nests are all moved to an egg hatchery that is on an open area of beach well above the high tide line which results in elevated incubation temperatures (Wibbels, 2007). Although many would argue that a strong female-bias at first would help the population recover faster, others might argue that maintaining the natural sex ratio would be better. Regardless, if males are not a limiting factor, the female bias could temporarily enhance the recovery of this species (Wibbels, 2003) and once they are recovered and the turtles are allowed to lay nests in their natural locations, the sex ratio would gradually return to the natural ratio.

Artificial incubation of sea turtle eggs has several advantages. The primary reason hatcheries have been used in the past is to prevent poaching and predation of the eggs, thus increasing hatching success. The use of egg hatcheries also provides the unique opportunity to manipulate sex ratios in order to enhance the recovery of an endangered population (Wibbels, 2003). However, the effects of specific sex ratios on the recovery of a population is not well understood (Wibbels, 2003) and requires knowledge of naturally occurring sex ratios and their effects on the reproductive ecology of a given population.

If eggs are moved to an egg hatchery, conservationists have to decide whether to duplicate the naturally occurring sex ratio or, alternatively, to manipulate the sex ratio in an effort to enhance the recovery of the population. In either case, a number of questions

arise. For example, what are the optimal temperatures for producing a particular sex, or a specific sex ratio? A wide variety of incubation temperatures for producing males, females, or mixed sex ratios have been reported in most sea turtles (reviewed by Mrosovsky, 1994; Wibbels, 2003). However, do all temperatures producing a given sex produce individuals of equal quality? Further, if an overall mixed sex ratio is desired, is it better to use an intermediate temperature producing that sex ratio, or is it better to put a proportion of the eggs at a female-producing temperature and the remainder of the eggs at a male-producing temperature?

The optimal method for addressing such questions is to incubate eggs at a variety of temperatures, allow the turtles to grow to sexual maturity and then evaluate their reproductive output. However, such studies might take a decade or more with most turtle species, and are thus, not feasible for short term studies. A short term approach is possible, however, via the evaluation of the morphology, histology, and endocrine physiology of the gonads and reproductive tracts of hatchlings produced from different incubation temperatures.

The other basic question confronting hatchery programs is the question of which sex ratio should be produced. Natural sex ratios produced from nesting beaches are very rarely 1:1 in sea turtles. Most nesting beaches that have been monitored produce biased ratios one way or the other. When incubating eggs, researchers have to decide to go with the natural ratio, a 1:1 ratio predicted by evolutionary theory (Fisher, 1930), or to bias the population to increase reproductive success in years to come. Unfortunately, information on naturally occurring sex ratios in sea turtle populations is relatively scarce. Furthermore, sex ratios are rarely monitored in conservation programs. This is due to the

logistical problems associated with capturing large numbers of immature turtles, and/or the lack of simple sexing techniques for hatchling and immature sea turtles. A prerequisite to studying sex ratios is the availability or development of accurate sexing techniques for sea turtles.

Sex Ratio Methodology

Hatchling and immature sea turtles cannot be sexed based on external morphology (Wibbels et al, 2000). In adults, males have long, muscular tails to enclose the penis (Miller, 1997; Wibbels, 2003) and can thus be easily identified, but hatchlings, juveniles, and sub-adults have no external secondary sexual characteristics (Wibbels et al., 2000) and therefore cannot be sexed by external appearance. Researchers caution against using external morphology as a sex determinant in the field for large immature animals due to the ease of mistaking a large immature male for a small mature female, so other methods are needed (Limpus, 1985; Larios, 1999).

Wibbels et al. (2000) evaluated several techniques for sexing immature sea turtles, including karyotyping, H-Y antigen, Bkm DNA fingerprinting, laparoscopy and serum testosterone levels. Other techniques include histology and nest temperature evaluation.

Karyotyping, H-Y antigen and DNA fingerprinting. In many animals, karyotyping for the sex chromosomes is an easy and practical method for sexing; however, sea turtles do not have sex chromosomes, so this method is not possible. Assays for H-Y antigen (an antigen reputed to be sex specific in some animals) did show higher levels in male

turtles than in females in small samples; however, this method is labor intensive, requires blood to be freshly sampled, and has shown mixed results in other studies (Standora and Spotila, 1985).

Bkm (banded krait minor) DNA fingerprinting is a method based on DNA isolation from the W chromosome of the snake, the banded krait (*Bungarus fasciatus*) (Singh et al., 1981) and was first used for identifying sex in sea turtles by Demas and Wachtel (1989). These researchers found that there were sex specific regions in the DNA of sea turtles, but the cost and logistics make this method very difficult to use for large sample sizes.

Histology. Determining gender through histology is effective and accurate (Mrosovsky et al., 1984a) but far from ideal since it requires the hatchlings to be sacrificed in order to section the gonadal tissue. Many research projects have used histological procedures to estimate the sex ratio of nests and entire nesting beaches by using the hatchlings that were found dead in the nest or were killed and recovered on the beach or by sacrificing a few hatchlings from each nest.

Histological procedures have been in place for many years and have not varied much from the original procedure (Humason, 1967).

Laparoscopy. One of the more accurate methods for sexing sea turtles is the use of laparoscopy. This method involves a minor surgery for the animal, in which the laparoscope is inserted into the body wall of the animal near the rear flipper (Wood et al., 1983). The morphology of the gonad can be seen clearly and gender determined fairly

easily. However, this method requires veterinary training, expensive equipment, a recovery period for the animals, and is time consuming and difficult to use in the field (Wibbels et al., 2000). Many researchers suggest using laparoscopy to validate other methods, due to the accuracy of results.

Testosterone radioimmunoassay. Serum testosterone levels can be tested using radioimmunoassay (RIA) techniques (Wibbels et al., 2000). Many studies have used this method (Owens et al., 1978; Gregory and Schmid, 2001; Wibbels 1988; Casale et al., 1998; Geis et al., 2003; 2005; Witzell et al., 2005) and have found it to be an accurate method for determining gender as long as the assay is validated with animals of known gender.

Nest temperatures. A variety of studies have monitored nest incubation temperatures or beach temperatures in an effort to predict hatchling sex ratios produced from a given nesting beach (Mrosovsky et al., 1984a; Mrosovsky and Provancha, 1989 and 1992; Godfrey et al, 1996; Marcovaldi et al., 1997; Hanson et al., 1998; Mrosovsky et al, 1999; Godley et al, 2001). This method alleviates the need for sacrificing hatchlings, but is dependent upon background data indicating which temperatures produce each sex in a given species. For this technique, small temperature data loggers sealed in water proof plastic bags are buried either within a nest at mid nest depth or on nesting beaches in areas of high nesting numbers at an average mid-nest depth. The data loggers are launched by computer prior to being buried and are set to record the temperature at specific intervals (e.g. every hour). Upon recovery, the average

temperatures during the middle third of incubation (the TSP) is calculated and used to predict the sex of the hatchlings.

Summary of Thesis Research

There were two primary projects involved in this research. The first study investigates the sex ratios of the leatherback sea turtle, *Dermochelys coriacea*. The leatherback occurs throughout the world's oceans, but is endangered throughout its range and is on the verge of extinction in the Pacific. The Atlantic leatherback nests in various locations from Central Africa in the southern hemisphere to the mid-Atlantic coast of the U.S. in the northern hemisphere. In North America, the primary nesting location is along the central and southeast Atlantic coast of Florida, with approximately 75-100 nests per year.

Due to its foraging and nesting migrations, the leatherback has the largest distribution of any reptile, since it has been well-documented to travel from tropical nesting areas to cold water feeding areas such as the waters off of Nova Scotia in the North Atlantic. A part of the first project was in collaboration with the Canadian Sea Turtle Research Project (CSTRP) and the Southwest Fisheries Science Center (SWFSC) in an effort to examine the sex ratio in the immature and adult portions of the leatherback population feeding off the coast of Nova Scotia. This is the only group in the world that captures relatively large numbers of leatherbacks each year at sea. Blood samples were obtained from each captured turtle and sex was predicted based on their tail length or circulating testosterone levels (as determined by RIA).

Another part of this first project investigated the hatchling sex ratios in the Atlantic leatherback. This part of the project was made possible through collaboration with Florida Atlantic University (FAU), which has one of the few permits in the U.S. that allows the manipulation of leatherback nests (i.e. insertion of data loggers, etc.). This sex ratio study included an evaluation of temperatures in leatherback nests from a major nesting beach in southeast Florida (Juno Beach). Data loggers were placed into nests and recorded temperature at one hour intervals for the duration of incubation. The middle third of the incubation period is the thermosensitive period for sea turtles, so the data in that time frame was used to predict the sex ratio.

The results of this project represent one of the few studies to examine hatchling sex ratios in the leatherback, and the first study to examine immature and adult sex ratios in this species. The results provide insight into the reproductive ecology of the Atlantic leatherback, and also suggest a natural sex ratio which will be considered when developing management strategy for this endangered sea turtle.

The second project in this thesis addresses the effects of specific incubation temperatures on the gonads and reproductive tracts of a turtle with TSD. As indicated previously, such information is of importance to sea turtle conservation programs that utilize egg hatcheries. That is, which incubation temperatures are best for producing males or females in hatchery programs? Additionally, this study may provide insight on evolutionary aspects of TSD in regard to how a specific incubation temperature could instill a greater fitness to a particular sex.

To address these subjects, the red-eared slider turtle, *Trachemys scripta*, was used as a model for sea turtles because they have the same pattern of TSD as sea turtles and

closely follow the same steps of development throughout the incubation time. Further, red-eared slider turtle eggs are commercially available in large numbers and their TSD has been well documented. The effects of specific incubation temperatures were examined relative to the gross morphology and histology of the gonads and reproductive tracts of pre-hatchling turtles. Additionally, the hormone content of the gonads was examined relative to incubation temperature. The results provide information that can be used to develop optimal management strategy for turtle hatchery programs. Further, the findings provide a potential physiological basis for the differential fitness hypothesis regarding the evolutionary significance of TSD. As such, the results support the hypothesis that TSD may persist in many reptiles because it provides the selective advantage of maximizing fitness.

EVALUATION OF LEATHERBACK SEX RATIOS IN THE NORTH ATLANTIC

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In preparation for Chelonian Conservation and Biology

Format adapted for thesis

ABSTRACT

The endangered leatherback sea turtle, *Dermochelys coriacea*, possesses temperature-dependent sex determination and, thus, can produce varied sex ratios due to the many factors that can affect nest incubation temperature. Biased sex ratios can be produced in natural settings as well as in egg hatcheries and those sex ratios can significantly affect the recovery of an endangered population. Therefore, there is a basic need to evaluate natural and artificial sex ratios in order to provide baseline data for generating effective management strategy for the recovery of the leatherback.

The current project examined sex ratios of hatchling, immature, and adult leatherback sea turtles from the North Atlantic. Hatchling sex ratios were estimated from a major nesting beach in Florida (Juno Beach) based on nest incubation temperatures. This was in collaboration with Florida Atlantic University for the 2006 and 2007 nesting seasons with seven nests per season being examined (due to permit limitations). To do this, data loggers were placed within a nest during egg disposition and the temperature was recorded every hour for the duration of the incubation time. The predicted sex ratio was based on the average daily temperature during the middle third of incubation, using previous pivotal temperature estimates for leatherbacks from Suriname and Costa Rica. The results suggest that both male and female biased sex ratios were produced in individual nests, but overall, a female bias was predicted.

Sex ratios were also estimated for immature and adult leatherback sea turtles captured in the North Atlantic in foraging grounds off of Nova Scotia, Canada. Turtles were captured in collaboration with the Canadian Sea Turtle Network. This is the only project world-wide which is currently capturing relatively large numbers of leatherback sea turtles at sea. Blood samples were taken from each turtle and plasma testosterone levels were assayed using a radioimmunoassay. A total of 26 turtles were captured during the late summer and fall of 2007. The turtles ranged in size from 134.2 to 171 cm in curved carapace length. The distribution of testosterone levels for the immature leatherbacks was similar to that reported for immature turtles in other sea turtle species, in which laparoscopy has been used to verify sex. Since laparoscopy was not possible in this study (due to permit limitations), previously reported data for other sea turtles were used as benchmarks for determining male versus female ranges of plasma testosterone levels. The results suggest a sex ratio of approximately 1.5:1.0. (F:M) for the immature turtles captured in this study (n =10). Additionally, tail length was used as a method of predicting sex in the adult turtles, and the results indicate an approximate 0.6:1.0 (F:M) sex ratio (n =16). Neither of these sex ratios differed significantly from a 1:1 sex ratio.

The results suggest sex ratios ranging from an approximate 1:1 sex ratio for adult and immature turtles captured in the waters off of Nova Scotia, to a predicted female bias for hatchling leatherbacks produced from Juno Beach during both 2006 and 2007. The results provide one of the first estimates of hatchling leatherback sex ratios from a major nesting beach in North America. Additionally, although samples size was limited, this study provides the first report of an immature sex ratio for the leatherback sea turtle and one of the few estimates of an adult sex ratio. Data of this sort is a prerequisite to

developing effective management strategy for the endangered leatherback sea turtle in the North Atlantic.

INTRODUCTION

The leatherback sea turtle, *Dermochelys coriacea*, is currently one of the most endangered sea turtles species in the world, with estimates that they may become completely extinct in some ranges during the next few decades if drastic measures are not taken (Spotila et al., 1996). The leatherback is the largest living turtle (Plotkin, 1995) and has the widest geographic range of any reptile (Pritchard and Trebbau, 1984) (Figure 1). Its survival status is currently threatened mostly by long line fishing practices (Thompson et al., 1998; Crowder, 2000; Lewison et al., 2004) with an estimate of 50,000 leatherbacks taken as long line by-catch in the year 2000 alone (Lewison et al., 2004). Population estimates of this species are difficult because they are so wide spread and live in open oceans, but it has been estimated that nesting numbers have declined 80-95% over the last 20 years in some areas, giving an indication of their critical numbers (Spotila et al., 1996; Crowder, 2000; Spotila et al., 2000; Martínez et al., 2007).

Figure 1. Major, minor and incidental nesting beaches of the leatherback sea turtle.

Due to these declining numbers, the leatherback sea turtle is the focus of many conservation projects around the world, many of which include the artificial incubation of eggs. While artificial incubation can boost hatchling numbers by protecting the eggs from poaching and predation and provide the advantage of being able to manipulate sex ratios in order to enhance the recovery of a population (Wibbels, 2003), there are potential disadvantages to this process. It is not completely understood, for instance, how manipulating the sex ratios of a population can affect the population in the long run (Wibbels, 2003), and the skewing of sex ratios may be done unintentionally if incubation temperatures are not monitored carefully. It is also necessary to have an understanding of the naturally occurring sex ratios of the populations in order to develop effective

management strategy for a hatchery program and this information can be difficult to obtain.

Like all other species of sea turtles, the leatherback possesses temperature-dependent sex determination (TSD) in which the incubation temperature determines the gender of the hatchlings (Rimblot et al., 1985; Mrosovsky, 1994; Binkley et al., 1998). With this type of TSD, males are produced at lower incubation temperatures and females are produced at higher incubation temperatures and a mixture of the two is produced at intermediate temperatures. This range of temperatures is called the TRT, or the transitional range of temperatures (Wibbels, 2003) and within it is the pivotal temperature, which will produce a 1:1 sex ratio (Mrosovsky and Pieau, 1991; Wibbels, 2003). Data suggest that the middle third of the incubation is when sex determination occurs for sea turtles (Yntema and Mrosovsky, 1982; Mrosovsky, 1994) so incubation temperature during this time period is of vital importance.

There are many factors that can affect nest temperature and the sex ratios produced over a single beach can vary widely (Mrosovsky et al., 1984) from producing a strong male bias to a strong female bias. Strong biases could enhance or hinder the recovery of a population. For example, biases could have detrimental effects on a population (Mrosovsky and Yntema, 1980; Mrosovsky, 1983), as one gender may become limiting and thus slow or even halt the propagation of the species. Alternatively, it is plausible that a female bias could enhance egg production (Wibbels, 2003). Due to these possibilities, it is important to understand what sex ratios are being produced naturally and artificially in endangered populations.

Naturally occurring sex ratios can be estimated in several ways but some require the ability to sex the turtles. Unfortunately, immature sea turtles cannot be sexed using external morphology (Wibbels, 1999; Wibbels et al., 2000), so other ways of estimating gender must be used. The most accurate method to sex sea turtles is through histology of the gonad, which requires sacrificing the animal. This would seem to be more detrimental to the population, so conservationists usually opt for other methods. Many studies have monitored nest incubation temperature, beach temperature, or incubation duration in an effort to predict natural hatchling sex ratios (Mrosovsky et al., 1984; Mrosovsky and Provancha, 1989 and 1992; Godfrey et al., 1996; Marcovaldi et al., 1997; Hanson et al., 1998; Mrosovsky et al., 1999; Godley et al., 2001) without sacrificing the animal.

Several studies have specifically evaluated incubation temperatures during the thermosensitive period of sex determination in order to predict hatchling sex ratios (Georges et al., 1994; Hanson et al., 1998; Kaska et al., 1998; Godley et al., 2001; Broderick et al., 2000; Binkley et al., 1998). To use this method, information about TSD in the species of interest needs to be determined. Specifically, the pivotal temperature and the transitional range of temperatures (TRT) must be known in order to predict the sex of the hatchlings based on temperature. Such information has been reported in most sea turtle populations, including the leatherback. A previous incubation study with the Pacific leatherback sea turtle from Costa Rica suggests a pivotal temperature of 29.4ºC, with temperatures above 30.0ºC producing 100% females and temperatures below 29.0ºC producing 100% males (Binckley et al., 1998). Other studies with the Atlantic

leatherback suggest a pivotal temperature of approximately 29.5ºC with the same 1ºC TRT as noted in the Pacific leatherback (Rimblot et al., 1985; Rimblot-Baly et al., 1986; Desvages et al., 1993; Godfrey et al., 1996; Girondot, 1999).

Other studies have used histology, beach temperatures, and incubation temperatures to estimate sex ratios of hatching leatherbacks. These studies estimated strong female biases in Costa Rica (Binckley et al., 1998), and moderate female biases to 1:1 ratios on the beaches of Suriname (Mrosovsky et al., 1984; Mrosovsky, 1994; Godfrey et al., 1996).

In addition to hatchling sex ratio studies, some previous studies have examined sex ratios in the immature portion of sea turtle populations (reviewed by Wibbels, 2003). A variety of studies have shown that plasma testosterone level is an accurate indicator of the sex of juvenile and adult sea turtles (reviewed by Wibbels et al., 2000). Testosterone levels can be determined by using a radioimmunoassay (RIA) technique on the serum or plasma fraction of a blood sample. Sex ratios have been examined in many sea turtle populations in this way (Owens et al., 1978; Morris, 1982; Wibbels et al., 1987; Wibbels, 1988; Wibbels et al., 1991; Bolten et al., 1992; Casale et al., 1998; Coyne, 2000; Wibbels et al., 2000; Geis et al., 2005; Witzell et al., 2005). From this information, sex ratios of populations can be extrapolated and used in population dynamic studies and in conservation efforts.

The current study evaluated sex ratios produced in the Atlantic leatherback population. Specifically, hatching, immature, and adult sex ratios were examined in this unique and endangered sea turtle. While a few hatchling sex ratios have been reported previously, this is the first report of an immature sex ratio and one of the few reports of

an adult sex ratio in the leatherback sea turtle. The immature sex ratio is of particular importance because it represents a condensation of many years of hatching production, thus providing insight into the history of sex ratios produced from the population's nesting beaches. Information of this sort is a prerequisite for generating effective management strategy for the endangered leatherback sea turtle. Knowledge of naturally occurring sex ratios can also provide insight when choosing incubation temperatures in egg hatchery programs and these data can indicate if naturally occurring leatherback sex ratios conform to those predicted by evolutionary theory (Fisher, 1930).

MATERIALS AND METHODS

Nest Temperature Project

Data Logger Preparation, Placement, and Calibration

Incubation temperatures were measured in 7 nests during both the 2006 and 2007 nesting seasons on Juno Beach, Florida. The number of nests examined was limited by permitting regulations due to the endangered status of the leatherback. Temperatures were monitored with Hobo data loggers (Onset Computer Corporation, Pocasset, MA) (Figure 2) which contain a micro-processor with attached thermister temperature sensor that is precise to approximately 0.3 to 0.5^{o}C. Data loggers were calibrated by placing them into incubators set at specific temperatures which approximated the range of temperatures that the data loggers would experience during the nest incubation time. Temperatures within the incubators were verified by pre-calibrated mercury thermometers and thermister probes. The data loggers were kept inside the incubators for at least one day, and the temperatures recorded by the data loggers were then compared to

actual temperatures. If necessary, a correction factor was assigned to a specific data logger.

Figure 2. A HOBO temperature data logger like those used in the nest temperature project for Juno Beach, Florida.

The data loggers were programmed with the Box Car Pro software to record the temperature at one hour intervals. They were then individually sealed in waterproof, plastic bags along with small vials of desiccant to protect the unit from water damage. After sealing, the data loggers were shipped to collaborators at Florida Atlantic University, where they were then placed in the approximate middle of the clutch while the turtles were nesting. The data loggers were removed when the nest had hatched. Once retrieved, data was analyzed using the Box Car program and exported to Microsoft Excel for evaluation. The dates for the middle third of incubation for each nest were calculated based on the total incubation duration. For all nests, the average, minimum,

and maximum temperatures during the middle third of incubation were calculated. The average temperature during the middle third of incubation was used to predict the hatchling sex ratio as described below.

Predicting Hatchling Sex Ratios

In order to predict hatchling sex ratios, the average incubation temperatures during the thermosensitive period of sex determination were compared to the pivotal temperatures and transitional range of temperatures (TRT) previously published for the leatherback sea turtle (Rimblot et al., 1985; Rimblot-Baly et al., 1986; Binckley et al., 1998; Chevalier et al., 1999; Girondot, 1999). A conservative approach was adopted that included 5 general sex ratio categories (Figure 3). These categories were: 1) average temperatures above the TRT were predicted to produce all females, 2) average temperatures above the pivotal, but below the upper range of the TRT were predicted to produce a female bias, 3) average temperatures that approximated pivotal were predicted to produce an approximate 1:1 sex ratio, 4) average temperatures below the pivotal but above the lower range of the TRT were predicted to produce a male bias, and 5) average temperatures below the TRT were predicted to produce all males.

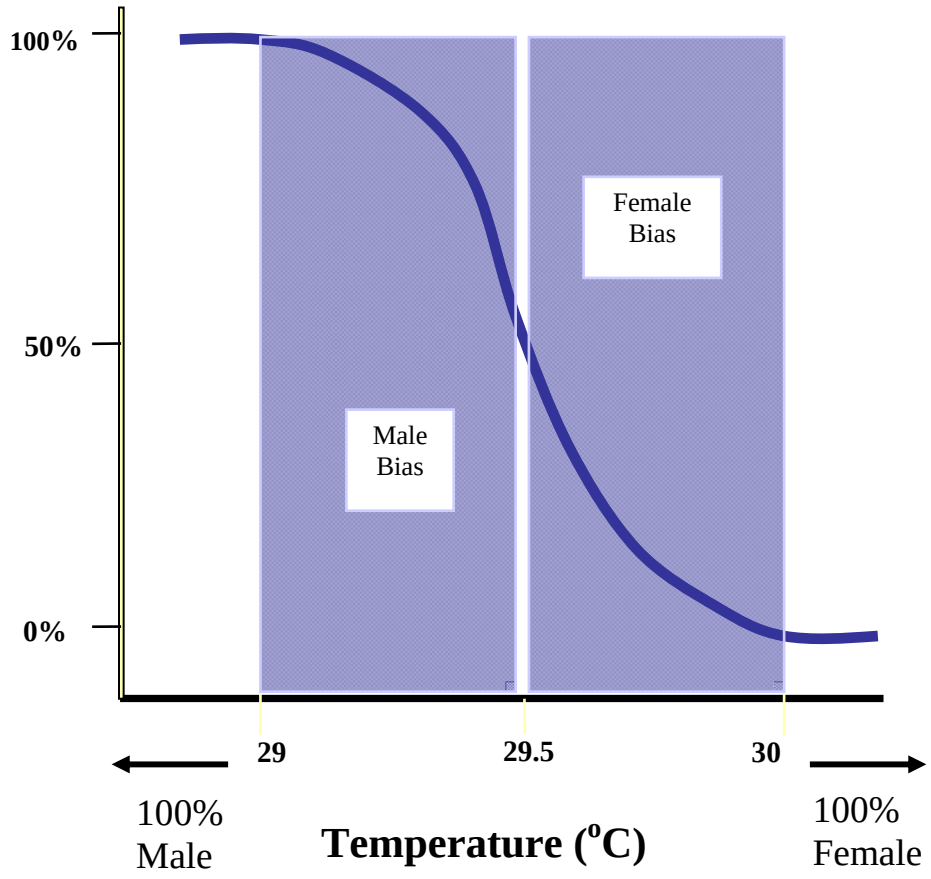

Figure 3. Sex ratio categories used for the leatherback sea turtle sex ratio analysis. A pivotal temperature of 29.5ºC was used and the TRT used was a one degree interval between 29º and 30ºC.

Immature and Adult Sex Ratio Project

In order to investigate immature and adult sex ratios of leatherbacks, collaboration was initiated with the Canadian Sea Turtle Network (CSTN) and the Southwest Fisheries Science Center (SWFSC). Blood samples were obtained from leatherback sea turtles captured in the coastal waters off of Nova Scotia. These animals were captured using a breakaway hoop net operated from a bowsprit that was attached to a commercial fishing

boat (James et al., 2005). All animals were guided to the stern of the boat and hoisted up a stern ramp onto the boat. Blood samples were typically taken within 20 minutes of capture and were taken from the bilateral dorsal sinus of the neck. Samples were placed on ice until they could be centrifuged, separated into plasma and blood cell fractions, and then frozen. The curved carapace lengths, (CCL), were taken from each turtle from the nuchal scute to the posterior tip of the carapace. Additionally, tail length measurements were taken ventrally from the cloaca to the tip of the tail and dorsally from the posterior tip of the carapace to the tip of the tail.

The plasma samples were analyzed in a radioimmunoassay (RIA) for testosterone. Approximately 300 µl aliquots of plasma were assayed in duplicate for each animal. The samples were extracted with 3 ml of anhydrous diethyl ether, dried under nitrogen gas, and reconstituted in 500 µl Tris-gel buffer (pH 7.0). To estimate extraction efficiency, 100 µl of a 1:10 trace dilution of tritiated testosterone (DuPont New England Nuclear, MA) was added to each sample prior to extraction. From each reconstituted sample, 2 samples of 200 µl were assayed by adding 100 µl of testosterone antisera (Fitzgerald Industries International Inc. lot #01916) and approximately 10,000 counts per minute (cpm) of testosterone trace in 100 µl volume.

The samples were allowed to incubate over night at 4ºC, and then 3 ml of dextran-coated charcoal suspension was added to each assay tube. The tubes were incubated for an additional 15 minutes after vortexing, then centrifuged for 15 minutes to separate bound and unbound fractions. The bound fractions were then decanted into 7-ml polyethylene scintillation vials (Fisher Scientific, GA), 3 ml of scintiverse cocktail

(Fisher Scientific, GA) was added, and samples were counted using a Beckman LS 6500 scintillation system.

A standard curve was run along with the samples by making serial dilutions of a testosterone standard, from 2000 pg/tube to 15.6 pg/tube. Also added to this were two control samples (run in duplicate) of known gender that had been assayed in this lab previously. These provided the means to generate inter- (17.46%) and intra-assay (13.26%) coefficients of variation.

The RIA data was analyzed with RIAMenu software in order to generate testosterone levels for each sample (in pg/ml).

RESULTS

Nest Temperature Project

A total of 14 nests were examined from Juno Beach, Florida, 7 during both the 2006 and 2007 nesting seasons. The lay dates of nests sampled in the current study ranged from April 22 to June 5 and are shown in Table 1 along with the minimum, maximum, and average temperatures during the middle third of incubation. The lay dates represent the portion of the nesting season for this beach when the majority of nests are laid. Additionally, the incubation duration and the predicted sex ratio are also shown in Table 1 (sex ratio is based on average temperature during the middle third of incubation). The average daily temperatures for the entire incubation time in each nest are shown in Figures 4a and 4b (2006 and 2007 respectively), along with the estimated pivotal temperature. The majority of the nests were estimated to produce female biases or 100% females.

Table 1

Data from the middle third of incubation from leatherback nests on Juno Beach, Florida. Average temperature column represents the average temperature during the middle third of incubation ± the standard deviation. Predicted sex ratio was based on average temperature.

Turtle #	Lay Date	Incubation Duration	Minimum Temp.	Maximum Temp.	Average Temp.	Predicted Sex Ratio
1	5/8/06	67	28.51	31.12	29.71 ± 0.78	female-bias
2	4/22/06	67	26.73	30.17	28.31 ± 0.92	all male
3	5/1/06	66	27.88	29.90	28.81 ± 0.73	all male
4	5/17/06	64	28.92	31.93	30.27 ± 1.01	all female
5	5/10/06	65	28.92	33.10	30.49 ± 1.11	all female
6	5/31/06	62	29.77	32.26	31.03 ± 0.71	all female
7	5/3/06	61	28.37	31.44	29.84 ± 0.97	female-bias
8	6/4/07	64	28.69	34.76	31.23 ± 1.89	all female
9	6/5/07	61	29.50	32.20	30.60 ± 0.76	all female
10	6/1/07	63	28.82	32.00	30.38 ± 0.98	all female
11	5/11/07	64	26.39	31.10	29.00 ± 1.23	male-bias
12	5/17/07	66	28.47	32.76	30.83 ± 1.35	all female
13	5/3/07	66	25.69	29.65	27.98 ± 1.07	all male
14	5/16/07	66	28.09	32.15	30.07 ± 1.35	all female

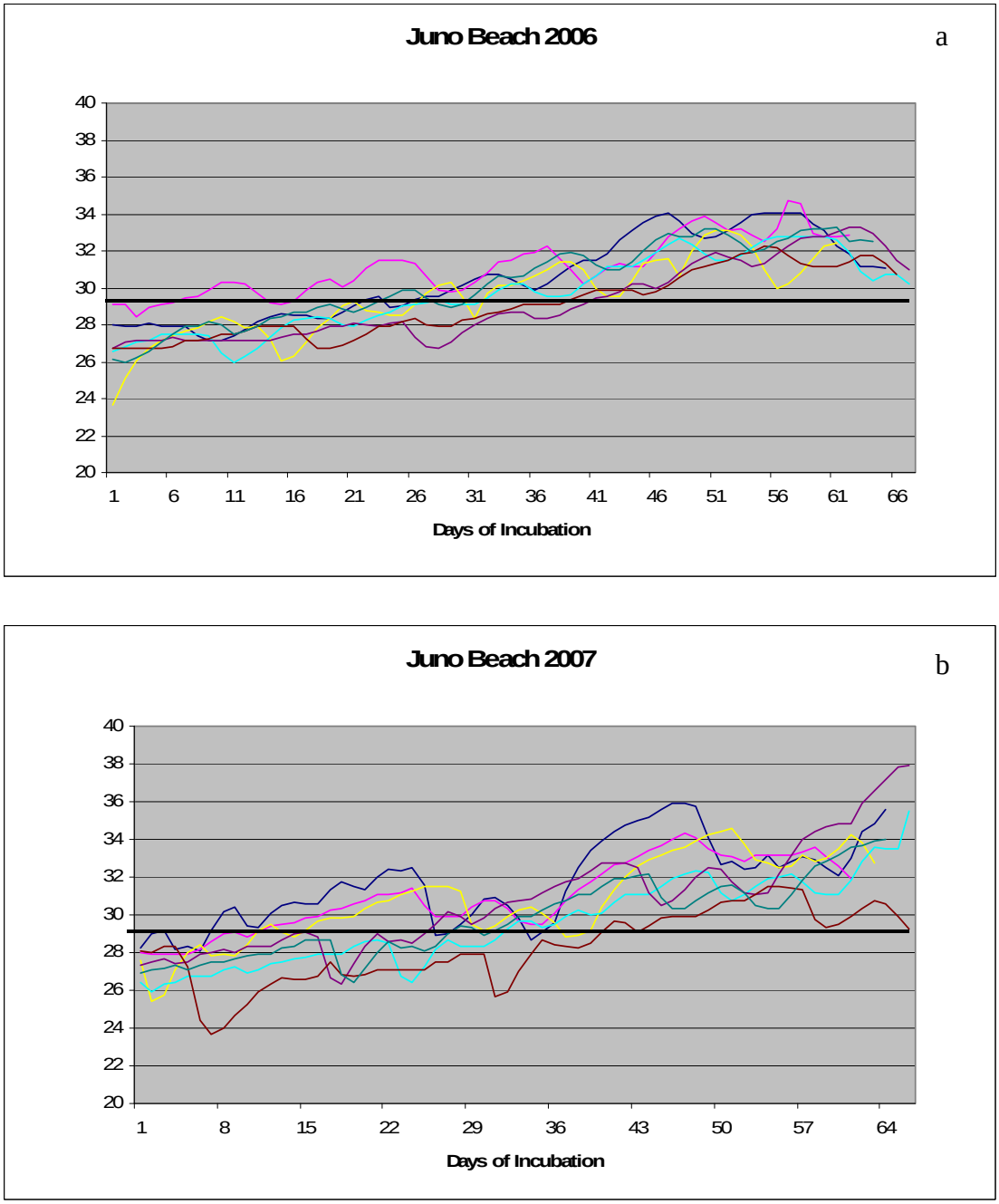

Figure 4. Average daily incubation temperature for the entire incubation duration for the 14 nests monitored on Juno Beach, Florida. Data for the 2006 nesting season (a) and for the 2007 season (b). The pivotal temperature, as shown by the black line, is 29.5ºC.

Immature and Adult Sex Ratios

Twenty six leatherback sea turtles were captured off the coast of Nova Scotia during the late summer and fall of 2007. Measurements and blood samples were obtained from each turtle. Turtles ranged in size from 134.2 cm to 171.0 cm with an average of 150.0 ± 9.61 (Tables 2 and 3). Previous studies on leatherback nesting beaches indicate that the typical nesting females in the Caribbean and north Atlantic average approximately 156 cm CCL (Leslie et al., 1996; Campbell et al., 1996; and Boulon et al., 1996), with the great majority of females being approximately 150 cm CCL or greater. Therefore, individuals in the current study were considered to be immature if their CCL was less than 150 cm. Table 1 and 2 lists CCL, tail lengths, and the testosterone levels of the immature and adult sea turtles sampled in the current study.

Table 2

Data from adult leatherback sea turtles captured off the coast of Nova Scotia, Canada (n = 16). Turtle ID number, curved carapace length measurements, total tail lengths, and plasma testosterone levels are shown.

Turtle ID#	CCL (mm)	Tail Length (cm)	Testosterone (pg/ml)
Z73652-DC-7	157.2	15.4	150.85
Z73654-DC-18	155.2	36.1	91.84
Z73655-DC-20	161.9	53.8	409.35
Z73656-DC-23	171.0	NA	1416.67
Z73657-DC-24	141.9	49.0	3535.98
Z73658-DC-26	152.0	63.3	1469.79
Z73660-DC-29	145.0	57.2	351.41
Z73661-DC-30	151.2	41.2	541.67
Z73664-DC-35	156.2	65.0	672.80
Z73665-DC-37	162.6	31.4	57.13
Z73666-DC-38	142.9	47.5	191.67
Z73667-DC-40	146.5	50.4	953.17
Z73668-DC-41	152.0	55.5	2266.93
Z73672-DC-52	166.2	35.8	416.00
Z73673-DC-56	163.4	56.5	2290.67
Z73676-DC-62	154.0	27.8	171.12

Table 3

Data from immature leatherback sea turtles captured off the coast of Nova Scotia, Canada (n =10). Turtle ID number, curved carapace length measurements, total tail lengths, and plasma testosterone levels are shown.

Turtle ID#	CCL (mm)	Tail Length (cm)	Testosterone (pg/ml)
Z73651-DC-6	139.0	11.7	331.08
Z73653-DC-9	146.0	38.9	124.37
Z73659-DC-28	146.6	38.2	4138.21
Z73662-DC-31	143.6	28.4	533.92
Z73663-DC-32	143.1	28.0	287.30
Z73669-DC-44	145.4	33.4	595.50
Z73670-DC-49	137.2	35.0	672.22
Z73671-DC-51	148.1	29.6	466.37
Z73674-DC-57	137.6	33.1	1387.55
Z73675-DC-59	134.2	33.7	1883.33

Tail length is commonly used as a secondary sexual characteristic to sex adult sea turtles (Wibbels, 2000), including the leatherback (James et al., 2007), but tail length does not appear to be a reliable indicator of sex in immature sea turtles (Wibbels, 2000). In general, the tails of adult female sea turtles normally extend to, or slightly past the posterior edge of the carapace, whereas male's tails extend well beyond the carapace. The sex of adult turtles (CCL > 150 cm) captured in the current study were based on tail lengths, with males being predicted for turtles with tail lengths (cloaca to tip of tail) of 45 cm or greater. (Figure 5 shows the adult sized turtles captured in the current study in order from shortest to longest tail lengths.) Additionally, four of the turtles that were under 150 cm CCL had tail lengths that were clearly indicative of males and were therefore placed into the adult group. A tail length measurement was not recorded for one adult turtle (Z73658-DC-26), but its testosterone level was high, thus indicating it was a male.

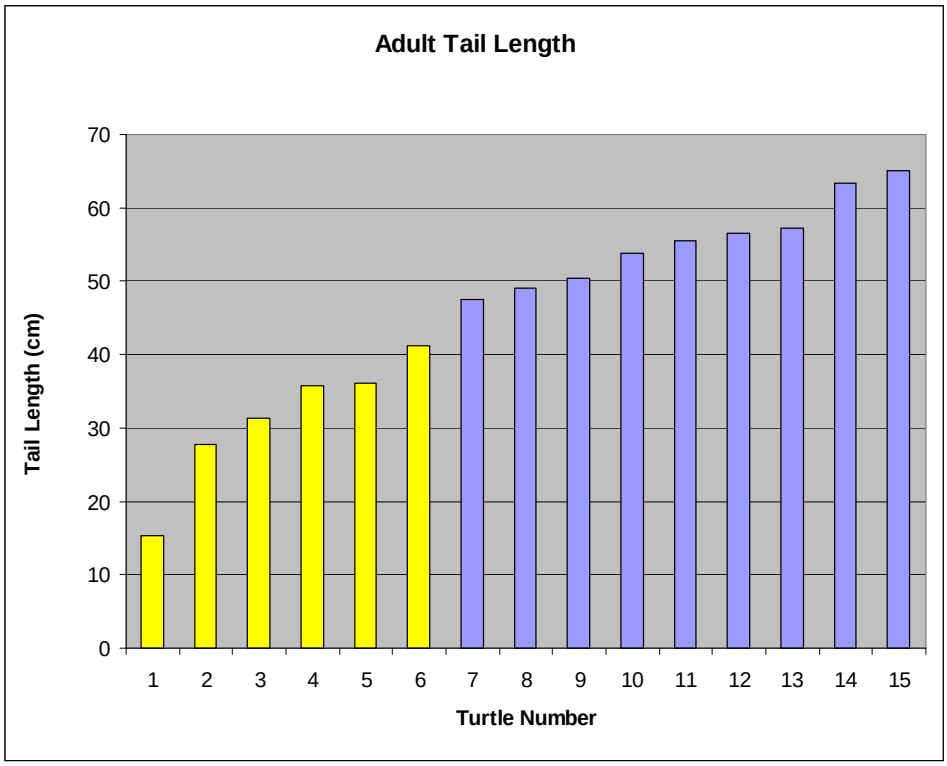

Figure 5. Tail length measurements for the adults sampled. Yellow bars represent predicted females and blue bars represent predicted males.

The distribution of plasma testosterone levels in the immatures is shown in Figure 6 ranging from the lowest to highest level. Testosterone levels for each of these turtles are shown in Figure 7 relative to carapace length and in Figure 8 relative to tail length. Analysis revealed no significant correlation between testosterone level and carapace length of immature turtles ($r = -0.01$, $p > 0.05$) but did show a significant correlation between testosterone level and tail length ($r = 0.59$, $p < 0.05$). The latter correlation could be due to males that are at or nearing puberty considering the sample represents large subadults. However, tail length alone does not appear to be a good indicator of sex because the ranges of the predicted males and females overlap.

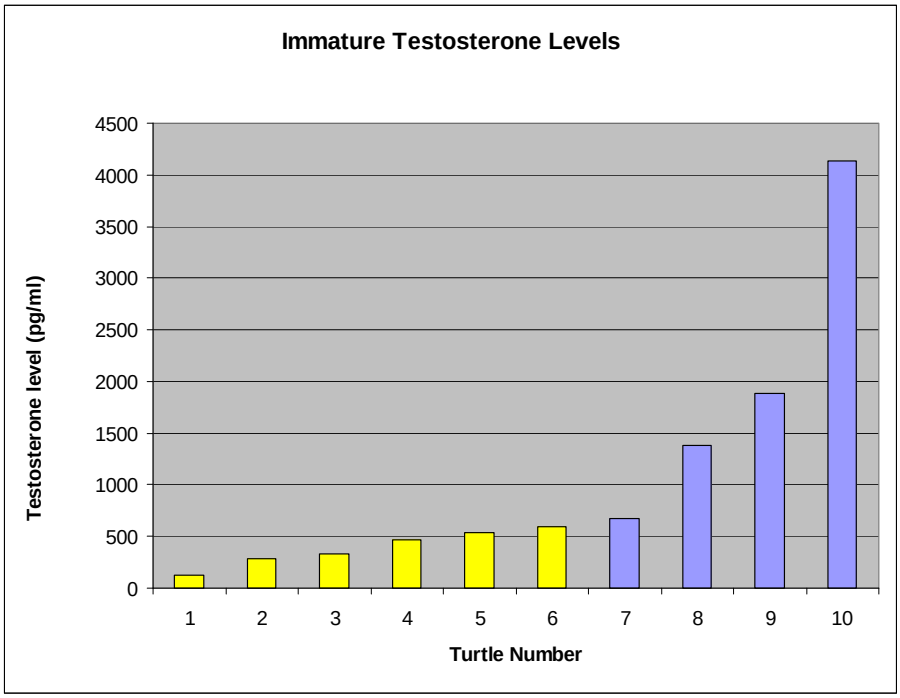

Figure 6. Plasma testosterone levels in immature leatherback sea turtles in order from lowest to highest pg/ml levels. Yellow bars represent predicted females and blue bars represent predicted males.

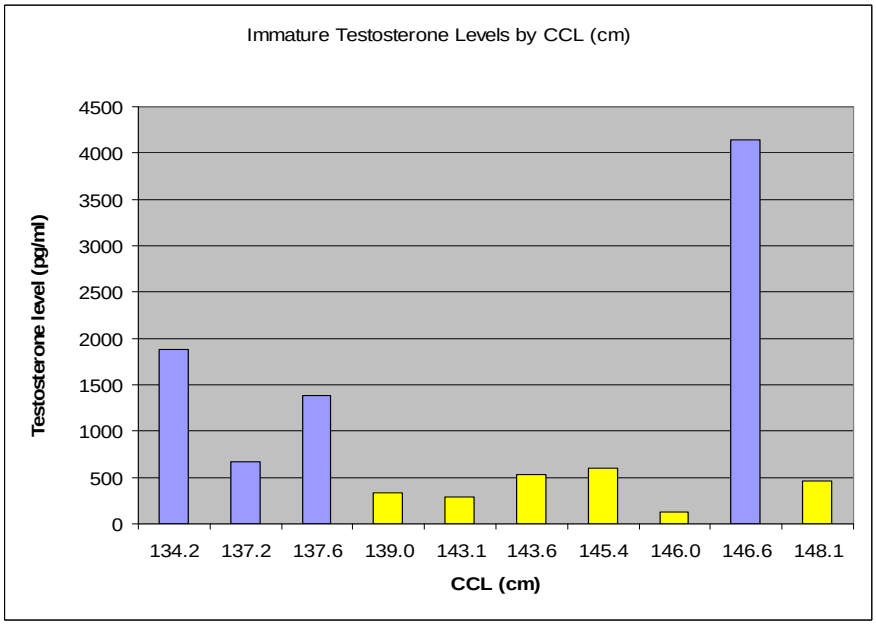

Figure 7. Plasma testosterone levels compared to the CCL (curved carapace length) measurements of the immature animals sampled. Yellow bars represent predicted females and blue bars represent predicted males.

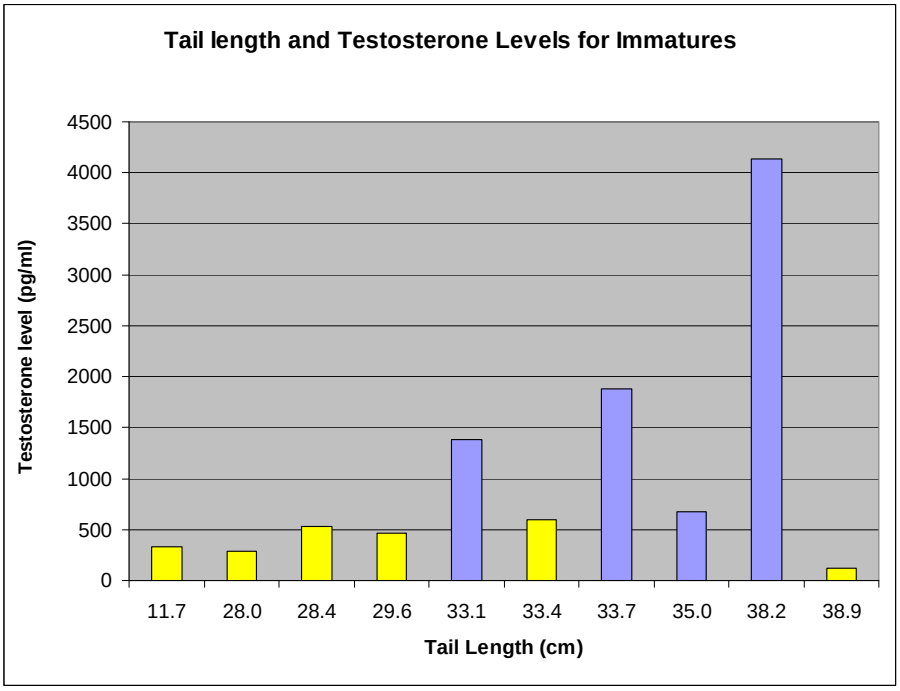

Figure 8. Testosterone levels of immature turtles compared to tail length. Yellow bars represent predicted females and blue bars represent predicted males.

Since laparoscopy was not possible to verify sex (due to permit limitations), previously reported testosterone levels from other sea turtle species were used to predict sex. Based on the data from previous studies, immature turtles with less than 600 pg/ml of testosterone were predicted to be female and those with levels greater than 600 pg/ml were predicted to be males (reviewed by Wibbels et al., 2000). The sex ratio of immature turtles (based on testosterone levels) was 1.5:1.0 (F:M, n = 10). This sex ratio did not differ significantly from a 1:1 ratio (Fisher's exact test, $p > 0.05$). Using, the predicted sexes, tail lengths of immature males were compared to immature females. The tail lengths of males were significantly longer than females (t test, $p < 0.05$), but the ranges overlapped (Figure 8).

The sex of adult turtles (based on tail length as described above) was 0.6:1.0 (F:M, n = 16). This sex ratio did not differ significantly from a 1:1 ratio (Fisher's exact test, $p > 0.05$). The adult sex ratio also did not differ significantly from the immature sex ratio (Fisher's exact test, $p > 0.05$)

DISCUSSION

Leatherback sea turtles are among the most critically endangered animals in the world (Spotila et al., 2000). Conservation programs have been put in place in many areas to help this population recover. In order to develop effective management strategy for this species, conservationists need to have a basic understanding of its biology and ecology. The leatherback possesses temperature-dependent sex determination which can significantly affect its reproductive ecology and survival status. Therefore, it is of conservational and ecological interest to monitor and evaluate sex ratios produced in leatherback populations.

The current study represents two collaborative projects that were initiated to investigate hatchling, immature, and adult sex ratios of leatherbacks. Several methods were used to estimate sex ratios in the various life stages of the leatherback. Incubation temperatures were used to estimate hatching sex ratios from Juno Beach, Florida, one of the most important nesting beaches for leatherback sea turtles in the U.S. Additionally, sex ratios were examined in the adult and immature portion of the population in a foraging area off the coast of Nova Scotia, Canada, based on tail length of adults and plasma testosterone level of immatures.

The nest temperature data suggests that both male and female biases were produced in the nests examined on Juno Beach during the 2006 and 2007 nesting seasons. However, female biases predominated with 10 of the 14 nests predicted to produce all females or a female bias (Table 1). These nests were laid from late April to early June which represents the middle portion of the leatherback nesting season during which the majority of eggs are laid. Thus, they may well be representative of a typical seasonal sex ratio produced from that beach. Juno Beach is one of the most important nesting beaches for leatherbacks in the U.S. and may be representative of several other leatherback nesting beaches in southwestern Florida. A few past studies have investigated sex ratios produced from leatherback nests. Previous studies from nesting beaches at Tortuguero in Costa Rica, Sandy Point in St. Croix, and in Suriname have reported overall female biases or near unbiased sex ratios (Mrosovsky et al., 1984; Leslie et al., 1996; Boulan et al., 1996). Thus, a female biased sex ratio from a leatherback beach is not unique, and such biases have frequently been reported for other sea turtle species (reviewed by Wibbels, 2003).

The significance of a female-biased sex ratio to the reproductive ecology in sea turtles is not completely understood at this time. A female bias may be beneficial to the population in that there would be more reproductively active females in the population. More reproductively active females means more eggs and hatchlings, and therefore a boost in the population numbers. However, a female bias also indicates a low number of males in the population and it is unknown at what percentage the males may become limiting and would result in low fertility and a loss of genetic diversity within the population. Thus, a female bias may be advantageous if males do not become a limiting

factor. From a conservational viewpoint, the sex ratio produced from Juno Beach appears appropriate and there is no need for nest manipulations at this time in order to alter sex ratios.

This study also examined adult and immature sex ratios by tail length and plasma testosterone levels, respectively. Studies of adult sex ratios have been rare in the past (James et al., 2007) and studies of immature sex ratios have not been reported. The current collaborative study provided the unique opportunity to investigate sex ratios in the adult and immature population of leatherbacks foraging in the waters off Nova Scotia. This region appears to be a foraging ground for both adult and immature leatherbacks (James et al., 2007). Previous studies suggest that smaller immature individuals may not frequent this area because of problems associated with maintaining increased body temperatures due to the smaller size of the turtles.

The adult sex ratio (with sex being based on tail lengths) was predicted to be 0.6:1.0 (F:M) and was not significantly different from a 1:1 sex ratio. A previous study in the north Atlantic reported sex ratios ranging from 1.8:1.0 (F:M) to 0.8:1.0 (F:M) for adult turtles captured from 1998 through 2006. It is possible that the relatively small sample size in the current study could be affected by sampling bias. The sampling was conducted after the nesting season (late summer and fall of 2007), so it is possible that the adult sex ratio may have been affected by sex specific migration patterns. That is, males may frequent this area more or return to it earlier after the nesting season than females. Regardless, the results extend the knowledge of adult sex ratios for this animal.

The immature turtles evaluated in this study were on the upper end of the immature size range (134 cm CCL or greater). A previous study examining migration

routes of leatherback turtles suggested that immature leatherbacks of less than 100 cm CCL may not venture into the colder waters that are frequented by larger individuals due to problems associated with maintaining an elevated core temperature (Eckert, 2002). As such, the immature turtles sampled in the current study represent a "subadult" portion of the population. The distribution of testosterone levels is similar to that previously reported for several other species of sea turtles (Wibbels et al., 2000). In those previous studies, sex was verified through laparoscopy. Since no laparoscopies were performed in this study (due to logistics and permit limitations when working with endangered leatherbacks in offshore waters), it was not possible to verify the sex of any of these individuals. However, the range of testosterone levels was consistent with those of other sea turtle species, and the results suggest a 1.5:1.0 sex ratio, which was not significantly different from a 1:1. This is the first report of a predicted sex ratio for immature leatherbacks.

Collectively, the results provide insight into the range of sex ratios that occur in the leatherback population inhabiting the north Atlantic. The sex ratios ranged from female biased for the hatchlings and immatures to male biased for adults. This information provides a foundation for making management decisions regarding the leatherback recovery strategy for this population. For example, programs at several nesting beaches move eggs to protected egg hatcheries and these results provide potential sex ratios that those hatcheries could adopt.

Finally, the sex ratios predicted in this study are consistent with those reported for other sea turtle populations (reviewed by Mrosovsky, 1994, Wibbels, 2003). Although significantly male biased sex ratios have been reported, unbiased and female biased sex

ratios tend to predominate in sea turtle sex ratio studies. Thus, the female biased hatchling sex ratio predicted in the current study is not unique. The reason for biased sex ratios is not clear, but could relate to the ecological and evolutionary significance of TSD (Shine, 1999). In particular, these biased ratios could reflect that TSD is advantageous because it allows the manipulation of sex ratios in order to optimize the fitness of the offspring (Shine, 1999; Warner and Shine, 2008).

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EFFECT OF INCUBATION TEMPERATURE ON THE MORPHOLOGY AND ENDOCRINOLOGY OF THE REPRODUCTIVE TRACT IN A TURTLE WITH TSD

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ABSTRACT

A variety of reptiles have temperature-dependent sex determination (TSD), where incubation temperature determines gender. This form of sex determination has significant conservational implications since it has the potential of producing highly biased sex ratios which can affect the reproductive output of a population. TSD is of particular interest to conservation programs that use hatcheries to artificially incubate the eggs of endangered sea turtles. Such programs need to select specific incubation temperatures in an effort to produce a desired sex ratio. This type of sex determination also has evolutionary implications because it may be advantageous for an animal to produce a specific gender under certain circumstances. The theory of differential fitness in regard to TSD implies that certain incubation temperatures may produce individuals of a particular sex due to a fitness advantage. Although the theory has been addressed in terms of the reproductive success of short lived species with TSD, actual physiological mechanisms have received little or no attention.

To address the evolutionary and conservational implications of TSD, this study evaluated the effect of specific incubation temperatures on the morphology and endocrinology of the gonads and reproductive tracts in a turtle with TSD. The red-eared slider turtle (*Trachemys scripta*) was used as a model because its TSD is similar to that of endangered sea turtles, its eggs are commercially available, and it TSD has been well-described. An experimental protocol was utilized in which the gonads and reproductive

tracts were compared between late-stage embryos incubated at temperatures that produced either 1) all females, 2) mostly females, 3) mostly males, or 4) all males. The gross morphology and histology of the reproductive tracts of these four groups were compared and the testosterone and estradiol content was measured. The results indicate significant variation between the females incubated at different temperatures and moderate differences in the males. The results suggest that all-male and all-female producing temperatures may be optimal for producing a desired sex ratio in an egg hatchery. Additionally, the results provide a potential physiological basis for the differential fitness hypothesis.

INTRODUCTION

A variety of reptiles, including sea turtles, possess temperature-dependent sex determination, TSD, where the incubation temperature during development determines the gender of the animal (reviewed by Bull, 1980; Janzen and Paukstis, 1991). This sort of sex determination has significant implications for the ecology, conservation and evolution of these reptiles. For example, the sex ratios resulting from TSD can affect the reproductive ecology and survival status of a particular population (Mrosovsky, 1983; Hanson et al., 1998). Therefore, the sex ratios produced from natural nests or from egg hatcheries in conservation programs can have a significant impact on the reproductive ecology and survival status of a population.

Conservation programs attempting to enhance the recovery of endangered reptiles often include a variety of methods aimed at protecting nests in order to enhance hatching success. For example, in the case of critically endangered sea turtle populations such as

the Kemp's ridley (*Lepidochelys kempi*) and the Pacific leatherback sea turtles (*Dermochelys coriacea),* the nesting females are protected on the beach and eggs are often moved to hatcheries to prevent predation and poaching. In some cases, nests are protected in their natural location because eggs will experience natural temperatures and conditions (Morreale et al., 1982). However, this is not always possible, and may not be the best method for optimizing hatching success. Therefore, many programs utilize egg hatcheries. Programs have been in place since 1968 for the Kemp's ridley on their main nesting beach in Rancho Nuevo, Mexico, in which the majority of the nests are transplanted into guarded nesting corrals immediately after deposition (Chavez et al, 1968; Marquez, 1994).

Artificial incubation also provides the unique opportunity to manipulate sex ratios in order to enhance the recovery of an endangered population (Wibbels, 2003). If eggs are moved to an egg hatchery, conservationists have to decide whether to duplicate the naturally occurring sex ratio or to manipulate it to produce a different sex ratio. In either case, a number of questions arise. For example, 1) what is the optimal sex ratio, and 2) what are the optimal temperatures for producing a particular sex, or a specific sex ratio?

In regards to the first question, animals with TSD can exhibit a wide range of sex ratios in natural settings (Mrosovsky, 1994; Wibbels, 2003). This range of sex ratios is due to factors such as the incredible variety of beach conditions, environmental temperatures, and species-specific variability in TSD. Depending on the sex ratio being produced, the propagation of the species could be hindered or enhanced. For example, the sex ratios could have detrimental effects on a population (Mrosovsky and Yntema, 1980; Mrosovsky, 1983), as one gender may become limiting and thus slow or even halt

the propagation of the species. Alternatively, it is plausible that a female bias could enhance egg production (Wibbels, 2003), so conservation programs typically attempt to produce either an unbiased or a female-biased hatching sex ratio. Unfortunately, the impact of such sex ratios on the reproductive ecology and recovery of an endangered population is currently speculative.

Once a program decides to produce a specific sex ratio, the second question must be addressed: which temperatures are best for producing the desired sex ratio? It is well documented that different incubation temperatures produce different genders (Bull, 1980; Wibbels, 2003) and these temperatures are known for many species of sea turtle. However, it has not been determined if the different temperatures have an effect on the quality (fitness) of the individual produced. For instance, are females from an all-female producing temperature the same as females produced from a temperature that produces both males and females? Further, if it is decided to produce a female-biased sex ratio, is it better to use a temperature that produces that sex ratio, or is it better to put a proportion of the eggs at a female-producing temperature and the remainder of the eggs at a male-producing temperature?

The optimal method for addressing this question is to incubate eggs at a variety of temperatures, allow the turtles to grow to sexual maturity and then evaluate their reproductive success. However, such studies might take a decade or more with most turtle species, and are thus not feasible for short term studies. As an alternative, this study utilized a short term approach by evaluating the morphology, histology, and endocrine physiology of the gonads and reproductive tracts of hatchlings produced from different incubation temperatures. Due to the endangered status of sea turtles, the red-

eared slider turtle, *Trachemys scripta*, was used as an alternative model for examining this subject because the pattern of sex determination in this turtle is similar to that in sea turtles and its TSD has been well-described (Wibbels et al., 1991).

This study also has implications regarding the evolution of TSD. Shine (1999) reviews a series of hypotheses regarding the possible advantages of TSD over other sex determination systems, including the possibility of differential fitness of offspring related to incubation temperature and sex. The differential fitness hypothesis was originally proposed by Charnov and Bull (1977) and predicts that TSD enhances the fitness of offspring by matching offspring sex to incubation conditions. That is, eggs should produce males when conditions are favorable for males and females when conditions are favorable for females. Due to this, sex ratios may not always approximate a 1:1 sex ratio in reptiles with TSD, as predicted by evolutionary theory (Fisher, 1930).

A recent study by Warner and Shine (2008) provides evidence supporting the differential fitness hypothesis in a short-lived lizard with TSD, the jacky dragon (*Amphibolurus muricatus*). That study evaluated the reproductive success of individuals produced at different temperatures, including experimentally sex-reversed individuals. The results indicated that fitness was sex-specific at a given temperature. That is, certain temperatures appeared to optimize the fitness of males, whereas others optimized the fitness of females. Similar results were suggested for the leopard gecko (*Eublepharis macularius*) in which specific temperatures produced individuals of greater reproductive fitness (Gutzke and Crews, 1988; Rhen and Crews, 1999). These animals exhibit very diverse body weights, hormone levels, and reproductive success depending on the incubation temperatures in which they were raised (Crews and Bull, 1987; Tousignant

and Crews, 1995; Rhen and Crews, 1999; Rhen et al., 2005). It has been documented that incubation temperature permanently influences adult reproductive behavior (Rhen and Crews, 1999) in this animal.

Thus, there is evidence supporting the differential fitness hypothesis in reptiles with TSD. However, the mechanism underlying the differential fitness has not been provided. This study addresses this subject by evaluating the quality of the gonad and reproductive tracts produced at different incubation temperatures in the red-eared slider turtle, as a possible physical mechanism for fitness differences.

MATERIALS AND METHODS

Obtaining Eggs and Egg Incubation

Red-eared slider (*Trachemys scripta*) eggs were obtained from Kliebert/Clark Turtle and Alligator Farm in Hammond, Louisiana. Eggs were collected daily in relatively large numbers so the experimental groups of eggs used in this study were all laid on the same day. The eggs were placed in incubators that were maintained at constant incubation temperatures. The incubation temperatures used were based on the known male and female producing temperatures for this species. 31°, 29.1°, 28.5° and 26°C were chosen because they represent all-female, female bias, male bias, and all-male temperatures respectively. Eggs were divided randomly into baskets for each incubator in order to avoid clutch effects (Dodd et al., 2006). A second set of eggs were incubated at 26ºC and treated with 10 µg of exogenous estradiol on the egg shell at approximately embryonic stage 16 (Yntema, 1968; Greenbaum, 2002) in order to sex reverse the

embryos from male to female. This method of sex reversal has been well documented in previous studies (Crews et al., 1991; Wibbels and Crews, 1992).

The incubators were custom-built and used a large water reservoir as heat sink to stabilize the temperature and maintain high humidity (Lang and Andrews, 1994). A mercury-contact thermostat was used to control temperature to approximately 0.1ºC. Smaller trays were placed over the water tray to hold the eggs, which were kept close to one another in similar fashion to a natural nest. Temperature was monitored by temperature probes that were placed on level with the eggs and by a mercury thermometer that was placed directly above the eggs. Temperature was monitored from outside the incubator and recorded several times a day throughout incubation. The incubators were only opened once weekly to add more water to the reservoir and to check for and remove eggs that were not developing. The temperature probes were calibrated with the mercury thermometer in order to judge the accuracy of the measurements.

All eggs were incubated at constant temperatures and one egg from each group was taken occasionally in order to stage the development. Staging was based on Yntema's guide for *Chelydra serpentina* (1968) and Greenbaum's guide for *Trachemys scripta* (2002). The stages were determined in order to judge the time to the pre-hatch stage, or stage 26, when the embryos would be euthanized and processed (IACUC Protocol No. 071007370)

Processing of Tissues

At stage 26, the eggs were removed from the incubator, weighed, opened, and the hatchlings were euthanized. Hatchling weight was recorded as well as shell length and

shell width. With a subset of hatchlings, a dissecting microscope was utilized for the dissection of the adrenal-kidney-gonad (AKG) complex. These AKGs were flash frozen in liquid nitrogen and then placed in a -60°C freezer to be used in hormone analysis at a later date. Another subset of hatchlings was dissected but the intact reproductive tracts were preserved in 10% buffered formalin. These hatchlings were used for gross morphology and histology analysis.

Gross Morphology and Histology

Preserved specimens were placed under a dissecting microscope equipped with a digital camera for morphological analysis. With each hatchling, multiple pictures were taken of the entire reproductive tract, the right and left gonads, and the right and left oviducts if seen. For a size reference, a five millimeter section of a ruler was included in each gonad and oviduct photo and was placed as close to and as level with the gonad as possible to get an accurate measurement. The photos were analyzed using Image J software (NIH).

During analysis, measurement tools of the Image J program were used to obtain a length, width and area of the gonad. Three separate readings were taken for every measurement and these were averaged.

After measurements were taken, a portion of the left gonad was dissected for histology. The right gonad was taken if there were any subsequent problems when histologically processing the left gonad. Sections of the middle third of the gonad were used in order to standardize the portion of the gonad that was histologically evaluated. To accomplish this, an incision was made one third of the way up the gonad from the caudal

end of the animal. A triangular wedge of tissue that included a portion of the gonad, kidney and oviduct was then removed (Figure 1).

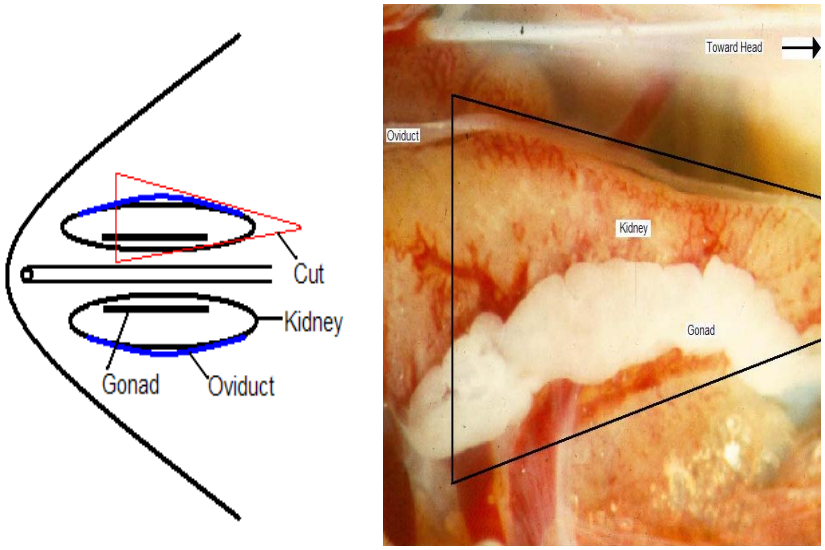

Figure 1. Diagram and photo of the adrenal-kidney-gonad (AKG) complex, showing the portion that is dissected for histology.

A standard paraffin histology procedure was used to process the tissue (Humason, 1967). The tissue samples were infiltrated with paraffin, embedded in paraffin blocks, and then sectioned at 8 µm with a microtone. Ribbons of tissue were transferred to microscope slides and dried overnight on a drying plate. Tissues were stained with hematoxylin and eosin (Humason, 1967).

Slides were examined with a compound microscope equipped with a digital camera. Photos were taken of the gonad and the oviduct (if present). The images of the gonads and oviducts were analyzed with Image J software. Overall gonad area was taken as well as cortex and medullary areas if the distinction could be made. Cortex width was

also taken at three set locations in the gonad. If the oviduct was seen, the overall area and lumen area were recorded. As with morphology, measurements were taken in groups of three and averaged. Pictures of the grid of a hemacytometer were taken at various magnifications and used to calibrate measurements from the Image J software.

For all morphology and histology measurements, a gonadosomatic index was preformed in order to compensate for the size of the hatchling, so that the differences could not be attributed to hatchling size.

Hormone Analysis

In order to obtain detectable levels of steroid hormones, AKGs were pooled into groups of 5 based on their incubation temperature and sex. The pools were thawed and homogenized in 2 ml of deionized water using a polytron homogenizer for 30 seconds. Aliquots of 1 ml were taken for both testosterone and estradiol radioimmunoassay (RIA). The 1 ml aliquots were extracted with 3 ml of anhydrous diethyl ether, dried under nitrogen gas, and reconstituted in 500 µl Tris-gel buffer (pH 7.0). To estimate extraction efficiency, 100 µl of a 1:10 trace dilution of tritiated testosterone or estradiol (DuPont New England Nuclear, MA) was added to each sample prior to extraction. After reconstitution, the estradiol samples were pipetted into 1.5 ml conicals and frozen until the estradiol RIA could be run. With the reconstituted testosterone samples, 2 sets of 200 µl were assayed by adding 100 µl of testosterone antisera (Fitzgerald Industries International Inc. lot #01916) and approximately 10,000 counts per minute (cpm) of tritiated testosterone in 100 µl volume.

The samples were allowed to incubate over night at 4ºC, and then 3 ml of dextran-coated charcoal suspension was added to each assay tube. The tubes were incubated for an additional 15 minutes after vortexing, then centrifuged for 15 minutes to separate bound and unbound fractions. The bound fractions were then decanted into 7-ml polyethylene scintillation vials (Fisher Scientific, GA), 3 ml of scintiverse cocktail (Fisher Scientific, GA) was added, and samples were counted using a Beckman LS 6500 scintillation system.

At a later date, the extracted samples for the estradiol analysis were thawed and examined by an estradiol RIA using the same protocol as the testosterone RIA. 100 µl of estradiol antisera was used (Fitzgerald Industries International Inc. lot #03381) along with approximately 10,000 cpm of tritiated estradiol.

A standard curve was run in both RIAs using standards that were prepared by serial dilutions of the respective hormone standard: from 2000 pg/tube to15.6 pg/tube for the testosterone standard curve and from 2000 pg/tube to 3.9 pg/tube for the estradiol standard curve. In addition, two control samples (each run in duplicate) with known hormone concentrations were run in each of the RIAs. These provided the means to generate inter- (17.46% and 2.88% for testosterone and estrogen respectively) and intra-assay (13.26% and 3.22%) coefficients of variation.

The RIA data was analyzed using RIAMenu software to generate the standard curve and the hormone levels for each sample (in pg/mg AKG).

RESULTS

Morphology

Ovaries of turtles are generally long, thin, and have a white appearance, whereas the testes are shorter, wider, and are well-vascularized. Definite gross morphological differences were seen in the gonads from the different temperatures, with those at intermediate temperatures looking less like those from the all-male and all-female temperatures. (Figure 2).

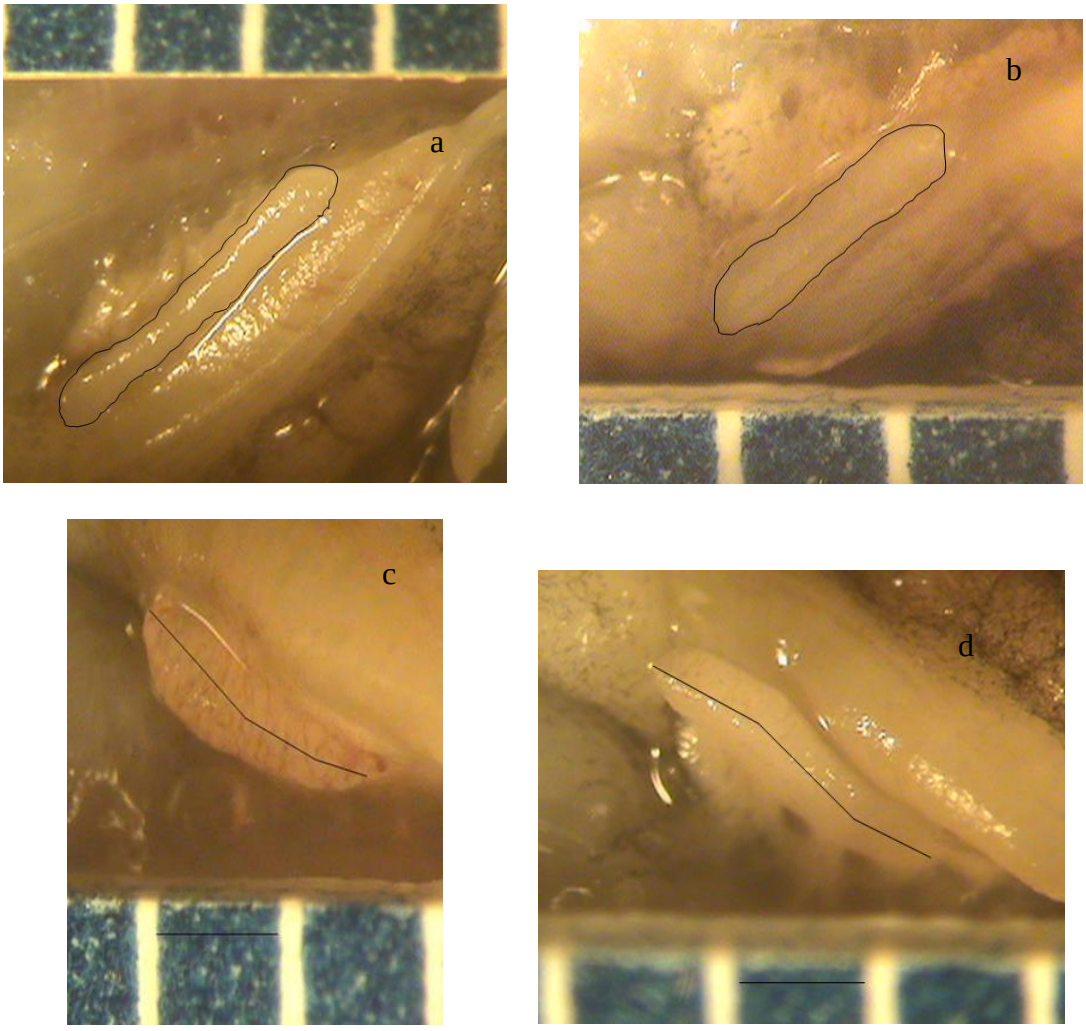

Figure 2. Morphological analysis of gonad length. A typical length ovary from 31ºC (a) and a short ovary from 28.5ºC (b). A typical sized, vascular testis from 26ºC (c) and a long testis with less vascularization from 29.1ºC (d). The black lines show how gonad area (a and b) and gonad length (c and d) were taken.

The average lengths, widths, and areas of the gonads from the four temperature groups for both males and females are shown in Table 1 and in graphical comparison in Figure 3. The gonads of females from the three separate incubation temperatures and the estradiol treated eggs from 26º were compared and found to have significant differences. After accounting for the size of the hatchling, the ovaries from 31º females were significantly longer and had a larger area than those from the other temperatures ($p=3.93x10^{-8}$ and $4.92x10^{-6}$ respectively). Differences in ovary width were not as distinct, but the individuals from 29.1ºC were significantly wider than the other groups ($p=0.01$).

The males in this experiment showed a significant difference in only one measurement. The testis from animals incubated at the temperatures producing a female-bias (29.1ºC) were significantly longer than those incubated at the all male-producing temperature, 26ºC ($p=0.036$). Otherwise, no significant variation was detected among the males produced at different temperatures.

Table 1

Morphological data for gonads from each temperature. All measurements are represented as the mean ± standard deviation. Gonad width was taken at the mid-gonad length.

Temp.	Sex	n	Average Gonad Length	Average Gonad Width	Average Gonad Area
31ºC	F	15	2.58 ± 0.48	0.28 ± 0.10	0.93 ± 0.34
29.1ºC	F	8	1.90 ± 0.17	0.41 ± 0.07	0.78 ± 0.23
28.5ºC	F	5	1.83 ± 0.17	0.35 ± 0.04	0.67 ± 0.21
26ºC + E	F	13	1.51 ± 0.41	0.27 ± 0.09	0.35 ± 0.24
26ºC	M	15	1.67 ± 0.36	0.47 ± 0.08	0.66 ± 0.33
28.5ºC	M	10	1.72 ± 0.54	0.44 ± 0.11	1.01 ± 0.38
29.1ºC	M	6	2.31 ± 0.23	0.57 ± 0.08	1.24 ± 0.15

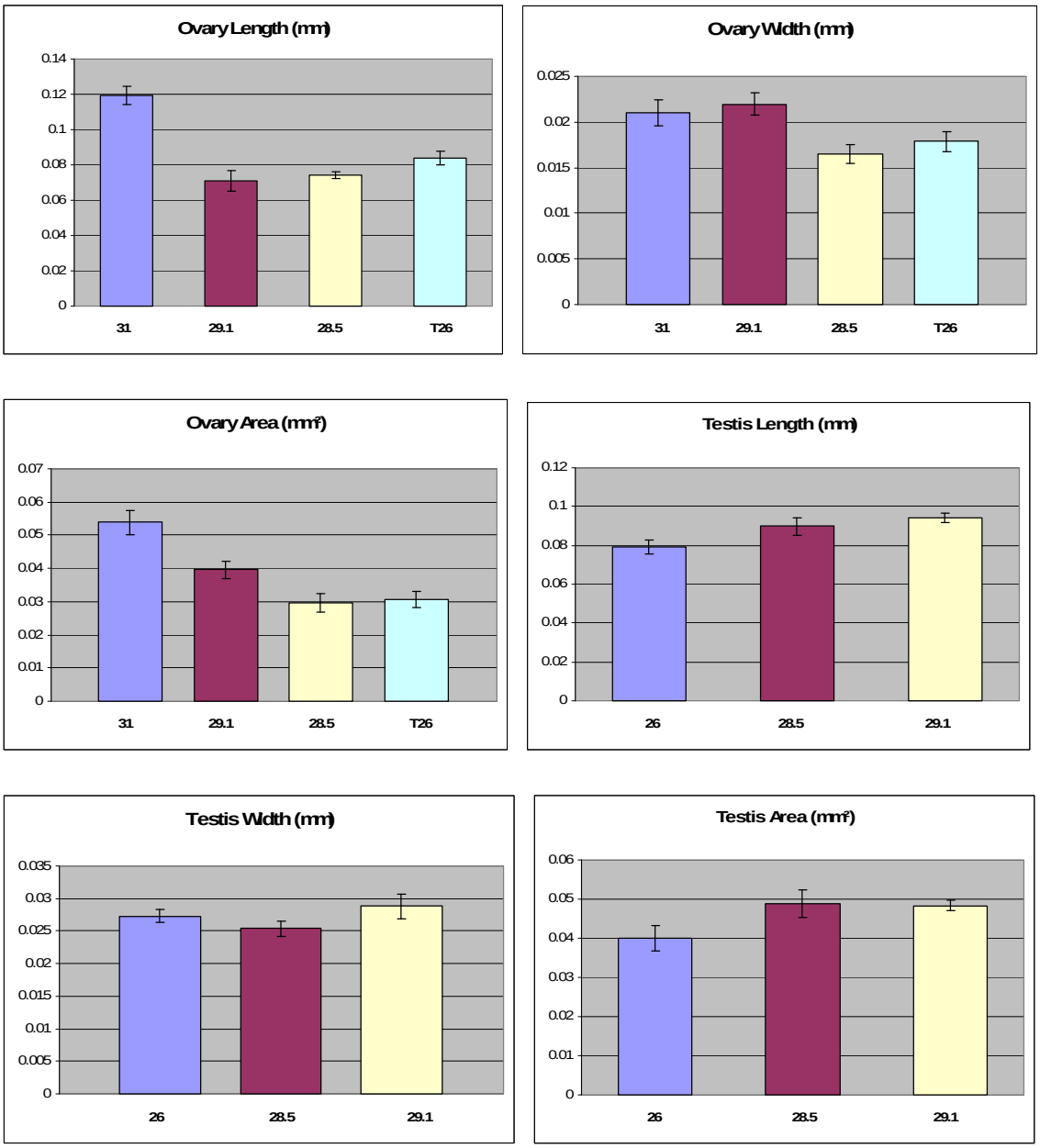

Figure 3. Comparison of length, width and areas for gonads from the four incubation temperatures. (T26 represents those treated with estradiol at 26ºC for male to female sex reversal.)

Histology

The ovaries of turtles show a well-developed cortex (outer region) and poorly organized medulla (inner portion), whereas males show little or no cortex and have a

well-organized medulla (Wibbels, 2003). The ovaries and testis from individuals from different temperatures were measured and described. The ovaries from 31ºC had distinct cortex and medulla areas as expected, but many of those from cooler temperatures showed no distinction between the two. The testis from 26ºC had organization and distinct tubules within the medullary region, but many of those from warmer temperatures did not (Figure 4). Some of the testes from warmer temperatures also showed a developed cortex region, although not as thick as in females.

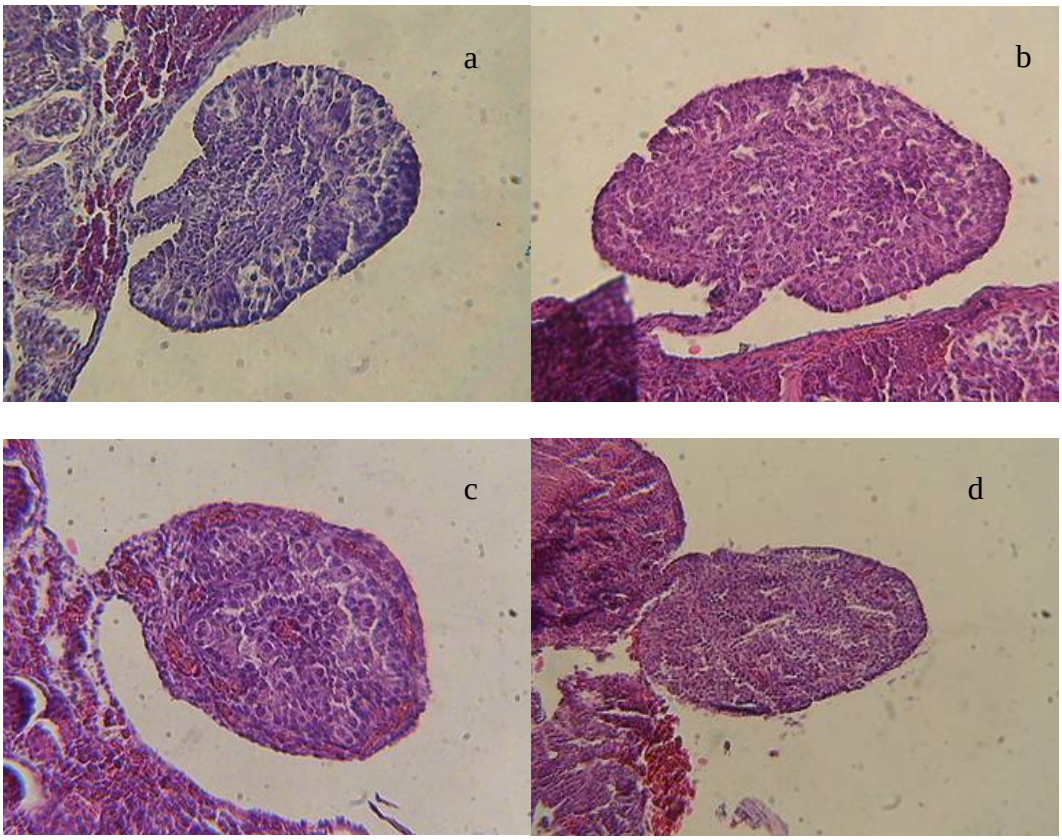

Figure 4. Examples of ovaries and testis from different incubation temperatures. An ovary from 31ºC (a) shows a thick cortex and distinct medulla whereas an ovary from 28.5ºC is less distinct (b). A testis from 26ºC (c) shows a well organized medulla with tubules whereas a testis from 29.1ºC (d), has less distinct tubules and a thin cortex is visible.

Histological analysis showed significant differences between the females from different temperatures. After accounting for the overall size of the hatchling, there were significant differences in females for the overall area of the gonad, cortex width, cortex area, and medulla area ($p= 4.3x10^{-7}$, $1.93x10^{-5}$, $9.61x10^{-6}$, and 0.03 respectively). The males showed no significant differences in the area of the gonad and cortex and medulla areas were not obtained. The average measurements from the histological analysis are shown in Table 2 and in graphical comparison in Figure 5.

Table 2

Average gonad measurements (± standard deviation) for males and females, from histological analysis. The number in the group analyzed is included in parentheses if it was different from the one listed for gonad area.

Temp.	Sex	n	Average Gonad Area	Average Cortex Width	Average Cortex Area	Average Medulla Area
31ºC	F	13	4.15 ± 0.87	0.55 ± 0.12	2.25 ± 0.42	1.73 ± 0.89
29.1ºC	F	8	2.60 ± 0.66	0.38 ± 0.09	1.53 ± 0.47	1.11 ± 0.51
28.5ºC	F	5	2.48 ± 1.18	0.31 (1)	1.51 (1)	0.74 (1)
26ºC + E	F	12	1.96 ± 0.80	0.28±0.05 (6)	0.98±0.39 (6)	0.79±0.51 (6)
26ºC	M	12	5.57 ± 1.95			
28.5ºC	M	8	4.32 ± 1.20			
29.1ºC	M	6	4.92 ± 1.54			

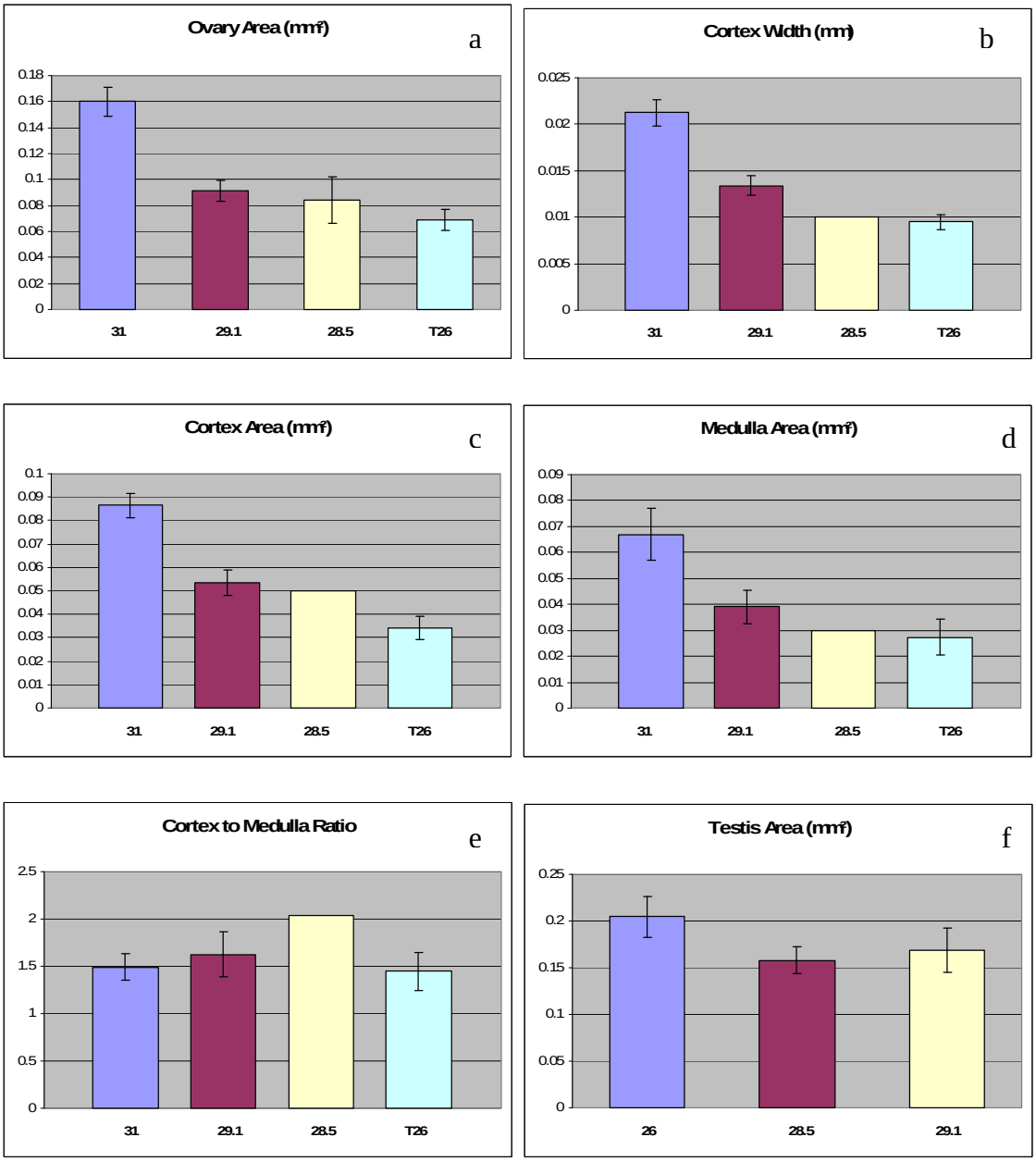

Figure 5. Comparisons between temperatures for the histological measurements for females (graphs a-e) and males (graph f).

In addition to the differences in the gonads, the oviducts in females from 31ºC had distinct lumens and cellular organization, whereas those from cooler temperatures were more regressed and often had no lumen. Oviducts in males were regressed at 26ºC or not

present at all, and those from warmer temperatures were forming, but too indistinct to be measured accurately (Figure 6).

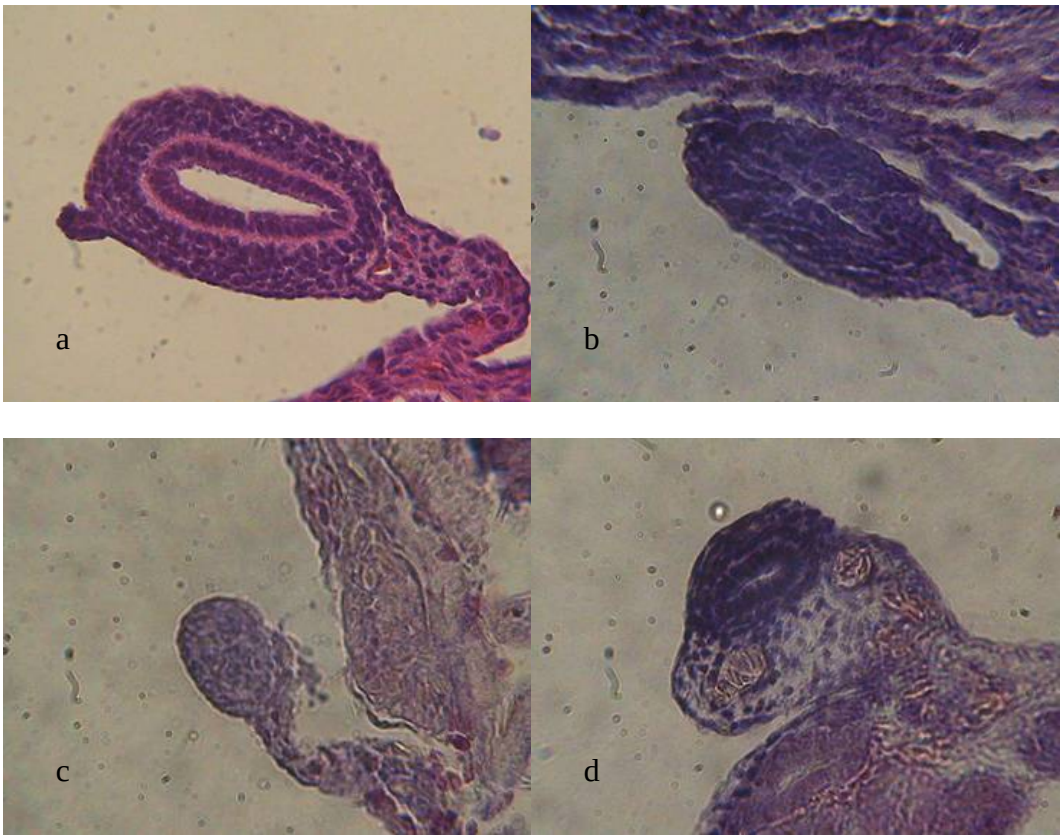

Figure 6. Examples of oviducts from different temperatures. Oviducts of females from 31ºC (a) and a regressed oviduct from a female from 28.5ºC (b). A regressed oviduct from a male at 26ºC (c) compared to a formed oviduct from a male at 29.1ºC (d).

The average areas for the oviducts and lumens and the oviduct to lumen ratio in females are shown in Table 3 and graphically in Figure 7. Females showed significant differences for the oviduct area ($p=0.04$) but not for the factors. These results are not listed for males, because values were often not obtained for more than 1 individual per temperature group.

Table 3

Oviduct measurements of females from histological analysis. Measurements are presented as mean ± standard deviation. The number sampled is included in parentheses if it was different from that originally listed.

Temp.	n	Oviduct Area	Lumen Area	Oviduct to Lumen Ratio
31ºC	15	0.74 ± 0.19	0.05 ± 0.06	0.06 ± 0.08
29.1ºC	6	0.56 ± 0.28	0.02 ± 0.02	0.07 ± 0.03
28.5ºC	3	0.69 ± 0.29	0.06 ± 0.05 (2)	0.09 ± 0.08 (2)
26ºC + E	7	0.47 ± 0.16	0.02 ± 0.02	0.05 ± 0.04

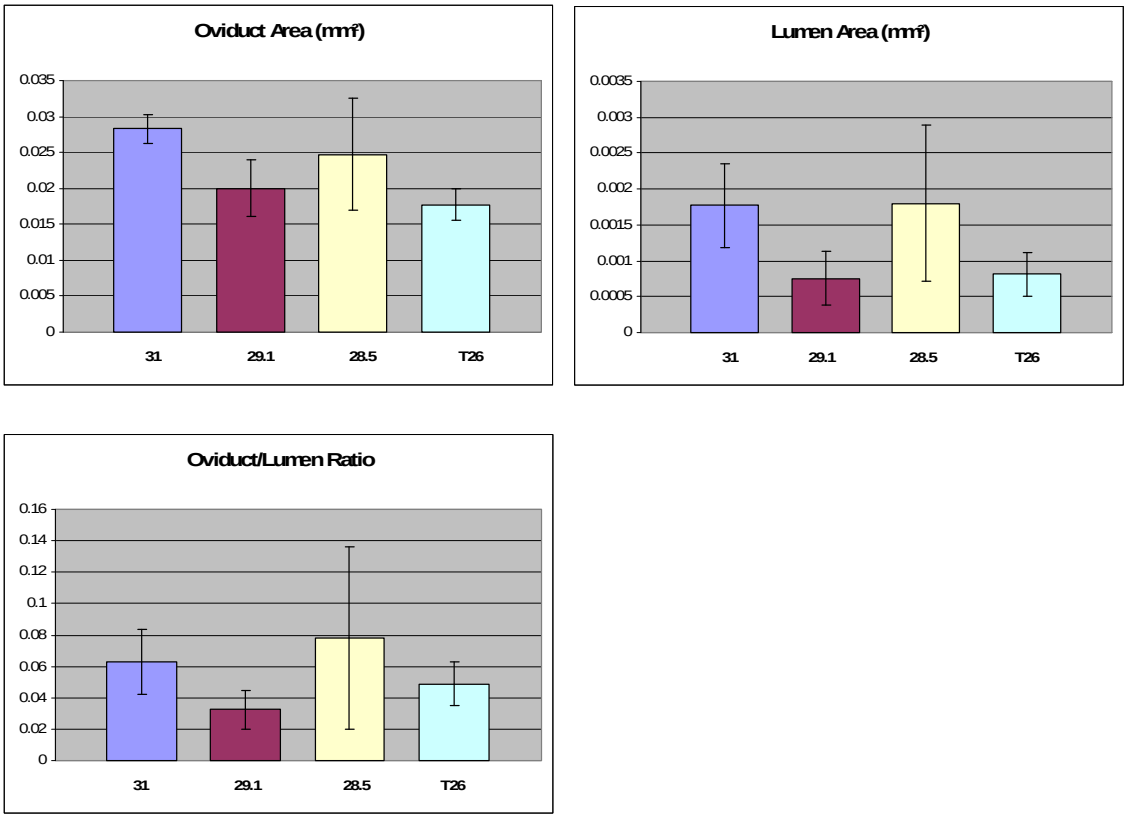

Figure 7. Comparison of the areas of the oviduct and lumen from the female-producing temperatures.

Hormone Analysis

Testosterone

The testosterone levels for females and males from the various temperatures were compared after RIA analysis. Females from 31ºC had significantly higher levels of testosterone when compared to those from intermediate temperatures and those that were sex reversed ($p=2.25x10^{-5}$). Although this may seem counterintuitive, the enzyme aromatase converts testosterone to estradiol in females (Pieau et al., 1995), so this may account for the differences. Table 4 shows the average pg content of testosterone in the AKGs of the animals from the different temperatures. All AKGs were pooled into groups of five in order to obtain readable levels. Figure 8 shows the graphical comparison of the testosterone levels in males and females.

As is other tests, males did not show significant differences in testosterone levels.

Table 4.

Plasma testosterone levels in male and female AKGs, pooled in groups of five. N = number of pools analyzed.

Temperature	Sex	n	Average pg T / mg AKG
31ºC	F	6	619.26 ± 82.55
29.1ºC	F	4	307.27 ± 88.37
28.5ºC	F	3	345.93 ± 58.06
26ºC + E	F	3	242.59 ± 30.92
26ºC	M	6	337.13 ± 112.24
28.5ºC	M	5	382.18 ± 64.06
29.1ºC	M	4	390.29 ± 85.04

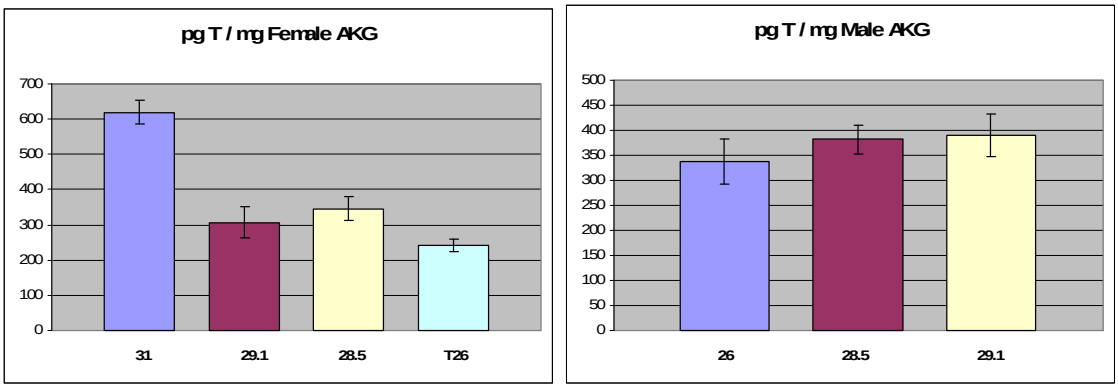

Figure 8. Plasma testosterone levels in the AKGs of males and females from different temperatures. Values indicated are in pg/mg AKG.

Estradiol

Due to the high standard deviations found, females from 31ºC did not have significantly higher levels of estradiol than the other groups. However, the highest levels recorded were from 31ºC and lower levels were recorded in the 29.1º and 28.5ºC groups. (Table 5 and Figure 9).

Males from the female-biased temperature (29.1ºC) had significantly higher levels of estradiol than those from the other temperatures ($p=0.03$).

Table 5.

Plasma estradiol levels in males and female AKGs, pooled in groups of five. N = number of pools analyzed.

Temperature	Sex	N	Average pg E / mg AKG
31ºC	F	5	65.38 ± 108.94
29.1ºC	F	4	11.88 ± 11.05
28.5ºC	F	3	28.75 ± 7.97
26ºC + E	F	3	25.50 ± 25.74
26ºC	M	6	8.11 ± 4.18
28.5ºC	M	5	4.33 ± 2.18
29.1ºC	M	4	18.17 ± 12.25

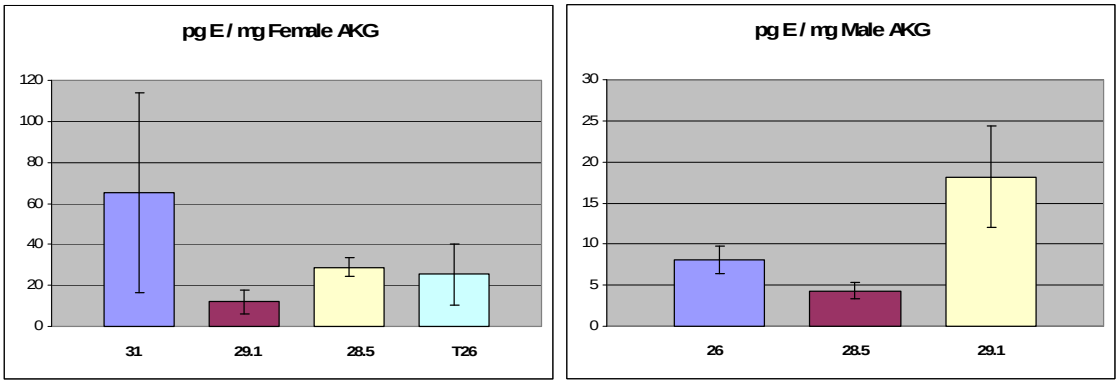

Figure 9. Plasma estradiol levels in AKGs of males and females from different temperatures. Values indicated in pg/ mg AKG.

DISCUSSION

Sea turtle conservation programs are in place around the world to help endangered sea turtle populations, and many of these utilize artificial hatcheries to incubate and protect eggs. These programs must monitor the sex ratios that they are producing in order to determine the effect the program will have on the reproductive success of the population in years to come. It is well documented that different incubation temperatures produce animals of different gender in species with TSD, but it is not understood if these animals are of the same fitness. A recent study with a lizard indicates that temperature affects adult reproductive fitness, but the mechanism behind this is unknown (Warner and Shine, 2008).

In an effort to determine which temperatures should be used in artificial hatchery programs and to test a possible mechanism for differential fitness, this study compared the gonads, reproductive tracts, and hormone levels of males and female hatchlings incubated at different temperatures. The results indicate that the females incubated at different temperatures are different in gross morphology, gonad and oviduct histology,

and endocrine physiology. The differences found in the reproductive tracts and gonads of individuals from different temperatures were significant in many categories evaluated. It was obvious that, for females, differences in incubation temperature produced variations in the morphology and endocrinology of the animal. The ovaries at the all-female temperature of 31ºC were longer, thinner, bigger overall, and produced more hormones than those from the intermediate temperatures and those that were sex reversed. The ovaries in females from intermediate temperatures were shorter than those from 31ºC and shorter than the testes of the males from the same temperatures. Likewise, the testes from intermediate temperatures were longer and less vascular than those from the all-male temperature. The gonads from 29.1º and 28.5ºC were less distinct and more difficult to classify as male or female, because they often shared characteristics of both ovaries and testes. Histologically, these gonads also often shared characteristics of both genders.

TSD could potentially have several advantages over other forms of sex determination. As discussed, this form of sex determination may have evolutionary significance because it allows a flexibility to produce males when the fitness of an animal is enhanced by being male, and vice versa when it is advantageous to be a female. For example, the results suggest that certain incubation temperatures are better for producing large ovaries, and those temperatures produce all females. In contrast, temperatures which produce smaller ovaries, produce very few females. Thus TSD could potentially be producing the most fit individual for a given incubation temperature.

It is plausible that the differences in morphology and endocrinology recorded in this study may be an indication of the future reproductive fitness of individuals. For

example, a larger ovary could produce higher hormone levels which could enhance fitness. A previous study suggested that egg incubation temperature correlated to higher adult hormone levels and adult behavior in the leopard geckos (Gutzke and Crews, 1988). Thus, these results potentially provide a mechanism by which TSD could afford a greater fitness to a particular sex.

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GENERAL CONCLUSIONS

This study addressed several aspects of TSD. The first project evaluated sex ratios produced in the Atlantic leatherback population. Specifically, hatching, immature, and adult sex ratios were examined in this unique and endangered sea turtle. This is one of the few reports of sex ratios in the leatherback sea turtle, and is the only study to examine an immature sex ratio. The immature sex ratio is of particular importance because it represents a condensation of many years of hatching production, thus providing insight on the history of sex ratios produced from the population's nesting beaches. Information of this sort is a prerequisite for generating effective management strategy for the Atlantic leatherback. For example, knowledge of naturally occurring sex ratios can provide insight into what sex ratios should be produced in egg hatchery programs. Further, these data can indicate if naturally occurring leatherback sex ratios conform to those predicted by evolutionary theory (Fisher, 1930).

Hatchling sex ratios were estimated from a major nesting beach in Florida (Juno Beach) based on nest incubation temperatures. This was in collaboration with Florida Atlantic University for the 2006 and 2007 nesting seasons with seven nests per season being examined (due to permit limitations). To do this, data loggers were placed within a nest during egg disposition and the temperature was recorded every hour for the duration of the incubation time. The predicted sex ratio was based on the average daily

temperature during the middle third of incubation, using previous pivotal temperature estimates for leatherbacks from Suriname and Costa Rica. The results suggest that both male and female biased sex ratios were produced in individual nests, but overall, a female bias was predicted.

Sex ratios were also estimated for immature and adult leatherback sea turtles captured in the North Atlantic in foraging grounds off of Nova Scotia, Canada. Turtles were captured in collaboration with the Canadian Sea Turtle Network. This is the only project world-wide which is currently capturing relatively large numbers of leatherback sea turtles at sea. Blood samples were taken from each turtle and plasma testosterone levels were assayed using a radioimmunoassay (RIA). A total of 26 turtles were captured during the late summer and fall of 2007. The turtles ranged in size from 134.2 to 171 cm in curved carapace length (CCL). Based on data from previous studies, turtles with CCL of less than 150 cm were considered immature. The distribution of testosterone levels for the immature leatherbacks was similar to that reported for immature turtles in other sea turtle species, in which laparoscopy has been used to verify sex. Since laparoscopy was not possible in this study (due to permit limitations), previously reported data for other sea turtles were used as benchmarks for determining male versus female ranges of plasma testosterone levels. The results suggest a sex ratio of approximately 1.5:1.0. (F:M) for the immature turtles captured in this study (n =10). Additionally, tail length was used as a method of predicting sex in the adult turtles, and the results indicate an approximate 0.6:1.0 (F:M) sex ratio (n =16). Neither of these sex ratios differed significantly from a 1:1 sex ratio.

The results of the first project suggest sex ratios ranging from an approximate 1:1 sex ratio for adult and immature turtles captured in the waters off of Nova Scotia, to a predicted female bias for hatchling leatherbacks produced from Juno Beach during both 2006 and 2007. The results provide one of the first estimates of hatchling leatherback sex ratios from a major nesting beach in North America. Additionally, although sample size was limited, this study provides the first report of an immature sex ratio for the leatherback sea turtle and one of the few estimates of an adult sex ratio. Data of this sort is a prerequisite to developing effective management strategy for the endangered leatherback sea turtle in the North Atlantic.

The second project in this study addressed a subject that is of importance to the evolution and conservation of reptiles with TSD: Is there an evolutionary advantage of TSD? It has been hypothesized that different incubation temperatures could result in altered sex and fitness of individuals (Shine, 1999; Warner and Shine, 2008). TSD may provide a flexibility that allows the production of males at a time when the fitness of an individual is enhanced by being a male, and vice versa when it is advantageous to be a female. If this is the case, TSD may provide a distinct advantage over systems such as the XX/XY and ZZ/ZW systems seen in mammals and birds respectively.

This project addressed the evolutionary and conservational implications of TSD by evaluating the effect of specific incubation temperatures on the morphology and endocrinology of the gonads and reproductive tracts in a turtle with TSD. The red-eared slider turtle (*Trachemys scripta*) was used as a model because its TSD is similar to that of endangered sea turtles, its eggs are commercially available, and its TSD has been well-described. An experimental protocol was utilized in which the gonads and reproductive

tracts were compared between late-stage embryos incubated at temperatures that produced either 1) all females, 2) mostly females, 3) mostly males, or 4) all males. The gross morphology and histology of the reproductive tracts of these four groups were compared and the testosterone and estradiol content was measured. The results indicate significant variation between the females incubated at different temperatures and moderate differences in the males from those same temperatures. The results suggest that all-male and all-female producing temperatures may be optimal for producing a desired sex ratio in an egg hatchery. Additionally, the results provide a potential physiological basis for the differential fitness hypothesis, thus providing information addressing the evolutionary basis of TSD.

Collectively, the results of these projects provide insight into two key topics concerning TSD. First, the natural sex ratios that were evaluated in the Atlantic leatherback are a prerequisite for understanding the ecology of this critically endangered sea turtle and for developing conservation strategies for its recovery. Second, the effects of incubation temperature on the morphology and endocrinology of the gonads and reproductive tracts of a turtle with TSD provide a possible link between temperature and reproductive fitness thus providing a potential mechanism for the evolutionary advantage of TSD. Both findings have significant implications for the conservation, ecology, and evolution of reptiles with TSD.

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APPENDIX: IUACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: October 23, 2007

TO: Thane Wibbels, Ph.D.
CH 255 1170
FAX: 975-6097

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Temperature-Dependent Sex Determination
Sponsor: Internal
Animal Project Number: 071007370

On October 23, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Turtles	A	60

Animal use is scheduled for review one year from October 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 071007370 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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