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Temperature-Dependent Sex Determination in Manouria Emys Emys, The Asian Forest Tortoise

Sherri Ann Emer

TEMPERATURE-DEPENDENT SEX DETERMINATION IN *MANOURIA EMYS EMYS*, THE ASIAN FOREST TORTOISE

by

SHERRI EMER

Under the Direction of Matthew Grober

ABSTRACT

Captive husbandry programs in zoos have documented nesting behavior and have successfully hatched *Manouria emys emys*, but data on sex determining mechanisms and sex ratios are absent. A total of 30 *M. e. emys* eggs were artificially incubated at five different temperatures in constant humidity. Mean incubator temperatures were 24.99°C, 25.06°C, 27.18°C, 28.00°C, and 30.79°C. Incubation duration ranged from 60 days to 92 days, and hatching success was 50%. Sex determined by histology and laparoscopy resulted in male differentiation at low temperatures (24.99°C, 27.18°C) and female differentiation at high temperatures (30.79°C). Pivotal temperature was estimated to be 29.29°C. The following investigation into temperature-dependent sex determination (TSD), including its presence or absence, pattern, and pivotal temperature, has implications for studies of adaptive significance of reproductive behaviors and of chelonian phylogenetic history. Additionally, the proposed study can provide foundations for conservation management decisions, and for captive breeding programs.

INDEX WORDS: *Manouria emys*, Burmese brown tortoise, Asian forest tortoise, Temperature-dependent sex determination, Pivotal temperature, Sex ratio, Reproduction, Hatching success, Conservation.

TEMPERATURE-DEPENDENT SEX DETERMINATION IN *MANOURIA EMYS EMYS*, THE ASIAN FOREST TORTOISE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

TSD	Temperature-dependent sex determination
IUCN	International Union for the Conservation of Nature
KG	Kidney-gonad
SD	Standard deviation

1 Introduction

1.1 *Manouria emys emys* natural history

First described by Schlegel and Müller in 1844, *Manouria* belongs to the class Reptilia, order Tesudines, family Testudinidae. There are two species in the genus, *M. impressa* and *M. emys*; *M. emys* is divided into two subspecies, *M. emys phayrei* (Blyth 1853) and *M. emys emys*. The tortoise described in this study is the latter, *M. emys emys*. Considered the most primitive of extant tortoises (Pritchard 1979), *M. e. emys* exhibits a lack of derived morphological characteristics (Crumly 1982; Crumly 1984), early stages of tortoise-like shells (Highfield 1990), and modified carpal bones (Auffenburg 1966). Ecologically, *M. e. emys* is important to forest maintenance as it contributes to the cleanup of leaves, fungi, and fruit on the forest floor and to fruit seed dispersal via its feces (Alderton 1988; McKeown et al. 1991; Moll 1989). Because of its decreasing numbers and inconspicuous habits, little is known regarding the tortoise's biology. *M. e. emys* is listed as endangered by International Union for the Conservation of Nature (IUCN), and data contributing to its biology is crucial for future management recommendations.

1.1.1 Geographic distribution and habitat

Manouria emys emys is native to the upland mesic habitats of Southeast Asia (McKeown et al. 1991) including Malaysia, Sumatra, Borneo and India (Moll 1989). These areas experience a tropical monsoon climate characterized by two monsoon seasons. The wetter, Northeast monsoon that occurs between November and March, and

the drier Southwest monsoon which occurs between June and July. Average annual rainfall is approximately two meters (~80 inches) (Figure 1.1.1) and humidity is high throughout the year. Høybye-Mortensen (2004) found that humidity measurements at the forest floor during the daytime rarely fell below 90%.

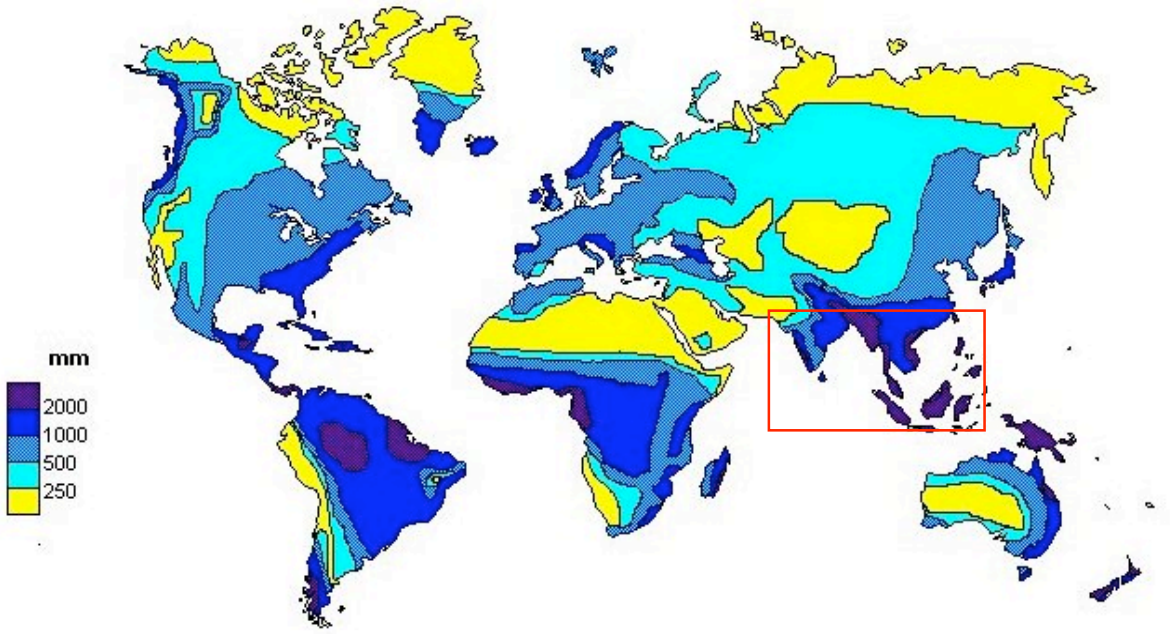

Figure 1.1.1 Annual global rainfall in millimeters. *M. emys* distribution indicated in red, area receiving 1000-2000 mm of rain annually (Jones 2002).

In a recent study of 14 adult individuals within the Tabin Wildlife Reserve in Northern Borneo, wild animals equipped with radio transmitters were observed in deep gullies, on the steep sides of ravines, and near the top of the hills (Høybye-Mortensen 2004). Of the study animals, tortoises were found to prefer activities like walking and

foraging at ambient temperatures between 25.8°C and 34.6°C. When nighttime temperatures dropped, and when daytime temperatures increased, the tortoises hid under debris or tree falls for insulation (Høybye-Mortensen 2004).

1.1.2 Morphology

Manouria emys emys often lacks the common sexually dimorphic characteristics that many other tortoises possess. There are subtle differences between males and females, however either can reach approximately 50 cm carapace length and 31 kg (Wirot 1979). Suggested male characteristics include longer, wider tails (Auffenburg and Iverson 1979; Ernst and Barbour 1989; Schaffer and Morgan 2002), a concave plastron (Ernst and Barbour 1989), and a bulging fifth central scute (Morgan and Schaffer 2001). Various scute lengths and widths have been used to distinguish males and females (Aranyavalai 1996), but the most dependable method to determine adult sex is likely by gonad identification via laparoscopy. Steroid hormone assays have been suggested (Høybye-Mortensen 2004), but records of basal levels do not exist in peer-reviewed literature.

Believed to be the most primitive of extant tortoises, *M. e. emys* lack some derived morphological characteristics of tortoises such as early stage tortoise-like shells (Crumly 1982; Pritchard 1979; Highfield 1990). Additionally, Crumly and colleagues (2004) examination of the carpal bones reveals that *Manouria* possess a single reduced phalangeal formula, which is thought to be a primal condition (Figure 1.1.2).

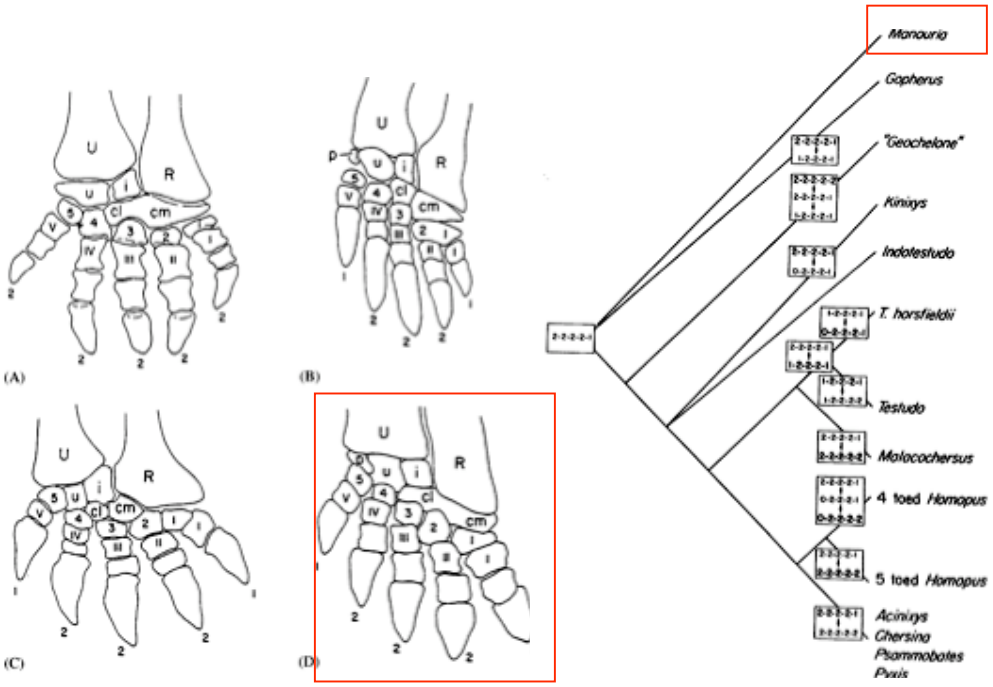

Figure 1.1.2 Difference in carpal bones in different Testudines and their phylogenetic relationships (Crumly and Sanchez-Villagra 2004).

1.1.3 Reproduction

Reproductive behavior in captive *M. e. emys* occurs throughout the year (Morgan and Schaffer 2001). Accounts of mating in the wild are limited. The record of two males attempting to mate with a single radio-tagged female in Borneo (Lambert and Howes 1994) and the June 2002 record of radio-tagged male and female study animals (Høybye-Mortensen 2004) may be the only documented observations. Behaviors associated with courtship and mating in captivity include head bobbing (McKeown et al. 1991), trailing (Auffenburg 1977), and vocalizations (McKeown et al. 1991), all of which have also been observed in the wild (Lambert and Howes 1994; Høybye-Mortensen 2004).

M. e. emys exhibits unique nesting behavior, similar to that of the American alligator (Deitz and Hines 1980) and of megapode birds, such as the brush turkey (Vleck et al. 1984), where the female constructs an above ground mound from leaf litter (Figure 1.1.3). Mound building, which is characterized by back sweeping leaf litter into a mound, begins several days prior to nesting. Just prior to oviposition, the female burrows head first into the mound, and excavates a cavity. Following oviposition, the female back sweeps to fill in the egg chamber; she continues this method increasing the size of the mound over the next several days (McKeown et al. 1991). The mound can reach heights of 91 cm and diameters of 2.5 m (McKeown 1999). The female may also guard the completed nest containing the eggs. This is accomplished by either pushing the intruder or by laying flat on top of the mound (McKeown et al. 1991). According to McKeown (1991) and colleagues' observations using mock predators, this behavior ceases a few days following oviposition; however, Eggenschwiler (2003) reported six weeks of nest guarding. Captive females nest once annually, between April and October, and clutches range from 21 to more than 53 eggs, which incubate for 63 to 84 days (McKeown et al. 1991).

Figure 1.1.3 Adult female from which eggs were obtained for this study, sitting on the side of the mound nest.

In July 2002, Høybye-Mortensen (2004) documented a wild female nesting in Borneo on top of a hill in secondary forest with dense canopy cover. The female stood up on all limbs with an outstretched neck, likely a display of aggressive/defensive behavior, then proceeded to lay on top of its mound. Although oviposition was already complete, the female continued to add twigs and leaves to the mound. In just one hour of observation, the female exhibited repeated sniffing and back sweeping behaviors, as well as lying on top of the nest. The behaviors continued for 2 days following the initial discovery. The nest was predated 10 days later, presumably by a civet, at which point a temperature logger was placed in the egg chamber. Temperature was recorded every 20 minutes for 9 days (Figure 1.2.2). A heavy rain during the course of measurements

caused egg chamber temperature to drop, but temperatures appeared to maintain within the range of ± 1°C thereafter (Høybye-Mortensen 2004).

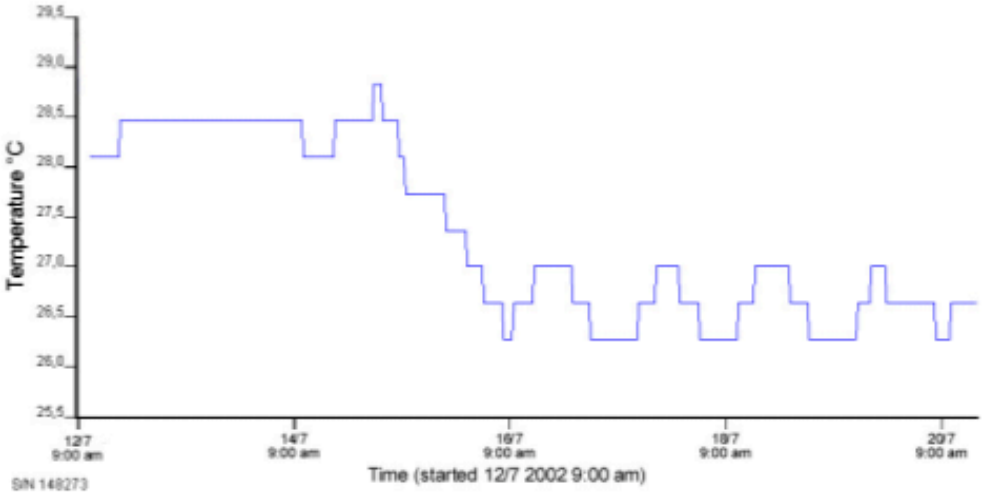

Figure 1.2.1 Nest temperatures within the egg chamber of a wild *M. e. emys* in Borneo (Høybye-Mortensen 2004).

1.1.4 Status

Manouria emys emys is listed with the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List as endangered, as defined by:

- population reduction in the form of an observed, estimated, inferred or suspected reduction of at least 50% over the last 10 years or three generations, whichever is the longer, based on (and specifying) a decline in area of

occupancy, and extent of occurrence and/or quality of habitat actual or potential levels of exploitation;

- population reduction of at least 50%, projected or suspected to be met within the next 10 years or three generations, whichever is the longer, based on (and specifying) a decline in area of occupancy, and extent of occurrence and/or quality of habitat actual or potential levels of exploitation (http://www.redlist.org).

The decline is attributed to exploitation for food, medicine, and pet trading as well as habitat destruction for palm oil plantations (McKeown, 1991).

1.2 Temperature-dependent sex determination

Some reptiles and most chelonians exhibit temperature-dependent sex determination (TSD) where the temperature at which the eggs are incubated determines gonad differentiation. Reptiles that possess TSD exhibit different patterns as defined by Ewert and Nelson (1991) (Figure 1.2.1). Pattern Ia (*Trachemys scripta*), the most common pattern, produces female-biased ratios at warmer temperatures and male-biased ratios at cooler temperatures (Ewert and Nelson 1991). Pattern Ib (*Sphenodon guntheri*) is characterized by increased male production at warmer temperatures and increased female production at cooler temperatures (Mitchell et al. 2006). Female-biased sex ratios at both cooler and warmer temperatures (*Chelydra serpentina*), and male-biased ratios at intermediate temperatures is characteristic of Pattern II (Ewert and Nelson 1991). Gonad

differentiation occurs during a thermosensitive period in embryogenesis, usually the middle third of development (Bull 1980).

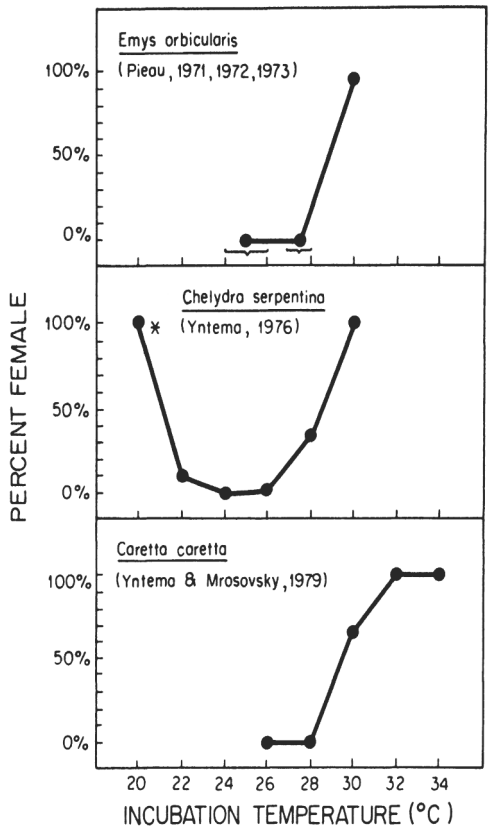

Figure 1.2.2 Percent female hatchlings yielded at various incubation temperatures in three different turtle species (Mrosovsky and Yntema 1980).

The mechanism of action of incubation temperature is poorly understood, but multiple models have been proposed (Crews et al. 1995; Pieau and Dorizzi 2004). A direct model suggests that temperature-sensitive promoters are associated with genes encoding for steroidogenic enzymes such as aromatase and reductase. These enzymes

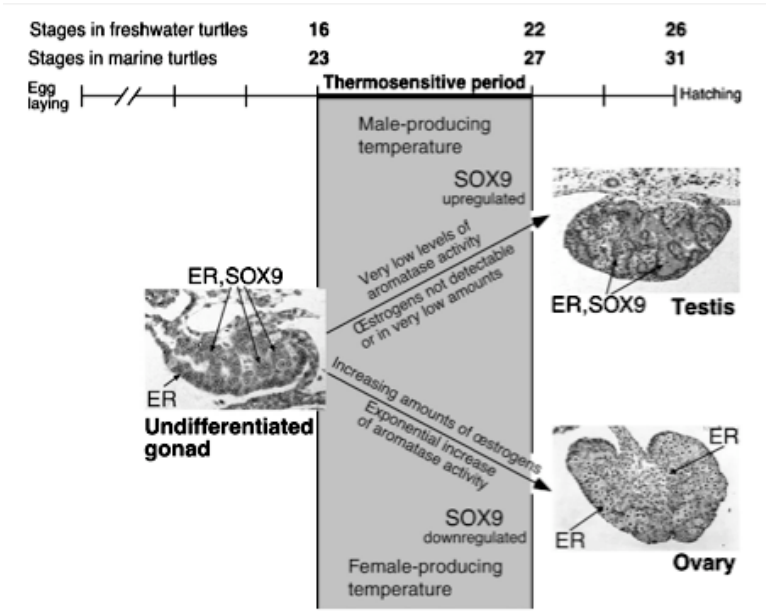

Figure 1.2.3 Molecular and physiological events involving SOX9 gene regulation and estrogen levels during gonad differentiation (Pieau and Dorizzi 2004).

convert testosterone (T) to estradiol (E_2) or dihydrotestosterone (DHT), respectively. In this scenario, aromatase genes would be activated and reductase genes inhibited at female-producing temperatures. Similarly, at male-producing temperatures, reductase genes would be activated and aromatase genes inhibited. Pieau and colleagues (1994) found a positive feedback between E_2 and aromatase at female-producing temperatures in a pond turtle. On the contrary, an indirect model suggests feedback control of product formation, as opposed to activation or inhibition of the genes encoding for the products. At female-producing temperatures, androgens would be metabolized to E_2, causing a positive feedback on androgen conversion, and negative feedback that would inhibit reductase (Crews et al. 1995). Most recently, SOX9 expression, an autosomal gene

involved in mammalian testicular formation (Yashi et al. 2005) was detected in the differentiating gonads of the marine turtle, *Lepidochelys olivacea*. Expression was upregulated in gonads incubated at male-producing temperatures, and was downregulated in those incubated at female-producing temperatures (Moreno-Mendoza et al. 2001) (Figure 1.2.3).

1.3 Objectives

TSD has not been previously examined in *M. e. emys*, but Zoo Atlanta has documented female biased ratios of eggs incubated at 28°C. It is hypothesized that *M. e. emys* will exhibit Pattern Ia TSD, with a threshold temperature around 27°C. Hatchlings incubated at 23-25°C are expected to be male biased, and those at 29-31°C female biased. It is anticipated that sex ratio at 27°C will be mixed. Knowledge of sex ratios produced at various temperatures provides insight into captive breeding and conservation practices. Additionally, the proposed study will be the first to verify TSD, define TSD pattern and estimate pivotal temperature in this species. Nest temperature can be affected by air temperature, rainfall, and decomposition; therefore, mound nesting may be an adaptive strategy that influences sex ratio, hatching success, mortality rates and hatchling performance.

2 Methods

2.1 Incubator design

Traditional incubation methods for TSD research involves the use of commercial incubators, however, in this scenario, commercial incubators would not suffice as they are designed to maintain higher temperatures than what this species can withstand. Additionally, some may not maintain humidity, and if they do, it is by a water reservoir on the inside, which requires opening/closing the instrument. Initially, a single incubator was designed, constructed, and tested prior to preparation of additional units. A tightly fit cover was constructed from Styrofoam™ with grooves for the electrical cords. The aquarium was filled with approximately six inches of water to maintain humidity. A 50-watt submersible heater with a thermostat (Rena Cal Top Light Excel by RENA®) was secured on the bottom, completely submerged, and the thermostat was set to 80°F (~27°C) to maintain constant heat. A wire rack was placed in the water. A plastic container containing a 1:1 vermiculite:water mixture and a digital probe thermometer (Coralife® Digital Thermometer by Energy Savers Unlimited) was placed on top of the rack. A digital hygrometer-thermometer (Fluker Farms) was attached to the inside wall near the top of the chamber. The substrate temperature was monitored externally twice daily via the probe digital thermometer, and humidity was recorded once daily. Monitoring was conducted in February 2006. The system maintained humidity, but temperature was poorly maintained at a large range. The incubator was modified by adding double-sided reflective insulation (R value = 3.5) around the perimeter and to the

bottom. Temperature readings were recorded again for a month and were maintained ±2°C.

Three additional modified incubators (Figure 2.1.1**)** were constructed following examination of initial temperature data. A 10-gallon aquarium was modified by attaching double-sided reflective insulation (R value = 3.5) around the perimeter and to the bottom. The plastic container was replaced with vented critter cages, each of which still contained the probe thermometer, and a LogTag data logger (LogTag Recorders Ltd.) was added to each. Each data recorder was configured to record temperature hourly and each was placed in a watertight case (ROC Gear Inc.) containing eight-mesh desiccant $CaSO_4$ (Drierite, W. A. Hammond Drierite Company Ltd.) to prevent corrosion due to high humidity. Thermostats were set to 23°C, 25°C, 27°C, 29°C and 31°C. An air pump was also added to each unit to circulate air for the prevention of hot spots. Hygrometers were not used in the final incubator design as high humidity (≥90%) eventually caused complete failure to operate. The complete incubators were arranged in a temperature-controlled (68°F) closed room at Zoo Atlanta, Herpetology Department.

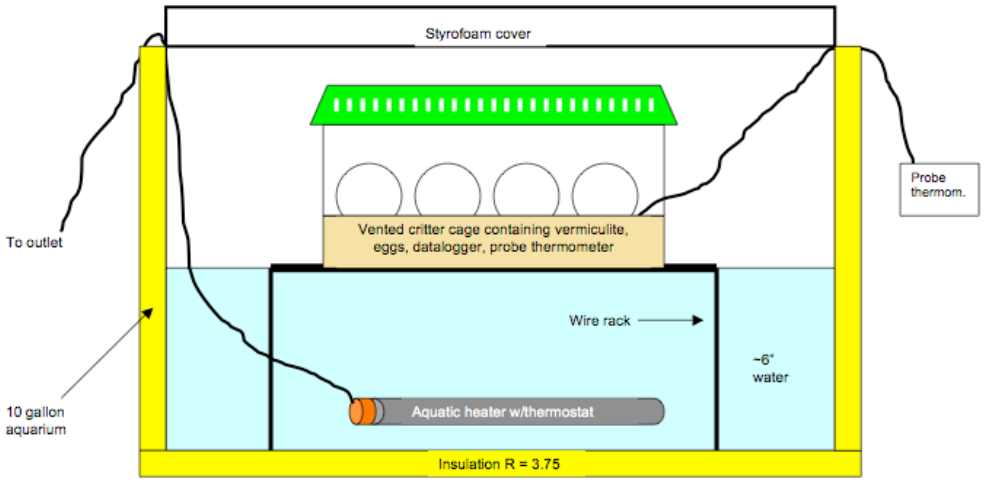

Figure 2.1.1 Final design used to incubate *M. emys* at constant temperatures.

2.2 Egg collection and study animals

Eggs for the following study were obtained from a single female 26 August 2006. Eggs were immediately relocated from the mound to the incubators located at Zoo Atlanta. Six eggs were placed in each of the five incubators. Additional eggs from a different gravid female were anticipated for use in the study, however, despite mound construction and administration of pitocin, oviposition never occurred.

2.3 Incubation monitoring

Although substrate temperatures were recorded hourly for the duration of incubation, the external probe thermometers were checked daily to confirm temperature maintenance. Visual observation of the eggs began at 50 days. At the first signs of pipping, the number of pipped eggs, completely hatched eggs, unhatched eggs, and rotten eggs were notated for each treatment. Unhatched eggs remained in the incubators until

the conclusion of the experiment, which was marked by the final hatching in the lowest temperature treatment. All remaining eggs were relocated to the freezer for later examination.

2.4 Post-hatching procedures

Hatchling tortoises remained in the critter cages within the incubators for 3-4 days post-hatching to allow for complete digestion of the yolk sac. They were then marked with fingernail paint according to treatment. The dorsal side of the left marginal scutes indicated the first digit of the temperature and the dorsal side of the right marginal scutes indicated the second digit of the temperature (Figure 2.4.1).

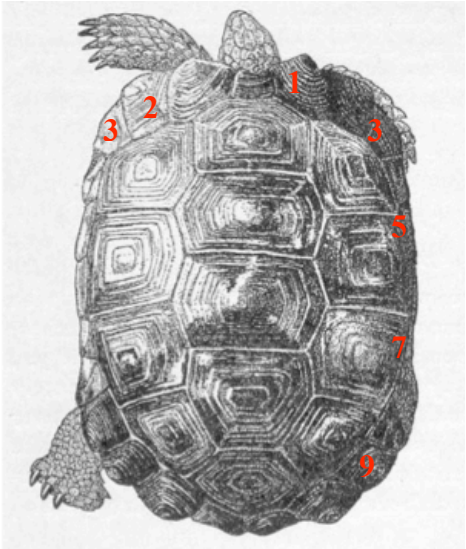

Figure 2.4.1 Marking system used to distinguish hatchlings incubated in five, constant- temperature chambers (illustration from Schaffer 2002).

2.5 Histology

Gonadal histologies of deceased embryos were examined to determine sex. The previously frozen, unhatched eggs were thawed in hot water and then dissected. Partially developed embryos were removed from the egg and were further examined under a dissecting microscope. The caudal plastron was removed to reveal internal organs, specifically, the dorsally situated kidney-gonad complex (KG). Once identified, a single KG was removed by cutting transversely midway down the kidney, caudal to the gonad, so that the KG specimen was cone-shaped (Figure 2.5.1). The KG was placed in a plastic cassette and added to jar containing a 4% paraformaldehyde solution. The hatchling containing the other intact KG was also added to the jar.

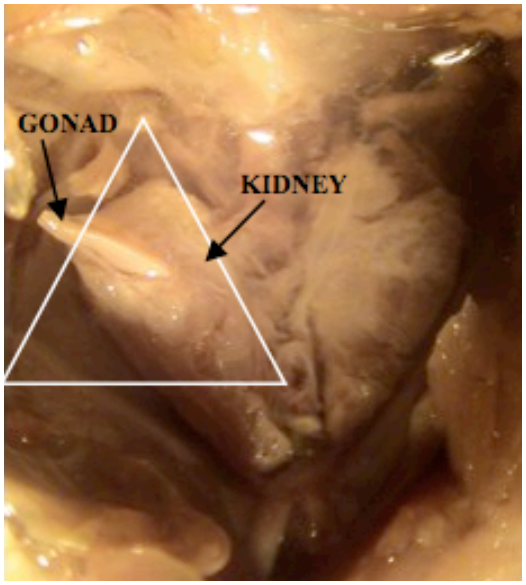

Figure 2.5.1 Incision made to kidney-gonad complex.

Tissue dehydration was conducted in a Shandon Citadel™ 1000 tissue processor (Thermo Fisher Scientific, Inc.) on program B for 24 hours. Once removed from the final paraffin infiltration step, KGs were embedded in paraffin using a Shandon Histocentre™ 2 tissue embedding system (Thermo Fisher Scientific, Inc.). Embedded AKGs were sliced on a microtome and then mounted on slides. Slides were stained in hematoxlin and eosin.

Gonad characterization was based on histology described by Wibbels (unpublished laboratory manual). Ovaries were identified by the presence of a dark cortex and a disorganized medulla, as well as a pronounced oviduct extending away from the kidney. Testes were identified by the lack of a dark cortex and a degenerating, or lack of an oviduct. The medulla is highly organized into seminiferous tubules.

Two deceased specimens were submitted to the University of Georgia Infectious Disease Laboratory at the College of Veterinary Medicine for necropsy and gonad histology.

2.6 Laparoscopy

Tortoise sex was determined via laparoscopy when hatchlings reached five months of age as described by S. Rivera (personal communication, March 2007). 0.02 mL 2% lidocaine was administered subcutaneously to each tortoise at the incision site. Each was placed in lateral recumbency with the caudal end at a 60° angle to displace the bladder and intestinal tract from the incision site. The right hindlimb was extended and a stab incision was made on the skin in the cranial inguinal area. Blunt dissection, with a

curved mosquito hemostat, was then used to enter the coelomic cavity. Each animal was turned upright and the telescope (Diameter 2.7 mm) was inserted through the incision to identify the gonads visually. Gonad identification was based on morphology described by Rostal and colleagues (1994); testes were characterized as lobular structures while ovaries were characterized as granular structures with developing follicles.

2.7 Pivotal temperature estimation

Pivotal temperature was estimated using the traditional method of determining sex ratio closest to the 50% level (Mrosovsky and Pieau 1991). Incubation duration (days) was determined for each hatchling. Hatchlings were then grouped by duration. The sex ratio was calculated for each duration group and a non-linear curve was fitted to the duration-ratio data. The duration corresponding to the point where the regression line crossed 50% of the ratio axis was equivalent to the pivotal incubation duration. Incubation temperature was plotted against incubation duration and the pivotal incubation duration was located. The temperature corresponding to the pivotal incubation duration defined the pivotal temperature.

2.8 Statistical analyses

Descriptive statistics (mean, standard deviation, and range) on the temperature data for each incubator were determined using LogTag Analyser software by LogTag Recorders Ltd. for Windows™. Sex differentiation data for each incubator was tested for significance using a chi-square analysis conducted in Microsoft® Excel® for Mac. The

non-linear curve for the ratio-duration data and the linear regression for the temperature-duration data were fitted in Prism 4 for Macintosh by GraphPad Software, Inc.

3 Results

3.1 Incubator trials

Data manually recorded from the probe thermometer resulted in maintenance of temperatures between 24.9°C and 28.4°C, with a mean of 26.7°C (SD = ± 0.7°C). Humidity was ranged from 68% and 99%, with a mean of 84.4% (SD = ± 9.8%). The addition of insulation resulted in the maintenance of temperatures within a ±2°C range, and humidity levels from 90-98%. Manually recorded results prompted incubator modifications and hourly temperature monitoring. The three additional incubators in which temperature was monitored hourly via data recorders resulted in mean temperatures of 26.4°C, SD = ±0.51°C, 32.1°C, SD = ±1.38°C, and 32.7°C, SD = ±1.76°C. Percent humidity was not recorded through the entire course of the experiment because the digital hygrometer ceased to function as a result of extreme condensation.

3.2 Incubation temperatures

Incubator temperatures varied slightly throughout the duration of incubation. Incubator temperatures (mean ± SD, range) were 30.79°C ± 0.44°C (26.5°C-31.6°C) (Figure 3.2.1), 28.00°C ± 0.40°C (26.9°C-29.6°C) (Figure 3.2.2), 27.18°C ± 0.66°C

(25.4°C-28.6°C) (Figure 3.2.3), 25.06°C ± 1.20 (21.2°C-28.0°C) (Figure 3.2.4), and 24.99°C ± 1.58°C (20.0°C-28.7°C) (Figure 3.2.5).

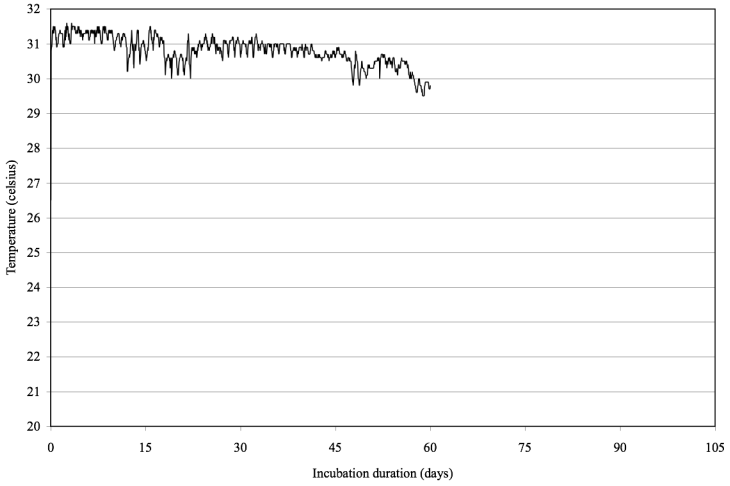

Figure 3.2.1 Hourly temperature recordings for incubator with a mean of 30.79°C, duration of 60 days.

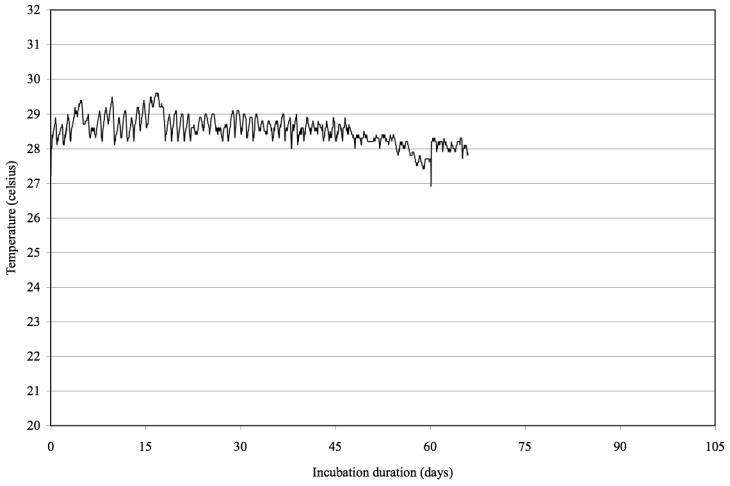

Figure 3.2.2 Hourly temperature recordings for incubator with a mean of 28°C, duration of 65 days.

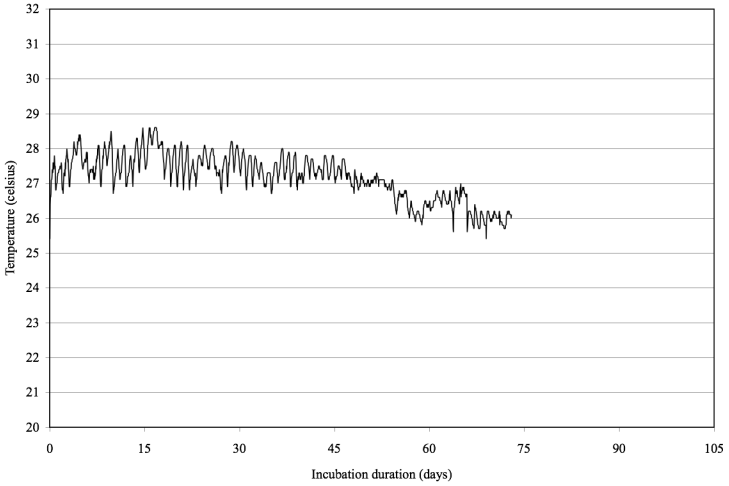

Figure 3.2.3 Hourly temperature recordings for incubator with a mean of 27.18°C, duration of 72 days.

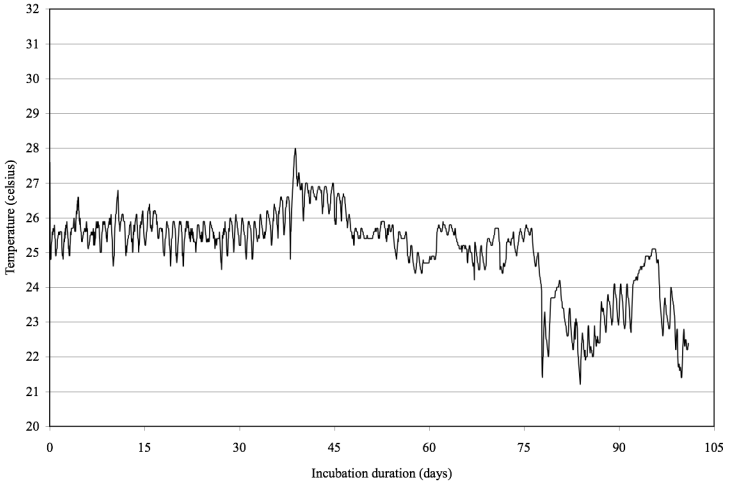

Figure 3.2.4 Hourly temperature recordings for incubator with a mean of 25.06°C.

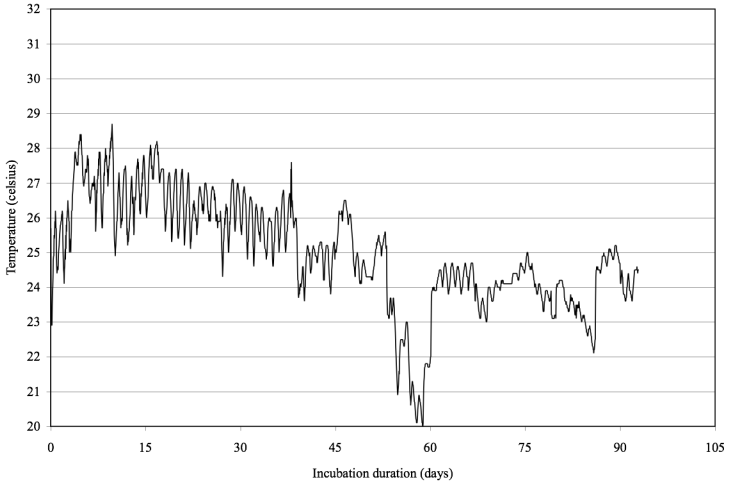

Figure 3.2.5 Hourly temperature recordings for incubator with a mean of 24.99C, duration of 92 days.

3.3 Incubation duration and hatching success

Of the 30 eggs incubated, 15 hatched yielding a hatching success of 50%. Incubation duration for eggs within the mean temperature 30.79°C was 60 days. Incubation duration was 65 days for eggs within the mean temperature 28.00°C, 72 days for eggs within the mean temperature 27.18°C, and 92 days for eggs incubating at a mean temperature of 24.99°C (Table 3.3.1). Of the six eggs in each of the incubators, four hatched from each. The incubator with a mean temperature of 25.06°C malfunctioned on day 11, indicated by a decrease in temperature and humidity (lack of condensation on the inside). All eggs from this incubator were visibly damaged thereafter, but eggs remained in the incubator an additional 60 days, and no hatching occurred.

Table 3.3.1 Mean temperature ± SD, range, hatching success, sex ratio, and incubation duration for five incubators.

	Temperature °C				
mean ± SD (range)	24.99 ± 1.58 (20.0-28.7)	25.06 ± 1.20 (21.20-28.00)	27.18 ± 0.66 (25.4 - 28.6)	28 ± 0.40 (26.9-29.6)	30.79 ± 0.44 (26.5-31.6)
No. hatched eggs	3	0	4	4	5
Percent male	100	n/a	100	n/a	0
Incubation duration (days)	92	n/a	72	65	60

Table 3.3.2 Numbers of hatched animals, deceased hatchlings, embryos, and undeveloped eggs.

	Temperature °C				
mean	24.99	25.06	27.18	28	30.79
No. hatched eggs	3	0	4	4	4
Deceased hatchlings	1	n/a	1	0	2
Unhatched embryos	1	n/a	1	0	1
No development	2	n/a	1	2	1

3.4 Histology

Histological examination was conducted on seven specimens. Two specimens, both deceased hatchlings from the 30.79°C incubator, were sent (for necropsy) to the University of Georgia Infectious Disease Laboratory at the College of Veterinary Medicine, which determined both specimens to be female. The remaining five were kept for in-house processing and consisted of three well-developed embryos and two deceased hatchlings. Tissue from the embryos was severely degraded therefore sex was unable to be determined. However, tissue from the deceased hatchlings was in better condition, and sex was determined as male for both specimens. Both exhibited seminiferous tubules, a characteristic of testis (Figure 3.4.1).

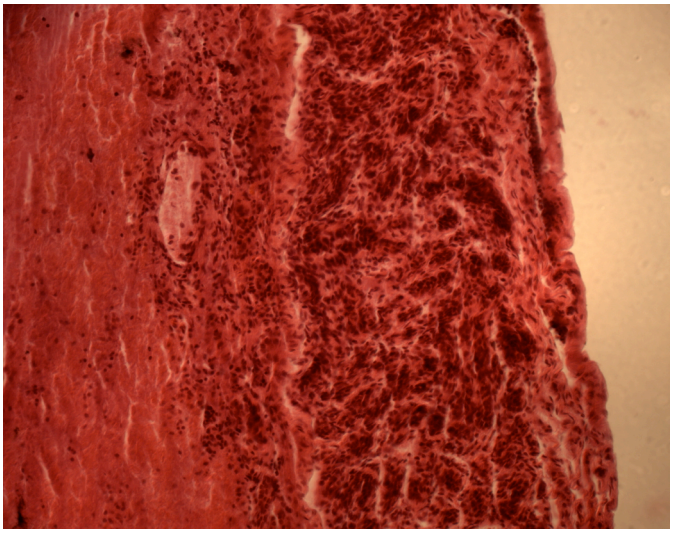

Figure 3.4.1 Transverse section through hatchling *M. e. emys* gonad incubated at 27.18°C.

3.5 Laparoscopy

Laparoscopic examinations of six *M. emys* hatchlings resulted in four males and two females (Figure 3.5.1). Ovaries were opaque with granular follicles and a large blood vessel. Testes were yellow lobes with many small vessels. One male hatchling was incubated at 24.99°C and the other three males were incubated at 27.18°C. Both females were incubated at 30.79°C.

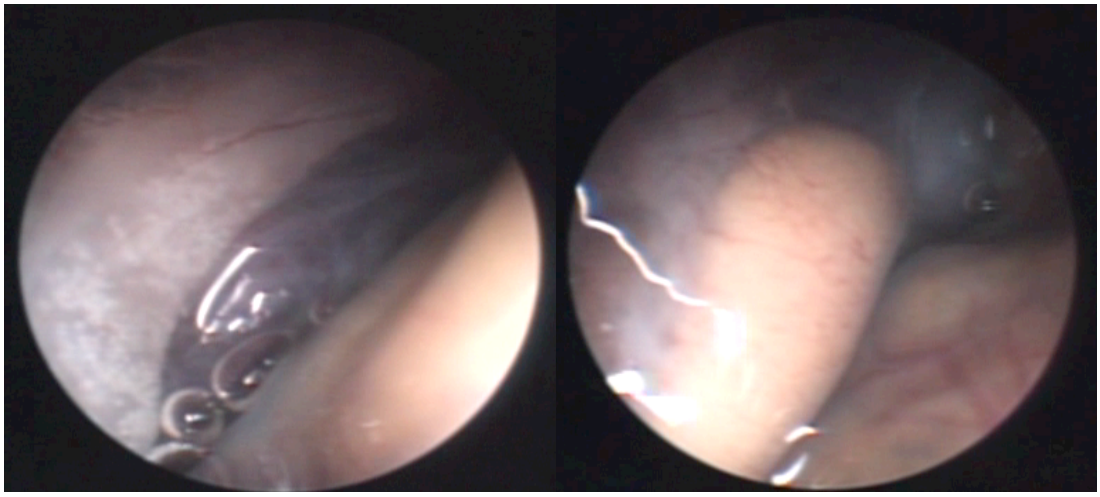

Figure 3.5.1 Laparoscopic images of an ovary (left) with developing follicles and a large vessel, and of a highly vascularized testis (right).

3.6 Sex ratio and pivotal temperature

The proportion of male hatchlings was 100% at 24.99°C and at 27.18°C and 0% at 30.79°C (Table 3.3.1). Sex ratio in this study was affected by temperature ($p = 0.035$; χ^2 test: $\chi^2 = 10$, d.f. = 2), male-biased at 24.99°C and 27.18°C and female biased at 30.79°C ($p = 0.13$; χ^2 test: $\chi^2 = 4.06$, d.f. = 2).

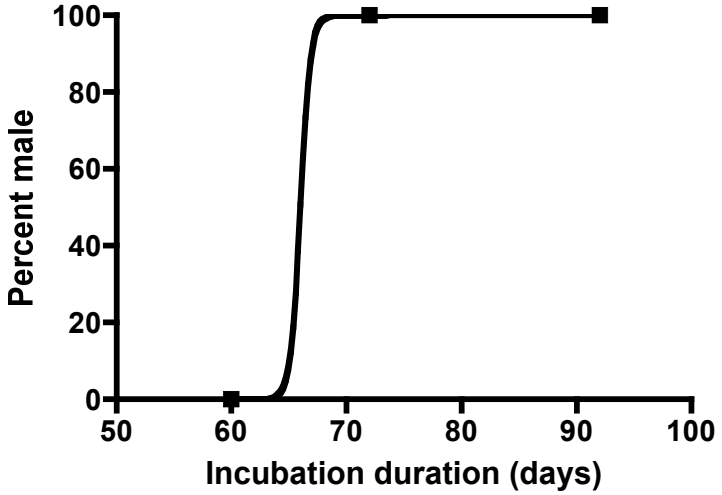

Figure 3.6.1 Relationship between sex ratio and incubation duration. Pivotal incubation duration was determined by locating the day corresponding to 50% males. The non-linear curve was fitted using Prism 4 software.

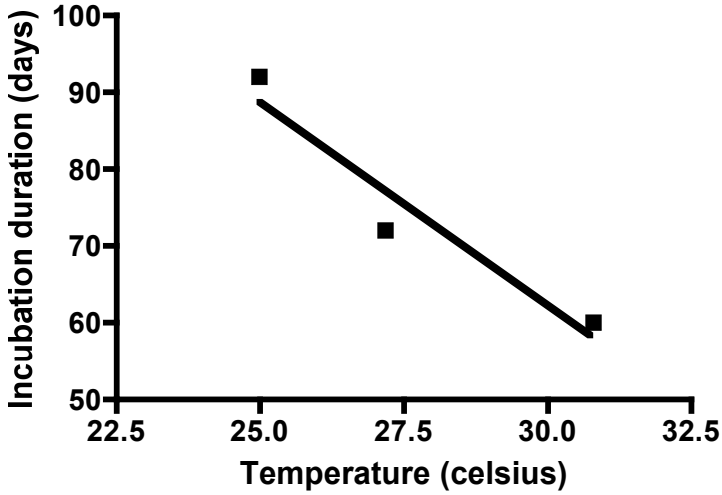

Figure 3.6.2 Relationship between incubation duration and temperature. Pivotal temperature was determined by locating the temperature corresponding to the pivotal incubation duration. The linear regression was fitted using Prism 4 software.

Pivotal incubation duration was estimated to be 66 days (Figure 3.6.1) and the pivotal temperature was estimated to be 29.29°C for this experiment (Figure 3.6.2).

4 Discussion

Investigations into the TSD of *M. e. emys* have not been conducted previous to this experiment. Zoos and breeders have documented successful artificial incubation of this species; however, hatchling sex has not been reported. Temperatures for artificial incubation have ranged from 23°C to 33°C (McKeown et al. 1991); however, temperature throughout incubation has not been reported, nor is it clear if eggs were housed in commercial incubators, homemade chambers, or enclosures. Furthermore, reported temperature settings resulting in successful hatching are confusing because of incorrect temperature conversions. The results of this study suggest that *M. e. emys* exhibits TSD where hatchlings incubated above 29°C differentiate into females, and those incubated below 29°C differentiate into males. Additionally, the results suggest that temperature may also contribute to hatching success and post-emergence hatchling survival. Incubation conditions, TSD evolution, TSD adaptive significance, and conservation implications are discussed.

4.1 Incubation conditions, duration and success

Incubation conditions contribute to incubation duration, hatching success, hatchling size (Spotila et al. 1994), locomotive performance (Miller et al. 1987), reproductive behavior, endocrine physiology (Gutzke and Crews 1988), and ultimately survival

(Spotila et al. 1994). The data demonstrate an inverse relationship between temperature and incubation duration, which is typical for amniote eggs (Deeming and Ferguson 1991). Warmer temperatures cause increases in new tissue synthesis and maintenance of previously formed tissue (Booth et al. 2000). Long incubation durations may result in higher metabolic expenditure because of the costly maintenance of existing tissue (Booth et al. 2000). The incubation durations in this study exhibit large differences for small differences in incubation temperature, for example, the 5.8°C difference between the warmest and coolest incubators resulted in more than a month difference in incubation duration. This is consistent with a 52-day difference in duration over a 7°C temperature difference in artificially incubated gopher tortoise eggs (Spotila et al. 1994). This may provide an advantage to embryos incubating at warmer temperatures, because they grow faster *in ovo*, hatch sooner, are able to feed sooner, allowing a head start on post-hatching growth. However, Miller and colleagues (1987) argue that embryos incubated at cooler temperatures have an advantage since they have more time to absorb the yolk, therefore emerging as larger hatchlings. Early hatching in fish (*Menidia menidia)* allows individuals to grow larger before breeding, consequently increasing lifetime reproductive success (Shine 1999; Conover and Kynard 1981; Conover 1984); however, *M. e. emys* produces only a single clutch, whereas *M. menidia* produces multiple clutches throughout a season. Depending upon ambient conditions at hatching (ie. monsoon vs. dry spell), a difference in incubation duration may be disadvantageous or advantageous to *M. e. emys* hatchlings.

It has been suggested that rate of development using incubation duration provides a good estimate of hatchling sex in a turtle with TSD because long durations imply cool temperatures and short durations imply warm temperatures (Mrosovsky et al. 1999; Mrosovsky and Yntema 1980). This technique has been validated using expected and actual field and laboratory data, and furthermore it provides a completely non-invasive method of sex estimation (Mrosovsky et al. 1999). While using incubation duration to estimate sex is not recommended for individual clutches, it can be applied to groups of nests such as those in hatcheries, or to multiple captive populations (Mrosovsky et al. 1999).

Hatching success in this study was maximized at incubation temperatures between approximately 27°C and 31°C. *M. e. phayrei* artificially incubated at the Honolulu Zoo had hatching successes ranging from 78.4% - 84.4% over a five year period (McKeown et al. 1991); however, *M. e. emys* artificially incubated at the Wassenaar Zoo had a hatching success of 21% (Louwman 1982). The broad temperature range and high humidity utilized in the study, especially at the lower temperatures, likely contributed to the hatching success of 50%, which is the approximate average of the former reports. Spotila and colleagues (1994) found that both temperature and substrate moisture affect hatching success in *Gopherus agassizii*. Increased substrate moisture content at cooler temperatures is lethal and decreased moisture content at extreme cool and/or warm temperatures is lethal, which is expected of a desert tortoise that possesses rigid, hard-shelled eggs. In this experiment, both temperature and humidity contributed to hatching success as demonstrated by successful hatching in the 24.99°C incubator that maintained

humidity, and by completely unsuccessful hatching in the 25.06°C incubator that experienced a large decrease in humidity. Because *M. e. emys* possesses soft-shelled eggs, they are expected to lose water more rapidly than hard-shelled eggs and they require substrates with higher water potentials (Ackerman 1992). Without protection of the nest mound, a soft-shelled egg like that of *M. emys* could lose water three times faster than that of a hard-shelled egg (Ackerman 1997).

Although hatching success was greatest between 27°C and 31°C, hatchling mortality was greatest at approximately 31°C, and was minimized between 27°C and 28°C. It is possible that high humidity at high temperatures is lethal for this species, as the hatchlings from the 31°C incubator were deceased within 6 days of hatching. Despite the possibility of lethal humidity levels, moisture content of the substrate can contribute to hatchling locomotor performance. *C. serpentina* hatchlings incubated in dry vermiculite and water (-850 kPa) perform poorly on locomotor tests that measure walking and swimming compared to their counterparts incubated in wet vermiculite and water (-150 kPa) (Miller et al. 1987). Performance was not measured in this study, but it would be beneficial to do so in future experiments, especially at facilities that incubate multiple clutches obtained from multiple females.

4.2 Gonadal sex

The appearance of the laparoscopically examined gonads was consistent with the descriptions provided by Rostal and colleagues (1994) regarding six-month-old gopher tortoises. The *M. e. emys* hatchlings were five months old upon laparoscopy, yet testes

and ovaries with primordial follicles were easily distinguished from one another. Upon gross anatomical examination, the gonads of the deceased and unhatched tortoises appeared to be testes; however, histological examination indicates otherwise, as exemplified by the 31°C females. The results of this experiment suggest that *M. e. emys* exhibits temperature-dependent sex determination, specifically Pattern Ia, where males are primarily produced at cooler temperatures and females at warmer temperatures. It was anticipated that males would be produced in the incubators set to approximately 23°C and 25°C. Although this hypothesis was supported, a mixed sex ratio was expected in the incubator set to approximately 27°C; however, four out of four hatchlings from this incubator were determined as male. It is possible that a slightly higher temperature, perhaps closer to 28°C would yield a mixed ratio. Hatchlings from the 28°C incubator were inadvertently relocated from the institution prior to sex determination, hence the lack of data for that group. A group of hatchlings not included in this study, originating from a different clutch from a different female, were also sexed during the laparoscope procedure. Those four individuals were artificially incubated at approximately 29°C, and all were female. The incubation temperatures used in this study and the resulting gonadal sex associated with each can be applied to artificial incubation methods currently practiced by captive breeding programs. Since 100% males are produced at approximately 27°C, and 100% females are produced at 29°C, captive breeding programs have the potential to maintain balanced sex ratios. Unfortunately, data regarding sex ratios of wild populations are limited, as Høybye-Mortensen (2004) recorded 11 wild

individuals, composed of two females, one male, and eight unknowns, which were likely juveniles lacking obvious sexually dimorphic characteristics.

4.3 Pivotal temperature

The pivotal temperature estimated for this study was 29.29°C, but this temperature can be refined with the addition of sex data from additional incubation temperatures. As previously mentioned, there was an incubator set to 29°C that maintained a mean of 28°C, but the hatchlings from this treatment were inadvertently relocated to another institution prior to sex determination. It is possible that this incubator may have yielded a mix of males and females, since the temperature approaches the estimated pivotal. The addition of those sex data would definitely provide a more accurate estimate of the pivotal temperature. During the laparoscopy, sex was determined for four additional hatchlings that were not study animals. These four females were incubated in a commercial incubator set to 28.89°C. If these data are incorporated in the data from the experiment, the pivotal incubation duration becomes 68 days, therefore refining the pivotal temperature to 28.73°C. Eggs incubated at or around the pivotal temperature may produce mixed ratios, but it is important to consider the possibility of ovotestes, where gonads exhibit characteristics of both ovaries and testes, as the long-term effects of this condition are unknown. Additionally, it is worthwhile to consider the temperatures that produce 100% males and 100% females that also maximize hatching success and hatchling survival. While incubation at 23°C would most likely yield 100% males, hatching success and post-emergence hatchling survival may be

low. Similarly, incubation at 33°C would likely result in 100% females; however, it may also compromise hatching success and hatchling survival. Refinement of the pivotal temperature and of the lower and upper temperature limits indicated by hatching success could more accurately suggest the preferential incubation conditions.

4.4 Adaptation and evolution

Manouria reproduction coincides with monsoon season (Wirot 1979; Høybye-Mortensen 2004). Nests can be exposed to increased rainfall and high air temperatures, which thereby affect nest temperatures and sex ratios. Rhodes and Lang (1996) report biased sex ratios in *A. mississpiensis* during years characterized by higher than average rainfall, which produces cooler nest temperatures. The tropical monsoon climate in the geographic range of *M. e. emys* is characterized by periods of heavy rainfall with intermittent dry spells (Høybye-Mortensen 2004). The mound nest maintains temperature ±1°C and likely serves to maintain humidity (Høybye-Mortensen 2004). In addition to climatic effects on nest temperature, the nest components influence temperature and humidity; for instance, leaf litter decomposition generates large amounts of heat. Nest properties such as location, size and materials are known to affect thermoregulatory and hydric conditions in mammals (King et al. 1964), birds (Møller 1984) and reptiles (Lutz and Dunbar-Cooper 1984). Because the results suggest that *M. e. emys* exhibits TSD, it is possible that the extent of investment that a female makes to mound construction can affect incubation conditions, consequently contributing to biased sex ratios, hatching success, offspring growth rate and fitness.

Auffenberg (1966) interpreted the high levels of variation in the carpus of tortoises as a failure of the component elements to serve a definite mechanical need, with respect to structure and interaction with the locomotory substrate. The aboveground nesting behavior of *Manouria* is unique. All other tortoise groups exhibit in ground nesting. In ground nests constructed by derived species may require modified bone structure compared to *Manouria*. Because nesting behavior has a direct effect on the thermal properties within the nest (King et al. 1964; Møller 1984; Lutz and Dunbar-Cooper 1984), and consequently TSD and hatching success, it is worthwhile to consider the evolution of mound nesting. It is unlikely that nesting behavior in *Manouria* evolved as an adaptation to flooding resulting from the tropical monsoon climate as other species of turtles inhabiting the same environment nest underground. It is more likely that primitive anatomical structure in *Manouria* (Auffenberg 1966) posed constraints on the ability to excavate an underground nest, resulting in above ground nest construction via sweeping of leaf litter. With the addition of sufficient materials, a mound nest may maintain thermal and hydric conditions that are optimal for hatching success and sex ratio. Additionally, conditions could be maintained within a narrow range as demonstrated by Høybye-Mortensen's (2004) recordings within a wild nest. It is possible that protection from flooding during monsoons was a secondary benefit of the mound nest, and that other species coped with climatic effects by adjusting reproductive season, as in *Indotestudo forsteni* which breeds from November to January, and in *Geochelone platynota* which nests in February in the same habitat (Ernst and Barbour 1989).

It is suggested that the ancestral condition of limited, large clutches is favored by natural selection in seasonal climates with short suitable reproductive periods (Shine and Greer 1991). Additionally, life-history theory predicts that costly behavior is associated with reproduction and that single clutches reduce energetic costs of reproduction (Doody et al. 2003). In *Carettochelys insculpta* it is suggested that turtles produce larger, heavier and more eggs following the wettest portion of the wet season because of high energy accumulation (Doody et al. 2003). Because *Manouria* produces a single clutch in a season, possibly due to energetically expensive reproductive behavior, it would be advantageous to provide optimal incubation conditions for offspring through mound construction. Consequently, the nest should be protected against predators while the female continues adding the quantity of materials necessary for homeostasis.

The most widely accepted theory on the adaptive significance of TSD is the differential fitness hypothesis (Lovich 1996; Shine 1999). It assumes that increased temperatures influence organismal fitness. If a female's offspring grow faster and, therefore, mature earlier under warmer conditions then mothers benefit by producing more females. Additionally, if male offspring incubated at cooler temperatures are more successful, then mothers benefit from male production (Lovich 1996; Shine 1999). Warmer nests hatch earlier than cooler nests; therefore individuals incubated at warmer temperatures can grow larger before breeding, consequently, increasing lifetime reproductive success (Shine 1999; Conover and Kynard 1981; Conover 1984). Although a relationship between sex, incubation temperature and growth rate is demonstrated in gekkonid lizards (Tousignant and Crews 1995) and in snapping turtles (Rhen and Lang

1995), because turtles are long-lived (Gibbons 1987) and age at reproductive maturity is proportional to lifespan (Shine and Iverson 1995), it is unlikely that hatching early provides lifetime reproductive advantages.

While *M. e. emys* has been shown to exhibit TSD, the presence of sex chromosomes remains unknown, therefore karyotyping would be beneficial. Although sex determination in reptiles can involve genotypic sex determination (GSD) with XX (female) and XY (male) chromosomes, or with ZZ (male) and ZW (female) chromosomes, or TSD, recent research has shown that high incubation temperatures reverse genotypic males to phenotypic females (Quinn et al. 2007). It is suggested that in a reptile with GSD via ZZ and ZW chromosomes, the W chromosome in unnecessary for female differentiation, but that the dosage of a gene on the Z chromosome drives sex determination, and gene activity sufficient for male differentiation only occurs at certain temperatures (Quinn et al. 2007). Quinn and colleagues (2007) suggest that selection for broad thermosensitive ranges could result in the evolution of TSD from GSD, and that frequency-dependent selection as a result of genotypic sex reversal could account for TSD patterns, especially that exhibited by *M. e. emys.*

4.5 Conservation

The discovery of TSD in threatened and endangered species like *M. e. emys* has serious implications for conservation programs. Knowledge of pivotal temperatures allows for artificial incubation at feminizing or masculinizing temperatures for conservation purposes. Although this can be used as a tool to produce a greater number

of females, and thereby increase the quantity of reproductively viable females, it would have a decidedly negative impact on the population structure and mating systems (Lovich 1996). Lovich (1996) argues that sex ratio manipulation changes the process of sexual selection and that it destabilizes resources utilized by both sexes. Additionally, sex ratio manipulation simulations performed by Girondot and colleagues (1998) indicate that artificial feminization of turtle embryos in a population can result in the selection of masculinizing alleles, thereby shifting the pivotal temperature to a male-producing temperature; a process that would otherwise occur by mutation over a period of time likely longer than that of a conservation plan. Alternatively, artificial incubation at or around the pivotal temperature could be utilized to produce males and females as both sexes are commonly found together in many natural nests (Mrosovsky and Godfrey 1995). Although gonad intersexuality (ovotestes) has been observed in hatchlings incubated at pivotal temperatures under artificial and natural conditions (Pieau 1982; Pieau and Dorizzi 1981), degeneration continues and ovotestes evolve into typical testes (Girondot et al. 1998). Currently, TSD appears the most logical application for feminizing and/or masculinizing *M. e. emys* embryos for conservation since manipulation via endogenous sex steroids is associated with several problems, including morphological abnormalities, and unknown physiological and behavioral consequences (Girondot et al. 1998). In agreement with Girondot and colleagues (1998), conservation plans should focus on protection of existing animals and habitats, protection against introduced predators, and against human exploitation.

5 Conclusion

Since *M. e. emys* is believed to be the most primitive of living tortoises (Pritchard 1979), this research can contribute to the study of the phylogenetic history of sex determining mechanisms in other chelonians and in other reptiles, while providing insight into the adaptive significance of reproductive behaviors of other turtles and tortoises. The presence of Pattern Ia TSD and an estimated pivotal temperature of 29.29°C in *M. e. emys* demonstrates a delicate balance between organism structures and behaviors, microhabitat conditions, and regional climatic conditions that can contribute to ecosystem maintenance. According to the IUCN, it is suspected that *M. e. emys* populations will experience a reduction of at least 50% within the upcoming 10 years or three generations (http://www.redlist.org/info/categories_criteria1994.html#categories). Information on conservation status and distribution provides a foundation for decisions regarding local and global preservation. Although little is known regarding *M. e. emys* in its natural habitat, investigations into its reproductive biology, especially TSD, can contribute to decisions regarding habitat protection planning, population transplanting, artificial incubation, and hatchery design.

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7 Appendices

Appendix A Statistical analyses

Chisquare test on 1:1 ratio of males to females.

Observed		
	Males	Females
23	2	0
27	4	0
31	0	4
Expected 1:1		
	Males	Females
23	1	1
27	2	2
31	2	2
df	p	
2	.00673795	

Temp	Sex	Observed	Expected	Obs-Exp	(Obs-Exp)^2	(Obs-Exp)^2/Exp
23	m	2	1	1	1	1
27	m	4	2	2	4	2
31	m	0	2	-2	4	2
23	f	0	1	-1	1	1
27	f	0	2	-2	4	2
31	f	4	2	2	4	2
					CHISQ	10

Chisquare test on hypothesized 99% males, 1% females at 31°C; equal ratio at 27°C; 1% males, 99% females at 31°.

Observed		
	Males	Females
23	2	0
27	4	0
31	0	4
Expected 99:1		
	Males	Females
23	1.98	0.04
27	2	2
31	0.02	3.96
df	p	
2	.13129573	

Temp	Sex	Observed	Expected	Obs-Exp	(Obs-Exp)^2	(Obs-Exp)^2/Exp
23	m	2	1.98	0.02	0.0004	0.00020202
27	m	4	2	2	4	2
31	m	0	0.02	-0.02	0.0004	0.02
23	f	0	0.04	-0.04	0.0016	0.04
27	f	0	2	-2	4	2
31	f	4	3.96	0.04	0.0016	0.00040404
					CHISQ	4.060606061

Appendix B Pivotal temperature estimation

TSD.pzf:Nonlin fit of my duration vs males - Equation - Sat Mar 31 17:13:03 2007

Equation

Equation:Sigmoidal dose-response
Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)))

;X is the logarithm of concentration. Y is the response
;Y starts at Bottom and goes to Top with a sigmoid shape.
;

TSD.pzf:Nonlin fit of Data 1:Table of results - Thu Mar 29 19:33:39 2007

		A
		Percent male
		Y
1	Sigmoidal dose-response	
2	Best-fit values	
3	BOTTOM	0.0
4	TOP	100.0
5	LOGEC50	66.00
6	EC50	Value too large
7	Std. Error	
8	LOGEC50	0.3071
9	95% Confidence Intervals	
10	LOGEC50	64.68 to 67.32
11	EC50	
12	Goodness of Fit	
13	Degrees of Freedom	2
14	R squared	1.000
15	Absolute Sum of Squares	2.000e-008
16	Sy.x	10.000e-005
17	Constraints	
18	BOTTOM	BOTTOM = 0.0
19	TOP	TOP = 100.0
20	Data	
21	Number of X values	3
22	Number of Y replicates	1
23	Total number of values	3
24	Number of missing values	0

TSD.pzf:Nonlin fit of Data 1:Curve - Thu Mar 29 19:32:44 2007

	X Seq.	A
	Incubation duration (days)	Percent male
	X	Y
1	60.000000	0.0001
2	60.214760	0.0002
3	60.429530	0.0003
4	60.644290	0.0004
5	60.859060	0.0007
6	61.073830	0.0012
7	61.288590	0.0019
8	61.503360	0.0032
9	61.718120	0.0052
10	61.932880	0.0086
11	62.147650	0.0140
12	62.362420	0.0230
13	62.577180	0.0378
14	62.791950	0.0619
15	63.006710	0.1015
16	63.221480	0.1662
17	63.436240	0.2723
18	63.651010	0.4457
19	63.865770	0.7288
20	64.080540	1.1894
21	64.295300	1.9356
22	64.510070	3.1350
23	64.724830	5.0394
24	64.939600	8.0050
25	65.154370	12.4864
26	65.369130	18.9596
27	65.583890	27.7254
28	65.798660	38.6130
29	66.013420	50.7726
30	66.228190	62.8415
31	66.442960	73.4960
32	66.657710	81.9720
33	66.872480	88.1735
34	67.087250	92.4386
35	67.302020	95.2484
36	67.516780	97.0474
37	67.731540	98.1783
38	67.946310	98.8811
39	68.161070	99.3146
40	68.375840	99.5809
41	68.590610	99.7440
42	68.805370	99.8437
43	69.020130	99.9046
44	69.234900	99.9418
45	69.449660	99.9645
46	69.664430	99.9784
47	69.879200	99.9868

TSD.pzf:Lin Reg of Data 2:Tabular results - Thu Mar 29 19:33:09 2007

		A
		Incubation duration (days)
		Y
1	Best-fit values	
2	Slope	-5.299 ± 1.545
3	Y-intercept when X=0.0	221.2 ± 42.89
4	X-intercept when Y=0.0	41.74
5	1/slope	-0.1887
6	95% Confidence Intervals	
7	Slope	-24.93 to 14.34
8	Y-intercept when X=0.0	-323.8 to 766.2
9	X-intercept when Y=0.0	
10	Goodness of Fit	
11	r squared	0.9216
12	Sy.x	6.401
13	Is slope significantly non-zero?	
14	F	11.76
15	DFn, DFd	1.000, 1.000
16	P value	0.1807
17	Deviation from zero?	Not Significant
18	Data	
19	Number of X values	3
20	Maximum number of Y replicates	1
21	Total number of values	3
22	Number of missing values	0