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Seasonal Reproductive Cycle of the Galápagos Tortoise (*Geochelone nigra*) in Captivity

David C. Rostal,[1]* Todd R. Robeck,[3] Janice S. Grumbles,[1] Patrick M. Burchfield,[4] and David Wm. Owens[2]

[1]*Department of Biology, Georgia Southern University, Statesboro, Georgia*
[2]*Department of Biology, Texas A&M University, College Station, Texas*
[3]*Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas*
[4]*Gladys Porter Zoo, Brownsville, Texas*

The reproductive physiology of nine Galápagos tortoises (*Geochelone nigra*) was studied from February 1988 to May 1989. The study encompassed the annual reproductive cycle to include complete mating and nesting sequences. Male (n = 4) and female (n = 5) seasonal reproductive changes were determined throughout the study with endocrine analysis and ultrasonographic examinations. Males displayed a prenuptial rise in serum testosterone ($\bar{x} \pm SE = 6.62 \pm 0.92$ ng/ml in August) during which gonadal maturation and spermatogenesis are thought to occur. The male reproductive cycle appears consistent with the prenuptial spermatogenic pattern exhibited by other tropical turtles. In the females, testosterone rose during the mating period ($\bar{x} \pm SE = 499.3 \pm 124.6$ pg/ml in October) prior to ovulation and is probably related to receptivity in the females. Progesterone was more variable, but also peaked during the mating period ($\bar{x} \pm SE = 1{,}017.2 \pm 220.6$ pg/ml in October) and appears related to ovulation. Estradiol rose several months prior to mating ($\bar{x} \pm SE = 75.5 \pm 11.9$ pg/ml in July) and was correlated with increased serum calcium levels. This increase in estradiol is thought to stimulate vitellogenesis several months prior to mating. Nesting occurred from November 1988 to April 1989, during which six clutches were laid. Clutch size ranged from eight to 17 eggs. Both male and female Galápagos tortoises display seasonal physiological changes that function to regulate annual reproductive patterns. Zoo Biol 17:505–517, 1998.

Key words: Reptilia; Testudines; Testudinidae; gonadal steroids; ultrasonography; total calcium; vitellogenesis

*Correspondence to: David C. Rostal, Department of Biology, Georgia Southern University, P.O. Box 8042, Statesboro, GA 30460. E-mail: Rostal@gsvms2.cc.gasou.edu

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INTRODUCTION

The reproductive biology of Galápagos tortoises (*Geochelone nigra*) has received limited study to date. Studies on their reproductive ecology have been conducted in Galápagos, both on the various islands and at the Charles Darwin Research Station [MacFarland et al., 1974a,b]. Bacon [1980] reviewed the history of captive propagation attempts outside Galápagos and common practices of captive reproduction of these giant tortoises at the San Diego Zoo. Captive breeding programs have had limited success over the years (Honolulu Zoo, Philadelphia Zoo, San Diego Zoo). Most of the available data pertains to the incubation of eggs, egg fertility, and rearing of young.

Wild Galápagos tortoises appear to display a seasonal reproductive cycle. Mating occurs from December to August, with a peak period from February to June. Nesting then appears to occur from July through November [MacFarland et al., 1974b]. Bacon [1980] discussed the possible proximate cues involved and dismissed photoperiod due to the equatorial location of Galápagos. He noted, however, the predictable annual cycle of precipitation and temperature variation documented by Alpert [1963] and Palmer and Pyle [1966]. Thus, the onset of mating activity would appear to coincide with the rainy season when temperatures are highest, followed by nesting during the dry season.

Captive reproduction of Galápagos tortoises (*G. nigra*) appears to be seasonal in at least the San Diego colony, where mating occurs from August to October when temperatures are highest, and nesting occurs from January through April [Bacon, 1980]. However, Throp [1975] found that males in the Honolulu Zoo colony were reproductively active all year round. Throp [1975] also stated that short separation of males from females is essential for successful reproduction.

The breeding colonies of Galápagos tortoises at the Gladys Porter Zoo were studied to answer physiological questions essential to understanding their reproductive biology. 1) Do Galápagos tortoises display seasonal patterns of hormone secretion and gonadal cycles? 2) Are both males and females seasonal? 3) Does separation of males have a physiological effect on their reproductive cycle? 4) What proximate cues may function to regulate seasonality?

METHODS

The breeding groups at the Gladys Porter Zoo, Brownsville, Texas, were studied from February 1988 to May 1989. Endocrinology, ultrasonography, and behavioral observations were used to evaluate reproductive seasonality and to uncover potential proximate cues for these seasonal changes. The mating and nesting activity of the group was monitored by the zoo staff and recorded in the reptile log book when observed. Blood sampling and ultrasound examinations were conducted throughout the study to determine seasonal reproductive changes in both males and females.

Animals and Housing

The study group was composed of nine tortoises (four adult males and five adult females) maintained in two separate enclosures. Each enclosure contained two males and one or four females. The division of animals into groups was based on size and suspected origin of collection (i.e., Microphyes, Vicina, and unknown origin). Females with unknown origins were larger than the two males from Vicina, therefore they were placed

in the enclosure with the larger males. Male curved carapace measurements ranged from 101 to 152 cm (n = 4). Female curved carapace measurements ranged from 85 to 118 cm (n = 5). The tortoises were maintained in 10×20-m outdoor enclosures year round. Each enclosure contained 2.6×4.0-m concrete watering pools with a sloping ramp and a maximum depth of 1.3 m. The enclosures also had a 3-m^2 mud wallow, mounds for exercise, and available shade trees. A heated barn was available in each enclosure during winter months (December, January, and February) when night temperatures dropped below 10°C. The tortoises were fed a special diet containing produce (carrots, celery, apples, and melons) and Purina Horse Chow (Purina Mills Inc., St. Louis, MO) supplemented with Vet-A-Mix Ethyodide (Vet-A-Mix Inc., Shenandoah, IA) once weekly. Alfalfa was fed daily. During the nesting period, produce was offered twice weekly [Hairston and Burchfield, 1989].

Behavioral Observations and Population Manipulations

The mating and nesting activity of the group was monitored by the zoo staff and recorded in the reptile log book when observed. To test the hypothesis that male separation increases mating activity, one male was repeatedly separated and reintroduced to the group at 4-month intervals to test for physiological effects of this captive breeding practice on tortoises. The same male was removed and held separate from the larger breeding group (two males/four females) for 2–3 weeks during the pre-mating (May), mating (September), and nesting periods (January). The male was then released back into the breeding group ~1 week prior to the normally scheduled monthly bleeding period.

Blood Collection, Steroid Radioimmunoassay, and Calcium Analysis

Blood sampling was conducted on a monthly basis from February 1988 to May 1989. Blood samples (20 ml) were collected from a venous plexus on the ventral surface of the foreleg [Dr. Elliott Jacobson, pers. comm.] using a 20-cc syringe with 21-gauge needle. Blood samples were collected from both males and females. The tortoise was placed on its back on an examination table [Robeck et al., 1990], and the forelimb was restrained by an assisting person. The depth and location of the sampling site were confirmed using ultrasound. Blood samples were centrifuged using a portable centrifuge at ~3,000 rpm for 10 min and the serum was collected. Serum was stored frozen until analysis.

Serum testosterone and progesterone were measured using a H^3 radioimmunoassay (RIA) similar to that described by Wibbels et al. [1990]. Tritiated testosterone and progesterone steroids were obtained from New England Nuclear and diluted with Tris/gelatin buffer to yield ~7,000 cpm per 100 µl and 10,000 cpm per 100 µl, respectively. Testosterone antiserum was obtained from Cambridge Medical Diagnostics and diluted 1/4,500 in Tris/gelatin assay buffer. Progesterone antiserum was obtained from Endocrine Sciences and diluted 1/1,500 in Tris/gelatin assay buffer. Testosterone and progesterone standards were obtained from Steraloids Inc. Four to six control samples were added to each assay. For males, 10–100 µl of serum was extracted. For females, 250–500 µl of serum was extracted. Serum was extracted using anhydrous ether. Samples were run in duplicate. Extraction efficiencies averaged 93.2%. Sensitivity of the assays were 2.3 and 21 pg/tube, respectively. Intra-assay coefficients of variation were 4.8 and 2.4%, respectively. Inter-assay coefficients of variation were 17.5 and 19.6%, respectively.

Serum estradiol was measured using an iodine kit provided by Diagnostic Products Corporation (Los Angeles, CA). For estradiol, 100 μl of serum was extracted using anhydrous ether. Samples were run in duplicate. Extraction efficiencies for estradiol averaged 99.1%. Sensitivity of the estradiol assay was 0.1 pg/tube. Intraassay coefficient of variation for the estradiol assay was 5.1% and interassay coefficient of variation for estradiol assay was 13.6%.

Total calcium was determined throughout the study for both males and females. Total calcium was demonstrated to be an effective indicator of vitellogenesis [Ho, 1987; Heck et al., 1997] and follicular growth in turtles [Callard et al., 1978; Rostal et al., 1994, 1998]. Serum total calcium was measured by flame atomic absorption spectrophotometry using a SpectrAA-20 atomic absorption spectrophotometer (Varian Techtron Pay, Ltd., Australia). Serum was diluted (1:26) in a 1% lanthanum oxide (La_2O_3) solution at room temperature. Total calcium (μg/ml) was compared to a standard curve (1, 5, 10, 20 μg/ml, Fisher Scientific AA Standard) at the time of assay.

Ultrasound Examinations

Ultrasound examinations were conducted at 1–2-month intervals to determine the reproductive status of females (ovarian follicular development) and for the detection of shelled oviductal eggs [Robeck et al., 1990]. Two ultrasound units were used, a linear array EQ 300 scanner with 5.0-MHz probe (Equisonics Inc., Bensenville, IL), and a mechanical sector ATL 4600 scanner with variable 3.0-, 5.0-, and 7.5-MHz probe (Advanced Technology Laboratory Inc., Bellevue, WA). The procedure involved placing the females in dorsal recumbency on a restraining platform. The hindlimbs were then pulled back using large nylon ropes and secured. This provided an accessible ultrasound "window" in the inguinal region cranial to the hindlimb. H-R jelly (H-R Lubricating Jelly; Carter-Wallace Inc., New York, NY) was used as a coupling gel and applied liberally to the inguinal region. The probe was then oriented in a cranial-medial direction in the inguinal region and reproductive structures (ovaries and oviducts) were imaged. Both sides were scanned independently. Structures imaged (i.e., follicles and eggs) were measured using the ultrasound unit's electronic calipers and recorded on videotape for later review [Robeck et al., 1990].

Data Analysis

Seasonal changes in hormone levels and serum calcium of both males and females were determined using repeated-measures analysis of variance by ranks ($p \leq 0.05$). Correlations between estradiol and serum calcium levels were determined using Pearson product-moment correlation coefficient (r, $p \leq 0.05$).

RESULTS

The breeding group of Galápagos tortoises displayed a distinct seasonal cycle with a mating period (August to November) and a nesting period (November to April). All males were observed mounting females during the mating period. Six clutches of eggs were laid from November to April with a total of 67 eggs. Seasonal changes in steroid levels were observed in both males (testosterone) and females (testosterone, progesterone and estradiol).

Male Testosterone

The males displayed a significant prenuptial rise in testosterone from May to July that continued into the mating period ($\chi^2 = 54.923$, df = 15, $p < 0.001$; Fig. 1A). Male testosterone levels ranged from a mean low of 0.15 ± 0.06 ng/ml (± SE, n = 4) during the nesting period (February) to a mean high of 6.62 ± 0.92 ng/ml (± SE, n = 4) during the mating period (August). The prenuptial rise observed in male testoster-

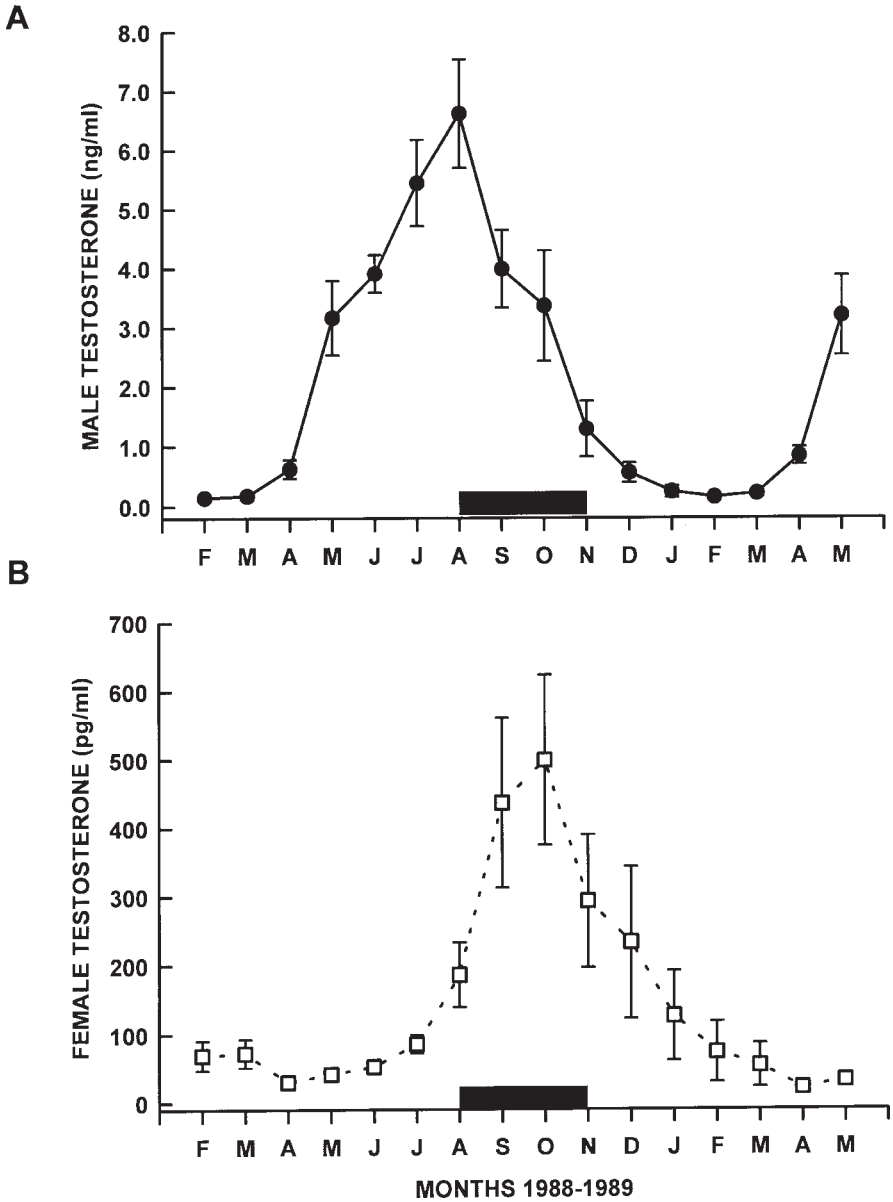

Fig. 1. Seasonal pattern of serum testosterone from captive adult Galápagos tortoises. **A:** Males (n = 4). **B:** Females (n = 5). Solid line represents mating period (August to November).

one occurred during a period when seasonal gonadal maturation and spermatogenesis would be expected to occur prior to the onset of mating activity.

Female Testosterone, Progesterone, and Estradiol

The females displayed a significant rise in testosterone (August to December), which coincided with the onset of mating activity (χ^2 = 49.266, df = 15, $p < 0.001$; Fig. 1B). Female testosterone levels ranged from a mean low of 21.8 ± 3.0 pg/ml (± SE, n = 5) during the late nesting period (April) to a mean high of 499.3 ± 124.6 pg/ml (± SE, n = 5) during the peak mating period (October). Female estradiol levels ranged from a mean low of 11.8 ± 2.3 pg/ml (± SE, n = 5) during the early nesting period (February) to a mean high of 75.5 ± 11.9 pg/ml (± SE, n = 5) during the pre-mating period (July). Estradiol levels were significantly elevated during the pre-mating period when vitellogenesis is thought to be maximal (χ^2 = 51.4, df =15, $p < 0.001$; Fig. 2A) . Females also displayed a significant increase in progesterone during the mating period (χ^2= 30.7, df = 15, $p < 0.01$; Fig. 2B). Female progesterone levels ranged from a mean low of 116.8 ± 63.7 pg/ml (± SE, n = 5) during the late nesting period (May) to a mean high of 1,017.2 ± 220.6 pg/ml (± SE, n = 5) during the peak mating period (October).

Ultrasound Results and Nesting

Ultrasound examinations of the females revealed large vitellogenic follicles ranging from 28 to 42 mm in diameter in all five females from February 1988 to May 1989. Shelled eggs were detected repeatedly in the oviducts of two females from October 1988 to April 1989. Oviductal eggs were normally observed on both sides of the tortoise. Oviductal eggs measured 55–68 mm in external diameter. Yolks of shelled eggs measured 34–43 mm in diameter.

Nesting occurred from November 1988 to April 1989 during which six clutches were laid. Clutch size ranged from 8 to 17 eggs with one female clutching four times (mean clutch size = 9 eggs) and the other female clutching two times (mean clutch size = 15.5 eggs). The number of days that eggs were retained was monitored from when eggs were first detected using ultrasound and when the eggs were finally laid. Duration of egg retention was highly variable (mean ± SE = 40.2 ± 9.7 days, n = 5) and ranged from 18 to 76 days.

Vitellogenesis and Total Calcium

Female serum calcium levels (indicative of vitellogenin) increased from the late nesting season (February 1988, mean ± SE = 217.5 ± 46.6 μg/ml, n = 4) to the mating season (August 1988, mean ± SE = 303.2 ± 37.8 μg/ml, n = 4) and then declined into the nesting season (December 1988, mean ± SE = 245.5 ± 37.5 ; Fig. 3); however, this increase was not statistically significant (χ^2 = 23.907, df = 15, p = 0.067) . Male serum calcium levels remained low and constant (χ^2 = 21.70, df = 15, NS) throughout the study (mean, 97.8–116.5 μg/ml; n = 4; Fig. 3).

Seasonal changes in female serum calcium was positively correlated with estradiol over the course of the reproductive cycle with elevated levels of both serum calcium and serum estradiol observed from May to October (r = 0.810 , df = 14, $p < 0.0001$; Fig. 4).

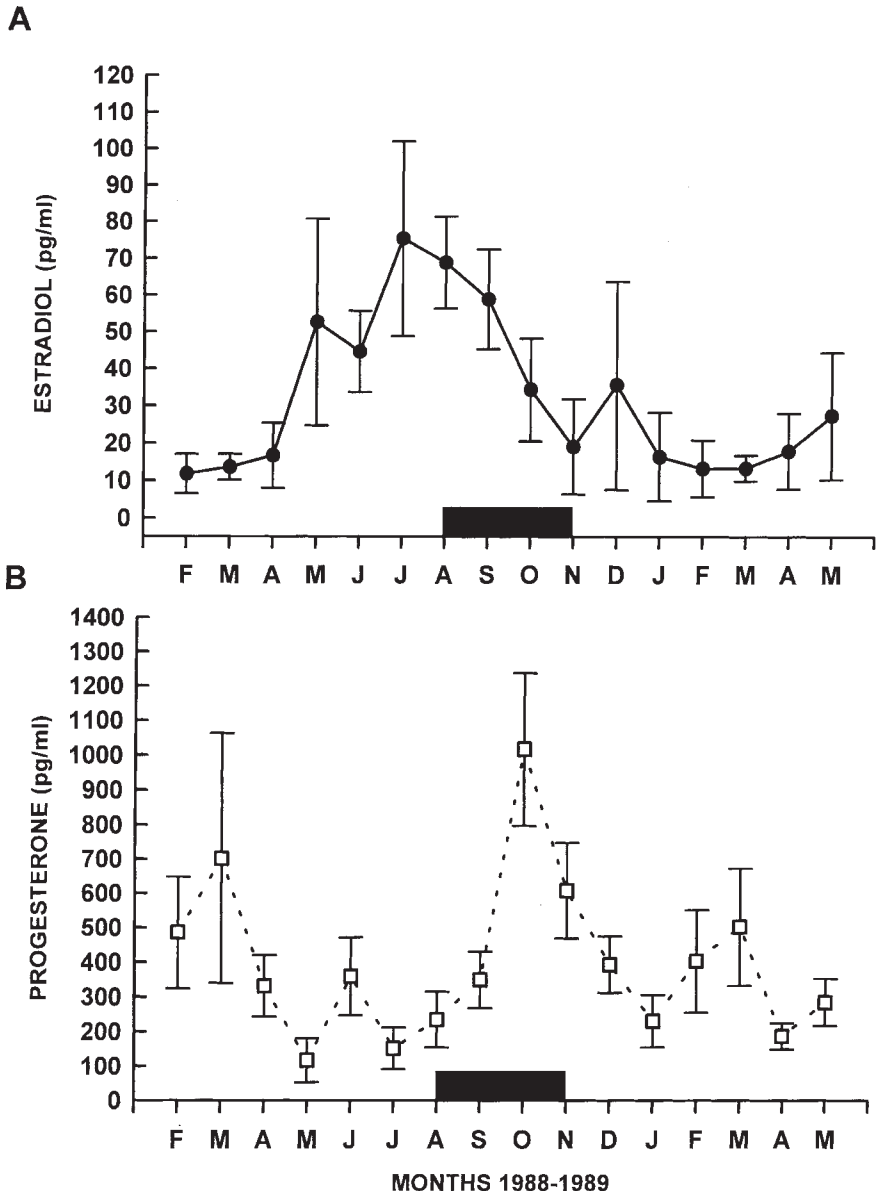

Fig. 2. Seasonal pattern of serum estradiol **(A)** and progesterone **(B)** from captive adult female Galápagos tortoises (n = 5). Solid line represents mating period (August to November).

Male Separation Experiment

Separation of one male from the larger breeding group (two males/four females) during the pre-mating (May), mating (September), and the nesting periods (January) did not result in any detectable physiological changes in reproductive cyclicity. Males displayed similar patterns in serum testosterone throughout the study period.

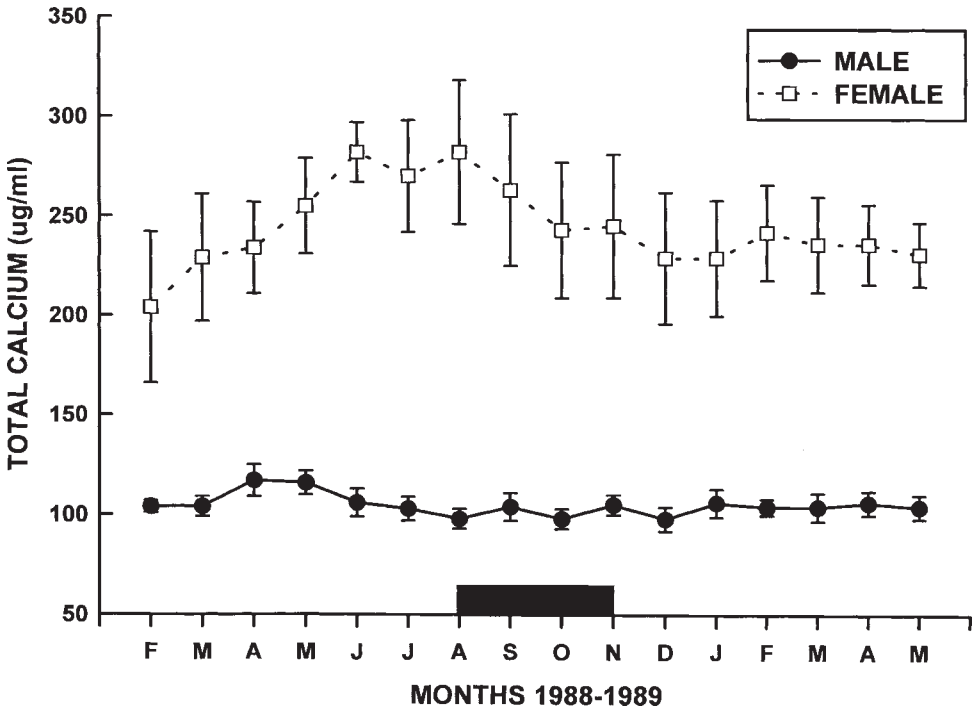

Fig. 3. Seasonal pattern of serum total calcium from captive adult male (n = 4) and female (n = 5) Galápagos tortoises. Solid line represents mating period (August to November).

Nesters vs. Non-nesters

Nesting females (n = 2) displayed a distinct surge in testosterone during the mating period, however, non-nesting females (n = 3) displayed erratic elevated levels of testosterone during the mating period and maintained elevated testosterone levels well into the nesting period. Similarly, nesting females (n = 2) displayed a distinct prenuptial rise in estradiol during the pre-mating period, whereas non-nesting females (n = 3) displayed less predictable elevated levels of estradiol during the pre-mating period and tended to maintain elevated estradiol levels well into the nesting period. Finally, nesting females (n = 2) displayed a distinct surge in progesterone during the mating period, while non-nesting females (n = 3) displayed erratic elevated levels of progesterone throughout the study. One nesting female displayed a distinct surge in progesterone during October suggesting a blood sampling near the time of ovulation.

DISCUSSION

The breeding colony of Galápagos tortoises (*G. nigra*) at the Gladys Porter Zoo, Brownsville, Texas, displayed seasonal physiological changes that coincided with the observed seasonal reproductive cycle. The prenuptial rise in male serum testosterone from May to August prior to mating is similar to that observed in the desert tortoise (*Gopherus agassizii*) [Rostal et al., 1994]. In male *G. agassizii*, this

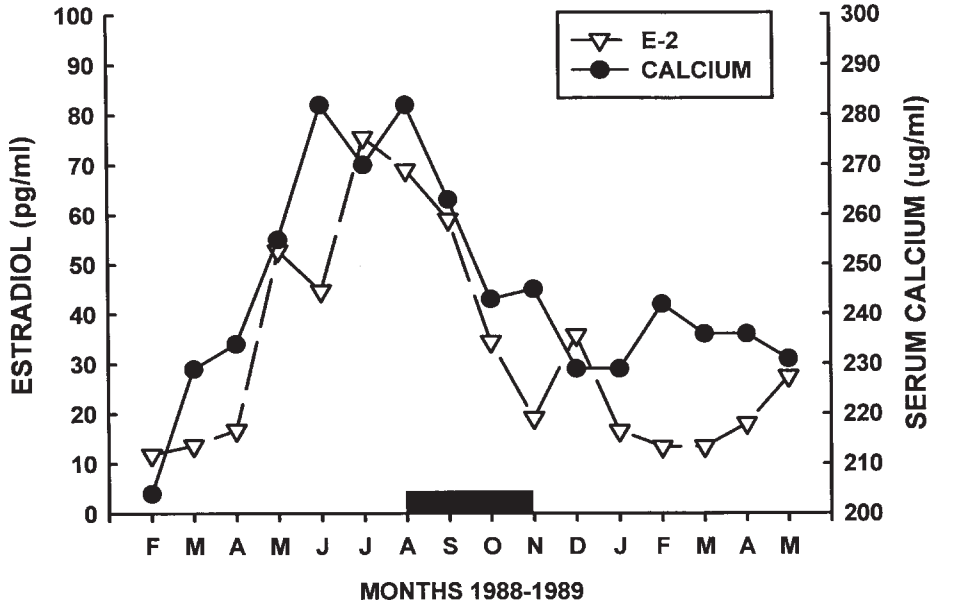

Fig. 4. Comparison of seasonal pattern for mean serum estradiol and mean serum calcium levels from captive adult female Galápagos tortoises (n = 5). Solid line represents mating period (August to November).

rise in testosterone is related to increased testicular maturation and spermatogenesis. During the mating period, female serum testosterone was also observed to rise significantly prior to ovulation and is thought to be related to receptivity in the female turtle [Rostal et al., 1998]. Estradiol levels were elevated during the pre-mating period when ovarian follicular growth was observed. Serum calcium levels were also correlated with elevated estradiol levels, indicative of vitellogenesis [Rostal et al., 1994, 1996, 1998]. Calcium binds to the vitellogenin molecule during vitellogenesis and total calcium levels can be used as an indicator of vitellogenesis in a variety of reptiles. In nesting tortoises, progesterone was more dramatic in its seasonal cycle, with a sharp increase observed in October after which shelled oviductal eggs were observed. The absence of shelled oviductal eggs prior to October in both nesting females supports the conclusion that progesterone is involved with ovulation in turtles and that ovulation had not occurred prior to the observed mating period. The variability observed in the number of days shelled eggs were retained in the oviduct elucidates the plasticity of the female tortoise's reproductive physiology.

Seasonal reproductive studies on tortoises are limited and have focused on more temperate and subtropical species [the desert tortoise, *G. agassizii*; Rostal et al., 1994; the gopher tortoise, *Gopherus polyphemus*, Iverson, 1980; Landers et al., 1980; Hermann's tortoise, *Testudo hermanni*, Kuchling et al., 1981]. The seasonal reproductive cycle observed in the captive Galápagos tortoises is more like that reported for several of the tropical sea turtle species (the green turtle, *Chelonia mydas*, Licht et al., 1979; the loggerhead turtle, *Caretta caretta*, Wibbels et al., 1990; and the Kemp's ridley sea turtle, *Lepidochelys kempi*, Rostal, 1991; Rostal et al., 1998]. Testosterone, progesterone, and estradiol fluctuate seasonally, similar to that observed in

the Galápagos tortoise. Testosterone levels increase significantly in both male and female as the mating season approaches. In the female, this increase is associated with ovarian maturation and follicular growth during vitellogenesis. Increased testosterone levels peak in the female during the mating season and then decline throughout the nesting season as clutches of follicles are ovulated. Female estradiol levels actually increase several months prior to mating and appear to stimulate vitellogenin production by the liver [Licht et al., 1979; Wibbels et al., 1990; Rostal et al., 1998]. Female progesterone levels are observed to surge both during mating and nesting as subsequent clutches of eggs are ovulated [Licht et al., 1982; Wibbels et al., 1992].

Seasonal ovarian maturation and follicular growth were monitored using ultrasonography and serum calcium levels. Limited follicular growth was observed during the post-nesting/pre-mating period and coincided with elevation in female serum calcium levels during this period. Vitellogenesis and follicular growth have been correlated with increased total serum calcium levels in the cobra, *Naja naja* [Lance, 1976], the painted turtle, *Chrysemys picta* [Callard et al., 1978], the American alligator, *Alligator mississippiensis* [Lance et al., 1983], the desert tortoise, *G. agassizii* [Rostal et al., 1994], the Kemp's ridley sea turtle, *Lepidochelys kempi* [Rostal, 1991; Rostal et al., 1998], and the tuatara, *Sphenodon punctatus* [Cree et al., 1991]. Similar to the observations of Casares et al. [1997], we observed large vitellogenic follicles throughout the 16-month study period.

The comparison of nesting and non-nesting female endocrine cycles revealed distinct differences in seasonal patterns. The cause of these differences is not clear, but it should be noted that the two females that did not reproduce in the larger breeding group were both smaller than the two females that did reproduce. To what degree these two smaller females may have been stressed from social interactions with the larger females is unknown. The third female that did not reproduce was housed only with two males on exhibit. Her endocrine cycle was similar to that of the females that did reproduce, however, hormone levels were much lower for all three measured. This female had produced eggs in previous years.

We were unable to detect any physiological changes in reproductive cyclicity in relation to repeated separation and reintroduction of the male Galápagos tortoise. One male was separated from the group during the premating (May), mating (September), and nesting periods (January). Although limited mounting activity may result from this practice in other colonies outside the normal mating period, the potential success of these matings is unclear and unlikely to result in viable offspring. Male and female Galápagos tortoises housed together throughout the year, however, did display a distinct mating period (July to November) immediately followed by a distinct nesting period (late November to early April). The increased mating activity observed following the reintroduction of the male into the group is probably an artifact of novel stimulus.

The seasonal cycle observed in the Brownsville colony compares closely to that reported for the San Diego colony, with a distinct mating and nesting sequence [Bacon, 1980]. As with the San Diego colony, the Brownsville colony begins mating during the hottest period of the year (July and August). Moll [1979] noted that the proximate environmental cues that influence reproductive cycles in reptiles appear to be temperature, light, and moisture. Temperature has been linked to the reproductive cycles of other chelonian species [Licht, 1984; Owens and Morris, 1985; Whittier and Crews, 1987; Rostal et al., 1994; Sarkar et al., 1996; Owens, 1997]. Temperature

may play an important role in regulating reproductive cycles of tropical turtles in which shifts in photoperiod are minimal. MacFarland et al. [1974b] noted that there are two major seasons in the Galápagos islands, the garúa or dry season (June–December) characterized by frequent cloud cover and misty rain, and the hot season (January–May) characterized by infrequent cloud cover, occasional heavy rains in some years, and intense solar radiation. Gonadal maturation and mating in both the male and female Galápagos tortoise appears to coincide with the hot season whereas ovulation and nesting coincides with the garúa or dry season. The shift in the timing of gonadal maturation, mating, and nesting observed in the Brownsville colony is probably a result of the shift in latitude and delay in temperature changes observed in south Texas. The corresponding garúa season in Texas would be October to March when ovulation and nesting were observed and the hot season would be April to September when gonadal maturation and mating were observed (Fig. 5). This delay represents a 4-month shift in timing of reproduction. Therefore, temperature would appear to be a proximate environmental cue worthy of further investigation.

CONCLUSIONS

1. Galápagos tortoises displayed seasonal physiological changes that coincided with the observed seasonal reproductive cycle.
2. Males displayed a prenuptial rise in serum testosterone during which gonadal maturation and spermatogenesis is thought to occur.
3. Females displayed a seasonal rise in serum testosterone that coincided with ovarian maturation and the mating period.

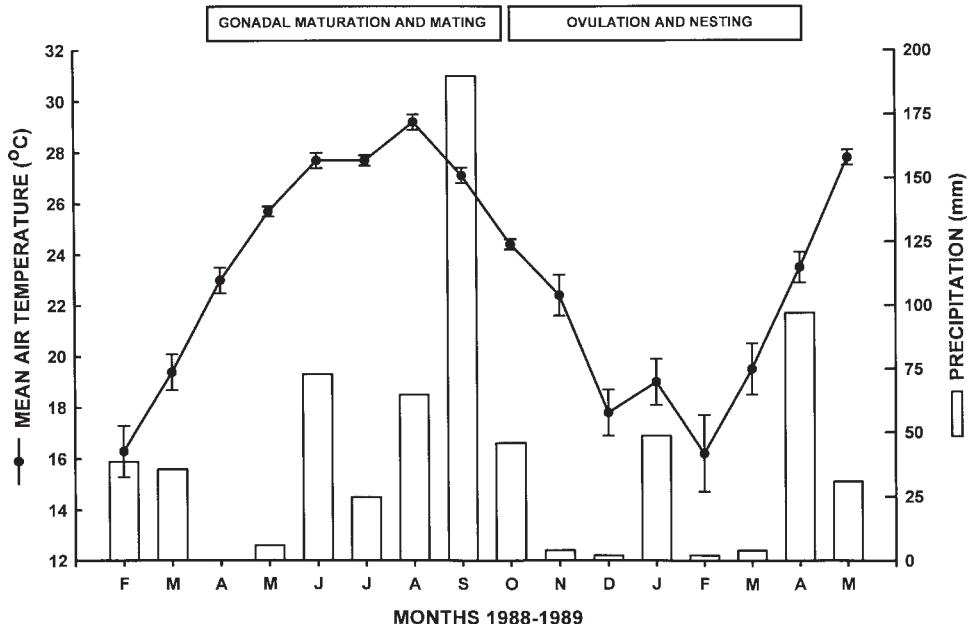

Fig. 5. Mean air temperature (degrees centigrade) and total precipitation (millimeters) per month for Brownsville, Texas, from February 1988 to May 1989.

4. Ovarian maturation and follicular growth coincided with elevated estradiol levels during the post-nesting and pre-mating periods prior to mating.
5. Serum calcium levels increased during the post-nesting period and coincided with ovarian maturation in females. Serum calcium levels were relatively stable in males throughout the study.
6. Nesting females displayed a sharp surge in progesterone during the mating period (October) that coincided with the timing of ovulation.
7. Repeated separation of a male Galápagos tortoise during the study did not result in any detectable physiological changes in reproductive cyclicity.

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