

LINNAEUS FUND RESEARCH REPORTS

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Reproductive Cycles of Male and Female Giant Tortoises (*Geochelone nigra*) on the Galápagos Islands by Plasma Steroid Analysis and Ultrasound Scanning. Linnaeus Fund Research Report

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The populations of giant tortoises of the Galápagos Islands, *Geochelone nigra*, have been severely decimated by human exploitation. Eight of the eleven surviving subspecies are threatened due to predation and competition from introduced mammals (MacFarland et al., 1974). They are also vulnerable to natural and man-made disasters (Pritchard, 1996). Since the 1990s, one of the largest and most stable tortoise populations on volcano Alcedo (Isabela Island; 3000–5000 individuals) has been strongly impacted by goats (CDRS, pers. comm.). The species is classified in Appendix I by CITES (U.S. Fish and Wildlife Service, 1997a) and is considered Endangered (U.S. Fish and Wildlife Service, 1997b). Of major importance in studies of natural populations, and for establishing efficient breeding programs, is an understanding of the basic reproductive biology of the species. However, knowledge of the reproductive physiology of the Galápagos tortoise remains extremely limited mainly because its endangered status restricts techniques for studying reproductive patterns.

Endocrinological evaluation of reproductive cyclicity is essential for assessing reproductive function. Blood samples have been used to monitor hormonal cycles in a number of species of turtles (Garstka et al., 1991; Lance et al., 1995;

Whittier et al., 1997; Rostal et al., 1998a). In order to interpret hormonal fluctuations, hormone levels should be correlated with actual reproductive activity, which can be validated by gonadal visualization. Ultrasound scanning allows direct observation of the reproductive organs without the necessity of using invasive procedures and is free of any radiation side-effects (Baker and Dalrymple, 1978; Kuchling, 1998). Ultrasonography has been applied to chelonian species for evaluating follicular activity and clutch formation (Kuchling, 1989; Robeck et al., 1990), as well as for long-term monitoring of the ovarian cycle (Kuchling and Bradshaw, 1993; Rostal et al., 1994; Rostal et al., 1997).

Most turtles show a postnuptial maturation of gametes (Altland, 1951; Ernst, 1971; White and Murphy, 1973; McPherson et al., 1982; Parmenter, 1985). A prenuptial cycle has been described only in tropical and subtropical species such as *Lissemys punctata* (Singh, 1977), *Chelonia mydas* (Licht et al., 1985) and *Caretta caretta* (Wibbels et al., 1990). In this study the annual reproductive cycle of male and female Galápagos tortoises will be shown, focusing on: (1) monitoring the timing of the ovarian and testicular cycle by analyzing sex hormones in plasma using radioimmunoassays (RIA), (2) monitoring the timing of the ovarian cycle and vitellogenesis in female *G. nigra* using ultrasound scanning of reproductive structures, and (3) recording environmental cues which are hypothesized to be important parameters influencing the reproductive cycle.

Materials and Methods

Between November 1995 and November 1996 blood samples ($n = 191$) were collected from adult Galápagos tortoises and ultrasound investigations were performed in females. The animals were kept under seminatural conditions at the Charles Darwin Research Station (CDRS) on Santa Cruz Island, Galápagos. Compared to wild animals the tortoises are housed in large corrals, which contain original vegetation (cactus, bushes, shrubs) and are restricted by walls of lava stones. The corrals include artificial water pools and several nesting sides, with the exception of the "Varias Islas" enclosure (see below) where the CDRS did not construct artificial nesting sites. The tortoises are fed three times a week with native plants. Most of the time they are left to fend for themselves and their contact with humans



Figure 1. Collection of blood from an adult male Galápagos tortoise (*Geochelone nigra*).

is restricted to when the pool is cleaned, when food is brought, or when eggs are collected.

Collection of Samples. — Blood samples were taken from 16 adult Galápagos tortoises (8 females, 8 males) at monthly intervals. The animals were kept in an enclosure named “Varias Islas” since the tortoises originated from different islands and belonged to several subspecies. To stabilize the tortoise during the procedure and to minimize movements of the extremities, the animal was turned on its back and placed in a car tire. Sampling took approximately 15 min per animal and was conducted at the end of each month.

Blood (5 ml) was collected from the foreleg (vena brachialis) with a heparinized syringe (Fig. 1). The sample was immediately placed into a heparin tube and centrifuged for 10 min at 3000 rpm within the next two hours. The plasma was separated from the blood cells and transferred into a cryotube containing sodium azide as a preservative. All tubes were immediately stored in a freezer until shipped to the Center for Reproduction of Endangered Species in San Diego, USA.

Ultrasonography. — Between November 1995 and April 1996 twenty adult females were examined monthly by ultrasound scanning. During the nesting season (May –



Figure 2. Ultrasound examination of an adult female Galápagos tortoises (*Geochelone nigra hoodensis*).

November) examinations were conducted once a week or every other week. Using a portable real-time ultrasound unit (Aloka SSD-500, 5 MHz convex transducer) the tortoises were scanned through the inguinal shell opening (Fig. 2). The animals were turned on the back and scanned on both sides to allow examination of both ovaries. Developing, preovulatory, and atretic ovarian follicles, as well as eggs at various stages of shell deposition were recorded and measured. Ovarian follicular activity was evaluated by monitoring the size of the largest group of developing follicles.

Eight females investigated belonged to the “Varias Islas” group, 7 females to the “Hood C2” group, and 5 females to the “Hood C1” group. The animals described as the “Hood” group belong to the subspecies *G. n. hoodensis* (Fig. 3) and are housed in two different enclosures (C1 and C2). Between 1963 and 1974 they had been removed from the island Española to save them from extinction. For decades they have been part of the captive breeding and repatriation program of the CDRS and the Galápagos National Park Service (GNPS) and have successfully reproduced (Cayot and Morillo, 1997). The “Hood C2” group consists of 9 tortoises (7 females and 2 males), the “Hood C1” group of 6 tortoises (5 females and 1 male).

Environmental Conditions. — All aspects related to the management of the Galápagos tortoises at the CDRS were recorded, including feeding regimes and environmental conditions. Air temperature and light intensity were moni-



Figure 3. Male saddle-back tortoise from Española Island (*Geochelone nigra hoodensis*).

tored daily at 0600, 1200, and 1800 hrs. Temperature was measured using a digital meter (UNITEST No. 93420 D). The tool was moved in the shade of a tree for 5 min, after which the temperature was leveled and the value was recorded. At 25°C the precision of the measurements is $\pm 0.8^\circ\text{C}$. Photointensity was measured with a lux meter (UNITEST No. 93408 D, range: 0–200,000 lx) which had a precision of $\pm 3\%$ from the measurement value + 5% from the end value) at 20°C, 80% relative humidity and at a color temperature of 2856°K. The sensor of the lux meter was horizontally placed on the surface of an elevated lava rock in an area where no shade could interrupt the sunlight. Direct photointensity was measured for 1 min and the highest value was recorded. All tools were positioned in the “Varias Islas” corral.

Analysis of Samples. — The steroids in the blood were analyzed by radioimmunoassay (RIA) procedures. Plasma corticosterone (B), testosterone (T), and progesterone (PRO) levels were measured using tritium (^3H) labeled steroids; estradiol (E2) was measured using a modified ^{125}I -kit.

Statistics. — An unpaired t-test ($p \leq 0.01$) was used to describe annual differences in plasma hormone levels between male and female Galápagos tortoises.

Results

All hormones showed seasonal changes in male and female *G. nigra*. Progesterone and estradiol levels were investigated in females only.

Corticosterone. — Male corticosterone showed low levels (means around 0.7 ng/ml) in November and December (postnesting). Hormone levels reached a maximum in February (mean = 1.5 ng/ml); they remained elevated until they rapidly declined in June (nesting), reaching their lowest point in July.

Corticosterone levels in females were low (means around 0.2 ng/ml) in November and December (postnesting). They began to rise in January and reached their highest level in April (mean = 1.1 ng/ml) during the mating season. Hormone levels remained elevated until they declined between August and October while the animals were nesting. Annual male and female corticosterone levels were significantly different.

Testosterone. — Male testosterone levels were low (mean = 3.8 ng/ml) in November during the postnesting season. They started to rise in December (prebreeding) until they reached their maximum in February (mean = 29.0 ng/ml) during the mating season. Hormone levels started to decline in May reaching lowest values in September (nesting).

Females showed low testosterone levels (means around 0.3 ng/ml) in November and December (postnesting). Between January and March testosterone levels increased and reached their maximum (mean = 1.7 ng/ml) during the mating season in April. In May testosterone levels started to decrease and reached a minimum in September (nesting season). Annual testosterone levels were significantly different in males and females.

Estradiol. — Female estradiol levels started to increase in November (postnesting) and reached highest values (mean = 160.5 pg/ml) in February during the mating season. Hormone levels started to drop in June and reached a minimum (mean = 33.0 pg/ml) in July when the nesting season peaked.

Progesterone. — Female progesterone showed low levels between November (postnesting) and March (mating). The hormone levels began to rise in April (breeding season) and reached a maximum in June (mean = 1.6 ng/ml) during the nesting season. Between July and September (nesting) progesterone levels decreased and reached a minimum (mean = 0.3 ng/ml) in October at the end of the nesting season.

Ultrasonography. — Ovarian follicles at various stages of development, atretic follicles, and oviductal eggs were identified by ultrasound. The time between the deposition and calcification of the shell and the laying of the eggs were recorded (oviductal period), as well as the interclutch interval and retention of eggs.

Vitellogenic follicles are echogenic and appear on the screen of the ultrasound as spherical and dense structures. Atretic follicles consist of echogenic and non-echogenic material, which shows irregular densities. Eggs are mostly spherical in shape and have a highly echogenic shell which is followed by an non-echoic albumin layer and an echogenic yolk.

From all examinations of both the right and left ovaries and oviducts of 20 *Geochelone nigra* females, 98% have clearly shown reproductive structures. In 2% of the examinations no follicles could be visualized in either one or both ovaries due to the oviduct being filled with eggs blocking the view of other structures. In 96% of cases vitellogenic follicles were observed in either one or both ovaries and 1% of the examinations showed only atretic follicles. Preovulatory follicles were found in the ovaries of all females throughout the year with large follicles ranging between 21 to 42 mm in diameter. At the CDRS the tortoises mate during the rainy season from January through June when the sizes of the follicles increased. The largest preovulatory follicles were recorded from May to September (nesting) where most of them ranged between 40 and 42 mm. Atretic follicles were observed during the entire year and they usually showed sizes similar to those of vitellogenic follicles.

At the CDRS *G. nigra* nests from May to October. Females of the “Hood C1” group produced one to three clutches between June and October with each clutch containing 5 to 10 eggs. The oviductal period ranged between 17 and 41 days. The interclutch interval ranged between 30 and 55 days. In the “Hood C2” group females laid one or two clutches between July and September. Each clutch contained 5 to 12 eggs. The oviductal period and the interclutch interval ranged between 23 and 50 days and 38 and 64 days, respectively. The females of the “Varias Islas” group showed different patterns in their nesting behavior with only 4 out of 8 females depositing their eggs in constructed nests. The remaining animals laid their eggs on the surface or other unusual places such as among rocks. Between May and

October females nesting "typically" produced one to two clutches which contained 5 to 14 eggs. The oviductal period ranged between 8 and 74 days and the interclutch interval between 40 and 89 days. The remaining 4 animals laid between 1 and 2 eggs on the surface in irregular time periods.

Retention of eggs was only observed in the "Varias Islas" females. Some females retained eggs for only a few days and then laid their clutches intermittently, dropping one to two eggs over several days until the whole clutch was laid completely. Other females retained eggs for several weeks, whereas one female showed hard-shelled eggs almost all year round. Females from the subspecies *G. n. hoodensis* ("Hood C1" and "Hood C2" group) always laid complete clutches throughout the whole investigation period and never retained eggs.

Environmental Conditions. — Three times a week the tortoises were fed fresh herbs, leaves, and grasses which had been collected in the highlands of Santa Cruz. The tortoises also actively searched for food within their corrals and fed on various seasonal plants including leaves, flowers, and fruits. No additional minerals or fruits were offered. During 5 to 6 days a week the tortoises had access to water *ad libitum*. When it rained, mud pools naturally formed and were used abundantly by the animals.

Highest air temperatures were recorded during the mating season in February and March with values between 24 and 31°C (monthly means). Lowest temperatures were found during the nesting season in August and September (means between 20 and 24°C). Photointensity reached its maximum at noon showing monthly means between 60,150 and 152,258 lx.

Discussion

Previously the reproductive physiology of Galápagos tortoises has been studied in captive animals only (Robeck et al., 1990; Casares, 1995; Casares et al., 1995, 1997; Rostal et al., 1998b). This study provides data from successfully breeding male and female Galápagos tortoises which hatched in the wild (Schramm et al., 1999). As adults they were transported to the breeding center on Santa Cruz Island where they now live under seminatural conditions and are exposed to their natural habitat and environmental conditions such as nutrition, temperature, photoperiod, light intensity, humidity, and precipitation. Male and female Galápagos tortoises at the CDRS show a distinct seasonal reproductive cycle with mating season peaking from January to June during the rainy season when temperatures are elevated. The nesting season begins in May and ends in October during the dry (*garúa*) season when temperatures are relatively low.

Male Reproductive Cycle. — In male reptiles androgen concentrations are elevated when spermiogenesis occurs (Lance, 1984). At the CDRS male testosterone levels start to rise in December, one month before the onset of the mating season, which appears to be related to testicular maturation

and spermiogenesis. They remain elevated during the mating season and decrease in May when regular copulations are still observed while the nesting season starts. The increase in testosterone just before copulation occurs suggests that male Galápagos tortoises produce their gametes before the onset of the mating season, indicating prenuptial testicular activity.

Female Reproductive Cycle. — In female chelonians testosterone levels rise during vitellogenesis and peak before ovulation occurs (Lance and Callard, 1978). At the CDRS, female Galápagos tortoises show high plasma testosterone levels during the mating season in April when large preovulatory follicles are found in the ovaries. Testosterone remains elevated until May – June when the first eggs are laid.

In turtles estradiol levels rise when vitellogenesis occurs (Lance and Callard, 1978; Ho, 1987). Females at the CDRS show a rise in estradiol in January at the beginning of the mating season when the sizes of follicles start to increase. In May, at the onset of the nesting season, estradiol drops when preovulatory follicles have reached their maximal diameter. Preovulatory follicles are found throughout the year but show their largest sizes during nesting when estradiol levels decrease between April and August.

In reptiles progesterone levels are elevated prior to and around the point of ovulation coinciding with the activity of periovulatory follicles and postovulatory follicles or corpora lutea (Lance and Callard, 1978; Licht, 1984). Turtles, which produce multiple clutches, show several ovulatory surges within one season (Licht et al., 1979; Owens and Morris, 1985). Females at the CDRS lay between one to three clutches per season but only show one clear progesterone peak during the nesting season in June. The detection of only one peak can be explained by the sampling frequency (one blood sample per animal per month) and other peaks could have been missed. Some weeks before and after the peak evolves, progesterone levels are elevated, indicating that ovulation occurred. This was validated by ultrasound investigations when albumin layers and thin-shelled eggs were detected a few days after a clutch of preovulatory follicles had ovulated. No corpora lutea could be detected during our examinations, due to the fact that they look very similar to atretic follicles in ultrasonography. This agrees with the findings from Robeck et al. (1990) where laparoscopy confirmed the observations from ultrasound but could not clearly distinguish atretic follicles from corpora lutea either. Only Swingland and Coe (1978) clearly described follicles and corpora lutea at various stages of development or regression in giant tortoises, obtaining their results from freshly killed Aldabra tortoises (*G. gigantea*).

Corticosterone Cycles. — Corticosterone is an important indicator of stress in reptiles since hormone levels increase while animals are manipulated (Lance and Lauren, 1984; Gregory et al., 1996). In *Chelydra serpentina* stress may influence sex steroids and can lower or even suppress them (Mahmoud et al., 1989). Mendonça and Licht (1986) observed fourfold lower magnitudes in the temporal pattern of circulating androgen in captive *Sternotherus odoratus* than in the field, suggesting that the animals could be

chronically stressed or stimulatory factors could be missed. Since testosterone, estradiol, progesterone, and corticosterone levels in male and female Galápagos tortoises show seasonal patterns matching the physiological conditions, dramatic stress-induced hormone changes can be excluded. It can be assumed that the handling of Galápagos tortoises for less than 15 min does not suppress the steroid levels although the animals were obviously stressed during the bleeding procedure. In other reptiles, such as alligators, response to stress is more rapid since stress-induced changes in hormone levels have been detected after 10 min (Lance and Lauren, 1984).

Male tortoises at the CDRS show high corticosterone levels during the mating season, which follow the cycle of testosterone. Both hormones peak in February and again in April and decrease during the nesting season. In female Galápagos tortoises corticosterone and testosterone levels also seem to be correlated. Both hormones show high levels during the mating season and decrease during the nesting season. In female *Caretta caretta* corticosterone levels seem to be correlated with ovarian development, yolk deposition, and testosterone levels (Whittier et al., 1997). Further investigations are needed to clarify the role of corticosterone regarding reproduction.

"Varias Islas" Group vs. "Hoodensis" Group. — The differences in reproductive patterns between the "Varias Islas" and the "Hoodensis" females can be explained by their habitat. CDRS personnel only collect and incubate eggs from the "Hoodensis" group and therefore did not construct any nesting facilities in the corral of the "Varias Islas" group. Many times females were observed seeking a nesting site for days (sometimes for weeks) and digging several false nests in the rocky soil before they finally deposited their eggs. This accounts for the frequent retention of eggs and their deposition on the surface of the soil, which was observed in females of the "Varias Islas" group only.

Environmental Conditions. — Temperature, moisture, and light seem to play an important role regarding the sexual cycles of male and female reptiles (Licht, 1972; Kar, 1987). In turtles, temperature and photoperiod is thought to influence testicular and ovarian activity (Moll, 1979; Kuchling, 1982; Mendonça and Licht, 1986).

On the Galápagos Islands temperatures are highest during the mating season when male tortoises show high testosterone and corticosterone levels and spermiogenesis is thought to occur. During the same period of time females show an increase in estradiol, while ovarian follicles mature and reach large preovulatory sizes. Female corticosterone levels are elevated when temperatures begin to decrease during the mating season in April. Between July and October (low temperatures) male testosterone and female estradiol reach their minimum. The Galápagos Islands are located on the Equator and have a relatively constant day and night length. On Santa Cruz Island the differences in photoperiod vary approximately ± 30 min in the mornings and evenings during the entire year. Previously, prenuptial gonadal patterns have only been described in turtles living in tropical or

subtropical climate zones (Singh, 1977; Licht et al., 1985; Wibbels et al., 1990), which agrees with our findings in the Galápagos tortoise. At the CDRS females lay their eggs during the dry season when humidity is relatively high and drizzle (*garúa*) occurs almost every day. The tortoises hatch between November and April during the rainy season when temperatures reach their maximum and heavy rainfalls may occur.

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