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SEASONAL REPRODUCTIVE CYCLE OF THE DESERT TORTOISE (*GOPHERUS AGASSIZII*) IN THE EASTERN MOJAVE DESERT

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ABSTRACT: The seasonal reproductive cycles of male and female desert tortoises (*Gopherus agassizii*) were studied under semi-natural conditions. Tortoises were maintained in outdoor pens subject to ambient weather conditions and received supplemental food and water. Heparinized blood samples were collected monthly using jugular puncture. Ovarian follicular growth and egg development were monitored using ultrasonography. Mating was observed in the fall (following nesting) and the spring (prior to nesting). Vitellogenesis occurred during the fall prior to hibernation. Nesting was observed from May–early July with females producing one or two clutches. Clutches ranged from 2–7 eggs. Both males and females displayed seasonal testosterone cycles.

Key words: *Gopherus agassizii*; Reproduction; Seasonal cycles; Testosterone; Ultrasonography

THERE are four species of tortoises found in North America (the gopher tortoise, *Gopherus polyphemus*; the Texas tortoise, *G. berlandieri*; the Bolson's tortoise, *G. flavomarginatus*; and the desert tortoise, *G. agassizii*), and all appear to be threatened due to human activities and habitat destruction. In 1989, *G. agassizii* was officially listed as a threatened species under the Endangered Species Act.

The reproductive biology of *G. agassizii* has been relatively unstudied (Spotila and Standora, 1986). *Gopherus polyphemus* has received the most study to date (Iverson, 1980; Landers et al., 1980; Palmer and Guillette, 1990; Taylor, 1982). More recently egg production has been studied in both *G. agassizii* and *G. berlandieri* (Turner et al., 1982, 1986; Judd and Rose, 1989). Information on the reproductive biology of *G. agassizii* needed for proper management and conservation plans is lacking.

In the current study, the reproductive biology of *G. agassizii* was studied over a 14 month period at the Desert Tortoise Conservation Center in Las Vegas, Nevada. Seasonal testosterone cycles, vitellogenesis, ovarian follicular growth, egg production and nesting were monitored and the reproductive cycles were delineated.

MATERIALS AND METHODS

Subjects

Fifty tortoises were maintained in ten reproductive groups composed of 2 males and 3 females. Male mean straight carapace length was 261.25 ± 3.93 mm (SE; n = 20). Female mean straight carapace length was 241.07 ± 2.32 mm (SE; n = 30). The tortoises were housed in 15 m × 30 m pens. Each pen had five artificial burrows, two sod plots, and two watering stations. Natural vegetation was present in the pens. The pens were also supplemented with alfalfa hay. The tortoises were exposed to ambient lighting and weather conditions (i.e., air temperature, humidity and rainfall).

Blood Collection and Radioimmunoassay

Heparinized blood samples (3–5 cc) were collected via jugular puncture (Jacobson et al., 1992). Blood samples were collected once a month on all 20 males and all 30

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females from August–October 1991 and April–September 1992. Blood samples were centrifuged and the plasma removed and frozen for later analysis.

Plasma testosterone levels were measured using a radio-immunoassay (Lance et al., 1985). Duplicate samples of plasma (2.5–100 μl) were extracted with 2.0 ml of ethyl acetate : n-hexane (3:2 V/V). Samples were vortexed for 30 seconds, snap frozen in a dry ice-methanol bath and the organic phase decanted into clean 12 × 75 mm culture tubes. The organic solvent was dried under a stream of nitrogen gas and the dried extract reconstituted in 0.5 ml assay buffer. To each tube was added approximately 10,000 cpm of tritiated testosterone in 0.1 ml of assay buffer and 0.1 ml of antibody. The tubes were vortexed briefly and then incubated overnight at 4 C. The unbound steroid was separated from the free with ice cold dextran charcoal and the supernatant decanted directly into a scintillation vial. In the case of female tortoises, duplicate samples of 100 μl of plasma were extracted; in the case of male tortoises, 100 μl of plasma was diluted 1:20 with assay buffer and aliquots equivalent to 2.5, 5 and 10 μl were extracted. The antibody to testosterone was obtained from ICN Biomedicals, Inc., Costa Mesa, California. At a working dilution of 1:56,000 this antibody showed a cross reactivity to dihydrotestosterone of 19% (testosterone 100%). No other steroid tested showed significant cross reactivity. The sensitivity of the assay was 5 pg. Two pools of tortoise plasma were prepared for internal standards and run with each assay. For further details, see Lance et al. (1985).

Total plasma calcium, monitored as an indicator of vitellogenesis and follicular growth, was measured using an automated Boehringer Mannheim diagnostic analyzer (Boehringer Mannheim Corp., Indianapolis, Indiana 46250, USA).

Ultrasound Examinations

The reproductive status of the females was monitored using ultrasonography similar to that described by Rostal et al. (1990). Females were scanned at two month intervals during the pre- and post-nesting periods to track ovarian follicular growth. During the nesting season, females were scanned every two weeks to monitor oviductal egg development and to track nesting. An Aloka 500V portable real-time ultrasound scanner (Corometrics Medical Systems, Inc., Wallingford, CT 06492, USA) with a 7.5 convex linear transducer was used. The procedure involved manually restraining the females in an upright position. The hindlimbs were then extended allowing access to the inguinal area. This provided an accessible ultrasound "window" in the inguinal region cranial to the hindlimb. Parker Aquasonic Ultrasound Gel (Parker Laboratories, Inc., Orange, NJ 07050, USA) was used as a coupling gel and applied liberally to the inguinal region. The probe was then oriented in a craniomedial direction and reproductive structures (ovaries and oviducts) were imaged. Both sides of the tortoise were scanned independently. Ovarian follicles, ovulated follicles (i.e., egg yolks), and egg shell dimensions were measured using the built-in electronic calipers (Rostal et al., 1990).

Histology

Male reproductive tissues were provided from animals sacrificed for upper respiratory tract disease (URTD) studies conducted by Dr. E. Jacobson, College of Veterinary Medicine, University of Florida. Tissues were fixed in 10% buffered formalin or Bouin's solution. Fixed tissues were trimmed, dehydrated through a series of ethanol, then embedded in Paraplast. Sections of 4 μm were prepared on glass slides and routinely stained with hematoxylin and eosin or Masson's trichrome.

Statistical Analyses

Seasonal changes in plasma testosterone, plasma calcium, and body mass of males and females were determined using repeated measures ANOVA followed by the Scheffe test ($P \leq 0.05$).

RESULTS

The desert tortoise (*G. agassizii*) displays a distinct seasonal reproductive cycle

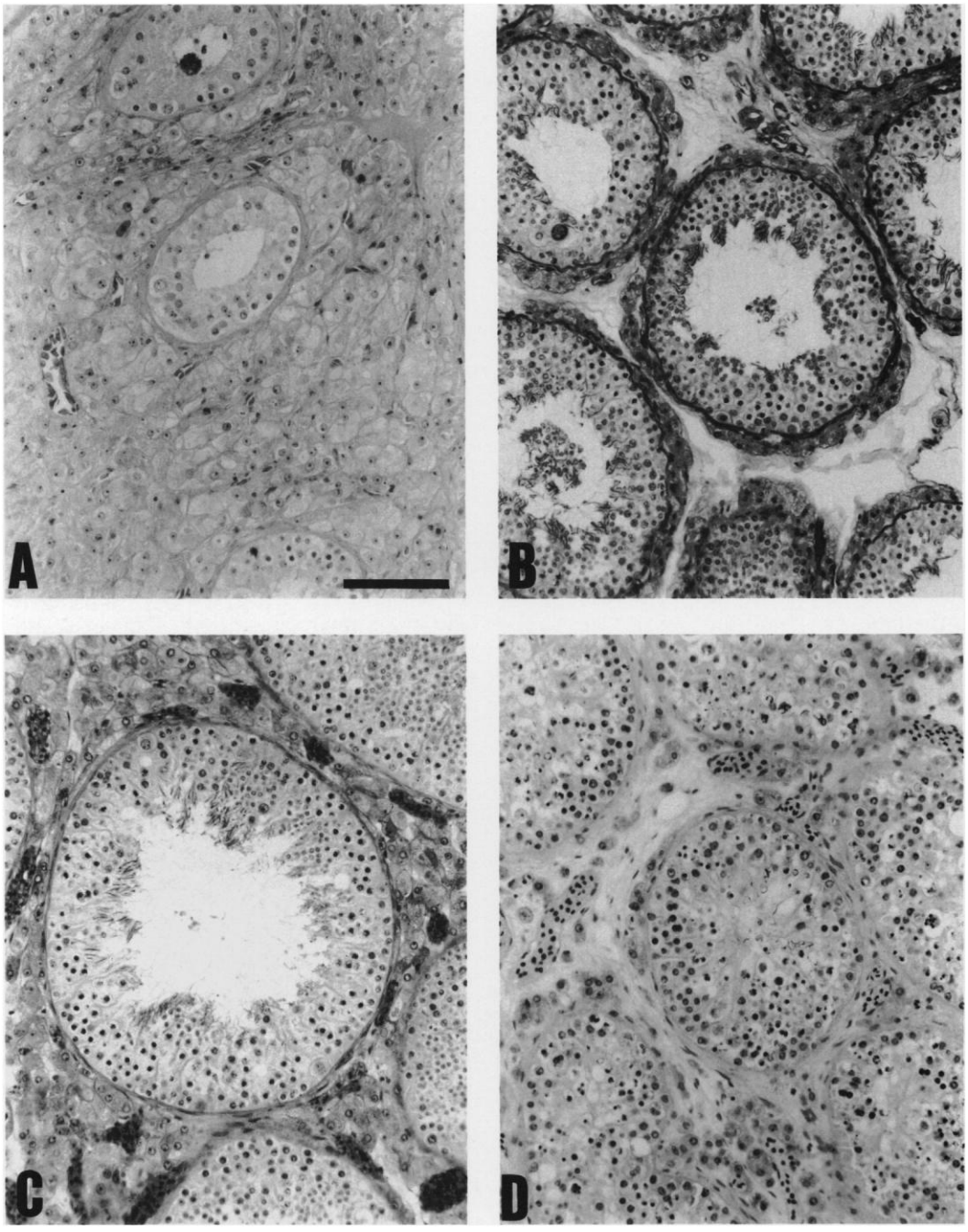

FIG. 1.—Histology of desert tortoise testis during a spermatogenic cycle. Bar in A = 100 μm. All four micrographs are at the same magnification. A. Section from a testis of an animal sacrificed in May. Seminiferous tubules are completely regressed and contain only spermatogonia and sertoli cells. The leydig cells appear greatly hypertrophied and fill the interstitial area. B. Section from a testis of an animal sacrificed in July. Spermatogenesis has progressed to stage 4–5 of McPherson et al. (1982). Spermatocytes and spermatids are abundant and a few mature spermatozoa are present. The leydig cells are also smaller in May. C. Section from a testis from an animal sacrificed in October. Seminiferous tubules are at their greatest diameter and

with intense mating activity occurring in the spring (April and May) and a second period in the fall (August–November). Male-male combat shows similar periods of activity. Nesting was observed during the spring and early summer (May–July).

Male Testicular Cycle and Plasma Testosterone

A seasonal testicular cycle was observed in males used in the upper respiratory tract disease (URTD) study. We were unable to obtain the complete reproductive tract or testis weight, so the condition of the epididymis remains speculative. In May, seminiferous tubules were completely regressed and contained only spermatogonia and sertoli cells. The leydig cells appeared greatly hypertrophied and filled the interstitial area (Fig. 1A). By July (Fig. 1B), spermatogenesis had progressed to stage 4 and 5 of McPherson et al. (1982). Spermatocytes and spermatids were abundant and a few mature spermatozoa were present. Prior to hibernation in October, diameter of seminiferous tubules and spermatogenesis appears maximal. The leydig cells again appeared hypertrophied (Fig. 1C). In April following emergence from hibernation, the lumen of the seminiferous tubules was filled with debris from the previous cycle. Large numbers of spermatogonia and spermatocytes were still present. The leydig cells were not particularly abundant and were only moderately developed (Fig. 1D).

Male tortoises displayed a significant rise in plasma testosterone during the summer from May to August ($F = 35.50$, df = 8, 152, $P < 0.001$) which continued into the fall mating period (Fig. 2). Plasma testosterone levels were then observed to decline prior to hibernation. Upon emergence from hibernation in April, testosterone levels were significantly reduced during the spring mating period (April and May).

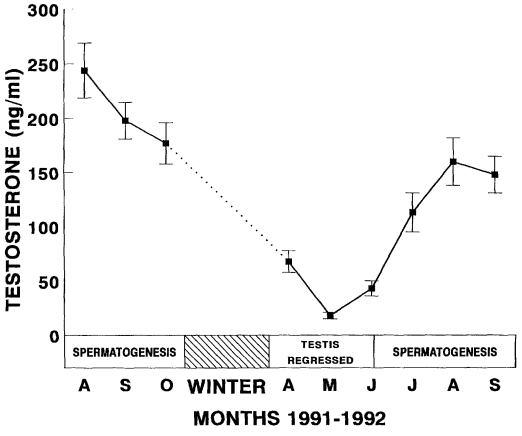

FIG. 2.—Mean monthly levels of plasma testosterone from 20 male desert tortoises from August 1991–September 1992. Shaded area represents hibernation period. Values are means ± SE.

Male testosterone levels ranged from a mean high of 243.60 ± 24.61 ng/ml (SE, $n = 20$) during the fall mating period (August) to a mean low of 18.37 ± 3.14 ng/ml (SE, $n = 20$) during the nesting period (May). High testosterone levels observed in August 1991 relative to August 1992 occurred three weeks after introduction of the animals into their respective breeding groups and were probably the result of increased novel interactions with conspecifics. During this period, males were attempting to establish territories and dominance relationships.

Female Ovarian Cycle and Plasma Testosterone

Female tortoises also displayed a significant rise in testosterone (July–October) prior to hibernation ($F = 48.38$, df = 8, 224, $P < 0.001$; Fig. 3). This increase coincided with the onset of the fall mating period. Female testosterone levels were highest following emergence from hibernation prior to ovulation in April and May. Female testosterone levels ranged from a

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spermatogenesis is at a maximum. The leydig cells again appeared hypertrophied. D. Section from a testis of an animal sacrificed in April. The lumen of the seminiferous tubules is filled with debris from the previous cycle. Large numbers of spermatogonia and spermatocytes are still present. The leydig cells are not particularly abundant and are only moderately developed.

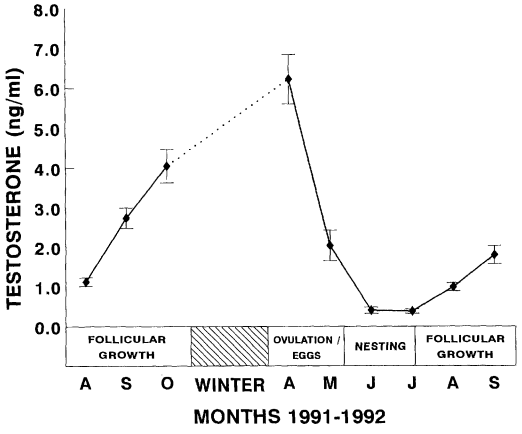

FIG. 3.—Mean monthly levels of plasma testosterone from 30 female desert tortoises from August 1991 to September 1992. Shaded area represents hibernation period. Values are means ± SE.

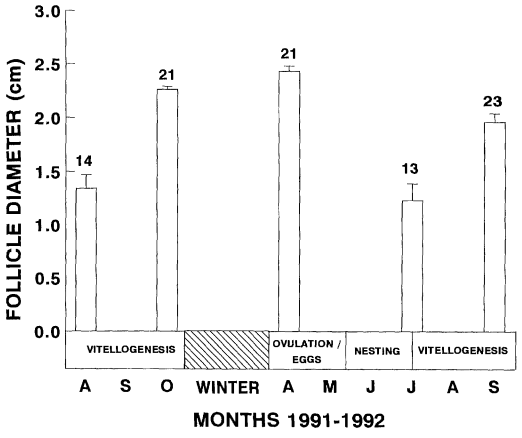

FIG. 4.—Mean diameter of largest ovarian follicles measured bimonthly from 30 female desert tortoises from August 1991–September 1992. Shaded area represents hibernation period. Values are means ± SE.

mean high of 6.22 ± 0.62 ng/ml (SE, n = 30) during the spring mating period (April) to a mean low of 0.37 ± 0.05 ng/ml (SE, n = 29) during the late nesting period (July).

Vitellogenesis and ovarian follicular growth were observed using ultrasonography during the late summer and fall (July–October) following the completion of nesting (May–July; Fig. 4). Ovarian follicles had matured to ovulatory size range ($\bar{x}$ ± SE = 2.43 ± 0.05 cm in diameter, n = 22) prior to hibernation (Fig. 5A). Shelled eggs were first observed in the oviducts of females in mid-April using ultrasonography. Recently ovulated follicles had a thin shell which allowed the ultrasound waves to pass and permitted accurate measurement of the yolk for the verification of ovulatory size (Fig. 5B). As the shell was further calcified, the ultrasound waves were reflected and resolution was reduced (Fig. 5C). Eggs had moved further down the oviduct and could be manually palpated at this stage. Ninety percent of the females had ovulated by April 30th and were shelling eggs in their oviducts. Nesting first occurred in May. Following the completion of nesting, atretic follicles were frequently observed (Fig. 5D). Atretic follicles were generally smaller than preovulatory vitellogenic follicles ranging from 0.7–1.9 cm in diameter, had a dark nonechoic core and varied in shape.

Plasma Calcium

Female plasma calcium levels (indicative of vitellogenin levels) were significantly elevated during the fall (July–September) when follicular growth was observed ($F = 14.52, \text{df} = 8, 72, P < 0.001$). Plasma calcium levels ranged from 8.2 ± 1.28 mg/dl (n = 10) in April following hibernation to 16.38 ± 0.7 mg/dl (n = 10)

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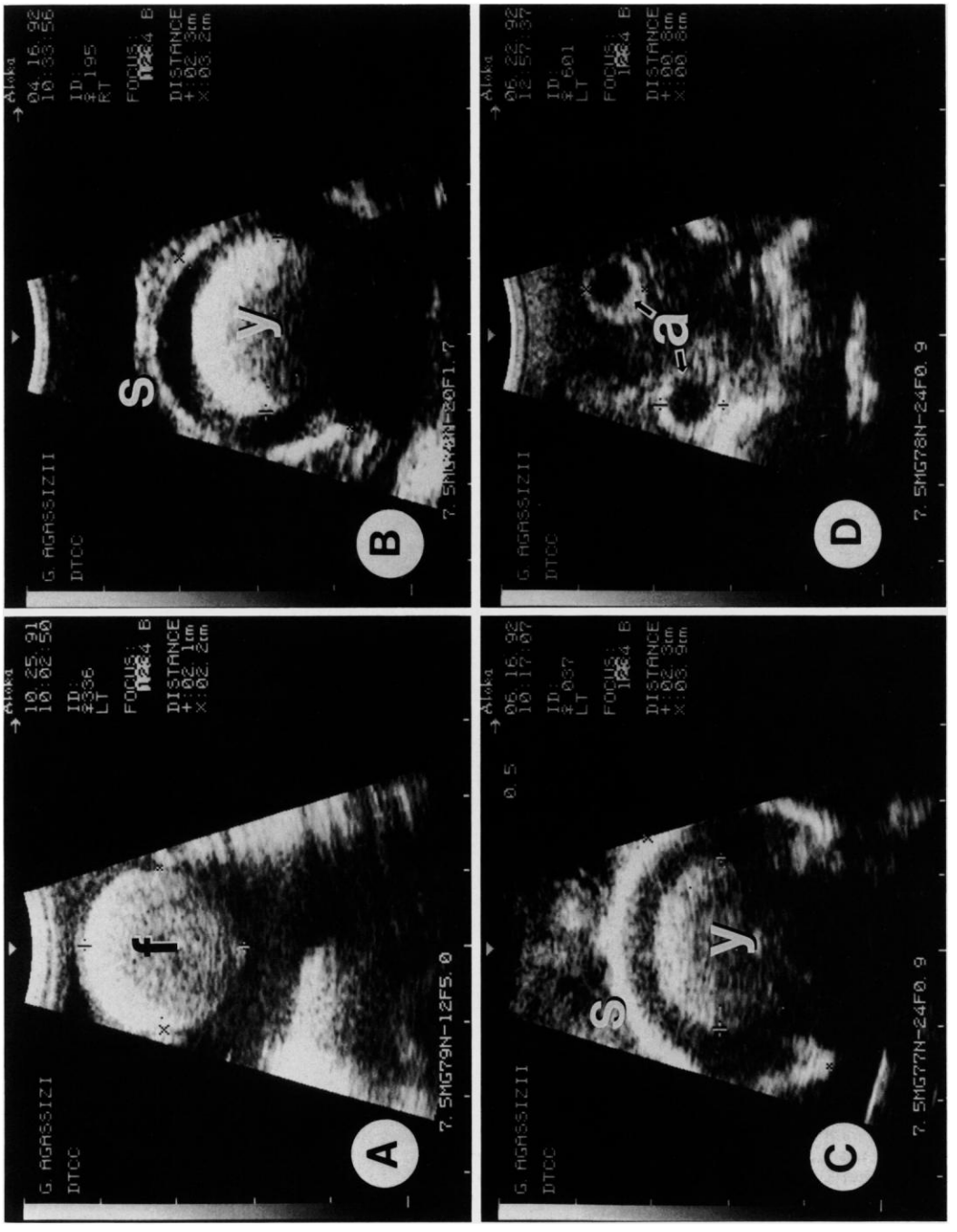
FIG. 5.—Ultrasonography of desert tortoise ovaries and oviductal eggs. A. Ultrasound image of a large vitellogenic follicle (f; 2.1 cm diameter) nearing ovulatory size prior to hibernation (October) in the ovary. B. Ultrasound image of a recently ovulated oviductal egg (less than 10 days postovulation) showing a well defined yolk (y; 2.3 cm diameter) and a thinly calcified shell (s). C. Ultrasound image of a fully developed oviductal egg (between 20 and 30 days postovulation) showing a less defined yolk (y; 2.3 cm diameter) and a well calcified shell (s). Resolution is poorer at this stage due to the heavily calcified shell. Oviductal eggs were normally observed on both sides of the tortoise and could be palpated manually at this stage. D. Ultrasound image of atretic ovarian follicles (a; 0.8 cm diameter) observed following the completion of the nesting cycle.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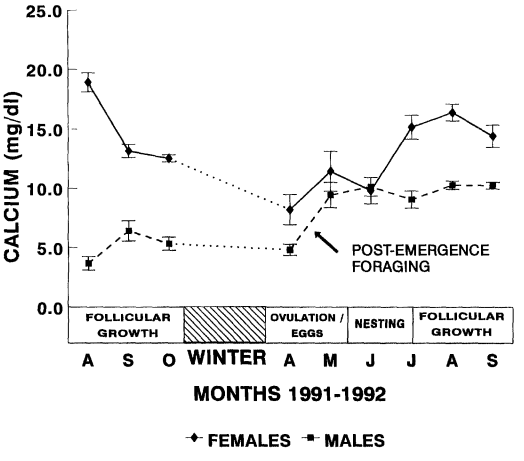

FIG. 6.—Mean monthly levels of plasma calcium from 10 male and 10 female desert tortoises from August 1991–September 1992. Shaded area represents hibernation period. Values are means ± SE.

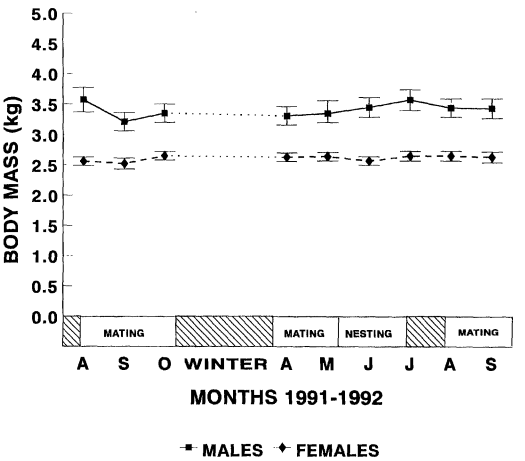

FIG. 7.—Mean monthly values of body mass from 20 male and 30 female desert tortoises from August 1991 to September 1992. Shaded area represents hibernation period. Values are means ± SE.

in August 1992 following the nesting season (Fig. 6). Male plasma calcium levels were significantly lower (F = 15.68, df = 8, 72, $P < 0.001$) in the fall of 1991 prior to hibernation (3.66 ± 0.57 mg/dl, n = 10), increased from April–May following spring emergence, and then remained relatively constant from May (9.44 ± 1.07 mg/dl, n = 10) to September (10.24 ± 0.29 mg/dl, n = 10; Fig. 6).

Body Mass

Males displayed seasonal changes in body mass which coincided with the seasonal recrudescence of the testes with significantly higher body mass from June–September (F = 6.17, df = 8, 152, $P < 0.001$; Fig. 7). Females displayed a seasonal change in body mass with significantly lower body mass (F = 3.82, df = 8, 225, $P < 0.001$) observed during the spring nesting period (June 1992) and the fall vitellogenic period (September 1991 and 1992).

Nesting and Clutch Size

Nesting was observed in late spring and early summer (12 May–3 July, 1992). A total of 33 clutches were laid. Mean clutch size was 4.68 ± 0.3 eggs (SE, n = 19). Nests were laid in natural burrows, artificial burrows and under vegetation. Clutch size ranged from 2–7 eggs with 22 females laying one clutch and 6 females laying 2 clutches. Primary or first clutches (mean clutch size = 5.07 ± 0.35 eggs, SE, n = 13) tended to be larger than second clutches (mean clutch size = 3.75 ± 0.63 eggs, SE, n = 4). Several nests were predated before they were located and collected or protected. The interval from ovulation to nesting was determined using ultrasonography and the time between first and second nestings. The time from ovulation to nesting during which the albumin layer and egg shell formation occurred was approximately 30 days.

Air Temperature and Precipitation

Average air temperature (C) and total precipitation (mm) per month from August 1991 to September 1992 (Las Vegas, NV: 36°05′ N latitude) are presented in Fig. 8 (data from the National Weather Service). Nesting occurred from mid-May to early-July when average monthly air temperatures ranged from 25.4–31.5 C. Vitellogenesis and ovarian maturation was observed following the nesting season and coincided with peak air temperatures. Male testicular maturation, increased testosterone levels and spermatogenesis coincided with increasing air temperatures from June–August. The tortoises were inactive in their burrows from November–March

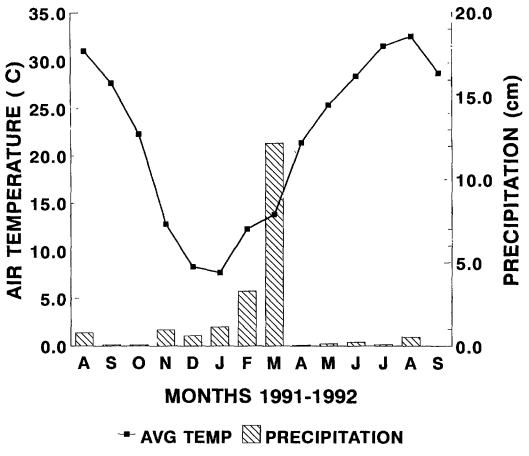

FIG. 8.—Average air temperature (C) and total precipitation (mm) per month collected at Las Vegas, Nevada (36°05′ N), from August 1991 to September 1992.

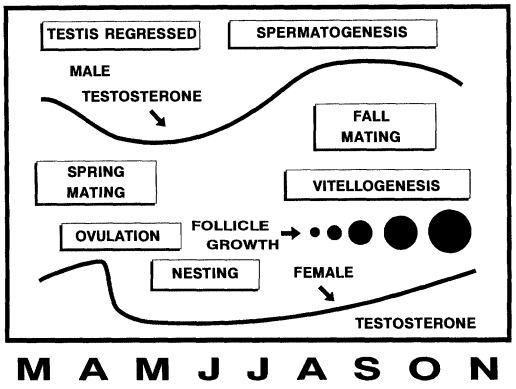

FIG. 9.—Seasonal reproductive cycles of male and female desert tortoises (*G. agassizii*) at the Desert Tortoise Conservation Center, Las Vegas, Nevada.

when average air temperatures ranged from 13.4 C in November to a low of 7.7 C in January. Monthly precipitation was variable with peak precipitation occurring in March. Air temperature appears to be a relatively constant environmental cue which coincides with physiological changes in reproduction.

DISCUSSION

The colony of desert tortoises (*G. agassizii*) at the Desert Tortoise Conservation Center displayed a seasonal reproductive cycle (Fig. 9). The reproductive cycle of *G. agassizii* shows similarities to both the gopher tortoise, *G. polyphemus* (Iverson, 1980; Landers et al., 1980) and the European tortoise, *Testudo hermanni* (Kuchling et al., 1981).

Male Reproductive Cycle

Males displayed a similar testicular cycle to other chelonian species studied to date (Lance, 1984; Licht, 1982; Moll, 1979; Mendonça and Licht, 1986; Risley, 1938). Spermatogenesis begins in late spring as temperature increases, reaches a peak in late summer and appears completed by early fall when mature spermatozoa move into the epididymides prior to hibernation. The testis is fully regressed in April upon emergence from hibernation. The increase in male plasma testosterone from May to August coincides with gonadal recrudescence. The seasonal pattern in testosterone levels is similar to that reported by Kuchling et al. (1981) for *T. hermanni*, but the levels are an order of magnitude higher in *G. agassizii* (1–20 vs. 20–400 ng/ml, respectively). McPherson et al. (1982) reported testosterone levels as high as 150 ng/ml in *Sternotherus odoratus*. Similar seasonal patterns of male testosterone and testicular maturation have been observed in a variety of other turtle species (Licht, 1982).

Normal plasma calcium levels reported for male reptiles range from 9–11 mg/dl (Dessauer, 1970, 1974). The decreased calcium levels observed in males during the fall of 1991 prior to hibernation may be indicative of the stressed condition of the males following their collection from the field that summer. The females used in this study had been in captivity for several months prior to their introduction into the study groups and displayed normal levels. The increase in calcium levels observed in males from April to May following spring emergence were also observed in the field population of tortoises studied near the Desert Tortoise Conservation Center and were probably the result of increased forage following heavy winter rains.

Female Reproductive Cycle

Moll (1979) identifies the first stage in the female annual cycle as follicular en-

largement resulting from yolk accumulation during vitellogenesis beginning in late summer. In female *G. agassizii*, follicular growth was monitored using ultrasonography and serum calcium levels. Follicular growth was observed during the post-nesting period and coincided with elevation in female serum calcium levels during this period. Elevations in total calcium levels have been associated with vitellogenesis (Ho, 1987) and follicular growth 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 Kemp's ridley sea turtle, *Lepidochelys kempi* (Rostal, 1991), and the tuatara, *Sphenodon punctatus* (Cree et al., 1991).

Ovarian follicles matured to ovulatory size from July (mean diameter = 1.23 ± 0.16 cm, SE, n = 13) to October (mean diameter = 2.26 ± 0.03 cm, SE, n = 21) prior to hibernation. Plasma testosterone rose during the fall and coincided with ovarian follicular growth prior to hibernation. Upon emergence from hibernation in March and April, ovarian follicles were at maximum ovulatory size (mean diameter = 2.43 ± 0.05 cm, SE, n = 22) which coincided with peak testosterone levels prior to ovulation. Iverson (1980) observed that vitellogenesis in *G. polyphemus* began in September following the nesting season and continued until the nesting season the following spring at which point ovarian follicles had matured to ovulatory size. Taylor (1982) observed increased estradiol levels during vitellogenesis in wild caught *G. polyphemus* from Florida (from Palmer and Guillette, 1990). We expect that a similar increase in plasma estradiol occurs in *G. agassizii* during the fall when vitellogenesis is observed. Moll (1979) reviewed the literature on turtles and noted that certain species complete vitellogenesis prior to hibernation (the snapping turtles, *Macroclemys* and *Chelydra*). In particular, it appears that in *Chrysemys picta* the first clutch of follicles enlarge to near ovulatory size before hibernation in its northern range, but in its southern range most follicular enlargement occurs between spring emergence and the nesting season (Moll, 1979). This latitudinal effect may also occur in *G. agassizii* and the animals studied here probably represent a northern population adapted to a longer winter. In *G. polyphemus*, vitellogenesis and follicular growth may slow during colder months (Iverson, 1980). Female plasma testosterone levels peaked in April following emergence from hibernation in April. This peak in testosterone coincided with ovulation and spring mating activity.

Shelled oviductal eggs were observed as early as 15 April, 1992 in six females, approximately two weeks following the spring emergence from their burrows. Nesting was observed from 12 May–3 July, 1992. Most females produced only one clutch of eggs during the season. Six females produced a second clutch. Clutch size and nesting were similar to that reported for other *G. agassizii* populations (Turner et al., 1986). The timing of ovulation and nesting was similar to that reported for *G. polyphemus* (Iverson 1980; Landers et al., 1980). Moll (1979) notes that nesting seasons commonly fall between late April and late July over much of the range of north temperate species.

Finally, there is a latent period between the end of nesting and follicular enlargement (Moll, 1979). This period appears to be relatively brief in our study population due to the long winter period which lasts four to five months. During this period, follicles remaining from previous cycles may undergo atresia. Atretic follicles were observed in several females following the nesting season.

Mating Activity and Sperm Storage

Mating activity was observed in the fall prior to hibernation, as well as in the spring following emergence from winter burrows. Testosterone levels of male *G. agassizii* varied between the fall and spring mating periods. Variation between fall and spring mating periods have been suggested for other turtle species (Licht, 1982). Licht (1982) suggests that elevated testosterone levels in the summer may "prime" sexual behavior for the fall or spring mating period. The peak observed in testosterone levels of female *G. agassizii* in the spring

may be related to increased receptivity and ovulation. Similar increases in female testosterone levels during the mating period have been observed in a variety of turtle species, *Chrysemys picta* (Callard et al., 1978), *Chelonia mydas* (Licht et al., 1979), *Sternotherus odoratus* (McPherson et al., 1982), *Caretta caretta* (Wibbels et al., 1990), *Lepidochelys kempi* (Rostal, 1991).

The fall mating period may play a more important function in northern populations of desert tortoises than previously thought. Sperm storage has been demonstrated in this genus as well as in other turtle species (Gist and Jones, 1987, 1989; Gist and Fischer, 1993). During 1992, heavy rains in March delayed the spring emergence of tortoises until April. Although two periods of mating activity are apparent in *G. agassizii*, it has yet to be demonstrated during which of these periods insemination occurs. The observation of shelled eggs in the oviducts of several females on 15 April, before any spring mating had occurred, supports the conclusion that mating in the fall may function as an alternative to spring mating in northern ranges. Gist et al. (1990) have suggested that fall mating prior to hibernation plays an important role in *C. picta*. Large quantities of sperm were only recovered from the oviducts of female *C. picta* during the fall and electroejaculation of male *C. picta* yielded sperm only during the fall.

Conclusion

Both male and female desert tortoises display distinct seasonal reproductive cycles. These cycles appeared to be adapted to the environmental conditions that *G. agassizii* encounters in its northern range. Mating and nesting occurred when environmental conditions were most favorable for extended activity and foraging. Activity declined during July and August when ambient temperatures were highest and resources were most limited. Nesting is timed such that hatchlings emerge at a favorable time for survival when summer rains may occur and resources become more available.

It is not clear which environmental cues may be influencing the reproductive cycle of *G. agassizii*. Moll (1979) notes that temperature, light, and moisture are the most important exogenous "proximate causes" influencing reproductive cycles. Temperature appears to be an important environmental cue influencing general cycles in reptiles and has been linked to the reproductive cycles of other chelonian species (Licht, 1984). Turner et al. (1986) noted that mean clutch frequencies were correlated with winter rainfall, however, clutch size did not differ between years. Further research is needed on other populations throughout the range of this species to understand its reproductive biology and to develop proper management plans.

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