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TESTOSTERONE AND REPRODUCTIVE ACTIVITY IN THE MALE TEGU LIZARD, *TUPINAMBIS MERIANAE*

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***Abstract.*—In this study, we examined plasma testosterone levels in male *Tupinambis merianae* lizards and their relation to the seasonal reproductive activities observed in this species. We found that the highest androgen levels were associated with reproductive activity in spring, comprising sperm production, reproductive behaviors (territoriality, courtship, and matings), and secretory activities of femoral glands and kidney sexual segment. We observed an additional rise of testosterone in the fall that correlated with a secondary phase of testicular recrudescence, but which was not accompanied by reproductive behaviors or by secretory activities of the femoral glands and kidney sexual segment. The absence of sperm in the seminiferous tubules and epididymis during the cold months indicates that spring matings of *T. merianae*, as in most lizards, are supported by prenuptial spermatogenesis. Besides its association with high levels of circulating androgens, the spring breeding events coincide with an increase in environmental temperatures. Both spermatogenic waves also coincide temporally with the equinoxes. Further studies are necessary to establish the relative contributions of endocrine and environmental factors in triggering reproductive events in this species. We believe that this second non-functional spermatogenic wave may be a remnant of an ancestral bimodal reproductive cycle in this species.**

Key Words.—femoral glands; renal sexual segment; reproductive behavior; spermatogenesis

INTRODUCTION

Reptiles, probably because of their ectothermal condition, have a wide variety of reproductive strategies that have evolved to address environmental challenges. These strategies include: (1) genetic and environmental sex determination mechanisms; (2) oviparous development and viviparity; (3) bisexuality and parthenogenesis; and (4) associated and dissociated reproductive cycles (Moore and Lindzey 1992; Whittier and Tokarz 1992). In species inhabiting environments with prolonged periods suitable to reproduction, associated cycles occur in which gametogenesis and steroidogenesis, temporally coincide with the mating period (Crews and Ganz 1992). In other reptiles, occupying extreme environments with only brief breeding periods, dissociated cycles occur in which there is a considerable gap between gametogenesis and matings (Crews and Ganz 1992). The existence of associated and dissociated reproductive cycles, in which processes such as gametogenesis, reproductive behaviors, and the expression of secondary sexual characters coincide or differ temporarily, offers interesting possibilities to study the differential role of endocrine and environmental factors in controlling each component of reproductive activity.

The genus *Tupinambis* (Squamata: Teiidae) comprises a group of large South American lizards (Presch 1973) that exhibit a widespread distribution from the Amazon to the north of Patagonia (including tropical to temperate climates). Of the seven currently recognized species (Ávila-Pires 1995; Fitzgerald et al. 1999; Vitt and Caldwell 2009), *T. merianae* (Fig. 1) and *T. rufescens* are the southernmost representatives, reaching the northern portion of Patagonia (Cei and Scolaro 1982). These species, which can attain a total adult length of 1.2 m and a body weight of 7 kg , were traditionally used by indigenous communities as a source of meat, fat, and leather (Donadío and Gallardo 1984; Norman 1987). However, given the current intense exploitation for their hides (Chardonnet et al. 2002), they are now listed on CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II (restricted trade). Captive breeding programs, rather than extraction from nature, have been proposed for the sustainable use of these lizards (Mercolli and Yanosky 1990; Noriega et al. 1996). These conservation efforts raise the need for a proper understanding of the reproductive biology of these saurians.

In typically seasonal climates, as found in Argentina, *T. merianae* and *T. rufescens* experience a five to six month torpor (between April and September) that circumscribes their main reproductive activities almost entirely to spring. They have a single annual oviposition and a relatively large clutch size, ranging from 20 to 50 eggs (Donadío and Gallardo 1984; Mercolli and Yanosky 1990; Noriega et al. 1996). After an incubation of 65–75 d, the active and independent offspring hatch in late spring and early summer (Mercolli and Yanosky 1990; Noriega et al.

FIGURE 1. Territorial marking by a male *Tupinambis merianae*, performed by dragging the pelvic girdle and hind legs on the ground. Bar = 10 cm. (Photographed by Valeria Garcia Valdez).

1996; Manes et al. 2003).

Both genders exhibit characteristic reproductive behaviors. Females display complex nesting, incubation, and egg protection behaviors (Noriega et al. 1996; Manes et al. 2003), while males exhibit territorial behavior associated with an increase in testicular weight (Noriega et al. 1996; 2002) and secretory activity of the femoral glands (Chamut et al. 2009) and renal sexual segment (Noriega et al. 2002). In addition to the various spring reproductive manifestations, males exhibit a second nonfunctional activation of spermatogenesis in late autumn (Noriega et al. 2002). In this study, we examined plasma testosterone levels in male lizards of *T. merianae* and their relation to the various reproductive events described for the species.

MATERIALS AND METHODS

Animals and study conditions.—We conducted this study during the seasonal/reproductive cycles of 2007–2008 and 2008–2009 with *T. merianae* specimens raised in captivity in the province of Tucumán, Northern Argentina (26º51´S and 65º17´W). This region is characterized by a temperate climate with a dry winter season. We kept the animals, with a male to female ratio of 1 to 4, in outdoor enclosures of approximately 60 m^2 fenced by masonry walls (1.2 m tall) and equipped with shelters and shade. This design, based on our previous experience, was suitable for animal welfare. We fed the lizards *ad libitum* a previously designed farm diet (Vega Parry and Manes 2000), which consisted of about 85% ground chicken heads and feet (1:2), 15% soybean meal, 0.25% vitamin-mineral supplement for broilers (Micromix, Biofarma, Córdoba, Argentina), 0.25% sodium chloride, and 0.1% butyl hydroxy toluene.

From the breeding groups, we selected nine adult males of at least 15 months of age and > 40 cm snout-vent length. We implanted these individuals with a MicroTransponder (ID-100A, Trovan Electronic Identification, Rosenbusch, Buenos Aires, Argentina) under the skin for individual monitoring. We monitored animals for the expression of reproductive behaviors, such as territoriality, courtship, and mating (Noriega et al 1996) during the periods of testicular recrudescense. Monitoring occurred by an observer located outside the enclosures.

Hormone assays.—We determined plasma testosterone concentrations by radioimmunoassay (RIA) using a [I-125]-Testosterone RIA DSL-4100 kit (Diagnostic Systems Laboratories, Webster, Texas, USA) in the Laboratorio de Reproducción y Lactancia, IMBECU, CONICET of Mendoza, Argentina. We proceeded by extracting 1.5 to 2 mL blood samples at key stages of the seasonal/reproductive cycle: torpor, torpor emergence, sexual interactions, postmating, gonadal involution, gonadal quiescence, and pretorpor between 0800 and 1000, for two consecutive years. The stages of gonadal involution and gonadal quiescence had been previously established thorough morphometric and histological studies (Noriega et al. 1996). We considered the pretorpor stage as the period (previous to the entrance to torpor) when the animals exhibit a decrease in activity and feeding, while spending longer periods in their shelters. We collected the blood from the caudal vein of unanesthetized males, using a heparinized syringe. We centrifuged the blood and separated and stored the plasma at −20 °C for hormone determinations.

To validate the testosterone RIA for *T. merianae* plasma, we assayed in parallel samples of: (1) plasma with no treatment; (2) plasma acidified with addition of 10% v:v of HCl 0.1 N to dissociate steroids from circulating SHBG (sex hormone-binding globulin); and (3) plasma extracted twice with 10 volumes ethylether, evaporated, and redissolved in assay buffer.

The untreated plasma gave lower values, whereas treatments 2 and 3 gave similar values, so we decided to carry out all determinations using acidified plasm. Serial dilutions of some samples gave values that were parallel to the standard curve. The efficiency of the acidification procedure was higher than 95% for the concentrations assayed, which were 2 and 50 ng/mL testosterone added to charcoal extracted plasma.

We used a mixed model approach to analyze the data (Pinheiro and Bates 2000). We considered the cycle and animals nested within the cycle as model random components because we were studying the same animals during two cycles. We compared means with Fisher's LSD test, corrected for multiple comparisons. We ran these models using the R software (R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.R-project.org/ [Accessed 22 December 2011]) and Infostat (Di Rienzo, J.A., F. Casanoves, M.G. Balzarini, L. Gonzalez, M. Tablada, and C.W. Robledo. 2011. InfoStat Versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. Available from http://www.infostat.com.ar [Accessed 19 September 2011]).

Histological studies.—We sacrificed four animals during the hormonal peaks of the second year of study to confirm previous observations on the activity of testes, kidney (Noriega et al 2002), and femoral glands (Chamut et al 2009). We euthanized them by cranial concussion followed by immediate spinal cord transection; this two-phase physical method of euthanasia is similar to that described by the AVMA Panel on Euthanasia (AVMA 2001; AAZV 2006). We found that this method, performed by experienced personnel, was the fastest and most humane form of euthanasia for these animals. The samples were fixed with 4% formaldehyde or in Duboscq-Brazil solution for 24–48 h and embedded in paraffin according to standard procedures. We cut 7 µm sections and stained them with hematoxylin-eosin and Heidenhain aniline azocarmin (Azan) for microscopic examination.

RESULTS

Reproductive behaviors and testicular activity.—Males began displaying territorial behaviors a few days after emergence from torpor (Fig. 1). After the emergence of the females, about two weeks later, they incorporated courtship and copulatory behaviors. Sexual interactions lasted about a month (October), after which males ceased displaying reproductive behaviors. Reproductive activities resulted in the expected rate of ovipositions by about half of the females.

Simultaneously with reproductive behavior displays, spermatogenic activity in the seminiferous tubules was intense (Fig. 2A), resulting in the accumulation of large sperm masses at the epididymal level (Fig. 2B). Furthermore, the testicular interstice showed conspicuous Leydig cells (Fig. 2C). The renal sexual segment (Fig. 2D) and the femoral glands (Fig. 2E) also showed evidence of a great deal of secretory activity.

We observed a second spermatogenic wave during autumn (April; Fig. 3A), which also resulted in a considerable accumulation of sperm in the epididymis (Fig 3B) and active Leydig cells in the testicular interstice (not shown). However, this autumn gonadal recrudescence was not associated with reproductive behaviors, nor with secretions from the femoral gland or renal sexual segment. Testicular activity was brief and we did not find sperm in the seminiferous tubules or the epididymis during the cold months (June-July).

Plasma testosterone concentrations.—Because the nine animals showed similar endocrine changes, we grouped the individual results as means with standard error. The highest levels of plasma testosterone were associated with spring (September - October) reproductive processes (sperm production and storage, reproductive behaviors, and secretory activity from femoral glands and renal sexual segment), which were significantly higher than other time periods (Fig. 4). A second increase in plasma testosterone concentration occurred in April, the pretorpor stage, coincident with the autumnal spermatogenic wave. Although, this androgen peak was markedly lower than the spring one, it was significantly higher than the values measured during the preceding gonadal involution and gonadal quiescence stages.

DISCUSSION

Spring reproductive activity in male *T. merianae* lizards shows a typically associated reproductive pattern, in which testicular activity with abundant sperm production coincides with various reproductive behaviors (matings included), secretions from the femoral gland and kidney sexual segment, and the highest plasma testosterone level. Subsequently, testes gradually regress to a nadir in late summer (February; Noriega et al. 2002). The simplicity of this reproductive scheme is, however, altered by the presence of an additional testicular recrudescence, which in spite of its brevity, is intense enough to cause sperm cell accumulation at epididymal level (Noriega et al. 2002; present study).

The non-functional character of this secondary spermatogenesis is indicated by the absence of sperm in seminiferous tubules and epididymis during the cold months. Thus, spring matings of *Tupinambis merianae*, as in most lizards, are supported by prenuptial spermatogenesis, as opposed to snakes and turtles, which rely mainly on postnuptial spermatogenesis (Lofts 1987; Lorenz et al. 2011). As with the spring reproductive activity, we observed that

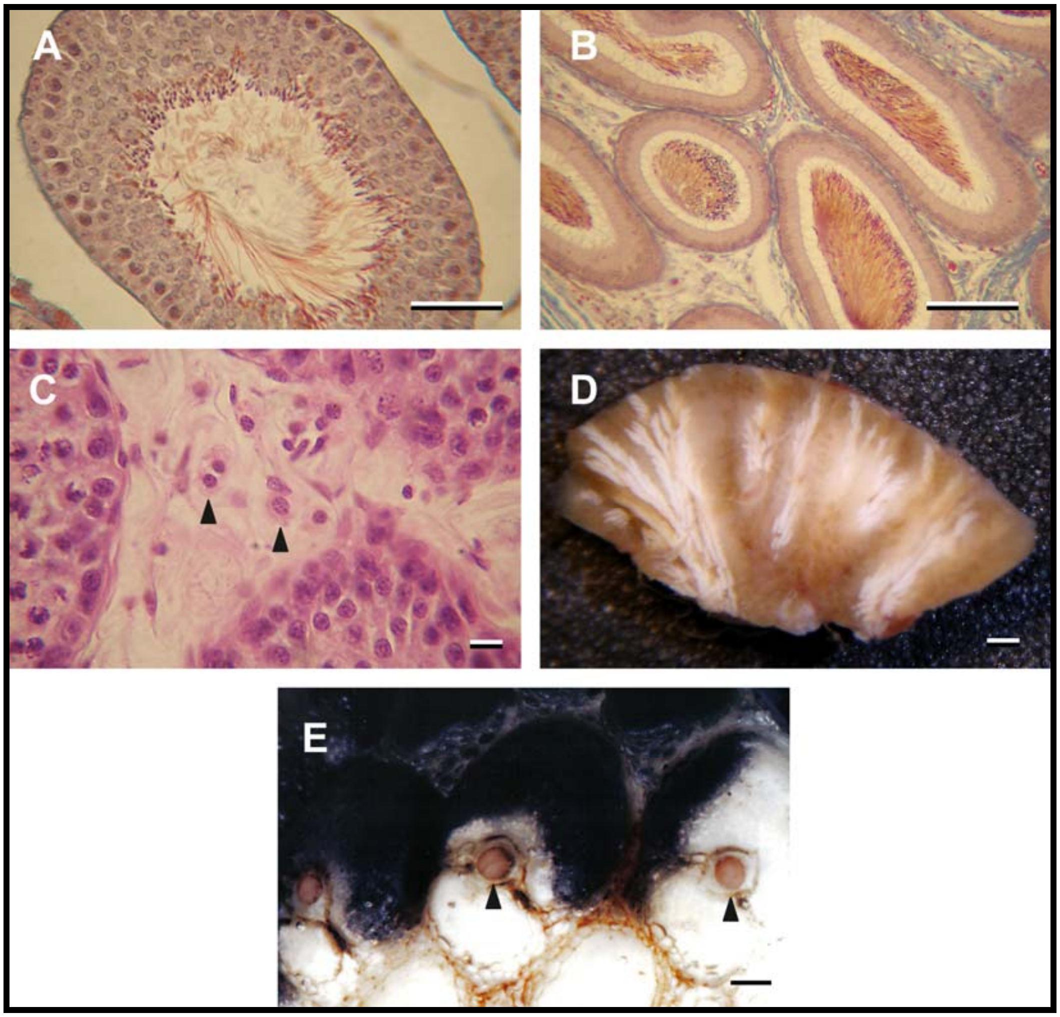

FIGURE 2. Spring reproductive events in male *Tupinambis merianae.* A: Active sperm production in a seminiferous tubule. Bar = 100 µm. B: Epididymal section showing dense sperm accumulations. Bar = 100 µm. C: Leydig cells (arrowheads) in the testicular interstice. Bar = 10 µm. D: Whitish sexual segments in a kidney cut, highlighted by intracellular accumulation of secretory granules. Bar = 1 mm. E: Femoral gland secretions (arrowheads) protruding at the surface of modified scales. Bar = 1 mm. (Photographed by Silvia Chamut).

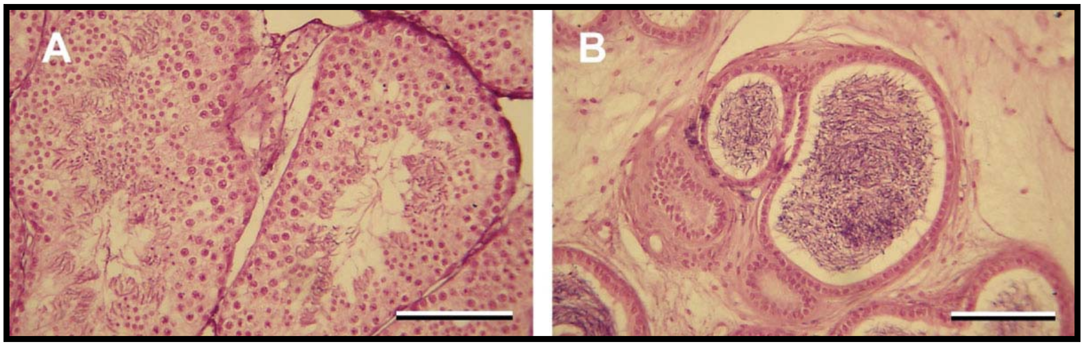

FIGURE 3. Autumn testicular recrudescence in *Tupinambis merianae.* A: Seminiferous tubule in active spermatogenesis. Bar = 100 µm. B: Section of epididymis, showing bulky sperm masses. Bar = 100 µm. (Photographed by Silvia Chamut)

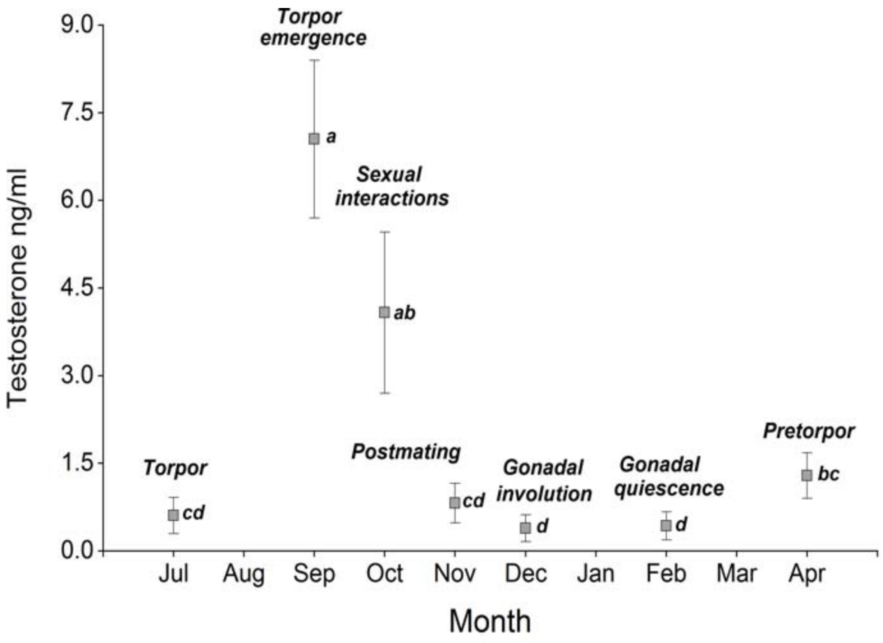

FIGURE 4. Plasma testosterone levels during male *Tupinambis merianae* reproductive / seasonal cycle. Results are expressed as mean ± standard error for groups of nine animals for each reproductive state. Stages with different letters indicate significant differences ($P \leq 0.05$).

the pretorpor secondary gonadal reactivation was associated with a lesser magnitude of increase in plasma testosterone levels, and both androgen peaks coincided with the presence of active Leydig cells in the testicular interstice. Secondary hormonal peaks, such as the one we found in this study, have been observed in other reptiles (Callard et al. 1976; Schuett et al. 1997; Lorenz et al. 2011), but they were not so clearly associated with an accessory gonadal recrudescence, as we observed in *Tupinambis merianae*.

The absence of reproductive behaviors and secretions from the femoral glands and renal sexual segment during the secondary testicular recrudescence could be attributed simply to the plasma testosterone levels, which may have been enough to induce spermatogenesis but not other reproductive events. However, spermatogenesis and reproductive behaviors observed in reptiles having dissociated reproductive cycles show species-specific sensitivity to circulating androgens (Jones et al. 1997), suggesting a more complex relationship between these processes and their endocrine regulation. Moreover, it should be noted that, besides its association with high levels of circulating androgen, the spring breeding period also coincides with an increase in environmental temperatures. Therefore, we cannot exclude a concurrent role of this climatic factor in triggering *T. merianae* reproductive behaviors, as happens in certain other reptiles, particularly those with dissociated reproductive cycles (Moore and Lindzey 1992; Whittier and Tokarz 1992).

Similarly, the temporal coincidence of both spermatogenic waves with the equinoxes could also involve day length or its rate and direction of variation as determinants of gonadal recrudescence. Therefore, further studies are necessary to establish the relative contributions of endocrine and environmental factors in triggering reproductive events in this species. The presence of two spermatogenic waves during the *T. merianae* testicular cycle could derive from an ancestral bimodal reproductive pattern, which is suggested by the completeness of the autumnal spermatogenic process and its hormonal support (implying an underlying genetic programming). Bimodal reproductive cycles, common in reptiles (Licht 1984), can exemplify expanded breeding situations, resulting from favorable environmental conditions, such as those found in tropical regions.

For instance, *T. teguixin*, an inhabitant of various tropical environments in South America, unlike the subtropical and temperate zone species *T. merianae* and *T. rufescens*, seems to be active throughout the year (Herrera and Robinson 2000). *Tupinambis teguixin* nests several times and lays fewer eggs at each nesting (Beebe 1945). We could explain the characteristically unimodal reproductive pattern of *T. merianae* with a relictual gonadal recrudescence in autumn, as derived from a bimodal reproductive cycle. This interpretation would be consistent with the migration of reptiles towards higher latitudes over time, as proposed by Darlington (1957).

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