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Plasmatic Estradiol and Progesterone Variations During the Reproductive Cycle of Captive Female Argentine Red Tegu Lizards, *Tupinambis rufescens*

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***Abstract.*—We analyzed the plasmatic profiles of 17 β-estradiol and progesterone during the reproductive cycles of captive female Argentine Red Tegus (*Tupinambis rufescens*). We studied adult females, some of which oviposited and subsequently incubated their eggs (fertile females), and some of which did not oviposit and, therefore, did not incubate (infertile females). Regardless of oviposition status, circulating levels of 17 β-estradiol were elevated at the end of hibernation, then diminished gradually and reached the minimum level in the pre-ovulatory stage. After oviposition, hormonal levels increased, reached their peak during incubation, and stayed elevated throughout reproductive quiescence. Progesterone showed the reverse pattern to 17 β-estradiol, increasing progressively from the end of hibernation to reach maximum concentration during the pre-ovulatory phase, at which point it started to decrease. We also found higher values of 17 β-estradiol and progesterone in incubating females compared with those that did not incubate. These results suggest a possible association between hormonal cycles and maternal behavior in this species, although the small sample size limits our ability to draw conclusions.**

Key Words.—follicular development; nidification behavior; sexual steroids; temperate zone lizard

Introduction

Tegus (*Tupinambis* spp.; Squamata, Teiidae) comprise a group of large oviparous lizards found exclusively in the South American plains that have had a long history of exploitation by humans. Both the Red Tegu (*Tupinambis rufescens*) and the Black and White Tegu (*T. merianae*), which are the largest and the southernmost species of the group, were traditionally hunted by indigenous communities that used their skin, meat, and fat (Donadio and Gallardo 1984; Norman 1987). During the last century until the present, these species began to suffer intense and sustained exploitation in pursuit of leather (Fitzgerald et al. 1991; Chardonnet et al. 2002; Vieites et al. 2007), which led to their inclusion in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II. Currently, the most promising option for the conservation of these animals appears to be through captive breeding programs for their sustainable use (Mercolli and Yanosky 1989; Noriega et al. 1996). There is currently a need to understand the reproductive biology of tegus to assist with conservation and to facilitate captive husbandry, and captive colonies have allowed us to begin this process. It is important to understand the roles of the main hormones that control the reproductive process, particularly in female tegus, which have more complex hormonal cycles than the males. For instance, 17 β-estradiol and progesterone hormones are involved in several critical aspects of female reproduction in reptiles: sexual receptivity and pheromone production (Tokarz and Crews 1980; Mendonça and Crews 1996; Parker and Mason 2012); vitellogenesis (Callard et al. 1978; Gavaud 1986; Bonnet et al. 1994); ovulation (Bentley 1998), and oviductal development (Gavaud 1986; Paolucci et al. 1992; Girling et al. 2000),

In temperate and subtropical regions, the reproduction of tegus is highly seasonal. The lizards hibernate for five or six months (April to September), limiting the main reproductive events almost entirely to spring (Noriega et al. 1996; Manes et al. 2007; Chamut et al. 2012). Both species show a wide repertoire of reproductive behaviors (i.e., territoriality, courtship, and copulation), which end in a single oviposition per year, with an average clutch size of 36 eggs (range 20–54 eggs; Donadío and Gallardo 1984). In addition, the females show complex and unusual behaviors regarding construction of the nest and incubation of the eggs (Mercolli and Yanosky 1989; Noriega et al. 1996; Manes et al. 2003). The eggs hatch at the end of spring or be-

ginning of summer after an incubation period of about 70 d (Mercolli and Yanosky 1989; Noriega et al. 1996; Manes et al. 2003).

When female *Tupinambis* are isolated, there is an arrest of follicular development in the pre-vitellogenic phase, indicating that copulation may trigger vitellogenesis and posterior ovulation (Manes et al. 2007). Subsequent ultrasound studies (García-Valdez et al. 2011), confirmed a close relationship between copulation and ovulation. However, we observed that not all matings concluded successfully in oviposition, as a large number of females showed anovulatory cycles associated with processes of follicular atresia (García-Valdez et al. 2011). Here, we describe a study on plasmatic variations of the ovarian hormones 17 β-estradiol and progesterone and their relationship to the follicular cycle and reproductive events of captive female Argentine Red Tegus (*Tupinambis rufescens*) within a temperate climate.

Materials and Methods

Animals and conditions for the study.—We conducted the study at El Manantial, in the province of Tucuman, northwestern Argentina. This region has a warm, temperate climate, with a dry period in the cold season. We bred *Tupinambis rufescens* specimens in captivity in the experimental hatchery of the Facultad de Agronomía y Zootecnia of Universidad Nacional de Tucumán. This hatchery includes common enclosures for breeding and reproduction as well as individual ones for nesting and egg incubation (Manes et al. 2003). All areas are in the open air, provided with refuge and shade, and enclosed by masonry walls. We have successfully used this design to maintain these animals in good condition as demonstrated by the higher growth rates and earlier onset of reproductive activity that they display compared with wild animals (Vega Parry and Manes 2004).

We fed lizards *ad libitum* with a previously designed hatchery diet, consisting of 85% ground chicken heads and feet (1:2), 15% soy flour, 0.25% vitamin and mineral supplement for chickens (Micromix; Biofarma, Cordoba, Argentina), 0.25% sodium chloride, and 0.1% butyl hydroxyl toluene (Vega-Parry and Manes 2000). We organized a reproductive group, consisting of 10 adult females and two adult males, with minimum snout-vent lengths of 38 and 37 cm, respectively. Selected females were about 3–5 y old and had experienced at least one oviposition in captivity.

We identified each animal using a radio frequency identification device with a transponder and electronic reader (Trovan, Ltd., Electronic Identification Systems, East Yorkshire UK). We implanted the microtransponder (ID-100A) under the skin using a disposable needle and applicator. At a desired moment, we read the microtransponder code using the electronic reader (LID 570 Pocket Reader; Trovan, Ltd., Electronic Identification Systems, East Yorkshire UK). An observer situated outside the enclosures verified the display of reproductive behaviors, which began a few days after lizards had emerged from their hibernation shelters (Noriega et al. 1996). When the females showed a characteristic abdominal distention related to follicular growth (Manes et al. 2007), which we verified by sonogram, we moved them to individual nesting areas (Manes et al. 2003) where they remained until their eggs hatched.

Hormonal studies.—We took blood samples in different stages of the reproductive / seasonal cycle: hibernation emergence, courtship, advanced vitellogenesis, oviposition, incubation, and reproductive quiescence (gonadal nadir, corresponding to the month of February; Noriega et al. 1996). In females with anovulatory cycles, we collected samples equivalent to the oviposition phase after the last registered egg-laying, when the sonogram showed a slight drop in follicular diameter. We obtained the samples corresponding to the incubation phase simultaneously with the females that were incubating eggs. We collected blood samples (1.5–2.0 ml) from the caudal vein with a heparinized syringe. We obtained the plasma by centrifugation, and stored it at -20º C. We determined the plasmatic concentrations of 17 β-estradiol and progesterone by radioimmunoassay (RIA) using commercial kits for total hormones (DSL-4800 and DSL-3400, double antibody RIAs, respectively; Diagnostic Systems Laboratories, Webster, Texas, USA) at the Laboratory for Reproduction and Lactation, Intituto de Medicina y Biología Experimental de Cuyo (IMBECU), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Mendoza, Argentina.

To validate the RIA for plasma of *Tupinambis*, we used several tests. To eliminate possible interference by plasmatic steroid binding proteins, we extracted plasma samples twice with 10 volumes of ethylic ether, then evaporated the samples, re-suspended them in 1 volume of PBS-Gelatine, and measured them alongside samples of the same protein with unextracted plasma. The results showed similar values between the extracted and non-extracted samples, which indicated that there was no significant interference of plasmatic proteins. Extraction efficiency was > 90% for both hormones (17 ß-estradiol and progesterone) as determined by adding known quantities (50 pg for estradiol and 20 ng for progesterone) to 1 ml of charcoal-extracted plasma that we extracted and measured in parallel with aliquots of the same non-extracted plasma. Therefore, we used plasma without extraction. Measuring hormone concentrations in different dilutions of plasma samples produced a

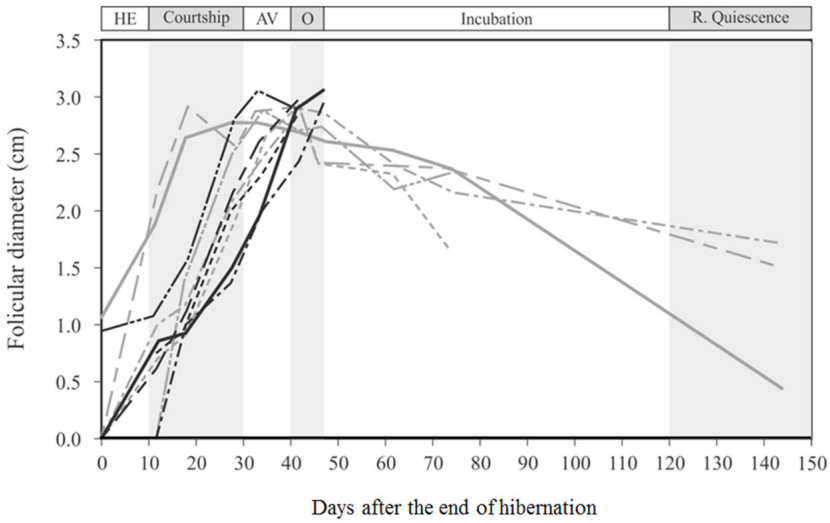

Figure 1. Follicular development in ovulatory (black lines) and anovulatory (gray lines) cycles of *Tupinambis rufescens* females. Abbreviations are HE = hibernation emergence; AV = advanced vitellogenesis; O = oviposition; R. Quiescence = reproductive quiescence.

dose-response curve parallel to the standard curve, indicating a linear response. The sensitivity of RIAs was of 5 pg/ml for estradiol and 0.3 ng/ml for progesterone. The intra-assay coefficients of variation were 7.5% and 5.1% for estradiol and progesterone, respectively, and we measured all the samples in duplicate in one assay for each hormone. According to the manufacturer, the antibodies were highly specific; the estradiol antibody showed cross-reactivity of 3.40% with D-equilenin, 2.5% with estradiol glucuronide, 0.64% with estriol, and < 0.5 % with the other estrogens, progestagens, or corticoids tested. The progesterone antibody had a cross-reactivity of 5% with 5α-Dihydroprogesterone and < 1% with the other progestagens or corticoids tested.

Sonograms.—We determined the follicular state of females by ultrasound, every 1–2 weeks, with an ultrasound scanner (LC 2010 Electromedicina; Berger Argentina S.A., Buenos Aires, Argentina) with a 5/7 MHz micro-convex transductor. In the case of females that oviposited, we suspended observation during incubation to reduce the possibility of interference with their behavior.

Statistical analysis.—We analyzed the data using mixed models (Vonesh and Chinchilli 1997; Pinheiro and Bates 2000). The fixed component was the seasonal / reproductive cycle and the random part of the model was the lizard because we took repeated measurements over time for each specimen. We used R software to run the analyses (R Development Core Team 2013) and checked the assumptions of the models as necessary. We also contrasted alternative models by means of likelihood ratio tests and compared means using Fisher's LSD test corrected for multiple comparisons. We compared Ovulatory-Anovulatory mean values within oviposition stage by means of one degree of freedom contrasts. We contrasted follicles maximum diameters using t-tests. For all analyses, we used a significance level of 5%.

Results

Reproductive behavior and follicular evolution.—A few days after emergence from hibernation, all of the females engaged in spring reproductive activities, showing the typical behavior of courtship and copulation. Approximately two weeks after copulation, we moved the females showing enlarged abdomens to the nesting individual areas. Only five of the 10 females in the study oviposited, displaying the characteristic behaviors of nesting, building the nest, and then incubating the eggs.

In general, follicles were undetectable by ultrasound (< 3 mm diameter) at the emergence of hibernation, although some females already showed developing follicles (Fig. 1). Simultaneously, with sexual interactions, the follicles began a sustained growth. These were anechogenic (dark images) up to about 10 mm in diameter, and then turned echogenic (bright images), probably due to the transition from a previtellogenic state to a vitellogenic one. Approaching ovulation, follicles reached approximately 30 mm in diameter. All of the females showed a similar follicular growth rate; however, five females that did not ovulate entered a process of massive follicular atresia. The pre-ovulatory follicles reached a diameter bordering on significantly greater diameter than those that underwent the atretic process 28.4–31.7

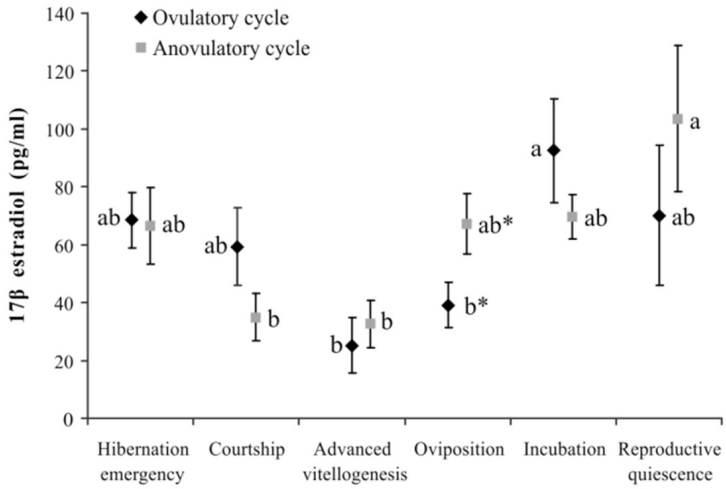

Figure 2. Circulating 17-β estradiol during the reproductive / seasonal cycle of *Tupinambis rufescens* females, measured by RIA. Symbols represent adjusted measurements with standard errors. Different superscript letters indicate significantly different values between reproductive stages within the same group. An asterisk (*) indicates significant differences (present only for ovulatory-anovulatory mean values within oviposition stage).

vs 27.5–28.7 mm ($t = 2.12$, df = 8, $P = 0.067$). Unlike the post-ovulatory ovaries, which were undetectable for the remainder of the reproductive / seasonal cycle, the ovaries of the females with anovulatory cycles showed atretic follicles of decreasing size, which were detectable by ultrasound up to 140 d after their maximum development (reproductive quiescence; Fig. 1).

Hormonal variations during the reproductive/ seasonal cycle.—Estrogen and progesterone showed variations in their plasmatic levels throughout the reproductive / seasonal cycle, with significant differences at the oviposition stage between individuals with ovulatory versus anovulatory cycles (Fisher's LSD test, all $P < 0.050$; Figs. 2 and 3). The levels of 17 β-estradiol were high at the end of hibernation and they tended to decrease during courtship and advanced vitellogenesis (Fig. 2). After oviposition, the estradiol levels increased, reaching the highest values during incubation and reproductive quiescence. These levels did not differ significantly from those found at the end of hibernation. During the incubation stage, the levels of estradiol in fertile females that oviposited rose, while they remained stable in infertile females, during the same period (Fig. 2).

The plasmatic concentrations of progesterone (Fig. 3) showed an inverse profile to that of 17 β-estradiol throughout the reproductive / seasonal cycle (compare Figs. 2 and 3). Thus, the levels of this hormone were low at the end of hibernation, increased during sexual interactions, reaching their highest during the advanced vitellogenesis phase. Afterwards, progesterone showed a tendency to decline, which was more pronounced in infertile females than in those that oviposited and incubated. We found the lowest levels of this hormone during the period of reproductive quiescence.

Discussion

Follicular development in *Tupinambis rufescens* occurred in parallel with reproductive activities. Similar to *Tupinambis merianae* (Garcia-Valdez et al. 2011), a closely related species, it consisted of a widespread event affecting both ovaries and all of the recruited oocytes (Garcia-Valdez et al. 2011). The presence of developing follicles in some of the studied females at the hibernation emergence suggest that these may have experienced some degree of physiological reactivation within their hibernation shelters. In females with ovulatory cycles, oocytes showed a steady growth of approximately 50 d ending with ovulation of the developed follicles. In contrast, half of the studied females did not ovulate, showing a massive follicular atresia of vitellogenic follicles with diameters similar to those of preovulatory oocytes. This type of atresia, rather than its classic role in the physiological regulation of the number of oocytes to ovulate, seems associated with ovulatory failure.

The high concentration of plasmatic 17 β-estradiol found at the hibernation emergence in female *Tupinambis rufescens* possibly indicates the requirements of an incipient reproductive cycle, constrained by a long hibernation period (Noriega et al. 1996; Manes et al. 2007; Chamut et al. 2012). The hormone is implicated in several early female reproductive activities, such as the establishing of sexual receptivity (Tokarz and Crews 1980; Mendonça and Crews 1996), the production of

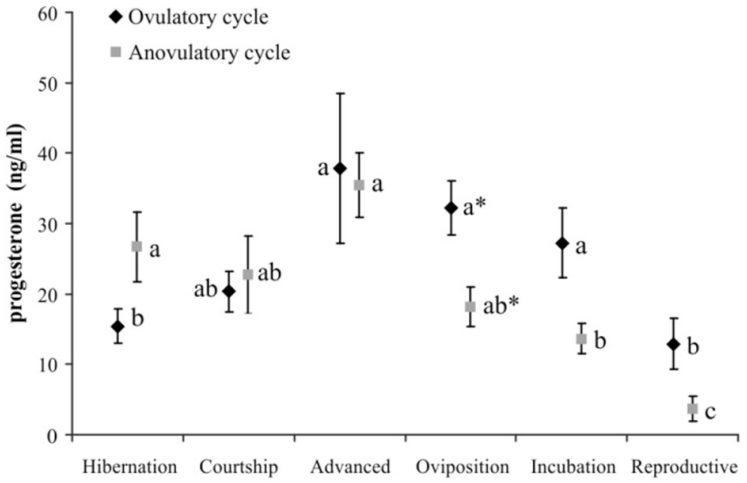

Figure 3. Variations of progesterone during reproductive / seasonal cycle of *Tupinambis rufescens* females, measured by RIA. Symbols represent adjusted measurements with standard errors. Different superscript letters indicate significantly different values between reproductive stages within the same group. An asterisk (*) indicates significant differences (present only for ovulatory-anovulatory mean values within oviposition stage).

pheromones (Mendonça and Crews 1996); Parker and Mason 2012), the mobilization of vitellogenic reserves (Callard et al. 1978; Gavaud 1986; Bonnet et al. 1994), and the development of oviducts (Gavaud 1986; Paolucci et al. 1992; Girling et al. 2000). The high availability of 17 β-estradiol at the hibernation emergence is probably in direct relation to the hormonal levels found at the end of the previous summer, which may be produced by steroidogenically active immature follicles (Sadjia et al. 2007) that persist during hibernation or become active immediately after emergence. Supporting this idea, we found high winter levels of 17 β-estradiol in *Tupinambis merianae*, species with a similar reproductive cycle (unpubl. data).

The decline of 17 β-estradiol with increasing follicular development in *Tupinambis rufescens* seems to run counter to the observations made for most reptiles in which follicular growth accompanies increasing levels of estrogen, promoting the vitellogenic process (Callard et al. 1978; Jones et al. 1997; Edwards and Jones 2001; Radder et al. 2001; Taylor and Denardo 2010). However, there are several exceptions that also show a variable relation between the hormone levels and the vitellogenic process. These include the Bearded Dragon Lizard (*Pogona barbata*), Green Sea Turtles (*Chelonia mydas*), and Loggerhead Turtles, (*Caretta caretta*; Etches and Petitte 1990; Wibbels et al. 1992; Amey and Whittier 2000; Jones 2011). It is possible that such inconsistencies are due to species-specific differences related to the timing of the reproductive processes, and their sensitivity to hormonal levels. Therefore, vitellogenesis would have a lower stimulus threshold than the preceding sexual behaviors. Alternatively, 17 β-estradiol could have a long-term vitellogenic effect, as has been noticed in species such as the Asp Viper (*Vipera aspis*), Painted Turtle (*Chrysemys picta*), and Atlantic Ridely Turtle (*Lepidochelys kempii*; Bonnet et al. 1994; Smelker et al. 2014).

Another important consideration is the complex multi-hormonal control of the vitellogenic process, wherein 17 β-estradiol interacts with progesterone, testosterone, and growth hormone (Ho et al 1982; Polzonetti-Magni et al. 2004; Custodia-Lora et al. 2005). Unbalanced levels of any of these hormones could also explain the absence of this process in other stages of the reproductive cycle that proceed with high levels of circulating estrogen. For progesterone, the increase of plasmatic levels during follicular development in *Tupinambis rufescens* is similarly observed in the preovulatory phase of the majority of reptiles (Ciarcia et al. 1993; Amey and Whittier 2000; Custodia-Lora and Callard 2002a). Depending on the inhibiting or stimulating function of vitellogenesis, defined by the amount of receptor isoforms, the hormone would act synergistically with estrogen during this phase (Duggan and Callard 2003; Custodia-Lora et al. 2004). Progesterone peaks in some reptiles right before ovulation, and it has been suggested that the hormone takes part in an ovulatory mechanism similar to that of birds (Bentley 1998). In addition, progesterone controls oviductal transit, reducing myometrial activity, and facilitating egg retention and the secretion of egg layers (Custodia-Lora and Callard 2002b). As expected, because of the oviparous condition of *Tupinambis rufescens*, the plasmatic levels of progesterone showed a post-ovulatory drop. However, the persistence of higher levels of this hormone in females that incubated compared with those that did not

may be attributed to a cooperative effect with estrogen in nesting and incubation behaviors, similar to that observed in birds (Silver 1978).

Although the low sample size limits statistical analysis, the differences in the values of estradiol and progesterone between females that laid their eggs and those who did not are suggestive. The highest levels in fertile females could relate to the complex behaviors of nesting and incubation of the species (Mercolli and Yanosky 1989; Noriega et al 1996; Manes et al 2003), rare in the life history of reptiles (Shine 1988). However further studies are required to confirm this possibility. Both of the hormones we studied, 17 β-estradiol and progesterone, showed a clear opposite relation in their plasmatic levels, at least during most of the reproductive cycle of *Tupinambis rufescens*. It could well be that many of the endocrine actions that we mention here depend on the balance of both hormones, rather than on their independent effects. We are confident that endocrine studies such as this one will contribute to a better understanding of the reproductive process of tegus and, through future endocrine experimental manipulations, will enable the development of useful tools for their conservation.

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