

## Dynamics of ovarian follicles in *Tupinambis meriana*e lizards

M. VALERIA GARCÍA VALDEZ<sup>1</sup>, SILVIA CHAMUT<sup>1</sup>, GONZALO VALDEZ JAEN, OSVALDO E. A. ARCE<sup>1</sup>, MARIO E. MANES<sup>1,\*</sup>

<sup>1</sup> Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, F. Ameghino s/n, El Manantial, (4105), Tucumán, Argentina. \*Corresponding autor. E-mail: mmanes@faz.unt.edu.ar

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**Abstract.** Follicular dynamics of *Tupinambis meriana*e lizards was analyzed by means of ultrasound examination and radio frequency identification, during three consecutive reproductive periods, both in reproduction and in sexual isolation. Series of follicular development which ended in ovulation, and series of follicular development without ovulation, followed by a regression process, were observed, as well as a combination of both. Ovulation was found to be strongly dependent on mating consummation. In 34% of the cases in which follicular development ended with ovulation, and hence oviposition, follicles showed a steady and uniform growth up to the periovulatory period, also acquiring a mild echogenicity. In the absence of ovulation (66% of cases), follicular development showed two patterns: a short and a long-term anovulatory follicular cycle. In the first pattern, follicles showed limited growth and a non-echoic aspect, which suggests a previtellogenic condition. In the second pattern, follicles exhibited a growth and echogenicity similar to periovulatory ones, presumably attaining a vitellogenic stage. In addition, follicular development in reproducing females was superior to that of sexually isolated females. Follicular development, ovulation, and follicular regression appeared to constitute generalized events affecting most, if not all, of the recruited follicles.

**Keywords.** *Tupinambis*, follicular dynamics, sexual interaction, induced ovulation, follicular regression.

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### INTRODUCTION

Reptiles exhibit a variety of reproductive strategies as response to different environmental conditions. In species with seasonal reproduction, gonadal cycle phases (recrudescence, climax, and gonadal quiescence) appear temporarily organized according to their thermic and energy demands, and possibly their duration (Saint Girons, 1984; 1985).

Consequently, differentiated gamete maturation requirements lead to gonadal cycle dissociation between sexes, a widely reported event in reptile reproduction (Moore and Lindzey, 1992; Whittier and Tokarz, 1992).

Therefore, apart from adequately adjusting to physical and climatic conditions, reptiles also use socio-sexual signals to coordinate their reproductive activity with that of individuals of the opposite sex. This type of intersexual regulation is mentioned by numerous reports of gonadal recrudescence induction, and concomitant hormonal changes (Crews and Garrick, 1980; Garstka et al., 1985; Mendoça and Crews, 1990; Summers et al., 1995; Shanbhag et al., 2002). The all-female parthenogenetic lizard *Cnemidophorus uniparens* constitutes an interesting evolutive phenomenon, since gonadal recrudescence is actually promoted by pseudo-male individuals (Crews et al., 1986). Furthermore, recent studies revealed cases of mating-induced ovulation in the loggerhead sea turtle, *Caretta caretta* (Manire et al., 2008) and possibly in the brown tree snake, *Boiga irregularis* (Mathies et al., 2004), as seen in mammals having reflex ovulation.

The South American oviparous lizards *Tupinambis rufescens* and *T. merianae* (Presch, 1973) which live in temperate and subtropical climates, show a lethargy period of approximately 6 months, which restricts main reproductive events (mating, oviposition, and incubation) to spring and early summer (Donadío and Gallardo, 1984; Mercolli and Yanosky, 1990; Noriega et al., 1996).

Studies on these species have also shown that gonadal activity is asynchronic between sexes. In fact, whereas the highest testicular activity coincides with mating events (Noriega et al., 2002), follicular development is only completed approximately 20 days after mating (Manes et al., 2007). Moreover, the short and intense vitellogenesis observed at the postnuptial stage pointed towards mating as the stimulus that triggers vitellogenesis and subsequent ovulation (Manes et al., 2007).

In this article, we analyzed the follicular dynamics of *Tupinambis merianae* lizards by means of ultrasound examination and identification of individuals through microchips. Animals in reproduction or sexually isolated were studied comparatively, in order to evaluate the influence of sexual interactions on follicular development and ovulation.

## MATERIALS AND METHODS

### *Animals and study conditions*

The study was carried out using adult specimens of *Tupinambis merianae*, raised in the experimental hatchery of Facultad de Agronomía y Zootecnia of Universidad Nacional de Tucumán. Female specimens had a 35 cm snout-vent length or more; male specimens were 39 cm or more. Besides their adult size, selected females have oviposited at least once.

The study was conducted during 2007-08, 2008-09 and 2009-10 reproductive seasons, in El Manantial, province of Tucumán, North of Argentina. This site has a warm temperate climate with dry season in the cold period.

The animals were kept in open-air enclosures, provided with shelters and shades. The enclosures were surrounded by 1.2 m tall masonry walls, and allowed each individual an area of 2 m<sup>2</sup>. They were fed ad libitum on a hatchery diet (Vega Parry and Manes, 2000).

In order to facilitate individual follow up, each specimen was implanted with a subcutaneous microchip of 11.5 x 2.1 mm (ID-100A microtransponder, Trovan Electronic Identification Devices LTD), on the left abdominal flank.

### *Experimental design*

To determine the influence of sexual interactions on follicular dynamics, the animals were subjected to two experimental conditions: under reproductive conditions and in sexual isolation. Breeding groups were formed with three to five females and one male. Sexually isolated groups were formed with three to five females in absence of male. New groups were created for each reproductive season, randomly redistributing the individuals.

To verify reproductive cycle events in the reproductive group (courtship, mating, oviposition, incubation, reproductive quiescence) (Noriega et al., 1996; Manes et al., 2007), the male specimens were placed daily with the females between 9 and 12 AM, and their activities were checked by an observer.

To allow comparisons, sexually isolated females examination was synchronized with that of females in reproductive conditions.

### *Ultrasound studies*

The ovaries were examined with an ultrasound scanner Berger LC 2010, with a micro convex probe of 5/7 MHz. Ultrasonographic observations were made every 7 to 14 days, since their departure from hibernation until oviposition. These were resumed after the incubation period, at the reproductive quiescence. Images of representative follicles in both ovaries were recorded both, on paper and digitally.

### *Statistical analyses*

The statistical modeling was carried out by using the statistical software R (R Development Core Team, 2009), packages nlme (Pinheiro and Bates, 2000; Pinheiro et al., 2009) and lattice (Sarkar, 2008; 2009). The mixed models methodology approach was applied to the data (Pinheiro and Bates, 2000; Vonesh and Chinchilli, 1997). Data was considered to be generated from a factorial experiment with repeated measures in time. Factor under analyses were: sexual interaction (in reproduction or in sexual isolation), follicular development (with ovulation, without ovulation: short and long term anovulatory cycles) and days after hibernation emergence. Data for oviposition were analyzed separately.

The minimal adequate model (Crawley, 2007), i.e. a model with all significant effects, was reached after backward selection of variables. Comparisons of models with alternative fixed parts and the same random structure were accomplished by means of likelihood ratio tests and the Akaike information (AIC) and Bayesian criterions (BIC). Estimation procedure was maximum likelihood (ML). Estimations for comparison of models with the same fixed part and alternative random structure were accomplished by means of restricted maximum likelihood (REML). The significance of the fixed effects was tested by means of conditional F tests (Pinheiro and Bates, 2000).

The maximum of each curve was obtained setting the first derivative to zero. The growth rate in the initial increasing growth phase was obtained evaluating the first derivative in the point corresponding the half the time necessary to reach the maximum follicular size. In the case of oviposition, a straight line model was fitted so the growth rate was just the estimated slope parameter.

## RESULTS

Twenty female specimens were observed, throughout three consecutive reproductive cycles. Six individuals were studied throughout one reproductive cycle, seven were observed during two cycles, and seven during three cycles, making a total of 41 cases. One of these developed an ovarian tumor (not described in this study).

Ultrasound examinations were performed from the end of hibernation (September) until reproductive quiescence (February). At the end of hibernation, ovaries appeared consistently undeveloped, as two small elongated formations, without detectable follicles. Afterwards, follicles started to grow, and they could be detected when they reached 3 mm in diameter. Ultrasound examinations revealed uniform follicular growth in both ovaries, after which follicles were either ovulated or went into regression.

### *Follicular development with ovulation*

Thirty four percent of the observed follicular cycles (14/41) ended in ovulation followed by oviposition (Fig. 1). Lack of oviductal egg retention in this species, makes oviposition a safe indicator of ovulation (unpublished observations).

In this variant, the follicles showed a steady, uniform growth throughout approximately 50 days (between 35 and 68 days). Few days before ovulation, follicles of mated females reached an average diameter of 2.66 ( $\pm$  0.16) cm, against 2.33 cm of follicular diameter of the only female, which happened to ovulate in the sexually isolated group. During development, follicles acquired a smooth echogenicity, irregularly distributed throughout the follicular mass (Fig. 2).

Since follicular discharge was massive, there were no remaining grown follicles in the ovaries at the end of ovulation. In a few cases, we observed small non-identifiable postovulatory formations, ephemeral and anechogenic, which ranged between 5 and 9 mm in diameter.

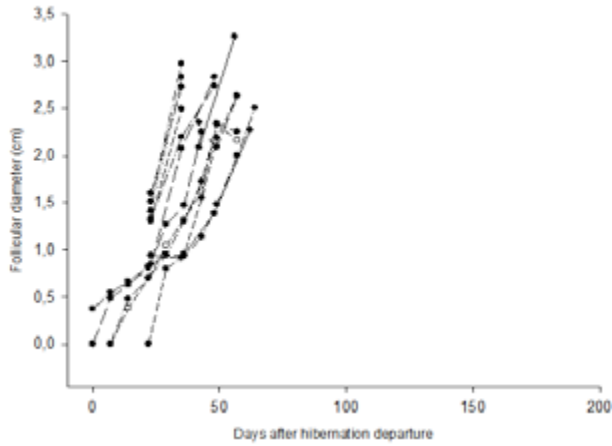
We also succeeded in observing oviductal eggs which appeared slightly elongated and aligned, although ultrasonography did not allow estimating their number, nor distinguishing whether they were shelled or unshelled.

### *Follicular development without ovulation*

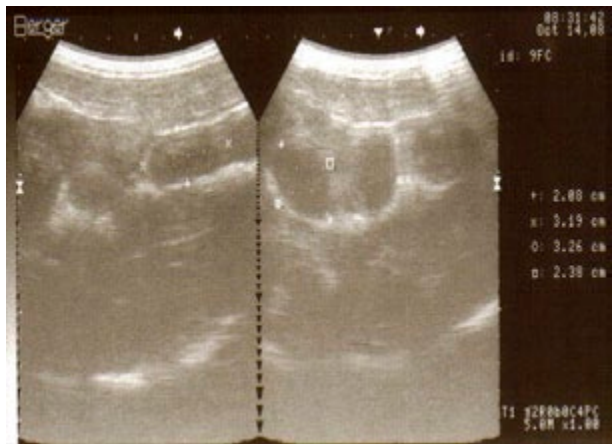
In the remaining 66% of cases (27/41), follicles also showed a noticeable growth, but unlike what was noticed in the previous situation, failed to ovulate, and underwent an extensive involution process. According their duration, we recognized two anovulatory cycle patterns (Fig. 3):

#### *Short term anovulatory cycle*

Follicles involved in this cycle grew poorly, reaching an average diameter of 1.05 ( $\pm$  0.06) and 0.91 ( $\pm$  0.2) cm in reproducing females and in sexually isolated ones, respectively (Fig. 3). Subsequently, they underwent regression, turning undetectable 80 days after



**Fig. 1.** *Tupinambis merianae* follicular development followed by ovulation. (●) Reproductively active females; (○) The only case of ovulation among sexually isolated females.

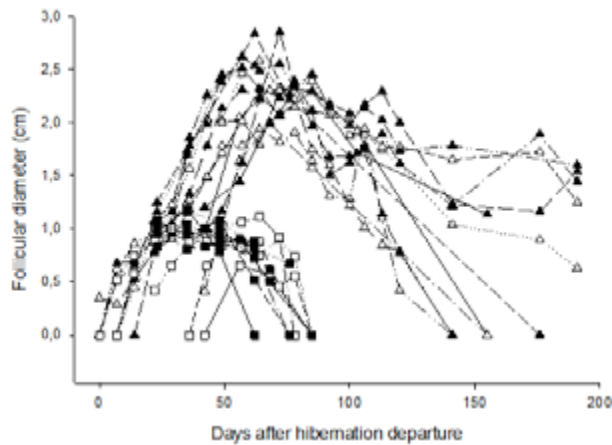


**Fig. 2.** *Tupinambis merianae* preovulatory follicles showing a mild irregular echogenicity.

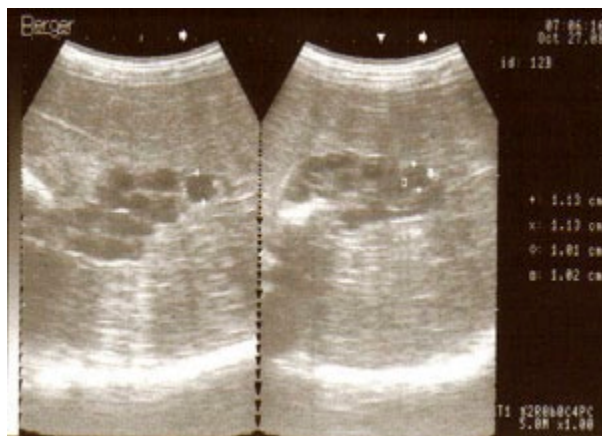
the end of hibernation. Throughout the entire process, follicles had a cystic appearance, and were characteristically anechogenic (Fig. 4).

*Long term anovulatory cycle*

Average follicular size in this cycle almost equaled that of periovulatory follicles: 2.58 ( $\pm 0.16$ ) and 2.32 ( $\pm 0.3$ ) cm in diameter, in reproducing females and in sexually isolated ones, respectively (Fig. 3). The follicles as those destined to ovulation acquired echogenicity mainly concentrated at the follicular cortex (Fig. 5).



**Fig. 3.** *Tupinambis merianae* anovulatory follicular cycles. Short term cycle: (■) reproductively active females; (□) Sexually isolated females. Long term cycle: (▲) reproductively active females; (△) Sexually isolated females.

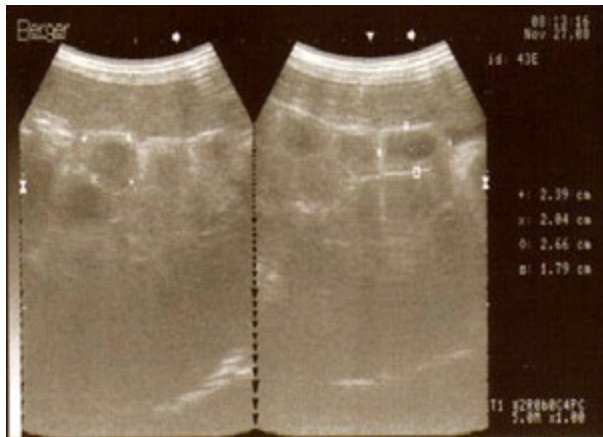


**Fig. 4.** Maximally grown *Tupinambis merianae* follicles from a short term anovulatory follicular cycle. Observe their anechogenic aspect.

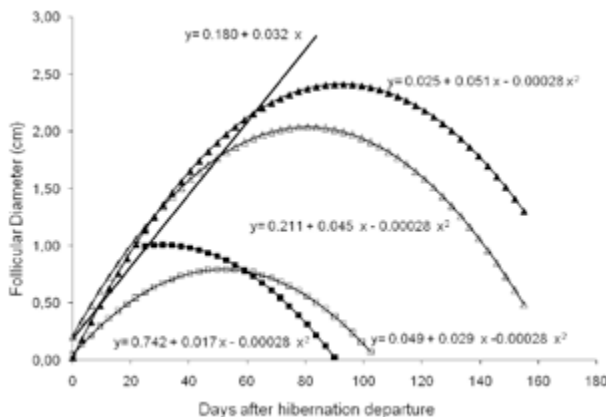
The subsequent involution period was longer, so it was possible to find follicles 200 days after the end of hibernation. Involution in this case was also characterized by an intensified echogenicity of the whole follicle.

The involution process in both anovulatory cycles, seemed to affect the whole of recruited follicles from both ovaries.

Mating took place between 2 and 4 weeks after the end of hibernation, involving females with varying degrees of follicular development (between 0.9 and 2.8 cm in diameter). The average time between mating and oviposition was 22 days, ranging from 7 to 33 days.



**Fig. 5.** Maximally grown *Tupinambis merianae* follicles from a long term anovulatory follicular cycle. Observe the echogenic aspect of the follicular cortex.



**Fig. 6.** Male effect on *Tupinambis merianae* follicular growth. (—) Follicular development with ovulation,  $r^2= 0.92$ ; Anovulatory follicular cycles ( $r^2= 0.86$ ): Short term cycle: (■) reproductively active females; (□) Sexually isolated females. Long term cycle: (▲) reproductively active females; (△) Sexually isolated females.

*Effects of sexual interactions on follicular behavior*

Ovulation rate, based on ovipositions, was considerably higher in sexually interacting females as compared to those sexually isolated: 45% (13 out of 29) versus 8% (1 out of 12) (Table 1).

Ovulation frequency within the reproducing group was clearly related to mating consummation (Table 1): verified mated females (21) presented 13 ovipositions, whereas non-mated ones (8) had none. Curiously, the only case of ovulation without mating involved a sexually isolated female (Table 1). In absence of ovulation, the two anovulatory follicular cycles were observed (Table 1), without either being favored by mating.

**Table 1.** Mating incidence on *Tupinambis merianae* ovulation/oviposition.

| Experimental condition        |         | Ovulation / oviposition | Follicular involution |
|-------------------------------|---------|-------------------------|-----------------------|
| Reproductively active females | Mated   | 21                      | 13                    |
|                               | Unmated | 8                       | -                     |
| Sexually isolated females     |         | 12                      | 1                     |
|                               |         |                         | 11 <sup>(*)</sup>     |

(\*) Including a female which developed an ovarian tumor.

**Table 2.** Effect of the male presence on *Tupinambis merianae* follicular growth parameters.

| Experimental condition        | Follicular cycle type                        | Maximum follicular diameter (cm) | Time to maximum follicular diameter (days) | Growth (cm/day) |
|-------------------------------|--|----------------------------------|--|-----------------|
| Reproductively active females | With ovulation                               | 2.83 ( $\pm$ 0.59)               | 84   | 0.032           |
|                               | Short term anovulatory follicular cycle      | 0.998 ( $\pm$ 0.04)              | 30   | 0.0086*         |
|                               | Long term anovulatory follicular cycle       | 2.347 ( $\pm$ 0.03)              | 91   | 0.026*          |
| Sexually isolated females     | Short term anovulatory follicular cycle      | 0.7998 ( $\pm$ 0.04)             | 52   | 0.014*          |
|                               | Long term anovulatory cycle follicular cycle | 2.046 ( $\pm$ 0.06)              | 81   | 0.023*          |

\* Estimated during the increasing phase of the slopes.

Follicular growth was also superior in reproducing females compared to sexually isolated ones (Fig. 6, Table 2).

Such extra follicular growth derived from male presence rather than mating occurrence, since no differences were observed between mated and unmated females.

In addition, sexually isolated females exhibited sexual behaviors similar to the reproducing ones.

Among females that joined reproductive groups during two or three consecutive years (14), we found some individuals that exhibited recurrence of oviposition cycles, some others, recurrence of anovulatory cycles, and finally others, combining both cases.

## DISCUSSION

Follicular growth in *Tupinambis merianae* began at the end of hibernation (September), leading either to ovulation or involution. Growth and ovulation processes appear to be generalized events, affecting both ovaries, and all or most recruited follicles in each reproductive cycle. This seem suitable for a once-a-year clutch production with high number of eggs (Donadío and Gallardo, 1984; Mercolli and Yanosky, 1990; Noriega et al., 1996), which can, in turn, be associated to a compact reproductive schedule for a limited



activity period in subtropical and temperate climates (Donadío and Gallardo, 1984; Mercolli and Yanosky, 1990; Noriega et al., 1996).

The strong correspondence between mating and oviposition frequency seems to indicate a mating increased level of stimuli required for the follicular cycle to end up with ovulation. However, not all matings led successfully to ovulation. Mating-induced ovulation is a widespread phenomenon in mammals, but has very few examples among reptiles (Manire et al., 2008). However, it may be more usual than expected, though masked by an interval between mating and ovulation, as seen in reptilian dissociated gonadal cycles (Moore and Lindzey, 1992; Whittier and Tokarz, 1992). Such asynchronicity would also require sperm retention in female genital ducts, which is a common reptilian strategy (Cuellar, 1966; Gist and Jones, 1987; Sever and Hamlett, 2002). A sperm retention mechanism in *Tupinambis merianae* females was proposed to overcome the gap of about 20 days between copulation and ovulation (Manes et al., 2007 and present results). We believe that such sperm retention does not extend beyond one reproductive period, since animals that copulated without ovipositing in a cycle, did not oviposit in the following cycle either, when regrouped into sexually isolated lots. In addition, the extra follicular growth shown by females kept in reproduction as compared to sexually isolated ones, reveals that the sole male presence without mating induces a certain degree of gonadal recrudescence, as previously observed in other reptiles (Crews and Garrick, 1980; Garstka et al., 1985; Mendonça and Crews, 1990; Summers et al., 1995; Shanbhag et al., 2002). Likewise, cases of spontaneous vitellogenesis and ovulation may reflect endocrine stimulation levels, similar to those achieved through sexual interactions. In this sense, we can not rule out pheromones interacting between reproducing and sexually isolated individuals, as suggested by sexual behaviors in isolated females.

The absence of ovulation in *Tupinambis merianae* was clearly related to an extensive follicular atresia. Considering its general physiological role as regulator of the oocyte number to ovulate, massive follicular atresia in this species looks more like the sign of a failure in follicle maturation or ovulation. Follicular atresia in short term anovulatory cycles, presumably involving previtellogenic follicles, could represent a timely departure of the follicular cycle to prevent a great vitellogenic effort considering the huge periovulatory ovarian mass (about 400 grams, Manes et al., 2007). On the contrary, follicular atresia in long term anovulatory cycle would be a rather late step back, when the vitellogenic process has already taken place. As indicated by tumor formations (Apichela et al., 2002 and present study), ovarian recovery does not always end up with satisfactory results. As reviewed by Saidapur (1978), an endocrine production by atretic follicles cannot be dismissed either.

Apart from the two general types of follicular involution described here, a previous sexual isolation essay (Apichela et al., 2002), showed the release of large follicular aggregates in the coelomic cavity. This phenomenon may represent a different type of "bursting atresia", as referred by Saidapur (1978) for some reptiles and birds.

Finally, from the present results, we can conclude that *Tupinambis merianae* lizards are able to oviposition every year, although for still unknown reasons, anovulatory cycles may appear.

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