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Testicular cycle of *Amphisbaena mertensii* Strauch, 1881 (Squamata: Amphisbaenidae) in northeastern Argentina

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Introduction

The perpetuation of species depends mainly on their reproductive success, thereby the study of reproductive biology is an important matter for understanding the evolution of life-history strategies (Seigel and Ford, 1987; Holycross and Goldberg, 2001).

Knowledge of amphisbaenian reproduction is scarce and fragmentary because the diggers and fossorial habits of worm lizards decrease the probability of encounter in nature (Andrade et al., 2006). Reproductive data are available for only 12% out of 197 species (Andrade et al., 2006; Uetz and Hošek, 2017). Published information consists of clutch and eggs sizes, oviposition season, and changes in gonadal sizes (Andrade et al., 2006). The testicular cycles known are based in the external morphology of the vas deferens and variation of testes weight and size (Andrade et al., 2006).

The use of histological and hormonal techniques allows to determine more precisely the minimum size at sexual maturity using a small sample, and the timing of the gametogenesis and its correlation with macroscopic variation of the gonads (Boretto and Ibargüengoytía, 2009; Boretto et al., 2012).

In amphisbaenians, histological studies related to the reproductive activity of males and females are restricted to *Blanus cinereus* Vandelli, 1797 (Blanidae) and *Trogonophis wiegmanni* Kaup, 1930 (Trogonophiidae) of Morocco (Bons and Saint-Girons, 1963) and *Diplometopon zarudnyi* Nikolsky, 1907 (Trogonophiidae) of Saudi Arabia (Al-Dokhi et al., 2013; Al-Sadoon et al., 2014).

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In the present study we determined the testicular cycle of *Amphisbaena mertensii* (Amphisbaenidae) by morphological and histological analysis of gonads and epididymis in order to provide additional data about its reproductive biology. The information known is based on isolated observations of specimens from southeastern Brazil and Paraguay (Pramuk and Alamillo, 2003; Andrade et al., 2006). *Amphisbaena mertensii* is an oviparous worm lizard that occurs in northeastern Argentina, southeastern Brazil and Paraguay (Ribeiro et al., 2007). Females lay six to eight soft-shelled eggs during spring and the incubation period lasts 59 days (Andrade et al., 2006).

Materials and methods

We analysed 16 preserved males of *A. mertensii* deposited in the herpetological collection of the Universidad Nacional del Nordeste (UNNEC) and collected during the four seasons between 1984 and 2013 in Chaco and Corrientes provinces.

We examined the gonads and the morphology of the epididymis under a stereomicroscope (Leica® ES2). Arbitrarily, we selected the right testicle and measured its length (TL) and width (TW) with digital caliper to calculate the testicular volume (TV) from the formula of the spheroid: $TV = 4/3\ \pi\ (TL/2)\ (TW/2)^2$. We registered the degree of epididymis folding and classified it as coiled (with sperm in lumen) and not coiled (without sperm in lumen) (López et al., 2009).

To investigate the gonadal activity, we obtained cross sections of testis and epididymis (5–7 μm thick) with a rotary microtome Spencer type (Arcano® KD 1508A) and used the conventional histological techniques of hematoxylin and eosin to describe the basic histological appearance, and PAS reaction (periodic acid-Schiff) to identify secretory activity of mucopolysaccharides in the epididymis. We observed and photographed the preparations under a light microscope (Olympus® CH30).

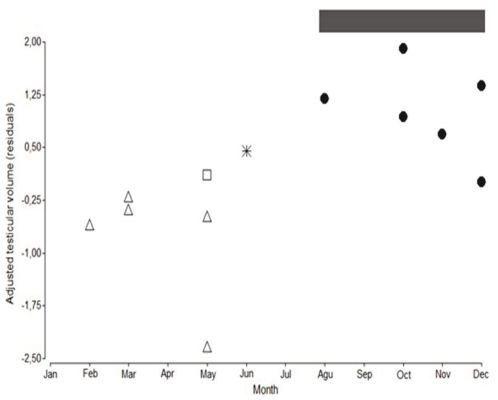

Figure 1. Monthly variation of testicular volume and spermatogenic stages in adults of *Amphisbaena mertensii*. Testis with spermatogenic stage 2 (squares), stage 3 (asterisks), stage 4 (circles black), and stage 5 (triangles). Gray bar above indicates the presence of sperm in epididymis.

We followed Gribbins (2010) for identifying types of germ cells and recognized five spermatogenic stages considering the type of germ cell more advanced into the seminiferous epithelium: (1) seminiferous epithelia with only spermatogonia, and seminiferous tubule without defined lumen, (2) primary and secondary spermatocytes at the luminal margin, (3) spermatids in different degrees of differentiation, (4) sperm in the lumen of the seminiferous tubule, and (5) testicular regression characterized by seminiferous epithelial with only Sertoli cells and spermatogonia and the tubule lumen open (modified from Mayhew and Wright, 1970).

We measured the snout-vent length (SVL) of males using a digital caliper Essex® (accuracy 0.1 mm) and established the minimum SVL at sexual maturity based on the smallest specimen with spermatogenic activity (stages 2–5) or spermatozoa in the epididymis.

We transformed data to their natural logarithms (ln) to meet the assumptions of normality and homogeneity of variance (Zuur et al., 2010). We performed a simple linear regression analysis between the SVL and TV, and we used the residuals of this regression to avoid the effect of size (Ramirez-Bautista and Vitt, 1997), using a significance level of 0.05. All statistics were performed using the software InfoStat (version 2011).

Results

Of the 16 males analyzed, 3 were found to be juveniles and 13 adults. The SVL in juvenile males varied between 147 and 195 mm ($\bar{x}$ = 179 mm ± 27.71 SD, n = 3) whereas SVL in adults ranged between 228 and 448 mm ($\bar{x}$ = 278.38 mm ± 58.51 SD, n = 13). The smaller adult male had testes in the spermatogenic stage 5.

There was a positive relationship between SVL and TV (r^2 = 0.52, P = 0.001, n = 16). The adjusted TV of adult males varied according to the months with the highest values recorded in October (spring; Fig. 1).

Juvenile males were captured in spring (September, October and November). They exhibited spermatogenic stage 1; the epididymis were not coiled and the epididymal epithelium had cubic cells, with no signs of secretory activity (negative PAS).

In adults individuals the folding of the epididymis increased from winter to spring. In mid-autumn (May) the testes were characterized by stage 2 (n = 1), and the cuboidal epididymal epithelium had not signs of secretory activity (negative PAS). Stage 3 (n = 1) and epididymal epithelium without secretory activity occurred in late autumn (June). From late winter (August) until late spring (December) seminiferous tubules and epididymis shown lumens full of spermatozoa (stage 4, n = 6). The epididymal epithelium was constituted by columnar cells with basal nuclei showing signs of secretory activity of mucopolysaccharides (positive PAS). Stage 5 (n = 5) occurred from mid-summer (February) to mid-autumn (May); the epididymis showed a cubic epithelium and a negative PAS.

Discussion

The testicular size changes in Amphisbaenidae are related to the reproductive cycle of individuals (Navega-Gonçalves, 2009). However, it is known that testicular enlargement is not always associated with sperm production, rather to both the gametogenesis and the activity of Leydig cells to produce hormones (Boretto et al., 2007). Thus, a reproductive cycle determined as seasonal, on the basis of testicular volume may be continuous when analysing the gonadal histology, as in *Sceloporus bicanthalis* (Hernández-Gallegos et al., 2002).

According to the increase of the testicular volume and the folding of epididymis we suggest a reproductive activity during mid-autumn to late spring in *A. mertensii,* as reported to neotropical Amphisbaenidae *Anops kingii* and *Amphisbaena munoai* in the central-east of Argentina and south-east of Brazil, respectively

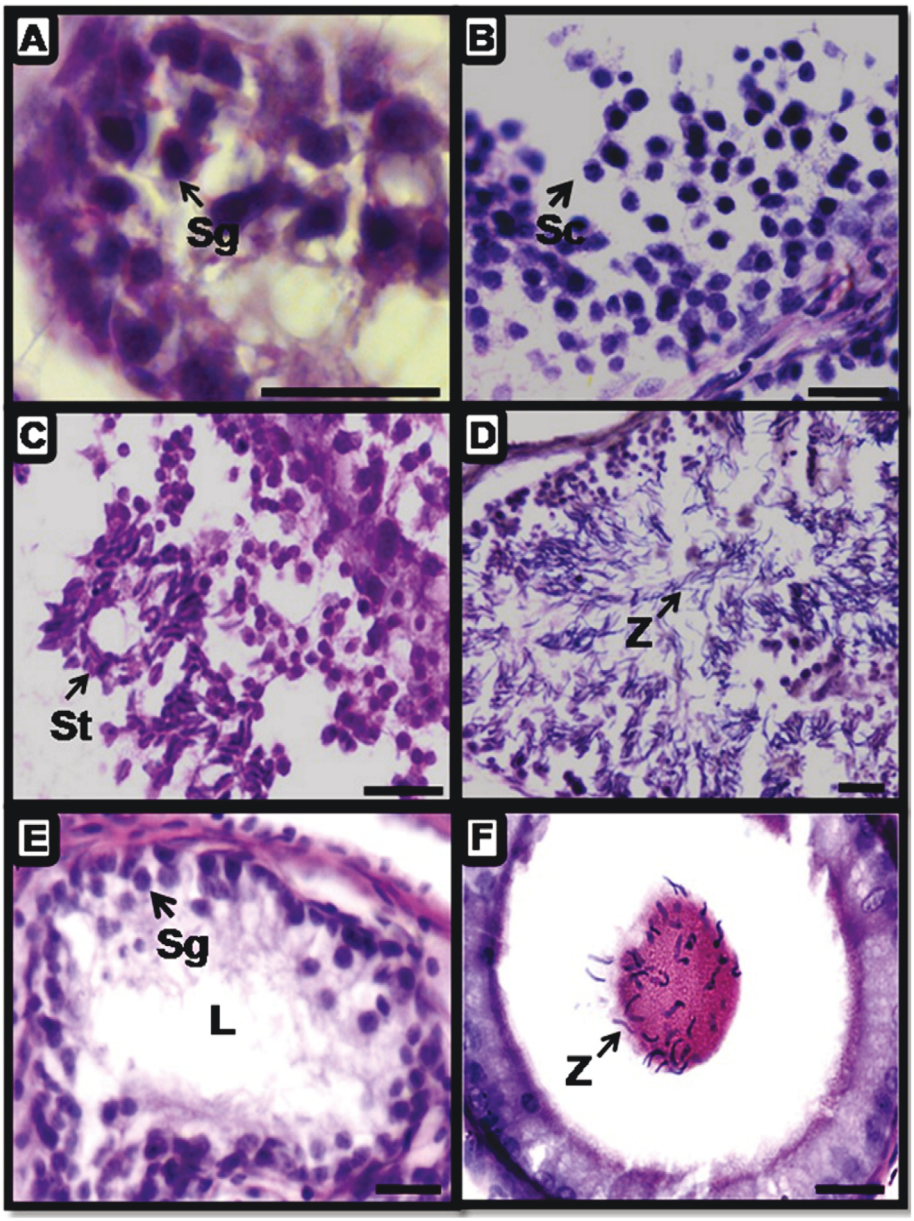

Figure 2. Photomicrographs of cross-section of seminiferous tubules and epididymis of *Amphisbaena mertensii*. (A) Stage 1, seminiferous tubule with spermatogonia. (B) Stage 2, showing spermatocytes. (C) Stage 3, with spermatids at the luminal margins. (D) Stage 4, with lumen filled by spermatozoa. (E) Stage 5, corresponding to testicular regression. (F) Epididymis showing columnar epithelium with a central mass of spermatozoa with a positive PAS reaction. Spermatogonia (Sg), spermatocytes (Sc), spermatids (St), spermatozoa (Z), and lumen of the seminiferous tubule (L). Scale bar = 10 μm.

(Vega, 2001; Balestrin and Cappellari, 2011). The histological analysis performed support the seasonality of the spermatogenic cycle in *A. mertensii* throughout the year.

In *A. mertensii* higher values of testicular volume occur in males with spermatogenic stage 4, when the seminiferous tubules are full of spermatozoa, indicating a positive relationship between an increase in testicular

Table 1. Spermatogenic activity in different family of Amphisbaenia. Sm: summer; A: autumn; W: winter; S: spring.

Species	Spermatogenic stages				Source
	2	3	4	5	
Amphisbaena mertensii (Amphisbaenidae)	A	A	W, S	Sm, A	This study
Blanus cinereus (Blanidae)	S	S	S	Sm, A, W	Bons and Saint-Girons, 1963
Diplometopon zarudnyi (Trogonophidae)	S	S	S	Sm, A, W	Al-Sadoon et al., 2014
Trogonophis wiegmanni (Trogonophidae)	S	S	S	Sm, A, W	Bons and Saint-Girons, 1963

size and gametogenic activity. In addition, the presence of sperm in the epididymis is associated exclusively with this stage.

The timing of spermatogenic activity in *A. mertensii* differs from *B. cinereus*, *T. wiegmanni* and *D. zarudnyi* (Table 1). In these worm lizards spermatogenesis (stages 2, 3 and 4) occurs in spring and testicular regression from summer to winter (Bons and Saint-Girons, 1963; Al-Sadoon et al., 2014). In *A. mertensii*, spermatogenesis begins in mid autumn to late spring (stage 2, 3 and 4) and the testicular regression (stage 5) occurs from summer to early autumn (Table 1). Most studies indicate that amphisbaenians have a seasonal reproductive activity during the high productivity season (Andrade et al., 2006). *Blanus cinereus*, *T. wiegmanni* and *D. zarudnyi* have the maximum sexual activity shortly after the end of hibernation, when environmental conditions become optimal (Al-Sadoon et al., 2014). In subtropical and tropical regions, the breeding season can be more variable and prolonged (Andrade et al., 2006; Mathies, 2011). This reproductive pattern was observed in *A. mertensii* whose spermatogenic activity extends between autumn and spring (seven months).

In *A. mertensii* from south-eastern Brazil and Paraguay, oviposition was recorded in spring, during the rainy season (Andrade et al., 2006). This information, along with our results suggests that probably, the cycles of both sexes are seasonal and synchronic. However, a detailed study of the reproductive activity of females from the northeast of Argentina is necessary. Seasonal and synchronous cycles were reported in *A. kingii*, *A. munoai* and *D. zarudnyi* (Vega, 2001; Balestrin and Cappellari, 2011; Al-Sadoon et al., 2014).

Further morphological and histological gonadal analysis and sex hormone surveys are needed to a comprehensive approach on the reproductive biology of Amphisbaenia and to determine the reproductive patterns of the group.

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Appendix 1. Specimens examined.

Corrientes province: Puerto Arazá, Isla Apipé Grande (27°28’42’’S, 56°56’34’’W) UNNEC 10184, 10414, 10128, 10183, 9779; Puerto San Antonio, Isla Apipé Grande (27°31’12’’S, 56°44’32’’W) UNNEC 10397, 10399; Rincón Santa María, Ituzaingó Department (27°31’56’’S, 56°38’2’’W) UNNEC 9552; Corrientes Department (27°28’16’’S, 58°50’22’’W) UNNEC 12648, 794, 1050, 856, 4910, 9844; Gobernador Ing. Valentín Virasoro, Santo Tomé Department (28°3’4’’S, 56°1’13’’W) UNNEC 538.

Chaco province: Puerto Tirol, Libertad Department (27°22’30’S, 59°7’16’’W) UNNEC 1052.

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