JOURNAL OF MORPHOLOGY 274:215–228 (2013)

The Reproductive Cycle of the Male Sleep Snake *Sibynomorphus mikanii* (Schlegel, 1837) From Southeastern Brazil

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ABSTRACT This study describes the male reproductive cycle of *Sibynomorphus mikanii* from southeastern Brazil considering macroscopic and microscopic variables. Spermatogenesis occurs during spring–summer (September–December) and spermiogenesis or maturation occurs in summer (December–February). The length and width of the kidney, the tubular diameter, and the epithelium height of the sexual segment of the kidney (SSK) are larger in summer–autumn (December–May). Histochemical reaction of the SSK [periodic acid-Schiff (PAS) and bromophenol blue (BB)] shows stronger results during summer–autumn, indicating an increase in the secretory activity of the granules. Testicular regression is observed in autumn and early winter (March–June) when a peak in the width of the ductus deferens occurs. The distal ductus deferens as well as the ampulla ductus deferentis exhibit secretory activities with positive reaction for PAS and BB. These results suggest that this secretion may nourish the spermatozoa while they are being stored in the ductus deferens. The increase in the Leydig cell nuclear diameter in association with SSK hypertrophy and the presence of sperm in the female indicate that the mating season occurs in autumn when testes begin to decrease their activity. The peak activity of Leydig cells and SSK exhibits an associated pattern with the mating season. However, spermatogenesis is dissociated of the copulation characterizing a complex reproductive cycle. At the individual level, *S. mikanii* males present a continuous cyclical reproductive pattern in the testes and kidneys (SSK), whereas at the populational level the reproductive pattern may be classified as seasonal semisynchronous. J. Morphol. 274:215–228, 2013.

KEY WORDS: spermatogenesis; sexual segment of the kidney; ampulla ductus deferentis; mating; neotropical Colubridae

INTRODUCTION

Reproductive cycles may be more plastic in tropical areas due to their climatic complexity and variable prey availability (Seigel and Ford, 1987; Santos et al., 2005; Brown and Shine, 2006; Barros et al., 2012). Reproductive patterns of snake species from the neotropical region are not completely understood (Pizzatto and Marques, 2002; Almeida-Santos and Salomão, 2002; Pizzatto et al., 2008a,b; Marques et al., 2009) and detailed studies on snake reproductive biology are lacking in comparison to the temperate region, where some species are well studied (Krohmer et al., 1987; Schuett et al., 2002; Siegel et al., 2009). Data on the reproduction of neotropical snakes differ among the sexes. Reliable data on the timing of female reproductive events are available for some lineages (Almeida-Santos and Salomão, 2002, Barros et al., 2012). On the other hand, most studies that include male reproductive events do not include any microscopic analysis (Pizzatto and Marques, 2007; Pizzatto et al., 2008a,b; Scartozzoni et al., 2009; Pinto et al., 2010; Orofino et al., 2010) and may present misleading conclusions for this reason (Mathies, 2011).

According to Mathies (2011), only 15.5% of the literature on reproductive biology of Central and South American snake species used histological methods to examine testicular condition and to characterize the spermatogenic cycle. In vertebrates, three strategies of germ cell development are recognizable: (a) an anamniote pattern (fish and amphibians); (b) an amniote pattern (sauropsids and mammals); and (c) a new strategy within amniotes exclusive to reptilian sauropsids that

Contract grant sponsor: CNPQ; Contract grant number: 134719/2007-7; Contract grant sponsor: FAPESP; Contract grant number: 07/51969-0.

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Received 4 July 2012; Revised 20 August 2012; Accepted 30 September 2012

Published online 29 November 2012 in Wiley Online Library (wileyonlinelibrary.com)
DOI: 10.1002/jmor.20099

show seasonal reproduction (Lebonde and Clermont, 1952; Lofts, 1977; Kumar, 1995; Gribbins and Gist, 2003; Gribbins et al., 2003; 2005).

In some squamates, gamete production by males is not associated directly with the mating season and an increase of sex hormone levels (Clesson et al., 2002). Squamates present a secondary sexual androgen-dependent structure which is known as the sexual segment of the kidney (SSK; Bishop, 1959). As SSK hypertrophy is under the control of testosterone, a correlation may be established with the mating period (Volsøe, 1944; Bishop, 1959; Fox, 1977; Schuett et al., 2002; Krohmer et al., 2004; Graham, 2006; Aldridge et al., 2011).

The SSK is characterized by a single layer of columnar epithelium with basal nuclei and a cytoplasm full of secretory granules (Sever et al., 2002; Krohmer et al., 2004). The number and density of these granules in the cytoplasm vary according to the hormonal status of the species and from one individual to another (Bishop, 1959; Fox, 1977). The SSK secretory product is always rich in proteins and presents low levels of mucin, but the reaction to periodic acid-Schiff (PAS) is variable: weak or absent in amphisbaenids and most lizards; highly positive in Anguidae, and highly variable (positive to absent) in snakes (Saint-Girons, 1972). Many functions have been proposed for the SSK secretions, such as sperm nutrition and activation (Bishop, 1959), or a role in female sperm storage processes especially in the formation of the copulatory plug observed in Natricinae and Viperidae (Fox, 1977; Almeida-Santos and Salomão, 1997; Almeida-Santos et al., 2004; Marinho et al., 2008b; Aldridge et al., 2011).

In male squamates, after gametes are produced in the testes, they may be used right away during copulation or may be stored in the ductus deferens (Fox, 1977; Almeida-Santos et al., 2004; Sever, 2004; Almeida-Santos et al., 2006). However, the mechanisms that allow sperm storage for an undetermined length of time are still unknown (Marinho et al., 2008a,b). The ampulla ductus deferentis might play a role in the sperm storage process (Trauth and Sever, 2011). The ampulla differs from the rest of the ductus deferens because it presents a deeply folded epithelium with secretory cells (Setchell et al., 1994). The ampulla is related to maturation, nutrition, storage, and phagocytosis of the spermatozoa in mammals (Cooper and Hamilton, 1977; Bergerson et al., 1994) and has been recently described in lizards (Akbarsha and Meeran, 1995; Akbarsha et al., 2005) and snakes (Sever, 2004; Siegel et al., 2009; Trauth and Sever, 2011).

The sleep snake, *Sibynomorphus mikanii* (Fam: Colubridae), is a nocturnal and terrestrial species that occurs only in South America (Laporta-Ferreira et al., 1986; Franco, 1994; Pyron et al., 2011). Vitellogenesis occurs from July to December (Pizzatto et al., 2008b) and the mating season occurs in autumn (Rojas, 2009). Eggs in the oviduct were recorded from late August to February (Pizzatto et al., 2008b). Pizzatto et al. (2008b) studied the reproductive ecology of Dipsadinae snakes and presented information on the sleep snake. They used testes length and ductus deferens diameter to infer timing of spermatogenesis and sperm storage, respectively. As these parameters did not differ significantly between the seasons, they concluded that male *S. mikanii* exhibit continuous sperm production throughout the year and absence of sperm storage in the ductus deferens (Pizzatto et al., 2008b). The validity of these hypotheses was tested. To our knowledge, this is the first study on the male reproductive cycle of a neotropical snake species that emphasizes the relationship between the different reproductive components, such as the spermatogenic cycle, sperm storage strategies, and the secretory cycle of the SSK.

MATERIAL AND METHODS

Animal collection—original data of 139 adult male *Sibynomorphus mikanii* (Schlegel, 1837) from the southeastern region of Brazil were obtained after dissection of specimens deposited between 1930–2005 in the collections of the Museu Nacional do Rio de Janeiro (MNRJ), Coleção Herpetológica "Alphonse Richard Hoge" (CHARH), and Coleção Município de São Paulo (MSP). These last two collections are located at the Instituto Butantan (IB). Climate in the sampling area is tropical. Rainfall is concentrated in summer when temperatures are higher (mean temperature = 24.9°C). During the winter, temperatures are lower (mean temperature = 15.5°C) and rainfall is reduced. Mean annual precipitation in São Paulo city is 1,517 mm (Mendonça and Danni-Oliveira, 2007). Austral seasons may be characterized as summer (late December–late March), autumn (late March–late June), winter (late June–late September), and spring (late September–late December). For each snake, the following data were collected: (1) snout-vent length (SVL), (2) length, width, and thickness of the right testes, (3) width of the right ductus deferens in the distal portion (between the kidney and the cloaca), (4) length of the right kidney, and (5) width of the right kidney in the proximal region. Volume of testes was calculated by the ellipsoid formula 4/3 Π a.b.c, in which a = length/2, b = width/2, c = thickness/2 (Pleguezuelos and Feriche, 1999).

Forty-six specimens received at IB (São Paulo city) between 2005 and 2007 were also analyzed. Individuals were euthanized by an intracoelomic injection of thionembutal (30 mg/Kg), followed by a 0.2 ml intracardiac injection of potassium chloride (KCl; Rojas, 2009). After dissection, the right testes were removed and weighed (precision balance—Marte AL 500C) and the Gonadosomatic Index (GSI) was calculated by the formula: right testis mass/body mass × 100 (Clesson et al., 2002). This work is in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation, and it was approved by the Ethical Committee for Animal Research of Butantan Institute (protocol number 370/07).

Histology—Recently euthanized animals (N = 24) were used for histological analysis. As a standard procedure, only organs of the right side were used in this study. Organs were fixed in 10% formalin solution and preserved in 70% alcohol for histological analysis.

From each specimen, a section was obtained from the midregion of the testis, the distal region of the ductus deferens (portion between the kidney and the cloaca), and the proximal region of the kidney. Samples were processed for light microscopy by historesin (Glycol methacrylate—Leica) and paraffin methods. Sections 2 and 5 μm thick were cut for historesin and

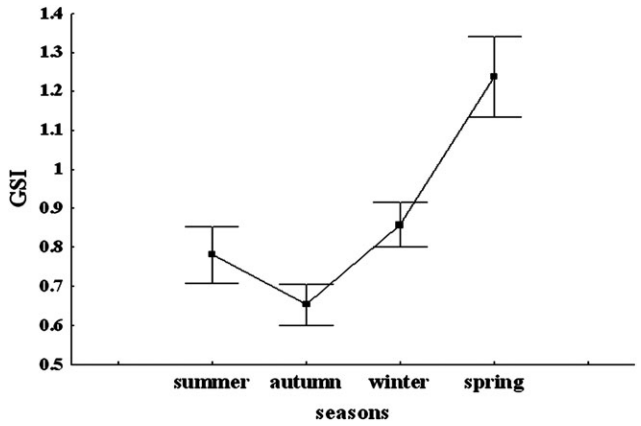

Fig. 1. GSI of the right testes of *Sibynomorphus mikanii* with an increase in spring suggesting an intense spermatogenic activity. Middle point represents average values and whiskers represent standard errors.

paraffin, respectively. Histological staining methods used were hematoxylin and eosin (H/E) in paraffin (Junqueira et al., 1979) and toluidine blue-fuchsin (T/F) for sections in historesin (Junqueira, 1995). Sections were submitted to the following reactions: bromophenol blue (BB) for protein identification, PAS for identification of neutral carbohydrates, and alcian blue (AB) pH 2.5 for carboxylated glycosaminoglycans. Slides were viewed using an Olympus BX51 microscope. Morphometric measurements and images of testes, kidneys, and ductus deferens were obtained via Image—Pro Express Olympus Program.

Morphological and structural variations in the seminiferous tubules and their interstitium were used to characterize the spermatogenic cycle. We followed Goldberg and Parker (1975) in delineating spermatogenic stages in snakes: Stage I (complete regression), II (early recrudescence), III (late recrudescence), IV (early spermiogenesis), V (spermiogenesis), and VI (early regression). Structural changes in the testes were verified by the following measurements: diameter and epithelial height of the seminiferous tubules and Leydig cell nuclear diameter. Measurements recorded for the SSK were: tubular diameter and epithelial height. Ten measures of each variable per individual were obtained using the software Image-Pro Express. Only seminiferous and uriniferous tubules presenting circular forms in transverse sections were measured.

Analysis of the density and staining intensity (BB and PAS) of the granules of the SSK was performed. Changes related to granule density were characterized by visible comparison (Krohmer et al., 1987) on a scale from 0 to 4 following Krohmer et al. (2004): 0 = no observable hypertrophy, 1 = hypertrophy with a few granules, 2 = hypertrophy with granules evident throughout the cytoplasm of all epithelial cells, 3 = secretory granules visible in the apical region of the cytoplasm, 4 = maximum density of secretory granules within the cytoplasm. Alterations in the staining intensity of the granules in the SSK were classified as Stages I to III according to Aldridge and Brown (1995): I- some granules present, lightly stained, II—many granules present, moderately stained, and III—many granules, intensely stained.

Statistical analysis—Seasonal variation in right testes volume, length, and width of the right kidney was compared between the seasons of the year. These data were tested by analysis of covariance using SVL as the covariable. Differences on width of the ductus deferens among the seasons were tested by analysis of variance (ANOVA). Post-hoc tests (Tukey test for parametric data and comparisons by Dunn method for nonparametric data or data with unequal variances) were used to identify differences between seasons.

The GSI was determined by ANOVA and significant results were analyzed by the Tukey post-hoc test. Differences in diameter and epithelium height of the seminiferous and SSK tubules among seasons were tested by Kruskal-Wallis. Seasonal variations observed in the Leydig cell nuclear diameter were also tested by Kruskal-Wallis. Significant differences between seasons were analyzed by a post-hoc test (comparisons by Dunn method). All statistical analyses were performed using Statistica version 7, assuming $P < 0.05$ as the criterion for significance. The analyses were carried out according to Zar (1999) and all variables were tested for normality and homoscedasticity before analysis.

RESULTS

Spermatogenic and Ductus Deferens Cycle

Seasonal variation in some parameters of the male reproductive cycle was identifiable during this study. The GSI differed between the seasons with an increase in testicular mass during the spring ($F = 9.37$, $n = 46$, $P = 0.00008$; Fig. 1). Post-hoc analysis (Tukey test) showed that testes were heavier during spring in comparison to other seasons (summer $P < 0.01$; autumn $P < 0.01$ and winter $P < 0.01$; Fig. 1). Testes volume showed an increase during spring and summer (Table 1), although statistical results were not significant ($F = 1.94$, $n = 139$, $P = 0.12$). The width of the distal ductus deferens differed between the seasons ($F = 4.04$, $n = 139$, $P = 0.009$). A post-hoc test (Tukey test) showed that the width was larger in autumn than in winter and spring (both, $P < 0.05$; Table 1).

Considering the data obtained using histological techniques, the diameter of the seminiferous tubules slightly increased in the spring ($H = 7.52$, $n = 240$, $P = 0.057$), although no significant differences were observed (Table 2). Variation in the seminiferous epithelium height was seasonal ($H = 52.84$, $n = 240$, $P < 0.0001$; Table 2) and a post-hoc test (comparisons

TABLE 1. Macroscopic measurements of snakes deposited in collections

Season	N	SVL (mm)	Testes volume	Ductus deferens width (mm)	Kidney lenght (mm)	Kidney width (mm)
Summer [(A)]	40	355.70 ± 32.2	117.63 ± 56.26	1.84 ± 0.45	45.50 ± 10.25	4.14 ± 0.65[C]
Autumn [(B)]	38	362.39 ± 31.5	105.38 ± 45.33	1.96 ± 0.44[C, D]	49.86 ± 9.59[C, D]	4.38 ± 0.71[C, D]
Winter [(C)]	30	340.26 ± 41.1	85.16 ± 38.19	1.68 ± 0.46[B]	41.36 ± 7.59[B]	3.63 ± 0.68[A, B]
Spring [(D)]	31	347.78 ± 31.7	110.11 ± 62.94	1.67 ± 0.51[B]	41.47 ± 8.53[B]	3.82 ± 0.54[B]

Post hoc analysis: significant differences ($P < 0.05$) between seasons are indicated by letters. Date are expressed as mean ± standard errors. N = specimens examined; SVL = snout-vent length. Each letter represents a season. For example, in table 1 autumn is represented by letter B and no differences in testes volume among seasons were found, but the width of the ductus deferens, length and width of the kidney are different (larger) in autumn (B) from winter (C) and spring (D).

TABLE 2. Specimens examined and structural variation of the testes of S. mikanii between different seasons

Season	N	Seminiferous tubule diameter (μm)	Seminiferous epithelial height (μm)	Leydig cell nuclear diameter (μm)
Summer [(A)]	4	292.73 ± 67.0	94.41 ± 16.3[B, C]	4.99 ± 1.54[B, C]
Autumn [(B)]	7	300.20 ±71.1	76.84 ± 19.5[A, D]	5.96 ± 1.22[A, D]
Winter [(C)]	5	315.90 ± 78.6	83.22 ± 28.9[A, D]	5.70 ± 1.08[A]
Spring [(D)]	8	323.57 ± 51.5	113.79 ± 39.4[B, C]	5.22 ± 0.99[B]

Post hoc analysis: significant differences ($P < 0.05$) between seasons are indicated by letters. Date are expressed as mean ± standard errors. N = specimens examined. Each letter represents a season. For example, in table 1 autumn is represented by letter B and no differences in testes volume among seasons were found, but the width of the ductus deferens, length and width of the kidney are different (larger) in autumn (B) from winter (C) and spring (D).

by Dunn method) showed that it was larger in spring and summer compared to autumn and winter ($P < 0.05$). Leydig cell nuclear diameter differed between seasons ($H = 24.11$, $n = 240$, $P < 0.0001$) and a post-hoc test (comparisons by Dunn method) showed that it was larger in autumn than in summer and in spring ($P < 0.05$) and also larger in winter than in summer ($P < 0.05$; Table 2).

Different developmental stages were characterized by cellular changes in the testes of *Sibynomorphus mikanii* (Table 3). Complete regression of the testes (Stage I, characterized by the presence of spermatogonia and Sertoli cells only) was not observed. Early recrudescence (Stage II) occurred in early winter (June). At that time, the epithelium was composed of five to six cell layers with a few spermatozoa in the lumen, remaining from a previous spermatogenic cycle. Cellular division began at this stage when many primary spermatocytes were observed (Fig. 2A). Late recrudescence (Stage III) occurred during late winter and spring (September to November) with 10 to 12 cell layers in the seminiferous epithelium and intense activity of cellular division (meiosis). The seminiferous epithelium was composed of many primary spermatocytes and some spermatids at an early developmental stage (Fig. 2B). By the latter part of spring, early spermiogenesis (Stage IV) began and the epithelium consisted of 10 to 12 cell layers with spermatids being the most prevalent cell type (Fig. 2C). In the summer, with spermiogenesis (Stage V) at its peak, the epithelium was composed of 8 to 10 cell layers with spermatids and spermatozoa being the dominant cell type (Fig. 2D). Early regression (Stage VI) began in autumn (March) and continued until early winter. At this stage, a reduced germinal epithelium with few remaining spermatids and spermatozoa in the lumen was observed. This last stage resembled Stage II (Fig. 2A).

TABLE 3. Stages of the spermatogenic cycle in Sibynomorphus mikanii

Stages	Spermatogenic condition	Months of occurrence
I	Complete regression: not observed	/
II	Early recrudescence: division of spermatogonia and primary spermatocytes	June
III	Late recrudescence: primary spermatocytes and spermatids	September–November
IV	Early spermiogenesis: spermatids in metamorphosis	December
V	Spermiogenesis: mature spermatoza in the lumen	December–February
VI	Early regression: decrease of the seminiferous epithelium (five to six layers)	March–June

Adapted from Goldberg and Parker (1975), Herpetologica, Allen Press Publishing Services.

The distal ductus deferens of *S. mikanii* exhibits a pseudostratified columnar epithelium consisting of a mixture of medium height principal cells and low basal cells (Fig. 3A,B). The epithelium rests on a thick muscular wall and it is surrounded by an external serosa (Fig. 3A,B). Sperm was present throughout the year in the distal ductus deferens and spermatozoa density did not differ among the seasons in this portion of the organ. Histochemical analysis of the distal ductus deferens presented positive reactions to PAS and BB in the apical cytoplasm of epithelial cells throughout the year (Fig. 3C,D). The BB reaction was less intense than that for the basement membrane of the distal ductus deferens (Fig. 3D). Secretory globular material associated with sperm mass was BB and PAS positive (Fig. 3C,D). The distal portion of the ductus deferens presented negative reactions to AB.

The ampulla ductus deferentis exhibited the same layers as the rest of the distal ductus deferens, but with a taller epithelium with irregular projections and formation of crypts (Fig. 4A). The nuclei of the spermatozoa seemed to be oriented toward these crypts (Fig. 4A). Spermatozoa density is lower in winter than in other seasons in the ampulla ductus deferentis (Fig. 4B). Secretory granules in the cytoplasm were positive to PAS and BB only during the winter in the ampulla (Fig. 4C,D). Positivity to PAS was also observed in the apical cytoplasm of epithelial cells in the ampulla throughout the year (Fig. 4C). The ampulla ductus deferentis presented negative reactions to AB.

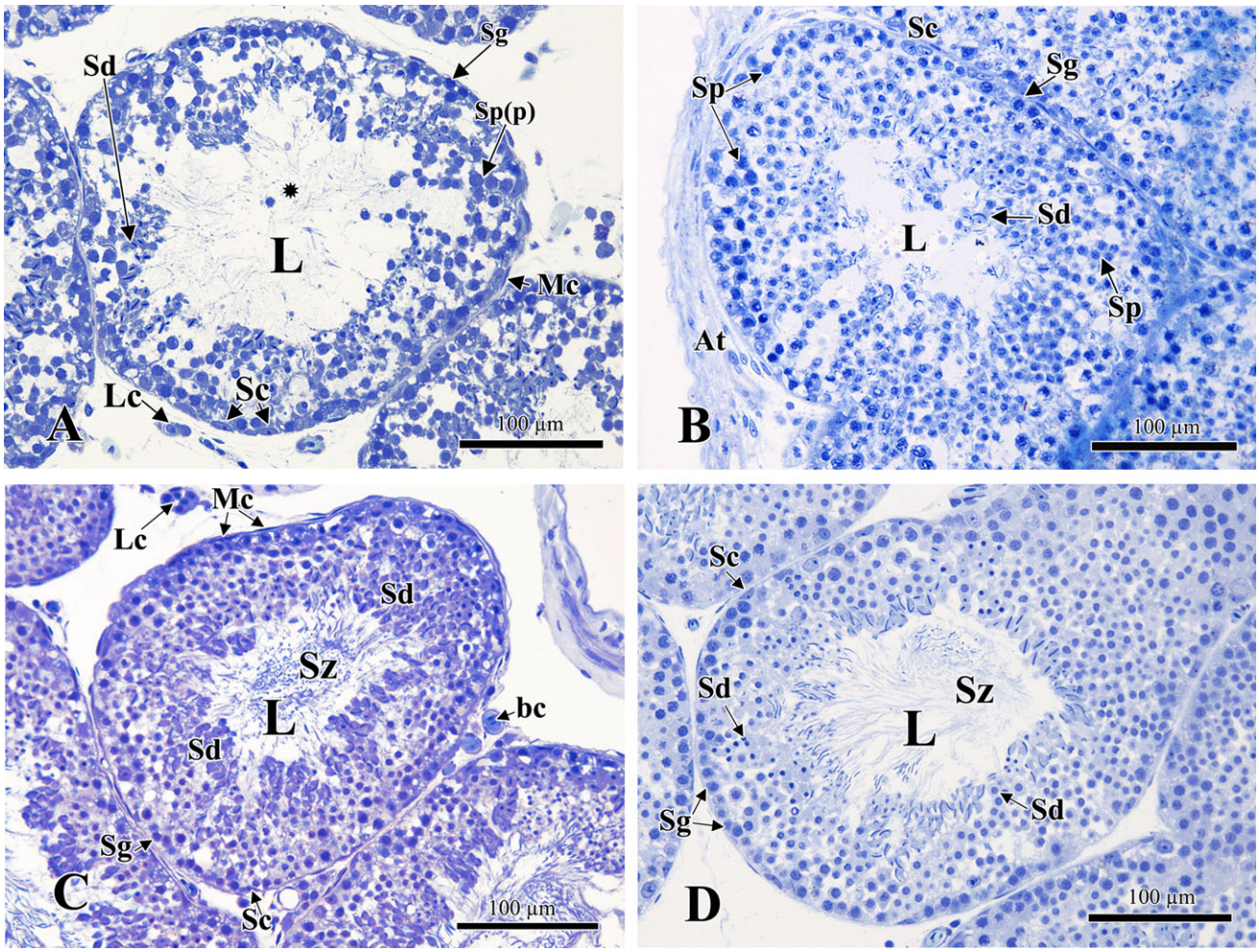

Fig. 2. Transverse sections of the testes of *Sibynomorphus mikanii* during the reproductive cycle. (**A**) A specimen in winter (June) showing different stages of cellular division in the seminiferous epithelium; (**B**) Light micrograph of an individual in spring (November) with the peak of cellular division and an increase in the seminiferous epithelium; (**C**) summer (December), lots of spermatids in metamorphosis; (**D**) late summer and early autumn (March), final phase of the spermiogenesis process. At: tunica albuginea; bc: blood capillaries; Lc: Leydig cells; Mc: myoid cells; Sc: Sertoli cells; L: lumen; Sd: spermatid; Sg: spermatogonia; Sp: primary spermatocyte; Sp (p): primary spermatocyte at pachytene stage; Sz: spermatozoa; (*) remaining spermatozoa. Toluidine blue-fuchsin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

SSK Secretory Cycle

Macroscopic measurements of the kidneys of *S. mikanii* presented significant differences between the seasons, with an increase in the length ($F = 3.56$, $n = 139$, $P < 0.01$) and width ($F = 5.14$, $n = 139$, $P < 0.002$). A post-hoc test (Tukey test) showed that kidney length was larger in autumn than in winter and in spring ($P < 0.01$; Table 1). Considering kidney width, a post-hoc test (Tukey test) showed that it was larger in autumn than in winter ($P < 0.0001$) and in spring ($P < 0.001$) and also larger in summer than in winter ($P < 0.009$; Table 1). The tubular diameter of the SSK differs among the seasons ($H = 63.2$, $n = 240$, $P < 0.0001$) and a post-hoc test (comparisons by Dunn method) showed that it was larger in summer than in autumn, winter, and spring ($P < 0.05$) and also larger in autumn than in winter and spring ($P < 0.05$; Fig. 5A). The epithelial height of the SSK exhibited a seasonal pattern ($H = 38.79$, $n = 240$, $P < 0.0001$; Fig. 5B) with a significant increase during summer and autumn in relation to winter and spring (comparisons by Dunn method, $P < 0.05$).

Histological and histochemical analysis showed that winter was the period of lowest SSK activity with a few weakly stained granules, concentrated in the basal region of the cells (Fig. 6A,B). The lumen was narrow and exhibited vacuoles on the medial region of the tubule which suggests a postsecretory pattern (Fig. 6B). Considering that the detection of proteins (BB) reacted positively, but weakly, this indicates that the secretory activity of granules during this season was low (Fig. 6C; Table 4, scale 1; Table 5, Stage I). However, SSK reacted positively to

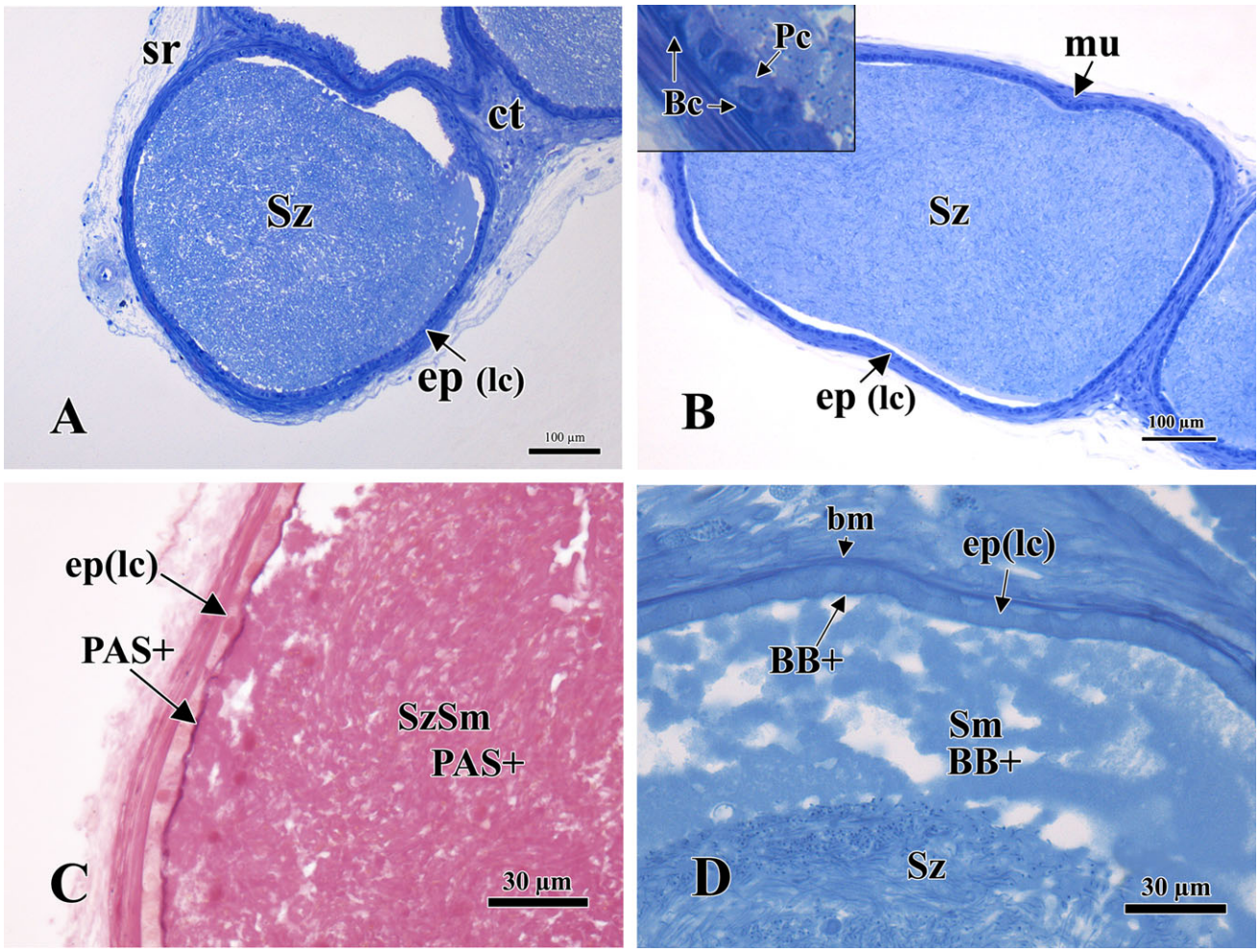

Fig. 3. Transverse sections of the distal ductus deferens in *Sibynomorphus mikanii* during seasons of the year. (**A**) Specimen in spring with lots of spermatozoa in the lumen. Toluidine blue-fuchsin. (**B**) A male collected in early summer (December) presents the same pattern observed in spring exhibiting an epithelium composed of principal cells and basal cells. Toluidine blue-fuchsin. (**C**) Light micrograph with positive reaction to PAS in the apical portion of the cells and sperm mass (**D**) Positive reaction to BB in the apical region of epithelial cells and secretory material. BB+: positive reaction to bromophenol blue; Bc: basal cells; bm: basement membrane; ct: connective tissue; ep (lc): pseudostratified epithelium with low cells; mu: muscular layer; PAS+: positive reaction to periodic- acid-Schiff; Pc: principal cells; Sm: secretory material; sr: serosa; Sz: spermatozoa; SzSm: sperm in secretory material.

PAS, thus it contains neutral carbohydrates (Fig. 6D; Table 4, scales 1 and 2; Table 5, Stage II).

During spring, the SSK was characterized by the presence of several moderately stained granules spread throughout the cytoplasm of the cells, including the apical region (Fig. 7A). The lumen of the tubule became wider and basal nuclei were present (Fig. 7B). The SSK was lightly positive to BB and the proximal convoluted tubule was totally negative (Fig. 7C; Table 4, scale 2; Table 5, Stage I). PAS reacted positively during this season as it was observed in the winter (Fig. 7D; Table 4, scales 2 and 3; Table 5, Stage II).

During summer, SSK exhibited a high density of granules all over the cytoplasm but mainly in the apical region of the cell (Fig. 8A,B). Little protrusions in the apical region of the cells indicate a sexual presecretory phase when the granules would be ready to be released into the lumen (Fig. 8B). The granules also stained and reacted more intensively to BB and PAS when compared with the reactions in winter and spring (Fig. 8C,D; Table 4, scales 3 and 4; Table 5, Stage III). A positive reaction to AB was observed in the collecting ducts in all seasons (Fig. 8D).

At the beginning of autumn, SSK cytoplasm was characterized by lots of intensively stained granules and a distended lumen (Fig. 9A). Granules were also observed in the lumen of the tubule (Fig. 9B). During the autumn, granules reacted strongly to BB and PAS as they did in the summer, suggesting an increase in the production of secretory granules (Fig. 9C,D; Table 4, scale 4; Table 5, Stage III). Secretory granules of the SSK exhibited negative reactions to AB in all seasons.

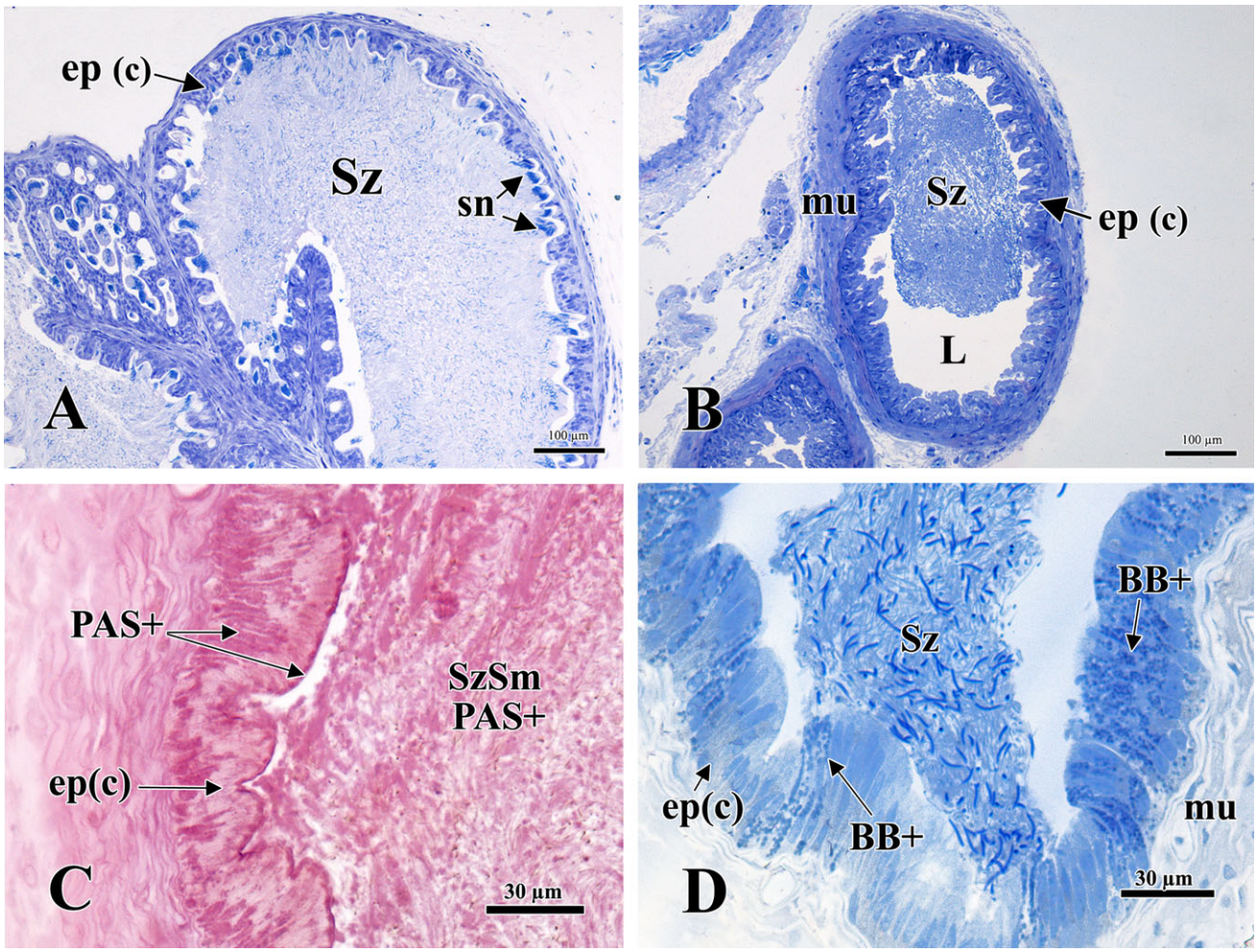

Fig. 4. Cross-sections of the ampulla ductus deferentis (**A**) Ampulla observed in a male collected during the mating season in autumn (April) with clusters of sperm nuclei intimately associated with the apical ampullar epithelium. Toluidine blue-fuchsin. (**B**) Ampulla of a male collected in winter (July) with a decrease of spermatozoa in the lumen, indicating a postmating stage. Toluidine blue-fuchsin. (**C**) Positivity to PAS in the ampulla with production of secretory granules during winter (**D**) Light micrograph of ampulla with secretory granules in the cytoplasm which were positive to BB during the winter. BB+: positive reaction to bromophenol blue; ep (c): pseudostratified epithelium with formation of crypts; L: lumen; mu: muscular layer; PAS+: positive reaction to periodic- acid-Schiff; sn: spermatozoa nuclei; Sz: spermatozoa; SzSm: sperm in secretory material.

DISCUSSION

Histological analysis revealed that spermatogenesis is not continuous throughout the year and that the epithelium of the distal ductus deferens and the ampulla of the ductus deferentis possibly present adaptations to sperm storage in *Sibynomorphus mikanii*. The use of seasonal differences in relative testes length and ductus deferens diameter to infer timing of spermatogenesis and presence/absence of sperm storage (Pizzatto et al., 2008b) failed to determine the timing of these reproductive events in *S. mikanii*. Thus, conclusions about reproductive patterns of squamate reptiles based on these methods (Pizzatto and Marques, 2002; Scartozzoni et al., 2009; Marques et al., 2009; Orofino et al., 2010; Pinto et al., 2010) should be confirmed by histological analysis. Although in some snake species the testes may be larger during spermatogenesis (Volsøe, 1944; Fox, 1952; Gribbins and Rheubert, 2011), this rule does not apply for every species. For example, testes of the pitviper *Crotalus scutulatus* are larger during the regression phase (Schuett et al., 2002).

In *S. mikanii*, a peak in the GSI during spring coincided with an increase in the seminiferous epithelium height, characterizing the spermatogenic process in this season. Thus, testes mass may be considered a reliable indicator of spermatogenesis in *S. mikanii*. Testes volume also increased during spermatogenesis although it was not statistically significant. However, only the histological analysis was able to reveal that the spermiogenesis (maturation process) occurs in summer when a higher density of spermatids and mature spermatozoa were observed. The autumn is the period of quies-

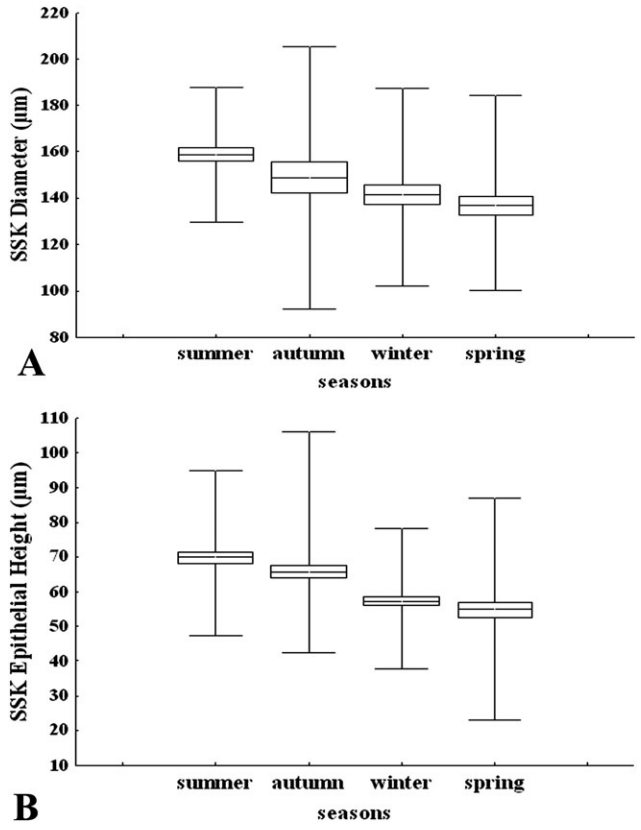

Fig. 5. Relationship between the seasons of the year and the average of (**A**) tubular diameter and (**B**) epithelial height of the SSK in *Sibynomorphus mikanii*. Middle line represents average values, boxes show standard errors and whiskers represent minimum and maximum values.

cence and reduction of the testicular activity, characterizing an annual spermatogenic cycle.

The beginning of spermatogenesis occurs during late winter in *S. mikanii.* This reproductive pattern is similar to that presented by *Leptodeira maculata* and *Leptodeira punctata* which are Dipsadinae snakes from New Mexico that present long spermatogenic cycles with production of spermatozoa during spring, summer, and autumn (Goldberg, 2004). Thereby, spermatogenesis may begin earlier in snakes from meridional regions than it does in snakes from northerly localities with colder temperatures (Fox, 1954).

Recently, Mathies (2011) summarized data on reproductive cycles of tropical snakes and proposed a new classification for snake reproductive cycles. Considering the individual level, *S. mikanii* males have a continuous cyclical reproductive pattern with reduction of gonadal activity during autumn and early winter. At the populational level, the reproductive cycle is seasonal semisynchronous with a recognizable peak of reproductive activity in summer–autumn (Fig. 10). Total regression of the testes was not observed in *S. mikanii*. This testicular stage is usually observed in snakes from temperate areas (Gartska et al., 1982). However, total regression in the testes was also described for the neotropical rattlesnake (*Crotalus durissus*; Salomão and Almeida-Santos, 2002; Barros et al., 2012).

The sleep snake shows an amniote pattern of germ cell development with the retention of anamniote characteristics. The germ cells proceed through the phases of spermatogenesis as a single population which leads to a single spermiation at the end of this process. This pattern has already been described for some squamate species from temperate regions, such as *Podarcis muralis* (Gribbins and Gist, 2003) and *Seminatrix pygaea* (Gribbins et al., 2005).

After gametes are produced in the gonads, they pass through genital accessory ducts and are finally deposited in the ductus deferens. The ductus deferens is the known site for sperm storage in reptiles and the epididymis does not play a role in the maturation and storage of spermatozoa as observed in mammals (Jones, 1998; Sever, 2004). Aldridge and Duvall (2002) studied viperid snakes from temperate regions and determined that mating is independent of spermatogenesis and that the males actually adapt to the female reproductive cycle. It is also true for *S. mikanii*, a neotropical species: spermatozoa is found in the ductus deferens throughout the year, which indicates a potential for copulation at any season of the year (Almeida-Santos and Salomão, 2002; Almeida-Santos et al., 2004).

In the sleep snake, the width of the ductus deferens showed a peak in the autumn which might suggest a spermiation process with posterior sperm storage. We also observed that the ampulla ductus deferentis is not macroscopically different from other portions of the ductus deferens and is microscopically similar to that observed in *Seminatrix pygae*a (Sever, 2004).

Histochemical analysis of the distal ductus deferens and ampulla ductus deferentis of the sleep snake showed some similarities with the secretory pattern of a few snake species that have already been studied (Sever, 2004; Siegel et al., 2009). Most of the cellular cytoplasm of the distal ductus deferens of *S. mikanii* is apparently nonreactive to histochemical tests as observed for *Seminatrix pygaea* (Sever, 2004). However, in all seasons, PAS- and BB-positive material is located in the apical cytoplasm of the ductus deferens, similarly to that found in *Agkistrodon piscivorus* (Siegel et al., 2009).

The ampulla ductus deferentis of the sleep snake presents projections of the epithelium and crypts with clusters of sperm nuclei intimately associated with the apical ampullar epithelium. The presence of cytoplasmic granules rich in protein and neutral carbohydrates suggests that this secretion assists in the nutrition of spermatozoa during storage. However, it is not possible to

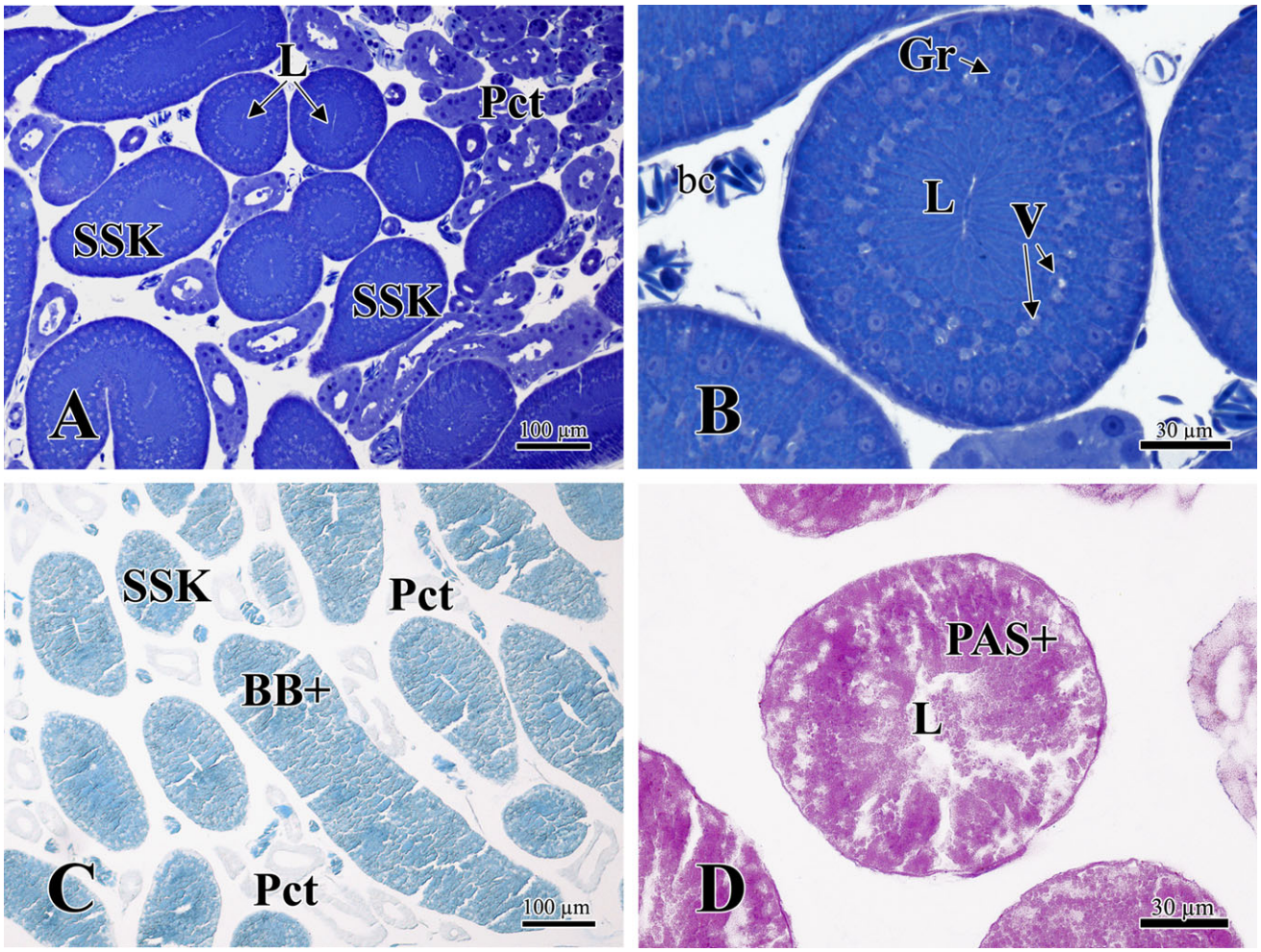

Fig. 6. Transverse sections of the kidney of *Sibynomorphus mikanii* during the postsecretory phase in winter. (**A** and **B**) June, narrow lumen and presence of vacuoles, characteristic of this phase. Fuchsin-toluidine blue. (**C**) June, light positive reaction to BB. (**D**) July, PAS positive reaction with granules rich in neutral carbohydrates. BB+: Positive for bromophenol blue; bc: capillary blood vessels; Gr: secretory granules; L: lumen; PAS+: Positivity to the periodic acid-Schiff; Pct: proximal convoluted tubule; SSK: sexual segment of the Kidney; V: cytoplasmic vacuoles.

understand how spermatozoa are attracted by the ampulla epithelium and what are its mechanisms of secretion because the techniques used in this study (light microscopy) are not ideal to deal with these questions. An ultrastructural study of the different regions of the ductus deferens of *S. mikanii* may reveal the existence of phagocytosis and the contribution of seminal plasma, as has been observed in the lizard *Sitana ponticeriana* (Akbarsha et al., 2005). The function and morphology of the ampulla differ along the squamate lineage (Trauth and Sever, 2011). To our knowledge, this is the first record of the occurrence of the ampulla ductus deferentis in a neotropical snake species. Thus, more research on this topic is necessary for a better understanding of the male snake's mechanisms of sperm storage. On the other hand, sperm storage in the female reproductive tract probably occurs during winter in *S. mikanii* because the mating season (autumn) is not synchronous to ovulation/fertilization (late winter).

TABLE 4. Density of sexual granules in the SSK epithelium of Sibynomorphus mikanii

Scale	Visible differences categorized	Months
0	No hypertrophy	Not observed
1	Hypertrophy with a few granules	June–September
2	Hypertrophy with granules evident throughout the cytoplasm	September–December
3	Secretory granules visible in the apical region of the cytoplasm	September–March
4	Maximum density of secretory granules within the cytoplasm	March–June

Adapted from Krohmer et al. (2004), Comp Biochem Physiol

Seasonal changes in the SSK of *Sibynomorphus mikanii* presented a similar pattern to that

TABLE 5. Seasonal variation in SSK granule staining intensity in Sibynomorphus mikanii

Stages	Staining intensity of granules	Months	
		Bromophenol blue (BB)	Periodic acid-Schiff (PAS)
I	some lightly stained granules	June-September	not observed
II	many moderately stained granules	September- December	June-December
III	many intensely stained granules	December- June	December-June

Adapted from Aldridge and Brown (1995), J Herpetol.

observed in the testes: continuous cyclical and seasonal semisynchronous (Fig. 10). However, these processes occur in different seasons. The period of minor activity of the germinative epithelium of the testes (autumn) occurs simultaneously to the period of major activity of the SSK epithelium. In *S. mikanii*, the increase of the tubular diameter and epithelium height of the SSK during summer–autumn occurred due to the maximum density and staining intensity of the granules produced in those seasons. The secretory granules produced by the SSK are of glycoprotein and mucoprotein nature. Histochemical analysis showed a light reaction to BB and a moderate reaction for PAS during winter. However, during summer and autumn, SSK exhibited a strong reaction for proteins (BB+) and neutral carbohydrates (PAS+), indicating an intense secretory ac-

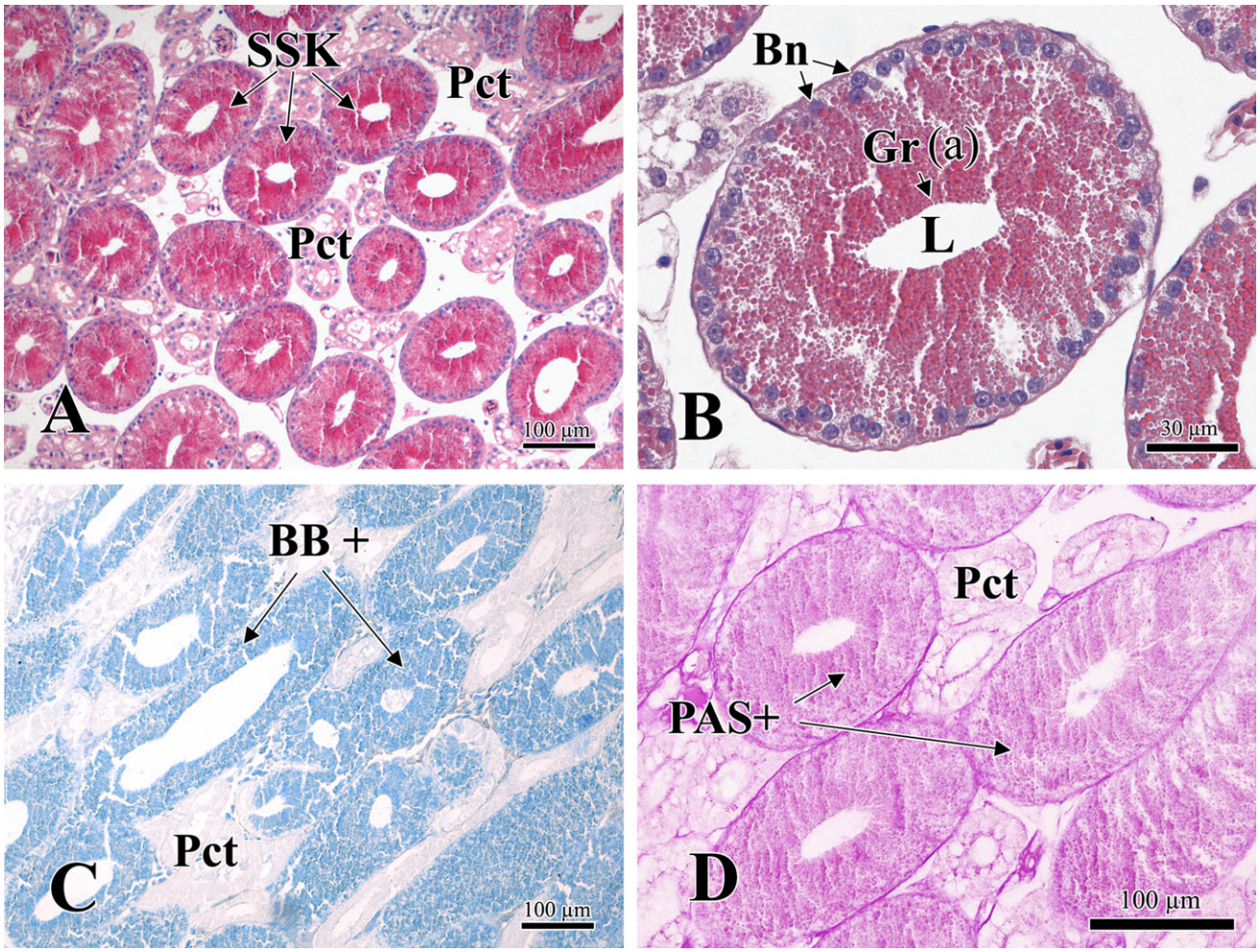

Fig. 7. Histology of the kidney of *Sibynomorphus mikanii* during the beginning of the presecretory phase in spring. (**A** and **B**) kidney of a male from November showing an intense staining in the cytoplasm and the presence of basal nuclei (H/E). (**C**) Specimen of September with SSK positive reaction to BB. (**D**) Section of October with SSK positive reaction to PAS. Bn: basal nuclei; BB+: positive reaction to bromophenol blue; Gr(a): secretory granules at the apical region; L: lumen; PAS+: positive reaction to periodic acid-Schiff; Pct: proximal convoluted tubule, SSK: sexual segment of the kidney.

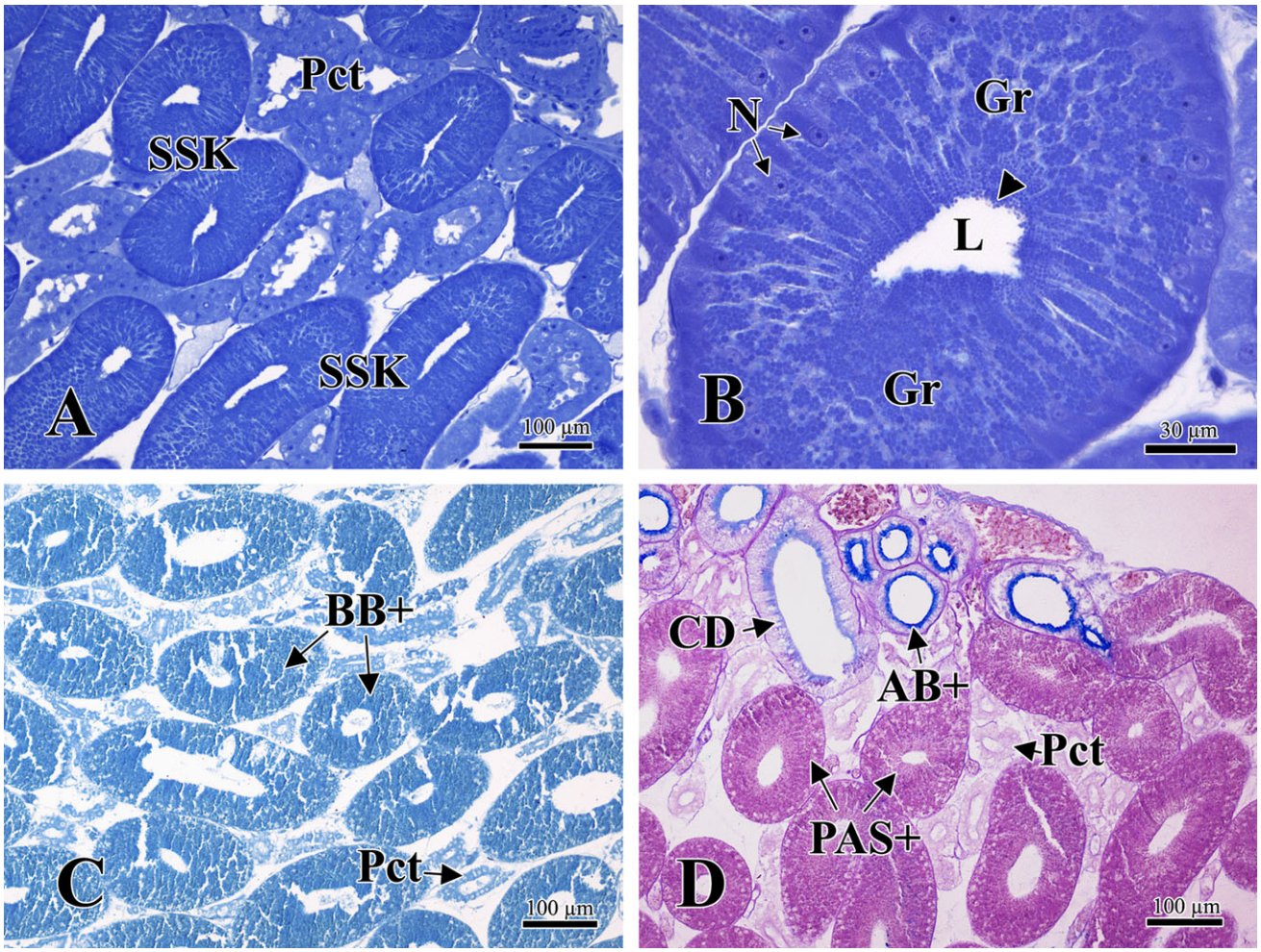

Fig. 8. Transverse sections of the kidney of *Sibynomorphus mikanii* during the presecretory phase in summer. (**A,B**) February, protrusions in the tubular luminal border (arrows) and granules in the apical region of the cells. Toluidine blue-fuchsin. (**C**) February, very strong reaction to BB, indicating a higher activity in the secretory granules. (**D**) Positive reactions of the SSK to PAS and of the collecting ducts to AB (pH 2, 5). BB+: positive reaction to bromophenol blue; AB+: positive reaction to alcian blue; PAS+: positive reaction to periodic acid-Schiff; CD: collecting ducts; Gr: secretory granules; L: lumen; N: nuclei, SSK: sexual segment of the kidney; Pct: proximal convoluted tubule. Arrowhead indicates apical protrusions.

tivity of the granules. This increase of secretory granules production coincides with a macroscopic hypertrophy of the kidneys in summer–autumn. In most species studied to date, with some exceptions, macroscopic variations of the kidneys were not observed among the different phases (active phase or mating season versus the quiescent phase; Fox, 1977; Sever et al., 2007).

During summer, the protrusion at the apical region of the SSK indicates a presecretory phase when the granules should be ready to be released in the lumen, as described for *Natrix natrix* (Kühnel and Krisch, 1974). This kind of protrusion was observed in the lumen of an individual collected in summer but the presence of granules in the lumen of the tubule was observed only during the autumn, indicating that the mating season really does occur during autumn. The increase in the Leydig cells nuclear diameter in autumn also suggests that mating occurs in this season. According to Banks (1992), Leydig cells are responsible for the main endocrine function of the testes: to synthesize and secrete the main circulating androgen known as testosterone. These cells are situated in the interstitium of the seminiferous tubules and changes of the nuclear diameter are associated to the season of major endocrine activity (testosterone production; Volsøe, 1944). After mating in autumn, the SSK of the sleep snake regresses and it is characterized by the presence of cytoplasmic vacuoles which indicate a postsecretory phase. This kind of change was described by Volsøe (1944) and it indicates a process of regression.

In the sleep snake, sperm was observed in the vagina of females during autumn. It is another evidence of mating during this season (Rojas, 2009). Thus, the copulation in *S. mikanii* is independent of spermatogenesis, characterizing the

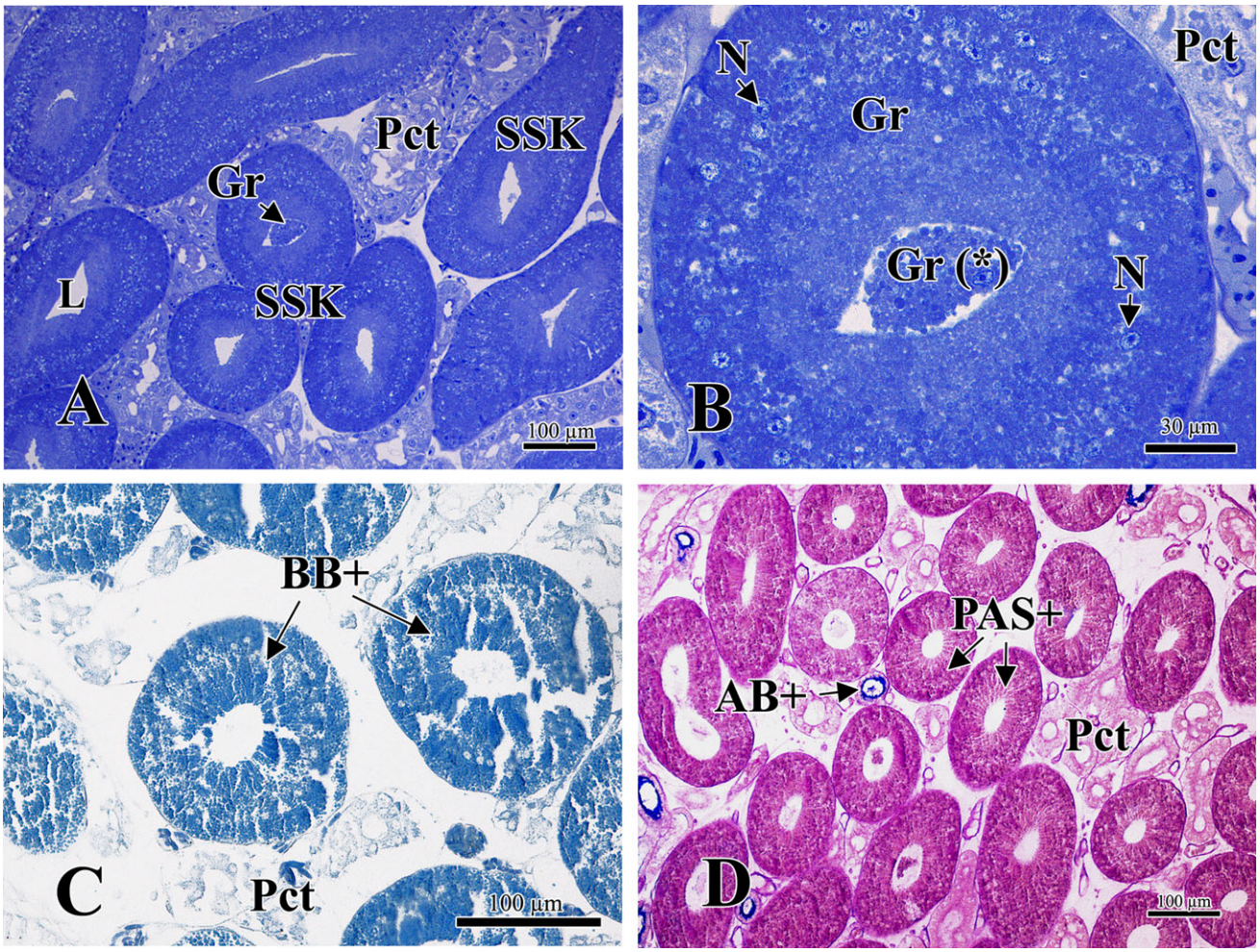

Fig. 9. Light micrographs of transverse sections of the kidney of *Sibynomorphus mikanii* during the secretory phase in autumn. (**A,B**) specimen in April with secretory granules being released in the lumen. Toluidine blue-fuchsin. (**C**) March, strong positive reaction to BB. (**D**) April, strong reaction to PAS. AB+: positive reaction to alcian blue; BB+: positive reaction to bromophenol blue; Gr(*): secretory granules in the lumen; L: lumen; N: nuclei; PAS+: positive reaction to periodic acid-Schiff; SSK: sexual segment of the kidney; Pct: proximal convoluted tubule.

reproductive cycle as dissociated (cf. Crews, 1984). However, two exceptions need to be highlighted: the increase of Leydig cell nuclear diameter (indicative of sex hormone secretion), and the SSK hypertrophy is coincident with the mating season in autumn. These are characteristics of an associated reproductive cycle which reinforces the idea that reproductive strategies are complex in snake species (cf. Crews, 1984; Shine, 2003). In summary, the endocrine activity of the testes (secretion of sex steroid hormone by Leydig cells) is associated with the mating season, whereas the exocrine activity (sperm production) is dissociated in *S. mikanii*.

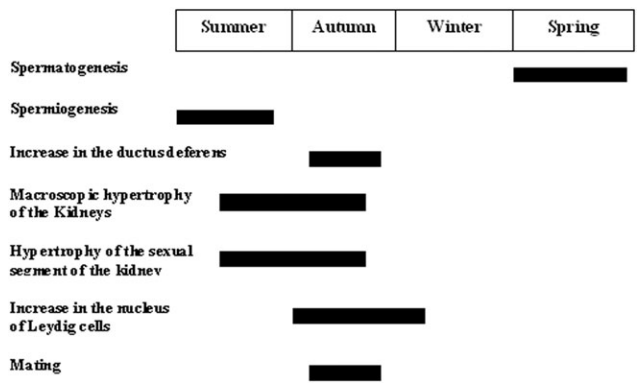

Fig. 10. Phases of the annual reproductive cycle of male *Sibynomorphus mikanii*.

Data presented herein described, for the first time, the SSK secretory cycle and its relationship with the testicular cycle of a snake from South America. Nevertheless, this study is not exhaustive: studies using other microscopic techniques such as scanning and transmission electron microscopy may reveal important details of *S. mikanii* reproductive strategies. We hope that this study may open new perspectives and perhaps

stimulate research on squamate reptiles in the neotropical region, which is still poorly known.

ACKNOWLEDGMENTS

The authors thank Carlos Jared and Marta Antoniazzi for kindly allowing use of material and equipments in the Laboratory of Cell Biology (IB), Simone Jared and Leonardo de Oliveira for their technical assistance. Francisco L. Franco (IB) and Ronaldo Fernandes (MNRJ) allowed access to collections. This study is based on a MSc thesis undertaken by Claudio A. Rojas.

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