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ANIMAL SCIENCE

Reproductive Biology, Sperm storage, and Sexual Maturity of *Thamnodynastes strigatus* (Serpentes: Dipsadidae)

LUIZA LOEBENS, TIAGO F. THEIS, SELMA M. ALMEIDA-SANTOS & SONIA Z. CECHIN

Abstract: Life history strategies determine and influence many aspects of species fitness. In this study, we describe the reproductive biology - reproductive cycle, sperm storage, and sexual maturity - of *Thamnodynastes strigatus* in South Brazil. We analyzed 49 individuals (25 males and 24 females) from herpetological collections. The reproductive cycle of males and females was described considering the morpho-anatomical and histological changes in the testes, ductus deferens, and kidney, as well in the ovary and oviduct. The age at the onset of sexual maturity was determined by skeletochronology of the caudal vertebra. The reproductive cycle is seasonal semi-synchronous and most individuals have a reproductive peak in spring and summer. The seasonal biennial reproductive cycle and viviparity are two phylogenetically conserved characters in Tachymenini snakes. *Thamnodynastes Strigatus* females store sperm in the utero-vaginal junction furrows during autumn. There were no differences between the ages of sexual maturity of males (4-11y) and females (4-12y). Females reach sexual maturity at larger body sizes, and this may confer an adaptive advantage due to a higher fecundity potential. Herein, we confirmed the previously described seasonal biennial reproductive cycle of *T. strigatus* through histological analysis.

Key words: reproduction, reproductive cycle, skeletochronology, spermatogenesis, vitellogenesis.

INTRODUCTION

Life history strategies determine the different ways in which organisms obtain and expend resources and influence many aspects of species fitness (Bonnet et al. 1998). The life history of snake species is highly variable but can be classified primarily into three categories: (1) oviparous and single-brooded species (2) viviparous species that breed annually and (3) viviparous species that reproduce biennially (Dunham et al. 1988, Shine 2003). The reproductive mode of snakes determines their life history reproductive traits (Bellini et al. 2018). The frequency of the biennial reproduction of some viviparous snakes is a consequence of the elevated costs of embryo retention (Edwards et al. 2002). These costs result from the high maternal investment in providing optimal conditions to embryogenesis (Shine 2004). Moreover, reproduction may reduce the survival and future fecundity of females (Shine 1980).

Although most snakes are oviparous (Sites et al. 2011), viviparity is a common mode of reproduction in snakes and evolved multiple times in different lineages (Blackburn 2015). There are two main hypotheses to explain the evolution of viviparity: the cold climate hypothesis, which states that viviparity is selected for in cold climates (Shine 1983), and the climatic predictability hypothesis that assumes that viviparity is advantageous

in seasonal climates (Shine 2014). The cold climate hypothesis is well supported therefore, the geographic distribution of viviparous snakes in warm climates is probably a result of phylogenetic conservatism (Feldman et al. 2015, Bellini et al. 2017).

The reproductive cycle of tropical snake populations is classified as seasonal or non-seasonal (Mathies 2011), and most species reproduce seasonally (Brown & Shine 2006). In seasonal reproduction, the gametogenesis of males and females may or not be synchronized (Saint Girons 1982). Therefor, females of many species of neotropical snakes can store sperm in the utero-vaginal junction (Almeida-Santos & Salomão 1997, 2002, Barros et al. 2012, Rojas 2013, Barros et al. 2014a, b, Rojas et al. 2015, Loebens et al. 2018, 2020). Sperm-storage structures in females evolved independently in different lineages of Squamata, increasing their reproductive success (Sever & Hamlett 2002).

Tachymenini (Dipsadidae) snakes are mainly distributed in South America, and most species are viviparous, since they probably originated in colder regions and dispersed into warmer areas (Zaher et al. 2009). The phylogenetic conservatism may also explain the absence of variability in the reproductive cycles of this tribe (Barros et al. 2012) since most of the studied populations have a seasonal biennial reproductive cycle (Bizerra 1998, Oliveira et al. 2003, Scartozzoni & Marques 2004, Bizerra et al. 2005, Rojas 2013, Bellini et al. 2014, Rebelato et al. 2016, Loebens et al. 2020). Currently, the reproductive biology of South American Xenodontine snakes is mainly explained by phylogenetic aspects rather than by ecology (Bellini et al. 2017).

Thamnodynastes strigatus (Günther 1858) belongs to the Neotropical monophyletic tribe Tachymenini Bailey 1967 (Zaher et al. 2009) and occurs in South and Southeast Brazil, Argentina, Uruguay and Paraguay (Cei 1993). It is a semi-arboreal nocturnal species that inhabits both terrestrial and semiaquatic environments (Bernarde et al. 2000). The reproductive cycle of *T. strigatus* was described based on the morphological analysis of individuals from eastern Argentina, Southern Paraguay, Southeastern Brazil, and Uruguay (Bellini et al. 2014). The species is a viviparous snake with a seasonal biennial reproductive cycle and female-biased sexual size dimorphism (Bellini et al. 2014, Loebens et al. 2019). We evaluated individuals from two herpetological collections to describe the reproductive biology of *T. strigatus* in South Brazil through morpho-anatomical and histological analyses. These types of analyses are a simple alternative method to describe many fundamental reproductive traits (e.g . reproductive cycle and sexual maturity) (Shine et al. 2014). Aims of this study was to test the following hypotheses: (i) *T. strigatus* have a seasonal pattern of reproduction in the subtropical region of Brazil influenced by climate seasonality and phylogenetic conservatism (ii) females store sperm in the oviduct during the colder season; and (iii) females reach sexual maturity later than males and at larger body sizes because of fecundity selection.

MATERIALS AND METHODS

Data collection

We analyzed 49 individuals of *T. strigatus* (25 males and 24 females) available in the herpetological collections of the Santa Maria Federal University (ZUFSM) and Pontifical Catholic University of Rio Grande do Sul (MCP-PUCRS) (Appendix). The exanimated individuals have geographical distribution throughout South Brazil (Rio Grande do Sul, Paraná, and Santa Catarina States Supplementary Material - Figure S1) where the climate is humid subtropical (Alvares et al. 2013).

We obtained the following data from each individual: month of death, snout-vent length (SVL, digital caliper to the nearest 0.01) and body mass (precision scale to the nearest 0.01 g, after draining). To describe the reproductive cycle, we examined the morpho-anatomical and histological changes in the reproductive system of males (testes, ductus deferens, and kidney) and females (ovary and oviduct). As a standard procedure, only right-sided organs were examined. The reproductive stages were described according to austral seasons: summer (late December-late March), autumn (late Marchlate June), winter (late June-late September) and spring (late September-late December).

Reproductive biology

We obtained morpho-anatomical variables using a digital caliper to the nearest 0.01 mm, and a precision scale to the nearest 0.01 g. The following morpho-anatomical data were collected to describe the reproductive cycle of males: mass (after draining), length, width, and thickness of the testes, width of the distal portion of the ductus deferens, and mass (after draining), length and width of the proximal region of the kidney (Roja et al. 2013). We calculated the testicular volume (TV) by the ellipsoid formula: $\mathbf{V = (4/3)\pi abc}$, where a = half the length, b = half the width, and c = half the testes thickness (Pleguezuelos & Feriche 1999). The following morpho-anatomical data were evaluated to examine the reproductive cycle of females: the number of vitellogenic follicles and/or embryos, the diameter of the largest follicle, and the ovary mass (both, after draining). Enlarged and yellowish oocytes were considered vitellogenic follicles, which appear interspersed with primary follicles, which are smaller and whitish (Almeida-Santos et al. 2014). We calculated the Gonadosomatic Index (GSI) as an indirect measure of the reproductive effort for both sexes using the formula: gonad mass/body mass x 100 (Clesson et al. 2002). In males, we considered the gonad mass as the total mass of both testes. The Renalsomatic index (RSI) was calculated for males by the formula: kidney mass/body mass * 100 (Htun-Han 1978).

We obtained samples from 46 individuals (24 males and 22 females) to conduct the histological analysis. The following male structures were prepared for the histological examination: proximal region of the testes, distal region of the ductus deferens, and proximal region of the kidney (Rojas et al. 2013). The histological examination of females was based on samples of the anterior and posterior infundibulum, uterus, utero-vaginal junction (UVJ), and vagina (Rojas et al. 2015). The histological samples were processed for light microscopy using the historesin method and sectioned (2 µm thick) with a Leica RM2245 microtome. We examined the slides with a ZEISS Axio Scope. A1 microscope attached to an Axiocam MRc5 camera. As a standard procedure, we obtained 10 measurements of the histological variables from each individual.

Different regions of the oviduct (distal, medial, and proximal) were examined to identify the presence of spermatozoa and sperm storage glands/tubules. The histological seasonal variation was evaluated on the: infundibulum epithelial height, uterus epithelial height, UVJ epithelial height, vaginal epithelial height, and on the uterus glands (shell glands) diameter.

We searched for the presence of spermatozoa in the seminiferous tubule and ductus deferens of males. We also evaluated the histological seasonal variation in the seminiferous tubule diameter and epithelial height, Leydig cell nuclear diameter, sexual segment of the kidney (SSK) tubular diameter and epithelial height (Rojas et al. 2013), as well

as ductus deferens diameter and epithelial height. The spermatogenic cycle was described according to the classification proposed by Goldberg & Parker (1975). The cycle of the SSK was analyzed according to Krohmer et al. (2004). The SSK is a sexually dimorphic structure with secretory activity under the control of testosterone (Krohmer et al. 2004).

We classified males and females reproductive cycle of *T. strigatus* at the individual and population level according to Mathies (2011). The main criterion to establish the sexual maturity of males was the presence of spermatozoa in the testes or ductus deferens (Shine 1977a). The sexual maturity of females was determined by the presence of spermatozoa in the vagina or oviduct (post-copulating) and/or the presence of vitellogenic follicles in the ovary or embryos in the oviduct (Shine 1977b).

The age at which males and females reach sexual maturity was estimated by skeletochronology of the caudal vertebra. We obtained bone samples, by clipping the tails close to the distal end (near 2 cm), in a way that the exact vertebrae taken from each snake varied (Waye & Gregory 1998). The bone samples were decalcified in 10% EDTA for seven days, processed in historesin, transversely sectioned (5 µm thick) with a Leica RM 2245 rotary microtome, and stained with toluidine blue. We obtained four transversal sections of the middle region of each sample. The histological bone tissues were independently analyzed by two different researchers with a ZEISS Axio Scope. A1 microscope attached to an Axiocam MRc 5 to count the number of lines of arrested growth (LAG`s) used to estimate specimen age (Castanet 1994). Age of sexual maturity was estimated based on the younger mature male and female, and longevity was determined considering older male and female.

Statistical analysis

To investigate the seasonal (monthly) variation in morpho-anatomical and histological data of males and females we used circular statistical analyses performed in Oriana 4.0 software (Kovach Computing Services 2004). Months were converted to angles varying from 0º (January) to 330º (December). Each angle (month) was associated with the mean value of the measured parameter or to the total occurrence frequency of individuals within each month. Months with the highest mean value or frequency correspond to the reproductive peaks. For the analysis, we considered: (1) mean vector (µ), which corresponds to the mean period of the year in which there is a higher activity rate of a given parameter (2) circular standard deviation (SD) and (3) the r vector (r), which corresponds to the mean data concentration around the circle (year), ranging from 0 (dispersed data) to 1 (data concentrated in the same direction). A significant result of the Rayleigh test (Z) indicates that the data are not uniformly distributed indicating a significant seasonality in the reproductive parameter.

Size-fecundity relationships in females were investigated using a multiple linear regression using body mass as the predictor variable, against dependent variables: ovary mass, follicles diameter, and number of follicles. A linear regression was performed to investigate the relationship between female SVL and clutch size (number of embryos in the oviducts). The potential fecundity (number of vitellogenic follicles) and the observed fecundity (number of embryos in the oviducts) were compared using a *t* test. We compared the reproductive effort (GSI) of males and females by a *t* test.

We investigated the differences between the ages at sexual maturity of males and females by an *t* test. The relationship between age and body size was evaluated using linear regressions

between age vs. SVL and age vs. body mass to investigate the relationship between age and body size of males and females separately. Statistical analyses were performed using Statistica version 10. All variables were tested homogeneity of variance and normality in the model residuals.

RESULTS

Male reproductive cycle

Testes volume (TV) varied seasonally (R = 78.95 P < 0.001 N = 25) with highest values in November and the lowest values in the autumn (μ = 298.84° SD = 96.73° r = 0.24 Fig. 1a). Gonadosomatic Index (GSI) was also higher in the spring (R = 3.59 P

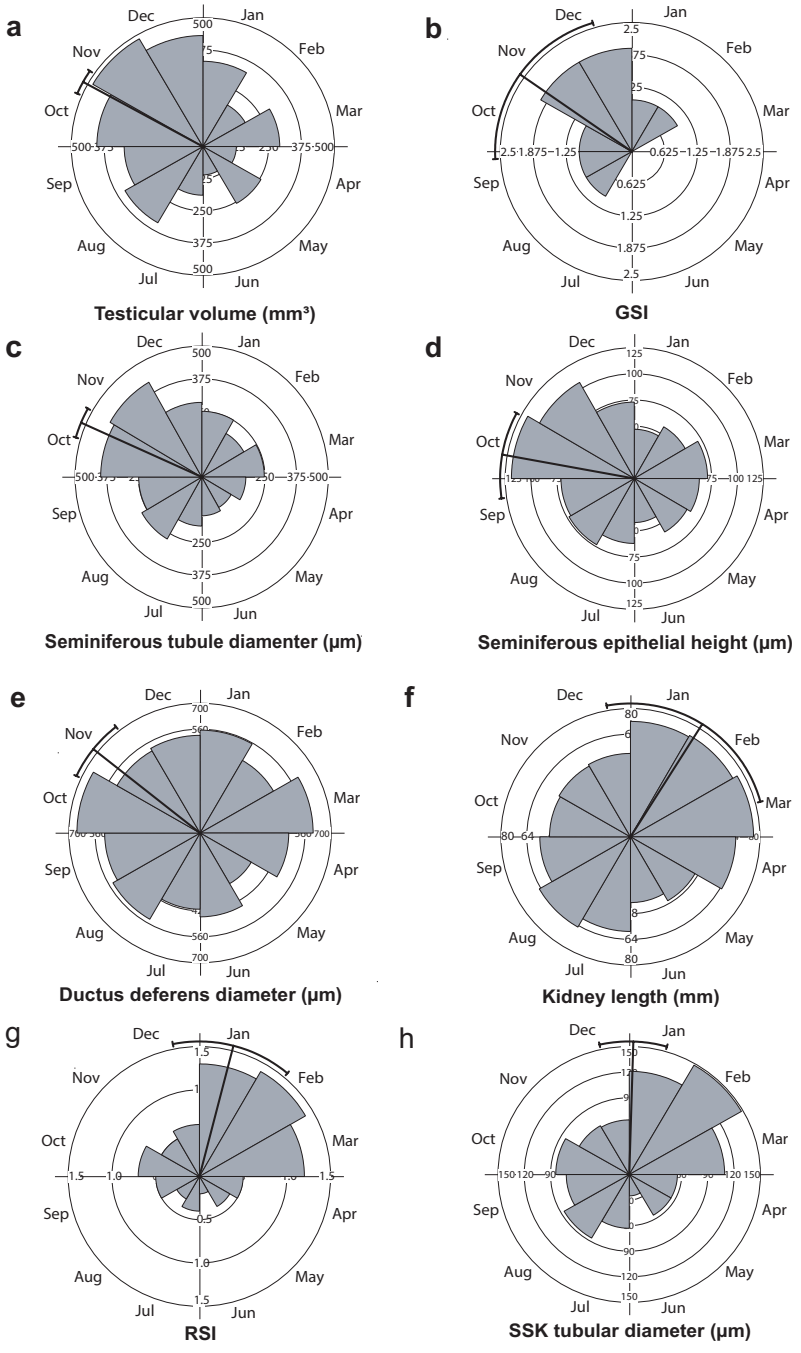


Figure 1. Seasonal variation in TV (testicular volume, mm³, a), GSI (gonadosomatic index b), seminiferous tubule diameter (c) and epithelial height (μm, d), ductus deferens diameter (μm, e) kidney length (mm, f), RSI (Renalsomatic index, g), and SSK (sexual segment of the kidney) tubular diameter (μm, h). Rose diagrams show mean monthly variation (bars), mean vector length (μ), and circular standard deviation (SD).

< 0.05 N = 25) with a peak in November and December (μ = 305.10° SD = 54.92° r = 0.63 Fig. 1b). The mean GSI of males was 1.17 ± 1.09 (range of 0.24-2.55).

Considering the microscopic data (Supplementary Material - Table SI), seminiferous tubule diameter was larger in the spring (R = 139.31 P < 0.001 N = 24) with a peak in October (μ = 293.55° SD = 100.15° r = 0.22 Fig. 1c). The seminiferous epithelium also varied seasonally (R = 18.10 P < 0.001 N = 24) with highest values in October and the lowest values in the autumn (μ = 280.14° SD = 112.30° r = 0.15 Fig. 1d). Leydig cell nuclear diameter had no significant variations between the seasons (R = 0.02 P = 0.98 N = 24) although there was tendency of larger diameter in spring and summer.

Spermatogenesis was a seasonal event in *T. strigatus*, with a peak in the spring and summer and an inactive period in autumn (Fig. 2a, Table SII). Sperm production was not continuous, and no sperm remnant were observed in the

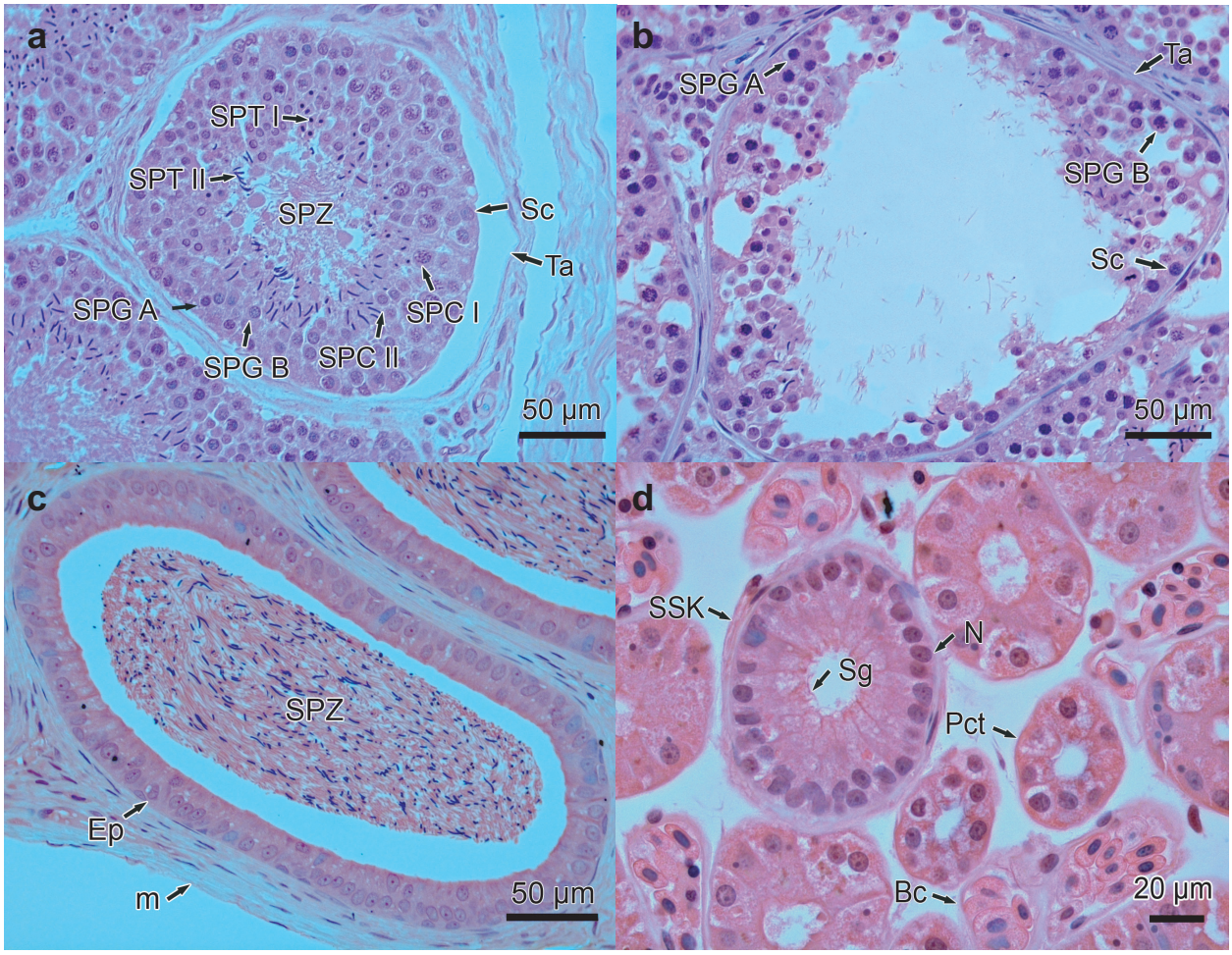


Figure 2. Transverse section of the testes (a, b), ductus deferens (c) and kidney (d) of *Thamnodinastes strigatus* from South Brazil. Transverse section of: testes in spermiogenesis (a) and regression (b) phases ductus deferens during spermiogenesis (c) and SSK in secretory peak (d). Ta, tunica albuginea SPG A, spermatogonia A SPG B, spermatogonia B SPC I, primary spermatocyte SPC II, secondary spermatocyte SPT I, spermatid I SPT II, spermatid II SPZ, spermatozoa Sc, Sertoli cell Ep, epithelium m, muscular layer Bc, blood capillaries N, nucleus Sg, secretory granules Pct, proximal convoluted tubule and SSK, sexual segment of the kidney. Light microscopy of samples stained with hematoxylin-eosin.

seminiferous tubules during the regression stage (Fig. 2b).

The ductus deferens diameter was larger in the spring (R = 34.71 P < 0.001 N = 24) with a peak in October (μ = 308.49° SD = 130.06° r = 0.08 Fig. 1e). There was no seasonal variation in the ductus deferens epithelial height (R = 2.73 P = 0.06 N = 24). The presence of sperm in the ductus deferens was detected throughout the year (Fig. 2c), except during the regression stage.

The kidney length was larger in the summer (R = 3.49 P < 0.05 N = 25) with a peak in February (μ = 32.27° SD = 132.03° r = 0.07 Fig. 1f). The kidney width had no significant variations between seasons (R = 0.36 P = 0.69 N = 25). Renalsomatic Index (RSI) was larger in the summer (R = 9.99 P < 0.001 N = 25) and the highest values occurred in January (μ = 14.45° SD = 84.68° r = 0.34 Fig. 1g).

The tubular diameter of the sexual segment of the kidney (SSK) was larger in the summer (R = 30.32 P < 0.001 N = 24) and the highest values occurred in January (μ = 1.70° SD = 106.31° r = 0.18 Fig. 1h). The SSK epithelial height had no variations between the seasons (R = 2.43 P = 0.09 N = 24). The histological investigation revealed a seasonal variation in the SSK activity (Table SIII). The SSK peak occurs in summer, when the cytoplasm of SSK cells is full of secretory granules being released (Fig. 2d).

Female reproductive cycle

The diameter of vitellogenic follicles was larger in the summer (R = 7.33 P < 0.001 N = 24) and the highest values occurred in January (μ = 1.05° SD = 81.70° r = 0.36 Fig. 3a). The GSI also increased in the summer (R = 4.15 P < 0.05 N = 24) with the highest values in January (μ = 3.44° SD = 50.44° r = 0.68, Fig. 3b).

Considering the microscopic data (Table SI), there was no seasonal variation in the following parameters: infundibulum epithelial height (R = 2.08 P = 0.12 N = 22) uterus epithelial height (R = 2.09 P = 0.13 N = 22), uterus glands (R = 2.69 P = 0.07 N = 22 Fig. 4a), and UVJ epithelial height (R = 2.05 P = 0.13 N = 22). The vaginal epithelial height was higher in the spring (R = 11.33 P < 0.001 N = 22) and the highest values occurred in November (μ = 329.21° SD = 16.45° r = 0.13 Fig. 3c). Females with sperm in the deep furrows of the UVJ (Figure 4b) were recorded in autumn (N = 6).

Females with vitellogenic follicles (larger than 2.99 mm) were found during all seasons, except for autumn. However, vitellogenesis peak occurs between spring and summer and ovulation in summer (larger follicles). The occurrence of pregnant females varied seasonally (R = 8.46 P < 0.001 N = 12) and the highest concentration was in November (μ = 323.54° SD = 43.34° r = 0.75 Fig 3d). Females simultaneously presenting mature follicles and embryos were not recorded. Neonates were recorded from late summer (March) to early winter (June). We raised the evidence to suggest the biennial reproductive frequency of T. strigatus: the absence of pregnant females simultaneously presenting vitellogenic follicles (Bellini et al. 2013). However, according to Blem (1982), evidence to support biennial cycles in squamates is only circumstantial.

Size-fecundity and reproductive effort

The linear model with body mass as predictor variable and dependent variables ovary mass, diameter of follicles, and number of follicles was significant (F = 2.80 DF = 3.21 r^2 = 0.20 P < 0.05 N = 24). Body mass was related to ovary mass (r = 0.17 P < 0.05 Fig. 3e). The mean ovary mass was 1.73 ± 1.38 g (range of 0.18-4.76 g). Body mass was also related to follicles diameter (r^2 = 0.15 P < 0.05 Fig. 3f). The mean length of vitellogenic follicles was 4.89 ± 3.85 mm (range of 2.99-13.16). The number of follicles was not significantly related to body mass (r^2= 0.06 P > 0.05). There was no correlation between female snout-vent length (SVL) and

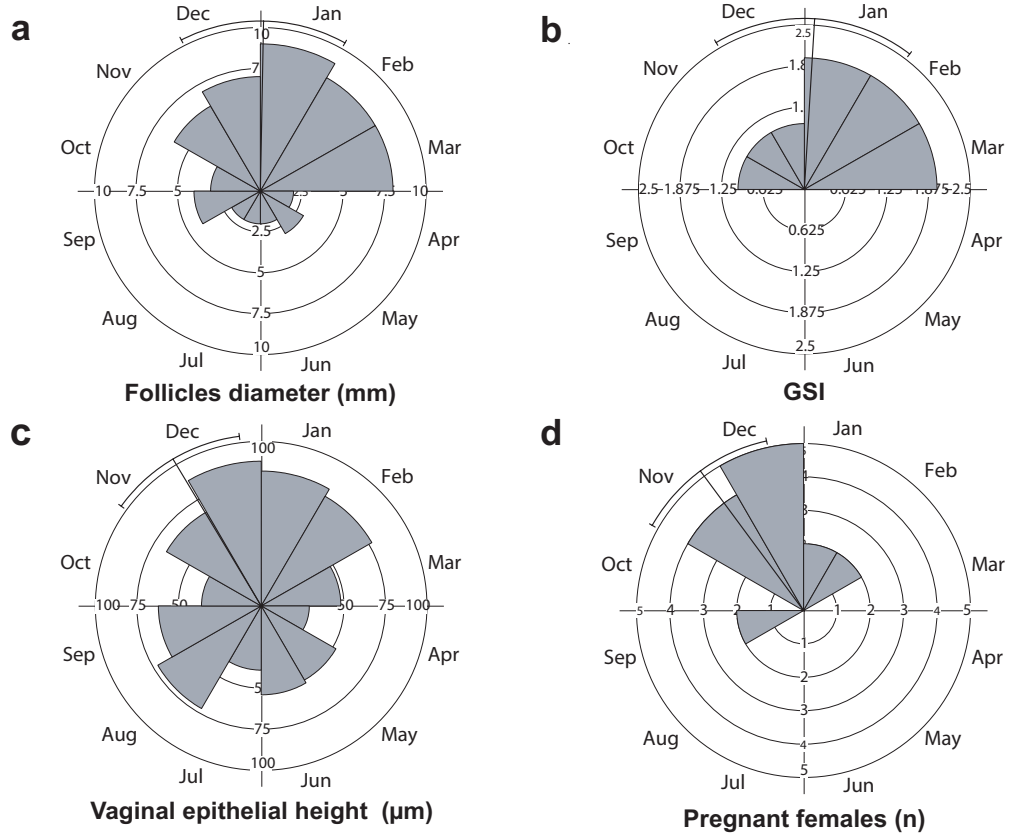
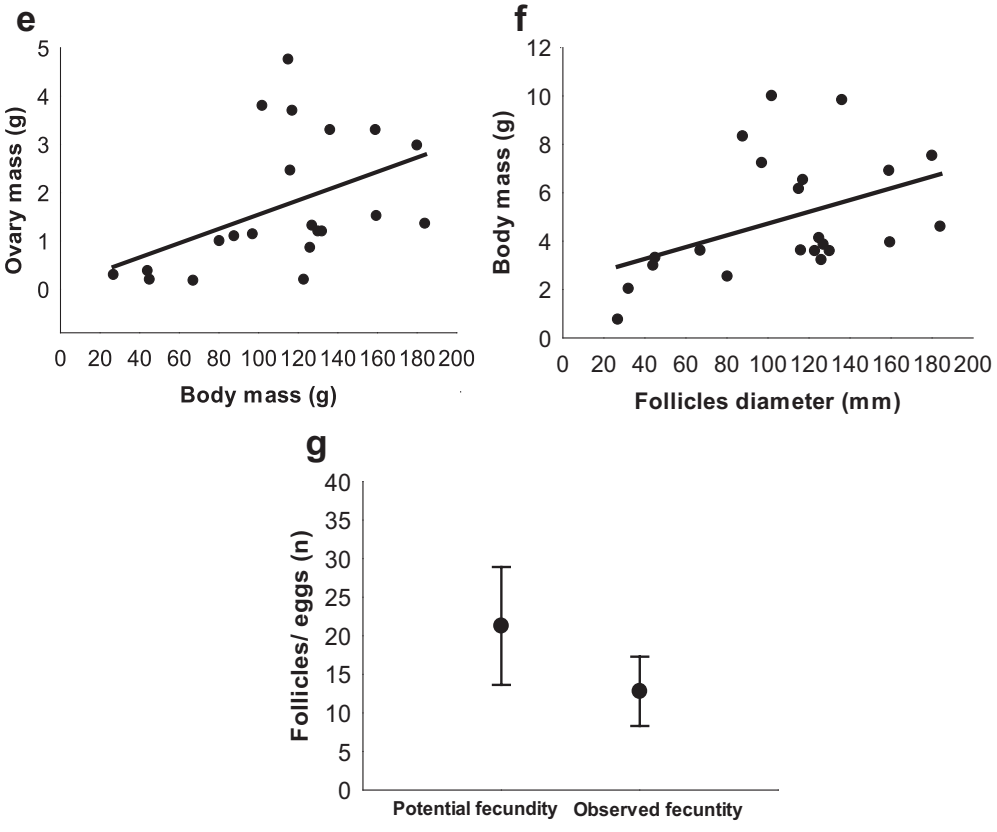


Figure 3. Seasonal variation in follicles diameter (a), GSI (gonadosomatic index b), vaginal epithelial height (µm, c), and pregnant females (n, d). Relationship between ovary mass and body mass (e) body mass and follicles diameter (f) and Potential vs. observed fecundity (g). Rose diagrams show mean monthly variation (bars), mean vector length (µ), and circular standard deviation (SD).. Scatter plot: middle point represents mean value, and whiskers represent standard deviation.



the clutch size (r = 0.09 P > 0.05). The potential fecundity (vitellogenic follicles) was significantly higher than the observed fecundity (embryos) (t = 4.24 P < 0.001 Fig. 3g). The mean clutch size (number of embryos in the oviducts) was 12.80 ± 4.49 (range of 4-21), while the average number of vitellogenic follicles in the ovary was 21.27 ± 7.64 (range of 11-36).

The mean GSI of females was 1.54 ± 1.71 (range of 0.12-6.68). The mean GSI of males was 1.17 ± 1.09 (range of 0.24-2.55). There was no difference between reproductive effort (GSI) of males and females (t = 0.89 P > 0.05 N = 25)

Sexual maturity

Based on the evaluated individuals, the estimated size at the onset of sexual maturity is 339 mm of SVL for males (size of the smallest mature male) and 406 mm of SVL for females (size of the smallest mature female). The younger mature male and female were 4 years old (males:4-11 y and females: 4-12 y), so both sexes had the same estimated age at the onset of sexual maturity (t = 1.68 P > 0.05). A transverse section of a twelve-year old individual is shown in Figure 5. There was no correlation between the age and the SVL for either males (F = 0.55 DF = 1 P > 0.05) or females (F = 0.42 DF = 1 P > 0.05). The body mass was also not related to the age of both males (F = 0.01 DF = 1 P > 0.05) and females (F = 0.63 DF = 1 P > 0.05).

DISCUSSION

Individually, males and females of *Thamnodynastes strigatus* have a discontinuous cyclic reproductive mode in South Brazil, with reduced gonadal activity in autumn. Hence, the reproductive cycle at the population level was seasonal semi-synchronous, with a reproductive peak in spring and summer (Mathies 2011). Our results agrees with previous description of the seasonal biennial reproductive cycle of *T. strigatus* (Bellini et al. 2014 see Table I) which we complemented with the description of the spermatogenic and oviductal cycle through histological analysis. Morphological analysis may be effective in determining the reproductive peak, but only histological analysis ensures the accurate description of reproductive events (Mathies 2011, Rojas et al. 2013, Loebens et al. 2017).

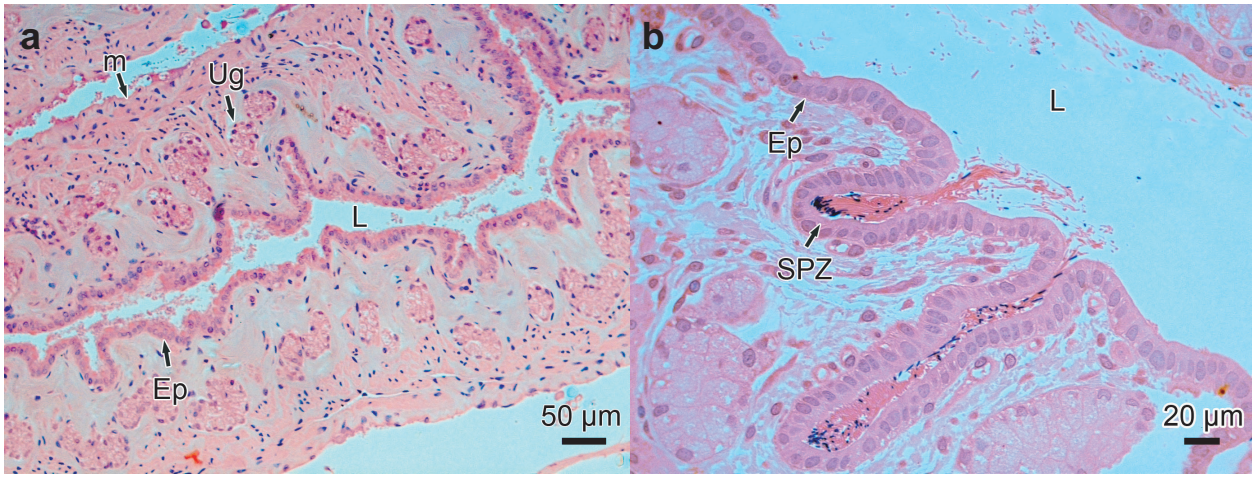


Figure 4. Histology of the uterus (a) and UVJ (utero-vaginal junction b) of *Thamnodynastes strigatus* from South Brazil. Transverse section of the uterus during the reproductive season (a) and UVJ in autumn exhibiting sperm storage in furrows (b). Ep, epithelium m, muscular layer L, lumen SPZ, spermatozoa Ug, uterine glands. Light microscopy of samples stained with hematoxylin-eosin.

The seasonal reproductive cycle of males was confirmed by the seasonal variation in the morpho-anatomical and histological attributes of testes (TV, GSI, and seminiferous tubule), ductus deferens (diameter), and kidney (length, RSI, and SSK tubular diameter). In *T. strigatus*, the testicular volume (TV) and gonadosomatic index (GSI) were higher in spring, coinciding with the increase in the seminiferous tubular diameter and epithelium height during the spermatogenic peak. However, only the histological analysis was able to reveal that spermiogenesis is not restricted to spring but extends into summer. The complete regression of the testis occurs briefly in autumn before a new spermatogenesis cycle starts. Although the regression phase is commonly seen in snakes from temperate areas, it was previously recorded in the tropical and subtropical snakes *Crotalus durissus* (Barros et al. 2012), *Bothrops erythromelas* (Barros et al. 2014b), and *Philodryas patagoniensis* (Loebens et al. 2017). Simultaneously with the period of testicular regression in autumn, the ductus deferens diameter reduces, and there is no evidence of sperm storage. Diameter of the ductus deferens increased in size after spermiation when more sperm were present. It has been shown previously that during storage in the ductus deferens, sperm complete maturation (Liang et al. 2011). The secretory activity of sexual segment of the kidney (SSK) was also higher in summer. Previous studies

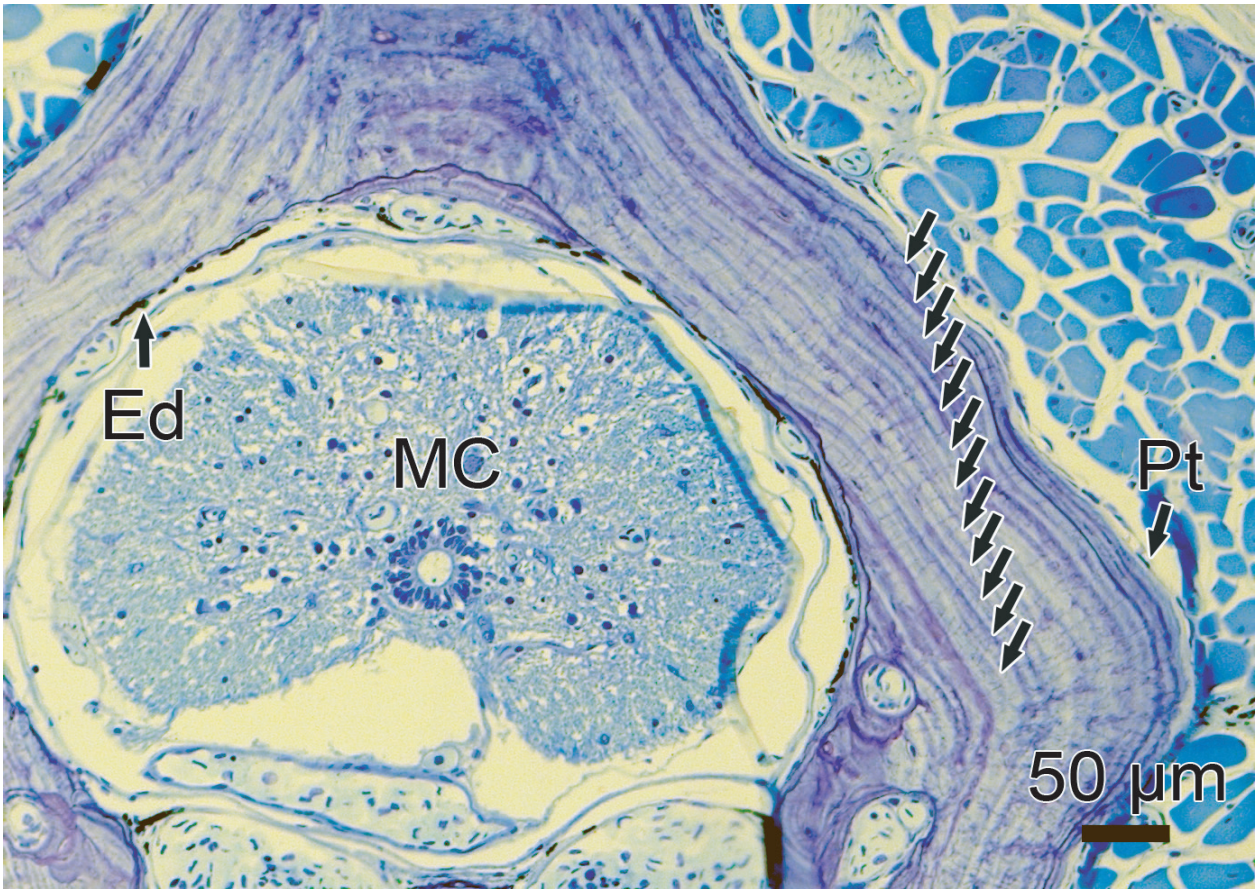


Figure 5. Transverse section of caudal vertebrae of a twelve-year (12) old *Thamnodynastes strigatus*. Arrow indicates individual growth layers. Ed: endosteum Pt: periosteum MC: spinal cord. Light microscopy of samples stained with toluidine blue.

demonstrated that the increase in size of the SSK is coincident with higher blood testosterone levels during the mating season (Krohmer et al. 2004, Mathies 2011).

The seasonal reproductive cycle of females was confirmed by the seasonal variation in the morpho-anatomical (follicles diameter and GSI) and histological attributes (vaginal epithelial height). The follicles diameter and GSI were higher in spring and summer, coinciding with the increase in the vaginal epithelial height. Although the UVJ epithelium was higher in autumn, this difference was not statistically significant. The UVJ activity after the mating season can be related to the embryo transport and sperm storage (Blackburn 1998). We could identify sperm in the UVJ sperm-storage in furrows during autumn, which provides geographic and temporal flexibility for females to fertilize the follicles in the absence of males (Almeida-Santos & Salomão 1997 Blackburn 1998). The mating season of *T. strigatus* is associated with secondary vitellogenesis and ovulation and, thus the sperm storage would not be mandatory. In many species of snakes from seasonal climates, sperm storage is an important mechanism that allows fertilization to be delayed (Schuett 1992), consequently parturition may occur in the most favorable time of the year for offspring survival (Shine 1977b). Other species of neotropical snakes also use the UVJ as a long-term sperm storage site, such as *Bothrops* spp. (Almeida-Santos & Salomão 2002, Nunes et al. 2010, Barros et al. 2014a, b), *Crotalus durissus* (Almeida-Santos & Salomão 1997, Barros et al. 2012), *P. patagoniensis* (Rojas et al. 2015, Loebens et al. 2018), and *Tomodon dorsatus* (Rojas 2013, Loebens et al. 2020).

The reproductive cycle of *T. strigatus*, in the subtropical-temperate region of South

Table I. Stages of the annual reproductive cycle of males *Thamnodynastes strigatus* in South Brazil.

	Summer	Autumn	Winter	Spring
Testes hypertrophy (volume)				X
GSI increases				X
Spermatogenesis				X
Spermiogenesis	X			X
Seminiferous tubule hypertrophy				X
Leydig cell activity	X			X
SSK hypertrophy	X			
SSK secretory peak	X			
Testicular regression		X		
Secondary vitellogenesis peak	X			
GSI increases	X			
Ovulation	X			
Mating	X			X
UVJ sperm storage		X		
Pregnancy	X			X
Neonates	X	X		

GSI, gonadosomatic index; SSK, sexual segment of the kidney; UVJ, utero-vaginal junction.
X correspond to the season of activity.

America, begins with vitellogenesis in summer and autumn and females with embryos in the oviduct are observed in winter and spring (Bellini et al. 2014). In Southeastern Brazil, *T. strigatus* exhibits vitellogenesis in late summer and autumn and females with embryos in the oviduct are observed during autumn, winter, and spring (Bizerra et al. 2005). Thus, in these two regions, females probably copulate in the autumn and then ovulate, and the embryonic development begins in late autumn and early winter (Bizerra et al. 2005, Bellini et al. 2014). We suggest that this is likely to occur due to the milder climate of these two regions. In the subtropical-temperate region of South America the climate in the cooler months in autumn and winter (mean temperature: 10 to 15 °C) is much higher than in South Brazil at the same time (mean temperature: -8 to 4 °C) (Alvares et al. 2013). This low temperature may influence females to store sperm during autumn and winter, and ovulation only occurs when temperatures are higher. *Thamnodynastes strigatus* females store sperm in the UVJ furrows during autumn and winter because pregnant females are only observed in spring and summer. This sperm storage in autumn may be an adaptation to the cold climate of the South Brazil, where females would store sperm in furrows and embryonic development would only occur in the warmer months of the year (summer and spring). Thus, the reproductive cycle of *T. strigatus* would be more restricted in South Brazil due to environmental factors.

Generally, snakes with a seasonal reproductive pattern evolved in environments where the ecological factors varied seasonally (Shine 2003, Barros et al. 2014a). Temperature, rainfall, photoperiod, lunar cycle, and food availability are some of the ecological factors that may constrain the reproductive cycle of snakes (Houston & Shine 1994, Sun et al. 2001, Bellini et al. 2017). Brown and Shine (2006) investigated the ecological factors influencing the reproductive seasonality of tropical keelback snakes (*Tropidonophis mairii*) in Australia and classified these factors into biotic (e. g. food availability and predation risk for hatchlings) or abiotic factors (e.g., incubation conditions). Their results indicated that the selective forces driving the reproductive seasonality are mainly the abiotic factors related to the optimal incubation conditions to produce larger hatchlings, that have an improved survival rate. Nevertheless, reproduction is a life-history trait of snakes that is not only shaped by ecological factors, but also by their phylogenetic history (Barros et al. 2012). Bellini et al. (2017) found that the reproductive biology of South American Xenodontine snakes is more strongly shaped by ancestry than by ecology since reproductive traits (e.g. reproductive mode, mean fecundity, reproductive potential, and frequency) have a strong phylogenetic signal in this clade.

The phylogenetic conservatism may explain the absence of variability between the reproductive traits of species of the same clade (Barros et al. 2012). Although snakes of the Tachymenini tribe have highly diverse morphology, habitat, activity pattern, and feeding habits, the reproductive features are less variable. They have viviparous reproductive mode and most populations have a seasonal biennial reproductive cycle. Examples of such reproductive cycle include *Gomesophis brasiliensis* females from Southeast and South Brazil (Oliveira et al. 2003) *Ptychophis flavovirgatus* females from Southeast and South Brazil (Scartozzoni & Marques 2004) *Thamnodynsates chaquensis* females from subtropical-temperate South America (Bellini et al. 2014), *Thamnodynsates hypoconia* from subtropical-temperate South America (Bellini et al. 2014), and females from Brazilian

subtemperate wetlands (Rebelato et al. 2016), *Thamnodynastes strigatus* from subtropical-temperate South America (Bellini et al. 2014) and *Tomodon dorsatus* from Southeast (Bizerra et al. 2005, Rojas 2013) and South Brazil (Rojas 2013, Loebens et al. 2020). A continuous reproductive cycle was suggested for males of *T. chaquensis* (Bellini et al. 2014), *T. hypoconia* (Rebelato et al. 2016), and *T. strigatus* (Bellini et al. 2014). However, these conclusions were based on the absence of seasonal variation in the testicular volume, which is not a good indicator of the spermatogenic activity (Mathies 2011, Rojas et al. 2013).

Most species of the Xenodontinae subfamily are oviparous (Sites et al. 2011), but there are viviparous species in the Tachymenini and Hydropsini tribes (Zaher et al. 2009). Viviparity evolved multiple times in different lineage of snakes as a result of progressive increases in the period of intrauterine retention of eggs (Blackburn 2015). The advantage of viviparity is that females can provide optimal conditions to embryogenesis regardless of the variation in the abiotic factors (Shine 2004). However, viviparity places on females a high demand of time and energy, in a way that it is almost impossible for a single female to reproduce in two consecutive years, which explains their biennial reproductive frequency (Edwards et al. 2002, Bellini et al. 2014). Another consequence of viviparity observed in *T. strigatus* is the relatively low number of embryos per female and per year since the selective advantages of viviparity are higher for species producing small clutches (Brown & Shine 2009).

Allometric relationships between maternal size and fecundity may provide evidence of selective pressures that drive the evolution of reproductive strategies (Tanaka & Mori 2011). We did not find a positive correlation between mother size (snout-vent length, SVL) and clutch size in *T. strigatus*, unlike the pattern observed in several species of snakes (Shine 1994a). This lack of correlation between the mother size and a higher neonate production was also demonstrated in other viviparous snakes (Bellini et al. 2018). However, the correlation between the ovary mass and follicles diameter and the female size (body mass) was positive, which probably influenced the evolution of the female-biased sexual size dimorphism in *T. strigatus* (Gregory 2004). However, there is a trade-off between the amount and the size of the embryos produced and this may vary between years within a single population (Olsson & Shine 1997). The largest ovarian follicles occur in summer, and this can produce larger embryos with higher survival rates in an unpredictable environment with lower food availability and higher juvenile density. According to Brown & Shine (2009), the increased size of embryos may enhance the progeny fitness in some circumstances for example, a larger body size may reduce the vulnerability to predation and enhance the survival rates. In our evaluation, the potential fecundity (follicles) was higher than the observed fecundity (embryos) since snakes commonly produce large number of follicles to increase the chances of fertilization, and the clutch size actually depends on the mother size and body condition (Brown & Shine 2009). The reproductive effort (GSI) was not related to the body size of either females or males and did not vary between sexes. According to Shine & Schwarzkopf (1992), in many reptiles, the reproductive effort is evolutionary determined by the survival costs of reproduction.

Females of *Thamnodynastes strigatus* reach sexual maturity at a larger SVL than males and this size difference is maintained later in life. Females grow larger than males in snake species in which males do not engage in physical battles for mating (Shine 1994a). The reproductive maturation of females at large body sizes may be

an adaptation related to a fecundity advantage since a large abdominal volume accommodates more embryos (Shine 1994a). Differently, the age at sexual maturity and the longevity did not variate between sexes. In most snake species, males reach sexual maturity earlier than females, once females delay sexual maturation to invest in growth (Shine 1994a). In *T. strigatus*, although females are larger than males, there is no delay in the sexual maturity. According to Shine (1994b), the offspring size is related to how much females grow after sexual maturity, and it is not affected by the reproductive mode (oviparity/viviparity). However, in reptiles, the growth rate is slower after sexual maturity (Waye 1999) and it may explain the absence of relationship between age and body size.

In conclusion, our results indicate that *T. strigatus* have a seasonal pattern of reproduction in subtropical Brazil, with a strong peak in spring and summer. Females developed a sperm-storage strategy and reach sexual maturation at a larger body size than males. Our histological analysis confirmed the previously described seasonal biennial reproductive cycle of *T. strigatus* (Bellini et al. 2014). We focused on the description of the spermatogenic and oviductal cycle, the sperm storage in females, and the age at sexual maturity. Finally, we encourage more research on the reproductive aspects of neotropical snakes to help us answer questions about the sexual dimorphism patterns, evolution of viviparity, selective forces shaping the reproductive cycles, and sperm storage mechanisms in squamates.

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SUPPLEMENTARY MATERIAL

Figure S1.
Tables SI, SII, SIII.

Appendix

Voucher specimens of *T. strigatus* analyzed in this study housed in the herpetological collections of the Federal University of Santa Maria (ZUFSM) and Pontifical Catholic University of Rio Grande do Sul (MCP- PUCRS)

Males (n = 24): ZUFSM0241, and 0404, 0698, 0716, 0823, 0867, 0991, 1222, 1227, 1246, 1279, 1285, 1448, 1647, 1657, 1661, 1712, and 2078. MCP01334, 01476, 02000, 03124, 03885, and 05649.

Females (n = 22): ZUFSM0167, 0197, 0632, 0714, 0809, 1302, 1734, 1735, 1890, 2069, 2070, 2212, 2243, 2280, and 2936. MCP04530, 05646, 05775, 06006, 06595, 07343, and 11503.

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LUIZA LOEBENS[1]
https://orcid.org/0000-0003-3217-6759

TIAGO F. THEIS[1]
https://orcid.org/0000-0003-2151-5524

SELMA M. ALMEIDA-SANTOS[2]
https://orcid.org/0000-0002-3928-1183

SONIA Z. CECHIN[1]
https://orcid.org/0000-0003-2632-1681

[1]Universidade Federal de Santa Maria, Departamento de Ecologia e Evolução, Laboratório de Herpetologia, Avenida Roraima, 1000, Camobi, 97105-900 Santa Maria, RS, Brazil

[2]Instituto Butantan, Laboratório de Ecologia e Evolução, Avenida Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil

Correspondence to: **Luiza Loebens**
E-mail: loebens.luiza@gmail.com

Author contributions

Luiza Loebens conceived and designed the analysis, collected and analyzed data, and wrote the paper. Tiago F. Theis collected and analyzed data, and wrote the paper. Selma M. Almeida Santos conceived and designed the analysis, and wrote the paper. Sonia Z. Cechin conceived and designed the analysis, and wrote the paper.