Acta Zoologica

Published for the Royal Swedish Academy of Sciences and the Danish Academy of Sciences and Letters

Acta Zoologica (Stockholm) doi: 10.1111/azo.12172

Reproductive biology of *Philodryas patagoniensis* (Snakes: Dipsadidae) in south Brazil: male reproductive cycle

Luiza Loebens,[1] Sonia Zanini Cechin,[1] Tiago Felipe Theis,[1] Livia Bataioli Moura[1] and Selma Maria Almeida-Santos[2]

[1]Laboratory of Herpetology, Santa Maria Federal University, 1000 Roraima Avenue, Camobi, Santa Maria, Rio Grande do Sul, 97105–900, Brazil; [2]Laboratory of Ecology and Evolution, Butantan Institute, 1500 Vital Brazil Avenue, São Paulo, São Paulo, 05503-900, Brazil

Keywords:
spermatogenesis, sexual segment of the kidney, ductus deferens, sperm storage

Accepted for publication:
18 July 2016

Abstract

Loebens, L., Cechin, S.Z., Theis, T.F., Moura, L.B. and Almeida-Santos, S.M. 2016. Reproductive biology of *Philodryas patagoniensis* (Snakes: Dipsadidae) in south Brazil: male reproductive cycle. — *Acta Zoologica* (Stockholm) **00**: 1–11.

The male reproductive cycle of *Philodryas patagoniensis* in south Brazil was described through morpho-anatomical and histological analysis of individuals deposited in zoological collections. Spermatogenesis occurred during late autumn–winter (June–September) and spermiogenesis occurred in spring–summer (October–March). The volume of the testes was smaller (quiescent) in winter, while the tubular diameter and the epithelial height of the seminiferous tubule were larger in summer (January–March). The ductus deferens presented spermatozoa all over the year and had no seasonal variation in diameter. The length of the kidney was larger in winter–spring (July–December), although the tubular diameter and epithelium height of the sexual segment of the kidney (SSK) were larger only in winter (July–September). Total testicular regression was observed in late autumn (May), simultaneously with the peak in SSK. Therefore, at the individual level, males exhibit a discontinuous cyclical reproduction. Considering the population level, the reproductive cycle is seasonal semisynchronous, with most of the individuals showing a reproductive peak in spring–summer (October–March). Here, we present evidence to support the importance of the microscopic approach to reproductive cycle studies. Finally, we discuss the intrinsic and extrinsic factors influencing *P. patagoniensis* reproductive patterns.

Luiza Loebens, Laboratory of Herpetology, Santa Maria Federal University (UFSM), Santa Maria, 1000 Roraima Avenue, Camobi, Santa Maria, Rio Grande do Sul (RS) 97105–900, Brazil. E-mail: loebens.luiza@gmail.com

Introduction

Reproduction is a central aspect of the life history of organisms, and information on reproductive strategies of a substantial amount of snake taxa is essential to investigate the evolution of reproductive traits and its patterns (Almeida-Santos and Salomão 2002). Although the reproduction is not crucial for the immediate survival, it is the currency of an individual's fitness, as it is essential for the species persistence. Nevertheless, reproduction is a critical event in the life of an individual because it represents a substantial energetic cost, especially to ectotherm animals which have low maintenance costs (Vitt and Caldwell 2014). Therefore, reproduction is not a continuous endeavour, and an appropriate timing of reproductive effort is critical (Brown and Shine 2006).

Reproductive cycles of snake populations vary from highly seasonal to aseasonal (Mathies 2011). While the majority of snakes from temperate zones present seasonal reproduction, in tropical and subtropical snakes the cycles trend to be more plastic: species reproduce in the dry season, wet season, over extended periods or even almost continuously (Seigel and Ford 1987). Summarizing, reproductive cycles of many snakes may vary over time, among populations, and even among individuals, being not easy to classify into discrete categories. Hence, the currently known diversity of reproductive

patterns of snakes suggests that a single explanation on this matter is not enough.

In squamates, the male spermatogenetic cycle may or may not coincide with the female reproductive cycle. In this context, the reproductive cycle may be defined as associated when gonadal and hormonal events in males and females coincide with the mating season. However, in species that the sperm production is not synchronous with the females ovulation and fertilization, the cycle is defined as dissociated (Volsøe 1944; Saint Girons 1982; Seigel and Ford 1987; Aldridge *et al.* 2009). In these cases of desynchronization between the timing of spermatogenesis, ovulation and mating, sperm storage appears to be obligatory (Sever and Hamlett 2002; Almeida-Santos *et al.* 2004). In male squamates, the ductus deferens evolved gradually through the phylogeny of this group as the main and long-term storage organ (Almeida-Santos *et al.* 2004; Sever 2004; Liang *et al.* 2011). Therefore, sperm storage is a reproductive strategy that established as a necessary stage in the reproductive cycle of squamates.

Furthermore, we should take account of the synchrony of reproductive cyclicality at the individual level and how this contributes to the seasonality of reproduction at the population level. For example, if individual males are seasonally reproductive, the male population cycle might be continuous if there are always a few males in spermatogenic condition in the population. In the opposite situation, if males in spermiogenesis condition occur in a restricted season of the year, the population cycle might be seasonal (Mathies 2011).

Another feature to be analysed in the reproductive cycle of male squamates is the sexual segment of the kidney (SSK), a sexually dimorphic structure that has secretory activity under the control of testosterone (Krohmer *et al.* 2004; Aldridge *et al.* 2011; Rojas *et al.* 2013). The SSK secretions are mixed with the semen and transmitted to the female during copulation (Aldridge *et al.* 2011); therefore, several functions have been proposed for this secretion; it may compose the seminal fluid, nurturing and activating the sperm (Bishop 1959). It may prevent or reduce subsequent remating by the female (Nilson and Andrén 1982) and act in the formation of the copulatory plug to maintain sperm stored in the oviduct (Almeida-Santos and Salomão 1997; Almeida-Santos *et al.* 2004). In addition, it may form a non-coagulated copulatory plug that acts as a viscous barrier to reduce the likelihood or speed of sperm transmission (Shine *et al.* 2000).

In this respect, until recently, the reproductive patterns of neotropical snakes were relatively less understood than those from temperate zones (Mathies 2011). Nevertheless, in the past two decades, Brazilian researchers have contributed considerably to the knowledge on the reproductive biology of neotropical snakes (Almeida-Santos *et al.* 2014). For a long time, reproductive data were only available for females (Barros *et al.* 2012), and several studies about male reproduction showed ambiguous results (Mathies 2011). However, recent researches had also included histological analysis to describe the spermatogenic and oviductal cycle of neotropical snakes (Rojas *et al.* 2013*;* Barros *et al.* 2014*a*,*b*; Braz *et al.* 2014; Resende and Nascimento 2015).

The *P. patagoniensis* (Girard 1858) is widely distributed in open areas of Brazil, Bolivia, Paraguay, Argentina and Uruguay (Peters and Orejas-Miranda 1970). Available information on its reproductive biology concerns aspects of female reproductive cycle, sexual dimorphism and oviductal sperm storage (Fowler and Salomão 1994; Fowler *et al.* 1998; Pontes 2007; López and Giraudo 2008; Rojas *et al.* 2015). The species is oviparous, reproducing seasonally. Vitellogenesis occurs from August to February (autumn–summer); clutches were recorded from November to January (spring–summer) and births from January to March (summer–early autumn; Pontes 2007; López and Giraudo 2008). Although direct evidence of the timing of mating (e.g. observations in the wild) is not available for *P. patagoniensis*, there is a single report of a probable mating in spring–summer (S.Z. Cechin 2014, pers. comm.).

Here, we describe the male reproductive biology of the snake *P. patagoniensis* in the south region of Brazil. The aims of this study were as follows: (i) to describe the male reproductive cycle through morphological and histological analyses of the testes, SSK and ductus deferens; and (ii) to investigate the size–fecundity relationships and the reproductive investment.

Methods

Data collection

We analysed 85 *P. patagoniensis* males from the south region of Brazil (Rio Grande do Sul, Paraná and Santa Catarina States) available in the herpetological collections of the Santa Maria Federal University (ZUFSM) and Pontifical Catholic University of Rio Grande do Sul (MCP–PUCRS) (Appendix 1). For each individual, we obtained information on month of death, snout–vent length (SVL) and body mass (BM).

The climate in the south region of Brazil is classified as humid subtropical (Köppen's climate classification Cfa-Cfb). The rainfall is well distributed throughout the seasons (mean annual precipitation 1.000–2.000 mm). However, the region shows well-defined temperature seasonality, with temperatures ranging from 0 °C (winter) to 40 °C (summer; Alvares *et al.* 2013).

Reproductive events were described according to austral seasons. The reproductive cycle of mature males was analysed considering morphological, macroscopic, and microscopic changes of the testes, ductus deferens and kidney.

Macroscopic data

For each specimen, the following macroscopic data were collected: mass, length, width, and thickness of the testes, width of the distal portion of ductus deferens, and length and width of the proximal region of kidney (Rojas *et al.* 2013). Testicular volume (TV) was calculated by the ellipsoid formula: $V = (4/3)\ \pi abc$, where a = half of length, b = half of width and c = half thickness of the testes (Pleguezuelos and Feriche 1999). The Gonadosomatic Index (GSI) was calculated by the formula according to: testes mass/body mass × 100 (Clesson *et al.* 2002).

Histology

We dissected 61 specimens to obtain histological samples of the proximal region of the testes, distal region of the ductus deferens and proximal region of the kidney (Rojas *et al.* 2013). As a standard procedure, only right-side organs were used, preserving the contralateral. The tissue samples were processed for light microscopy by Historesin (Leica) method. Sections of 2 μm thickness (Leica RM2245 microtome) were stained with haematoxylin–eosin. Slides were analysed using a ZEISS Axio Scope.A1 microscope with Axiocam MRc 5. We obtained 10 measurements of the microscopic variables of each individual: seminiferous tubule diameter and epithelial height, Leydig cell nuclear diameter, and tubular diameter of SSK and epithelial height (Rojas *et al.* 2013). Only seminiferous and SSK tubules presenting circular form in the transverse section were considered to the morphometric analysis.

To determine the sexual maturity, we used the presence of spermatozoa in the testes or ductus deferens as the main criterion, and the convoluted or non-convoluted aspect of the ductus deferens as a secondary criterion (Shine 1977). We analysed the spermatogenic cycle according to stages classification: (I) complete regression; (II) early recrudescence; (III) late recrudescence; (IV) early spermiogenesis; (V) spermiogenesis; and (VI) early regression (Goldberg and Parker 1975). The male reproductive cycle was classified at the individual and population level according to Mathies (2011). The cycle of the sexual segment of the kidney (SSK) was classified into stages: (0) SSK not hypertrophied; (1) SSK hypertrophied with a few granules; (2) SSK cytoplasm full of secretory granules; (3) SSK secretory granules apically in the cytoplasm; and (4) maximum density of SSK secretory granules (Krohmer *et al.* 2004).

Statistical analysis

The correlation between the macroscopic measurements and the body length (SVL) was tested by linear regression (Shine 1977). Therefore, analysis of covariance (ANCOVA) was employed to test the seasonal variation in testes volume, ductus deferens width, and kidney length and width, using SVL as the covariate. The GSI was determined by ANOVA, and the significant results were analysed by the Tukey's test. Aiming to investigate the seasonal variation in seminiferous tubule diameter and epithelial height, Leydig cell nuclear diameter and SSK tubular diameter and epithelial height were used in the analysis of variance (ANOVA). *Post hoc* tests (Tukey) were used to identify differences between seasons.

To explore the synchronism between SSK and spermatogenic cycles, we performed a correlation analysis (Pearson's coefficient) using the TV versus kidney length, seminiferous tubule diameter versus SSK tubule diameter, and seminiferous epithelial height versus SSK epithelial height.

To investigate size–fecundity relationships, we performed linear regressions using only BM as the predictor variable, because SVL and BM were strongly correlated ($r = 0.80$, $P < 0.0001$). Therefore, we analysed the relationship between the following variables: BM versus TV, and BM versus testicular mass. Correlations between body size (SVL) and the values of GSI were tested through the Pearson's correlation coefficient.

Statistical analyses were performed using Statistica version 10 (Statsoft 2011). All variables were tested for normality and homogeneity of variances prior to analysis, and data were log-transformed when necessary. The plots were created on Sigmaplot 12 (Systat 2011).

Results

Spermatogenic cycle

Seasonal variation in some macroscopic parameters of the male reproductive organs was statistical identifiable. Testes volume showed a decrease during winter ($F = 17.1645$, $P < 0.0001$, Fig. 1A) in comparison with summer, autumn and spring ($P < 0.001$ for the three seasons, Table 1). The GSI was different between the seasons ($F = 11.5245$, $P < 0.0005$, Fig. 1B), with an increase in testicular mass during the spring and summer, in comparison with autumn ($P < 0.05$ for both seasons, Table 1), and winter ($P < 0.05$ and $P < 0.005$, Table 1). The ductus deferens width was larger in spring, although no significant differences were observed in this feature between the seasons ($F = 0.4387$, $P = 0.7287$, Table 1). Sperms in the ampulla ductus deferens were observed all over the year, but in autumn and winter, the spermatozoa density was visually reduced (Fig. 2A), when compared with spring and summer (Fig. 2B).

Considering the microscopic data, the variation in the seminiferous tubules diameter was seasonal ($F = 4.6791$, $P = 0.0059$, Fig. 1C) and was larger in summer, compared with winter and spring ($P < 0.05$ and $P < 0.01$, respectively, Table 2). Accordantly, the variation in the seminiferous epithelium height was seasonal ($F = 5.0417$, $P = 0.0042$, Fig. 1D) and it increased in summer, when compared with winter and spring ($P < 0.01$ and $P < 0.05$, respectively, Table 2). Leydig cell nuclear diameter differed between seasons ($F = 5.5430$, $P = 0.0024$, Fig. 1E), and it was larger in

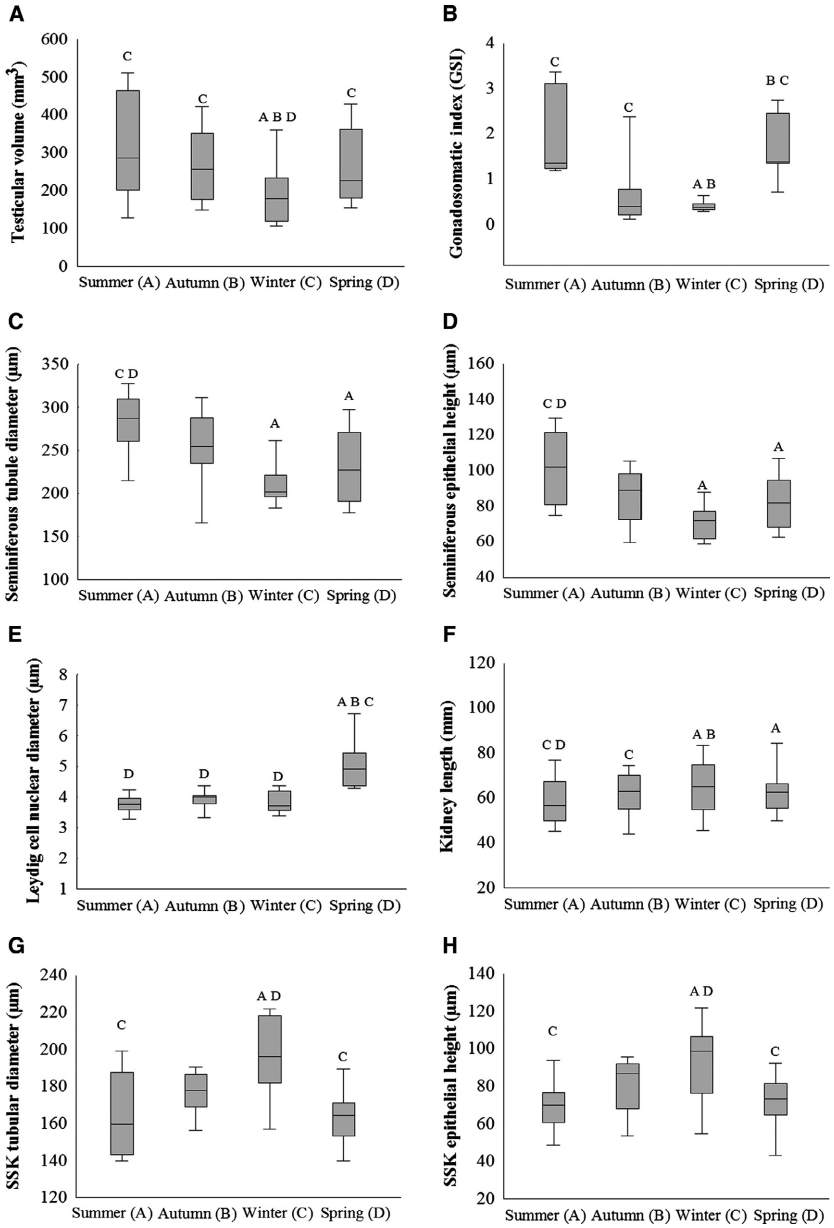

Fig. 1—Seasonal variation in—**A.** Testicular volume,—**B.** Gonadosomatic index—**C.** Seminiferous tubule diameter,—**D.** Seminiferous epithelial height,—**E.** Leydig cell nuclear diameter,—**F.** Kidney length, —**G.** SSK tubular diameter and—**H.** SSK epithelial height for males of *P. patagoniensis* in south Brazil. Middle line represents mean values, boxes show standard deviation, and whiskers represent minimum and maximum values.

spring than in summer, autumn and winter ($P < 0.05$ for the three seasons, Table 2).

Therefore, different developmental stages were characterized by cellular changes in the testes of *P. patagoniensis* (Table 3). Complete regression of the testes (Stage I, Fig. 3A) occurred in autumn (May), and it was characterized by the presence of spermatogonia A and B and Sertoli cells only. Early recrudescence (Stage II, Fig. 3B) was observed in late autumn (June) and early winter (July), when proliferation of spermatogonia, primary spermatocytes and first meiosis cells began the growth of the epithelium. Late recrudescence (Stage III, Fig. 3C) occurred in spring (July–September), with predominance of primary spermatocytes and emergence of round spermatids. This was the phase of maximum cellular division (meiosis); thus, the number of spermatogonia decreased substantially. Early spermiogenesis (Stage IV) lasted from spring to early summer (October–January), when a few spermatogonia and spermatocytes were seen, but spermatids were the most prevalent cell type in the epithelium. At this time, many of the round spermatids had advanced to the elongating spermatid stage. The spermiogenesis (Stage V, Fig. 3D) occurred from spring (November) to early autumn (March), the epithelium was high and composed by late spermatids and many mature spermatozoa were released into the lumen (spermiation). Early regression (Stage VI) occurred in autumn (April–May), presenting epithelium atrophy and few

Table 1 Macroscopic measurements of male *P. patagoniensis* snake in south Brazil

Season	SVL (mm)	Testes volume (mm^3)	GSI	Ductus deferens width (mm)	Kidney length (mm)	Kidney width (mm)
Summer (A)	653.58 ± 38.30	295.46 ± 48.97^{C}	1.32 ± 0.28^{C}	1.62 ± 0.20	$58.80 \pm 7.04^{C,D}$	5.64 ± 1.84
Autumn (B)	644.30 ± 39.78	274.17 ± 47.61^{C}	0.65 ± 0.18^{C}	1.44 ± 0.24	62.02 ± 2.69^{C}	6.23 ± 1.96
Winter (C)	636.86 ± 26.28	$183.00 \pm 62.40^{A,B,D}$	$0.41 \pm 0.08^{A,B}$	1.64 ± 0.25	$64.09 \pm 3.48^{A,B}$	5.50 ± 2.07
Spring (D)	649.46 ± 23.22	256.49 ± 22.87^{C}	$1.88 \pm 0.45^{B,C}$	1.85 ± 0.22	63.57 ± 5.10^{A}	5.79 ± 1.70

Adapted from Rojas *et al.* (2013).
Post hoc analysis: significant differences ($P < 0.05$) between seasons are indicated by letters. Each letter represents a season. Data are expressed as mean ± standard errors. SVL, snout–vent length; GSI, Gonadosomatic Index.

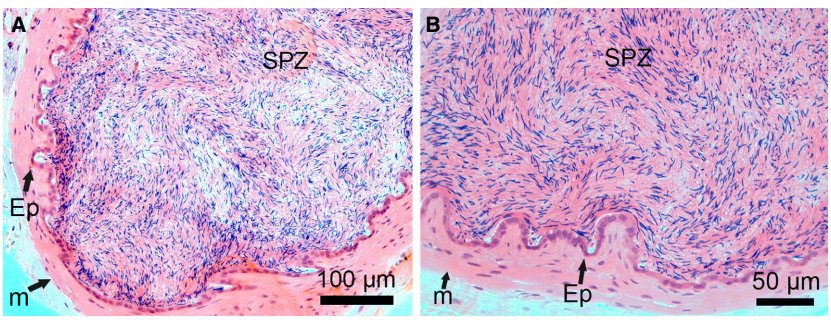

Fig. 2—Histology of the ductus deferens of *P. patagoniensis* during—**A.** non–reproductive period with low density of spermatozoa (long–term storage) and —**B.** the reproductive period with a high density of spermatozoa. SPZ, spermatozoa; Ep, epithelium; and m, muscular layer.

Table 2 Structural variation of *P. patagoniensis* testes between different seasons

Season	Seminiferous tubule diameter (µm)	Seminiferous epithelial height (µm)	Leydig cell nuclear diameter (µm)
Summer (A)	$287.82 \pm 13.50^{C,D}$	$105.45 \pm 6.53^{C,D}$	3.73 ± 030^{D}
Autumn (B)	253.29 ± 13.35	90.29 ± 4.47	3.93 ± 0.27^{D}
Winter (C)	218.29 ± 12.32^{A}	75.14 ± 4.21^{A}	3.82 ± 0.23^{D}
Spring (D)	233.23 ± 9.19^{A}	86.13 ± 3.43^{A}	$4.92 \pm 0.24^{A,B,C}$

Adapted from Rojas *et al.* (2013).
Post hoc analysis: significant differences ($P < 0.05$) between seasons are indicated by letters. Each letter represents a season. Data are expressed as mean ± standard errors.

Table 3 Stages of the spermatogenic cycle in *P. patagoniensis* in south Brazil

Stages	Months of occurrence
(I) Complete regression	May
(II) Early recrudescence: division of spermatogonia and primary spermatocytes	June–July
(III) Late recrudescence: primary spermatocytes and spermatids	July–September
(IV) Early spermiogenesis: spermatids in metamorphosis	October–January
(V) Spermiogenesis: mature spermatozoa in the lumen	November–March
(VI) Early regression: decrease in the seminiferous epithelium	April–May

Adapted from Goldberg and Parker (1975).

spermatids and the remainder of spermatozoa in the lumen. At this point, spermatocyte and spermatid leftovers underwent degeneration, so cellular debris were evidenced in the lumen.

SSK cycle

Macroscopic measurements of *P. patagoniensis* kidneys presented significant differences in length values between the seasons ($F = 8.2871$, $P = 0.0008$, Fig. 1F), but not in width values ($F = 0.9216$, $P = 0.4552$, Table 1). The kidney length was larger in winter than in summer and autumn ($P < 0.001$ for both seasons, Table 1), and larger in spring than in summer ($P < 0.05$, Table 1).

The tubular diameter of the SSK differed among the seasons ($F = 4.0781$, $P = 0.012$, Table 4, Fig. 1G), and it was larger in winter than in spring and summer ($P < 0.05$ for both seasons, Table 4). The epithelial height of the SSK exhibited a seasonal variation ($F = 3.7512$, $P = 0.0164$, Fig. 1H), with a significant increase during winter in relation to spring and summer ($P < 0.05$ for both season, Table 3).

Although macroscopic differences in the kidney size were hardly detected, histological investigation revealed clear seasonal variation in SSK activity (Table 5). The histology of kidney showed that the SSK was not hypertrophied in

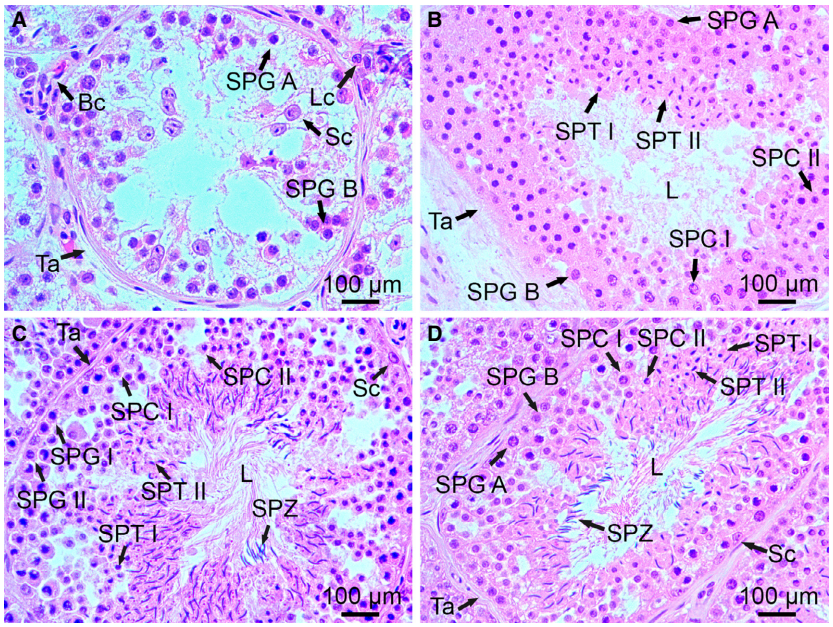

Fig. 3—Transverse sections of the testes of *P. patagoniensis* from south Brazil. In —**A.** autumn (May), with seminiferous epithelium in regression, —**B.** in winter (July), showing different stages of cellular division in the seminiferous epithelium, —**C.** in spring (October), with the peak of cellular division and spermatid in metamorphosis, and —**D.** in late summer (March), final phase of the spermiogenesis process. Ta, tunica albuginea; Bc, blood capillaries; Lc, Leydig cells; Sc, Sertoli cells; L, lumen; Sd, spermatid; SPG A, spermatogonia A; SPG B, spermatogonia B; SPC I, primary spermatocyte; SPC II, secondary spermatocyte; SPT I, spermatid I; SPT II, spermatid II; and SPZ, spermatozoa.

Table 4 Structural variation of *P. patagoniensis* kidney between different seasons

Season	SSK tubular diameter (µm)	SSK epithelial height (µm)
Summer (A)	160.11 ± 9.18^{C}	68.77 ± 3.95^{C}
Autumn (B)	176.62 ± 7.75	81.14 ± 5.06
Winter (C)	$197.78 \pm 9.75^{A,D}$	$92.33 \pm 7.55^{A,D}$
Spring (D)	169.30 ± 4.7^{C}	73.00 ± 3.21^{C}

Adapted from Rojas *et al.* (2013).
Post hoc analysis: significant differences ($P < 0.05$) between seasons are indicated by letters. Each letter represents a season. Data are expressed as mean ± standard errors. SSK, sexual segment of kidney.

late summer–early autumn, which was the period of regression (Stage 0, Fig. 4A). At this phase, the SSK lumen was narrow and with vacuoles on the medial region of the tubule. Autumn was the period of the start of the activity with a few granules weakly stained in the basal region of the cells (Stage 1).

In winter, the SSK was characterized by the presence of several granules spread throughout the cytoplasm of the cells, wider tubule lumen and basal nuclei (Stage 2, Fig. 4B). Subsequently, on late winter–spring, SSK cells showed a large amount of granules in the cytoplasm and secretory vesicles apically positioned prepared to be released into the lumen of the SSK tubule (Stage 3; Fig. 4C).

The secretory phases occurred in the spring and extended to summer. At this period, the number of serous granules in the SSK cytoplasm increased, causing a hypertrophy in the epithelium and a decrease in luminal volume. Secretory granules were observed filling the apical and basal portions of the SSK cells and in the lumen of the tubule (Stage 4; Fig. 4D). Secretory activity was reduced in late summer, when granules were seen aggregated only towards the apical end of the cells, when secretory phase was resumed again.

Comparison of SSK and spermatogenic cycles

The SSK demonstrated maximum activity in winter, preceding the spermatogenic peak (late spring–summer). Thus, the correlation analysis confirmed that SSK and spermatogenic cycles exhibited negative relationship between the following measurements: TV versus kidney length ($r = -0.80$, $P < 0.05$, Fig. 5A), seminiferous tubule diameter versus SSK tubule diameter ($r = -0.82$, $P < 0.05$, Fig. 5B), and seminiferous epithelial height versus SSK epithelial height ($r = -0.87$, $P < 0.05$, Fig. 5C).

Size–fecundity relationships

Body mass had a positive and significant effect on TV ($r = 0.79$, $P < 0.0001$). BM had a significant relationship with TV ($R^2 = 0.61$, $F = 95.71$, $P < 0.0001$), but not with testicular mass ($R^2 = -0.011$, $F = 0.35$, $P = 0.56$, Fig. 6A). The overall mean of the GSI for males was 1.13 ± 0.22 (range 0.11–3.8). The male SVL and reproductive effort (GSI) were positively correlated ($r = 0.30$, $P < 0.05$, Fig. 6B). The smallest mature male presented 61.1 g of mass, SVL of 440 mm and total length of 690 mm.

Discussion

P. patagoniensis exhibits spatial germ cell development of the testes, which is a common strategy seen within most of the amniotes (Granados-González *et al.* 2015). In this kind of

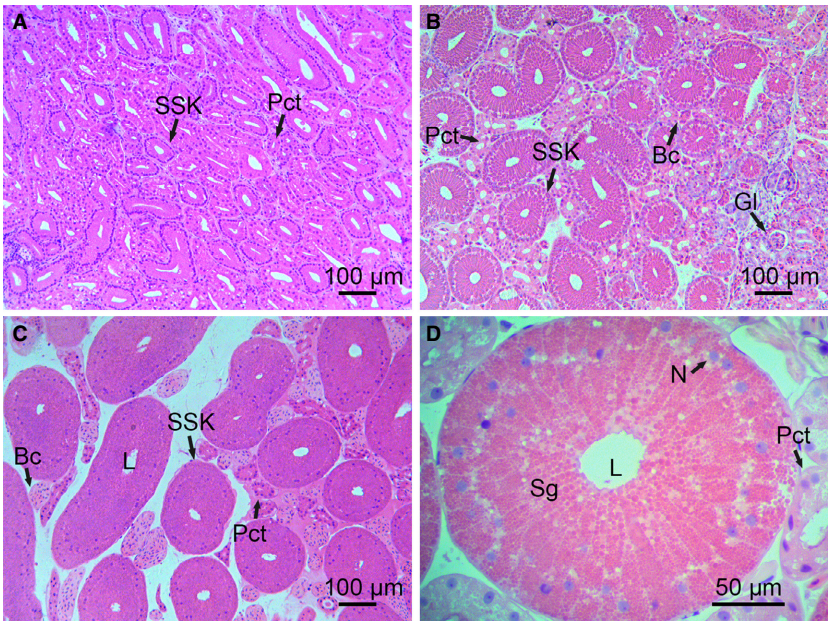

Fig. 4—Histology of the kidney of *P. patagoniensis* males from south Brazil. —**A.** SSK regressed (late summer–early autumn), —**B.** SSK in hypertrophy with granules evident throughout the cytoplasm (winter), —**C.** SSK with secretory granules visible in the apical region of the cytoplasm (late winter–spring), —**D.** detail of SSK cells full of maximum density of secretory granules within the cytoplasm (late spring–summer). Bc, blood capillaries; Sg, secretory granules; Gl, kidney glomerulum; L, lumen; Pct, proximal convoluted tubule; SSK, sexual segment of the kidney.

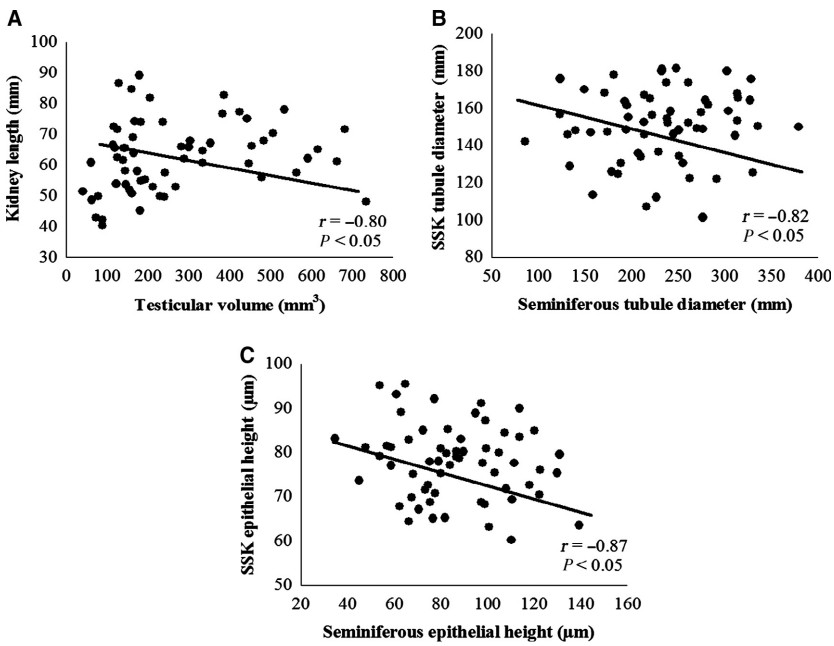

Fig. 5—Relationship between —**A.** TV and kidney length, —**B.** seminiferous tubule diameter versus SSK tubule diameter and —**C.** seminiferous epithelial height versus SSK epithelial height for *P. patagoniensis* males in south Brazil.

gamete production, the germ cell populations are seen layered together in the seminiferous epithelium and progress uniformly through the phases of spermatogenesis as a single cohort (Gribbins 2011).

The spermatogenic cycle of *P. patagoniensis* was extended but not continuous throughout the year; the spermatogenesis occurred during late autumn–winter (June–September), and spermiogenesis was completed in spring–summer (October–March; Table 6). The regression of the seminiferous epithelium occurred only for a short period in autumn (May). In other words, the timing of seasonal spermatogenesis was interleaved with quick episodes of testicular total regression. This phase is commonly seen in snakes from temperate areas. Despite this fact, the neotropical snakes *Crotalus durissus* (Barros *et al.* 2012) and *Bothrops erythromelas* (Barros *et al.* 2014*b*) also exhibit a phase of testicular regression. The main cause for testicular regression in temperate species is the temperature, while in tropical species is the rainfall (Krohmer and Lutterschmidt 2011). However, in subtropical environments of south Brazil, the seasonal changes in temperature resemble temperate regions.

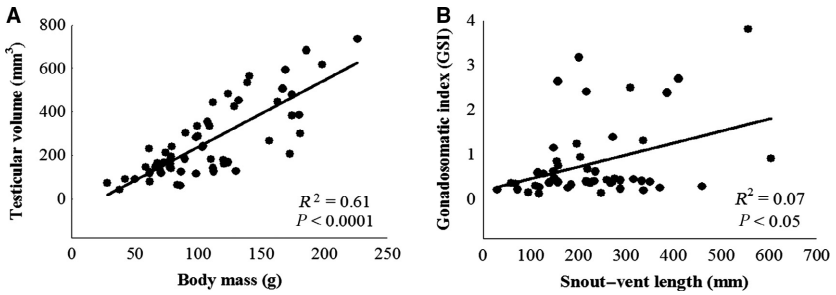

Fig. 6—Relationship between —**A.** body mass and TV and —**B.** body size and gonadosomatic index for *P. patagoniensis* males in south Brazil.

Table 5 Stages of the SSK cycle in *P. patagoniensis* in south Brazil

Stages	Months of occurrence
(0) SSK not hypertrophied	March–April
(1) SSK hypertrophied with a few granules	May–June
(2) SSK cytoplasm full of secretory granules	July–September
(3) SSK secretory granules apically in the cytoplasm	September–November
(4) Maximum density of SSK secretory granules	December–February

Adapted from Krohmer *et al.* (2004).

Table 6 Phases of the annual reproductive cycle of male *P. patagoniensis* in south Brazil

	Summer	Autumn	Winter	Spring
Testes hypertrophy (volume)	■	■		■
GSI increases				■
Spermatogenesis				■
Spermiogenesis	■			
Seminiferous tubule hypertrophy	■			
Leydig cell activity				■
Kidney hypertrophy (length)	■	■		
SSK hypertrophy			■	
Testicular regression		■		
Mating	■			■

Shades area correspond to the season of activity.

Therefore, the male reproductive cycle of *P. patagoniensis* may be classified according to Mathies (2011): at the individual level, males exhibit a discontinuous cyclical reproduction, with reproductively quiescence of gonads in late autumn. According to this classification, at the population level, the reproductive cycle is defined by the synchrony of reproduction observed within the male individuals. In this case, the cycles of individuals do not progress in close synchrony, but tend to be more coincident at a particular time of year (spring–summer), identifiable as a peak period of reproduction. Because of this, at the population level, we classify the reproductive cycle of *P. patagoniensis* as seasonal semisynchronous, with most of the individuals showing a reproductive peak in spring–summer. The reproductive cycle is complexly influenced by intrinsic and environmental factors. In this sense, cold winter temperatures are considered the main restriction on the duration of the reproductive season, because only in summer the temperature and insolation are high enough to allow the embryonic development (Gregory 2009).

The ductus deferens demonstrated no seasonal variation in diameter and presented spermatozoa in the lumen throughout the year. Hence, the ductus deferens is probably playing a role of long-term sperm storage, which points out to the fact that males may be capable of copulating at any time (Almeida-Santos and Salomão 1997). In this case, mating may occur independently of spermatogenesis because the male can adjust to the female reproductive cycle, being able to provide viable spermatozoa during early spring matings. The sperm storage is a synapomorphy of squamata that contributed to their successful invasion of the terrestrial environment (Gribbins *et al.* 2005).

In *P. patagoniensis*, a peak in the GSI occurred during spring; nevertheless, TV appeared to be almost constant from spring to autumn, being quiescent only in winter. Meanwhile, the seminiferous tubules showed an increase in the tubule diameter and epithelial height during spring–summer. These events coincide with the time of highest activity of the testes in the spermiogenesis stage. Thus, separately, neither GSI nor testes volume should be considered a reliable indicator of spermatogenesis in *P. patagoniensis*. The histological analysis is crucial to determine the reproductive cycle (Mathies 2011).

The Leydig cells, which bordered the basal lamina of the germinal epithelium, showed an increase in the nuclear diameter in spring, typical of the mating season (Rojas *et al.* 2013). Hypertrophy in the Leydig cells nuclear diameter is associated with its endocrine activity peak of testosterone synthesis, coinciding with the spermatogenesis (Volsøe 1944). The increase in steroidogenic activity is closely associated with the transformation of peritubular cells from fibroblast to myoid-like appearance. This phenomenon suggests the involvement of Leydig cells in the sperm transport, aiding the contraction of seminiferous tubules (Kumar *et al.* 2011).

The SSK cycle follows a temporal strategy (Aldridge *et al.* 2011), in which synthesis and secretory phases are separated into non-reproductive and reproductive seasons, respectively. The increase in the tubular diameter of SSK and epithelium height during winter occurred due to the height of the secretory cells that were full of secretory granules in those seasons (Sever

et al. 2007). Probably, the activity peak in the winter prepares the SSK for its immediate use in the mating season (spring–summer; Aldridge *et al.* 2011; Rojas *et al.* 2013). The presence of secretory granules in the SSK tubule lumen in spring occurred associated with the mating season. After mating in spring–summer, the SSK of the *P. patagoniensis* regressed.

The smaller activity period of the spermatogenic epithelium (winter) occurred simultaneously to the period of the most activity of the SSK epithelium. The SSK secretory cycle and its relationship with the testicular cycle indicate that the development of the SSK represents a substantial energetic cost that may be equal to or greater than the cost of development of the testes (Aldridge *et al.* 2011). So we assume that SSK is also an energetically expensive structure of vital importance to reproductive biology of the *P. patagoniensis*.

Size–fecundity relationships and reproductive investment may vary even among closely related species (Shine 1988). In the case of *P. patagoniensis*, body mass had a positive relationship with TV, but not with testicular mass. In addition, the male SVL and reproductive effort (GSI) were positive correlated. The testes size is commonly hypertrophied in species with sperm competition and/or in populations with high prey availability (Møller and Briskie 1995). In this context, ecologists have classically considered that the reproductive investment is extremely different between the sexes. Even though, reproduction is energetically expensive for both males and females (Olsson *et al.* 1997). Despite this, the energy costs of sperm production are just a small component of reproduction when compared with the energy costs of reproductive behaviours (Winne and Hopkins 2006).

The *P. patagoniensis* is a widely distributed species, although information on its reproductive cycle is not available for males from other populations. But, taking account of the female reproductive cycle (Fowler *et al.* 1998; Pontes 2007; López and Giraudo 2008; Rojas *et al.* 2015), all the populations analysed show seasonal reproduction and some variation is recognized just in the time of reproductive events, such as vitellogenesis, ovulation, oviposition and hatchling of newborns. Interpopulational variation in reproductive traits is often attributed to different climatic conditions (Pizzatto and Marques 2006; Mathies *et al.* 2010), while the absence of variability in reproductive patterns among populations that live under different climatic conditions, like observed to *P. patagoniensis*, may be attributed to phylogenetic conservatism (James and Shine 1988). *P. patagoniensis* females from south Brazil show seasonal reproductive cycle with ovulation in spring and oviposition in summer (Pontes 2007; López and Giraudo 2008), but clearly occurs some asynchrony with the male cycle. Because of this, the ability to sperm storage in the male ductus deferens and in the female oviduct (Rojas *et al.* 2015) evolved to allow the reproductive success of *P. patagoniensis*.

In conclusion, our results indicate that *P. patagoniensis* males have a seasonal pattern of reproduction in subtropical Brazil. The use of morpho-anatomical analysis allowed to infer a reproductive peak, but we presented evidence to support the argument that the histology was essential to reveal the precise time of some reproductive events, such as sperm storage. After all, conclusions about reproductive patterns of squamate reptiles based just on morpho-anatomical methods are not very reliable. To analyse completely the male reproductive strategies of *P. patagoniensis*, more studies using electron microscopy and histochemistry techniques are required, as well as laboratory experiments. Therefore, we expect that this study increases the knowledge about reproduction of neotropical squamate reptiles and stimulates further research.

Acknowledgements

We are grateful to Dr C. A. Rojas and Dr P. A. Hartmann for providing comments on an earlier version of this manuscript, and to G. M. F. Pontes for allowing us to examine specimens under their care. We also thank the editor and anonymous reviewers for their helpful comments and suggestions.

References

Aldridge, R. D., Goldberg, S. R., Wisniewski, S. S., Bufalino, A. P. and Dillman, C. B. 2009. The reproductive cycle and estrus in the colubrid snakes of temperate North America. – *Contemporary Herpetology* **4**: 1–31.

Aldridge, R. D., Jellen, B. C., Siegel, D. S. and Wisniewski, S. S. 2011. The sexual segment of the kidney. In: Aldridge, R. D. and Sever, D. M. (Eds): Reproductive Biology and Phylogeny of Snakes, pp. 477–509. Science Publishers, Enfield.

Almeida-Santos, S. M. and Salomão, M. G. 1997. Long–term sperm storage in the female Neotropical Rattlesnake *Crotalus durissus*. – *Japanese Journal of Herpetology* **17**: 46–52.

Almeida-Santos, S. M. and Salomão, M. G. 2002. Reproduction in neotropical pitvipers, with emphasis on species of the genus *Bothrops*. In: Schuett, G., Hoggren, M., Douglas, M. E. and Greene, H. W. (Eds): Biology of the Vipers, pp. 445–462. Eagle Mountain, Carmel, IN.

Almeida-Santos, S. M., Laporta-Ferreira, I. L., Antoniazzi, M. M. and Jared, C. 2004. Sperm storage in males of the snake *Crotalus durissus terrificus* (Crotalinae: Viperidae) in southeastern Brazil. – *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **139**: 169–174.

Almeida-Santos, S. M., Braz, H. B., Santos, L. C., Sueiro, L. R., Barros, V. A., Rojas, C. A. and Kasperoviczus, K. N. 2014. Biologia reprodutiva de serpentes: Recomendações para a coleta e análise de dados. – *Herpetologia Brasileira* **3**: 14–24.

Alvares, C. A., Stape, J. L., Sentelhas, P. C., Gonçalvex, J. L. M. and Sparovek, G. 2013. Köppen's climate classification map for Brazil. – *Meteorologische Zeitschrift* **22**: 711–728.

Barros, V. A., Sueiro, L. R. and Almeida-Santos, S. M. 2012. Reproductive biology of the neotropical rattlesnake *Crotalus durissus* from northeastern Brazil: A test of phylogenetic conservatism of reproductive patterns. – *The Herpetological Journal* **22**: 97–104.

Barros, V. A., Rojas, C. A. and Almeida-Santos, S. M. 2014a. Is rainfall seasonality important for reproductive strategies in viviparous Neotropical pit vipers? A case study with *Bothrops leucurus* from the Brazilian Atlantic Forest. – *The Herpetological Journal* **24**: 69–77.

Barros, V. A., Rojas, C. A. and Almeida-Santos, S. M. 2014b. Reproductive biology of *Bothrops erythromelas* from the Brazilian Caatinga. – *Advances in Zoology* **2014**: 1–11.

Bishop, J. E. 1959. A histological and histochemical study of the kidney tubule of the common garter snake, *Thamnophis sirtalis*, with special reference to the sexual segment in the male. – *Journal of Morphology* **104**: 307–357.

Braz, H. B., Kasperoviczus, K. N. and Almeida-Santos, S. M. 2014. Reproductive ecology and diet of the fossorial snake *Phalotris lativittatus* in the Brazilian Cerrado. – *The Herpetological Journal* **24**: 49–57.

Brown, G. P. and Shine, R. 2006.Why do most tropical animals reproduce seasonally? Testing hypotheses on an Australian snake. – *Ecological Society of America* **87**: 133–143.

Clesson, D., Bautista, A., Baleckaitis, D. D. and Krohmer, R. W. 2002. Reproductive biology of male eastern garter snakes (*Thamnophis sirtalis sirtalis*) from a denning populations in Central Wisconsin. – *American Midland Naturalist* **147**: 376–386.

Fowler, I. R. and Salomão, M. G. 1994. A study of sexual dimorphism in six species from the colubrid snake genus *Philodryas*. – *The Snake* **26**: 117–122.

Fowler, I. R., Salomão, M. G. and Jordão, R. S. 1998. A description of the female reproductive cycle in four species from the neotropical colubrid snake *Philodryas* (Colubridae: Xenodontinae). – *The Snake* **28**: 71–78.

Goldberg, S. R. and Parker, W. S. 1975. Seasonal testicular histology of the colubrid snakes, *Masticophis taeniatus* and *Pituophis melanoleucus*. – *Herpetologica* **31**: 317–322.

Granados-González, G., Rheubert, J. L., Villagrán-SantaCruz, M., González-Herrera, M. E., Dávila-Cedillo, J. V., Gribbins, K. M. and Hernández-Gallegos, O. 2015. Male reproductive cycle in *Aspidoscelis costata costata* (Squamata:Teiidae) from Tonatico, Estado de México, México. – *Acta Zoologica* **96**: 108–116.

Gregory, P. T. 2009. Northern lights and seasonal sex: The reproductive ecology of cool–climate snakes. – *Herpetologica* **65**: 1–13.

Gribbins, K. M. 2011. Reptilian spermatogenesis. A histological and ultrastructural perspective. – *Spermatogenesis* **1**: 250–269.

Gribbins, K. M., Happ, C. S. and Sever, D. M. 2005. Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). V. The temporal germ cell development strategy of the testis. – *Acta Zoologica* **86**: 223–230.

James, C. and Shine, R. 1988. Life history strategies of Australian lizards: A comparison between the tropics and the temperate zone. – *Oecologia* **75**: 307–316.

Krohmer, R. W. and Lutterschmidt, D. I. 2011. Environmental and neuroendocrine control in snakes. In: Aldridge, R. D. and Sever, D. M. (Eds): Reproductive Biology and Phylogeny of Snakes, pp. 289–346. Science Publishers, Enfield.

Krohmer, R. W., Martinez, D. and Mason, R. T. 2004. Development of the renal sexual segment in inmature snakes: Effect of sex steroid hormones. – *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **139**: 55–64.

Kumar, S., Roy, B. and Umesh, R. 2011. Hormonal regulation of testicular functions in reptiles. In: Norris, D. and Lopez, K. (Ed.): Hormones and Reproduction of Vertebrates–Reptiles, pp. 89–115. Academic Press, San Diego, CA.

Liang, G., Liu, Q., Yu, H. and Wang, Q. 2011. Histological and immunocytochemical study of deferens ducts in the Chinese rat snake (*Zaocys dhumnades*). – *Zoological Research* **32**: 663–669.

López, M. S. and Giraudo, A. R. 2008. Ecology of the snake *Philodryas patagonensis* (Serpentes: Colubridae) from Northeast Argentina. – *Journal of Herpetology* **42**: 474–480.

Mathies, T. 2011. Reproductive cycles of tropical snakes. In: Aldridge, R. D. and Sever, D. M. (Eds): Reproductive Biology and Phylogeny of Snakes, pp. 511–550. Science Publishers, Enfield.

Mathies, T., Cruz, J. A., Lance, V. A. and Savidge, J. A. 2010. Reproductive biology of male brown tree snakes (*Boiga irregularis*) on Guam. – *Journal of Herpetology* **44**: 209–221.

Møller, A. P. and Briskie, J. V. 1995. Extra–pair paternity, sperm competition and the evolution of testis size in birds. – *Behavioral Ecology and Sociobiology* **36**: 35–365.

Nilson, G. and Andrén, C. 1982. Function of renal sex secretion and male hierarchy in the adder, *Vipera berus*, during reproduction. – *Hormones and Behavior* **16**: 404–413.

Olsson, M., Madsen, T. and Shine, R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, Vipera berus. – *Proceedings of the Royal Society of London B: Biological Sciences* **264**: 455–459.

Peters, J. A. and Orejas-Miranda, B. 1970. Catalogue of the neotropical Squamata. Part I – Snakes. – *United States National Museum Bulletin* **297**: 1–347.

Pizzatto, L. and Marques, O. A. V. 2006. Interpopulational variation in reproductive cycles and activity of the water snake *Liophis miliaris* (Colubridae) in Brazil. – *The Herpetological Journal* **16**: 353–362.

Pleguezuelos, J. M. and Feriche, M. 1999. Reproductive ecology of the horseshoe whip snake (*Coluber hippocrepis)* in the Southeast of the Iberian Peninsula. – *Journal of Herpetology* **33**: 202–207.

Pontes, G. M. F. 2007. História natural de *Philodryas patagoniensis* (Serpentes: Colubridae) no litoral do Rio Grande do Sul, Brasil. PhD Thesis. Pontífica Universidade Católica, Porto Alegre, Rio Grande do Sul, Brazil,

Resende, F. C. and Nascimento, L. B. 2015. The female reproductive cycle of the neotropical snake *Atractus pantostictus* (Fernandes and Puorto, 1993) from South-eastern Brazil. – *Anatomia Histologia e Embryologia* **44**: 225–235.

Rojas, C. A., Barros, V. A. and Almeida-Santos, S. M. 2013. The Reproductive Cycle of the Male Sleep Snake *Sibynomorphus mikanii* (Schlegel, 1837) in Southeastern Brazil. – *Journal of Morphology* **274**: 215–228.

Rojas, C. A., Barros, V. A. and Almeida-Santos, S. M. 2015. Sperm storage and morphofunctional bases of the female reproductive tract of the snake *Philodryas patagoniensis* from southeastern Brazil. – *Zoomorphology* **134**: 1–10.

Saint Girons, H. 1982. Reproductive cycles of male snakes and their relationships with climate and female reproductive cycles. – *Herpetologica* **38**: 5–16.

Seigel, R. A. and Ford, N. B. 1987. Reproductive ecology. In: Seigel, R. A., Collins, J. T. and Novak, S. S. (Eds): Snakes: Ecology and Evolutionary Biology, pp. 210–252. Macmillan Publishing, New York, NY.

Sever, D. M. 2004. Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). IV. Occurrence of an ampulla ductus deferentis. – *Journal of Morphology* **262**: 714–730.

Sever, D. M. and Hamlett, W. C. 2002. Female sperm storage in reptiles. – *Journal of Experimental Zoology* **292**: 187–199.

Sever, D. M., Siegel, S. D., Bagwill, A., Eckstut, E. M., Alexander, L., Camus, A. and Morgan, C. 2007. Renal sexual segment of the cottonmouth snake *Agkistrodon piscivorous* (Reptilia, Squamata, Viperidae). – *Journal of Morphology* **269**: 640–653.

Shine, R. 1977. Reproduction in Australian elapid snakes. II. Testicular cycles and mating seasons. – *Australian Journal of Zoology* **25**: 647–653.

Shine, R. 1988. Constraints on reproductive investment: A comparison between aquatic and terrestrial snakes. – *Evolution* **42**: 17–27.

Shine, R., Olsson, M. M. and Mason, R. T. 2000. Chastity belts in gartersnakes: The functional significance of mating plugs. – *Biological Journal of the Linnean Society* **70**: 377–390.

Statsoft, Inc. 2011. Statistica for Windows (data analysis software system), version 10.

Systat, Inc. 2011. Sigmaplot for Windows, version 12.0. Systat Software.

Vitt, L. J. and Caldwell, J. P. 2014. Reproduction and life histories. In: Vitt, L. J. and Caldwell, J. P. (Eds): Herpetology: An Introductory Biology of Amphibians and Reptiles, 4th edn, pp. 117–155. Academic Press, San Diego, CA.

Volsøe, H. 1944. Structure and seasonal variation of the male reproductive organs of *Vipera berus* (L.). – *Spolia Zoologica Musei Hauniensis* **5**: 1–157.

Winne, C. T. and Hopkins, W. A. 2006. Influence of sex and reproductive condition on terrestrial and aquatic locomotor performance in the semi–aquatic snake *Seminatrix pygaea*. – *Functional Ecology* **20**: 1054–1061.

Appendix 1. Voucher specimens of *P. patagoniensis* males analysed 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)

Macroscopic and anatomical analysis (n = 85): ZUFSM0352, 0353, 0356, 0391, 0482, 0543, 0640, 0649, 0689, 0696, 0794, 0835, 0842, 0968, 0978, 0990, 1117, 1303, 1419, 1430, 1435, 1437, 1455, 1601, 1675, 1684, 1703, 1725, 1748, 1853, 2229, 2347, 2427, 2430, 2470, 2566, 2633, 2737, 2743, 2746, 2766, 2913, 3004, and 3041; MCP02503, 05499, 05753, 05759, 06474, 10992, 11043, 12525, 14263, 14277, 14336, 14482, 14774, 14878, 14897, 15679, 15680, 15681, 15682, 15847, 16722, 16946, 16947, 16948, 16949, 16951, 16961, 16962, 16963, 16964, 16969, 16970, 16971, 17018, 17859, 17955, 17966, 17988, 17989, 18065, and 18368.

Histology (n = 61): ZUFSM0353, 0356, 0482, 0640, 0649, 0689, 0696, 0794, 0835, 0842, 0968, 0978, 0990, 1117, 1419, 1430, 1435, 1437, 1455, 1601, 1675, 1684, 1725, 1748, 1853, 2229, 2347, 2430, 2470, 2566, 2743, 2746, 2766, and 2913; MCP14277, 14482, 14878, 14897, 15679, 15681, 15682, 15847, 16722, 16946, 16947, 16948, 16949, 16951, 16961, 16962, 16963, 16964, 16969, 16970, 16971, 17018, 17859, 17955, 17966, 17988, and 17989.

