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Asynchronic steroid activity of Leydig and Sertoli cells related to spermatogenic and testosterone cycle in *Phymaturus antofagastensis*

J.M. Boretto [a,*], N.R. Ibargüengoytía [a], G.A. Jahn [b], J.C. Acosta [c], A.E. Vincenti [d], M.W. Fornés [d]

[a] *INIBIOMA (Universidad Nacional del Comahue – CONICET), Quintral 1250, Bariloche, 8400 Río Negro, Argentina*
[b] *LARLAC-IMBECU, CC 855, 5500, Mendoza, Argentina*
[c] *Departamento de Biología e Instituto y Museo de Ciencias Naturales, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de San Juan, Avenida Ignacio de la Roza y Meglioli, San Juan (5400), Argentina*
[d] *Laboratorio de Investigaciones Andrológicas de Mendoza (LIAM) del Instituto de Histología y Embriología de Mendoza, Facultad de Ciencias Médicas, UNCuyo-CCT, Mendoza – CONICET, Argentina*

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ABSTRACT

The severe environments where *Phymaturus* lizards inhabit in the Andes highlands and in Patagonia, Argentina, impose restrictions on their reproduction, offering a framework for the development of life history strategies to overcome hard weather conditions. Among them, prolonged female cycles, asynchrony between sexes in receptivity, and sperm storage in males, were described. Asynchrony in the reproductive timing between males and females is a consequence of different energy requirements for gametogenesis, and often imply the existence of cellular mechanisms to enhance fertilization, such as the asynchronic steroid synthesis between testicular compartments, allowing gametogenesis independently of mating. In the present study ultrastructural and hormone assays were combined for the first time in liolaemids. Specifically, morphological features of steroid activity in Leydig and Sertoli cells, and serum testosterone concentrations have been studied in the lizard *Phymaturus antofagastensis*. Leydig and Sertoli cells presented morphological features characteristic of steroid synthesis during the spermatogenesis, and evident asynchronic steroid production between testicular compartments. Active Sertoli cells and inactive Leydig cells were observed in spring and autumn, while in mid-summer their steroid activity was synchronic in coincidence with maximal abundance of spermatozoa in epididymis. Serum testosterone concentration was at its maximum in mid-summer (126–230 ng ml^{-1}), and minimum in late spring (4–24 ng ml^{-1}) and early autumn (2–17 ng ml^{-1}). In view of these results, *P. antofagastensis* males show an original approach to adjust their reproductive activity to physiological and environmental constraints at high latitudes and altitudes in the Andean highlands of Argentina.

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1. Introduction

The study of the ecophysiological responses to severe climatic conditions in reptiles inhabiting at high latitudes and altitudes, offers an opportunity to understand reproductive and evolutive diversity (Tinkle and Gibbons, 1977; Shine, 1985; Blackburn, 1993). Reptiles that inhabit cold climates are constrained by the need to reproduce during short activity seasons since they hibernate long periods when physiological activity is almost nil (Saint Girons, 1985; Gotthard, 2001). Furthermore, several species from harsh environments have shown different adaptations to develop successful reproduction in the short span from spring to autumn developing reproductive styles that favor male/female encounters, nourishment by viviparity, birth in warmer periods of the activity seasons, and larger offspring (e.g. Bull and Shine, 1979; Saint Girons, 1985; Bonnet et al., 1992; Cree et al., 1992; Cree and Guillette, 1995; Olsson and Shine, 1999; Wapstra et al., 1999; Edwards et al., 2002; Ibargüengoytía and Casalins, 2007).

The genus *Phymaturus* represents an appealing model for the study of life history adaptations to harsh environments, because a significant part of the year is unsuitable for growth and reproduction, as a consequence of the lower mean annual temperatures. The genus *Phymaturus* which is entirely viviparous and mostly herbivorous comprises 23 species distributed in cool and harsh environments of the Andean and Patagonian habitats in Argentina and Chile (Cei, 1986, 1993; Lobo and Quinteros, 2005; Scolaro and Ibargüengoytía, 2007; Pincheira-Donoso et al., 2008). Reproductive biology of the genus *Phymaturus* has shown the existence of biennial female reproductive cycles caused by either prolonged pregnancy (*Phymaturus vociferator*, Habit and Ortiz, 1996; previously called *Phymaturus flagellifer*, see Pincheira-Donoso, 2004), pro-

* Corresponding author.
E-mail addresses: jboretto@crub.uncoma.edu.ar (J.M. Boretto), norai@crub.uncoma.edu.ar (N.R. Ibargüengoytía), gjahn@mendoza-conicet.gov.ar (G.A. Jahn), jcacostasanjuan@gmail.com (J.C. Acosta), mfornes@fcm.uncu.edu.ar (M.W. Fornés).

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longed vitellogenesis (*Phymaturus tenebrosus*, Ibargüengoytía, 2004; *Phymaturus antofagastensis*, Boretto and Ibargüengoytía, 2006; *Phymaturus punae*, Boretto et al., 2007; *Phymaturus cf. palluma*, Cabezas Cartes, 2008) or annual to biennial cycles by years of skipped reproduction (Boretto and Ibargüengoytía, 2009). As a consequence of the prolonged female cycles, the availability of reproductive females in the entire population, is reduced, influencing male reproductive cycles (Ibargüengoytía, 2004; Boretto and Ibargüengoytía, 2006, 2009; Boretto et al., 2007; Cabezas Cartes, 2008).

Males of the *Phymaturus* genus, instead, seem to try to adapt to female cycles developing several different strategies such as prenuptial cycles with spermatogenesis from spring to mid-summer, in synchrony with the follicular development in females (*P. vociferator*, Habit and Ortiz, 1996; *P. punae*, Boretto et al., 2007); postnuptial cycles showing spermatogenesis in mid-summer and spermiogenesis from early autumn to the next spring, when mating and ovulation also occur (*P. tenebrosus*, Ibargüengoytía, 2004; *Phymaturus zapalensis*, Boretto and Ibargüengoytía, 2009); or continuous cycles with sperm availability throughout the activity season (*P. antofagastensis*, Boretto and Ibargüengoytía, 2006; *P. cf. palluma*, Cabezas Cartes, 2008).

The asynchrony between males and females in the timing of reproduction has been found to be accompanied by the asynchronic steroidogenic activity in Leydig and Sertoli cells (Callard et al., 1976; Lofts and Tsui, 1977; Callard and Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988; Mahmoud and Licht, 1997). The Sertoli cells appear to have the potential to synthesize a variety of steroids, their contribution to the circulating androgen pool is minimal, and restricted to the seminiferous tubules influencing functions in the synchronization and maintenance of spermatogenesis (Callard et al., 1976; Bardin et al., 1988; Dubois et al., 1988). Instead, androgens produced by the Leydig cells are considered to enter the peripheral circulation influencing courting and mating behaviors (Callard et al., 1976; Callard and Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988). These differences in the testosterone bioavailability and distribution between Sertoli and Leydig cells allow the inter-independence of spermatogenesis and mating (Mahmoud et al., 1985; Dubois et al., 1988; Mahmoud and Licht, 1997).

The steroidogenic activity has been characterized by histochemical and ultrastructural studies, mainly detecting the presence of Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD), and occasionally 17β-HSD. The secretion of hormones may be detected by the marked development of smooth endoplasmic reticulum (SER), the presence of mitochondria with tubular cristae and a reduction of cytoplasmic lipid droplets (e.g. Lofts and Tsui, 1977; Mori, 1984; Mahmoud et al., 1985; Dubois et al., 1988; Mahmoud and Licht, 1997; Ibargüengoytía et al., 1999; Ferreira and Dolder, 2003). The accumulation of cholesterol rich in lipid droplets in Leydig and Sertoli cells has also been recognized as an indicator of steroidogenic inactivity, and their gradual decrease of active conversion of cholesterol to androgens (Callard et al., 1976; Lofts and Tsui, 1977; Mahmoud et al., 1985; Dubois et al., 1988). In Sertoli cells, the presence of 3β-HSD varied according to the season in turtles (Callard and Ho, 1980; Dubois et al., 1988) and the presence of organelles related to steroidogenic activity in Sertoli cells has been documented in the lizard *Liolaemus darwini* (Gutierrez and Yapur, 1983), and in the snake *Eryx jayakari* (Al-Dokhi et al., 2004), although the possible steroid synthesis and its relationship with spermatogenesis was not discussed.

There are no studies at present, of the steroidogenic dynamic activity in the testicular compartments related to the reproductive endocrinology in the genus *Phymaturus*. There is only one study of histological characterization of the interstitial tissue that describes the testosterone cycle in Liolaemidae; the study was done on the viviparous *Liolaemus gravenhorsti*. This species shows seasonal variations in Leydig cell morphology related to morphological changes in the seminiferous epithelium, and fluctuations of testosterone concentrations (Leyton et al., 1977).

The aim of this study is to establish the role of Sertoli and Leydig cells during the spermatogenic cycle of *P. antofagastensis*, and to characterize the testosterone cycle and the mating period of the species. *P. antofagastensis* is restricted to the high mountains of the Department of Antofagasta de la Sierra and Tinogasta, in Catamarca Province, located at 4000 m above sea level (Cei, 1993). In this locality, the climate is cool and semiarid with broad daily thermal amplitude, high solar radiation and irregular precipitation. *P. antofagastensis* shows biennial female reproductive cycles, and males show intrasexual asynchrony in the spermatogenic stages and storage of spermatozoa in the epididymis regardless of the season, of the presence of active spermatogenesis or of gonadal involution, increasing the chances of fertilizing females at any time (Boretto and Ibargüengoytía, 2006). The characteristics of the reproductive cycles of *P. antofagastensis* lead to an asynchrony between sexes in receptivity, to the existence of sperm storage observed only in males (Boretto and Ibargüengoytía, 2006; Boretto, 2009), and raise questions about the male sex steroid cycle and the dynamics of steroidogenesis during the active season.

Herein we characterized the seasonal variations in the functional state of the testicular compartment by ultrastructural studies of organelles involved in testosterone synthesis and determined the variations of serum testosterone levels during the reproductive season. These results are discussed in reference to the hypothesis that testosterone produced by Leydig cells is related mainly to mating behavior, while testosterone produced by Sertoli cells trigger spermatogenesis and sperm maturation allowing independence in the timing of these two events.

2. Materials and methods

2.1. Specimens and environment

Male specimens of *P. antofagastensis* ($n = 13$) were collected during late spring (December 2003), in a location known as "Paso San Francisco" in the northeast of Catamarca Province, Argentina (27°02′00″S; 68°04′11″W, 4200 m), and during mid-summer and early autumn (February–March 2005, respectively), at 150 km near to Fiambalá city (Catamarca; 27°72′S; 68°15°, 4200 m). Land is characterized by high plains known as the "Puna" or "Altiplano" of Andean Mountains in central and northern Argentina. The soil is sandy and rocky and vegetation is shrub steppe, mostly *Poa* and *Festuca* grasses (Cabrera, 1976). The climate is cool with broad daily thermal amplitude, high solar radiation and irregular precipitation occurring mostly in summer (103–324 mm). Maximum and minimum mean annual temperatures are between 21 and −3 °C, respectively. Maximum and minimum absolute temperatures are approximately between 30 and −18 °C, respectively (Cabrera, 1994). In winter there is an impenetrable snow barrier (Belver, pers. com.).

2.2. Blood samples

Lizards were weighed (g), anesthetized with an intraperitoneal dose of sodium thiopental (0.03 mm^3/10 g of body weight), and immediately a blood sample was taken from the tail artery with an insulin syringe (1 mm^3). Blood samples were clotted in a microtube at ambient temperature, spun at 1500 rpm for 15 min, and stored at −20 °C until their analysis.

2.3. Tissue samples, SVL and gonadal index

After blood extraction, males were killed by a lethal i.p. dose of sodium thiopental. The left testis and epididymis were isolated and fixed by immersion in a fixative solution. This solution was prepared with 4% glutaraldehyde (v/v), 2% of freshly prepared paraformaldehyde (v/v) in saline phosphate buffer (Saline phosphate buffer was prepared diluting a Sigma tablet in 200 ml of bi distilled water. Working concentration: 0.01 M phosphate buffer, 0.137 M NaCl and 0.0027 M KCl, pH 7.4). Males were then kept in the fixative Bouin's solution for 24 h and preserved in 70% ethanol until used.

Snout-vent length (SVL) and antero-posterior diameter of each right testis was measured with a Vernier caliper on a camera lucida scheme (error was ±0.1 mm). Right testis and epididymis of all males were removed and processed using routine histological techniques.

2.4. Histology analysis – light microscopy

Sections (7 μm) of each right testis and epididymis were stained with classical Hematoxylin and Eosin method and examined with an Olympus BX40 microscope following the description of Mayhew and Wright (1970). Cell associations (Stages of seminiferous epithelium cycle) were defined as (1) only spermatogonia, (2) primary or/and secondary spermatocytes, (3) spermatids, (4) spermatozoa in tubular lumen and in the epididymis, and (5) regression with scarce spermatozoa in tubular lumen and spermatozoa in epididymis. The development of interstitial tissue lying between seminiferous tubules was qualitatively classified as scarce, medium, or highly developed.

2.5. Testosterone measurements

Frozen serum samples were defrosted, and aliquots (50 μl) were used to determine Testosterone concentration in sera extracted with ethanol 100%. Serum aliquots (50 μl) were mixed with 500 μl ethanol 100% and the precipitated proteins separated by centrifugation at 1000×*g* for 15 min. The precipitate was re-extracted with 250 μl ethanol 100%, centrifuged and the pooled supernatants evaporated overnight at 36 °C. The residues were dissolved 150 μl in PBS gelatin by incubation for 60 min at 37 °C in a Dubnoff shaker. Aliquots (25 μl) were used for testosterone determination by RIA. Radioimmunoassay was performed using the commercial kit DSL-4100 Testosterone RIA.

2.6. Ultrastructural analyses

Samples for ultrastructural studies ($n = 12$) were washed in PBS postfixed in 1% osmium tetroxide overnight at 4 °C. Then, the osmified material was dehydrated through a graded alcohol–acetone series and finally embedded in Epon 812® (Ted pella). Ultrathin sections were obtained with a LEICA (Ultracut) ultramicrotome and stained with lead citrate and uranyl acetate. Observations were made using a Zeiss EM (900 series) microscope. Micrographics' of Leydig ($n_{\text{total Leydig cells}} = 60$) and Sertoli cells ($n_{\text{total Sertoli cells}} = 42$) of adult males were made and these pictures were analyzed using a stereoscopic microscope (Olympus SZ-PT40). Specifically, absence/presence and abundance (scarce, medium or abundant) of mitochondria, smooth endoplasmic reticulum (SER), glycogen granules and lysosomes were recorded. Additionally, the following were considered: presence of residual bodies in Sertoli cells, morphology of the mitochondria (with lamellar cristae or tubulo-vesicular cristae) and nuclear morphology, such as chromatin condensation and nucleolus morphology (absence, presence or the presence of different nucleolus regions) in Sertoli and Leydig cells. The diameter of each lipid droplet and the cytoplasm area were quantified with Image Pro Plus (4.0 version) software. The lipid droplets area in relation to the total cellular area was established (and expressed as a percentage) in each cell of each male, and the mean value for each individual was calculated.

2.7. Statistical analyses

For statistical analyses we used the statistical software SigmaStat 3.5®, Sigma Plot 10.0®, SPSS 9.0® and Table Curve. Analyses of variance (ANOVA), Simple and Multiple regression (Stepwise) analyses, and Spearman Correlation were used to test the significant dependence of the variables. Assumptions of normality and homogeneity of variance were tested with the one-sample Kolmogorov–Smirnov test and with the Levene test, respectively. When normality or variance-homogeneity assumptions were broken, Mann–Whitney rank sum and Kruskal–Wallis one-way analysis of variance on ranks (KW) were used for means comparisons (Sokal and Rohlf, 1969).

3. Results

3.1. Body condition of males

Snout-vent length of adult males of *P. antofagastensis* ranged from 86.9 to 99.5 mm, and body weight ranged from 27.5 to 37.0 g. The testis size in adult males varied from 4.0 to 9.6 mm. The juvenile male exhibited a 76.9 mm of SVL, 19.5 g of body weight and 2.16 mm of testis diameter.

3.2. Serum testosterone concentrations

In late spring, males exhibited spermatocytes in testes with scarce spermatozoa stored in epididymis or spermatids stage without spermatozoa in the epididymis. The serum testosterone concentrations found, ranged from 4.13 to 24.05 ng ml^{-1} (Fig. 1A). Instead, in mid-summer maximum peaks of testosterone (125.61–229.99 ng ml^{-1}) were observed and all males exhibited abundant spermatozoa in testis and in epididymis (Fig. 1A). In early autumn, minimum testosterone values were observed (2.31–17.18 ng ml^{-1}) in males with abundant spermatozoa in testis and epididymis as well as in males with testicular regression, and scarce spermatozoa in epididymis (Fig. 1A). Serum testosterone concentration of a juvenile male was determined, and exhibited a minimum value of 0.874 ng ml^{-1} (Fig. 1B).

There were significant differences in serum testosterone concentration between adult males captured in different months, with maximum value in mid-summer ($\text{mean}_{\text{spring}} = 14.06 \pm 5.75$ ng ml^{-1}; $\text{mean}_{\text{summer}} = 167.98 \pm 31.70$ ng ml^{-1}; $\text{mean}_{\text{autumn}} = 7.48 \pm 2.55$ ng ml^{-1}; Kruskal–Wallis, $X^2 = 6.30$, $df = 2$, $P < 0.043$; Fig. 1A). There were no significant differences in serum testosterone concentration among adult males when they were grouped by spermatogenic stages (Kruskal–Wallis, $X^2 = 2.23$, $df = 3$, $P > 0.525$; Fig. 1B), or by the abundance of spermatozoa in epididymis (ANCOVA, $F_{2,11} = 0.40$, $P > 0.684$), with body weight as a significant co-variable ($F_{1,11} = 9.79$, $P < 0.017$).

3.3. Ultrastructural analysis

3.3.1. Nuclei and nucleoli

In males captured in spring and summer, Leydig cells exhibited spherical nuclei containing heterochromatin in gross and fine granules (Fig. 2A–D), and in autumn exhibited convoluted nuclei (Fig. 2G). In spring and autumn Leydig cells showed electron-dense and larger nucleoli (Fig. 2B and E), more than the nucleoli of males captured in summer (Fig. 2D). Throughout the activity season

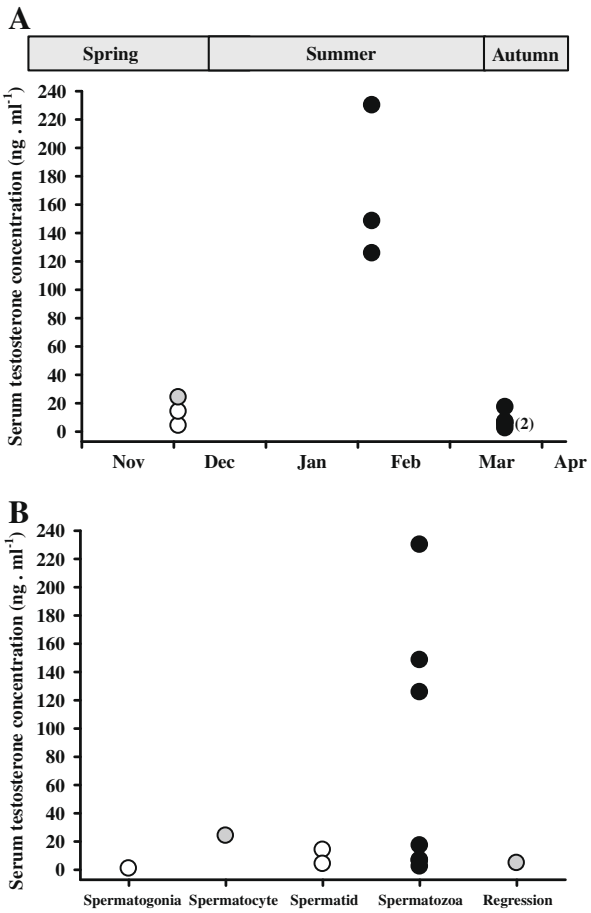

Fig. 1. Testosterone cycle in males of *P. antofagastensis*. Serum testosterone concentration (ng ml^{-1}) versus date (A) or spermatogenic stage (B) of each male are presented. Males with abundant (point), scarce (gray point) or absent (circle) spermatozoa in the epididymis are differentiated. Brackets indicate the number of observations.

males presented Sertoli cells with polymorphic and irregular nuclei, some of them with prolongations toward the tubular lumen (Fig. 3A–F). In males captured in spring, Sertoli cells exhibited small, spherical and electron-dense nucleoli (Fig. 3A), whereas in autumn electron-dense nucleoli with irregular morphology prevailed (Fig. 3D).

3.3.2. Mitochondria and SER

In all adult males captured in spring (spermatocytes or spermatids stages) Leydig cells showed similar proportions of mitochondria with lamellar or with tubular cristae, while in males of summer (spermatozoa stage) and autumn (spermatozoa or regression) principally mitochondria with tubular cristae were observed (Fig. 2 and Table 1). In males captured in spring, Sertoli cells exhibited principally (70%) mitochondria with lamellar cristae, whereas in summer all Sertoli cells exhibited both types of mitochondria. In autumn males, 42% of Sertoli cells studied presented both types of mitochondria, while in the rest of cells only mitochondria with lamellar cristae were observed (Fig. 3 and Table 1). In Leydig cells of summer, maximum peak in the abundance of mitochondria and SER development were observed in males with spermatozoa testicular stages and maximum serum testosterone concentrations (mitochondria: $mean_{spring} = 1.61 \pm 0.25$; $mean_{summer} = 2.10 \pm 0.10$; $mean_{autumn} = 1.90 \pm 0.19$; SER: see Figs. 2 and 4).

Males captured in spring and summer, beginning the spermatogenic cycle, exhibited Sertoli cells with abundant mitochondria, whereas autumn males that were finishing the cycle, showed a lesser abundance ($mean_{spring} = 2.61 \pm 0.11$; $mean_{summer} = 2.63 \pm 0.13$; $mean_{autumn} = 1.87 \pm 0.34$). Although in spring, SER of Sertoli cells exhibited great development, the general abundance of this organelle was scarce throughout the active season (Figs. 3 and 5). There were no significant differences in SER development of Leydig cell among males with different dates of capture (Kruskal–Wallis, $X^2 = 2.82$, $df = 2$, $P > 0.244$) or in Sertoli cell SER ($X^2 = 1.65$, $df = 2$, $P > 0.439$). Throughout the active season Leydig cells exhibited moderate to high SER development, without a significant correlation with serum testosterone concentration (Spearman Correlation, $r = -0.08$, $n = 10$, $P > 0.827$), or with lipid percentage ($r = 0.03$, $n = 12$, $P > 0.931$), even when the lipid percentage exhibited important fluctuations during the active season (Fig. 4).

3.3.3. Lipid content

Leydig cell lipid content, measured as the percentage of cytoplasm area covered by lipid droplets, was highest in males captured in spring and autumn, and lowest in summer (Figs. 2 and 3). There was a significant negative relationship between serum testosterone concentration and lipid content of Leydig cells (Lineal Regression, $F_{1,9} = 17.45$, $P < 0.003$), and there were significant differences in Leydig cell lipid content between males captured in spring, summer or autumn (ANOVA, $F_{2,12} = 5.73$, $P < 0.025$; $P_{spring-summer} < 0.009$; $P_{summer-autumn} < 0.018$; Fig. 4). However, there were no significant differences in the lipid percentage of Leydig cell of males grouped by spermatogenic stages (ANCOVA, $F_{3,12} = 0.661$, $P > 0.556$; co-variable body weight, $P < 0.048$; Fig. 6).

Sertoli cells did not exhibit a significant correlation between SER development and lipid content (Spearman Correlation, $r = -0.44$, $n = 12$, $P > 0.149$), and the lipid content was reduced and inferior to the values observed in Leydig cells, throughout the active season, although the highest values were observed in spring (Figs. 3 and 5). There were no significant differences in the lipid percentage of Sertoli cells of males grouped by capture date (ANOVA, $F_{2,12} = 0.43$, $P > 0.662$; Fig. 5) or by the spermatogenic stages ($F_{3,12} = 0.51$, $P > 0.685$; Fig. 6), and there was no significant relationship between Sertoli cells lipid percentage and male's SVL, body weight or date of capture (Multiple Regression Stepwise, $P > 0.05$).

3.3.4. Presence and abundance of other organelles

Residual bodies (Fig. 7A) were observed in few Sertoli cells of spring males (spermatocytes or spermatids stages), and in half of Sertoli cells of summer (spermatozoa stage), probably as a consequence of cytoplasmic material absorption activity. Residual bodies were observed in 33% of Sertoli cells of males captured in autumn, and these were generally small. Primary and secondary lysosomes were observed in half of Sertoli cells of spring and autumn, and in the majority of Sertoli cells of summer. Primary lysosomes were scarce, while secondary lysosomes were scarce to moderate in Sertoli cells of spring males, scarce in summer and moderate in autumn. In all males studied, Sertoli cells presented disperse glycogen granules in the cytoplasm (Fig. 7A). Additionally, the presence of multillamelar bodies in all Leydig cells of males captured in summer, and in some Leydig cells of autumn males was observed. Multillamelar bodies, also denominated multillamelar liposomes, are structures of variable size that consist on ordered concentric membrane caps that encapsulate lipids (Figs. 2F and 7B).

3.4. Analysis of the existence of temporal asynchrony in steroid activity between interstitial and tubular compartments of the testes

In *P. antofagastensis* indications of asynchronic steroid activity between Sertoli and Leydig cells were observed in the majority of

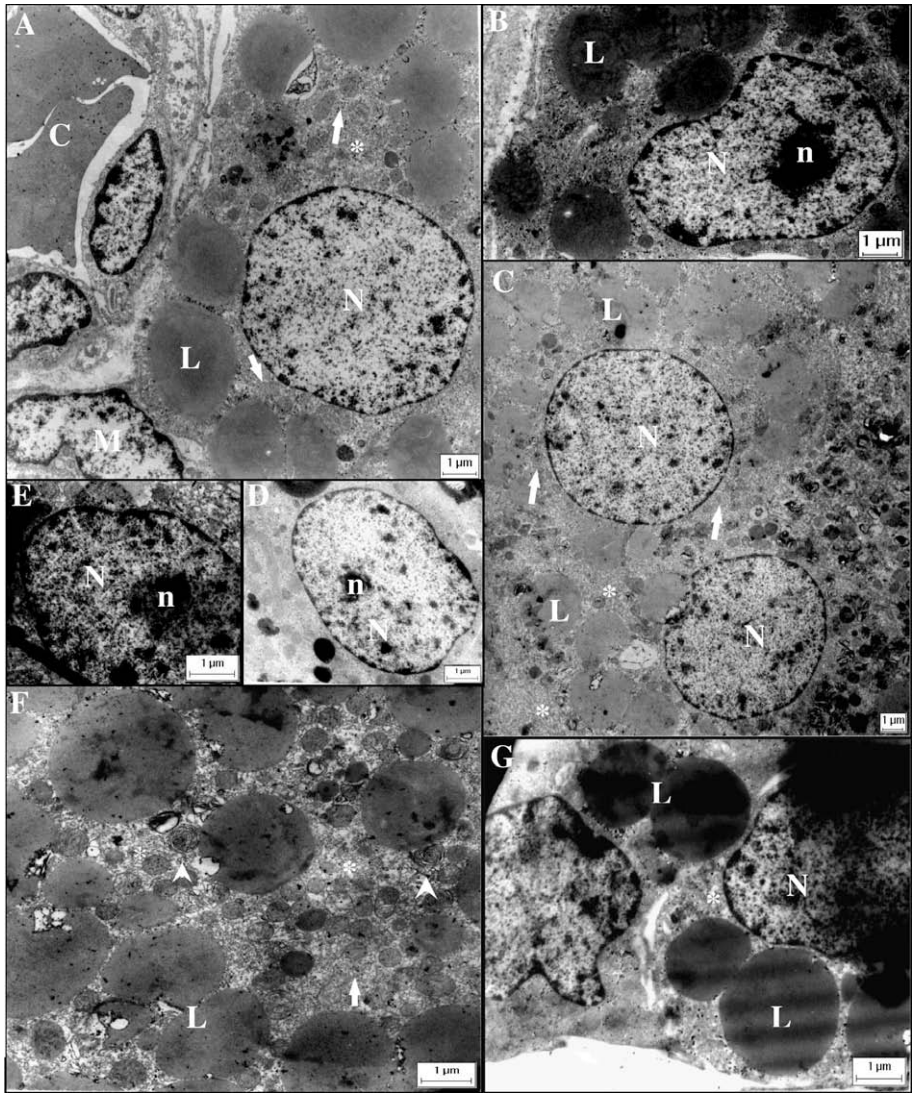

Fig. 2. Leydig cells of adult males of *P. antofagastensis* during the active season. (A) and (B) Leydig cell of male captured in spring; (C) and (D) in summer; (E–G) in autumn. N, nuclei; M, macrophage; C, blood duct (capillary); L, lipid droplet; ∗, smooth endoplasmic reticulum; arrow indicates the presence of mitochondria and arrow head indicates the presence of multillamelar bodies.

the adult males studied during the active season (Table 1). Males captured in spring exhibited Sertoli cells with abundant mitochondria, some of them with tubular cristae, scarce to moderate SER abundance, and generally scarce lipid content, indicative of active steroid synthesis. These males exhibited early spermatogenic stages and lower serum testosterone concentration. In all males captured in spring, Leydig cells were inactive in relation to the steroid synthesis and showed maximum abundance of lipids (Table 1). In summer, males exhibited steroid synchrony between testicular compartments, because Sertoli as well as Leydig cells showed morphologic signs of steroid activity. All males in this season exhibited higher serum testosterone concentration and abundant spermatozoa in testes and epididymis. In autumn, asynchrony in steroid activity between compartments was observed again, characterized by the presence of active Sertoli cells, with scarce lipid content and abundant organelles related to the steroid synthesis, as was observed in males studied in spring. Leydig cells were inactive, with maximum abundance of lipid content (Table 1). Most males studied in autumn exhibited spermatozoa in testes and epididymis and lower serum testosterone concentration, indicative of the end of the mating period and the end of the spermatogenic cycle. Additionally, one male exhibited testicular regression and scarce spermatozoa in the epididymis (Table 1).

4. Discussion

Males of *P. antofagastensis* perform spermatogenic cycle and mating in different periods of the activity season characterized by the temporal asynchrony of testosterone secretion in Leydig and Sertoli cells, principally in spring and autumn. Leydig and Sertoli cells of adult males of *P. antofagastensis* showed morphological evidences of steroidogenic activity, such as the presence of SER development, temporal variations in lipid content, and mitochondria with tubular cristae. Sertoli cells exhibited ultrastructural features of steroidogenic activity in most of the adult males studied, independently of their reproductive or hormonal state. Leydig cells instead, were active only in males captured in mid-summer with spermatozoa in the epididymis and maximum concentrations of serum testosterone. *P. antofagastensis* showed spermatogenesis and low plasmatic testosterone levels in spring and autumn, in coincidence with steroidogenic activity in Sertoli cells and inactiv-

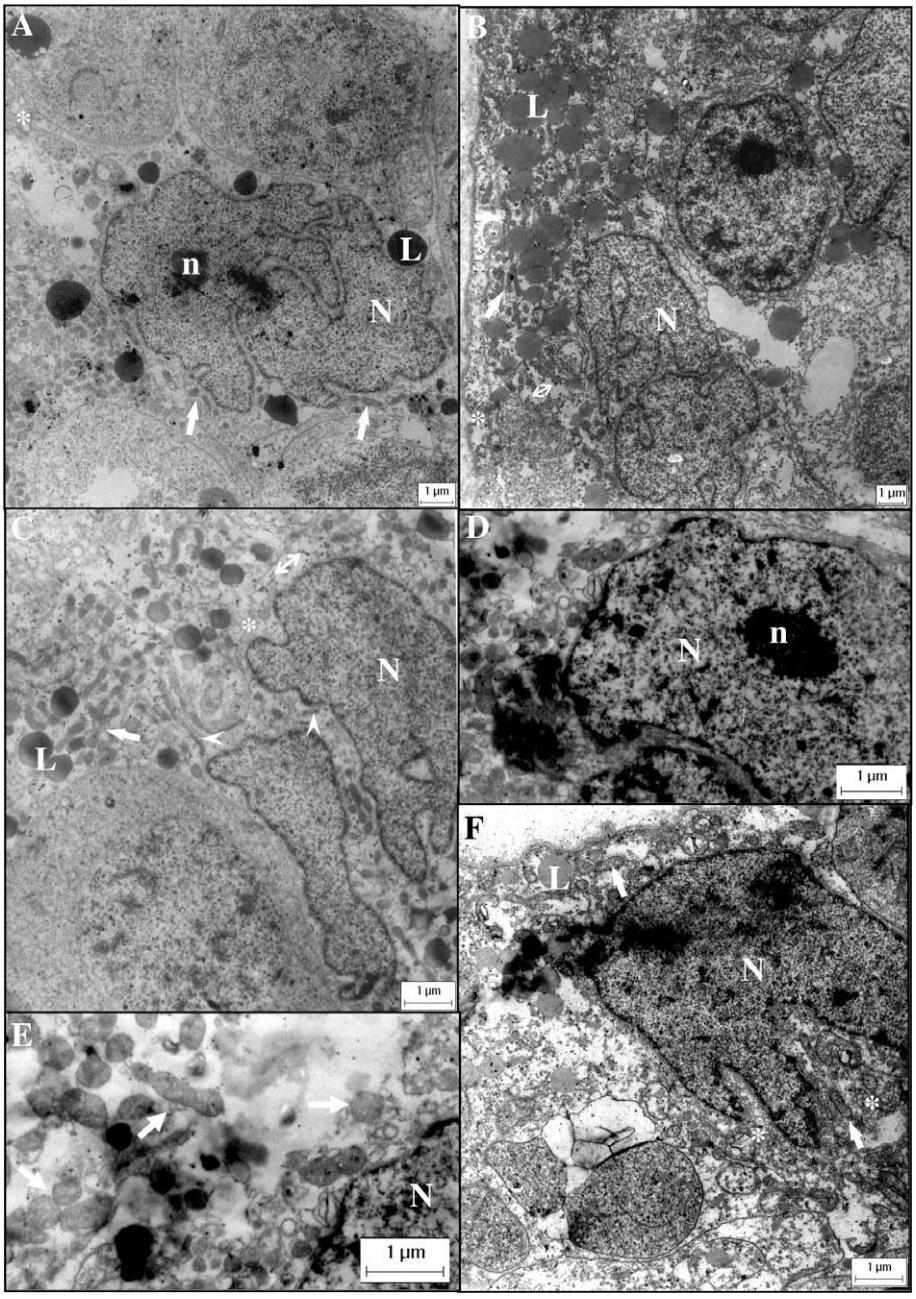

Fig. 3. Sertoli cells of adult males of *P. antofagastensis* during the active season. (A) and (B) Sertoli cells of males captured in spring; (C) in summer; and (D) in autumn. N, nucleus; n, nucleolus; L, lipid droplet; ∗, smooth endoplasmic reticulum; arrow, mitochondria; arrow head, rough endoplasmic reticulum; arrows with double head, glycogen granules.

ity in Leydig cells. This alternation of steroidogenic activity has been found also in the turtle *Chrysemys picta* (Callard et al., 1976; Callard and Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988) and *Chelydra serpentina* (Mahmoud et al., 1985; Mahmoud and Licht, 1997). In coincidence with the observations performed in *P. antofagastensis*, Mahmoud and Licht (1997) described for *C. serpentina*, the existence of a short reproductive activity season (95–100 days, from mid spring to the end of summer), with a unique maximum peak of testosterone in mid-summer when spermiation occurs, pointing out that to begin a new spermatogenic cycle in spring, a steroid synthesis by Sertoli cells is necessary.

The presence of SER development in Sertoli cells was described in males of lizards *L. darwini* (Gutierrez and Yapur, 1983), *Eumeces laticeps* (Okia, 1992), in the fish *Tilapia rendalli* (Van Vuren and Soley, 1990), in the turtle *Pseudameys scripta* (Sprando and Russell, 1987), and in the snake *E. jayakari* (Al-Dokhi et al., 2004) among others, but only in *T. rendalli*, was the possible steroidogenic activity of these cells suggested (Van Vuren and Soley, 1990). The presence of SER, lipid content and mitochondria with tubular cristae in Sertoli cells of *P. antofagastensis* point out that there is steroidogenic activity, but determination of enzymes 3β-HSD or 17β-HSD, is necessary to support these findings.

The Leydig cells of *P. antofagastensis* males showed a negative relationship between serum testosterone concentration and lipids content, as is expected when an active conversion of cholesterol to androgens occurs in steroid cells (Callard et al., 1976; Lofts and Tsui, 1977; Mahmoud et al., 1985; Dubois et al., 1988). A synchronic reduction in lipid content between Leydig and Sertoli cells

Table 1
Steroid activity analysis in the interstitial and tubular compartments of the testes in *Phymaturus antofagastensis*. Capture season; spermatogenic stage; presence or absence of spermatozoa in the epididymis; serum testosterone concentration (T, ng ml^{-1}), lipid percentage (lipids; X = 0.1–20%; XX = 21–40%; XXX = 41–67%) SER and mitochondria abundance (X = 0.1–1.4; XX = 1.5–2.4; XXX = 2.5–3), and morphology of mitochondria cristae (tubular = T; lamellar = L) in Leydig and Sertoli cells of each male, were indicated. Finally, the Leydig and Sertoli cells were classified (activity) as active (A) or inactive (I) in accordance with all steroid features mentioned above, and synchronic (S) or asynchronic (A) steroid activity was indicated.

Season	Spermatogenic stage	Epididymis	T	Leydig cells				Sertoli cells				Steroid
				Lipids	SER	Mitochondria	Activity	Lipids	SER	Mitochondria	Activity	Activity
Spring	Spermatids	—	—	XXX	XX	XX/T	I	XX	X	XXX/L	I	S
	Spermatids	Scarce	—	XXX	XXX	XX/T	I	XX	XX	XXX/L	I	S
	Spermatocites	Scarce	24.1	XXX	XXX	X/T	I	X	X	XXX/L	A	A
	Spermatids	Absent	14.0	XXX	XX	X/L	I	X	X	XXX/L	A	A
	Spermatids	Absent	4.1	XXX	XXX	XX/T	I	X	XX	XX/L–T	A	A
Summer	Spermatozoa	Abundant	229.9	XX	XX	XX/T	A	X	X	XXX/L–T	A	S
	Spermatozoa	Abundant	125.6	XX	XXX	XX/T	A	X	XX	XXX/L–T	A	S
Autumn	Spermatozoa	Abundant	17.2	XXX	X	X/T	I	X	X	XX/L–T	A	A
	Spermatozoa	Abundant	6.4	XXX	XX	XX/T	I	X	X	XXX/T–L	A	A
	Spermatozoa	Abundant	2.3	XXX	XXX	XX/L	I	X	XX	XX/L	A	A
	Regression	Scarce	4.7	XXX	XX	XX/L	I	X	—	X/—	I	S
	Spermatozoa	Abundant	6.8	XXX	XX	XX/T	I	XX	—	XX/—	I	S

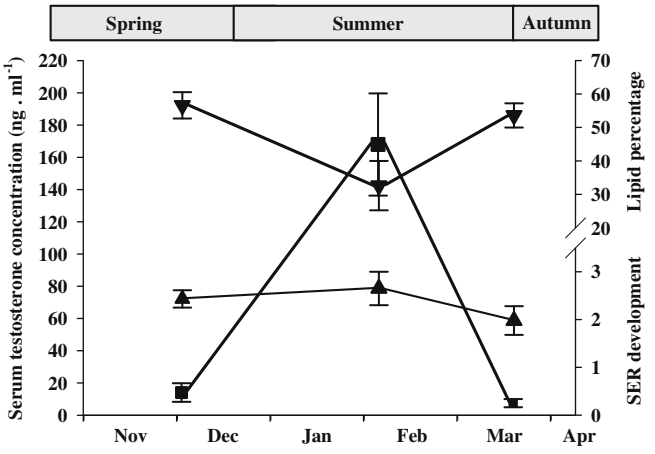

Fig. 4. Activity state of Leydig cells in males of *P. antofagastensis* in relation to the steroidogenic synthesis of testosterone during the active season. The mean values and standard error of serum testosterone concentration (ng ml^{-1}; square), of smooth endoplasmic reticulum development (SER, triangle up) and of the lipids percentage (triangle down) versus date, are presented.

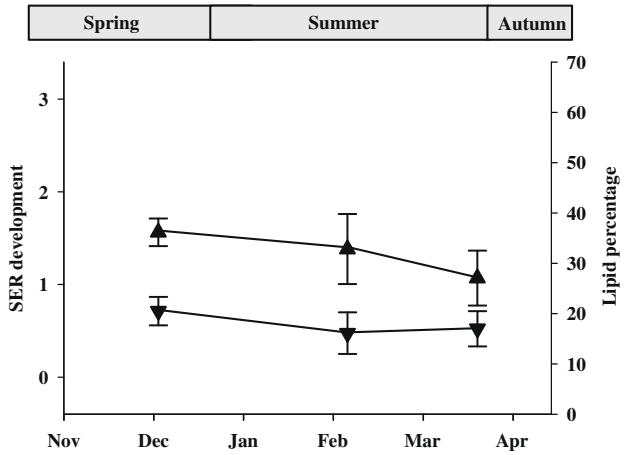

Fig. 5. Activity state of Sertoli cells in males of *P. antofagastensis* during the active season. Mean and standard error of smooth endoplasmic reticulum abundance (SER, triangles up) and the lipid percentage (triangles down) versus date are represented.

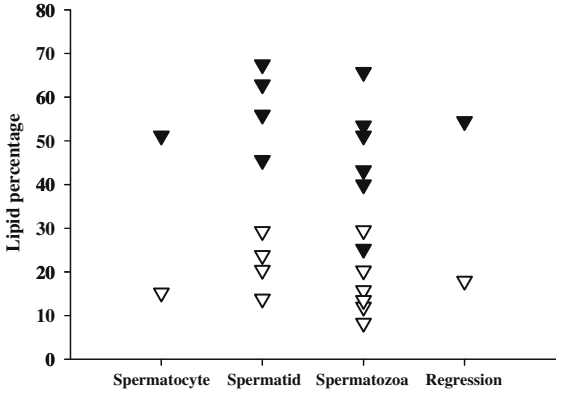

Fig. 6. Lipids dynamic in Leydig cells (black triangles) and Sertoli cells (white triangles) during the reproductive cycle of adult males of *P. antofagastensis*. Mean value of lipid percentage versus the spermatogenic cycle of each adult male studied are presented.

was observed in males captured in mid-summer, whereas in spring and in autumn, abundant lipid content in Leydig cells and a reduction in Sertoli cells was observed. Similarly, an asynchrony in lipid contents between interstitial tissues and the seminiferous tubules and a reduction of lipid droplets in the seminiferous tubules coincident with the spermatogenesis was observed in the snake *Nerodia sipedon* (Weil and Aldridge, 1981). The results presented here support the hypothesis that in males of *P. antofagastensis* the androgens produced by Sertoli cells remain in the seminiferous tubules, without entering the general circulation, as previously suggested for *C. picta* (Callard et al., 1976; Callard and Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988), *C. serpentina* (Mahmoud et al., 1985; Mahmoud and Licht, 1997) and *Testudo graeca* (Ibargüengoytía et al., 1999).

In previous studies, it has not been possible to define if ovulation occurs in *P. antofagastensis* in coincidence with the two peaks of maximum abundance of spermatozoa in males in spring or in autumn, or in both, especially because courtship and mating behaviors were not observed in the field (Boretto and Ibargüengoytía, 2006). In the present work, we were able to confirm that the mating period occurs in mid-summer in coincidence with the maximum peak of testosterone (February = 230 ng ml^{-1}), while in early autumn (March = 2–17 ng ml^{-1}) and in late spring (December = 4–24 ng ml^{-1}) serum testosterone concentration

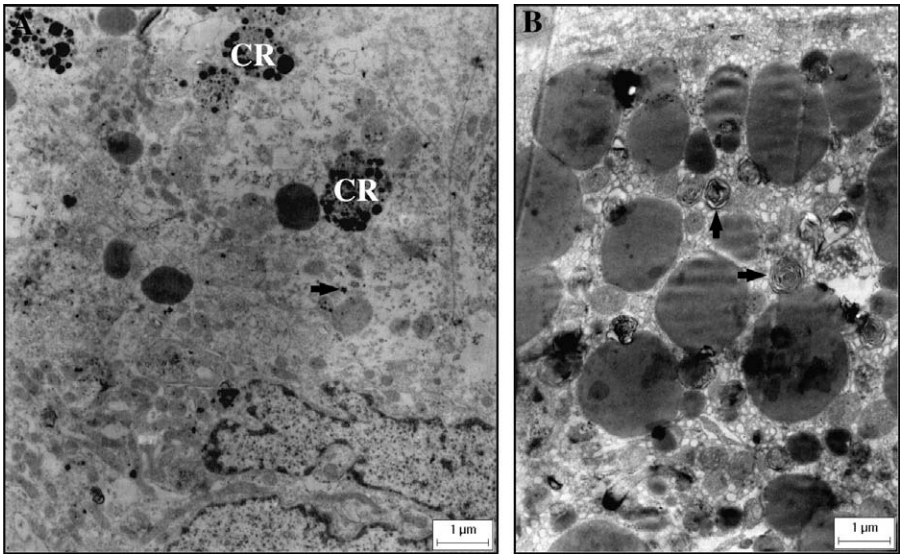

Fig. 7. Additional structures observed in cells of *P. antofagastensis* males during the active season. (A) Residual bodies (RB) and glycogen granules (arrows) in Sertoli cells; (B) multillamelar bodies (arrows) in Leydig cells.

was minimum. In the genus *Sceloporus*, it was proposed that the mating period changed from spring to autumn, with prolonged gestation over winter and birth in the next spring, in order to favor maximal newborn survival (Méndez-de la Cruz et al., 1998). This reproductive model was observed in populations at high altitudes in tropical latitudes, where environmental temperatures during winter were more benign than in summer (Méndez-de la Cruz et al., 1998). Nevertheless, this pattern may not be possible at higher latitudes where cool and snowy winters could strongly affect the embryonic development and offspring survival (Méndez-de la Cruz et al., 1998), so it is expected that females of *P. antofagastensis* mate in mid-summer, but reserve sperm in oviducts during winter, until the following spring when ovulation probably occurs.

The maximum serum testosterone concentrations found in *P. antofagastensis* during the activity season (230 ng ml^{-1}) were similar to other *Phymaturus* studied (*P. cf. palluma* maximum peak = 199 ng ml^{-1}, unpublished data) and different from others (*P. punae* maximum peak = 165 ng ml^{-1}; *P. zapalensis* maximum peak = 152 ng ml^{-1}; Boretto, 2009). The maximum peak in *P. antofagastensis* was higher than the maximum levels found in other species, as the lizards *L. gravenhorsti* (approx. 60 ng ml^{-1}; Leyton et al., 1977), *Niveoscincus metallicus* (approx. 70 ng ml^{-1}; Swain and Jones, 1994), *Niveoscincus ocellatus* (approx. 20 ng ml^{-1}; Jones et al., 1997), *Urosaurus ornatus* (approx. 80 ng ml^{-1}; Moore et al., 1991), the turtle *C. serpentina* (approx. 55–60 ng ml^{-1}; Mahmoud et al., 1985; Mahmoud and Licht, 1997), the sea turtle *Caretta caretta* (approx. 8 ng ml^{-1}; Wibbels et al., 1990) or the snakes *Crotalus atrox* (approx. 70 ng ml^{-1}; Taylor et al., 2004), *N. sipedon* (approx. 23 ng ml^{-1}; Weil and Aldridge, 1981), *Thamnophis sirtalis concinnus* (approx. 90 ng ml^{-1}; Moore et al., 2000) and the cottonmouth *Agkistrodon piscivorus* (approx. 60 ng ml^{-1}; Graham et al., 2008) among other reptiles. Testosterone levels higher than the concentrations found in *P. antofagastensis* were reported in males of the freshwater turtle *C. picta* (maximum peak = 740 ng ml^{-1}; Callard et al., 1976) reinforcing the specificity of hormone levels and peaks in reptiles.

Males of *P. antofagastensis* show asynchrony among males in the spermatogenic stages with two maximum peaks of abundance of spermatozoa in the epididymis, one in late spring and the other in mid-summer (Boretto and Ibargüengoytía, 2006), but a unique peak of maximum serum testosterone concentration was found in mid-summer, showing asynchrony between male and female reproductive cycles and indicating that mating occurs in mid-summer. In relation to this, in some lizards living in temperate climates of Australia, with asynchronic cycles between sexes, two mating periods in spring and in autumn were described (e.g. *N. metallicus* and *N. ocellatus*; Swain and Jones, 1994; Jones and Swain, 1996; Jones et al., 1997). In *P. antofagastensis* a second mating period is not expected due to the low serum testosterone concentrations found in males captured in late spring (4–24 ng ml^{-1}). Nevertheless, reproductive behavior associated with courtship and mating does not necessarily depend on high levels of circulating steroids, and can be triggered by the increment of environmental temperature as it happens in the snakes *N. sipedon* (Weil and Aldridge, 1981) and *T. sirtalis* (Moore and Lindzey, 1992), and in the turtle *Trionix sinensis* (Lofts and Tsui, 1977). Thereby, due to the presence of spermatozoa stored in the epididymis of *P. antofagastensis* in spring (Boretto and Ibargüengoytía, 2006), we cannot discard a possibility of a second mating period stimulated by the increment of environmental temperature at this time.

This present work represents an advance in the understanding of the physiological and behavioral adaptations of males to the female cycle in *Phymaturus*, under cool environments in the Andean Highlands of Argentina, characterized by the existence of cellular mechanisms that allow the development of the spermatogenic cycle independently of the mating period.

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References

Al-Dokhi, O.A., Al-Onazee, Y.Z., Mubarak, M., 2004. Light and electron microscopy of the testicular tissue of the snake *Eryx jayakari* (Squamata, Reptilia) with a reference to the dividing germ cells. J. Biol. Sci. 4 (3), 345–351.

Bardin, C.W., Cheng, C.Y., Musto, N.A., Gunsalus, G.L., 1988. The Sertoli cell. In: Knobil, E., Neill, J. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 933–974.

Blackburn, D.G., 1993. Standardized criteria for the recognition of reproductive modes in squamate reptiles. Herpetologica 49 (1), 118–132.
Bonnet, X., Naulleau, G., Mauget, R., 1992. Cycle sexuelle de la femelle de *Vipera aspis* (Squamata, Viperidae), importance des reserves et aspects metaboliques. Bull. Soc. Zool. Fr. 117, 279–290.
Boretto, J.M., 2009. Ecofisiología reproductiva del género *Phymaturus* (Liolaemidae): estudio histológico, ultraestructural y bioquímico. Ph.D Thesis. Universidad Nacional del Comahue, Argentina.
Boretto, J.M., Ibargüengoytía, N.R., 2006. Asynchronous spermatogenesis and biennial female cycle of the viviparous lizard *Phymaturus antofagastensis* (Liolaemidae): reproductive responses to high altitudes and temperate climate of Catamarca, Argentina. Amphibia-Reptilia 27, 25–36.
Boretto, J.M., Ibargüengoytía, N.R., Acosta, J.C., Blanco, G.M., Villavicencio, H.J., Marinero, J.A., 2007. Reproductive biology and sexual dimorphism of a high-altitude population of the viviparous lizard *Phymaturus punae* from the Andes in Argentina. Amphibia-Reptilia 28, 427–432.
Boretto, J.M., Ibargüengoytía, N.R., 2009. *Phymaturus* of Patagonia, Argentina: reproductive biology of *Phymaturus zapalensis* (Liolaemidae) and a comparison of sexual dimorphism within the genus. J. Herpetol. 43 (1), 96–104.
Bull, J.J., Shine, R., 1979. Iteroparous animals that skip opportunities for reproduction. Am. Nat. 114, 296–316.
Cabezas Cartes, F., 2008. Estudio morfológico e histológico de la biología reproductiva del lagarto vivíparo *Phymaturus palluma* (Liolaemidae) de San Juan, Argentina. Undergraduate Thesis. Universidad Nacional de Córdoba, Argentina.
Cabrera, A.L., 1976. Regiones Fitogeográficas de la República Argentina. In: ACME (Ed.), Enciclopedia Argentina de Agricultura y Jardinería 2. Buenos Aires, Argentina.
Cabrera, A.L., 1994. Regiones Fitogeográficas Argentinas. In: Kugler, W. (Ed.), Enciclopedia Argentina de Agricultura y Jardinería. Editorial Acme S.A.C.I. Buenos Aires, Argentina.
Callard, I.P., Ho, M.S., 1980. Seasonal reproductive cycles in reptiles. In: Progress in Reproductive Biology: Seasonal Reproduction in Higher Vertebrates. Karger, Basel, pp. 5–38.
Callard, I.P., Callard, G.V., Lance, V., Eccles, S., 1976. Seasonal changes in testicular structure and function and the effects of gonadotropins in the freshwater turtle, *Chrysemys picta*. Gen. Comp. Endocrinol. 30, 347–356.
Cei, J.M., 1986. Reptiles del centro, centro-oeste y sur de la Argentina: Herpetofauna de las zonas áridas y semiáridas. Museo regionale di Scienze Naturali Torino, Torino, Italy.
Cei, J.M., 1993. Reptiles del noroeste, nordeste y este de la Argentina: Herpetofauna de las selvas subtropicales, Puna y Pampas. Museo regionale di Scienze Naturali Torino, Torino, Italy.
Cree, A., Guillette Jr., L.J., 1995. Biennial reproduction with a fourteen-month pregnancy in the gecko *Hoplodactylus maculatus* from Southern New Zealand. J. Herpetol. 20, 163–173.
Cree, A., Cockrem, J., Guillette, L., 1992. Reproductive cycles of male and female tuatara (*Sphenodom punctatus*) on Stephens Island, New Zealand. J. Zool. 226, 199–217.
Dubois, W., Pudney, J., Callard, I.P., 1988. The annual testicular cycle in the turtle, *Chrysemys picta*: a histochemical and electron microscopic study. Gen. Comp. Endocrinol. 71, 191–204.
Edwards, A., Jones, S.M., Wapstra, E., 2002. Multiennial reproduction in females of a viviparous, temperate-zone skink, *Tiliqua nigrolutea*. Herpetologica 58, 407–414.
Ferreira, A., Dolder, H., 2003. Cytochemical study of spermiogenesis and mature spermatozoa in the lizard *Tropidurus itambere* (Reptilia, Squamata). Acta Histochem. 105 (4), 339–352.
Gotthard, K., 2001. Growth strategies of ectothermic animals in temperate environments. In: Atkinson, D., Thorndyke, M. (Eds.), Environment and Animal Development. BIOS Scientific Publishers, Oxford, pp. 287–304.
Graham, S.P., Earley, R.L., Hoss, S.K., Schuett, G.W., Grober, M.S., 2008. The reproductive biology of male cottonmouths (*Agkistrodon piscivorus*): do plasma steroid hormones predict the mating season? Gen. Comp. Endocrinol. 159, 226–235.
Gutierrez, L.S., Yapur, L.B., 1983. Spermiogenesis of the lizard *Liolaemus darwini*: an ultrastructural and cytochemical study. Microsc. Electron. Biol. Cell. 7 (2), 57–71.
Habit, E.M., Ortiz, J., 1996. Ciclo reproductivo de *Phymaturus flagellifer* (Reptilia, Tropiduridae). B. Soc. Biol. Concepción 67, 7–14.
Ibargüengoytía, N.R., 2004. Prolonged cycles as a common reproductive pattern in viviparous Lizards from Patagonia, Argentina: reproductive cycle of *Phymaturus patagonicus*. J. Herpetol. 38 (1), 73–79.
Ibargüengoytía, N.R., Casalins, L., 2007. Reproductive biology of the southernmost gecko *Homonota darwini*: convergent life-history patterns among southern hemisphere reptiles living in harsh environments. J. Herpetol. 41, 71–79.
Ibargüengoytía, N.R., Pastor, L.M., Pallares, J., 1999. A light microscopy and ultrastructural study of the testes of tortoise *Testudo graeca* (Testudinidae). J. Submicrosc. Cytol. Pathol. 31 (2), 221–230.
Jones, S.M., Swain, R., 1996. Annual reproductive cycle and annual cycles of reproductive hormones in plasma of female *Niveoscincus metallicus* from Tasmania. J. Herpetol. 30, 140–146.
Jones, S.M., Wapstra, E., Swain, R., 1997. Asynchronous male and female gonadal cycles and plasma steroid concentrations in a viviparous lizard, *Niveoscincus ocellatus* (Scincidae), from Tasmania. Gen. Comp. Endocrinol. 108, 271–281.
Leyton, V.C., Morales, B.C., Bustos-Obregón, E., 1977. Seasonal changes in testicular function of the lizard *Liolaemus gravenhorsti*. Arch. Biol. (Bruxelles) 88, 393–405.
Lobo, F., Quinteros, S., 2005. A morphology-based phylogeny of *Phymaturus* (Iguania: Liolaemidae) with the description of four new species from Argentina. Pap. Avulsos. Zool. 45, 143–177.
Lofts, B., Tsui, H.W., 1977. Histological and histochemical changes in the gonads and epididymides of the male Soft-shelled turtle, *Trionyx sinensis*. J. Zool. (London) 181, 57–68.
Mahmoud, I.Y., Licht, P., 1997. Seasonal changes in gonadal activity and the effects of stress on reproductive hormones in the common snapping turtle, *Chelydra serpentina*. Gen. Comp. Endocrinol. 107, 359–372.
Mahmoud, I.Y., Cyrus, R.V., Bennett, T.M., Woller, M.J., Montag, D.M., 1985. Ultrastructural changes in testes of the snapping turtle, Chelydra serpentina, in relation to plasma testosterone, Δ^5-3b-hydroxysteroid dehydrogenase, and cholesterol. Gen. Comp. Endocrinol. 57, 454–464.
Mayhew, W.W., Wright, S., 1970. Seasonal changes in testicular histology of three species of the lizard genus *Uma*. J. Morphol. 130, 163–186.
Méndez-de la Cruz, F.R., Villagrán-Santa Cruz, M., Andrews, R.M., 1998. Evolution of viviparity in the lizard genus *Sceloporus*. Herpetologica 54, 521–532.
Mori, H., 1984. Ultrastructural and stereological analysis of Leydig cells. In: Motta, P.M. (Ed.), Ultrastructural of Endocrine Cells and Tissues. Martinus Nijhoff Publishers, Boston, pp. 225–237.
Moore, M.C., Lindzey, J., 1992. The physiological basis of sexual behavior in male reptiles. In: Gans, C., Crews, D. (Eds.), Biology of the Reptilia. University of Chicago Press, Chicago, pp. 70–113.
Moore, M.C., Thompson, C.W., Marler, C.A., 1991. Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 81, 217–226.
Moore, I.T., Lerner, J.P., Lerner, D.T., Mason, R.T., 2000. Relationships between annual cycles of testosterone, corticosterone, and body condition in male red-spotted garter snakes, *Thamnophis sirtalis concinnus*. Physiol. Biochem. Zool. 73 (3), 307–312.
Okia, N.O., 1992. The Sertoli cell membrane body in the skink. Tissue Cell 24, 283–289.
Olsson, M., Shine, R., 1999. Plasticity in frequency of reproduction in an alpine lizard *Niveoscincus microlepidotus*. Copeia 1999, 794–796.
Pincheira-Donoso, D., 2004. A new species of the genus *Phymaturus* (Iguania: Tropiduridae: Liolaemini) from Central-Southern Chile. Multequina 13, 57–70.
Pincheira-Donoso, D., Scolaro, J.A., Sura, P., 2008. A monographic catalogue on the systematics and phylogeny of the South American iguanian lizard family Liolaemidae (Squamata, Iguania). Zootaxa 1800, 1–85.
Saint Girons, H., 1985. Comparative data on lepidosaurian reproduction and some time tables. In: Gans, C. (Ed.), Biology of the Reptilia. John Wiley & Sons Inc., New York, pp. 35–58.
Scolaro, J.A., Ibargüengoytía, N.R., 2007. A new species of *Phymaturus* from rocky outcrops in the central steppe of Río Negro province, Patagonia Argentina (Reptilia: Iguania: Liolaemidae). Zootaxa 1524, 47–55.
Shine, R., 1985. The evolution of viviparity in reptiles: an ecological analysis. In: Gans, C., Billet, F. (Eds.), Biology of Reptilia. John Wiley & Sons, New York, pp. 605–694.
Sokal, R.R., Rohlf, F., 1969. Biometry. The Principles and Practice of Statistics in Biological Research. W.H. Freeman & Co., USA.
Sprando, R.L., Russell, L.D., 1987. A comparative study of Sertoli cell ectoplasmic specializations in selected non-mammalian vertebrates. Tissue Cell 19 (4), 479–493.
Swain, R., Jones, S.M., 1994. Annual cycle of plasma testosterone and other reproductive parameters in the Tasmanian skink, *Niveoscincus metallicus*. Herpetologica 50 (4), 502–509.
Taylor, E.N., DeNardo, D.F., Jennings, D.H., 2004. Seasonal steroid hormone levels and their relation to reproduction in the Western Diamond-backed Rattlesnake, *Crotalus atrox* (Serpentes: Viperidae). Gen. Comp. Endocrinol. 136, 328–337.
Tinkle, D.W., Gibbons, J.W., 1977. The Distribution and Evolution of Viviparity in Reptiles, vol. 154. Miscellaneous Publications. Museum of Zoology, University of Michigan, pp. 1–55.
Van Vuren, J.H.J., Soley, J.T., 1990. Some ultrastructural observations of Leydig and Sertoli cells in the testis of *Tilapia rendalli* following induced testicular recrudescence. J. Morphol. 206, 57–63.
Wapstra, E., Swain, R., Jones, S.M., O'Reilly, J., 1999. Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata: Scincidae). Aust. J. Zool. 47, 539–550.
Weil, M.R., Aldridge, R.D., 1981. Seasonal androgenesis in the male water snake, *Nerodia sipedon*. Gen. Comp. Endocrinol. 44, 44–53.
Wibbels, T., Owens, D., Limpus, C., Reed, P., Amoss, M., 1990. Seasonal changes in gonadal steroid concentrations associated with migration, mating, and nesting in loggerhead sea turtles. Gen. Comp. Endocrinol. 79, 154–164.