How males synchronize their reproductive cycles with females to cope with seasonal climate: An endocrinal and ultrastructural study of *Phymaturus zapalensis* lizards (Liolaemidae)

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Phymaturus zapalensis inhabits harsh thermal environments in the steppe of Patagonia, Argentina, characterized by climate conditions that impose constraints on reproduction, providing an appealing model to study the role of steroid hormones in the regulation of seasonal reproductive events. Males of *P. zapalensis* exhibited a postnuptial spermatogenic cycle with spermiation in mid-spring in synchrony with female ovulation time when mating occurs, followed by testicular recrudescence, but do not show sperm reservoir during hibernation period in winter. Females of *P. zapalensis* can reproduce annually or biennially. Here, we studied the steroidogenic functions of testicular compartments of *P. zapalensis* by analysing serum testosterone and ultrastructure related to steroidogenic activity in Sertoli and Leydig cells, as a possible mechanism for the synchronization of male and female reproductive cycles. The testosterone cycle resembles the gonadal cycle in *P. zapalensis* previously described by morphology and histology of testes. Testosterone concentration is highest in mid-spring and lowest in early summer, with an initial recovery at the beginning of a new spermatogenic cycle in late summer and early autumn. Ultrastructural morphological features indicative of steroidogenic activity in Leydig and Sertoli cells were observed during the spermatogenic cycle. Evidence of temporal asynchrony in steroidogenic activity between compartments were found in males captured in summer and autumn, while synchronous activity was found during mating in spring. Temporal separation of steroidogenic activity serves to synchronize male and female cycles in *P. zapalensis* and assures the adjustment of reproductive activity to physiological and environmental constraints.

Key words: Leydig and Sertoli cells, lizard, steroidogenesis, testicular function, testosterone

INTRODUCTION

Postnuptial male spermatogenic cycles, characterized by the occurrence of testicular recrudescence after mating in summer and a sperm reservoir in the epididymis throughout winter (Pudney, 1995), are a common pattern in reptiles such as snakes and turtles that inhabit regions with cold temperate or unpredictable climates (Moore & Lindzey, 1992). In these species, males have restricted time to resume spermatogenesis (Moore & Lindzey, 1992; Pudney, 1995). As a result, spermatogenesis and mating occur at different times, and high levels of plasma androgens do not co-occur with reproductive behaviours such as courting and mating, resulting in dissociated cycles (Saint Girons, 1985; Moore & Lindzey, 1992; Whittier & Tokarz, 1992). Nevertheless, depending on the species and habitats, spermatogenesis may not be completed before overwintering and within the same population some males copulate in autumn while others do it in the following spring as with some snakes (Saint Girons, 1985) and lizards (e.g. *Niveoscincus ocellatus*, Jones *et al*., 1997). The plasticity in spermiation time, and the possibility to store sperm in the epididymis, assures that males are ready to mate in synchrony with the ovulation time of females in spring. 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 favour male-female encounters (e.g. Bull & Shine, 1979; Saint Girons, 1985), nourishment by viviparity (Saint Girons, 1985; Bonnet et al., 1992), birth in warmer periods of the activity seasons and larger offspring (Cree et al., 1992; Cree & Guillette, 1995; Wapstra et al., 1999; Edwards et al., 2002; Ibargüengoytía & Casalins, 2007).

All species in the genus *Phymaturus* are viviparous and inhabit the cold and harsh environments of the Andean Highlands of Argentina and Chile and the Patagonian steppe of Argentina (Cei, 1986, 1993). Females of the genus *Phymaturus* exhibit an extended reproductive cycle, taking at least one activity season to perform either vitellogenesis or pregnancy (Habit & Ortiz, 1996;

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Ibargüengoytía, 2004; Boretto & Ibargüengoytía, 2006, 2009; Boretto et al., 2007; Cabezas Cartes et al., 2010). This reproductive strategy observed in most viviparous females (Duvall et al., 1982; Callard et al., 1992; Custodia-Lora & Callard, 2002) has been attributed to the physiological constraint of undergoing vitellogenesis while pregnant, and because they are also constrained by the severe climate and long hibernation period from mid-autumn to early spring. As a result, the operational sex ratio of reproductive individuals is biased towards males (Ibargüengoytía, 2004; Boretto & Ibargüengoytía, 2006, 2009; Boretto et al., 2007; Cabezas Cartes et al., 2010). Females of *P. zapalensis* reproduce annually or biennially, because they often skip a year of reproduction to feed and store energy for reproduction the following year, while other females of the population perform vitellogenesis in spring and pregnancy from late spring to midsummer (Boretto & Ibargüengoytía, 2009). Males feature a postnuptial cycle, with a long spermatogenesis starting after copulation in mid-summer, with arrest of spermatogenesis during hibernation in winter, which is completed by mid-spring when the epididymis contains spermatozoa in synchrony with the females ovulation period (Boretto & Ibargüengoytía, 2009). Males of *P. zapalensis*, in contrast with the general pattern for postnuptial cycles, do not reserve sperm during the winter months in the epididymis, and spermiation and spermatozoa in the epididymis occur entirely after the hibernation period, even though some individuals show incipient spermiation features in autumn (Boretto & Ibargüengoytía, 2009). In summary, males of *P. zapalensis* exhibit a dissociated reproductive cycle, with a long spermatogenesis temporally dissociated from mating, providing an appealing model to study environmental endocrinology and to explore the role of steroid hormones in the regulation of seasonal reproductive events.

Postnuptial dissociated cycles have evolved numerous times in several reptile families (Whittier & Tokarz, 1992) and in some species this results in alternated steroidogenic activity between the Leydig cells in the interstitial compartment of the testis and the Sertoli cells in the tubular compartment of the testis (Lofts & Tsui, 1977; Callard & Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988; Mahmoud & Licht, 1997). Sertoli cells appear to have the potential to synthesize a variety of steroids, but their contribution to the circulating androgen pool is minimal and limited to the seminiferous tubules, influencing functions in the synchronization and maintenance of spermatogenesis (Bardin et al., 1988; Dubois et al., 1988). In contrast, androgens produced by Leydig cells enter the peripheral circulation, influencing courtship behaviour and mating events (Dubois et al., 1988). These differences between Sertoli and Leydig cells in testosterone bio-availability and distribution allow the independence or temporal dissociation between spermatogenesis and mating (Mahmoud et al., 1985; Dubois et al., 1988; Mahmoud & Licht, 1997). This mechanism is expected to have particular importance in species with postnuptial reproductive cycles, such as *P. zapalensis*, because spermatogenesis occurs temporally dissociated from spermiation and mating time.

Seasonal variations in male serum testosterone concentration in the family Liolaemidae have been studied in the viviparous lizard: *Liolaemus gravenhorsti* which exhibits a postnuptial spermatogenic cycle (Leyton et al., 1977), in *P. antofagastensis* (Boretto & Ibargüengoytía, 2006; Boretto et al., 2010) and *P. cf palluma* (Cabezas Cartes et al., 2010) with a continuous and asynchronous spermatogenic cycle, and in *P. punae*, a species with a prenuptial male cycle (Boretto et al., 2007; Boretto, 2009). *Phymaturus punae* and *P. antofagastensis* show asynchronous steroidogenic activity between testicular compartments in relation to the serum testosterone cycle and the spermatogenic cycle (Boretto 2009; Boretto et al., 2010, respectively). For adult males of *L. gravenhorsti,* Leyton et al. (1977) report seasonal variation in the activity of the interstitial tissue related to morphological changes in the seminiferous epithelium and testosterone fluctuations.

Herein we study the steroidogenic activity in Leydig and Sertoli cells by analysing serum testosterone concentration and the ultrastructural features of the organelles during the activity season of *P. zapalensis* males. The results are discussed in relation to the evolutionary adaptations of males to synchronize with the female reproductive cycle in a species with low frequency of reproduction in a cold temperate environment.

MATERIALS AND METHODS

Specimens

Adult males of *P. zapalensis* (n=23) were collected monthly from December 2004 to March 2005 and from September to December 2005, near the National Park Laguna Blanca in Zapala, Occidental District (Neuquén province, Argentina 39°44'S and 70°22'W, 824 to 1312 m). For the ultrastructural studies we utilized a sample (n=8) representative of the activity season and the spermatogenic stages observed in previous studies (Boretto & Ibargüengoytía, 2009).

Environment characteristics

Phymaturus zapalensis inhabit the Patagonian steppe, in the phyto-geographic province Patagónica, Occidental District, characterized by dry and cold climates, intense winds from the west (especially in summer), and mean annual precipitation of 176 mm (Cabrera, 1976). Besides the capture site there is snow in winter, freezing temperatures most of the year when the Laguna Blanca lagoon can be partially frozen. The landscape is characterized by plateaus with steppe vegetation over sandy soil (Cabrera, 1976).

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 microtubes at ambient temperature, spun at 1,500 rpm for 15 min, serum separated and stored at -20 °C until analyses were performed.

Tissue samples, Snout-Vent Length and gonadal index

After blood collection, 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 a saline phosphate buffer (Saline phosphate buffer -PBS- was prepared by diluting a Sigma tablet in 200 ml of double-distilled water. Working concentration: 0.01M 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.

Data of snout-vent length (SVL), antero-posterior diameter of the right testis, and spermatogenic stages of the same individuals used in the present study were obtained from Boretto and Ibargüengoytía (2009). The spermatogenic stages considered were: (1) primary or/and secondary spermatocytes, (2) spermatids, (3) spermatozoa in tubular lumen and in the epididymis, and (4) regression with scarce spermatozoa in the tubular lumen and spermatozoa in the epididymis (*sensu* Boretto & Ibargüengoytía, 2009).

Serum Testosterone measurement

Frozen serum samples were thawed, and aliquots (50 µl) were used to determine testosterone concentration in sera extracted with 100% ethanol. Serum aliquots (50 µl) were mixed with 500 µl 100% ethanol and the precipitated proteins were separated by centrifugation at 1000 x *g* for 15 min. The precipitate was re-extracted with 250 µl 100% ethanol, centrifuged and the pooled supernatants evaporated overnight at 36 ºC. The residues were dissolved in 150 µl of phosphate buffered saline with gelatin (PBSG) by incubation for 60 min at 37 ºC in a Dubnoff shaker. Aliquots (25 µl) were used for testosterone determination by Radioimmunoassay (RIA), performed using the commercial kit DSL-4100 Testosterone RIA (Diagnostic Systems Laboratories, Webster, TX), and the assay was performed by duplicate. To the standard curve points provided by the manufacturer one additional point was added, of 50 ng ml^{-1}. The curve was linear at least to this point.

Extraction efficiency was greater than 90% for the concentrations assayed, that were 2 and 50 ng ml^{-1} testosterone added to the charcoal extracted serum and assayed. Serial dilutions of one sample each from males in spring, early and late summer were parallel to the standard curve. All samples were measured in the same assay and the intra-assay coefficient of variation was 7%. The minimum detectable concentration for the assay was 7.5 pg per tube. Cross reactivity, as informed by the manufacturer was 6% with 5alpha dihydrotestosterone, 2% with 5-androstane-3beta, 17beta diol and 11-oxotestosterone and less than 1% or non-detectable with all other assayed androgens, corticoids, estrogens or progesterone.

Ultrastructural analyses

Samples for ultrastructural studies were washed in PBS postfixed in 1% osmium tetroxide overnight at 4°C. Then, the fixed 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). Micrographs of Leydig and Sertoli cells of each adult males of *P. zapalensis* were obtained (2−10 Leydig cells per male, $n_{\text{total Leydig cells}}$=32; 2−5 Sertoli cells per male, $n_{\text{total Sertoli cells}}$=27) and these micrographs were analyzed using a stereoscopic microscope (Olympus SZ-PT40). Specifically, absence/presence and abundance (scarce=1; medium=2; abundant=3) of mitochondria, and smooth endoplasmic reticulum (SER) were recorded. Additionally, the morphology of the mitochondria (with lamellar cristae or tubulo-vesicular cristae), and the nuclear morphology, such as chromatin condensation and the nucleolus morphology (absence, presence or the presence of different nucleolus regions) in Sertoli and Leydig cells were considered. The diameter of each lipid droplet and the cytoplasm area were quantified with Image Pro Plus (4.0 version) software. The percentage of the total cellular area occupied by lipid droplets was established for each cell and the mean value for each individual was calculated.

Statistical analyses

For statistical analysis we used the statistical software programs Sigma Stat® 3.5 and SPSS® 11.0. Analyses of variance (ANOVA) were used for means comparisons, and 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 not met, Mann-Whitney rank sum and Kruskal-Wallis one-way analysis of variance on ranks (KW) were used for means comparisons. The significance level used for all statistical tests was 5% (Sokal & Rohlf, 1969; Norusis, 1986). The software program Table Curve was used for quadratics functions. Data are presented as means±standard errors.

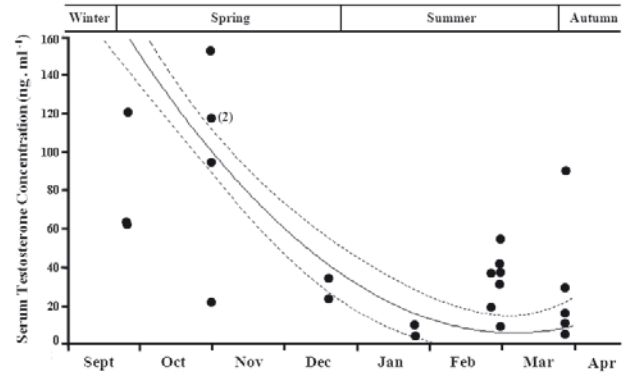

Fig. 1. Annual testosterone cycle of *Phymaturus zapalensis* males. Serum testosterone concentration (ng ml^{-1}) versus date with the linear regression and 95% confidence intervals are represented.

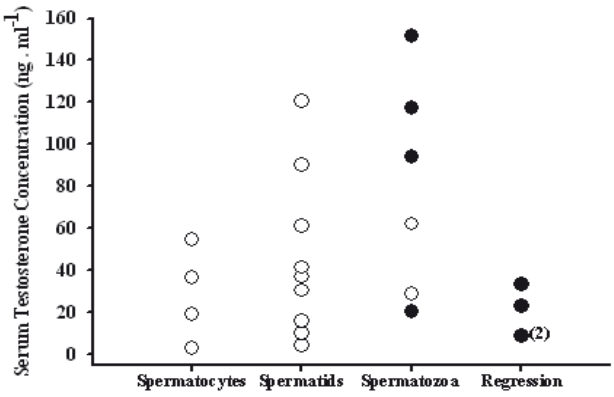

Fig. 2. Testosterone levels and the spermatogenic cycle of *Phymaturus zapalensis.* Serum testosterone concentration (ng ml^{-1}) versus the spermatogenic stage of each male are represented. The presence (black symbol) and the absence (white symbols) of spermatozoa in the epididymis are indicated.

RESULTS

Serum Testosterone concentration

The fitted curve of serum testosterone concentrations reveals that adult males *P. zapalensis* show the highest values during early spring and the lowest values in early summer ($y=a+bx^{0.5}lnx+c(lnx)^2$; r^2=0.819; F=45.48; P<0.001; Fig. 1). The serum testosterone concentration of males captured at different seasons showed significant differences (ANOVA, $F_{2,22}$=5.063, P=0.017), specifically between males captured in spring and summer (Holm-Sidak, t=2.948, P=0.008; $mean_{spring}$= 75.98±15.76 ng ml^{-1}; $mean_{summer}$= 26.65±5.84 ng ml^{-1}; $mean_{autumn}$ = 29.84±15.59 ng ml^{-1}), and between males of spring and autumn (t=2.331, P=0.030). Testosterone levels of males captured in the summer and autumn did not differ (t=0.161, P=0.874). There were not significant differences in serum testosterone concentration between males grouped according to the spermatogenic stage (ANOVA, $F_{3,23}$=2.53, P=0.088; Fig. 2), or grouped according to the presence of spermatozoa in the epididymis (Mann-Whitney, Z=-0.87, n=23, P=0.413; Fig. 2).

Ultrastructural features of cellular organelles

Nuclei: Males of *P. zapalensis* captured in early spring, that show either spermatid or spermatozoa stages and high serum testosterone levels, exhibited Leydig cells with irregular and undulate nuclei with dispersed heterochromatin (Fig. 3A; Table 1). In contrast, Sertoli cells were characterized by larger and more regularly shaped nuclei (Fig. 4A). In those males sampled later in the spring with early testicular regression, Leydig cell nuclei were more regular shaped and nucleoli were larger and more spherical, with reduced electron density (Fig. 3B, C). However, Sertoli cells were characterized by deep membrane folding and irregularly shaped nuclei (Fig. 4B). Males sampled in summer, when a new spermatogenic cycle starts (spermatocyte or spermatid stages), and serum testosterone level increases, exhibited Leydig cells with regular and spherical shape nuclei (Fig. 3D-F), and Sertoli cells with compact and small nuclei, and irregular and electron-dense nucleoli (Fig. 4C). The males captured in autumn with spermatid or spermatozoa stages, and at the first peak of serum testosterone concentration show Leydig cells irregular nuclei and peripheral heterochromatin (Fig. 3G, H). In autumn only a few males exhibited Sertoli cells with irregular-shaped nuclei with deep membrane folding and larger and high electron-dense nucleoli (Fig. 4D). The rest of the Sertoli cells exhibited compact and small nuclei and irregular and electron-dense nucleoli.

Mitochondria and SER: Males of *P. zapalensis* showed in Leydig cells a pick of tubular cristae mitochondria in early spring (Fig. 3A), changing in late spring to mainly with lamellar cristae mitochondria (Fig. 3B). In summer only mitochondria with tubular cristae were observed (Fig. 3E-F), and in autumn all the Leydig cells showed mitochondria with both types of cristae, predominating tubular cristae in 50% of the cells (Fig. 3G, I; Table 1). Adult males did not show differences in the abundance of mitochondria in Leydig cells among seasons ($mean_{spring}$=2.30±0.64; $mean_{summer}$=2.75±0.25; $mean_{autumn}$=2.29±0.41; Kruskal-Wallis, H=1.462, df=2, P=0.539). The abundance of mitochondria in Sertoli cells was low throughout the activity season ($mean_{spring}$=1.47±0.41; $mean_{summer}$=1.40±0.53; $mean_{autumn}$= 1.29±0.06; Kruskal-Wallis, H=0.112, df=2, P=0.968). In spring, mitochondria with only lamellar cristae were observed in 82% of Sertoli cells, in 56% of Sertoli cells in summer and in all Sertoli cells in autumn. The rest of the Sertoli cells showed both types of mitochondrial cristae (Table 1).

The Leydig and Sertoli cell SER development did not show significant differences among seasons (Kruskal-$Wallis_{Leydig}$, H=2.25, df=2, P=0.368; $mean_{spring}$=0.47±0.19; $mean_{summer}$=2.13±0.23; $mean_{autumn}$=1.83±0.25; P=0.368; Fig. 5; Kruskal-$Wallis_{Sertoli}$, H=2.63, df=2, P=0.269; $mean_{spring}$=0.73±0.24; $mean_{summer}$=1.00±0.17; $mean_{autumn}$=0.36±0.18; Fig. 6). Additionally, there was no significant relationship between SER development and lipid content of Leydig cells (Spearman Correlation, r=0.35, n=8, P=0.200) and between SER development and serum testosterone concentration (r=-0.46, n=8, P=0.129; Fig. 5).

Lipids percentage: Statistical analysis indicate that there were no significant differences in the lipid content of Leydig or Sertoli cells of males when they were grouped according to the capture season ($ANOVA_{Leydig}$, $F_{2,8}$=0.99, P=0.432, $mean_{spring}$=30.43±9.53; $mean_{summer}$=36.15±5.62; $mean_{autumn}$=50.26±15.36, Fig. 5; $ANOVA_{Sertoli}$, $F_{2,8}$=0.37, P=0.709, $mean_{spring}$=18.38±3.30; $mean_{summer}$=22.80±3.97; $mean_{autumn}$=20.89±4.44, Fig. 6), or by spermatogenic stages (Leydig: $F_{3,8}$=3.39, P=0.135; Sertoli: $F_{3,8}$=0.80, P=0.554; Fig. 7). The Leydig cells lipid percentage and serum testosterone concentrations did not exhibit a significant relationship, either with SVL, body weight of males or capture date (Multiple Regression Stepwise, P>0.05). In Sertoli cells there were no significant relationships between SER development and lipid content

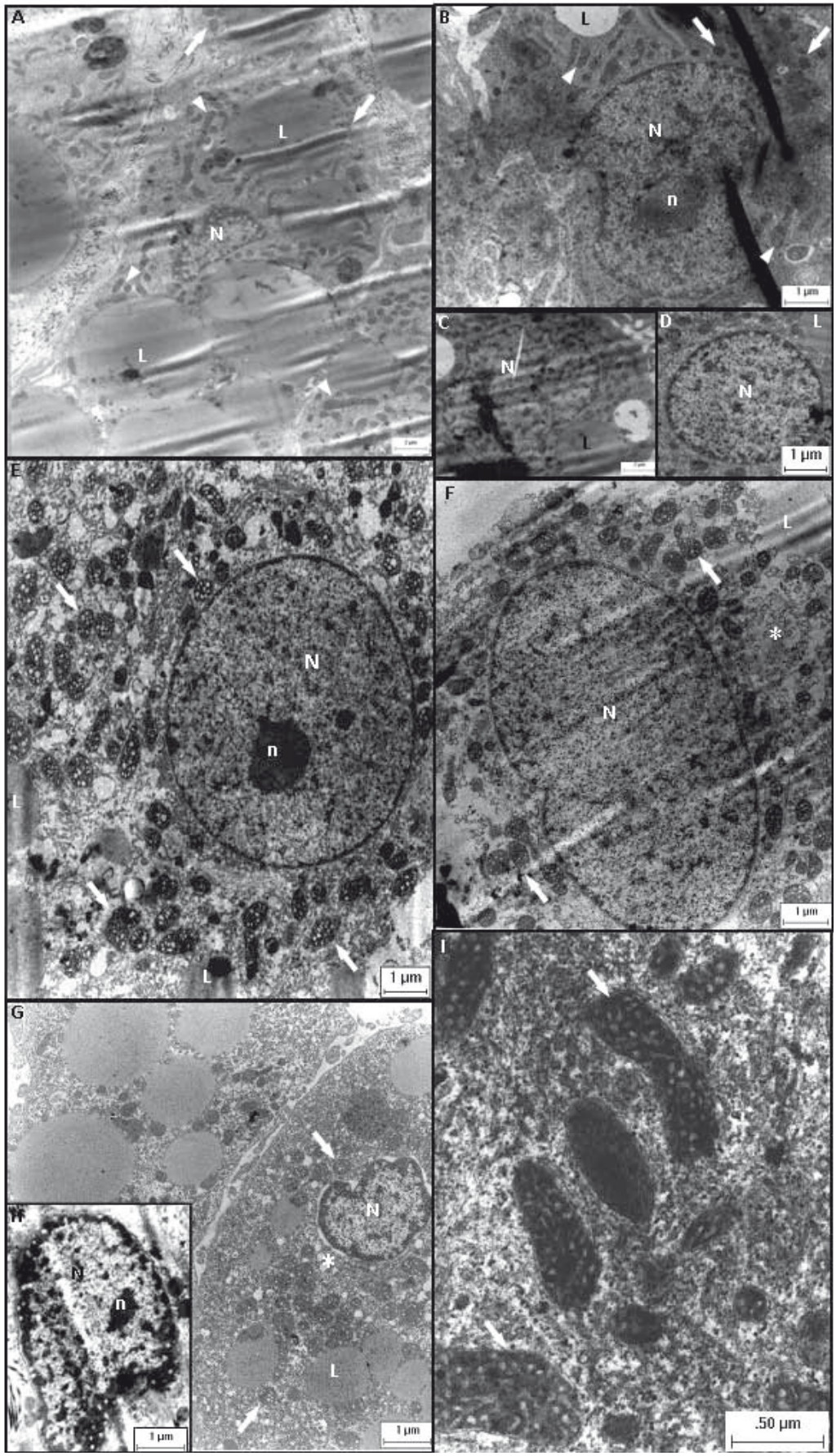

Fig. 3. Leydig cells of males of *Phymaturus zapalensis* during the activity season. (A-B-C) Leydig cells in spring; (D-E-F) summer; and (G-H-I) early autumn. Details of mitochondrial morphology in Leydig cells captured in summer (E) and in autumn (I), with predominance of mitochondria with tubular cristae (arrows), were exhibited. N: nucleus; n: nucleolus; L: lipid droplet; *: smooth endoplasmic reticulum; arrows head: mitochondria with lamellar cristae.

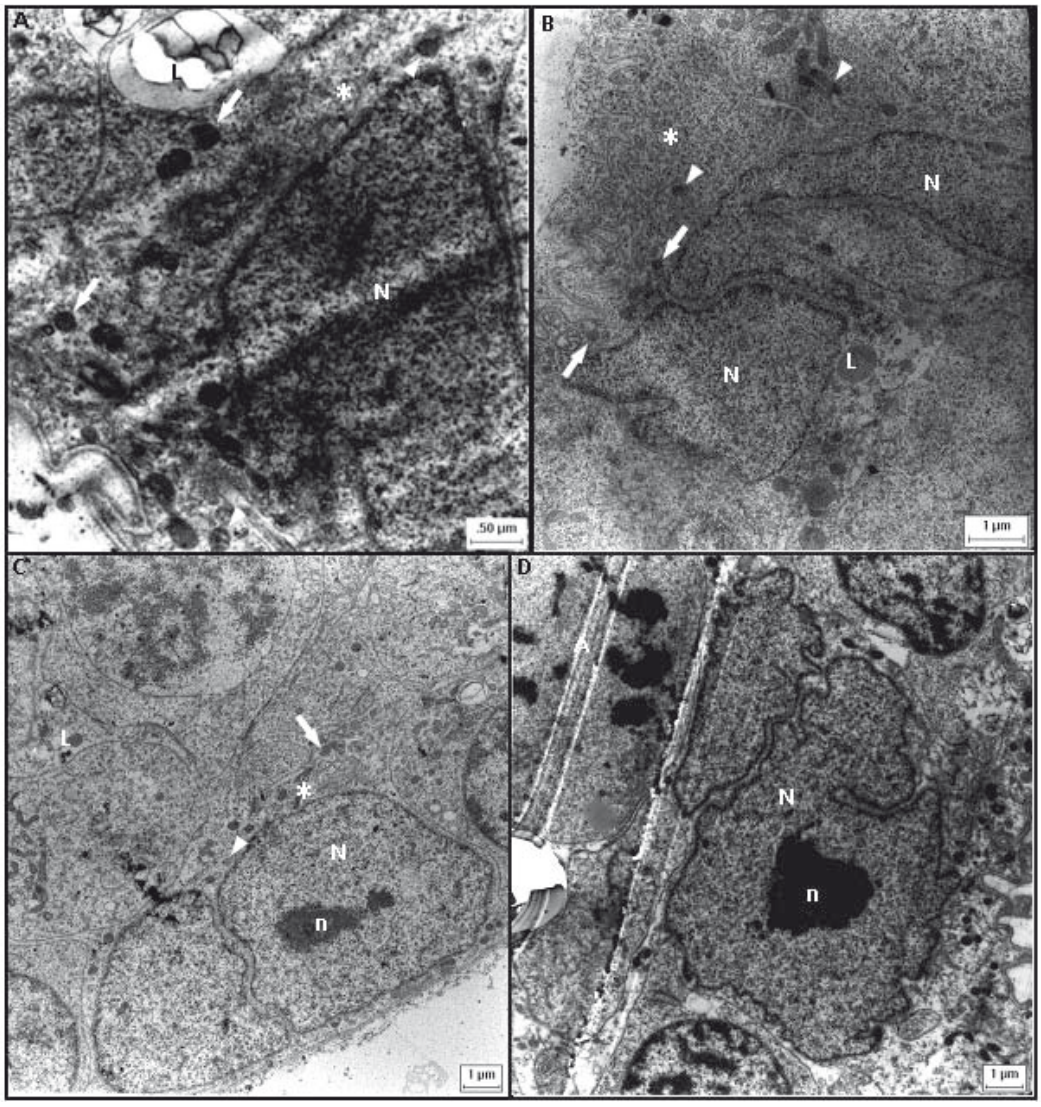

Fig. 4. Sertoli cells of adult males of *Phymaturus zapalensis* during the activity season. (A) Sertoli cells in early spring; (B) late spring; (C) mid-summer; and (D) early autumn. N: nucleus; n: nucleolus; L: lipid droplet; *: smooth endoplasmic reticulum; arrows: mitochondria with tubular cristae; arrows head: mitochondria with lamellar cristae.

(Spearman Correlation, r=-0.03, n=8, P=0.476). There were no significant relationships between Sertoli cells lipid content and SVL, body weight of males, or capture date (Multiple Regression Stepwise, P>0.05).

Analysis of the existence of temporal asynchrony in steroidogenic activity between interstitial and tubular compartments of the testis

Some males of *P. zapalensis* captured in summer and autumn that exhibited spermatid or spermatozoa stages, without spermatozoa in the epididymis and moderate serum testosterone concentration, exhibited asynchronous steroidogenic activity between testis compartments, characterized by the presence of Sertoli cells with morphological signs of steroid synthesis, whereas Leydig cells were inactive. Only one male that possessed spermatid stage and moderate serum testosterone level exhibited steroidogenic activity in Leydig cells and inactivity in Sertoli cells (Table 1).

DISCUSSION

In reptiles with postnuptial male cycles, spermatogenesis may not be completed before overwintering, and depending on the species and habitat, result in varying degrees of synchrony between interstitial cell activity, behavioural and spermatogenic activity (Callard & Ho, 1980). Postnuptial male cycle in *P. zapalensis* is not completed before hibernation, and there are not spermatozoa stored in the epididymis (Boretto & Ibargüengoytía, 2009), with Leydig and Sertoli cells showing morphological features characteristic of steroidogenic activity coinciding with an evident asynchrony in testosterone production between testicular compartments. Only during spring, when testosterone peaks and mating occurs, the steroidogenic activity of Leydig and Sertoli cells was synchronous, suggesting that Sertoli cells are responsible for spermiation, while Leydig cells are mainly responsible for mating behaviour. Serum testosterone levels then decrease abruptly, and the testicular recrudescence of the next spermatogenic

cycle is accompanied by a gradual increase in serum testosterone concentrations. In summer and autumn, some of the males exhibited steroid synthesis features in Sertoli cells and spermatid or spermatozoa stages, whereas Leydig cells were inactive, supporting the hypothesis that Sertoli cells androgens act locally in the seminiferous tubules to synchronize and maintain the spermatogenic cycle. Testosterone synthesized by Leydig cells in spring could be the necessary stimulus to initiate activity in the seminiferous tubules and a new spermatogenic cycle in early summer. Histochemical studies are necessary to determine the presence of key enzymes, such as 3β-HSD or 17β-HSD to validate the hypotheses of steroidogenic activity in Sertoli cells and their temporal asynchrony with the steroidogenic activity of Leydig cells in *P. zapalensis*. However, the presence of SER, lipid droplets and mitochondria with tubular cristae in Sertoli cells of *P. zapalensis* males is indicative of the existence of steroidogenic activity in these cells, as has been described in *P. antofagastensis* (Boretto et al., 2010).

Two species of turtles also exhibit a postnuptial spermatogenic cycle and express decoupled functional activity of seminiferous tubules and interstitial tissues similar to what we found in *P. zapalensis* (the Chinese softshell turtle, *Trionyx sinensis*, Lofts & Tsui, 1977, and the painted turtle, *C. picta*, Callard & Ho, 1980). As with *P. zapalensis*, in *T. sinensis* the period of courtship and mating occurs in spring, the interstitial cells exhibit morphologic evidence of steroidogenic activity and a strong positive reaction for the 3β-HSD, whereas the seminiferous tubules were in regression, inactive and with high quantities of lipids and males inseminate females with the spermatozoa stored in the epididymis (Lofts & Tsui, 1977). In *P. zapalensis* instead, Sertoli cells seem to be an active site of steroid synthesis during spring, when mating occurs. Sertoli cells are active after the mating period in *T. sinensis*, and this steroidogenic activity is attributed to a spermatoquinetic effect on the adjacent germinal tissue. In this case, also reported in the turtle *C. picta* (Callard & Ho, 1980; Dubois et al., 1988), the androgens produced by the interstitial cells did not stimulate the spermatogenic cycle directly, given that the seminiferous tubules were inactive at the time that the interstitial cells expressed maximal steroidogenic activity (Lofts & Tsui, 1977).

Males of the genus *Phymaturus* exhibit different strategies to coordinate their reproductive cycles with those of females. Particularly in *P. zapalensis* males, the testosterone cycle described in the present work parallels the annual and postnuptial spermatogenic cycle described previously (Boretto & Ibargüengoytía, 2009). The only liolaemid with a postnuptial cycle studied was the viviparous lizard *L. gravenhorsti* (Leyton et al., 1977), and the testosterone profile differed from that of *P. zapalensis*. Males of *L. gravenhorsti* had maximum testosterone concentrations in winter when spermatogenesis had ceased, low concentrations during the mating period in spring and medium concentrations during spermatogenesis in summer and autumn (Leyton et al., 1977). In contrast, testosterone concentrations in *P. zapalensis* males shows a maximum in mid-spring, the

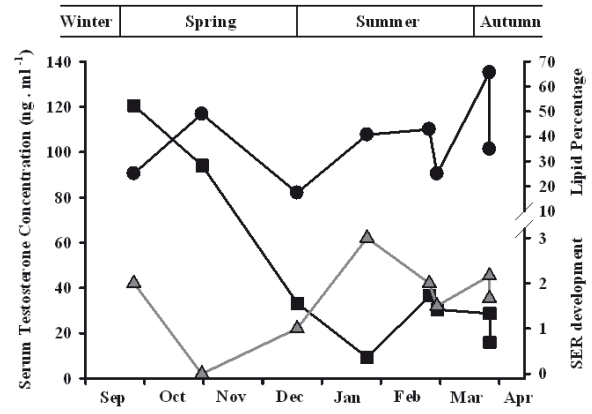

Fig. 5. Variations in steroid activity of *Phymaturus zapalensis* Leydig cells, in relation with testosterone levels during the activity season. Mean serum testosterone concentration (ng ml^{-1}; squares), mean SER development (SER, triangles), and mean lipid percentage (points) of Leydig cells of each male versus date, were indicated.

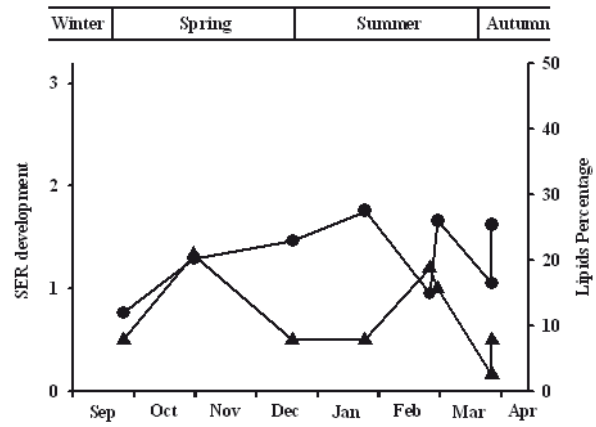

Fig. 6. Variations in steroid activity of *Phymaturus zapalensis* Sertoli cells, during the activity season. The mean SER development (SER, triangles) and the mean lipid percentage (points) of Sertoli cells for each adult male versus date are indicated.

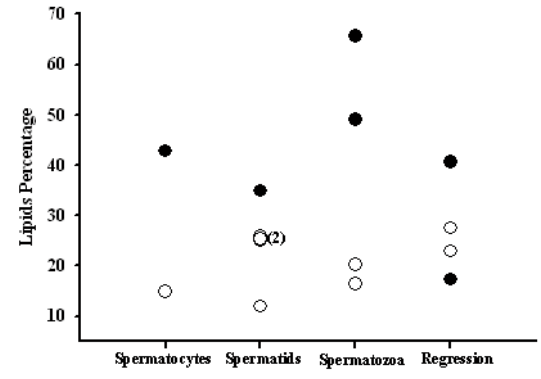

Fig. 7. Lipid dynamics of Leydig (black) and Sertoli cells (white) of *Phymaturus zapalensis* adult males during the activity season. The mean lipid percentage versus the spermatogenic stages of each male is exhibited. Brackets indicate the number of individuals.

Table 1. Steroidogenic activity in the interstitial and tubular compartments of the testis in *Phymaturus zapalensis*. Capture season (Season); spermatogenic stage; presence or absence of spermatozoa in the epididymis (Sperm in Epid); serum testosterone concentration (T, ng ml^{-1}); mean and standard errors of the lipid percentage (Lipids), SER and mitochondria abundance; predominant morphology of mitochondria cristae (tubular=T; lamellar=L); in Leydig and Sertoli cells of each male are indicated. Leydig and Sertoli cells were classified (Activity) as active (A) or inactive (I) in accordance with all steroid features mentioned above, and synchronous (S) or asynchronous (A) steroid activity between compartments is indicated.

Season	Spermatogenic Stage	Sperm in Epid	T	Leydig cells				Sertoli cells				Steroid Activity
				Lipids	SER	Mitochondria	Activity	Lipids	SER	Mitochondria	Activity	
Spring	Spermatids	absent	120.6	25.04±8.35	2±0	3±0 /T-L	A	11.97±2.73	0.5±0.29	1±0 /L	A	**S**
	Spermatozoa	present	94.2	48.96±3.67	0±0	2.15±0.26 /L	A	20.23±7.11	1.33±0.67	1.67±0.33 /L	A	**S**
	Regression	present	33.2	17.30±8.30	1±0	1.75±0.75 /L	I	22.94±5.44	0.5±0.25	1.75±0.25 /L	I	**S**
Summer	Regression	present	9.2	40.65±2.74	3±0	3±0 /T	I	27.48±2.64	0.5±0.5	1±0 /L	I	**S**
	Spermatocytes	absent	36.5	42.82±2.54	2±0	2.75±0.25 /T	I	14.91±3.65	1.2±0.2	1.2±0.2 /L	A	**A**
	Spermatids	absent	30.4	24.99±3.04	1.5±0.5	2.5±0.5 /T	A	26.0±0.43	1±0	2±1 /L-T	I	**A**
Autumn	Spermatozoa	absent	28.7	65.62±12.38	2.17±0.6	2±0.58 /T	I	16.45±4.08	0.17±0.17	1.33±0.33 /L	A	**A**
	Spermatids	absent	15.9	34.90±3.30	1.67±0.25	2.58±0.20 /L	I	25.33±2.19	0.5±0.29	1.25±0.25 /L	I	**S**

lowest levels in early summer and a restart of a new cycle in late summer and early autumn. This testosterone cycle ensures the synchrony of males and females reproductive cycles in *P. zapalensis*, given that females ovulate in mid-spring.

Bimodal patterns of testosterone secretion have been found in the northern water snake *Nerodia sipedon*, in the snapping turtle *Chelydra serpentina* (Weil & Aldridge, 1981; Mahmoud et al., 1985; respectively) and in the viviparous Tasmanian lizard, *Niveoscincus ocellatus* (Jones et al., 1997) that inhabit environments with temperate and seasonal climates that could be comparable with environments of *P. zapalensis*. But the activity of *N. ocellatus* males is inverse to *P. zapalensis*. Males of *N. ocellatus* have a high peak of testosterone in late summer and early autumn and a lower peak in the spring after emergence from hibernation, attributed to another period of mating behaviour in the spring. The elevated levels of testosterone found in males of *P. zapalensis* that were captured at the end of their activity season could be maintained throughout winter when metabolism is almost arrested during hibernation, reaching the maximum peak in the next spring, as is the case in the northern water snake *Nerodia sipedon* (Weil & Aldridge, 1981). The spermatogenic and testosterone cycles observed in males of *P. zapalensis* are possible due to the temporal separation of steroidogenic activity of Leydig and Sertoli cells. The maximum serum testosterone concentrations found in males of *P. zapalensis* during the activity season (152 ng ml^{-1}) were similar to the levels found in other *Phymaturus* species (maximum peak $_{P.\ punae}$=165 ng ml^{-1}, Boretto, 2009; maximum peak $_{P.\ antofagastensis}$=230 ng ml^{-1}, Boretto et al., 2010; maximum peak $_{P.\ cf.\ palluma}$=199 ng ml^{-1}, Cabezas Cartes et al., 2010). During the activity season in *P. zapalensis*, the serum testosterone cycle were similar to the cycle observed in the snapping turtle, *C. serpentina* (Mahmoud et al., 1985). In the snapping turtle as in *P. zapalensis*, the Leydig cells were the principal site of androgen synthesis, becoming active just after the end of the hibernation period (Mahmoud et al., 1985; Mahmoud & Licht 1997).

Phymaturus males have shown different responses to thermal limitations, increasing the chances of male and female encounters, as was observed in the present work. Hormonal and ultrastructural studies in *P. zapalensis* suggest that Leydig and Sertoli cell steroidogenic activity serves to synchronize male and female reproductive cycles and assure that reproduction occurs when conditions within the constraining environment of the Patagonian steppes are most suitable for rearing young.

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