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Testosterone Cycle and Regulation of Reproductive Events in the Lizard *Phymaturus punae* (Liolaemidae) from the Highlands of the Andes, Argentina

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ABSTRACT.—**The genus *Phymaturus*, entirely viviparous and mostly herbivorous, inhabits the cold and harsh environments of the Andean highlands of Argentina and Chile, and the Patagonian steppe of Argentina. *Phymaturus punae* is a vulnerable lizard endemic to the Biosphere Reserve San Guillermo (National Park and Provincial Reserve) in San Juan (Argentina) that inhabits high altitudes of 3,100–4,200 m. The reproductive cycles of males and females of *P. punae* have been described previously. Males perform a prenuptial and annual cycle of spermatogenesis, and females reproduce once every 2 yr. As a consequence, the adjustment in the timing of males to the reproductive cycles of females must be very precise to ensure reproductive success. We elucidate the time of mating and the asynchrony of male and female reproductive events in *P. punae* based on endocrine and ultrastructural studies. Present hormonal results support the idea that copulation in *P. punae* occurs at the end of the activity season. Ultrastructural features observed in Sertoli and Leydig cells indicate that both types of cells have the potential to synthesize steroid hormones, to support the spermatogenic cycle and the mating period, respectively. In *P. punae* the cases of temporal asynchrony in steroid activity suggest that this mechanism must be important to start the spermatogenesis in spring, supported by the steroid activity of Sertoli cells, as Leydig cells are inactive. Nevertheless the asynchronic steroid mechanism seems to be more necessary in *Phymaturus* species with continuous or postnuptial cycles than in species with prenuptial cycles, like *P. punae*.**

RESUMEN.—**El género *Phymaturus*, enteramente vivíparo y mayormente herbívoro, habita ambientes fríos y rigurosos de la Cordillera de los Andes en Argentina y Chile, y de la estepa Patagónica Argentina. *Phymaturus punae* es un lagarto vulnerable, endémico de la Reserva de la Biósfera San Guillermo (Parque Nacional y Reserva Provincial) en San Juan (Argentina), que habita a altas altitudes entre los 3.100 y 4.200 metros. Los ciclos reproductivos masculinos y femeninos de *P. punae* fueron descriptos. Los machos realizan un ciclo espermatogénico anual prenupcial, y las hembras se reproducen una vez cada dos años. En consecuencia, el ajuste de los tiempos de los machos al ciclo reproductivo femenino debe ser muy preciso para asegurar los sucesos reproductivos. Dilucidamos el período de cópula y la asincronía de los eventos reproductivos de machos y hembras de *P. punae*, en base a estudios endócrinos y ultraestructurales. Los resultados hormonales obtenidos apoyan la idea que la cópula en *P. punae* ocurre al final de la temporada de actividad. Las observaciones ultraestructurales en células de Sertoli y de Leydig indican que ambos tipos celulares tienen el potencial para sintetizar hormonas esteroideas, para mantener la espermatogénesis y la cópula, respectivamente. En *P. punae* los casos de asincronía temporal en la actividad esteroidea, sugieren que este mecanismo es importante para iniciar la espermatogénesis en primavera, sostenida por la actividad esteroidea de las células de Sertoli, mientras las células de Leydig están inactivas. No obstante, el mecanismo de asincronía esteroidea parece ser más necesario en especies de *Phymaturus* con ciclos continuos o postnupciales, que en especies con ciclos prenupciales, como *P. punae*.**

In reptiles living in harsh and cold environments, the timing of male and female reproductive cycles is under strong selection to increase the offspring's probability of survival during its first year (Saint Girons, 1985; Olsson and Shine, 1998; Gotthard, 2001). In this sense, lizards are constrained by the need to reproduce during short activity seasons, from midspring to early autumn. Brumation occurs from autumn to spring, and during this long period, physiological activity is almost nil (Saint Girons, 1985; Gotthard, 2001). Lizards living in cold climates often show reproductive styles that favor male/female encounters (Saint Girons, 1985), nourishment by viviparity (Olsson and Shine, 1998, 1999), birth during warmer periods of activity seasons, and larger offspring (Cree and Guillette, 1995; Edwards et al., 2002; Ibargüengoytía and Casalins, 2007). Lizards of the genus *Phymaturus* inhabit cold regions at high altitudes in the Andes highlands of Argentina and Chile, or at high latitudes in the Patagonian steppe of Argentina (e.g., Cei, 1986, 1993; Etheridge, 1995; Scolaro and Pincheira-Donoso, 2010; Lobo et al., 2012; Morando et al., 2013). In these geographical areas, a large part of the year is unsuitable for growth and reproduction (Boretto and Ibargüengoytía, 2006; Boretto et al., 2007; Ibargüengoytía et al., 2008; Boretto and Ibargüengoytía, 2009; Cabezas Cartes et al., 2010). The ambient temperatures and the extensions of the activity seasons are neither high enough nor long enough to allow females to complete vitellogenesis, ovulation, gestation, and parturition in a single season, and as consequence, the female cycle is prolonged to two or more years, as was observed in all *Phymaturus* species studied up to date (Habit and Ortiz, 1996; Ibargüengoytía, 2004; Boretto and Ibargüengoytía, 2006; Boretto et al., 2007; Boretto and Ibargüengoytía, 2009; Cabezas Cartes et al., 2010). As a consequence of the female prolonged cycles, all *Phymaturus* species exhibited low mean annual reproductive output and a low proportion of receptive females in the populations (Habit and Ortiz, 1996; Ibargüengoytía, 2004; Boretto and Ibargüengoytía, 2006; Boretto et al., 2007; Boretto and Ibargüengoytía, 2009; Cabezas Cartes et al., 2010). Males have developed different paths to coordinate with female cycles, showing interspecific differences. Males of *Phymaturus tenebrosus* (Ibargüengoytía, 2004) and *Phymaturus zapalensis* (Boretto and Ibargüengoytía, 2009) showed the same annual postnuptial cycle; *Phymaturus antofagastensis* (Boretto and Ibargüengoytía, 2006) and *Phymaturus aguanegra* (Cabezas Cartes et al., 2010)

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exhibited an asynchronous spermatogenic cycle among males, continuous in terms of the presence of males with spermatozoa in the epididymis and high serum testosterone concentrations during the entire length of the activity season (Boretto et al., 2010; Cabezas Cartes et al., 2010). *Phymaturus vociferator* (Habit and Ortiz, 1996) and *Phymaturus punae* (Boretto et al., 2007) present the same male prenuptial annual cycle. Previous ultrastructural and endocrine studies have showed that males of *P. antofagastensis* and *P. zapalensis* develop physiological mechanisms that allow them to synchronize the female reproductive cycle (Boretto et al., 2010, 2012). These mechanisms consist of asynchronous steroid hormone synthesis between interstitial and tubular compartments of the testes (Leydig and Sertoli cells, respectively) making gametogenesis temporally independent of mating, as described previously in turtles (Callard and Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988; Mahmoud and Licht, 1997). The steroidogenic activity of cells has been characterized by ultrastructural studies, by the observations of marked development of smooth endoplasmic reticulum, the presence of abundance of mitochondria with tubular cristae, and a reduction of cytoplasmic lipid droplets (e.g., Lofts and Tsui, 1977; Dubois et al., 1988; Ibargüengoytía et al., 1999; Ferreira and Dolder, 2003). The accumulation of cholesterol rich in lipid droplets in Leydig and Sertoli cells, a minor development of smooth endoplasmic reticulum, and a minor abundance of mitochondria with tubular cristae, have also been recognized as an indicator of steroidogenic inactivity (e.g., Callard et al., 1976; Lofts and Tsui, 1977; Mahmoud et al., 1985; Dubois et al., 1988).

We studied the serum testosterone cycle and the role of Leydig and Sertoli cells in the steroid hormone synthesis and in the regulation of reproductive events in *Phymaturus punae*, a lizard species with a prenuptial and annual spermatogenic cycle. *Phymaturus punae* is an endemic and vulnerable viviparous lizard that inhabits the Biosphere Reserve San Guillermo (National Park and Provincial Reserve), located at 3,100–4,200 m a.s.l. in the Andes Mountains in San Juan (Argentina). In *P. punae* spermatogenesis starts in spring and ends in midsummer when abundant spermatozoa appear in both testicles and epididymis (Boretto et al., 2007). Females, with a litter of only one or two offspring, exhibit a biennial reproductive cycle based on the simultaneous presence of females performing either advanced vitellogenesis or late pregnancy in mid-summer (Boretto et al., 2007). However, we were not able to determine when mating, ovulation, and fecundity occur in *P. punae* because the high risk of avalanche is a considerable safety concern and makes access to the study site almost impossible in early spring and autumn. In consequence, Boretto et al. (2007) hypothesized two possibilities: first, ovulation and mating occur in autumn, and females develop a prolonged gestation over winter that ends in midsummer when births occur, or females store the sperm in the reproductive tract during the brumation period and ovulation occurs in spring. Second, mating occurs in spring and males store sperm in the epididymis and females develop a prolonged 1-yr vitellogenesis, from spring to autumn, which is arrested over winter, and continues and ends the following spring when ovulation and mating occur (Boretto et al., 2007). Histological studies of the reproductive tract of vitellogenic females, captured in late spring and midsummer, revealed the absence of sperm storage tubules in *P. punae*, and in consequence Boretto et al. (2007) suggested that the mating period probably occurs in spring, and males store the sperm during winter.

In the present work, based on endocrine and ultrastructural studies in adult males of *P. punae*, we define 1) when mating occurs, 2) the serum testosterone cycle, and 3) whether *P. punae* has a testicular asynchronous steroid mechanism that allows males to perform spermatogenesis and mating at different times during the activity season, like the asynchronous steroidogenic activity between Leydig and Sertoli cells observed in *P. antofagastensis* (Boretto et al., 2010) and *P. zapalensis* (Boretto et al., 2012).

Materials and Methods

Specimens.—Adult males ($n = 8$) of *P. punae* were collected in spring (December) 2004 and summer (February) 2005 in the Provincial Reserve San Guillermo, San Juan (Argentina, 28°59′–29°02′S; 69°29′–69°05′W; 3,100–4,200 m a.s.l.). The number of specimens examined in this study was necessarily small because of the protected conservation status of species that inhabit the Biosphere Reserve Park of the Park San Guillermo, the *P. punae* endemism in this site, and the recent recategorization as Vulnerable, that exclude the possibility of capturing more lizards (Abdala et al., 2012).

Environmental Characteristics.—The Provincial Reserve San Guillermo in San Juan province (Argentina) is in the highlands of the Andes; it extends over 170,000 hectares (Haene et al., 2000) and has a cold climate with low temperatures most of the year and low precipitation, including during the activity period of lizards (Fig. 1). Mean annual temperature is −5°C, but the absolute minimum temperature registered in July was −15°C (Cajal et al., 1981). The phytogeographic region is characterized by Nanophanerophytas zigophyllaceae as *Larrea nitida*, *Larrea divaricada*, *Bulnesia retamo*, *Lycium*, *Adesmia*, and *Senecio*, and rockrose xerophytes and the grass family (Gramineae) as *Stipa* (Cajal et al., 1981).

Blood Samples.—Lizards were weighed (g) and anesthetized with an intraperitoneal dose of sodium thiopental (0.03 mm^3/10 g of body weight); 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, lizards 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: 0.01 M phosphate buffer, 0.137 M NaCl and 0.0027 M KCl, pH 7.4). The specimens were kept in the fixative Bouin's solution for 24 h and preserved in 70% ethanol until use. The specimens are kept in the Department of Biology and Institute and Museum of Natural Science, Universidad Nacional de San Juan.

Data of snout–vent length (SVL), anteroposterior diameter of the right testis, and spermatogenic stages of the same individuals used in the present study were obtained from Boretto et al. (2007). The spermatogenic stages considered were 1) spermatids, 2) spermatozoa (in tubular lumen and in the epididymis), and 3) regression with scarce spermatozoa in the tubular lumen and spermatozoa in the epididymis (*sensu* Boretto et al., 2007).

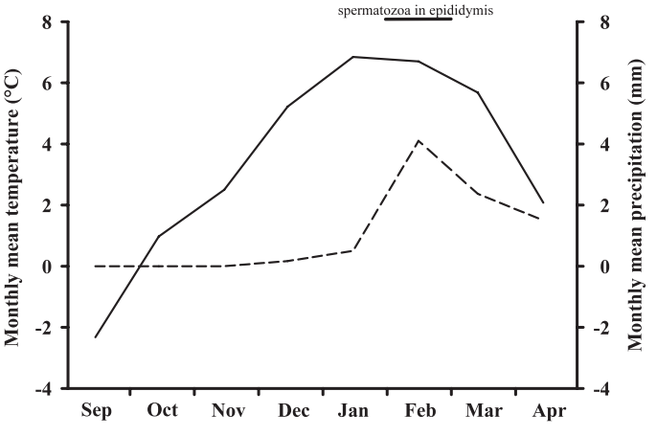

FIG. 1. Climatic variables at capture site of *Phymaturus punae*. The average monthly accumulated rainfall (mm, dashes), and ambient temperature (°C, solid line) during the activity season, spring to autumn, in the southern hemisphere, are presented. The black line points out the presence of males with spermatozoa in the tubular lumen and/or in the epididymis (Boretto et al., 2007).

Testosterone Measurements.—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 1,000 × *g* for 15 min. The precipitate was re-extracted with 250 μl 100% ethanol and centrifuged, and the pooled supernatants were evaporated overnight at 36°C. The residues were dissolved in 150 μl of PBS with gelatin (PBSG) by incubation for 60 min at 37°C in a Dubnoff shaker. Aliquots (25 μl) were used for testosterone determination by RIA, performed with the use of 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, which 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 and 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 nondetectable with all other assayed androgens, corticoids, estrogens, or progesterone.

Ultrastructural Analysis.—Samples for ultrastructural studies were washed in PBS and postfixed in 1% osmium tetroxide overnight at 4°C. Then, the fixed material was dehydrated through a gr[illegible] alcohol-acetone series and finally embedded in Epon 812® [illegible]ed pella). Ultrathin sections were obtained with a LEICA (Ultracut) ultramicrotome and stained with lead citrate and uranyl acetate. Observations were made with the use of a Zeiss EM (900 series). Micrographs of Leydig and Sertoli cells of each adult male *P. punae* were obtained (two–nine Leydig cells per male, $n_{totalcells} = 37$, one–four Sertoli cells per male, $n_{totalcells} = 16$), and these micrographs were analyzed with the use of 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 qualitatively recorded, and independently verified by two authors. 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 Analysis.—For statistical analyses we used the statistical software programs Sigma Stat 3.5®, SPSS 15.0®, Sigma Plot 10.0®, and analysis of variance (ANOVA) and covariance (ANCOVA), linear regression, multiple regression (stepwise), Spearman correlation, Mann–Whitney rank sum and Kruskal–Wallis one-way analysis of variance on ranks (KW). Assumptions of normality and homogeneity of variance were tested with the one-sample Kolmogorov–Smirnov test and with the Levene test, respectively (Sokal and Rohlf, 1969; Norusis, 1986). Means are given +/[illegible]tandard error (SE).

RESULTS

Serum Testosterone Concentration.—Males of *P. punae* captured in spring did not show significant differences in SVL or body mass with males captured in summer (ANOVA, SVL: $F_{1,8} = 0.001$, $P = 0.971$; body mass: $F_{1,8} = 0.022$, $P = 0.886$). Additionally, there was no significant relationship between serum testosterone concentration and SVL or body mass (linear regression, SVL: $F_{1,7} = 0.868$, $P = 0.388$; body mass: $F_{1,7} = 3.532$, $P = 0.109$). Males showed significantly different testosterone concentration between seasons (ANCOVA, $F_{1,8} = 17.00$, $P = 0.009$; Table 1) considering body mass as a significant covariable in the model ($F_{1,8} = 11.17$, $P = 0.021$). The lowest serum testosterone levels were exhibited by males captured in spring with either spermatids, spermatozoa, or regression testicular stages, but without spermatozoa in the epididymis. Maximum testosterone levels were exhibited in males captured in summer with spermatozoa or regression stages, although with adundant spermatozoa in epididymis (Table 1).

Testosterone concentrations were significantly different between males grouped according to the presence or absence of spermatozoa in the epididymis (ANCOVA, $F_{1,7} = 17.34$, $P = 0.014$, Table 1; body mass as a significant covariable, $F_{1,7} = 11.54$, $P = 0.027$), although there were no significant differences in testosterone levels among males grouped according to the spermatogenic stage (Kruskal–Wallis, $H = 3.00$, df = 2, $P = 0.223$).

Ultrastructural Features.—Nuclei: Males of *P. punae* captured in spring, showing either spermatids or spermatozoa stages and low serum testosterone levels, exhibited 75% of Leydig cells with regular, spherical, and compact-shaped nuclei with peripherical condensated heterochromatin. The nucleoli was electron dense and exhibited medium size in the majority of cells, whereas it was big and highly electron dense only in a few cells. In summer, in males with spermatozoa or early regression stages and high testosterone levels, 66% of the Leydig cells exhibited regular-shaped nuclei with dispersed chromatin (Fig. 2). The rest of the Leydig cells exhibited irregular and undulate nuclei with hetechromatin condensed in the periphery. The Leydig cells of males captured in summer also exhibited big and

TABLE 1. Steroidogenic activity in the interstitial and tubular compartments of the testis in *Phymaturus punae* in spring and summer. Snout–vent length (SVL, mm), body weight (BW, g), spermatogenic stage (SS, spermatids: Sp, spermatozoa: Sz, segression: Rg), presence (P) or absence (A) of spermatozoa in the epididymis (SEpid), serum testosterone concentration (T, ng · ml^{-1}), mean and standard errors of the lipid percentage (Lipids), smooth endoplasmic reticulum (SER) and mitochondria abundance, and the predominant morphology of mitochondria cristae (tubular = T; lamellar = L) in Leydig and Sertoli cells of each male are indicated. The mean and standard errors of some variables for each season are presented in bold font. Leydig and Sertoli cells were classified (A) 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.

					Leydig cells				Sertoli cells				Steroid
SVL	BW	SS	SEpid	T	Lipids	SER	Mitochondria	Activity	Lipids	SER	Mitochondria	Activity	activity
Spring													
95.9	**29**	**Sp**	**A**	**2.1**	**20.3 ± 5.0**	**2.5 ± 0.1**	**0.8 ± 0.2/L**	**I**	**5.2 ± 3.3**	**0.7 ± 0.3**	**1.2 ± 0.2/L**	**A**	**A**
102.4	**38**	**Sp**	**A**	**32.4**	**25.2 ± 4.2**	**2.1 ± 0.3**	**2.0 ± 0.1/T**	**A**	**5.0 ± 2.6**	**1.3 ± 0.3**	**2.5 ± 0.5/L**	**A**	**S**
103.4	**38**	**Rg**	**A**	**35.3**	**16.6 ± 4.3**	**2.6 ± 0.2**	**2.4 ± 0.4/T**	**A**	**11.3 ± 1.4**	**0.0 ± 0.0**	**1.7 ± 0.3/L**	**I**	**A**
100.6 ± 2.4	35 ± 3			23.3 ± 10.6	20.7 ± 2.5	2.4 ± 0.1	1.7 ± 0.5		7.2 ± 2.1	0.7 ± 0.4	1.7 ± 0.4		
Summer													
95.3	**29.5**	**Sz**	**P**	**53.5**	**32.1 ± 7.8**	**3.0 ± 0.0**	**2.3 ± 0.8/T**	**A**	**8.4 ± 0.0**	**0.0 ± 0.0**	**2.0 ± 0.0/L**	**I**	**A**
95.2	**29**	**Sz**	**P**	**78.2**	**31.9 ± 10.7**	**2.0 ± 0.6**	**1.5 ± 0.0/T-L**	**A**	**2.4 ± 0.5**	**0.5 ± 0.3**	**0.8 ± 0.2/L**	**A**	**S**
95.8	**34**	**Sz**	**P**	**62.9**	**16.0 ± 12.1**	**2.0 ± 1.0**	**2.0 ± 0.6/T**	**A**	**4.9 ± 2.2**	**1.5 ± 0.5**	**2.5 ± 0.0/L**	**A**	**S**
100.2	**42**	**Rg**	**P**	**164.9**	**73.9 ± 1.8**	**1.8 ± 0.3**	**2.0 ± 0.2/T**	**I**	**20.9 ± 0.0**	**2.0 ± 0.0**	**2.5 ± 0.0/L**	**I**	**S**
96.6 ± 1.2	33.6 ± 3			89.9 ± 25.5	38.5 ± 12.4	2.2 ± 0.3	1.9 ± 0.2		9.2 ± 4.1	1 ± 0.5	1.9 ± 0.4		

highly electron-dense nucleoli. All spring and summer males exhibited Sertoli cells with deep membrane folding and irregularly shaped nuclei and big and electron-dense nucleoli (Fig. 3).

Mitochondria and SER: All Leydig cells exhibited great variability in SER development (Figs. 2, 4A) and mitochondria abundance, and a similar proportion of mitochondria with lamellar or tubular cristae (Fig. 2; Table 1). There were no significant differences in the abundance of mitochondria in Leydig cells between seasons (Mann–Whitney, $Z = -1.528$, $n = 6$, $P = 0.127$), being scarce to moderate in spring, and moderate in summer (Table 1). All males captured, independently of seasons, showed most of the Sertoli cells with only lamellar cristae mitochondria, and only few cells showed both types of cristae (Fig. 3; Table 1). There was high variation in the mitochondria abundance (scarce to moderate) in Sertoli cells and there were no significant differences between seasons ($Z = -0.367$, $n = 7$, $P = 0.714$; Table 1).

The SER development in Leydig and Sertoli cells did not show significant differences between seasons ($Z_{\text{Leydig}} = -1.07$, $n = 7$, $P = 0.400$; $Z_{\text{Sertoli}} = -0.54$, $n = 7$, $P = 0.629$). The SER development was moderate in all Leydig cells. In Sertoli cells the SER development was absent or scarce in males of spring, and moderate in summer (Fig. 4A,B; Table 1). Additionally, the SER development and the lipid content did not show a significant correlation in Leydig cells (Spearman correlation, $r = -0.18$, $n = 7$, $P = 0.350$); neither did the SER development show a significant relationship with the testosterone concentration ($r = -0.67$, $n = 7$, $P = 0.051$; Fig. 4A).

Lipids percentage: In Leydig and in Sertoli cells there were no significant differences in the lipid content between seasons ($\text{ANOVA}_{\text{Leydig}}$, $F_{1,7} = 1.44$, $P = 0.284$; $\text{ANOVA}_{\text{Sertoli}}$, $F_{1,7} = 0.15$, $P = 0.715$; Fig. 4A,B), or among spermatogenic stages (Kruskal–Wallis, $H_{\text{Leydig}} = 0.214$, $df = 2$, $P = 0.898$; $H_{\text{Sertoli}} = 3.929$, $df = 2$, $P = 0.140$; Fig. 5). There was a significant and positive relationship between the testosterone concentration and the lipid content of Leydig cells (linear regression, $F_{1,6} = 20.69$, $P = 0.006$; Fig. 4A). In Sertoli cells there was no significant relationship between SER development and lipid content (Spearman correlation, $r = -0.02$, $n = 7$, $P = 0.485$), or between lipid content and SVL or body mass of males, or between seasons (multiple regression stepwise, $P > 0.05$).

Other organelles present only in sertoli cells: Rough endoplasmic reticulum and Golgi apparatus were observed in males from both seasons. Residual bodies were observed in 50% of the males captured in spring, but were not observed in males captured in summer. In all Sertoli cells primary and secondary lysosomes and variations in the abundance of glycogen granules were also observed in males from both seasons (Fig. 3).

Analysis of the Existence of Temporal Asynchrony in Steroid Activity Between Interstitial and Tubular Compartments of the Testes.—Ultrastructural features indicative of the existence of asynchronous steroidogenic activity between testicular compartments were observed in three males of *P. punae* from the seven analyzed. One of the males captured in spring with testicular regression and without sperm, and another one captured in summer in spermatozoa stage and with sperm in the epididymis, exhibited Leydig cells with ultrastructural features characteristic of active steroid synthesis, whereas Sertoli cells were inactive. These two males showed moderate serum testosterone concentration.

The third male with ultrastructural evidences of asynchronous steroidogenic activity between testicular compartments

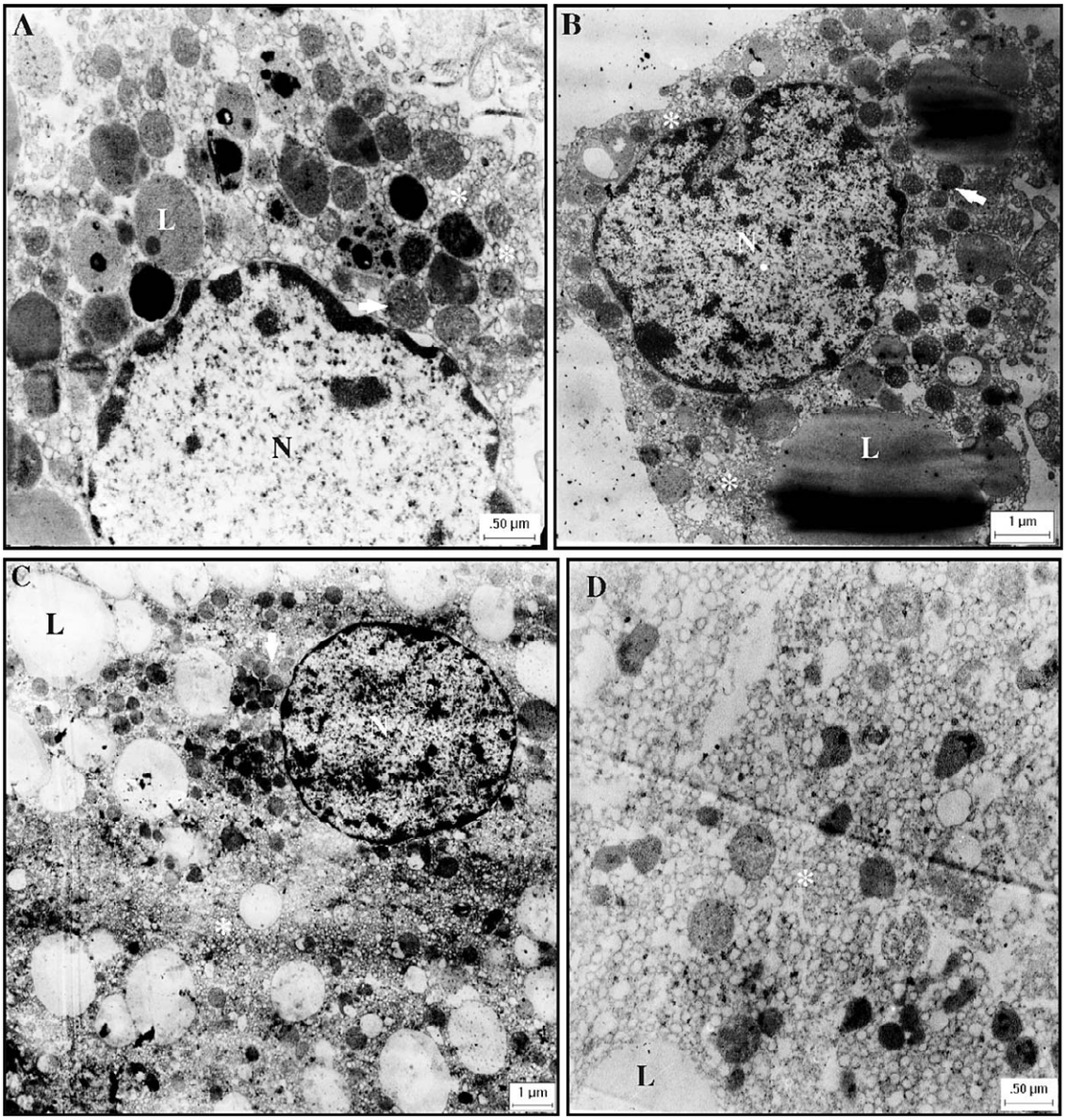

FIG. 2. Changes in ultastructural features of Leydig cells of *Phymaturus punae* males during the activity season. Note the regular-shaped nuclei (N) with dispersed chromatin, the moderate development of smooth endoplasmic reticulum (*), and mitochondria (arrows) abundance, in spring (A,B) and in summer (C,D). Lipid content (L: lipid droplet) was less in males captured in spring and an increase can be noted in summer.

was captured in spring, and showed inactive Leydig cells and Sertoli cells with ultrastructural features of active steroid synthesis. This male exhibited spermatid testicular stage without spermatozoa in the epididymis, and the lowest serum testosterone concentration registered (Table 1).

DISCUSSION

The Andes highlands impose restrictions on *P. punae* reproduction, and lizards have developed life-history strategies to overcome hard weather conditions. The study of this species offers an opportunity to understand reproductive diversity and evolutionary processes. We found evidence to confirm that mating occurs in late summer or early autumn in *P. punae*. This is supported by high serum testosterone concentrations exhibited by all males captured in summer, in contrast with males captured in spring that exhibited low testosterone levels. All males captured in summer showed spermatozoa in the epididymis, whereas in spring no male presented sperm in the epididymis independently of the testicular stages (Boretto et al., 2007). In addition, ultrastructural features observed in Sertoli and Leydig cells indicate that both types of cells have the potential to synthesize steroid hormones, to support the spermatogenic cycle and the mating period, respectively. Cases of temporal asynchrony in steroid activity suggest that this mechanism must be important to start the spermatogenesis in spring in *P. punae*, an idea that is supported by the steroid activity of Sertoli cells, because Leydig cells are inactive.

Autumn mating, at the end of the activity season, is exhibited by other Liolaemids that inhabit cold and harsh environments (Leyton et al., 1977, 1982; Ramírez-Pinilla, 1991; Habit and Ortiz, 1996). It has been proposed that the evolution of

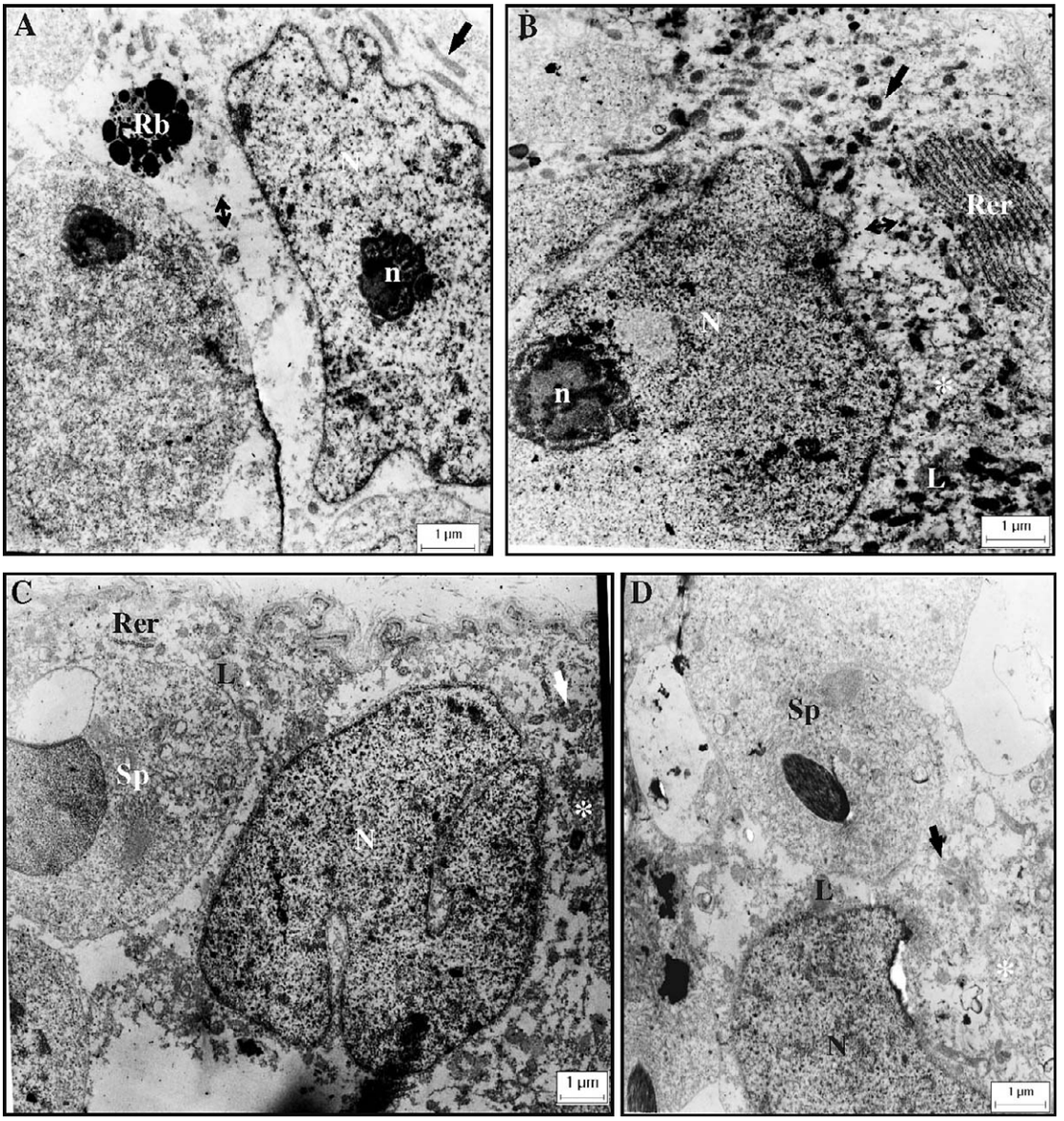

FIG. 3. Changes in ultrastructural features of Sertoli cells of *Phymaturus punae* males during the activity season. Note the irregularly shaped nuclei (N) with a deep membrane folding and a big and electron-dense nucleoli (n) of males in spring (A,B) and summer (C,D). Note the predominance of lamellar cristae mitochondria (arrows), and the constant development of smooth endoplasmic reticulum (*) and lipid droplets (L) across the seasons. Rough endoplasmic reticulum (Rer), residual body (Rb), and glycogen granules (double arrows) are also indicated. Males captured in summer showed spermatids (Sp) in their seminiferous tubules.

viviparity was accompanied by a shift from spring to autumn gametogenesis (Guillette, 1982; Ramírez-Pinilla, 1991; Méndez-de la Cruz et al., 1998; Mouton et al., 2012). The duration of the brumation period, as well as the cold temperatures during that period, have been shown to be necessary to complete vitellogenesis and ovarian growth in several reptiles living in harsh environments (*Lacerta vivipara*, Gavaud, 1983; *Thamnophis sirtalis parietalis*, Whittier and Tokarz, 1992; *Chrysemys picta*, Duvall et al., 1982, among others).

In this scenario, and considering the extreme climate restrictions for embryo development during winter at 3,100–4,200 m, we propose that female *P. punae* reserve sperm in the reproductive tract after mating in late summer–autumn, and that the ovulation and fertilization occurs in spring, when vitellogenesis is completed and environmental conditions are more benign for embryo development. The same reproductive cycle was hypothesized for *P. antofagastensis* (Boretto et al., 2010) and *P. aguanegra* (Cabezas Cartes et al., 2010). Nevertheless, we cannot totally discard gestation over winter, given that other viviparous Liolaemids that live at high altitudes, such as *Liolaemus multiformis multiformis* (4,600 m; Pearson, 1954), *Liolaemus huacahuasicus* (3,700 m; Ramírez-Pinilla, 1991), and *Phymaturus vociferator* (Habit and Ortiz, 1996) exhibit vitellogenesis and ovulation in autumn, followed by pregnancy during the winter, and births in spring (Pearson, 1954; Ramírez-Pinilla, 1991) or in summer months (Habit and Ortiz, 1996).

Reproductive male cycles in genus *Phymaturus* with high levels of testosterone in late summer have been observed in *P. punae* and in *P. antofagastensis* (Boretto et al., 2010). The

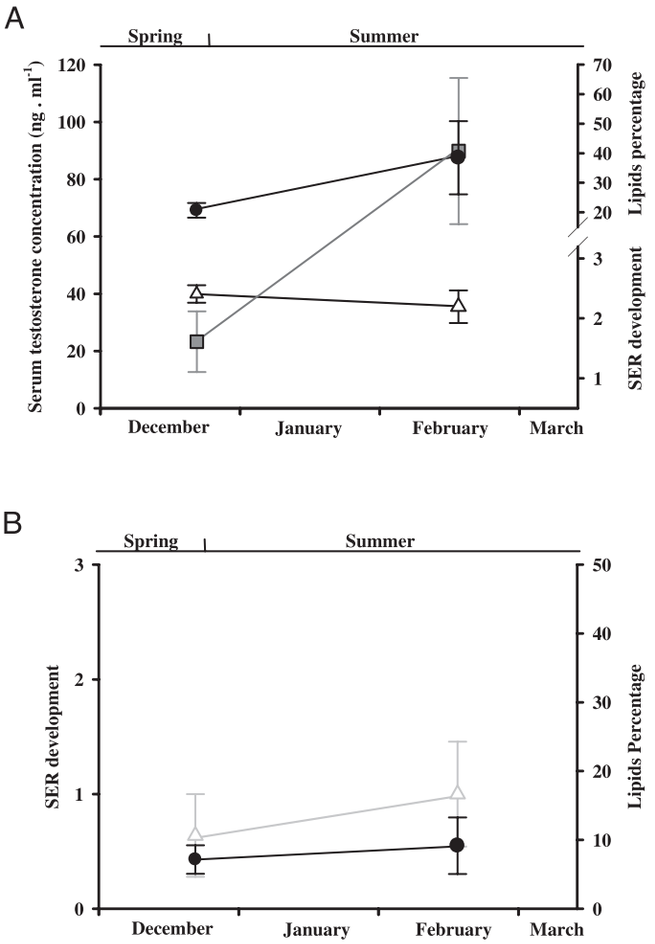

FIG. 4. Variations in steroid activity of *Phymaturus punae* Leydig cells (A) and Sertoli cells (B), during the activity season. Mean serum testosterone concentration (ng ml^{-1}, squares), mean smooth endoplasmic reticulum development (SER, triangles), and mean lipid percentage (circles) of Leydig cells (A) and Sertoli cells (B) are indicated.

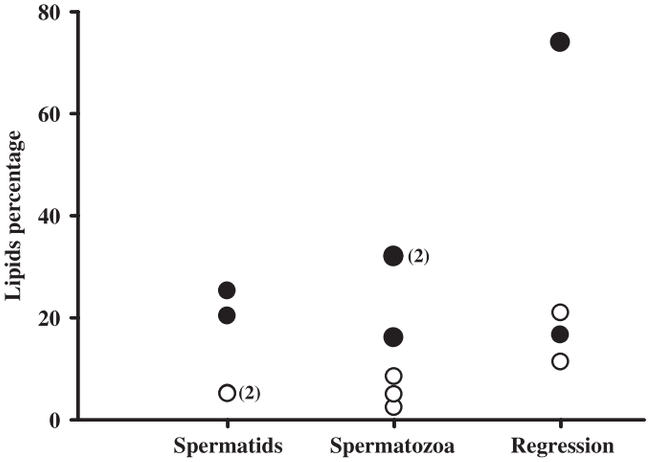

FIG. 5. Lipid dynamics of Leydig (black) and Sertoli cells (white) of *Phymaturus punae* males during the activity season. The mean lipid percentage according the spermatogenic stages of each male is exhibited. Parentheses indicate the number of individuals.

prenuptial male cycle proposed for *P. punae* (Boretto et al., 2007), and confirmed in this work, was considered exceptional by Duvall et al. (1982) when referring to *Sceloporus grammicus microlepidotus* (Guillette and Casas-Andreu, 1980). Later studies described prenuptial cycles with spermiation and mating in late summer or autumn in New Zealand and Australian species like *Niveoscincus ocellatus* (Jones et al., 1997), and *Niveoscincus metallicus* (Swain and Jones, 1994) that inhabit environments with temperate or cold climate winters at similar latitudes as the *Phymaturus* species. In New Zealand and Australian species, fertilization occurs in the next spring with the sperm stored in females during winter (e.g., Swain and Jones, 1994; Cree and Guillette, 1995; Jones et al., 1997; Olsson and Shine, 1998, 1999; Wilson and Cree, 2003).

Various factors may have led to the evolution of sperm storage, and it is clear that this capability alters the relationship between sexual behavior, copulation, and fertilization (Saint Girons, 1985; Whittier and Tokarz, 1992). Sperm storage may also lead to dissociation between reproductive behavior and the synthesis of gonadal steroid hormones, because sperm storage permits the separation of the evolutionary factors that regulate the timing of sexual behavior from those that regulate the timing of the production of young (Whittier and Tokarz, 1992). It is expected that sperm storage in females is confirmed for *P. punae*, and if so, males and females of this species reproduce asynchronously, like *P. antofagastensis* (Boretto et al., 2010) and *P. aguanegra* (Cabezas Cartes et al., 2010), that also live at more than 3,000 m in the Andes Mountains.

The asynchrony between males and females in the timing of reproduction can be a consequence of different requirements of energy for gametogenesis. Lizards with asynchronic male and female cycles could have mechanisms to assure fertilization, such as the asynchronic steroid hormone synthesis between testicular compartments that allow gametogenesis independently of mating (Callard et al., 1976; Lofts and Tsui, 1977; Callard and Ho, 1980). We found that both Leydig and Sertoli cells presented ultrastructural features indicative of potential steroidogenic activity in *P. punae* males. In addition, three cases of temporal asynchrony in steroidogenic activity between testicular compartments were observed. This steroid mechanism would allow *P. punae* males to start spermatogenesis in spring, supported by the steroidogenic activity of Sertoli cells, whereas Leydig cells were inactive. In addition, the steroid hormone synthesis of Leydig cells supported spermiation and mating behaviors in summer, when Sertoli cells were inactive. Results from *P. punae* add support to the statement that androgens synthesized by Sertoli cells have a minimal contribution to the circulating androgen pool and are limited 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). Moreover, the steroidogenic activity of Leydig cells of *P. punae* support the hypothesis that their testosterone synthesis enters into the peripheral circulation, influencing courtship behavior and mating events (Callard et al., 1976; Callard and Ho, 1980; Mahmoud et al., 1985; Dubois et al., 1988), and that the *P. punae* mating period occurs in summer or autumn, and not in spring.

Future studies will be performed to explore the effect of body mass on the serum testosterone concentrations, and its influence on intrasexual competition, dominance, and reproductive behavior. For example, in *Anolis carolinensis* populations lizards with greater body size ("heavyweight") exhibited 50% higher testosterone levels than males with smaller body sizes ("lightweight"), during the reproductive season (Husak et al., 2007). In

our study, body mass of adult males *P. punae* was a significant covariable when comparing testosterone levels between seasons, and between males with or without spermatozoa in the epididymis. It would be interesting to study the relationship of testosterone concentration and body size, and how it affects dominance among males.

Our study reveals how environmental and physiological constraints can have different effects on the male reproductive cycle in different species within a genus. At present, *P. punae* is the only species of this interesting genus with a prenuptial male cycle, with ultrastructural features indicative of steroidogenic activity in Leydig and Sertoli cells and cases of asynchronous steroid production between testicular compartments. Previous studies demonstrate the importance of temporal asynchrony in steroidogenic activity in reptilian species with postnuptial male reproductive cycles (such as *P. zapalensis* [Boretto et al., 2012], *Trionyx sinensis* [Lofts and Tsui, 1977], *Chrysemys picta* [Dubois et al., 1988], *Chelydra serpentina* [Callard and Ho, 1980; Mahmoud et al., 1985; Mahmoud and Licht, 1997]), or continuous male cycles with sperm availability throughout the activity season (such as *P. antofagastensis* [Boretto et al., 2010]), because in these species spermatogenesis occurs temporally dissociated from spermiation and mating time. We found that because this steroid mechanism is present in *P. punae*, the testosterone cycle and the steroidogenic activity of testicular compartments suggest that females store sperm in the reproductive tract during the winter, thus dissociating the mating period and the time of ovulation and fertilization.

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