Seasonal changes of testicular parameters in southern pudu *Pudu puda* in relationship to circannual variation of its reproductive hormones

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Seasonal variation of testicular and epididymal parameters in the smallest deer, the southern pudu *Pudu puda* Molina, 1782 were related to the circannual fluctuation of plasma levels of its reproductive hormones. The diameter and the length of testes, the diameter of epididymis as well as the diameter and the heights of epithelium of seminiferous and epididymal tubules were measured. Morphological evaluation of spermatogenesis were performed on histological sections of testicular and epididymal tissues. Most of these gonadal parameters exhibit a seasonal variation which is in accordance with the reproductive pattern as well as the variation of reproductive hormones. Seasonal fluctuations of LH and testosterone indicate two seasonal activations of the reproductive system, one in the spring (antler mineralization), the other in the fall (rut). FSH exhibit one prolonged peak (summer to fall) whereas prolactin follows the expected, photoperiodically-dependant time course with peak levels around the summer solstice. The measurements of testicular parameters indicate a prolonged period of gonadal activity in southern pudu but the period of maximal reproductive stimulation is limited to the time around the rut.

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Introduction

In wild ungulates of temperate zones, photoperiodicity regulates the important life-sustainable activities such as feeding, locomotion, sleep and reproduction. Cervid species inhabiting temperate zones are seasonal breeders and therefore their reproductive organs and concentrations of reproductive hormones exhibit annual changes which are synchronized by light. In male cervids, these changes were studied in red deer *Cervus elaphus* (Jaczewski 1954, Lincoln 1971, Hocherau-de

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Riviers and Lincoln 1978), fallow deer *Dama dama* (Chaplin and White 1972, Rolf and Fischer 1990), roe deer *Capreolus capreolus* (Stieve 1949, Bubenik 1971, Schams and Barth 1982, Sempere 1990), sika deer *Cervus nippon* (Suzuki *et al.* 1992), wapiti *Cervus elaphus canadensis* (Haigh *et al.* 1984), white-tailed deer *Odocoileus virginianus* (Wislocki *et al.* 1947, Mirarchi *et al.* 1977, 1978) and several other species.

Circannual changes of reproductive hormones were also investigated in males of subtropical and tropical deer, such as Rusa deer *Cervus rusa timorensis* (van Mourik and Stelmasiak 1990), axis deer *Axis axis* (Loudon and Curlewis 1988, Bubenik *et al.* 1991), muntjac *Muntiacus reevesi* (Chapman and Harris 1991) and Eld's deer *Cervus eldi thamin* (Monfort *et al.* 1993). Most recently, our group determined the rate of development of reproductive organs and circannual variation of luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone (T), and prolactin (PRL) in plasma of male southern pudu *Pudu puda* Molina, 1782, a small subtropical deer living in Chile (Reyes *et al.* 1988, Reyes-Toledo *et al.* 1993, Bubenik *et al.* 1996).

In most of the previously mentioned cervid species, the testicular volume, the size of epididymis and the amount of spermatozoa in the seminiferous tubules and the epididymal canals were undergoing distinct seasonal changes which more or less correspond to circannual variation of levels of reproductive hormones (Lincoln 1971, Mirarchi *et al.* 1977, 1978, Haigh 1984, Sempere 1990, Monfort *et al.* 1993). In pudu, however, we have detected one seasonal elevation for prolactin and FSH, but two other hormones, LH and T exhibited two peaks of almost equal size, one of them in the spring and the other in the fall. What is mostly remarkable, the pudu exhibit only one fall rut followed by a parturition period after approximately 203 days of pregnancy (Reyes *et al.* 1988).

In order to elucidate this obvious discrepancy between the seasonal variation of reproductive hormones and the reproductive pattern of this species, we measured the size of testes and the epididymis, the diameters of their lumens as well as the thickness of the epithelial cells during the four seasonal periods and compared them with the circannual variation of reproductive hormones.

Material and methods

The studies were carried out during approximately one year period at the animal care facility of University of Concepcion, Chile. The measurements were performed in six adult males which were immobilized by 1:1 mixture of Rompun (xylazine hydrocholoride) and Ketamine (ketamine hydrochloride) delivered by an injection dart. The details of this technique are described in our previous publication (Bubenik and Reyes-Toledo 1994). The measurements of testes and epididymides (length and diameter) were performed by caliper on immobilized deer. The diameter of each caput epididymis was calculated by subtracting the values of testes length without epididymis from the values of testis length with the epididymis. Tissue samples were obtained either from animals which were sacrificed because of injuries or from healthy males in which a needle biopsy was performed. Tissue samples from the testes were available only from January (*n* = 6), March (*n* = 6), August (*n* = 6), September

(n = 6) and November (n = 6) and the tissue samples from the epididymis were available only from March (n = 6), August (n = 6) and September (n = 6). The measurements of testes and epididymis were performed in January, March, July, September, October and November.

The gonadal tissues were fixed in Bouin solution, embedded in paraffin, section to 20 μ and then stained with hematoxylin and eosin. The diameter of seminiferous tubules and the epididymal tubules as well as the thickness of epithelial cells of the seminiferous tubules and the ductus epididymis were measured by an ocular micrometer in 10–12 sections magnified 125 or 200 times.

Blood samples were taken from the jugular vein into pre-heparinized tubes and after centrifugation, the plasma was frozen to –20°C until further radioimmunoassays (RIA) for the reproductive hormones was performed. The details of these techniques were reported in Bubenik *et al.* (1996).

The data of testicular parameters were subjected to a multiple range analysis of variance (ANOVA) using Quattropro software, and the Mann-Whitney U-test. Significant differences were expressed at the 95% confidence level. The data on seasonal changes in plasma concentration of hormones were subjected to General Linear Model Procedure for Unbalance ANOVA (SAS) and the differences between means were determined by t-test. Because no significant differences in testicular parameters were detected between the right and the left testes, both measurements were combined.

Results

The largest average value of the testicular length (32.4 mm) was observed in March (rut) but only slightly smaller value (31.3 mm) were found in November or January (31.1 mm) (Fig. 1). The smallest average lengths were detected in July (winter; 24.7 mm) and September (26.2 mm). Statistical calculation showed no significant differences between November and March (late spring, early fall) and between July and October (winter and spring). However, a significant increase ($p < 0.05$) was detected in November as compared to October.

Testicular diameter peaks in January and March (both months 21.8 mm) and the minimal value (14.8 mm) was found in July (Fig. 1). There were no significant differences between July, September and October but a significant increase ($p < 0.05$) occured between October and November as well as between November and January.
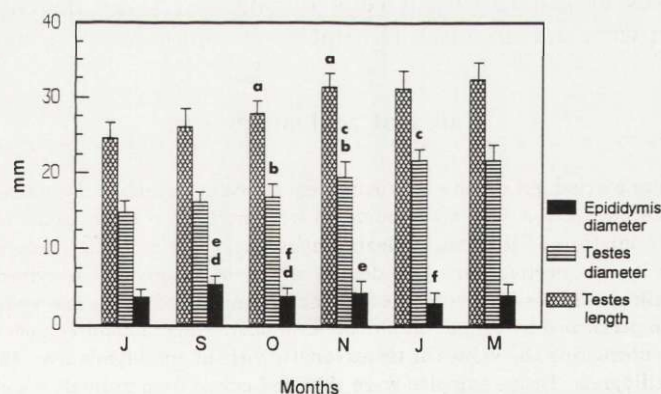

Fig. 1. Seasonal variation of length and diameter of testes and the diameter of epididymis in pudu (n = 6). Values marked with the same letter are significantly different ($p < 0.05$).

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The variation of average diameter of epididymis exhibited only a minimal seasonal variation with highest values (5.4 mm) observed in September (spring) and lowest value (2.8 mm) detected in January (summer; Fig. 1). A significantly higher levels ($p < 0.05$) were detected in September as compared to October. September values were also higher than those of November and October levels were higher than January values.

A pronounced seasonal variation in diameters of seminiferous tubules (S.T.) was detected (Fig. 2) with peak values (36.9 µ) observed in March (rut) and the lowest values (19.0 µ) detected in September. The largest variance in lumen diameter (ranging from 17.7 to 63.0) was observed in March. The values for both months, January and March differed significantly ($p < 0.05$) from August and September values, and September differed from November.

The seasonal variation of epithelial height of S.T. followed closely the pattern established for the diameter of S.T. (Fig. 2). Peak values (10.7 µ) were observed in March and the lowest values (3.7) were detected in September. Significant differences ($p < 0.05$) were detected between January and September, March and August, March and September, and also between November and January.

The highest value of the diameter of the epididymis (30.8 µ) was obtained from March and the lowest (14.6 µ) was observed in September (Fig. 2). The March value differed significantly ($p < 0.05$) from the values in August and September.

The epithelial height exhibited the lowest value (2.3 µ) in August and the highest (6.7 µ) in March (Fig. 2). The March values were significantly different ($p < 0.05$) from values in August. A fully developed seminiferous tubules with
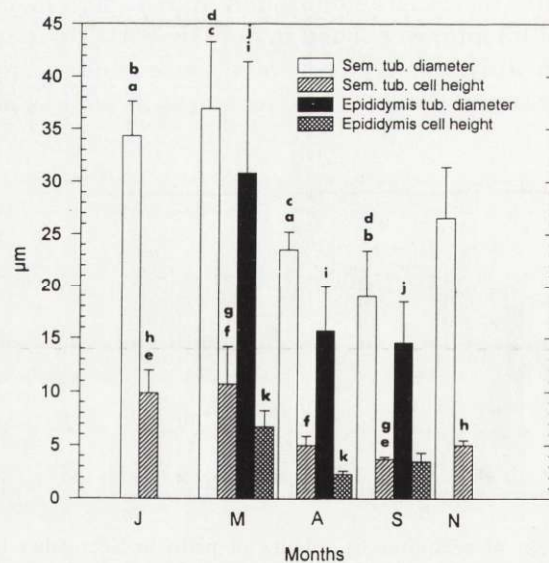

Fig. 2. Seasonal variation of diameter and epithelial height of seminiferous and epididymal tubules in pudu ($n = 6$). Values marked with the same letter are significantly different ($p < 0.05$).
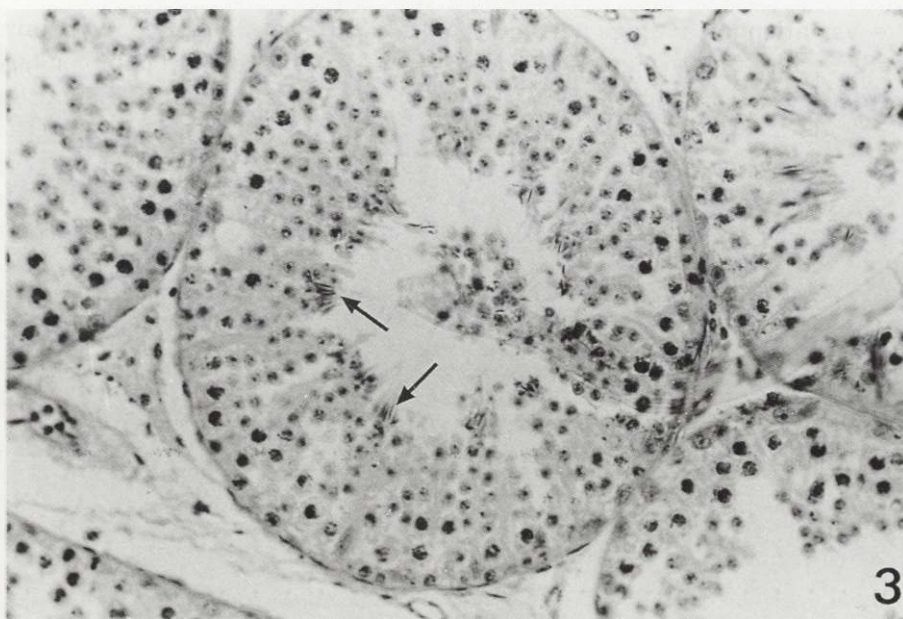
Fig. 3. Transverse section of seminiferous tubules of pudu in March (rut). Note the wide thickness of the germinative epithelium and the presence of mature spermatozoa in the central canal. Magnification 200×.
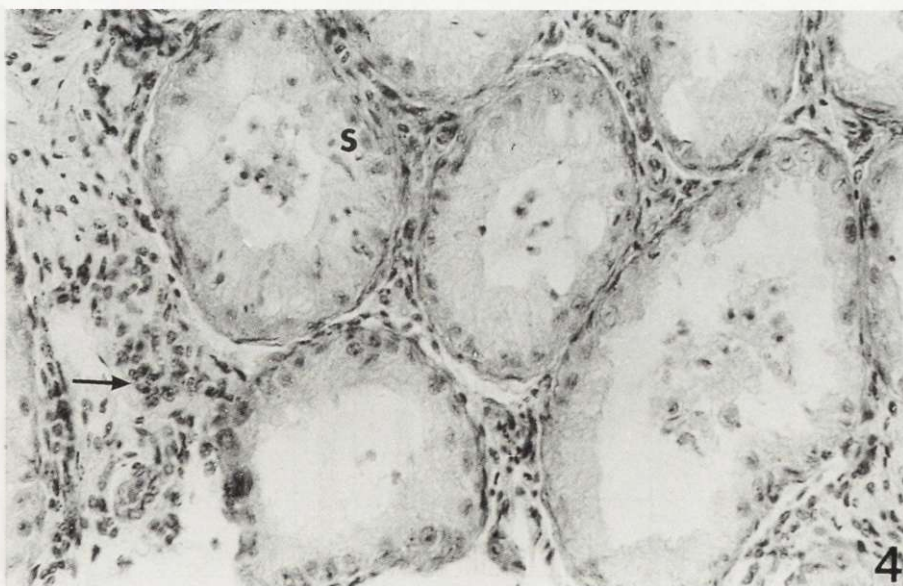

Fig. 4. Transverse section of seminiferous tubules of pudu in September (spring). Note the thin germinative epithelium consisting mostly from spermatogonia and the few undifferentiated cells seen in the lumen. Compare the large size of the interstitial tissues in this section containing numerous Leydig cells with the much smaller number of these cells seen in the Fig. 3. Magnification 125×.
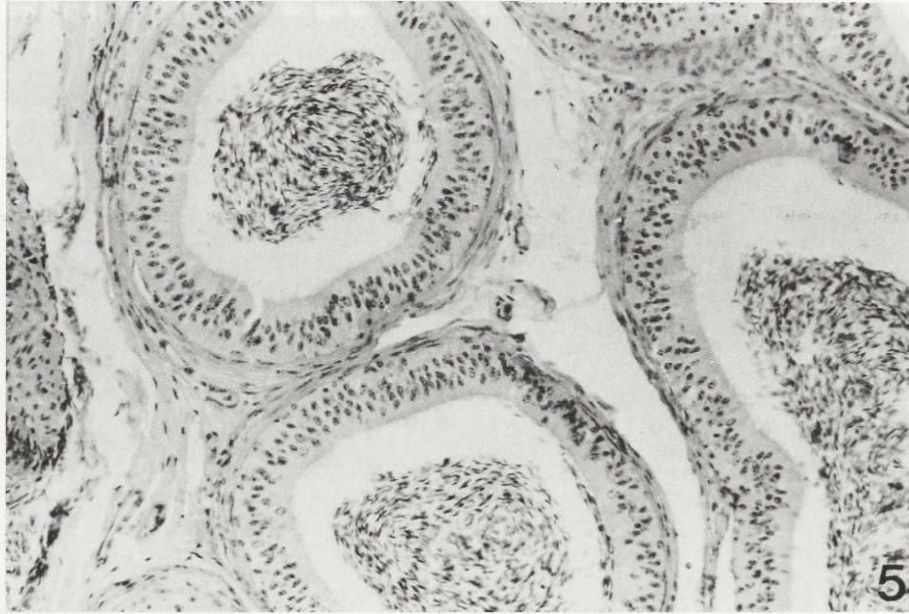
Fig. 5. Transverse section of the epididymal tubules of pudu in March. Note the thick epithelium of the tubular walls and the large lumens filled with great quantity of spermatozoa. Magnification 125×.
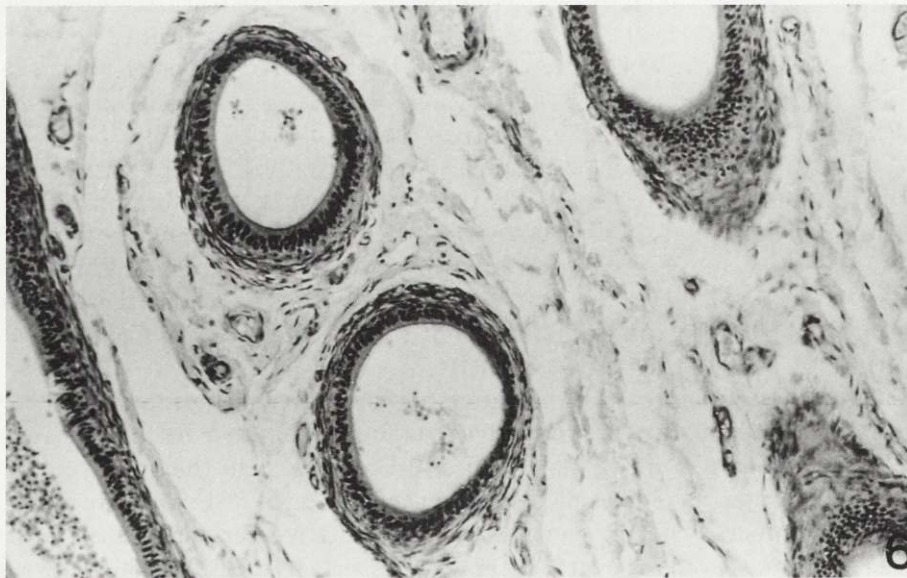

Fig. 6. Transverse section of the epididymal tubules of pudu in September. Note the thin tubular wall and the mostly empty lumen containing occasionally few spermatocytes. Note also the massive amount of intertubular connective tissue in this section as compared to Fig. 5. Magnification 125×.

mature spermatozoa present in the lumen and large interstitial spaces with well developed Leydig cells were observed in March (Fig. 3). In September mostly only spermatogonia were visible inside the seminiferous tubules but paradoxically, large interstitial spaces were filled with densely compacted Leydig cells (Fig. 4).

An enlarged epididymal tubules filled with numerous, closely compacted spermatozoa, were observed in March (Fig. 5). In September, the tubules were wide open and only infrequently few precursor cells (most probably spermatocytes) were encountered inside the lumen (Fig. 6).

Average values of LH exhibited two peaks ($p < 0.05$), one in August (winter) (1.78 ng/ml), the other in March (fall) (1.90 ng/ml). The levels of testosterone showed also two peaks of almost equal size ($p < 0.001$), one in March (3.18 ng/ml), the other in October (3.11 ng/ml). FSH levels exhibited a prolonged peak period from December (summer) (58 ng/ml) to March (63 ng/ml) ($p < 0.01$). Finally, average prolactin (PRL) concentration reached peak values (28.3 ng/ml) in December (summer solstice) ($p < 0.001$) and the lowest one (2.8 ng/ml) in April (fall). The two seasonal peaks of LH and T, as well as the single peaks of FSH and PRL were significantly higher than the minimal values of the year (Fig. 7).

Discussion

Most cervids living in the boreal or temperate regions are strongly influenced by a photoperiod and exhibit a clearly-defined rutting period, restricted to only few weeks (Goss 1969, Lincoln 1985). Conversely, there is a general rule that the populations living closer to equator exhibit a longer rutting season than populations found in the more boreal regions (Zuckerman 1953, Bubenik et al. 1990). The northern limit of southern pudu's distribution (35°S; Hershkowitz 1982) would suggest that this species could accommodate an extended rutting period. This indeed was the case of the reproductive pattern of captive pudu bred in Cologne Zoo in Germany. Although 55% of fawns were born in May (spring) and another 15% were born in June, the rest of fawns were born in July, August, September, January, and April (Hershkovitz 1982). A similar pattern of parturition was also observed in the breeding captive colony kept at the University of Concepcion in Chile (Reyes et al. 1988).

The seasonal parameters of reproductive hormones in southern pudu indicates a bi-seasonal activation of the reproductive axis (Reyes-Toledo et al. 1993, Bubenik et al. 1996) with equal peak levels of LH and T observed in the spring and the fall. In addition, an extended period of high concentration of FSH was also detected (see Fig. 7). A temporary spring elevations of reproductive hormones is not unique to pudu as it was observed in several other cervid species (Bubenik 1986). However, these out of rut peaks do not come close to the rutting values, as was the case in pudu (Fig. 7). The only exception is the roe deer, where a regular bi-annual elevation of testosterone was reported (Schams and Barth 1982, Sempere 1990).
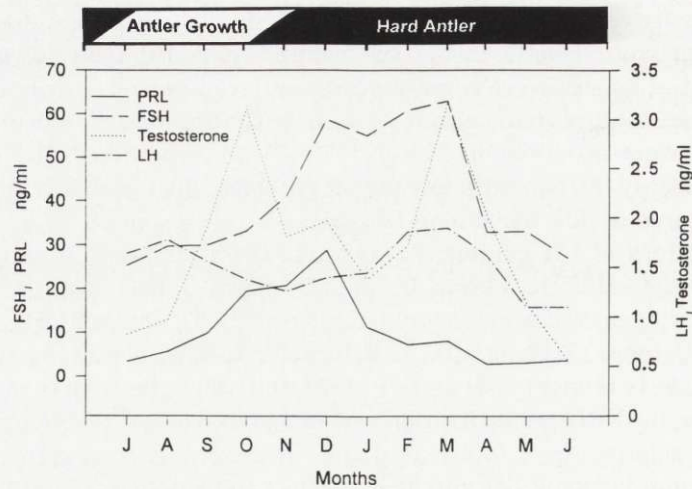
Fig. 7. Circannual variation of LH, testosterone, FSH, and prolactin in southern pudu. Note the close correspondence of bi-annual elevation of LH and testosterone ($n = 6$).

Whereas some testicular parameters in pudu, such as the diameter of seminiferous and epididymal tubules (Fig. 2), exhibit well pronounced seasonal variation, other parameters, such as the length and diameter of testes (Fig. 1) reflect an extensive (several months long) period of reproductive activation. In addition, the histological picture of testes and epididymides indicate a maximal reproductive activation around the rut. On the contrary, the seasonal variation, in testis length and the diameter, is minimal and the diameter of the epididymis is wider in September than during the rut in March (Fig. 1). This discrepancy of seasonal variation was also noticed in the testicular morphology. It is interesting to note, that the rather remarkably enlarged testicular interstitia observed during the month of September follows the preceding elevation of LH, seen in August (Fig. 7). In addition, the subsequent elevation of T levels (peaking in October) may have maintained the activation of the reproduction, as evidenced by the 5 month-long elevation of FSH (Fig. 7). In that respect, the pudu appears to be somewhat similar to roe deer (Schams and Barth 1982). Because of the restricted time available to reactivate the reproductive axis after the winter lull, the LH rises in the spring, followed by testosterone and FSH. The rising LH levels activate the Leydig cells and induce the increase of testes size. Similarly to roe deer, the first T peak, observed in pudu in October causes the antler mineralization; the second T peak (in March) coincides with the rut (Fig. 7) (Sempere 1990). (It should be pointed out that October in the southern hemisphere corresponds to April in the northern hemisphere; March corresponds to September). The role of FSH appears to be limited to the activation and maintenance of spermatogenesis. The distinct sea-

sonal variation of prolactin, which follows the circannual variation of day-length indicated that pudu's reproduction is photoperiodically-controlled (Bubenik 1986).

The discrepancy between the usually sharp circannual peak of testosterone and the limited period of the rutting behaviour (only few weeks) and the prolonged period of fertility reported in most male cervids (lasting up to 6 months) is not restricted to temperate or tropical deer. Such extended period of male fertility was reported not only in the tropical Eld's deer (Monfort *et al.* 1993) and Reeve's muntjac (Chapman and Harris 1991), temperate axis deer (Loudon and Curlewis 1988) and the atypically breeding cervids such as the roe deer (Stieve 1949), but was also observed in the typically short-day breeding cervids, such as the red deer (Lincoln 1971, Bubenik *et al.* 1985), wapiti (Haigh *et al.* 1984), white-tailed deer (Mirarchi *et al.* 1977), and sika deer (Suzuki *et al.* 1992).

In his review of seasonal breeding of deer Lincoln (1985) suggested, that a circannual decrease in testis weight of at least 75% is necessary to assure a complete cessation of spermatogenesis. This hypothesis is supported by data from several boreal cervids in which there is a concise seasonal rut followed by a limited parturition period. In fallow deer, the maximal seasonal decline of testicular weight was around 85% (Chaplin and White 1972), in roe deer the testicular volume decreased by 84% (Stieve 1949) and in white-tailed deer and red deer the testes weight declined by 76% (Mirarchi *et al.* 1977) and 75%, respectively (Lincoln 1971). In the tropical Eld's deer however, the scrotal circumference declined seasonally by 50% (Monfort *et al.* 1993), but the temperate sika deer, the testes size decreased by only 36% (Suzuki *et al.* 1992). The smallest decline of testicular size were then observed in the tropical axis deer (27%), (Loudon and Curlewis 1988), temperate pudu (24%) (this study) and the tropical Reeve's muntjac (23%) (Chapman and Harris 1991). Interestingly, these cervids with low seasonal variation of testicular size differ in their reproductive and antler cycle patterns. Whereas axis deer exhibit no interindividual seasonal synchronization of the antler or the reproductive cycle (Loudon and Curlewis 1988), in muntjacs only the antler cycle is seasonally synchronized but not the reproduction (Chapman and Harris 1991). In pudu, both the antler cycle and the reproductive pattern are more or less synchronized (Reyes *et al.* 1988, Bubenik *et al.* 1996).

In view of these facts, the seasonal pattern of testicular activation and the circannual variation of reproductive hormones, as described here in pudu, is not entirely surprising. As the ancestral pudu originated in the subequatorial regions of South America (Hershkovitz 1982) it can be speculated that its original reproductive pattern was aseasonal. Only when the southern pudu move to his current location, his rut and the parturition period stabilized in the most optimal periods of the year.

Our data provides the first evidence, that seasonal changes of testicular parameters in southern pudu are corresponding to the circannual variation of its reproductive hormones. We have concluded, that the reproductive system of male

pudu exhibits a prolonged period of gonadal activation but the maximal stimulation is clearly limited to the time around the rut.

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