Influence of melatonin and sexual hormones on the proliferating cell nuclear antigen expression in the adrenal cortex of a seasonal breeder (*Lagostomus maximus*)

Luis Ezequiel Gallol [1, 2], Fabricio Busolini [1, 2] and Fabian Heber Mohamed [1]

[1]*Cátedra de Histología, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Av. Ejército de los Andes 950-2° Piso- CP (5700) San Luis, Argentina.*

[2] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), San Luis, Argentina.*

Correspondence should be addressed to Dr. Fabian Heber Mohamed.

Corresponding author:

Dr. Fabian H. Mohamed

Cátedra de Histología

Área de Morfología

Facultad de Química, Bioquímica y Farmacia

Universidad Nacional de San Luis

Av. Ejército de los Andes 950-Bloque I, 1º Piso (5700) San Luis, Argentina

E-mail: fhmo@unsl.edu.ar

TEL: +54-0266-4520300 int 1628

FAX: +54-0266-4430224

Orcid ID: 0000-0003-3196-8922.

Running title: **Proliferation in the viscacha adrenal cortex**

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ar.24457

Grant sponsor(s): Secretaría de Ciencia y Técnica, Universidad Nacional de San Luis; Grant number: Project 2-3118.

ABSTRACT

The viscacha (*Lagostomus maximus*) is a rodent of nocturnal habits, whose physiology and behavior vary according to modifications of environmental signals. The objective of this study is to assess the influence of melatonin and sexual hormones on the viscacha adrenal cortex proliferative activity through the immunohistochemical detection of proliferating cell nuclear antigen (PCNA) along with hormonal determinations. PCNA expression was studied in male viscachas to assess the effect of melatonin administration, castration and the annual reproductive cycle. In female viscachas, PCNA was studied in nonpregnant and pregnant viscachas. PCNA expression was observed in adrenocortical cells (PCNA-A) and endothelial cells (PCNA-E). Melatonin-administered animals showed a significantly lower number of PCNA-A compared to the control group. No significant difference could be established in the number of PCNA-A and PCNA-E between castrated and control animals. However, the morphometric analysis showed an increase in the size of the cortex of castrated animals, along with other cytological features. Significant differences in serum testosterone levels were observed during the male viscacha reproductive cycle, with the lowest levels encountered during the regression period (winter). Male viscachas exhibited a significantly high number of PCNA-A during late autumn and a high number of PCNA-E during winter. In females, hormonal determinations showed a peak of progesterone and estrogen during mid-pregnancy, along with a notably high number of PCNA-A and an increase in the number of PCNA-E. Our results suggest that proliferation in the adrenal cortex of the viscacha varies in relation to melatonin, sexual hormones and environmental conditions.

Key words**:** adrenal cortex-proliferation-melatonin-androgen-environmental stressors.

INTRODUCTION

In order to survive, animals need to adapt constantly to environmental changes. The hypothalamic-pituitary-adrenal (HPA) axis is crucial for physiological adaptation and improvement of survival chances when facing homeostatic challenges. The adrenal cortex is a major source of steroid hormones. In mammals, proper adjustment of the adrenal gland functionality is crucial to achieve a healthy pregnancy as disruptions in the HPA axis activity have been reported to lead to preterm labor (Kramer et al., 2009), low birth weight in the offspring (Amugongo and Husklo, 2014; Fan et al., 2018), hypertension (Gardner et al., 1997), glucose intolerance (Shen et al., 2003; Lesage et al., 2004) and mental illness (Kleinhaus et al., 2010). Disruptions in the HPA axis activity could also lead to maternal and/or fetal demise during gestation or puerperium (Lindsay and Nieman, 2005). The adult mammal adrenal cortex is composed of three zones that are morphologically and functionally distinct. These zones are arranged in concentric layers and produce specific steroid hormones. The outermost layer is called the zona glomerulosa (ZG) and lies beneath the fibrous capsule that envelopes the adrenal glands. In this region, cells are arranged in tube-shaped structures separated by connective tissue. Cells in the ZG secrete mineralocorticoid aldosterone. Underneath the ZG lies the zona fasciculata (ZF), where adrenocortical cells are arranged into radial fascicles in close relation with fenestrated capillaries. Due to the content of numerous lipid droplets in their cytoplasm that give ZF cells a foamy aspect, they are often referred to as ‘spongiocytes’. Cells in the ZF produce glucocorticoids which have diverse effects on metabolism, development, immunity and behavior. The third and innermost layer of the adrenal cortex is the zona reticularis (ZR), located between the ZF and the adrenal medulla. In this region, cells are arranged in cords that anastomose into an irregular network separated by sinusoid capillaries. Cells in the ZR are responsible for the production of adrenal androgens. (Willenberg and Bornstein, 2017).

In basal conditions, the adult mammalian adrenal cortex has low proliferative activity and cellular replacement is quite slow. This feature makes it difficult to study the dynamics of their proliferation, which is why various experimental stressful conditions and pharmacological

manipulation have been used to increase adrenocortical cell turnover and gland remodeling (Bozzo et al., 2011; Zaki et al., 2018). Three theories have been proposed to understand the dynamics of cell proliferation and remodeling of the adrenal cortex: the migration theory, the transformation theory and the zonal theory (Wolkersdörfer and Bornstein, 1998; Chang et al. 2013). The proliferating cell nuclear antigen (PCNA) is a nuclear nonhistone protein that is necessary for DNA synthesis and is an accessory protein for DNA polymerase alpha, which is elevated during the G1/S phase of the cell cycle. PCNA expression may be used as a marker of cell proliferation because cells remain in the G1/S phase for a longer time when proliferating (Kelman, 1997).

The experimental model used in the present study is the viscacha (*Lagostomus maximus maximus*), a hystricomorph South American rodent belonging to the Chinchillidae family (Llanos and Crespo, 1952). The viscacha lives in burrows and has nocturnal habits. Its physiology and behavior vary along the year according to modifications of environmental factors such as the natural photoperiod length, temperature, rainfall pattern, food composition, and social interactions. In its habitat, the adult male viscacha follows an annual reproductive cycle that is mainly characterized by higher gonadal activity in summer and lower gonadal activity during winter (Fuentes et al., 1991; Muñoz et al., 1999; Aguilera-Merlo et al., 2004). Previous studies conducted in our laboratory have shown that this reproductive cycle is synchronized by the environmental photoperiod through the pineal gland and its main hormone, melatonin (Piezzi et al., 1984; Cruceño et al., 2013). To date, only one study has dealt with the adrenal cortex of the viscacha and the possible influence of the natural photoperiod on some morphometric characteristics of the adrenocortical cell nuclei (Ribes et al., 1999). On the other hand, the female viscacha is a monoestral animal whose reproductive characteristics, such as polyovulation and implantation, and birth of its offspring, have been described in previous studies (Weir 1971; Gil et al., 2007; Filippa and Mohamed, 2010). Viscachas usually carry out their pregnancy during autumn and winter, and give birth in spring when environmental conditions are better suited for the survival of

both mother and offspring. Gestation lasts approximately 150 days, which is a particularly long gestation period for a rodent.

. The objective of this work is to study the influence of melatonin and sexual hormones on the proliferative activity of the adrenal cortex of the *Lagostomus maximus maximus* using PCNA as cell proliferation marker.

MATERIALS AND METHODS

All animals used in this study were captured in their natural habitat near San Luis, Argentina (33°20′ S, altitude 760 m), using a conveniently placed system of rectangular traps. Environmental conditions such as solar irradiation values, expressed as heliophany, and seasonal mean values of precipitations and temperature were provided by the Red de Estaciones Meteorológicas de San Luis and the Servicio Meterológico Nacional (Table 1). In all cases, the animals were intramuscularly anesthetized with a combined solution of ketamine (ketamine 50; Vetanarcol; König S.A., Barcelona, Spain) and xylazine (Xylazine 20, PharmaVet, Laboratorios PharmaVet S.A. Rosario, Santa Fe, Argentina) at a dose of 12 and 0.4 mg/kg bodyweight, respectively. The adrenal glands were quickly excised under anesthesia and put into Bouin's fixative solution. The time between anesthesia injection and tissue collection was approximately five minutes. When necessary, blood samples were obtained by cardiac puncture. Animals were sacrificed by intracardiac injection of Euthanyl (0.25 ml kg−1 body weight, sodium pento-barbitone, sodium diphenylhydantoin, Brouwer S.A., Buenos Aires, Argentine). This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of San Luis (Resolution No. 001-19-DC) and was in accordance with the National Institute of Health (NIH, USA) guidelines for the use of experimental animals. Additionally, a study protocol for conducting scientific research within the territory of this province has been approved by the Biodiversity Control Area of the San Luis Environment Ministry (Resolution No. 18 PMA-18).

Melatonin administration experiment

Eight adult male viscachas captured during summer were used (body weight > 5 kg). The animals were kept in isolated boxes under a 14L: 10D photoperiod at 24 ± 2 °C with ad libitum access to food and water. The experimental group (n = 4) received two daily subcutaneous injections of melatonin (Sigma, 100 μg.kg−1 body weight in peanut oil solution) at 9:00 A.M and 5:00 P.M. The control group (n = 4) received only the diluent. After 9 weeks of melatonin

administration all animals were sacrificed and the adrenal glands were excised. This experimental design has already been used in previous experiments for this species (Rosales et al., 2016; Gallol and Mohamed, 2018).

Castration experiment

Eight adult male viscachas (body weight > 5 kg) captured during summer were used for this experiment. The animals were randomly divided into an experimental group and a control group: Those in the experimental group (n = 4) were subjected to surgical castration, while those in the control group (n = 4) underwent sham-castration. All animals were kept in isolated boxes under a 14L: 10D photoperiod at 24 ± 2 °C with ad libitum access to food and water. After 6 weeks, all animals were sacrificed and the adrenal glands were excised. This time length has been used in previous castration experiments conducted in this species (Filippa et al., 2014; Gallol and Mohamed, 2018), and it is similar to the castration time length in which major histological changes could be observed in the adrenal gland of other rodents (Hall and Korenvchesky, 1938; Benmouloud et al., 2014). Gonadal activity in the control group was assessed through histological examination of testes.

Male reproductive cycle study

Sixteen adult male viscachas (body weight > 5 kg) were used for this study. These animals were obtained during the following periods: summer (February – March, reproductive period; n=4), late autumn (late April - early June; n=4), winter (July – August, regression period; n=4) and spring (November – December, recovery period; n=4). Captured animals were immediately transferred to the animal facility and sacrificed at 8:00 a.m. Gonadal activity in all four groups was assessed through careful histological examination of testes according to the observations of Fuentes et al. (1991).

Pregnancy study

Sixteen female viscachas were used for this study. The reproductive condition of the viscachas was carefully assessed on the basis of the following criteria: **(1)** Uterine horn examination to determinate the presence of embryos and fetuses. **(2)** Histological assessment of the ovaries to evaluate sexual maturity of nonpregnant animals. Nonpregnant viscachas were captured during summer (February - March; n=4). Pregnant viscachas were classified into three different groups according to the number and size of embryos or fetuses: early-pregnancy (captured during autumn, April - June, with two or more embryos from 1 to 3 cm; n=4), mid-pregnancy (captured during winter, July - August, with two fetuses from 9 to 11 cm; n=4) and late-pregnancy (captured in spring, September - December, two fetuses measuring more than 19 cm; n=4). This classification was performed according to previous reports by Gil et al. (2007), Filippa and Mohamed (2010), Busolini et al. (2018) and Rosales et al. (2019).

Serum testosterone determination

Whole blood samples were first incubated at 4∘ C for 30 min and then centrifuged at 5,000 g for 5 min for separation and serum obtainment. Serum testosterone concentrations were quantified using the total testosterone test, which is a solid phase competitive chemiluminescent enzyme immunoassay performed in a Siemens Medical IMMULITE 1000 Immunoassay Analyzer (Siemens Medical Solutions Diagnostics).

Serum estradiol and progesterone determination

Whole blood samples were incubated at 37° C for 30 min in a thermostatic water bath and centrifuged at 5000 g for 10 min for separation and serum obtainment. Serum estradiol and progesterone levels were quantified using the RIA Estradiol (A21854, Immunotech s.r.o., Prague, Czech Republic) and RIA Progesterone (IM1188, Immunotech s.r.o., Prague, Czech Republic) assay kits from Beckman Coulter®, respectively. These tests are competitive radioimmunoassays.

Immunohistochemistry

Adrenal glands were maintained in Bouin's solution during 24 h for proper fixation and then dehydrated in an increasing ethanol series, embedded in paraffin and sagittally sectioned (5 μm thick) using a rotary microtome (MICROM HM-325, Microm International Gmbh, Walldorf, Baden-Württemberg, Germany). Tissue sections were first deparaffinized with xylene, hydrated through decreasing concentrations of ethanol and rinsed with distilled water and phosphate-buffered saline (PBS, 0.01 M; pH 7.4). Heat-mediated antigen retrieval was performed by microwaving the sections for 6 min at full power in a 900-W microwave in sodium citrate buffer (0.01 M, pH 6.0). The immunohistochemical staining was carried out using the streptavidinbiotin-peroxidase complex method. Endogenous peroxidase activity was inhibited with 3% H2O2 for 20 min, and nonspecific binding sites for immunoglobulins were blocked incubating for 20 min with normal serum in PBS containing 1% bovine serum albumin, 0.09% sodium azide and 0.1% Tween-20. Primary antibody incubation was performed in a humidified chamber at 4 °C overnight. The anti-PCNA monoclonal primary antibody used in the present study (proliferating cell nuclear antigen, Catalog N° AM 252-5M; BioGenex, San Ramon, CA, USA) has already been used with successful results in viscacha (Rosales et al., 2016; 2019). After rinsing the sections in PBS for 10 min, the immunohistochemical visualization was achieved using the Super Sensitive Ready-to-Use Immunostaining Kit (BioGenex, San Ramon, CA, USA) as follows: sections were incubated for 30 min with biotinylated anti-IgG, washed in PBS and then incubated for 30 min with horseradish peroxidase-conjugated streptavidin. The reaction sites were visualized using a freshly prepared solution of 100 μl 3, 3′-diaminobenzidine tetrahydrochloride and 50 μl H2O2 in 2.5 ml PBS (0.01 M; pH 7.4). Finally, sections were subsequently dehydrated and mounted. Positive controls (Figure 1A) for PCNA immunohistochemical staining were carried out on intestine sections as recommended by the supplier. Negative controls for antibody specificity were performed (Figure 1B, C): (1) primary antibody replacement by 10% non-immune serum diluted in PBS, and (2) absorption of primary antibody with homologous antigen. No positive structures or cells were found in these sections.

Cell count and morphometric analysis

PCNA-positive adrenocortical cells (PCNA-A) and PCNA-positive endothelial cells (PCNA-E) were counted using a computer-assisted image analysis system consisting of an Olympus BX-40 binocular microscope interfaced with a computer. Images were captured by a Sony SSC-DC5OA camera and processed using the Image Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD, USA). The microscopy cell-counting procedure was carried out as follows: three regularly spaced (50 μm) serial tissue sections (4 μm thick) were used, the whole extension of the sections was analyzed, and the average number of PCNA-A and PCNA-E were expressed related to a reference area of 305.602 μm2. Castration experience morphometric parameters were measured on Hematoxylin-eosin (H-E) stained slides using the Image Pro Plus 5.0 software after performing a distance calibration using a stage micrometer (Reichert, Austria).

Statistical analysis

Means and standard errors for all data sets were calculated. Differences between multiple groups were evaluated using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Differences between two groups were evaluated using Mann-Whitney U test. A P value of <0.05 was considered statistically significant.

RESULTS

Melatonin administration

A statistically significant difference ($p<0.05$) in the number of PCNA-A in the adrenal cortex could be observed between the experimental (0.51 ± 0.07) and control groups (1.40 ± 0.45). In the latter, many PCNA-A could be observed mainly in the outer adrenal cortex (Figure 2A), most of them in the outer ZF and the ZG/ZF boundary (Figure 2B). PCNA-E were also observed. Both PCNA-A and PCNA-E in the inner ZF region were less frequent. No PCNA-positive cells could be observed in the ZR. In melatonin administered animals, both PCNA-A and PCNA-E

could also be observed in the outer cortex, but in smaller numbers (Figure 2C, D). Both PCNA-A and PCNA-E in the inner ZF were scarce. The average number of PCNA-E was slightly higher in the control group and exhibited more variability between animals (0.71 ± 0.16) compared with the experimental group (0.47 ± 0.08), although the statistical analysis did not show a significant difference between them. Raw histological/statistical data regarding PCNA-positive adrenocortical cells distribution available in S1-section of Supplementary Material.

Castration experiment

The average number of both PCNA-A and PCNA-E in the adrenal cortex was low in both experimental (0.44 ± 0.06, 0.19 ± 0.04 respectively) and control groups (0.35 ± 0.05, 0.30 ± 0.08 respectively), and did not show any statistically significant difference between them (Figure 3E, I). On the other hand, the morphometric analysis showed a notable widening of the ZF and ZR of castrated animals compared to control animals (Figure 3A, F). In these zones, adrenocortical cells showed hypertrophy (Figure 3B, D) compared to control animals (Figure 3G, H). In addition, vacuolization and signs of lipid depletion (Figure 3C) could be observed in the inner ZF cells. Furthermore, spongiocytes located in the deepest portions of the ZF exhibit an evident lipid depletion that make it difficult to distinguish ZF cells from hypertrophied ZR cells. Raw histological/statistical data regarding PCNA-positive adrenocortical cells distribution available in S2-section of Supplementary Material.

Male reproductive cycle study

The adrenal cortex of animals captured during summer exhibited a scarce number of both PCNA-A and PCNA-E, mainly found in the outer cortex regions (Figure 4A). The number of PCNA-A and PCNA-E was significantly lower when compared with animals captured during late autumn and winter, and no significant difference could be established compared to spring-captured animals (Table 2).

Late autumn adrenals showed a high number of PCNA-A. In some animals, PCNA-A could be observed in the outer cortex, mainly in the ZG/ ZF boundary (Figure 4B, E). However, there were late autumn-captured animals in which most PCNA-A were not observed in the outer cortex but found in the inner ZF (Figure 4C). PCNA-E were frequently observed in relation with PCNA-A (Figure 4G). PCNA-A in the ZR were only occasionally found (Figure 4H). The number of PCNA-A was significantly higher compared to all other seasons. The number of PCNA-E was significantly higher compared to summer and spring-captured animals, and it was significantly lower than those captured in winter (Table 2).

In winter-captured animals, PCNA-A could still be frequently observed mainly in the outer cortex, but some labeled cells were also eventually found in deeper regions of the ZF (Figure 4I, K). During this season, a notably high number of PCNA-E was observed throughout the adrenal cortex (Figure 4I, L, M). The number of PCNA-A was significantly higher than that observed in summer and spring-captured animals, but significantly lower than that seen in late autumn-captured animals. The number of PCNA-E observed was the highest of all seasons (Table 2).

During spring, PCNA-A and PCNA-E were sparsely found throughout the adrenal cortex (Figure 4N), and they were significantly lower in number compared to animals captured during late autumn and winter. No significant differences could be established in relation to summer-captured animals (Table 2).

Raw histological/statistical data regarding PCNA-positive adrenocortical cells distribution available in S3-section of Supplementary Material.

Significant variations in serum testosterone levels were found during the annual reproductive cycle (Figure 5).

Pregnancy study

Significant variations in serum hormone levels were found between nonpregnant and pregnant viscachas, exhibiting a peak during mid-pregnancy, followed by a decline towards the late-pregnancy period (Fig. 6).

In nonpregnant (Fig. 7A, B) and early-pregnancy viscachas (Fig. 7C, D), PCNA-positive cells in the adrenal cortex were scarce and were most frequently found in the outer cortex around the zona Glomerulosa and zona Fasciculata boundary (ZG/ZF). No significant differences in the number of PCNA-A and PCNA-E could be established between these two groups (Table 3).

In mid-pregnancy viscachas, the adrenal cortex presented a large number of PCNA-A. In some animals, the PCNA-A were located mainly in the outer cortex, mostly around the ZG/ZF boundary region (Fig. 8A, D), while in other viscachas, they were found throughout the ZF (Fig. 8B, E). In addition, there were some mid-pregnancy animals in which PCNA-A were observed mainly in the inner ZF (Fig. 8C). PCNA-A in the ZR could be frequently observed in this group. Mid-pregnancy viscachas showed the highest number of PCNA-A and PCNA-E, exhibiting statically significant differences in relation to all the other groups (Table 3).

Late-pregnancy viscachas presented enlarged adrenals with few PCNA-positive cells scattered throughout the outer cortex (Fig. 9). The number of PCNA-A and PCNA-E was significantly lower compared to mid-pregnancy viscachas. No statically significant differences in the number of PCNA-A and PCNA-E could be established between the late-pregnancy group and the nonpregnant and early-pregnancy groups (Table 3).

Raw histological/statistical data regarding PCNA-positive adrenocortical cells distribution available in S4-section of Supplementary Material.

DISCUSSION

In 1972, Vaughan et al. reported notable modifications on the weight of adrenal glands of mice subjected to pinealectomy and/or melatonin administration, suggesting an influence of the pineal hormone on the function of the adrenal gland. Since then, studies in rats (Yamada, 1990), primates (Torres-Farfan et al., 2003) and humans (Campino et al., 2011) have shown an inhibitory effect of melatonin on the adrenal cortex of those species. Melatonin exerts its effects via high affinity G protein-coupled membrane receptors (MT-1 and MT-2) and a cytosolic quinone reductase enzyme (MT-3) (Dubocovich et al., 2010). The presence of the G protein–coupled melatonin receptor 1 (MT-1) in the adrenal cortex was established by Torres-Farfan et al. (2003) in primates, and afterwards reported in rats (Richter et al. 2008) and humans (Campino et al., 2008). Our results demonstrate that melatonin administration inhibits the proliferation of adrenocortical cells in the male viscacha adrenal cortex under stressful conditions of daily manipulation and subcutaneous injections. Although the specific mechanism through which melatonin could exert a direct antiproliferative effect on adrenocortical cells is currently unknown. Many studies on other cell lines have demonstrated the capacity of this hormone to exert an antiproliferative effect through binding to the MT-1 receptor. This receptor activation inhibits the protein kinase A (PKA) pathway and CREB phosphorylation resulting in cAMP decrease. The MT-1 receptor also modulates phosphorylation of mitogen-activated protein kinases (MAPKs). Additionally, melatonin is a small lipophilic molecule that can cross cell membranes exerting several receptor-independent effects that include activation of different cascades and ion channels resulting in cAMP decrease, PLC, PKC, MAPK and PI3K/Akt pathway modulations (Kadekaro et al. 2004, Gatti et al. 2017). On the other hand, an indirect action of melatonin over adrenocortical cells cannot be disregarded, as previous studies conducted in our laboratory have shown that melatonin administration produces a decrease in the percentage area occupied by ACTH cells in the male viscacha pituitary gland (Filippa and Mohamed, 2006). ACTH is a well-known adrenocortical cell mitogen that exerts its effects through genomic and rapid non-genomic mechanisms (Lotfi and Mendoca, 2016, Spiga et al. 2017, Russel and Lightman, 2019). Therefore,

its diminished pituitary production in the male viscacha might also explain the reduction in the proliferation of adrenocortical cells observed in the melatonin administered animals of our study. Even though there is relatively little information about the mechanism underlying the androgen regulation of the HPA axis, it has been reported that androgens seem to have an inhibitory effect on the hypothalamic CRH (Handa et al., 1994; Lund et al., 2004; Weiser et al., 2008). Moreover, a possible androgenic modulation downstream of the HPA axis cannot be discarded as Benmouloud et al. (2014) reported variations in the adrenal cortex AR expression of sand rats, suggesting a direct action of gonadal steroids on the adrenal gland. Studies in different species have also reported modifications on the adrenal cortex physiology and morphology induced by gonadectomy (Hall and Korenchevsky, 1938; Bélanger et al., 1992; Benmouloud et al., 2014).

A prior immunohistochemical study performed in our laboratory revealed that steroidal gonadal hormones affect the activity of the male viscacha pituitary ACTH-cells, and thereby, the activity of the HPA-axis (Filippa and Mohamed, 2006). No statistically significant difference in the number of PCNA-A and PCNA-E between castrated and control animals could be established in the present study. However, histomorphological changes were observed, which are well-known signs of a hyperstimulated adrenal cortex (Ferreira et al., 2010; Gannouni et al., 2014). In addition, the observed vacuolar degeneration of many ZF cells is a nonspecific lesion associated with stress states (Howard, 1983). It is interesting to note that this apparent special sensitivity to the gland functionality of the cells of the innermost portion of the ZF had been pointed out many years ago by Zwemer (1936), who described that cells in the inner half of the ZF present various states of discharge when subjecting the adrenals to different stimuli. The fact that the male viscacha is a strictly seasonal breeder with profound variations of testosterone levels along its reproductive cycle, androgen modulation over the adrenal gland functionality might be crucial to guarantee environmental adaptation with the consequent reproductive success and species survival.

Summer constitutes the male viscacha reproductive period. During this season, the adrenal cortex exhibits low proliferative activity. A long photoperiod, abundant rainfall and mild

temperatures during this period guarantee increased food and water availability, which contributes to meeting the energetic demands of reproduction. Absence of major environmental stressors like low temperatures, a short photoperiod and nutrient restrictions in combination with high testosterone levels results in an overall decrease in HPA axis activity. It is noteworthy that stress response hormones produce an important suppression of gonadal function and reproduction (Whirledge and Cidlowski, 2010; Andric et al., 2013; Kheirabad et al., 2016). Considering that serum testosterone levels during the reproductive period of the male viscacha are almost five-fold higher than those in the regression period, its modulatory effect on HPA axis activity must be key to prevent stress-mediated disruption of the reproductive axis.

Late autumn is a period characterized by rapidly changing environmental conditions which demand an adequate adaptive response. A progressively shorter photoperiod signal implies an increase in the pineal gland activity of the male viscacha (Busolini et al., 2017). In this rodent, melatonin exerts an antigonadotropic function that causes a reduction of testosterone levels (Chaves et al., 2015). Furthermore, the influence of other environmental stressors, such as decreasing temperatures, lower water and food availability, starts to have a major impact on HPA axis functionality. Our results show that during this period adrenocortical cells exhibit the highest proliferative activity. PCNA-E were also observed more frequently. PCNA expression in endothelial cells has been considered as a parameter of neovascularization (Plasswilm et al., 1999; Imura et al., 2004). This increase in the proliferative activity of the adrenal cortex can be explained by the decrease in testosterone levels and the consequent relieving of the androgenic inhibitory modulatory effect on the HPA axis. Moreover, changing environmental conditions require an adaptive response of the energy metabolism. The winter season corresponds to the regression period of the male viscacha reproductive cycle During this season, the number of PCNA-E was the highest and could be observed in the ZG and mainly in the sinusoidal endothelial cell-lined channels of the ZF. This marked increment in proliferating endothelial cells might indicate neovascularization processes necessary to cope with the gland nutrient and substrate requirements. Ongoing ultrastructural studies in our laboratories revealed that during winter, adrenocortical cells

exhibit a decreased lipid content and a highly developed smooth endoplasmic reticulum (L Gallol & F Mohamed, unpublished observations) compatible with an enhanced steroidogenic activity. These findings in the adrenal cortex can be explained by the lack of the androgenic modulatory effect over the HPA axis during the regression period in conjunction with the impact of winter environmental stressors. Conversely, the average number of PCNA-A was significantly lower than in autumn and these cells could be observed mainly in the outer cortex. This finding might be explained by the above-discussed antiproliferative effect of melatonin. Seasonal changes in night length produce parallel changes in the duration of melatonin secretion, which is secreted for longer periods and at higher levels in winter than in summer (Wehr, 1997). Previous studies in our laboratory have demonstrated seasonal histomorphological changes in cells of the male viscacha pineal gland in relation to a higher glandular activity, which produces maximum serum levels of melatonin during the male viscacha regression period (Dominguez et al., 1987; Pelzer et al., 1999; Busolini et al., 2017).

During spring, the male viscacha undergoes a gradual reactivation of its reproductive capacities, constituting the gonadal recovery period of the cycle. In this season, PCNA-positive cells in the adrenal cortex were scarcely found. This reduction could be explained by a decreased impact of environmental stressors over the HPA axis. Moreover, serum testosterone concentrations during the recovery period are the highest of the whole reproductive cycle, indicating that the androgenic modulatory effect over the HPA axis must be important for an adequate recovery of the reproductive axis activity of the male viscacha.

During pregnancy, the regulation of the maternal HPA axis in mammals undergoes profound changes. Even though the regulation of adrenal cortex activity during pregnancy varies between different species, they share a common fact: a reduced response of the HPA axis to stressors (Windle et al., 2010; Brunton and Russell, 2011). In humans and primates, corticotrophin releasing hormone (CRH) is produced and released from the placenta into the bloodstream. On the other hand, it has been postulated and sustained over many years that the rodent placenta does not

produce CRH, which remains a matter of discussion. Apart from differences in the regulation of adrenal cortex activity between species, the functional importance of corticosteroids during pregnancy is undeniable (Fajer, 1971, Sheriff et al., 2018).Our study revealed no differences in the proliferative activity of the viscacha adrenal cortex between early-pregnant and non-pregnant viscachas. Plasma estrogen and progesterone levels start to increase during early-pregnancy, probably due to ovarian input. The adrenal cortex of mid-pregnancy viscachas exhibits a striking increase in PCNA expression. Cell proliferation predominates in the outer cortex: the ZG, outer ZF and especially in the ZG/ZF boundary. In addition, PCNA-A are observed in the inner ZF and even in the ZR. It is during mid-pregnancy when progesterone and estrogens reach the highest levels in the viscacha. This latter observation is noteworthy because the proliferative effect of estrogens on the H295R adrenal cell line has been confirmed by Montanaro et al. (2005) through thymidine incorporation studies, and there is also significant evidence that progesterone could exert a proliferative effect on adrenal cells (de Cremoux et al., 2008). A previous study conducted in our laboratory showed that during mid-pregnancy, viscacha ovaries undergo conspicuous ultrastructural changes compatible with a high steroidogenic activity from the interstitial tissue and corpus luteum, which led us to postulate that these organs are the source of the female hormone increment observed during pregnancy (Gil et al., 2007). However, the adrenal cortex is capable of synthetizing estrogens (Barakat et al., 2016), and progesterone is a precursor of most of the steroids synthesized by adrenocortical cells. Furthermore, the fetal and maternal adrenal glands are the major sources of dehydroepiandrosterone-sulfate and androstenedione, which are precursors used by ovaries and placenta in the production of estrogens and progesterone (Kaludjerovic and Ward, 2012). Thus, future studies are necessary to determine whether the female viscacha adrenal gland is a source of female hormones during pregnancy. Moreover, in mid-pregnancy viscachas PCNA expression in endothelial cells was significantly higher in relation to all the other groups. This latter observation might indicate a process of neovascularization (Plasswilm et al., 1999; Imura et al., 2004).

To our knowledge, this is the first study to assess adrenal cortex proliferative activity on a seasonal breeder rodent captured in its natural habitat during different periods of its annual reproductive cycle and pregnancy. The results obtained in the present study suggest that proliferation in the adrenal cortex of the viscacha is influenced by melatonin, sexual hormones and environmental stressors, participating in the complex mechanisms that regulate reproduction and adaptation to achieve survival of the species when faced with adverse environmental conditions.

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Angelina Bernardi for her technical participation. We also thank the members of GAECI-UNSL for their linguistic advice.

REFERENCES

Aguilera-Merlo C, Muñoz E, Dominguez S, Scardapane L, Piezzi R. 2005. Epididymis of viscacha (*Lagostomus maximus maximus*): morphological changes during the annual reproductive cycle. Anat Rec A Discov Mol Cell Evol Biol 282(1):83-92.

Amugongo SK, Hlusko LJ. 2014. Impact of maternal prenatal stress on growth of the offspring. Aging Dis 5(1):1-16.

Andric SA, Kojic Z, Bjelic MM, Mihajlovic AI, Baburski AZ, Sokanovic SJ, Janjic MM, Stojkov NJ, Stojilkovic SS, Kostic TS. 2013. The opposite roles of glucocorticoid and alpha1-adrenergic receptors in stress triggered apoptosis of rat Leydig cells. Am J Physiol Endocrinol Metab 304(1):51-59.

Barakat R, Oakley O, Kim H, Jin J, Ko CJ. 2016. Extra-gonadal sites of estrogen biosynthesis and function. BMB Rep 49(9):488-496.

Bélanger B, Caron S, Boudou P, Fiet J, Bélanger A. Adrenal steroidogenesis in the guinea pig: effects of androgens. 1992. Steroids 57(2):76-81.

Benmouloud A, Amirat Z, Khammar F, Patchev AV, Exbrayat JM, Almeida OF. 2014. Androgen receptor-mediated regulation of adrenocortical activity in the sand rat, *Psammomys obesus*. J Comp Physiol B 184(8):1055-1063.

Bozzo AA, Soñez AC, Monedero Cobeta I, Rolando NA, Romanini MC, Lazarte M, Gauna HM, Mugnaini MT, Ávila R. 2011. Chronic Stress Effects on Adrenal Cortex Cellular Proliferation in Pregnant Rats. Int. J. Morphol [online] 29(4):1148-1157.

Brunton PJ, Russell JA. 2011. Allopregnanolone and suppressed hypothalamo-pituitary-adrenal axis stress responses in late-pregnancy in the rat. Stress 14(1):6-12.

Busolini FI, Rosales GJ, Filippa VP, Mohamed FH. 2017. A Seasonal and Age-Related Study of Interstitial Cells in the Pineal Gland of Male Viscacha (*Lagostomus maximus maximus*). Anat Rec (Hoboken) 300(10):1847-1857.

Campino C, Valenzuela F, Arteaga E, Torres-Farfan C, Trucco C, Velasco A, Guzman S, Seron-Ferre M. 2008. [Melatonin reduces cortisol response to ACTH in humans]. Rev Med Chil 136(11):1390-1397.

Campino C, Valenzuela FJ, Torres-Farfan C, Reynolds HE, Abarzua-Catalan L, Arteaga E, Trucco C, Guzman S, Valenzuela GJ, Seron-Ferre M. 2011. Melatonin exerts direct inhibitory

actions on ACTH responses in the human adrenal gland. Horm Metab Res 43(5):337-342.

Chang SP, Morrison HD, Nilsson F, Kenyon CJ, West JD, Morley SD. 2013. Cell proliferation, movement and differentiation during maintenance of the adult mouse adrenal cortex. PLoS One 8(12): e81865.

Chaves M, Aguilera-Merlo C, Cruceno A, Fogal T, Mohamed F. 2015. Morphological study of the prostate gland in viscacha (*Lagostomus maximus maximus*) during periods of maximal and minimal reproductive activity. Anat Rec (Hoboken) 298(11):1919-1931.

de Cremoux P, Rosenberg D, Goussard J, Bremont-Weil C, Tissier F, Tran-Perennou C, Groussin L, Bertagna X, Bertherat J, Raffin-Sanson ML. 2008. Expression of progesterone and estradiol receptors in normal adrenal cortex, adrenocortical tumors, and primary pigmented nodular adrenocortical disease. Endocr Relat Cancer 15(2):465-474.

Cruceño AM, de Rosas JC, Fóscolo M, Chaves EM, Scardapane L, Dominguez S, Aguilera-Merlo C. 2013. Effects of Photoperiod on Epididymal and Sperm Morphology in a Wild Rodent, the Viscacha (*Lagostomus maximus maximus*). ISRN Anat 2013:128921. doi: 10.5402/2013/128921

Dominguez S, Piezzi RS, Scardapane L, Guzman JA. 1987. A light and electron microscopic study of the pineal gland of the viscacha (*Lagostomus maximus maximus*). J Pineal Res 4(2):211-219.

Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. 2010. International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. Pharmacol Rev 62(3):343-380.

Howard EB. 1983. Miscellaneous diseases. In: Pathobiology of Marine Mammal Diseases, Volume II. CRC press. p 163-225.

Fajer AB, Holzbauer M, Newport HM. 1971. The contribution of the adrenal gland to the total amount of progesterone produced in the female rat. J Physiol 214(1):115-126.

Fan F, Zou Y, Zhang Y, Ma X, Zhang J, Liu C, Li J, Pei M, Jiang Y, Dart AM. 2018. The relationship between maternal anxiety and cortisol during pregnancy and birth weight of chinese neonates. BMC Pregnancy Childbirth 18(1):265.

Ferreira M, de Abreu LC, Valenti VE, Meneghini A, Murad N, Ferreira C. 2010. Electric countershock and cold stress effects on liver and adrenal gland. Clinics (Sao Paulo) 65(3):291-296.

Filippa V, Godoy D, Perez E, Mohamed F. 2014. Effects of castration on androgen receptors and gonadotropins in the pituitary of adult male viscachas. Reprod Fertil Dev 26(7):991-1000.

Filippa V, Mohamed F. 2006. ACTH cells of pituitary pars distalis of viscacha (*Lagostomus maximus maximus*): immunohistochemical study in relation to season, sex, and growth. Gen Comp Endocrinol 146(3):217-225.

Filippa V, Mohamed F. 2010. The pituitary of non-pregnant and pregnant viscachas (*Lagostomus maximus maximus*): a comparative study by immunohistochemistry and morphometric analysis. Zoology (Jena) 113(6):361-372.

Fuentes LB, Caravaca N, Pelzer LE, Scardapane LA, Piezzi RS, Guzman JA. 1991. Seasonal variations in the testis and epididymis of vizcacha (*Lagostomus maximus maximus*). Biol Reprod 45(3):493-497.

Gallol LE, Mohamed FH. 2018. Immunomorphometric variations of sustentacular cells of the male viscacha adrenal medulla during the annual reproductive cycle. Effects of androgens and melatonin. Acta Histochem 120(4):363-372.

Gannouni N, Mhamdi A, El May M, Tebourbi O, Rhouma KB. 2014. Morphological changes of adrenal gland and heart tissue after varying duration of noise exposure in adult rat. Noise Health 16(73):416-421.

Gardner DS, Jackson AA, Langley-Evans SC. 1997. Maintenance of maternal diet-induced hypertension in the rat is dependent on glucocorticoids. Hypertension 30(6):1525-1530.

Gatti G, Lucini V, Dugnani S, Calastretti A, Spadoni G, Bedini A, Rivara S, Mor M, Canti G, Scaglione F, Bevilacqua A. 2017. Antiproliferative and pro-apoptotic activity of melatonin analogues on melanoma and breast cancer cells. Oncotarget 8(40): 68338–68353.

Gil E, Forneris M, Dominguez S, Penissi A, Fogal T, Piezzi RS, Scardapane L. 2007. Morphological and endocrine study of the ovarian interstitial tissue of viscacha (*Lagostomus maximus maximus*). Anat Rec (Hoboken) 290(7):788-794.

Hall K, Korenchevsky V. 1938. Effects of castration and of sexual hormones on the adrenals of male rats. J Physiol 91(4):365-374.

Handa RJ, Nunley KM, Lorens SA, Louie JP, McGivern RF, Bollnow MR. 1994. Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors. Physiol Behav 55(1):117-124.

Howard EB. Miscellaneous diseases. 1983. In: Howard EB, editor. Pathobiology of marine

mammal diseases, Volume II. CRC press. p 163-225.

Imura S, Miyake H, Izumi K, Tashiro S, Uehara H. 2004. Correlation of vascular endothelial cell proliferation with microvessel density and expression of vascular endothelial growth factor and basic fibroblast growth factor in hepatocellular carcinoma. J Med Invest 51(3-4):202-209.

Kadekaro AL, Andrade LN, Floeter-Winter LM, Rollag MD, Virador V, Vieira W, Castrucci AM. 2004. MT-1 melatonin receptor expression increases the antiproliferative effect of melatonin on S-91 murine melanoma cells. J Pineal Res 36(3):204-211.

Kaludjerovic J, Ward WE. 2012. The Interplay between Estrogen and Fetal Adrenal Cortex. J Nutr Metab 2012:837901.

Kelman Z. 1997. PCNA: structure, functions and interactions. Oncogene 13;14(6):629-640.

Kheirabad MK, Khodabandeh Z, Rahmanifar F, Tamadon A, Jahromi BN, Owjfard M, Koohi-Hosseinabadi O. 2016. Testicular germ cells apoptosis following exposure to chronic stress in rats. Asian Pacific Journal of Reproduction 5(5):371-375.

Kleinhaus K, Steinfeld S, Balaban J, Goodman L, Craft TS, Malaspina D, Myers MM, Moore H. 2010. Effects of excessive glucocorticoid receptor stimulation during early gestation on psychomotor and social behavior in the rat. Developmental psychobiology 52(2):121-132.

Kramer MS, Lydon J, Seguin L, Goulet L, Kahn SR, McNamara H, Genest J, Dassa C, Chen MF, Sharma S, Meaney MJ, Thomson S, Van Uum S, Koren G, Dahhou M, Lamoureux J, Platt RW. 2009. Stress pathways to spontaneous preterm birth: the role of stressors, psychological distress, and stress hormones. Am J Epidemiol 169(11):1319-1326.

Lesage J, Del-Favero F, Leonhardt M, Louvart H, Maccari S, Vieau D, Darnaudery M. 2004. Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. J Endocrinol 181(2):291-296.

Lindsay JR, Nieman LK. 2005. The hypothalamic-pituitary-adrenal axis in pregnancy: challenges in disease detection and treatment. Endocr Rev 26(6):775-799.

Llanos AC, Crespo JA. 1952. Ecología de la vizcacha ("*Lagostumus maximus maximus*" Blainv.) en el nordeste de la provincia de Entre Ríos. Revista de Investigaciones Agrícolas Extra Nueva Serie 10:5-95.

Lotfi CF, de Mendonca PO. 2016. Comparative Effect of ACTH and Related Peptides on Proliferation and Growth of Rat Adrenal Gland. Front Endocrinol (Lausanne) 7:39.

Lund TD, Munson DJ, Haldy ME, Handa RJ. 2004. Androgen Inhibits, While Oestrogen Enhances, Restraint-Induced Activation of Neuropeptide Neurones in the Paraventricular Nucleus of the Hypothalamus. J Neuroendocrinol 16(3):272-278.

Montanaro D, Maggiolini M, Recchia AG, Sirianni R, Aquila S, Barzon L, Fallo F, Ando S, Pezzi V. 2005. Antiestrogens upregulate estrogen receptor beta expression and inhibit adrenocortical H295R cell proliferation. J Mol Endocrinol 35(2):245-256.

Muñoz E, Calderón C, Fogal T, Domínguez S, Scardapane L, Guzmán J, Piezzi RS. 1999. Melatonina testicular: Papel en la ciclicidad reproductiva. Boletín Informativo SAA 8:55-67.

Pelzer LE, Calderón CP, Guzmán J. 1999. Changes in weight and hydroxyindole-O-methyltransferase activity of pineal gland of the plains viscacha (*Lagostomus maximus maximus*). Mastozool Neotrop 6:31–38.

Piezzi RS, Guzmán JA, Pelzer LE, Scardapane L, Domínguez S. 1984. Biological role of the pineal. Responses to the environmental photoperiod. Arch Biol Med Exp (Santiago) 17(3-4):273-282.

Plasswilm L, Hoper J, Cordes N, Tannapfel A. 1999. Investigation of microvessel density after irradiation. Radiat Res 151(4):454-460.

Ribes AC, Mohamed F, Dominguez S, Delgado M, Scardapane L, Guzman J, Piezzi R. 1999. Probable effect of photoperiod on seasonal variation in the nuclear volume of the adrenal cortex of viscacha (*Lagostomus maximus maximus*). Braz J Med Biol Res 32(9):1115-20.

Richter HG, Torres-Farfan C, Garcia-Sesnich J, Abarzua-Catalan L, Henriquez MG, Alvarez-Felmer M, Gaete F, Rehren GE, Seron-Ferre M. 2008. Rhythmic expression of functional MT1 melatonin receptors in the rat adrenal gland. Endocrinology 149(3):995-1003.

Rosales GJ, Busolini FI, Mohamed FH, Filippa VP. 2016. Effects of melatonin and gonadal androgens on cell proliferation in the pituitary of viscachas (*Lagostomus maximus maximus*). Cell Prolif 49(5):644-653.

Rosales GJ, Perez E, Rodriguez GB, Filippa VP, Mohamed FH. 2019. Variations in the adenohypophysis of the expression of proliferating cellular nuclear antigen, oestrogen and androgen receptors in relation to gonadal steroids during pregnancy of viscachas (*Lagostomus maximus maximus*). Reprod Fertil Dev. doi: 10.1071/RD18423.

Russell G, Lightman S. 2019. The human stress response. Nat Rev Endocrinol 15(9):525-534.

Shen CN, Seckl JR, Slack JM, Tosh D. 2003. Glucocorticoids suppress beta-cell development and induce hepatic metaplasia in embryonic pancreas. Biochem J 375(1):41-50.

Sheriff MJ, Dantzer B, Love OP, Orrock JL. 2018. Error management theory and the adaptive significance of transgenerational maternal-stress effects on offspring phenotype. Ecol Evol 8(13):6473-6482.

Spiga F, Zavala E, Walker JJ, Zhao Z, Terry JR, Lightman S. 2017. Dynamic responses of the adrenal steroidogenic regulatory network. Proc Natl Acad Sci U S A 114(31):6466–6474.

Torres-Farfan C, Richter HG, Rojas-Garcia P, Vergara M, Forcelledo ML, Valladares LE, Torrealba F, Valenzuela GJ, Seron-Ferre M. 2003. mt1 Melatonin receptor in the primate adrenal gland: inhibition of adrenocorticotropin-stimulated cortisol production by melatonin. J Clin Endocrinol Metab 88(1):450-458.

Vaughan MK, Vaughan GM, Reiter RJ, Benson B. 1972. Effect of melatonin and other pineal indoles on adrenal enlargement produced in male and female mice by pinealectomy, unilateral adrenalectomy, castration, and cold stress. Neuroendocrinology 10(3):139-154.

Wehr TA. Melatonin and seasonal rhythms. 1997. J Biol Rhythms 12(6):518-527.

Weir BJ. 1971. The reproductive organs of the female plains viscacha, *Lagostomus maximus*. J Reprod Fertil 25(3):365-373.

Weiser MJ, Goel N, Sandau US, Bale TL, Handa RJ. 2008. Androgen regulation of corticotropin-releasing hormone receptor 2 (CRHR2) mRNA expression and receptor binding in the rat brain. Exp Neurol 214(1):62-68.

Whirledge S, Cidlowski JA. 2010. Glucocorticoids, stress, and fertility. Minerva Endocrinol 35(2):109-125.

Willenberg HS, Bornstein SR. 2017. Adrenal Cortex; Development, Anatomy, Physiology. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, Dungan K, Grossman A, Hershman JM, Kaltsas G, Koch C, Kopp P et al., editors. Endotext. South Dartmouth (MA).

Windle RJ, Wood SA, Kershaw YM, Lightman SL, Ingram CD. 2010. Reduced stress responsiveness in pregnancy: relationship with pattern of forebrain c-fos mRNA expression. Brain Res 1358:102-109.

Wolkersdörfer GW, Bornstein SR. 1998. Tissue remodelling in the adrenal gland. Biochem Pharmacol 56(2):163-171.

Yamada K. 1990. Effects of melatonin on adrenal function in male rats. Res Commun Chem Pathol Pharmacol 69(2):241-244.

Zaki SM, Abdelgawad FA, El-Shaarawy EAA, Radwan RAK, Aboul-Hoda BE. 2018. Stress-induced changes in the aged-rat adrenal cortex. Histological and histomorphometric study. Folia Morphol (Warsz) 77(4):629-641.

Zwemer RL. 1936. A Study of Adrenal Cortex Morphology. Am J Pathol 12(1):107-114.

FIGURE LEGENDS

Fig. 1. Technique controls for PCNA immunohistochemistry. A) Rat gut. Positive control. Many PCNA-positive cells with labeled nuclei can be observed. B) Rat gut. Negative control. No immunoreactive cells are observed. C) Adrenal cortex section of a female viscacha during the mid-pregnancy period used as a negative control. No immunoreactive cells are observed. Arrow: PCNA-positive cells. ZG: zona glomerulosa, ZF: zona fasciculata. IHC followed by hematoxylin counterstaining. (A, B) Scale bar: 250 µm, Inset: 12.5 µm. (C) Scale bar: 25 µm.

Fig. 2. Melatonin administration experiment. Adrenal cortex. IHC-PCNA. A) Control. Many PCNA-A are observed. B) Control. PCNA-positive adrenocortical cells in the ZG/ZF boundary C) Melatonin administration. A significantly lower number of PCNA-positive adrenocortical cells are seen compared with control animals. D) Melatonin administration. A PCNA-positive adrenocortical cell and a PCNA-positive endothelial cell in the ZG/ZF boundary. Arrowhead: PCNA-positive adrenocortical cell. C: capsule, ZG: zona glomerulosa, ZF: zona fasciculata. (A-B) Scale bar: 250 µm. (C-D) IHC followed by hematoxylin counterstaining. Scale bar 25 µm.

Fig. 3. Castrated animals (A-E). A) Scale mosaic micrograph covering the full width of a castrated male viscacha adrenal cortex. H-E. B) Micrograph showing enlarged ZF cells (arrow) in comparison to control animals. v: blood vessels. H-E. C) Innermost ZF cells exhibiting cytoplasmic lipid depletion (compare with Figure 3G) and vacuolization (arrowheads). Some cells show an intense acidophil cytoplasm (arrow) indicative of a marked lipid depletion. H-E. D) Castration. Micrograph showing enlarged ZR cells in comparison to control animals (arrows). ZG: zona glomerulosa, ZF: zona fasciculata. IHQ-PCNA. E) Adrenal cortex of a castrated animal. Few PCNA-positive cells can be observed (arrows). ZG: zona glomerulosa, ZF: zona fasciculata, ZR: zona reticularis, M: medulla. (A) Scale bar: 100 µm. (B-D) Scale bar: 25 µm. (E) Scale bar 250 µm.

Control animals (F-I). F) Scale mosaic micrograph covering the full width of a control male viscacha adrenal cortex. G) ZF cells (spongiocytes) of a control animal. H-E. H) ZR cells of a control animal exhibiting typical small nuclei and acidophile cytoplasm. H-E. I) Adrenal cortex of a control animal. Few PCNA-positive cells can be observed (arrows). ZG: zona glomerulosa, ZF: zona fasciculata. IHQ-PCNA. ZG: zona glomerulosa, ZF: zona fasciculata, ZR: zona reticularis, M: medulla. (F) Scale bar: 100 µm. (G-H) Scale bar: 25 µm. (I) Scale bar 250 µm. * $p<0.01$ ZF(A) versus ZF(F). ** $p<0.05$ ZR(A) versus ZR(F). Mann-Whitney U test.

Fig. 4. Male viscacha adrenal cortex. IHC-PCNA. A) Summer. Mosaic micrograph of a section of the adrenal cortex. Few PCNA-A are observed scattered throughout the cortex. B) Late autumn. Mosaic micrograph of a section of the adrenal cortex. Several PCNA-A are observed in the outer cortex, mainly around the ZG/ZR boundary. C) Late autumn. Mosaic micrograph of a section of the adrenal cortex. Only a few PCNA-A can be seen in the outer cortex, but many are observed in the innermost portion of the ZF. D) Late autumn. Subcapsular PCNA-positive cell whose localization matches that proposed for adrenocortical stem cells. E) Autumn. A group of PCNA-A in the ZG and in the ZG/ZF boundary. F) Late autumn. Many PCNA-A located around the ZG/ZF boundary. Inset: adrenocortical cell exhibiting a mitotic figure. G) Late autumn. High magnification micrograph of a PCNA-A in close relation with a PCNA-E. H) Autumn. A PCNA-A in ZR. I) Winter. Mosaic micrograph of a section of the adrenal cortex. PCNA-A can be seen mainly in the outer cortex, and many PCNA-E can be observed lining blood vessels throughout the cortex. J) PCNA-A and PCNA-E observed in the ZG. K) A group of PCNA-A and a PCNA-E located around the ZG/ ZF boundary. L) Longitudinal section showing a blood vessel lined by PCNA-E. M) Cross section showing several adrenal cortex blood vessels exhibiting PCNA-E. Inset: endothelial cell exhibiting a mitotic figure. N) Spring. Mosaic micrograph of a section of the adrenal cortex. Few PCNA-A are observed throughout the cortex. Arrow: PCNA-positive adrenocortical cells. Arrowhead: PCNA-positive endotelial cells. White arrow: mitotic figure. C: capsule, ZG: zona glomerulosa, ZG/ZF: zona glomerulosa-zona fasciculata boundary, ZF: zona

fasciculata, IZF: innermost zona fasciculata. (A, B, C, I, N) Scale Bar: 100 µm. (D-H, J-M) IHC followed by hematoxylin counterstaining. Scale bar: 25 µm.

Fig. 5. During the male viscacha regression period, serum testosterone levels (1.27 ± 0.30 ng/ml) are significantly lower a ($p<0.01$) than those found during the reproductive period (4.92 ± 0.44 ng/ml) and recovery period (5.94 ± 0.49). No statistically significant difference could be established between the reproduction period and recovery period. Values are expressed as mean ± SEM (n = 4).

Fig. 6. Mid-pregnancy viscachas exhibit the highest values of estradiol (75.02 ± 2.5 pg/ml) and progesterone (53.75 ± 2.39 ng/ml). [a] A statistically significant difference ($p<0,05$) could be established when comparing mid-pregnancy estrogen levels versus nonpregnant (18.01 ± 3.19 pg/ml), early-pregnancy (27.50 ± 2.5 pg/ml) and late-pregnancy levels (24.25 ± 2.17 pg/ml). No statistically significant difference in estradiol levels could be established between nonpregnant, early-pregnancy and late-pregnancy viscachas. [b] Progesterone levels show significant differences ($p <0,05$) when comparing mid-pregnancy viscachas with nonpregnant (0.72 ± 0.11 ng/ml), early-pregnancy (4.64 ± 0.95 ng/ml) and late-pregnancy viscachas (18.61 ± 2.25 ng/ml). [c] Statistically significant differences in progesterone levels could also be established between early-pregnancy and nonpregnant viscachas ($p<0,05$). [d] Late-pregnancy progesterone levels also exhibit statistically significant differences compared with early-pregnancy and nonpregnant viscachas ($p<0,05$). Values are expressed as mean ± SEM (n = 4). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Fig. 7. Female viscacha adrenal cortex. IHC-PCNA. A, B) Nonpregnant. Some PCNA- positive adrenocortical cells can be observed mainly in the outer cortex comprising the ZG and ZF. C, D) Early-pregnancy. PCNA-positive adrenocortical cells can be observed in the ZG and ZF. Arrows: PCNA-positive adrenocortical cells. ZG: zona glomerulosa, ZF: zona fasciculata. A, C) Scale bar: 250 µm. B, D) IHC followed by hematoxylin counterstain, scale bar: 25 µm.

Fig. 8. Female viscacha adrenal cortex. Mid-pregnancy. IHC-PCNA. A) Several PCNA-positive adrenocortical cells can be seen around the zona glomerulosa-zona fasciculata boundary. B) Several PCNA-positive adrenocortical cells can be observed throughout the outer cortex. C) PCNA-positive adrenocortical cells in the outer cortex are scarce, but many can be observed in the innermost zona fasciculata. D) Several PCNA-positive adrenocortical cells can be seen in the zona glomerulosa-zona fasciculata boundary. A mitotic figure can also be observed. E) PCNA-positive adrenocortical cells in the zona fasciculata. Inset: High magnification micrograph of a PCNA-positive endothelial cell lining a blood vessel. A PCNA-positive adrenocortical cell can also be observed nearby. F-H) Mid-pregnancy. High magnification micrographs of mitotic figures that can be frequently observed in the adrenal cortex during mid-pregnancy. Arrows: PCNA-positive adrenocortical cells, white arrows: mitotic figures, arrowhead: PCNA-positive endothelial cell. C: capsule, ZG: zona glomerulosa, ZG/ZF: Zona glomerulosa-zona fasciculata boundary, ZF: zona fasciculata, IZF: innermost zona fasciculata. A-C) Scale bar 250 µm. D-E) IHC followed by hematoxylin counterstain, scale bar 25 µm, inset scale bar 5 µm. F-H) IHC followed by hematoxylin counterstain, scale bar 5 µm.

Fig. 9. Female viscacha adrenal cortex. Late-pregnancy. IHC-PCNA. A, B) Some PCNA-positive cells can be observed in the outer cortex. Arrows: PCNA-positive adrenocortical cells, arrowhead: PCNA-positive endothelial cell, ZG: zona glomerulosa, ZF: zona fasciculata. A) Scale bar 250 µm. B) IHC followed by hematoxylin counterstain, scale bar 25 µm.

TABLES

Table 1. Seasonal environmental conditions.

	SUMMER	AUTUMN	WINTER	SPRING
PRECIPITATION (mm)	313	67	26	144
AVERAGE TEMPERATURE (^{o}C)	23	13	10	21
HELIOPHANY (h)	9.5	7.6	6.83	9.8

Average values of precipitations, temperatures and heliophany in San Luis, Argentina.

Table 2. Seasonal study of PCNA-positive adrenocortical and endothelial cells in the adrenal cortex.

	nPCNA-A	nPCNA-E
SUMMER	0.32 ± 0.07	0.34 ± 0.15
LATE AUTUMN	1.96 ± 0.27[a]	0.91 ± 0.20[c]
WINTER	0.78 ± 0.05[b]	2.96 ± 0.90[d]
SPRING	0.39 ± 0.07	0.48 ± 0.10

The values are expressed as mean ± SEM (n=4). nPCNA-A: average number of PCNA-positive adrenocortical cells per reference area.[a] $p<0.05$ late autumn versus summer, spring and winter. [b]$p<0.05$ winter vs summer and spring. No statistically significant difference could be established between summer-early autumn and spring. Kruskal-Wallis test followed by Dunn's multiple comparisons test. nPCNA-E: average number of PCNA-positive endothelial cells per reference area. [c]$p<0.05$ late autumn vs summer-early autumn and spring. [d]$p< 0.01$ winter versus summer-early autumn, spring and autumn. No statistically significant difference could be established between summer-early autumn and spring. Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Table 3. PCNA-positive adrenocortical and endothelial cells in the adrenal cortex of pregnant and nonpregnant viscachas.

	nPCNA-A	nPCNA-E
NONPREGNANT	0.67 ± 0.13	0.28 ± 0.05
EARLY-PREGNANCY	0.76 ± 0.07	0.33 ± 0.04
MID-PREGNANCY	9.53 ± 2.83 [a]	0.80 ± 0.08 [b]
LATE PREGANANCY	0.46 ± 0.04	0.48 ± 0.06

The values are expressed as mean ± SEM (n=4). nPCNA-A: average number of PCNA-positive adrenocortical cells per reference area. nPCNA-E: average number of PCNA-positive endothelial cells per reference area. [a]$p<0.01$ mid-pregnancy versus nonpregnant, early-pregnancy and late-pregnancy. [b]$p<0.05$ mid-pregnancy versus nonpregnant, early-pregnancy and late-pregnancy. Groups were evaluated using Kruskal-Wallis test followed by Dunn's multiple comparisons test.