

Morphological and biochemical study of the pineal gland of pregnant and non-pregnant female vizcachas (*Lagostomus maximus maximus*)

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Abstract

OBJECTIVES: The morphological and biochemical aspects of the pineal gland of pregnant and non-pregnant female vizcachas were investigated to examine whether there is a relationship between the pregnancy and this gland.

METHODS: Pregnant and non-pregnant adult female vizcachas were used. The ultrastructure of type I cell (dark and light pinealocytes) was studied. Pineal and serum melatonin were investigated by radioimmunoassay. Radiometric methods were applied to investigate the arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) activities.

RESULTS: The morphological study showed the existence of dark and light pinealocytes. The differences between both cells are slight. However, the appearance of dark pinealocytes suggests that they have a high activity. These cells were more abundant in the pineal gland of pregnant females. The pineal melatonin levels and the AA-NAT activity were higher in the pregnant females. Serum melatonin and HIOMT activity did not show significant differences.

CONCLUSION: Our results suggest that the morphological and biochemical changes in the pineal gland of pregnant vizcachas are associated with the gestation. However, the relation between the pineal gland and the pregnancy is complex and further studies are necessary to clearly establish it.

Introduction

The participation of the pineal gland in the reproduction is well documented. However, reports about the pineal gland and pregnancy are scanty. To date, histochemical, morphological and biochemical changes in the pineal gland during the pregnancy have been informed. Studies in the pineal gland of guinea pig have shown a strong

increase of NADH diaphorase and NADPH diaphorase activities in the second half of the pregnancy [1] and a major number of "synaptic" ribbons in pregnant animals [2]. Karasek et al. examined the ultrastructure of rat pinealocytes during the last phase of pregnancy [3]. They observed an increase in the number of Golgi profile and dense-core

vesicles at day 21 and 22 and a decrease in the number of vacuoles containing a flocculent material in the last day of gestation. Studies in mole [4], sow [5] and rat [6] have also described ultrastructural changes in the pineal gland in the pregnancy. Similarly, biochemical studies have associated the pineal activity with pregnancy in human [7] and rat [8]. Furthermore, other authors have suggested the rat pineal gland may be modifying the synthesis and release of gonadotropin and the levels of gonadal hormones during the last phase of gestation [9, 10]. Most of the investigations suggest an enhanced metabolism in the pineal organ during the gestation, but the relationship between this gland and this physiological condition remains to be perfectly established [11].

The vizcacha (*Lagostomus maximus maximus*), the largest member of the family Chinchillidae, inhabits in the southern hemisphere from Paraguay through central Argentina [12]. This rodent exhibits a nocturnal behaviour, lives in extensive burrows and emerges at dawn and dusk to feed. Due to the restricted exposition to the light, vizcacha has resulted to be an interesting model to study the pineal gland and the processes in which it is involved. Morphological [13, 14] and biochemical [15, 16] studies of the pineal gland of male vizcachas have been carried out in the authors' laboratory. Conversely, the pineal gland of female vizcachas has never been studied heretofore. In its natural habitat, the vizcacha is a seasonal breeder. A large number of pregnant animals are present in winter; however, pregnant females have been found in other seasons [17]. The gestation period is 154 days [18].

Because of these antecedents, the aim of the present study was to examine the morphological and biochemical aspects in the pineal gland of pregnant and non-pregnant female vizcachas to establish a probable relation between the pineal activity and the pregnancy in this rodent.

Material and methods

Animals

Pregnant and non-pregnant adult female vizcachas, weighing 2–4 kg, were captured in their habitat near San Luis, Argentina (33° 20' south latitude, 769 m altitude) during 2000 and 2002. In San Luis, in summer there is up to 14 hr light daily with an average temperature of 25°C. In winter, the light phase decreases to 10 hr and average temperature to 10°C. The rainfall is 206 mm in summer and 18 mm in winter.

In the present investigation, 4 pregnant and 4 non-pregnant animals were used for morphological study and 31 pregnant and 31 non-pregnant vizcachas for the biochemical assays. The reproductive condition of the vizcachas was carefully assessed on the following basis: a) the examination of the uterine horns, b) the analysis of the ovaries by light microscopy, c) body weight [19, 20] and d) lens weight [21].

The animals were anaesthetized with Nembutal (pentobarbital) and blood samples were obtained by cardiac puncture. The serum was separated by centrifugation, transferred to glass vials and stored at –20°C until assay-

ing. After that, the animals were killed by decapitation in the morning (08:00–10:00). The brains were exposed and the pineal gland were quickly removed and used for electron microscopy or biochemical assays.

Microscopy

For electron microscopy, the pineal glands were fixed "in situ" with formaldehyde-glutaraldehyde in phosphate buffer [22] for 10 min, removed and placed in the same fixative for an additional 6 hr at room temperature, post fixed in cold 2% OsO₄ for 12 hr, dehydrated in acetone, and embedded in Spurr's resin. One-micrometer-thick sections were obtained with a Porter Blum ultramicrotome and dyed with toluidine blue for the light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate [23] and were observed under a Siemens Elmiskop I electron microscope.

Pineal and serum melatonin

For biochemical assays, the pineal glands were weighed, placed in vials on dry ice and kept frozen at –70°C until they were used in assays.

Pineal glands were homogenized in 1.0 ml of ice-cold 0.05 M sodium borate buffer (pH 10.0). The homogenates (1 ml) and the serum (0.5 ml) were mixed, for 30s, with 4 and 2 ml of methanol, respectively, and centrifuged at 1500 g at 4°C for 10 min [24]. The supernatants were transferred to another tube and dried in a rotary-vacuum evaporator under reduce pressure. The residues were stored at –20°C until melatonin determination by radioimmunoassay. The dried residues of pineal and serum were reconstituted in 1.0 and 0.5 ml of 0.1M tricine buffer (pH 5.5), respectively. Melatonin estimates were done by duplicate using a radioimmunoassay as described Fraser et al. [25] with a lower limit of sensitivity of 2.5–5.0 pg/tube. 500 µl for each standard or sample in buffer tricine and 200 µl of a specific antiserum to melatonin raised in sheep (Stockgrand Ltd.) were mixed and incubated at room temperature for 30 min. 100 µl of ³H-melatonin were added to all tubes and incubated for 18 h at 4°C. Antibody-bound melatonin was separated from the free fraction by incubation for 15 min at 4°C in ice with 500 µl dextran-coated charcoal. The tubes were spun at 1500 g for 15 min at 4°C. 700 µl aliquots of the supernatant were removed into vials containing 3.0 ml scintillation fluid. Vials were shaken at room temperature for 1h. Radioactivity was counted in all tubes. The melatonin concentration in the samples were determined from standard curve and expressed as pg/mg of wet weight in pineal and pg/ml in serum.

Arylalkylamine-N-acetyltransferase Activity

Pineal glands were homogenized with 0.25 ml of potassium phosphate (pH 6.5). The homogenates were incubated with 0.1 µmol of tryptamine and 3.4 nmoles of acetyl-l-¹⁴C coenzyme A (50 mCi/nmol) in a 1 ml glass vial. The reaction was carried out at 37°C for 10 min and stopped by the addition of 0.5 ml of 0.5 M borate buffer (pH 10). The reaction mixture was transferred into a test

tube containing 6 ml of toluene/isoamyl alcohol (97:3), and shaken for 30 s. After centrifugation at 3500 rpm for 10 min, 5 ml of the organic phase were transferred and evaporated to dryness. The residue was dissolved with 1 ml of ethanol, 10 ml of scintillation solution was added, and the radioactivity was measured [26]. Results were expressed as pmoles/mg tissue/10 min.

Hydroxyindole-O-methyltransferase activity

The hydroxyindole-O-methyltransferase activity was determined according to the method of Axelrod et al. [27]. The pineal were homogenized in ice-cold 0.05 M phosphate buffer (pH 7.9). 200 μ l aliquots of each homogenate were incubated with N-acetylserotonin (Sigma, Chemical Co.) and 14 C-Methyl-S-Adenosyl-methionine (54 mCi/nmol), for 30 min at 37°C. The reaction stopped by adding 1 ml of 0.2 M borate buffer (pH 10). 14 C-melatonin was extracted with 6 ml of chloroform. A 4 μ l aliquot of the chloroform phase was transferred to a scintillation vial and evaporated to dryness. After, 1 ml of ethanol and 10 ml of scintillation solution were added to each vial, and the radioactivity was measured. The results were expressed as pmoles 14 C-melatonin /mg tissue/hr.

Statistical analyses

Means and standard errors were calculated for all data set. Student's t-test was performed. A value of $p < 0.05$ was accepted as statistically significant.

Results

Light microscopy

The parenchyma of the pineal gland of female vizcachas consisted primarily of two types of cells. Most cells possessed a round or oval nucleus with prominent nucleolus (type I cells or pinealocytes of population I), and some others presented an irregular-shaped nucleus (type II cells or pinealocytes of population II). Light and dark pinealocytes were distinguished within type I cell. Some interstitial or glial cells and pigment-containing cells were also observed.

The analysis of the semithin section revealed that the pineal gland of pregnant and non-pregnant vizcachas showed different aspects (Fig. 1 a, b). The pineal parenchyma of the pregnant females presented a major amount of dark pinealocytes.

Electron microscopy

The same type cells described at light microscopy level were identified at ultrastructural level. However, we only considered the type I cells because its cytological features can be related to the objective of the present investigation.

Light and dark pinealocytes showed different nuclear and cytoplasmic electron-density. They were irregular in shape with cytoplasmic processes emerging from their perikarya. In both type of cells the nucleus was large and round/oval in shape with prominent nucleolus. The chromatin was finely dispersed in the nuclear matrix.

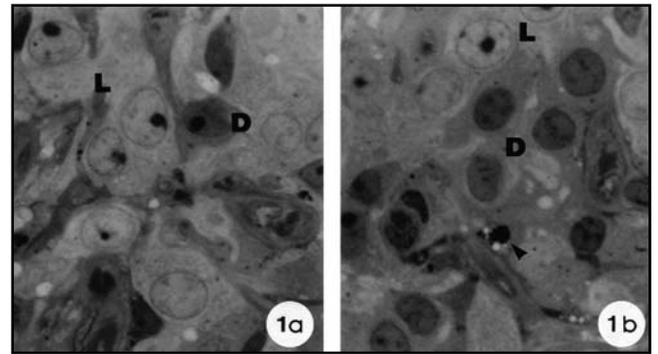


Figure 1. Semithin sections showing the parenchyma of pineal glands obtained from non-pregnant female (a) and pregnant female (b). Dark (D) and light (L) pinealocytes are distinguished in both samples, but the dark pinealocytes are abundant in pregnant vizcachas. An aggregation of pigments can be observed (arrowhead). Stain: toluidine blue. Original magnification: $\times 1000$.

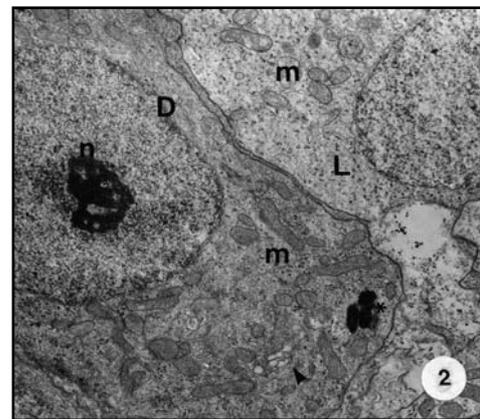


Figure 2. Electron micrograph of the pineal gland in a pregnant vizcacha. The nucleus of dark pinealocyte (D) contains an evident nucleolus (n) and the cytoplasm has numerous mitochondria (m) and dilated cisterns of endoplasmic reticulum (arrowhead). L: light pinealocyte. Asterisk: premelanosomes. Original magnification: $\times 6000$.

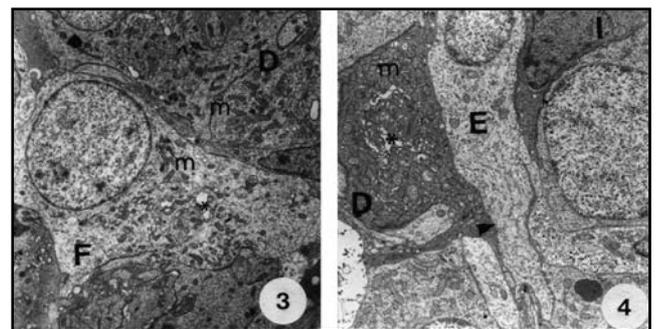


Figure 3. Electron micrograph shows a "full" light pinealocyte (F). Its cytoplasm contains numerous mitochondria (m) and dilated cisterns of endoplasmic reticulum (asterisk). The cytoplasmic portions of two dark pinealocytes (D) can be also observed. The sample corresponds to a pregnant vizcacha. Original magnification: $\times 2000$.

Figure 4. Electron micrograph shows the pineal parenchyma in a non-pregnant vizcacha. The "empty" light pinealocyte (E) has few organelles, but the cytoplasm of the dark pinealocyte (D) contains abundant mitochondria (m) and dilated cisterns of endoplasmic reticulum (asterisk). Arrowhead: pineal process. I: interstitial cell. Original magnification: $\times 3000$.

The cytoplasm of dark pinealocytes was always full of organelles. The organelles consisted principally of abundant mitochondria and dilated cisterns of the endoplasmic reticulum. Dense bodies, developed Golgi apparatus, ribosomes, glycogen granules and subsurface cisterns were observed (Fig. 2).

The appearance of light pinealocytes presented marked variations. Some light pinealocytes (“full” light pinealocytes) had an aspect similar to the dark pinealocytes (Fig. 3).

The cytoplasm of other light pinealocytes was scarce in organelles (“empty” light pinealocytes) (Fig. 4). Stages intermediate in electron-density were also present.

The dark and “full” light pinealocytes were always present in pregnant and non-pregnant animals. The dark pinealocytes were more abundant in the pregnant females. The “empty” light pinealocytes were only observed in non-pregnant animals.

Biochemical assays

Figures 5 and 6 show the levels of the pineal and serum melatonin of pregnant and non-pregnant females, respectively. The values of AA-NAT and HIOMT activities of pregnant and non-pregnant vizcachas are shown in Figures 7 and 8.

Pineal and serum melatonin

The higher levels of the pineal melatonin were found in pregnant females (Student’s t-test, $p < 0.05$, Fig. 5).

No statistical differences in serum melatonin between pregnant and non-pregnant animals were determined (Student’s t-test, n.s., Fig. 6).

AA-NAT and HIOMT activities

The AA-NAT activity in pregnant vizcachas was higher than in non-pregnant vizcachas. (Student’s t-test, $p < 0.001$, Fig. 7).

The HIOMT activity did not show significant differences (Student’s t-test, n.s.; Fig. 8).

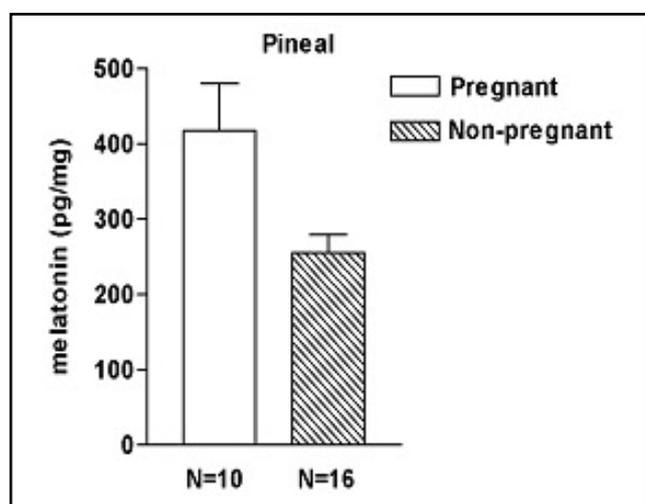


Figure 5. Pineal melatonin levels in pregnant and non-pregnant female vizcachas. Each bar represents means \pm SEM. (Student’s t-test, $p < 0.05$)

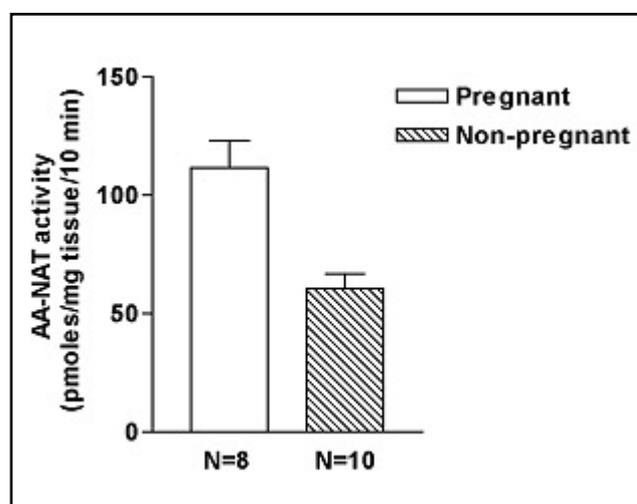


Figure 7. AA-NAT activity in the pineal gland of pregnant and non-pregnant female vizcachas. Values are means \pm SEM. (Student’s t-test, $p < 0.001$).

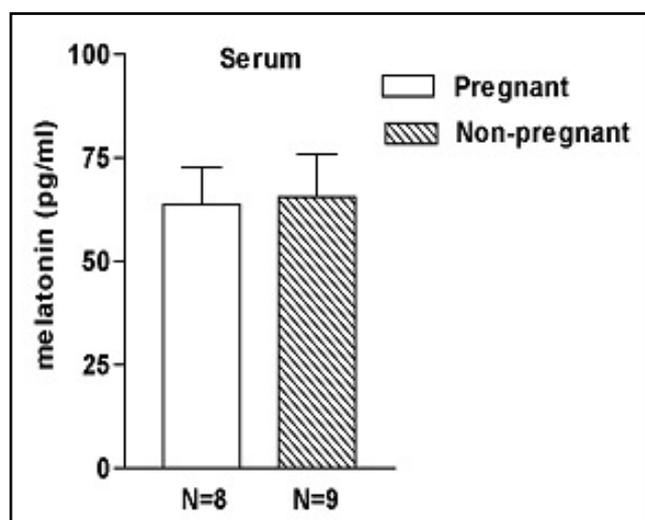


Figure 6. Serum melatonin levels in pregnant and non-pregnant female vizcachas. Values are means \pm SEM. (Student’s t-test, n.s.)

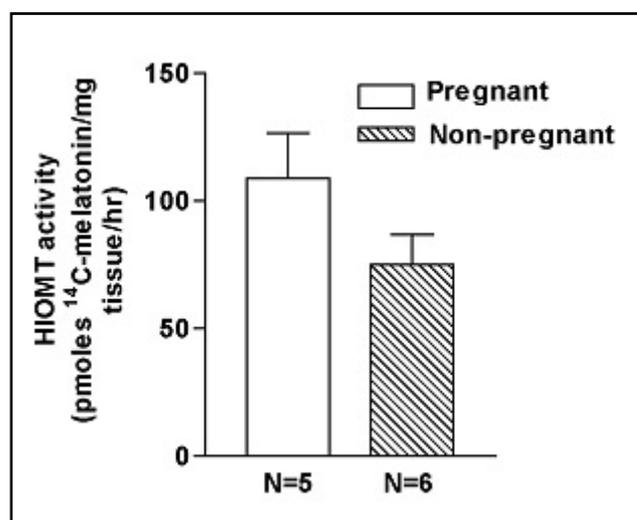


Figure 8. HIOMT activity in the pineal gland of pregnant and non-pregnant female vizcachas. Values are means \pm SEM. (Student’s t-test, n.s.)

Discussion

The involvement of the pineal gland in the regulation of reproduction in seasonal breeders has been well demonstrated [28, 29]. On the other hand, the pregnancy is a reproductive state characterized by a high hormonal activity in females. However, few reports describe the morphology and physiology of the pineal gland in pregnancy. The results of the present study show that morphological and biochemical changes in the vizcacha pineal gland might be associated with pregnancy.

The histology and ultrastructure of the pineal gland of vizcacha have been previously described in males [13, 14]. The same cellular populations of male vizcacha pineal were observed in females. In both sexes, there were differences in degree but not in type. Comparative histological and ultrastructural observations between samples obtained from pregnant and non-pregnant animals showed morphological differences in the type I cell. Light and dark pinealocytes have been also observed in numerous other mammals [30]. This distinction is based only on a difference in electron density. Nevertheless, most authors consider that light and dark pinealocytes are different stages of the same cell type that may be reflected in a differential susceptibility to the fixative [31]. Lues observed an increased number of dark pinealocytes with more mitochondria, endoplasmic reticulum and agranular vesicles in the second half of pregnancy [32]. The cytological features of type I cells of female vizcachas suggest different functional stages. The dark and "full" light pinealocytes were characterized by the appearance of active cells. In contrast, the "empty" light pinealocytes showed scanty organelles. Likewise, the amount of dark pinealocytes was increased in the pregnant females and the "empty" light pinealocytes were only observed in non-pregnant females. These results might indicate a probable increase in the pineal activity during the pregnancy.

In our study, the levels of pineal melatonin were higher during gestation. Similarly, other authors have found changes in the levels of melatonin in the pregnancy [7, 33]. Most investigations indicate an increase of melatonin, and a progonadal effect of the indole has been suggested in pregnant rodents [34]. In contrast, we observed that the circulating melatonin remained unaltered in both groups. The differences between the glandular and serous compartments suggest that the hormonal synthesis was increased but the secretion was probably unmodified during gestation. In addition, Calderón et al. found that in vizcacha the serum diurnal melatonin variation was delayed in relation to the one in the pineal [35]. These authors interpret that the melatonin may be stored or released slowly in vizcacha pineal. Thus, in the female vizcachas, the hormonal factors of pregnancy might contribute to regulate the synthesis, the storage or the delayed release of the indole during gestation.

According to high level of pineal melatonin, we observed an increase in the AA-NAT activity of pregnant vizcachas. Contrarily, the HIOMT activity was unvaried in both groups. Furthermore, the AA-NAT activity remained higher in pregnant females when it was com-

pared in different seasons, whereas the HIOMT activity increased in autumn and decreased in spring in samples obtained from pregnant animals (results not shown). In a recent review, Simonneaux and Ribelayga conclude that the pineal gland is a junction of various neural and endocrine inputs [36]. These authors remark "the extraordinary capacity of the pineal gland to integrate numerous hormonal and neural messages via several signal transduction pathways". Based on this concept, we suggest that the changes in AA-NAT activity might be related to the physiological state of females. Probably, this enzyme is sensible to the endocrine cues during the gestation. In contrast, the behaviour of HIOMT may be explained because its activity has been related to seasonal photoperiodic variations [37]. Consequently, HIOMT might not be associated to the endocrine variations of the pregnancy.

The pineal-pituitary-gonadal axis of adult male vizcachas has been exhaustively studied in our laboratory [38, 39, 40, 41, 42]. The morphological and biochemical results indicate that the adult male vizcacha is a photoperiod-dependent seasonal breeder. For example, a high activity of the pineal gland is parallel with a testicular regression period during the winter (short day-length). However, the role of photoperiod in female vizcachas remains to be well established. In our study, the pineal variations appear to be more related to the pregnancy and less related to the photoperiod. Similarly, Bishnupuri and Halder have reported that the constant light, long day-length and short day-length condition did not significantly affect the pineal activity during gestation and lactation [43]. Moreover, Mohamed et al. studied the colloid in the pituitary pars distalis of vizcacha [44]. In male vizcachas, their results showed seasonal variations associated with the photoperiod and the pineal gland. Conversely, they observed that in adult females, the number of colloid accumulations does not vary in relation to the season, but the mean follicular diameters were higher in pregnant animals when they were compared with non-pregnant animals. Thus, we may consider the hypothesis that the pineal gland of female vizcachas might be most susceptible to endocrine cues during the pregnancy, or that the gestation might decrease its response to photoperiod.

The pregnancy is a special physiology stage that exhibits a complex interplay of endocrine facts. Furthermore, the hormonal variations are drastic during the gestation. Consequently, the relation between the pregnancy and the pineal gland is not simple. This is the first study carried out in the pineal gland of female vizcachas. Our results suggest that a relation between the pineal gland and pregnancy can be established. Nevertheless, more studies are necessary to clearly establish the mechanisms involved in this relation.

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REFERENCES

- 1 Vollrath L, Schmidt DS. Enzymhистоchemische untersuchungen an der zirbeldrüse normaler und trächtiger meerschweinchen. [(Enzyme histochemical investigations on the pineal gland of normal and pregnant guinea pig) (In Germany with English abstract)]. *Histochemie* 1969; **20**:328–337.
- 2 Vollrath L, Huss H. The synaptic ribbons of the guinea-pig pineal gland under normal and experimental conditions. *Z Zellforsch* 1973; **139**:417–429
- 3 Karasek M, Lewinska I, Lewinski A, Hansen JT, Reiter RJ. Ultrastructure of rat pinealocytes during the last phase of pregnancy. *Cytobios* 1982; **33**(130):103–10.
- 4 Pevet P, Smith AR The pineal gland of the mole (*Talpa europaea* L.). II. Ultrastructural variations observed in the pinealocytes during different parts of the sexual cycle. *J Neural Transm* 1975; **36**:227–48.
- 5 Wyrzykowski Z, Przybylska B and Wyrzykowski K. Morphology of the pineal gland in pregnant and nursing sows. *J Pineal Res* 1987; **4**:125–134.
- 6 Satodate R, Sasou S, Ota M, Takashio M and Yoshida Y. An electron microscopical morphometrical study on pinealocytes of pregnant rats. *Endokrinologie* 1980; **75**(3):285–291.
- 7 Pang SF, Tang PL, Tang GWK, Yam AWC, Ng KW. Plasma levels of immunoreactive melatonin, estradiol, progesterone, follicle stimulating hormone, and β -human chorionic gonadotropin during pregnancy and shortly after parturition in humans. *J Pineal Res* 1987; **4**:21–31.
- 8 Tigchelaar PV, Nalbandov AV. The effect of the pineal gland on ovulation and pregnancy in rat. *Biol Reprod* 1975; **13**:461–469.
- 9 Nir I, Hirsschmann N, Goldhaber G, Shani J. Pinelectomy-induced changes in blood and pituitary luteinizing hormone and prolactin levels during the last phase of pregnancy in rats. *Neuroendocrinology* 1979; **28**:44–51
- 10 Nir I, Hirsschmann N. The effect of pinelectomy on serum progesterone and oestradiol levels during the last phase of pregnancy in rats. *Experientia* 1980; **36**:259–260.
- 11 Lew G.M. Morphological and biochemical changes in the pineal gland in pregnancy. *Life Sci*. 1987; **41**:2589–2596.
- 12 Redford KH, Eisenberg JP. Mammals of the Neotropics. The southern Cone. Volume 2 (Chile, Argentina, Uruguay, Paraguay). The University of Chicago Press. 1992.
- 13 Domínguez S, Piezzi RS, Scardapane L, Guzmán J. A light and electron microscopic study of the pineal gland of the viscacha (*Lagostomus maximus maximus*). *J Pineal Res*. 1987; **4**:211–219.
- 14 Cernuda-Cernuda R, Piezzi RS, Domínguez S, Alvarez-Uría M. Cell populations in the pineal gland of the viscacha (*Lagostomus maximus maximus*). Seasonal variations. *Histol Histopathol* 2003; **18**:827–836.
- 15 Pelzer L, Calderón C, Guzmán J. Changes in weight and hydroxyindole-O-methyltransferase activity of pineal gland of the plains viscacha (*Lagostomus maximus maximus*). *Mastozool Neotrop* 1999; **6**(1):31–38.
- 16 Fuentes L, Møller M, Muñoz E, Calderón C and Pelzer L. Seasonal variations in the expression of the mRNA encoding β_1 -adrenoceptor and AA-NAT enzyme, and the AA-NAT activity in the pineal gland of viscacha (*Lagostomus maximus maximus*). Correlation with serum melatonin. *Biol Rhy Res* 2003; **34** (2):193–206.
- 17 Jackson J.E. Reproductive parameters of the plains viscacha (*Lagostomus maximus*) in San Luis province, Argentina. *Vida Sylv Neotrop* 1989. **2**(1):57–62.
- 18 Weir BJ. The reproductive physiology of the plains viscacha, *Lagostomus maximus*. *J Reprod Fertil* 1971; **25**:355–363.
- 19 Llanos AC y Crespo JA. Ecología de la viscacha (*Lagostomus maximus maximus* Blainv.) en el Nordeste de la Provincia de Entre Ríos. [Ecology of viscacha (*Lagostomus maximus maximus* Blainv.) in the northeast of Entre Ríos province] (In Spanish)] *Revista de Investigaciones Agrícolas*. 1954. Extra Nueva Serie N° 10:5–95.
- 20 Branch LC, Villareal D, Fowler GS. Recruitment, dispersal, and group fusion in a declining population of the plains viscacha (*Lagostomus maximus*; Chinchillidae). *J Mammal* 1993; **74**:9–20.
- 21 Jackson J.E. Determinación de la edad en la viscacha (*Lagostomus maximus*) en base al peso del cristalino. [(Determination of the age in viscacha (*Lagostomus maximus*) by lens weight) (In Spanish with summary in English)] *Vida Silv. Neotrop*.1986; **1**(1):41–44.
- 22 Karnovsky MJ. A formaldehyde-glutaraldehyde fixate of high osmolarity for use in electron microscopy. *J Cell Biol* 1965; **27**:49A.
- 23 Millonig G. A modified producer for lead staining of thin sections. *J Biophys Biochem Cytol* 1961; **11**:736–739.
- 24 Di WL, Kadva A, Djahanbakhch O, Silman R. Radioimmunoassay of bound and free melatonin in plasma. *Clin Chem* 1998; **44**(2):304–310.
- 25 Fraser S, Cowen P, Franklin M, Franey C, Arendt J. Direct radioimmunoassay for melatonin in plasma. *Clin Chem* 1983; **29**(2):396–397.
- 26 Deguchi T, Axelrod J. Sensitive assay for serotonin N-Acetyltransferase activity in rat pineal gland. *Anal Biochem* 1972; **50**:174–179.
- 27 Axelrod J, Wurtman RJ, Snyder S. Control of Hydroxyindole-O-methyltransferase activity in the rat pineal gland by environmental lighting. *J Biol Chem* 1965; **240**:949–954.
- 28 Cagnacci A, Volpe A. Influence of melatonin and photoperiod on animal and human reproduction. *J Endocrinol Invest* 1996; **19**:382–411.
- 29 Arendt J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. *Rev Reprod* 1998; **3**:13–22.
- 30 Pévet P, Racey PA. The pineal gland of nocturnal mammals. II. The ultrastructure of the pineal gland in the pipistrelle bat (*Pipistrellus pipistrellus* L.): Presence of two populations of pinealocytes. *Cell Tissue Res* 1981; **216**:253–271.
- 31 Karasek M, Reiter RJ. Morphofunctional aspect of the mammalian pineal gland. *Microsc Res Tech* 1992; **21**:136–157.
- 32 Lues G. Die Feinstruktur der Zirbeldrüse normaler, trächtiger und experimentell beeinflusster Meerschweinchen. [(Fine structure of the pineal gland in normal, pregnancy and experimentally influenced guinea pig) (In Germany)] *Z. Zellforsch*. 1971; **114**(1):38–60
- 33 Kennaway DJ, Matthews CD, Seamark RF. Pineal function in pregnancy: Studies in sheep and man. In: *Pineal Function*. Matthews CD, Seamark RF (eds.) Elsevier Biomedical Press, Amsterdam, 1981; p. 123–136.
- 34 Bishnupuri KS, Haldar C. Profile of organ weights and plasma concentrations of melatonin, estradiol and progesterone during gestation and post-parturition in female Indian palm squirrel *Funambulus pennanti*. *Indian J Exp Biol* 2000; **38**:974–981.
- 35 Calderón CP, Muñoz EM, Pelzer LE. Effect of lithium on the rhythms of melatonin in the pineal gland, serum and retina of viscacha (*Lagostomus maximus maximus*). *Biol Rhy Res* 2001; **32** (2):179–189.
- 36 Simonneaux V, Ribelayga C. Generation of the melatonin endocrine message in mammals: A review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacol Rev* 2003; **55**(2):325–395.
- 37 Ribelayga C, Pévet P, Simonneaux V. HIOMT drives the photoperiodic changes in the amplitude of the melatonin peak of the Siberian hamster. *Am J Physiol* 2000; **278**:R1339–R1345.
- 38 Guzmán J, Piezzi RS, Pelzer L, Scardapane L, Domínguez S. Hydroxyindole-O-methyltransferase (HIOMT) and serotonin in the pineal gland of the viscacha (*Lagostomus maximus maximus*). *Acta Physiol Lat Am* 1977; **27**:31–36.
- 39 Fuentes L, Caravaca N, Pelzer L, Scardapane L, Piezzi RS and Guzmán J. Seasonal Variations in the testis and epididymis of viscacha (*Lagostomus maximus maximus*). *Biol Reprod* 1991; **45**:493–497.
- 40 Fuentes L, Calvo JC, Charreau EH, Guzmán JA. Seasonal variations in testicular LH, FSH and PRL receptors in vitro testosterone production; and serum testosterone concentration in adult male viscacha (*Lagostomus maximus maximus*). *Gen Comp Endocrinol* 1993; **90**:133–141.
- 41 Muñoz E, Fogal T, Domínguez S, Scardapane L, Guzmán J. A, Piezzi RS. Seasonal changes of the Leydig cells of viscacha (*Lagostomus maximus maximus*): a light and electron microscopy study. *Tiss Cell* 1997; **29**:119–128.
- 42 Muñoz E, Fogal T, Domínguez S, Scardapane L, Guzmán J. A, Piezzi RS. Stages of the cycle of the seminiferous epithelium of the viscacha (*Lagostomus maximus maximus*). *Anat Rec* 1998; **252**:8–16.
- 43 Bishnupuri KS, Haldar C. Impact of photoperiodic exposures during late gestation and lactation periods on the pineal and reproductive physiology of the Indian palm squirrel *Funambulus pennanti*. *J Reprod Fertil* 2000; **118**:295–301.
- 44 Mohamed F, Fogal T, Domínguez S, Scardapane L, Guzmán J and Piezzi RS. Colloid in the pituitary pars distalis of viscacha (*Lagostomus maximus maximus*): Ultrastructure and occurrence in relation to season, sex, and growth. *Anat Rec* 2000; **258**:252–261.