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Day-night changes in plasma melatonin levels, synaptophysin expression and ultrastructural properties of pinealocytes in developing female sheep under natural long and short photoperiods

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Summary. The aim of the present study was to analyze the 24-h rhythm in plasma melatonin concentration and the day-night differences in synaptophysin expresion and ultrastructural characteristics of the pinealocytes in developing female sheep. Ewes of three different ages were examined: infantile (1-6 months old), pubertal and early fertile age (9-24 months old) and adult (36-60 months old). Experiments were conducted under natural non-stimulatory (long) and stimulatory (short) photoperiods. The obtained results were similar for both analyzed photoperiods. Plasma melatonin concentration, measured in samples obtained every 4 h, showed a similar pattern in the three age groups, with peak values at 02:00 h and troughs at 14:00 h. Mean value of plasma melatonin levels in 9-24 month-old sheep was significantly greater than that in younger or older sheep. The weight of pineal glands obtained at night (02:00 h) was significantly higher than in daylight (14:00 h). Pubertal and early fertile sheep had the largest pineal glands. The pineal volume, and the total number of pinealocytes per gland of 9-24 months-old sheep differed significantly from that of younger or older sheep. The pineal volume, and the mean volume of pinealocytes was significantly greater in animals killed at night. Number of pinealocytes did not vary between animals killed during daylight or at night. The mean volumen of pinealocytes did not show statistical differences between the age groups. In quantitative ultrastructural analysis of pinealocyte cells, the relative volume of mitochondria, rough endoplasmic reticulum and Golgi complexes was significantly greater in 9-24 month-old sheep and in animals killed at night. The relative volume of lipid droplets was highest in older sheep. Collectively, the data support the existence of developmental changes in pinealocyte morphology and quantity, partially in coincidence with a higher melatonin secretion rate.

Key words: Sheep, Melatonin, Pinealocytes, Ultrastructure, Radioimmunoassay, Long and short photoperiods

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Introduction

The sheep is a mammalian specie from temperate latitudes whose breeding activity exhibits seasonal variations controlled by the annual photoperiodic cycle (Karsch et al., 1984; Lincoln and Richarson, 1998). Photoperiodic information is conveyed through several neural relays from the retina to the pineal gland where the light signal is translated into a daily cycle of melatonin secretion, with maximal values at night. The length of the nocturnal secretion of melatonin is a chemical code for the duration of the night (Bittman and Karschet al., 1984).

Nighttime melatonin rise mediates the ewe's reproductive responses to inhibitory (long), as well as stimulatory (short) photoperiods (Malpaux et al., 1997). Indeed the regulation by melatonin of hypothalamic-pituitary events in the ewe to advance seasonal estrous activity, with no undesirable effects upon fertility, and its induction of those seasonal responses associated with short days, support a place for melatonin in controlled-breeding programs in major sheep-producing countries (Kennaway et al., 1982).

Histological studies do not provide evidence for functional inactivity of the mammalian pineal gland in old age. In fact, the mammalian pineal gland seems to be active in old age and few age-related morphological changes have been described in this gland (Cernuda-Cernuda et al., 2000). Very few studies have been

published on the developmental changes of the pineal glands of sheep. In ovine embryos at different stages of development we reported that the growth of the pineal gland is biphasic, with an ontogenic-proliferative phase beginning at 30 days with an invasion of ependymal cells and the proliferation of the pinealoblasts, and a hypertrophic-differentiation phase including differentiation of pinealoblasts into pinealocytes (Redondo et al., 1996; Regodon et al., 1998). A primary cell type (pinealoblasts) was observed from 54 days until birth. A second cell population, classified as interstitial cells, appeared at 78 days gestation and persisted until birth (Redondo et al., 1996; Regodon et al., 1998).

The aim of the present study was to extend these observations to the postnatal development period of the sheep pineal gland. Animals were examined under natural non-stimulatory and stimulatory photoperiods and at three different ages: infantile (1-6 months old), pubertal and early fertile age (9-24 months old) and adult (36-60 months old). Twenty- four hours changes in plasma melatonin concentration and day-night differences in immunocytochemical, morphometrical and ultrastructural features of pinealocytes were recorded.

Materials and methods

Animals

Forty-eight female Merino ewes were divided into three age groups (n=8/group): infantile (1-6 months old), pubertal and early fertile age (9-24 months old) and adult (36-60 months old) for each analyzed photoperiods. Each group of animals was kept in an 18 square meter pen under natural illumination with free access to food and water. The study was conducted during June, for non-estimulatory (long) photoperiod, at this time of the year the photoperiod length was about 16 h (sunrise: 06:00 h; sunset: 21:55 h); and december, for estimulatory (short) photoperiod (sunrise: 08:00 h; sunset: 17:55 h), at the Faculty of Veterinary Sciences, University of Cáceres, Spain (latitude: 40° 25' North).

After 7 days of acclimatization to the research facilities, blood samples (3 ml) were obtained every 4 h starting at 06:00 h; samples at night were obtained under a 2 lux red light. Blood samples were collected with heparin as an anticoagulant, the plasma being separated by centrifugation at 4.500 g and stored at –20 ºC until further assay.

On the next day, groups of 3 animals were sacrificed at the middle of light or night periods, i.e., at 14:00 h or 02:00 h, respectively. Sheep were tranquilized by injecting propionyl phenothiazine (0.5 mg/100 kg b.w., given intramuscularly) and anesthesia was induced by the intravenous injection of sodium thiopental (4 g in 20% aqueous solution). The pineal gland was quickly taken out, weighed and fixed either in 10% buffered formol (immunocytochemistry) or in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) (ultrastructure).

Radioimmunoassay (RIA)

Plasma melatonin concentrations were measured by a direct RIA using a specific antiserum against melatonin raised in sheep (No. G/S/704-6483, Stockgrand Ltd, School of Biological Sciences, University of Surrey, Guilford, UK) and ^{3}H-melatonin as a radioligand (Fraser et al., 1983). All samples were measured in the same RIA with an intraassay coefficient of variation of 6%. Sensitivity of the assay was 10 pg/ml.

Immunocytochemical and morphometrical analysis

An ExtrAvidin Peroxidase Staining (EAS) was carried out on deparaffinized pineal samples taken from the distal, intermediate and proximal areas of each gland to detect the pinealocyte marker synaptophysin (Redecker et al., 1990). Tissue was deparaffinized, hydrated and treated sequentially with 15 % hydrogen peroxide for 30 min in order to block endogenous peroxidase activity. Non-specific tissue binding sites were blocked by incubation in 1% normal goat serum for 30 min. Samples were incubated with monoclonal anti/human synaptophysin (1:200 dilution) (Sigma/ Aldrich Química, Madrid, Spain, no. S5768) for 3 hours at 20 °C. Biotinylated goat anti-mouse IgG (1:200 dilution) (Sigma/Aldrich Quimica, Madrid, Spain no. B7151) was then added to the sections for 30 min. Finally sections were incubated with diluted (1:50) ExtrAvidin-Horseradish Peroxidase (Sigma/Aldrich Quimica, Madrid, Spain no. E2886) for 1 h. After diaminobenzidine reaction, a nuclear counterstaining with Mayer haematoxylin was applied.

The specificity of the staining reaction was determined in control experiments. They comprised prior absorption of the primary antibody, substitution of the primary antibody by PBS or normal mouse serum 1:100, or omission of both primary and secondary antibodies.

To measure the pineal volume, sections were cut at 1 μm and stained with EAS to detect the pinealocyte marker synaptophysin. The sections were taken with a separation of 100 μm until the entire pineal gland was sectioned. The area of the sections (S) was estimated by calculating the area of the profile drawn using the semiautomatic image analizing system (Vids IV). The total pineal volume was calculated by means of Cavalieri's direct estimator (Gundersen et al., 1988; Cernuda-Cernuda et al., 2000): $V_{pineal} = t \Sigma Si$; (t = 100 μm).

The total number of pinealocytes per gland was calculated by multiplying the numerical density of pinealocytes by the volume of the gland. The numerical density of pinealocytes (NV) was calculated according to the formula of López and Alvarez-Uria (1994) and Cernuda-Cernuda et al. (2000): NV = NA / (T+D-2h), in

which:

NA is the number of pinealocyte nuclei per unit area of sections. Five sections from each pineal gland, separated each other by a distance of roughly 50 mm, were used. Five fields, measuring 10,000 mm^2 each, were randommly selected per sections; a total of 100 fields per age group were evaluated.

T is the average thickness of the sections (1 mm).

D is the average of maximal nuclear diameter. 25 random pinealocyte nuclei per gland (100 nuclei per age groups) were measured.

h is the height of the smallest the of recognisable cap section, which was arbitrarily estimated to be 10% of the nuclear diameter.

The average volume per pinealocyte was estimated by dividing the absolute volume by the total number of pinealocytes.

Ultrastructural analysis

Pineal glands fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 12 h at room temperature, were post-fixed for 2 h in 1% osmium tetroxide in cacodylate buffer. Samples were dehydrated in graded ethanol solution (50, 70, 90 and 100%) and finally embedded in epoxy resin. Ultrathin sections were cut, stained with lead citrate and uranyl acetate, and examined under a Jeol Jem 100 S-X electron microscope.

Quantitative ultrastructural analysis was performed as described by Karasek and Zielinska (2000) and Lewczuk and Przybylska-Gornowicz (2000). Ten pictures (x10,000) from distal, intermediate and proximal areas of each pineal gland were analyzed using a systematic random sampling method. A digital semiautomatic analyzer (Vids IV, Leica Spain) was used to obtain the morphometric data. Relative volume (as percent of cytoplasm) of mitochondria, rough endoplasmic reticulum, Golgi complex, lysosomes and lipid droplets were computed using point count analysis (Weibel, 1979). For estimation of relative volumen of cell organelles the pictures were enlarged photographically to 15,000.

Statistical analysis

Results were statistically analyzed by using a one-way analysis of variance (ANOVA), a factorial ANOVA or unpaired Student's t test, as stated.

Results

Table 1 shows the 24-hour cycle of plasma melatonin concentration in infantile (1-6 months), pubertal and early fertile age (9-24 months) and adult (36-60 months) female sheep kept under non-stimulatory and stimulatory natural photoperiods. The general pattern was the same in the three groups of sheep, with peak values at 02:00 h and at 14:00 h for each analyzed photoperiod. A factorial ANOVA indicated that the mean value of melatonin levels found in 9-24 month-old sheep was significantly greater than that in younger or older sheep ($F=14.68$, Tukey test: $p<0.0001$) and ($F=15.45$, Tukey test: $p<0.0002$) for non-stimulatory and stimulatory photoperiods, respectively.

Immunocytochemical findings in pineal glands of the three groups of sheep studied are summarized in Figs. 1-6. Immunolabeling of the pinealocyte marker synaptophysin was similar for long and short photoperiods. The immunoreaction was intense in all groups of sheep examined. Morphology of synaptophysin-positive cells was similar that of pinealocytes. The perivascular space exhibited a very heavy immunolabeling, with no positive staining being observed in blood vessels.

Table 2 summarizes data on the weight of the gland,

Table 1. Plasma melatonin concentrations (pg / ml) of female sheep of different ages kept under natural non-stimulatory and stimulatory photoperiods.

	STIMULATORY PHOTOPERIOD						
	Light Period				Dark Period		
Clocktime (h)	06:00	10: 00	14: 00	18: 00	22: 00	2: 00	06: 00
1-6 months	180±7	64±9[a]	46±6[a]	68± 9	124±12[a]	192±12[a]	182±7
	80±6	66±8[a]	44±7[a]	76±8	104±9[a]	194±9[a]	86±7[a]
9-24 months	191±10[b]	75±10[a,b]	56±9[a,b]	81±8[b]	138±14[a,b]	215±1[5a,b]	193±8[b]
	90±9[b]	77±8[a,b]	53±9[a,b]	87±9 [b]	112±11[a,b]	213±12[a,b]	94±10[b]
36-60 months	179± 8	67±8[a]	45± 5[a]	72±8	121±13[a]	189±12[a]	183±8
	80± 5	69±8[a]	43± 5[a]	77± 7	101± 8[a]	91±15[a]	85±9
Clocktime (h)	06:00	10: 00	14: 00	18: 00	22: 00	2: 00	06: 00
	Light Period				Dark Period		
	NON STIMULATORY PHOTOPERIOD						

Radioimmunoassay was made as described in Methods. Shown are the means ± SEM (n= 24 in each hours). [a]: p<005 vs 6:00, in the corresponding groups. [b]: p<005 vs groups I and III in the corresponding hours.

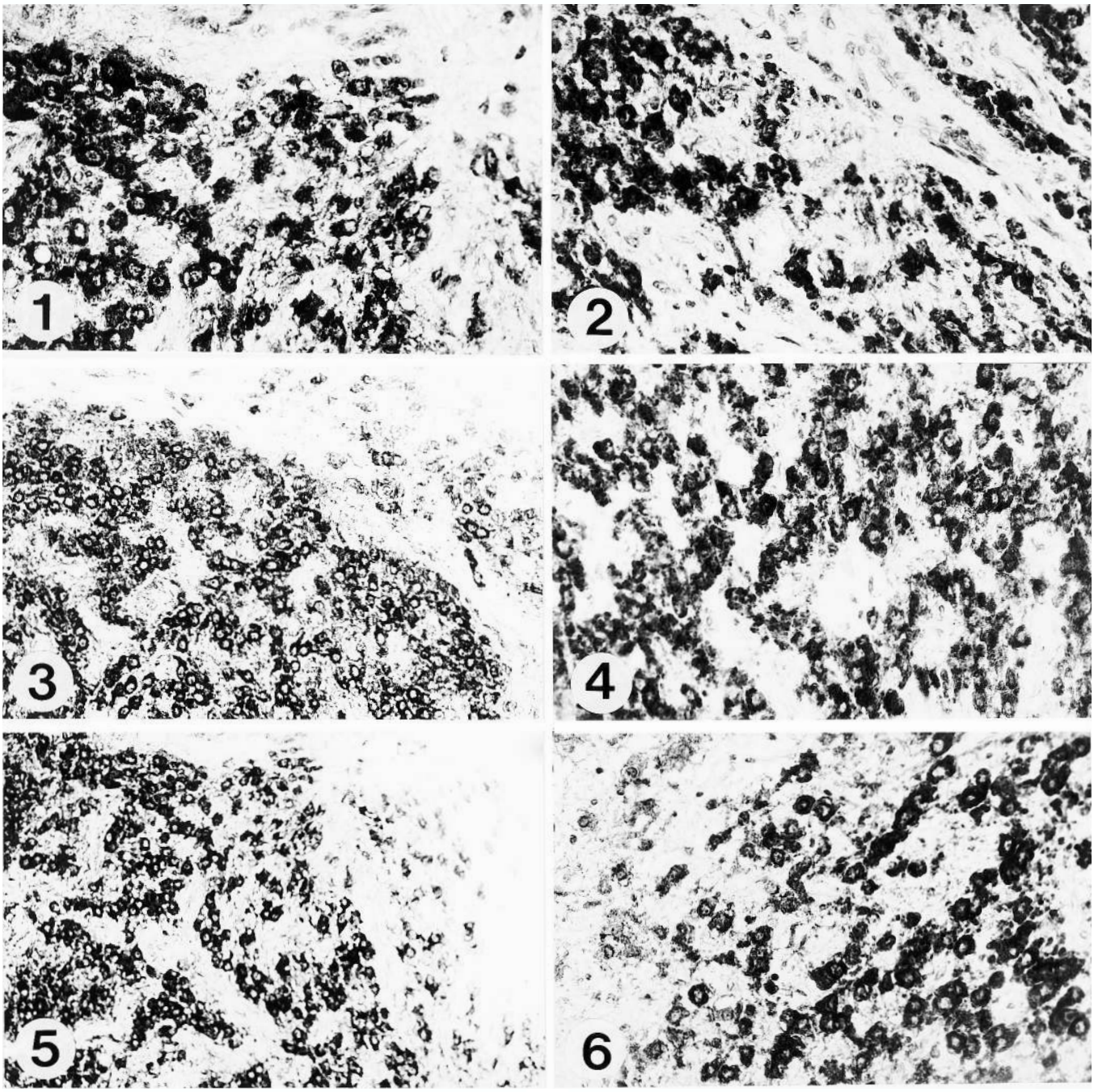


Fig. 1. Pineal gland of a 1-month-old female sheep, killed during daylight (14:00 h) under non stimulatory photoperiod. Some SYNP-positive cells are seen in the cortical region. EAS. x 350

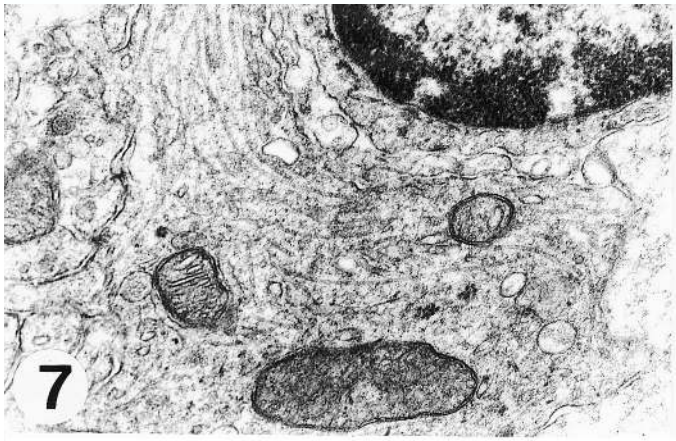
Fig. 2. Pineal gland of a 9-month-old female sheep, killed at night (02:00 h) under non stimulatory photoperiod. Some SYNP-positive cells are seen in the medular region. EAS. x 350

Fig. 3. Pineal gland of a 24-month-old female sheep, killed during daylight (14:00 h) under non stimulatory photoperiod. Numerous SYNP-positive cells are seen in the cortical region. EAS. x 250

Fig. 4. Pineal gland of a 24-month-old female sheep, killed at night (02:00 h) under stimulatory photoperiod. Numerous SYNP-positive cells are seen in the medullar region. EAS. x 350

Fig. 5. Pineal gland of a 36-month-old female sheep, killed during daylight (14:00 h) under stimulatory photoperiod. Some SYNP-positive cells are seen in the cortical region. EAS. x 350

Fig. 6. Pineal gland of a 60-month-old female sheep, killed at night (02:00 h) under stimulatory photoperiod. Some SYNP-positive cells occur in the medullar region. EAS. x 350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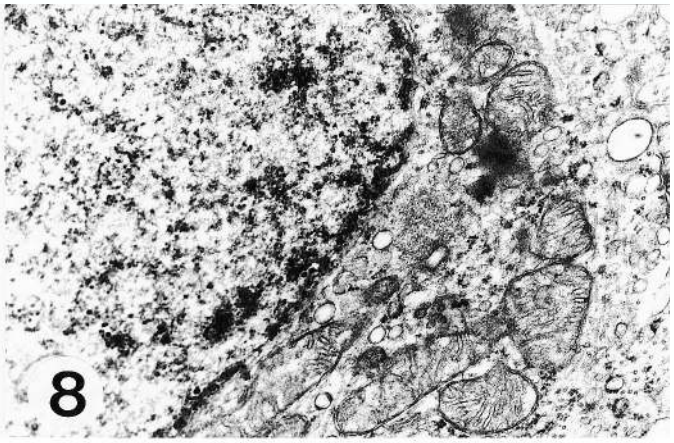
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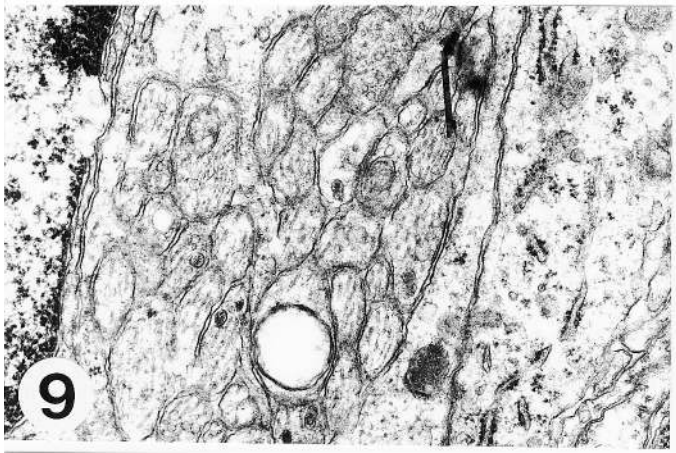
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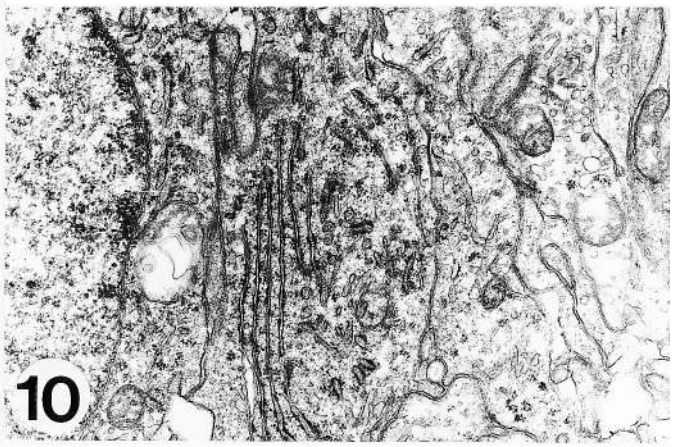
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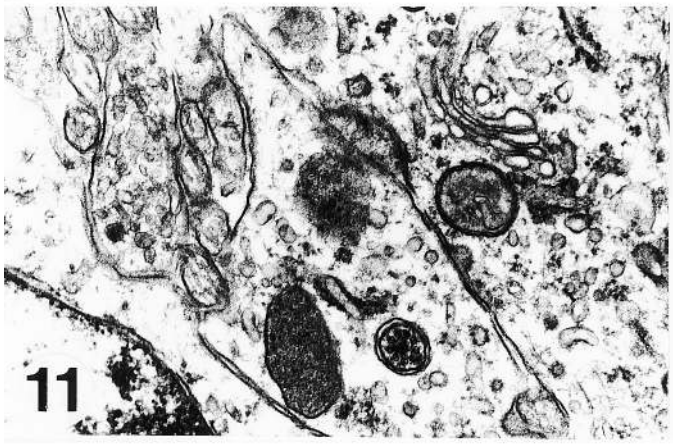
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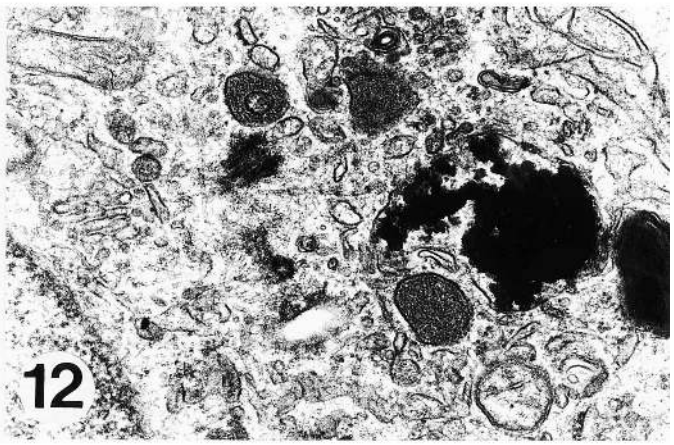
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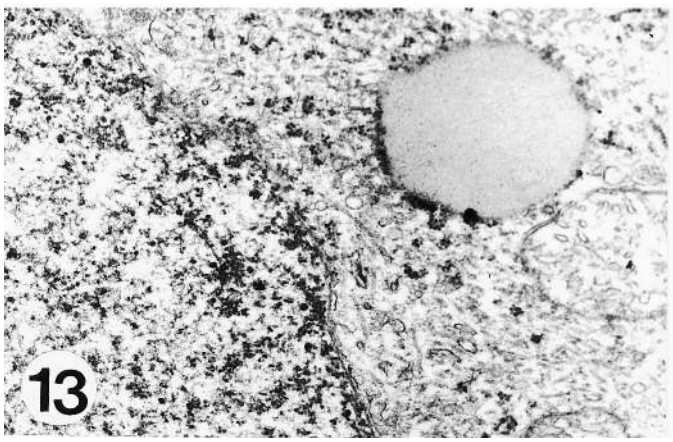
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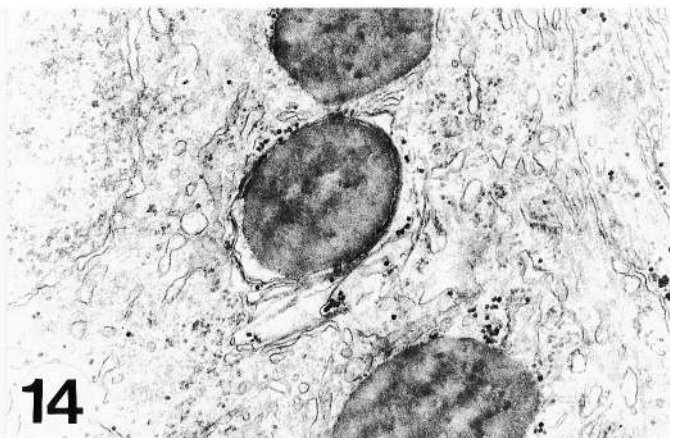
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Figs. 7 and 8. Cytoplasm of pinealocytes in a 1-month-old sheep killed during daylight or at night, respectively, under non-stimulatory photoperiod. Mitochondria. x 15000

Figs. 9 and 10. Cytoplasm of pinealocytes in a 1-month-old sheep killed during daylight or at night, respectively, under non-stimulatory photoperiod. Rough endoplasmic reticulum. x 15000

Figs. 11 and 12. Cytoplasm of pinealocytes in a 24-month-old sheep killed during daylight, or at night respectively, under non-stimulatory photoperiod. Golgi Complexes. x 15000

Figs. 13 and 14. Cytoplasm of pinealocytes in 24-month-old and 60-month-old sheep killed during daylight respectively, under non-stimulatory photoperiod. Lipid droplets. x 15000

pineal volume, total number of pinealocytes per gland and average volume per pinealocyte (x100 mm^3) of female sheep of different ages kept under natural non-stimulatory and stimulatory photoperiods and killed during daylight (14:00 h) or at night (02:00 h). As indicated by main factor analysis in a factorial ANOVA, 9-24 month-old sheep had larger pineals than those of younger or older sheep ($F=12.5$, $p<0.0003$) and ($F=14.6$, $p<0.0002$) for long and short photoperiods, respectively. Analyzed as main factor, pineal glands were heavier at night than day ($F=9.5$, $p<0.001$, and $F=8.4$, $p<0.002$). The volume of pineal glands of animals sacrificed at 2:00 h was significantly greater than that of sheep killed at 14:00 ($F=7.9$, $p<0.003$) and ($F=8.7$, $p<0.003$) for non-stimulatory and stimulatory photoperiods, respectively. On the other hand, the pineal volume of 9-24 month-old sheep was significantly higher when compared to younger or older animals ($F=15.7$, $p<0.0002$ and $F=16.2$, $p<0.0003$). Pineal glands of 9-24 month-old sheep showed more pinealocytes than younger and older sheep ($F=15.5$, $p<0.0002$ and $F=13.9$, $p<0.0004$). Number of pinealocytes did not vary between animals killed during daylight or at night (Table 2). The mean volumes of pinealocytes were generally larger in sheep killed at night as compared to daylight ($F=6.8$, $p<0.002$ and $F=7.6$, $p<0.003$ for long and short photoperiods) with no

Table 2. Pineal weight (mg), pineal volume (x10^6 mm^3), total number of pinealocytes per gland (x10^3) and average volume per pinealocyte (x10^2 mm^3) of female sheep of different ages kept under natural non-stimulatory and stimulatory photoperiods and killed during daylight (14:00 h) or at night (02:00 h).

	1-6 MOTHS OLD SHEEP		9-24 MOTHS OLD SHEEP		36-60 MOTHS OLD SHEEP	
	14:00	2:00	14:00	2:00	14:00	2:00
Pineal weight (mg)						
Non Stimul. Photop.	48±2[a,b]	55±3[a]	62±4[b]	72±4	50±3[a,b]	56±2[a]
Stimul. Photop.	50±3[a,b]	57±3[a]	64±5[b]	75±5	51±4[a,b]	59±2[a]
Pineal Volume (x10^6 mm^3)						
Non Stimul. Photop.	627±16[a,b]	873±18[a]	891 ±12[b]	1252 ±2 3	663±14[a,b]	894±11[a]
Stimul. Photop.	635±12[a,b]	892±168[a]	905 ±11[b]	1288 ±25	643±12[a,b]	875±10[a]
Total number of pinealocytes/gland						
Non Stimul. Photop.	352791±3912[a]	355643± 2913[a]	419285±4915	423211±5212	359184± 4823[a]	360664±4515[a]
Stimul. Photop.	360834±3537[a]	374556± 2817[a]	432192±4017	455232±5113	348591± 4124[a]	351606±4489[a]
Average volume per pinealocyte (mm^3)						
Non Stimul. Photop.	1483± 47[b]	1985± 49	1512± 36[b]	2014± 41	1520± 35[b]	1981± 38
Stimul. Photop.	1448± 37[b]	2003± 38	1546± 29[b]	2024± 31	1530± 31[b]	2001± 29

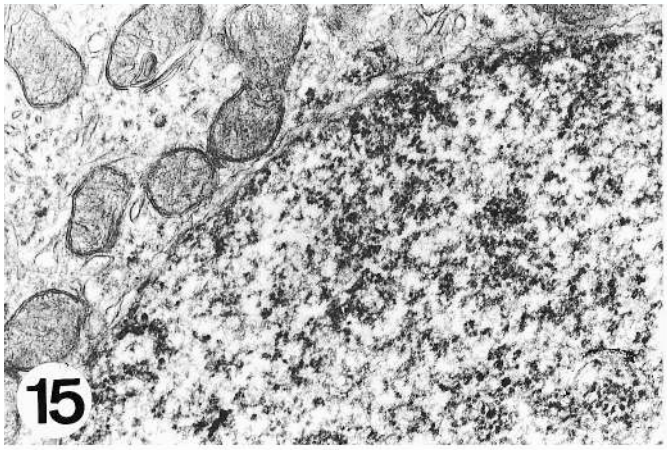
Values represent the means±SEM (n= 4 sheep/group for each photoperiod). [a]: $p<0.05$ vs group II in the corresponding light or dark cycles. [b]: $p<0.05$ vs dark in the corresponding groups. [c]: $p<0.05$ vs group III in the corresponding light or dark cycles.

Figs. 15 and 16. Cytoplasm of pinealocytes in a 1-month-old sheep killed during daylight or at night, respectively, under stimulatory photoperiod. Mitochondria. x 15000

Figs. 17 and 18. Cytoplasm of pinealocytes in a 1-month-old sheep killed during daylight or at night, respectively, under stimulatory photoperiod. Rough endoplasmic reticulum. x 15000

Figs. 19 and 20. Cytoplasm of pinealocytes in a 24-month-old sheep killed during daylight, or at night respectively, under non-stimulatory photoperiod. Golgi Complexes. x 15000

Figs. 21 and 22. Cytoplasm of pinealocytes in 24-month-old and 60-month-old sheep killed during daylight respectively, under non-stimulatory photoperiod. Lipid droplets. x 15000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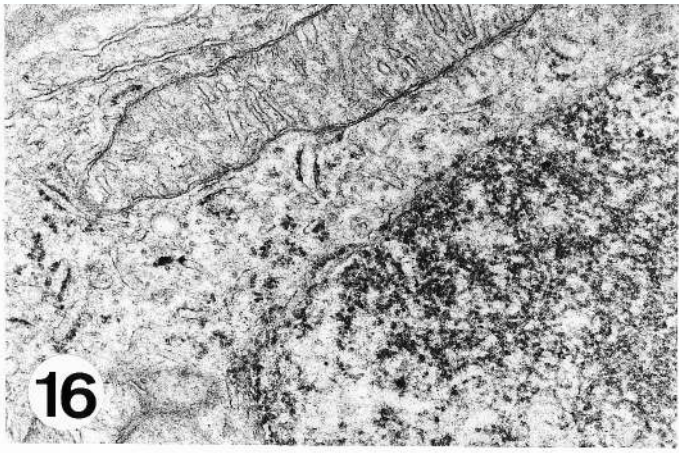
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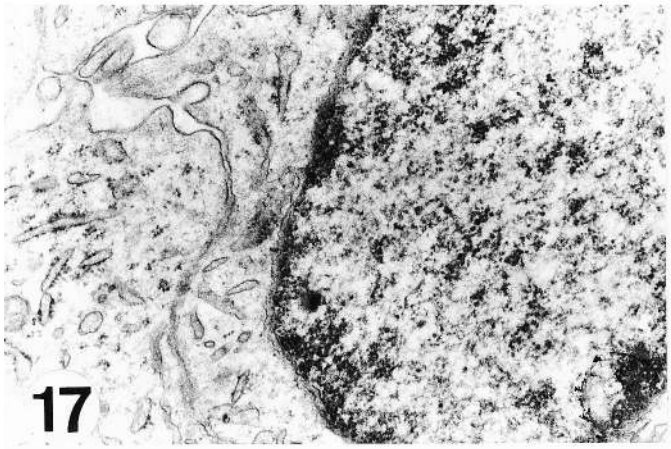
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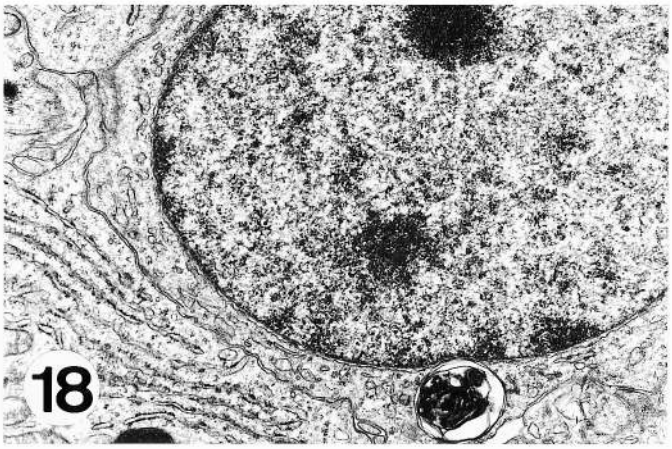
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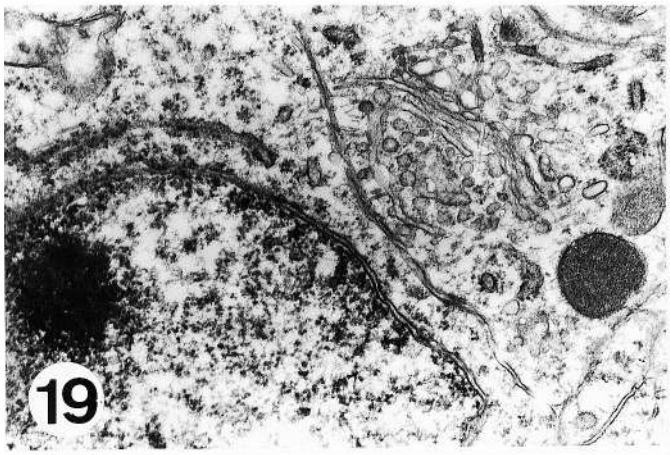
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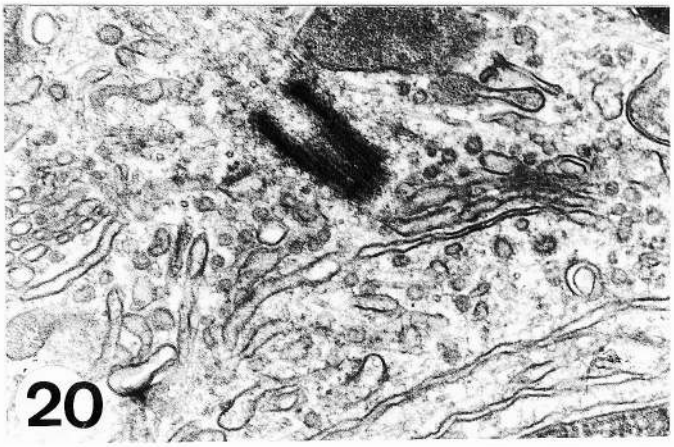
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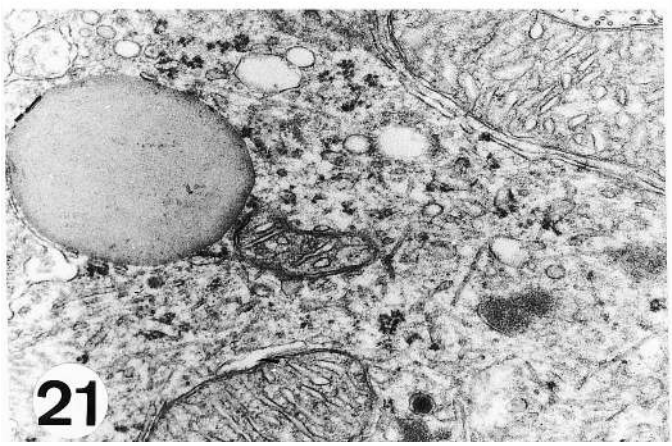
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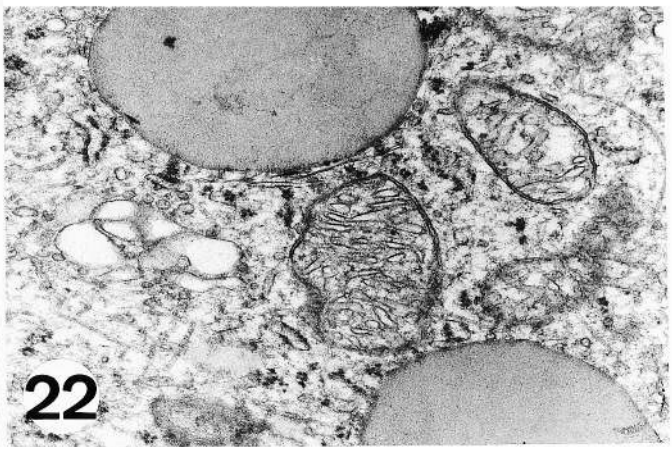
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differences among the three age groups.

Figures 7-14 and 15-22 depict ultrastructure of female sheep pineal during non stimulatory and stimulatory photoperiods, respectively. The pinealocytes showed similar characteristics in the three age groups, and in animals killed at light or night periods under natural non-stimulatory and stimulatory photoperiods. They had ovoid or elongated nuclei in which small granule clumps of chromatin were visible within a moderate dense nucleoplasm. Nucleoli, which were also ovoid in shape, presented a diffuse nucleolonema surrounding a fibrillo-granular core. Perinuclear Golgi complexes exhibited either cisternae with associated vesicles or small, elongated and moderately dilated saccules. Diplosomes were observed in the vicinity of Golgi complexes. Larger electron-dense mitochondria and rough endoplasmic reticulum with moderated saccule dilation and abundant ribosomes were scattered throughout the cytoplasm. Pinealocyte processes had bulbous endings containing numerous microtubules and electron-lucent vesicles that outnumbered dense core vesicles, together with gap junctions.

Quantitatively, a number of differences among pinealocytes of the three age groups became apparent (Table 3). Mitochondrial, rough endoplasmic reticulum and Golgi complex relative volume was significantly higher in 9-24 month-old sheep when compared to younger or older animals ($F=16.8$, $p<0.0001$ and $F=17.5$, $p<0.0002$) . Within each age group relative volumes of these organelles were greater in animals killed at night ($F=12.8$, $p<0.0002$ and $F=13.6$, $p<0.0001$) . Lysosomal relative volume did not differ among groups while that of lipid droplets was higher in elder sheep ($F=16.4$, $p<0.0002$ and $F=16.8$, $p<0.0001$ for long and short photoperiods) (Table 3).

Discussion

The above mentioned results demonstrate biochemical, cytochemical and ultrastructural changes in pineal glands of ewes from the infantile period to adulthood. The experiment was conducted under natural non-stimulatory and stimulatory photoperiods to minimize and maximize any difference that could derive from the estrous cycle in fertile sheep. Plasma melatonin concentration showed a similar pattern in the three age groups examined, i.e., infantile (1-6 months old), pubertal and early fertile age (9-24 months old) and adult (36-60 months old), with a maximum at 02:00 h and a nadir at 14:00 h. These results essentially agreed with a number of observations in literature (Rollag and Niswender, 1976; McMillen and Nowak, 1991; Arendt, 1995; Picazo and Lincoln, 1995; Chemineau et al., 1996; Ravault and Chesneau, 1999; Martínez-Soriano et al., 2002a).

As repeatedly reported (Chemineau et al., 1996; Zarazaga et al., 1998; Santiago-Moreno et al., 2000) interindividual differences in the amplitude and duration of melatonin rhythm in sheep are relatively high as compared to individual variability. This variability has a strong genetic basis and was attributed to variations in pineal weight rather than to variations in pineal enzyme activities (Arendt, 1995; Coon et al., 1999). The Merino ewes studied in our work derived from a relatively homogeneous genetic lineage with 50% of genetic homology at least, this probably explains the small variation found in circulating melatonin levels. In any event, the increase in melatonin at night correlated with

Table 3. Relative volume (% of cytoplasm) of mitochondria, rough endoplasmic reticulum, Golgi complex, lysosomes and lipid droplets in pinealocytes of female sheep killed during daylight or at night under natural non-stimulatory and stimulatory photoperiods.

RELATIVE VOLUME (%)	1-6 MONTHS OLD SHEEP		9-24 MONTHS OLD SHEEP		36-60 MONTHS OLD SHEEP	
	14:00	2:00	14:00	2:00	14:00	2:00
Mitochondria						
Non Estimul. Photop.	6±2^{a,b}	12±3^{a}	18±4^{b}	25±4	7±3^{a,b}	13±2^{a}
Estimul. Photop.	5±3^{a,b}	13±3^{a}	20±5^{b}	27±3	8±3^{a,b}	14±3^{a}
Rough endoplasmic reticulum						
Non Estimul. Photop.	4±1^{a,b}	7±1.5^{a}	10±2^{b}	15±3	3±1^{a,b}	7±1.5^{a}
Estimul. Photop.	5±1.5^{a,b}	9±2.5^{a}	12±3^{b}	16±2	4±1^{a,b}	7±1^{a}
Golgi Complex						
Non Estimul. Photop.	3±1.2^{a,b}	6.5±1.1^{a}	8±1.5^{b}	11±1.5	3.5±1.2^{a,b}	6±1.5^{a}
Estimul. Photop.	4±1.5^{a,b}	7.5±1^{a}	9±1.5^{b}	13±2.5	4.5±1.5^{a,b}	7±1^{a}
Lysosomes						
Non Estimul. Photop.	1.2± 0.2	1.1± 0.3	1.4± 0.3	1.1± 0.2	1.3± 0.1	1.4± 0.2
Estimul. Photop.	1.5± 0.3	1.4± 0.3	1.3± 0.2	1.3± 0.2	1.2± 0.2	1.3± 0.1
Lipid Droplets						
Non Estimul. Photop.	6.5±1.2^{a,c}	7±1.1^{c}	7.1±1.2^{c}	6.8±0.9^{c}	12 ±1.5	13± 1.1
Estimul. Photop.	7.5±1.5^{a,c}	8±1 c	8±1 c	7.5±1.5^{c}	13 ±1.5	15± 1

Quantitative ultrastructural analysis was made as described in Methods. Shown are the means±SEM (n= 80 in each group). ^{a}: $p<0.05$ vs group II in the corresponding light or dark cycles. ^{b}: $p<0.05$ vs dark in the corresponding groups. ^{c}: $p<0.05$ vs group III in the corresponding light or dark cycles.

greater pineal weights (Coon et al., 1999).

The mean value of plasma melatonin levels found in pubertal and early fertile ewes was significantly higher than that found in younger or older animals. This presumably indicated a higher melatonin secretion at this age, since pinealectomy suppressed day-night differences and depressed circulating melatonin levels in sheep (McMillen and Nowak, 1989). As reported in many species including man (Arendt, 1995; Cornelissen et al., 2000), blood melatonin levels decline with age.

Plasma melatonin levels correlated with some morphometric parameters in the pineal of sheep killed at times of maximal and minimal melatonin levels (i.e., 02:00 h and 14:00 h). These include an increased pineal weight and significantly larger the mean volume of pinealocytes as well as an increased relative volume of mitochondria, rough endoplasmic reticulum and Golgi complexes in pineal cells of sheep killed at night. These ultrastructural findings agreed with those reported in sheep exposed to natural short photoperiods (Lewczuk et al., 1993; Karasek and Zielinska, 2000) and artificial long photoperiods (Lewczuk et al., 1993). In contrast, larger mitochondria in pinealocytes were observed in pigs during daylight as compared to nighttime (Lewczuk and Przybylska-Gornowicz, 2000).

The raised mean plasma melatonin levels found in pubertal and early fertile ewes coincided with larger pineal glands and a higher number of pinealocytes per gland, as well as with an increase in relative volume of mitochondria, rough endoplasmic reticulum and Golgi complexes in pinealocytes of ewes of the same age. The finding that the number of synaptophysin-positive cells was higher in 9-24 month-old sheep than in 1-6 month-old sheep indicated an increase in the number of pinealocytes from birth to puberty. This finding, not reported before in sheep pineal gland, was similar to that which was reported in rats (Feng et al., 1998) and in CBA mice (Cernuda-Cernuda et al., 2000). Distribution and localization of synaptophysin-positive cells were homogeneous in this study, thus not supporting the existence of the regional morphological and biochemical differences found in the rat and bovine pineal gland (Chuluyan et al., 1990; Sato et al., 1994; Hira et al., 1998; Martínez-Soriano et al., 2002b).

A last aspect of the present results deserves comment. The relative volume of lipid droplets in pinealocytes (presumably an index of pineal aging process) was highest in older sheep (Karasek and Zielinska, 2000). Lysosomes showed no changes in relation to environmental light conditions, a findings also described in pigs pinealocytes (Lewczuk and Przybylska-Gornowicz, 2000).

Collectively, the results support the existence of developmental morphological changes in sheep pineal gland, partially in coincidence with a greater melatonin secretion rate. Our results suggest that pineal melatonin might play an important role in the increase of pinealocyte activity.

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