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Postnatal development of female sheep pineal gland under natural inhibitory photoperiods: an immunocytochemical and physiological (melatonin concentration) study

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Summary. The purpose of this study was to determine structural and immunocytochemical changes taking place during the day and at night in developing sheep pineal gland under natural non-stimulatory photoperiods (summer solstice). Additionally, the diurnal cycle of plasma melatonin levels was charted and differences between diurnal and nocturnal pineal melatonin concentrations were analyzed. 36 ewes of three different ages were examined: infants (1-6 months old), pubertal and early fertile age (9-24 months old) and adults (36-60 months old). Plasma and pineal gland melatonin levels were higher in pubertal sheep than in infants or adults. Pubertal sheep pineal glands were also heavier, contained a larger number of pinealocytes and interstitial cells and displayed more evident innervation and vascularisation than infants or adults. There was no difference in the number of pinealocytes and interstitial cells between animals killed during daylight or at night. Gland weight, pinealocyte nuclear profile areas and plasma melatonin concentrations were all significantly higher at night than during the day.

Key words: Pineal gland, Sheep, Melatonin, Postnatal development, Long photoperiod

Introduction

The timing of reproductive activity in seasonal breeding sheep relies on daily photoperiodic signals being relayed to provide information on the time of year (Lincoln et al., 1998; Guerin et al., 2000). 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 pineal gland, through its nocturnal melatonin secretions, mediates the effects of inhibitory (long) and stimulatory (short) photoperiod on reproduction in female sheep. The length of the nocturnal secretion of melatonin is a chemical code for the duration of the night (Bittman et al., 1984).

Although the literature contains numerous descriptions of sheep pineal gland morphology (Redondo et al., 1996, 2001; Franco et al., 1997; Regodón et al., 1998a,b, 2001), few studies address the influence of natural lighting conditions on sheep pineal gland structure (Lewczuk et al., 1993). No analyses have been performed of plasma and pineal gland melatonin content in developing sheep.

The aims of the present experiment were: 1) to describe qualitative and quantitative changes taking place in sheep pineal gland structure during postnatal development; 2) to measure plasma and pineal gland melatonin levels in developing sheep.

Materials and methods

Animals

36 female Merino ewes were divided into three age groups (n= 12/group): infants (1-6 months old), pubertal and early fertile age (9-24 months old) and adults (36-60 months old). Each group was kept in an 18-square-meter pen under natural lighting with free access to food and water. The study was conducted in the month of June at the Faculty of Veterinary Sciences, University of Cáceres, Spain (latitude: 40\25' North). At this time of the year, photoperiod length was about 16 h (sunrise: 06:0 h; sunset: 22:55 h).

After 7 days of acclimatization to the research facilities, blood samples (3 ml) were obtained every 4 h starting at 0600 h (06:00, 10:00, 14:00, 18:00, 22:00, 02:00, 06:00); samples were obtained at night under a 2-lux red light. Blood samples were collected with heparin

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as anticoagulant, the plasma being separated by centrifugation at 4500 g and being stored at – 20 °C until further use.

On the following day, animals from all 3 groups (n=12 in each age group; n=6 for light and dark period respectively) were slaughtered at the middle of daylight or night periods, i.e., at 14:00 h or 2:00 h, respectively. Sheep were tranquilized by intramuscular injection of propionyl phenothiazine (0.5 mg/100 kg b.w.) and anesthesia was induced by intravenous injection of sodium thiopental (4 g in 20% aqueous solution) . The pineal gland was immediately removed and weighed.

Radioimmunoassay (RIA)

Plasma melatonin concentrations were measured by 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, Guildford, UK) and ^{3}H-melatonin as 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.

High-performance liquid chromatography(HPLC)

Pineals were removed and homogenized by sonication (100 W 6 sec) in 250 ml of ice-cold 0.1 M perchloric acid. After centrifuging at 6000 g for 6 min, supernatants were frozen and stored at -80 °C until assay. Immediately before assay, samples were thawed at room temperature, and 25 ml aliquots were injected into the HPLC system with electrochemical detection (Coulochem, 5100A, ESA; USA). The system was run at a flow rate of 1.2 ml/min, at 2300 psi . The mobile phase (pH 3.4) was 0.1 M phosphate-potassium buffer containing 50 mM citric acid, 50 mM sodium acetate, and 0.05 mM EDTA. Melatonin was separated in a Spherisorb ODS II reversed phase column (100x4.6; 3 mm) and oxidized at a potential of 0.9V versus a platinic (Ag/AgCL) reference electrode. The minimum detectable sensitivity with signal-noise ratio set was 15 pg. Intra- and interassay coefficients of variance were 5% and 6%.

The protein assay was measured following the method of Lowry et al. (1951).

Immunocytochemical and morphometrical analysis

ExtrAvidin Peroxidase Staining (EAS) was performed on deparaffinized pineal samples taken from the distal, intermediate and proximal areas of each gland to detect the pinealocyte marker [non neuron enolase (NNE), (Sato et al., 1995)]; glial cells markers [glial fibrillary acidic protein (GFAP) and vimentin (VIM), (Redondo et al., 2001b)] and markers of peptidergic innervation [neuropeptyde Y (NPY) and vasointestinal peptide (VIP) (Cozzi et al., 1994; Mikkelsen and Moller, 1999)].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 the following primary antisera: 1:200 monoclonal anti/human NNE (Sigma/Aldrich Química, Madrid, Spain, no. S5768); 1:400 monoclonal anti-human GFAP (Sigma/Aldrich Química, Madrid, Spain, no. G-3893); 1:20 monoclonal anti-human VIM (Sigma/Aldrich Química, Madrid, Spain, no. V-5255); 1:200 monoclonal anti-human NPY (Sigma/Aldrich Química, Madrid, Spain, no. N9528); and 1:20 monoclonal anti-human VIP (Sigma/Aldrich Química, Madrid, Spain, no. V3508) 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. Sections were finally incubated with diluted (1:50) ExtrAvidin-Horseradish Peroxidase (Sigma/Aldrich Quimica, Madrid, Spain no. E2886) for 1h. After diaminobenzidine reaction, nuclear counterstaining with Mayer hematoxylin was applied.

The specificity of the staining reaction was determined in control experiments. These 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.

For morphometrical analysis, samples were taken from the following pineal areas: proximal, intermediate and distal (close to the recessus pinealis), and from the dorso- and ventroperipheral and dorso- and ventrocentral pineal aspects. The dorsoperipheral and ventroperipheral areas corresponded to the glandular cortex, and the dorsocentral and ventrocentral regions to the medulla (Regodon et al., 1998a). Two sections from each portion, separated from each other by a distance of roughly 50 mm, were used. Two fields, measuring 10000 mm^2 each, were randomly selected per section. Nuclear density of NNE-, GFAP-, and VIM-positive cells (an index of number of pinealocytes and interstitial cells, respectively) was expressed as the number of nuclei/ 10000 μm^2 (Sakai et al., 1996; Regodon et al., 1998a). The nuclear profile area was determined in 100 nuclei of NNE-positive cells selected from fields and sections.

Structural analysis

Pineal glands were fixed by immersion in Bouin's solution and embedded in paraffin. Seven- micron sections were stained with H-E, phosphotungstic acid hematoxylin (PTAH), Masson's Trichrome (MT), Gomori's silver for Reticulin Fibers (GR), Masson Fontana (MF) for identification of melanin, and Von Kossa (VK) for detection of calcium.

Results

Table 1 shows the 24-hour cycle of plasma

melatonin levels, pineal protein content and pineal melatonin concentrations in infant (1-6 months old), pubertal and early fertile age (9-24 months old) and adult (36-60 months old) female sheep slaughtered by day and by night under natural light and dark cycles in summer. Plasma melatonin levels displayed the same diurnal pattern in the three age-groups, with peak values at 02:00 h and at 14:00 h. Factorial ANOVA showed that mean melatonin levels in pubertal sheep were significantly higher than those of infants and adult sheep ($F=17.25$, Tukey test: $P< 0.0003$).

ANOVA also indicated a significant increase in pineal protein content in pubertal animals ($F=16.8$, $P<0.0001$) with respect to adults and infants, with no significant difference between the latter groups. In all three age-groups, protein content was significantly higher at night than during the day ($F=15.8$, $P<0.0001$). Pineal melatonin levels rose significantly in pubertal sheep ($F= 17.6$, $P<0.0003$) compared to infants and adults. No significant differences in pineal melatonin content were recorded between infants and adults. In all groups, melatonin concentrations were significantly higher by night than by day ($F=18.8$, $P<0.0002$).

Table 2 summarizes data on gland weight; density of NNE-, GFAP-, and VIM-positive cells; and nuclear profile areas of NNE-positive cells for the three age-groups, killed by day and by night under natural inhibitory photoperiods in summer.

As indicated by main factor analysis in a factorial ANOVA, pubertal sheep displayed larger pineals than infants or adult sheep ($F=15.7$, $P<0.0002$). Analyzed as main factor, pineal glands weighed more at night than during daylight ($F=9.5$, $P<0.002$).

Pubertal pineal glands contained more pinealocytes and interstitial cells than infant or adult glands, as indicated by an increased number of NNE-positive cells ($F=16.5$, $P<0.0001$), GFAP-positive cells ($F=14.5$, $P<0.0002$), and VIM positive cells ($F=13.4$, $P<0.0002$).There was no difference in the number of pinealocytes and interstitial cells between animals killed during daylight or at night. In all age-groups, pinealocyte and interstitial cell densities were greater in the medulla than in the cortex ($F= 8.4$, $P<0.002$ and $F=9.4$, $P<0.001$, respectively).

Immunocytochemical findings in the pineal glands of the three age-groups were similar in daylight-slaughtered and night-slaughtered sheep. Immunocytochemical tests are summarized in Figs. 1-8. Cells staining positively to NNE displayed the morphological characteristics of pinealocytes. No intercellular differences were observed in terms of the degree of immunostaining between cells in the same age-group. Intercellular areas, which were full of pinealocyte processes, also stained positively. Interstitial cells adjacent to positively-staining cells were negative; vascularized tissue stroma was also negative.

GFAP+ cells were observed in the pineal parenchyma in infants, distributed uniformly throughout the gland, mainly in perivascular locations. These cells displayed small, dense, ovoid nuclei, and an intensely-staining rim of cytoplasm bordering negative nuclei together with a small number of processes, of varying diameters, arranged longitudinally and transversally amongst pinealocytes. GFAP reactivity increased in the pubertal group, where clusters of GFAP+ cells were frequently observed in the vicinity of blood vessels. In the adult group, GFAP+ somata and cell processes were clearly less numerous than in pubertal animals. No significant differences in distribution or location were noted with respect to the other groups. VIM+ cells showed a lower degree of vascular tropism than GFAP+ cells. Staining to VIM was intense in the glands of pubertal sheep. Positive cells were located close to pineal connective tissue stroma and blood vessels.

Structural analysis

No structural differences were recorded between pineal glands from day-slaughtered and night-slaughtered sheep; the two are therefore described

Table 1. Plasma melatonin levels (pg / ml); Pineal protein contents (mg); and melatonin protein levels (ng/mg.prot) in ewes killed during daylight or at night under natural inhibitory photoperiods.

	LIGHT PERIOD				DARK PERIOD		
Clocktime (h)	06:	10:	14:	18:	22:	02:	06:
1-6 months old	68±6	55±7[a]	37±4[a] 1.3±0.1[d] 10±4d	59±9	94±10[a] 1.7±0.2[c,d] 18±4cd	183±13[a]	74±6
9-24 months old	77±8[b]	65±8[a,b]	46±7[a,b] 2.5±0.3 18±3	71±7[b]	108±12[a, b] 2.9±0.2[c] 26±4[c]	205±14[a,b]	83±8[b]
36-60 months old	67±8	58±7 [a]	35±5[a] 1.4±0.2[d] 11±3[d]	61±7	91±12 a 1.9±0.1[c,d] 17±3[c,d]	179±12[a]	72±7

RIA and HPLC were performed as described in Methods. Shown are means±SEM (n=36 and 4 for RIA and HPLC respectively, at each hour). [a]:$P< 0.05$ vs 6:00, in the corresponding groups. [b]: $P< 0.05$ vs groups I and III at the corresponding hours. [c]: $P< 0.05$ vs 14:00, in the corresponding groups. [d] $P< 0.05$ vs groups I and III at the corresponding hours.

together.

Group 1 (infants; 1-6 months old)

On sagittal sections, glands displayed an ovoid-rounded morphology, and lay rostral to the cerebellum and caudal to the cerebral hemispheres. The dorsal surface bordered the 3rd ventricle wall. The pineal gland stretched along roughly 70% of the wall, lined by a cuboid-cell ependyma. The gland was bordered laterally by the habenular and posterior comissures.

The surface of the gland was surrounded by a capsule (Figs. 9-10) consisting of a simple squamous epithelium and subepithelial connective tissue containing occasional fibroblasts, sparse collagen and elastic fibers, and a moderate number of reticular fibers (Fig. 10). Vascular structures were visible within the capsule. Connective-vascular septa radiated from the capsule into the parenchyma, dividing it into pseudolobules containing pinealocyte clumps forming follicular-glomerular structures.

The gland was divided into two clearly distinct areas: a dorso- and ventroperipheral area, the cortex (Fig. 9), displaying fewer cells; and a dorsomedual and ventromedial area, the medulla (Fig. 11), containing more cells (Table 2). Cells were randomly arranged, with no clear orientation; this diffuse arrangement was occasionally interrupted by rosettes (Fig. 9, 11), which were observed in both the cortex and the medulla and comprised a rounded or irregular lumen surrounded by cell groups of varying abundance.

Pineal parenchyma contained two main cell types: pinealocytes and interstitial cells. Pinealocytes (Fig. 9, 11), which were more numerous (Table 2), were visible as large cells with pale-chromatin nuclei and prominent nucleoli. Interstitial cells (Fig. 9, 11) were less abundant (Table 2) and were found mainly in perivascular locations; nuclei were ovoid, with dense homogeneous chromatin. Lymphocytes were also observed, chiefly in the medullary area (Fig. 12). Pigmented cells stained strongly silver to the Masson Fontana stain (Fig. 13) and some degenerated cells with pyknotic nuclei were

Table 2. Pineal weight (mg);nuclear density of NNE-, GFAP-, VIM-positive cells (number of nuclei/10.000 mm^2); and nuclear profile area of NNE-positive cells (mm^2) in ewes killed during daylight or at night under natural inhibitory photoperiods.

	1-6 MONTHS OLD				9-24 MONTHS OLD				36-60 MONTHS OLD			
	Light		Dark		Light		Dark		Light		Dark	
Pineal weight (mg)	$54\pm6^{a,b}$		63 ± 6^{a}		66 ± 6^{b}		77 ± 8		$55\pm5^{a,b}$		64 ± 6^{a}	
	COR^{c}	MED	COR^{c}	MED	COR^{c}	MED	COR^{c}	MED	COR^{c}	MED	COR^{c}	MED
NNE + cells In 10.000 μm^2	$82\pm6^{a,b}$	$92\pm5^{a,b}$	81 ± 5^{a}	90 ± 5^{a}	95 ± 6^{b}	104 ± 6^{b}	94 ± 6	103 ± 6	$84\pm6^{a,b}$	$93\pm4^{a,b}$	80 ± 5^{a}	91 ± 4^{a}
GFAP + cells In 10.000 mm^2	$10\pm2^{a,b}$	$15\pm4^{a,b}$	11 ± 3^{a}	16 ± 3^{a}	20 ± 4^{b}	25 ± 4^{b}	19 ± 5	26 ± 3	$11\pm3^{a,b}$	$16\pm3^{a,b}$	10 ± 3^{a}	15 ± 2^{a}
VIM + cells In 10.000 μm^2	$8\pm3^{a,b}$	$13\pm3^{a,b}$	9 ± 3^{a}	14 ± 4^{a}	18 ± 4^{b}	25 ± 4^{b}	17 ± 4	24 ± 4	$9\pm3^{a,b}$	$14\pm3^{a,b}$	10 ± 2^{a}	15 ± 4^{a}
Nuclear profile areas NNE + (μm^2)	29.7 ± 0.5^{b}		34.2 ± 0.7		28.9 ± 0.4^{b}		35.3 ± 0.5		30.2 ± 0.4^{b}		34.9 ± 0.5	

Pineal weight (means ± SEM; n=12 in each group). Morphometrical analysis was performed as described in Methods. Shown are means±SEM (n=100 in each animal). COR=Cortical regions. MED = Medullary regions. [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 Medullary regions.

Fig. 1. Sheep pineal gland, 1 month old, slaughtered during day. NNE-positive cells in cortex. EAS, x 250

Fig. 2. Sheep pineal gland, 9 months old, slaughtered during day. NNE-positive cells in medulla. EAS, x 350

Fig. 3. Sheep pineal gland, 9 months old, slaughtered during night. NNE-positive cells in medulla. EAS, x 180

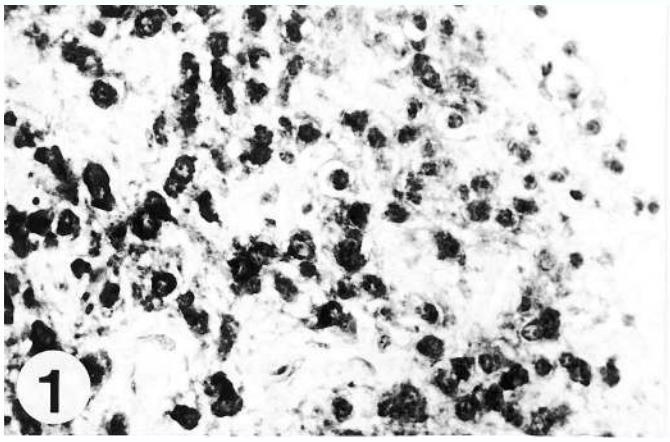
Fig. 4. Sheep pineal gland, 60 months old, slaughtered during night. NNE-positive cells in medulla. EAS, x 350

Fig. 5. Sheep pineal gland, 12 months old, slaughtered during day. GFAP-positive cells in perivascular arrangement. EAS, x 350

Fig. 6. Sheep pineal gland, 24 months old, slaughtered during night. VIM-positive cells in perivascular arrangement. EAS, x 450

Fig. 7. Sheep pineal gland, 24 months old, slaughtered during. VIPergic nerve fibers. EAS, x 180

Fig. 8. Sheep pineal gland, 24 months old, slaughtered during night. NPY-positive nerve fibers. EAS, x 250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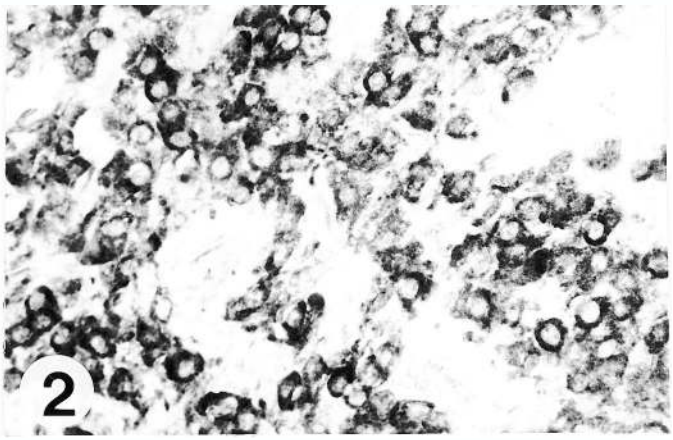
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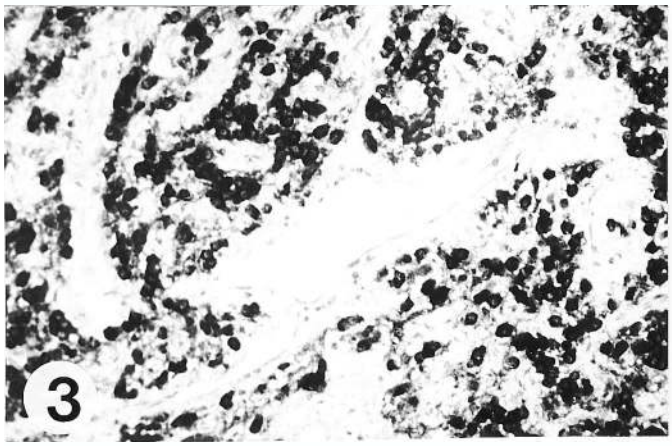
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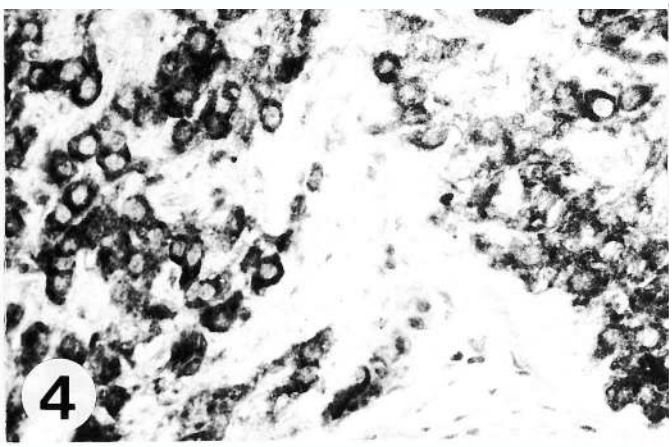
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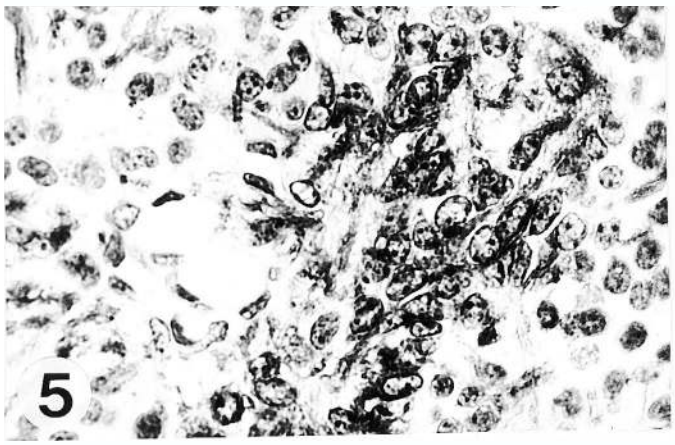
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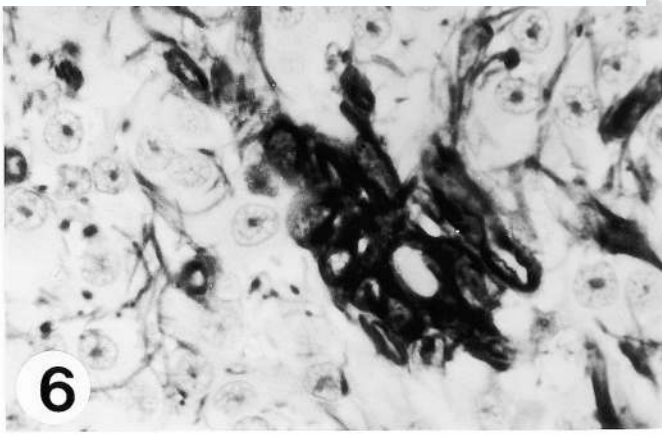
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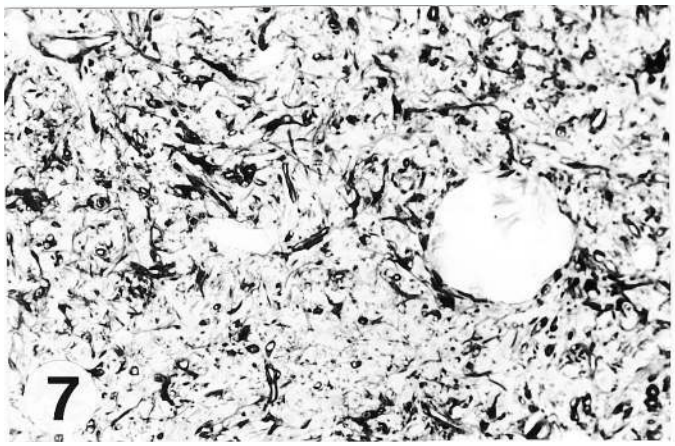
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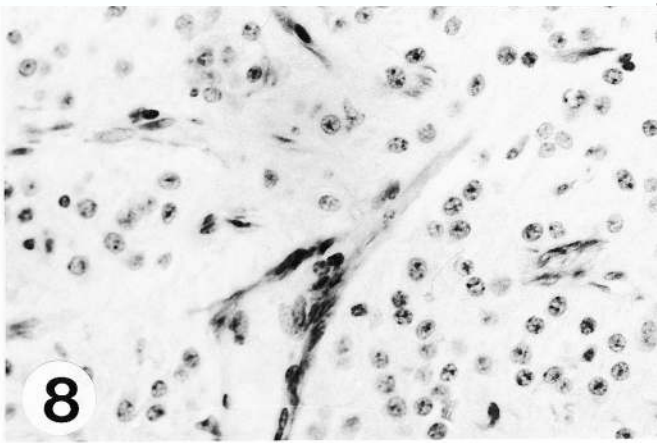
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observed below the capsule. Mitoses were frequently observed.

Gland stroma was composed of moderate numbers of collagen and reticular fibers (Fig. 10). Stroma radiated from the capsule into the parenchyma, forming trabecular septa in interstitial and perivascular spaces (Fig. 12). Vascular structures were smaller but more numerous in the cortex than in the medulla. PTAH staining revealed occasional glial fibers, which were more abundant in peripheral locations.

Group II (pubertal animals; 12-24 months old)

In pubertal/early fertile sheep, the pineal gland extended along 85% of the 3rd ventricle wall between the habenular and posterior commisures. The capsule comprised a simple squamous epithelium (Fig. 14) and a greater abundance of collagen and reticular fibers. Pseudolobulation was more marked than in the infant gland (Fig. 14), and trabecular septa contained larger amounts of collagen and reticular fibers; rosettes, however, were far fewer in number, and in many cases virtually non-existent.

Glands in this age-group displayed an extraordinary wealth of cells (Table 2). Pinealocytes and interstitial cells continued to predominate, as in the younger age-group (Figs. 14-17), but there was a notable fall in the number of pigmented cells, and neither lymphocytes nor degenerated cells were any longer apparent. Pineal gland parenchyma retained its cortico-medullary structure.

Collagen and reticular fibers were more abundant than in the earlier group, both in perivascular and interstitial stroma (Fig. 18). Vascular structures in cortex and medulla (Fig. 15) displayed larger lumina and a greater number of component cells than in infant pineal glands.

PTAH staining revealed abundant glial nerve fibers. Calcium was present in all 24-month-old animals, forming small concentric spheres (Fig. 19).

Group III (adult animals; 36-60 months old)

All morphological characteristics of the pineal gland in adult sheep were as reported for pubertal sheep, differences being quantitative rather than qualitative. These included: greater abundance of collagen and reticulin fibers in the capsule (Fig. 20), and capsular presence of calcium (Fig. 21); greater abundance of collagen and reticulin in trabecular septa and pseudolobules; reduced density of pinealocytes and interstitial cells in both cortex and medulla (Table 2); more abundant collagen and reticulin in intercellular and perivascular interstitial connective tissue (Figs. 20-23); reduced vascularisation in both cortex and medulla; and finally greater abundance of calcium in the parenchyma, where it occupied large irregular areas from which glandular cytology was absent (Fig. 24).

Discussion

Pineal and plasma melatonin concentrations showed a similar diurnal pattern in the three age groups examined (i.e. infant, pubertal/early fertile and adult) with peaks at 02:00 and troughs at 14:00. These results essentially agree with a number of observations in the literature (McNulty et al., 1990; Miguez et al., 1996; Chemineau et al., 1996; Ravault et al., 1999; Hamase et al., 2000). The mean value of plasma and pineal melatonin levels found in pubertal/ early fertile ewes was significantly higher than that recorded in younger or older animals (Zarazaga et al., 1998). Adult ewes displayed significantly lower concentrations. As reported in sheep (Cornelissen et al., 2000), as well as in many other species including man (Arendt, 1988), blood melatonin levels decline with age.

The highest pineal melatonin levels were recorded in pubertal animals, which also displayed the heaviest glands. In all age-groups, nocturnal plasma and pineal melatonin concentrations were higher than diurnal levels. Similar findings have previously been reported for various species, including sheep, using RIA (Chemineau et al., 1996; Ravault et al., 1999) o HPLC (McNulty et al., 1990; Hong et al., 1993; Miguez et al., 1996; Hamase et al., 2000). Similarly, pineal glands were heavier at night than during the day. Coon et. al. (1999), correlate the increase in melatonin at night with

Fig. 9. Sheep pineal gland, 1 month old. Glandular cortical area. Capsule of simple cuboid epithelium. Rosettes (arrow). H-E, x 250

Fig. 10. Sheep pineal gland, 3 months old. Note reticular fibers in capsule and vascular structures. RG, x 250

Fig. 11. Sheep pineal gland, 3 months old. Glandular cytology: pinealocytes, interstitial cells and rosettes (arrow). H-E, x 250

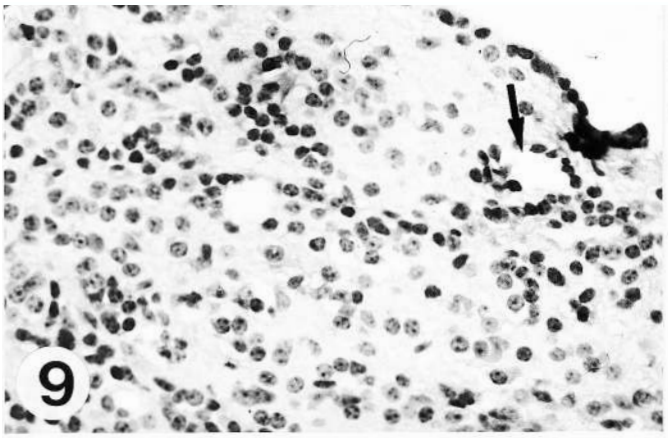
Fig. 12. Sheep pineal gland, 6 months old. Lymphocyte niche in medullary area. H-E, x 250

Fig. 13 . Sheep pineal gland, 6 months old. Pigmented cells in cortical area. MF, x 350

Fig. 14. Sheep pineal gland, 9 months old. Capsule, trabecular septa and pseudolobules. H-E, x 180

Fig. 15. Sheep pineal gland, 12 months old. Vascular structures in cortex. H-E, x 180

Fig. 16 . Sheep pineal gland, 12 months old. Vascular structures in medulla. H-E, x 250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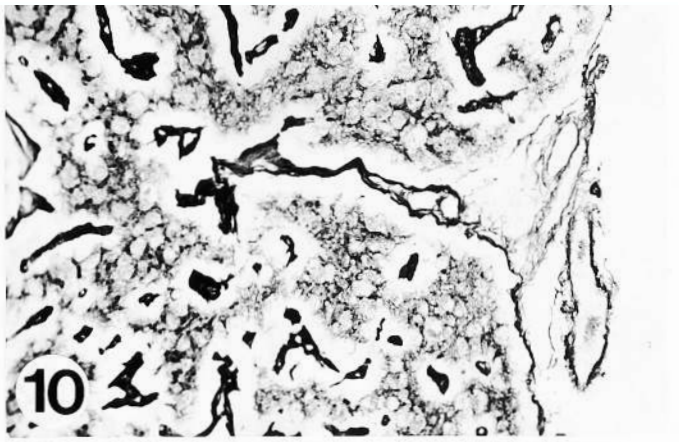
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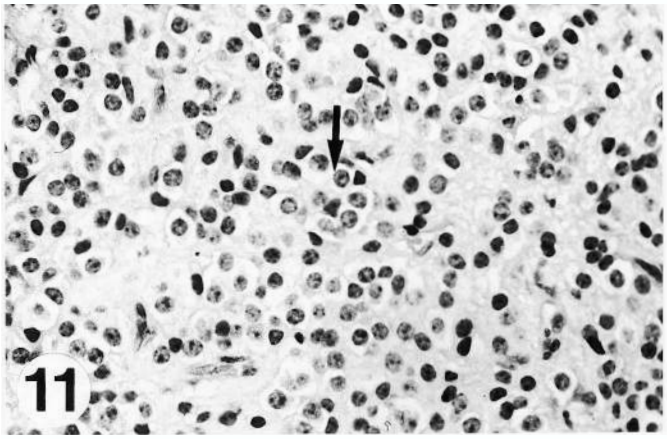
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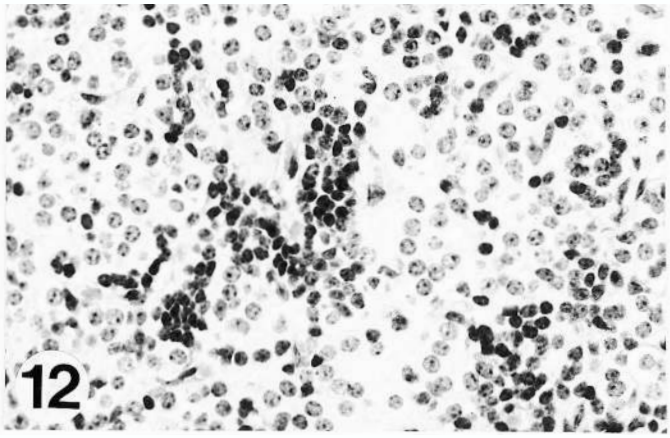
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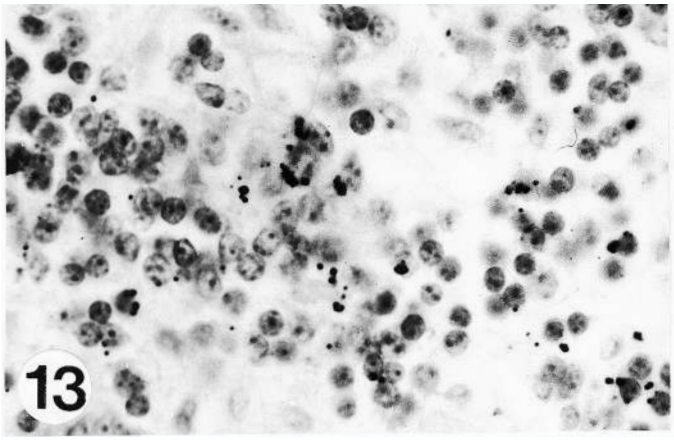
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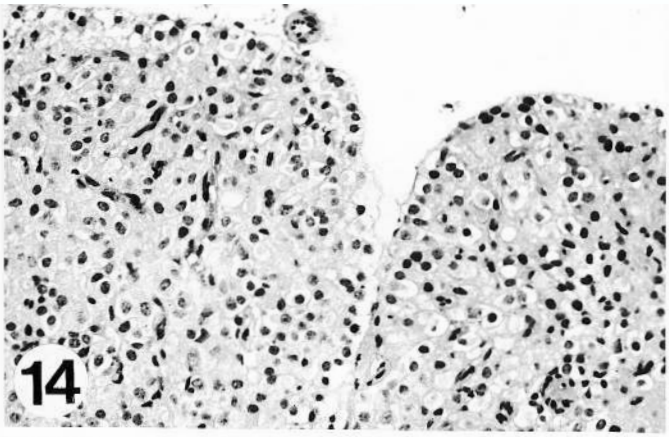
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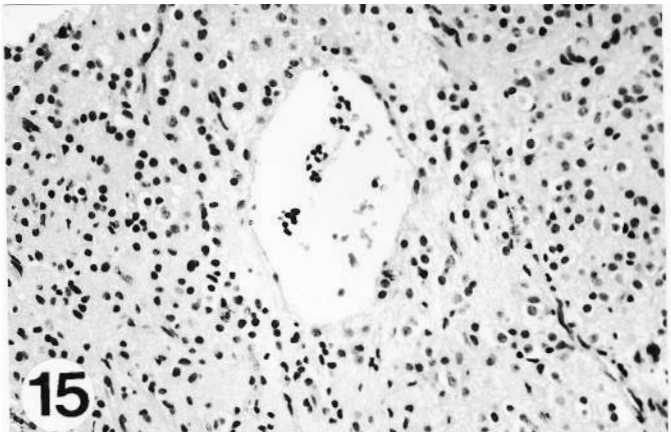
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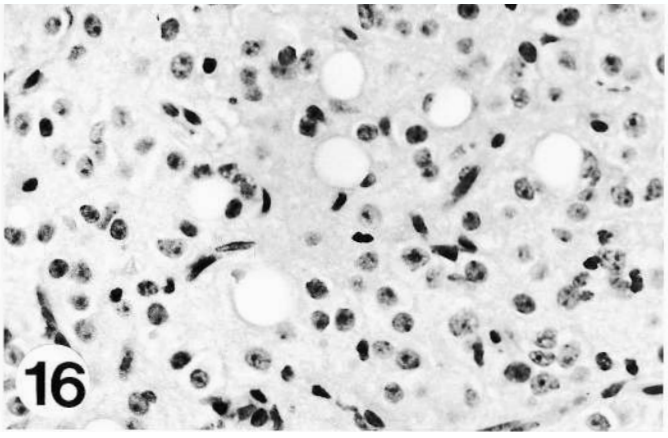
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greater pineal weights.

Plasma and pineal melatonin levels correlated with some morphometric parameters in the pineal glands of sheep killed at times of maximal and minimal melatonin levels (i.e. 02:00 and 14:00). These included increased pineal weight and significantly larger nuclear profile area of NNE-positive cells in the pineal glands of night-slaughtered sheep. The increase in mean plasma and pineal melatonin levels found in pubertal/early fertile ewes coincided with larger pineal glands and a higher number of nuclei of NNE-positive cells in ewes of the same age. The fact that the number NNE-positive cell nuclei was higher in sheep 9-24 months old than in infants indicated an increase in the number of cells from birth to puberty. This finding, not hitherto documented in sheep pineal gland, has been reported in rats (Feng et al., 1998).

The present results indicate heterogeneous pinealocyte distribution, with higher density values recorded in central than in peripheral areas of the gland, both by day and at night; this would point to a division of pineal zones into cortex and medulla. A similar division has been reported in prenatal sheep development (Regodón et al., 1998a), and in both rat and bovine pineal gland, where the division was found to be both morphological and biochemical (Chuluyan et al., 1990; Sato et al., 1994; Sakai et al., 1996; Hira et al., 1998).

Like pinealocytes, interstitial cells displayed no numerical variation between day and night. Age, in contrast, influenced the numerical density of these cells: numbers increased from 9 to 24 months old, coinciding with increased pineal weight and higher plasma and pineal melatonin levels. As during prenatal development (Franco et al., 1997; Regodón et al., 1998a), cells were chiefly located in perivascular areas; cortex and medulla displayed differing cell densities. The histochemical reaction to PTAH, coupled with immunoexpression of glial cell markers, suggest that these are glial cells-astrocytes, a finding previously reported in sheep during both intrauterine development (Franco et al., 1997; Regodón et al., 1998a) and postnatal growth (unpublished observations).

Previous experiments during sheep prenatal development (Regodón et al., 1998b) suggest that pigmented cells are ultrastructurally different from pinealocytes and interstitial cells, which also contain melanin pigment. Regodón et al. (2001), in ultrastructural studies of postnatal sheep development, did not encounter pigmented cells as distinct from pinealocyte and interstitial cells. These findings agree with the results of the present study, in which melanin pigment was observed only in sheep between 1 and 6 months old.

Glandular architecture, characterised by the formation of glomerule-containing pseudolobules, represented a continuation of that observed during embryonic growth (Franco et al., 1997; Regodón et al., 1998a). Rosettes were still visible, though fewer, and numbers clearly declined with age. The results obtained here suggest that rosettes are formed by pinealocytes, as indicated in studies of prenatal growth (Regodón et al., 1998a); in contrast, García-Mauriño et al. (1992a,b), in studies of rabbit pineal gland, report that the rosettes comprise undifferentiated 3rd ventricle ependymary cells. The presence of rosettes in prepubertal animals, and their absence in pubertal and adult sheep, suggests evidence of a glandular growth mechanism commencing prior to birth and thereafter disappearing as the gland develops.

Intercellular and perivascular stroma was formed by collagen and reticulin fibers, which became more abundant with age. Calvo and Boya (1984), and López-Muñoz et al. (1992a,b) report similar findings in rat and rabbit pineal glands, respectively.

Vascularisation was greater in pubertal pineals than in infant or adult. Electron microscopy revealed non-fenestrated capillaries (Regodón et al., 2001), as observed during intrauterine development (Regodón et al., 1998a).

PTAH staining detected glial nerve fibers, chiefly in perivascular locations, throughout postnatal development. Immunohistochemistry confirmed sympathetic (Mikkelsen and Moller, 1999) and parasympathetic peptidergic innervation (Cozzi et al., 1994; Regodón et al., 1998a). Vascularisation and

Fig.17. Sheep pineal gland, 12 months old. Abundant interstitial cells in perivascular arrangement. PTAH, x 180

Fig. 18. Sheep pineal gland, 12 months old. Perivascular and interstitial collagen fibers. TM, x 180

Fig. 19. Sheep pineal gland, 24 months old. Concentric calcium spheres. H-E, x 350

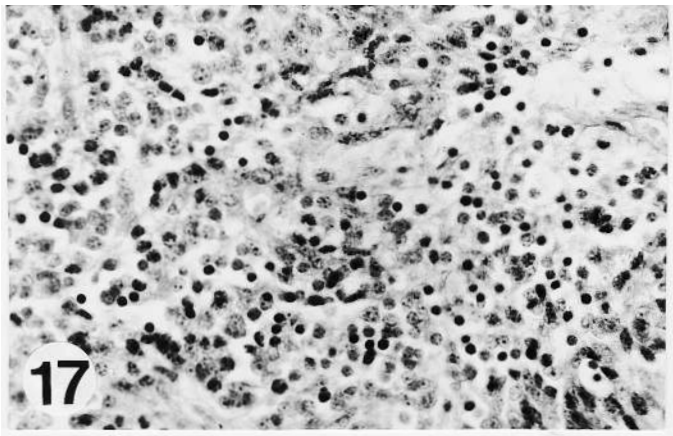
Fig. 20 . Sheep pineal gland, 36 months old. Abundant reticulin in capsular and perivascular connective tissue. RG, x 250

Fig. 21 . Sheep pineal gland, 36 months old. Capsule calcification. H-E, x 180

Fig. .22 . Sheep pineal gland, 48 months old. Abundant interstitial collagen. TM, x 250

Fig. 23 . Sheep pineal gland, 48 months old. Medullary vascularisation. H-E, x 250

Fig. 24 . Sheep pineal gland, 60 months old. Calcium in cortex. VK, x 250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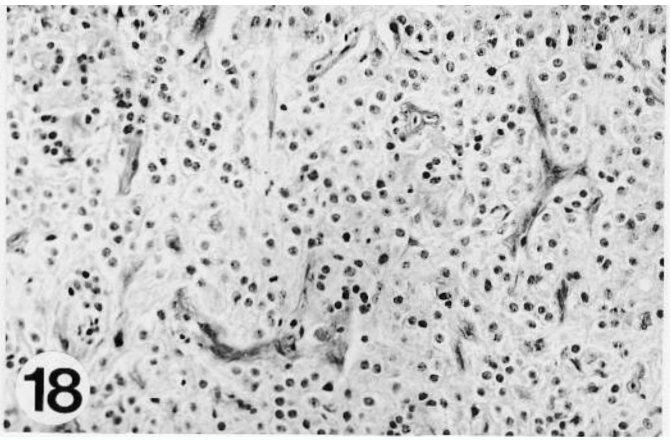
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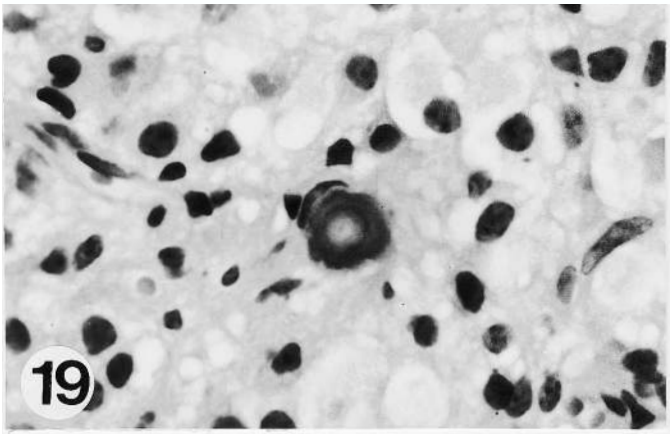
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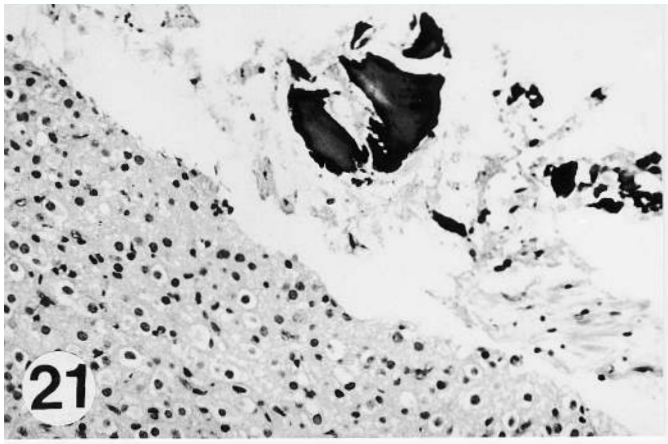
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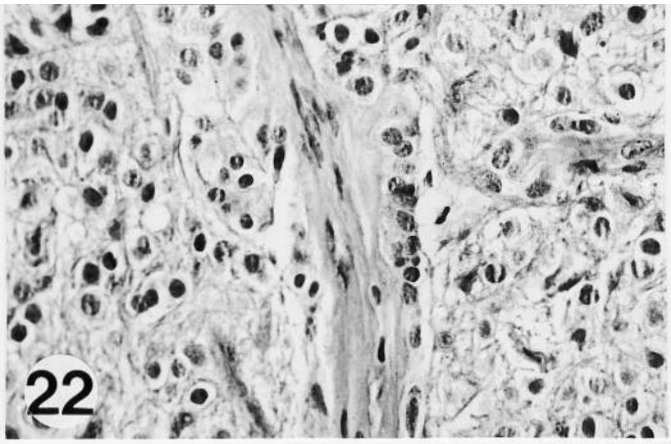
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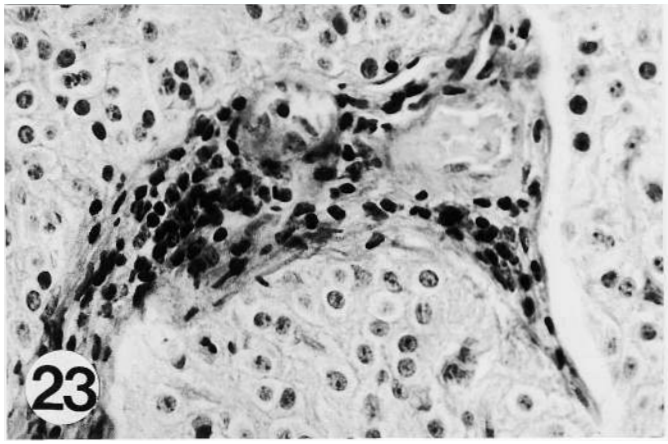
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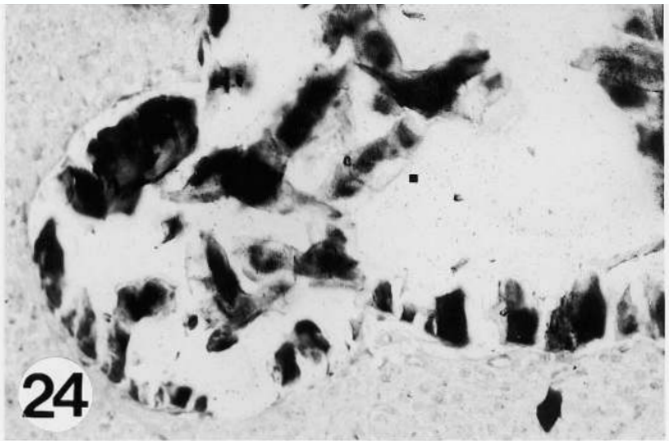
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innervation were most developed from 9 to 24 months, i.e. the pubertal group which at the same time displayed the highest plasma and pineal gland melatonin levels.

Calcium was detected from 9 months onwards, with increasing deposition until 60 months. A correlation was observed between age and the number of acervular layers. Layer formation may be related to circumstantial failures in calcium homeostasis in the pineal gland (Schmid et al., 1994; Vigh et al., 1998), or to pinealocyte secretory activity (Galliani et al., 1990). No correlation was established here between calcium deposition and melatonin secretion; the highest melatonin levels were detected in pubertal/early fertile sheep, while the largest calcium deposits were found in adult animals.

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