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Circadian and seasonal cortico-medullary variations in pinealocyte nuclear size. A comparative statistical analysis

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Summary. Circadian and seasonal variations were observed in the karyometric index of pinealocytes in the cortical and medullary regions of the distal pineal body. The study involved 70 Wistar rats over a 24-hour interval (0:6, 10:00, 14:00, 18:00, 22:00, 02:00, 06:00 h) during two natural photoluminous periods, i.e. late summer (Long photoperiod) and Winter (Short photoperiod). The results show a difference between the high and low points of both photoperiods. Cortico-medullary differences are found at different times of day during long photoperiod (0:6; 10:00; 14:00 and 18:00 h.) and short photoperiod (14:00; 22:00 and 02:00 h.). The varianza annalysse between nuclear volume and point-time and between nuclear volume, point-time and location are significative. A high correlation between circadian rhythms and volumetric variations in both layers and photoperiod are found. The results also show significant differences in cortico-medullary karyometric indices between both seasons as well as between the diurnal and nocturnal hours of both photoperiods. It is suggested that the pineal body of the rat is influenced by circadian and seasonal photoperiod and may have groups of cells with different functional characteristics, depending on their location within the gland.

Key words: Pineal body, Circadian changes, Seasonal changes, Cortico-medullary pinealocyte karyometric index

Introduction

The relationship between luminosity and pineal gland physiology has been known for a long time (Wurtman and Axelrod, 1964; Axelrod et al., 1965; Merrit and Sulkowski, 1969; Wurtman and Ozaki, 1978). The pineal gland has been studied from different morphological viewpoints in an attempt to establish links with the corresponding physiological rhythmic parameters.

Variations in pineal body weight, pinealocyte nuclear volume and mitotic activity have been observed over 24-hour cycles (Becker and Vollrath, 1983).

Karyometric studies by Quay and Renzoni (1966) and Renzoni and Quay (1964) on rodent pinealocytes revealed variations in nuclear sizes during the 24-hour cycle. Such size variations were also established between the cortical and medullary gland regions.

On the other hand, a number of morphological and physiological animal studies suggest a possible division of the pineal gland parenchyma into an external «cortex» and central «medulla». Among other parameters, this is evidenced by variations in pinealocyte nuclear size (Vollrath, 1981).

Such cortico-medullary circadian differences have not been confirmed by all authors (Welsh et al., 1979; Heidbuchel and Vollrath, 1983), though they have been suggested by others - specially in relation to rodents (Miline et al., 1968; Blumfield and Tap, 1970; López Iglesias et al., 1987).

Recently, some authors (Diehl et al., 1984) have reported cortico-medullary differences, though these were found to depend on the pineal region considered. In turn, Becker and Vollrath (1983) reported rhythmic in pinealocyte nuclear size within the peripheral-gland region, but not in the central zone.

Studies in different seasons (Popova et al., 1975) have shown that the central and peripheral regions of the gland differ in responsive capacity.

This study attempts to establish whether these karyometric differences may be a consequence of varying conditions in natural luminosity.

Materials and methods

Seventy male Wistar rats weighing 275±18 g were used in the study. The animals were divided into two groups of 35 and were housed for 3 months in our

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laboratory for acclimatization before starting the sacrifice. They were kept under routine laboratory conditions (5 animals per cage, 18-20° C, relative humidity 45-50%) and under natural circadian and seasonal luminosity (light-dark cycle); i.e., 08.00-18:30 pm during the short photoperiod and 07:00-21:00 pm for the long photoperiod; as established from Valencia Metereological centre information.

A standard diet was supplied, along with tap water ad libitum.

The animals were sacrificed with 10% Nembutal (intraperitoneally) in group of five, every four hours (06:00, 10:00, 14:00, 18:00, 22:00, 02:00, 06:00).

This was carried out from September 11 to 12, (long photoperiod; and was repeated from February 2 to 3, 1984 (short photoperiod).

All animals were subjected to intracardiac 5% glutaraldehyde perfusion following saline cleansing. Once removed, the pineal bodies were fixed and refixed in osmium tetroxide.

Dehydration followed in a graded acetone series. The pieces were then contrasted with uranyl acetate and embedded in Epon.

The karyometric indices were determined from sections stained with toluidine blue.

Previous studies (De la Guardia et al., 1988) had shown that 100 nuclei are sufficiently representative, the values differing by not more than 10± from the mean of 500 measurements. The 100 nuclei measured came from four sections taken from the pars distalis (C) of the pineal body (Vollrath, 1981).

Nuclear size measurements were made in two layers of the gland; i.e., centre (medulla) and distal peripheral zone (cortex), which have different staining aspect (Figs. 1-4). Only clearly visible pinealocyte nuclei were considered. For each animal and region 25 x 4 nuclei from different sections were measured. The selection of subsequent sections was made so that each section was at least 15 µm away from the preceding one, so as to avoid including more than one section of each nucleus.

The major (A) and minor (B) diameters of the nuclei were determined under high magnification (x 100) with a micrometer fitted to the eyepiece. The nuclear volume (Vn) was calculated from the measured values using the Jacobj formula (1935) $Vn=\pi/6$ AxB2 multiplied by a microscope constant (K).

As it was not the aim of the present study to obtain absolute values for pinealocyte nuclear volume, but rather to carry out comparisons in relation to different seasonal photoperiods, no attempt was made to correct the data and compensate for shrinkage.

The statistical evaluation of the data was made after a descriptive study, using the usual centralization and dispersion parameters to provide an initial impression of the distribution of the variables. This was then completed by determining limits, range, variation coefficient, standard error, Kurtosis and skewness.

Comparative analysis of the variables was carried out by contrast and significance tests, accepting an error alpha of under 5%. As continuous, quantitative variables were involved in the study, paired comparisons were made with a measurement comparison test: the Student t-test was chosen for its reliability.

Homocedasticity conditions were previously verified using the F Snedecor test, and the normality hypothesis of the variables was systematically contrasted by non-parametric testing of data fitting-essentially through the Kolmogorov-Smirnov test for single samples, obtained by accepting the alternative hypothesis.

In simultaneously comparing the means of more than two variables, we used analysis of variance (ANOVA) - a fundamentsl statistical method to analyze the effect produced by any data classification of the mean value of a variable. Occasionally, a study of the relation between certain variables was desirable; classical correlation techniques were used in such cases.

The true determination of the degree of relationship between the chosen variables was obtained by the Pearson correlation test.

The data processing was done with the statistical program Stat-work and systat, supported by a graphic package (Cricket Graph) and performed on an Apple Macintosh Plus Computer.

Results

Descriptive analysis

The evolution of the karyometric indices determined for the cortical and medullary pineal regions of both groups, (Figs. 1-4) are given in graphs 1 and 2. It is seen that the cortico-medullary differences were not significant for either photoperiod, though the graphs do show a difference between the high and low points of both photoperiods.

The highest peak corresponding to the long photoperiod, was observed at 06:00 hours for the cortical region, and at 10:00 hours for the medullary zone and the lowest values around 22:00 hours in both layers (Graph 1).

These peak values reversed during the short photoperiod and showed maximum values at 06:00 hours for the cortical region; a low point being observed at 14:00 hours for the cortical region and at 18:00 hours for the medulla (Graph 2).

Significant point-time differences (*$p>0.005$, **$p>0.0005$) were found on comparing the cortico-medullary indice in both short and long photoperiods, and between different photoperiods (Graphs 1-4).

Finally, the mean global karyometric index in both photoperiods was greater during the long photophase ($p>0.002$) (Diagram 1).

These results point to a clear circadian and seasonal evolution of nuclear volume in both layers, with oscillations throughout the 24-hour cycle, that in turn varied according to the length of the photoperiod. In order to confirm these data we performed a unidirectional analysis of variance, taking point-hour as

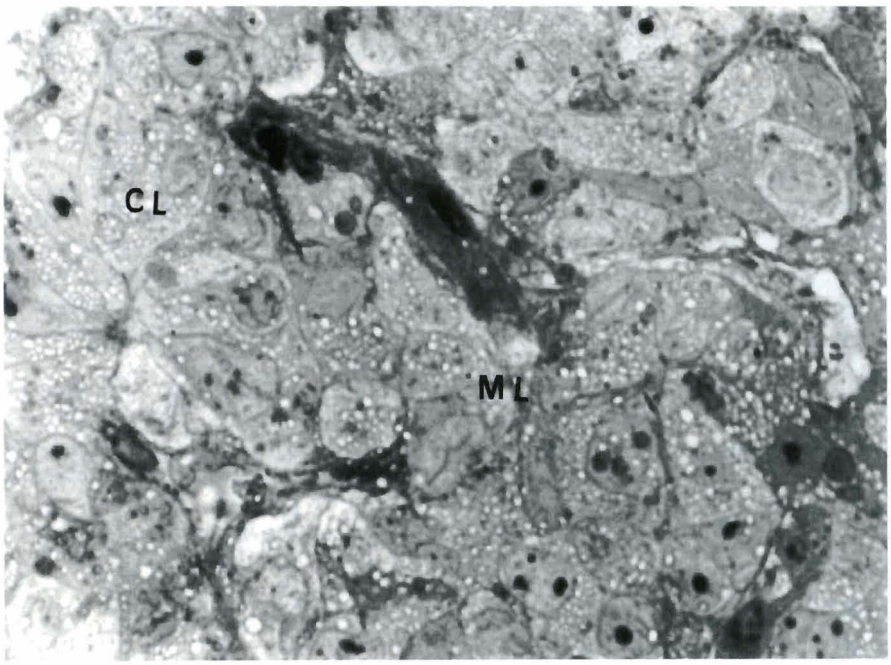


Fig. 1. Cortico-medullar layers. 02:00 hour. Long photoperiod. CL: Cortical layer. ML: Medullar layer. x 100

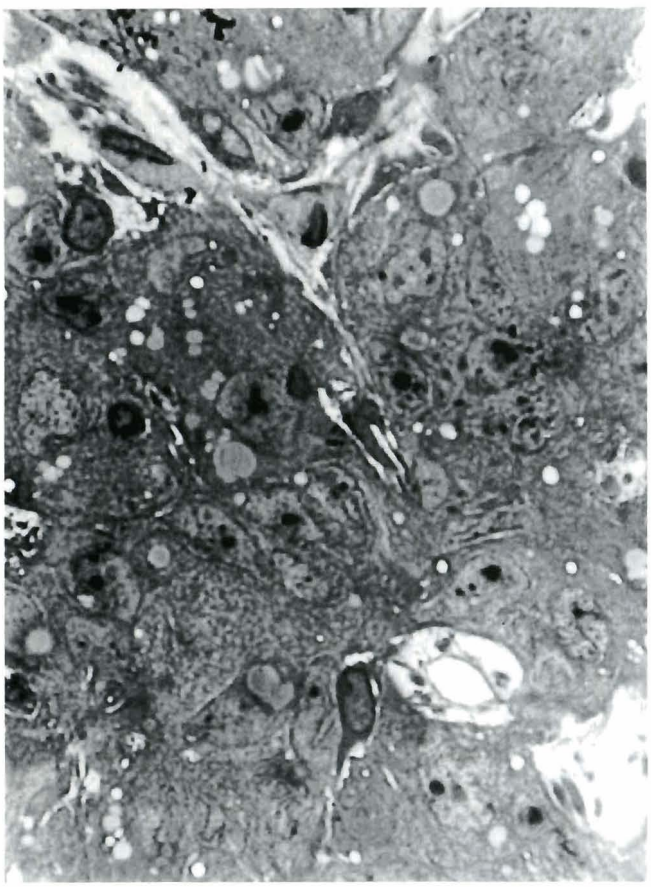


Fig. 2. Medullar layer. 02:00 hour. Long photoperiod. x 100

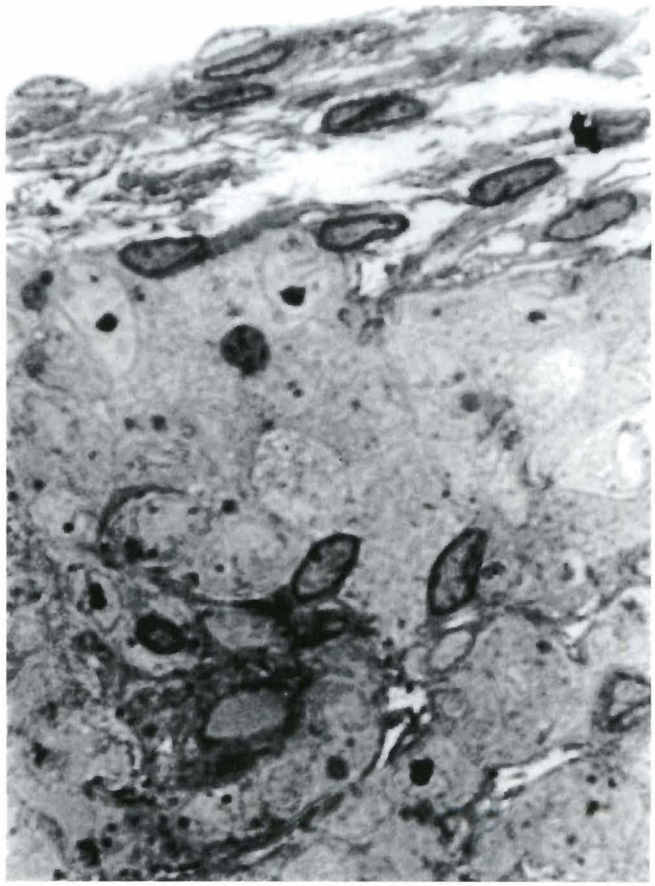


Fig. 3. Cortical layer. 06:00 hour. Short photoperiod. x 100

the factor influencing mean karyometric index.

Statistical analysis

The results, shown in Table 1 (a,b), confirmed the existence of a statistically significative influence of the point-time on the karyometrix index variation in both cortical (p>0.0001) and medullar (p>0.0001) layer during short and long photoperiod.

The correlation and regression studies between nuclear volume and hour distribution -the latter being taken to represent the independent variable- are indicated in Table 2: a) short photophase; b) long photophase. The nature of the variables led us to perform a polynomial fit -grade 4 and grade 3 respectively- being the most appropriate. Graphs 1 and 2 reflect the different circadian evolution of the karyometric indices in the peripheral and central zones.

As a result, we decided to study the influence of location (qualitative variable) on mean nuclear volume (quantitative variable). The results of this analysis (Table 3) showed a statistically significant (p>0.01) difference

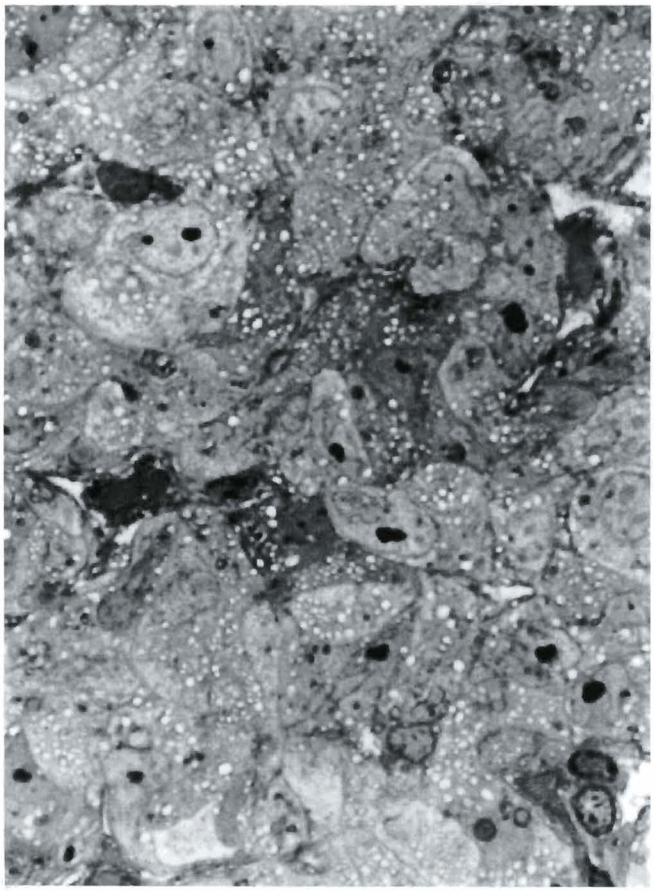
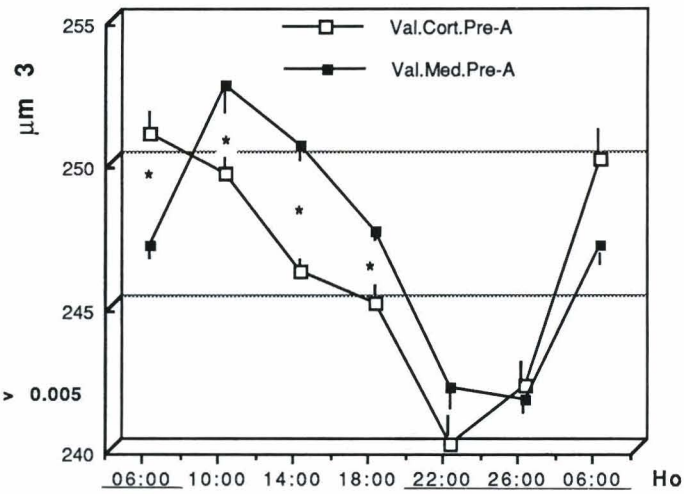
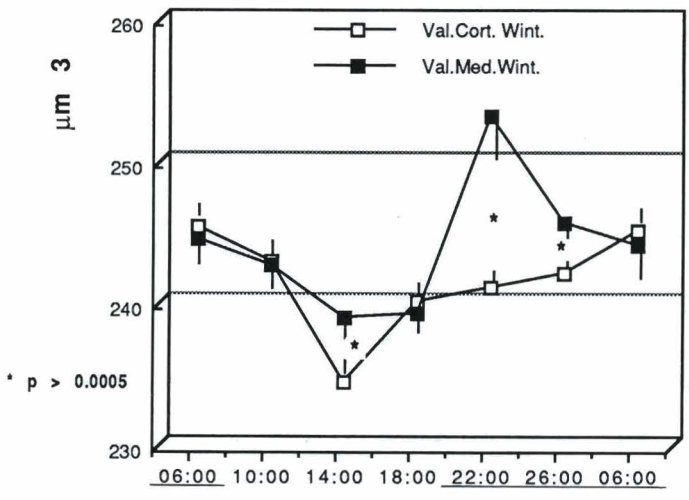


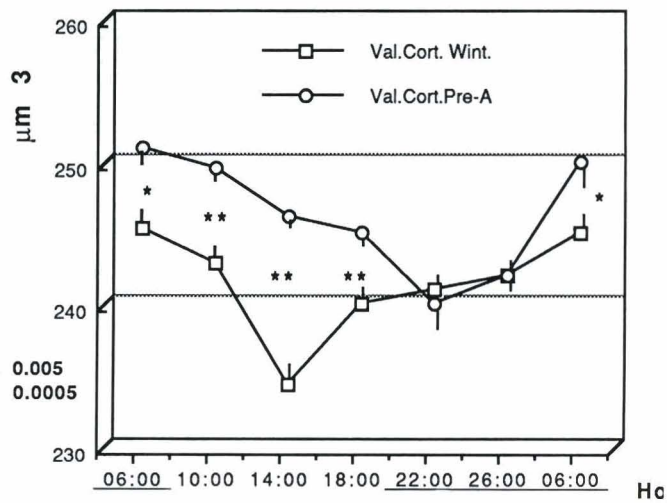
Fig. 4. Medullar layer 06:00 hour. Short photoperiod. x 100



Graph 1. Circadian cortico-medullary evolution. Long photoperiod (Pre Autum). Verticals bars indicate standard deviation and black-point statistical point-time significance.



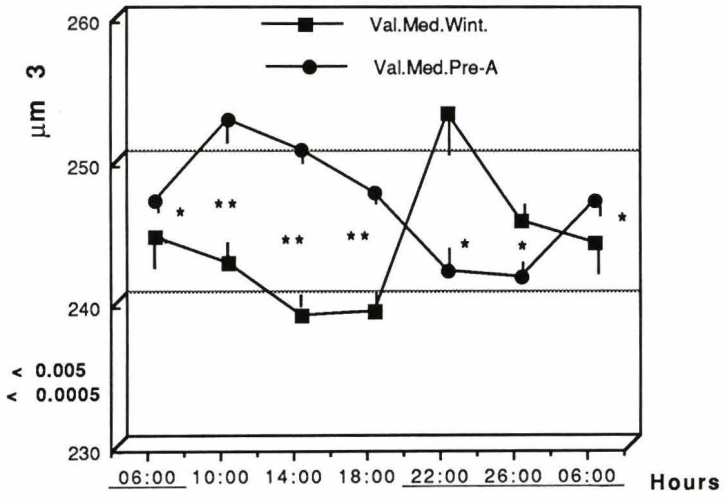
Graph 2. Circadian cortico-medullary evolution. Short photoperiod (Winter). The horizontal bars indicate the dark-hours.



Graph 3. Comparative circadian evolution between cortical layers in both photoperiods.

during the winter photophase; this could be interpreted as an indication that during this phase location indeed exerts a significant effect on nuclear volume variation. On the contrary, during the long photophase, location did not significantly ($p>0.1$) determine nuclear variation.

The next step in the statistical analysis of the results was to determine the influence of the seasons and hour rhythms on each layer, employing point hours and photoperiod as qualitative variables; an analysis was thus made of their effects on mean karyometric index and location (quantitative variables). The results obtained are shown in Table 4, where a high degree of significance ($p>0.0001$) and interaction is appreciated.



Graph 4. Comparative circadian evolution between medullar layers in both photoperiods.

Discussion

The results of the karyometric index variations show a clearly significant circadian difference between the karyometric indices of the cortical and medullary zones considered separately. These variations in rhythmic circadian karyometric activity of the cortico-medullary regions were more pronounced during the diurnal hours in animals sacrificed during the long-photoperiod and viceversa (Graph 1). This points to a difference in circadian nuclear activity depending on seasonal period.

The analysis of variance showed that the point hour cycle exerted a significant effect on the changes in cortical and medullary nuclear volume corresponding to both photophases (Table 1).

Graphs 1 and 2 reflect this result, lending support to the idea of a rhythmic cycle with limits established by the solar day, in which environmental luminosity plays a decisive role. These results are in agreement with those obtained in rats by Diehl (1981), Becker and Vollrath (1983), Quay and Renzoni (1966), Renzoni and Quay (1964), and Matshusima et al. (1983, 1989) in hamster and mouse.

The regression and correlation studies comparing volumetric index and hour distribution -taking the latter an an independent variable- also pointed in this direction (Table 2). The fact that both variables correlated was confirmed by the values indicated in the tables. In accordance with the polynomial correlation coefficient (Table 2a), they showed that in the cortical zone during

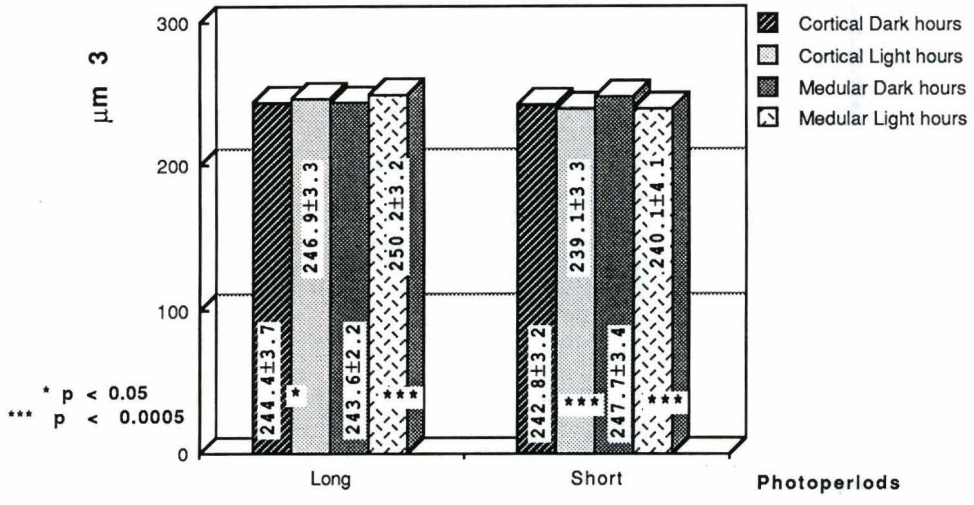


Diagram 1. Cortical and medullar dark-light differences during the same photoperiod.

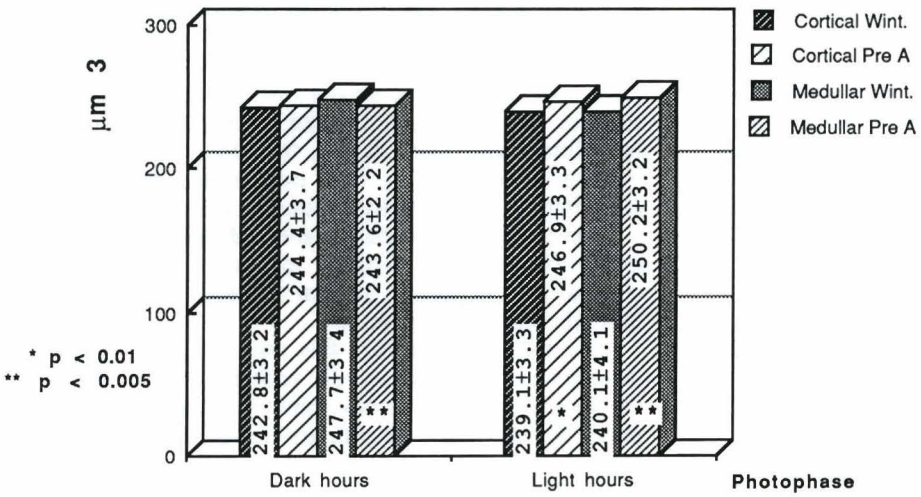


Diagram 2. Cortical and medullar dark-light differences during different photoperiods.

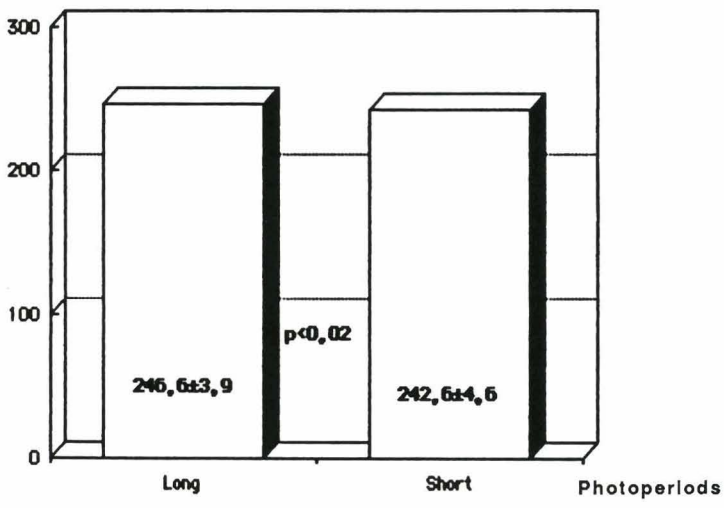


Diagram 3. Seasonal photoperiod comparison.

the short photoperiod, the hour rhythm exerted an 84.1% influence on the karyometric variations.

The determination coefficient in turn showed that 70.8% of the cortical variations during this photophase were explained by the circadian variations, whereas the remaining 29.2% corresponded to chance and/or other variables. On applying the same reasoning to the central region, the hour rhythm was found to exert a much greater influence (97.7%). In reality, the variations depended almost entirely on the hour rhythm, bearing in mind that the circadian rhythm may involve other

Table 1. Varianza analysis between variation nuclear volume and Poin-Time.

a) Short photoperiod (Winter).

Cortical layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between Point-times	340.782	5	68.156	39.889	0.000
Error	41.008	24	1.709		
Total	381.790	29			

Medullar layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between Point-times	674.908	5	134.982	213.410	0.000
Error	15.180	24	0.633		
Total	690.088	29			

b) Long photoperiod (Pre-Autum)

Cortical layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between Point-times	434.174	5	86.835	349.905	0.000
Error	5.956	24	0.248		
Total	440.130	29			

Medullar layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between Point-times	488.703	5	97.741	252.886	0.000
Error	9.276	24	0.386		
Total	497.979	29			

Table 2. Polinomial correlations between circadian rhythms and volumetric variations.

a) Short photoperiod (Winter).

Cortical layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Model	270.302	4	67.576	15.153	0.000
Error	111.487	25	4.459		
Total	381.790	29			

Coefficient of determination	0.708
Coefficient of correlation	0.841
Standard error of estimate	2.112
Durbin-Watson statistic	0.977

Medullar layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Model	658.860	3	219.16	182.851	0.000
Error	31.228	26	1.201		
Total	690.088	29			

Coefficient of determination	0.955
Coefficient of correlation	0.977
Standard error of estimate	1.096
Durbin-Watson statistic	0.419

b) Long photoperiod (Pre-Autum)

Cortical layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Model	397.270	3	132.423	80.332	0.000
Error	42.860	26	1.648		
Total	440.130	29			

Coefficient of determination	0.903
Coefficient of correlation	0.950
Standard error of estimate	1.284
Durbin-Watson statistic	0.803

Medullar layer

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Model	470.532	2	235.266	231.437	0.000
Error	27.447	27	1.017		
Total	497.979	29			

Coefficient of determination	0.945
Coefficient of correlation	0.972
Standard error of estimate	1.008
Durbin-Watson statistic	1.628

Table 3. Variance analysis between localization and karyometric index variations.

a) Short photoperiod (Winter)

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between localization	131.128	1	131.128	7.095	0.010
Error	1071.878	58	18.481		
Total	1203.006	59			

b) Long photoperiod (Pre-Autum)

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between localization	24.194	1	24.194	1.496	0.121
Error	938.108	58	16.174		
Total	962.302	59			

Table 4. Variance analysis between nuclear volume, point-time and localization.

a) Short photoperiod (Winter).

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between localization	131.128	1	131.128	112.020	0.000
Between point-time	699.955	5	139.991	119.591	0.000
Interaction	315.735	5	63.147	53.945	0.000
Error	56.188	48	1.171		
Total	1203.006	59			

b) Long photoperiod (Pre-Autum)

Source	Sum of Squares	Degree of Freedom	Mean Squares	F-Ratio	Prob>F
Between localization	24.194	1	24.194	76.240	0.000
Between point-time	810.609	5	162.122	510.888	0.000
Interaction	112.267	5	22.453	70.757	0.000
Error	15.232	48	0.317		
Total	962.302	59			

variables, such as luminous intensity, predominance of certain wavelengths, geomagnetic variations, endogenous rhythms, etc.

The determination coefficient, in turn, showed that 95.5% of the nuclear volume variations in the central zone during the winter photoperiod corresponded to the circadian variations, and only 4.5% to other factors mentioned.

In the case of the long photophase, the correlation coefficient showed that the circadian factor exerted a 95% influence on the cortical nuclear volume (Table 2b) and a 97.2% influence on the medullary zone -the determination coefficients being 90.3% and 94.5%, respectively.

This clearly shows that the volumetric variations during the day are markedly different for the cortical and medullary layers during the short photophase, the differences decreasing during the long photoperiod.

Thus, our results show that nuclear volume in the central zone of the pineal gland is greater than in the peripheral region, and since nuclear volume is directly

related to cell function, we may state that the pineal gland presents two functionally distinct regions -as reflected by graphs 1 and 2. On the other hand, there are clear differences between location and the hour and seasonal factor.

Bidirectional analysis of variance has made it possible to combine factors and study their joint influence. The results of this analysis, showed that nuclear volume variation was significantly ($p>0.0001$) influenced by cortical or medullary location, and by the hour evolution during the 24-hour cycle ($p>0.0001$) during both seasons -although these effects appear to condition hour influence on nuclear volume.

In any case, it seems clear from the results of our study that a circadian and seasonal difference exists in the karyometric index of the pinealocyte in the C or distal part of the pineal body of the Wistar Rat.

These differences between both photoperiods and hour rhythm were most marked during the short photoperiod, and agree with the results expressed by McNulty (1982) in fish. As suggested previously, these results support the idea that the cortico-medullary differences at karyometric level are also seasonal.

On the other hand, the fact that the significant cortico-medullary differences appeared at different precise hours in each photoperiod analyzed, together with the greater variation sensitivity of the medullary nuclei suggest a certain degree of individually and specifity in terms of the response encountered in both pineal layers. This may be conditioned by amplitude of luminosity and by luminous wavelength, which, through the accesory optic tract and sympathetic nerve fibres, influence the metabolic activity of pinealocyte (Minneman et al., 1975; Moore, 1978; Balemans et al., 1982). On the other hand, it has even been suggested that the Earth's magnetic fields acts through circadian fluctuations as a biological pacemaker and the luminosity factors might play an important role in the greater or lesser degree of such magnetic pineal sensitivity (Bliss and Heppner, 1976; Welker, 1983; Demaine and Semm, 1985; De la Guardia et al., 1988; Giménez-González et al., 1991).

These data, together with the possible existence of functionally and topographical different pineal regions (Jung and Vollrath, 1982) and supported by the different electrophysiological pineal responses according to the recorded zone and stimulus applied (Semm and Vollrath, 1979, 1980; Reuss and Vollrath, 1984), suggest that the pineal body may present groups of cells with different functional characteristics, depending on their location within the gland.

These cells would respond differently and specifically according to the physiological status of the animal, and to external factors.

In relation to the above mentioned discrepancies observed in the literature among the works of different authors, some interesting considerations should be made.

To our knowledge, and in agreement with Diehl (1981), karyometric indices are indeed reliable parameters for the evaluation of pinealocyte activity. Although these parameters follow a marked circadian rhythm, it has to be considered carefully since, as already suggested by Diehl (1981), a possible overlap of infradian and seasonal rhythms may exist. Contrary to Heidbüchel and Vollrath (1983) it seems unlikely that the discrepancies observed in the literature would arise from an artefactual fixing.

The results shown are the first ones from experiments in which circadian and seasonal conditions have been controlled and compared. No previous results, to our knowledge, demonstrate the differences observed in karyometric indices between short and long natural photoperiods, performing the experiments at the same time of day.

Finally, it can be established that a significantly different evolution in the nuclear size of cortical and medullar pinealocytes of the distal part of the rat pineal gland exists. This activity is different between both seasons (different natural photoperiods). The nuclear size mesured during light and dark period is also statistically different in both cell layers and both seasons considered.

Therefore, it can be concluded that the circadian and seasonal photoperiods are determinant factors in the functional activity of the distal pinealocyte with marked differences between cortical and medullar layers.

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