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ORIGINAL ARTICLE

The effects of periodic alteration of the temperature on the rhythmic melatonin release of explanted chicken pineals

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Abstract

The melatonin rhythm of cultured chicken pineal cells can be synchronized by cyclic environmental effects. Unlike the effects of light on the melatonin secretion, those of the temperature changes are much less known. Similarly, only a few data are available on the interactions between environmental illumination and periodic temperature changes and on the sensitivity of the pineal gland to temperature changes in different ages of animals.

We monitored the effects of temperature on chicken pineals for several days *in vitro*, in a perifusion system under different illumination patterns. The effects of temperature on pineals from chicken of different age were also compared.

The phase of the melatonin rhythm was controlled by periodic elevations of temperature under both constant darkness and continuous illumination. These results show that rhythmic changes of temperature prevent desynchronization induced by constant light. Following elevation of the temperature, the melatonin rhythm of pineals of young chickens (less, than 14 weeks old) was altered for 16–18 hours. Similar changes in melatonin rhythm were not found in older animals. It is concluded that the sensitivity for temperature changes of the pineal cells is varying with age.

Introduction

Melatonin secretion from chicken pineal shows circadian rhythm. Previous studies indicated that this rhythm is maintained even *in vitro* [3, 21, 27]. It was concluded that the avian pineal organ contains a melatonin synthesizing apparatus and a complete circadian clock [6, 7, 22]. The phase of the melatonin rhythm can be shifted by the alteration of environmental illumination [3, 8, 9, 27]. It was also observed that light is not the only stimulus, that entrains the melatonin rhythm [9]. Temperature can be another environmental factor.

It has been described, that periodic, slight changes of the temperature could entrain circadian clocks in prokaryote [19], plants [4], fungi [15], insects [18, 31], poikilothermic [12, 16] and homoeothermic vertebrates [17]. Studies related to this topic were reviewed by Rensing & Ruoff [25].

In birds, the pineal body is located directly under the thin calvaria, about 3 millimeters from the surface. Thus, the environmental temperature can possibly affect the pineal cells more than the body-temperature. Rhythmic alteration of the temperature (between 39 and 41°C) in the chicken pineal body was described *in vivo* [11].

In vitro studies showed that temperature pulses can shift the phase and temperature cycles can entrain the melatonin rhythm of the chicken pineal cells [1, 30]. In these studies, results were obtained from static cultures or from dynamic systems utilizing sampling time as long as two hours. Dynamic system with frequent sampling (less than one hour) would provide more details on subtle changes in the melatonin secretion.

Although under *in vivo* circumstances the melatonin rhythm is influenced by several factors simultaneously, most studies investigated only one factor (light, temperature, drugs, etc.) at a time [1, 8, 29]. Barrett & Takahashi described temperature dependent changes in the sensitivity of the melatonin rhythm to light and to anisomycin [2]. Further details on the interactions between the effects of different environmental factors on the melatonin rhythm were not available.

Several authors described that melatonin production of the pineal gland seems to change with aging [14, 20, 28]. Selmaoui described age related differences in serum melatonin and pineal N-acetyl-transferase (NAT) activity and in the response of rat pineal to a 50-Hz magnetic field [26]. However, Nathan found no age dependent changes in the sensitivity of the pineal gland to dim light [23]. The contradiction of these data raised the question of the possible age related differences in the sensitivity of pineal body to periodic environmental temperature changes.

In this paper, we planned to collect more data on the interactions between the effects of environmental illumination and periodic temperature changes and on the sensitivity of the pineal gland to temperature changes in chicken of different age by monitoring the melatonin secretion of the pineal gland.

Materials and methods

Animals and housing conditions

White Leghorn chickens of both sexes were used. Animals were kept at 25 °C and fed with maize. Water and food was accessible *ad libitum*. The chickens were kept under constant, standardized light cycles; 14 hours light (L; from 06.00 to 20.00) and 10 hours dark (D) for at least two weeks before our experiments. The animals were sacrificed between 13.00 and 14.00 p.m. Animal housing, care and application of experimental procedures were carried out in accordance with institutional guidelines under approved protocols (No: BA02/2000-31/2001, University of Pécs).

Perifusion system

Perifusion technique was applied as described in details earlier [10, 24]. Briefly: Fragments of two chicken pineals were mixed with Sephadex G-10 (Sigma) and distributed into two glass columns. A Medium 199 (Sigma) based tissue culture medium, equilibrated with a mixture of 95% air and 5% carbon dioxide was passed through the columns at a flow rate of 0.1 ml/min, under controlled temperature conditions (37°C). Samples were collected at 30 minutes intervals.

To ensure complete darkness, the perifusion system was placed in a dark room. In the experiments under constant illumination (LL), the columns were exposed to 20W white fluorescent light from 25 cm (600 lux). The temperature of the medium and that of the water jackets around the columns were controlled by the same circulating thermostat (Braun Thermomix BM and/or Lauda Ecoline 211).

Melatonin assay

Melatonin content from 20 µl-s of the collected perifusion fluid fractions was determined with radioimmunoassay (RIA) developed in our laboratory [24]. The assay sensitivity was at 80 pmol/l. The intra- and interassay coefficients of variation were 5–6% and 7–8%, respectively.

Statistical analysis

Each figure shows data from one of 3–4 similarly designed experiments which resulted in virtually identical graphs. The results of RIA of duplicate samples were performed with the aid of a computer program, written in our laboratory. Perifusion results were analyzed by our computer program (for principles see: [10]).

The figures show melatonin values of all the fractions collected during the experiment. To exclude the effects of explantation of the pineal gland, however, the mathematical analysis of perifusion data was started from the second evening of the experiments. The averages of melatonin secretion during 8 hours of the objective night (22:00–06:00, 16 samples) and the objective day (10:00–18:00, also 16 samples) were calculated. From these averages, the ratios of the nighttime melatonin secretion to that of the daytime (N/D ratio, respectively) were specified. From the last two days of each experiment, four ratios were calculated (*Table 1*). When results of two experiments were to be compared, these N/D ratios (n=4) were analyzed with Student's T-test (with t-Test: Two-Sample Assuming Unequal Variances). In one part of the experiments, the variances of melatonin contents of samples, collected from 10:00 to 20:00 (20 samples), were analyzed with F-test. Significant difference was considered at $p<0.05$.

Results

Melatonin secretion under constant conditions

Chicken pineals, explanted into an *in vitro* perifusion system and kept under continuous darkness and constant temperature, showed unequivocal signs of circadian melatonin rhythm (*Fig. 1.*). This secretion pattern was similar to that seen under normal light-dark-cycle. The maximal concentration values (peaks) appeared between 23:00 and 01:00, while

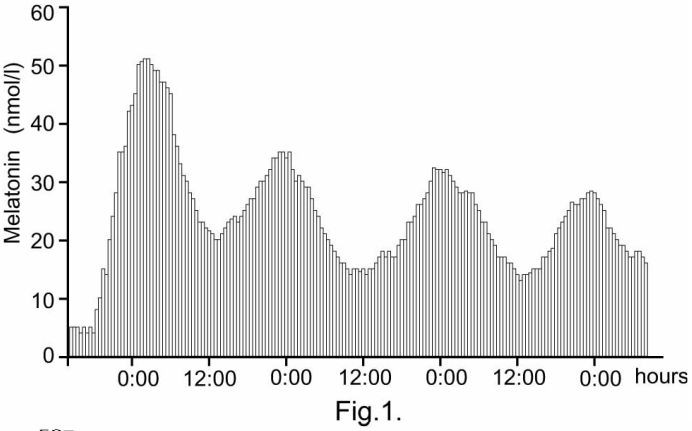

Fig.1.

Figure 1. Melatonin secretion under constant conditions
The chicken pineal gland was kept under constant temperature and continuous darkness in the perifusion system. The columns indicate melatonin contents of 30 minute, consecutive fractions. On the horizontal axis, absolute time is indicated. A regular, daily rhythm of the melatonin production was observed. From the second experimental day, the maximal concentration values (peaks) appeared between 23:00 and 01:00, while the secretion was minimal between 11:00 and 13:00.

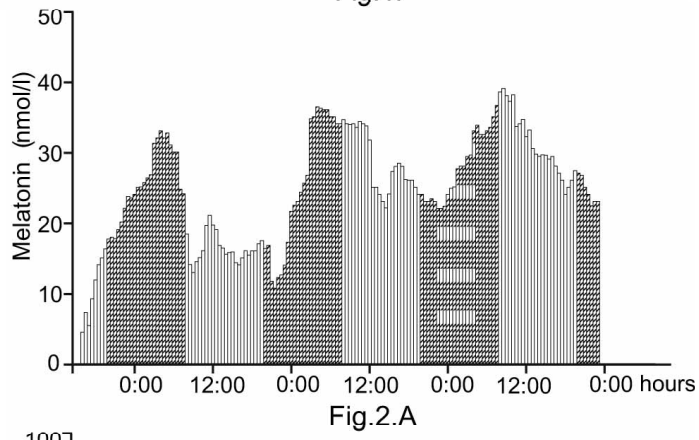

Fig.2.A

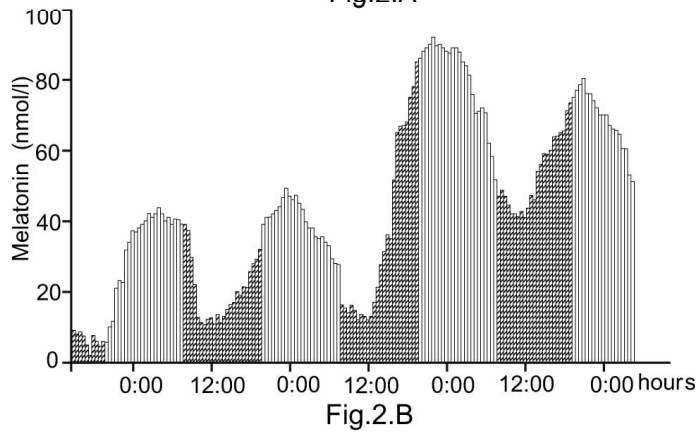

Fig.2.B

Figure 2. Melatonin secretion under 12 hour long periods of temperature elevation
The effects of periodic 12 hour long elevations of temperature (41 °C, hatched bars) on the perifused chicken pineal glands under continuous darkness were monitored. The structures of the plot in this, and the following figures are the same as that of Figure 1. The melatonin secretion decreased during the first hour of the elevated temperature then started increasing gradually. The phase of the melatonin rhythm was inverted by daily application of 41 °C between 20:00 and 08:00 (**A**). By the third day, nighttime melatonin peaks gradually decreased, and appeared at 10:00. The phase of the circadian melatonin secretion was not altered under rhythmic, daytime elevation of temperature (08:00 – 20:00) (**B**). The peaks still appeared around midnight. The N/D ratios were significantly smaller in experiment A than that in experiments B and in the experiments under constant temperature.

Table 1: Numeric values of the nighttime/daytime MT production ratios (N/D) of the experiments. For details see the results chapter and the figures.

		Constant temperature and darkness	41 °C by night	41 °C by day	C.I.*	C.I.*+ 41°C by day	C.I.*+ 41°C by night
		Fig.1.	Fig. 2.A.	Fig. 2.B.	Fig. 3.A.	Fig. 3.B.	**Fig. 3.C.**
	N/D ratios	1.8483	0.9768	2.8654	1.2521	3.3835	0.7465
		1.9055	0.8862	1.8621	1.2349	1.8217	0.6828
		1.4593	0.8141	2.0875	0.93	3.6939	0.8553
		1.5044	0.7387	1.3566	0.9173	1.9888	0.7824
	Average	1.6794	0.8539	2.0429	1.0836	2.722	0.7668
	SEM**	0,1150	0,0508	0,3139	0,0924	0,477	0,036
p-values***	Fig.1.	–	0,0028	–	0,0068	–	0,0016
	Fig. 2.A.	0,0028	–	0,0334	–	–	–
	Fig. 2.B.	–	0,0334	–	–	–	–
	Fig. 3.A.	0,0068	–	–	–	0,0433	0,0331
	Fig. 3.B.	–	–	–	0,0433	–	0,0265
	Fig. 3.C.	0,0016	–	–	0,0331	0,0265	–

Note. *C.I. stands for under continuous illumination. ** SEM stands for standard error of mean
*** The comparisons were carried out with T-test. Comparisons were made only in pairs of values with expected biological reasons.

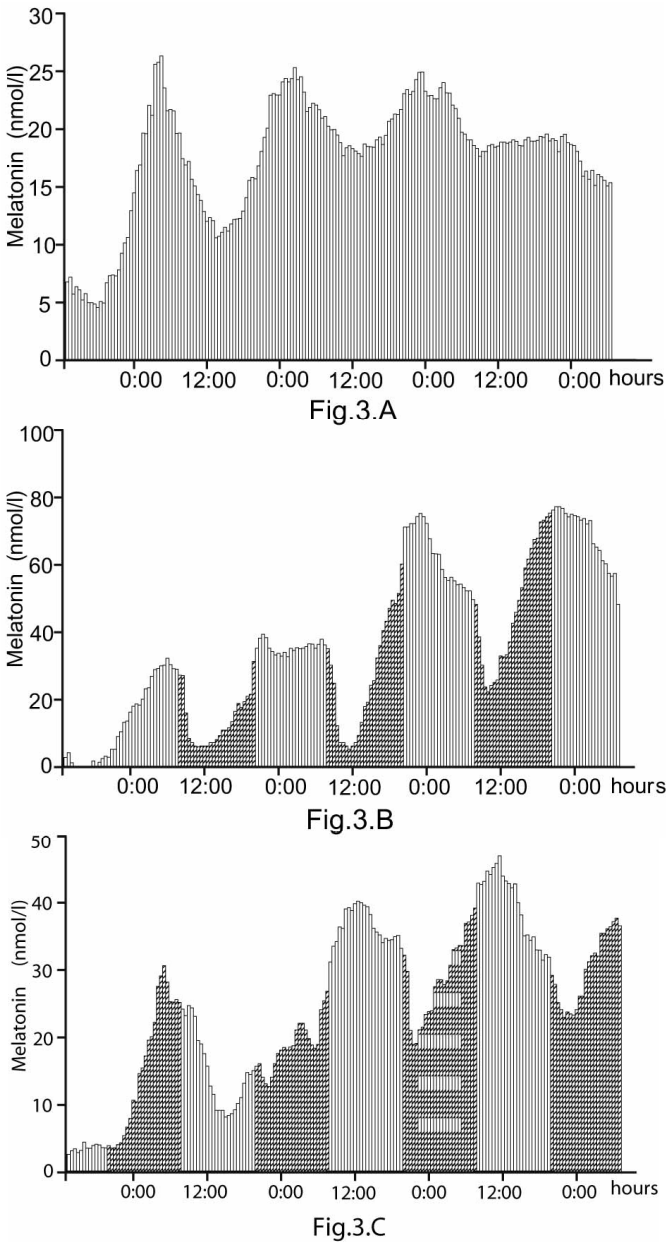

Figure 3. Melatonin secretion under continuous illumination
The chicken pineal glands were kept under continuous illumination in the perifusion system. In one part of the experiments the temperature was constant (white columns, A), in other cases it was elevated (41 °C, hatched bars) between 08:00 and 20:00 (B) or between 20:00 and 08:00 (C). Under combination of continuous illumination and constant temperature, the peaks became wider and the amplitude of the curve decreased (A), the N/D ratios were significantly smaller than that in the experiments under constant darkness (Fig. 1). In spite of the continuous illumination, the amplitude of the melatonin rhythm was not reduced when temperature was altered (compare A with B and C). When temperature was elevated during daytime, peaks appeared around midnight (B). The N/D ratio in experiment B was significantly different from that of experiment A. The phase of the melatonin rhythm was inverted by the nighttime elevation of the temperature (20:00 – 08:00) (C) similarly to that seen on Figure 2.A.

the secretion was minimal between 11:00 and 13:00. The first peak was always higher and appeared later (around 02:00) than the consecutive ones.

Melatonin secretion under constant darkness and 12 hour long periods of temperature elevation

The phase of the circadian melatonin secretion could be inverted by warming up the column periodically (from 37 °C to 41 °C), from 20:00 to 08:00 in just two days (*Fig.2.A*). During the last two days, the N/D ratios in these groups became smaller than one, and significantly smaller than the similar ratios in the control groups (*Table 1*, p=0.0028). In contrast, the phase could not be influenced by increasing the temperature from 08:00 to 20:00 (*Fig. 2.B*). The N/D ratios in the experiments shown on *Fig. A and B* were also significantly different from each other (*Table 1*, p=0.0334). Following an initial decrease, melatonin secretion started increasing during the warm period in both experiments.

Melatonin secretion under continuous illumination

When continuous illumination was applied under constant temperature (37 °C), the peaks became wider and started in early afternoon from the second day. The amplitude of the curve decreased due to a higher daytime melatonin secretion (*Fig 3.A*). The N/D ratios were around 1 (*Table 1*), and were significantly smaller than the ratios in the experiments under constant darkness (p=0.0068).

Experiments were also carried out under continuous illumination (*Fig. 3.B–C*). Daily, 12 hour long application of 41 °C on the perifused pineals abolished the effect of the continuous illumination; the amplitude of the melatonin rhythm did not become reduced (compare *Fig. 3.A with B and C*). The N/D ratios in experiments B and C were significantly different from those of experiment A (*Table 1.*, B vs. A: p=0.0433 and C vs. A: p=0.0331). Also, warming up the column during the night (20:00 – 08:00) inverted the phase of the melatonin rhythm (*Fig. 3.C*). The N/D ratio became smaller than 1 and significantly smaller than that in experiment B or in the control (constant temperature and darkness) experiments (C vs. B: p=0.0265 and C vs. control: p=0.0016).

Melatonin secretion under constant darkness with short (less than 12 hour) periods of temperature elevations

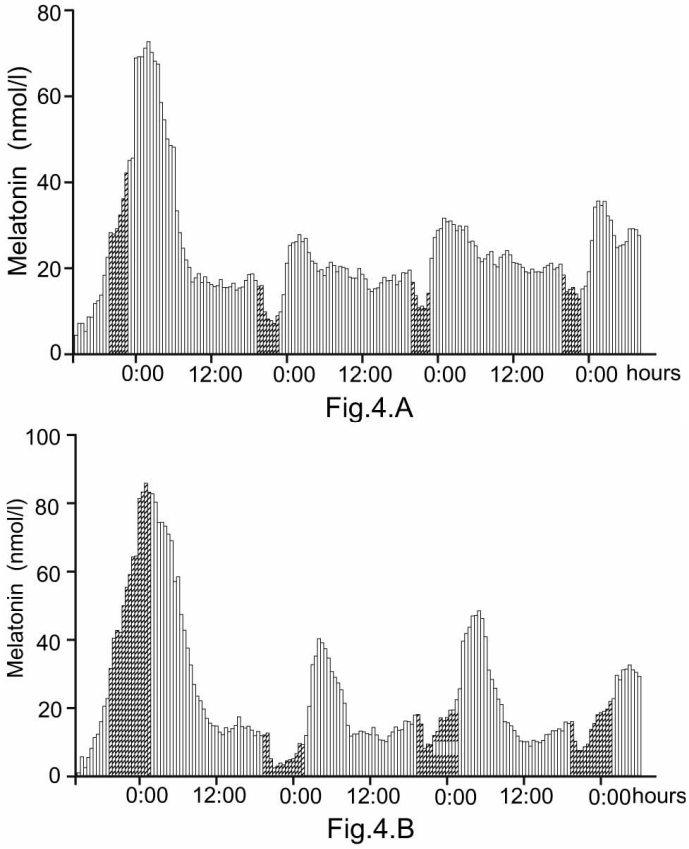

Figure 4. The effects of temperature elevations on the pineals of chickens younger than 14 weeks
In vitro melatonin secretion under periodic, 3 (A) or 6 (B) hour long elevation of temperature (41°C, hatched bars) and continuous darkness from the pineal gland of 9 weeks old chicken were monitored. 3 hour long (20:00 – 23:00) pulses of elevated temperature had no effect on the melatonin secretion on the first day. From the second day, the melatonin secretion decreased during the 41 °C period and melatonin did not increase in the afternoon. The nighttime peaks became reduced (A). Similar effects, with a slightly higher nighttime peaks, were observed under daily, 6 hour long (20:00 – 02:00) elevation of temperature on the pineal gland of 10 weeks old chicken (B). The variances of the melatonin contents in the samples, collected between 10:00 and 20:00, were significantly smaller in the graphs shown on Fig. 4. than in those presented on Fig. 5. and on Fig. 1 (constant temperature).

Table 2: Changes in the MT concentrations from10:00 to 20:00

		Constant temperature and darkness	From pineals younger than 14 weeks		From pineals older than 14 weeks	
			41°C for 3 hours	41°C for 6hours	41°C for 6hours	41°C for 8 hours
		Fig.1.	Fig. 4.A.	Fig. 4.B.	Fig. 5.A.	**Fig. 5.B.**
MT secretion 10:00–20:00 (n=20)	Average	17,4524	17,1908	12,8187	45,5136	44,1981
	Variance	9,2976	2,2598	2,8887	112,2567	54,1686
p values**	SEM*	0.6854	0.3361	0.3899	2.3691	1.6457
	Fig.1.	–	0,0016	0,0079	–	–
	Fig. 4.A.	0,0016	–	–	$2,5899*10^{-12}$	$1,8353*10^{-09}$
	Fig. 4.B.	0,0079	–	–	$7,0541*10^{-11}$	$3,2729*10^{-08}$
	Fig. 5.A.	–	$2,5899*10^{-12}$	$7,0541*10^{-11}$	–	–
	Fig. 5.B.	–	$1,8353*10^{-09}$	$3,2729*10^{-08}$	–	–

Note. * SEM stands for standard error of mean
** The analysis was carried out with F-test. Comparisons were made only in pairs of values with expected biological reasons.

The pineals of the chickens were exposed to elevations of temperature (from 37 °C to 41 °C) for three or six hour (from 20:00 to 23:00 or 02:00) on successive days (*Fig. 4.A and Fig. 4.B*). After the first peak, melatonin secretion decreased under the warm period, and increased thereafter. In pineals of chickens younger than 14 weeks, melatonin secretion did not increase in the afternoon, the alteration of the melatonin contents in the samples, collected during the day, decreased. Nighttime peaks were reduced and delayed.

Neither six nor eight hour long alterations of temperature could produce the same effect on the pineals of the chickens older than 14 weeks (*Fig. 5.A and B*). In these experiments, application of elevated temperature (41 °C) for 6 or 8 hours did not abolish the elevation of melatonin secretion at the afternoon, only temporary interrupted the peaks. In both cases, the peaks were shifted to between 06:00–07:00. The variances of the melatonin contents in the samples, collected between 10:00 and 20:00, were significantly smaller in pineals of young chicken (younger than 14 weeks) (*Fig. 4.A and B*) than in those from older

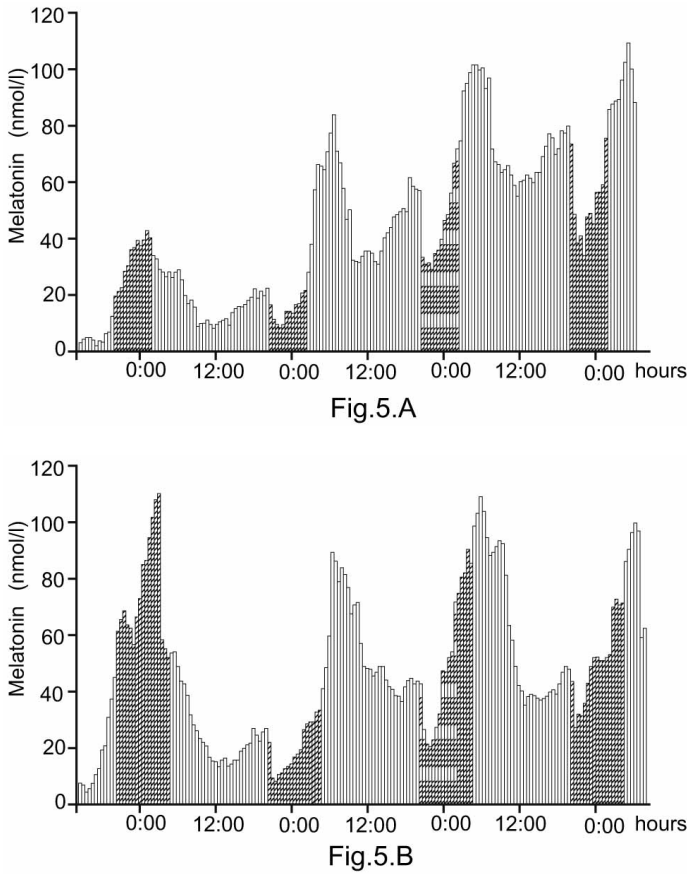

Figure 5. The effects of temperature elevations on the pineals of chickens older than 14 weeks
The temperature was elevated periodically for 6 (A) or 8 (B) hours (hatched bars). *In vitro* melatonin secretion from the pineal gland of 28 (A) and 15 (B) weeks old chicken is shown. The peaks were only interrupted and delayed, but not reduced, by the elevations of temperature. The daytime increase of the melatonin secretion was not abolished. The variances of the melatonin contents in the samples, collected between 10:00 and 20:00, were significantly higher in these experiments than in those shown on Fig. 4.

animals (*Fig. 5.A and B*) (*Table 2*, p<0.001 in each cases) or in the experiments under constant temperature (*Table 2*, p=0.0016 and p=0.0079).

Discussion

In accordance with our earlier data, chicken pineal gland continued circadian melatonin secretion *in vitro* under continuous darkness and constant temperature for several days (*Fig. 1.*) [9]. The first peak was regularly higher and appeared later in most of our experiments than the rest of the peaks. This is probably due to physicochemical influences affected the cells during explantation.

Periodic elevation of temperature for 12 hour during nighttime (started at 20.00 h), but not during daytime, slowly inverted the phase of the melatonin rhythm under constant darkness (*Fig. 2.A–B*). Barrett & Takahashi also found complete phase inversion if the 6 hours long elevation of temperature started at circadian time 14:00 [1]. Single temperature pulses in static culture, under continuous illumination with red light, did not induce complete phase inversion [30]. Based on these findings, we suggest that melatonin rhythm can be entrained by periodic changes in the environmental temperature under constant darkness.

An increase of average melatonin concentration was found in our experiments with more than 6 hour long periods of higher temperature (*Figures 2.A–B, 3.B–C, 5.A–B*). This can be due to nonspecific enzyme stimulation by the alterations of temperature. An increase of melatonin secretion under the second halves of both 6 and 12 hour long applications of elevated temperature may be explained by an adaptive mechanism of the pineals (*Figures 2.A–B, 3.B–C, 4.B, 5.A–B*). In most of our experiments, the elevation of temperature had no effects on the first peak. This can be explained by a transient decrease in the sensitivity of the pineal cells after the physicochemical influences during explantation (*Figures 2.A, 3.C, 4.A–B*).

Under continuous illumination and constant temperature, the amplitude of the melatonin rhythm decreased and the overall melatonin secretion increased (*Fig. 3.A*). It is suggested that continuous illumination either has desynchronizing effect on the clock mechanism, or results in continuous stimulation to the melatonin synthesizer apparatus [see also: 9].

Results of the experiments presented on *Figure 3 B–C* indicate that temperature and light can mutually influence each others effects. This is in accordance with the findings of Barrett & Takahashi, who showed a temperature dependent change in the sensitivity of the melatonin rhythm to light [2]. Our data show that melatonin rhythm can be entrained by periodic changes in the environmental temperature also under constant illumination. The rhythmic alteration of temperature may control the circadian oscillator in the avian pineal gland even under desynchronizing environmental effects. We also suggest that periodic environmental stimuli (in an appropriate phase) have an amplitude increasing effect on the rhythmic melatonin secretion. In the nature, the effects of various synchronizing factors are combined, may result in differences between the *in vivo* and *in vitro* data.

Inversion of the phase of the melatonin rhythm can also be due to an inhibition of the melatonin

synthesizing enzyme apparatus under 41 °C or to direct regulation of the circadian oscillator (*Fig. 2*). To achieve more information, we decreased the duration of elevated temperature to eight, six or three hour (*Fig. 4.–5.*). The type of the response was a function of the age of the animals. In perifused pineals of chickens younger than 14 weeks, the rhythm was also altered 16–18 hour after the elevation of temperature (*Fig. 4.A–B*). In contrast, three hour pulses of 41 °C were found ineffective by other authors [1]. It is possible that the changes in the melatonin secretion, presented on *Fig. 4.A–B*, indicate the affection of the circadian oscillator by the periodic elevation of temperature in pineals of young (less than 14 weeks) chickens. It was also suggested by Zatz that the effects of elevated temperature on the melatonin secretion are not mediated merely by changing the activities of the melatonin synthesizing enzymes [30].

Similar changes of melatonin rhythm shown on *Fig. 4.A–B* were not found if the pineal glands were removed from animals older than 14 weeks (compare *Fig. 4.* with 5.). The effects of elevated temperature on the pineals of these chickens can be a consequence of an acute inhibition of the synthesizing enzymes. Changes were described in the activity of aryl-amin-N-acetil-transferase (AA-NAT) following the alterations of the temperature [5, 13]. The presented data suggest that in chickens, younger than 14 weeks, temperature can be a synchronizing factor. On the other hand, temperature seems to have minor role in older animals. It is concluded that the sensitivity for temperature changes of the pineal cells is varying with age.

The calculated N/D ratios were found to be useful indicators of changes in the amplitudes of the curves. Generally, if the secretion is synchronized to the day, the N/D ratio was found over 1.4. If the secretion is desynchronized and the amplitude decreased, the ratio is around 1. If the phase shifted by at least 7 hours or is completely inversed, the ratio became smaller than 1. To compare the gradient of the peaks, the changes in the concentrations of the released melatonin during a specific period were analyzed with F-test. It is concluded that the statistical analyses, suggested by us, proved to be easy to use and describe adequately the changes in rhythmic graphs.

From these data we conclude that the *in vitro* melatonin rhythm can be entrained by periodic alteration of temperature under both constant darkness and continuous illumination. The rhythmic changes of the environmental temperature modifies the desynchronizing effects of constant light on the avian pineals in an age independent way. Melatonin rhythm of explanted chicken pineals can be synchronized by the periodic alterations of temperature only in young (less than 14 weeks) animals. In older chickens, changes of temperature seem not to modify directly the clock mechanism only show metabolic inhibitory effect. The sensitivity of pineal gland to temperature alteration changes around the 14th week of life, weeks before the sexual maturation. The alterations of the environmental temperature (primary the body temperature of the hen) may play important role in the synchronization of the melatonin rhythm before hatching. By aging daily changes in the illumination possibly take over this role of temperature.

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