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CONTRIBUTION OF FACTORS REGULATING MELATONIN RELEASE FROM PINEAL GLAND OF CARP (*Cyprinus carpio* L.) IN NORMAL AND IN POLLUTED ENVIRONMENTS

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A B S T R A C T. The study concerned the following issues: source and circadian melatonin concentration in blood of mature carp females during the spawning period, influence of a several days' decrease in light intensity, influence of long term fish feeding with zinc added fodder on the system: pineal gland - hypothalamus - pituitary gland - gonads.

It was demonstrated that the pineal gland was the only organ responsible for the presence of melatonin in the blood and that the sense of sight was indispensable only for the circadian synchronisation of the changes in hormone concentration. The melatonin level oscillated regularly over 24 h showing low values during day and high over night. A drop in light intensity evoked disturbances in the circadian rhythm of melatonin and of GtH2, resulting in a diminished efficiency of hormonal stimulation of spawning. Also a long term encumbrance of zinc salt perturbed the circadian rhythm of the two hormones, evoking changes in the central nervous system and, in the effect, retardation of gonadal activity during the spawning period.

Key words: PINEAL, MELATONIN, RHYTHMS, GONADOTROPIN, ZINC, CARP

INTRODUCTION

The pineal gland plays an important role as an endocrine organ in modulating functioning of the path: hypothalamus - pituitary gland - gonads (Reiter 1986). Melatonin in lower vertebrates, and in fish in particular, adapts and synchronises the organism to environmental rhythms (Vight et al. 1975), and - through hypothalamus - can to a greater or lesser degree inhibit or stimulate the processes related to maturation and reproduction (Joss 1973, De Vlaming 1975, Sundararaj and Kashavanath 1976, Popek 1991).

The pineal gland is considered to be the main source of melatonin synthesis; there are, however, published data indicating that in the case of mammals this hormone can be synthesised in other parts of the organism (Quay and Ma 1976, Gern and Ralph 1979, Mhatre et al. 1988, Bubenik and Dhanvantari 1989). It was estimated that the amount of extra-pineal melatonin in birds could reach from 9% (Reppert and Sagar 1983) to 33 % (Underwood et al. 1984). With regard to fish no unequivocal data exist

on the origin of melatonin circulating in the blood. Due to this the first research task was to identify the source of this hormone in blood as well as to investigate the circadian profile of melatonin concentration in the blood of mature carp females during the spawning period. The results of this study have given a basis for further investigations which should assess to what an extent an adversely altered environment could disturb the physiological equilibrium of an organism.

The available literature indicates that heavy metal salts (e.g. copper, cadmium, zinc) in mammals inhibit synthesis of the enzymes (HIOMT, NAT) responsible for melatonin metabolism (Morton 1987) and for its receptor affinity (Cardinali et al. 1979, Vacas and Cardinali 1980). These findings suggest that presence of metal cations (at a given concentration) in the environment can exert an immediate and indirect negative effect on melatonin metabolism and, as indicated in the earlier findings on carp (Popek 1991), also on gonadotropin level. In view of this the second research task was to find out whether a continuous influx of zinc salt administered with fodder can disturb physiological level of the two hormones in the blood of mature carp females and, consequently, affect the final phases of maturation and reproduction of this species.

Deterioration of water environment increases continuously; beside typical chemical contamination, waters carry more and more physical pollutants originating from earthwork, land reclamation, storms, etc., which considerably diminish water transparency. That is why the third research task was to check if a several days black-out (simulated in the laboratory) could adversely affect functioning of the system hypothalamus - pituitary gland - gonads, through changes in circadian melatonin concentrations and of gonadotropin (GtH2) in carp during the spawning season.

MATERIAL AND METHODS

All experiments were carried out during the spawning season using 100 sexually mature carp (*Cyprinus carpio* L.) females divided into groups and placed in concrete 2 m^3 tanks with flowing water. Aeration provided oxygen at the level of not less than 4 mg O_2 per litre. Temperature of water (22°C) and illumination (1500 lux at tank bottom) were electronically controlled and complied with the conditions in ponds in which the fish had been kept. The photoperiod L:D was 16:8 (light was switched on at 4 a.m. sharp).

Three research tasks were accomplished. In the first one, which aimed at the determination of melatonin circulating in the blood, 5 experimental groups were used as follows:

- fish after pineal gland removal (Px) using the method of Popek et al. (1994),
- fish after bilateral disruption of optical nerves and of eye blood vessels (Opthx),
- fish after the same intervention as above together with pinealectomy (Opthx + Px),
- control females subjected to a pseudo-operation (Sham), and
- intact fish.

In addition to this, the circadian profile of melatonin concentration in the blood of mature carp females was investigated during the spawning period.

In the second undertaking, light intensity was measured at the bottom of the fattening and of spawning ponds during sunny days (1500 lux) as well was after longer rainfalls (5 - 10 lux) using a waterproof probe and a luxmeter. The obtained results were simulated in the laboratory in order to evaluate influence of a 4 days' black-out (10 lux at the tank bottom during day) on the circadian profile of melatonin and GtH2 levels, and to assess female response to a stimulation of spawning by means of pituitary homogenate injections (0.8 mg/kg body weight).

The third task involved carp females which had been fed bruised barley mixed with 2.5 g of zinc ($ZnSO_4$) per kg of the fodder. Circadian changes of melatonin and GtH2 concentrations in blood were determined in these fish.

Blood samples were taken from the caudal vein of all treated fish using a heparinised syringe. Night sampling was performed in total darkness. For melatonin content determination, the blood was taken at 12 o'clock, then at 2 h intervals during the dark period, 2 h before the end of the lighting period, every 2 h during the dark period, 2 h after beginning of lighting, terminating at 12 o'clock the next day. (i. e. at 12, 18, 20, 22, 24, 2, 4, 6, and 12 h sharp). As to the GtH2 determination, blood samples were collected every 4 h, i.e. at 12, 18, 24, 6, and 12 hours. Melatonin level was estimated by means of the radioimmunologic method (RIA) using the sets of DRG 125 J-MELATONIN RIA (DRG Instruments GmbH). GtH2 levels were estimated using the enzymatic method (ELISA) (Kah et al. 1989).

The obtained data were subjected to a single classification analysis of variance and to Duncan's test and the results were presented in graphs showing mean values

and standard errors. In addition, the data were analysed by means of cosinor circles and ellipse of errors (Halberg 1967).

RESULTS AND DISCUSSION

During the first undertaking, the aim of which was to determine the source of melatonin, it was unequivocally shown that the pineal gland was the only organ responsible for the presence of melatonin in the peripheral blood, as the pinealectomy alone entirely eliminated melatonin from the circulating blood (below 0.2 pg/ml - the RIA method sensitivity). This result confirms the findings of Kezuka (1988) in goldfish, when the pineal gland removal eliminated circadian cycles of melatonin levels in blood. In the fish with bilaterally disrupted nervous and blood vascular contact with the eyes (preventing infiltration of melatonin to blood through the retina), a shift was disclosed of acrophase by 6 h towards the beginning of the dark phase (night). Maximum melatonin level in these fish (95.98 pg/ml) occurred at 22 h, but in the case of both control groups the maximum (average 145 pg/ml) was found at 4 h. The amplitude, i.e. deviations from the circadian average, in the fish deprived of the nervous and of blood-vascular contact with the eyes was substantially lower (Fig.1); there was no statistically significant difference between the control groups ($p<0.01$).

Experiments on the 24 h level of GtH2 in carp blood demonstrated that pinaelectomy had no effect on the circadian concentration of this hormone, and the main rhythm parameters like acrophase and amplitude were similar to those in the control groups. Disruption of nervous and blood-vascular connections of eyes with the organism brought about flattening of the amplitude, that is of the circadian curve of GtH2 release to blood. Lack of distinct differences between GtH2 levels at various times of the 24 h period may reflect a disappearance of the circadian rhythm. This conclusion was confirmed by the statistical analysis. The discussed intervention together with pinealectomy resulted in a shift of acrophase from noon hours (as in the control groups) to 18 h. Also, no statistically significant difference between the means ($p<0.01$) was revealed in these fish (Fig. 2).

Results of this experiment indicate unequivocally that the pineal gland in carp is the only place responsible for the dynamics of melatonin concentration in blood, whereas the retina of eyes synthesises melatonin for local needs and participates in synch-

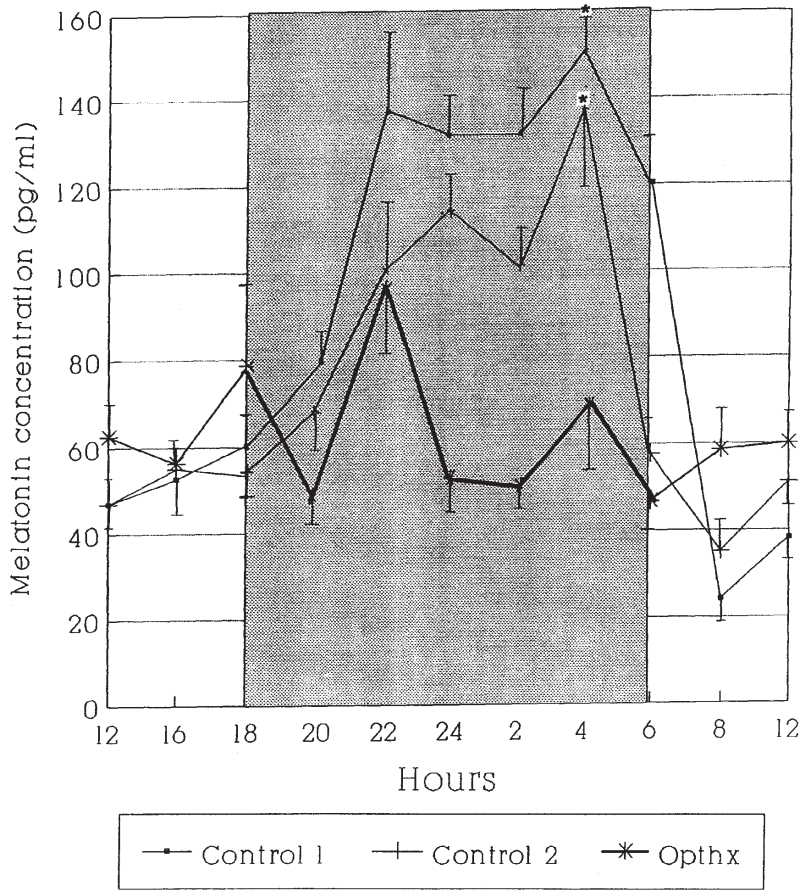
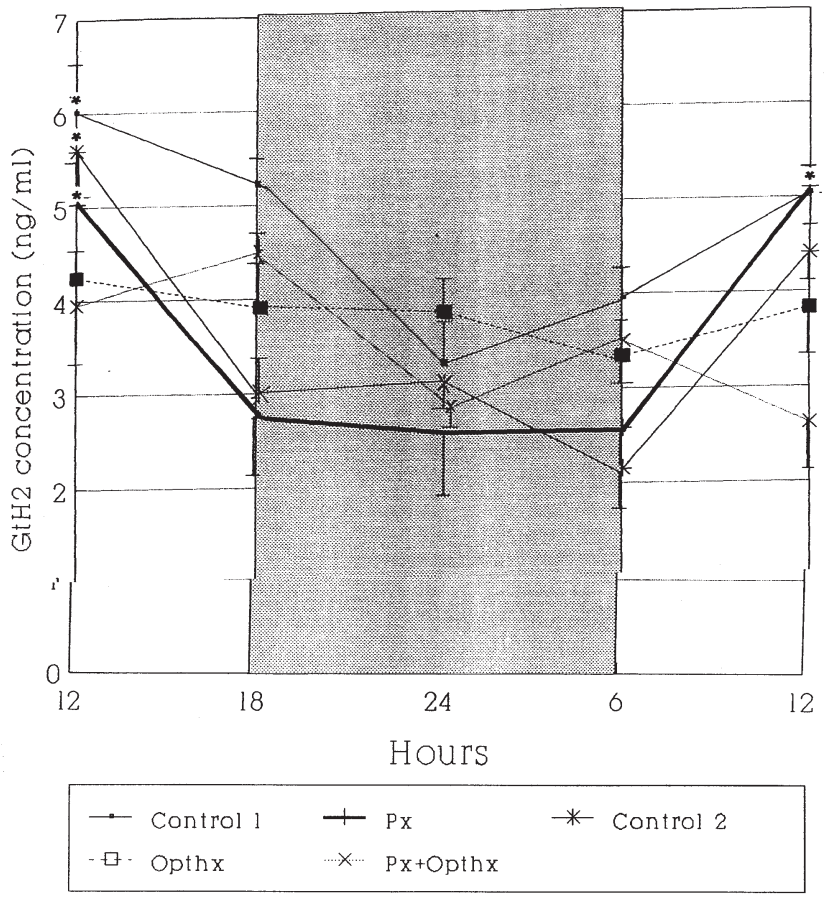


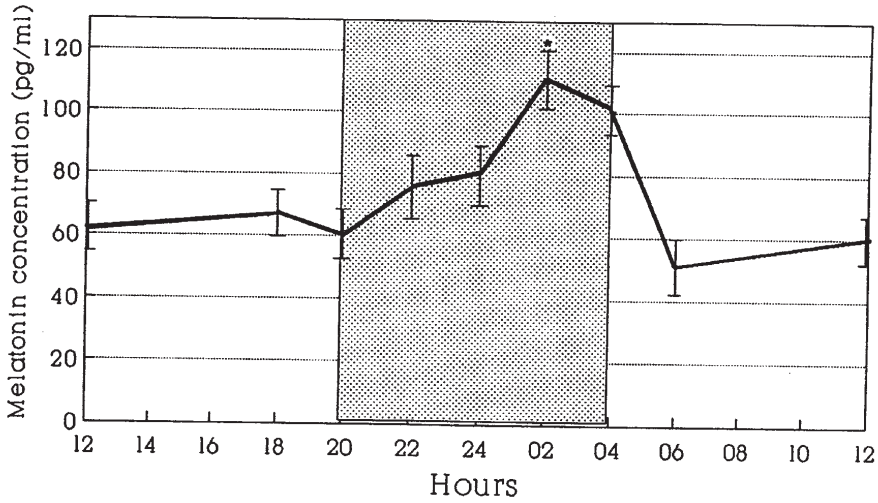
Fig. 1. Circadian changes of melatonin level in the blood of mature carp females (± standard error). Thin lines - control group (1) and pseudo-operated group (Sham)(2). Thick line - fish after bilateral disruption of optical nerves and of eye blood vessels (Opthx). In fish without the pineal gland there was no melatonin in blood. Shaded area - night time. Asterisks (*) indicate averages that are significantly ($p<0.01$) higher than those of day time.

ronising melatonin releasing from pineal gland, similarly to the same process in stickleback (Ekström 1987).

It was further demonstrated during the same undertaking that light, rhythmical changes of its intensity in particular, were the main factor controlling circadian fluctuations of melatonin concentration. In mature carp females during the spawning period, the fluctuations ranged from 50.43 (± 12.82) pg/ml during day time (6 o'clock) to 110.53 (± 15.67) pg/ml at night (2 o'clock a.m.). The two values differed significantly



($p<0.01$), and the analysis by means of cosinor circle and of ellipse of errors showed a circadian rhythm with the acrophase at 2^{15} o'clock (Fig.3). The average concentrations of melatonin in carp females during day were similar to those found in rainbow trout (Duston and Bromage 1986) and goldfish (Kezuka et al. 1992), whereas they were lower in carp during night hours. It follows that the pineal gland in fish can function as an internal clock and melatonin creates its hands; most of all its circadian levels in the organ itself, in the blood, as well as in the cerebrospinal fluid. The circadian changes of melatonin rhythm, reflecting the photoperiod changes, carry information



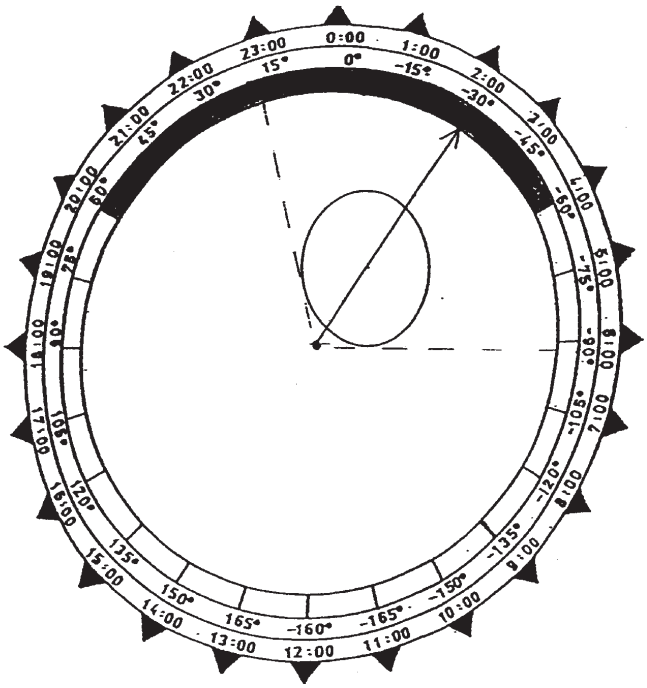
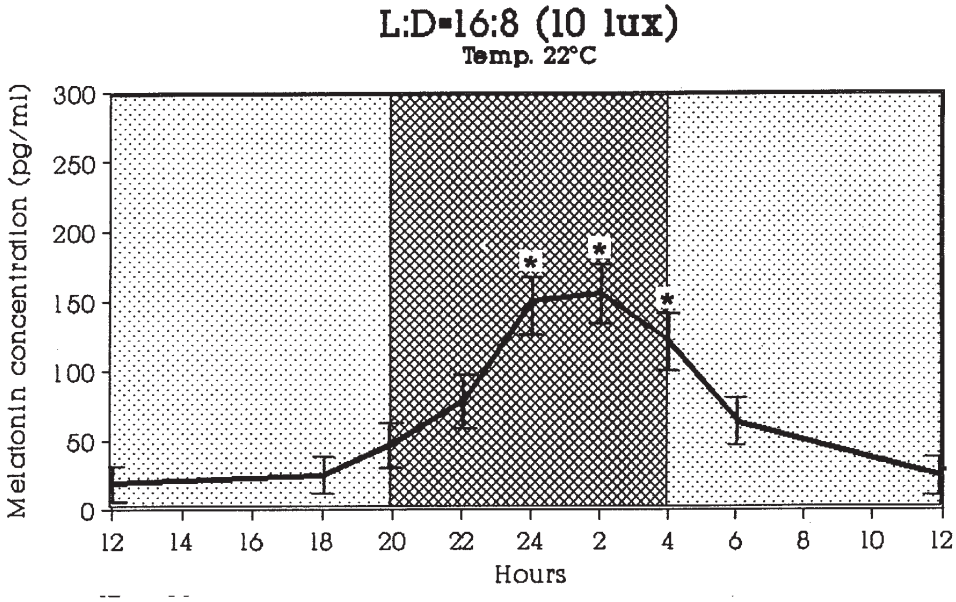
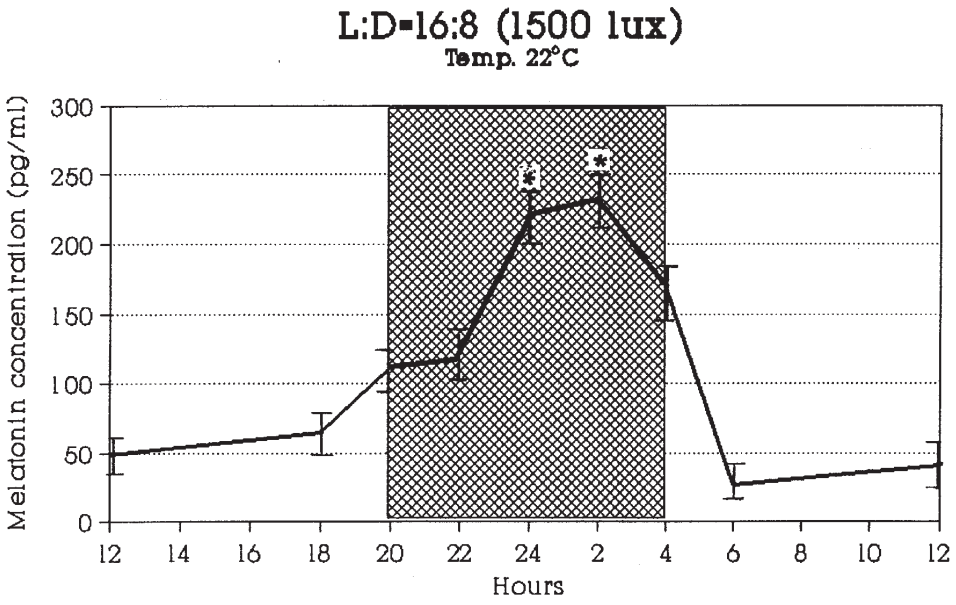


Fig. 2. Circadian changes of gonadotropin (GtH2) levels in the blood of mature carp females (± standard error). Thin lines - control group (1) and Sham (2). Thick line - Px. Broken line - (Opthx). Dotted line - Px + Opthx. Shaded area - night time. Asterisks (*) indicate averages that are significantly ($p<0.01$) higher than those of night time.


L:D=16:8 (10 lux)
Temp. 22°C
Melatonin concentration (pg/ml)
300
250
200
150
100
50
0
12 14 16 18 20 22 24 2 4 6 8 10 12
Hours


L:D=16:8 (1500 lux)
Temp. 22°C
Melatonin concentration (pg/ml)
300
250
200
150
100
50
0
12 14 16 18 20 22 24 2 4 6 8 10 12
Hours

on the time of day and of night, and determine time of the year (season) and synchronise the proper somatic and sexual development.

The sun light, as a "Zeitgaber", synchronises the internal melatonin rhythm, whereas lack of light causes the so-called rhythm drift, viz. shifting of the acrophase (maximum hormone level) resulting from shrinking or extending of the rhythm period. (Reiter 1991). In the second research undertaking, no shift of melatonin rhythm phases was found as the result of several days black-out (10 lux) of the fish tanks, as maximum hormone level (156.36 pg/ml) appeared at 2 h. However, there were significant differences between amplitudes of the two rhythms (Fig. 4). A limited light penetration brought about lower (by about 30 %) amplitude of melatonin changes than in the controls, especially during the dark period. The black-out resulted in desynchronisation of the gonadotropin rhythm (Fig. 5 and 6). Because melatonin is able to block the hypothalamic dopamine in neurones (Zisapel and Laudon 1983, Alexiuk and Vriend 1991), and dopamine plays the role of a hypothalamic factor which inhibits GtH2 release (Chang and Peter 1983), one can conjecture that the noted diminishing of the melatonin rhythm amplitude by almost 30 % in blood of the black-out group could generate the desynchronisation of GtH2 rhythm through a change of hypothalamic aminergic system. This was probably the cause of a total lack of response to the stimulation of females with a pituitary homogenate. On the other hand, ovulation occurred in the control group in 4 out of 7 (57%) females injected with the c.h.h.

These findings indicate that a decrease of light intensity during several days acts on the central nervous system through eyes and pineal gland, resulting in disturbances of circadian rhythms of melatonin and gonadotropin. Moreover, a decrease of oocyte response to gonadotropin and less efficient control of the reproduction of fish follows.

Data related to melatonin level in fish fed zinc added fodder (the third undertaking) indicate a desynchronising effect of zinc on circadian changes of the hormone in blood, because a maximum melatonin level (183.24 pg/ml) did not occur during dark hours (as in normal conditions) but during the second half of day (at 6 h). The second, though lower, peak of the hormone level (average 170 pg/ml) occurred in the middle of the dark period, between 22 h and 2 h. Lack of the circadian rhythm of these changes was confirmed by the analysis of mean errors in a cosinor circle (Fig. 7). With regard to the circadian GtH2 changes in fish fed zinc added feeds, disturbances of this hormone rhythm were also noted, as in the case of melatonin. Maximum GtH2 levels (average 14 ng/ml) was found over night (at midnight) as well as during daytime (at 6

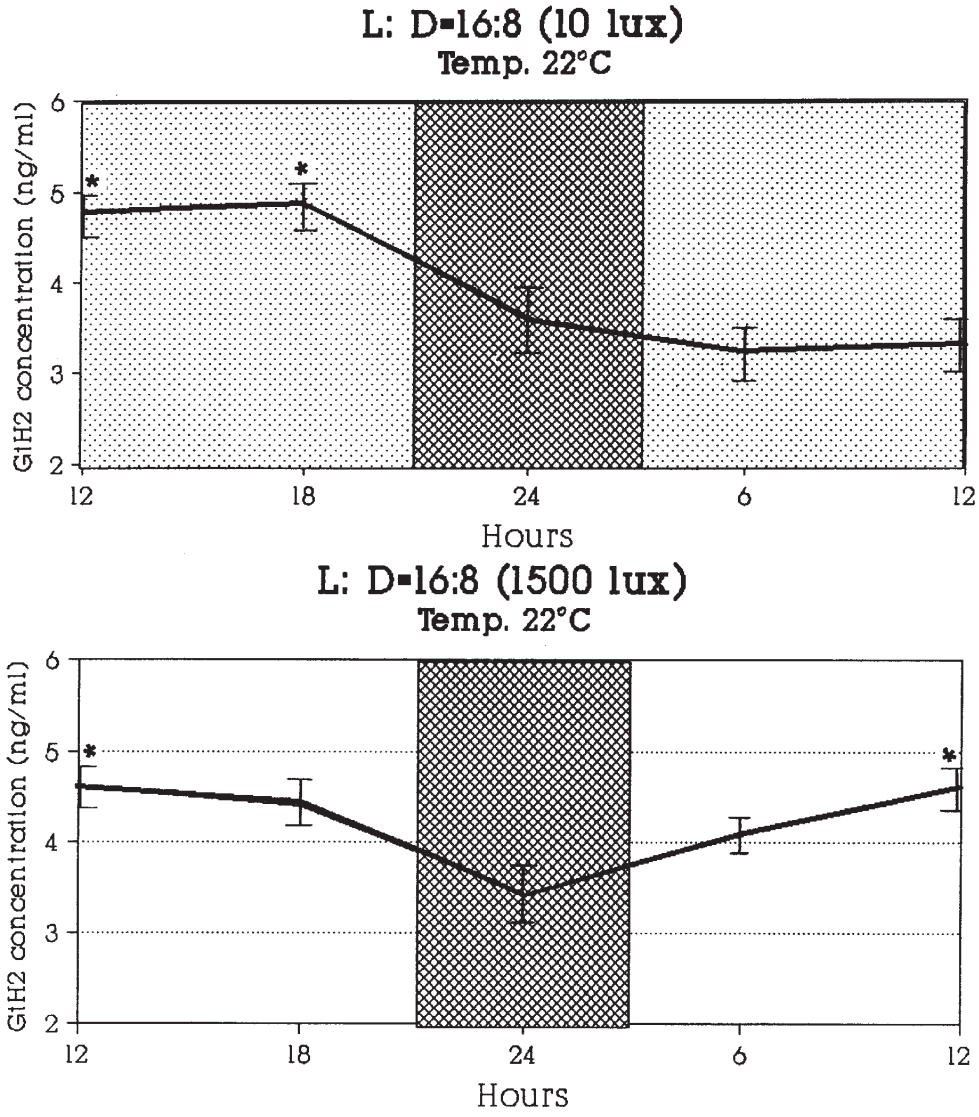


Fig. 3. Circadian changes of melatonin level in the blood of mature carp females. Upper part: - lines show averages (± standard error). Shaded area - night time. Asterisk (*) indicates averages that are significantly ($p<0.01$) higher than those of day time. Lower part: results are shown by means of cosinor circles. Location of the ellipse beyond the geometric center of the circle indicates a circadian rhythm. Shaded part of the circle - night hours. Arrow shows acrophase.

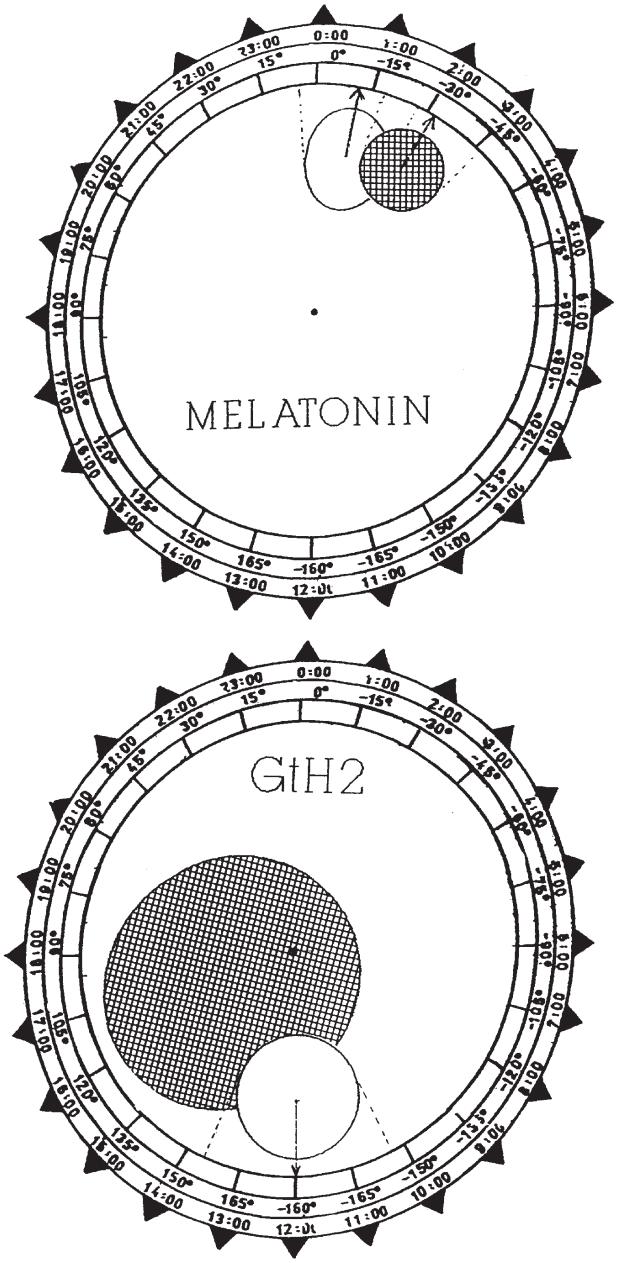


Fig. 4. Circadian changes of melatonin level in the blood of mature carp females, kept over 4 days in darkness (10 lux) - upper part, and in control fish (1500 lux) - lower pert. Lines show averages (± standard error). Light shaded area in the upper part - the black-out time, dark shaded area in both parts - night ti-

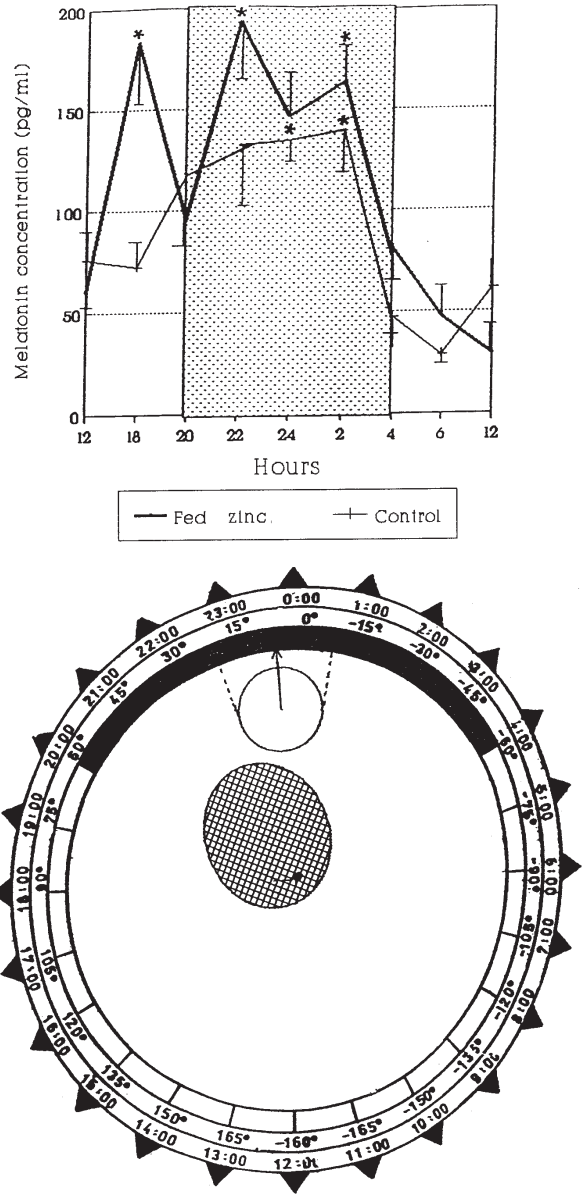


Fig. 5. Circadian changes of gonadotropin (GtH2) levels in the blood of mature carp females, kept in darkness (10 lux) over 4 days - upper part. The changes in control fish (1500 lux) - lower part. Lines - averages, ± standard error. Asterisks (*) indicate averages that are significantly ($p<0.01$) higher than the remaining ones. Other explanations as in Fig. 4.

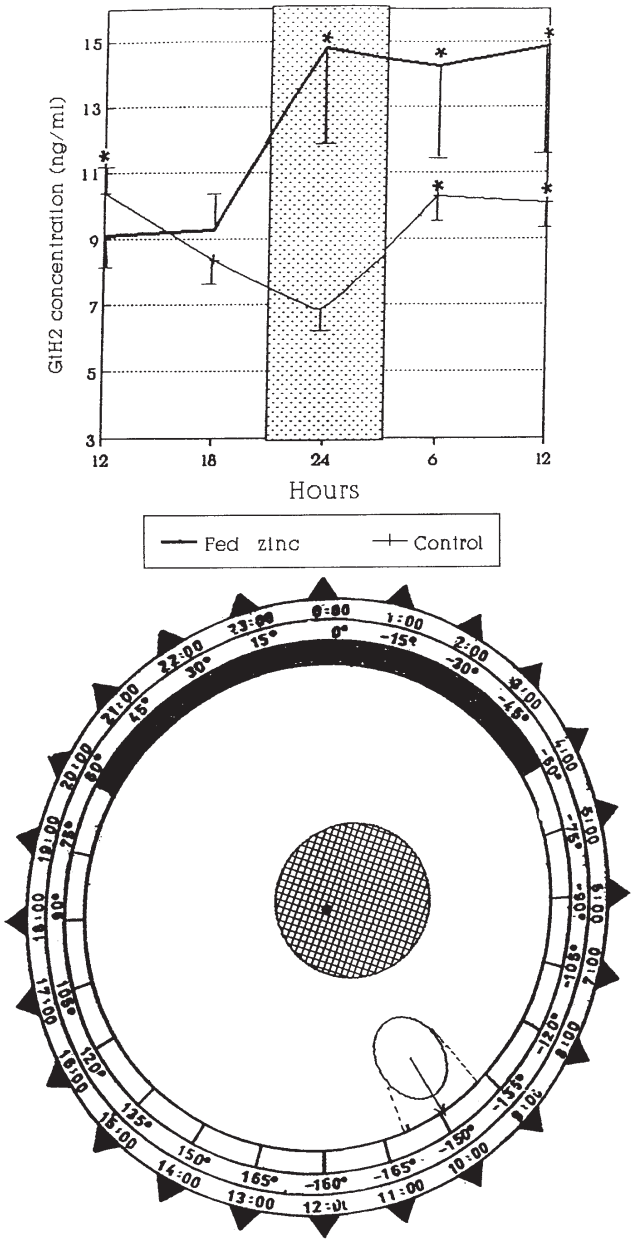


Fig. 6. Circadian changes of melatonin (upper circle) and of gonadotropin (lower circle) levels in the blood of mature carp females after 4 days in darkness (10 lux) - hatched ellipses, and in control fish (1500 lux) - empty ellipses. Other explanations as in Fig. 3.

h and at noon). It should be added that high GtH2 level at night was twice higher than in the control fish. Also the use of cosinor circle confirmed desynchronisation of this hormone circadian rhythm in females fed zinc added feeds (Fig. 8). These findings show that environment deterioration, apart from direct effects, can result in changes of the central nervous system by disturbing the endogenic rhythms of the hormones participating in hypothalami-hypophyseal control of the fish reproduction.

SUMMARY

The studies concentrated on two problems. The first, of a basic character, concerned identification of the place where melatonin was produced and the circadian changes of its concentration in the blood of mature carp females during the spawning period. Results of these investigations constituted a basis for further experiments.

The second problem referred to the effects of adversely altered environment on the central nervous system - the likely desynchronisation of synthesis and release of melatonin and gonadotropin during the spawning period.

It was demonstrated for the first time in carp that the pineal gland was the only gland responsible for the presence of melatonin in circulating blood. The sense of sight is indispensable only for a full synchronisation of rhythms and for maintaining their amplitude. Further experiments enabled to discover, also for the first time, the circadian rhythm of melatonin levels in the blood of mature carp females during the spawning period. They supplied evidence that the pineal gland could play a role of a biological clock, similarly as in mammals, which delimited the time of day and of night. A decrease of light intensity during the daytime disturbs this whole sensitive device, and if this occurs during the spawning period, it can diminish the efficiency of controlled fish reproduction. The stress evoked by the presence of heavy metals in fish body makes unstable the system pineal gland- hypothalamus-pituitary gland. Considering that it is a long term event, the stress can inhibit gonad functioning and subsequently result in a decrease of fish population abundance in polluted environments.

These findings have a definite practical aspect. They allow to formulate certain recommendations for the fishery practice, with a positive effect on cyprinid fish culture:

- particular care should be taken with regard to water purity (transparency) in ponds for keeping maturing fish and in spawning ponds in order to provide illumination of fish of no less intensity than 2000 lux, irrespective of adverse atmospheric conditions (e.g. storm water) or phytoplankton blooms,
- if the spawners are kept in indoor tanks before hormonally induced spawning, the possibility of installing a temperature control device should be considered in order to keep lower temperature at night and higher during daytime, as it is in nature, because such changes support the endogenic rhythm of the reproductive hormones,
- natural photoperiod in the indoor tanks, resembling that of the spawning period (for cyprinids the most often L:D = 16:8), should be maintained and illumination should be of no less than 1500 lux,
- injections to induce spawning should be made before noon because maximum gonadotropin concentrations and minimum melatonin levels occur at that time,
- water with zinc concentrations above 1 mg/l is not suitable for fish breeding because of an inhibiting effect of this element on maturation and reproduction; fattening only can be carried out in such waters.

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STRESZCZENIE

UDZIAŁ CZYNNIKÓW REGULUJĄCYCH UWALNIANIE MELATONINY Z SZYSZYNKI KARPII (*Cyprinus carpio* L.) W WARUNKACH PRAWIDŁOWEGO I ZANIECZYSZCZONEGO ŚRODOWISKA WODNEGO

W przeprowadzonych badaniach określono: źródło oraz dobowe stężenia melatoniny krążącej w krwi dojrzałych samic karpia w okresie tarłowym, wpływ kilkudniowego spadku natężenia światła oraz długotrwałego żywienia ryb konwencjonalną paszą z dodatkiem soli cynku na układ: szyszynka - podwzgórze - przysadka mózgowa - gonady.

W badaniach wykazano, że jedynym organem odpowiedzialnym za obecność melatoniny w krwioobiegu jest szyszynka, a zmysł wzroku konieczny jest jedynie do dobowej synchronizacji zmian stężenia hormonu. Poziom melatoniny w krwi nie jest stały, lecz zmienia się rytmicznie w ciągu doby, z niskim stężeniem hormomonu w dzień i wysokim w nocy. Spadek natężenia światła w dzień powoduje zaburzenia w dobowym rytmie melatoniny i GtH2, zmniejszając efektywność hormonalnej stymulacji tarła. Również długotrwałe obciążenie organizmu solami cynku zaburza dobowe rytmy obu badanych hormonów, prowadząc do zmian w obrębie centralnego systemu nerwowego, a w konsekwencji do hamowania aktywności gonad w okresie tarłowym.

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