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EFFECTS OF PINEAL GLAND AND MELATONIN ON MATURATION GONADOTROPIN (GtH2) SECRETION FROM PERIFUSED PITUITARY GLANDS OF MATURE CARP DURING SPAWNING

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ABSTRACT

The study, carried out during the spawning season, involved 30 mature carp females aged 5 years. The fish were divided into two experimental groups, those belonging to one of them being injected pimozide, a dopamine antagonist. The pituitaries and pineals were removed from all the females and perifused. Perifusion was carried out in pure medium alone, in the medium in the presence of the pineal gland, and in the medium enriched with dissolved melatonin. The perifusion proceeded in 2000 lux intensity light and in total darkness. Maturation gonadotropin (GtH2) concentration in the perifusion medium pumped through columns was determined with ELISA, an immunoenzymatic technique. The experimental treatments within each group showed no significant differences ($p>0.01$) with respect to the GtH2 concentrations in the perifusion medium. This result can be regarded as an evidence of a lack of any direct effect of the pineal gland and/or melatonin on GtH2 secretion from the carp pituitary during spawning.

Key words: pineal, melatonin, endocrine maturation control, pituitary, perifusion, carp.

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INTRODUCTION

The discovery and cloning of melatonin receptors [26] present on the mammalian hypothalamic neuroendocrine cell membranes and on those of the frontal pituitary secretion cells allowed to gain some insight into the role of those receptors in the reproduction physiology processes in which melatonin is involved [25]. As the mammalian brain contains a relatively low number of melatonin receptors, their numbers being much higher in the hypothalamus and in the frontal part of the pituitary gland [31], it is at present suggested that it is most likely the hypothalamus-pituitary axis that functionally connects melatonin and the endocrine system [14]. However, it has not been revealed so far where precisely the interaction takes place [11, 12, 13]. Similarly, the mechanism of melatonin action in the hypothalamus has not been hitherto elucidated. With regard to teleost fish, evidence on the presence of sites in the rainbow trout central nervous system, showing high affinity to 2-[^{125}I] iodinemelatonin, which signal the presence of melatonin receptors there, was reported in 1999 [15].

The pituitary is the major gland controlling reproduction in fish (as it does in mammals as well). The pituitary synthesises and releases gonadotropic hormones (GtH1, GtH2) which stimulate gonad growth, development, and maturation. Maturation gonadotropin (GtH2) secretion from the teleost pituitary is controlled by both stimulatory and inhibitory factors. The major hypothalamus-related stimulant of GtH2 release is GnRH [16]. The remaining stimulants include, i.a., noradrenalin [4, 17], serotonin [29, 30], neuropeptide Y [2], and opiates [27]. The GtH2 secretion inhibitors include mainly dopamine (DA) [3] and GABA [22].

The pineal melatonin, too, is associated with the photoperiod-dependent sexual cycle and reproduction in fish. As shown by the long-term research on carp, the hormone is important in controlling the reproductive seasonality by stimulating the final stages of sexual maturation and by synchronising the oocyte maturity with optimal timing of spawning [19]. Melatonin was also found to affect estradiol levels in mature carp females [21] and to indirectly influence the GtH2 levels [24].

As early as in the 1980's, pinealectomy was demonstrated to have a potential to stimulate or inhibit, depending on the photoperiod, gonadotropin secretion in goldfish [7]. However, as demonstrated in carp, the absence of the gland during spawning did not affect the blood serum GtH2 concentration, nor did it alter the circadian rhythm of the hormone's secretion [24]. In addition, other *in vitro* studies demonstrated that melatonin affected neither steroideogenesis nor, directly, oocyte maturation [20]. As can be then concluded, similarly to the case in higher vertebrates, the relationship between melatonin and GtH2 secretion is not clear, nor has the site of melatonin action been revealed. It is, however, suspected that melatonin affects GtH2 secretion via hypothalamic stimulatory (GnRH) or inhibitory (DA) centres [1, 18]. The neurohormones mentioned exert a very strong controlling influence on the secretory activity of the pituitary gland and, perhaps, interfered with attempts to unveil, *in vivo,* a direct relation of the pineal gland and/or melatonin with GtH2 secretion.

To corroborate or rule out any direct action of the pineal gland and melatonin on the maturation gonadotropin (GtH2) secretion from the mature carp female pituitary during spawning, *in vitro* experiments were performed. This approach was selected because it is only perifusion of the entire pituitaries in the presence of melatonin or the pineal gland that can rule out any indirect melatonin effect, i.e., its mediation in the action of other hypothalamic stimulants or inhibitors on the pituitary.

MATERIALS AND METHODS

The study involved 30 adult carp (*Cyprinus carpio* L.) females aged 5 years, originating from a culture kept at the Agricultural University in Cracow Fisheries Experimental Station. The experiments were carried out in June 1998. After capture, the fish were placed in 6 concrete flow-through 2 m^3 tanks (5 fish in each). Water in each tank was artificially aerated to maintain the dissolved oxygen content at no less that 4 mg O_2 dm^{-3}. Water temperature (22°C) and light regime (L:D=16:8; light of intensity>1500 lux switched on at 4:00 a.m.) were electronically controlled and adjusted to natural conditions in ponds the fish had been kept at. The 15 females in Group I were injected with pimozide (Sigma Chemical Co.) at a concentration of 5 mg kg^{-1} body weight; pimozide was dissolved in physiological salt solution acidified with 0.1 n CH_3COOH. The 15 females in Group II were injected with physiological salt solution only. After 4 days, the fish were narcotised with 1% ethylene glycol monophenyl ether (Merck-Schuchardt Co.) and decapitated; subsequently, their pineals and pituitaries were removed as rapidly as possible (within about 2 min) and placed on ice. Once the entire material had been collected, the pituitaries were at first placed in the physiological salt solution, to be subsequently transferred to biogel (P-2 Gel Fine, Bio-Rad Lab., France) in the perifusion column.

To perifuse the pituitaries, a special prototype continuous tissue perifusion device, consisting of 3 modules, was constructed. Each module contained five 2 ml perifusion columns to house the pituitaries on biogel. The perifusion medium consisted of buffered salt solution [8]. The 15-channel peristaltic pump used made it possible to maintain a continuous controlled flow (8 ml per hour flow rate) of the perifusion medium. Each set contained also a luxmeter sensor placed next to the perifusion columns, whereby the light regime during the experiment could be controlled. Two 11 W "Lival" halogene lamps provided uniform illumination of a set, producing light of a constant 2000 lux intensity. Wrapping a set in aluminium foil allowed to keep it in total darkness (0 lux).

Each of the 5 columns of the first set contained unaccompanied pituitaries (control treatment, PitC). Pituitaries accompanied by pineals were placed in the second set, each column containing one pituitary gland and 3 pineals (PitP treatment). The melatonin-enriched medium (300 pg ml^{-1}) was pumped through the third set columns containing pituitaries (PitMt treatment). Prior to the experiment, melatonin (Sigma Chemical Co.) was dissolved first in 5µl 96% ethanol and then in the physiological salt solution.

The pituitaries were rinsed with pure perifusion medium for 90 min. to establish a basal level of gonadotropin secretion; thereupon, they were incubated for 180 min. at 22°C. Illumination of 200 lux intensity was applied for the initial 60 min; the light was switched off during the subsequent 120 min during which time the columns were kept wrapped in aluminium foil to ensure total darkness (0 lux). The medium flowing through the column was sampled every 15 min. The medium samples were kept in the freezer (-20°C) before gonadotropin (GtH2) concentration was assayed with the ELISA immunoenzymatic technique [9]. The results obtained were subjected to the 1-way analysis of variance. Significance of differences between means was tested with Duncan's multiple range test.

RESULTS

Group I (pimozide-injected fish)

The GtH2 concentration in filtrates collected from the perifusion columns in which pituitaries were incubated in the pure medium (control treatment, PitC) was found to range from 30.22 (± 6.47) to 48.74 (±4.62) ng ml^{-1}.
The filtrates collected from those perifusion columns in which pituitaries were incubated in the presence of pineals (PitP treatment) were found to contain GtH2 in mean concentrations ranging from 24.69 (±6.68) to 34.91 (±4.75) ng ml^{-1}.

The filtrates collected from the perifusion columns in which pituitaries were incubated in the melatonin-enriched medium (PitMt treatment) were found to contain GtH2 in mean concentrations of 22.35 (±5.17) to 47.78 (±9.69) ng ml^{-1}.

No significant differences ($p>0.01$) in GtH2 concentrations between different treatments were observed.

Group II (non-injected fish)

The GtH2 concentration in filtrates collected from the perifusion columns in which pituitaries were incubated in the pure medium (control treatment, PitC) was found to range from 26.88.22 (± 7.45) to 42.71 (±13.17) ng ml^{-1}.
The filtrates collected from those perifusion columns in which pituitaries were incubated in the presence of pineal glands (PitP treatment) were found to contain GtH2 in mean concentrations ranging from 24.26 (±5.29) to 42.46 (±9.52) ng ml^{-1}.

The filtrates collected from the perifusion columns in which pituitaries were incubated in the melatonin-enriched medium (PitMt treatment) were found to contain GtH2 in mean concentrations of 13.53 (±2.98) to 28.6 (±5.83) ng ml^{-1}.

No significant differences ($p>0.01$) in GtH2 concentrations between different treatments were observed.
In Group I (pimozide-injected fish) treatment involving pituitary incubation in the melatonin-enriched medium (PitMt), the GtH2 concentrations recorded after 60 and 75 minutes of perifusion were found to be statistically significantly different ($p<0.01$) from the corresponding GtH2 concentrations in PitMt treatment of Group II (non-injected fish).

All the results (±SE) are shown in Figure 1 and Figure 2.

Figure 1. Mean (±SE) GtH2 concentrations in medium during perifusion of pituitaries alone (PitC), pituitaries in the presence of pineals (PitP), and pituitaries in the presence of melatonin (PitMt); the pituitaries were removed from pimozide-injected carp females (Group I). Perifusion was carried out under illumination (2000 lux) during the initial 60 min. and thereafter in the dark (60 to 180 min.; 0 lux).

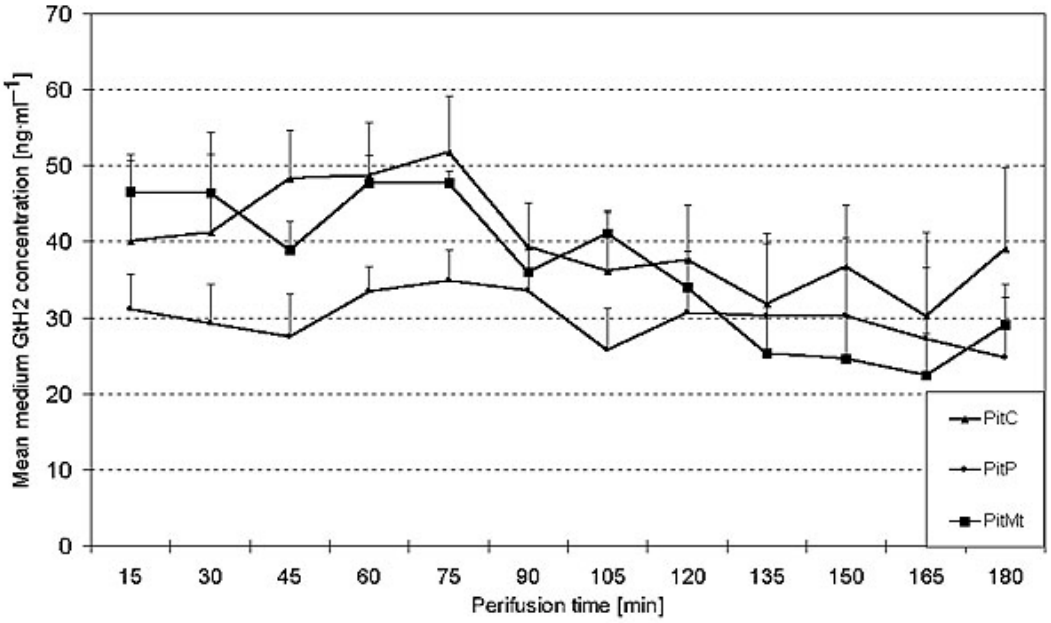
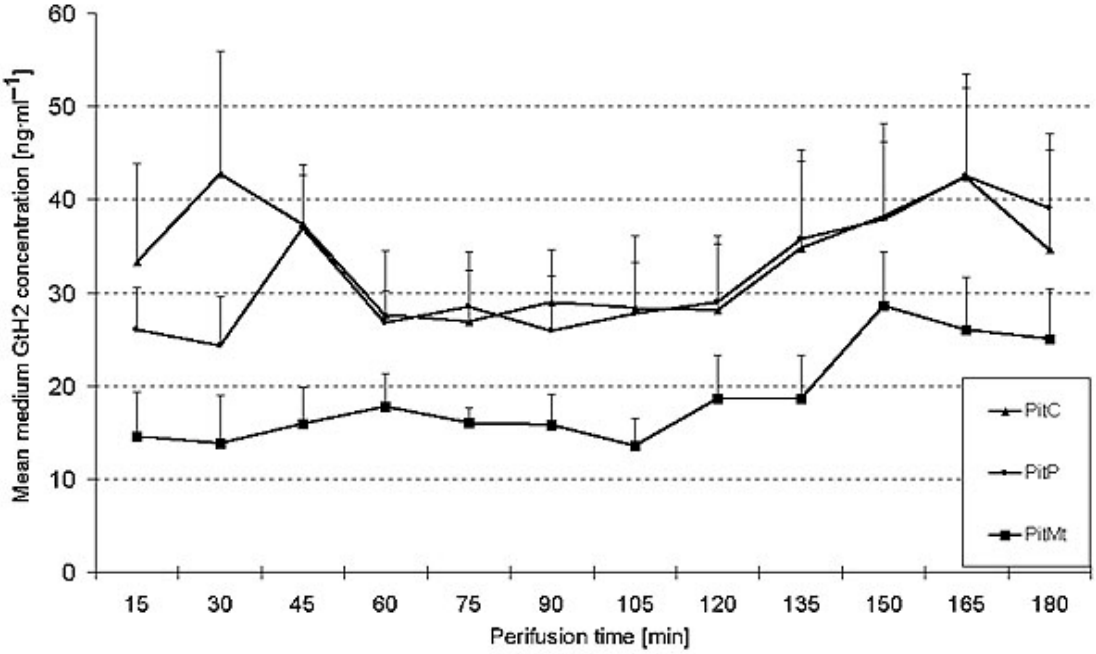


Figure 2. Mean (±SE) GtH2 concentrations in medium during perifusion of pituitaries alone (PitC), pituitaries in the presence of pineals (PitP), and pituitaries in the presence of melatonin (PitMt); the pituitaries were removed from non-pimozide injected carp females (Group II). Perifusion was carried out under illumination (2000 lux) during the initial 60 min. and thereafter in the dark (60 to 180 min.; 0 lux).



DISCUSSION

Perifusion of the entire pituitaries was used in this study as the method of choice because, as opposed to incubation if dispersed pituitary cells, the functional connections between dopaminergic and GnRH-releasing neurons on the one hand and gonadotropes on the other are left intact, which is of a key importance. In addition, as already mentioned in the Introduction, the selection of an *in vitro* method was dictated by the fact that it allows to observe in detail the behaviour of a tissue not controlled by an organism. In this way, a gland can be subjected to "stimulus-response" treatment, the response being independent of additional modifications resulting from, e.g., metabolism or feedback from gonadal steroids.

In the experiment described, the Group I females had been injected, a week before the actual observations, with pimozide, a specific DA antagonist. The injection was intended to block the D-2 DA receptors located in the pituitary gonadotropes, as dopamine is known to inhibit GtH1 secretion and release to the blood by blocking the adenomonophosphate acid (cAMP) formation [3]. Thus application of pimozide cut short the inhibitory effect of the hypothalamic DA which could have been maintained even during perifusion of the pituitary.

This was in fact corroborated by the results obtained. During the initial 120 minutes of perifusion, the GtH2 concentrations in those perifusion media which passed through the columns (PitC, PitP, and PitMt) containing pituitaries from the pimozide-injected fish were higher, by 13 ng ml^{-1} on the average, from the corresponding GtH2 concentrations in Group II (non-injected fish), the differences being statistically significant ($p<0.01$) after 60 and 75 minutes of perifusion. The data show that the inhibitory activity of the hypothalamic dopamine may continue even after hypophysectomy and that it is stronger than the stimulatory effect of GnRH.

However, the basic problem addressed in this work was the participation of melatonin in maturation gonadotropin secretion from the perifused carp pituitary. Owing to its photoreceptor cells, the pineal gland, when removed from the body, responds to changes in light intensity by changing melatonin secretion, i.e., acts identically as it does under normal physiological conditions [6, 10]. Thus during the second (dark) phase of the experiment, the pituitaries were perifused in the presence of endogenous melatonin. Analysis of the results demonstrated that, in the presence of the pineals (PitP), the pituitary GtH2 secretion profile in neither group was significantly altered either during the first (light) or the second (dark) phase of the experiment. No significant differences in GtH2 concentrations were revealed with respect to the control treatments (PitC). This is an evidence that endogenous melatonin at concentrations at least equal to the natural ones does not affect the *in vitro* GtH2 synthesis and secretion, regardless of the fact whether or not the fish had been earlier injected with pimozide (Figs 1 and 2).

Melatonin dissolves well in aqueous solutions and remains stable down to very low concentrations [28]. The ease with which melatonin is dissolved in body fluids may explain its presence in the cellular cytoplasm and its reaction with nuclear membrane receptors [5]. This facility was used to follow the pituitary reaction to exogenous melatonin.

The perifusion medium used in the PitMt treatments contained melatonin dissolved to the concentration of 300 pg ml^{-2}. In their earlier work, Popek et al. [23] showed that the concentration in question corresponded to the doubled maximum level of the hormone in the carp blood during summer. At the same time, the absence of melatonin-metabolising hormones in the perifusion medium allowed to maintain the constant melatonin concentration in the medium throughout the experiment. The perifusion medium mean GtH2 concentration in Group I (blocked dopaminergic system), amounting to 47 ng ml^{-1} at the beginning of the experiment, was shown to drop to 36 ng ml^{-1} after 90 min. and to 2 ng ml^{-1} at the end (after 180 min.). The corresponding GtH2 concentration in Group II (non-injected fish) was lower (ranging from 14 to 28 ng ml^{-1}), but – similarly to Group I – the mean concentrations did not differ significantly ($p>0.01$) from the mean GtH2 concentrations in both control treatments (PitC) (Figs 1 and 2).

As can be concluded from the above, melatonin cannot be, in all likelihood, associated with GtH2 secretion from the perifused pituitaries. The lack of association is perhaps related to the absence, or a very low number, of melatonin receptors on the gonadotrope membranes. There are no data on the location of those specific receptors. On the other hand, detailed studies on their distribution in the rainbow trout revealed their presence in the central nervous system (the thalamic region, pretectal area, and the optic tectum) and a total absence in the pituitary [15].

To sum up, the lack of exo- and endogenous melatonin-induced changes in GtH2 secretion regardless of whether or not the dopaminergic system was blocked points to the lack of any direct melatonin effect on GtH2 secretion from the carp pituitary during spawning. This hypothesis is supported by results of earlier studies [24] in which pinealectomy failed to alter the GtH2 blood level circadian rhythmicity. It should be then inferred that it is most probably in the hypothalamus where melatonin actually affects the fish gonadotropic activity.

CONCLUSIONS

Melatonin shows no direct effect on maturation gonadotropin (GtH2) secretion from the perifused carp pituitary during spawning.

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REFERENCES

1. Breton B., Mikolajczyk T., Popek W., 1993, The neuroendocrine control of the gonadotropin (GtH2) secretion in teleost fish. In: Aquaculture: Fundamental and Applied Research (B. Lahlou and P. Vitiello Eds.) pp. 199-215. American Geophysical Union. U.S.A.
2. Breton B., Mikolajczyk T., Popek W., Bieniarz K. Epler P., 1991, Neuropeptide Y stimulates *in vivo* gonadotropin secretion in teleost fish. Gen.Comp.Endocrinol. 84, 277-273.
3. Chang J.P., Peter R.E., 1983, Effects of dopamine on gonadotropin release in goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 49, 22-31.
4. Chang J.P., Van Goof F., Acharya S., 1991, Influences of norepinephrine, and adrenergic agonist and antagonist on gonadotropin secretion from dispersed pituitary cells of goldfish, *Carassius auratus*. Neuroendocrinol. 54, 202-210.
5. Costa E.J.X., Lopes R.H., Lamy-Freund M.T., 1995, Permeability of pure lipid bilayers to melatonin. Pineal Res. 19, 123-126.
6. Cwioro E., Popek W., Luszczek-Trojnar E., 1998, The effects of temperature on the melatonin secretion from superfused carp pineals in spawning time. 7th International Symposium of Polish Network of Molecular and Cellular Biology UNESCO/PAS "Molecular and Physiological Aspects of Regulatory Processes of the Organism". Krakow, 22 - 23 June 1998. Materials, 63 - 64.
7. Hontela A., Peter R.E., 1980, Effects of pinealectomy, blinding and sexual condition on serum gonadotropin levels in the goldfish. Gen. Comp. Endocrinol. 40, 168-179.
8. Jalabert B., Szollosi D., Fostier A., 1973, Étude comparé de l' action des hormones hypophysaires et steroides sur la maturation *in vitro* de ovocytes de la Truite et du Carassin (Poissons téléosténs). Ann. Biol. Anim. Biochim. Biophys. 13, 59-72.
9. Kah O., Pontet A., Rodriguez J., Calas A., Breton B., 1989, Development of an enzyme-linked immunosorbent assay for goldfish gonadotropin. Biol. Reprod. 40, 68-73.
10. Kezuka, H., Furukawa, K., Aida, K., Hanyu, I., 1988, Daily cycles in plasma melatonin under long or short photoperiod in the common carp, *Cyprinus carpio*. Gen. Comp. Endocrinol. 72, 296-302.
11. Lincoln G.A., 1992, Administration of melatonin into the mediobasal hypothalamus as a continuous or intermittent signal effects the secretion of follicle stimulating hormone and prolactin in the ram. J. Pineal Res. 12, 135-144.
12. Malpaux B., Daveau A., Maurice F., Locatelli A., Thierry J.C., 1994, Evidence that melatonin binding sites in the pars tuberalis do not mediate the photoperiodic actions of melatonin on LH and prolactin secretion in ewes. J. Reprod. Fertil. 101, 625-632.
13. Maywood E.S., Hastings M.H., 1995, Lesions of the iodomelatonin-binding sites of the mediobasal hypothalamus spare the lactotropic, but block the gonadotropic response of male Syrian hamsters to short photoperiod and to melatonin. Endocrinology 136, 144-153.
14. Maywood E.S., Bittman E.L., Hastings M.H., 1996, Lesions of the melatonin - and androgen-responsive tissue of the dorsomedial nucleus of the hypothalamus block the gonadal response of male Syrian hamsters to programmed infusions of melatonin. Biol. Reprod. 54, 470-477.
15. Mazurais D., Brierley I., Anglade I., Drew J., Randall C., Bromage N., Michel D., Kah O., Williams L.M., 1999, Central melatonin receptors in the rainbow trout: Comperative distribution of ligand binding and gene expression. J. Comp. Neurol. 409, 313-324.
16. Peter R.E., Trudeau V.L., Sloley B.D., 1991, Brain regulation of reproduction in teleost. Bull. Inst. Zool., Academia Sinica, Monograph 16, 89-118.
17. Peter R.E., Trudeau V.L., Sloley B.D., Peng C., Nahorniak C.S., 1991: Actions of catecholamines, peptides and sex steroids in regulation of gonadotropin-II in the goldfish. In: "Reproductive Physiology of Fish". (A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe Eds.), 30-34, Sheffield.
18. Popek W., Epler P., 1999, Effects of intraventricular melatonin microinjections on hypothalamic catecholamine activity in carp females during a year. Electronic Journal of Polish Agricultural Universities (www.ejpau.media.pl), 2 (1) Fisheries.
19. Popek W., Bieniarz K., Epler P., 1991, Role of pineal gland in sexual cycle in common carp. In: "Chronobiology and Chronomedicine" (J. Surowiak and M.H. Lewandowski Eds.), 99-102. Verlag Peter Lang, Frankfurt.

20. Popek W., Epler P., Sokolowska-Mikolajczyk M., 1996, Melatonin does not affect steroidogenesis or maturation of carp oocytes *in vitro* during the prespawning period. Pol. Arch. Hydrobiol. 43(3), 379-385.
21. Popek W., Galas J., Epler P., 1997, The role of pineal gland in seasonal changes of blood estradiol level in immature and mature carp females. Arch. Pol. Fish. 5(2), 259-265.
22. Popek W., Breton B., Sokolowska-Mikolajczyk M., Epler P., 1994, The effects of bicuculline (a GABA receptor antagonist) on LHRH-A and pimozide stimulated gonadotropin (GtH2) release in female carp (*Cyprinus carpio* L.). Comp. Biochem. Physiol. 108/1, 129-135.
23. Popek W., Epler P., Bieniarz K., Sokolowska-Mikolajczyk M., 1997, Contribution of factors regulating melatonin release from pineal gland of carp (*Cyprinus carpio* L.) in normal and in polluted environments. Arch. Pol. Fish. 5(1), 59-75.
24. Popek W., Breton B., Piotrowski W., Bieniarz K., Epler P., 1994, The role of the pineal gland in the control of a circadian pituitary gonadotropin release rhythmicity in mature female carp. Neuroendocrinol. Lett. 16(3), 183-193.
25. Reiter R.J., 1995, Functional pleiotropy of the neurohormone melatonin: antioxidant protection and neuroendocrine regulation. Frontiers in Neuroendocrinology 16, 383-415.
26. Reppert S.M., Weaver D.R., Ebisawa T., 1994, Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 13, 1177-1185.
27. Rosenblum P.M., Peter R.E., 1989, Evidence for the involvement of endogenous opioids in the regulation on gonadotropin secretion in male goldfish *Carassius auratus*. Gen. Comp. Endocrinol. 73, 21-27.
28. Shida, C.S., Castrucii, A.M.L., Lamy-Freund, M.T., 1994, High melatonin solubility in aquas medium. J. Pineal Res. 16, 198-201.
29. Somoza G.M., Peter R.E., 1991, Effects of serotonin on gonadotropin and growth hormone release from *in vitro* perifused goldfish pituitary fragments. Gen. Comp. Endocrinol. 82, 103-110.
30. Somoza G.M., Yu K.L., Peter R.E., 1988, Serotonin stimulates gonadotropin release in female and male goldfish, *Carassius auratus* L. Gen. Comp. Endocrinol. 72, 374-382.
31. Stankov B., Fraschini F., Reiter R.J., 1993, Melatonin receptors: distribution, biochemistry and pharmacology. In: "Melatonin: Biosynthesis, Physiological Effects and Clinical Application". (H.-S. Yu, R.J. Reiter, Eds), 155-186. CRC Press, Boca Raton, London.

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