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Effects of Photoperiod, Pinealectomy and Ophthalmectomy on Circulating Melatonin Rhythms in the Goldfish, *Carassius auratus*

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ABSTRACT—Effects of photoperiod, pinealectomy and ophthalmectomy on circulating melatonin rhythms were studied in the goldfish, *Carassius auratus*. Under light-dark (LD) 16:8 or LD 8:16 photoperiod, plasma melatonin levels exhibited diurnal rhythms with high titers during the scotophase and low titers during the photophase. When the fish were transferred from LD 12:12 to continuous dark conditions, plasma melatonin levels exhibited circadian changes during the first 3 days under continuous dark-ness (DD). The rhythms, however, became indistrinct during days 7–8 and 14–15. When the fish were transferred to continuous light conditions, plasma melatonin remained at low levels. Pinealectomy abolished high melatonin levels in the plasma at mid-dark, but ophthalmectomy did not. These results clearly indicate that circulating melatonin levels are photoperiod-dependent and shows circadian rhythms under DD conditions, and that the plasma melatonin rhythm is mainly generated by the pineal gland.

INTRODUCTION

The pineal gland of vertebrates synthesizes and secretes its indole hormone, melatonin (N-acetyl-5-methoxytryptamine) into the blood and cerebrospinal flud. Melatonin has been considered as the time-keeping hormone because of its cyclic appearance: melatonin levels in the pineal gland, blood, and cerebrospinal fluid fluctuate in a rhythmic fashion that is coincident with a given photoperiod. Under light-dark (LD) cycles, high meatonin titers were observed during the scotophase while low values were seen during the photophase in all vertebrate classes including fishes [1–13].

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The pineal gland of fishes is considered to be a circadian oscillator, since behavioral analysis revealed that the pineal gland is implicated in the control of circadian organization and rhythmicity in locomotor activities [14, 15]. In addition, endogenous rhythmicity in melatonin secretion from the pineal gland under continuous dark (DD) conditions *in vitro* has been reported in three teleost species, the pike (*Esox lucius*) [16], the gold-fish (*Carassius auratus*) [17, 18], and the white sucker (*Catostomus commersoni*) [19]. These results suggest that melatonin secreted from the pineal gland is playing an important role as an internal zeitgeber in controlling bioligcal rhythms in fishes.

Photoperiod is considered to be the most important environmental factor which modulates melatonin rhythms. Reports, however, on the effects of long or short photoperiod on circulating melatonin thythms in fishes were restricted to the rainbow trout *Oncorhynchus mykiss* [20] and the common carp *Cyprinus carpio* [12], and no report concerning endogenous rhythms of melatonin *in vivo*

under continuous dark (DD) or continuous light (LL) conditions is available.

Melatonin and the enzymes responsible for melatonin biosynthesis (i.e. serotonin N-acetyltransferase and hydroxyindole-O-methyltransferase) have been reported to localize in the retina (for review, see [21]). These results indicate that melatonin is synthesized not only in the pineal gland but also in the retina. Pinealectomy and ophthalmectomy experiments revealed that contributions of the pineal gland and the retina to circulating melatonin rhythms exhibited inter-species differences. In the Japanese quail, for example, the retina contributes to the blood level of melatonin and daily cycles of circulating melatonin in pinealectomized animals [22]. In the chicken, on the other hand, pinealectomy abolished circulating melatonin rhythms [23]. In case of teleost fish, melatonin synthesis in the pineal gland and the retina have been reported in several species [11, 13, 16–19, 24–26], but how these two tissues contribute to the circulating melatonin level is not fully understood.

The present study was conducted to examine the effects of photoperiod on circulating melatonin rhythms, and to confirm the main source of circulating melatonin in the goldfish.

MATERIALS AND METHODS

Experimental fishes Two-year old goldfish (*Carassius auratus*) were purchased from a local dealer. They were reared in indoor stock tanks at 24°C under natural photoperiod at The Fisheries Laboratory, The University of Tokyo (Maisaka, Shizuoka, Japan) until used. Fish were fed commercial trout pellets *ad libitum*.

Experiment 1 Ninety male goldfish weighing 50–150 g were used in this experiment. In October, 45 fish were transferred into each of 2 indoor experimental tanks, and acclimated under LD 16 : 8 (lights on 0400–2000 hr) or LD 8 : 16 (lights on 0800–1600 hr) at 24°C for 2 weeks. Blood samples were taken at 2–4 hr intervals (n=5) for twenty-four hr.

Experiment 2 Three hundred goldfish weighing 38–174 g were used in this experiment. In July, 150 fish were transferred into each of two indoor experimental tanks, and acclimated under LD 12 : 12 (lights on 0600–1800 hr) at 24°C for 2 weeks. Blood samples were taken at 1200 hr on Day 0 (57–97 g females, n=7; 53–110 g males, n=5) and at 0400 hr on day 1 (47–112 g females, n=6; 47–100 g males, n=5) to examine sexual differences in plasma melatonin levels. The light conditions were changed to DD or LL conditions from 0600 hr on day 1. Blood samples were taken every 4 hr (n=5) at 0800 hr on day 1 to 0800 hr on day 3 (days 1–3), at 1200 hr on day 7 to 1200 hr on day 8 (days 7–8), and at 1200 hr on day 14 to 1200 hr on day 15 (days 14–15).

Experiment 3 Forty-two glodfish weighing 72–154 g were used in this experiment. After the acclimation under LD 12 : 12 (lights on 0600–1800 hr) at 24°C for 3 weeks in August, they were pinealectomized (PINX, n=9), sham-pinealectomized (Sham, n=8), ophthalmectomized (EYEX, n=8), or pinealectomized and ophthalmectomized (PINX+EYEX, n=9). Pinealectomy and sham-pinealectomy were accomplished according to the method of De Vlaming [27] and ophthalmectomy was performed following the procedure of Fenwick [28]. Animals were identified by fin-clips. Intact control (INTACT, n=6) were only fin-clipped. One week after operation, blood samples were taken at middark (2300–0100 hr) and mid-light (1100–1300 hr, 36 hr after the sampling for middark).

Sample collection and RIA Blood samples were taken according to the procedure by Kezuka *et al.* [12] under anesthesia with 0.06% 2-phenoxyethanol in Exp. 1 or with 0.05% ethyl-*p*-aminobenzoate in Exps. 2 and 3. The anesthesia did not interfere with the RIA. Blood samples were centrifuged at 3000 rpm for 20 minutes, and plasma was stored at −20°C until the assay.

Melatonin levels in the plasma were measured by the RIA after a partial purification with Sep-pak C_{18} cartridge as previously described and validated for the goldfish plasma [12]. The minimum detectable level of the RIA was 32 pg/ml plasma in these experiments.

Statistics The difference of means was analyzed by ANOVA and Duncan's multiple range test, or by paired *t*-test.

RESULTS

Circulating melatonin rhythms under long or short daylength

Plasma melatonin levels exhibited distinct daily rhythms under both long (LD 16:8) and short LD 8:16) daylength as shown in Figure 1. The lowest levels were seen at 1900 hr under LD 16:8 and at 1500 hr under LD 8:16. Levels were observed to increase after lights-off, and remained elevated during the scotophase. The highest levels were observed at 0000 hr under LD 16:8 and at 0400 hr under LD 8:16. Subsequently, melatonin levels started to decrease, although the fish were still in the scotophase, and returned to the basal levels after lights were turned on.

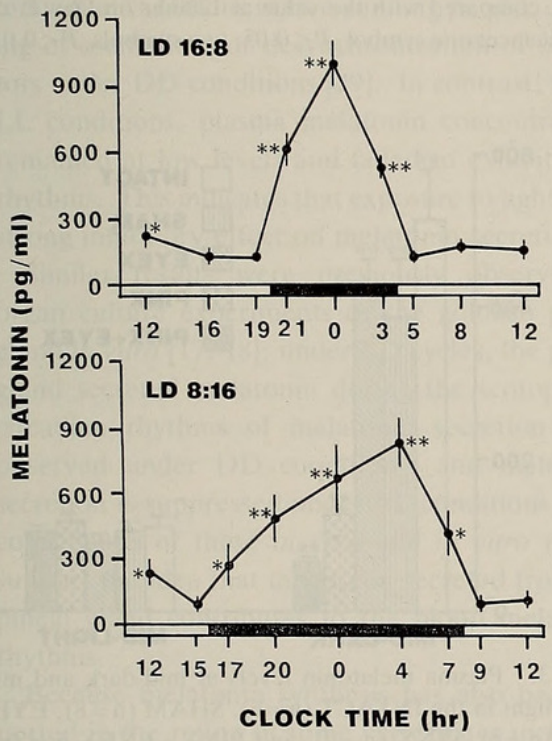

FIG. 1. Circulating melatonin rhythms under long (LD 16:8, upper) or short (LD 8:16, lower) photoperiod. Each point represents the mean ± SE (n = 5). Solid bars and open bars along the X-axis indicate the scotophase and the photophase, respectively. Significance: under LD 16:8, *, compared with the values during the photophase (1200, 1600 and 1900 hr on Day 1 and at 0500, 0800 and 1200 hr on Day 2); under LD 8:16, *, compared with the values at 1500 hr on Day 1 and at 0900 and 1200 hr on Day 2; ★, compared with the values at 1200 and 1700 hr on Day 1. Levels of significance: one symbol, $P<0.05$; two symbols, $P<0.01$.

Under LD 16:8, the values during the scotophase (2100 hr on Day 1 and 0000 and 0300 hr on Day 2) were significantly higher than those during the photophase (1200, 1600 and 1900 hr on Day 1 and 0500, 0800 and 1200 hr on Day 2) ($P<0.01$). Under LD 8:16, the values at 2000 hr on Day 1 and 0000 and 0400 hr on Day 2 were significantly higher than those at 1500 hr on Day 1 and 0900 and 1200 hr on Day 2 ($P<0.01$), and the values at 0000 and 0400 hr on Day 2 were significantly higher than those at 1200 and 1700 hr on Day 1 ($P<0.01$). The value at 0700 hr on Day 2 was significantly higher than those at 1500 hr on Day 1 and 0900 and 1200 hr on Day 2 ($P<0.05$).

Sexual difference in circulating melatonin levels

Plasma melatonin levels in females and males were 154 ± 18 and 154 ± 38 pg/ml at 1200 hr on Day 0, and 460 ± 48 and 667 ± 121 pg/ml at 0400 hr on Day 1 (mean ± SE), respectively. There was no detectable difference in plasma melatonin levels between sexes either during the photophase (at 1200 hr on Day 0) or during the scotophase (at 0400 hr on Day 1). Therefore, female and male fish were not separately dealt with in the following experiments.

Circulating melatonin rhythms under DD or LL conditions

Changes in plasma melatonin levels under DD or LL conditions are shown in Figure 2. Under the LD cycles used for the acclimation, melatonin levels exhibited day-night fluctuations: The values during the scotophase (0400 hr on Day 1) were significantly higher than those during the photophase (1200 hr on Day 0, $P<0.01$). When the fish were transferred into DD conditions, plasma melatonin levels exhibited circadian changes during the first 3 days with high levels during the subjective scotophase and low during the subjective photophase. The level at 0400 hr on Day 2 was significantly higher than tose at 1200 hr on Day 1 and those at 1200 on Day 2 ($P<0.05$), and the levels at 2000 hr

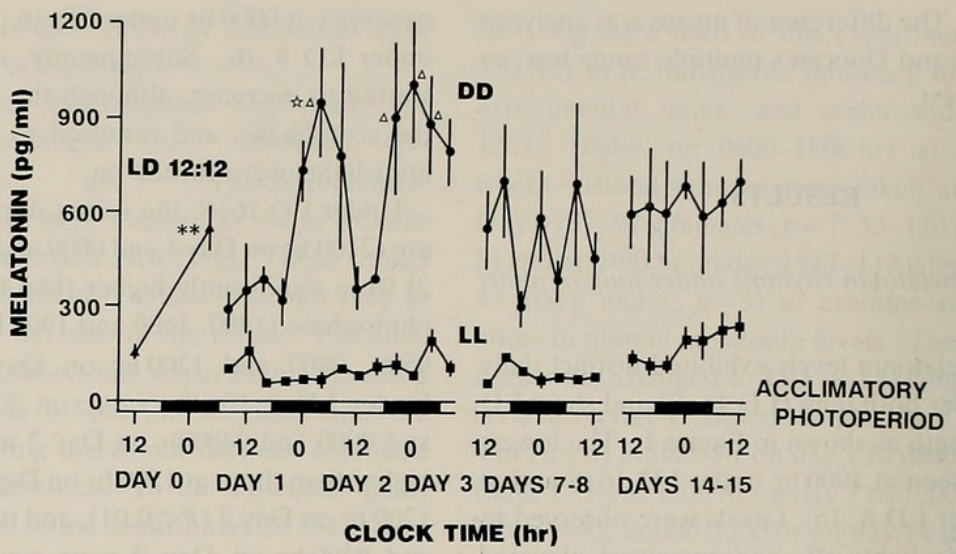

FIG. 2. Circulating melatonin rhythms under DD (●) or LL (■) conditions. Each point represents the mean ± SE (1200 hr on Day 0, n = 12; 0400 hr on Day 1, n = 11; under DD or LL, n = 5 each). Solid bars and open bars along the X-axis represent the scotophase and the photophase of the acclimatory photoperiod, respectively. Significance: *, compared with the value at 1200 hr on Day 1; ☆, compared with the value at 1200 hr on Day 1; △, compared with the value at 1200 hr on Day 2. Levels of significance: one symbol, $P<0.05$; two symbols, $P<0.01$.

on Day 2 and at 0000 and 0400 hr on Day 3 were significantly higher than those at 1200 hr on Day 2 ($P<0.05$). During Days 7–8, circadian-like changes in plasma melatonin levels disappeared. During Days 14–15, melatonin levels remained at elevated levels. In contrast, under LL conditions, plams melatonin levels remained at low titers throughout the experiment.

Effects of PINX and/or EYEX on circulating melatonin rhythms

Plasma melatonin levels at mid-dark and mid-light one week after the operation are shown in Figure 3. At mid-dark, melatonin levels in the PINX and PINX+EYEX fish were significantly lower than those in the Intact, Sham, and EYEX groups ($P<0.01$). No significant difference was observed among mid-light values of all groups.

In the Intact, Sham, and EYEX groups, melatonin levels at mid-dark were significantly higher than those at mid-light ($P<0.01$). There was no significant difference between values at mid-dark and mid-light in the PINX, whereas in the PINX+EYEX the value at mid-dark was significantly lower than those at mid-day ($P<0.01$).

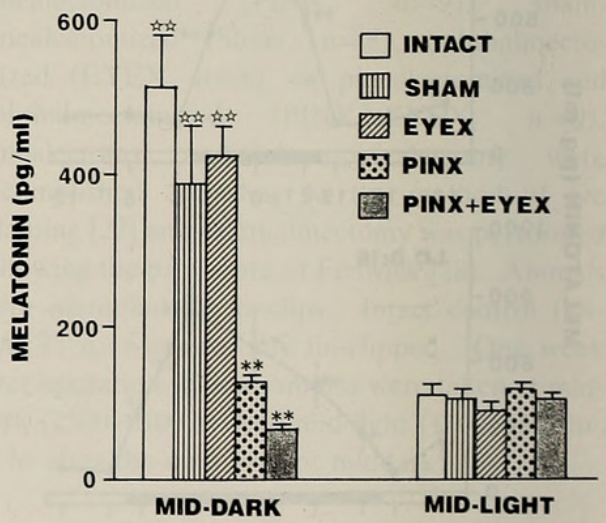

FIG. 3. Plasma melatonin levels at mid-dark and mid-light in the INTACT (n=6), SHAM (n=8), EYEX (n=8), PINX (n=9), and PINX+EYEX fish (n=9). Each point represents the mean ± SE. Significance: *, compared with the value in Sham at mid-dark; ☆, compared with the value at mid-light in each group. Levels of significance: two symbols, $P<0.01$.

DISCUSSION

In the goldfish, plasma melatonin levels exhibited clear daily rhythms both under long and short daylength; low during the photophase and

high during the scotophase with peak near mid-dark phase. Our results were basically the same as those reported in other vertebrate species [1–13]. These results indicate that daily fluctuations of melatonin in the body fluid are a common phenomenon in vertebrates and that photoperiod is one of the most important factors controlling melatonin rhythms.

Under DD conditions, plasma melatonin levels exhibited endogenous rhythms during Days 1–3; high melatonin levels were observed during the subjective scotophase and low titers were seen during the subjective photophase. This suggests that circulating melatonin rhythms are driven by a circadian oscillator. These rhythms disappeared during Days 7–8 and 14–15. Several explanations can be presented for this observation such as: large individual variations in free-running period, damping of oscillation, or desynchronization of oscillators under DD conditions [29]. In contrast, under LL conditions, plasma melatonin concentrations remained at low levels and failed to exhibit daily rhythms. This indicates that exposure to light has a strong inhibitory effect on melatonin secretion.

Similar results were previously observed in organ culture experiments of the goldfish pineal gland *in vitro* [17, 18]: under LD cycles, the pineal gland secreted melatonin during the scotophase; circadian rhythms of melatonin secretion were observed under DD conditions; and melatonin secretion is suppressed under LL conditions. The coincidence of these *in vivo* and *in vitro* results support the idea that melatonin secreted from the pineal gland contributes to the blood melatonin rhythms.

Because melatonin synthesis has also been reported in the retina of some vertebrates including fishes in addition to the pineal gland [5, 8, 13, 21–25, 30–33], we examined effects of pinealectomy and/or ophthalmectomy on circulating melatonin levels to confirm the main source of circulating melatonin in the goldfish. In the PINX and PINX+EYEX groups, high values at mid-dark disappeared, whereas in the EYEX group significantly higher values were maintained at mid-dark. These results clearly indicate that the pineal gland, and not the retina, is the main organ which secretes melatonin into the circulatory system in this species. We cannot, however, exclude the possibility that a small amount of melatonin is secreted from the retina. Although the difference is not significant, plasma melatonin levels at mid-dark were lower in the PINX+EYEX group that those in the PINX group. In addition, we recently found in the goldfish that melatonin contents in the eye also show daily fluctuations (Iigo and Aida, in preparation).

Interestingly, plasma melatonin in the goldfish was still at detectable level after pinealectomy and ophthalmectomy, and the plasma melatonin levels at mid-light was significantly higher than those at mid-dark in PINX+EYEX fish. This residual melatonin may be simply due to secretion from remnants of the pineal tissue after pinealectomy, and/or secretion from other parts of the body. The Harderian gland and the intestine have been suggested at the extrapineal-extraretinal source of melatonin in mammals and birds [31–33].

The pineal gland plays an important role as the photoneuroendocrine transducer in vertebrates, and melatonin secreted from the pineal gland into the blood is thought to serve as an internal zeitgeber. In seasonally breeding mammals, melatonin mediates photoperiodic information and the modulation of melatonin secretory profiles is involved in the determination of the reproductive season (for review, see [34]). In temperate-zone fishes, photoperiod is one of the most important factors determining the spawning season [35–37]. Photoperiod controls daily melatonin cycles in teleost fishes [10–13, 20] as well as in mammals [1–3], however, the relationship between seasonal reproduction and melatonin rhythms is still unknown. Further investigations will reveal the exact role of the pineal gland and circulating melatonin rhythms in teleost fish.

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