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Seasonal Variation in the Activity of Melatonin Synthesis in Cultivated Pineal Body of Fresh Water Teleost: *Zacco temmincki*.

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Abstract The seasonal changes in the activity of the melatonin synthsis were studied with the cultivated pineal body of *Zacco temmincki*. The activity of melatonin synthesis was measured by the radioactivity of the labeled melatonin secreted into culture medium using ^{14}C-5-hydroxy-tryptamine as a precursor.

Gonad somatic index of the female fish showed a big value from June through August and that of the male became maximum in June and had become small after that. The fishes that used in this experiment showed the delaied spawning season for one month than ordinary year.

The synthetic activity of melatonin in *Zacco temmincki* pineal body synchronized with light-dark cycle (LD 12 : 12) with low levels in the light period and high in the dark one. The difference of the activity between the light and dark was not seen from October through March. Seasonal change was detected in the synthetic activity of melatonin low in winter and high in summer. The rhythms of the melatonin synthetic activity in constant darkness were seen in only August and September, but not in other months through the year. These results indicate clearly that the circadian rhythm exists in the melatonin synthesis of the pineal body in *Zacco temmincki*.

Keywords: melatonin, seasonal variation, pineal body, teleost

Introduction

In all vertebrate species investigated so far, melatonin synthesis in the pineal body is active during the dark phase and low during the light phase (Armstrong, 1989; Bolliet et al., Cahill, 1996; 1994; Deguchi, 1979; Falcon et al., 1989, Filadelfi and Delaurocastrucci 1996; Iigo et al., 1991; Kezuka et al., 1989; Pang and Ralph, 1975; 1996; Underwood, 1985, 1989; Zachmann et al., 1992). An increase in the activity of the serotonin *N*-acetyltransferase (NAT) which converts serotonin to *N*-acetylserotonin results the nocturnal rise in melatonin synthesis (Klein and Weller, 1972; Falcon et al., 1989; Binkley et al., 1973). In the ectothermic animal, the effects of temperature on the melatonin production were studied, resulting in elevation of melatonin synthesis by raising temperature within a proper range (Iigo and Aida, 1995; Tilden and Hutchinson, 1993, Falcon et al., 1994).

In the fishes, pineal bodies possesses typical ciliary photoreceptor cells and are susceptible to light (Morita et al., 1989; Tabata and Minh-Nyo, 1989; Dodt and Meissl, 1982; Tabata, 1982), so that the sunlight affects directly on the melatonin synthesis in the pineal body of wild fish. Also, many species of fish have annual reproductive cycle, and the plasma levels of gonadal hormones and GtH are changed by season (Bromage et al., 1982; Kobayashi et al., 1986; Lou et al., 1984; Shimizu et al., 1987). It is conceivable that these hormone are exerting influences on melatonin synthesis in the pineal body (Preslock, 1977; Begay et al., 1994).

Although there are many reports about the circadian

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rhythm in the melatonin synthesis in the pineal body (Cahill, 1996; Bolliet et al., 1996; Molinaborja et al., 1996; Iigo et al., 1991; McNulty, 1982) and seasonal rhythms of melatonin production (Wehr, 1997; Iigo and Aida, 1995), there are few reports on the rhythmical variation through 1 year. It is speculated that the circadian rhythm of melatonin biosynthesis in the pineal body changes by the season. It was investigated whether the activity of the melatonin synthesis changes by the season, and also whether the circadian rhythm itself changes by the season in the present study using the cultured pineal body of fresh-water fish *Zacco temmincki*.

MATERIALS AND METHODS

Animal

In the experiment, teleost fish (*Zacco temmincki*) with body weights ranging from 10g to 50 g, were used. These fishes were collected from the brook of the Matsue-shi suburbs in the middle of every month (from April, 1997 to March, 1998). They were maintained for no more than 2 hours in oxygenated water in the laboratory room not air conditioned. The pineal organs were dissected from the fish sacrificed by decapitation during 16：00 ～ 18：00. And also, the gonad weight in each individual was measured in order to calculate gonad somatic index (GSI: gonad weight x 100 / body weight) as the guideline of the natural maturity of *Zacco temmincki*.

Organ culture

Pineal bodies dissected from fish were made to adhere individually to the small piece of the filter (pore size : 0.8 μm, filter type:AA, Nihon Millipore Ltd.), immediately placed in 100μl of culture medium GIT (Wako Pure Chemical Industries, Ltd.) supplemented with 1μl of antibiotics (penicillin : 5U, streptomycin : 0.5μg), and kept at water temperature of the collection river (the mean value of the water temperature for 3 days before the experiment in each month) (Fig. 1) until the commencement of organ culture at 18：00. Pineal bodies were kept at that temperature with continuous shaking in an incubator during organ culture.

The light condition was performed under light－dark cycle (lights on 6：00, lights off 18：00) during 72 hours from the commencement of organ culture, and then kept constant darkness for 84 hours until light on at 6：00. A fluorescent light (10 W) illuminated the surface of culture medium with 500lx was used for the lighting. The incubation for 2 days (48hr) from the start of organ culture was regard as preincubation. During preincubation, the culture medium was exchanged with fresh medium at 12：00 and 24：00. The measurements of melatonin synthetic activity was started from the 3rd day.

Each sample was referred to as LD (n), LD (d), DD 1 (n), DD1 (d), DD2 (n), DD2 (d), DD3 (n), DD3 (d), DD4 (n) and LL4 (d) in order from the beginning. LD, DD and LL mean that light-dark cycle, constant darkness and constant light respectively, and (d) and (n) is showing that time is the 6：00～18：00 and 18： 00～6：00 each.

Measurement of the synthetic activity of the melatonin

The melatonin synthetic activity was measured by the radioactivities of ^{14}C-melatonin secreted into the culture medium after addition of ^{14}C-5HT (5-hydroxy [side-chain -2-^{14}C] tryptamine creatinine sulphate, Amersham, specific activity : 2.07 GBq/mmol) as the melatonin precursor. Through all the experiments, 5μl (1.85MBq/ml) of radiolabelled serotonin was given into the each culture fluid at 11：00 and 23：00.

The pineal bodies were cultured for additional 2 hours in the presence of ^{14}C-5HT. The medium of each culture tubes was collected, before being replaced with fresh GIT 100μl containing 1μl of antibiotics. These samples were kept at -30 ℃ in the freezer until extraction of melatonin. Dim red light was used to perform these procedures in the dark period.

Melatonin kept in freezer was extracted into the 1 ml chloroform by vigorous shaking. After removal of the aqueous phase, the organic phase was evaporated to dryness by centrifugal evaporator. The evaporated residue was dissolved with 50 μl chloroform and spotted on thin-layer chromatography (Silicagel 70FM Plate : Wako Pure Chemical Industries, Ltd.). This plate was developed in a solution of ethyl acetate and dried in air. Synthesized ^{14}C-melatonin was quantitated using TCL linear analyzer (TCL Multi Tracemasters LB284 : Berthold Co. Ltd.).

All results are expressed as radioactivity (cpm) of ^{14}C-melatonin synthesized in every 1mg of pineal body for

2 hours.

Statistical analysis

Results were expressed as mean ±SE. The difference between mean values was statistically analyzed with one -tailed paired sample by Student t-test.

RESULTS

Seasonal changes of GSI

The relative values of weights of gonad to whole body expressed as GSI were very high in female during May to August (Fig. 2). Although the figure in July decreased but not significantly different from that of May, June and August. The high values in these months would reflect the continuous growth of eggs in ovaries. The high index decreased drastically in September. This indicates that these female fishes laid eggs before the end of August. On the other hand, the values of male fishes were high during summer but not so much. The GSI values of female and male were low and near constant during September to April. Anatomical judgement of sex was difficult in September and October but became possible after December.

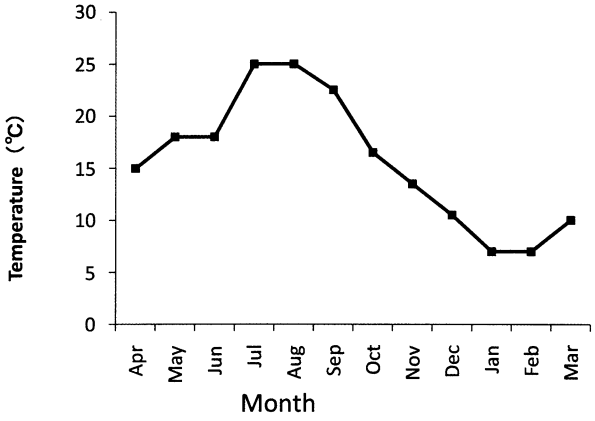

Fig. 1. Change of the water temperature at the river of the collection point. The mean value of the water temperature for 3 days before the experiment in each month is shown.

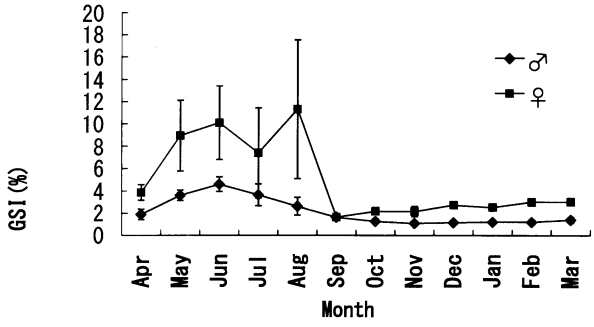

Fig. 2. Seasonal changes in gonad somatic index (GSI) of male and female Zacco temmincki during the reproductive cycle. Data are represented as mean of 5 fish ± SE.

Activity of the melatonin synthesis

Activity of the melatonin synthesis became high from July and maintained a high level until September (Fig. 3, Fig. 4). The level became low in October and maintained

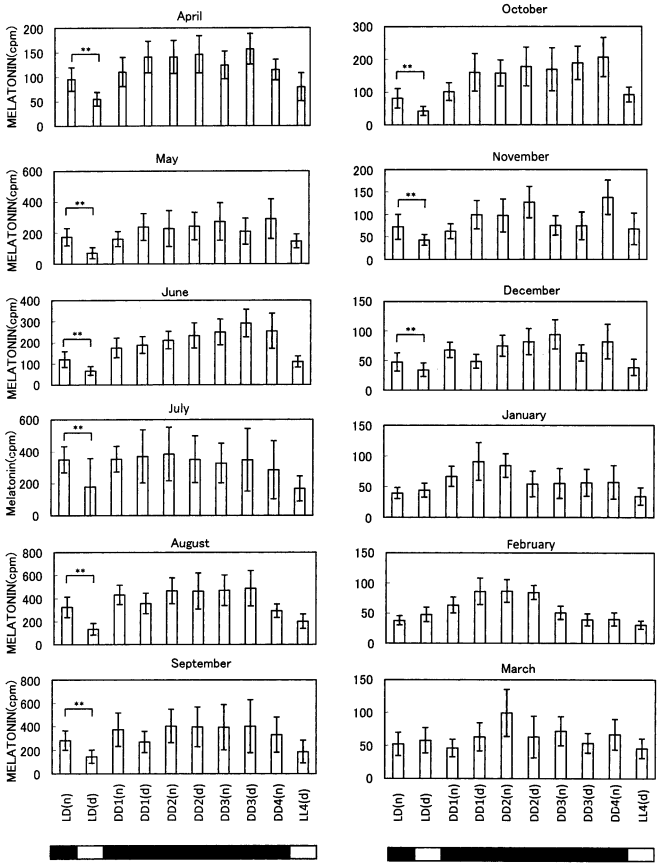

Fig. 3. Seasonal change of the melatonin synthetic activity. Pineal bodies were isolated during the light period and preincubated in light-dark cycle for 2 days at river temperature. After preincubation, experiments were started at the beginning of the dark period. From after 24 hours the pineals were exposed under the DD condition, and finally to the light condition. The melatonin synthetic activity is expressed with radioactivity (cpm) of ^{14}C-melatonin produced for 2 hours per 1 mg of pineal body. Values represent the mean ± SE (n=10). The lighting conditions are diagrammed at the bottom of the figures; the filled bars correspond to the darkness. LD (d) values compared with the LD (n) values by Student t-test gave *p<0.05, **p, 0.01.

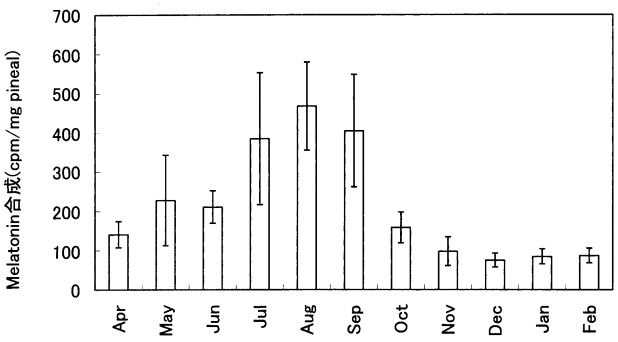

Fig. 4. Seasonal change of melatonin synthetic activity. The values (mean ± SE; n=10) represent with radioactivity of ^{14}C-melatonin that was produced during 2 hours after 29 hours from the start of continuous darkness.

a low value after that. This change was similar to that of cultivation temperature (the water temperature of the river) (Fig. 1).

The melatonin synthesis in the pineal body of this fish became active at night (scotophase) and was suppressed in daytime (photophase) (Fig. 3). There were not big fluctuation on the suppression of the activity of the melatonin synthesis to light (showing in the figure as proportion of the melatonin synthetic activity in light period to it in dark period during light-dark cycle) until November (Fig. 3, Fig. 5). In the period from January through March, this suppression by light was not obvious because the activity of melatonin synthesis with the dark period (DD) became very low (Fig. 3, Fig. 5).

The circadian rhythms of melatonin synthetic activity were observed in August and September, but not in other months (Fig. 3, Fig. 6). The observed rhythms of melatonin synthetic activity succeeded to no more than one 24hr cycle (Fig. 3).

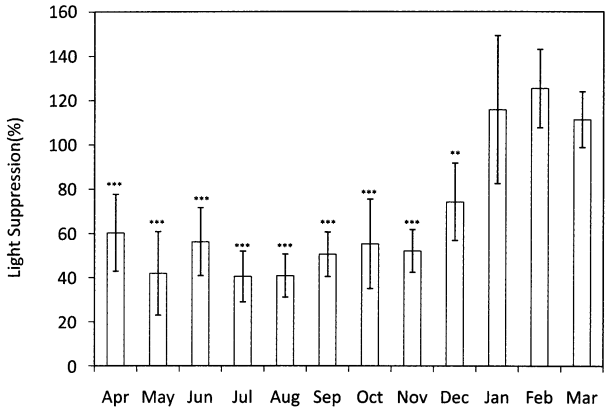

Fig. 5. Seasonal change of light suppression during light-dark cycle. The light suppression [LD (d) x 100 / LD (n)] is shown with the proportion of the value in the light period to in the darkness period. Values represent the mean ± SE (n=10). LD (d) values compared with the LD (n) values by paired sample t-test gave $^{**}p<0.005$, $***p, 0.001$.

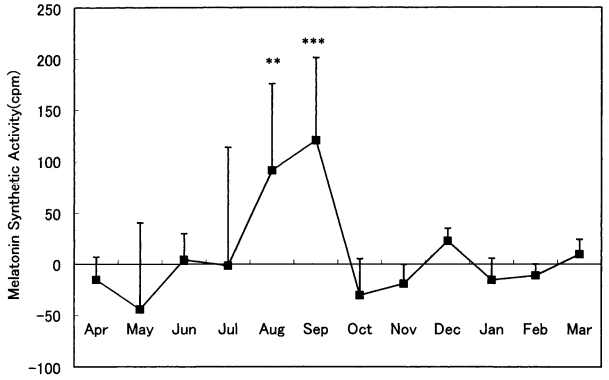

Fig. 6. Seasonal change of rhythmicity in the melatonin synthetic activity during constant darkness. The values of rhythmicity were calculated from the following formula: { DD1 (n) + DD2 (n) } / 2 − DD1 (d). Values represent the mean ± SE (n=10). { DD1 (n) + DD2 (n) } / 2 values compared with the DD1 (d) values by paired sample t-test gave $^{**}p<0.005$, $***p, 0.001$.

DISCUSSION

Radioactive tryptophan was used as melatonin precursor to study the melatonin synthesis (McNulty, 1986; Morton, 1990). They studied melatonin synthesis using the homogenate of the tryptophan incorporated pineal bodies by HPLC and thin layer chromatography. In this experiment, however, radiolabelled ^{14}C 5-HT was used to study quantitatively the synthetic activity of melatonin measuring the radioactivities in the culture medium secreted from the pineal body. The rate limiting enzyme of the melatonin synthesis is NAT which converts serotonin to N-acetylserotonin (Klein and Weller, 1972; Binkley et al., 1973; Deguchi, 1979). When ^{14}C 5-HT was used as precursor material, few radioactive metabolic product was detected in the culture medium. Therefore ^{14}C 5-HT is the sufficient and excellent material to study the biosynthetic activity of melatonin. Furthermore, one-dimension thin layer chromatography with

ethyl acetate as a solvent is enough to isolate newly synthesized melatonin secreted into culture medium. The method used in this experiment is more direct and easier than that using tryptophan and radioimmunoassay to examine the change and comparison of the melatonin synthetic activity.

The GSI values of female were very high in this experiment from May to August (Fig. 2). The big fluctuation of GSI in July is suggesting that some female fishes had ended egg-laying and the ovaries become small. After August, GSI values decreased sharply in September. It is said that the breeding season of *Zacco temmincki* in Matsue district is from June to July every year. But these results in this experiment indicate that the female fishes spawned from July through August. This means that the spawning season of *Zacco temmincki* was late one month from ordinary year. This might be caused by the lower temperature of the river from June to August than that of ordinary year (Fig. 1). On the other hand, GSI of the male fish was the highest value in June as same as ordinary year. This suggests that the effect of temperature on the development of reproductive cells is different between male and female of *Zacco temmincki*.

Iigo and Aida (1995) have presented the results that plasma melatonin concentrations at mid-dark changed seasonally and was influenced by environmental temperature in the goldfish reared under natural condition. In our experiment also, it was shown that the activities of the melatonin synthesis of the *Zacco temmincki* pineal body were high during the period from July to September (fig. 3, Fig. 4). This seasonal change was similar to that of the water temperature of the river (Fig. 1). It is conceivable that the melatonin synthesis in the cultivated pineal body is dependent on cultivation temperature. It has been reported that the activity of N-acetyltransferase is depend on the temperature in the pineal organ of other fish (Falcon J et al., 1996). It is accepted, however, the melatonin synthesis in the cultivated pineal body is not only depend on a temperature but also on a light and an endogenous rhythm (Underwood, 1985; Zachmann et al., 1992; Tilden and Hutchinson, 1993). Furthermore, the melatonin synthetic activity in *Zacco* would be effected by ganadal hormones (in preparation, Takabatake et al.).

In this experiment, although the melatonin production in the pineal body was high during the dark period of light-dark (LD) cycle, this difference of melatonin synthesis could not be detected in the winter season (Fig. 3, Fig. 5). These results are consistent with the reports obtained from other species (Delgado and Vivien-Roels, 1989; Firth et al., 1989). It is considered that the rhythm ability was not gone in winter, but it is due to the suppression of melatonin biosynthesis in the dark period by the low temperature. In fact, in the experiments of cultivating the pineal body at constant temperature, it has been shown that the melatonin synthetic activity of *Zacco temmincki* was high during dark phase and low in light one through the year (in preparation, Takabakake et al.).

The melatonin rhythmic secretion is governed by pineal oscillators in all species of fish so far investigated except the rainbow trout and masu salmon. The melatonin production rhythms of these fishes were not observed under constant darkness (Gern and Greenhouse, 1988; Iigo et al., 1997). It has been demonstrated that the rhythms of the melatonin production had damped out within several days in constant darkness even in the case that there are circadian oscillators in pineal bodies (Bolliet et al., 1994; Molinaborjia et al., 1996). Although typical circadian rhythm of the melatonin synthesis in the pineal body was not seen in all months through the year in this research, the rhythms were seen clearly in 1st day of the constant darkness in August and September (Fig. 3, Fig. 6). This is showing definitely that circadian rhythm exists in the melatonin synthesis of the pineal body in *Zacco temmincki*. The reason of disappearance of the circadian rhythm in all months except August and September might be a seasonal change in the degree of synchronization among the cell clocks and /or in the rhythmicity (the strength of the oscillator) in *Zacco temmincki*. It is necessary to study further on these possibilities.

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