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Authors: Masuda, Tomohiro, Iigo, Masayuki, Mizusawa, Kanta, Naruse, Mayumi, Oishi, Tadashi, et al.

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Variations in Plasma Melatonin Levels of the Rainbow Trout (*Oncorhynchus mykiss*) under Various Light and Temperature Conditions

Tomohiro Masuda[1,*], Masayuki Iigo[2,3], Kanta Mizusawa[1,4], Mayumi Naruse[4,5], Tadashi Oishi[5], Katsumi Aida[1] and Mitsuo Tabata[4]

[1]*Laboratory of Aquatic Animal Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan*
[2]*Department of Anatomy, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8511, Japan*
[3]*Department of Applied Biological Chemistry, Faculty of Agriculture, Utsunomiya University, 350 Mine-Machi, Utsunomiya, Tochigi 321-8505, Japan*
[4]*Department of Animal Sciences, Teikyo University of Science and Technology, 2525 Uenohara, Yamanashi 409-0193, Japan*
[5]*Graduate School of Human Culture, Nara Women's University, Kitauoya nishi-machi, Nara 630-8506, Japan*

ABSTRACT—Daily variations in plasma melatonin levels in the rainbow trout *Oncorhynchus mykiss* were studied under various light and temperature conditions. Plasma melatonin levels were higher at mid-dark than those at mid-light under light-dark (LD) cycles. An acute exposure to darkness (2 hr) during the light phase significantly elevated the plasma melatonin to the level that is comparable with those at mid-dark, while an acute exposure to a light pulse (2 hr) during the dark phase significantly suppressed melatonin to the level that is comparable with those at mid-light. Plasma melatonin kept constantly high and low levels under constant darkness and constant light, respectively. No circadian rhythm was seen under both conditions. When the fish were subjected to simulative seasonal conditions (simulative (S)-spring: under LD 13.1:10.9 at 13°C; S-summer: under LD 14.3:9.7 at 16.5°C; S-autumn: under LD 11.3:12.7 at 13°C; S-winter: under LD 10.1:13.9 at 9°C), melatonin levels during the dark phase were significantly higher than those during the light phase irrespective of simulative seasons. The peak melatonin level in each simulative season significantly correlated with temperature but not with the length of the dark phase employed. In addition, the peak melatonin level in S-autumn was significantly higher than those in S-spring although water temperature was the same under these conditions. These results indicate that the melatonin rhythm in the trout plasma is not regulated by an endogenous circadian clock but by combination of photoperiod and water temperature.

Key words: rainbow trout *Oncorhynchus mykiss*, melatonin, circadian rhythm, photoperiod, temperature

INTRODUCTION

Fish can detect daily and seasonal changes in the surrounding environment and adjust their physiological condition and behavior to the changes. Among the environmental factors, light and water temperature are considered to be important for fishes. One of the transducers of these environmental factors is melatonin synthesized in photoreceptor cells in the pineal organ and retina and then released into general circulation (Falcón *et al*., 1992; Iigo *et al*., 1994, 1997a, b; Ekström and Meissl, 1997; Falcón, 1999).

Melatonin production in the pineal organ and retina and its levels in the blood exhibit daily rhythms with low and high levels during the light and dark phases under light-dark (LD) cycles, respectively. The duration of the nocturnal elevation in melatonin production depends on the length of the dark

* Corresponding author: Tel. +81-3-5841-5289;
FAX. +81-3-5841-5288.
E-mail: masuda@marine.fs.a.u-tokyo.ac.jp

phase: longer under short photoperiod than under long photoperiod. In addition, exposure to light during the dark phase acutely suppresses melatonin production. Under constant light (LL), melatonin production is constantly suppressed as well. Thus, melatonin is considered as chemical expression of darkness (Iigo *et al*., 1994, 1997b; Ekström and Meissl, 1997; Falcón, 1999).

Although light is the principal environmental factor that regulates the melatonin rhythms, they are also regulated by temperature in ectothermic animals. In fishes, *in vivo* and *in vitro* experiments revealed that the nocturnal elevation in melatonin production is influenced by temperature (Zachmann *et al*., 1992; Max and Menaker, 1992; Iigo and Aida, 1995). Furthermore, temperature cycles could synchronize the rhythmic melatonin production in the pineal organ (Falcón *et al*., 1994).

The melatonin rhythms in fishes are also regulated by circadian clocks in most, if not all, species: melatonin rhythms in the pineal organ, retina, and blood persist even under constant darkness (DD) with high and low levels during the subjective-night and the subjective-day, respectively (Iigo *et al*., 1994, 1997b; Ekström and Meissl, 1997; Falcón, 1999). Thus, melatonin production is regulated by both internal (the circadian clock) and external factors (light and temperature).

The rainbow trout is a fish widely used for physiological research and could be a useful model to elucidate the regulatory mechanism of the melatonin rhythm in fishes. This fish is interesting because *in vitro* pineal culture experiments revealed that the pineal organ does not contain the circadian clock that regulates melatonin production (Gern and Greenhouse, 1988; Gern *et al*., 1992; Max and Menaker, 1992; Meissl and Brandstätter, 1992). Several *in vivo* and *in vitro* studies have examined the regulatory mechanisms of melatonin rhythms in this species (Falcón *et al*., 1991; Meissl and Brandstätter, 1992). However, the regulatory mechanisms of circulating melatonin rhythms by internal and external factors are not fully understood yet.

In this study, to elucidate the regulatory mechanisms by the circadian clock, photoperiod and temperature of the plasma melatonin rhythm in the rainbow trout in more details, we first examined the effects of acute changes in lighting conditions on plasma melatonin levels at mid-light and mid-dark. Then, we determined plasma levels of melatonin in the rainbow trout kept under LL and DD. Finally, we tested the effects of temperature and photoperiod on plasma melatonin rhythms in the fish kept under simulative seasonal conditions.

MATERIALS AND METHODS

Experimental fish

Rainbow trout were supplied from the Fuji Trout Hatchery, Shizuoka Fisheries Experimental Station, Shizuoka Prefecture, Japan. They were transferred and kept in stock tanks at St. Marianna University School of Medicine, Kawasaki, Kanagawa Prefecture (Exps. 1 and 2), or at Teikyo University of Science and Technology, Uenohara, Yamanashi Prefecture, Japan (Exp. 3). The tanks were kept in a controlled environment chamber, in which ambient temperature and photoperiod were regulated. Fish were fed commercial trout pellets by automatic feeders (Exps. 1 and 2) or the self-feeding devices (Exp. 3) as previously described (Sánchez-Vázquez *et al*., 1998) until use. White fluorescent bulbs were used as light source. The light intensity at the water surface was approximately 900 lx during the light phase.

Sampling procedure

The blood samples were collected from the trout under anesthesia with 0.04% 2-phenoxyethanol as previously described (Iigo *et al*., 1997a). Briefly, blood samples were taken from the caudal vasculature with a heparinized syringe from the anesthetized fish. For the sampling during the dark phase, fish were anesthetized in the dark. After the upper part of the body was covered with a black sheet to avoid photoreception by the pineal organ and eyes, a dim-red light was turned on and blood samples were taken. Blood samples were collected in test tubes on ice, centrifuged (3,000 rpm, 20 min, 4°C), and plasma was separated. Samples were stored at –80°C until analysis.

Determination of plasma melatonin contents by radioimmunoassay (RIA)

Melatonin levels in the plasma were determined by RIA as described by Fraser *et al.* (1983) and modified by Sánchez-Vázquez *et al.* (2000) using anti-melatonin serum (Stockground, Surrey, UK) and [*O*-methyl-^{3}H]melatonin (85 Ci/mmol; Amersham Pharmacia Biotech, Tokyo, Japan).

Experiment 1: Effects of an acute exposure to darkness during the light and to light during the dark phase on plasma melatonin levels

Twenty-eight fish were used in this experiment. Fish were reared in flow-through aquaria (30×32×60cm, 46 L, 5-10 fish/tank) under LD 12:12 (lights on 06:00–18:00 hr) at 15°C for a week. Blood samples from the control fish were collected at mid-light (12:00 hr) and mid-dark (00:00 hr) under LD 12:12 (n=5 each). Blood samples were also collected at 12:00 hr from the fish acutely exposed to darkness (10:00–12:00 hr) during the light phase, or at 00:00 hr from the fish acutely exposed to light (500 lx: 22:00–00:00 hr) during the dark phase (n=5 each).

Experiment 2: Variations in plasma melatonin levels in the trout kept under LL or DD

One hundred fish were used in this experiment. They were reared in flow-through aquaria as in Exp. 1. Blood samples were taken at 12:00 and 16:00 hr under LD 12:12. Then the fish were exposed to LL or DD from 18:00 hr and blood samples were collected 6 times under LL and 12 times under DD at 4 hr intervals (n=5) from 20:00.

Experiment 3: Plasma melatonin rhythms in the trout kept under simulative seasonal conditions

Three hundred and twenty fish were used in this experiment. Fish were reared in 8 circular tanks (50 cm in diameter, 40 cm in depth, 80 L, ~40 fish/tank). To investigate the influence of photoperiod and water temperature on plasma melatonin rhythms, we mimicked four seasons: simulative (S)-spring, under LD 13.1:10.9 at 13°C (light on 06:27–19:33 hr); S-summer, under LD 14.3:9.7 at 16.5°C (light on 05:51–20:09 hr); S-autumn, under LD 11.3:12.7 at 13°C (light on 07:21–18:39 hr); S-winter, under LD 10.1:13.9 at 9°C (light on 07:57–18:03 hr). These were based on the average photoperiod and water temperature in April (S-spring), July (S-summer), October (S-autumn), and January (S-winter) in Shiba River (longitude 138°35' E, latitude 35°15'N), near the Fuji Trout Hatchery from which the experimental fish were obtained.

These fish were kept in each simulative condition for at least 1 month before the samplings. The fish were fed only during the light phase using demand-feeders and feeding rhythms were recorded as described (Sánchez-Vázquez *et al.*, 2000). These data will be published elsewhere. The blood samples were collected 8 times at 3 hr intervals from 1600 hr for each simulative group (n=10 at each time point).

Statistics

Variations in plasma melatonin levels among experimental groups in Exp. 1, variations in plasma melatonin levels under LL or DD in Exp. 2, and daily variations in plasma melatonin levels in each simulative season in Exp. 3 were analyzed by one-way ANOVA followed by the Tukey's multiple comparison test. The effects of sampling time and simulative seasons in Exp. 3 were analyzed by two-way ANOVA. Correlation of peak melatonin levels in each simulative season (individual values at the peak time in each 24 hr cycle) and temperature or the length of the dark phase employed was analyzed by linear regression. Differences in the peak melatonin level in each simulative season were compared by one-way ANOVA followed by the Tukey's multiple comparison test.

RESULTS

Effects of an acute exposure to darkness during the light phase and to light during the dark phase on plasma melatonin levels

Effects of an acute exposure to darkness (10:00–12:00 hr) during the light phase on plasma melatonin levels at mid-light (12:00 hr) and to light (22:00–00:00 hr) during the dark phase on plasma melatonin levels at mid-dark (00:00 hr) are shown in Fig. 1. The results of one-way ANOVA indicated that the lighting conditions significantly affected plasma melatonin levels in the trout (P<0.0001). Tukey's multiple comparison test demonstrated that plasma melatonin levels under mid-dark and dark pulse during the light phase were significantly higher than those under mid-light and light

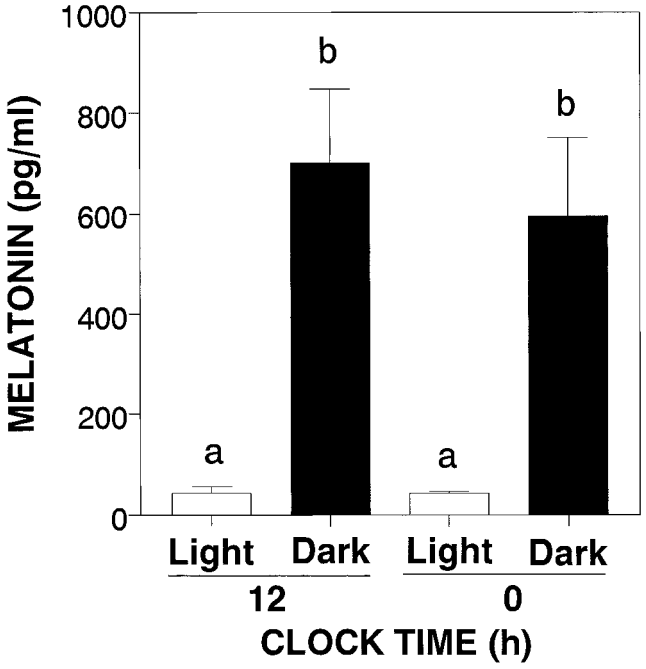

Fig. 1. Effects of acute changes in photic environment on plasma melatonin levels of the rainbow trout at mid-light and at mid-dark. Fish reared under LD 12:12 (light on 06:00–18:00 hr) and blood samples were collected at mid-light (12:00 hr) with (Dark) or without (Light) dark pulse (10:00–12:00 hr) or at mid-dark (00:00 hr) with (Light) or without (Dark) light pulse (22:00–00:00 hr). Values are mean±SEM (n=5). Each time point comes from different animals. Values displaying different symbols are significantly different from one another (P< 0.001).

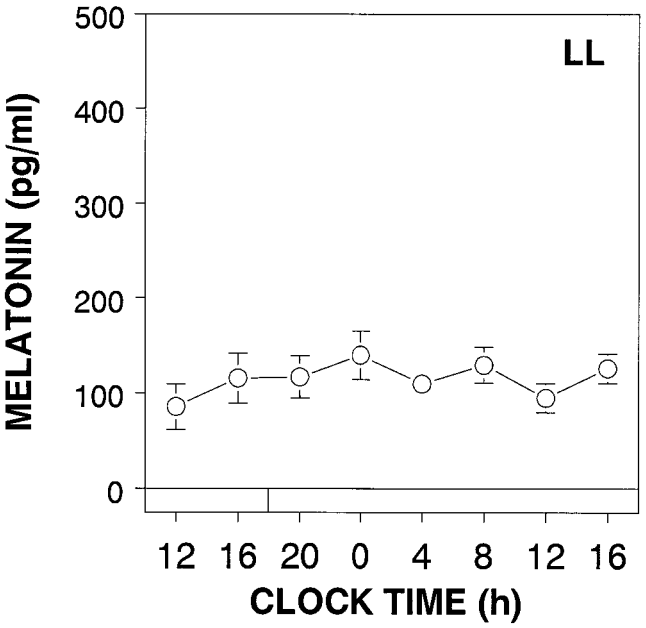

Fig. 2. Changes in plasma melatonin levels of the rainbow trout kept under LL. Fish reared under LD 12:12 (light on 06:00–18:00 hr) were exposed to LL from the normal light offset (18:00 hr) and then sampled 6 times at 4 hr intervals from 20:00 to 16:00 hr. An open bar along the X-axis represents the light phase. Values are mean ±SEM (n=5). Each time point comes from different animals.

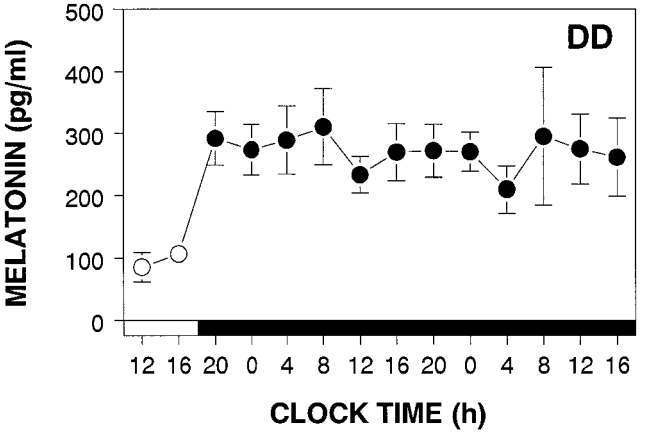

Fig. 3. Changes in plasma melatonin levels of the rainbow trout kept under DD. Fish reared under LD 12:12 (light on 06:00–18:00 hr) were exposed to DD from the normal light offset (18:00 hr) and then sampled 12 times at 4 hr intervals from 20:00 hr. Solid and open bars along the X-axis represent the dark and light phases, respectively. Values are mean ±SEM (n=5). Each time point comes from different animals.

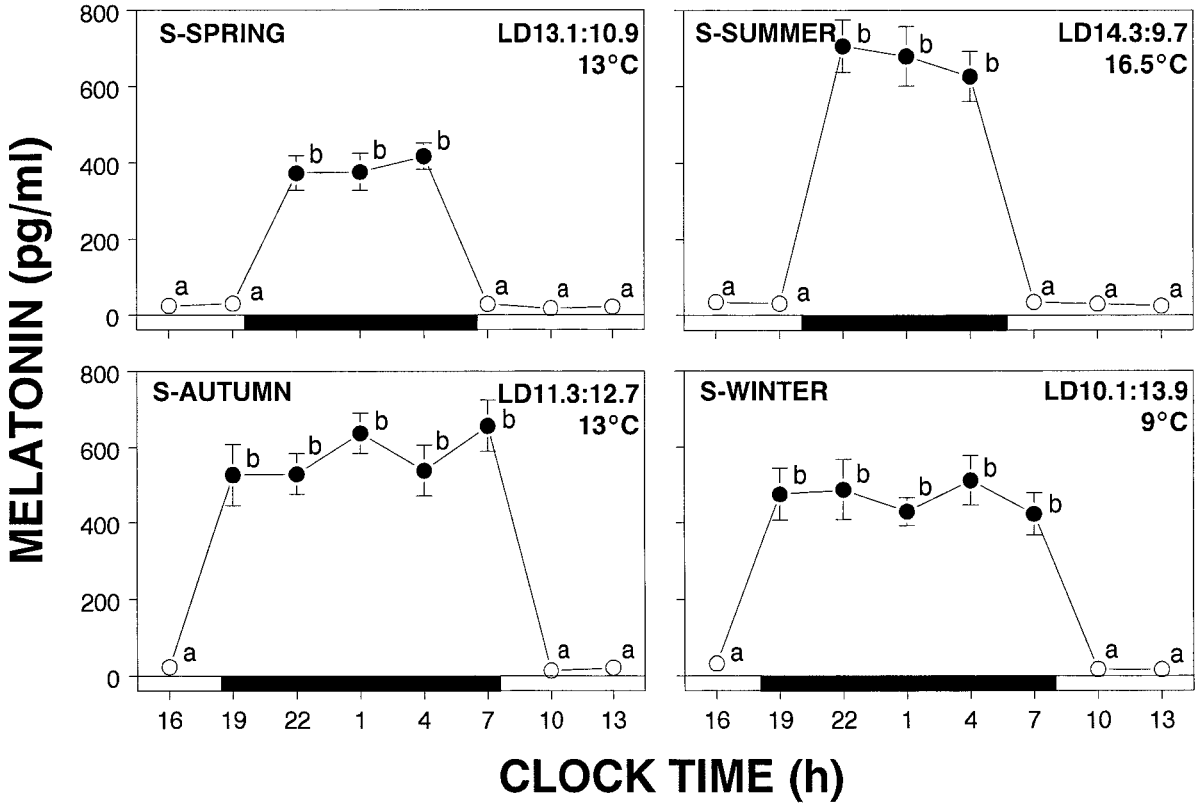

Fig. 4. Daily variations in plasma melatonin levels of the rainbow trout kept under simulative seasonal conditions. S-spring (LD 13.1:10.9, light on 06:27–19:33 hr, 13°C); S-summer (LD 14.3:9.7, light on 05:51–20:09 hr, 16.5°C); S-autumn (LD 11.3:12.7, light on 07:21–18:39 hr, 13°C); S-winter (LD 10.1:13.9, light on 07:57–18:03 hr, 9°C). Fish were reared under each simulative seasonal condition for at least 1 month and then blood samples were collected 8 times at 3 hr intervals from 16:00 to 13:00 hr. Solid and open bars along the X-axis represent the dark and light phases, respectively. Values are mean ±SEM (n=4–10). The SEM is not visible when it is smaller than the symbol. Each time point comes from different animals. Values displaying different symbols are significantly (P< 0.001) different from one another.

pulse during the dark phase (mid-dark vs. mid-light, light pulse, P<0.0001; mid-light vs. dark pulse, P<0.0001).

Variations in plasma melatonin levels in the trout kept under LL or DD

Changes in plasma melatonin levels in the rainbow trout reared under LL or DD are exhibited in Figs. 2 and 3. Plasma melatonin kept constantly low and high levels under LL and DD, respectively. No significant variations were seen under both conditions (ANOVA, P>0.05).

Plasma melatonin rhythms in the trout kept under simulative seasonal conditions

Daily variations in plasma melatonin levels in the rainbow trout kept under simulative seasonal conditions are shown in Fig. 4. Plasma melatonin levels exhibited significant daily variations regardless of temperature and photoperiod used for each simulative season (P<0.0001). Tukey's multiple comparison test demonstrated that plasma melatonin levels during the dark phase were significantly higher than those during the light phase (under S-spring and S-summer: 16:00, 19:00, 07:00, 10:00, or 13:00 hr vs. 22:00, 01:00 or 04:00 hr, P< 0.001; under S-autumn and S-winter: 16:00, 10:00, or 13:00 hr vs. 19:00, 22:00, 01:00, 04:00 or 07:00 hr, P< 0.001).

The results of two-way ANOVA indicated that both sampling time and simulative season affected plasma melatonin levels in the trout (P<0.0001). Linear-regression analyses demonstrated that the peak melatonin level in each simulative season significantly correlated with the change in water temperature (r=0.373, n=36, P<0.05) but not with the duration of the dark phase employed (r=0.205, n=36, P>0.05). When the peak melatonin level in each simulative season was compared, one-way ANOVA indicated a significant effect of simulative season (P<0.0001). Tukey's comparison test showed that the peak melatonin level in S-summer was significantly higher than that in S-spring (P<0.01) and S-winter (P<0.05), and the peak level in S-autumn was significantly higher than that in S-spring (P<0.05).

DISCUSSION

In this study, we determined daily variations in plasma melatonin levels of the rainbow trout reared under various light and temperature conditions to examine endogenous and exogenous regulation of the melatonin rhythm in the

plasma, which is mainly generated by the pineal organ (Gern *et al*., 1978b; Sánchez-Vázquez *et al*., 2000). We first examined whether melatonin levels in rainbow trout plasma is regulated by an endogenous circadian clock or not. Under LD cycles, plasma melatonin levels exhibited a significant day-night changes with higher levels at mid-dark than those at mid-light as expected. This is consistent with the reported changes in plasma melatonin levels in this species (Gern *et al*., 1978a, b; Sánchez-Vázquez *et al*., 2000). A light pulse during the dark phase decreased plasma melatonin concentration significantly to the level that is comparable with those at mid-light under LD. This is also consistent with a previous report that examined the effects of 2 hr light pulse during the early, middle or late dark phase on plasma melatonin levels in the trout (Alvariño *et al*., 1993). On the contrary, dark exposure during the light phase increased plasma melatonin significantly to the level that was comparable with those at mid-dark under LD. In the case of melatonin rhythms regulated by the circadian clock, there exists a refractory period when the dark-exposure does not induce full-amplitude increase in melatonin levels (Falcón *et al*., 1989; Iigo *et al*., 1997b). However, in the present results, the response of the plasma melatonin level was not refractory to acute exposure to darkness, suggesting that the plasma melatonin rhythm in the rainbow trout is not under the circadian control. Furthermore, circadian rhythms were observed neither under LL nor under DD: plasma melatonin levels kept constantly low and high titers under LL and DD, respectively. Altogether, we concluded that melatonin levels in the rainbow trout plasma are controlled directly by light and darkness, but not by an endogenous circadian clock. This is consistent with the previous reports on the plasma melatonin rhythms under constant conditions in this species (Randall *et al*., 1991; Gern *et al*., 1992) and also with lack of the circadian regulation of melatonin production in the pineal organ of salmonids including the rainbow trout (Gern and Greenhouse, 1988; Gern *et al*., 1992; Max and Menaker, 1992; Iigo *et al*., 1998).

Melatonin functions as chemical expression of darkness to convey not only the time of a day but also the time of a year. This idea is based on the fact that melatonin production exhibits a robust daily rhythm that is sensitive to the change in daylength: the duration of nocturnal increase in melatonin production is longer under short photoperiod than that under long photoperiod (Iigo *et al*., 1994; Randall *et al*., 1995). Furthermore, in ectothermic animals, temperature is also an important factor that influences the amplitude of the melatonin rhythm (i.e. the peak melatonin level during the dark phase) (Iigo and Aida, 1995). Therefore, in the present study, we determined daily variations in plasma melatonin levels in the rainbow trout reared under simulative-seasonal environment in order to investigate the effects of daylength, temperature and their interaction. The results indicate that plasma melatonin levels in the rainbow trout exhibit significant daily variations with higher levels during the dark phase than those during the light phase regardless of simulative seasons. The duration of the dark phase determines that of nocturnal increase in plasma melatonin levels. Peak melatonin levels also exhibited significant variations among simulative seasons, suggesting the presence of seasonal change in the amplitude of melatonin rhythms in the rainbow trout under natural environment. This should be examined in future experiments.

The influences of water temperature on plasma melatonin levels and on the activities of melatonin generating enzymes were reported in a few teleost species. In the goldfish, plasma melatonin levels at mid-dark exhibited a temperature-dependent increase with higher levels at higher temperature in the temperature range of 5–25°C (Iigo and Aida, 1995). In the pineal organ of the rainbow trout and pike, the activity of arylalkylamine *N*-acetyltransferase, the rate-limiting enzyme in melatonin synthesis, is temperature-sensitive with peak activity around 12–15 and 18–25°C, respectively (Falcón *et al*., 1992). In this study, there was a significant correlation between peak melatonin levels and water temperature, indicating that the amplitude of plasma melatonin rhythm in the rainbow trout is temperature-dependent. However, temperature is not the only factor that affects the amplitude because even at the same temperature, there was a significant difference in the peak melatonin levels: the peak melatonin level in S-autumn was higher than that in S-spring. A similar result was recently reported in European sea bass (Garcia-Allegue *et al*., 2001**)**. Thus, melatonin profiles in the plasma of the trout and sea bass are influenced by combination of photoperiod and temperature: the duration of nocturnal increase is determined by that of the dark phase, while the peak melatonin level may be determined by combination of photoperiod and water temperature.

From the previous and present studies, we can speculate that melatonin is an endocrine signal that informs both daily and calendar time, as in mammals (Reiter, 1980). In salmonid fish, locomotor and feeding activities exhibit robust daily rhythms, and maturation and smoltification occur during the specific time of a year (Iigo and Tabata, 1997; Sánchez-Vázquez and Tabata, 1998; Okumoto *et al*., 1989; Randall *et al*., 1998). Melatonin might be involved in the regulation of these daily and seasonal rhythms. Further investigation should be required to elucidate the mechanism of melatonin action.

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