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Lack of Circadian Regulation of Melatonin Rhythms in the Sockeye Salmon (*Oncorhynchus nerka*) *in vivo* and *in vitro*

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Melatonin profiles were determined in the plasma *in vivo* and in the pineal organ *in vitro* of the sockeye salmon (*Oncorhynchus nerka*) under various light conditions to test whether they are under circadian regulation. When serial blood samples were taken at 4-h intervals for 3 days via a cannula inserted into the dorsal aorta, plasma melatonin exhibited significant fluctuation under a light-dark cycle, with higher levels during the dark phase than during the light phase. No rhythmic fluctuations persisted under either constant dark or constant light, with constant low and high levels, respectively. Melatonin release from the pineal organ in flow-through culture exhibited a similar pattern in response to the change in light conditions, with high and low release associated with the dark and light phases, respectively. These results indicate that melatonin production in the sockeye salmon is driven by light and darkness but lacks circadian regulation.

Key words: melatonin, pineal organ, light, daily rhythm, circadian rhythm, salmon

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone produced in the pineal organ and the retina of vertebrates. Melatonin production is governed by environmental light-dark (LD) cycles, with higher amounts produced during the dark phase than during the light phase. Melatonin contents in the pineal organ, retina, and blood exhibit similar fluctuations. Furthermore, the duration of the nocturnal elevation of melatonin depends on the length of the dark phase. Thus, melatonin is considered as a chemical expression of darkness, and its nocturnal increase is used as an internal zeitgeber to regulate daily and seasonal rhythms in the physiology and behavior in a number of vertebrate species (Yu and Reiter, 1992).

In a large number of vertebrates, including fish, melatonin production is also regulated by a circadian-clock mechanism. Even under constant dark (DD), daily fluctuation in melatonin production persists, with higher levels seen during subjective night than during subjective day (Iigo *et al*., 1994,1997a; Ekström and Meissl, 1997; Falcón, 1999). However, several *in vitro* studies have demonstrated that the pineal organs of salmonids such as the rainbow trout, *Oncorhynchus mykiss*, and masu salmon, *O. masou*, do not exhibit circadian rhythms in melatonin release when maintained under DD (Gern and Greenhouse, 1988; Iigo *et al*., 1998), indicating either that the pineal organ does not contain a circadian clock that regulates melatonin production or that the coupling between the pineal circadian clock and melatonin synthesis is quite weak.

Evidence indicates that the pineal organ and its hormone melatonin play important roles as an internal synchronizer in the circadian organization of vertebrates (Cassone, 1990). Therefore, elucidation of *in vivo* melatonin profiles is important to investigating the roles of melatonin in the circadian system at the organismal level. However, little is known about the circulating melatonin profiles in individual fish, partly because of the difficulty of serial blood sampling for a long period. To overcome this difficulty, in the present study we applied a cannulation technique that allowed us to investigate plasma melatonin profiles individually. Here we report *in vivo* changes in plasma melatonin concentration, measured at 4-h intervals for 3 days, in the landlocked sockeye salmon (hime salmon, *Oncorhynchus nerka*) kept under LD, DD, and constant light (LL) conditions. Melatonin release from the pineal organ maintained in flow-through culture was also monitored to compare melatonin rhythms *in vivo* and *in vitro*.

MATERIALS AND METHODS

Sockeye salmon

Sockeye salmon were reared in an outdoor pond at the Freshwater Fisheries Research Division, National Research Institute of Fisheries Science (formerly the Nikko Branch of the National Research Institute of Aquaculture), Tochigi, Japan. Two- or three-

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year-old fish (410±50 g in body weight; mean±SEM, n=14) were used for the cannulation experiments, while one-year-old fish (80±9 g in body weight, n=4) were used for the pineal culture experiment. Experiments were carried out in accordance with the guidelines of The Physiological Society of Japan for the Use and Care of Experimental Animals and also with the current laws of Japan.

Cannulation and blood sampling

Fish were reared under LD 12:12 (light on 0600–1800 hr) for at least 2 days before surgery for cannulation. Illumination was supplied with white fluorescent bulbs for the light phase. Light intensity measured at the surface of the water was 700–1,300 lx during the light phase.

Fish were anesthetized in 0.06% 2-phenoxyethanol and placed on a surgical table with the ventral side upward. Water containing 0.06% 2-phenoxyethanol was continuously perfused over the gills during surgery. A polyethylene tube (size 3, 0.5 mm I.D., 1.0 mm O.D., 60 cm long; Hibiki, Tokyo, Japan) filled with 0.9% NaCl containing 100 U/ml heparin was implanted into the dorsal aorta under anaesthesia after the method of Iwama and Ishimatsu (1994) for later serial blood sampling. The incision was then sutured. The fish were returned to individual flow-through aquaria (340 × 530 × 90 mm; average flow rate 9.5 l/min) and reared under LD 12:12 at 9°C for an additional 1–3 days until sampling. Fish were not fed after the operation.

Serial blood samples (200–300 µl each) were collected 20 times from the sockeye salmon via cannula, at 4-h intervals starting at 1600 hr, under one of the following light conditions: (1) LD 12:12 for 3 days (designated as the LD group), (2) LD 12:12 for the first day followed by DD for 2 days (the LD/DD group), or (3) LD 12:12 for the first day followed by LL for 2 days (the LD/ LL group). Light conditions were changed from LD 12:12 to DD or LL at the normal light offset (1800 hr). A dim red light was turned on for sampling during the dark phase, but extreme care was taken to avoid photoreception by the pineal organ and lateral eyes. Blood samples were centrifuged at 3,000 rpm for 5 min. Plasma was separated, rapidly frozen at –80°C, and stored at –30°C until extraction.

Flow-through pineal culture

Fish were transferred to Utsunomiya University (Tochigi, Japan), where pineal culture was performed. Upon the arrival, the pineal organs were dissected out and individually maintained in flow-through culture at 15°C under LD 12:12 (lights on 0600–1800 hr) for 24 h and then under DD for 72 h, followed by LL for 24 h

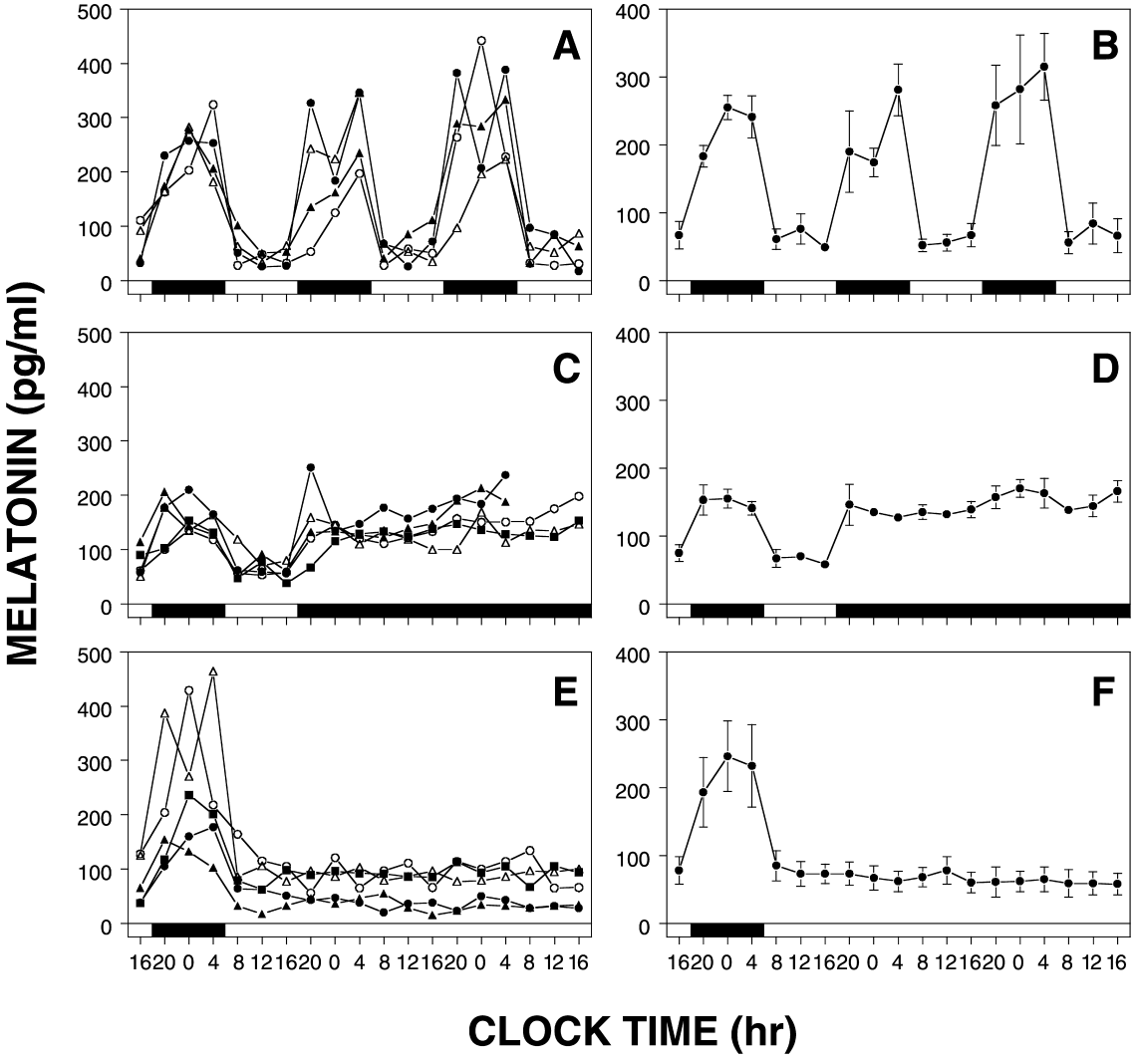

Fig. 1. Plasma melatonin profiles of the sockeye salmon under various light conditions. **(A, B)** LD 12:12; **(C, D)** LD 12:12 and DD; **(E, F)** LD 12:12 and LL. **(A, C, E)** Individual profiles; **(B, D, F)** mean profiles. Each point represents mean±SEM (n=4 for B, 4–5 for D, and for F). Solid and open bars along the X-axis represent the dark and light phases, respectively.

and then LD 12:12 for 24h, as previously described (Iigo *et al*., 1991, 1998, 2004). A white fluorescent bulb (20 W) was used as the light source. Light intensity at the surface of the incubation chamber was approximately 1,000 lx when the light was on. Perfusates were collected at 3-h intervals and stored at –30°C until radioimmunoassay.

Radioimmunoassay

Melatonin in the plasma was partially purified using Sep-pak C_{18} cartridges (Waters Associates, Milford, MA, USA) (Kezuka *et al*., 1988) and subjected to RIA as previously described (Iigo *et al*., 1997b). Melatonin contents in perfusates were determined directly by RIA as previously described (Iigo *et al*., 1998). Cross-reactivities of the anti-melatonin serum (HAC-AA92-03RBP86, kindly provided by Prof. K. Wakabayashi, Gunma University, Maebashi, Japan) with melatonin-related compounds were as follows: 6-hydroxymelatonin, 0.65%; *N*-acetylserotonin, 0.098%; other indole compounds tested,<0.025%. Parallelism of inhibition curves was demonstrated between serial two-fold dilutions of an authentic melatonin (Sigma Co., St. Louis) standard and the plasma extract or perfusates (data not shown). Intra- and inter-assay coefficients of variation were 4.9% (n=7) and 7.4% (n=5) for the plasma and 4.5% (n=3) and 3.5% (n=6) for the perfusates, respectively.

Statistics

Variation in plasma melatonin levels under LD, DD or LL were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test.

RESULTS AND DISCUSSION

Previous studies have demonstrated that melatonin production in the pineal organ exhibits a circadian rhythm under DD in many teleost species (Falcón *et al*., 1989; Iigo *et al*., 1991, 2004; Zachmann *et al*., 1992; Bolliet *et al*., 1996; Cahill, 1996; Okimoto *et al*., 1999a,b; Roberts *et al*., 2003). However, in salmonid fishes such as the rainbow trout and masu salmon, melatonin production is constantly activated under DD (Gern and Greenhouse, 1988; Iigo *et al*., 1998). In the present study, to test whether or not melatonin release from the pineal organ is under circadian control, as observed in teleosts other than salmonids, we examined melatonin rhythms in the sockeye salmon *in vitro* as well as *in vivo*.

In vivo cannulation experiments demonstrated melatonin profiles individually under LD, DD, and LL conditions (Fig. 1). Under LD, plasma melatonin concentration exhibited daily variation, with high values during the dark phase in all fish, although the absolute concentration differed among individuals (Fig. 1A, C, E). There was significant daily variation (one-way ANOVA, $P<0.0001$), and the plasma melatonin level during the dark phase was significantly higher than during the light phase (Tukey's multiple-range test, at least $P<0.05$). However, when fish were exposed to DD (Fig. 1C, D) or LL (Fig. 1E, F), plasma melatonin was maintained at high and low levels that were comparable to those during the dark and light phases under LD 12:12, respectively. There was no significant variation in plasma melatonin level under DD or LL (ANOVA, $P>0.05$).

The melatonin profile under each light condition was uniform, although the absolute melatonin concentration varied among individuals. Kulczykowska *et al*. (2001) also demonstrated individual melatonin profiles in chronically cannulated flounder (*Platichthys flesus*). It thus turned out that cannulation is a useful technique to demonstrate individual melatonin profiles. However, there remains a problem: haematocrit values of the blood decreased after frequent sampling. The replacement of red blood cells resuspended in the equivalent volume of saline should be required for longer sampling. Alternatively, *in vivo* microdialysis would provide a valuable tool for analyzing individual melatonin profiles (Nakahara *et al*., 2003).

In vitro flow-through culture demonstrated melatonin secretory profiles from individual pineal organs of sockeye salmon under various light conditions (Fig. 2). Although absolute release differed among individuals, the pattern of change was consistent, as in plasma melatonin profiles. Under LD 12:12, the pineal organs released melatonin according to the LD alternation. Low and high melatonin release was associated with the light and dark phases,

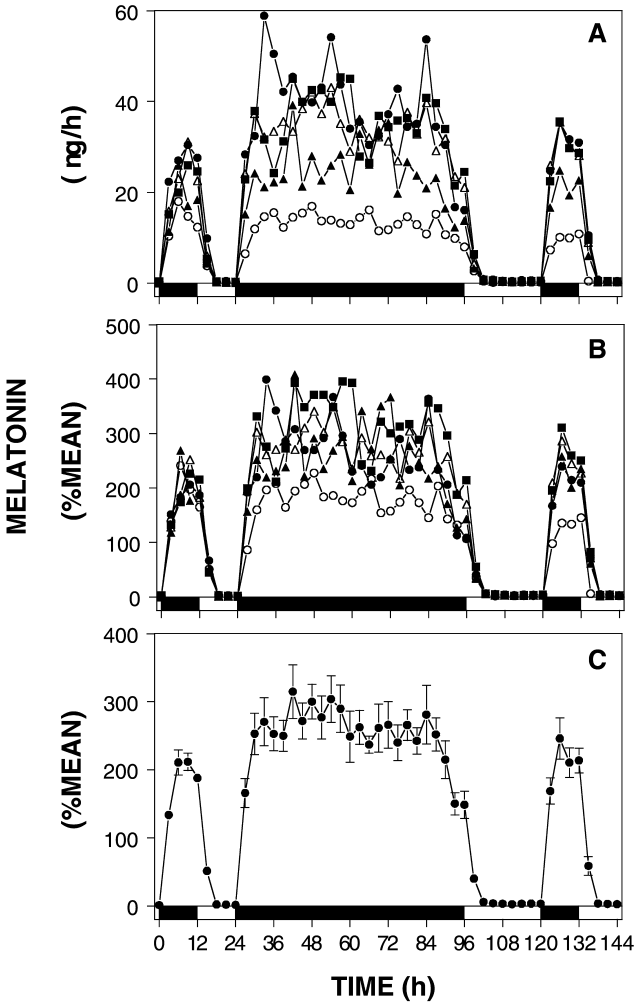

Fig. 2. *In vitro* melatonin secretory profiles of the pineal organ of the sockeye salmon maintained in flow-through culture under various light conditions. **(A)** Individual profiles. **(B)** Normalized profiles; melatonin release was normalized relative to the amount of melatonin release during the first 24 h under LD. **(C)** Average profiles. Each point represents mean±SEM (n=4). Data were plotted at the end of collection intervals. Melatonin release during the preincubation period is plotted at Time 0. Solid and open bars along the X-axis represent the dark and light phases, respectively.

respectively. However, no rhythmic melatonin release was observed under either DD or LL. No free-running rhythm in melatonin release was observed. Under DD, the pineal organ constantly released a large quantity of melatonin during both subjective day and subjective night. However, upon the exposure to LL, melatonin release was continually suppressed. Exposure to LD reestablished a rhythmic fluctuation.

Both *in vivo* and *in vitro*, the light phase is associated with low melatonin titers and the dark phase with high melatonin titers in the sockeye salmon. Thus, under LD cycles, melatonin concentration in the plasma and the amount of melatonin released from the pineal organ exhibited significant daily rhythms. However, no significant variation was seen under either DD or LL, with constant high and low levels, respectively. Therefore, the sockeye salmon is an additional example of a teleost in which melatonin production lacks circadian regulation.

Why these salmonids (rainbow trout, masu salmon, and sockeye salmon; all belong to *Oncorhynchus*) have lost circadian regulation of melatonin production is not known at present. However, it is interesting to note that the pineal organs of ayu (Osmeriformes) and pike (Esociformes), both close relatives of salmonids (Salmoniformes), use a circadian clock to regulate melatonin release (Falcón *et al*., 1989; Iigo *et al*., 2004). Taxonomically, all the three orders (Salmoniformes, Osmeriformes, and Esociformes) belong to Superorder Protacanthopterygii (Nelson, 1994). Thus, it is reasonable to speculate that the pineal organ of ancestral protacanthopterygians had a circadian clock, but that ancestral salmonids lost circadian regulation of melatonin production in the pineal organ during evolution, after divergence from Osmeriformes/Esociformes. Further comparative studies using salmonids other than *Oncorhynchus* as well as their related species will be required to substantiate this hypothesis.

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