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Disturbance of Plasma Melatonin Profile by High Dose Melatonin Administration Inhibits Testicular Maturation of Precocious Male Masu Salmon

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ABSTRACT—We have previously shown that the testicular development of underyearling male masu salmon *Oncorhynchus masou* reared under a long photoperiod was accelerated by oral melatonin treatment (0.5mg melatonin/kg body weight/day), suggesting that melatonin mediates photoperiodic signaling. In this study, we further examined the effects of a disturbance in the plasma melatonin profile on gonadal development in underyearling male masu salmon by administering a higher dose of melatonin. Fish randomly selected in June were divided into two groups. They were reared under a light:dark (LD) cycle of 16:8 (lights on 04:00–20:00 hr) and fed with pellets sprayed with melatonin or vehicle twice a day at 08:30 and at 15:30 hr (7.5mg melatonin/kg body weight/day) until October. Fish were sampled on Day 0, 25, 60, 90 and 120. The plasma melatonin levels were high in the dark phase and low in the light phase in the control group, while they were constantly high with no significant change in the melatonin-treated group. Melatonin treatment had inhibitory effects on the gonadosomatic index and plasma testosterone levels. Pituitary salmon gonadotropin-releasing hormone content and luteinizing hormone content were significantly lower in the melatonin-treated group on Day 60 and 90, respectively. These results indicate that the plasma melatonin profile is important for mediating photoperiodic signals that regulate brain-pituitary-gonadal axis in underyearling precocious male masu salmon.

Key words: melatonin, oral administration, photoperiod, testicular maturation, masu salmon

INTRODUCTION

Gonadal maturation in salmonid fish occurs in autumn, indicating that a short photoperiod or the change from a long to a short photoperiod accelerates gonadal maturation (see Bromage *et al*., 2001). The testicular maturation of underyearling precocious male masu salmon *Oncorhynchus masou* is accelerated by changing the photoperiod from natural to short (LD 8:16), whereas it is delayed by keeping a long photoperiod (LD 16:8) from June; spermiation occurred in August and September-October under short and long photoperiods, respectively (Amano *et al*., 1994, 1995). Changes in salmon gonadotropin-releasing hormone (sGnRH) mRNA levels in the brain and luteinizing hormone (LH) levels in the pituitary are well consistent with those of testicular development; they increased when fish spermiated in both photoperiodic groups (Amano *et al*., 1995). These results indicate that a short photoperiod stimulates testicular development via the activation of the brain-pituitary-gonadal axis in masu salmon.

Melatonin produced in the pineal organ is considered to mediate photoperiodic signaling in fish as in other vertebrate

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classes: circulating melatonin levels are high during the night and low during the day (see Iigo *et al*., 1994a), and the duration of nocturnal elevation is longer under a short photoperiod than long photoperiod in rainbow trout *Oncorhynchus mykiss* (Duston and Bromage, 1986), Atlantic salmon *Salmo salar* (Randall *et al*., 1995), common carp *Cyprinus carpio* (Kezuka *et al*., 1988), goldfish *Carassius auratus* (Kezuka *et al*., 1992; Iigo and Aida, 1995), and masu salmon (Iigo, 1996; Iigo *et al*., in preparation). Furthermore, melatonin is known to affect several seasonal parameters such as reproductive activity in fish (Zachmann *et al*., 1992). We have previously shown that testicular development in underyearling male masu salmon reared under a long photoperiod was accelerated by oral melatonin treatment which mimics plasma melatonin profile under short photoperiod: fish were fed with pellets sprayed with melatonin (0.5 mg melatonin/kg body weight/day) once a day at 11:00 hr (lights on 04:00–20:00 hr) from June to October (Amano *et al*., 2000). These results suggest that melatonin is involved in providing photoperiodic information and that the plasma melatonin profile after melatonin treatment is recognized the same as "a short photoperiod."

As Zachmann *et al*. (1992) has pointed out, the effects of melatonin are often contradictory, because they can depend on several factors, e.g., photoperiod, temperature, season, age of the animal, and dose and time of administration during the day and year. Therefore, to further clarify the roles of melatonin in mediating photoperiodic signaling in fish, we have examined the effects of a disturbance in the plasma melatonin profile by administering a higher dose of melatonin in underyearling male masu salmon reared under a long photoperiod from June. We have compared the gonadosomatic index (GSI), plasma testosterone levels and pituitary contents of sGnRH and gonadotropin (GTH) between the control and melatonin-treated groups.

MATERIALS AND METHODS

Fish

The experiment was conducted at the Nikko Branch of the National Research Institute of Aquaculture, Nikko, Tochigi Prefecture, Japan. Masu salmon eggs were fertilized by artificial methods in October 1998 and hatched in December 1998. Most male masu salmon of the strain used in this study mature precociously in the first and the second autumn, and females mature at 3 years of age (Amano *et al*., 1992). The fish were reared in indoor tanks under a natural photoperiod in spring water at 9–10°C before the study.

Experimental protocol

On June 21, 1999, underyearling masu salmon (mean body weight (BW), 8.5 g) were randomly selected and divided into control and melatonin-treated groups (125 fish each). Each group was transferred to a tank (0.5×2.0×0.2 m) in a light-proof room shielded with black cloth and reared in spring water at 9–10°C. Fish were reared under a long photoperiod (LD 16:8; lights on 04:00–20:00 hr) and fed normal commercial pellets for rainbow trout twice a day at 08:30 and 15:30 hr (0.75% of BW each) until June 23 using an automatic feeder. Melatonin sprayed pellets or control pellets were provided on June 24 (Day 0). Melatonin-containing pellets were prepared by spraying an ethanol solution of melatonin (1g/200 ml) on 2 kg of rainbow trout commercial pellets. The final melatonin content was 500 μg /g-dry pellets. The control pellets were sprayed with only ethanol. Ethanol was evaporated overnight at room temperature and the pellets were stored at –20°C until use. Fish were fed with the pellets sprayed with melatonin or vehicle twice a day at 08:30 and 15:30 hr (0.75% of BW each) using an automatic feeder. Thus, fish of the melatonin-treated group were fed with 7.5mg melatonin/kg-BW/day.

Sampling

The fish were sampled on June 24 (Day 0), July 19 (Day 25), August 23 (Day 60), September 22 (Day 90) and October 22 (Day 120). Sampling was done between 09:00 and 16:00 hr. Fish were anesthetized in 2-phenoxyethanol (0.05%), and body length (BL) and BW were measured. Spermiation was checked by gentle pressing of the abdomen. Blood was collected using a heparinized hematocrit tube by cutting the caudal fin to measure plasma testosterone levels. Blood samples were centrifuged at 2500 g for 10 min at 4°C and plasma was stored at –20°C until analysis. The pituitary was dissected out and frozen on dry ice to measure GTH and sGnRH contents. The bodies without brains were fixed with Bouin's fluid for 24 hr and gonads were dissected out and their weights were measured to calculate GSI. GSI was calculated as follows: gonadal weight/BW × 100. Males whose GSI was more than 0.15% were judged as precocious (see Amano *et al*., 2000). Then, gonads were embedded in paraplast (Monoject, Sherwood Medical, St Louis, MO) and sectioned at 5 μm. The sections were stained with hematoxylin and eosin for histological observation. Data for immature males and females were omitted for analysis.

Changes in plasma melatonin levels of the control and melatonin-treated groups were examined on August 3–4. Blood was sampled from 5 individuals of both sexes at 12:00, 18:00, 22:00, 00:00, 02:00, 06:00, and 12:00 hr. For the sampling during the dark phase, fish were caught and anesthetized in the dark. Then, a dim-red light was turned on during the blood sampling.

Measurements of melatonin, testosterone, sGnRH and GTH

Plasma melatonin levels were measured by radioimmunoassay (RIA) (Iigo *et al*., 1997a). Plasma testosterone levels were measured by time-resolved fluoroimmunoassay (TR-FIA) (Yamada *et al*., 1997). sGnRH and GTH were extracted from the pituitary as previously reported (Amano *et al*., 1992). sGnRH content was measured by RIA for sGnRH (Okuzawa *et al*., 1990). GTH content was measured by TR-FIA for salmonid follicle-stimulating hormone (FSH) and LH (Amano *et al*., 2000).

Statistics

Changes in plasma melatonin levels, BL, BW, GSI, plasma testosterone levels, pituitary sGnRH content and pituitary GTH content in each group were examined by one-way analysis of variance (ANOVA) followed by Scheffe's *F*-test. Student's *t* test was used to compare the value between both groups at each sampling. Two-way ANOVA was used for statistical analysis of the effects of melatonin treatment and sampling time on plasma melatonin levels, BL, BW, GSI, plasma testosterone levels, pituitary sGnRH content and pituitary GTH content. The difference in the spermiation rate between the two groups was analyzed by Fisher's exact probability test at each sampling.

RESULTS

Plasma melatonin profiles

Plasma melatonin profiles after the feeding of pellets sprayed with melatonin or vehicle are shown in Fig. 1. In the control group, plasma melatonin levels exhibited significant

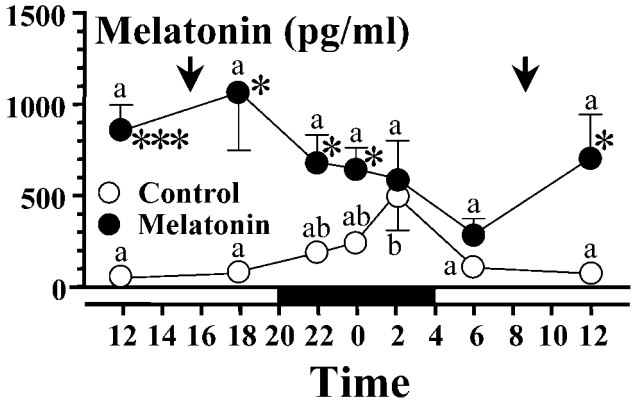

Fig. 1. Changes in plasma melatonin levels. Fish were fed pellets sprayed with melatonin or vehicle twice a day at 08:30 and 15:30 hr (arrows). Blood was sampled from 5 individuals of both sexes at 12:00, 18:00, 22:00, 00:00, 02:00, 06:00, and 12:00 hr. Each value is expressed as the mean and standard error. In each group, means with differing letters differ significantly (P<0.05). ***(P<0.001) and *(P<0.05) indicate the level of statistical difference between the two groups.

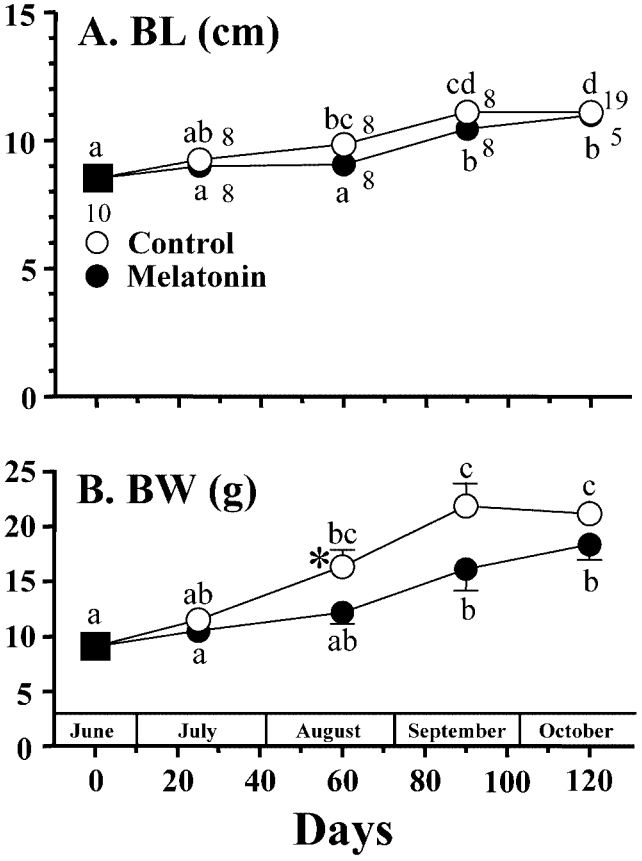

Fig. 2. Changes in (A) BL (cm) and (B) BW (g). Numbers beside each symbol indicate the number of fish employed. Each value is expressed as the mean and standard error. In each group, means with differing letters differ significantly (P<0.05). *(P<0.05) indicates the level of statistical difference between the two groups.

variation (one-way ANOVA, P<0.001) with higher levels during the dark phase, peaking at 02:00 (12:00 (first day) vs 02:00, P<0.01; 18:00 vs 02:00, P<0.01; 02:00 vs 06:00, P<0.01; 02:00 vs 12:00 hr (second day), P<0.01; Scheffe's F-test). In the melatonin-treated group, on the other hand, plasma melatonin levels were constantly high and did not change significantly (one-way ANOVA, P=0.2809). The results of two-way ANOVA indicated that the melatonin treatment had a stimulatory effect on plasma melatonin levels (P<0.001) and that the sampling time had no significant effect (P=0.2739). Interaction between melatonin treatment and sampling time was not significant (P=0.0892). Plasma melatonin levels were significantly higher in the melatonin-treated group than the control group at 12:00, 18:00, 22:00, 00:00, and 12:00 hr (second day) (t-test).

Somatic growth

BL continued to increase until Day 120 in both groups (Fig. 2A). No significant differences in BL were observed between the groups at each sampling (t-test). The results of two-way ANOVA indicated that the melatonin treatment had an inhibitory effect on BL (P<0.05) and that the sampling time had a significant effect (P<0.001). Interaction between melatonin treatment and sampling time was not significant (P=0.4534).

BW continued to increase until Day 120 in both groups (Fig. 2B). BW was significantly lower in the melatonin-treated group than the control group on Day 60 (t-test, P<0.05). The results of two-way ANOVA indicated that the melatonin treatment had an inhibitory effect on BW (P<0.01) and that the sampling time had a significant effect (P<0.001). Interaction between melatonin treatment and sampling time was not significant (P=0.2051).

GSI and testicular development

GSI significantly increased on Day 60 and 90 in the control and the melatonin-treated group, respectively (Fig. 3). No significant differences in GSI were observed between the groups at each sampling (t-test). The results of two-way ANOVA indicated that the melatonin treatment had an inhibitory effect on GSI (P<0.01) and the sampling time had a significant effect (P<0.001). The interaction between melatonin treatment and sampling time was not significant (P=0.3138).

Spermiation was observed on Day 90 and 120 in both groups. On Day 90, 2 fish spermiated out of 8 fish in both the groups (25.0%). On Day 120, 12 fish spermiated out of 19 fish in the control group (63.2%) and 4 fish spermiated out of 5 fish in the melatonin- treated group (80.0%): no significant differences were observed in the spermiation rate between the two groups (Fisher's exact probability test, P=0.6311). By histological observation, normal testes were seen in all the fish examined and no histological differences were seen in testis between the two groups in each sampling time.

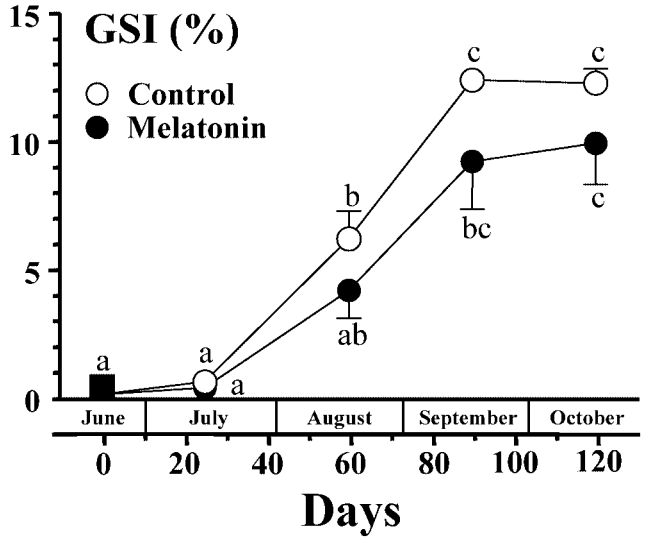

Fig. 3. Changes in GSI. Each value is expressed as the mean and standard error. In each group, means with differing letters differ significantly (P<0.05).

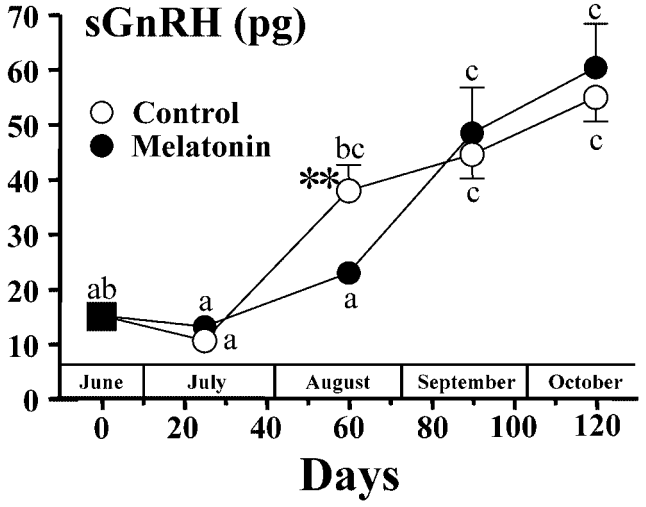

Fig. 5. Changes in pituitary sGnRH content. Each value is expressed as the mean and standard error. In each group, means with differing letters differ significantly (P<0.05). **(P<0.01) indicates the level of statistical difference between the two groups.

Plasma testosterone levels

Plasma testosterone levels continued to increase until Day 120 in the control group, while plasma testosterone levels did not show significant changes throughout the experiment in the melatonin-treated group (Fig. 4). Plasma testosterone levels were significantly lower in the melatonin-treated group than the control group on Day 60 (t-test, P<0.05). The results of two-way ANOVA indicated that the melatonin treatment had an inhibitory effect on testosterone levels (P<0.01) and that the sampling time had a significant effect (P<0.001). The interaction between melatonin treatment and sampling time was not significant (P=0.5162).

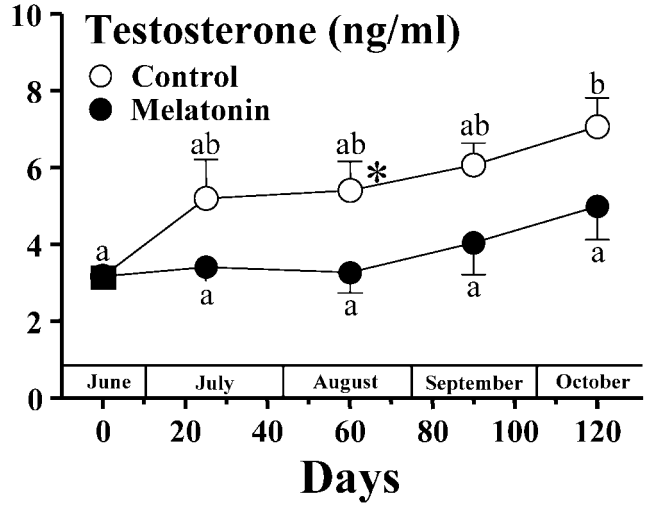

Fig. 4. Changes in plasma testosterone levels. Each value is expressed as the mean and standard error. In each group, means with differing letters differ significantly (P<0.05). *(P<0.05) indicates the level of statistical difference between the two groups.

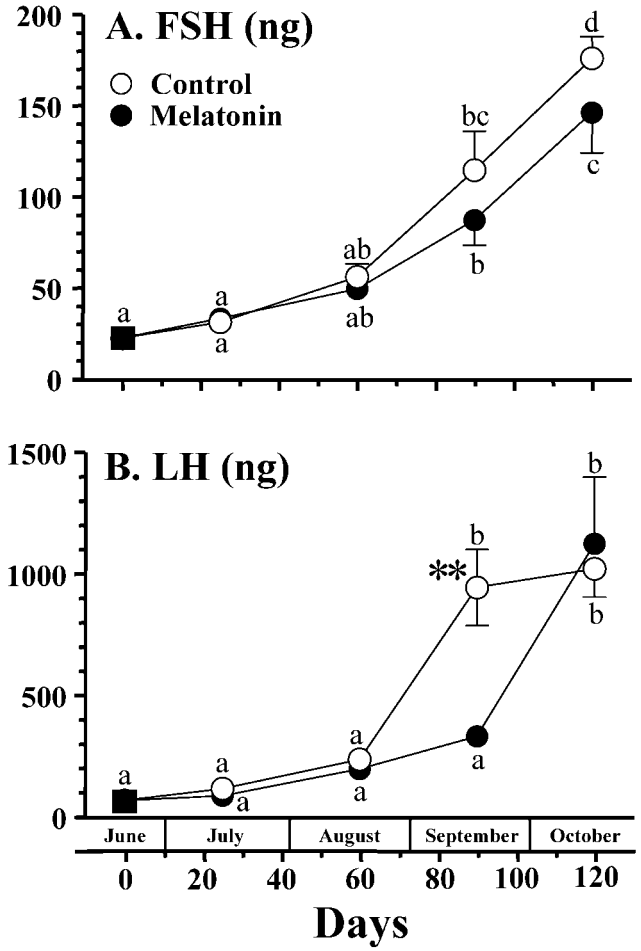

Fig. 6. Changes in pituitary (A) FSH content and (B) LH content. Each value is expressed as the mean and standard error. In each group, means with differing letters differ significantly (P<0.05). **(P<0.01) indicates the level of statistical difference between the two groups.

Pituitary sGnRH content

Pituitary sGnRH content significantly increased on Day 60 and 90 in the control and the melatonin-treated group, respectively (Fig. 5). Pituitary sGnRH content was significantly lower in the melatonin-treated group than the control group on Day 60 (*t*-test, $P<0.01$). The results of two-way ANOVA indicated that the melatonin treatment had no effect on sGnRH ($P=0.8409$) but the sampling time had a significant effect ($P<0.001$). The interaction between melatonin treatment and sampling time was not significant ($P=0.2453$).

Pituitary FSH and LH contents

Pituitary FSH content continued to increase until Day 120 in both groups (Fig. 6A). No significant differences in pituitary FSH content were observed between the groups at each sampling (*t*-test). The results of two-way ANOVA indicated that the melatonin treatment had no effect on FSH ($P=0.1310$) but the sampling time had a significant effect ($P<0.001$). The interaction between melatonin treatment and sampling time was not significant ($P=0.5967$).

Pituitary LH content significantly increased on Day 90 and 120 in the control and the melatonin-treated group, respectively (Fig. 6B). Pituitary LH content was significantly lower in the melatonin-treated group than the control group on Day 90 (*t*-test, $P<0.01$). The results of two-way ANOVA indicated that the melatonin treatment had no effect on LH ($P=0.1109$) but the sampling time had a significant effect ($P<0.001$). The interaction between melatonin treatment and sampling time was significant ($P<0.05$).

DISCUSSION

It is demonstrated that administering pellets sprayed with “high-dose” melatonin disturbed the typical plasma melatonin profile under light-dark conditions. In the melatonin-treated group, plasma melatonin levels were constantly high and did not change significantly during the 24-hr LD cycle, although they tended to be low in the early morning. Duration time of low melatonin levels is probably very short, because plasma melatonin levels might have increased immediately after eating pellets at 08:30 hr.

In the previous study, testicular development in underyearling male masu salmon reared under a long photoperiod was accelerated by oral low-dose melatonin treatment. The plasma melatonin profile in the masu salmon fed melatonin sprayed pellets once at 11:00 hr under a long photoperiod is similar to that expected under a short photoperiod, suggesting that the treatment was recognized as a short photoperiod (Amano *et al*., 2000). In the present study, by contrast, high-dose melatonin treatment had an inhibitory effect on GSI and plasma testosterone levels, suggesting that melatonin treatment was not recognized as a short photoperiod. It is reported that, in order for the melatonin signal to be interpreted correctly, a melatonin-free interval within each 24 hr cycle may be required as has been reported in hamsters (Maywood *et al*., 1991). In the present study, there seemed no melatonin-free interval or no period when plasma melatonin levels were below basal levels: the melatonin message might not be conveyed, resulting in a delay of testicular maturation. These results indicate that plasma melatonin profile is important for mediating photoperiodic signals that regulate gonadal maturation in underyearling precocious male masu salmon.

The melatonin treatment inhibited gonadal maturation possibly via inhibiting sGnRH and LH, since the levels of pituitary sGnRH on Day 60 and LH on Day 90 were significantly lower in the melatonin-treated group. However, in the previous study, it is suggested that low-does melatonin administration stimulates testicular development by stimulating pituitary FSH levels (Amano *et al*., 2000). Although plasma GTH levels were not measured because insufficient plasma was obtained from individual fish in both experiments, roles of FSH and LH in early developmental stage should be clarified in future.

Melatonin treatment also inhibited somatic growth. Thus, it is possible that a delay of gonadal maturation in the melatonin-treated group was, at least in part, due to the suppressed growth or reduced amount of food intake rather than the direct action of melatonin on the brain and/or the pituitary, although the precise mechanism is not clear at this stage.

Where is the site of melatonin action that regulates photoperiodism in masu salmon? We have recently examined the characteristics of melatonin binding sites in the masu salmon brain by radioreceptor assay (Amano *et al*., 2003). The specific binding of 2-[^{125}I]iodomelatonin was rapid, stable, saturable and reversible. Saturation experiments demonstrated that 2-[^{125}I]iodomelatonin binds to a single class of receptor sites. Competition experiments revealed that the binding sites are highly specific for melatonin and related analogues. Treatment with guanosine 5’-*O*-(3-thiotriphosphate) significantly reduced the specific binding, indicating that melatonin binding sites in the masu salmon brain are coupled to G protein. We also examined the changes of melatonin binding sites in the brain of precocious male masu salmon during testicular maturation induced by photoperiodic treatment. An affinity and a total binding capacity were high when testicular maturation began under short photoperiod (Amano *et al*., unpublished data). These results suggest that these changes in melatonin binding sites in the brain are of physiological relevance in the mediation of photoperiodic signaling via melatonin in precocious male masu salmon.

Melatonin binding sites have been localized to the preoptic-anterior hypothalamus and also in other brain areas in the goldfish (Martinoli *et al*., 1991; Iigo *et al*., 1994b), Atlantic salmon (Ekström and Vaněček, 1992), rainbow trout (Davies *et al*., 1994; Mazurais *et al*., 1999), and catfish *Silrus asotus* (Iigo *et al*., 1997b). A possible site is the preoptic area. sGnRH neurons are located in this area and considered to be involved in the secretion of GTH in masu salmon (see Amano *et al*., 1997). Recently, we have found

melatonin binding sites in the preoptic area and pituitary of masu salmon (Amano *et al*., unpublished data). Since it is unlikely that melatonin receptors are located on sGnRH neurons, melatonin may act through interneurons to influence the neural activity of sGnRH and thereby regulate the secretion of GTH from the pituitary. It is also possible that melatonin acts directly to the pituitary as in pike *Esox lucius* (Gaildrat and Falcon, 1999), because melatonin binding sites exist in the pituitary of masu salmon (Amano *et al*., unpublished data). Moreover, the possibility can not be ruled out that melatonin acts directly to the testis, although the existence of melatonin binding sites have not been reported in teleost fishes to date.

In conclusion, high dose melatonin administration twice a day (7.5mg melatonin/kg body weight/day) disturbed plasma melatonin profiles in precocious male masu salmon under long photoperiod; they were constantly high with no significant change. Furthermore, delay of testicular maturation and low levels of sGnRH and LH in the pituitary were observed by high dose melatonin administration. In our previous study, on the contrary, low dose melatonin administration once a day (0.5mg melatonin/kg body weight/day) stimulated testicular development but did not completely activate the brain-pituitary-gonadal axis (Amano *et al*., 2000). Taken together, these results support the idea that the plasma melatonin profile is important for mediating photoperiodic signals that regulate the brain-pituitary-gonadal axis. Further studies are required to elucidate how melatonin mediates photoperiodic signaling in fish.

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