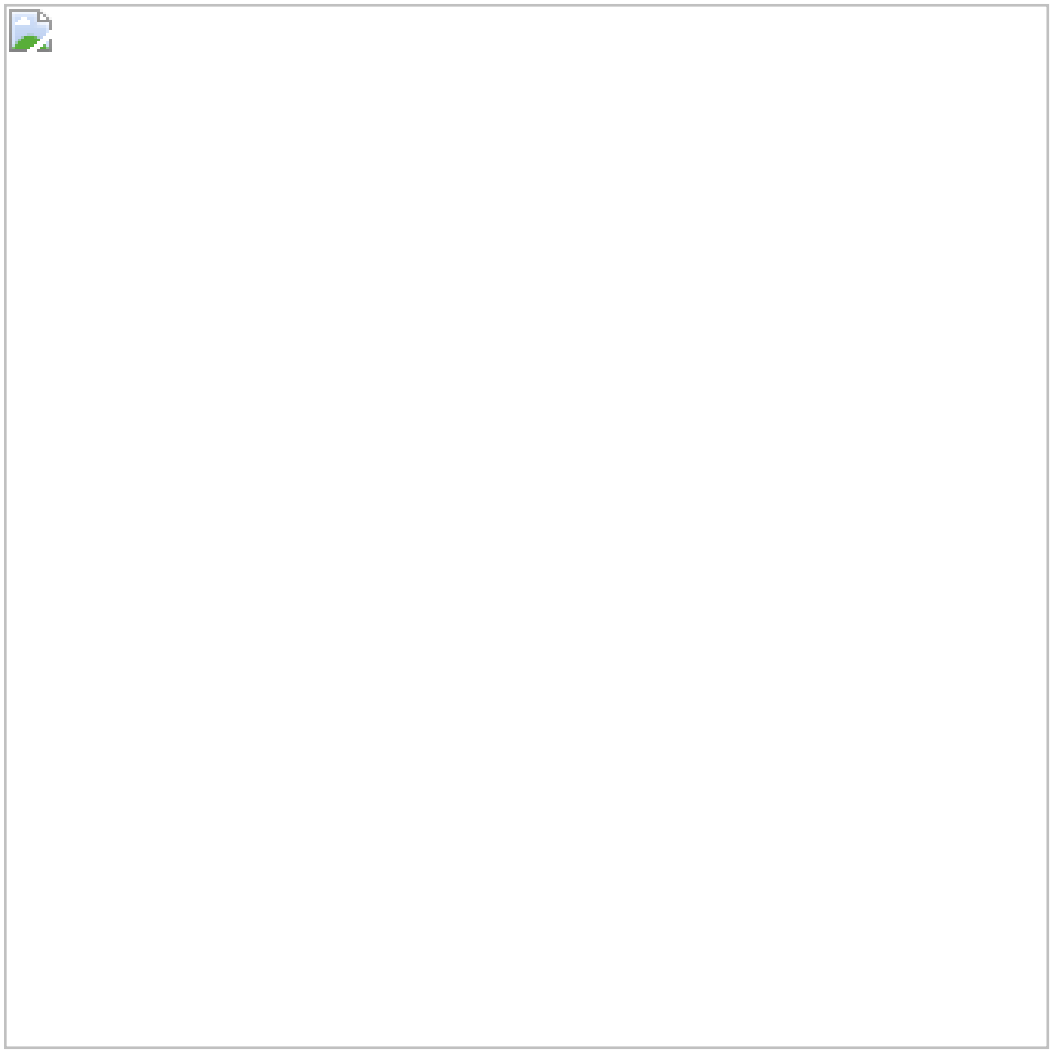

Circadian Changes in Serum Concentrations of Steroids in Japanese Char Salvelinus leucomaenis at the Stage of Final Maturation

Authors: Yamada, Hideaki, Satoh, Ri-ichi, Ogoh, Masashi, Takaji, Keigo, Fujimoto, Yasufumi, et al.

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Circadian Changes in Serum Concentrations of Steroids in Japanese Char *Salvelinus leucomaenis* at the Stage of Final Maturation

Hideaki Yamada[1]*, Ri-ichi Satoh[1], Masashi Ogoh[1], Keigo Takaji[1], Yasufumi Fujimoto[1], Takeshi Hakuba[1], Hiroaki Chiba[1], Akira Kambegawa[2] and Munehico Iwata[1]

[1]*School of Fisheries Sciences, Kitasato University, Sanriku, Iwate 022-0101, Japan*
[2]*The Kambegawa Laboratory, Komae, Tokyo, Japan*

ABSTRACT—Circadian changes in serum concentrations of testosterone (T), 11-ketotestosterone (11KT), estradiol-17β (E2), 17α,20β-dihydroxy-4-pregnen-3-one (DHP), 17α-hydroxyprogesterone (OHP), cortisol (F) and progesterone (P) were investigated in the spermiated/ovulated Japanese char *Salvelinus leucomaenis* for over three days using newly developed time-resolved fluoroimmunoassays. Testosterone and DHP in both sex and 11KT in male showed significantly ($P<0.05$) higher serum levels just before/after onset of darkness (15:00 or 18:00), and the levels during night and daytime were significantly ($P<0.05$) lower than those of the peak levels. Serum F levels in both sex during dark phase were significantly ($P<0.05$) higher than those levels during daytime. A surge of serum OHP concentrations in both sexes was observed at the time of twilight (03:00). The peak time of serum T, 11KT and DHP levels were approximately 6 hours prior to those of serum F and OHP levels. Serum E2 in female and P in both sex fluctuated intensely during sampling period, and did not show remarkable changes. These results strongly suggest the existence of circadian-like diel changes in serum T, DHP, F and OHP levels in both sex and 11KT in male, and no variations in serum E2 in female and P in both sex in spermiated/ovulated Japanese char under the stage of final maturation. Furthermore, relationship between circadian rhythms of steroid hormones and spawning behaviors are discussed in the present study.

Key words: testosterone, cortisol, progesterone, final maturation, spawning behavior

INTRODUCTION

In many of Pacific salmon, males and females generally die after spawning. Whereas, Japanese char *Salvelinus leucomaenis* are known to survive spawning and may reproduce several times before dying. In contrast to these life-historical information, physiological and endocrinological information around spawning time, especially after spawning, are little in repeatedly spawning species such as Japanese char. Investigating endocrinological changes in reproductive hormones around spawning time of Japanese char make not only providing endocrinological information but also being able to discuss relationship between those steroids and several behaviors related to feeding and spawning.

In salmonid species, sexual maturation and spawning are endocrinologically regulated by hypothalamus – pituitary – gonadal axis, and sex steroids such as 11-ketotestosterone (11KT) and estradiol-17β (E2) act on spermatogenesis and vitellogenesis in males and females, respectively. Blood sex steroids levels of these hormones are generally high during sexual maturation, but clearly low at the time of final maturation in both sexes (Campbell *et al*., 1980; Fostier *et al*., 1983). In contrast to 11KT and E2, the blood hormone levels of 17α,20β-dihydroxy-4-pregnen-3-one (DHP), which is well known and investigated to induce final maturation including spermiation or ovulation, are remarkably high around the time of final maturation (Goetz, 1983; Young *et al*., 1983). Although there are many of endocrinological reports on sexual and final maturation as above, changes in those hormones around/after spawning stage have not been well investigated.

It is well known that diel changes in blood cortisol concentrations are observed in juvenile Atlantic salmon *Salmo salar* (Nichols and Weisbart, 1984; Thorpe *et al*., 1987), immature rainbow trout *Oncorhynchus mykiss* (Bry, 1982; Rance *et al*., 1982; Laidley and Leatherland, 1988; Holloway

* Corresponding author: Tel. +81-192-44-1910;
FAX. +81-192-44-1910.
E-mail: hyamada@kitasato-u.ac.jp

et al., 1994), and brown trout *Salmo trutta* (Pickerling and Pottinger, 1983). Thyroid hormones (Eales *et al*., 1981; Laidley and Leatherland, 1988; Boujard and Leatherland, 1992; Boujard *et al*., 1993; Gomez *et al*., 1997) and growth hormone (Holloway *et al*., 1994; Reddy and Leatherland, 1994) also show diel variations in blood levels of rainbow trout. Lamba *et al*. (1983) have reported the diel changes in blood sex steroids (testosterone and E2) of catfish *Heteropneustes fossilis*, and Santos *et al*. (1986) showed diurnal changes in blood testosterone (T), E2, DHP, and 17α-hydroxyprogesteone (OHP) concentrations in carp *Cyprinus carpio*. However, no report on the circadian changes in sex steroids and other steroid hormones has been investigated in matured salmonid fish.

In the present study, we have investigated diel changes in serum reproductive steroids (11KT in male, E2 in female and DHP in both sexes) levels for over 3 days to understand exact pattern of circadian changes and biological roles of reproductive hormones around spawning time in Japanese char. In addition, cortisol, T, OHP and progesterone changes were also investigated. We have discussed those results concerning to relationship between reproductive/spawning behaviors and the circadian changes of steroid hormones in the spermiated/ovulated Japanese char after the stage of final maturation.

MATERIALS AND METHODS

Experimental fish

Two years old Japanese char *Salvelinus leucomaenis* were obtained from a local salmon hatchery, Iwate, Japan. The fish were reared in a FRP tank (2,000 liter) under natural photoperiod, and fed at 9:00, 13:00 and 17:00 (3% body weight commercial diet/day) for 6 months before sampling. The fish were not fed during sampling period for three days. Water temperature was 8.6±0.6°C throughout sampling period.

Sampling schedule

Fish were sampled every 3 hr for 3 days, starting at 12:00 on November 6 and finishing at 12:00 on November 9. At each sampling, ten fish were carefully collected with a hand net, and were quickly anesthetized with 0.05% 2-phenoxyethanol. After collecting blood from caudal blood vessels using 1 ml syringe with 26G needle, folk length (26.5±0.11 cm, n=250) and body weight (155±2.56 g, n=250) were measured. The blood was centrifuged at 3,000 x g for 15 min, and the obtained serum was stored at –30°C until further hormone assays. Testis and ovary were in stage of just after spermiation or ovulation. Mean gonadosomatic indices of all male and female fish were 0.58±0.05 (n=133) and 2.98±0.42 (n=117), respectively.

Chemicals

Europium (Eu) labeling reagent and enhancement solution were purchased from Perkin-Elmer (Finland). Testosterone (T), 11-ketoteststerone (11KT), estradiol-17β (E2), 17α,20β-dihydroxy-4-pregnen-3-one (DHP), 17α-hydroxypreogesterone (OHP), progesterone (P), cortisol (F) and other steroids for cross reactivity, and bovine serum albumin (BSA) were purchased from Sigma Chemicals (St. Louis, MO, USA). The anti-rabbit IgG goat IgG fraction was purchased from Seikagaku Kogyo (Tokyo, Japan). Antisera for T (FKA102), 11KT (FKA118E), E2 (FKA236E), DHP (FKA332), F (FKA402), OHP (FKA332), and P (FKA302) were purchased from Cosmo-Bio (Tokyo, Japan). Other chemicals were purchased from WAKO Pure Chemicals (Tokyo, Japan).

Time-Resolved Fluoroimmunoassay (TR-FIA)

Serum concentrations of steroid hormones were determined by time-resolved fluoroimmunoassay (TR-FIA) according to the method of Yamada *et al*. (1997). In brief, T-BSA, 11KT-BSA, E2-BSA, DHP-BSA, F-BSA, OHP-BSA, and P-BSA conjugates were prepared by the methods of Hosoda *et al*. (1979) and Asahina *et al*. (1995). Steroid-BSA conjugate was immobilized to the wells of microtiter plate (Nunc Immunoplate, Nunc, Denmark) at the concentrations of 0.05 μg/ml for T-BSA, 11KT-BSA and DHP-BSA, 0.02 μg/ml for E2-BSA, and 0.5 μg/ml for F-BSA, OHP-BSA and P-BSA. After three washes with 0.9% saline, the wells were blocked with 0.1% BSA, followed by three washes for immunoassays. Fifty μl of standard or extracted serum samples and 150 μl of anti-steroid sera were dispensed to the wells. Dilution factors of anti-steroid sera for T, 11KT, E2, DHP, F, OHP, and P were ×10,000, ×48,000, ×100,000, ×20,000, ×640,000, ×800,000, ×3,000, respectively. After the immunoreaction (4°C, overnight) and three washes, europium-labeled anti-rabbit IgG goat IgG (Eu-IgG) was added to the wells, and the plate was shaken for 1 hr at room temperature. Eu was dissociated from the complex of steroid, primary antibody and Eu-IgG by addition of enhancement solution, and the intensity of Eu was measured by Arcus 1234 fluorometer (Wallac Oy, Finland).

Cross reactivities of T (Yamada *et al*., 1997), 11KT, E2, DHP, F, OHP, and P TR-FIA system were satisfactorily low in each system (data not shown).

Hormone assays

Serum hormone concentrations of T, 11KT, E2, DHP, F, OHP, and P were time-resolved fluoroimmunoassayed. 11-ketotestosterone and E2 were measured and presented in male and female, respectively. Testosterone, DHP, OHP, F and P levels (n=10) were measured in both sexes, and presented in male and female separately. Serum levels of T, DHP, F, OHP and P of males and females were statistically the same at all samplings. Sample size of male and female were between 4 and 6 at each sampling, except for following samplings; November 6 [15:00 (male : female =7:3)], November 7 [12:00 (3:7), 18:00 (7:3)], November 8 [6:00 (7:3), 18:00 (7:3)], November 9 [0:00 (8:2), 12:00 (1:9)].

Statistical analysis

All hormone data presented are expressed as means± standard error. Serum levels of male and female were analyzed by one-way ANOVA and t-test. All serum hormone levels were statistically analyzed by one-way ANOVA, followed by Duncan's multiple range test.

RESULTS

Male

Circadian changes in serum testosterone and 11-ketotestosterone

Serum T levels showed slight increase at 18:00 on November 6 and 8 (Fig. 1A). Slight, but not significant increase in serum T levels was observed at 06:00 on November 7, and 18:00 (just before onset of darkness) on November 7. Remarkable and significant ($P<0.05$) changes in serum 11KT were observed throughout sampling period (Fig. 1B). The levels peaked at 15:00, followed by decreasing to basal levels during nighttime until noon.

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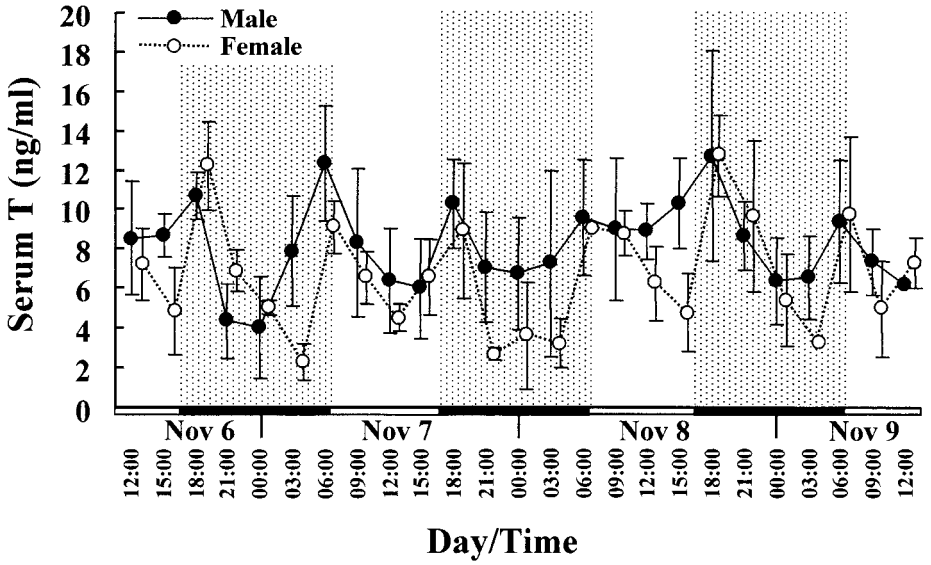

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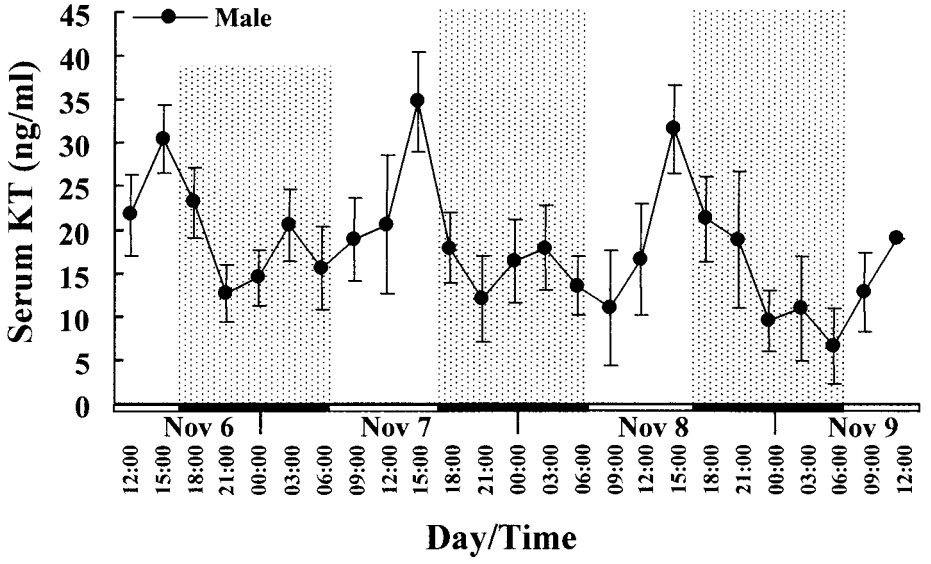

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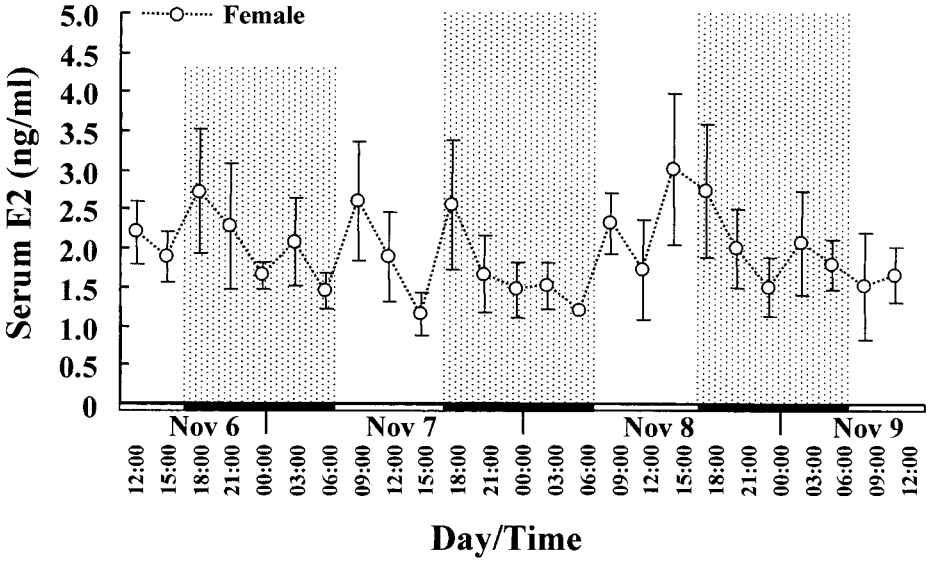

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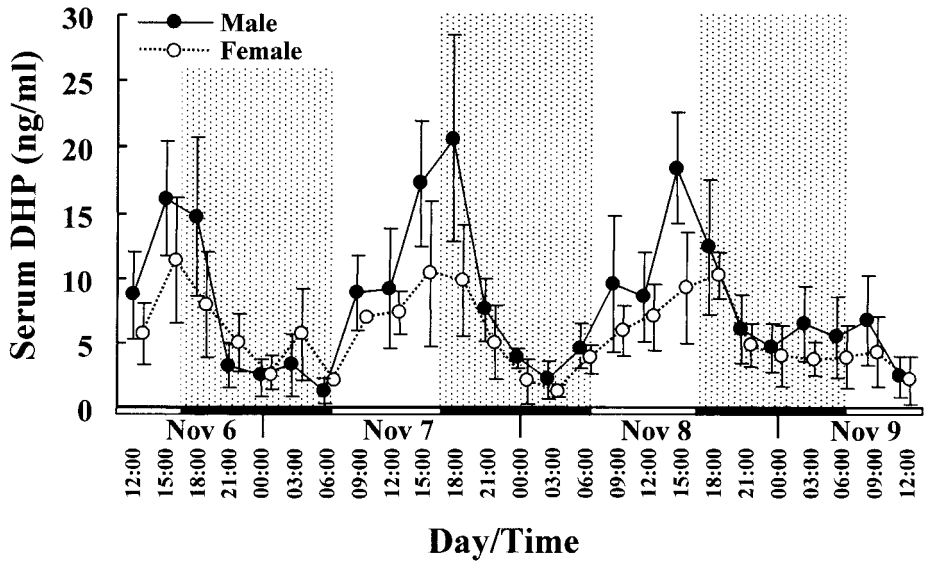

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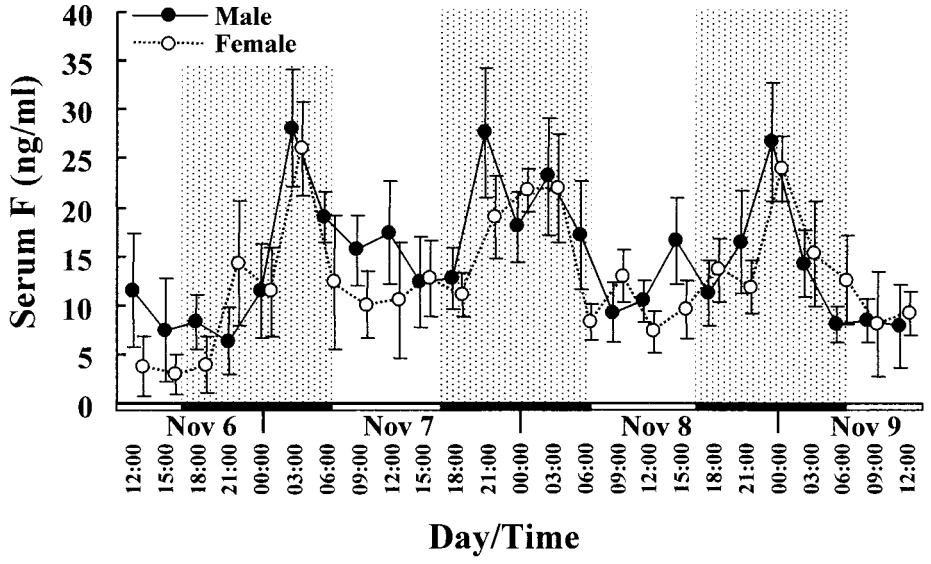

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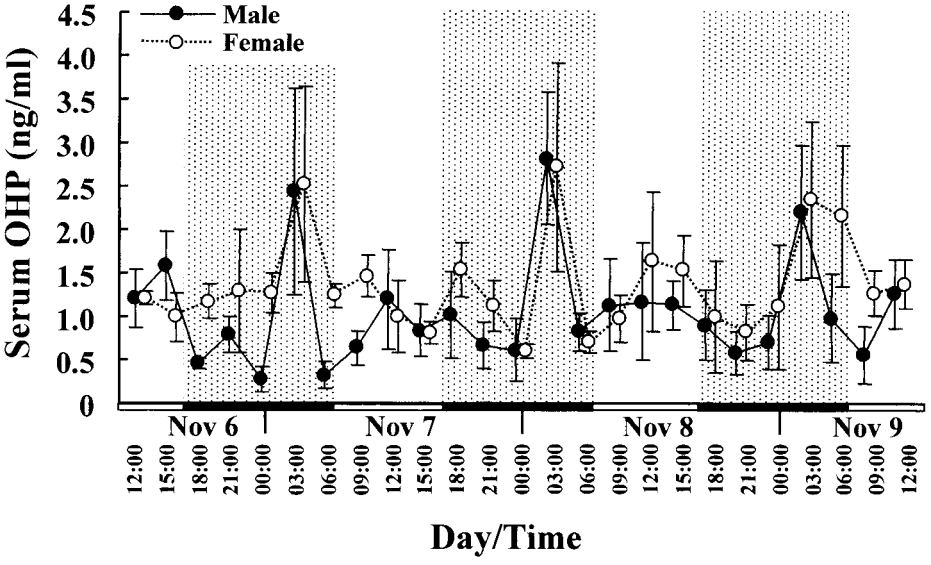

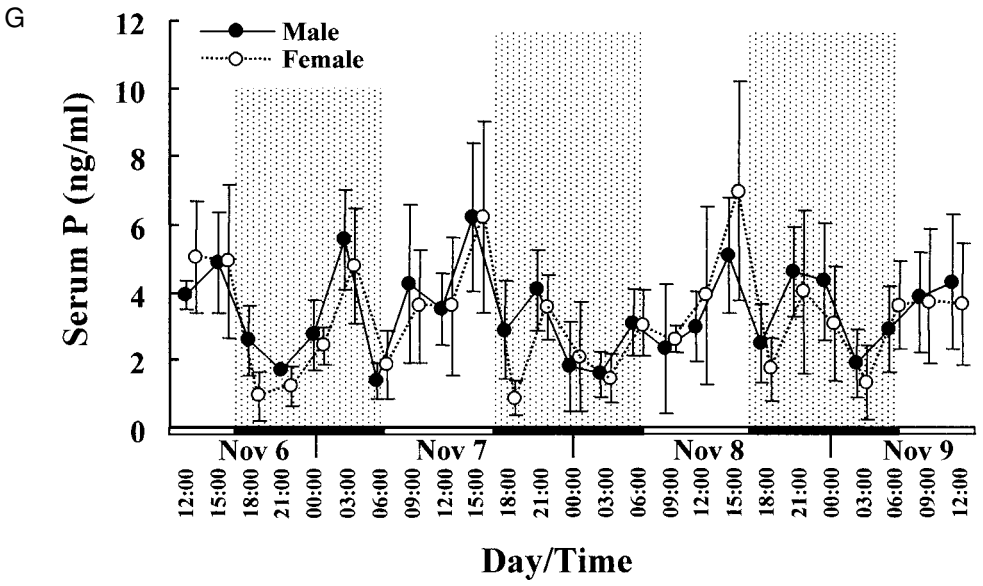

Fig. 1. Circadian changes in serum levels of various steroids. (A: testosterone in both sex, B: 11-ketotestosterone in male, C: estradiol-17β in female, D: 17α,20β-dihydroxy-4-pregnen-3-one in both sex, E: cortisol in both sex, F: 17α-hydroxyprogesterone in both sex, G: progesterone in both sex) in the Japanese char *Salvelinus leucomaenis* in the spawning season. Each point represents the mean±SE. Shade shows nighttime from sunset to sunrise in calendar.

Circadian changes in serum 17α,20β-dihydroxy-4-pregnen-3-one and cortisol

Male fish showed clear and rhythmical increase in serum DHP concentrations at 18:00 on November 7 and 8 (Fig. 1D). On November 6, peak was observed at 15:00, but the levels at 15:00 and 18:00 were the same. The levels during nighttime until noon were significantly ($P<0.05$) lower than peak levels. Relatively high serum F concentrations (Fig. 1E) were observed in males during nighttime (21:00~03:00), and those levels decreased to basal levels in daytime.

Circadian changes in serum 17α-hydroxyprogesterone and progesterone

A sharp peak of serum OHP levels (Fig. 1F) was observed at 03:00, and the level was significantly higher than other sampling times, however the maximum concentrations in a daytime was observed at 12:00 (Nov 7 and 9) and 15:00 (Nov 6 and 8). Although significant changes were not observed in serum P concentrations, the levels increased at 15:00 throughout sampling period (Fig. 1G).

Females

Circadian changes in serum testosterone and estradiol-17β

Serum T levels showed significant ($P<0.05$) peaks at 18:00 on November 6 and 6:00 on November 7 and 8 (Fig. 1A). Slight, but not significant increase in serum T levels was observed at 18:00 on November 7 and 8, and 6:00 on November 9. Although E2 level did not show remarkable change in serum concentrations (Fig. 1C), it tended to rise around sunset during the sampling period.

Circadian changes in serum 17α,20β-dihydroxy-4-pregnen-3-one and cortisol

The female fish showed clear increase in serum DHP concentrations at 15:00 on November 6 and 7, and 18:00 on November 8 (Fig. 1D). The levels during nighttime were significantly ($P<0.05$) lower than peak levels. Serum F concentrations during nighttime (21:00~03:00) were observed (Fig. 1E), and peak levels were observed at 3:00 on November 7 and 8, and at 0:00 on November 9. The serum F levels decreased to basal levels in daytime.

Circadian changes in serum 17α-hydroxyprogesterone and progesterone

Serum OHP levels (Fig. 1F) were peaked at 3:00 on November 7, 8 and 9, and the level was significantly higher than other sampling times. Significant changes were not observed in serum P concentrations, however slight increase was observed at 12:00 on November 6, at 3:00 and 15:00 on November 7, and at 15:00 on November 8 (Fig. 1G).

DISCUSSION

In many of reports on circadian changes of blood hormone concentrations (Eales *et al*., 1981; Rance *et al*., 1982; Lamba *et al*., 1983; Pickering and Pottinger, 1983; Santos *et al*., 1986; Thorpe *et al*., 1987; Laidley and Leatherland, 1988; Holloway *et al*., 1994; Reddy and Leatherland, 1994; Gomez *et al*., 1997), they have discussed the results, that were obtained from one day data. In contrast to those reports, we investigated circadian changes of blood hormone levels for over 3 days. Although the changing patterns were almost the same, slight different was observed among 3 days. Continual sampling over two or three days can normalize daily fluctuations of blood hormone levels, which may

be occur by the slight changes in rearing conditions or environmental stimuli, and the continual sampling can provide accurate patters of hormone levels.

In the present study, serum F peak was observed in nighttime regardless of male and female same as Atlantic salmon (Thorpe *et al*., 1987) and brown trout (Pickering and Pottinger, 1983; Laidley and Leatherland, 1988). On the other hand, a peak of F in daytime (Garcia and Meier, 1973; Singley and Chavin, 1975), in night (Redgate, 1974), and under both phases (Peter *et al*., 1978) has also reported in various fishes. This discrepancy of peak time of F may due to species differences, feeding schedules and developmental stage differences as suggested by Pickering and Pottinger (1983). According to Delahunty *et al*. (1978), single feeding in the morning causes F peak in dark phase. Though feeding had already stopped in the morning of November 6 in the present study, F peak was observed during nighttime for 3 days (Nov 6–9). The F peak observed in the present study may be induced by other biological and/or environmental stimuli.

Sampling fish from individual tank is better to obtain F results in the circadian study, because of negligible of sampling stress on the experimental fish (Pickerling and Pottinger, 1983). In the present study, the fish were sampled from a tank for 3 days with careful netting to avoid netting stress to the remaining fish, therefore the average of every serum sample was maintained in physiological levels. The experimental fish clearly indicated increase in serum F concentrations during nighttime for over three sampling days in the present study, suggesting the circadian rhythm in serum F concentration of Japanese char.

Until now, diel changes in blood sex steroids of salmonid fish have not been reported. In teleost, catfish *Heteropneustes fossilis* (Lamba *et al*., 1983) showed increase in blood E2 and T levels at the onset of darkness, and the carp *Cyprinus carpio* (Santos *et al*., 1986) exhibited diurnal changes in blood T, E2, DHP, and OHP concentrations. Serum T concentrations in both sex were the same, and increased around the onset of darkness in the present study as same as Lamba *et al*. (1983). In addition, changing pattern of serum 11KT concentrations in male was almost the same as that of serum T levels. The high level of serum T and 11KT levels was observed at same clock time for over 3 sampling days, strongly suggesting existence of circadian rhythms of serum T in both sex and 11KT in male in the Japanese char during spawning period.

The T concentrations in both sex at the time of twilight before sunrise and sunset were significantly ($P<0.01$) higher than the T levels in nighttime (21:00~03:00) in the present study. The increase in serum T levels at twilight time may be involved in the phenomenon to be common to male and female such as enhanced feeding activity and its related behaviors. In general, active feeding behavior of Japanese char was observed at twilight time (in our preliminary observation). In wild population of salmonid fishes, moreover, feeding and its related behavior such as aggressive behavior activity are high at the time of twilight before sunrise in Atlantic salmon *Salmo salar* (Kadri *et al*., 1997), and blood testosterone concentration of dominant fish is significantly higher than that of subordinate brown trout *S. trutta* (Cardwell *et al*., 1996). These reports strongly suggest close relationship between testosterone and feeding or its related behaviors in fishes. This is also supported by the data from T treatment in *Cyprinodon variegatus*, in which aggressive behaviors were enhanced by T administration (Higby *et al*., 1991). Moreover, environmental light and vision are important for recognizing other individuals in a school, and the feeding activities under relatively low illumination intensity (0.01~0.4 lx) in salmonid fish (Azuma and Iwata, 1996).

Peak of serum 11KT level in male may be involved in mating behavior including territorial and aggressive behaviors of male char at the time of spawning season. 11-ketotestosterone induces typical male-type spawning behavior in the male goldfish (Kobayashi and Nakanishi, 1999) and in female goldfish (Stacey and Kobayashi, 1996; Kobayashi *et al*., 1997). Male salmon defend the nesting females from other males, and 11KT levels of male kokanee salmon placed with females are higher than that of male without female (Liley *et al*., 1993). Moreover, 11KT levels of dominant male rainbow trout are higher than subordinate fish (Cardwell *et al*., 1996).

In the present study, spermiated male and ovulated female showed clear and rhythmical increase in serum DHP levels, suggesting that the DHP was synthesized in endocrine tissues of somewhere including gonad. We have no data related to origin of the circulating DHP. Further investigation is required to elucidate this phenomena. In addition, increase in serum DHP concentrations at 15:00 (November 6) and 18:00 (November 7, 8) may be involved in initiation of mating and spawning behaviors of both male and female, which will probably occur some time later. In the wild population, the spawning activities are strong during darkness in pink salmon (Smirnov, 1975; Chebanov, 1980) and in sockeye salmon and lake char (Martin and Olver, 1980). The spawning behavior of female salmon starts just after (within 30 min) onset of ovulation (Prof. Maekawa, personal communication). Furthermore, administration of DHP into sexually matured rainbow trout can induce spawning behavior (Mayer *et al*., 1994). Although no reports on spawning time were found in wild Japanese char, increase in serum DHP levels at the onset of darkness is possibly involved in spawning behavior of male and female Japanese char.

In the previous report, catfish shows E2 peak during dark phase of reproductive period (Lamba *et al*., 1983), however no E2 rhythm was observed in female in the present study. Because of that E2 is synthesized at ovarian follicles during reproductive period, and the serum level was high during vitellogenesis, but low in final maturation of char (Kagawa *et al*., 1981). The female char have ovulated already in the present study, suggesting less E2 synthesizing activities than vitellogenic stage. This may be the reason for low E2 concentrations and no remarkable changes in the

female char after ovulation.

A surge of serum OHP concentrations at 3:00 was observed. Whereas, serum 11KT, E2 and DHP levels in nighttime were relatively low compared to those of daytime. It may be related to the metabolic pathways of sex steroids and DHP, because the OHP is intermediate metabolite from progesterone in the sex steroid and DHP pathways. Serum P concentrations showed a tendency to rise at 15:00 in both male and female in the present study, thus biological meanings of the tendency were unknown.

In conclusions, we have investigated diel changes in serum T, 11KT, E2, DHP, F, OHP, and P, and firstly found the circadian variations in serum T, DHP, OHP and F in both sex and 11KT in male, but not found in E2 in female and P in both sex for over 3 sampling days in spermiated/ovulated Japanese char. Possible involvement of circadian changes of various steroids in spawning behavior such as mating, spawning, territorial and aggressive behaviors were discussed in the present study.

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