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Sex-specific Cortisol and Sex Steroids Responses in Stressed Sockeye Salmon during Spawning Period

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ABSTRACT—Response to stress during the spawning period of sockeye salmon (*Oncorhynchus nerka*) was investigated by measuring cortisol, selected steroids and glucose levels in the plasma of cultured fish under acute confinement conditions. Fish were individually placed in nets and sacrificed to obtain blood samples after each confinement period: 0, 3, 5,15 and 30 min, respectively. In males, acute stress increased cortisol levels in the plasma at 15 min and testosterone, 11-ketotestosterone and glucose levels in the plasma within 3 min. In females, the cortisol level was higher than that in males and did not significantly change at 15 and 30 min. Testosterone levels in females decreased at 15 min in confinement. Progesterone, 17-hydroxyprogesterone and 17,20 β-dihydroxy-4-pregnen-3-one levels did not change with confinement time in females. To know the reproductive stage of the fish used in the stress experiment we collected blood and measured sex steroid hormones in different fish from the same pond one week and one day before the experiment. In these fish, the sex steroids in males and females changed with the pattern of breeding. Cortisol levels were higher in females than in males for one week. This study demonstrates that the cortisol level in females is higher than in males during their spawning period, and that females might be less sensitive to changes in the level of cortisol caused by acute stress than are the males.

Key Words: Stress, Cortisol, Sex steroid, Breeding season, Salmon

INTRODUCTION

Collection of blood samples from wild salmon is necessary to study their physiological conditions by measuring hormones and other chemicals in the blood. However, capture, handling, and bleeding of fish cause stress, and the stress induces changes in the circulating hormone levels (Greenberg and Wingfield, 1987; Carragher and Sumpter, 1990). In teleosts, cortisol is a major stress hormone and its plasma level increases are positively correlated with stress levels (Billard and Gillet, 1981; Pickering *et al.*, 1982; Sumpter *et al.*, 1986). Many studies of adult salmon also reported a correlation between plasma cortisol concentration and stress (Billard and Gillet, 1981; Pickering *et al.*, 1982, Barry *et al.*, 1995; Pickering and Pottinger, 1987a,b; Carragher *et al.*, 1989; Carragher and Sumpter, 1990; Foo and Lam, 1993; Chopin *et al.*, 1996; Jardine *et al.*, 1996; Waring, *et al.*, 1997, Carruth *et al.*, 2000). Acute handling and confinement (stressors) of juvenile and immature Atlantic salmon for 3 hours is accompanied by an increase concentration of plasma cortisol (Carey and McCormick, 1998). During physiological changes such as a smoltification and sexual maturation, cortisol levels increase but androgen levels in plasma inversely decrease due to stress (Barry *et al.*, 1995; Pickering *et al.*, 1987). In mature salmon, stress caused by the administration of exogenous cortisol induced suppression of reproductive and gamete development (Carragher *et al.*, 1989; Carragher and Sumpter, 1990; Foo and Lam, 1993). In mammals, the effect of stress on reduction of gonadal maturation and reproductive activity is induced by a decline in plasma concentrations of reproductive hormones. Starvation is one of the inducers of stress and strongly induces the gonadal degradation. However, migrating salmon maintain their reproductive activity even when they are exposed to the physiological stressors of osmotic regulation and starvation during the spawning period (Robertson *et al.*, 1961; Fagerlund, 1967). Reproductive hormones for controlling reproduction in migrating salmon were produced and stimulated the reproductive phenomena of spawning during starvation (So *et al.*, 1985; Dye *et al.*, 1987; Truscott *et al.*, 1986; Ikuta, 1996). The physiological mechanisms responsible for the differences in the reproductive response to stress between mammal and migrating salmon have not been determined yet.

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In the present study, sockeye salmon were investigated to study the effects of acute stress on circulating levels of cortisol, sex steroids and glucose, which increase in relation to stress during the spawning period.

MATERIALS AND METHODS

Fish

Twenty-five mature male and 25 mature female sockeye salmon (*Oncorhynchus nerka*), reared in one of the ponds of the Nikko Branch of the National Research Institute of Aquaculture, Chugushi, Nikko City, were used for a stress experiment on 9 September 1993. The females were bearing ovulated eggs in the body cavity, and males contained milt, which could be stripped. Their body weights (mean ± S.E.M.) were 725.0±106.4 g and 613.6±81.3 g in males and females, respectively. All fish were bled without anesthesia immediately after the confinement time. These fish had ovulated or spermiated but had not spawned. In addition, 10 female and 10 male cultured sockeye salmon were similarly sacrificed to monitor levels of cortisol, sex steroids and glucose in plasma on 2 and 8 September 1993 before the stress experiment. Their body weights (mean±S.E.M.) were 775.2± 96.9 and 761.5±49.6 in males and females, respectively.

Stress experiment

A stress experiment was performed on cultured sockeye salmon on 9 September 1993. The experimental design is detailed in a previous paper (Kubokawa *et al.*, 1999). Briefly it is described below. A small pool, 2.0 m×0.5 m, was built near the pond. This size was large

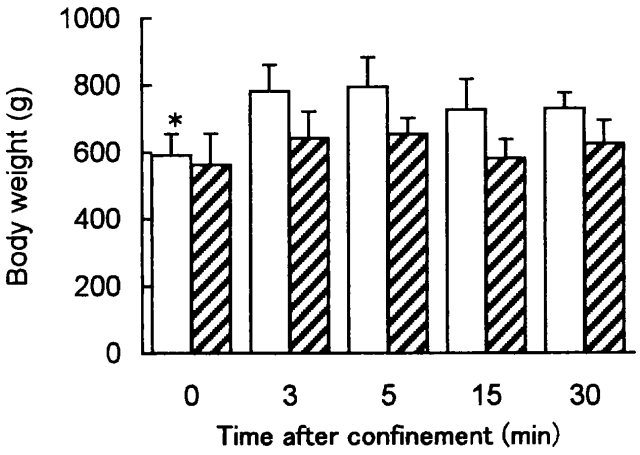

Fig. 1. Mean of body weights of sockeye salmon that were used in stress experiments at each confinement time. Males (open column) and females (shaded column) were used in groups of five. *Significantly different at P≦0.01 when compared between initial time and 3 min of confinement in males.

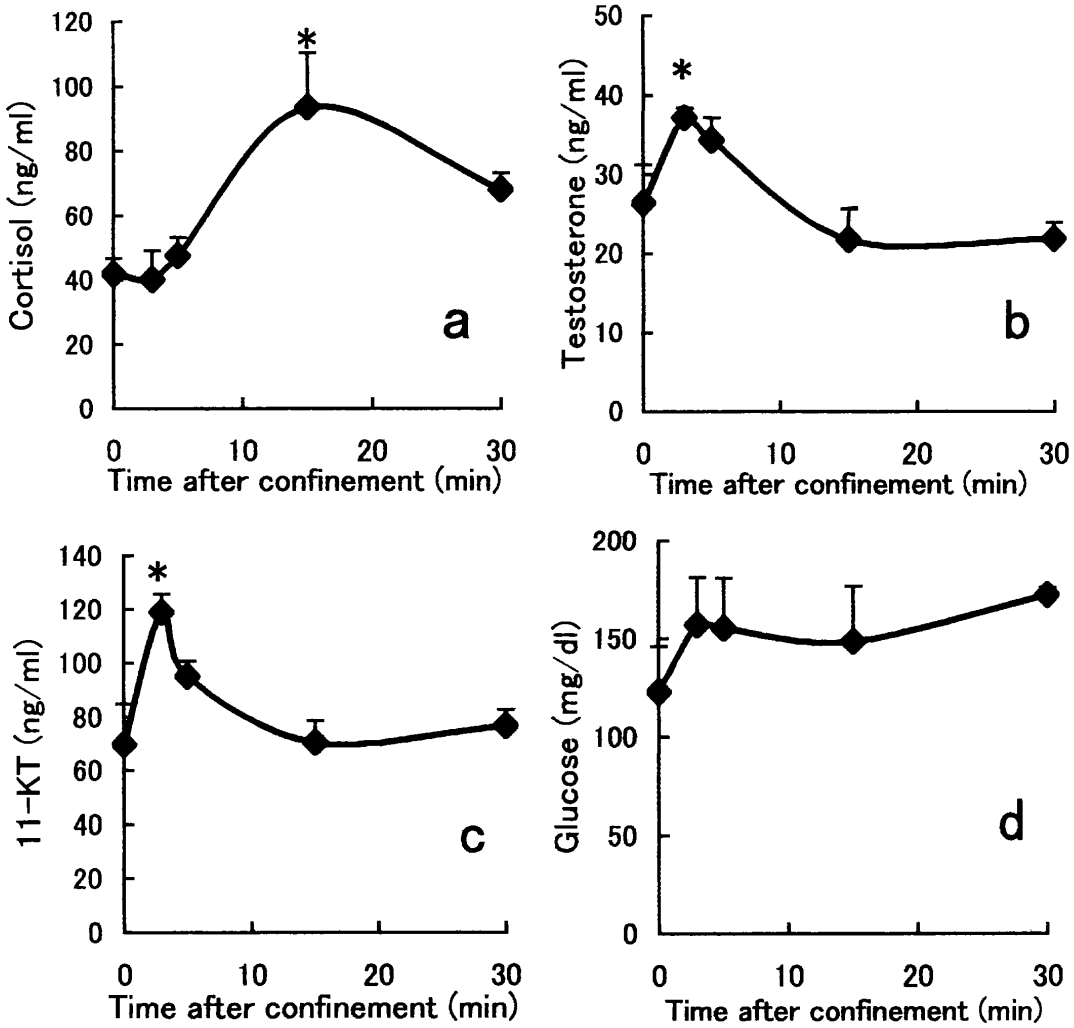

Fig. 2. Changes in plasma cortisol (a), testosterone (b), 11-ketotestosterone (11-KT) (c) and glucose (d) levels in male sockeye salmon exposed to the acute stress during 3, 5, 15 and 30 min. *Significantly different at P≦0.01 when compared with the hormone concentration at the initial time. Five fish were used for each confinement time.

enough to contain ten confinement nets used to hold the experimental fish. Pond water was circulated continuously through the pool. Bleeding of fish was performed at the edge of the pond. Soon after capture from the culture pond, each fish was confined in a small bag made of fishing net and put into the small pool. Blood was collected through the net within 0.5 min after the bag was removed from the pool. Five males and five females out of 50 fish were bled and then sacrificed to check for gonadal maturation after each confinement time, 0, 3, 5 15 and 30 min. One ml of blood was collected from the caudal vein of each fish with a 1 ml sterilized syringe. Blood samples were immediately centrifuged and the resulting plasmas were stored at −80°C until radioimmunoassay of hormones and glucose measurement could be made. Unhandled fish, controls for the stress experiment, were corresponded to fish that was sacrificed at time zero without the confinement stress.

Measurement of cortisol, sex steroids and glucose

Cortisol and steroid levels were measured at the University of Tokyo by using antibodies established in previously published papers. For males, concentrations of cortisol, testosterone and 11-

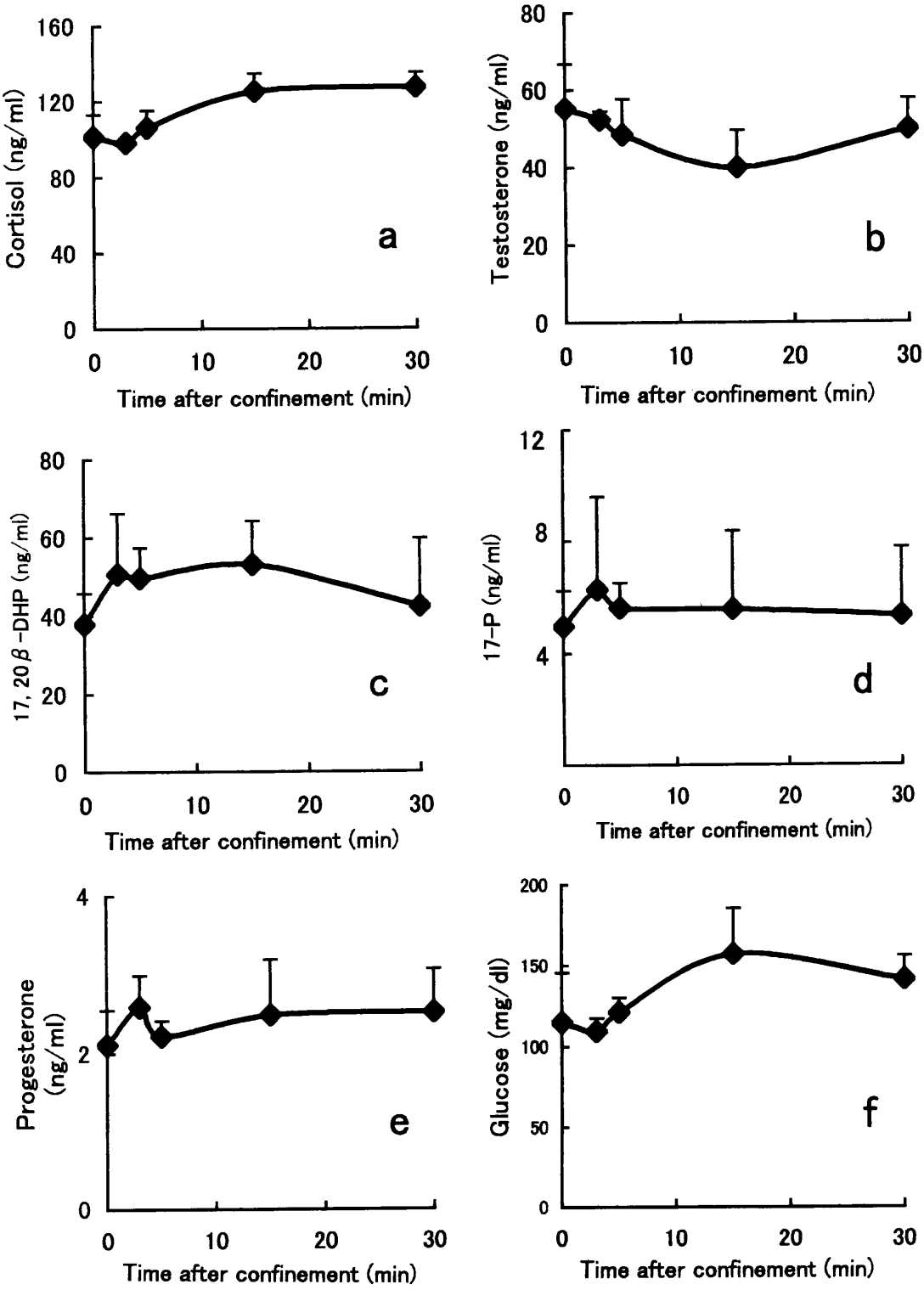

Fig. 3. Changes in plasma cortisol (a), testosterone (b), 17,20 β-dihydroxy-4 pregnen-3-one (17,20 β-DHP) (c), 17-hydroxyprogesterone (17-P) (d), progesterone (e) and glucose (f) levels in female sockeye salmon exposed to the acute stress during 3, 5, 15 and 30 min. *Significantly different at P≦0.01 when compared with the hormone concentration at the initial time. Five fish were used for each confinement time.

ketotestosterone (11-KT) were determined by radioimmunoassays. For females, concentrations of cortisol, estradiol-17 β (E_2), testosterone, 17-hydroxyprogesterone (17-P), and 17,20 β-dihydroxy-4-pregnen-3-one (17, 20 β-DHP) were determined by radioimmunoassays. The procedures of radioimmunoassays followed those in Takahashi *et al.* (1985) for cortisol, Lou *et al.* (1985) and Shimizu *et al.* (1985) for testosterone, E_2, progesterone, 17-P and 17,20 β-DHP, and Lou *et al.* (1986) for 11-KT. Plasma E_2 levels in females were less than the minimal detectable value, 30 pg/ml. Intraassay and interassay coefficients of sex steroid hormones were described previously (Zairin *et al.*, 1992). For both males and females, the glucose concentration in plasma was determined by using a commercial kit, Glucose CII-test Wako (Wako Pure Chemical Inc. Ltd., Osaka). The linear range of standard glucose was 0.5 mg/ml to 4 mg/ml.

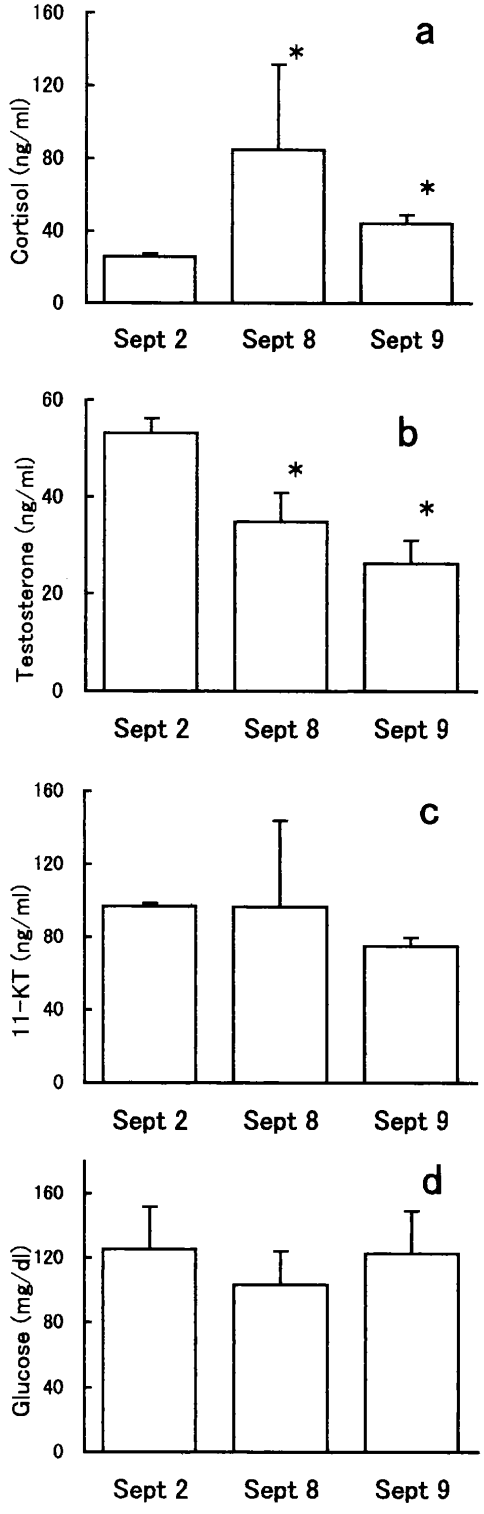

Fig. 4. Changes in mean plasma cortisol (a); testosterone (b), 11-ketotestosterone (11-KT) (c) and glucose (d) levels in unhandled male sockeye salmons on one day and one week before stress experiment. Results on 9 September correspond to values of time zero at the experiment. *Significantly different at P≦0.01 when compared with hormone levels on 2 September. The number of fish used was 10, 10 and 5 on September 2, 8 and 9, respectively.

Statistics

The analysis of variance with one-way layout followed by Duncan's multiple range tests was employed to compare body weight, hormone and glucose levels between animal groups in the confined-stress experiment, and for comparison between unhandled salmon captured on different days.

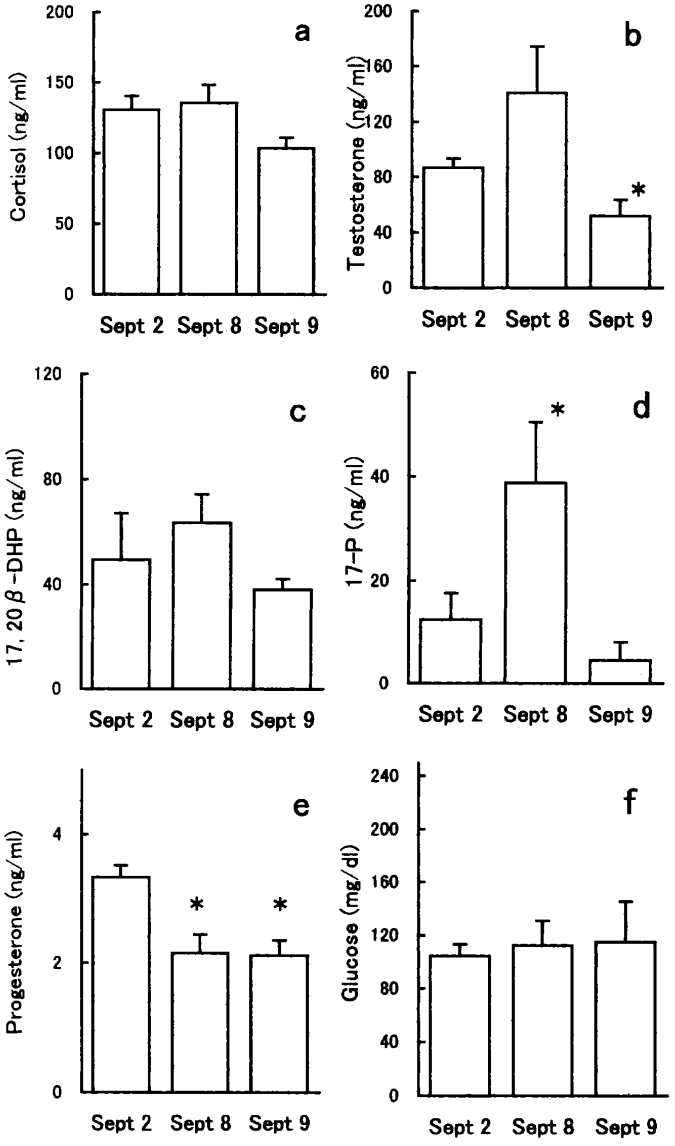

Fig. 5. Changes in mean plasma cortisol (a), testosterone (b), 17,20 β-dihydroxy-4 pregnen-3-one (17,20 β-DHP) (c), 17-hydroxyprogesterone (17-P) (d), progesterone (e) and glucose(f) levels in unhandled female sockeye salmons on one day and one week before stress experiment. Results on 9 September correspond to values of time zero at the experiment. *Significantly different at P≦0.01 when compared with hormone levels on 2 September. The number of fish was 10, 10 and 5 on 2,8 and 9 September, respectively.

RESULTS

Body weight

The body weight of males at initial time was significantly lower than for other groups of males ($P \leqq 0.01$, Fig.1). It is suspected that the smaller size fish were easier to catch than the larger fish using the net method. A similar tendency was shown for females although it was not significant (Fig.1).

Cortisol, sex steroids and glucose in stressed fish

Fish were collected from the culture pond and immediately measured to determine the cortisol, sex steroids and glucose levels in plasma for each confinement time. The mean initial cortisol level in males (41.9±10.6 ng/ml) was significantly lower than that in females (101.5±18.3 ng/ml) (Fig.2a and 3a) ($P \leqq 0.01$). The differences in cortisol levels between males and females appeared throughout the range of confinement times. Under the stress of confinement, cortisol levels increased at 15 min in males (increment at 5 min and 15 min being significant at $P \leqq 0.01$) (Fig.2a), while the level slightly decreased at 30 min. In females, cortisol levels slightly increased after 15 and 30 min. (Fig.3a). The testosterone levels in males were significantly lower than those in females throughout the confinement time (Fig.2b and 3b) ($P \leqq 0.01$). In males, the initial values of testosterone and 11-KT were 26.4 ±11.7 ng/ml and 69.7±35.1 ng/ml, respectively. Testosterone and 11-KT levels significantly increased at 3 min ($P \leqq 0.01$) and gradually decreased with time and were retained at 30 min after confinement (Fig.2b and c).

In females, initial values of sex steroids were 55.3±27.2 ng/ml in testosterone, 37.9±7.8 ng/ml in 17,20 β-DHP, 4.5± 3.6 ng/ml in 17-P, and 2.1±0.4 ng/ml in progesterone (Fig.3b, c, d and e). The increase of testosterone levels at 3 min in males was not observed in females, but the level gradually decreased with time as it did in the males. The mean levels of 17,20 β-DHP, 17-P, and progesterone did not show a significant increase nor decrease with time of the confinement stress (Fig. 3c, d and e). E_2 levels were lower than the detection limit of 30 pg/ml (data not shown). The plasma glucose levels increased at 3 min in males (Fig.2d), and gradually with time in females (Fig.3f).

Cortisol, sex steroids and glucose in unhandled fish

In blood samples were taken from unconfined fish, stress, cortisol, steroids and glucose levels were measured one week before and one day before in the acute stress experiment, in order to confirm the reproductive stage of these fish. The mean cortisol levels in males increased on 8 and 9 September (Fig. 4a). In females, no significant difference in the mean cortisol level was observed over three days. (Fig.5a). The mean cortisol level in females was higher than in males even on 8 September.

In males, the mean testosterone level was significantly decreased on 8 and 9 September ($P \leqq 0.01$)(Fig.4b). The mean 11- KT and glucose levels were almost constant over three days (Fig.4c and d). In females, testosterone increased on 8 September and decreased on 9 September (Fig. 5b). There was no significant difference in increase of testosterone. The mean level of 17-P was significantly increased on 8 September and decreased on 9 September ($P \leqq 0.01$)(Fig.5d). The mean levels of 17,20 β-DHP and glucose did not change throughout the three days (Fig.5c and f). The mean levels of progesterone significantly decreased on 8 and 9 September ($P \leqq 0.01$) (Fig.5e).

Correlation Analysis

To confirm the generality of a relationship between cortisol and sex steroid levels exhibited in the confinement experiment, simple correlation coefficients between hormone levels of all fish used in the experiment were calculated and the significance of these correlations was tested. In males, the cortisol level was significantly and negatively correlated with testosterone level, and weekly and negatively with 11-KT level ($P \leqq 0.01$). Testosterone and 11-KT levels were positively correlated well ($P \leqq 0.01$) (Table 1). However, in females, no significant correlation was found between levels of cortisol and any of the sex steroids measured. Positive and week correlation was obtained between levels of testosterone and progesterone, testosterone and 17-P, and progesterone and 17,20 β-DHP (Table 2).

Table 1. Correlation coefficiencies of changes among cortisol, sex steroids, glucose and body weight in male

	T	11-KT	Glucose	BW
Cortisol	–0.65	–0.54	0.2	0.006
T		0.84	–0.2	0.35
11-KT			–0.07	0.42
Glucose				0.45

T: testosterone, 11-KT:11-Ketotestosterone
BW: body weight

Table 2. Correlation coefficiencies of changes among cortisol, sex steroids, glucose and body weight in female

	T	DHP	P	17-P	Glucose	BW
Cortisol	–0.26	0.11	0.1	–0.06	0.3	–0.18
T		0	0.58	0.41	0.19	0.3
DHP			0.42	0.38	–0.05	0.05
P				0.34	0.35	0.13
17-P					–0.04	0.35
Glucose						0.08

T: testosterone, DHP:17, 20 β-dihydroxy-4-pregnen-3-one,
P: progesterone, 17-P: 17-hydroxyprogesterone, BW: body weight

DISCUSSION

In the present study, we demonstrated the sex-specific response to acute stress in migrating salmon during the spawning period. The plasma cortisol level in mature female landlocked sockeye salmon had already reached the high level when challenged by confinement stress, and did not increase for 30 min into the experiment. However mature male sockeye salmon responded well to the acute stress with increas-

ing cortisol concentrations. In addition, we measured plasma cortisol concentrations in fish from the culture pond and the cortisol level in males was lower than in females before spawning. It was considered that the plasma cortisol concentration in females increased faster than in males, and that the sensitivity to stress in females is lower than in males before spawning. The high levels of cortisol in females suggested that the mechanism for synthesis of cortisol might cause damage on a part of the hypothalamus – pituitary – interrenal (HPI) axis at the end of a spawning period.

Carruth *et al.* (2000) reported that the plasma cortisol concentration in a landlocked subspecies of sockeye salmon increased in association with upstream migration, and that there were no statistically significant differences between the sexes at any stage of gonadal development. His result is different from our result with respect to the sex-specific response to stress. Two possible explanations are considered in for the differing results. First, the fish used in his study were acclimated for one week after they were caught in the river. Second, the time period in which blood samples were collected in his study was longer than that in ours, which was 0.5 min. These differences in experimental design may have influenced the cortisol level in either males or females.

In order to measure the cortisol concentration without stress, a cannulation system and a large culture pond would be needed to collect blood samples over time. However, it is very difficult to keep fish that have been cannulated in a large pond and sample their blood using this system. Accordingly, the base value used to measure hormone level changes in the acute stress experiment corresponded to the value at time zero, because a control fish is only stressed by the collection of blood at the beginning of the experiment.

When migrating salmon die after spawning, the cortisol level in both sexes had increased to extraordinary high levels. However, there is no reports that female cortisol levels are higher than males before spawning except for those in our previous paper (Kubokawa, *et al.*, 1999). Many investigators have studied on the elevation of stress-induced cortisol in male and female teleost under various physiological and reproductive conditions (Bonga, 1997). The increase of plasma cortisol levels during a spawning migration is also considered to be cause by some sort of stress, for example, the stressors such as long-distance migration and change from sea water to fresh in ocean-run salmonid. However, Carruth *et al.* (2000) reported that landlocked sockeye salmon, which migrate in short-distance from lake to natal spawning river, showed the elevation of plasma cortisol levels during an upstream migration without the stressors described above. It is suggested that the elevation of cortisol in an upstream migration might be influenced greatly by an endogenous system. Our result, that is the different endocrine response to stress between females and males of landlocked sockeye salmon, also indicates the important role of an endogenous system. Females may have been stressed prior to the spawning by ovarian maturation and ovary maintenance. Another possible explanation may be that females are more severely stressed by the large volume of ovaries than that of testis. In the future, a study design to determine the effect of an artificial increase of gonadal volume on cortisol levels might be a productive approach.

It was reported that, when immature all-female salmon produced by triploid treatment and mature all-female salmon produced by diploid treatment were challenged with 2.5 hr of confinement stress, and cortisol and glucose levels in the immature salmon increased approximately twice as much as in the mature salmon (Sadler *et al.*, 2000). This result along with ours suggests that the bio-defense system against stress becomes insensitive in mature female salmon, and that an excess amount of stress stops the synthesis or secretion of additional cortisol internally.

Changes in plasma sex steroids from the beginning of sexual maturation to spawning were reported in upstream migrating sockeye salmon (Ikuta, 1996; Truscott *et al*, 1986). Moreover, Ikuta measured annual changes of steroid hormone levels in adult sockeye salmon at Nikko Branch of the National Research Institute of Aquaculture (unpublished data). In males, the testosterone level increased during the migration and the highest level was approximately 50 ng/ml when they gathered at the mouth of the stream. At the final stage of migration and before spawning, 11-KT elevated to approximately 80 ng/ml and testosterone level decreased at that time. In our study, the decrease of testosterone and the high and constant level of 11-KT consistent with his data. In females, maximum levels of testosterone, 17-P and progesterone were approximately 200 ng/ml, 25 ng/ml and 4 ng/ml, respectively before spawning, and then these steroids decreased in the spawning. In the spawning, 17,20 β-DHP was slightly increased to approximately 20 ng/ml. In our study, the significant increase of 17-P on 8 September may show the spawning condition, but no spent females were found on 8 and 9 September. Mean levels of the other steroids in females approximately consistent with his data. In comparison, males used in this study might be spermiated and females ovulated, but they were not spawned.

The inhibitory effects of stressors on reproductive performance have been established for many different teleost species (Bonga, 1997). However, the mechanism by which stress inhibits reproduction and levels in the endocrine cascade at which stress acts are less clear. In sexually mature males of brown and rainbow trout, acute confinement stress for 1 hr elevated plasma cortisol and ACTH levels and reduced plasma testosterone and 11-KT levels (Pickering *et al.*, 1987; Pankhurst and Van Der Kraak, 2000). In sexually mature females of brown and rainbow trout, plasma testosterone levels were depressed by acute stress or treatment with exogenous cortisol (Pottinger *et al.*, 1996; Pankhurst and Van Der Kraak, 2000). These androgen level findings are the same as those in other species. Furthermore, plasma levels of gonadotropin (GTH or GTH-II) were unaffected by stress, but the gonadotropin contents in the pituitary were decreased (Pickering *et al.*, 1987; Carragher *et al.*, 1989; Pankhurst and Van Der Kraak, 2000). On the other hands, implantation of testosterone or

11-KT reduced the elevation of the stress-induced plasma cortisol levels in both mature rainbow and brown trout (Pottinger, *et al.*, 1996; Young *et al.*, 1996). It is suggested that in mature males and females of salmonid the HPI axis regulates gonadal steroidogenesis, and gonadal steroids might regulate stress-induced activity of the HPI axis (Bonga, 1997; Pottinger *et al.*, 1996; Pankhurst and Van Der Kraak, 2000). However, our study in females indicated that the cortisol does not directly regulate gonadal testosterone secretion. Further experiment needs to reveal the direct effects of gonadal steroids on interrenal and indirect effects on interrenal function via the hypothalamus-pituitary axis.

Under stress, catecholamines act directly on the liver to stimulate glycogenolysis and result in mobilization of glucose (Axelrod and Reisine, 1984). Furthermore, glucose level is a correlate of corticosteroid level under an acute stress (Thomas and Neff, 1985; Vijayan *et al.*, 1994; Rotllant and Tort, 1997; Vijayan *et al.*, 1997). In our study, in females, testosterone and glucose levels increased without an increase in cortisol levels. It is more plausible that another stress hormone such as adrenaline is responsible for the glycogenolysis (Wingfield *et al.* 1992). About a manner of testosterone, we could discuss on the possibility of the existence of interrenal steroid, although there is no published data on it in teleost. In mammals, over 45 steroids including androgens have been isolated and chemically characterized from adrenal gland extracts, and studied on the function (Norman and Litwack, 1997). Recently, Fearon *et al.* (1998) reported that the differential control of adrenal androgens and cortisol in human. Thus, the existence of interrenal androgen may be needed to consider on the relationship between the function of HIP axis and stress in teleost.

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