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Physiological studies on somatic growth and sexual maturation in salmonids

**A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Fisheries
Science**

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General Introduction

Salmonids are important biological and economic resources in Northern Japan and are a unique group of fish possessing complex life histories. In Japan, there are four main salmonid species: pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*O. keta*), sockeye salmon (*O. nerka*), and masu salmon (*O. masou*). The former two salmonids have different life cycles with latter two. Pink and chum salmon juveniles make their downstream migrations to seawater environments within a few months after emergence, and adult maturing fish carry out their upstream migrations a few weeks before final sexual maturation. On the other hand, sockeye and masu salmon smolts stay for about a year and half in freshwater environments and make downstream migrations to seawater, and the adults undergo upstream migrations several months ahead of sexual reproduction. Though most of them are anadromous and some of them are also known to have a unique lacustrine form of life (Yamamoto et al., 2000). Both forms show similar physiological changes associated with somatic growth and gonadal maturation. In recent years, there has been a great interest in the study of gonadal steroidogenesis in commercially important fish species, aiming to improve reproduction and maximizing fingerling production (Venkatesh et al., 1992). With successful stock enhancement programs, it would have been possible to restore chum salmon stock resources successfully in Japan. In this fish along with stock enhancement programs, monitoring of the adult returnees is necessary from endocrinological viewpoint for an extended period of time. In contrast, the restoration of stocks of the other fish species such as sockeye and masu salmon, which have also a great market demand, still remains in low. In this regard, the development of efficient techniques for producing active smolt and improving adult returns is the key element in the propagation of these fish species. For this purpose, basic knowledge on the biology and physiology of each fish species is important.

Modern aquaculture lies on two main principles: enhancement of body growth and acceleration of sexual maturation. To achieve such goals, the manipulation of growth and gonadal maturation using different peptides, amino acids and alternation of environmental factors such as nutrition and photoperiod have been practiced. Before applying these technologies, accurate information is needed on the mechanisms involved in neuroendocrine regulation of growth and maturation in teleost fish, including salmonids. Thus, for better understanding of neuroendocrine

mechanisms involved in somatic growth and gonadal maturation, it is essential to examine seasonal changes in endocrine functions and their responsiveness to the external factors that applied for manipulation of somatic growth and gonadal maturation. However, only few reports have been documented on seasonal profiles of the endocrine functions and roles of the components that interact and regulate somatic growth and sexual maturation in salmonids.

Like other vertebrates, growth and reproduction in teleosts are coordinated by nervous and endocrine systems with the involvement of various hormones and other environmental factors in the two main axes (somatotropic and gonadotropic). Several lines of evidence showed the existence of interrelationship between growth and gonadal maturation (Le Bail, 1988; Moriyama and Kawauchi, 2001). This relationship occurs at different levels of gonadotropic and somatotropic endocrine axes.

Growth in fish is a complex function regulated by growth hormone (GH) and insulin-like growth factor (IGF) system (Reinecke et al., 2005; Wood et al., 2005). The general organization of this system is well conserved in fish, includes GH, GH receptor (GHR), IGF-I, IGF-II, IGF receptors (IGFRI and IGFRII), and IGF binding proteins (Gabillard et al., 2005). The somatotropic endocrine axis begins with receiving the external stimuli (temperature, photoperiod and food availability) and internal physiological conditions (hormones) in the central nervous system (CNS) and are processed, integrated and passed to endocrine organs including hypothalamus (HYP), pituitary and target organs. The hypothalamic hormones, growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone (GHIH), regulate the production of GH in pituitary. In addition, a newly identified peptide, ghrelin, regulates not only pituitary GH secretion but also feeding and drinking behavior in fish (Parhar et al., 2003; Unniappan and Peter, 2005). Then, GH is released into the bloodstream, binds to its specific receptors in the target organs mainly in the liver, and stimulates synthesis and release of IGF-I. A wide variety of tissues, such as brain, gill, muscle, kidney, and gut are also known to produce IGF-I locally in teleosts, although liver is the primary site of IGF-I production (Duan, 1997).

GH is a single chain polypeptide of about 190 amino acids synthesized by somatotrophs located in the proximal pars distalis (Nagahama et al., 1981). It is involved mainly in the regulation of somatic growth (Donaldson et al., 1979), and other metabolic functions such as maintenance of carbohydrate and osmoregulation

(Hirano, 1986). The widely accepted somatomedin hypothesis states that many of the growth promoting effects of GH are mediated by circulating IGF-I (Daughaday and Rotwein, 1989). IGF-I is a 7.5-kDa mitogenic polypeptide, belonging to the insulin superfamily because of its structural relation to proinsulin and its production is influenced by physiological conditions (Shimizu et al., 1999). The biological action of IGF-I are mediated through the IGF receptor. IGF-I circulated in the blood tightly bound to specific binding proteins that differ in the site of origin as well as in biological function (Moriyama and Kawauchi, 2001).

IGF-I has both metabolic and mitogenic activities. IGF-I, besides acting as a mediator of somatotrophic actions of GH, it activates directly the hypothalamic-pituitary-gonadal axis to promote onset of puberty (Hiney et al., 1991; Huang et al., 1998). IGF-I stimulated the release of gonadotropin-releasing hormone (GnRH) in vertebrates at puberty, and GnRH in turn elevated plasma levels of luteinizing hormone (LH) (Hiney et al., 1996). In addition, it functioned directly at the pituitary level. For instance, increased basal secretions of follicle stimulating hormone (FSH) and LH, and GnRH-induced LH secretion have been reported by adding IGF-I to rats pituitary cell cultures (Soldani et al., 1994, 1995). In teleosts, IGF-I increases the release and cell contents of LH in pituitary cells of European eels (*Anguilla anguilla*) in a time- and dose-dependent manner (Huang et al., 1999). In salmonids, co-administration of IGF-I and salmon GnRH (sGnRH) to coho salmon (*O. kisutch*) pituitary cells increased FSH and LH releases and contents (Baker et al., 2000). All the above research findings in vertebrates in general and fish in particular support the notion that IGF is involved in stimulating the pituitary-gonad axis. Therefore, it is thought to act as a signaling molecule that transmits growth and nutritional status to the gonadotropic axis at the onset of puberty. Several aspects on the structure and biological functions of GH and IGF-I in teleosts have been investigated due to their potential use as growth enhancer in aquaculture.

On the other hand, various components within the gonadotropic endocrine axis interact to determine sexual maturation. The main event of sexual maturation begins with the reception of the environmental stimuli such as food availability, photoperiod, temperature, salinity and presence of vegetation or substrate and the internal stimuli. It is mediated by the nervous system, which involves the passage of information from the sensory receptors to CNS. In the anterior part of the brain or HYP, this neural information regulates the activity of the pituitary gland through chemical substances

(releasing or inhibiting hormones). These releasing hormones stimulate the release of the hormone gonadotropin (GTH) from the pituitary gland into the blood. The target organ of GTH is the gonad, and its main effect is to stimulate the production of sex steroids that are synthesized by special theca cells found on the follicular envelope of the oocytes (Hoar and Nagahama, 1978) and are responsible for the development and maturation of gametes (Nagahama, 2000).

The hypothalamic decapeptide, GnRH represents the main step in the cascade of hormones participating in the coordination of reproductive physiology (Guilgur et al., 2006). This neuropeptide is synthesized by axon terminals of GnRH neurons and reaches pituitary by direct innervations of the gonadotrophs, or in close vicinity to them in teleostean fishes (Anglade et al., 1993; Mousa and Mousa, 2003). It stimulates the synthesis and release FSH and LH, and these two hormones travel through the blood stream to the target organs (gonads) where they trigger steroidogenesis, gametogenesis and other processes. Specifically, FSH is involved in synthesis of estradiol-17 β (E₂), vitellogenesis and spermatogenesis in early reproductive stages, whereas, LH appears late in the reproductive stages and regulates spermiation and ovulation. Sex steroids are responsible for the maturation of gametes, for instance E₂ in vitellogenesis, 11-ketotestosterone (11-KT) in spermatogenesis (Planas et al., 1993) and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) in final ovulation or spermiation (Nagahama, 1987). Steroids and other factors such as activin and inhibin feedback to HYP and pituitary, and thus up-regulating or down-regulating further transcription, translation and release of GnRH and gonadotropins (Nagahama, 1994). When this interlinked web functions properly, and given adequate environmental and behavioral cues, this hormone cascade culminates in oocyte ovulation and spawning in females, and in sperm maturation and release in males.

Based on the knowledge of these neuroendocrine functions, particularly the hormone cascades which are assumed to be exclusively the product of vertebrate endocrine organs, big achievements have been made in aquaculture such as enhancement of growth and maximizing production cost per output. However, in recent years, with successive filtration and powerful chromatography methods (e.g., HPLC) purified forms of physiologically active substances have been investigated from microorganisms such as fungi and algae (Lenard, 1992). Recently, research has been focused on peptide and steroid hormone signalling systems, and differences

between bacterial and vertebrate signal transduction systems have been widely discussed (Janssens, 1988a,b). Janssens (1987) suggested that the vertebrate hormone systems might have their origins in early eukaryotes, and Pertseva (1991) reported an additional evidence for the root of signaling mechanisms both in early eukaryotes and in prokaryotes. Consequently, considerable evidence for the presence of mammalian-like peptide hormones in microorganisms has appeared in the literature (Kincaid, 1991; Van Houten, 1992).

The various components of steroid metabolism such as testosterone (T), progesterone, 17 β -hydroxysteroid dehydrogenase (17 β -HSD), androgen binding proteins and endogenous substrates of 17 β -HSD have been identified in the mycelium of several fungus species, supporting the hypothesis that fungi possess an endocrine function (Kastelic-Suhadolc et al., 1994; Zakelj-Mavric et al., 1995; Itagaki and Iwaya, 1988; Plementias et al., 1999). These findings, mainly endogenous steroidogenesis and the identification of steroidal substrate or substrates of 17 β -HSD in the cytosols of the many fungi tested so far may indicate the possibility of other fungal species possessing similar properties in their mycelium (Pogacar et al., 1998; Lanisnik and Zakelj-Mavric, 2000).

Moreover, previous reports indicate that the physiologically active substances produced by the product of *Rhizopus* have various influences in many vertebrates including humans (Kikuchi et al., 1976; Higuchi et al., 1979). More recently, Bhandari et al. (2002) investigate the effects of long-term *Rhizopus* extract (RU) feeding on somatic growth and sexual maturation in fish. However, the endocrine mechanism of the physiologically active substances present in the mycelium of RU is not yet known.

In salmonids, at a particular stage of somatic growth, certain endocrinological changes cause fish to shift from feeding migration to spawning migration. This shift from feeding migration to homing migration has been thought to coincide with the onset of gonadal maturation (Ueda and Yamauchi, 1995). This gonadal maturation during homing migration is regulated by the brain-pituitary-gonadal (BPG) axis as described above. GnRHs, GTHs, steroids and other hormones have important roles in the control of homing behavior and gonadal maturation (Fitzpatrick et al., 1986). Therefore, the understanding of physiological and environmental mechanisms that govern sexual maturation in salmonids is very important. This requires elucidation of hormonal profiles and their interactions in brain, pituitary and serum during homing

migration of fish for an extended period. Changes in salmon GnRH mRNAs levels in discrete brain loci have been investigated in chum salmon during upstream migration (Onuma et al., 2005). Besides, changes in the plasma levels of steroid hormones during gonadal maturation and spawning migration are reported, e.g., in pink salmon (Dye et al., 1986), sockeye salmon (Truscott et al., 1986), masu salmon (Amano et al., 1995) and chum salmon (Ueda et al., 1984). All the above studies investigated the hormonal changes for only one spawning period. But, data on endocrinological changes from a single spawning period could not sufficiently depict the regular changes during homing migration. That means there is a gap that is missing from single season studies. Further, endocrine changes are under the influences of various factors such as body size, nutritional condition, gonadal maturity, and oceanographic conditions, and they are found to differ from year to year (Onuma et al., 2003). No studies have been conducted to investigate the hormone levels in brain, pituitary and serum for an extended period and clarify their year-to-year differences, except one report by Onuma et al. (2003) which investigated the year-to-year differences in plasma levels of steroid hormones during upstream migration of chum salmon.

Furthermore, the role of hatcheries in producing active smolts for release in stock enhancement programs is immense. In hatcheries, fish from early age to adult could be easily stressed at any stage of the routine activities unless appropriate care is taken. Management procedures such as capture, tank transference, counting and weighting are potential stressors and critical as stress inductor in fish (Barcellos et al., 2001). The effects of stress resulting from aquaculture practices on fish and methods of minimizing such effects have received considerable attention through the years (Cech et al., 1996). All the activities mentioned above cause a characteristic stress response, which is generally known, in primarily level, by measuring serum cortisol levels in stressed fish (Flos et al., 1988; Foo and Lam, 1993a). The mechanism through which stressors may act to affect reproductive capabilities is via the endocrine stress response. The activation of the hypothalamic-pituitary-interrenal (HPI) axis is a universal reaction of teleosts to stressors, and consists of a hormone cascade culminating in the release of the corticosteroid, from the interrenal cortisol into the bloodstream (Donaldson, 1981).

Cortisol and other hormones have been reported to be present in the freshly ovulated eggs of a number of teleost species, and environmental stressors have been implicated in the decrease of egg quality in salmonids (Stratholt et al., 1997). Thyroid

hormones [thyroxine (T₄) and triiodothyronine (T₃)] have been intensively examined in fish for their presence in unfertilized eggs, as well as their changes during development. Treatment of developing fish with thyroid hormones induces earlier development and accelerates yolk absorption, growth and morphological differentiation and metamorphosis (Leatherland, 1982). However, the exact effects of cortisol and thyroid hormones in the developing embryos are not well understood.

For the understanding of the effect of RU administration on somatic growth and sexual maturation, RU, in a refined form, was mixed with commercial pellets and fed to 0+ masu salmon. Monthly changes in fork length, body weight, and serum levels of steroid hormones and IGF-I were measured. Moreover, *in vitro* RU and RU fractions incubations of HYP, pituitary and gonads were carried out at different doses of RU and its fractions to investigate the possible direct effects of RU on the hormonal releases of these tissues. In addition, time-course incubations of RU were made on HYP, pituitary and gonadal tissues in masu salmon.

For the understanding of the hormonal profiles in brain, pituitary and serum, chum salmon were sampled at Bering Sea from late-June to mid-July 2003, 2004 and 2005. Besides, fish were caught at four to six points along their homing route from Ishikari Bay to their spawning ground near Chitose Branch of National Salmon Resource Center in Hokkaido from late-September to early-October 2003, 2004 and 2005. In all samplings, blood, brain and pituitary samples were collected and assayed for the respective hormones.

Furthermore, to search for the possible reasons behind the low eyed-egg percentage of the pond-reared masu salmon at Kumaishi station; first, serum cortisol levels of masu salmon during the spawning period at Kumaishi and Mori stations were measured and compared. Secondly, measurement of cortisol levels in serum and fertilized eggs was carried out from matured female masu salmon at Kumaishi station and these levels were related to the eyed-egg percentages. Finally, the thyroid hormones (T₃ and T₄) were measured from fertilized eggs.

Chapter 1.1

Effects of *Rhizopus* extract (RU) administration on somatic growth and gonadal maturation in masu salmon

Introduction

The hydroxylation of steroids by fungal and bacterial biocatalysts has been known for long period of time (Peterson et al., 1952). This procedure remains one of the most useful steroid hydroxylation methods and the value of microbial steroid hydroxylation in the preparation of pharmacologically active steroids is well established. Recently, the identification of steroid substrates and investigation of their roles in the steroidal biosynthesis of vertebrates in some fungi species suggests the possibility of other fungal species having similar properties in their mycelium (Lanisnik et al., 2001; Kristan et al., 2003).

Rhizopus is a water extract of *Rhizopus delemar*, which is a kind of filamentous fungi belonging to the family mucoraceae of group thallophyta. It is one of the most commonly used microorganisms for the production of pharmacologically active steroids through steroid hydroxylation (Charney and Herzong, 1967). Many studies indicate that the physiologically active substance produced by *Rhizopus delemar* and *Rhizopus nigricans* have various influences on reproductive function in vertebrates including fish. To mention some: the products of *Rhizopus* increase the rates of laying eggs, fertilization and hatching in hen and quail (Ushikoshi, 1963), pregnancy rates of cows (Umezu et al., 1973; Sato, 1976), ovarian steroidogenesis in rats (Saito et al., 1980; Horiuch et al., 1985), and enhances body growth in sockeye salmon, *O. nerka* (Bhandari et al., 2002). These findings in one way or another indicate that the physiologically active substances found in RU may play active roles in somatic growth and sexual maturation though the detailed endocrine mechanism of the bioactive substances is not established.

It is well known that somatic growth and sexual maturation are the results of the interaction of two factors: the internal genetic factor and the external environment. Sexual maturation is controlled by BPG axis, whereas, somatic growth is controlled by GH-IGF-I axis. The roles of the different factors that are involved in for each axis were extensively studied (Björnsson, 1997; Moriyama and Kawauchi, 2001). IGF-I is thought to mediate these axes. IGF-I is mainly involved in the regulation of metabolism in the cells, differentiation and proliferation of the cells, and ultimately body growth. Treatment of teleost fish with GH and IGF-I stimulates somatic growth (McCormick et al., 1992). A significant correlation was observed between GH levels and IGF-like immuno-reactivity in plasma with a 1.5 hour delay in trout (Niu et al.,

1993). Furthermore, administration of GH increases plasma IGF-I level in trout (Moriyama, 1995). Beckman et al. (1998) showed that higher plasma IGF-I levels were observed in fast-growing fish than in slow-growing fish. The same authors also reported that plasma IGF-I levels of chinook salmon were higher in warm-water fish than in cold-water fish. In sum, all these studies indicate that the changes in GH and IGF-I levels are closely correlated with the growth rate of the fish and reflect the coordination between the endocrine system and physiological responses when environmental cues are changing. Therefore, measuring the serum IGF-I levels would provide a valuable indicator of physiological condition and growth in fish.

Thus, in this study, long-term feeding of RU was conducted in masu salmon to examine the effect of RU administration on somatic growth and sexual maturation by *in vivo*. Monthly examination of the changes in fork length and body weight, and measurement of serum levels of sex steroid hormones and IGF-I were carried out.

Materials and Methods

Experimental animals

Lacustrine masu salmon of Toya strain were used at Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University. Fish were reared in 1400 L circular tanks under the natural photoperiod with continuous flow of spring water. The water temperature ranged from 8.7°C to 10°C. Underyearling (0+) masu salmon were divided into 3 groups as RU 200 mg/kg (RU 200), 20 mg/kg (RU 20) commercial pellets fed groups and control groups. The initial stocking densities of the fish were 500 fish/tank. RU, in a refined form, was mixed with commercial trout pellets (Oriental Feed Industry, Yokohama, Japan) at the rate of 20 and 200 mg/kg feed using feed oil (40 ml/kg of feed). Fish were fed daily at the rate of 2.5% from April to December and 1% from January to March, and feeding rates were adjusted according to the body weight measured at monthly sampling. The fish were reared from June 2002 to April 2004 for 2 years. When the experiment started, fish were 4.35 ± 0.074 cm in fork length and 0.69 ± 0.037 g ($n = 30$) in body weight.

In every sampling protocol, 15-20 fish from each treatment group were randomly selected and anaesthetized with 0.01% tricaine methanesulfonate (MS222, Nakalai tesque, Kyoto, Japan) buffered with equal amount of sodium bicarbonate. A

gentle pressure was applied on the abdomen to check whether spermiation or ovulation has occurred or not. Fork length and body weight were then measured. Blood samples were collected from caudal vasculature, kept on ice and later centrifuged at 3000 rpm for 15 minutes to obtain serum samples, which were stored at -30°C until assayed. Finally, gonads and liver were taken out and weighed. Gonadosomatic index (GSI) was computed as gonad weight \times 100/body weight.

Time-resolved fluoroimmunoassay

Serum levels of sex steroid hormones T, E₂, 11-KT and DHP were measured by time-resolved fluoroimmunoassay (TR-FIA) methods. Time-resolved fluoroimmunoassays have high sensitivity compared to radioimmunoassay techniques. The protocols for T, E₂, 11-KT and DHP assays followed those developed by Yamada et al. (1997). In brief, ether-extracted steroid hormones in the serum samples were evaporated in a 45°C water bath with a continuous flow of nitrogen gas, reconstituted into 600 μl of assay buffer containing 0.05 M Tris, 0.9% NaCl, 0.5% bovine serum albumin (BSA), 0.05% NaN₃, 0.01% Tween 40, 20 μM diethylenetriamine-N,N,N',N'',N'''-pentaacetic acid (DTPA), pH 7.75. The reconstituted samples were stored at -30°C until assayed. These samples were applied to 96-well microtiter plates (Wallac Oy, Turku, Finland) in which BSA-conjugated antigen was immobilized by physical adsorption.

In the assay, standards of hormones were applied in triplicate, whereas samples in duplicate. Following incubation with BSA-conjugated antigen at 4°C overnight, Eu-labeled IgG was added to each well, incubated at room temperature for 1 hour, and it was stringently washed to remove unbound Eu-labeled IgG. Then, enhancement solution (0.1 M acetate-phthalate buffer, pH 3.2, containing 0.1% Triton X-100, 15 μM 2-naph-thoyltrifluoroacetone, 50 μM tri-n-octylphosphine oxide; Perkin-Elmer, Wallac Oy, Turku, Finland) was pipetted and the intensity of fluorescence from dissociated Eu was measured with a time-resolved fluorometer (1234 DELFIA fluorometer; Wallac Oy) using DOS-based Multicalc software (Wallac Oy). Standard curves in each assay were plotted and values were calculated automatically by the time-resolved fluorometer. In each assay, standard samples prepared from the pooled masu salmon were used as sub-controls. The intra- and inter-assay coefficients of variations were 8.64 and 11.14 for T, 8.11 and 10.90 for 11-KT, 9.67 and 14.48 for E₂, 8.88 and 13.70% for DHP, respectively.

Extraction of IGF-I

Serum samples were extracted according to the method of Shimizu et al. (1999). Eighteen microliters of serum were thoroughly mixed with acid-ethanol (87.5% ethanol and 12.5% 2 N HCl, v/v) at a ratio of 1:4 and then incubated at room temperature for 30 min. The tubes were centrifuged at 15000 rpm for 10 min at 4°C. Fifty microliters of the supernatant was decanted into a new set of tubes and neutralized with 0.855 M Tris Base. The samples were stored at -30°C and then centrifuged at 15000 rpm for 10 min at 4°C during assay. The supernatant was used for IGF-I assay.

IGF-I assay

Serum IGF-I was measured by TR-FIA methods according to Andoh (2005). Briefly, microtiter plates (Wallac, Turku, Finland) were coated with 100 µl of affinity purified anti-rabbit IgG at the concentration of 10 µg/ml in 50 mM Tris-HCl (pH 7.8) containing 0.9% NaCl, and 0.05% NaN₃ (coating buffer) for 2 hours at 4°C. This antibody was purified according to a conventional method using rabbit IgG and NHS-activated HiTrap column (Amershambiosciences, Piscataway, NJ). After three washes with 20 mM Tris-HCl containing 150 mM NaCl, 0.05% Tween 20 (pH 7.8, wash buffer), 100 µl of biotinylated IGF-I solution containing 50 mM Tris-HCl, 150 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 25% BlockAce (Dainippon Pharmaceutical Co., LTD. Tokyo, Japan) and 0.05% NaN₃ (crossreaction buffer) at the concentration 1/1478 (v/v) was dispensed to each well. Ten µl of serially diluted standards and samples was dispensed and this was followed by addition of 8 µl of anti-barramundi IGF-I antiserum (anti-recombinant barramundi IGF-I, GroPep) diluted with cross reaction buffer at concentration of 1/9400 (v/v) and incubated for 18 hour at room temperature. After three washes with wash buffer, Eu-avidin conjugate (Wallac Oy, Turku, Finland) diluted with assay buffer (0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN₃, 0.01% Tween 40, 20 µM DTPA, pH 7.75) at 1/2500 (v/v) was dispensed at the volume of 100 µl to each well and incubated for 2 hours at room temperature. After 4 washes, 100 µl of enhancement solution (Perkin-Elmer) was pipetted and the microtiter plate was shaken for 5 min. The intensity of fluorescence from dissociated Eu was measured with a time-resolved fluorometer (1234 DELFIA fluorometer; Wallac Oy) using DOS-based Multicalc software (Wallac Oy). Standard curves in each assay were plotted and values were calculated

automatically by the time-resolved fluorometer. In each assay, standard samples prepared from the pooled masu salmon were used as sub-controls. Intra- and inter-assay variabilities were 6.98 and 9.02%, respectively.

Statistical analysis

All the data are expressed as means \pm standard error of mean (SEM). To assess significant differences between RU fed and control groups, data were subjected to one-way ANOVA analysis followed by Fisher's PLSD. Means were considered statistically significant when $P < 0.05$.

Results

Growth

Fork length and body weight of RU fed and control immature fish increased gradually from 0+ July 2002 (Fig. 1). RU 200 immature fish showed significantly higher fork length and body weight values from 0+ September to December; and from 1+ January to March. Fork length was significantly higher in 1+ April and June; and in 2+ from February to March in RU 200 immature males (Fig. 2). Body weight in RU 200 immature males were also significantly increased from 1+ April to June and from 2+ February to March. Maturing males were also found from 1+ June through December, and a significant increase in somatic growth was observed in September and October in RU 200 fed groups (data not shown). In RU 200 immature females, fork length was significantly higher in 1+ April, May, July, November and December; and in 2+ March and April (Fig. 3). Body weight in RU 200 immature females were also significantly increased from 1+ April to August and from October to December, and in 2+ March and April. All females in both treatment groups were immature.

Sexual maturation

GSI of immature and maturing males in both groups showed a similar increasing trend but low values in the controls (Fig. 4). In both sexes, RU fed fish did not show any significant difference in GSI as compared to the controls. In both sexes, RU administration did not accelerate the changes in GSI in 0+ and 1+. GSI peaked in 1+ August for maturing males in both groups.

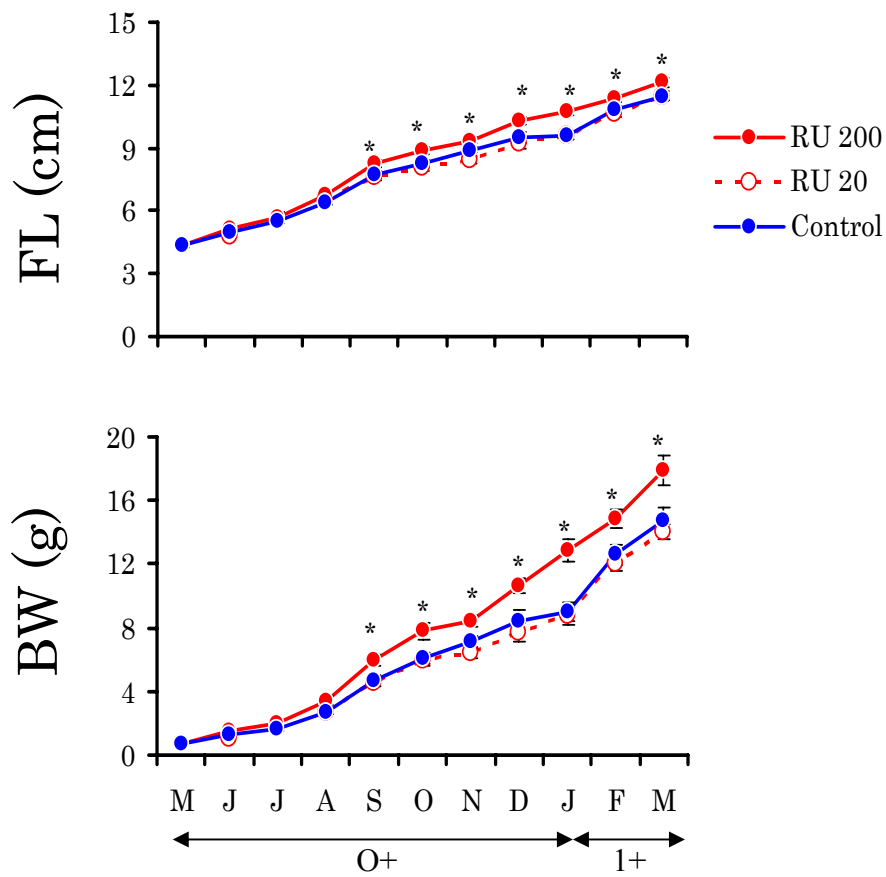


Fig. 1. Changes in fork length and body weight of immature (0+ - 1+) masu salmon of Toya strain by long-term RU feeding. Each point represents the mean \pm SEM. Asterisks indicate significance differences between RU 200 fed and control groups. *P < 0.05. Number of fish sampled, $n = 20$.

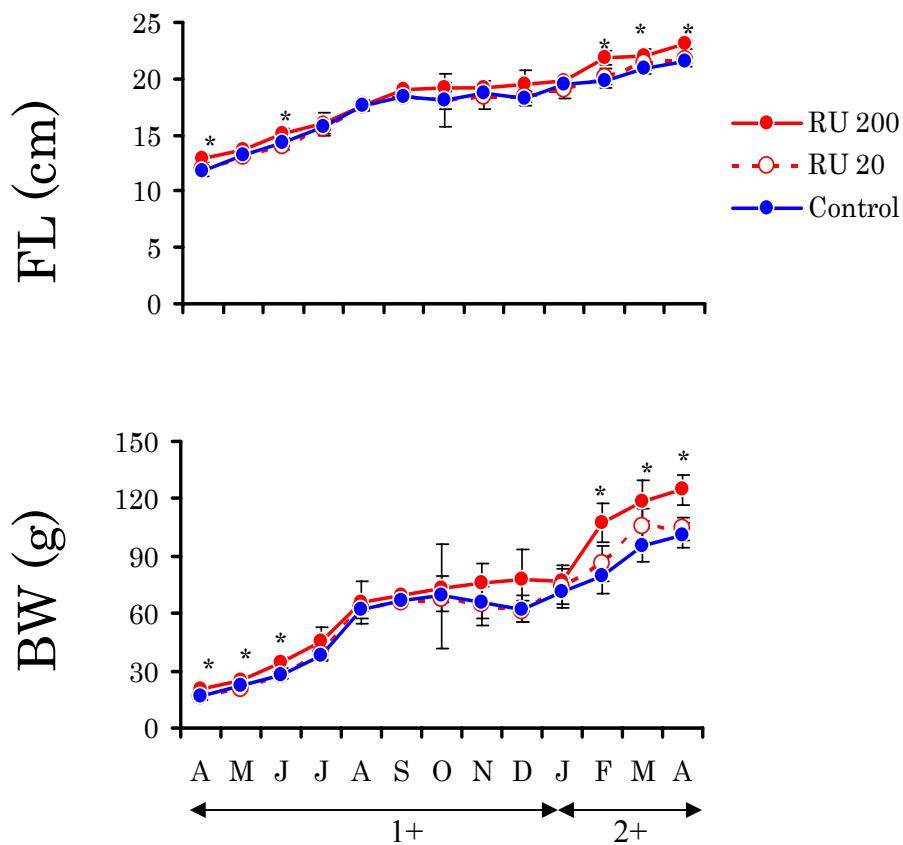


Fig. 2. Changes in fork length and body weight of immature (1+ - 2+) male masu salmon of Toya strain by long-term RU feeding. Each point represents the mean \pm SEM. Asterisks indicate significance differences between RU 200 fed and control groups. *P<0.05. Number of fish sampled, $n = 3-13$.

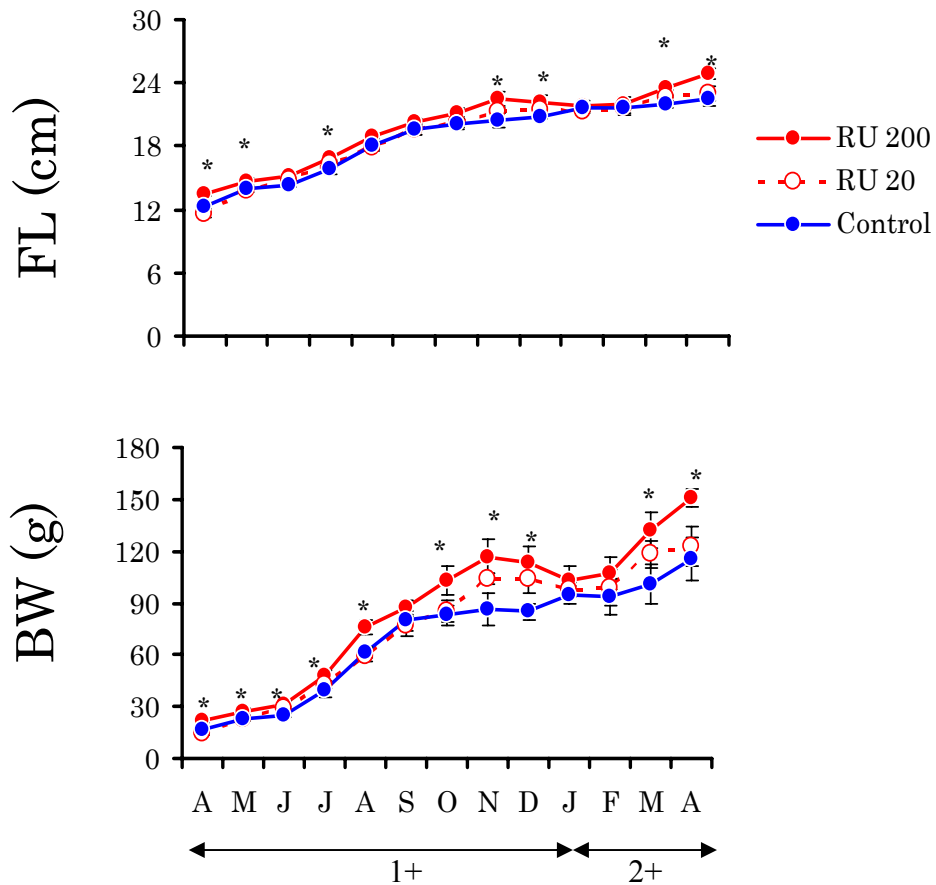


Fig. 3. Changes in fork length and body weight of female masu salmon of Toya strain by long-term RU feeding. Each point represents the mean \pm SEM. Asterisks indicate significance differences between RU 200 fed and control groups. *P < 0.05. Number of fish sampled, $n = 7-13$.

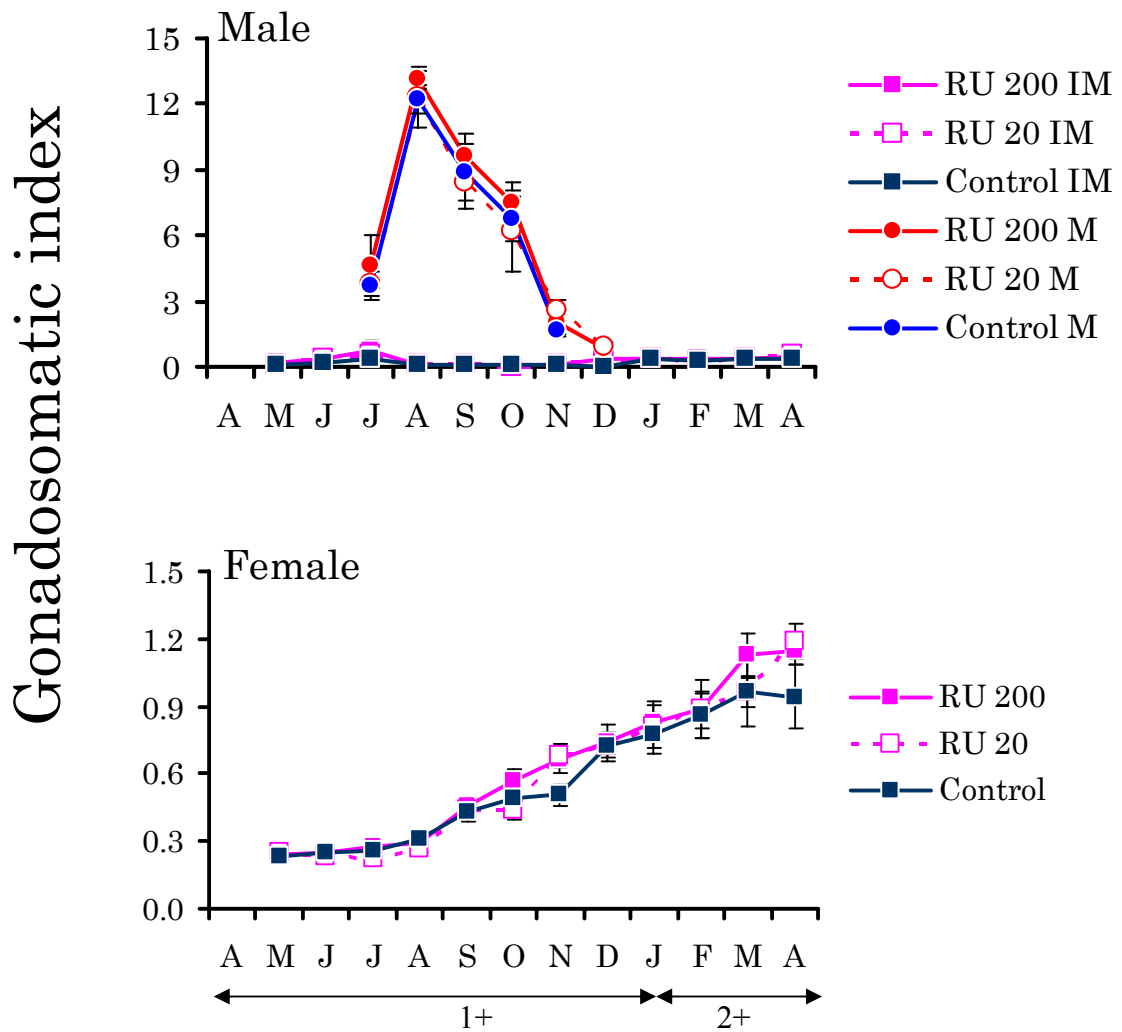


Fig. 4. Changes in gonadosomatic index (GSI) of female and male masu salmon of Toya by long term RU feeding. Each point represents the mean \pm SEM. IM: Immature, M: Mature.

Serum levels of steroid hormones in males

Serum T and 11-KT levels increased from 1+ June in males in both RU-fed and control groups (Fig. 5). Serum T levels in these groups were consistently elevated from 1+ June (where the presence of the first maturing males was detected) to August (peak) and showed a decreasing tendency after 1+ August. While the serum DHP levels were low till July but showed a surge increase in 1+ September. During this period, serum T levels in RU 200 fed groups increased significantly ($P < 0.05$) in 1+ June, August, September, and December; and 2+ March and April for males. In RU 200 males, serum levels of 11-KT increased significantly in 1+ June, July, September, and November; and 2+ March and April. In RU 200 males, serum DHP levels were significantly increased in 1+ September (Fig. 5).

Serum levels of steroid hormones in females

Serum T and E_2 levels showed a gradual increase from 1+ September in immature females in both RU-fed and control groups (Fig. 6). Serum T levels in females showed a relative increase from 1+ September to December and decreased in 2+ January and February, and again increased in 2+ March and April. Though gonadal observation indicated that all females were immature, there was an increase in DHP levels in July and September. During this period, serum T levels in RU 200 groups increased significantly ($P < 0.05$) in 1+ June and December; and 2+ March. In RU 200 females, serum levels of E_2 increased significantly in 1+ March, August, and November; and 2+ March. In RU 200 females, serum DHP levels did not show any significant increase as compared to controls (Fig. 6).

In August sampling, serum DHP levels in females were notably lower than the preceding or the following sampling month's values. It is hard to come up with a meaningful physiological explanation for this transient phenomenon. Although all females were found to be immature from physical examination of gonads and gonad weight, unexpectedly increased levels of DHP (final maturation hormone) were found in July and September for some of the sampled females in both treatment groups. The apparent increase in DHP levels at this time might be related to precociousness.

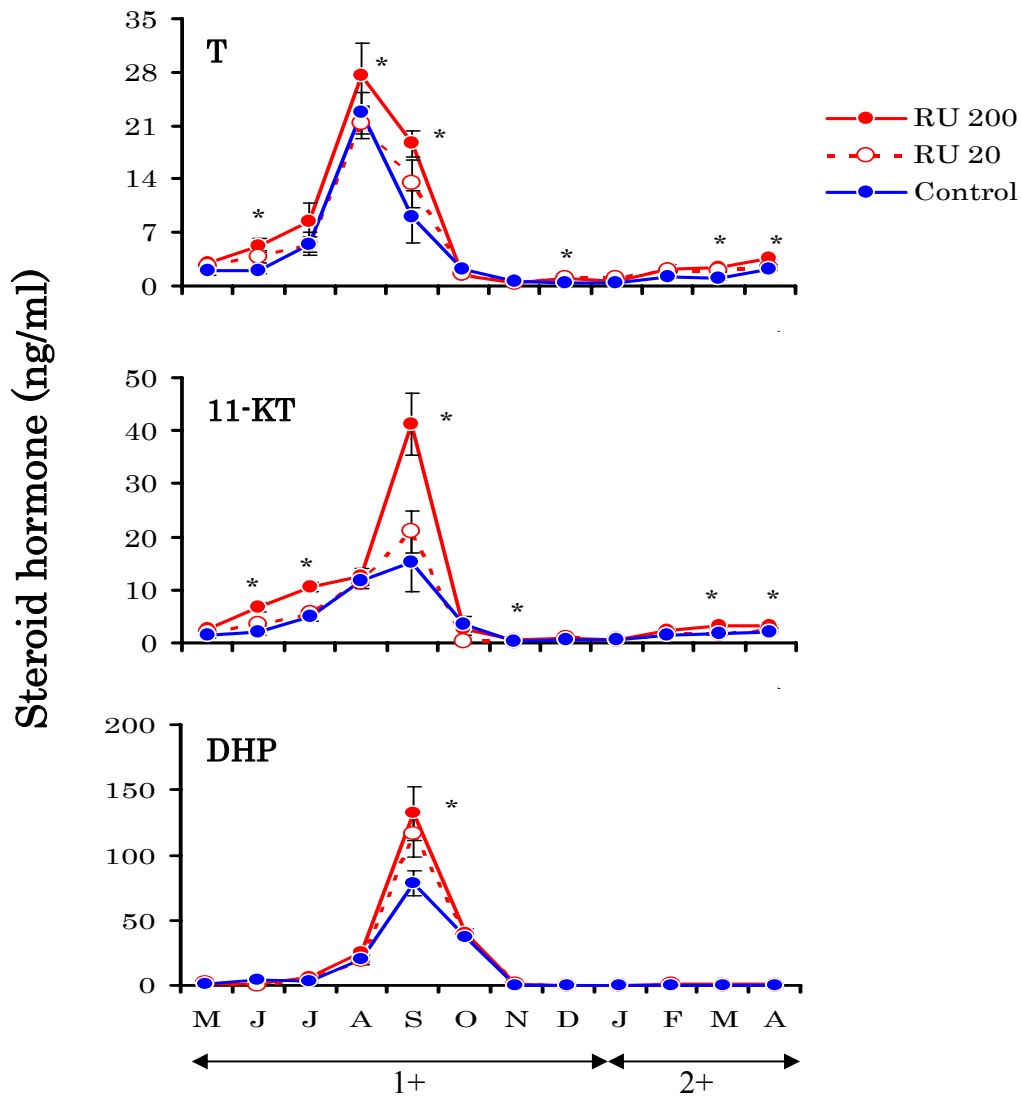


Fig. 5. Changes in serum testosterone (T), 11-ketotestosterone (11-KT) and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) levels of male masu salmon by long-term RU feeding. Asterisks indicate significant differences between RU 200 fed and control groups. * $P < 0.05$. Each point represents the mean \pm SEM. Number of fish sampled, $n = 6-13$.

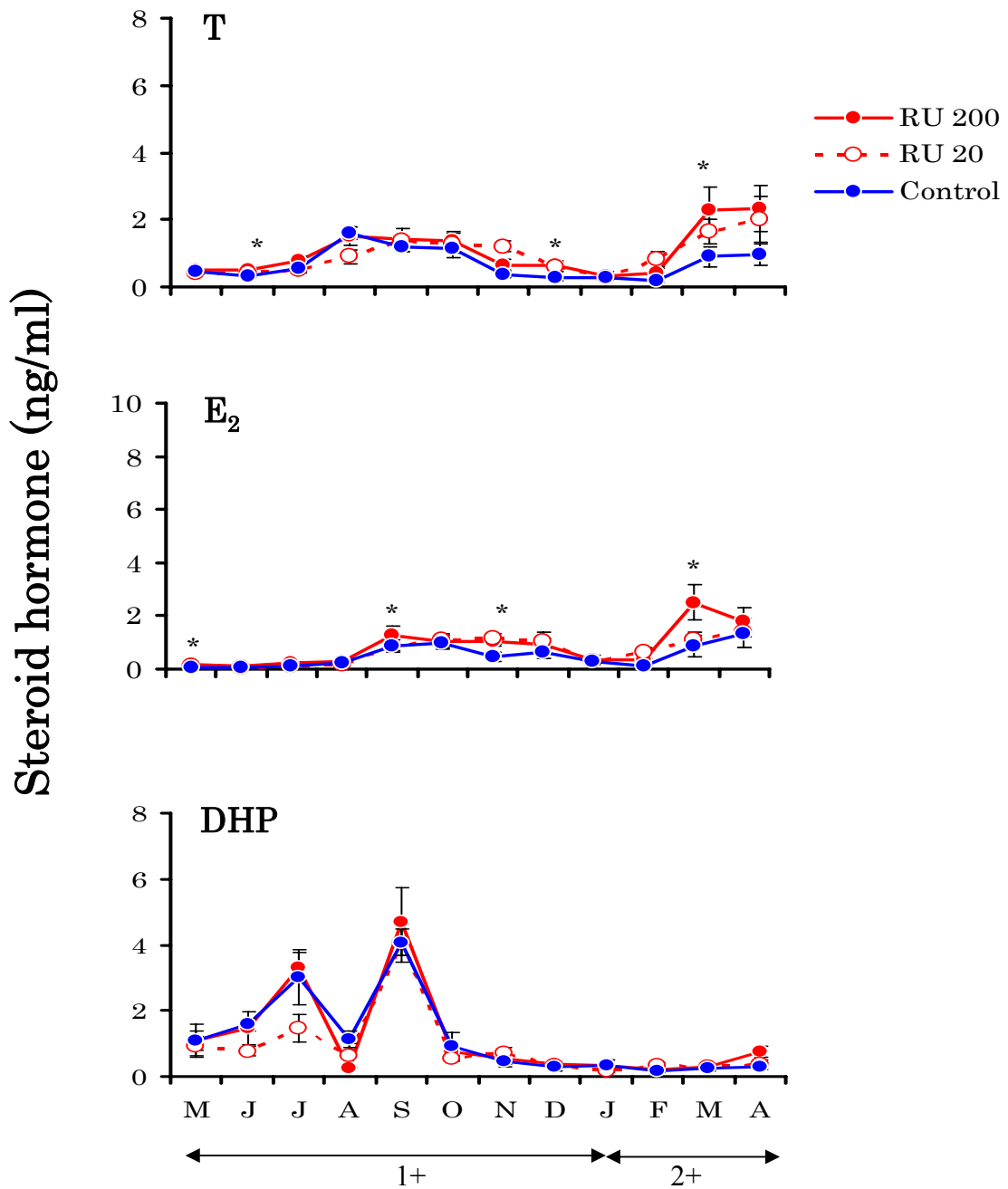


Fig. 6. Changes in serum testosterone (T), estradiol-17 β (E₂), and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) levels of female masu salmon by long-term RU feeding. Asterisks indicate significant differences between RU 200 fed and control groups. *P < 0.05. Each point represents the mean \pm SEM. Number of fish sampled, n = 6-13.

Serum IGF-I levels in males

Serum IGF-I levels increased consistently 1+ May in the treatment and control groups (Fig. 7). IGF-I levels peaked in August and started to decrease and remained relatively constant until 2+ February and again started to rise from February to April (Fig. 7). In RU 200 fed males, serum IGF-I levels were significantly increased 1+ June, September, October and December, and in 2+ March and April.

Serum IGF-I levels in females

Serum IGF-I levels showed a steadily increase 1+ May in both groups (Fig. 7). IGF-I levels showed a peak level in August and started to decrease and remained relatively constant until 2+ February and again showed a steadily increase till April (Fig. 7). In RU 200 fed females, serum IGF-I levels were significantly increased 1+ July, August, October and November, and in 2+ March and April.

Both sexes showed similar pattern of changes in serum IGF-I levels. There was poor relationship between body size and serum IGF-I ($r < 0.18$) in both sexes. In both sexes, there was a relatively good relationship between serum T and IGF-I levels ($r > 0.60$) in RU-fed and control groups. Serum IGF-I were more or less related to 11-KT levels ($r \approx 0.50$) and GSI ($r \approx 0.50$) in males but this relationship was not evident in females. On the other hand, significant increases in serum IGF-I levels were accompanied by significant increases in somatic growth. However, changes in growth over time were quite different from those in IGF-I.

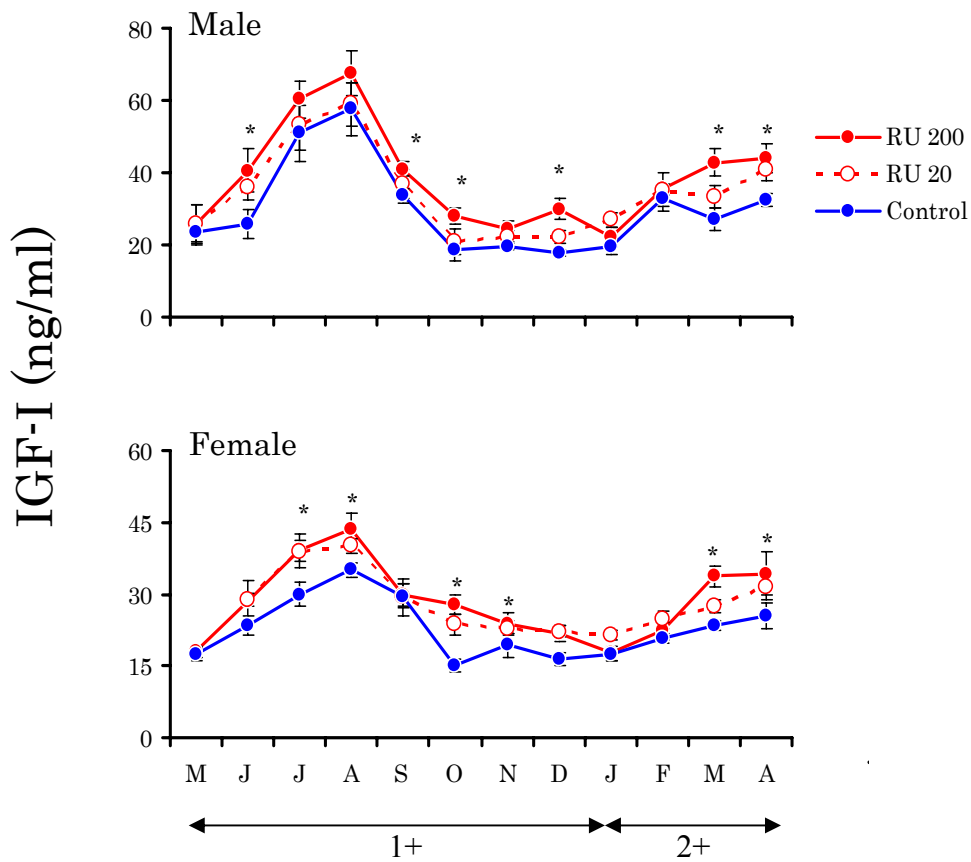


Fig. 7. Changes in serum insulin-like growth factor-I (IGF-I) levels of male and female masu salmon by long-term RU feeding. Asterisks indicate significant differences between RU 200 fed and control groups. *P < 0.05. Each point represents the mean \pm SEM. Number of fish sampled, $n = 6-13$.

Discussion

In this study, the effects of long-term RU administration on somatic growth and serum steroids and IGF-I were investigated. Significant body size increases in RU-fed groups were observed from 0+ September to 1+ March. In addition, somatic growth of RU-fed groups in both sexes was significantly higher than that of the control in 1+ spring, summer and autumn, and 2+ spring. The promotion of growth in both sexes might be due to the physiologically active substances present in the mycelium of RU. Bhandari et al. (2002) reported similar growth enhancement effects of RU in lacustrine sockeye salmon. They also suggested that somatic growth promotion by long-term feeding of RU might be brought by essential amino acids (leucine and isoleucine) that might be contained in RU, probably acting via amino acid metabolism. Amino acids are the essential nutrients for muscle growth in vertebrates including fish (Brown and Cameron, 1991). Arginine, one of the essential amino acids, is a powerful insulinotropin in fishes (Mommensen, 2001), which acts through GH-IGF axis being a secretagogue of insulin in pancreas. Intraperitoneal arginine injection also leads to a long and sustained increase in plasma levels of insulin (Carneiro et al., 1993), and secondarily in IGF-I (Banos et al., 1999). Not only arginine but also other amino acids such as glutamine and proline act concurrently in the biosynthesis of protein and then muscle growth. In this study, the higher growth rates in RU-fed groups might be justified by the direct involvement of amino acids contained in RU in protein synthesis or through the activation of GH-IGF axis.

GSI values increased to peak levels in 1+ August in males since maturing male fish were found, whereas, in females, it increased throughout the sampling period as none of them were found to be on maturing. Long-term RU feeding did not significantly increase the changes in GSI in both sexes and groups. Similar result has been reported by Bhandari et al. (2002). On the other hand, GnRH analog (GnRHa) implantations did not accelerate the changes in GSI in 1+ and 2+ male masu salmon (Bhandari, 2002).

RU 200 fed males showed acceleration of spermiation as well as significant elevation in the plasma levels of androgens in 1+ June, August, September and December and 2+ March and April, and DHP in 1+ September. RU 200 fed females showed significant elevation of serum T levels in 1+ June and December, and 2+ March and of serum E₂ levels in 1+ March, September and November, and 2+ March.

But, there was no significant increase in serum DHP levels. It seemed that the effect of RU administration on gonadal maturation was less pronounced in female masu salmon. With regard to this, it has been found that long-term RU feeding in sockeye salmon has significantly elevated plasma levels of androgens in males and accelerated spermiation, but with no effects in females (Bhandari et al., 2002). The reasons behind this less pronounced effect of RU in females are not clear. The fed RU may be too low to bring any effective physiological effect in females. In previous findings, endogenously synthesized molecules have been identified in the fungus *Pleurotus ostreatus*, which were mainly of T, androstenedione, progesterone and their metabolic products testosterone acetate and testololactone (Plementias et al., 1999). Lanisnik et al. (2001) investigate the physiological function of 17 β -HSD from the fungus *Cochliobolus lunatus* in inducing different estrogens and androgens. However, the stimulatory mechanism of the physiologically active substance in RU is still not clear.

In RU fed males, the serum level of DHP was significantly elevated in 1+ September as compared to control males. Serum levels of DHP in RU 200 and 20 groups of mature males reached 1.5 times higher compared to control mature males in 1+ September. This consistent increase in serum DHP at the final maturation indicates that the physiologically active substance present in RU could have stimulated the biosynthesis of DHP. Even though it has nothing to do with the biosynthesis of steroids, the enzyme glucoamylase, which readily converts starch to D-glucose, has been found in RU (Pazur and Okada, 1967). On the other hand, from other *Rhizopus* species like *Rhizopus nigricans*, progesterone, progesterone receptors and guanosine triphosphatease (GTHase) activity has been investigated (Bavec et al., 2000). During the onset of spermiation and ovulation the shift from progesterone to 17 α -hydroxyprogesterone and also from 17 α -hydroxyprogesterone to DHP occurs in the presence of the enzymes 17 α -hydroxylase/17,20-lyase (P450c17) and 20 β -hydroxysteroid dehydrogenase (20 β -HSD), respectively (Young et al., 1986; Nagahama, 1987). Taken together, there could be a possibility that the physiologically active substances in RU may participate in the biosynthetic pathway of steroids either by providing substrate to the pathway or accelerating the turnover rate of steroid production through the steroidogenic enzymes.

There was a considerable significant increase in serum IGF-I in RU-fed groups through out the feeding period though increases in winter seemed to a little bit low.

Interestingly, significant increases in serum IGF-I levels were accompanied by significant increases in somatic growth. This simultaneous increase was expected as IGF-I is the main mediator in somatic growth and it is highly correlated to growth rate in fish in general and salmonids in particular (Dickhoff et al., 1997; Duan, 1998; Moriyama and Kawauchi, 2001). IGF-I is also a multipurpose signaling molecule that addresses many metabolic and coordination events. The principal environmental regulator of GH-IGF-I axis is nutritional status (Pedroso et al., 2006). Amino acids are the building blocks of protein and are essential for muscle and body growth (Conlon et al., 1993). In this regard, the interaction of different amino acids leads to a consistent increase in serum IGF-I levels and activates GH-IGF-I system as described above. On the other hand, many fungal species including RU are believed to contain physiologically active substances, which could have active roles in body growth and gonadal maturation. Therefore, in this study, significant increases in serum IGF-I in RU-fed group may be related the direct effects of amino acids and enzymes on protein biosynthesis or through the activation of the complex GH-IGF system.

Serum IGF-I contents in this study were of similar order to circulating contents of other salmonids (Nordgarden et al., 2005; Campbell et al., 2006; Gabillard et al., 2006). Like our study, Campbell et al. (2003) reported similar pattern of IGF-I changes in chinook salmon. They described that the increase of plasma IGF-I levels from June to September of the previous year and decline of these levels to a low level over winter and increase to a maximum in September. But, maximum IGF-I levels were observed in August in our study. They also mentioned that maturing males tended to have higher plasma levels by April and the relationship between gonadal maturation and plasma IGF-I levels in maturing males became stronger after April. Serum IGF-I levels may vary among salmonids and are subjected to change by environmental factors. Increases in plasma IGF-I that occur with increased growth rate and body size in fish is one of the key components activating the reproductive axis (Campbell et al., 2006). IGF-I has direct effects on steroid production (Maestro et al., 1997; Kagawa et al., 2003) and gonadotropins (Ando et al., 2006). Thus, the increased serum levels of IGF-I in this study may consequently result in promotion of somatic growth and activation of BPG axis through direct and indirect effects.

In all treatments of this study, there was poor relationship between serum IGF-I levels and body size in both sexes. Previous finding reported controversial results on such relationships. Beckman et al. (1998) found a strong relation between mean

plasma IGF-I levels and mean growth rate, but little relation between mean IGF-I and mean body size independent of growth, suggesting that body size alone has little influence on plasma IGF-I levels. Little evidence of a strong relation was found between mean plasma IGF-I levels and mean growth rate of under-yearling chinook salmon (Silverstein et al., 1998). Besides, Nordgarden et al. (2005) reported that plasma GH, IGF-I and insulin hormones were not good indicators of growth because poor relationships were seen among them. As opposed to these reports, several other studies suggest that IGF-I is correlated to growth in salmonids (Prat et al., 1996; McCormick et al., 2000). Pierce et al. (2001) found a clear correspondence between feeding rate, growth rate and circulating IGF-I levels in coho salmon. The poor correlation observed in this study may be related to the multi-purpose function of IGF-I and capability of many tissues to produce IGF-I besides liver (Le Gac et al., 1996; Leibush et al., 1996). Further work is required to clarify the factual relationships in masu salmon.

There was a high positive relationship between serum IGF-I and 11-KT for males; and serum IGF-I and E₂ levels for females. A positive correlation was also observed between serum IGF-I levels; and GSI and HSI in males and females, respectively. These results agree with the reports in chinook salmon (Campbell et al., 2003) and coho salmon (Campbell et al., 2006). Both reports showed a consistent positive correlation among levels of plasma IGF-I, 11-KT, E₂ and also pituitary FSH, suggesting the importance of these factors in the link that body growth influences early stages of gametogenesis or sexual maturation. Further clarification of these relationships and its roles in the BPG axis and GH-IGF-I axis is essential.

In conclusion, it has been revealed that RU has growth promoting ability in masu salmon of both sexes. Considerable increases in serum IGF-I levels were found by long-term RU feeding. RU had also increased sex steroids and accelerated sexual maturation, especially in males. This consistent increases in body growth which was also accompanied by magnificent increase in serum IGF-I contents in RU-fed groups indicates that somatic growth in masu salmon may be brought by activation of GH-IGF-I axis, where the physiologically active substance contained in the mycelium of RU influences not only this axis but also steroidal biosynthetic pathways. Further *in vivo* and *in vitro* studies on the physiological functions of bioactive substances in RU are necessary to elucidate the roles of RU in body growth and sexual maturation in teleosts.

Chapter 1.2

***In vitro* effects of *Rhizopus* extract (RU) and its fractions on hormonal levels in brain, pituitary and gonads in masu salmon**

Introduction

The effects of gonadal steroids on the synthesis of gonadotropins have been recognized for a long time and are considered to be a part of the positive feedback mechanisms operating in immature fish. These effects may be exerted indirectly, through modulation of the neuroendocrine factors regulating gonadotropin release or directly at the level of GTH gene transcription (Trudeau and Peter, 1995). The onset of FSH and LH secretion could be initiated by a positive feedback by steroids (Crim and Evans, 1979; Borg et al., 1998; Swanson and Dickhoff, 1988). Dubois et al. (2001) investigated the role of T and E₂ in controlling the catfish GnRH system. Previous studies in teleosts indicate that gonadal steroids, including T and other androgens, can increase GTH II mRNA synthesis and/or *de novo* GTH II protein production in black carp *Mylopharyngodon piceus* (Gur et al., 1995), African catfish *Clarias gariepinus* (Rebers et al., 1997), European eel *Anguilla anguilla* (Montero et al., 1995) and goldfish *Carassius auratus* (Huggard et al., 1996).

Moreover, the functional development of the GnRH system in immature teleosts is under stimulatory control of gonadal steroids, especially T. This has been demonstrated for the masu salmon (Amano et al., 1994), rainbow trout *O. mykiss* (Breton and Sambroni, 1996), platyfish *Xiphophorus maculatus* (Schreibman et al., 1986) and African catfish (Dubois et al., 1998). Besides, it has been confirmed that T and E₂ could be able to exert a positive influence on the amounts of catfish GnRH (cfGnRH) during the later stages of pubertal development in African catfish (Dubois et al., 2000).

Recently, the steroid hormones, T, androstenedione, progesterone and their metabolic products testosterone acetate, androgen-binding proteins and 17 β -HSD were identified as endogenously synthesized molecules in the mycelium of some fungi (Lanisnik et al., 1992; Lanisnik and Zakelj-Mavric, 2000). Recently, the different physiologically active substances contained in the mycelium of RU have been characterized and their effects on mammalian steroid hormone synthesis have been investigated as describe in Chapter 1.1.

Many *in vivo* studies in RU administration have indicated promotion of growth and acceleration of the steroidogenesis in many vertebrates including fish. It is suggested that the physiologically active substance present in RU may play a great role in these promotion and/or acceleration effects. However, the detailed endocrine

mechanisms involved in these physiologically active substances are not known. In this chapter, dose-dependent and time course *in vitro* incubation of RU and its fractions on brain, pituitary and gonadal tissues was carried out to investigate the possible direct effects of RU on the hormonal release or production of these tissues.

Materials and Methods

Experimental Animals

Lacustrine masu salmon of the Mori and Toya strains were used at Toya Lake Station. Fish were transported from Mori Station in July 2003 and also fish reared at Toya Lake Station were used for the dose-dependent and time course RU and its fractions incubation experiments. Fish were transported a month ahead of the actual experiment. The fish were anaesthetized with 0.01% MS222 and then body length and weight were measured. Immediately, brain and pituitary of each fish were removed by decapitation and brain was divided into three regions as olfactory bulb (OB), telencephalon (TE) and HYP, and only HYP was used for *in vitro* experiments. HYP and pituitaries were put temporarily into 24 well microplates (Iwaki, Chiba prefecture, Funabashi, Japan) containing ice-cold Leibovitz's L-15 medium (Gibco, New York, U.S.A).

The gonads (testis and ovary) were carefully removed and transferred into large glass Petri dishes containing ice-cold Leibovitz's L-15 medium. Testes were minced with razors into small pieces, on average weighing 25 grams. Ovarian follicles were isolated and used for each treatment.

RU fractionation

The refined RU was further fractionated by synthetic adsorbent method 2 which mainly uses different eluants under chromatographic methods. RU was fractionated as follows: Fifteen grams of refined RU was dissolved in 100 ml of water. The dissolved solution was centrifuged at 3500 rpm for 15 min. The supernatant (upper phase) was filtered into 1 μ m membrane filter so that suspended and minute matter is removed. Then, the filtered solution had undergone chromatographic adsorption using synthetic adsorbent sepabeads-SP70 and different eluants. Water, 80% methanol (MeOH-H₂O) and 80% acetone (Acetone-H₂O) were used as eluants.

The RU fractionates which are eluted by water, 80% methanol and 80% acetone eluants were named as water fractionate (RU fraction A), methanol fractionate (RU fraction B) and acetone fractionate (RU fraction C), respectively. The water fractionate contains water and water-soluble active substances. The methanol fractionate is supposed to contain protein, amino acids and fats. The acetone fractionate may contain low molecular weight amino acids. Moreover, the water fractionate underwent further extraction using 80% ethanol. The upper phase of the solution had undergone reduced pressure filtration to remove ethanol and it is considered as one fractionate (RU fraction E). This fractionate mainly contains monosaccharide (sugar). On the other hand, the lower phase (ethanol precipitate) is named as ethanol fractionate (RU fraction D) and it is supposed to contain higher molecular weight carbohydrates (polysaccharides). Eight ml of eluates were collected for each fraction. After obtaining these fractionates, *in vitro* incubations were carried out on different tissues.

RU incubation

Based on the results from my Master's study and preliminary experiments, dose-dependent incubations of the HYP, pituitary, minced testis and ovarian follicles were made at 0, 1, 10, 100 and 1000 µg RU /ml L-15 medium for 18 hours in a humidified incubator at 15°C (late August 2004). Time-course incubations were carried out for 6, 12, 18, 24 and 36 hours in a humidified incubator at 15°C. Triplicate incubations were made for each treatment. After termination of the incubation period, the samples and the media were collected and stored at -80°C until TR-FIA analysis. Because the medium hormonal release from HYP was too low to detect, hormones were measured from tissue extractions.

RU fractions incubation

Dose-dependent incubations of the HYP, pituitary, minced testis and ovarian follicles were made at 0, 10, 100, 1000 and 10,000 µg RU fractions/ml L-15 medium for 18 hours in a humidified incubator at 15°C (September 2005). The RU fractions used in this experiment were A, B, C, D and E as well as original RU. Triplicate incubations were made for each treatment. After termination of the incubation period, the samples and the media were collected and stored at -80°C until TR-FIA analysis.

Extraction of hormones from brain and pituitary

Salmon GnRH (sGnRH) and gonadotropins (GTHs) from the HYP and pituitary were extracted according to the method of Okuzawa et al. (1990). In brief, the frozen HYP and pituitary tissues and media in 1 ml of 0.1 N HCl were homogenized by sonication, followed by addition of 0.1 ml 1 N NaOH. After centrifugation at 15,000 rpm for 30 min at 4°C, the supernatant was lyophilized under vacuum, reconstituted in assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 µM DTPA, 0.01% Tween 40, pH 7.2) for sGnRH and assay buffer (0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN₃, 0.01% Tween 40, 20 µM DTPA, pH 7.75) for GTHs. The reconstituted samples were frozen at -30°C until TR-FIA.

Extraction of steroids from gonad medium

Briefly, ether-extracted steroid hormones in the medium samples were evaporated in a 45°C water bath with a continuous flow of nitrogen gas, reconstituted into 600 µl of assay buffer containing 0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN₃, 0.01% Tween 40, 20 µM DTPA, pH 7.75 and stored at -30°C until assayed.

Time-resolved fluoroimmunoassay for sGnRH

The protocols for sGnRH assay followed those developed by Yamada et al. (2002). Antiserum for sGnRH was immobilized at the surface of the microtiter plates (Wallac Oy Turku, Finland) by physical adsorption for 18 hours at 4°C. After three washes with 0.9% saline, the plates were blocked with a 0.1% BSA solution (0.05 M Na₂HPO₄, 3% sucrose, 0.1% BSA, 0.05% NaN₃) for 1 hour at 4°C. The plate was washed with 0.9% saline three times for immunoassays. One hundred microliters of assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 µM DTPA, 0.01% Tween 40, pH 7.2), 50 µl of standards, diluted samples, and 50 µl of biotinylated sGnRH (0.1 ng/ml) were dispensed into the wells. All standards, samples, and biotinylated sGnRH were diluted with assay buffer. The plate was then incubated overnight at 4°C. After incubation and three washes with 0.9% saline, 200 µl of Eu-labeled streptavidin (50 ng/well) in assay buffer was added to the plate for detection of primary antibody-antigen complex on the surface of the wells. The plate was shaken for 1 hour at room temperature. After three washes with 0.9% saline, Eu was dissociated from the antibody-antigen complex on the surface of the wells with enhancement solution (0.1 M acetate-phthalate buffer, pH 3.2, containing 0.1% Triton

X-100, 15 μM 2-naph-thoyltrifluoroacetone, 50 μM tri-n-octylphosphine oxide; Perkin-Elmer). The plate was shaken for 5 min at room temperature, and the intensity of the dissociated Eu was measured by a time-resolved fluorometer (1234 DELFIA fluorometer; Wallac Oy) using DOS-based Multicalc software (Wallac Oy). Standard curves in each assay were plotted and values were calculated automatically by the time-resolved fluorometer.

Time-resolved fluoroimmunoassay for FSH and LH

The protocols for GTHs assays followed those developed by Yamada et al. (2002). Antiserum for GTHs (2nd IgG) was immobilized at the surface of the microtiter plates (Wallac Oy Turku, Finland) by physical adsorption for 18 hours at 4°C. After three washes with 0.9% saline, the plates were blocked with a 0.1% BSA solution. The plate was washed with 0.9% saline three times for immunoassays. One hundred microliters of assay buffer (0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN₃, 0.01% Tween 40, 20 μM DTPA, pH 7.75) and 100 μl of anti-LH and FSH with dilution rates of 100,000 and 5, 000 times, respectively, were pipetted and incubated for 18 hours at 4°C. Then, 50 μl of assay buffer, standards, and diluted samples were dispensed to the wells and incubated for 2 hours with low shaking at room temperature. Fifty microliters of Eu-GTHs (X600 dilution rates) in assay buffer was added to the plate and incubated overnight at 4°C. After three washes with 0.9% saline, Eu was dissociated from the antibody–antigen complex on the surface of the wells with enhancement solution. The intensity of the dissociated Eu was measured by a time-resolved fluorometer (1234 DELFIA fluorometer; Wallac Oy) using DOS-based Multicalc software (Wallac Oy). Standard curves in each assay were plotted and values were calculated automatically by the time-resolved fluorometer. BSA-conjugated antigens, antibodies and Eu-labeled IgG were kindly provided by Dr. M. Amano, Kitasato University.

Time-resolved fluoroimmunoassay for steroids in gonad tissues

The method for time-resolved fluoroimmunoassay for steroids in the gonadal media is the same as serum steroids as described in Chapter 1.1.

Statistical Analysis

Data obtained were expressed as means \pm SEM. To assess significant

differences between RU-treated and control groups, data were subjected to one-way ANOVA analysis followed by Fisher's PLSD or Dunnett's multiple comparison tests. Differences were considered statistically significant when $P < 0.05$.

Results

1.1 RU Dose incubations

Changes in sGnRH concentration of hypothalamus

Fig. 8 shows the ability of RU to stimulate sGnRH production of HYP by *in vitro*. HYP incubates of both sexes showed a dose-related increase in the production of sGnRH when they were incubated with RU for 18 hours. Salmon GnRH levels of the HYP of both sexes were significantly increased at higher doses of RU incubation ($P < 0.05$). This dose dependent increase of HYP sGnRH indicates that the physiologically active substances present in RU may potentiate sGnRH production *in vitro*.

Changes in GTHs of pituitary

Figs. 9 and 10 show the changes in the pituitary LH and FSH levels at different doses of RU incubation. LH levels of pituitary incubates in both sexes were increased in RU treatments as compared to control but not significant with RU incubation (Fig. 9). FSH levels of pituitary incubate in males showed a dose-dependent increase with RU incubation (Fig. 10). There was a significant increase ($P < 0.05$) at the highest dose RU incubation in both sexes. Generally, these findings suggest that RU incubation at higher doses may have had direct effects on the release of pituitary GTHs in both sexes.

LH levels at 10 μg RU/ml L-15 medium in females were notably lower than the preceding or the following RU dose incubations (indicated with red vertical arrow in Fig. 10). It is hard to come up with a reasonable physiological explanation for this transient phenomenon as some unknown factors could possibly affect the results of this incubation either during incubation or assay. The extremely low value obtained in this study was also confined to this RU incubation only. Though nothing indicates that a technical mishap had occurred, this cannot be excluded.

Changes in testicular steroid hormones

T, 11-KT and DHP release in the testis increased with RU dose increase (Fig. 11). There was a significant increase in T at all doses and 11-KT at 100 and 1000 μg RU /ml L-15 medium ($P < 0.05$). There was an increased DHP release in the medium of testis incubates with RU doses (Fig. 11). DHP was significantly increased on the testis incubates at 100 and 1000 μg RU/ml L-15 medium dose ($P < 0.05$). *In vitro* incubation of testis with RU increased the release of T, 11-KT and DHP in the testis medium as compared to the basal releases.

Changes in ovarian steroid hormones

Fig. 12 shows the ability of RU to enhance steroidal release in the medium of ovarian incubates. At higher doses of RU incubation, T and E_2 production in the ovarian incubates were significantly increased ($P < 0.05$). Though DHP releases in the ovarian incubates were higher than the control, significant differences were not observed. This significant increase in the production of T and E_2 in ovarian incubates may be due to the direct involvement of the physiologically active substances present in RU in the biosynthesis of ovarian steroid or the acceleration of the turnover rate of the biosynthetic pathway.

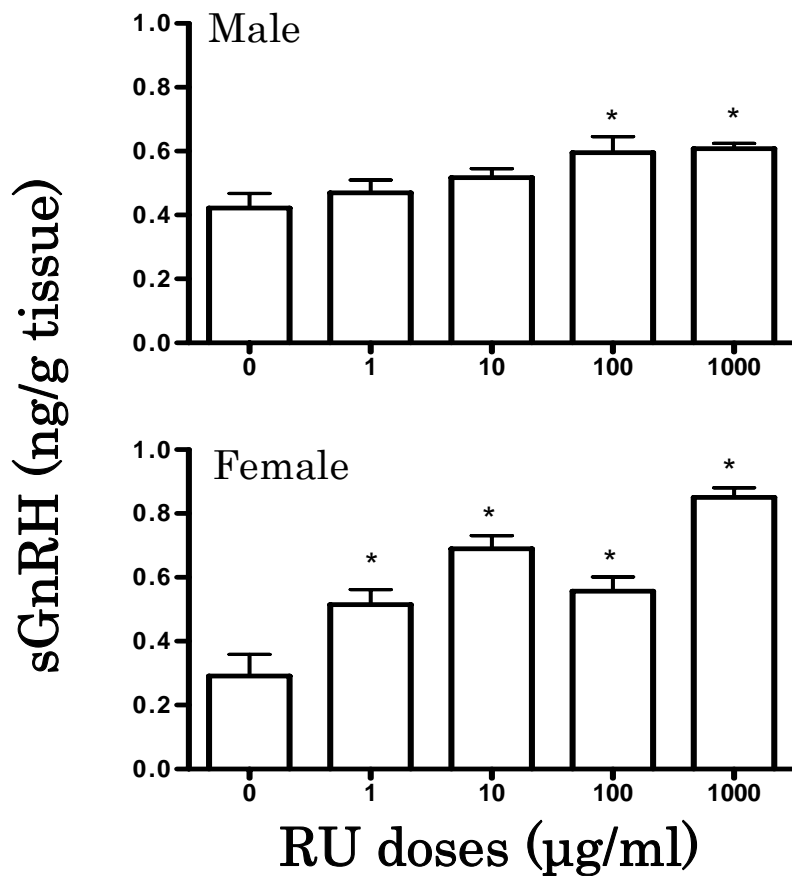


Fig. 8. Changes in the salmon gonadotropin-releasing hormone (sGnRH) concentrations in the HYP incubates of male and female masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU doses and control ($n = 3$). * $P < 0.05$.

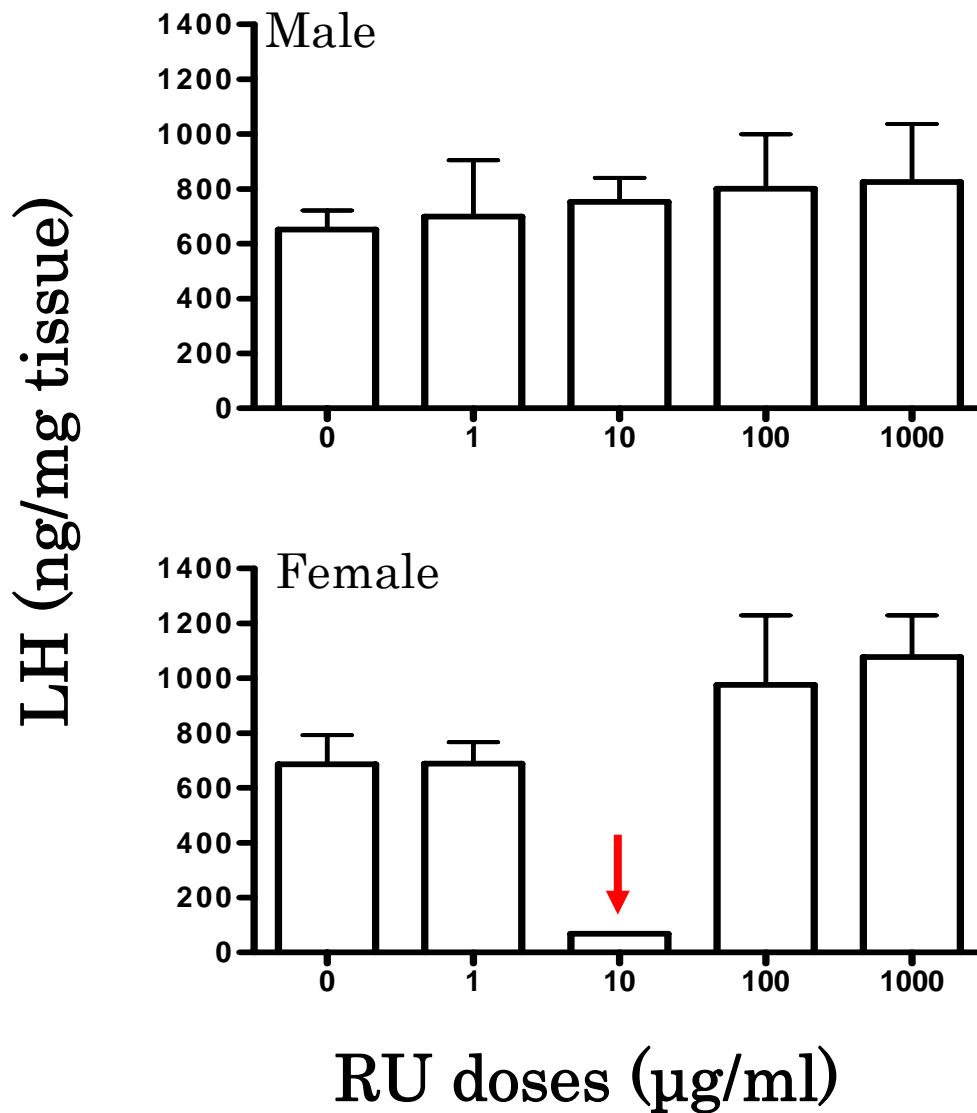


Fig. 9. Changes in the luteinizing hormone (LH) releases in the medium of pituitary incubates of male and female masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU incubates and control ($n = 3$). * $P < 0.05$. The vertical red arrow indicates the notably low values of LH at 10 $\mu\text{g/ml}$ RU dose.

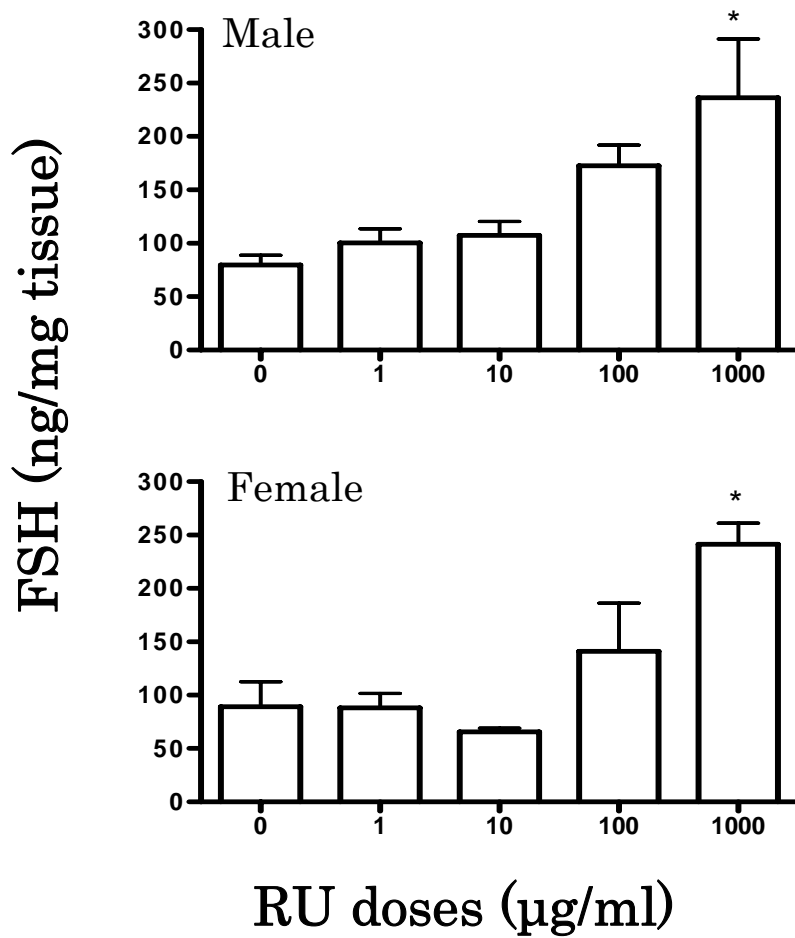


Fig. 10. Changes in the follicle-stimulating hormone (FSH) releases in the medium of pituitary incubates of male and female masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU incubates and control ($n = 3$). * $P < 0.05$.

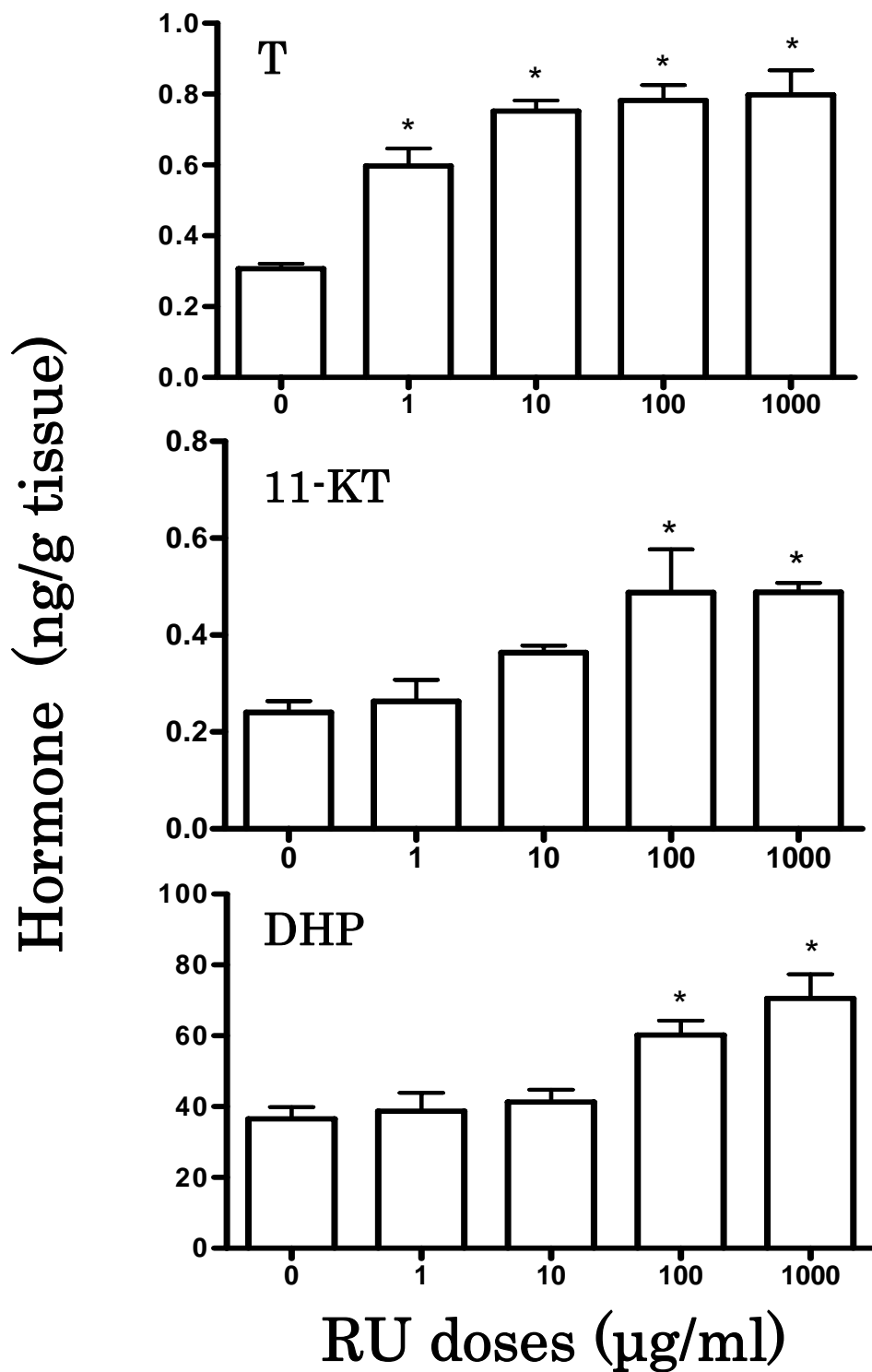


Fig. 11. Changes in testosterone (T), 11-ketotestosterone (11-KT), and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) releases in the medium of testis incubates of masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU incubates and control ($n = 3$). * $P < 0.05$.

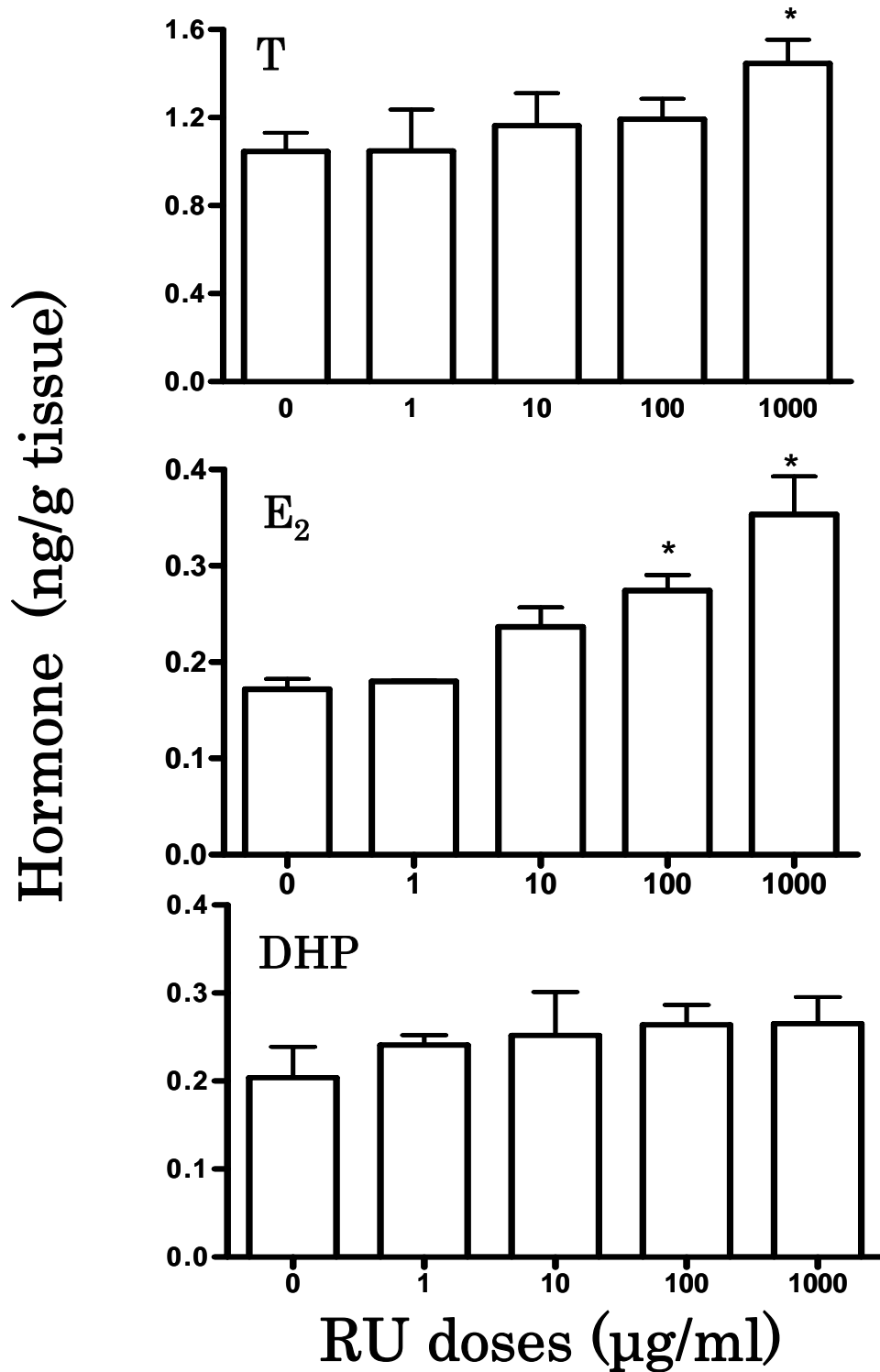


Fig. 12. Changes in testosterone (T), estradiol-17 β (E₂), and 17 α ,20 β -dihydroxy-4-regnen-3-one (DHP) releases in the medium of ovarian incubates of masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU incubates and control ($n = 3$). * $P < 0.05$.

1.2 Time-course incubations

Changes in sGnRH concentration of hypothalamus

Fig. 13 shows the sGnRH production in the HYP of masu salmon at different hours of RU incubation. Starting from 6 hours, sGnRH production in the HYP tissues seemed to increase till 18 and 24 hours for males and females, respectively and then started to decrease afterwards in both treatments. In both sexes, sGnRH levels in RU treated tissues were not statistically different from the counterpart controls. *In vitro* incubation of HYP was not carried out at 18 hours for females due to sample shortage. The higher values of sGnRH at early hours of incubation may probably indicate faster production of sGnRH in the HYP incubates.

Changes in GTHs concentration of pituitary

LH levels in the pituitary incubate increased throughout the incubation period (Fig. 14). RU incubation significantly increased LH release from pituitary at 24 hours of incubation for females. LH levels in the pituitary incubates of males increased within the first 24 hours of incubation and decreased afterwards. There was a significance difference between the RU incubates and control at 18 and 24 hours for males. FSH levels in the pituitary incubate of males and females constantly increased throughout the incubation period in RU treated and controls (Fig. 15). No significant difference was observed between the RU incubates and control in FSH release throughout the incubation period.

Changes in testicular steroid hormones

Fig. 16 shows T and 11-KT releases in the medium of testis incubate at different hours of incubation. These androgens showed similar increasing trends in the first 24 hours of incubation. There was time-dependent increase in T and 11-KT releases in the medium of testis incubate. They were increased significantly in the medium of RU incubated testis at 12, 18 and 24 hours of incubation. Like the androgens, DHP releases in the medium of testis incubates were increased in the first 24 hours of incubation. Significant increases in DHP were observed at 12, 18 and 24 hours of incubation.

Changes in ovarian steroid hormones

T and E₂ releases in the medium of ovary incubate at different hours of incubation were shown in fig. 17. T and E₂ releases in the medium increased gradually as the time progresses. T releases were significant at 6 hours of incubation, whereas E₂ releases were significant at all hours of incubation. The release of DHP in the medium of ovarian incubates showed some kinds of irregularities. They decreased from 6 hours to 12 hours of incubation and increased (peaked) at 18 hours of incubation and again showed a decreasing tendency at 24 and 36 hours of incubation. DHP release in the medium of ovarian incubates were significant at all hours of incubation in RU treated ones as compared to the control.

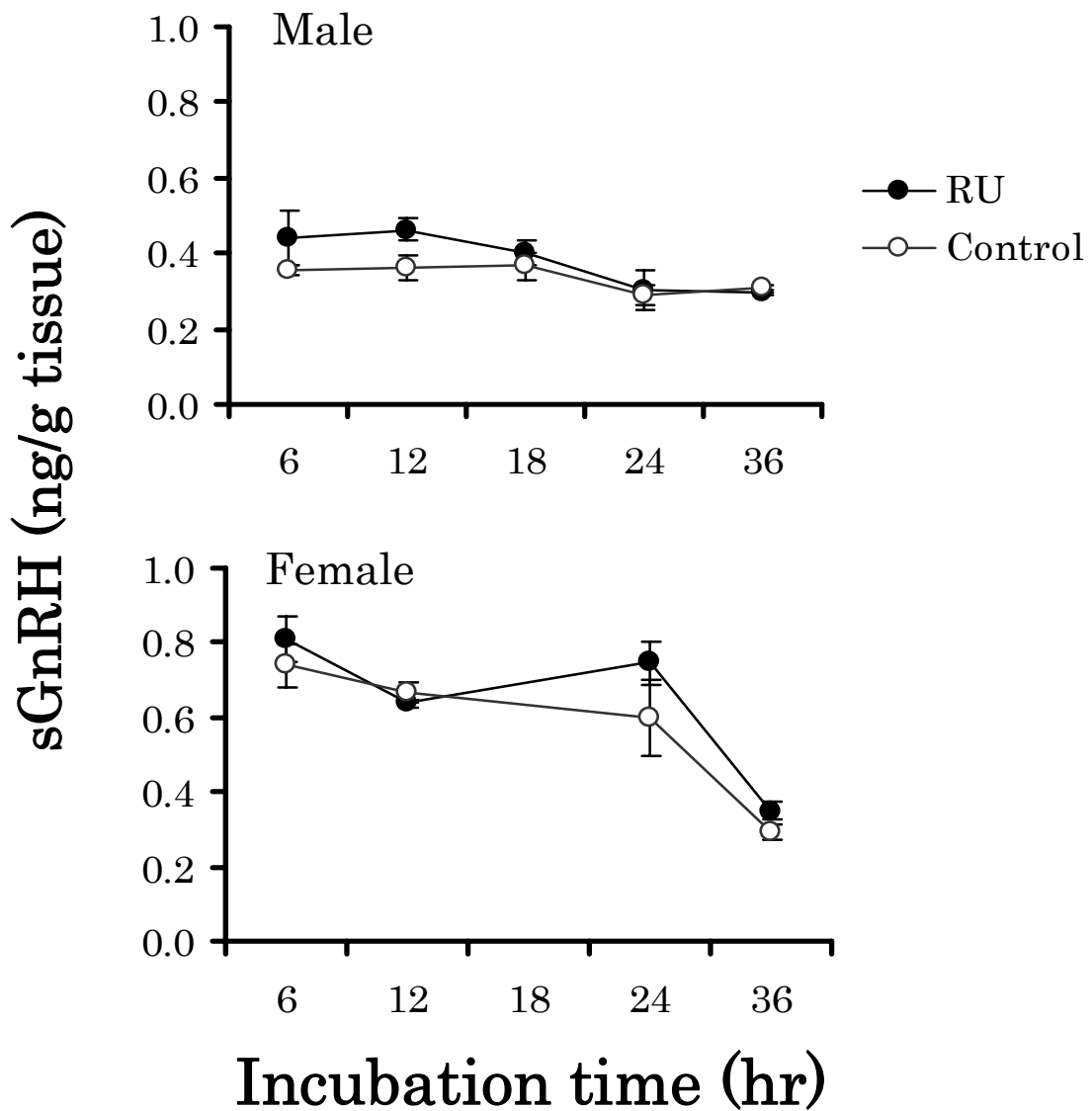


Fig. 13. Changes in the salmon gonadotropin-releasing hormone (sGnRH) concentrations in the HYP incubates of male and female masu salmon at different hours of RU incubation (100 μ g/ml RU dose) *in vitro*. Results are expressed as means \pm SEM.

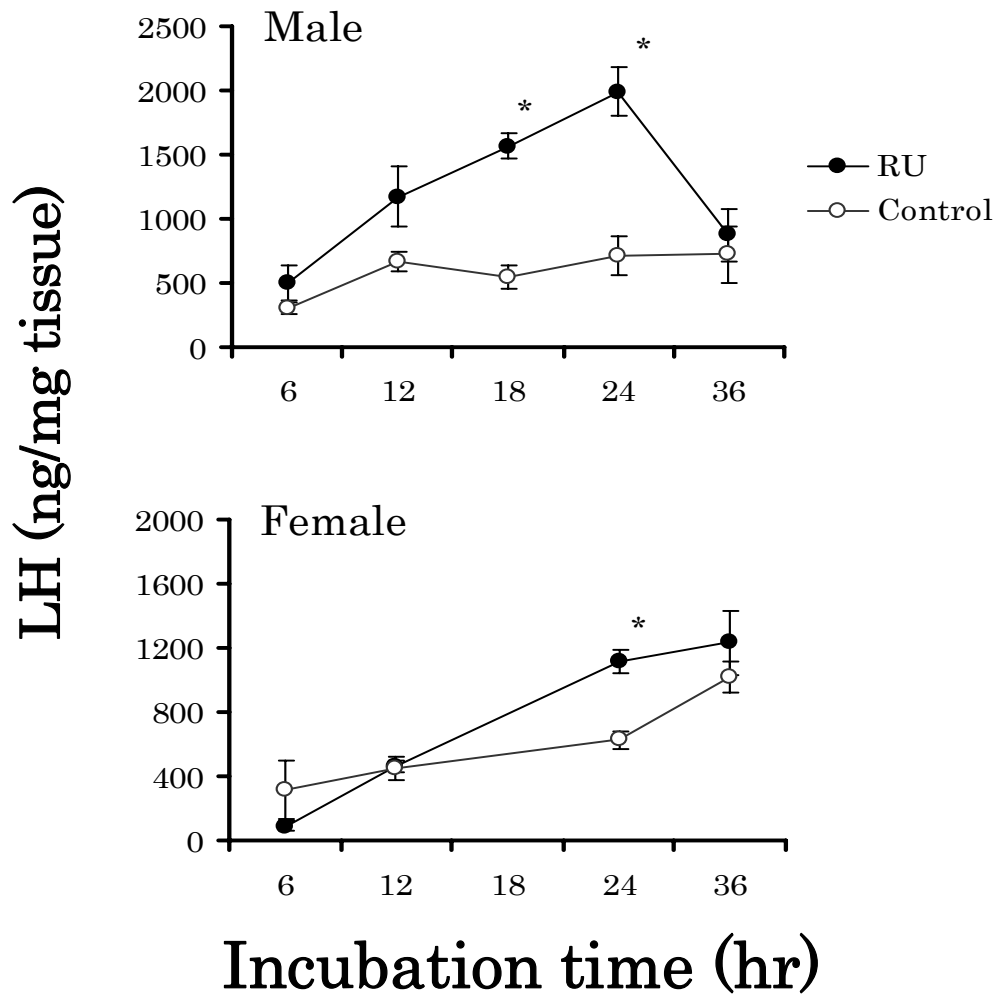


Fig. 14. Changes in the luteinizing hormone (LH) releases in the pituitary incubates of male and female masu salmon at different hours of RU incubation (100 µg/ml RU dose) *in vitro*. Results are expressed as means ± SEM. Asterisks indicate a significance difference between RU treatment and control ($n = 3$). * $P < 0.05$.

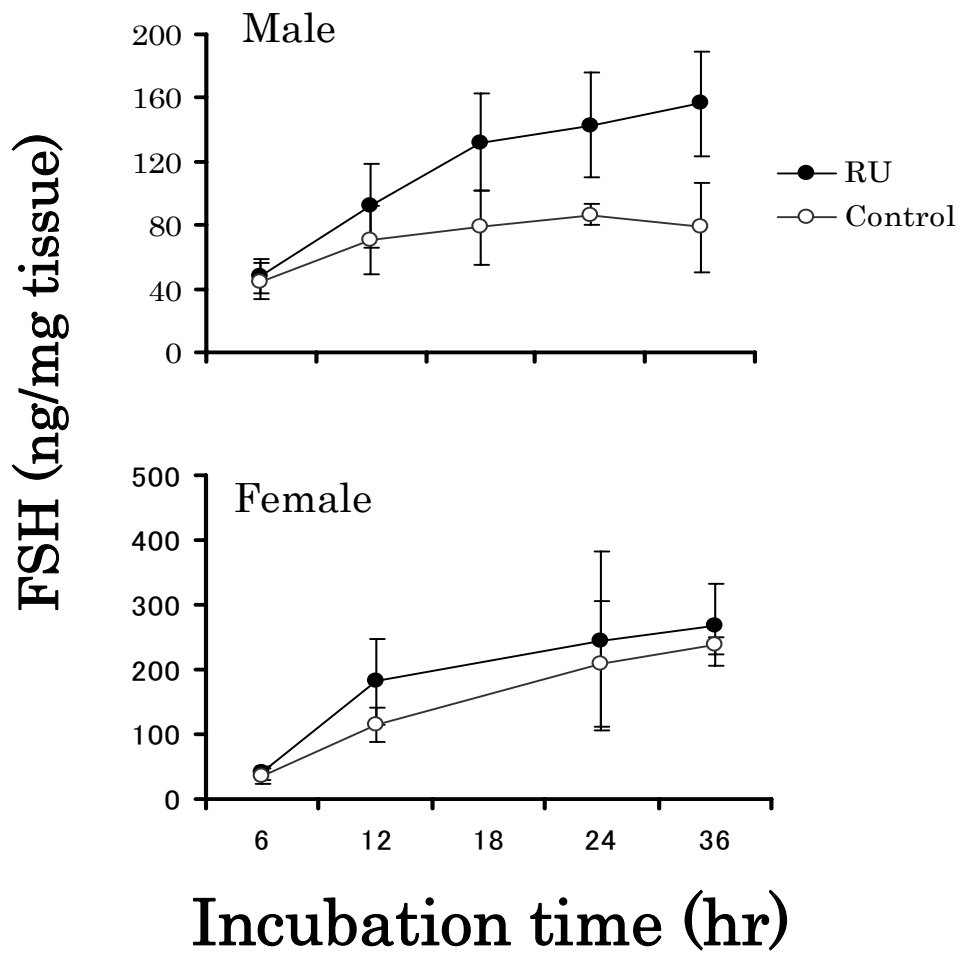


Fig. 15. Changes in follicle-stimulating hormone (FSH) releases in the pituitary incubates of male and female masu salmon at different hours of RU incubation (100 $\mu\text{g/ml}$ RU dose) *in vitro*. Results are expressed as means \pm SEM.

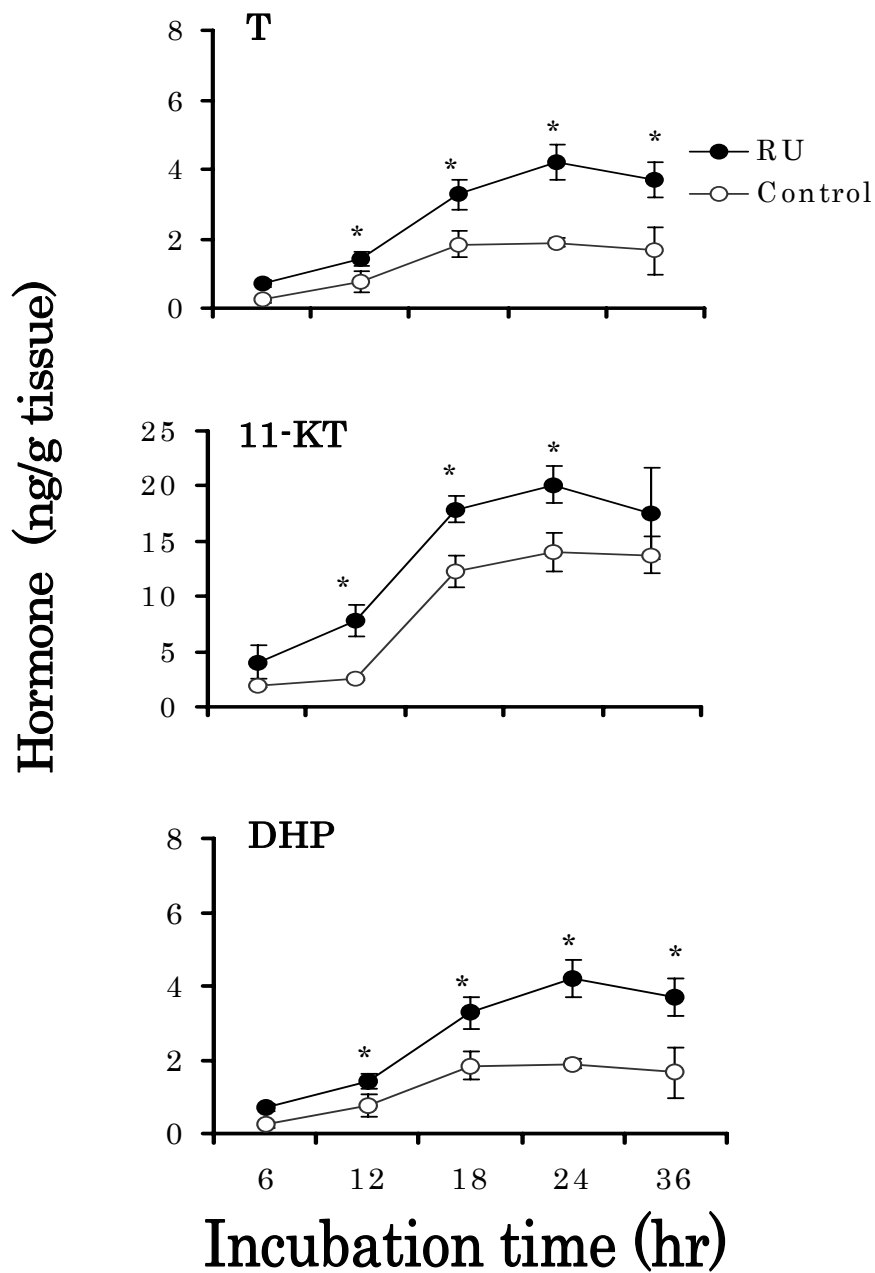


Fig. 16. Changes in testosterone (T), 11-ketotestosterone (11-KT), and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) releases in the testis incubates masu salmon at different hours of RU incubation (100 μ g/ml RU dose) *in vitro*. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU treatment and control ($n = 3$). * $P < 0.05$.

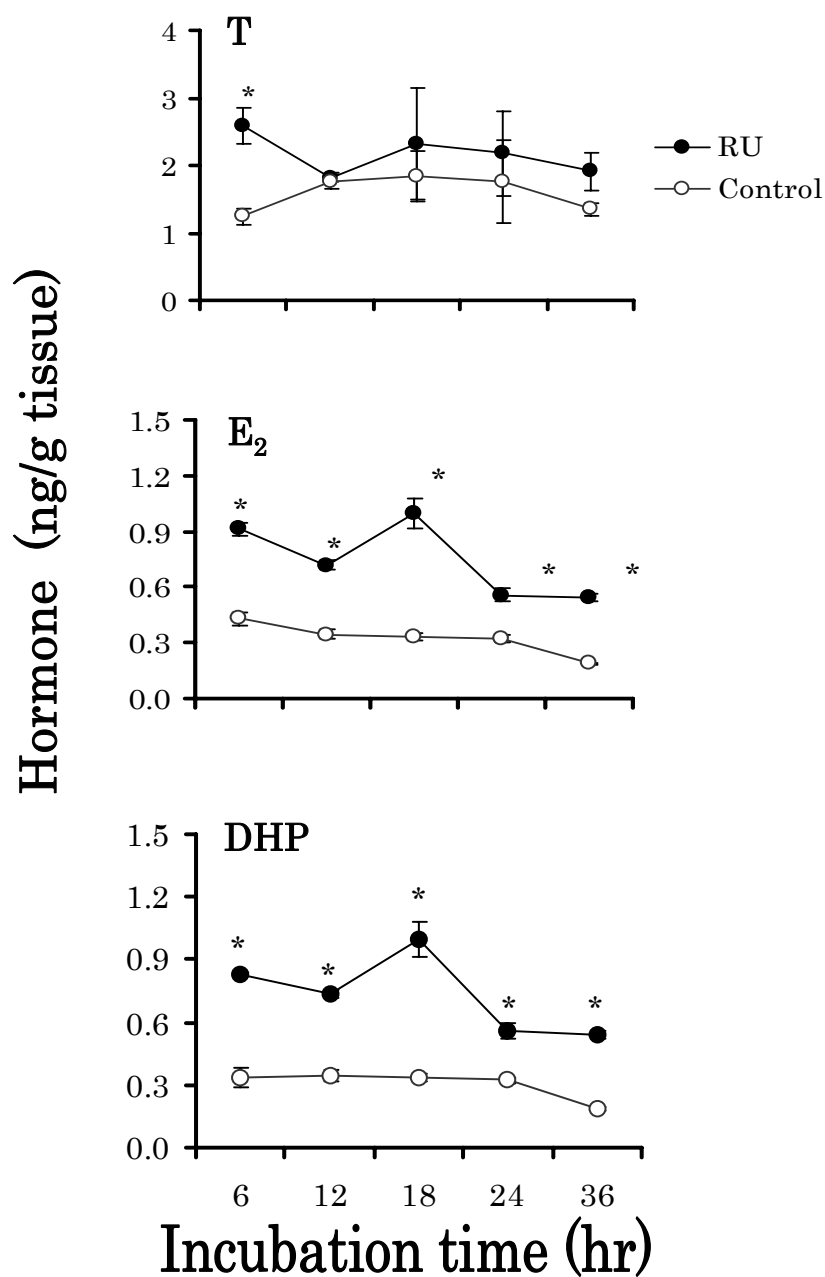


Fig. 17. Changes in testosterone (T), estradiol-17 β (E₂), and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) releases in the ovarian incubates masu salmon at different hours of RU incubation (100 μ g/ml RU dose) *in vitro*. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU treatment and control ($n = 3$). *P < 0.05.

1.3 Dose incubation of RU fractions

Changes in sGnRH concentration of hypothalamus

Figure 18 shows the ability of RU fractions to stimulate sGnRH production in the tissues of HYP in both sexes. Salmon GnRH production in the HYP incubates increased at all dose levels and the differences between the different doses seemed minimal in males. All RU fractions had significantly increased sGnRH production in the HYP at all dose levels as compared to control in males. In females, sGnRH production increased in most of RU fractions though the values at higher doses seemed to decrease (Fig. 18). Most of the RU fractions significantly increased sGnRH production in HYP tissues at different doses RU fractions.

Changes in GTHs of pituitary

Figure 19 shows the changes in the pituitary LH releases at different doses of RU fractions incubation. LH levels in both sexes showed greater increases in most of RU fractions. Typically, LH releases in males were significantly increased in most of RU fraction doses as compared to control. In females, significant increases in LH releases were less pronounced/common implying the presence sex variation in RU fraction effects. FSH releases in pituitary incubates of males showed a dose-dependent increase with most RU fractions (Fig 20). In both sexes, significant increases in FSH releases were observed at higher doses of RU fractions.

Changes in testicular steroid hormones

T and 11-KT releases in the testis incubate increased with RU fraction dose increase (Fig. 21). There was a significant increase in testis androgens release at the highest dose of original and RU fraction A ($P < 0.05$). In all RU fractions, T and 11-KT releases were higher as compared to control at the highest doses. There was also a significant increase in DHP release in the testis incubates at 100 μg RU fraction/ml L-15 medium dose for original and RU fraction E; and at 10000 μg RU fraction/ml L-15 medium dose for RU fraction A. *In vitro* incubation of testis with RU fractions increased release of T, 11-KT and DHP as compared to the basal production for some of the RU fractions.

Changes in ovarian steroid hormones

Figure 22 shows the capability of different RU fractions to enhance steroidal release in ovarian incubates. Increases in T and E₂ releases in ovarian incubates were observed at different doses of RU incubations. T releases in ovarian incubates were significantly increased at 100 µg RU fraction/ml L-15 medium dose (P < 0.05) for original, RU fraction A, B and E. Similar increases were observed at higher doses of RU fraction A incubation. Original, RU fractions A and C had significant effects in increasing E₂ releases in the medium of ovarian incubates at higher doses as compared to the basal production. Besides, original, RU fractions A and B had significantly increased DHP release in ovarian incubates (Fig. 22). This significant increase in the production of T, E₂ and DHP in ovarian incubates at different doses of RU fractions may probably indicate the direct involvement of the physiologically active substances dissolved in these fractions in the biosynthesis of ovarian steroid production.

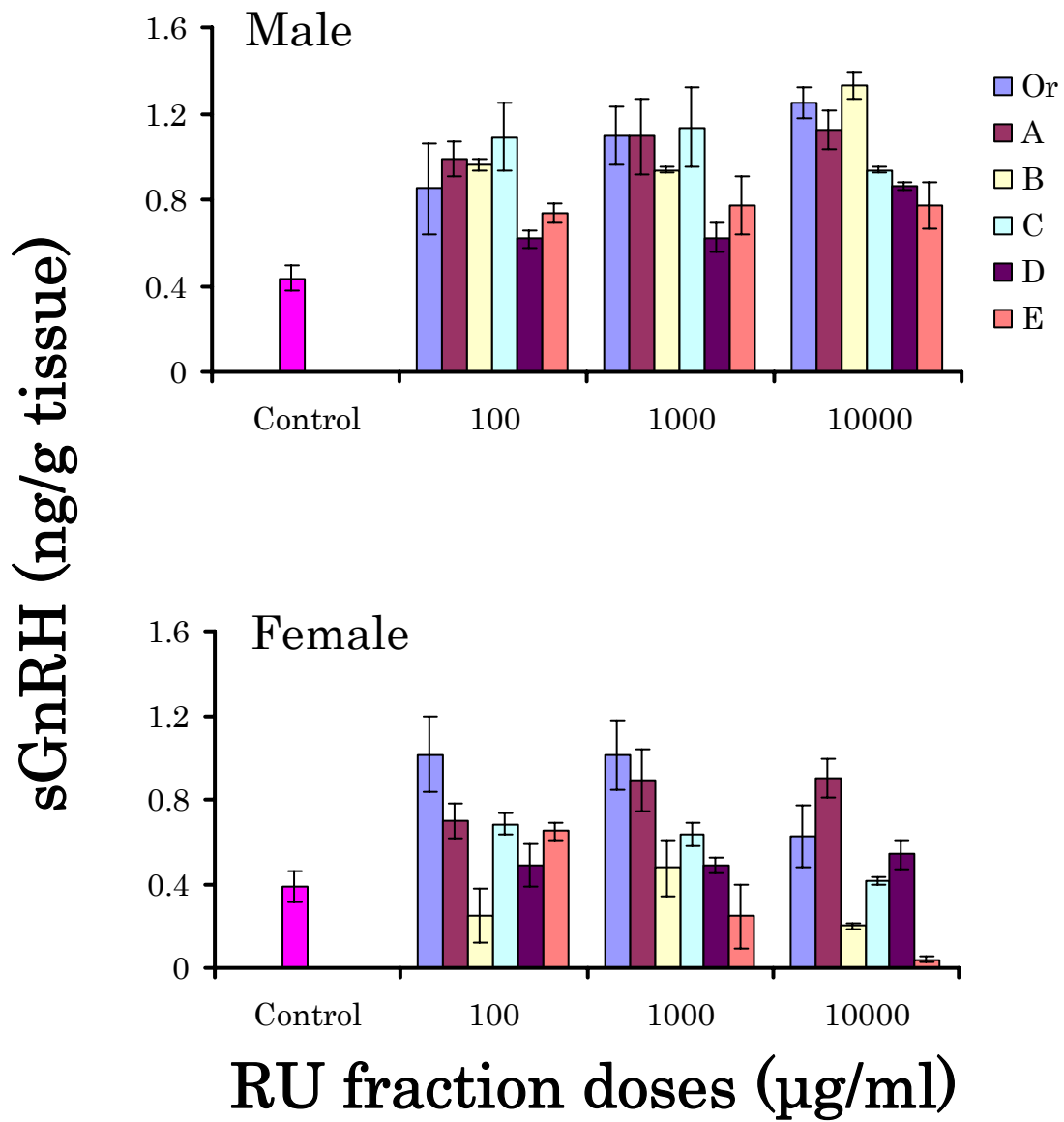


Fig. 18. Changes in the salmon gonadotropin-releasing hormone (sGnRH) concentrations in the hypothalamus (HYP) incubates of male and female masu salmon at different doses of RU fractions incubations for 18 hours. Results are expressed as means \pm SEM ($n = 3$).

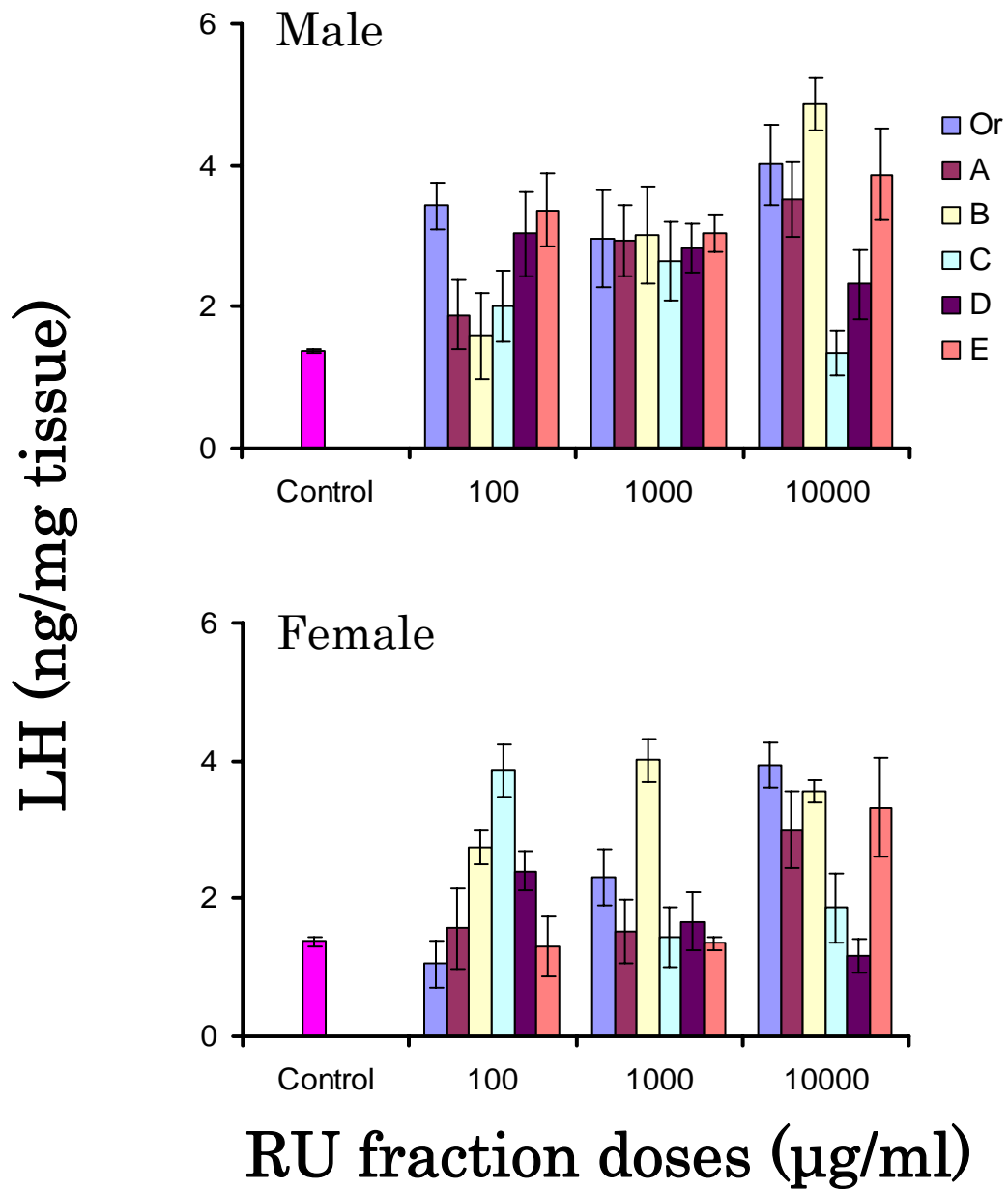


Fig. 19. Changes in the luteinizing hormone (LH) releases in the medium of pituitary incubates of male and female masu salmon at different doses of RU fractions incubations for 18 hours. Results are expressed as means \pm SEM ($n = 3$).

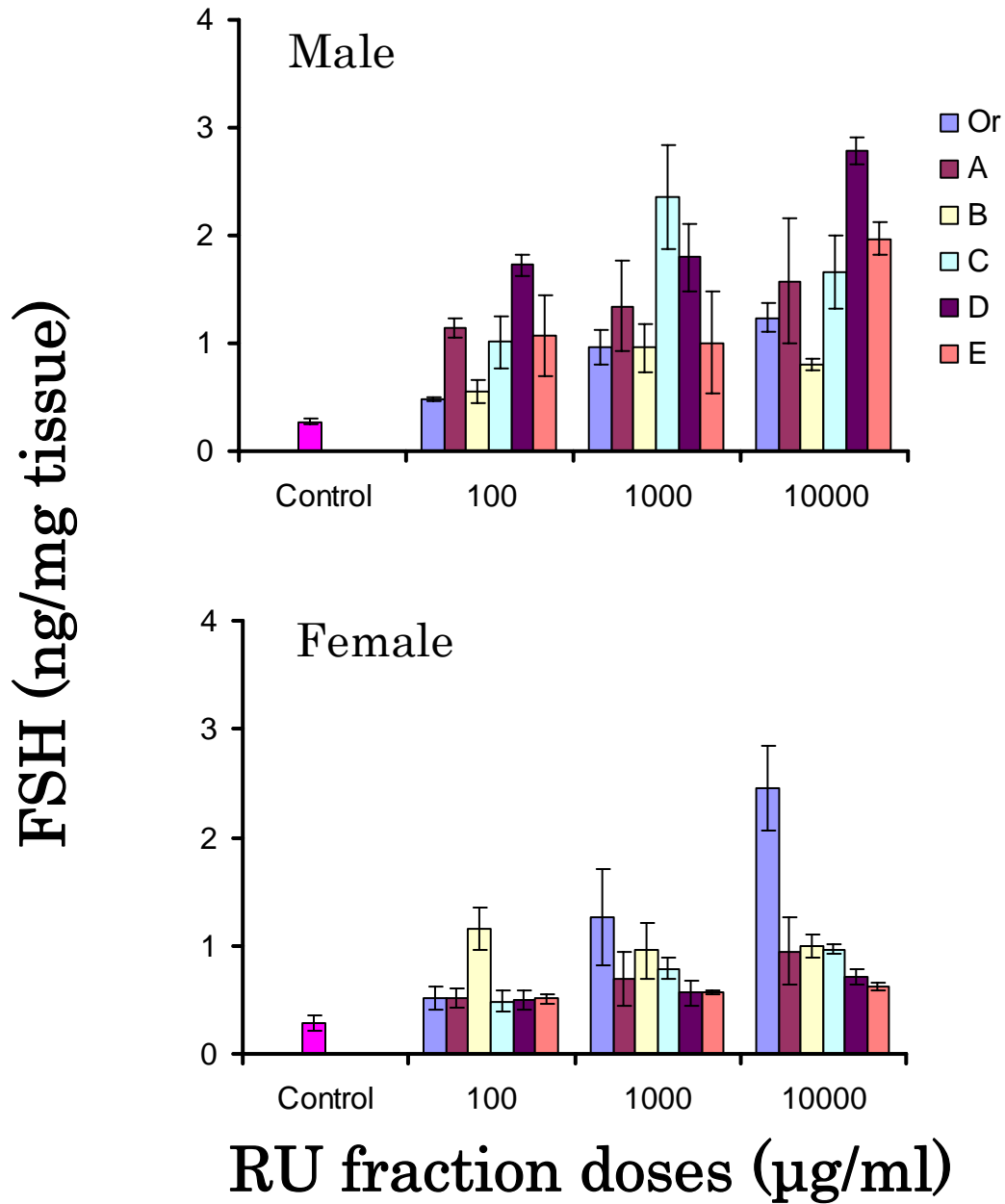


Fig. 20. Changes in the follicle-stimulating hormone (FSH) releases in the medium of pituitary incubates of male and female masu salmon at different doses of RU fractions incubations for 18 hours. Results are expressed as means \pm SEM ($n = 3$).

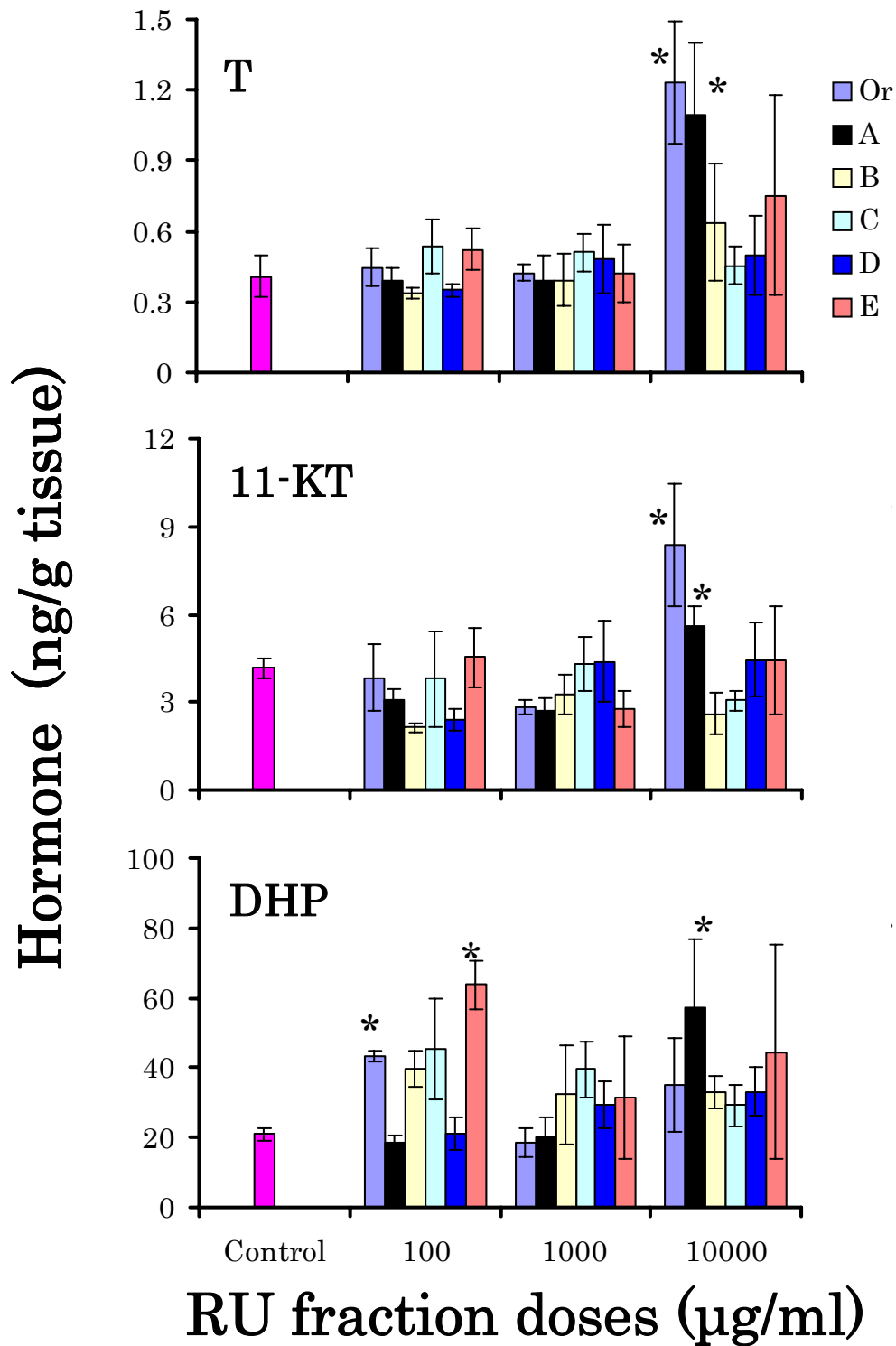


Fig. 21. Changes in testosterone (T), 11-ketotestosterone (11-KT), and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) releases in the medium of testis incubates of masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means \pm SEM. Asterisks indicate a significance difference between RU fractions incubates and control ($n = 3$). * $P < 0.05$.

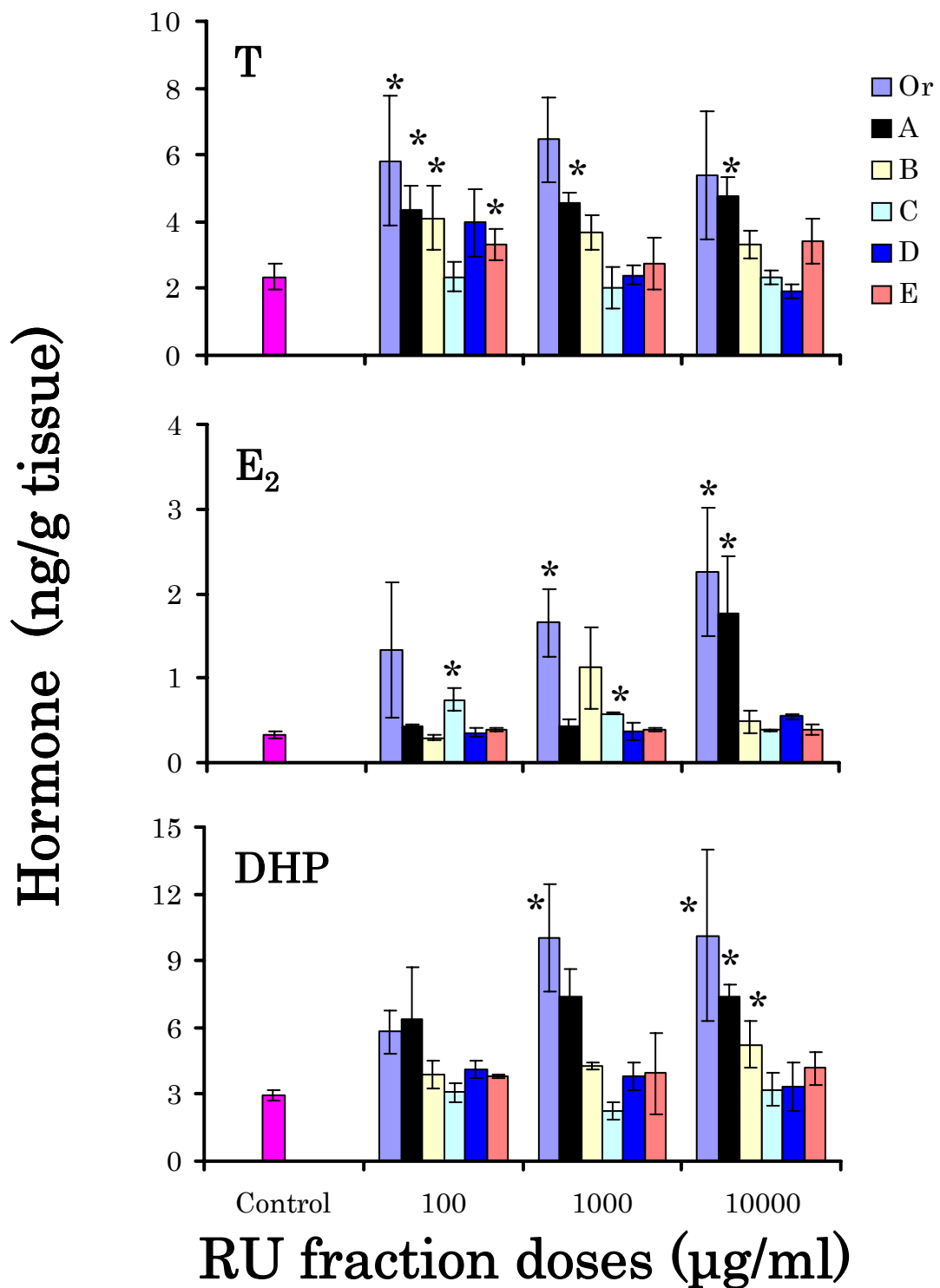


Fig. 22. Changes in testosterone (T), estradiol-17β (E₂), and 17α,20β-dihydroxy-4-regnen-3-one (DHP) releases in the medium of ovarian incubates of masu salmon at different doses of RU incubation for 18 hours. Results are expressed as means ± SEM. Asterisks indicate a significance difference between RU incubates and control (*n* = 3). **P* < 0.05.

Discussion

In the present study, dose-dependent and time course incubations of RU and its fractions were carried out on HYP, pituitary and gonadal tissues of maturing masu salmon at 2+ and 3+ to investigate the effect of RU and its fractions on the hormonal release and/or production of the formerly mentioned tissues. Dose-dependent RU and its fractions incubations of HYP and pituitary tissues significantly increased the hormonal releases and productions in the respective tissues at higher doses when compared to the control. In other words, the different RU fractions that are obtained by washing with different eluants had the capability to initiate hormonal releases from HYP and pituitary tissues, suggesting the presence of physiologically active substances in the eluates of RU fractions. The exact nature of these active substances present in RU could not be known by this study.

Like any other fungal species, RU is believed to contain physiologically active substances in their mycelium. One indication is that the enzyme glucoamylase, which readily converts starch into D-glucose, has been obtained from *Rhizopus delemar* in a high degree of purity by successive filtration and chromatography (Pazur and Okada, 1967). From *Rhizopus nigricans*, which is closely related to *Rhizopus delemar*, steroidogenic enzyme 17 β -HSD and progesterone receptors have been identified (Bavec et al., 2000). The steroid hormones T, androstenedione, progesterone and their metabolic products testosterone acetate and testololactone were also identified as endogenously synthesized molecules in the fungus *Pleurotus ostreatus* (Plementias et al., 1999). In addition, three components of the steroid hormone signalling system, 17 β -HSD, androgen binding proteins and steroid hormone signalling molecule T were determined in the filamentous fungus *Cochliobolus lunatus* (Zakelj-Mavric et al., 1995). It has also been shown that the physiologically active substances produced and/or contained by *Rhizopus* species, has various influences in the growth and reproductive function of many vertebrates. To this end, the physiologically active substances (T, androstenedione, progesterone and androgen binding proteins) that might be contained in the mycelium of *Rhizopus delemar* could be able to play an important role in potentiating the hormone signaling system of HYP and pituitary directly and indirectly.

As to the effects of exogenous steroids on brain and pituitary and gonad, many reports indicated that exogenous gonadal steroids accelerate the maturation of the

BPG axis in many teleost species (Xiong et al., 1994; Amano et al., 1997; Lee et al., 2004). Huggard and Habibi (1995) have reported a positive effect of T on the LH- β mRNA levels in perfused pituitary fragments of sexually mature fish. Similar effects of T were found on the LH β -subunit of black carp and rainbow trout (Gur et al., 1995; Rebers et al., 1997). Treatment with testosterone also activated the development of the hypothalamic GnRH system in African catfish, *Clarias gariepinus* (Cavaco et al., 1998; Dubois et al., 1998). Moreover, the ability of overnight *in vitro* T treatment to directly increase the efficacy of GnRH-stimulated LH response has been demonstrated in cultured goldfish pituitary cells prepared from females at all three stages of ovarian maturation (regressed, recrudescing, mature), as well as in cells from both males and females at sexually regressed and mature (pre-spawning) stages (Lo et al., 1995; Lo and Chang, 1998).

In mammals, steroid-positive feedback on gonadotrophs involves modulation of GnRH signal transduction components (Tobin et al., 1997). Furthermore, T has a stimulating effect on catfish GnRH (cfGnRH) immuno-reactivity in brain and pituitary in all three age groups (in pre-pubertal fish, at the onset of puberty and during later puberty) in African catfish (Dubois et al., 2000).

Taken together, the increased hormonal production in both the HYP and pituitary tissues by RU and its fractions incubations in this experiment may be explained by the direct involvement of the steroid hormones and enzymes contained in RU through modulation of the GnRH and GTH signal transduction component. Thus, these findings shall be cited as another possibility of direct effects of RU on sGnRH and GTHs production.

As it has been in the hormonal concentrations of HYP and pituitary tissues, higher doses of RU incubations of the gonads significantly increased the T, 11-KT, E₂ and DHP released in both sexes except females DHP in the gonad incubates. In addition, the significant increase in T, 11-KT, E₂ and DHP releases in the gonadal incubates was limited to a few RU fractions (RU fraction A and B) at higher doses. As mentioned in the materials and methods RU fraction A is supposed to contain water-soluble substances and water, while RU fraction B contains peptides, amino acids and fat as 80% MeOH-H₂O was used as an elute. Unfortunately, further fractionation, concentration and obtaining of the different peaks of these RU fractions were not made. At this stage, it would hard to pinpoint the exact bioactive substance

present in the eluates of RU fraction that brought the differences in the release of steroids.

Generally, the effects of RU and its fractions incubation might be due to the direct stimulation of the steroids in the gonadal incubate by the physiologically active substances contained in RU and its fractions. As discussed above, these physiologically active substances could be androstenedione, T, progesterone, androgen binding proteins and other unspecified steroids. Different steroid enzymes activators could be part of this stimulation processes. With regard to the effects of steroids in gonads, recent studies have shown that T, E₂ and 11-KT implantations in male African catfish has significantly increased the plasma levels of T, E₂ and 11-KT, respectively (Dubois et al., 2000).

In *in vitro* experiments, the administration of DHP, a final maturation inducing hormone of oocytes in salmonids (Nagahama, 1987), and 17-hydroxyprogesterone, a precursor of DHP, into the incubation medium induced final maturation of oocytes (Yamauchi and Yamamoto, 1982). Addition of DHP into the incubation medium induced germinal vesicle breakdown (GVBD) in the oocytes at the migratory nucleus stage (Ohta et al., 1997). Incubation of testis slices with oxytocin increases sperm concentration of the medium compared to the control incubations (Viveiros et al., 2003). Furthermore, *Rhizopus* incubation of rat gonads accelerated ovarian and testicular steroidogenesis upon pretreatment with steroids or gonadotropins (Seto et al., unpublished). Further *in vitro* studies are essential to confirm the reproducibility of RU fractions. It is essential to fractionate, concentrate and get different peaks of RU fractions A and B; and test their effects on HYP, pituitary and gonad tissues.

Chapter 2

Trends of hormonal changes in brain, pituitary and serum of chum salmon during homing migration

Introduction

The unique feature of anadromous salmonids is their precise ability to return to the natal river site for reproduction which is a biological process by which organisms create their descendants and ensures the survival of the young. Pacific salmonids spawn only once during each reproductive cycle and die after spawning. Homing behavior of salmonids is considered to be closely related to gonadal maturation (Ueda and Yamauchi, 1995; Sato et al., 1997) and regulated by neuroendocrine systems, mainly the BPG axis. Spawning migration of salmonids composed of traveling thousands of kilometers, rapid increase in gonadal maturation and migration behaviors such as the selection of their natal river, nest making and mating. In this regard, sGnRH is thought to play a central role in coordinating all the events in homing migration through hormone cascades. So, it seems that the activation of the GnRH system by different stimuli is the key event in the onset of puberty. The diverse functions of GnRHs include neuroendocrine, neurotransmitter, neuromodulator, autocrine and paracrine regulation (King and Millar, 1997; Okuzawa and Kobayashi, 1999). These diverse functions are possible due to the wide distribution sGnRH producing neurons in the brain as well as innervations of sGnRH immuno-reactive fibers into the pituitary (Amano et al., 1991). The GnRH system in fish shows species specific molecular types and localization in the brain.

Previous findings indicate that sGnRH neurons in the pre-optic area (POA) and ventral telencephalon (VT) are involved in gonadal maturation possibly by stimulating FSH and LH synthesis and release, whereas neurons in the olfactory bulbs and the terminal nerve (TN) ganglion may have different roles (Amano et al., 1995). TN GnRH neurons project their axon widely in the brain and GnRH released from TN controls the motivational state of various nervous actions in the animal, suggesting the neuromodulator actions of sGnRH (Ishizaki et al., 2004). Changes in sGnRH mRNA levels in the different brain loci have been investigated by *in situ* hybridization during the upstream migration of chum salmon (Kudo et al., 1996). They reported that strong signals of sGnRH mRNA in OB and olfactory nerve were revealed at coastal sea, but these signals were not observed at spawning ground. In contrast, sGnRH mRNA levels in POA increased at spawning ground. However, information on the peptide levels is limited during homing migration of chum salmon.

The next key gland that plays an important role in homing migration of chum salmon is the pituitary. It mediates the actions of brain on gonads by producing FSH and LH in salmonids (Sekine et al., 1989; Suzuki et al., 1998a). It has been clearly shown that FSH producing cells are located in close association with the somatotrophs, surrounding the nerve ramifications in the proximal pars distalis, while the FSH producing cells are more peripheral; the α subunit mRNA is found in both cell types (Naito et al., 1991). Studies on the pituitary contents and plasma levels (Suzuki et al., 1988b; Sumpter and Scott, 1989) of GTHs have clearly indicated that there is elevation of FSH levels during gonadal maturation, while the significant elevation of LH occurred at the final stage of sexual maturation. These studies are from a single spawning season. Extended data is scarcely available in chum salmon especially during homing migration.

On the other hand, there are several reports that investigated changes in serum steroid levels during spawning migration in salmonid fish (Ueda et al., 1984; Onuma et al., 2003). The function of these steroids during homing migration is also well described by many researchers as indicated in general introduction.

In teleosts, the roles of IGF-I in stimulating the BPG axis have been documented (Huang et al., 1998; Nordgarden et al., 2005). In salmonids, application of IGF-I and salmon GnRH concurrently to rainbow trout pituitary cells increased the FSH and LH releases (Weil et al., 1999). IGF-I is also thought to act as a signaling molecule that transmits growth and nutritional status to the gonadotropic axis at the onset of puberty as the plasma levels of IGF-I are highly correlated to growth rate in salmonids (Beckman et al., 2001; Campbell et al., 2003). However, no information is available on the serum profiles in chum salmon during homing migration.

In order to investigate the hormone profiles in the brain of chum salmon, sGnRH was measured from OB, TE, HYP and pituitary during homing migration for three consecutive years. sGnRH, FSH and LH hormones were also measured from pituitary and related to each other. Further, hormone profiles of serum T, 11-KT, E₂, DHP and IGF-I were investigated during homing migration of chum salmon, and correlations were made among peptides and steroids

Material and Methods

Field sampling

Each six to seven male and female adult chum salmon were sampled at seven points along their spawning migration from Bering Sea to spawning ground in 2003, 2004 and 2005. Chum salmon were sampled at Bering Sea from late-June to mid-July 2003, 2004 and 2005 during the cruise of research vessel *Wakatake Maru*. These fish were considered to be Japanese stocks and ready to return to some of the Japanese rivers in Hokkaido. Migrating chum salmon were also captured at offshore of Ishikari Bay, Ishikari Bay (coastal area), estuary of Ishikari river, branch point of the Chitose River from the Ishikari River, midstream of Chitose River (pre-spawning ground), and Chitose Branch of the National Salmon Resources Center (spawning ground) in September and October of the same years mentioned above (Fig. 23). Every year, millions of juveniles are released in this water system. Fish were caught by different fishing methods.

Blood and tissue collection

Fish were taken out of water and, immediately fork length and body weight were measured. Blood samples were collected from caudal vasculature, and kept on ice until centrifuged at 3000 rpm for 15 minutes to obtain serum samples, and then stored at -30°C until assayed. After blood collection, a gentle pressure was applied on the abdomen to check whether spermiation or ovulation has occurred or not. Upon decapitation, brains and pituitary were rapidly removed and brains were divided into three regions; OB, TE and HYP and immediately frozen at -80°C until assayed. Finally, gonads were dissected out and weighed. GSI was computed as indicated in chapter 1.1.

Extraction and measurement of serum steroids and IGF-I were carried out as described in chapter 1.1.

Extraction of sGnRH and GTHs

Salmon GnRH and GTHs from brain and pituitary were extracted according to the procedure of Okuzawa et al. (1990). In brief, the frozen HYP and pituitary tissues in 1 ml of 0.1 N HCl were homogenized by sonication, followed by addition of 0.1 ml 1 N NaOH. After centrifugation at 15,000 rpm for 30 min at 4°C the supernatant

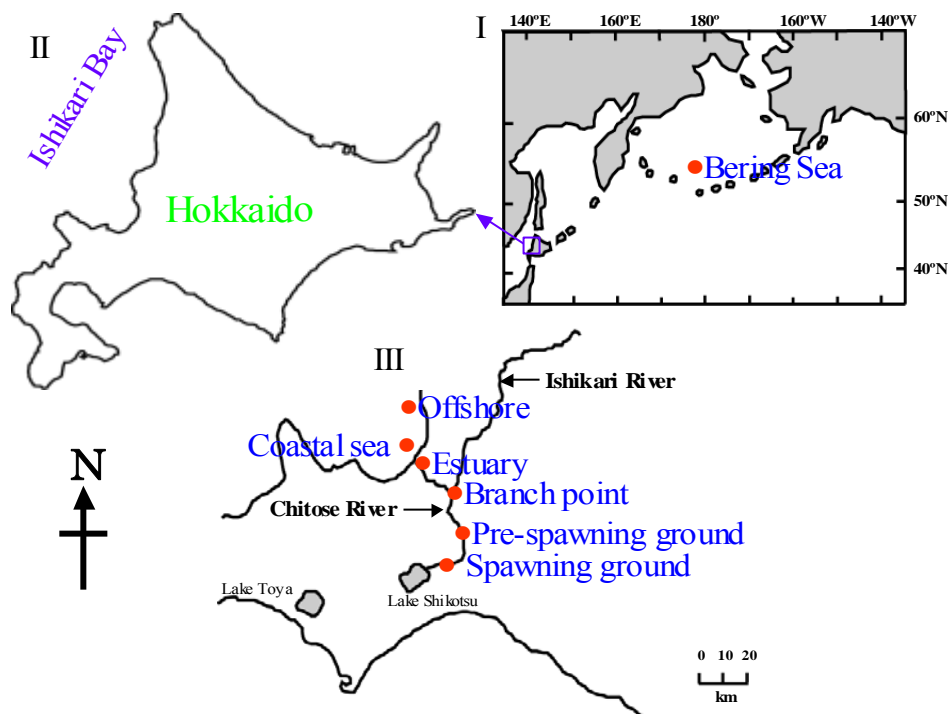


Fig. 23. Map of the North Pacific Ocean and the sampling sites (indicated in red circles with all the seven sampling points written next to it).

was lyophilized under vacuum, reconstituted in assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 μ M DTPA, 0.01% Tween 40, pH 7.2) for sGnRH. LH and FSH were extracted in the same way as described above, and were reconstituted in assay buffer (0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN₃, 0.01% Tween 40, 20 μ M DTPA, pH 7.75) for GTHs. The reconstituted samples were frozen at -30°C until assayed. The protocols for sGnRH and GTHs assay in the brain and pituitary followed those developed by Yamada et al. (2002). The rest of these assay procedures are similar to the one described in chapter 1.2. The intra- and inter-assay coefficients of variation were 3.6% ($n = 18$) and 6.1% ($n = 6$), respectively, at about 50% binding. Displacement curves of serially two fold-diluted brain extracts were parallel with the sGnRH standard curves (data not shown). In addition, intra-assay coefficients of variation were 4.8% ($n = 18$) and 6.8% ($n = 18$), for FSH and LH, respectively, whereas inter-assay coefficients of variation were 8.7% ($n = 6$) and 8.3% ($n = 6$) at about 50% binding. Displacement curves of serially two fold-diluted brain extracts were parallel with the LH and FSH standard curves.

Data Analysis

All the data are expressed as means \pm SEM. To assess significant differences among sampling points within the same year, data were subjected to one-way ANOVA analysis followed by Fisher's PLSD test. Correlations (correlation coefficient, r) among different parameters were calculated by Pearson's correlation test.

Results

Salmon GnRH levels in the olfactory bulb and telencephalon

In both sexes of all years examined, levels of sGnRH in the OB and TE were increased at offshore as the homing migration advances and decreased or remained constant at coastal sea, and again increased at estuary or branch point and decreased finally (Fig. 24). Since we missed sampling at estuary and branch point for 2005, the apparently high sGnRH levels observed in OB and TE at branch point for this year may be misleading. Peak levels of sGnRH were observed at estuary or branch point in both sexes as this is clearly seen in 2003 and 2004, and these levels were significant

when compared to the rest of sampling points. In all years examined, sGnRH levels in OB and TE showed similar patterns of changes in both sexes and sGnRH levels in TE tended to be higher than in OB. However, the absolute amounts of sGnRH in OB and TE varied among the years at some of the sampling points in both sexes. Absolute amounts of OB and TE sGnRH were higher at Bering Sea and offshore in 2003, whereas, in 2004, these values were higher at coastal sea, estuary and branch point. In 2003 males, levels of sGnRH at Bering Sea were more or less similar to offshore and coastal sea, and this may indicate the involvement of releasing hormones in the homing behavior of chum salmon.

Salmon GnRH in the hypothalamus

In both sexes of all years examined, levels of sGnRH in the HYP were increased at offshore during homing migration and decreased or remained constant at coastal sea and again increased to peak levels at estuary (Fig. 25). Peak levels of sGnRH were observed at estuary or branch point for both sexes as clearly shown in 2003 and 2004. These levels were significant when compared to the rest of sampling points. One exception is that in 2003 males, sGnRH levels at Bering Sea were comparable with those values at offshore and coastal sea and peak levels were observed at branch point. This may probably indicate the earlier of initiation of homing migration and the involvement of BPG axis in migration behavior of chum salmon.

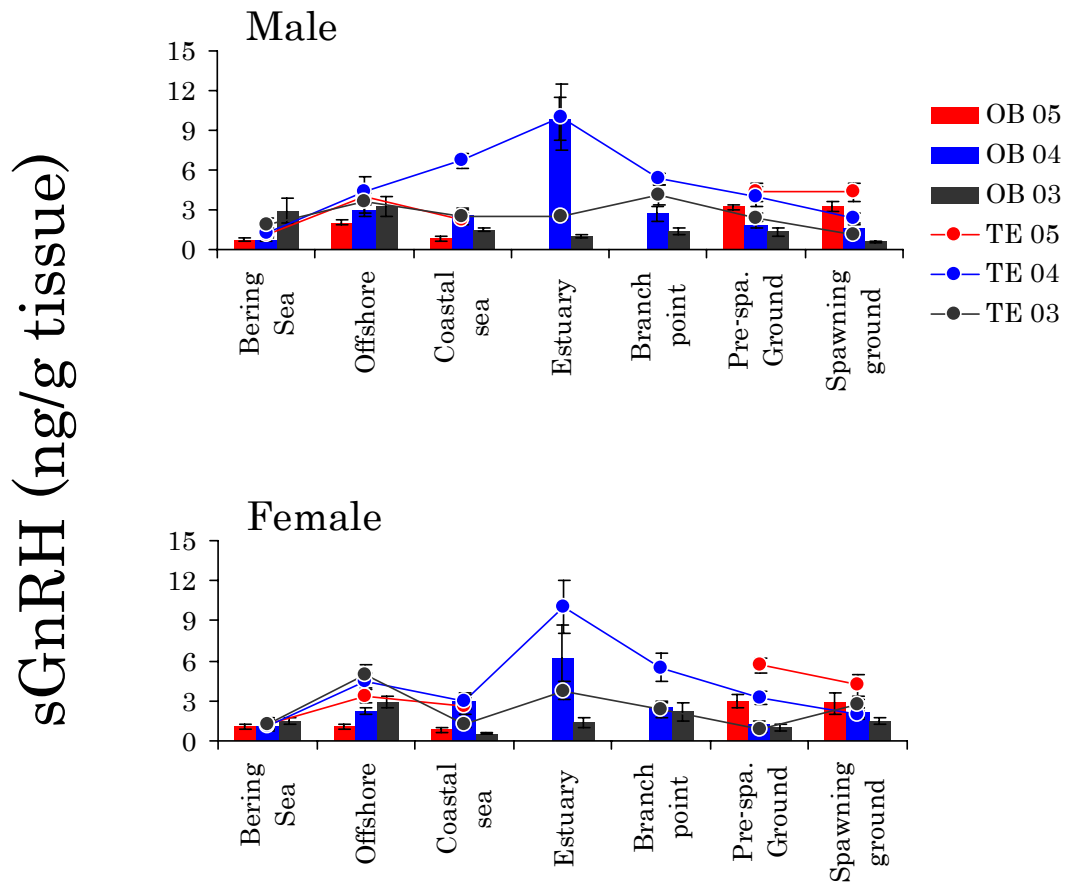


Fig. 24. Changes in salmon gonadotropin-releasing hormone (sGnRH) levels in olfactory bulb (OB) [bar] and telencephalon (TE) [line] of male and female chum salmon during homing migration from 2003 to 2005. Results are expressed as means \pm SEM ($n = 3-8$).

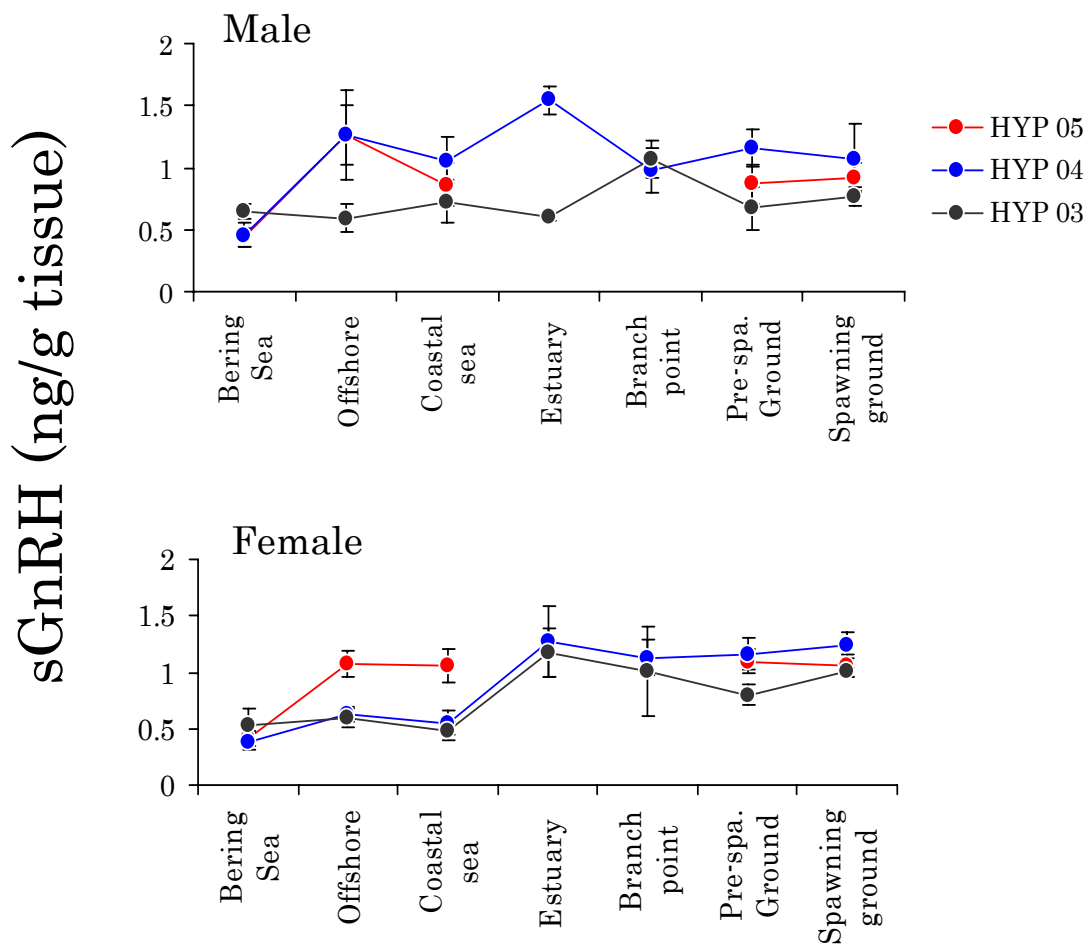


Fig. 25. Changes in salmon gonadotropin-releasing hormone (sGnRH) levels in hypothalamus (HYP) of male and female chum salmon during homing migration from 2003 to 2005. Results are expressed as means \pm SEM ($n = 3-8$).

Salmon GnRH and LH levels in the pituitary

Fig. 26 shows sGnRH (bar graphs) and LH (line graphs) levels in the pituitary during homing migration. Generally, in both sexes of all years examined, sGnRH and LH levels showed increased levels from Bering Sea to offshore, remained constant or decreased at coastal sea, and increased at estuary and branch point. In 2003 and 2004 males, peak levels sGnRH were observed at offshore, whereas LH levels peaked at pre- and/or spawning ground. In 2003 and 2004 females, both sGnRH and LH peaked at estuary. Generally, in both sexes of 2003 and 2004, sGnRH and LH in pituitary showed a decreasing and an increasing tendency, respectively towards the spawning ground. It is difficult to predict the peak levels of sGnRH and LH in pituitary for 2005 as the data lacks two sampling points (estuary and branch point). In 2005 of both sexes, sGnRH and LH levels in pituitary showed an increasing tendency at spawning ground. The absolute amounts of sGnRH and LH in 2003 were higher than those in 2004 and 2005 at all sampling points except some irregularities at pre-and spawning ground. In all years examined, LH levels showed similar pattern of changes with sGnRH and there was good relationship between them except in 2004 males. In all years examined, the peak levels of sGnRH and LH were statistically significant when compared to those values at Bering Sea.

FSH levels in the pituitary

The pattern of FSH changes in pituitary was shown in fig. 27 for both sexes. Compared to other hormones, changes in FSH levels were variable among the years. In 2005 of both sexes, FSH levels showed increased levels at offshore, then decreased at coastal sea, and again showed apparent increase at the pre-and spawning ground, although nothing is known for sampling points at estuary and branch point. For both sexes in 2004, FSH levels showed a minor increase till branch point and decreased at pre- and spawning ground. In 2003 males, FSH levels increased at offshore and remained constant between coastal sea and branch point and finally decreased at pre- and spawning ground, whereas in females, consistent increase in FSH were observed till coastal sea and remained fairly constant at estuary and sharply decreased afterwards. Oceanic environment might have contributed for this variation.

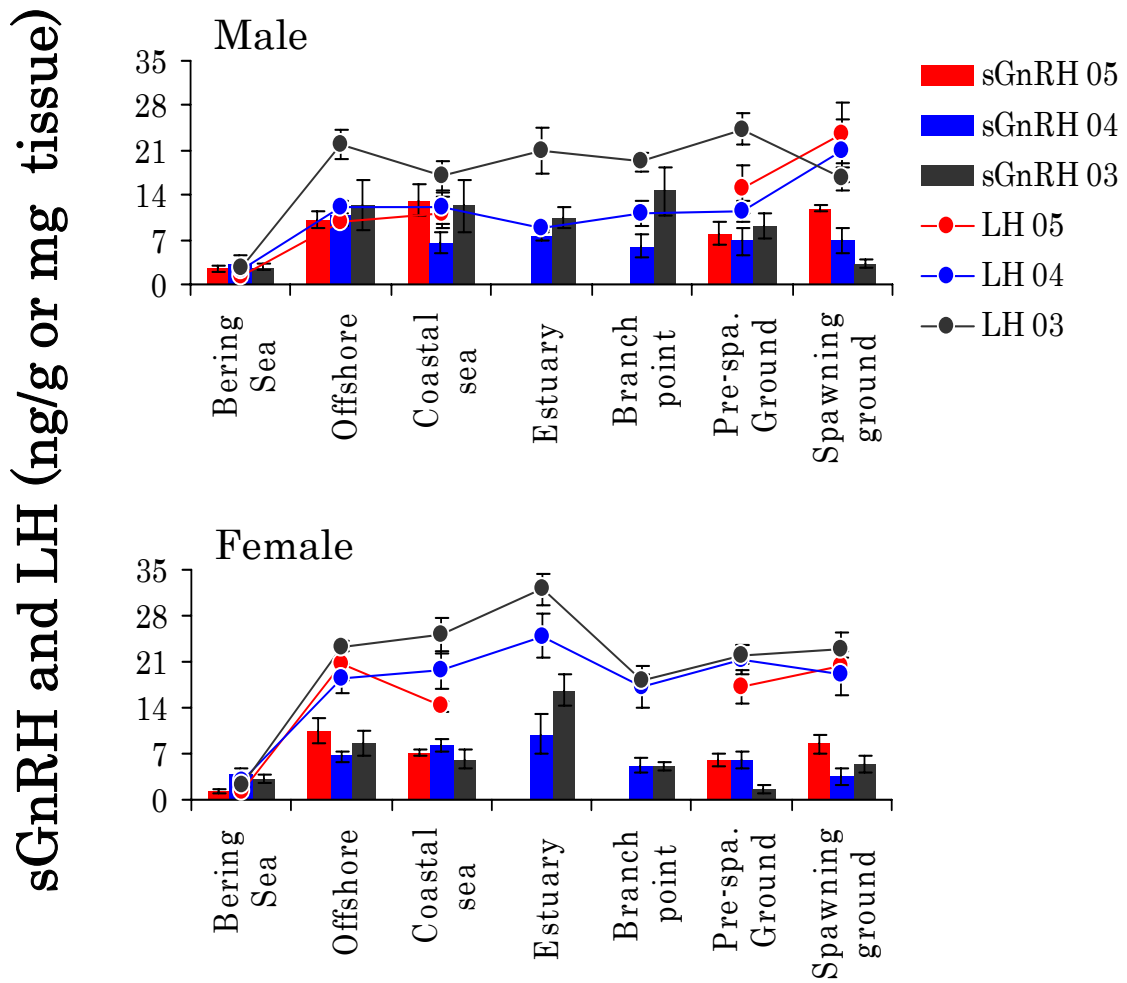


Fig. 26. Changes in salmon gonadotropin-releasing hormone (sGnRH) [bar] and luteinizing hormone (LH) [line] levels in pituitary of male and female chum salmon during homing migration from 2003 to 2005. Results are expressed as means \pm SEM ($n = 3-8$).

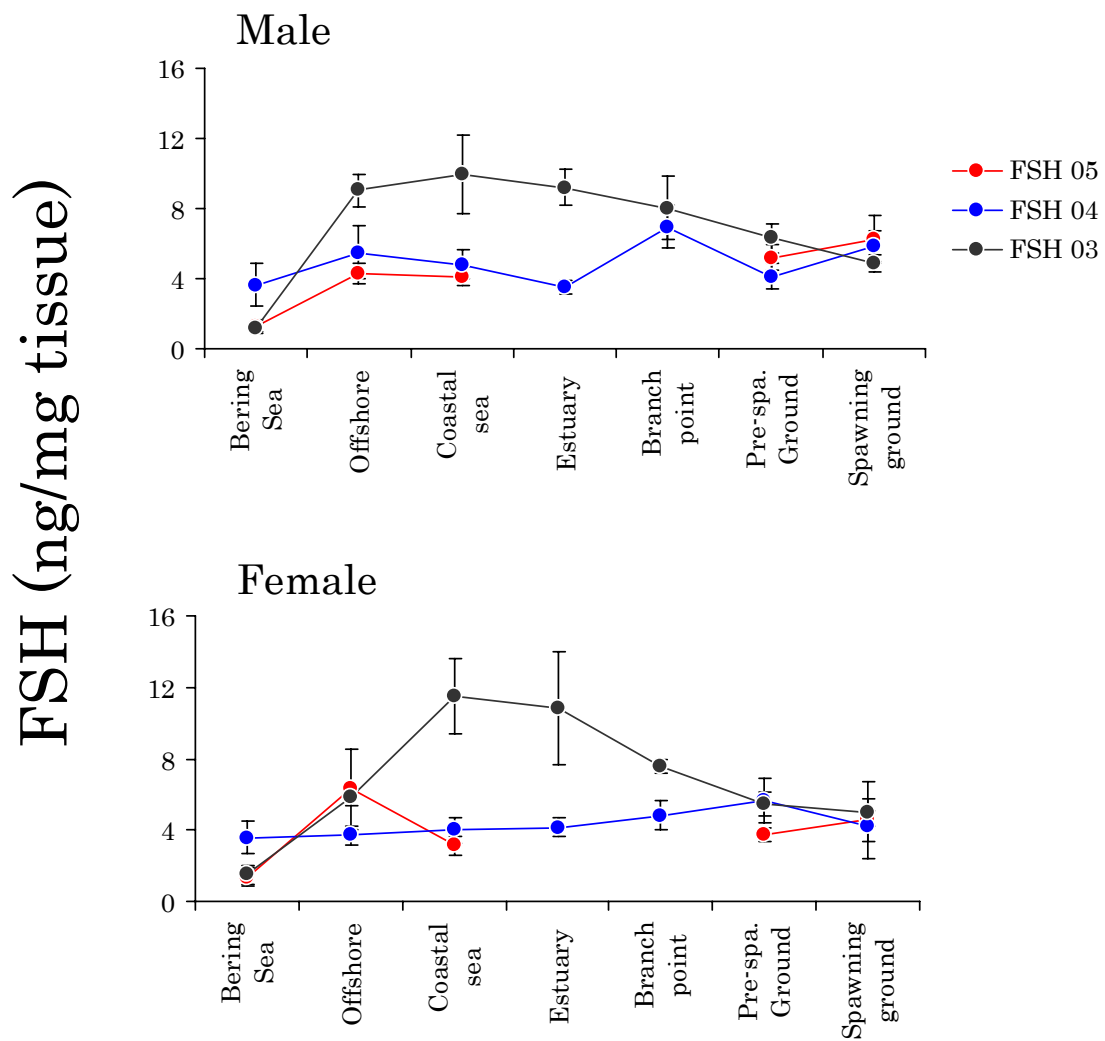


Fig. 27. Changes in follicle-stimulating hormone (FSH) levels in pituitary of male and female chum salmon during homing migration from 2003 to 2005. Results are expressed as means \pm SEM ($n = 3-8$).

Gonadosomatic index

GSI in both sexes were at comparable values and showed similar pattern of changes among the years examined (Fig. 28). In males, GSI values increased from Bering Sea to Estuary where the maximum was observed and decreased afterwards during upstream migration, whereas, in females, GSI values showed a sharp increase from Bering Sea to offshore, then gradually increased to spawning ground during homing migration. Development of secondary sex characteristics (nuptial color) were already evident. These data may indicate that the completion of spermatogenesis and vitellogenesis and success of final spawning.

Serum steroid hormones

In both sexes, serum T contents and pattern of changes were more or less similar among the years (Figs. 29 and 30). Serum levels of T in both sexes increased consistently and peaked at estuary, and showed a gradual decrease till pre-spawning ground and a sharp decrease at spawning ground for 2003 and 2004. Like 2003 and 2004, serum T levels in 2005 showed similar pattern of changes, but since sampling was not carried out at estuary, pinpointing of the peak levels for this year could not be made. Unlike the other sampling points, T levels at estuary and branch point in 2003 were low as compared to 2004 and 2005. In both sexes of all years examined, T levels at peak points were significant as compared to the other sampling points.

Like T, serum 11-KT levels of males were consistently increased and showed peak levels at estuary and branch point for 2003 and 2004, respectively, and decreased intermittently at pre-spawning ground and increased slightly at spawning ground (Fig. 29). In females, serum 11-KT levels were about 15 times lower than those in males. 11-KT levels in females showed considerable increase at offshore and gradually increased with advancement in upstream migration for 2003 and 2005 (Fig. 30). Unlike in 2003 and 2005, 2004 females attained peak 11-KT levels at branch point, and then decreased during further upstream migration to the hatchery. The absolute amounts of E_2 in female were about 20 times higher than those values in males (Figs. 29 and 30). In both sexes, serum E_2 levels were consistently increased and reached to peak levels at estuary and branch point for 2004 and 2003, respectively and then dropped off precipitously afterwards. E_2 levels in 2005 apparently showed similar pattern of changes as in 2004, although the data lacks samples from estuary. In both sexes of all years examined, serum E_2 levels at peak points were significant and also

showed a sharp decrease thereafter. Absolute amounts of E₂ were low at branch point in 2003 when compared to 2004 and 2005.

Serum DHP levels in both sexes of all years were low till branch point and showed a surge increase in the pre- and spawning ground (Figs. 29 and 30). One exception was that serum DHP levels in 2003 females were low at pre-spawning ground. Peak levels of DHP were observed at spawning ground in both sexes of all years examined and these levels were statistically significant when compared to other sampling points except the pre-spawning ground. Serum DHP levels of females were much higher than males (5-fold increase). The absolute amounts of serum DHP were variable among years at pre- and spawning ground where very low value was observed at pre-spawning ground in 2003 females, and a relatively low value was seen at spawning ground in 2004 males.

Serum IGF-I levels

In all years examined, serum IGF-I levels of males greatly increased from Bering Sea to offshore, peaked here and started to show a minor decrease towards branch point and finally showed a sharp decrease at pre- and spawning ground (Fig. 31). In males, serum IGF-I levels showed similar pattern of changes among the years. The high IGF-I levels at offshore (peak), coastal sea and estuary were significantly higher than those values at Bering Sea, pre- and spawning ground.

Generally IGF-I levels in females were high at Bering Sea, and the absolute amounts were more or less comparable to each other among the years examined (Fig. 31). Sharp decreases in serum IGF-I levels were observed at pre- and spawning ground. The higher IGF-I levels observed at Bering Sea, offshore, coastal sea, estuary and branch point were significantly higher than pre-spawning and spawning ground. Though the physiological implication is not clear, serum IGF-I levels in females showed low contents and different patterns of changes at Bering Sea as compared to males during homing migration.

There was a high positive relationship between serum IGF-I and 11-KT for males and serum IGF-I and E₂ levels for females. There was a positive correlation of serum IGF-I levels with GSI for males, and with HSI for females (data not shown). IGF-I was also positively correlated to condition factor in both sexes (data not shown).

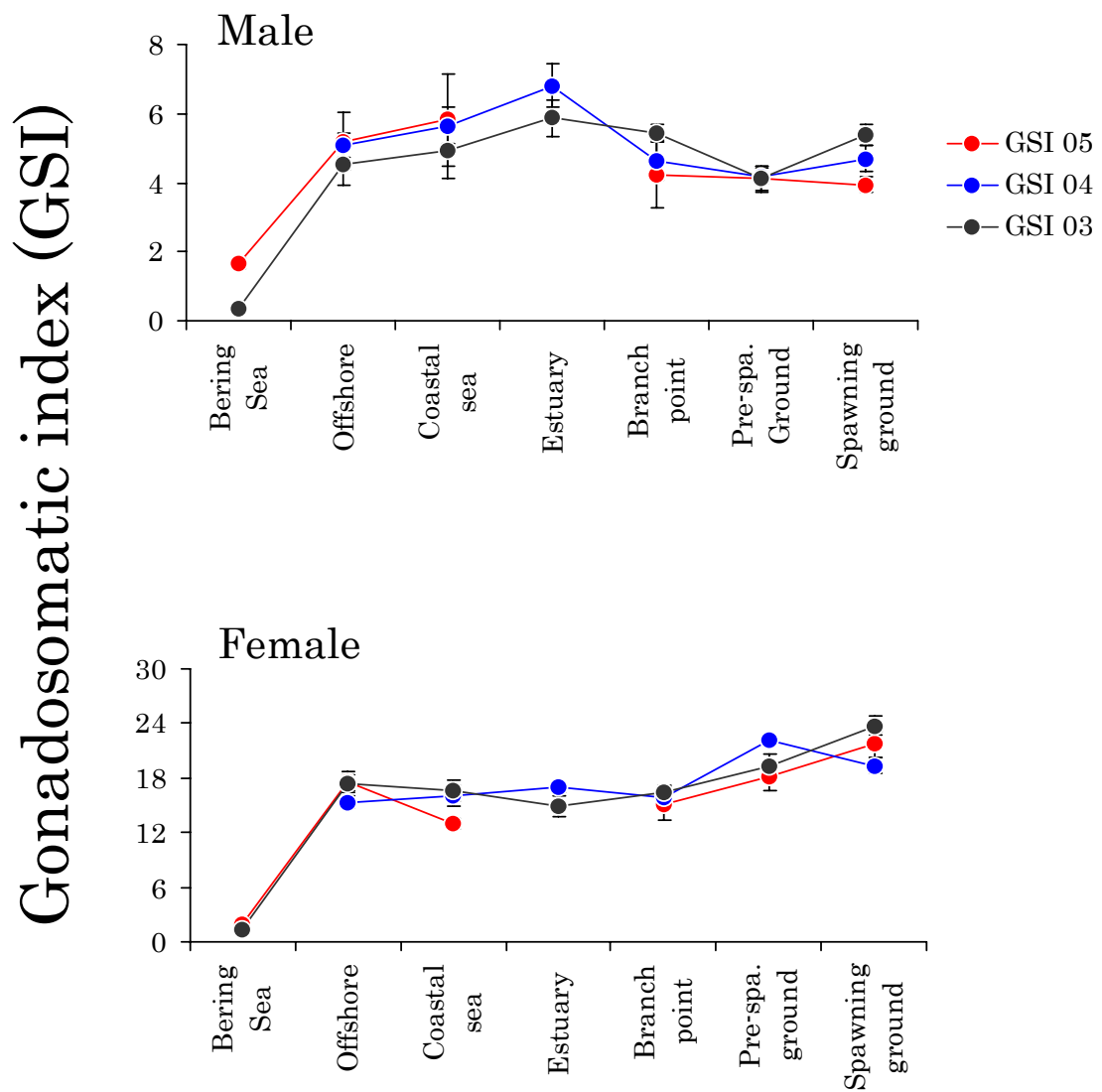


Fig. 28. Changes in gonadosomatic index (GSI) of male and female chum salmon during homing migration from 2003 to 2005. Results are expressed as means \pm SEM ($n = 10$).

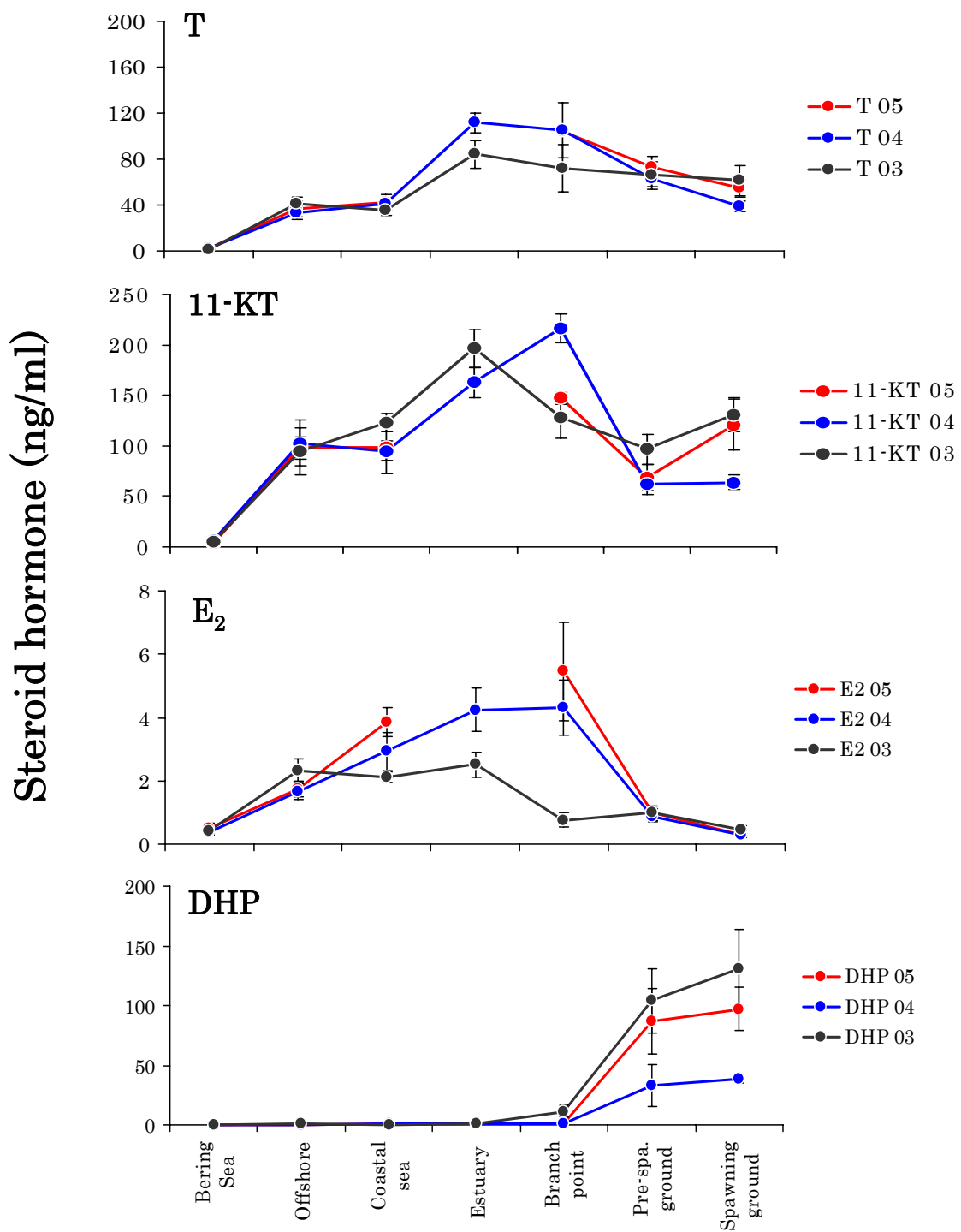


Fig. 29. Changes in serum testosterone (T), 11-ketotestosterone (11-KT), estradiol-17β (E₂), and 17α,20β-dihydroxy-4-regnen-3-one (DHP) levels of male chum salmon during homing migration from 2003 to 2005. Results are expressed as means ± SEM (*n* = 3-8).

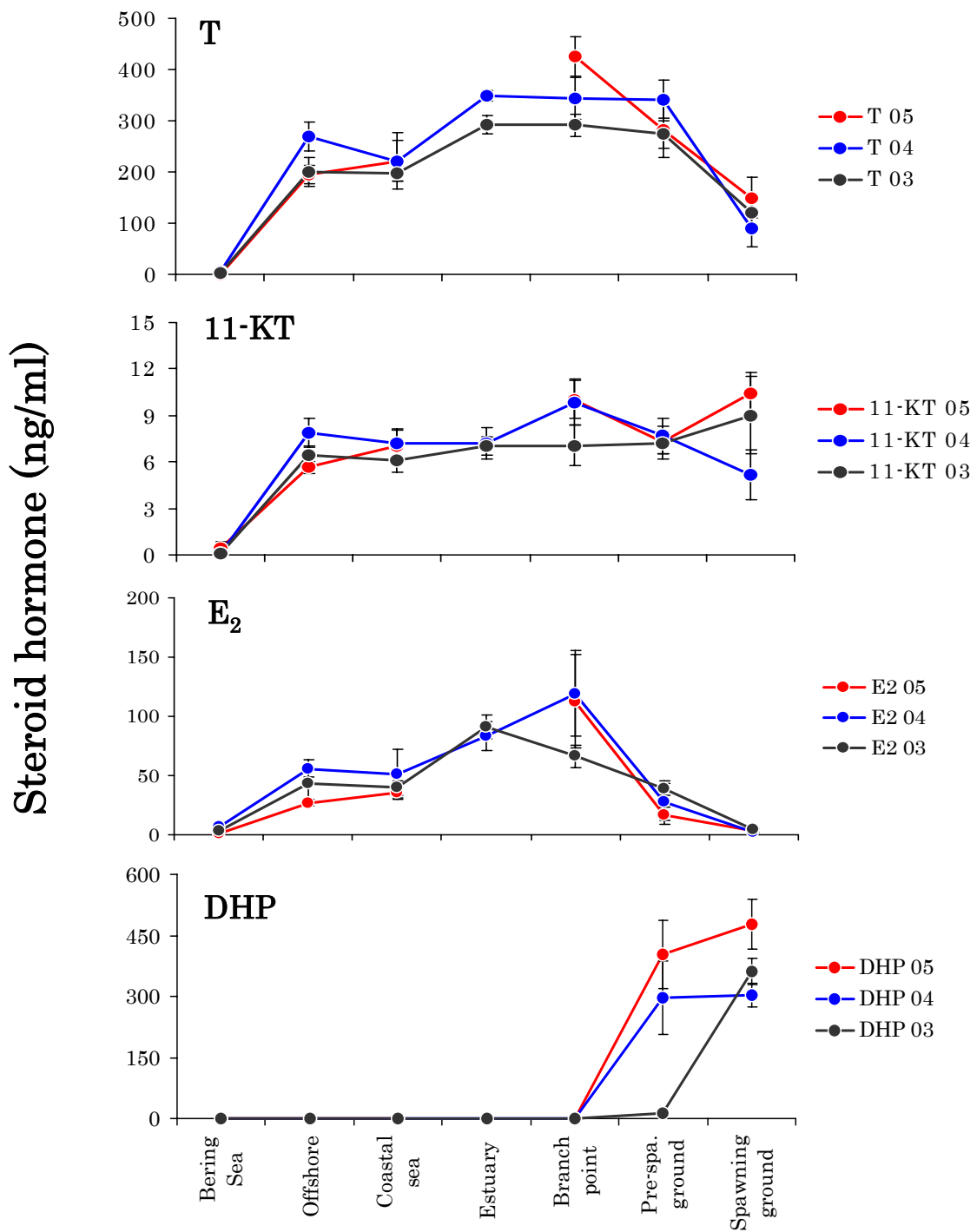


Fig. 30. Changes in serum testosterone (T), 11-ketotestosterone (11-KT), estradiol-17β (E₂), and 17α,20β-dihydroxy-4-regnen-3-one (DHP) levels of female chum salmon during homing migration from 2003 to 2005. Results are expressed as means ± SEM (*n* = 3-8).

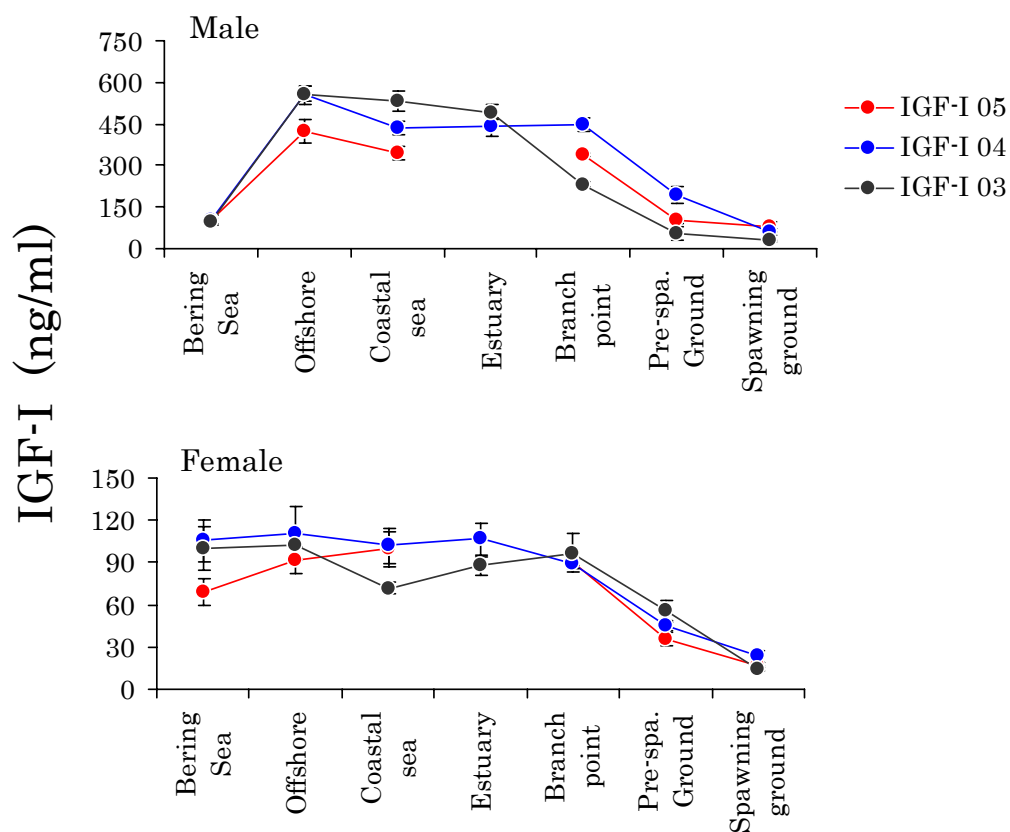


Fig. 31. Changes in serum insulin-like growth factor-I (IGF-I) levels of male and female chum salmon during homing migration from 2003 to 2005. Results are expressed as means \pm SEM ($n = 3-8$).

Discussion

The present study investigated the hormonal changes in brain, pituitary and serum of chum salmon in both sexes during homing migration for three consecutive years. In search of the basic neuroendocrine event occurring in naturally spawning fish, accumulation of qualitative and quantitative information over several years is needed, because gonadal maturation of fish is mostly affected by many factors and it has been found to vary from year to year particularly under the influence of oceanic temperature (Saito et al., 2001; Onuma et al., 2003).

In this study, sGnRH levels in OB and TE consistently increased at offshore and peaked at estuary or branch point in both sexes. sGnRH levels in OB and TE showed similar pattern of changes but the absolute amounts in TE constantly tended to be higher. Many reports are in line with these findings (Kudo et al., 1996; Ando et al., 2001; Kitani, 2003). Onuma et al. (2005) reported that the amounts of sGnRH mRNAs in the OB and TN elevated from the coast to the natal hatchery in chum salmon during upstream migration, whereas, Kudo et al. (1996) reported that strong signals of sGnRH mRNA in OB and olfactory nerve were observed at coastal sea but not at the spawning ground. sGnRH mRNA expression in OB increases during upstream migration of chum salmon (Parhar et al., 1994) and sGnRH mRNA expression was found to be high when the fish were residing in the estuary of their natal river and decreased as the fish went up the river to spawn (Kudo et al., 1994).

GnRH neurons in the OB and TN are not essential to gonadal development but function; instead as a neuromodulatory function is suggested (Oka, 1992; Kobayashi et al., 1994). In salmonids, it is believed that the olfactory and sensory systems could have important roles for the selection of natal river system during upstream migration. Fibers of sGnRH neurons in transitional area between olfactory nerve and OB project to the olfactory epithelium and the forebrain (Nevitt et al., 1995; Kudo et al., 1996). They were also localized in the retina (Zucker and Dowling, 1987; Wirsig-Wiechmann and Oka, 2002). Increased excitability of olfactory receptor neurons (Eisthen et al., 2000) and retinal cells (Umino and Dowling, 1991) were observed when sGnRH was applied to them. Therefore, the consistent elevation sGnRH peptides in our data may indicate that sGnRH hormone in the OB and TN could be involved in control of migratory and spawning behavior. This notion was clearly observed in pre-spawning chum salmon. GnRH administration of pre-spawning chum

stimulated preferences of the fish to fresh water, and shortened homing duration from river mouth to natal site (Kitahashi et al., 2001).

The region of TE is large and includes TN, and the transition areas between OB and TN, and between TN and POA. Besides, part of POA might be included in TE as clear anatomical identification of the TN- and POA-sGnRH system is difficult during cutting or isolation of these tissues in salmonids, since the GnRH neurons located in the ventral forebrain are consecutive and the GnRH form produced in these neurons is the same (Amano et al., 2003). So, such changes in the levels of sGnRH in TE might be brought by the activation of TN sGnRH and other neurons. Amano et al. (2003) reported increases in sGnRH mRNA levels in the VT and POA during gonadal maturation in masu salmon, suggesting the possible involvement of these areas in gonadal maturation.

In both sexes of all years examined, levels of sGnRH in the HYP were increased mainly at offshore during homing migration and showed peak levels at peak levels at estuary and/or branch point. Absolute amounts of sGnRH remained relatively high at pre- and spawning ground. In 2003, exceptionally high levels of sGnRH in HYP were observed at Bering Sea and they were comparable with those values at offshore and coastal sea. This may probably indicate the earlier of initiation of homing migration and the involvement of BPG axis in homing migration of chum salmon. In teleosts, sGnRH neurons in the VT and preopticus parvocellularis anterioris (PPa) are known as the hypophysiotropic GnRH neurons as these neurons project (send fibers) to the pituitary (Yamamoto et al., 1998; Lethimonier et al., 2004). Examination of GnRH content in the brain and pituitary of masu salmon from hatching until gonadal maturation revealed that sGnRH content in the TE (including the POA) and the pituitary increased in parallel with gonadal maturation, suggesting sGnRH produced in these regions stimulates GTH secretion for gonadal maturation (Amano et al., 1992, 1995). As opposed to this, sGnRH content of HYP tended to decrease with testicular maturation and ovulation in rainbow trout (Yamada et al., 2002) and goldfish (Yu et al., 1991). These results indicate that sGnRH contents in different brain regions may vary among species. In chum salmon, strong sGnRH immunoreactivity and hybridization signals have been consistently revealed from neurons in the OB, TE and POA, but the hybridization signals of sGnRH in TE and POA were much stronger in fish from the spawning ground than in those from the coastal sea (Kudo et al., 1996). Generally, considering the above results on gene

expression levels at different brain regions during upstream migration and gonadal maturation, the increases in peptide (sGnRH) levels in OB, TE and HYP of all years examined may indicate that neurons in these regions are associated with control of reproductive phenomenon in migrating chum salmon.

In both sexes of all years examined, sGnRH and LH levels showed increased levels from Bering Sea to offshore, remained fairly constant or decreased at coastal sea, and peaked at estuary as clearly seen in 2003 and 2004 females. Relatively high levels of sGnRH and LH levels were also at pre- and spawning ground. Besides, LH levels showed similar pattern of changes with sGnRH in all years examined and there was good relationship between them except for 2004 males, indicating the activation of LH synthesis and release in pituitary by sGnRH. The sex differences in peak levels of GnRH and LH are not clear. This may be due to the differences in the relative state of maturity of gonads and osmolality which influence some process in gonadal maturation. There is a lot of information on the sGnRH and LH peptide and gene levels in pituitary in salmonids and other teleosts (Schulz et al., 1997; Dickey and Swanson, 2000; Gur et al., 2001). sGnRH contents in the pituitary were higher in 3+ mature fish when compared to 1+ immature fish (Okuzawa et al., 1990; Yamada et al., 2002). Injection of goldfish with sGnRH stimulates FSH, LH and LH- β mRNA levels after 24 hours, and *in vitro* incubation of pituitary fragments with sGnRH continuously stimulated FSH- and LH-mRNA levels for 12 hours (Klausen et al., 2001). In salmonids, GnRH regulates biosynthesis and release of GTHs differently, depending on the reproductive stages (Ando and Urano, 2005).

Generally, FSH levels showed increased from offshore to estuary and showed a decreasing tendency at pre- and spawning ground. In 2003, absolute amounts of pituitary FSH at coastal sea and estuary were higher than those in 2004. In chum salmon, major gonadal development and gametogenesis occurs before pre-spawning ground. Our results agree with the previous suggestions that FSH, but not LH, is involved in regulating early stages of gonad development and spermatogenesis as it is detectable in pituitary, and then plasma in salmonids (Nozaki et al., 1990; Amano et al., 1993; Koyabashi et al., 1997). In contrast, LH is not detectable in plasma until final oocyte maturation in females and production of mature spermatozoa in males (Prat et al., 1996; Mylonas et al., 1997; Davies et al., 1999). Thus, our results support the notion that FSH is involved in early gonadal maturation in fish, possibly acting via the stimulation of androgen and/or estrogen production and other mechanisms. That

is why FSH levels in 2004 of this study at Bering Sea showed comparable values with the rest sampling points and there was a good relationship between pituitary FSH and serum androgens.

Generally, for most of the years examined, there was a high correlation between sGnRH in brain regions and serum steroids in both sexes. Serum androgens and E₂ were related to sGnRH levels in TE, HYP and pituitary ($r > 0.55$), indicating the involvement of steroid hormones in the homing migration of chum salmon. In this regard, intraperitoneal injection of T causes increase of sGnRH mRNA levels in TN ganglion in tilapia (Soga et al., 1998). Implantation of T, 11-KT, or E₂ induces upstream migratory behavior in masu salmon (Munakata et al., 2001). Positive and negative feedback effects of steroids on HYP and pituitary is a well established fact. Further studies are essential to clarify such complex relationships.

In males of all years examined, GSI values increased from Bering Sea to estuary of the natal river and decreased thereafter, whereas in females it showed a consistent increase from Bering Sea to spawning ground of the natal river during homing migration of chum salmon. These results are consistent with the previous data that GSI values are near the maximum at the midway of the river in males, but in females GSI levels increase gradually and reach to a peak level at spawning ground during upstream migration of chum salmon (Mastumoto, 2002; Onuma et al., 2003). Sharp increases from Bering Sea to offshore may indicate the initiation of homing at certain stage of feeding migration which is associated with gonadal maturation. In all years of our study, there was successful maturation of gonads and spawning.

In this study, serum levels of steroid hormones showed a minor difference among the years and all hormone levels measured varied within certain ranges during homing migration of chum salmon. Serum T, 11-KT and E₂ showed a consistent increase till estuary and branch point and decreased thereafter. As expected, serum contents of 11-KT (< 11 ng/ml) and E₂ (< 6 ng/ml) were low in females and males, respectively. The maturation inducing hormone, DHP, remained low till the branch point and showed a surge increase at the pre- and spawning ground, where the values at spawning ground were more than 50 and 300 times those at branch point and estuary for males and females, respectively.

Absolute amounts of steroid hormones and their trends of changes were comparable with other reports in chum salmon (Ueda et al., 1984; Matsumoto, 2002). Onuma et al. (2003) reported that plasma T and E₂ levels in both sexes increased

significantly on midway of the homing route, but 11-KT levels in females had the tendency to increase during upstream migration from coastal area to midway of the river, and then decreased near hatchery, followed by rapid increase at hatchery. Besides, they indicated that levels of DHP in both sexes were found to be low during upstream migration until the fish reached to midway of the river and those at hatchery were more than 100 times those on midway of the river. Similar pattern of changes of steroid hormones have been reported from sockeye salmon sampled at four different points during upstream migration in Adams River (Truscott et al., 1986).

In other salmonids as in our study, a decrease in serum T and 11-KT levels, and an increase in serum DHP levels during the onset of spermiation were observed (Scott and Sumpter, 1989). Barry et al. (1990) proposed that these differences in serum levels of androgens and DHP prior to or during spermiation reflect a shift in the steroidogenic pathway in the testis, from production of androgens to production of DHP (Barry et al., 1990). Furthermore, Planas and Swanson (1995) conducted *in vitro* incubation of maturing coho salmon testis at different stages of spermatogenesis with GTHs and reported that the decrease in serum androgen levels could be related to the decline in serum levels of FSH and other changes.

In this study, the absolute amounts of serum T in females was higher than in males, possibly because of the critical role of T as a substrate (precursor) for the production of E₂ (Kagawa et al., 1982). Similar results were reported in migrating chum salmon (Onuma et al., 2003). 11-KT levels at final spawning ground showed a rapid increase in females, but a minor increase in males even though the physiological significance is not clear (Lokman et al., 2002). 11-KT levels coincided in female and male longear fish, and it has been suggested that it may play a role in female spawning behavior (Fentress et al., 2006). In female Japanese eels, 11-KT level has been reported to play active roles in controlling pre-vitellogenic oocyte growth (Matsubara et al., 2003). In this study, whether 11-KT has some important function at final mating (spawning) or not needs to be verified. On the other hand, it may play steroid feedback action on pituitary (Crim et al., 1981; Breton and Sambroni, 1996).

Sharp decrease in serum E₂ of females at pre- and spawning ground was followed by sharp increase in serum DHP at pre-spawning ground which may indicate the occurrence of steroid shift from E₂ production to DHP production in chum salmon before spawning (Nagahama et al., 1995). The apparently low levels in androgens and E₂ at estuary and branch point, and DHP at pre-spawning ground in 2003 as

compared to 2004 and 2005 may indicate that sea surface temperature might have influenced the physiological data in this study. Onuma et al. (2003) reported that the levels of T and 11-KT at midway of the homing route in the warm years are significantly higher than those values in the cool years for both sexes. They also reported that males at hatchery in warm years were more sexually advanced than those fish in cool years. However, in our study such sexual advancement was not observed. In this study, data from quick bulletin of ocean condition, satellite images of NOAA and CTD (conductivity temperature-depth) recorder indicated that 2003 was a bit colder than 2004 and 2005. Year 2003 may be considered as intermediate year as the temperature difference was small.

This study was also aimed at investigating serum IGF-I profiles and its relationship with somatic growth and endocrine changes during homing migration of chum salmon. To our knowledge, no research has been done to examine the serum IGF-I profiles during homing migration of salmonids for such a longer period. In all years examined, serum IGF-I levels in males showed a consistent increase from Bering Sea to offshore and remained constant and/or started to show a minor decrease towards branch point, and finally showed a sharp decrease at pre- and spawning ground. Furthermore, serum IGF-I levels at offshore, coastal sea and estuary were significant as compared to those values at Bering Sea, pre- and spawning ground in males. Quite different from males in absolute amounts and pattern of changes, serum IGF-I levels in females at Bering Sea were comparable to those at offshore and coastal sea, and finally showed a sharp decline at spawning ground. Interestingly, serum IGF-I levels in males were more than double to those of females in all years examined, suggesting that there may be sex differences in IGF-I production during homing migration of chum salmon. This result is consistent with the report that plasma IGF-I concentrations were always higher in male sunshine bass, although female fish were always larger than male fish (Davis and McEntire, 2006). In contrast, Moriyama et al. (1997) reported similar absolute amounts of plasma IGF-I in both sexes of precociously maturing amago salmon. Generally, serum IGF-I levels obtained in this study were comparable with other reports (Moriyama et al., 1997; Congleton et al., 2003). Species difference and stage of development might have attributed for the apparently high serum IGF-I level in migrating chum of this study.

High serum IGF-I levels before final spawning and their sharp decrease after that; and similar pattern of changes for each sex may indicate that serum IGF-I levels

may have other important roles such as acceleration gonadal maturation beyond promotion of somatic growth. This is because upstream migration of chum occurs within a few weeks (Kitahashi et al., 2000), and increases in body sizes were low among sampling points except Bering Sea. Therefore, the role of IGF-I in mediating GH-IGF-axis at this state may be minimal.

Most of the earlier research studies conducted on IGF-I mainly focused on its roles as a mediator of growth and other physiological functions as described in the introduction section. However, recently Campbell et al. (2003, 2006) clearly indicate the interaction between BPG-axis and GH-IGF-I axis and the role of IGF-I as a signaling molecule in this cross talk. They reported that plasma IGF-I increases during gonadal maturation, especially in the early mitotic stages of steroidogenesis and obtained positive relationships among plasma IGF-I, 11-KT, E₂ and pituitary FSH. In our study, the same positive relationships among serum IGF-I, 11-KT, E₂, and pituitary FSH were as obtained in both sexes. On the other hand, body size did not seem to have positive correlation with IGF-I in both sexes in chum salmon during homing migration except 2005 males and this has been discussed in chapter 1.1.

Taken together, IGF-I may play active roles in the activation of gametogenesis or steroidogenesis either by its effects on gonadotropin and steroids. Whether the comparatively high IGF-I levels observed at Bering Sea of this study had any connection with earlier start of oocytes development or not is not known. Campbell et al. (2006) mentioned that a general increase of components of the FSH-ovarian steroid system, plasma IGF-I and intraovarian IGFs occurred as oocytes accumulated lipid droplets. However, the complex mechanisms involved in this process is unknown. Further studies are required to investigate the roles and sex differences in serum IGF-I and its receptors on gonadal maturation during homing migration of chum salmon.

In summary, this is the first study which investigates the hormonal profiles in the BPG axis for three years during the homing migration of chum salmon. The peptide levels in different brain regions were increased during homing migration of chum salmon. At the same time, elevations in pituitary GTHs were observed. More importantly, serum steroids were consistently elevated at the midway of the upstream migration. Serum IGF-I levels showed a magnificent increase before the final spawning, probably indicating their involvement in BPG axis. Taken together, the results of this study support the fact that activation of BPG axis occurs in coordination of final gonadal maturation and migratory behavior of chum salmon.

Chapter 3

Relationship between eyed-egg percentage and levels of cortisol and thyroid hormone in masu salmon

Introduction

Survival of fertilized eggs until the eyed-egg stage is critical for the success of hatcheries. Incubation of poor quality eggs could lead to heavy losses at the eyed and latter stages of development. The fact that variations in egg quality lead to variable post fertilization success has been demonstrated in rainbow trout *O. mykiss* (Bromage et al., 1992) and gilthead seabream *Sparus aurata* (Fernandez-Palacios et al., 1997). Many factors are thought to affect the quality and survival of eggs, including nutrition, genetics, stress and time after ovulation (Schreck et al., 2001).

In intensive rearing facilities, fish are often subjected to multiple stressors such as pollutants (Schreck and Lorz, 1978; Tomasso et al., 1981), electric shock (Schreck et al., 1976), transportation, anesthesia, temperature extremes (Strange et al., 1977; Barton and Peter, 1982) and handling, which could result in adaptive or maladaptive stress responses (Acerete et al., 2004). In teleosts, HPI axis is activated by stressors where the HYP receives input about stressors affecting the system and transfers this information in the form of a chemical called corticotropin-releasing hormone (CRH). CRH stimulates the release of adrenocorticotrophic hormone (ACTH). ACTH is then circulated to the adrenal cortex where it stimulates the production and release of cortisol.

Generally the role of cortisol during stressful stimuli is to induce overall organismal sequestration of energy reserves by regulating the metabolism of carbohydrates, proteins and lipids (Leach and Taylor, 1982). Elevated cortisol can have deleterious effects on the reproductive capabilities of teleosts as it has been linked with declines in body size, gonadosomatic index, egg size, gamete quality and pituitary gonadotropin content (Foo and Lam, 1993b; Kime and Nash, 1999). It also reduces hepatic estradiol-binding sites in salmonids (Pottinger and Pickering, 1990), suggesting mechanisms by which cortisol may affect egg quality.

Furthermore, significant amounts of T, E₂, cortisol, T₃ and T₄ are consistently found in vitellogenic, freshly ovulated and fertilized mature eggs of teleosts (de Jesus and Hirano, 1992; Deane and Woo, 2003) and they have been speculated to be of maternal origin (Greenblatt et al., 1989; Hwang et al., 1992). There is a large body of evidence indicating that exogenous thyroid hormones affect the patterns of growth and developmental processes in fish (See review by Brown and Bern, 1989). Treatment of developing fish with T₄ and T₃ hormones induces earlier development

and accelerates yolk absorption, growth and morphological differentiation (Leatherland, 1982). However, the physiological significance of these hormones in developing embryos is still unknown.

During recent years, low numbers of eyed eggs or eyed embryos in pond-reared masu salmon have been reported at Kumaishi station of Hokkaido Fish Hatchery, Japan, implying high mortality of fertilized eggs or failure of fertilization. Reaching to the eyed-egg stage is one of the critical stages in the early life history of fish as described above. The stage at which the eyes become visible as black spots within the developing embryo is a convenient landmark of development during embryogenesis, and this varies from species to species (mainly affected by incubation temperature). In 2002, the average hatching rate of masu salmon at Kumaishi and Mori stations was 71.5% and 90.0%, respectively (N. Koide, pers. comm.), suggesting that the difference may have been environmentally caused. Thus, this study examines cortisol levels and their possible relation with survival of fertilized eggs in pond-reared masu salmon at Kumaishi station. Firstly, serum cortisol levels of maturing masu salmon at Kumaishi and Mori were measured and compared. Secondly, serum and eyed-egg cortisol levels were measured and related to the eyed-egg percentages. Finally, the relationship between eyed-egg percentage and concentrations of T₃ and T₄ hormones in the eyed eggs was examined.

Materials and Methods

Experimental fish

Masu salmon were reared in outdoor tanks under natural photoperiod with a continuous flow of spring water at Kumaishi and Mori stations of the Hokkaido Fish Hatchery. The fish sampled at Kumaishi and Mori originated in Shiribetsu River and had been reared in intensive culture ponds for 20 and 35 years, respectively. The two stations belong in a similar agro-ecological zone. At Mori and Kumaishi the average water temperatures were $10.5 \pm 0.8^{\circ}\text{C}$ and $9.0 \pm 0.9^{\circ}\text{C}$, respectively, with Kumaishi station exhibiting relatively high temperature fluctuations. Six years of data indicate that water temperature at Kumaishi was higher than Mori by 0.2–2.5°C from June to October and lower than Mori by 0.4–4.3°C from December to April. The two stations

have almost the same natural photoperiod. Masu salmon at Mori and Kumaishi start spawning at the beginning and in the middle of September, respectively.

Fish were fed dry pellets at a rate of 1.7–2.0% of body weight every two days until the end of July, after which they were given dry pellets but did not take them readily. The tank sizes at Kumaishi and Mori cover respective areas of 263.0 m² and 265.6 m², are filled to respective depths of 71 cm and 70 cm, and were stocked at respective densities of 4245 and 5214 fish/tank. Groups of 20 fish were randomly sampled from March to September 2001, every two months at each station. The 20 fish sampled each month were different individuals of the same batches, and were anaesthetized with MS222. To minimize the effects of handling stress, blood was collected within 3 min of handling from caudal vessels, kept on ice and later centrifuged at 3000 rpm for 15 min at 4°C to obtain serum samples, which were stored at –30°C until assayed. Pituitaries were also taken out upon decapitation and those from Mori were used for another unrelated experiment. Finally, gonads were removed and weighed to calculate GSI as an estimate of gonadal maturity. Average fork length, body weight and GSI of experimental fish are shown in Table I.

To investigate cortisol concentrations in serum and eyed eggs in relation to the eyed-egg percentage, blood samples were collected from 33 mature female masu salmon at Kumaishi in September 2002 (fork length, FL = 38.4 ± 0.5 cm, body weight, BW = 674 ± 26 g and gonadosomatic index, GSI = 21.3 ± 0.50%). The eggs were taken from 33 mature females, kept separately by individual female, and were artificially fertilized by pooled sperm taken from mature males. Fertilized eggs were incubated in an artificial incubator at approximately 12°C with continuous flow of spring water under dark condition. The fertilized eggs were sampled at the eyed-egg stage (26 days after fertilization). Total egg count was conducted right away by counting dead eggs and subtracting them from the total to get the number of eyed eggs. Thus, the eggs were counted twice. Eyed-egg percentage (100 x total number of eyed eggs/total number of fertilized eggs) was calculated for all fertilized eggs. The sampled eyed eggs were stored at –80°C for later extraction and hormonal analyses.

Cortisol extraction from eyed eggs

Extraction of eyed eggs for cortisol measurement was done according to the method adopted by Hiroi et al. (1997). In brief, frozen eyed eggs weighing 100–200

mg were homogenized in a five-fold volume of ice-cold phosphate buffered saline (PBS). A total of 300 μ l of the homogenate was extracted twice with 3 ml of diethyl ether by mixing vigorously for 2 min. After freezing at -80°C for 10 min, the ether layer was collected by decantation and dried out at room temperature. To reconstitute the dried residue, 300 μ l of tetrachloromethane was added and mixed for 4 min. Then, 300 μ l of assay buffer was added and mixed for 2 min. Finally, the extract was centrifuged at 3000 rpm for 10 min at 4°C , and the upper layer was collected and stored at -30°C until cortisol analysis.

Assay of cortisol

Cortisol levels of serum and eyed eggs were measured using DELFIA Cortisol kit R060-101 (Wallac Oy, Turku, Finland) in a time-resolved fluorometer. A total of 25 μ l of standard in triplicate and samples in duplicate was pipetted into 96-well microtiter plates, and diluted Eu tracer/antibody solution was added and incubated for 1 hour at room temperature. After a stringent wash, enhancement solution was added, and the intensity of fluorescence from dissociated Eu was measured by a time-resolved fluorometer using DOS-based Multicalc software. The standard curve in each assay was plotted, and sample values were calculated. The intra- and interassay coefficients of variance were 9.8% ($n = 18$) and 18.3% ($n = 6$), respectively.

Assays of T_3 and T_4

The protocols for T_3 and T_4 assays followed those developed by Satoh et al. (2000). In brief, frozen eyed eggs weighing 100–200 mg were homogenized in a five-fold volume of ice-cold PBS, and 300 μ l of the homogenates were used for T_3 and T_4 assays. Antisera for T_3 and T_4 were immobilized at the surface of the microtiter plates by physical adsorption for 18 hours at 4°C . Antisera, antibodies and Eu-labeled thyroid BSA were kindly provided by Dr. H. Yamada, Kitasato University. Then, 50 μ l of assay buffer, 60 μ l standards and 10 μ l of samples were added. Next, 50 μ l of europium thyroid BSA (Eu- T_3 -BSA and Eu- T_4 -BSA) in assay buffer was added to the plates. Finally, 50 μ l of anti- T_3 and - T_4 were dispensed and incubated overnight at 4°C . After three washes with 0.9% saline, Eu was dissociated from the antibody–antigen complex on the surface of the wells with enhancement solution. The intensity of the dissociated Eu was measured by a time-resolved fluorometer. All

measurements were acquired in a single T₃ and T₄ assay. The intraassay coefficients of variance were 8.0% ($n = 11$) and 8.2% ($n = 11$) for T₃ and T₄, respectively.

Statistical analysis

To assess differences in cortisol levels of masu salmon at Kumaishi and Mori stations, two-way ANOVA, followed by Bonferroni Post-test, was used. Relationships between eyed-egg percentages and cortisol levels, and eyed-egg percentages and thyroid hormone levels were examined using linear regression analyses. Statistics and graphing were performed using GraphPad Prism 4.0 (San Diego, CA, USA).

Results

Comparison of serum cortisol levels

Serum cortisol levels were significantly higher in May and July for males at Kumaishi compared to males at Mori, but lower in September (Fig. 32). Serum cortisol levels of females were significantly higher (Two-way ANOVA, $n = 10$, $P < 0.01$) in May and July at Kumaishi than at Mori, but did not differ in March and September. Generally, comparison of cortisol levels in the two stations indicates that serum cortisol levels of masu salmon at Kumaishi were typically much higher (reaching 10 times higher levels) than those at Mori, especially in 1+ May and July.

Eyed-egg percentages and cortisol levels in serum and eyed eggs at Kumaishi

Figure 33 shows the relationship between eyed-egg percentage and cortisol levels in serum and eyed-eggs at Kumaishi. The eyed-egg percentage decreased with increasing cortisol levels in both the maternal serum ($r^2 = 0.689$, $P < 0.001$) [Fig. 33(a)] and eyed eggs ($r^2 = 0.760$, $P < 0.001$) [Fig. 33(b)], indicating an inverse relationship. There was a linear positive relationship ($r^2 = 0.647$, $P < 0.001$) between serum cortisol and the concentration of cortisol in fertilized eggs (Fig. 34). In contrast, as T₃ ($r^2 = 0.484$, $n = 33$, $P < 0.001$) [Fig. 35(a)] and T₄ ($r^2 = 0.564$, $n = 33$, $P < 0.001$) [Fig. 35(b)] levels increased in the eyed eggs, the eyed-egg percentage increased, indicating a positive relationship.

Table I. Fork length (FL), body weight (BW) and gonadosomatic index (GSI =100* gonad weight/BW) in masu salmon sampled at Mori and Kumaishi stations (mean \pm SEM).

Month	Station	Sex	<i>n</i>	FL (cm)	BW (g)	GSI (%)
March	Mori	Male	10	33.3 \pm 0.9	426 \pm 32	0.13 \pm 0.01
		Female	10	33.8 \pm 0.9	460 \pm 40	0.90 \pm 0.04
	Kumaishi	Male	10	26.9 \pm 0.6	226 \pm 16	0.16 \pm 0.01
		Female	10	30.6 \pm 0.9	345 \pm 28	0.88 \pm 0.03
May	Mori	Male	10	35.3 \pm 0.9	479 \pm 36	0.71 \pm 0.15
		Female	10	33.6 \pm 0.4	427 \pm 20	1.96 \pm 0.30
	Kumaishi	Male	10	30.8 \pm 0.7	342 \pm 24	0.59 \pm 0.08
		Female	10	34.1 \pm 1.0	529 \pm 50	1.54 \pm 0.09
July	Mori	Male	10	37.6 \pm 0.7	728 \pm 38	8.65 \pm 0.41
		Female	10	35.7 \pm 0.9	602 \pm 41	9.01 \pm 1.26
	Kumaishi	Male	10	40.4 \pm 0.7	871 \pm 50	7.17 \pm 0.32
		Female	10	38.2 \pm 0.9	765 \pm 63	8.82 \pm 0.90
September	Mori	Male	10	38.7 \pm 1.4	664 \pm 78	8.34 \pm 0.29
		Female	10	37.6 \pm 0.9	621 \pm 40	27.28 \pm 1.09
	Kumaishi	Male	10	37.8 \pm 0.7	634 \pm 37	7.59 \pm 0.28
		Female	10	36.0 \pm 0.9	571 \pm 49	24.68 \pm 1.12

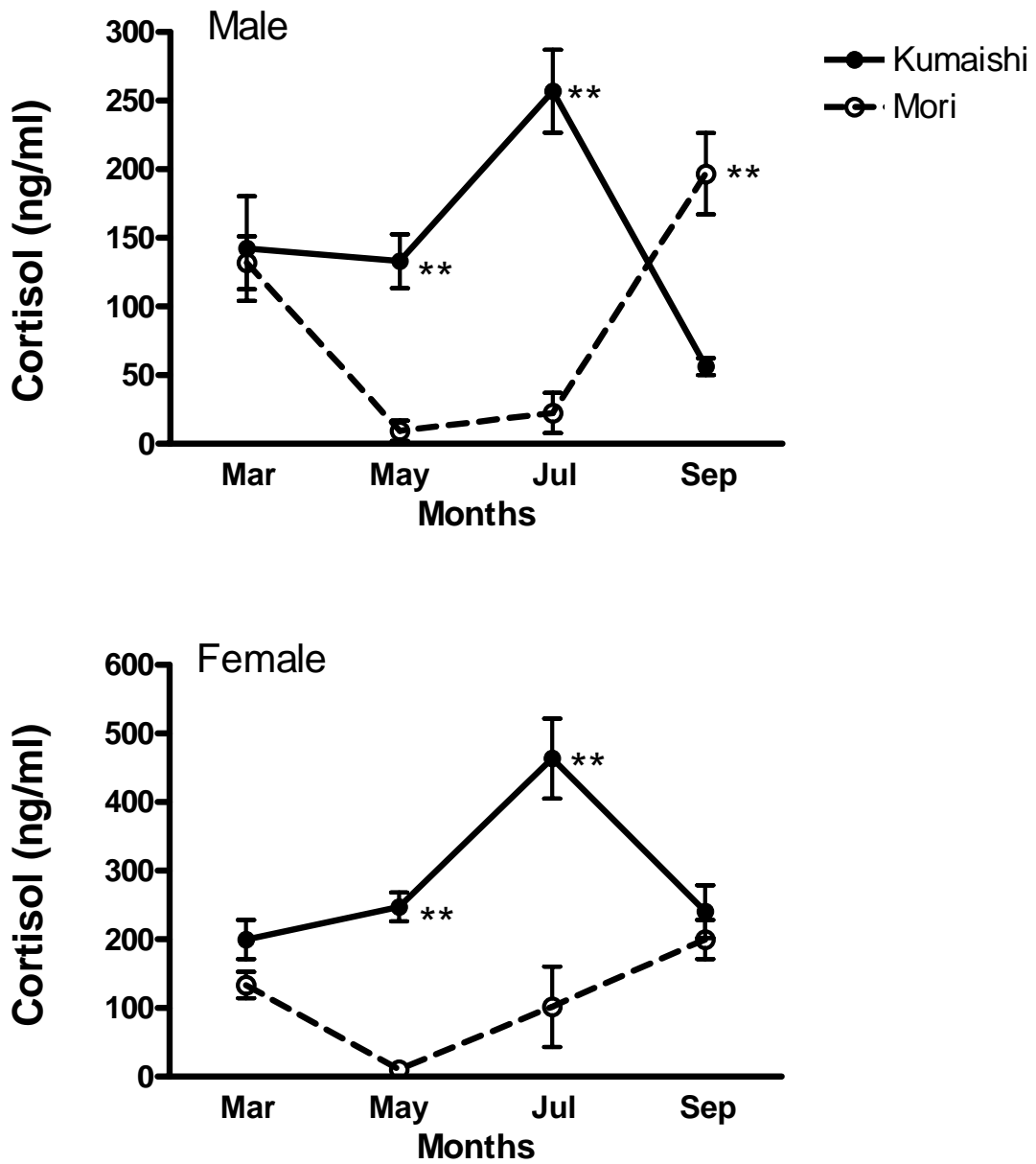


Fig. 32. Comparison of serum cortisol levels of male and female maturing masu salmon from March to September 2001 at Kumaishi and Mori stations. Values represent the mean \pm SEM ($n = 10$). Asterisks indicate significant differences between the two stations. ** $P < 0.01$.

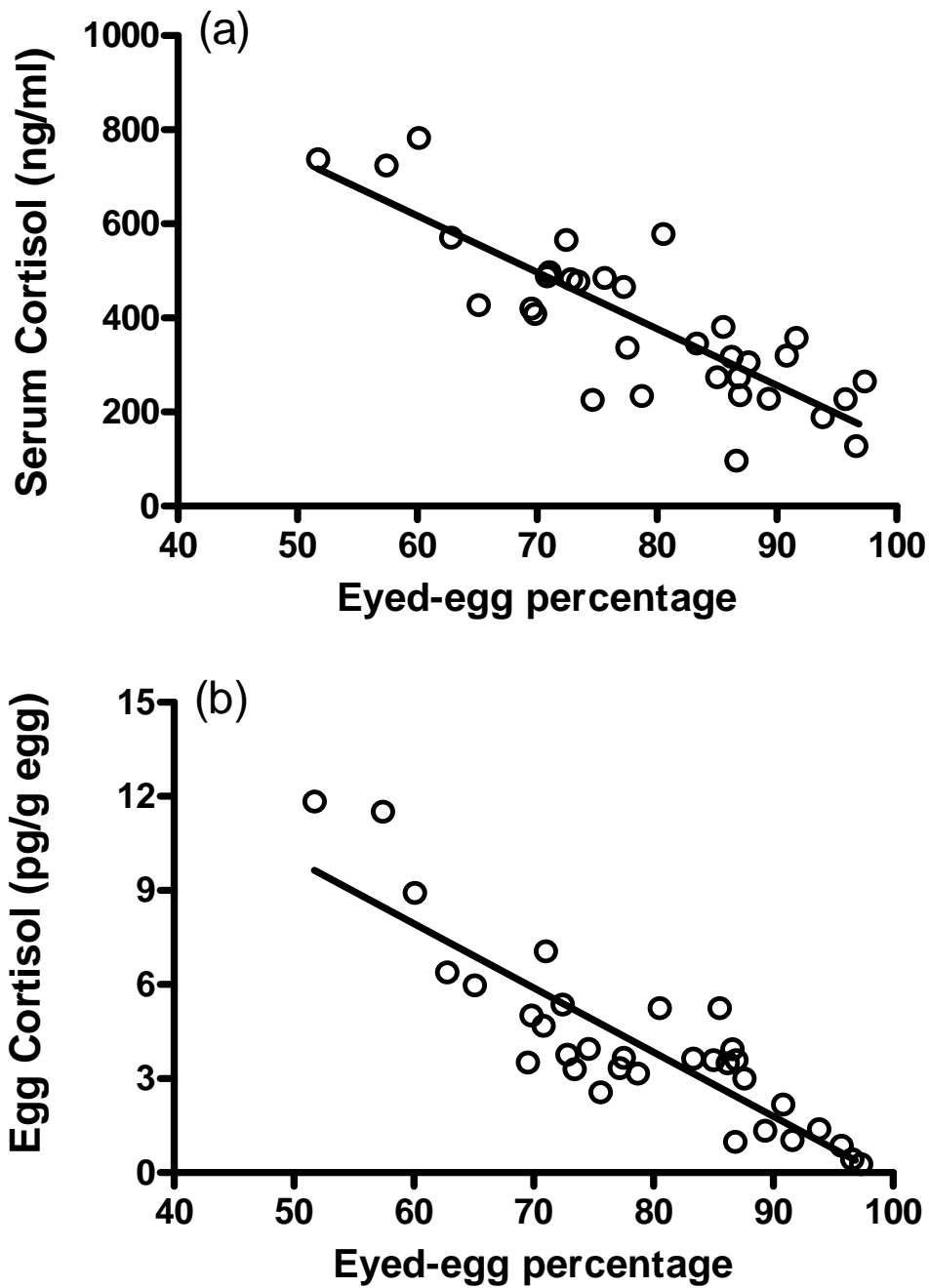


Fig. 33. Relationships between survival percentage of eggs at the eyed-egg stage and cortisol levels in serum (a) and in eyed eggs (b) from female masu salmon at Kumaishi station ($n = 33$). Each open circle (o) represents a batch of eggs measured from one individual. The curves were fitted by: $y = -12.018x + 1338.200$ and $y = -0.205x + 20.246$ for (a) and (b), respectively.

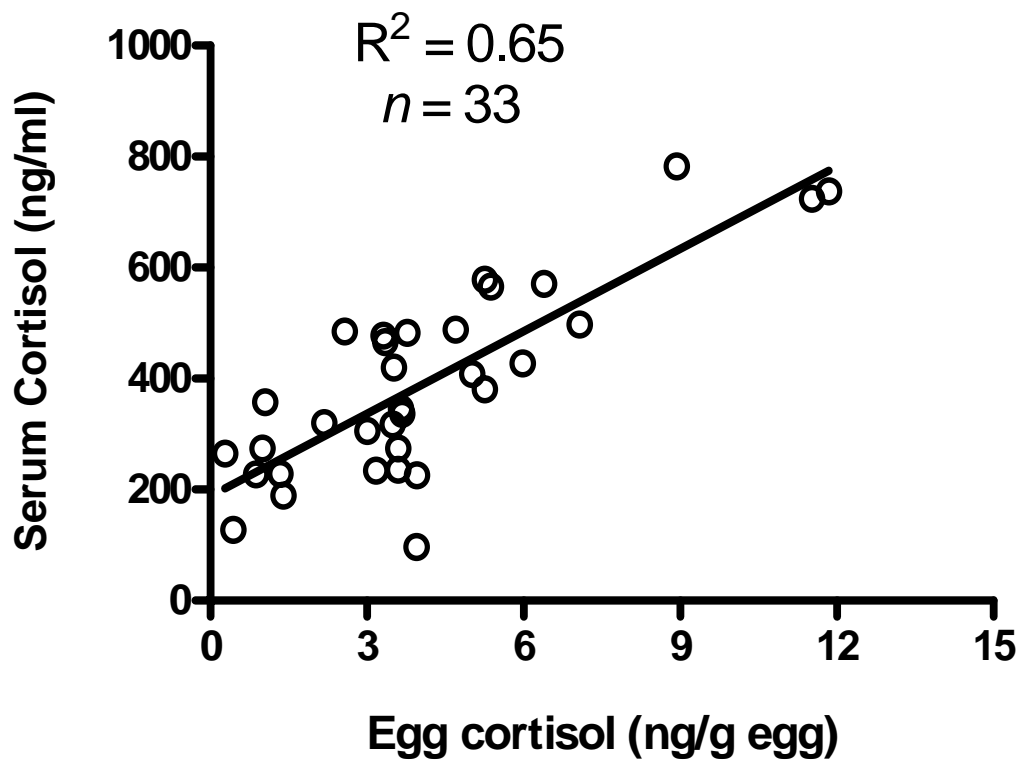


Fig. 34. Relationships between serum cortisol and eyed egg cortisol from matured female masu salmon at Kumaishi station. Each open circle (o) represents a batch of eggs measured from one individual. The curve was fitted by: $y = 49.54x + 188.09$.

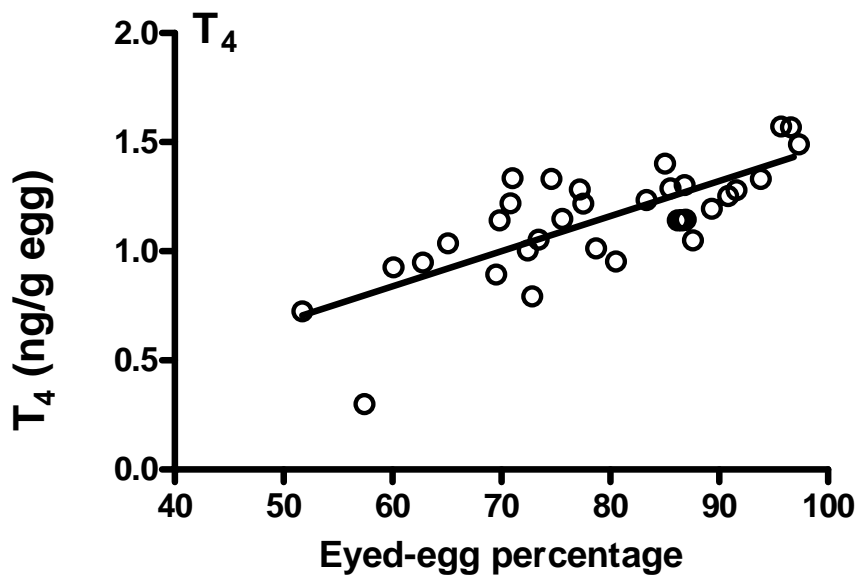
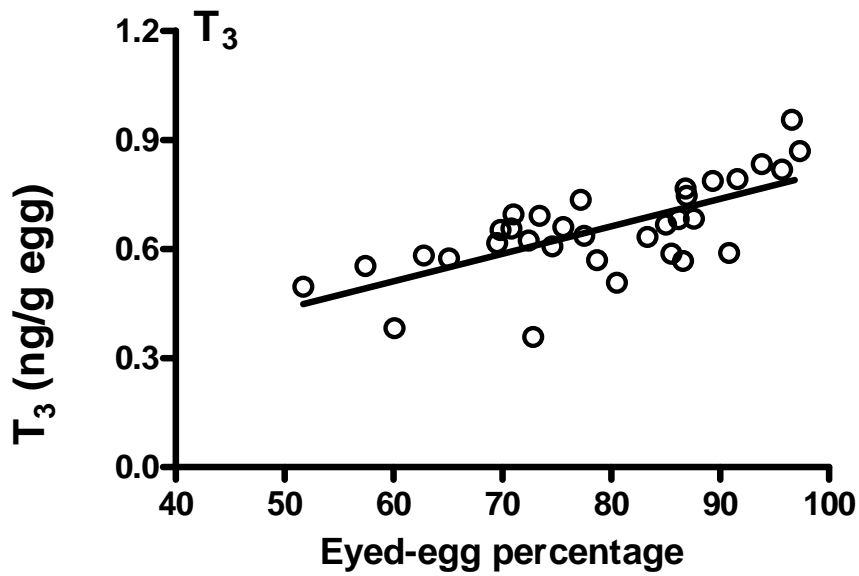


Fig. 35. Relationships between survival percentage of eggs at the eyed-egg stage, and T_3 and T_4 levels in eyed eggs from female masu salmon at Kumaishi station ($n = 33$). Each open circle (o) represents a batch of eggs measured from one individual. The curves were fitted by: $y = 0.008x + 0.057$ and $y = 0.016x - 0.126$ for T_3 and T_4 , respectively.

Discussion

Assessment of cortisol hormone levels during the gonadal maturation period of masu salmon at the two stations indicated that serum cortisol levels in males were much higher at Kumaishi (reaching approximately 11–14 times) than at Mori in May and July. Serum cortisol levels of female masu salmon were also very much higher at Kumaishi than at Mori in May (approximately 24 times) and July (approximately 4 times). Serum cortisol levels were not statistically different between the two stations in March and September.

Generally, these results indicate the serum cortisol levels were frequently higher at Kumaishi when compared to Mori. It seems that the differences in serum cortisol levels between the two stations might not be affected by the assay coefficient of variations (CVs) as variations of cortisol levels within and between assays were far lower than the station differences obtained in this study. Even though previously published data on masu salmon could not be found, serum cortisol levels measured in this study (especially those at Mori) were comparable to those of sexually mature and spawning Pacific salmon (Morrison et al., 1985; Onuma et al., 2003). However, serum cortisol levels were frequently elevated at Kumaishi. As opposed to Mori station and previous findings, the decreasing tendency of cortisol levels at Kumaishi during spawning period is not clear. In salmonids, previous findings indicate the consistent elevation of serum cortisol at the final spawning time (Carruth et al., 2000). Because of the differences in the start spawning between the stations, sampling was not done at the same time in September at both stations. Delayed sampling in September at Kumaishi might have contributed to insignificant differences in serum cortisol levels between the two stations, though the GSI observed at the two stations in September was similar. On the other hand, individual differences might have contributed for these insignificant differences in serum cortisol levels between the two stations during the spawning period.

The frequently high serum cortisol levels observed in masu salmon at Kumaishi during the pre-spawning period may indicate a cumulative corticosteroid response to environmental stressors through the activation of the HPI axis (Rivier and Rivest, 1991). Kubokawa et al. (1999) reported that male sockeye salmon *O. nerka* respond to confinement stress with elevated levels of cortisol during the breeding season. In our study, the exact source of the possible environmental stressor

responsible for the increased cortisol levels of masu salmon at Kumaishi is not known. It may be due to certain management practices that occurred in the rearing environment or the genetic variations that might have arisen from long-term rearing. Since the problem occurred frequently for years, the cause for low eyed-egg percentage at Kumaishi might be due to environmental stress. However, the genetic variability generated by long-term rearing of these stocks could not be ruled out.

In this study, we used standard egg extraction procedures proven to be effective for measuring cortisol contents in unfertilized, fertilized and eyed-eggs and in minimizing the interference of lipid substances in the egg extracts with assay procedure. There might be possible interference of lipid substances in the egg extracts with the assays. Nevertheless, the eyed-egg cortisol contents of masu salmon obtained in this study were comparable to those reported from other teleosts (Hwang et al., 1992).

The positive relationship between cortisol levels in maternal serum and eyed eggs documented in our study is consistent with previous reports. Intra-arterial injection of labeled cortisol in Pacific salmon has been shown to cause a transfer of cortisol to almost all tissues very shortly after application (Donaldson and Fagerlund, 1972). Stratholt et al. (1997) indicated that increased plasma cortisol caused by an applied stressor leads to increased deposition of cortisol in the oocytes in adult female coho salmon, but they also suggested that the deposited egg cortisol content does not seem to affect early development. Besides, the entry of cortisol into the oocytes is suggested to be nonspecific by *in vitro* incubation of oocytes with labeled cortisol (Tagawa et al., 2000).

The presence of a two-month lag-time between significantly elevated serum cortisol levels in females at Kumaishi and the spawning period seems to indicate a weak connection between stress in fish and cortisol in the eyed eggs. However, the elevated serum cortisol levels during the pre-spawning season can still have significant effects on gamete quality and survival rates of embryos. Serum cortisol levels of masu salmon which were correlated with eyed-egg cortisol content and eyed-egg percentage at Kumaishi (September 2002) were significantly higher than the serum cortisol levels measured in September 2001 at both stations, despite differences in batch and year.

Cortisol levels in serum and eyed eggs were both negatively related to eyed-egg percentage. It is known that many factors are involved in raising serum cortisol

levels in teleosts. The frequently high cortisol levels in serum and fertilized eggs, and their negative relationship with eyed-egg percentage at Kumaishi could indicate that these cortisol levels might have an impact in lowering the eyed-egg percentage in pond-reared masu salmon. With regard to this phenomenon, Campbell et al. (1992) reported that a direct application of an environmental stressor to maturing male or female salmonids during oogenesis could adversely affect the quality of gametes in terms of subsequent viability. They found that there were no differences in somatic weight or length between the repeatedly stressed and control groups at the end of the experiment, but exposure to repeated acute stress during reproductive development resulted in a significant delay in ovulation and reduced egg size in females, significantly lower sperm counts in males, and most importantly, significantly lower survival rates for progeny from stressed fish. Reproductive performance impairment has also been reported in male striped bass subjected to significant stress during maturation and spawning (Castranova et al., 2005). In addition, elevated plasma cortisol in juvenile and adult salmonids has been associated with reduced immunocompetence, increased mortality rates, and decreased egg size and quality (Fevolden et al., 1993). Moreover, experiments in mammals (guinea pigs and mice) indicate that a stress response in the mother, such as elevated levels of catecholamines and corticosteroids, can be reflected in the fetus and may affect the developing offspring (Dauprat et al., 1990; Takahashi et al., 1998). Assuming the cortisol in fertilized eggs to be of maternal origin and considering the highly variable nature of serum cortisol in response to the presence or absence of stressors, it can be suggested that increases in serum cortisol in an adult fish caused by environmental stressors during gonadal development and maturation might result in increased cortisol levels in ovulated, fertilized and eyed eggs. Thus, it is possible that stress and consequent increased cortisol levels have deleterious effects on fertilized eggs and lower the survival rate of embryos at Kumaishi.

In this study, fertilized eggs at Mori were unfortunately not measured in the same way as those from Kumaishi, but still there was a great variation in hatching success between the two stations in 2002. In 2002, the average eyed-egg percentages were 73.0% and 93.1% at Kumaishi and Mori, respectively (unpublished data). In the same year, the corresponding average hatch up rates were 65.8% and 80.0%. This difference was evident for years.

In our study, the T₃ and T₄ hormone concentrations in the eyed-eggs were similar to the findings previously reported in the oocytes of salmonids (Tagawa and Hirano, 1990; Raine and Leatherland, 2003). When the T₃ and T₄ levels increased in eyed eggs, the eyed-egg percentages also increased, suggesting that fertilized eggs with higher eyed-egg percentages had higher T₃ and T₄ levels. It has been reported that T₃ and T₄ hormones from maternal serum are transferred into fish eggs (Kobuke et al., 1987) and cross the placenta in mammals (Morreale de Escobar et al., 1985). Ayson and Lam (1993) found that T₄ injection of female rabbitfish *Siganus guttatus* caused T₄ and T₃ levels to increase in the plasma of females, and subsequently, to increase in their oocytes. Thus, the oocyte thyroid hormone appears to be related to maternal serum thyroid levels, suggesting the limited control of oocytes on their hormone content. Even though maternal serum T₃ and T₄ levels were not measured in our study, T₃ and T₄ levels observed in the eyed eggs of this experiment could possibly originate from the circulating maternal thyroid hormones.

The key role of thyroid hormones appears to be associated with metamorphosis or direct development as supplementation of water or larval feed with thyroid hormones is known to accelerate growth and developmental changes associated with the transition of teleost larvae to juveniles (Lam, 1980). Treatment of pre-spawning female striped bass *Morone saxatilis* with T₃ has been associated with accelerated larval development and enhanced larval survival (Brown et al., 1988). Tagawa and Hirano (1990) reported findings that suggest important roles of maternal thyroid hormones for developing salmon embryos during yolk absorption. In zebrafish *Brachydanio rerio*, the inhibition of thyroid hormone synthesis prevents the transition of larvae to juveniles (Brown, 1997). Raine and Leatherland (2003) suggested that the maternal thyroid hormones in oocytes represent a reserve of iodide that could be metabolized and used by the embryonic thyroid tissue for the controlled production of other endogenous hormones. In other vertebrates, enhanced growth and differentiation of embryos have been found, for example, in hens given the highest dose of T₄ (Wilson and McNabb, 1997). Thus, the positive relationship between eyed-egg percentage and T₃ and T₄ levels in eyed eggs may indicate the possibility of differential survival rates of fertilized eggs depending on the content of the thyroid hormones, which, in turn, are linked to maternal thyroid contents.

To summarize, the relationship between eyed-egg percentages and cortisol levels of serum and eyed eggs was revealed for the first time in masu salmon at

Kumaishi. The frequently higher cortisol levels observed in serum and in fertilized eggs may be the cause of lowered eyed-egg percentage observed at this station. Since many of the management practices used in hatcheries are potentially stressful, quantitative evaluation of the effects of such procedures on gamete quality could facilitate changes in the conditions employed in order to minimize stress and ensure production of viable offspring. Thus, it would be beneficial to consider and clarify such relationships in other teleosts and use this information to improve management practices in hatcheries.

☆ The following precautionary measures should be taken to avert this problem or to increase the eyed-egg percentage at Kumaishi station:

- It is essential to study the root cause for high cortisol levels in blood of pond-reared masu salmon before and during spawning season.
- Assessment of the breeding environment (water temperature, water quality, etc.) would be essential.
- Management practices that could possibly lead to stress should be improved.
- The accumulation of the egg cortisol as well as its effect on fertilization needs to be clearly understood.
- Even if the egg and blood cortisol is high, it is good to select eggs with high eyed-egg percentage for the next breeding.
- It is advisable to practice selective breeding.
- Further well-planned *in vivo* and *in vitro* studies should be carried out.

General Discussion

In the present study, effects of RU and endocrine changes have been investigated with special emphasis on hormone cascades in GH-IGF-I, BPG and HPI axes at different stages of salmonids life cycle (embryonic, growing and maturing salmonids). Study on the effects of RU and its fractions mainly focus on somatic growth and sexual maturation *in vivo*; and hormonal releases/productions in the HYP, pituitary and gonadal incubates *in vitro*. Study on endocrinological changes during homing migration mainly focuses on hormonal profiles in the BPG axis during homing migration of chum salmon. Besides, serum IGF-I profiles and the possible roles in BPG axis have been investigated. This is the first report ever made on IGF-I profiles during homing migration in chum salmon. Furthermore, relationship between eye-egg percentages and cortisol levels in serum and fertilized eggs were established.

To investigate the effect of long-term RU administration on somatic growth and gonadal maturation in masu salmon, fish were divided into three groups: control and RU-fed groups (RU 20 and RU 200); and monthly changes in somatic growth and gonadal maturation were measured. RU 200 fed groups showed higher growth responses as compared to other groups. RU 200 immature fish showed significantly higher body size increase throughout the sampling period. Fork length and body weights were also significantly higher in 1+ spring, summer and 2+ autumn in RU 200 immature males and females. Maturing males showed a significant increase in somatic growth during spawning time in RU 200 fed groups. In parallel with this, greater increases in serum IGF-I were observed in RU-fed groups. In RU 200 fed males, serum IGF-I levels were significantly elevated in 1+ June, September, October and December and in 2+ March and April. Besides, in RU 200 fed females, serum IGF-I levels were significantly increased 1+ July, August, October and November and in 2+ March and April. Interestingly, significant increases in serum IGF-I were accompanied by significant increase in somatic growth. This result is expected as IGF-I is the main mediator in GH-IGF-I axis or growth rate in salmonids (Moriyama et al., 1997; Duan, 1998). The simultaneous increase of serum IGF-I and body growth of RU fed groups observed in this study may be due to the involvement of physiologically active substances present in the mycelium of RU. Many fungal species are believed to contain physiologically active substances. Amino acids and

other active substances that may be contained in RU might activate GH-IGF-I axis in fish (Bhandari et al., 2002). Arginine and other essential amino acids are potential activators of somatic growth or GH-IGF-I axis (Mommsen, 2001). So, in this study, the significant somatic growth may be related to the direct effects of amino acids and other active substances such as steroids and enzymes through promotion protein anabolism or through the activation of the complex GH-IGF system.

Serum IGF-I was poorly related ($r < 0.1$) to either fork length or body weight and previous research results are controversial (in favor and against). This may be due to the various metabolic function of IGF-I. It seemed that serum IGF-I levels were seasonally changing. On the other hand, IGF-I was positively correlated with main androgen and estrogen which agrees with latest reports by Campbell et al. (2003, 2006), suggesting the possible link between of IGF-I and BPG axis.

RU 200 fed groups showed significantly higher serum T, 11-KT, E₂ levels in pre-spawning seasons and DHP in spawning time. Serum DHP levels in RU fed groups increased almost 1.5 times to controls. These results suggest that the physiologically active substance present might have stimulated T, 11-KT, E₂, and DHP production. In many other *Rhizopus* species, T, androstenedione, progesterone, progesterone receptors and others enzymes have been investigated (Plementias et al., 1999). Characterization of these active substances and their roles on mammalian steroid biosynthesis has been documented (Kristan et al., 2003). Thus, the physiologically active substances present in RU could have stimulated steroidogenesis in both sexes either by providing a substrate or accelerating the turn over rate.

To investigate *in vitro* effects of RU, dose-dependent and time-course incubation of HYP, pituitary and gonads were conducted, and hormone release and production from these tissues were measured. Besides, different RU fractions were obtained by synthetic adsorbent method and dose-dependent RU fractions incubations were made on the fore-mentioned tissues. Generally, RU incubation of HYP, pituitary and gonads showed a dose-dependent increase in their respective hormonal releases/productions of these tissues in masu salmon; and significant differences were observed at higher doses of RU incubation. Time-course RU incubations indicated that there was an increasing tendency in the respective hormonal levels of the incubated tissues as time progressed but showed a decreasing tendency after 24 hours of incubation. Moreover, almost all RU fractions at all dose levels had brought a higher increase in hormonal levels of HYP and pituitary incubates, and levels were

significant at higher doses for most of the RU fractions when compared to control. In testis and ovary incubates, mostly RU fractions A and B at higher doses had significant increases in steroid releases in the medium of gonadal incubates. The dose-dependent increase in hormonal levels of HYP, pituitary and gonad incubates with RU is similar to the results obtained during my Master's study. This consistent hormonal increase in HYP, pituitary and gonad incubates may indicate the capability of RU to stimulate hormonal releases in the medium of these incubates. As it has been described in the *in vivo* discussion, three different components of the steroid hormone signalling system, 17 β -HSD, androgen binding proteins and steroid hormone signalling molecule testosterone were determined in the filamentous fungus *Cochliobolus lunatus* (Zakelj-Mavric et al., 1995). T, androstenedione, progesterone and androgen binding proteins have been investigated in the mycelium of *Rhizopus* species as endogenously synthesized molecules (Plementias et al., 1999). All these physiologically active substances may be contained in the mycelium of RU, and thus, resulting significant increases in hormonal releases.

Effects of RU fractions on hormonal releases of HYP, pituitary and gonad incubates seemed tissue specific. While all most all RU fractions increased hormonal production in HYP and pituitary incubates, only few fractions brought significant increases in hormonal releases of gonadal incubates. These variations are not clear. The contents of the different RU fractions in the eluates might cause different responses in these tissues. Further fractionation, purification and isolation of RU fraction A and B would be essential. Generally, significant increases in the hormonal release were found from HYP, pituitary and gonad incubates when they were incubated with RU and its fractions. Thus, this finding could be cited as another possibility of direct effects of RU on BPG axis.

In summary, long-term RU administration enhanced somatic growth in both sexes and elevated serum steroids and IGF-I in masu salmon. *In vitro* incubation of HYP, pituitary and gonads with RU increased hormonal production of the respective tissues. This consistent increase in serum IGF-I which was followed by promotion of body growth suggests that somatic growth in masu salmon was achieved through activation of GH-IGF-I axis, where the physiologically active substances in RU might have played active roles in activation of this axis and steroid biosynthesis. Thus, RU could be a useful tool for the manipulation of somatic growth and sexual maturation in aquaculture.

In chapter 2, hormonal profiles in the BPG axis were investigated during homing migration of chum salmon for three years. Generally, sGnRH levels in OB and TE showed similar pattern of changes in both sexes, but absolute amounts in TE always tended to be higher. sGnRH in both brain loci showed a consistent increase at offshore and peaked at estuary and/or branch point. In one incident (2003 males) levels of sGnRH at Bering Sea were similar to those at offshore and coastal sea, indicating the activation of sGnRH neurons in the initiation of homing migration (Onuma et al., 2004). These results agree with the report that strong signals of sGnRH mRNA in OB and olfactory nerve were observed at coastal sea but not at spawning ground during upstream migration of chum salmon (Kudo et al., 1996). Because of their vicinity to the olfactory system and wide distribution of sGnRH neurons in OB and TN, sGnRH produced in these regions are believed to play neuromodulatory roles such as selection of the natal river (Kobayashi et al., 1997). TE is relatively large and contains many sGnRH producing neurons. This may be part of the reason for higher levels of peptides found in TE.

On the other hand, in all years examined, sGnRH profile in HYP showed a rough similarity with those in OB and TE, although the physiological implication of this similarity is not clear (Figs. 24 and 25). Consistent increases at offshore and peak levels at estuary or branch point, and also typical high values at Bering Sea may indicate in the involvement BPG axis in initiation of homing migration of salmonids. Previous reports indicate a consistent activation of sGnRH neurons in VT and HYP during sexual maturation; suggested the association of these neurons in controlling GTHs synthesis and release (Kobayashi et al., 1997). Thus, the sGnRH measured from VT an HYP of this study may involve in regulating GTHs release during homing migration of salmonids.

sGnRH and LH levels in pituitary showed similar pattern of changes whereby both showed increased levels at offshore and typical high levels at spawning ground especially LH. There was also a positive relationship between these peptides which may indicate induction of LH synthesis by sGnRH. sGnRH regulation of GTHs synthesis and release is well established by many studies (Ando et al., 2004). In rainbow trout, sGnRH pituitary contents in spermiating or ovulating fish have been found to be significantly higher than those in immature and postspermiated or postovulated fish (Yamada et al., 2002). A massive increase in pituitary LH content occurs during vitellogenesis and later stages of spermatogenesis reaching highest

levels in fully mature fish (Gomez et al., 1999; Hassin et al., 2000). Our data agree with these results and supports the notion that sGnRH stimulates LH synthesis which shows up-regulation with the progress of gametogenesis. On the other hand, pituitary FSH levels increased till estuary and showed a decreasing tendency at pre- and spawning ground. Initial up-regulation of pituitary and serum FSH during early gonadal growth, and decline just prior to ovulation and spermiation has been reported from different teleosts (Melamed et al., 1997; Hassin et al., 1999). Furthermore, FSH levels in pituitary were positively correlated with androgens. Therefore, our results support the idea that FSH is involved in early stages of gonadal maturation.

Serum androgens and estrogen showed more or less similar pattern of changes among the years, consistently increased till estuary and branch point and decreased thereafter. On contrary, serum DHP was at low level till the fish reached branch point and showed a sharp increase at pre- and spawning ground. There are many reports that are dealt serum steroids and are in agreement with our results (Fitzpatrick et al., 1986; Truscott et al., 1986; Onuma et al., 2003). These variations in serum levels of androgens/estrogens; and DHP before or during spawning may reflect a shift steroidogenic pathway from production of androgens/estrogen to the production of DHP (Barry et al., 1989; Nagahama et al., 1995). In female, serum T levels were higher than males may because of its critical role as a precursor of E₂ (Young et al., 1983).

Serum IGF-I profiles showed almost similar trends of changes and comparable contents among the years, but differ in absolute amounts and even trends of changes. However, the absolute values declined rapidly prior to final spawning. Variations in serum IGF-I levels may be true sex difference as CVs of the assays were low and measurements were made at the same time with same standards, antigen, antibodies, and Eu-avidin. Hence, higher serum IGF-I before final spawning and subsequent decrease during spawning might suggest that IGF-I could play active roles in BPG axis during gametogenesis. In this regard, there is considerable information (Huang et al., 1998; Nader et al., 1999). IGF-I could influence gonadotropin production and steroidogenesis directly as discussed above. Furthermore, there was a positive relationship between IGF-I and 11-KT in males, and between IGF-I and E₂ in females, suggesting the pivotal role of IGF-I on early stage gonadal maturation.

There was a positive relationship between serum steroids, and hormones in brain and pituitary. This is due to the fact that gonadal maturation is hormonally

controlled by the BPG axis (Urano et al., 1999). This axis initiates and accelerates the homing migration behavior of salmonids (Ueda and Yamauchi, 1995; Onuma et al., 2005). And the understanding of the basic endocrinological mechanisms in this axis would be of a great importance scientific application. To mention some: intramuscular implantation of T could induce upstream migratory behavior in masu salmon (Munakata et al., 2001), and GnRH analog accelerates gonadal maturation and shortens homing duration of sockeye salmon (Fukaya et al., 1998; Kitahashi et al., 1998). In sum, this study clearly indicates the involvement of BPG and GH-IGF-I axes in a coordinated manner during homing migration of salmonids.

In search for the possible reasons behind the low hatchability rate of pond-reared masu salmon at Kumaishi station; first, serum cortisol levels at early maturing season were investigated and compared with Mori station. Comparison between the two stations was possible because, these stations are found in similar agro-ecological zones and the fish were of the same type and origin. The result of this study indicated that serum cortisol levels at Kumaishi were about 12 times higher than those at Mori in May and July. Based on this result, the second experiment was conducted. Cortisol levels in serum of matured females and in the eyed eggs of the same fish were measured and correlated with the eyed-egg percentages. Eyed-egg percentage decreased with increasing cortisol levels in both the maternal serum and eyed eggs, indicating an inverse relationship. As opposed to this, when T_3 and T_4 levels increased in the eyed eggs, the eyed-egg percentage increased, indicating a positive relationship. It seems that fertilized egg cortisol originates from the serum cortisol of the maternal fish (Stratholt et al., 1997). The frequently high cortisol levels in serum and then in fertilized eggs, and their negative relationship with eyed-egg percentage at Kumaishi could indicate that these cortisol levels might have an impact in lowering the eyed-egg percentage in pond-reared masu salmon. Effects severe stress and the consequently production of cortisol in the HPI axis have been elaborated in the introduction and discussion of this chapter. One typical example, a direct application of an environmental stressor to maturing salmonids during gametogenesis could adversely affect the quality of gametes in terms of subsequent viability (Campbell et al., 1992). Thus, high cortisol levels in serum and fertilized eggs could have a negative impact on the eyed-egg percentage of pond-reared masu salmon at Kumaishi. This result could be good source information for hatcheries and they have to consider such relationships during the routine management practices.

Conclusion

The present study was conducted to obtain a basic knowledge on the *in vivo* and *in vitro* effects of RU administration and on endocrine changes at different stages of salmonids life cycle with special emphasis on hormone cascades in GH-IGF-I, BPG and HPI axes. Long-term RU feeding was conducted on underyearling lacustrine masu salmon, where body growth changes and sexual maturation were monitored. RU and its fractions incubations were conducted on HYP, pituitary and gonad; and hormonal releases/productions in the respective tissues were investigated by biochemical methods. Hormonal profiles in the BPG axis were investigated by immunoassays during homing migration of chum salmon. Further, assessment of serum and eyed-egg cortisol during gonadal maturation and its influence hatching rate were conducted by physical counting and biochemical methods. Results for each physio-endocrine parameter selected on the GH-IGF-I, BPG, and HPI axes were clarified as follows:

1. Long-term RU feeding promoted somatic growth and accelerated gonadal maturation especially in males. Serum levels of T, 11-KT, E₂ and DHP were significantly increased in RU 200 fed males. Serum IGF-I levels were significantly increased in RU-fed groups and these significant increase in serum IGF-I were accompanied by significant increase in body size, indicating the involvement of physiologically active substance present in RU in stimulating the GH-IGF-I axis and steroid biosynthetic pathways.
2. RU incubation of HYP, pituitary and gonadal tissues increased hormonal releases in the respective tissues in a dose-dependent manner. Higher doses of RU incubation (100 µg/ml and 1000 µg/ml) significantly increased hormonal release in the medium of HYP, pituitary and gonad incubates. Furthermore, incubation of HYP, pituitary and gonads with RU increased hormonal releases in the medium of these tissues in time-dependent manner. Significant increases in hormonal release were observed in RU treated tissues compared to control. These data indicates the direct effects of RU on the main tissues that involve in the BPG axis.
3. RU fractions incubation of HYP and pituitary significantly increased hormonal release in the respective tissues in most of RU fractions at all dose levels. On

the other hand, RU fraction A and B had significantly increased hormonal production in the medium of gonadal incubates, indicating the stimulatory effect of bioactive substances present in the eluants of these fractions.

4. Frequent stimulation of BPG axis was observed during the homing migration of chum salmon in all three years examined. Specifically, sGnRH in OB, TE and HYP increased at offshore and peaked at either estuary or branch point. Elevated levels of FSH in pituitary were observed at coastal sea, estuary and branch point. Pituitary sGnRH and LH showed similar trends of changes with consistent increases at offshore and estuary. Serum T, 11-KT and E₂ showed peak levels at estuary and branch point and decreased afterwards, whereas serum DHP was very low till branch point and showed a surge increase at pre- and spawning ground. Serum IGF-I levels were high at offshore, coastal sea and estuary for males and females. Further, there was a positive correlation between serum IGF-I and main androgen/estrogen, indicating the potential role of IGF-I in stimulating gonadal maturation. Generally, the positive relationship between hormonal profiles in brain, pituitary and serum suggests that the key roles of hormone cascades in the BPG and GH-IGF-I axes for successful homing migration of salmonids.
5. In search of the possible reasons for the low hatchability of pond-reared masu salmon, cortisol levels were found to be very high during pre-spawning period at Kumaishi. Besides, cortisol levels in serum and fertilized eggs were negatively related to eyed-egg percentage, where as thyroid hormones were positively related. Thus, the frequently high cortisol levels at Kumaishi might cause low hatchability at this station.

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