

Activity of the pituitary–gonadal axis is increased prior to the onset of spawning migration of chum salmon

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SUMMARY

The activity of the pituitary–gonadal axis (PG axis) in pre-migratory and homing chum salmon was examined because endocrine mechanisms underlying the onset of spawning migration remain unknown. Pre-migratory fish were caught in the central Bering Sea in June, July and September 2001, 2002 and 2003, and in the Gulf of Alaska in February 2006. They were classified into immature and maturing adults on the basis of gonadal development. The maturing adults commenced spawning migration to coastal areas by the end of summer, because almost all fish in the Bering Sea were immature in September. In the pituitaries of maturing adults, the copy numbers of FSH β mRNA and the FSH content were 2.5- to 100-fold those of the immature fish. Similarly, the amounts of LH β mRNA and LH content in the maturing adults were 100- to 1000-fold those of immature fish. The plasma levels of testosterone, 11-ketotestosterone and estradiol were higher than 10 nmol l⁻¹ in maturing adults, but lower than 1.0 nmol l⁻¹ in immature fish. The increase in the activity of the PG-axis components had already initiated in the maturing adults while they were still in the Gulf of Alaska in winter. In the homing adults, the pituitary contents and the plasma levels of gonadotropins and plasma sex steroid hormones peaked during upstream migration from the coast to the natal hatchery. The present results thus indicate that the seasonal increase in the activity of the PG axis is an important endocrine event that is inseparable from initiation of spawning migration of chum salmon.

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Key words: spawning migration, salmon, follicle-stimulating hormone, luteinizing hormone, steroid hormone, pituitary–gonadal axis.

INTRODUCTION

The spawning migration of chum salmon is an annual reproductive behavior that covers thousands of miles (Salo, 1991), and consists of several phases: initiation of the homing behavior, approach to the coast, adaptation to fresh water (FW), and upstream migration (Urano et al., 1999). The hypothesis for the route of oceanic migration of chum salmon indicates that winter chum salmon of the Japanese population leave the Gulf of Alaska for the Bering Sea in spring (Urawa, 2000). In summer before the commencement of spawning migration, chum salmon originating in Japan form mixed populations with stocks from other geographical areas of the Pacific Rim in the Bering Sea, although Japanese populations are distributed mainly in the central Bering Sea (Moriya et al., 2007; Sato et al., 2007). Homing adults, which leave the Bering Sea in early summer, require two to three months to reach their natal rivers in Japan (Tanaka et al., 2005). Then, they adapt to FW and complete their final maturation during upstream migration in autumn (Makino et al., 2007).

Gonadal maturation of salmonids is regulated primarily by pituitary gonadotropins (GTHs), i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and sex steroid hormones such

as testosterone (T), 11-ketotestosterone (11KT), estradiol (E2) and 17 α -20 β -dihydroxy-4-pregnen-3-one (DHP) (Swanson et al., 2003). It is also true in artificially reared masu salmon that served as a fish model to understand seasonal changes in the pituitary–gonadal axis (PG axis) of Japanese chum salmon (Kitahashi et al., 2004; Jodo et al., 2005). The absolute amounts of mRNAs encoding GTH subunits, i.e. glycoprotein (GP) α 2, FSH β and LH β , in the pituitary started to be elevated during gametogenesis from spring through early summer (Kitahashi et al., 2004). The plasma levels of T, 11KT, and E2 were elevated during the same period (Jodo et al., 2005). Afterward, they peaked during the spawning period in autumn, followed by an increase in the plasma levels of DHP and final maturation (Jodo et al., 2005). Therefore, we hypothesized that activity of the PG axis of oceanic chum salmon changes similarly during their spawning migration.

In homing salmon, activity of the PG axis increased during the final phases of the spawning migration. The amount of LH β mRNA in the pituitary of chum salmon has been shown to increase during upstream migration in the long Ishikari River (Kitahashi et al., 1998; Onuma et al., 2005) and the short Otsuchi River (Onuma et al., 2003b). The plasma levels of GTHs also increase during upstream

migration of pre-spawning chum salmon (Ueda et al., 1984), pink salmon (Dye et al., 1986) and sockeye salmon (Truscott et al., 1986), although these studies detected mainly LH because of a lack of antisera that specifically distinguishes salmon FSH from LH. The plasma levels of T, 11KT and E2 in pre-spawning chum salmon peak during upstream migration from the coast until the fish reach the midway to the upriver, then there is an increase in the plasma levels of DHP and final maturation when the fish arrived at the natal hatchery (Onuma et al., 2003a). However, little information is available regarding the activity of the PG axis during the period when chum salmon are migrating in the Gulf of Alaska and also during the phase when fish initiate their homing behavior from the Bering Sea. Understanding of the endocrine mechanisms, which govern the spawning migration, requires comprehensive information on changes in activity of the PG axis during the above-mentioned behavioral phases.

In the present study, we tried to clarify changes in the activity of the PG axis in chum salmon of Japanese populations at several locations along the presumed pathway of their spawning migration. Pre-migratory fish were sampled in the central Bering Sea in summer and autumn and the Gulf of Alaska in winter (Fig. 1A). Japanese populations were identified on the basis of mitochondrial DNA (mtDNA) haplotypes that were determined with DNA microarray (Moriya et al., 2004). In autumn, homing adults were collected along their migratory pathway in the Ishikari River–Ishikari Bay system in Hokkaido, Japan. Sampling sites includes coastal areas, the estuary, midway and the upstream in the river, and at or near the natal hatchery (Fig. 1B). The activity of the PG axis was assessed from the absolute amounts of GPO2, FSH β and LH β mRNAs, FSH and LH contents in the pituitary, and plasma levels of GTHs, T, 11KT, E2 and DHP. We here report increase in the activity of the PG axis prior to the initiation of spawning migration from the Bering Sea.

MATERIALS AND METHODS

Pre-migratory chum salmon

Pre-migratory chum salmon (*Oncorhynchus keta* Walbaum 1792) were caught in the Bering Sea during oceanic research cruises in the summer and autumn from 2001 to 2003, and in the Gulf of Alaska during an oceanic research cruise in the winter in 2006. The location of fishing stations is summarized in Fig. 1A. Populations of chum salmon in the Bering Sea are composed of many stocks from the rivers in the Pacific Rim. The previous stock identification of individuals from mtDNA haplotypes showed that Japanese chum salmon were the dominant stock distributed in the central Bering Sea in 2002, 2003 and 2004 (Moriya et al., 2007; Sato et al., 2007).

Summer fish in the central Bering Sea were fished by surface longline during cruises of the research vessel (RV) *Wakatake-maru* from late June to mid-July (Fig. 1A). The longline was set 30 min before sunset, and hauled 30 min after the sunset. After retrieval from the longline, salmon were transferred into a flow-through seawater (SW) tank, and actively swimming fish were sampled. A maximum of 10 individuals were sampled at each station. At the same stations, a series of gillnets was set in the evening and hauled in the morning. Chum salmon caught by the gill nets were used to obtain sufficient individuals for clarification of the proportion of immature and maturing fish from frequency distributions of the gonadosomatic index (GSI; gonad mass/body mass \times 100). In September, fish were caught with a 50 \times 50 m trawl net towed at the surface at 5 knots for 1 h usually starting at 9:00 h. Trawl operations were conducted in the Bering Sea by RV *Kaiyo-maru* of the Fisheries Agency of Japan in 2002 and 2003 (Fig. 1A). In 2001, fish on the presumed homing route to Japan were sampled using gill nets during

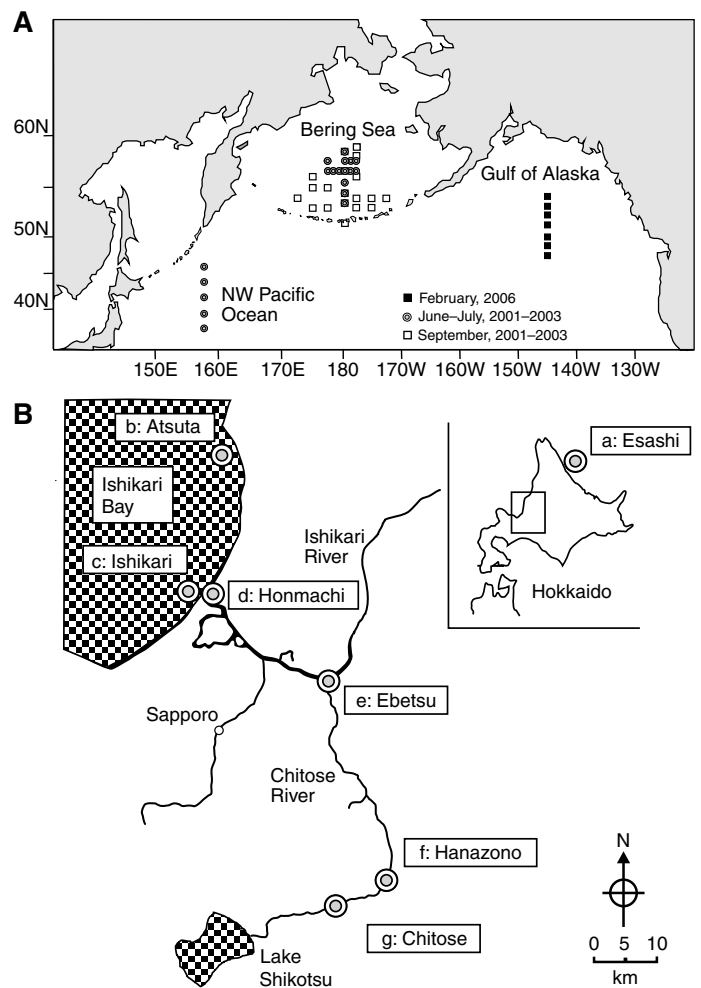


Fig. 1. Offshore, coastal and freshwater sampling locations where chum salmon were collected. (A) Sampling locations for immature and maturing chum salmon in the Gulf of Alaska, the central Bering Sea and the NW Pacific Ocean. In summer, chum salmon were caught in the Bering Sea along and near 180° longitude during cruises of the RV *Wakatake-maru* in 2001–2003, and at locations in the NW Pacific Ocean at 154°59'E during the cruise of the RV *Hokusei-maru* in 2001 (circles). In autumn, fish were caught in the central Bering Sea during cruises of the RV *Kaiyo-maru* in September 2001–2003 (open squares). In winter, chum salmon were caught at stations in the Gulf of Alaska during cruises of the RV *Kaiyo-maru* in February 2006 (filled squares). (B) Sampling locations for pre-spawning chum salmon in September–October, 2001–2003 in SW and FW in Hokkaido, Japan. a: Esashi on the NE coast; b, c: Ishikari Bay; d: Ishikari River estuary; e: midway between the coast and the hatchery; f: fish wheel located 4 km downstream from the hatchery; and g: the hatchery at the Chitose Field Station.

a cruise of the RV *Hokusei-maru* of Hokkaido University from 39°N to 44°N in the northwest (NW) Pacific Ocean (Fig. 1A).

Winter chum salmon in the Gulf of Alaska were also caught by a 50 \times 50 m trawl net towed at 5 knots for 1 h during the cruise of RV *Kaiyo-maru* that was operated under the auspices of the North Pacific Anadromous Fish Commission, in February, 2006. Live chum salmon were selected, and tissue and blood samples were collected.

We collected data from the Bering chum salmon for three years to confirm that reproducible or typical data were obtained from ocean-migrating chum salmon, because our previous endocrine analyses of homing chum salmon showed considerable year-to-year

variations (Onuma et al., 2003a; Onuma et al., 2003b; Onuma et al., 2005). We determined plasma levels of Cl^- and cortisol in all fish used in the present study and confirmed that the levels were not different between fish captured by the trawl and longline gear (data not shown).

Homing chum salmon

Homing adults were captured along their migratory route in the Ishikari River–Ishikari Bay system (Fig. 1B) to clarify changes in the activity of the PG axis during upstream migration. In this river, about 30 million juvenile chum salmon are released every year from the hatchery at the Chitose Field Station, hereafter referred to as the hatchery, which is located on a tributary of the Ishikari River (G in Fig. 1B). The number of naturally spawning chum salmon is much lower than the hatchery-released chum salmon, and more than 95% of hatchery-released fish around the Ishikari Bay are from the Ishikari River. Thus, almost all fish caught in the Ishikari River–Ishikari Bay water system can be regarded as those released from the hatchery. The distance from the mouth of the Ishikari River to the Chitose Field Station is about 70 km. Analyses of data loggers attached to homing adults in the Ishikari Bay showed that fish spent a few weeks to reach the hatchery from the mouth of the Ishikari River (Kitahashi et al., 2000; Makino et al., 2007).

In mid-September, fish were caught at Esashi on the NE coast of Hokkaido facing the Sea of Okhotsk (A in Fig. 1B) because tagging analyses of homing chum salmon showed a considerable portion of fish caught here migrated to the Ishikari Bay within a week. In addition, fish were sampled at six locations along the

homing pathway from the Ishikari Bay to the hatchery from late September to mid-October, when homing chum salmon were abundant in the Ishikari River. Fish in SW areas were captured in the Ishikari Bay using set-nets commercially operated at Atsuta (B in Fig. 1B), 20 km from the river mouth, and Ishikari (C), 1.5 km from the river mouth. Fish in the Ishikari River estuary were captured by a gill net at Honmachi (D), the area between the river mouth and a wharf 2.5 km upstream from the river mouth. Fish midway in the FW were captured by a gill net at Ebetsu (E) at the junction of the mainstream and the Chitose River. Homing adults near the hatchery were captured by the fish wheel at Hanazono (F) 4 km downstream of the hatchery (G). Fish captured at Hanazono were transported to the hatchery and kept in a rearing pond at 12°C under natural photoperiod for about a week until fully mature. Fully matured adults were sampled in the hatchery.

Blood and tissue sampling

All sampling procedures were conducted under the animal care guidelines of Hokkaido University. Following a blow to the head, fork length and body mass of the fish were measured (Table 1), and blood samples were collected from the caudal vasculature. Blood samples were temporarily kept on ice, centrifuged at 1870g for 15 min to obtain the plasma, and stored at –30°C until assays of hormones were conducted. A portion of the blood clot was stored at –30°C for later extraction of DNA to determine the mtDNA haplotypes. Pituitaries were collected and frozen in liquid nitrogen and stored at –80°C until total RNA could be extracted. In 2002 and 2003, the pituitaries were halved using a razor blade along the

Table 1. Fork length, body mass and gonadosomatic index of chum salmon in the central Bering Sea, the Northwest Pacific Ocean and the Gulf of Alaska

| Sex | Year | Months | Location | Stage of gonads | N | FL (cm) | BM (kg) | GSI (%) |
|--------|------|-----------|----------------|-------------------|----|-------------------------|--------------------------|--------------------------|
| Male | 2001 | Jun.–Jul. | Bering Sea | Immature | 15 | 52.2±1.3 ^a | 1.74±0.16 ^a | 0.24±0.04 ^a |
| | | | | Maturing adult I | 18 | 59.2±1.4 ^b | 2.70±0.21 ^{a,b} | 0.65±0.07 ^a |
| | | | | Maturing adult II | 4 | 63.2±1.9 ^b | 3.75±0.42 ^c | 4.67±0.88 ^b |
| | | Jun. | NW Pacific | Maturing adult II | 9 | 58.1±0.7 ^{a,b} | 2.90±0.10 ^{b,c} | 2.21±0.30 ^c |
| | 2002 | Jun.–Jul. | Bering Sea | Immature | 27 | 47.8±1.4 ^a | 1.32±0.12 ^a | 0.19±0.02 ^{a,b} |
| | | | | Maturing adult I | 15 | 60.7±1.7 ^b | 2.72±0.21 ^b | 0.61±0.08 ^a |
| | | | | Maturing adult II | 6 | 65.2±2.3 ^b | 3.66±0.49 ^c | 1.92±0.66 ^c |
| | | Sep. | – | Immature | 32 | 49.9±1.1 ^a | 1.61±0.14 ^a | 0.12±0.01 ^b |
| | 2003 | Jun.–Jul. | – | Immature | 31 | 47.2±0.9 ^a | 1.26±0.08 ^a | 0.24±0.02 ^{a,b} |
| | | | | Maturing adult I | 18 | 59.2±1.3 ^b | 2.69±0.24 ^b | 0.49±0.06 ^a |
| | | | | Maturing adult II | 9 | 63.5±2.1 ^b | 3.44±0.37 ^c | 0.89±0.14 ^c |
| | | Sep. | – | Immature | 37 | 50.7±0.9 ^a | 1.56±0.11 ^a | 0.07±0.01 ^d |
| | 2006 | Feb. | Gulf of Alaska | Immature | 26 | 46.4±1.4 ^a | 1.06±0.10 ^a | 0.05±0.00 ^a |
| | | | | Maturing adult I | 11 | 53.7±1.7 ^b | 1.44±0.09 ^b | 0.08±0.01 ^b |
| Female | 2001 | Jun.–Jul. | Bering Sea | Immature | 14 | 53.1±1.2 ^a | 1.80±0.12 ^a | 1.07±0.05 ^a |
| | | | | Maturing adult I | 19 | 62.0±0.7 ^b | 3.04±0.14 ^b | 2.22±0.14 ^{a,b} |
| | | | | Maturing adult II | 5 | 58.0±2.2 ^b | 2.78±0.57 ^b | 8.36±1.36 ^c |
| | | Jun. | NW Pacific | Maturing adult I | 9 | 57.2±1.5 ^{a,b} | 2.65±0.17 ^b | 3.04±0.43 ^b |
| | 2002 | Jun.–Jul. | Bering Sea | Immature | 23 | 48.3±1.3 ^a | 1.30±0.11 ^a | 1.16±0.05 ^a |
| | | | | Maturing adult I | 16 | 62.0±0.6 ^b | 2.74±0.15 ^b | 2.28±0.16 ^b |
| | | | | Maturing adult II | 4 | 64.1±2.3 ^b | 3.34±0.28 ^b | 7.55±1.18 ^c |
| | | Sep. | – | Immature | 27 | 48.5±1.2 ^a | 1.45±0.12 ^a | 0.71±0.03 ^a |
| | 2003 | Jun.–Jul. | Bering Sea | Immature | 30 | 47.5±0.9 ^a | 1.23±0.06 ^a | 0.98±0.05 ^a |
| | | | | Maturing adult I | 18 | 57.4±0.7 ^b | 2.30±0.09 ^b | 2.39±0.09 ^b |
| | | | | Maturing adult II | 7 | 60.7±1.7 ^b | 3.00±0.28 ^b | 5.01±0.61 ^c |
| | | Sep. | – | Immature | 37 | 50.1±0.6 ^a | 1.40±0.04 ^a | 0.73±0.03 ^a |
| | 2006 | Feb. | Gulf of Alaska | Immature | 20 | 45.2±1.3 ^a | 0.97±0.08 ^a | 0.82±0.05 ^a |
| | | | | Maturing adult I | 16 | 52.8±0.7 ^b | 1.51±0.07 ^b | 1.25±0.07 ^b |

FL, fork length; BM, body mass; GSI, gonadosomatic index.

Values are means ± s.e.m. They are a portion of the groups shown in Fig. 1A, and used for sampling of the pituitary, gonad and plasma.

Fish were divided into immature fish, maturing adult I and II on the basis of histological inspection of gonads. Values with different superscript letters indicate significant difference within the same year ($P<0.05$).

mid-sagittal plane and stored in two different tubes to determine the amount of GTH subunit mRNAs and GTHs from the same individuals. Spermiation and ovulation of homing adults were checked by gentle massage of the abdomen. Gonads were removed and weighed to calculate the GSI. A portion of the testes and ovaries were fixed in Bouin's solution for 1 day, then replaced with 70% ethanol and stored at 4°C for later histological analyses. Fish age was determined from the number of annuli in their scales, as previously reported (Davis et al., 1990).

Classification of immature fish and maturing adults

Pre-migratory fish were classified as immature or maturing adults by histological diagnosis of gonads. The testes were dehydrated in a graded series of ethanol, embedded in Paraplast, sectioned at 10 µm, and stained with Delafield's Hematoxylin and Eosin. The ovaries of immature and early maturing fish before initiation of vitellogenesis were sectioned as described above, whereas ovaries in vitellogenesis were halved with a fine razor to assess histological aspects, as previously described (Onuma et al., 2003a). Stages of gametogenesis

were determined according to Campbell et al. (Campbell et al., 2003; Campbell et al., 2006). Male chum salmon whose testes were mostly composed of primary A spermatogonia were regarded as immature. Males with testes containing germ cells of late B spermatogonia and spermatocytes were designated as maturing adult I fish. Males with testes containing spermatozoon were designated as maturing adult II fish. Female chum salmon whose ovaries contained oocytes with little yolk globules were designated as immature fish. Females with ovaries containing centrally located yolk globules were designated as maturing adult I fish. Females with ovaries whose yolk globules were in the peripheral regions of oocytes were designated as maturing adult II fish.

Identification of mitochondrial DNA haplotypes

The previously reported analyses from our group showed nucleotide sequence variations in the 5' half of the control region of mtDNA from 48 populations of chum salmon in the Pacific Rim including Japan, Russia and North America, and recognized 30 haplotypes on the basis of single nucleotide polymorphisms

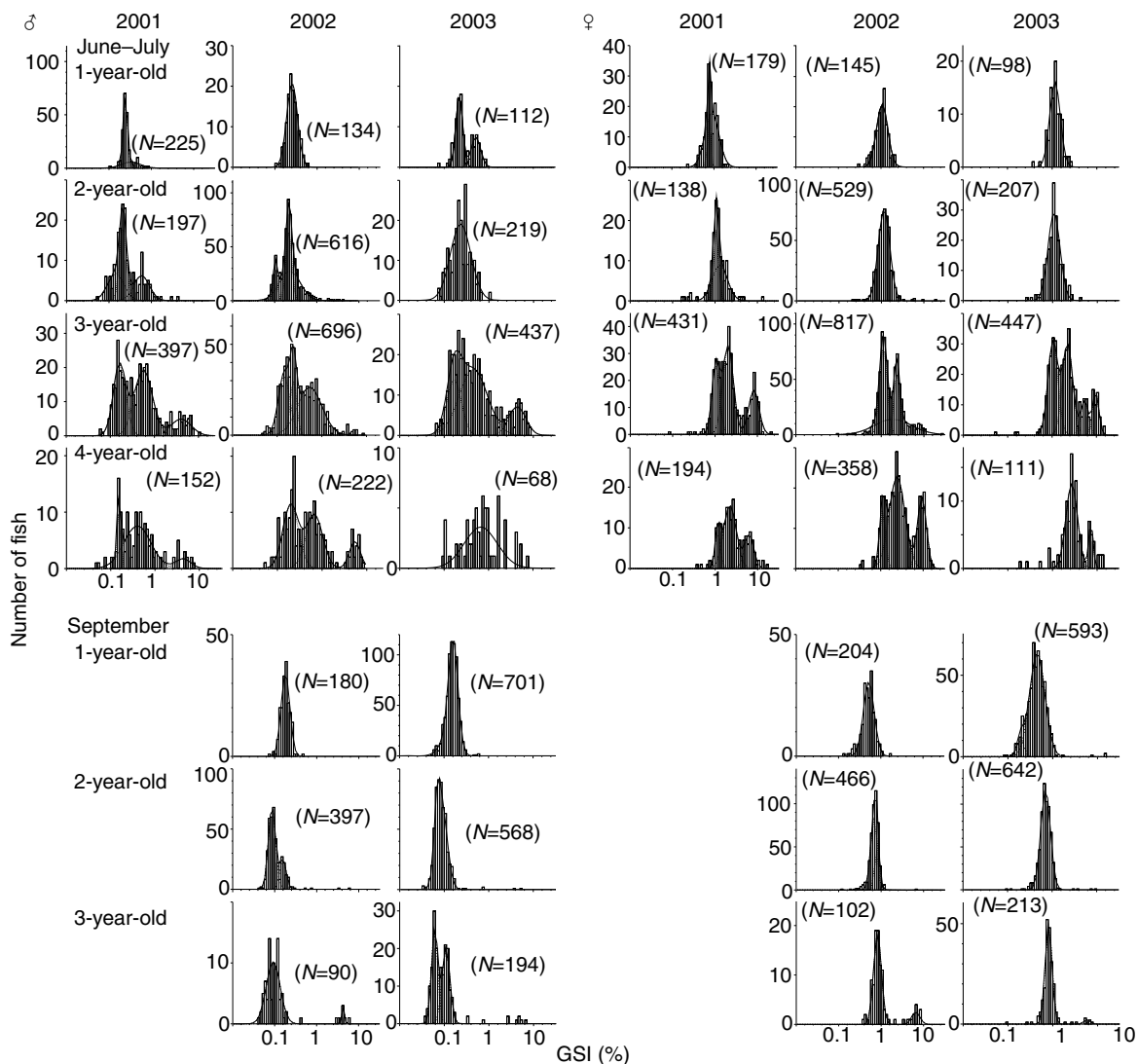


Fig. 2. Frequency distributions of the gonadosomatic index (GSI) for pre-migratory male and female chum salmon in the central Bering Sea, 2001–2003. The GSIs were determined from fish caught during summer (June and July, upper panel) and autumn (September, lower panel). Fitted curves were obtained using a Gaussian distribution with multiple peaks. The ages of fish were estimated from their scales. Data collected during summer show a sizable component of fish with a high GSI, whereas fish with a high GSI are nearly absent in September.

(SNPs) in this region (Sato et al., 2004). An oligonucleotide microarray that detects these SNPs was developed for rapid identification of these chum salmon haplotypes (Moriya et al., 2004). In the present study, the mtDNA haplotype of individual pre-migratory fish was identified by this technique. Samples (2 µl) of the blood clots were dissolved in 300 µl of Cell Lysis Solution (Gentra system, Minneapolis, MN, USA), and DNA was recovered by ethanol precipitation. The extracted DNA was used as a template for PCR amplification of about 500bp in the 5' half of the control region of mtDNA. Amplification was performed with a biotinylated reverse primer for labeling of PCR products. The PCR products were hybridized with oligonucleotide captures on a microarray, and hybridization signals were visualized by the conventional ABC method with tetramethylbenzidine as a chromogen. The mtDNA haplotypes of individuals were then identified by patterns of hybridization signals. The fish in the present study were then divided into the genealogical clade A (Japanese population), clade B (Japanese, Russian and North American populations) and clade C (Japanese and Russian populations) on the basis of the established category (Sato et al., 2004). The sequence of primer, condition of PCR and specificity

of hybridization have been described elsewhere (Moriya et al., 2004).

Quantitative real-time PCR assays of mRNAs encoding GTH subunits

Gene expression for GTH subunits was assessed by quantification of the absolute amount of mRNAs in the pituitary. We utilized the established protocol in which cDNAs transcribed from known copy numbers of particular mRNAs were used to obtain standard curves (Ando et al., 2004). Internal reference RNAs, such as those for β -actin and GAPDH, were not used to normalize the amounts of target RNAs, because expression of housekeeping genes can vary depending on physiological status (Bustin, 2000).

Total RNA was extracted from individual pituitary samples by the guanidium thiocyanate hot phenol–chloroform method (Chirgwin et al., 1979). Total RNA was treated with 1 i.u. of deoxyribonuclease I (TaKaRa Biochemicals, Shiga, Japan) to degrade genomic DNA, and then recovered by a phenol–chloroform–isoamyl alcohol extraction, chloroform extraction, and ethanol precipitation. Concentrations of total RNA were measured with a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies,

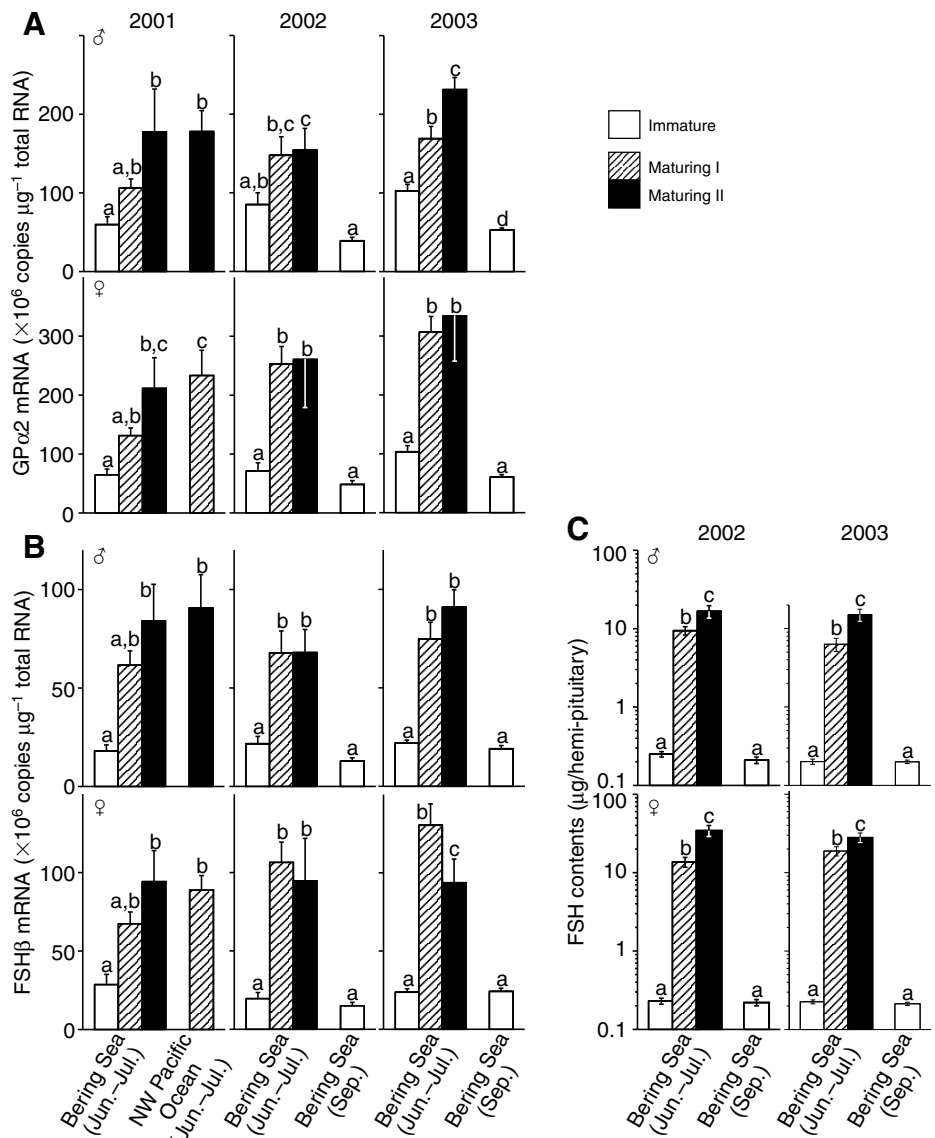


Fig. 3. (A) Amount of glycoprotein $\alpha 2$ (GP $\alpha 2$) mRNA, (B) follicle-stimulating hormone β (FSH β) mRNA and (C) FSH in the pituitaries of chum salmon in the central Bering Sea and the NW Pacific Ocean, 2001–2003. Fish were divided into immature fish and maturing adult I and II on the basis of histological observation of the gonads. Value are means \pm s.e.m. Significant differences among groups are indicated with different letters ($P < 0.05$ one-way ANOVA and Tukey's test). See Table 1 for the number of fish.

Wilmington, DE, USA). The integrity of total RNA was confirmed by agarose gel electrophoresis. In samples obtained in 2002 and 2003, one side of the hemisected pituitary was randomly selected and used for extraction of total RNA. The amount of all GP subunit mRNAs was not significantly different between the two sides of hemisected pituitary from a single fish in extra pituitary samples of pre-spawning fish that were collected for the use as the pooled standard sample through assays of mRNA. The recovery rate of these extraction and precipitation protocols was confirmed to be about 80% using known amounts of total RNAs from chum salmon livers and pituitaries (Onuma et al., 2005). The first strand cDNA was synthesized from 200 ng of total RNA as previously described (Onuma et al., 2005), diluted 1:10, and used as sample cDNAs.

Standard sense RNAs for GP α 2, FSH β and LH β sequences were synthesized to prepare standard curves as previously described (Ando et al., 2004). In addition, sense RNA having the thyroid stimulating hormone β (TSH β) sequence was prepared to assess gene expression for other GP subunits. In brief, plasmid DNAs containing a full cDNA insert for chum salmon GP α 2, FSH β , LH β [a gift from Kyowa Hakko (Sekine et al., 1989)] and TSH β [a gift from Kitasato University (Ito et al., 1993)] were digested with a restriction enzyme just after the 3' end of cDNA insert. Sense RNAs were synthesized with a MAXIscript™ kit (Ambion, Austin, TX, USA), and absolute amounts were quantified with the NanoDrop ND-1000 Spectrometer. Afterwards, serially diluted standard RNA (from 2×10^2 to 2×10^9 copies) were reverse transcribed to first strand cDNA, diluted 1:10, and used as the standards for quantitative real-time PCR assays.

Quantitative real-time PCRs were carried out with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster, CA, USA). The conditions of PCR and nucleotide sequences of primers and fluorogenic probes for GTH subunit mRNAs were described previously (Ando et al., 2004). For TSH β mRNA, the sequences of primers were 5'-GCTCAGCC ACCACGGTCAC-3' (forward) and 5'-GGAAGTGGCCGTA-GGCTGC-3' (reverse), and that of the probe was 5'-(Fam)-CCTTCGTCACGTACCCATAAGCATG-(Tamra)-3' (TaqMan probe, PE Applied Biosystems). A standard sample (pooled chum salmon pituitary cDNA) was used, in triplicate, in each assay to estimate coefficients of variation (CV) within and between assays.

In the present study, the ranges of intra-assay CVs were 0.8–14.2% for the GP α 2 mRNA assay, 3.2–14.4% for the FSH β mRNA assay, 3.2–15.8% for the LH β mRNA assay and 2.8–9.3% for the TSH β mRNA assay. The inter-assay CVs were 10.8% for the GP α 2 mRNA assay, 13.1% for the FSH β mRNA assay, 15.5% for the LH β mRNA assay and 6.9% for the TSH β mRNA assay. We confirmed that the single specific fragment was amplified in each assay system and the curves obtained from serially diluted pituitary cDNA were parallel to the standard curves, which were linear between 4×10^1 and 4×10^7 copies.

Radioimmunoassays of GTHs in the pituitary and plasma

The pituitary contents and plasma levels of FSH and LH were determined by heterologous radioimmunoassays (RIAs) for coho salmon GTHs, as described previously (Suzuki et al., 1988; Swanson et al., 1989). Cross-reactivity of FSH in the LH assay was less than 0.01% and that of LH in the FSH assay was 6.0%. Detectable cross-reactions with TSH, the growth hormone prolactin (PRL) and somatolactin were not found. The hemisected pituitaries, the other halves of which were used in mRNA assays, were sonicated on ice following addition of 1 ml phosphate buffer (0.05 mol l^{-1} , pH 7.4) which contained 1 mmol l^{-1} phenylmethylsulfonyl fluoride. They were centrifuged at $7700g$ for 10 min at 4°C , supernatants were diluted with phosphate buffer containing bovine serum albumin (BSA), and stored at 4°C until assayed serial dilution of the pituitary extract and plasma showed displacement curves parallel to those obtained from purified coho salmon GTHs in both the FSH and LH assays (data not shown). Plasma levels of LH in the pre-migratory fish were not assayed because preliminary tests showed that the levels were less than the detection limit. Purified coho salmon GTHs, which served as standard samples, were used to calculate assay CVs. The ranges of intra-assay CVs were 0.7–7.0% for the FSH assay and 0.6–14.4% for the LH assay. The inter-assay CVs were 7.5% for the FSH assay and 13.4% for the LH assay.

Enzyme immunoassay of plasma sex steroid hormones

Plasma levels of T, 11KT, E2 and DHP were determined by enzyme immunoassays, as previously described (Onuma et al., 2003a). Steroid hormones were extracted from plasma samples with diethyl ether, evaporated to dryness with a gentle flow of nitrogen gas, and

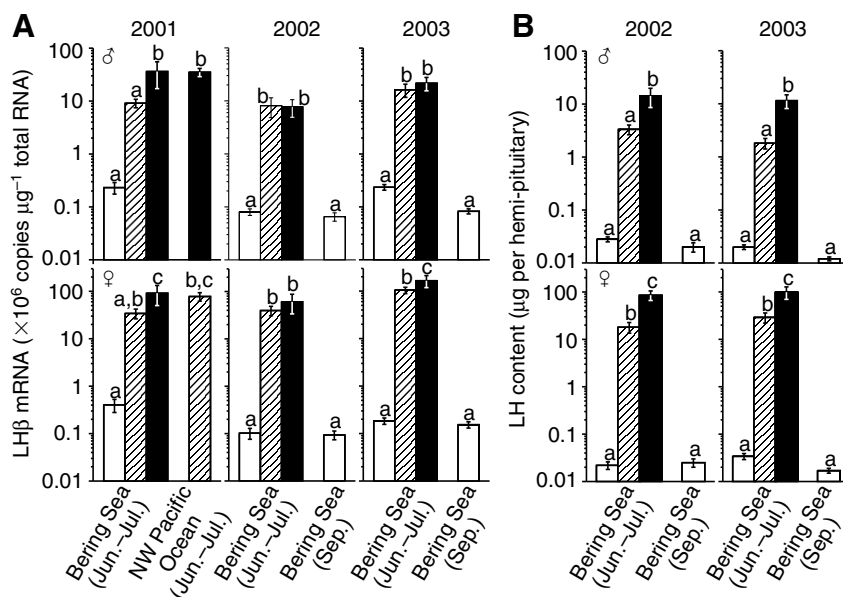


Fig. 4. (A) Amount of luteinizing hormone β (LH β) mRNA and (B) LH in the pituitaries of chum salmon in the central Bering Sea and the NW Pacific Ocean, 2001–2003. Fish were divided into immature fish and maturing adult I and II, as shown in the inset in Fig. 3. Values are means \pm s.e.m. Significant differences among groups are identified with different letters ($P < 0.05$ one-way ANOVA and Tukey's test). See Table 1 for the number of fish.

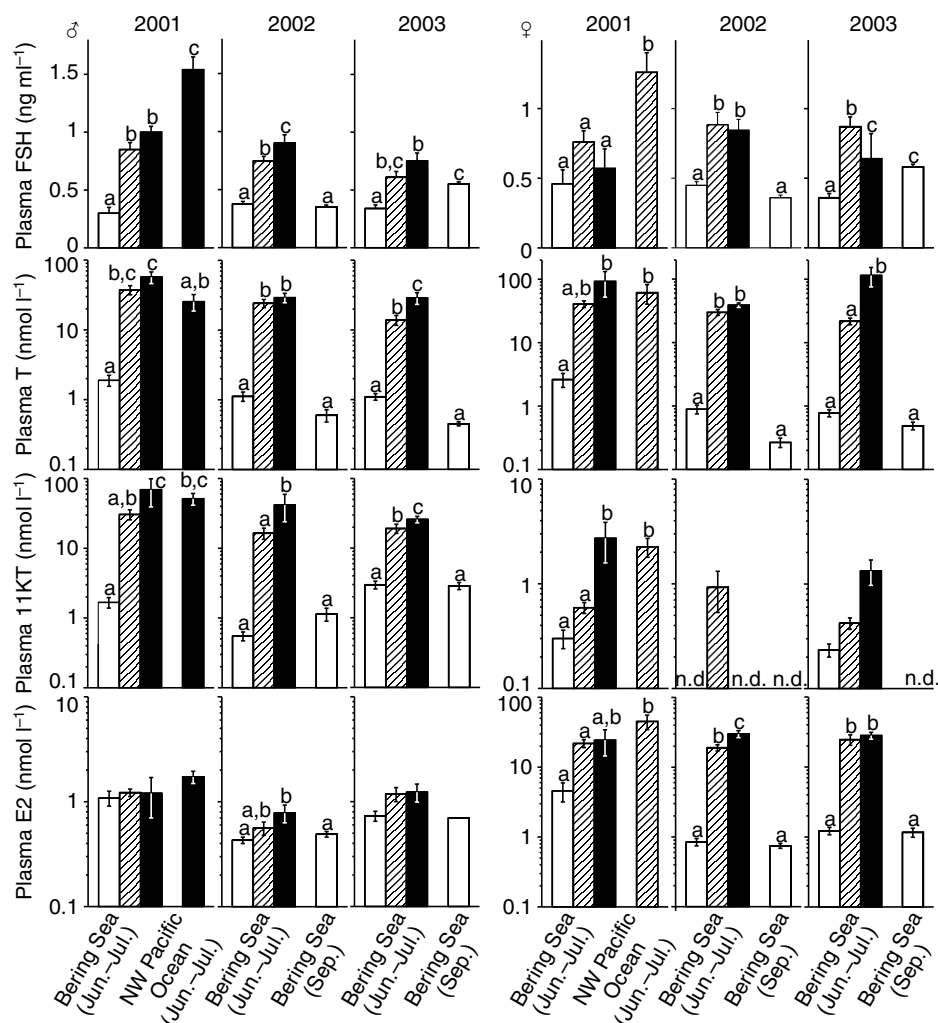


Fig. 5. Plasma levels of follicle-stimulating hormone (FSH), testosterone (T), 11-ketotestosterone (11KT) and estradiol (E2) in chum salmon in the central Bering Sea and the NW Pacific Ocean, 2001–2003. Fish were divided into immature fish and maturing adult I and II, as shown in the inset in Fig. 3. Values are means \pm s.e.m. n.d., not determined. Significant differences among groups are identified with different letters ($P < 0.05$ one-way ANOVA and Tukey's test). See Table 1 for the number of fish.

reconstituted with assay buffer containing 0.2% BSA and 0.01% thimerosal in 0.05 mol l⁻¹ borate buffer, pH 7.8. Microtiter plates (MS-8496F; Sumitomo Bakelite, Tokyo, Japan) were coated with 15 μ g ml⁻¹ of anti-rabbit IgG (ICN Pharmaceuticals, Aurora, CA, USA), 0.05% carbonate buffer, pH 9.7, and further coated with blocking solution containing 0.1% BSA and 3% sucrose in 0.05 mol l⁻¹ phosphate buffer, pH 7.4. The standards and samples were incubated with anti-steroid antiserum and horseradish peroxidase-labeled steroid (T: FKA 102 and FKA 101; 11KT: FKA 118 and FKA 117; E2: FKA 236 and FKA 235; DHP: FKA 332 and FKA 331; Cosmo Bio Co. Ltd, Tokyo, Japan) at 4°C overnight. A substrate solution (0.5 mg ml⁻¹ *o*-phenylenediamine, 0.01% H₂O₂ in 0.2 mol l⁻¹ citrate buffer, pH 4.5) was added and incubated at room temperature for 30 min. After washing with 0.9% NaCl, the absorbance at 492 nm was measured with a microplate reader (MTP-300, Corona Electric Company, Hitachinaka, Japan). A standard sample (pooled chum salmon plasma) was used to calculate assay CVs. In the present assays, the sensitivities of assays were 30 pg ml⁻¹ for T and 11KT, and 10 pg ml⁻¹ for E2 and DHP (Onuma et al., 2003a). The ranges of intra-assay CVs were 1.8–13.0% for the T assay, 1.7–10.0% for the 11KT assay, 1.8–8.7% for the E2 assay, and 1.8–12.0% for the DHP assay. The inter-assay CVs were 12.5% for the T assay, 7.7% for the 11KT assay, 15.1% for the E2 assay, and 6.2% for the DHP assay. Recovery of steroid hormone extractions from plasma and specificities of the assays were previously confirmed (Onuma et al., 2003a).

Statistics

Amounts of GTH subunit mRNAs were calculated as copies/ μ g total RNA. Pituitary contents of FSH and LH were calculated as μ g/pituitary to show stored amounts. Values were shown as mean \pm standard error of the mean. One or two outlier values were removed from each group using the Smirnov–Grubb's test, when significant. Data from different sampling stations in the Bering Sea were combined because differences among fishing stations were not significant. Since the maturing adults with elevated activity of the PG axis were found regardless of mtDNA haplotypes, data from all haplotypes were pooled and treated as immature or maturing groups to test relationships between gonadal development and the levels of the PG-axis hormones. One-way ANOVA followed by a *post-hoc* Tukey's test was applied to test for statistically significant differences among groups or among the sampling points. Data from pre-migratory fish in the Bering Sea were classified into genealogical clade A (Japanese population) and clade B (Common haplotypes in Japanese, Russian and North American populations), and correlations between the parameters were determined by Pearson's correlation test ($P < 0.05$ and 0.01). The outlier values detected by the Smirnov–Grubb's test did not bias the results because significant correlations were similarly found even when the outliers were included in the correlation test. Significant correlations were similarly detected when nonparametric Spearman's test was applied (data not shown).

Table 2. Correlation coefficients (*r*) among reproductive parameters collected from male chum salmon in the central Bering Sea

| Parameter | Testis mass | GSI | Plasma T | Plasma 11KT | Plasma FSH | Pituitary FSH | Pituitary LH | GP α 2 mRNA | FSH β mRNA | LH β mRNA |
|--------------------|--|--|--|--|--|--|----------------------------|--|--|-----------------|
| GSI | 0.91 [†] 0.98 [†] | | | | | | | | | |
| Plasma T | 0.73 [†] 0.73 [†] | 0.61 [†] 0.72 [†] | | | | | | | | |
| Plasma 11KT | 0.71 [†] 0.56 [†] | 0.51 [†] 0.64 [†] | 0.65 [†] 0.78 [†] | | | | | | | |
| Plasma FSH | 0.79 [†] 0.48 [†] | 0.73 [†] 0.51 [†] | 0.73 [†] 0.76 [†] | 0.61 [†] 0.66 [†] | | | | | | |
| Pituitary FSH | 0.85 [†] 0.68 [†] | 0.78 [†] 0.64 [†] | 0.75 [†] 0.76 [†] | 0.66 [†] 0.72 [†] | 0.84 [†] 0.52 [†] | | | | | |
| Pituitary LH | 0.86 [†] 0.69 [†] | 0.87 [†] 0.44 [†] | 0.63 [†] 0.68 [†] | 0.61 [†] 0.48 [†] | 0.70 [†] 0.36* | 0.81 [†] 0.95 [†] | | | | |
| GP α 2 mRNA | 0.56 [†] 0.31* | 0.54 [†] 0.31* | 0.43* 0.36 [†] | 0.47* 0.29* | 0.50 [†] 0.37 [†] | 0.63 [†] 0.56 [†] | 0.56* 0.44* | | | |
| FSH β mRNA | 0.52 [†] 0.26* | 0.54 [†] 0.38 [†] | 0.51 [†] 0.46 [†] | 0.49 [†] 0.44 [†] | 0.52 [†] 0.51 [†] | 0.53 [†] 0.69 [†] | 0.54* 0.33* | 0.88 [†] 0.84 [†] | | |
| LH β mRNA | 0.41* 0.45* | 0.50 [†] 0.58 [†] | 0.33 0.47 [†] | 0.33 0.47 [†] | 0.28 0.37 [†] | 0.51 [†] 0.62 [†] | 0.58* 0.56 [†] | 0.78 [†] 0.61* | 0.81 [†] 0.66 [†] | |
| TSH β mRNA | 0.22 0.20 | 0.23 -0.07 | 0.32 0.11 | 0.01 -0.05 | 0.15 0.00 | 0.33 0.17 | 0.32 0.28 | 0.59 [†] 0.36 [†] | 0.37* 0.10 | 0.25 0.14 |

The samples, which were collected in June and July, were grouped as clade A (upper value, *N*=17–34) and clade B (lower value, *N*=41–72) on the basis of mitochondrial DNA haplotypes. Clade A is the Japanese population, and clade B is a mixture of Japanese, Russian and North American populations.

**P*<0.05, [†]*P*<0.01.

RESULTS

Gonadal maturity of pre-migratory chum salmon in the Bering Sea

Chum salmon in the Bering Sea were a mixture of immature and maturing fish as is shown by frequency distributions of the GSI (gonadosomatic index) in June and July (Fig. 2). The GSI of most 1- and 2-year-old fish was about 0.1% in the males and about 1.0% in the females, whereas the GSI of 3- and 4-year-old fish ranged between 0.1% and 10% in the males, and 1.0% and 20% in the females. In September, the GSI of most 3-year-old males was about

0.1%, and that of females was about 1.0%. Histological analysis of gonads showed that chum salmon caught in June and July were a mixture of immature fish and maturing adults, while fish caught in September were almost exclusively immature (Table 1). Most of the 1- and 2-year-old fish were identified as immature fish (data not shown). The maturing adults with gonads of advanced developmental stages thus left the Bering Sea by the end of summer.

Identification of mtDNA haplotypes showed that about 30% of maturing adult I fish belonged to the genealogical clade A, i.e. the Japanese population (supplementary material Table S1). Other

Table 3. Correlation coefficients (*r*) among reproductive parameters collected from female chum salmon in the central Bering Sea

| Parameter | Ovary weight | GSI | Plasma T | Plasma E2 | Plasma FSH | Pituitary FSH | Pituitary LH | GP α 2 mRNA | FSH β mRNA | LH β mRNA |
|--------------------|--|--|--|--|----------------------------|--|----------------------------|--|--|-----------------|
| GSI | 0.96 [†] 0.94 [†] | | | | | | | | | |
| Plasma T | 0.72 [†] 0.74 [†] | 0.71 [†] 0.74 [†] | | | | | | | | |
| Plasma E2 | 0.50 [†] 0.70 [†] | 0.55 [†] 0.59 [†] | 0.61 [†] 0.65 [†] | | | | | | | |
| Plasma FSH | 0.33* 0.26* | 0.37* 0.21 | 0.62 [†] 0.29* | 0.53 [†] 0.37 [†] | | | | | | |
| Pituitary FSH | 0.92 [†] 0.85 [†] | 0.91 [†] 0.81 [†] | 0.85 [†] 0.44* | 0.59 [†] 0.64 [†] | 0.49* 0.47 [†] | | | | | |
| Pituitary LH | 0.81 [†] 0.81 [†] | 0.76 [†] 0.74 [†] | 0.59 [†] 0.50 [†] | 0.27 0.52 [†] | 0.01 0.40* | 0.78 [†] 0.79 [†] | | | | |
| GP α 2 mRNA | 0.42* 0.39 [†] | 0.44 [†] 0.40 [†] | 0.27 0.32 [†] | 0.52 [†] 0.35 [†] | 0.08 0.34 [†] | 0.65 [†] 0.72 [†] | 0.46* 0.54* | | | |
| FSH β mRNA | 0.42 [†] 0.39 [†] | 0.46 [†] 0.39 [†] | 0.35* 0.45 [†] | 0.56 [†] 0.36 [†] | 0.20 0.36 [†] | 0.69 [†] 0.69 [†] | 0.25 0.55* | 0.91 [†] 0.92 [†] | | |
| LH β mRNA | 0.54 [†] 0.41 [†] | 0.52 [†] 0.43 [†] | 0.36* 0.41 [†] | 0.49 [†] 0.36 [†] | 0.07 0.35 [†] | 0.73 [†] 0.70 [†] | 0.83* 0.45 [†] | 0.78 [†] 0.79* | 0.70 [†] 0.82 [†] | |
| TSH β mRNA | -0.08 -0.08 | -0.09 -0.10 | -0.06 -0.10 | 0.12 -0.02 | -0.03 -0.02 | -0.14 0.22 | -0.24 0.24 | 0.44 [†] 0.38 [†] | 0.37* 0.32* | 0.08 0.11 |

The samples that were collected in June and July were grouped as clade A (upper value, *N*=20–40) and clade B (lower value, *N*=32–67) on the basis of mitochondrial DNA haplotypes. Clade A is the Japanese population, and clade B is a mixture of Japanese, Russian, and North American populations.

**P*<0.05, [†]*P*<0.01.

maturing adult I fish were identified as clade B, i.e. a mixture of Japanese, Russian and North American populations, and clade C, i.e. a mixture of the Japanese and Russian populations. About half of the maturing adult I fish are thus considered to belong to the Japanese population. The proportion of maturing adult II fish that belonged to clade A was much lower than that of maturing adult I fish.

Increases in the amount of GTH subunit mRNAs and GTH in the pituitaries of pre-migratory chum salmon in the Bering Sea

The absolute amounts of mRNAs encoding GTH subunits in the pituitaries of the maturing adults were significantly higher than those of the immature fish in June and July (Figs 3 and 4). The amounts of $GP\alpha 2$ and FSH β mRNAs in the maturing adults were two- to fivefold those observed in the immature fish (Fig. 3A,B). Such differences were seen in the three years examined. In 2001, the amount of FSH β mRNA in the maturing adults in the Bering Sea was similar to that in the fish in the NW Pacific Ocean. There was also more FSH in the pituitary of the maturing adults than in the immature fish (Fig. 3C). In the immature fish, the FSH content in the fish caught in June and July did not significantly differ from that in the fish caught in September.

Pituitary LH β mRNA was about 100-fold higher in the maturing adults than in the immature fish in all three years examined (Fig. 4A). LH in the pituitary of the maturing adults was also about 1000-fold higher than that in the immature fish (Fig. 4B). Such differences were not observed in the amount of TSH β mRNA (data not shown). In the immature fish, the amount of LH β mRNA and LH in fish caught in June and July did not significantly differ from the amounts observed in fish caught in September.

Increases in the plasma levels of FSH, T, 11KT and E2 in pre-migratory chum salmon in the Bering Sea

The plasma levels of hormones related to the activity of the PG axis in the maturing adults were higher than those in the immature fish, in accordance with the above-mentioned higher synthesis of GTHs. In males, the plasma level of FSH in the maturing adults was two- to threefold that found in the immature fish (Fig. 5). T and 11KT in the plasma of the immature fish were both about 1.0 nmol l^{-1} , whereas in the maturing adults 10 nmol l^{-1} were recorded in the three years examined. In 2001, the levels of T and 11KT in the maturing adults caught in the NW Pacific Ocean were more than 10 nmol l^{-1} , which was the same level as those observed in fish caught in the central Bering Sea.

In females, the plasma level of FSH in the maturing adults were 1.5- to 2.5-fold the levels seen in the immature fish (Fig. 5). In 2001, the FSH level in the maturing females caught in the NW Pacific Ocean was higher than that observed in the immature fish. T and E2 levels in the plasma of the immature fish were both about 1.0 nmol l^{-1} , whereas in the maturing adults they were more than 10 nmol l^{-1} , in the three years examined.

The elevation of the PG-axis activity was observed regardless of the origins of chum salmon

There were positive correlations among the pituitary, plasma and gonadal parameters for the activity of the PG axis in pre-migratory chum salmon of both males (Table 2) and females (Table 3). Maturing adults that showed high pituitary GTHs were observed in both clade A and B (Fig. 6). The plasma levels of FSH, T and 11KT in males (Table 2) and FSH, T and E2 in females (Table 3) correlated positively ($P < 0.05$) with the gonad masses and the GSI in both clades. The plasma levels of T, 11KT or E2 correlated positively

with the amount of GTH subunit mRNAs and the contents of FSH and LH in the pituitaries. By contrast, the amount of mRNA encoding another GP, i.e. TSH β , did not correlate with the GSI and other parameters related to sexual maturation.

Elevation of the PG-axis activity was initiated in the winter chum salmon in the Gulf of Alaska

Maturing adults with higher activity of the PG axis were found even in the Gulf of Alaska in February 2006 (Fig. 7). About 30% of males and 45% of females were maturing adult I (Table 1). The levels of pituitary FSH and LH in the maturing fish were significantly higher

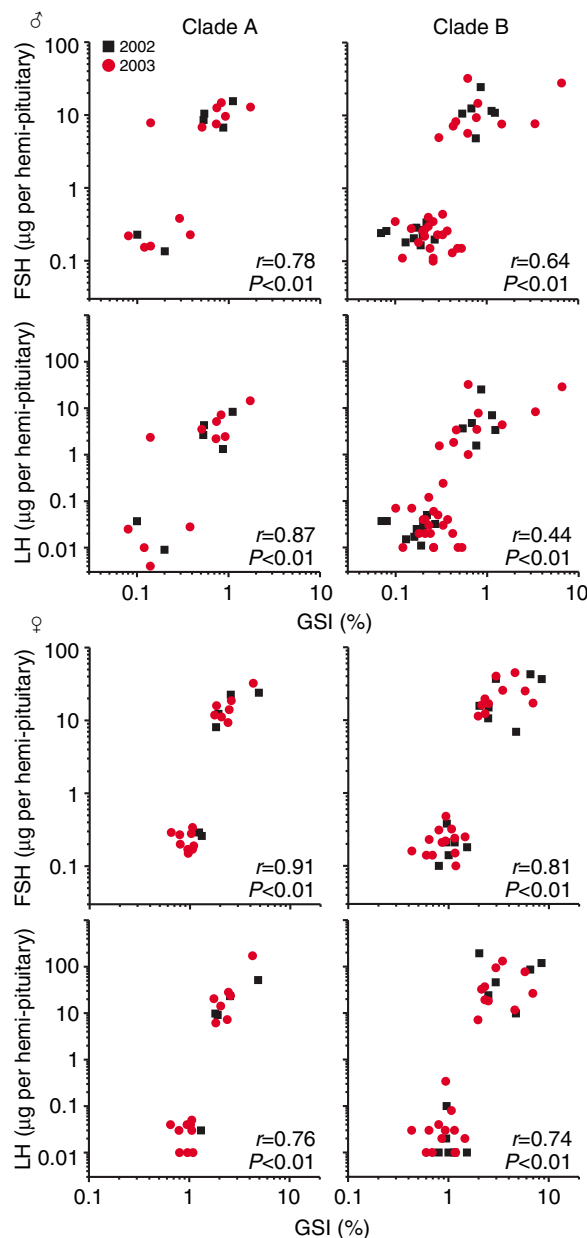


Fig. 6. Scatter plots showing the correlation between the gonadosomatic index (GSI) and the pituitary contents of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in pre-migratory male and female chum salmon in the central Bering Sea, 2002-2003. Clade A is made up of Japanese populations and clade B is a mixture of Russian, North American and Japanese populations. The maturing adults with increased FSH and LH were observed in both clades.

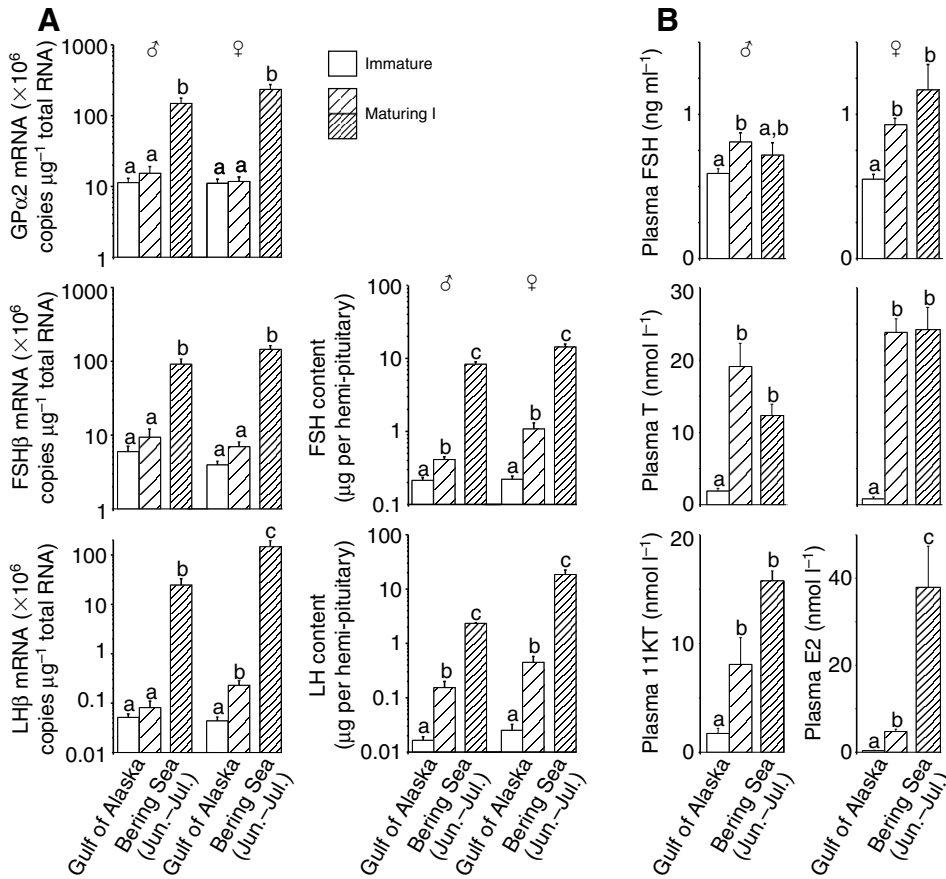


Fig. 7. Activity of the pituitary-gonadal axis (PG axis) in winter chum salmon in the Gulf of Alaska in terms of (A) the amounts of glycoprotein $\alpha 2$ (GP $\alpha 2$), follicle-stimulating hormone β (FSH β) and luteinizing hormone (LH β) mRNAs, FSH and LH in the pituitary and (B) the plasma level of FSH, testosterone (T), 11-ketotestosterone (11KT) and estradiol (E2). Fish were collected in February 2006, and divided into immature fish and maturing adult I on the basis of histological features of the gonads. Samples from the winter chum salmon were assayed together with those from the maturing Bering fish in 2003 ($N=5$ in each sex) for comparison of data with the summer fish in the Bering Sea. Value are means \pm s.e.m. See Table 1 for the number of fish.

than in the immature fish. The levels of plasma FSH and T in both sexes, 11KT in the males and E2 in females were several to 10-fold those in the immature fish. The levels of these PG-axis hormones correlated positively ($P<0.05$) with the gonad masses and the GSI in both sexes (data not shown).

The magnitude of increase in the activity of the PG-axis components in the maturing fish in the Gulf of Alaska were lower compared to those of the Bering Sea fish (Fig. 7). The absolute amounts of GTH subunit mRNAs and FSH and LH of the maturing fish in the Gulf of Alaska were less than one tenth those from the

Table 4. Fork length, body mass, gonadosomatic index and spermiation or ovulation ratio (ratio of fish that had spermiated or ovulated) of homing adults in 2002

| Sex | Point | Date of sampling | N | FL (cm) | BM (kg) | GSI (%) | Spermiation or ovulation (%) |
|--------|-------|------------------|----|-----------------|----------------------------------|-----------------------------------|------------------------------|
| Male | A | 19 Sep. | 10 | 67.3 \pm 1.8 | 3.84 \pm 0.24 ^{a,b} | 5.01 \pm 0.18* | 0.0 |
| | B | 30 Sep. | 10 | 69.0 \pm 1.3* | 3.83 \pm 0.22 ^{a,b} | 5.91 \pm 0.24* | 0.0 |
| | C | 1 Oct. | 10 | 72.3 \pm 1.1* | 4.50 \pm 0.22 ^{a*} | 5.07 \pm 0.11* | 0.0 |
| | D | 2 Oct. | 5 | 67.9 \pm 1.2 | 3.66 \pm 0.25 ^{a,b} | 5.24 \pm 0.16* | 0.0 |
| | E | 3 Oct. | 8 | 65.1 \pm 2.2 | 3.12 \pm 0.29 ^b | 5.25 \pm 0.59* | 0.0 |
| | F | 3 Oct. | 8 | 71.9 \pm 1.3* | 4.14 \pm 0.28 ^{a,b,*} | 4.64 \pm 0.48* | 75.0 |
| | G | 2 Oct. | 10 | 68.5 \pm 1.4* | 3.61 \pm 0.25 ^{a,b} | 4.97 \pm 0.38* | 100.0 |
| Female | A | — | 11 | 67.4 \pm 1.3* | 3.86 \pm 0.20* | 12.91 \pm 0.42 ^{a,*} | 0.0 |
| | B | — | 10 | 67.8 \pm 1.1* | 3.68 \pm 0.21* | 14.86 \pm 0.32 ^{a,b,*} | 0.0 |
| | C | — | 10 | 69.4 \pm 1.0* | 3.96 \pm 0.19* | 16.90 \pm 0.85 ^{b,c,*} | 0.0 |
| | D | — | 8 | 65.8 \pm 0.8 | 3.40 \pm 0.16 | 15.76 \pm 0.42 ^{a,b,*} | 0.0 |
| | E | — | 8 | 68.8 \pm 0.9* | 3.84 \pm 0.22* | 16.37 \pm 0.54 ^{b,*} | 0.0 |
| | F | — | 9 | 68.4 \pm 1.3* | 3.60 \pm 0.25 | 20.64 \pm 1.29 ^{c,d,*} | 33.3 |
| | G | — | 10 | 66.7 \pm 1.4 | 3.21 \pm 0.23 | 21.46 \pm 1.14 ^{d,*} | 100.0 |

FL, fork length; BM, body mass; GSI, gonadosomatic index.

A–G, correspond to the sampling points in Fig. 1B; A, Northeast coast of Hokkaido; B,C, coastal areas in Ishikari Bay; D, Ishikari River estuary; E, midway point of the upriver migration; F, fish wheel located 4 km downstream of the hatchery; and G, the Chitose salmon hatchery.

Values are means \pm s.e.m. Superscripts letters indicate significant difference ($P<0.05$) within the same sex and year. * $P<0.05$ compared to maturing adult I in the central Bering Sea.

Data in 2001 and 2003 are shown in Table S2 in supplementary material.

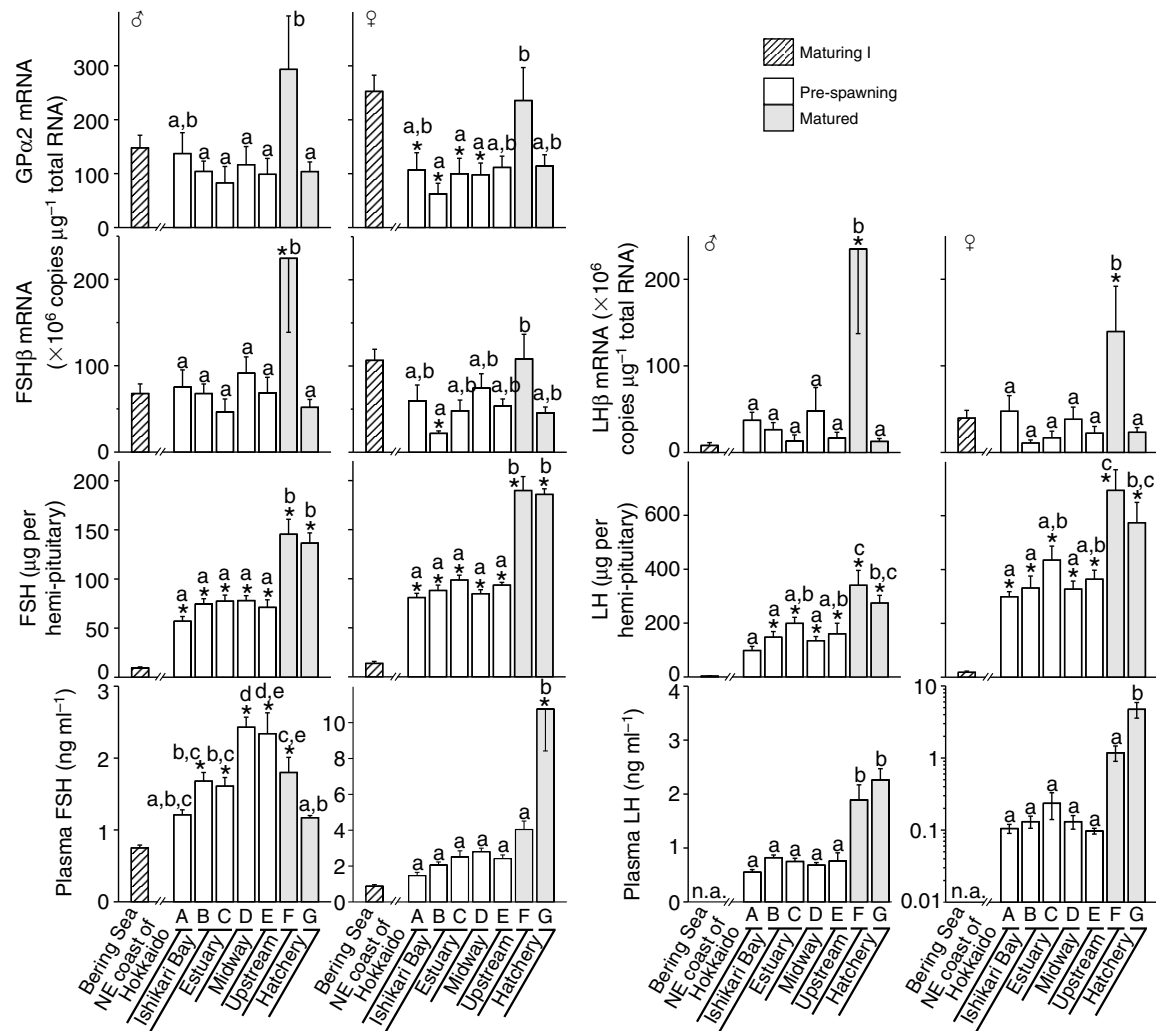


Fig. 8. Changes in the levels of gonadotropin (GTH) subunit mRNAs in the pituitary, and pituitary and plasma levels of follicle-stimulating hormone FSH and luteinizing hormone LH during upstream migration in 2002. Pre-spawning and mature adults were collected along their homing pathway from the coast to the hatchery, as shown in Fig. 1B. Fish near or at the hatchery almost completed final maturation, so that they were considered as mature adults. Furthermore, data are compared with those from maturing adult 1 in the Bering Sea. Date of sampling and the number of fish are shown in Table 4. Value are means \pm s.e.m. n.a., not applicable. Significant differences among sampling points are identified with different letters ($P < 0.05$ one-way ANOVA and Tukey's test). Asterisks indicate significant difference ($P < 0.05$) when compared with the maturing adult 1 in the central Bering Sea.

Bering Sea. The circulating levels of FSH, T and 11KT in the maturing adults in the Gulf of Alaska attained similar levels to those in the maturing fish in the Bering Sea.

Changes in the PG-axis activity in homing adults

Patterns of change in the activity of the PG axis in pre-spawning chum salmon homing to the natal Ishikari River were similar in 2001, 2002 and 2003 (supplementary material Table S2 and Figs S1–4). Therefore, the results of 2002 were described as a representative of the patterns for the three years examined. In both sexes, the GSI of pre-spawning adults caught off the NE coast of Hokkaido was significantly higher than that of maturing adult 1 in the central Bering Sea (Table 4). In males, the GSI attained the maximum value during upstream migration from the coast to the midway point, and then decreased during further upstream migration because of completion of spermatogenesis and release of milt into abdomen. In females, the GSI was 12–17% during upstream migration from the coast to the hatchery midway

point, then went up to 20% by the time they arrived at the fish wheel near the hatchery. Almost all males had spermiated by the time they arrived at the fish wheel, while all of the females ovulated at the hatchery. Homing adults completed final maturation at or near the hatchery, in accordance with the previous observations from 1995 through 2000 (Onuma et al., 2003a; Onuma et al., 2005).

In the homing adults, the amounts of GPα2, FSHβ and LHβ mRNAs peaked in fish caught at the fish wheel, followed by a decrease at the hatchery (Fig. 8). In both sexes, the pituitary FSH and LH contents in fully mature fish at or near the hatchery were about twofold those observed in pre-spawning adults. The plasma level of FSH in males peaked at the mid-point of the upstream river migration, and that in females peaked when they reached or were near the hatchery. The plasma LH level in fish at the hatchery showed three- to tenfold increases over fish caught at the midway point. Such increases were observed in 2001, 2002 and 2003 (supplementary material Figs S1 and S2).

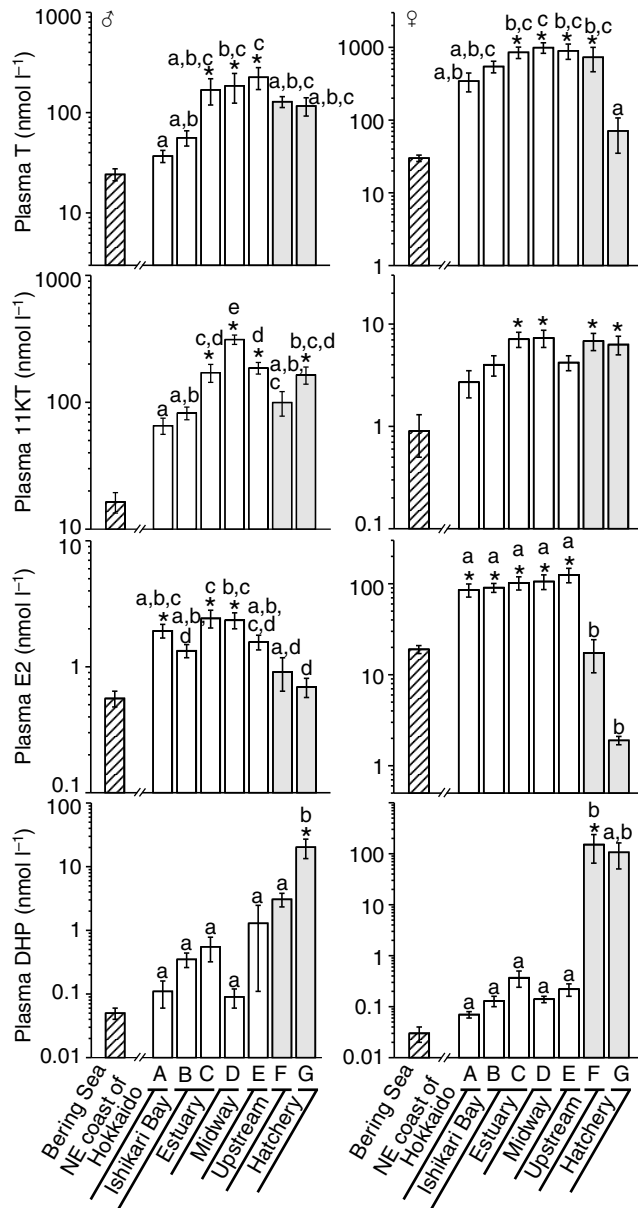


Fig. 9. Changes in plasma levels of testosterone (T), 11-ketotestosterone (11KT), estradiol (E2) and 17α - 20β -dihydroxy-4-pregnen-3-one (DHP) during upstream migration in 2002. Pre-spawning and mature adults were collected along their homing pathway from the coast to the hatchery, as shown in Fig. 1B. Fish at or near the hatchery almost completed final maturation, so that they were considered as mature adults. Furthermore, data are compared with those from maturing adult I in the Bering Sea. Date of sampling and the number of fish are shown in Table 4. Value are means \pm s.e.m. Significant differences among sampling points are identified with different letters ($P < 0.05$ one-way ANOVA and Tukey's test). Asterisks indicate significant difference ($P < 0.05$) when compared with the maturing adult 1 in the central Bering Sea.

The plasma levels of T and 11KT in males were elevated gradually during the spawning migration from the Bering Sea to the Ishikari Bay, and reached up to 100 nmol l^{-1} at the midway point of the upstream migration (Fig. 9). In females, the plasma levels of T and E2 increased to 100 nmol l^{-1} by the time the fish were caught at the midway point of the upstream migration. Thereafter, the levels decreased in mature females caught at or near the hatchery. The

plasma level of DHP, a final maturation-inducing hormone, in fish caught at or near the hatchery was more than 100-fold that of fish caught at the coast and at the midway point of the upstream migration. Such increases were similarly observed in the three years examined both in males (supplementary material Fig. S3) and females (supplementary material Fig. S4).

DISCUSSION

In the present study, we examined changes in the activity of the PG axis of chum salmon from the Bering Sea, about half of which were identified as Japanese stock, at several important locations along their spawning migration. Quantitative information was collected over three years because the activities of the PG axis in pre-spawning chum salmon showed year-to-year variation, which reflected influences of oceanic environments (Onuma et al., 2003a; Onuma et al., 2005). Our present study showed the general pattern of changes in the PG axis of homing chum salmon because the results were similar in the three years examined. The magnitudes of significant differences in the amounts of mRNAs and the levels of hormones were much greater than the assay CVs of 0.7–15.8%. The elevation in the amounts of GTH subunit mRNAs in the maturing adults was not due to changes in the sizes of pituitaries, because the magnitude of increases in copy numbers of mRNAs was much larger than that in body mass (Tables 1 and 4). The present results are thus reliable to discuss how the activity of the PG axis changes during important phases of homing, such as migration from the Gulf of Alaska to the Bering Sea, initiation of homing, entrance into FW, and upstream migration.

Chum salmon in the Bering Sea were a mixture of immature and maturing fish in June and July, whereas most fish were immature in September (Fig. 2). A concern is an influence of catching probability of immature and maturing fish as a result of different fishing methods, i.e. gill net and trawl net. Summer chum salmon in the Bering Sea were collected by the two different fishing methods in 2003, nonetheless the ratio of maturing adults were not different (data not shown). The patterns of distribution of the GSI were similar in the three years. Therefore, the present study showed the relationship of gonadal development to initiation of spawning migration. Chum salmon with gonads of advanced developmental stages left the Bering Sea for the natal river by the end of summer.

Activity of the PG axis during the spawning migration

In the Bering Sea, the maturing adults that were captured in June and July showed increases in the parameters of activity of the PG axis (Figs 3–5). The maturing adults thus left the Bering Sea after the increase in the activity of the PG axis. These increases were commonly observed in both the clade A, i.e. the Japanese population, and the clade B, i.e. a mixture of Japanese, Russian and North American populations (Fig. 6). Therefore, the increase in activity of the PG axis is considered as a general endocrine phenomenon that is inseparable from initiation of the spawning migration of chum salmon.

The increases in the endocrine parameters for activity of the PG axis had already initiated in fish in the Gulf of Alaska in February (Fig. 7). Such activation of the PG axis in winter coincides with seasonal changes seen in hatchery-reared male chinook salmon (Campbell et al., 2003), coho salmon (Campbell et al., 2006) and masu salmon (Furukuma et al., 2008). The previous studies showed that reproductive parameters such as GSI and the plasma levels of FSH, 11-KT and E2 started to elevate from winter to spring. The increase in the PG-axis activity should be triggered several months

before the initiation of spawning migration from the Bering Sea in summer. Future investigations will clarify factors in the immature fish that interact with the reproductive endocrine system and determine whether to mature or not to mature in the coming spawning season.

The increase in the PG-axis activity was reflected by increases in the amounts of mRNAs encoding GTH subunits and both FSH and LH content in the pituitary. In salmonids, FSH is involved in the earlier phases of gametogenesis, whereas LH is involved in the final maturation (Swanson et al., 2003), because LH was not detectable in plasma until the spawning period (Prat et al., 1996; Gomez et al., 1999). In rainbow trout, the amount of FSH receptor mRNA in the testis was higher until March, whereas LH receptor (LH-R) mRNA peaked in August (Kusakabe et al., 2006). These facts indicate that FSH is important for the increase in the GSI and levels of sex steroid hormones in the plasma of chum salmon prior to initiation of spawning migration.

The plasma levels of T, 11KT and E2 in maturing adults were about tenfold those in immature fish. In maturing adults, the concentration exceeded 10 nmol l^{-1} , which is higher than the equilibrium dissociation constants (K_d) of androgen receptors (ARs) and estrogen receptors (ERs). The K_d for ARs and [^3H]T was estimated as $2\text{--}5\text{ nmol l}^{-1}$ in cytosolic and nuclear extracts of goldfish forebrain (Pasmanik and Callard, 1988) and the K_d for ERs and [^3H]E2 was estimated as $1.5\text{--}2.5\text{ nmol l}^{-1}$ in extracts of rainbow trout forebrain (Allison and Omeljaniuk, 2000). Furthermore, implantation of T, 11KT or E2 induced migratory behavior in masu salmon in artificial raceways (Munakata et al., 2001). We consider that the levels of circulating sex steroid hormones in maturing chum salmon migrating from the Gulf of Alaska to the Bering Sea are sufficient to act on the forebrain to initiate migratory behavior, although it is difficult to confirm this by behavioral experiments using oceanic chum salmon.

The amount of LH β mRNA and the LH in the pituitary of maturing adults were more than 100-fold those of immature fish (Figs 4 and 7). Synthesis of LH is thus stimulated in the pituitary of pre-migratory chum salmon in the Bering Sea before summer. Nonetheless, the increase in the GSI and levels of steroid hormones in the plasma cannot be explained by LH, because our preliminary test showed that the plasma level of LH was below the detection limit in the plasma of the maturing adults. Afterwards, the plasma LH level increased in the homing adults when they were near or at the natal hatchery during upstream migration in the spawning period (Fig. 8). Therefore, the synthesized and stored LH in the pituitary of the high sea chum salmon may be released for the final maturation during the last phase of spawning migration.

In homing adults, the amounts of FSH β mRNA and FSH protein in the pituitary, and the plasma levels of FSH were elevated during upstream migration (Fig. 8). However, increases in FSH β mRNA were not apparent in the previous analyses of chum salmon in the Ishikari River–Ishikari Bay system from 1993 to 1999 (Kitahashi et al., 1998; Onuma et al., 2005). Although we do not have any lines of evidence to explain this discrepancy, there are several reports which showed increases in the pituitary FSH contents during the spawning season (Nozaki et al., 1990; Naito et al., 1991; Amano et al., 1992; Amano et al., 1993). The plasma FSH level also increased before spermiation and after ovulation in rainbow trout (Prat et al., 1996; Gomez et al., 1999). Cross reaction of FSH with LH-R was demonstrated by binding of ^{125}I -FSH and ^{125}I -LH to sections of the testis of coho salmon (Miwa et al., 1994), and binding of them to membranes of ovarian

theca-interstitial layers and granulosa cells of coho salmon (Yan et al., 1992). Stimulation of cAMP production by FSH was also seen in COS-7 cells that expressed amago salmon LH-R (Oba et al., 1999). These findings suggest an involvement of FSH in control of the final maturation.

The plasma levels of T, 11KT and E2 were increased in homing adults during migration from the coast to the natal river through the midway point or the junction of the main Ishikari River and the tributary (Fig. 9). Elevated levels of sex steroid hormones have been found to be associated with the adaptation to FW in pre-spawning chum salmon (Makino et al., 2007). Makino et al. found that the plasma levels of T and/or E2 were related to the amount of time spent in FW, when spontaneous behavior of pre-spawning chum salmon was monitored in an aquarium that allowed fish to migrate between separated SW and FW streams (Makino et al., 2007). An application of T, 11KT or E2 directly modulated prolactin (PRL) gene expression, a FW adaptation hormone (Hirano et al., 1987), in primary pituitary cells of pre-spawning and mature masu salmon (Onuma et al., 2007). In homing salmon, the endocrine systems that govern salt and water homeostasis are thus under the influence of activity of the PG axis.

Underlying mechanisms: season-dependent regulation of the PG axis

The amounts of GTH subunit mRNAs in the pituitary and the plasma levels of T, 11KT and E2 in maturing adults were more than tenfold those in immature fish in June and July. In masu salmon reared in aquaria, these parameters were elevated between March and June, and peaked by the spawning season in late September (Kitahashi et al., 2004). Seasonal changes in the activity of the PG axis of ocean-migrating chum salmon thus coincided with those in farmed masu salmon. The regulatory mechanisms of the PG axis in ocean-migrating chum salmon appears to be similar to those found in aquaculture condition (Ando and Urano, 2005).

The activity of the salmonid PG axis is regulated primarily by hypophysiotropic salmon gonadotropin-releasing hormone (sGnRH) neurons in the ventral telencephalon and the preoptic area (Amano et al., 1997). Salmon GnRH directly elevated the amounts of GP α 2 and FSH β mRNAs in pituitaries of maturing coho salmon *in vitro* in May (Dickey and Swanson, 2000). In masu salmon, the amount of sGnRH mRNAs in the forebrain loci were already high in the pre-pubertal stage from winter to spring, declined toward summer, and then increased in the spawning period in autumn (Ando et al., 2001; Kitahashi et al., 2004). Such changes corresponded to seasonal changes in the sGnRH content in the forebrain, whereas the pituitary sGnRH was gradually elevated from spring to autumn (Amano et al., 1992; Amano et al., 1993). We recently found that sGnRH mRNA increased in the ventral telencephalon and the preoptic area of maturing chum salmon in the winter in the Gulf of Alaska (our unpublished data). Since the amount of sGnRH mRNA was not different between the immature fish and maturing adults in the summer in the Bering Sea (Onuma et al., 2008), sGnRH gene expression seemed to be increased prior to the arrival of the chum salmon in the Bering Sea. The PG axis of pre-migratory chum salmon may be activated by sGnRH neurons until early summer, prior to initiation of spawning migration.

In terms of the neuroendocrine control of spawning migration, sGnRH neurons have the appropriate neuroanatomical features to coordinate functions of the PG axis and the central nervous system (Urano et al., 1999; Onuma et al., 2005). In salmonids, sGnRH immunoreactive fibers are localized in the neurohypophysis and the various brain loci including the optic tectum and the hypothalamic

neurosecretory neurons. In addition to its well-established function in controlling the PG axis, sGnRH has been shown to directly increased electric activities of magnocellular neurosecretory neurons in rainbow trout (Saito et al., 2003), and facilitated synaptic transmission of retinotectal neurons (Kinoshita et al., 2007). Behaviorally, administration of the GnRH analog (GnRHa) to pre-spawning chum salmon stimulated a preference for FW, and shortened the duration of the homing migration from the mouth of the Ishikari River to the hatchery (Kitahashi et al., 2001). These findings strongly support our hypothesis that the GnRH–GTH system is essential for the control of migratory events, including coordination of the initiation of gonadal development and the spawning migration.

The plasma levels of T, 11KT and E2 also increased in line with the increases in the amounts of GTH subunit mRNA (Table 2 and 3). In salmonids, there is an abundance of evidence to show that sGnRH and sex steroid hormones synergistically stimulate syntheses of GTHs from spring to early summer (Ando and Urano, 2005). Co-incubation with both sGnRH and E2 increased the amounts of LH β mRNA and released FSH and LH from primary pituitary cells of masu salmon (Ando et al., 2004). In March and May, sGnRH, T or E2 elevated expression of the salmon fushi tarazu factor 1 homolog (sFF1-I) gene and estrogen receptor (ER) α , which directly activated the salmon LH β promoter (Le Drean et al., 1996), in primary pituitary cells of masu salmon (Onuma et al., 2007). Furthermore, T and E2 elevated the amounts of LH β mRNA in primary pituitary cells of immature rainbow trout (Xiong et al., 1994), maturing coho salmon in February and April (Dickey and Swanson, 1998), and maturing masu salmon in May (Ando et al., 2004). Such stimulatory effects of sex steroids were attenuated after the late stages of gametogenesis in late July (Xiong et al., 1994; Ando et al., 2004; Onuma et al., 2007). Both transcription and translation of GTHs are thus season-dependently upregulated from early spring to summer before the period when chum salmon initiate spawning migration and leave the Bering Sea for their natal river.

In conclusion, the activity of the PG axis was increased in chum salmon during migration from the Gulf of Alaska to the Bering Sea from winter to summer. The increase in activity of the PG axis is one of the seasonal endocrine events that are inseparable from the onset of spawning migration.

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