HOKKAIDO UNIVERSITY

Title	Molecular biological study on imprinting related genes in salmon
Author(s)	Hino, Hiroshi
Degree Grantor	北海道大学
Degree Name	博士(水産科学)
Dissertation Number	甲第8241号
Issue Date	2007-03-23
DOI	https://doi.org/10.14943/doctoral.k8241
Doc URL	https://hdl.handle.net/2115/26199
Type	doctoral thesis
File Information	thesis.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Molecular biological study on imprinting related genes in salmon

Hiroshi Hino

Division of Marine Bioresource and
Environmental Science
Graduate School of Fisheries Sciences
Hokkaido University

2007

CONTENTS

I. Introduction 1

II-1. Isolation of olfactory imprinting related genes from lacustrine sockeye salmon by cDNA-representational difference analysis 7

Materials and methods

1) Fish 7

2) Construction of a forward subtractive cDNA library 7

3) Differential screening 8

4) Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) 13

Results

1) One-year-old fish specific cDNAs 15

2) Semi-quantitative RT-PCR analysis 17

II-2. Characterization of an olfactory imprinting related gene (SOIG) in adromous salmonids 19

Materials and methods

1) Fish 19

2) Northern blot analysis 26

3) *In situ* hybridization 26

4) Construction of cDNA library 27

5) Screening and cloning 28

6) Accession numbers 28

7) Cloning of cDNA fragments encoding SOIG from salmonids 29

8) Amplification of 5' and 3' ends of cDNA fragments encoding SOIG of masu salmon and chum salmon 29

9) Time-resolved fluoroimmunoassay for serum thyroid hormone 31

10) Real time PCR 32

11) Triiodothyronine (T_3) injection 33

12) Statistical analysis 33

Results

1) Northern blot analysis for SOIG mRNA expression in five salmonids ——— 35

2) Localization of SOIG mRNA in the olfactory system of lacustrine sockeye salmon ——— 35

3) Expression of SOIG mRNA in the olfactory bulb of one-year-old individual lacsutrine sockeye salmon ——— 39

4) cDNA cloning and characterization of SOIG ——— 39

5) Isolation of cDNA encoding SOIG from four salmonid species ——— 42

6) Changes in condition factor and serum T4 concentrations of one-year-old lacustrine sockeye salmon ——— 48

7) Sexual maturation of lacustrine sockeye salmon and chum salmon ——— 51

8) Changes in SOIG mRNA levels during ontogeny of lacustrine sockeye salmon ——— 51

9) Changes in SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon from emergence to post-PST ——— 51

10) Changes in SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon during sexual maturation ——— 53

11) Comparison of SOIG mRNA levels in one-year-old and three-year-old lacustrine sockeye salmon ——— 53

12) Changes in SOIG mRNA levels in the olfactory epithelium of chum salmon from early emergence to downstream migration ——— 56

13) Changes in SOIG mRNA levels of chum salmon during homing migration ——— 56

14) Influence of T_3 treatment ——— 59

Discussion ——— 61

III. Characterization of salmon glutamate carboxypeptidase (sGCP) in lacustrine sockeye salmon ——— 70

Materials and methods

1) Fish ——— 70

2) Semi-quantitative RT-PCR ——— 70

3) Construction of cDNA library and screening ——— 71

4) Accession numbers ——— 72

5) Real time PCR ——— 72

6) Statistical analysis ——— 74

Results

1) Tissue distributions of sGCP mRNA in lacustrine sockeye salmon ——— 75

2) cDNA cloning and characterization of sGCP ——— 75

3) Changes in the expression levels of sGCP mRNA in the olfactory bulb of lacustrine sockeye salmon from pre-PST to post-PST ——— 82

Discussion ——— 84

IV. General discussion ——— 88

V. Summary ——— 95

VI. Acknowledgements ——— 98

VII. References ——— 99

I. Introduction

In Japan, four species of anadromous salmonids (sockeye salmon, *Oncorhynchus nerka*; masu salmon, *O. masou*; chum salmon, *O. keta*; pink salmon, *O. gorbuscha*) mainly exist. These salmonids are divided into two types by the difference in the timing of downstream migration and upstream migration. In the first type such as chum salmon and pink salmon, the fry start downstream migration for ocean immediately after emergence, because they have seawater tolerance. After feeding migration for several years in the North Pacific Ocean, adult salmon start homing migration to their natal river for spawning and their gonads have almost ripened during migration in the North Pacific Ocean. In the second type such as sockeye salmon and masu salmon, the fly spends in the natal river for about 16-18 months, and individuals obtain the ability of seawater tolerance throughout the smoltification process and start downstream migration for ocean. Adult salmon carry out upstream migration to their natal river about five months prior to the gonadal maturation.

Anadromous salmonids are important resources for fisheries in Japan. Recently, the resources of chum and pink salmon are abundant and become very important food materials in fishing industry. On the other hand, the resources of masu and sockeye salmon are fluctuated year by year and are low levels in comparison with the former species. Hence, the propagation of these species needs the development of efficient techniques for both producing smolt and improving adult returns, and the elucidation of imprinting mechanisms in anadromous salmonids has become one of the most important subject in salmon propagation project.

The olfactory system of fish mediates prey and kin recognition, reproductive behaviors, and escape from danger. To accomplish these tasks, the olfactory system

detects many substance including amino acids, nucleotides, steroids, and prostaglandins. In teleost, the olfactory epithelium is composed of the olfactory receptor cell, basal cell, supporting cell, goblet cell, crypt cell and ciliated nonsensory cell. In mammals, two functionally distinct classes of chemicals, odorants and pheromones, are detected and processed through anatomically segregated neural pathways in the main olfactory system and the vomeronasal system, respectively (Buck, 2000; Mombaerts, 2004). Volatile odorants are received by a large repertoire of odorant receptors (ORs) expressed in the ciliated olfactory sensory neurons (OSNs) in the olfactory epithelium. In contrast, pheromones are received mostly by vomeronasal receptors (VRs) in the microvillous sensory neurons in the vomeronasal organ. On the other hand, both the ciliated and microvillous OSNs are equally distributed in the olfactory system of fish. Odorant substances dissolved in water are detected by these two types of OSNs in the olfactory epithelium.

The first ORs were identified in the rat, and now referred as the OR superfamily of odorant receptors (Buck and Axel, 1991; Mombaerts, 2004). Subsequent to the initial discovery of the OR superfamily of odorant receptors, two unrelated types of G protein-coupled receptors were identified in the mammalian vomeronasal organ, the V1R receptors (Dulac and Axel, 1995) and the V2R receptors (Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Herrada and Dulac, 1997).

The OR gene superfamily is the largest multigene superfamily described in mammalian genomes. The completion of the mouse genome confirmed the existence of about 1068 potential OR genes (Zhang and Firestein, 2002; Zhang et al., 2004). On the other hand, molecular cloning and genomic DNA blot hybridizations in fish species suggest an OR repertoire size approximately five- to ten-fold smaller than that of

mammalian species (Ngai et al., 1993; Barth et al., 1997). Recently, several odorant receptors have been isolated from the Atlantic salmon, *Salmo salar*, (Wickens et al., 2001; Dukes et al., 2004, 2006).

In general, olfactory transduction initiates the binding of odorants to G protein-coupled seven transmembrane domain receptors in the ciliary membrane of ciliated olfactory receptor neurons (cORNs) and then leads to the activation of adenylate cyclase or phospholipase C to produce the second messenger such as adenosine 3',5'-cyclic monophospate (cAMP) and inositol 1,4,5-triphosphate (IP_3; Lowe et al., 1989; Buck and Axel, 1991). Increase in intercellular levels of these second messengers opens cyclic nucleotide-gated channels and IP_3-activated channels in the membrane of cORNs, resulting in the membrane depolarization of action potentials (Nakamura and Gold, 1987). This depolarization further activates voltage-gated ion channels and generates spike activities, which are transmitted as olfactory information by the axons of cORNs to the olfactory bulb.

Anadromous salmonids are well known by the accurate homing migrations guided by odors imprinted on their natal stream. Behavioral studies have demonstrated that coho salmon (*O. kisutch*) are unable to return their home stream when their olfactory sense was blocked during their homing migration, suggesting that olfaction was crucial for homing migration (Wisby and Hasler, 1954). These studies led to the olfactory hypothesis that salmon memorize the odors of home stream water during their river life (olfactory imprinting) and return to the same home stream by recollecting natal odors. Cooper et al. (1974) reported that the home stream odors were non-volatile. Electrophysiological studies in masu salmon showed that the olfactory organ can discriminate the difference in composition of amino acids between

different river waters (Shoji et al., 2000).

For the timing of olfactory imprinting, Hasler and Scholz (1983) suggested with artificial odorants that juvenile coho salmon learn the odors of their home stream during smoltification, parr-smolt transformation (PST). Nevitt et al. (1994) demonstrated that the peripheral olfactory system of coho salmon is sensitized to a specific odorant after exposure to that odorant during the PST. Dittman et al. (1996) also confirmed the importance of the PST as a sensitive period for olfactory imprinting by coho salmon.

During the PST, the plasma concentration levels of several hormones such as thyroxine (T_4), cortisol, insulin-like growth factor-I, and growth hormone are changed. Morphological and physiological changes such as body silvering and osmoregulatory adaptation to seawater are controlled by these hormones. T_4 also influences the olfactory function in salmonids. Scholz (1980) demonstrated that pre-PST coho salmon with artificially elevated T_4 levels exposed to an odor are able to retain long-term memories as compared with untreated control fish. On the other hand, it is also known that T_4 depresses electro-encephalography (EEG) activity, with no measurable change in electro-olfactogram (EOG) activity, in response to nasal stimulation with L-alanine in Atlantic salmon (Morin et al., 1995). In addition, the triiodothyronine (T_3)-specific binding value of thyroid hormone receptor in the olfactory epithelium were increased markedly at the PST (Kudo et al., 1994). Moreover, thyroid hormone induced olfactory cellular proliferation during the PST (Lema and Nevitt, 2004).

The formation of memory has been intensively studied in mammals from several aspects such as biochemistry, molecular biology, pharmacology, and electrophysiology. Recently, much of the relevant experimental studies are concentrated on the possible

role of long-term potentiation (LTP) in learning and memory, and many of the studies have focused on *N*-methyl-$_{D}$-aspartate (NMDA) receptor which induces LTP and the expression of immediately early genes such as *c-fos*. LTP is formed in the brain of zebrafish, *Danio rerio* (Nam et al., 2004), rainbow trout, *O. mykiss* (Kinoshita et al., 2004), carp, *Cyprinus carpio* (Satou et al., 2006), and lacustrine sockeye salmon (Satou et al., 1996). *c-fos* is also reported to be expressed in the brain of lacustrine sockeye salmon (Fukaya, 1999) and rainbow trout (Matsuoka et al., 1998; Kinoshita et al., 2005). These findings support the possibility that olfactory imprinting of salmon is controlled at the gene level, and that the genes related to imprinting are expressed in the olfactory system (olfactory epithelium, olfactory bulb and olfactory nerve) of salmonid species during the PST.

In this context, the cDNA-representational difference analysis (cDNA-RDA) is available, because it can detect small differences between two samples from sequential stages of development, such as parr and smolt stages. This PCR-based was originally designed to identify genomic differences between two complex genomes (Lisitsyn et al., 1993). The cDNA-RDA was later adapted to enable the isolation of gene transcripts which are differentially expressed in one sample relative to another (Hubank and Schatz, 1994), allowing the detection of low-abundance transcripts in a sample (O'Neill and Sinclair, 1997).

In the present study, I attempted to elucidate the mechanism of olfactory imprinting in salmonids and to isolate the imprinting period related gene from the olfactory system of lacustrine sockeye salmon during the PST by subtractive hybridization technique of cDNA-RDA (chapter II-1). Using the olfactory bulb of one-year-old lacustrine sockeye salmon at a phase of the PST as a tester and

three-year-old fish at a phase of feeding migration stage as a driver, the cDNA-RDA was carried out. From the subtractive cDNA library, I succeed in isolating two clones, clone *2* (sockeye salmon olfactory imprinting related gene; SOIG) and clone *3* (salmon glutamate carboxypeptidase; sGCP), both of which show different mRNA expression levels between the olfactory bulbs of one- and three-year-old lacustrine sockeye salmon.

In chapter II-2, a cDNA clone encoding the full-length SOIG was isolated from the cDNA library constructed from the olfactory epithelium, and the sequence analysis of SOIG was performed. The distribution of SOIG expression in several tissues of lacustrine sockeye salmon, masu salmon, chum salmon, pink salmon, and rainbow trout were examined by Northern blot and *in situ* hybridization. Moreover, changes in the SOIG mRNA levels of the olfactory epithelium during the PST, downstream migration, sexual maturation, and homing migration in either lacustrine sockeye salmon or chum salmon was revealed by real time PCR.

In chapter III, the full-length sGCP cDNA was isolated from the cDNA library of whole brain and its sequence was analyzed. Moreover, expression patterns and changes in the sGCP mRNA levels in the olfactory bulb of lacustrine sockeye salmon during the PST were examined by semi-quantitative RT-PCR and real time PCR.

II-1. Isolation of olfactory imprinting related genes from lacustrine sockeye salmon by cDNA-representational difference analysis

Materials and methods

1) Fish

One- and three-year-old lacustrine sockeye salmon reared in the Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, were used. The fish were reared in 1400 L circular tanks under the natural photoperiod with continuous flow of spring water. Fish were fed on standard commercial pellets.

One-year-old smolt fish (Fork length: 9.5-16 cm, Body weight: 8.1-37.6 g) were sampled in May, 2002. Three-year-old fish (Fork length: 15.2-20.4 cm, Body weight: 37.9-91.8 g) were also sampled in June, 2002. The body color of one-year-old smolt fish was silver, and their fins revealed clear intense black pigments. The three-year-old fish did not show any smolt characteristics. Twenty fish were used from each age group. Fish were anesthetized with 0.005% eugenol (4-ally-2-methoxy-phenol; Wako, Osaka, Japan), and then the olfactory epithelia and olfactory bulbs were surgically isolated. Total RNA was isolated from each tissue using ISOGEN (Nippongene, Toyama, Japan) according to the manufacturer's instruction.

2) Construction of a forward subtractive cDNA library

Subtractive cDNA libraries were constructed using 2 μg of total RNA extracted from the olfactory bulbs of the one- and three-year-old fish (for each group, n=20). First and second strand of cDNA synthesis employed a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Subtraction was performed in two directions. In the forward subtraction

experiment, the olfactory bulb of one-year-old fish was the tester, and in reverse subtraction, the tester was three-year-old fish. The cDNA-RDA technique was performed according to the protocol of Niwa et al. (1997; Fig. 1). Briefly, double stranded cDNA was digested with *Mbo*I and ligated to R-Bgl-12 and R-Bgl-24 adaptors at 16˚C for 16 hr (for primer sequences see Table 1). Amplicons for both tester and driver were generated with PCR reactions using R-Bgl-24. *Mbo*I digestion was used to remove the R-Bgl-12/24 adaptors from both tester and driver amplicons before the tester was ligated to J-Bgl-12/24 adaptors. Subtractive hybridizations were performed at 67˚C for 20 hr. To generate a first difference product (DP1), 0.4 μg of J-Bgl-12/24 adaptors ligated tester was mixed with 40 μg of driver at a ratio of 1:100. DP1 was digested with *Mbo*I to remove J-Bgl-12/24 adaptors before ligation of N-Bgl-12/24 adaptors. To generate DP2, 50 ng of N-Bgl-12/24 adaptors ligated DP1 was mixed with 40 μg of driver at a ratio of 1:800 (DP2). To generate a third difference product (DP3), 100 pg J-Bgl-12/24 adaptors ligated DP2 was mixed with 40 μg of driver at a ratio of 1:400,000. Finally, DP3 was digested with *Mbo*I and the forward subtraction product was subcloned into the *Bam* HI site of pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA). The libraries were plated onto agar plates (50 μg/ml ampicillin) and incubated at 37˚C for 14 hr and then briefly at 4˚C to allow the blue/white staining to be clearly distinguishable.

3) Differential screening

Differential screening was carried out to identify the false positive clones in forward subtractive cDNA library. Approximately 1000 white colonies from the forward subtraction library were randomly picked. The insert of each colony was

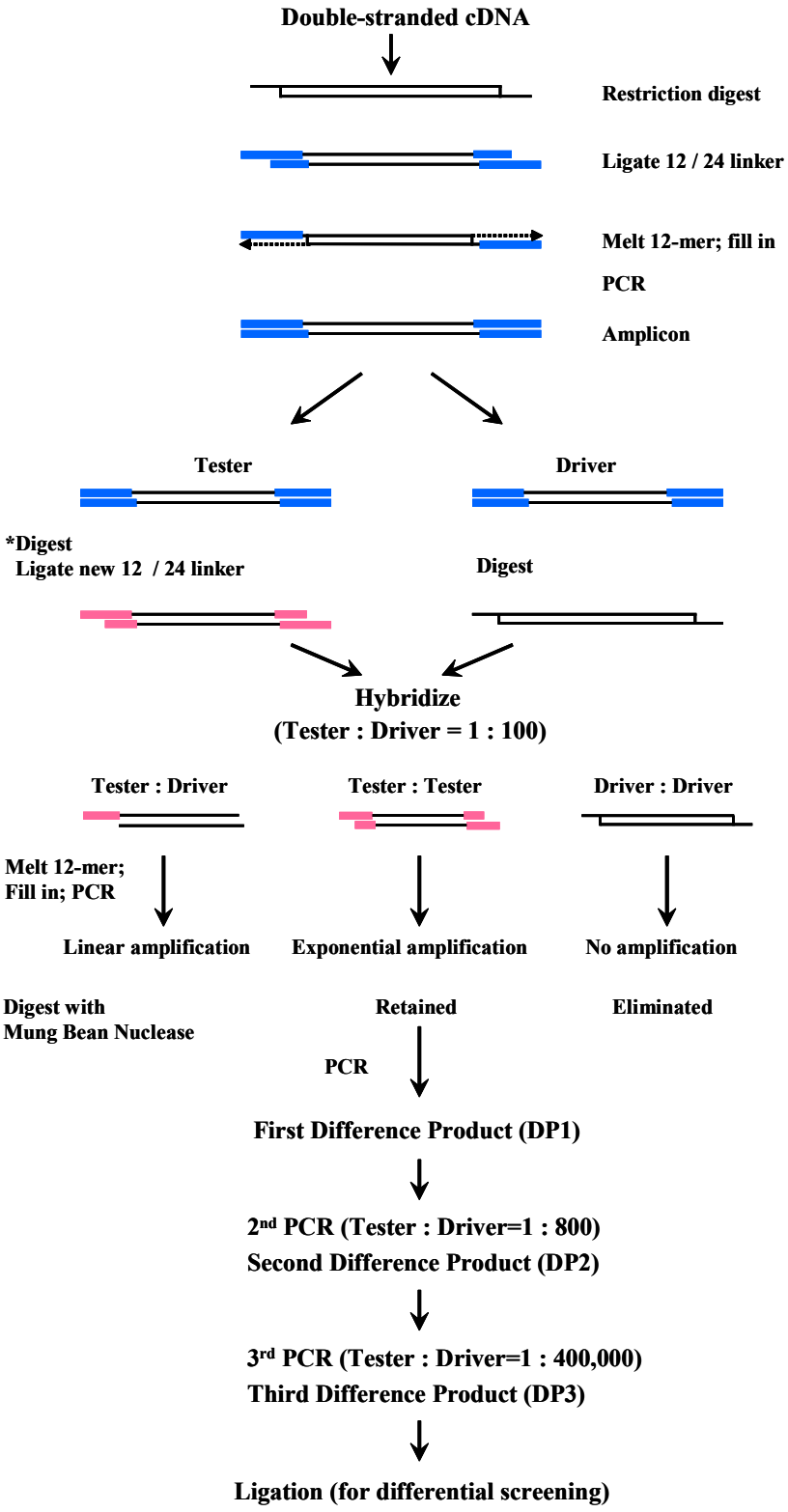


Fig. 1 Schematic protocol for cDNA-representational difference analysis (cDNA-RDA).

Table 1. Sequence of primers used for cDNA-RDA

Primer	Sequences
R-Bgl-12	5′-GATCTGCGGTGA-3′
R-Bgl-24	5′-AGCACTCTCCAGCCTCTCACCGCA-3′
J-Bgl-12	5′-GATCTGTTCATG-3′
J-Bgl-24	5′-ACCGACGTCGACTATCCATGAACA-3′
N-Bgl-12	5′-GATCTTCCCTCG-3′
N-Bgl-24	5′-AGGCAACTGTGCTATCCGAGGGAA-3′

amplified by PCR using T7 and T3 primers. The amplification profile was comprised of an initial cycle of 4 min at 94˚C followed by 30 cycles as follows: 94˚C for 30 sec (dissociation), 55˚C for 30 sec (annealing), and 72˚C for 1 min (extension). Two microliter aliquot of each amplifications was dot-blotted on to a Hybond-N^+ membrane (Amersham Biosciences, Buckinghamshire, UK). Two identical membranes were used for all the PCR amplifications. DIG High Prime (Roche, Mannheim, Germany) was used for generation of hybridization probes. After being treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl), neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA) and fixation solution (0.4 M NaOH), these membranes were prehybridized for 4 hr in a solution of 50% formamide, 5×standard saline citrate (SSC; 16.65 mM NaCl, 16.65 mM sodium citrate, pH7.0), 0.1% N-lauroylsarcocine, 0.02% sodium dodecyl sulfate (SDS) and 1% blocking reagent (Roche) at 42˚C. Membranes were then hybridized in the same buffer at 42˚C for 16 hr with forward or reverse probes from subtraction products. Membranes were washed twice in 2×SSC containing 0.1% SDS for 5 min, followed by 0.1×SSC containing 0.1% SDS for 15 min at 68˚C. Membranes were incubated for 30 min with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (Roche) at 37˚C. The signals were detected with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) reaction.

The clones that hybridized to the forward subtraction probe only were collected and purified (Fig. 2). Sequence was determined by ABI PRISM ^{TM} 377 DNA Sequencer (PerkinElmer Life Sciences, Tokyo, Japan) using the Big dye terminator RR mix (Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Warrington, UK). The sequences were submitted to the DNA Data Bank of Japan (DDBJ: http://www.ddbj

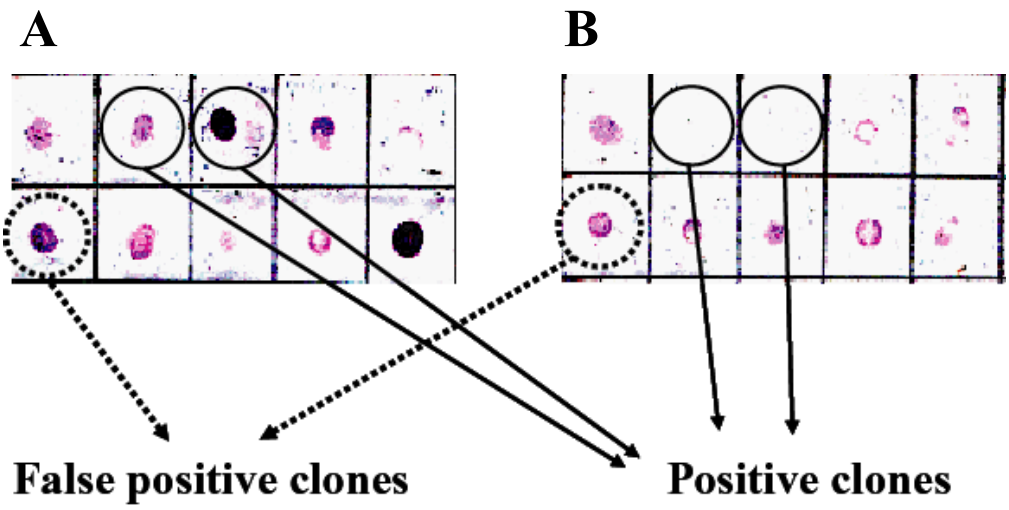


Fig. 2 Typical results of differential screening. The open circles show positive clones. The dotted circles indicate false positive clones. Forward and reverse subtraction product probes were indicated by A and B, respectively.

.nig.ac.jp).

4) Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was performed using the same olfactory bulb total RNA as that used for the cDNA-RDA. Single-stranded cDNAs were synthesized from 2.5 μg of the olfactory bulb total RNA from one- and three-year-old fish using a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. PCR amplification was performed with 0.5 μl of the first strand cDNA solution using primers for clone *1*, *2*, *3* and *4* (Table 2). The amplification profile was comprised an initial cycle of 2 min at 94˚C followed by 35 cycles as follows: 94˚C for 1 min, 58˚C for 1 min, and 72˚C for 1 min. Amplification of ß-actin was also performed with the same first strand cDNA using primers ß-actin (Table 2). The amplification profile was comprised an initiating cycle of 2 min at 94˚C followed by 35 cycles as follows: 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 30 sec. The amplified products were electrophoresed on a 3% agarose gel (Nippongene). A scanning densimeter was used to determine the ratio of intensity of each band relative to β-actin. Densimetric analysis of gel bands was performed by using the National Institutes of Health (NIH) image analysis program.

Table 2. Sequences of primers used for semi-quantitative RT-PCR. AS, Anti-sense primer; S, Sense primer.

Primer	Sequences
1-S	5´-ACAGCTGGTCCAGATGTTCA-3´
1-AS	5´-GCCTTCCATGTAGTCCTCAT-3´
2-S	5´-ACACTCAAGTCCATTGTGGG-3´
2-AS	5´-GGACGACCATTTTTGTCAGTC-3´
3-S	5´-TTGGCGTTACCCTACCGTTA-3´
3-AS	5´-ACTGATCATGGTCACCTTCAG-3´
4-S	5´-ATCCGTCGTATCATGAGACC-3´
4-AS	5´-TTGAGGAGCTTGCTGACCACAT-3´
ß -actin-S	5´-TGTAACATGGGCAGTATCCTT-3´
ß -actin-AS	5´-GATGTCGGAACACATGTGCACT-3´

Results

1) One-year-old fish specific cDNAs

I obtained six clones that hybridized to the forward subtraction probe from differential screening. Their sequences were compared with the data deposited in the DDBJ (Table 3). The clone *1* is 98% similar to TAT-binding protein-1 (TBP-1) of rainbow trout. TBP-1 was discovered as human immunodeficiency virus-related protein from human (Nelbock et al., 1990). In rodents, TBP-1 mRNA exists widely in the brain and is strongly expressed in the olfactory bulb. Moreover, TBP-1 is mainly expressed in the sensory nuclei and many cortical neurons (Nakamura et al., 1998), suggesting that clone *1* is one of the principal molecules in the olfactory bulb of lacustrine sockeye salmon.

The clone *3* shows 67% similarity to glutamate carboxypeptidase-like protein of African clawed frog (*Xenopus laevis*). Glutamate carboxypeptidase II (GCP II) is a metallopeptidase which cleaves *N*-acetylaspartylglutamate (NAAG) into *N*-acetylaspartate and glutamate in the central nervous system (Carter and Coyle, 1998). NAAG was discovered in the mammalian central nervous system (Curatolo et al., 1965) and its function as a neurotransmitter was demonstrated (Coyle, 1997). Recently, Puttfarcken et al. (1993) was reported that NAAG may act as a partial agonist/antagonist at NMDA receptor. The function of clone *3* may engage in the olfactory imprinting if the clone cleaved NAAG which affects as antagonist at NMDA receptor in the olfactory bulb at the PST.

The clone *2* is 60% similar to a mouse (*Mus musculus*) gene, and clone *4* shows 82% similarity to a zebrafish (*Danio rerio*) gene, but these genes showed no similarity to the known sequences in the Genbank database. However, it is likely that clones *2*

Table 3. Clones obtained from forward subtractive cDNA library using the olfactory bulbs of one-year-old lacustrine sockeye salmon.

Clone No.	Size of fragment	Identity	Gene name	Accession No	Species
1	204 bp	98%	TAT-binding protein-1 mRNA, partial cds.	AF281342	*Oncorhynchus mykiss*
2	170 bp	60%	DNA sequence from clone RP23-17616 on chromosome 11	AL596212	*Mus musculus*
3	397 bp	67%	Glutamate carboxypeptidase-like protein 1 mRNA, partial cds	AY188285	*Xenopus laevis*
4	167 bp	82%	cDNA clone MGC: 64169 IMAGE: 67974 00, complete cds.	BC053296	*Danio rerio*
5	193 bp	90%	40S ribosomal protein S3a mRNA	AF402811	*Ictalurus punctatus*
6	203 bp	99%	Beta globin subunit mRNA	AY026061	*Oncorhynchus nerka*

and *4* may represent untranslated regions of the known genes, because these clones have very short fragments (clone *2*: 170 bp, clone *4*: 167 bp; Table 3).

On the contrary, clone *5* is 90% similar to 40S ribosormal protein for catfish (*Ictalurus punctatus*) and clone *6* shows 99% similarity to beta globin subunit mRNA for sockeye salmon (*O. nerka*). These clones were therefore excluded from the candidates for further semi-quantitative RT-PCR analysis.

2) Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was performed with the aim of finding a difference in the expression levels of mRNA between the olfactory bulbs of one- and three-year-old fish (Fig. 3). No differences in the expression levels of clones *1* and *4* were detected in the olfactory bulbs between the two age classes of fish, but clones *2* and *3* mRNA expression levels in the olfactory bulb of one-year-old fish were tended to be higher than that of three-year-old.

In chapters II-2 and III, the characterization of clones *2* and *3* were performed, respectively.

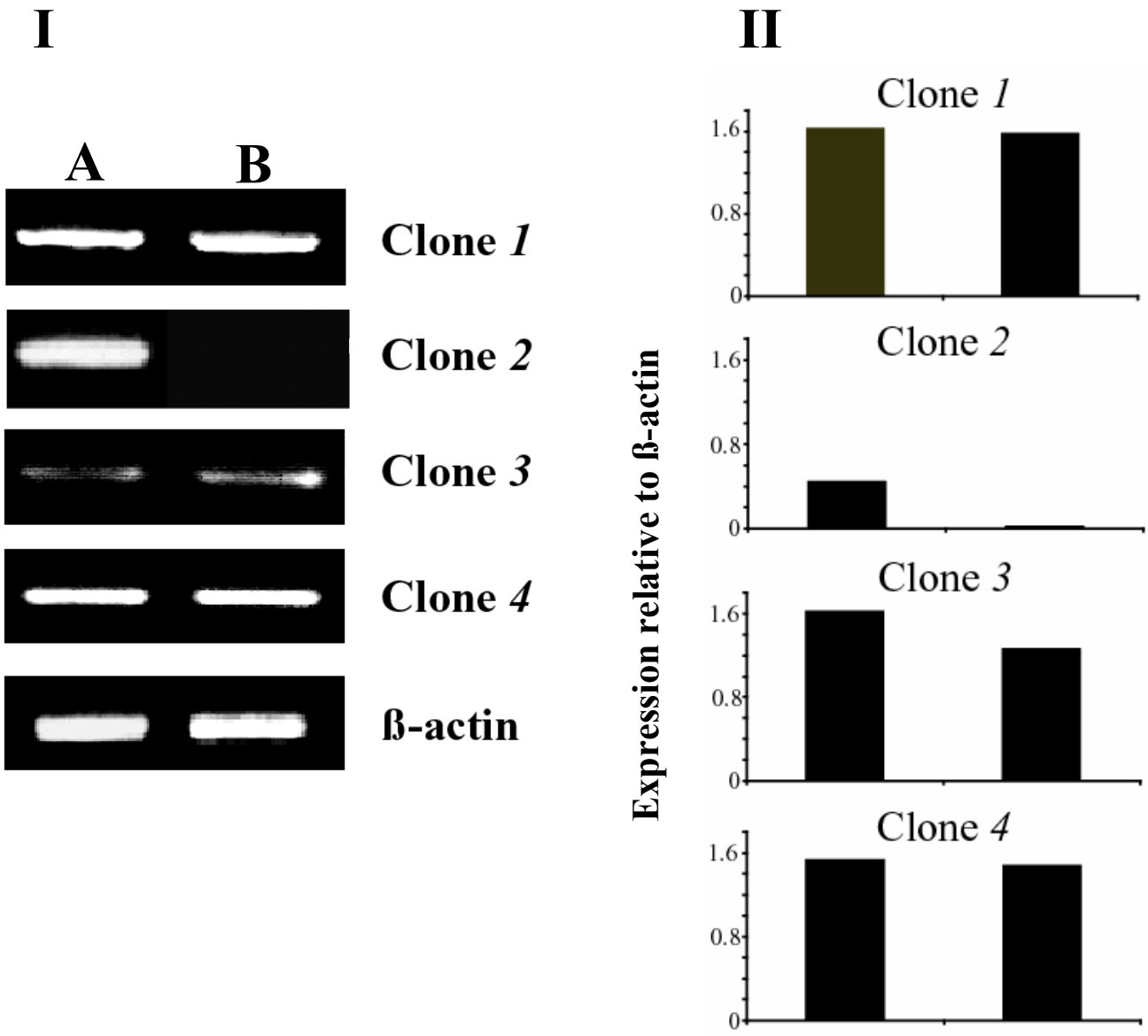


Fig. 3 Semi-quantitative RT-PCR amplification of clones *1*, *2*, *3* and *4*. (I) Results of 3% agarose gel electrophoresis of semi-quantitative RT-PCR products. Specific products for clones *1, 2, 3, 4* and ß-actin mRNAs were amplified from aliquots of the same mRNAs of the olfactory bulb of one- and three-year-old fish. RT-PCR was performed using pooled samples (20 fish per each group). (II) Expression levels of clones *1*, *2*, *3* and *4* in the olfactory bulb of one- and three-year-old relative to β-actin. A and B indicate the olfactory bulb of one- and three-year-old fish, respectively.

II-2. Characterization of an olfactory imprinting related gene (SOIG) in adromous salmonids

Materials and methods

1) Fish

For mRNA expression analysis and SOIG cloning, one-year-old lacustrine sockeye salmon, masu salmon, rainbow trout, under yearling chum salmon and pink salmon were used. Males of masu salmon (Fork length: 9.5-11.0 cm, Body weight: 6.25-12.49 g), rainbow trout (Fork length: 11.3-13.5 cm, Body weight: 11.40-16.47g), and chum salmon (Fork length: 6.7-8.1 cm, Body weight: 2.35-3.78 g) were reared in the Toya Lake Station in May, 2006, and lacustrine sockeye salmon in May, 2004 (Fork length: 10.1-11.6 cm, Body weight: 9.78-11.24g). Pink salmon were obtained from the Shibetsu Salmon Museum in May, 2006 (Fork length: 8.2-9.4 cm, Body weight: 2.87-4.01g).

For construction of cDNA library and T_3 injection, one-year-old male lacustrine sockeye salmon (Fork length: 9.6-10.4 cm, Body weight: 8.32-12.7 g) were obtained from the Toya Lake Station in May, 2005.

Fish were anesthetized with 0.005% eugenol and then the olfactory epithelium, gill, liver, heart, head kidney, spleen, muscle, intestine, testis and brain were surgically isolated. For Northern blot analysis, brains of lacustrine sockeye salmon were dissected into the olfactory bulb, telencephalon, hypothalamus, optic tectum, cerebellum, and medulla oblongata. Total RNA was isolated from each tissue using ISOGEN (Nippongene) according to the manufacturer's instruction.

For quantitative analysis of SOIG mRNA expression during ontogeny of

lacustrine sockeye salmon, embryos reared in the Toya Lake Station in 2005 were sampled at 43 and 60 days after fertilization. At each sampling, five embryos were collected. Alevin (5 fish, 65 days after fertilization) were sampled in the Toya Lake Station in 2005. Total RNA was isolated from the whole embryos and the whole body of alevin where the yolk was surgically removed.

For quantitative analysis of serum T_4 levels and SOIG mRNA expression levels in the olfactory epithelium, tissues of lacustrine sockeye salmon and chum salmon were sampled at several stages in their life history. Fly, one-year-old lacustrine sockeye salmon (from February to September) and three-year-old fish (from June to Octorber) were sampled in the Toya Lake Station in 2006 (5 fish of both sexes). Fork length (FL), body weight (BW), condition factor (CF), and gonadsomatic index (GSI) of one- and three-year-old lacustrine sockeye salmon are shown in Table 4 and Figure 4. CF was calculated by [BW (g)/FL $(cm)^3$] $\times 10^3$. GSI was calculated by [gonadal weight (g)/BW (g)] $\times 10^2$. Under yearling chum salmon were sampled from January to May, 2006 in the Toya Lake Station, and July, 2006 in the Chitose Salmon Aquarium (5 fish of both sexes, Table 5).

Chum salmon during feeding migration were caught by longline at sampling point 56˚ 30' N, 177˚ 00' E in the North Pacific Ocean (Bering Sea) as part of the research cruise of the Wakatake-Maru, and the fish during homing migration stage were caught at six locations along their homing route from offshore to spawning ground, the Chitose Salmon Hatchery in 2003 and 2004 (Fig. 5). FL, BW, and GSI of sampled chum salmon are shown in Table 5 and Figure 6. Fish were anesthetized with 0.005% eugenol and then blood samples of one-year-old lacustine sockeye salmon were collected from the caudal vasculature, and centrifuged at 3000 rpm for 15 min to obtain

Table 4. Fork length (FL), body weight (BW), condition factor (CF), and gonado-somatic index (GSI) of lacustrine sockeye salmon in 2006. (A) One-year-old lacustrine sockeye salmon from February to September. (B) Three-year-old lacustrine sockeye salmon from June to October during sexual maturation.

A

Year	Sex	Date	*n*	FL (cm)	BW (g)	CF (%)
2006	Male	2-Feb	5	7.94 ± 0.44	4.150 ± 0.693	8.05 ± 0.24
	Female		5	7.92 ± 0.29	4.208 ± 0.415	8.37 ± 0.17
	Male	23-Mar	5	8.70 ± 0.33	6.048 ± 0.581	9.11 ± 0.29
	Female		5	9.16 ± 0.17	7.283 ± 0.395	9.44 ± 0.17
	Male	18-Apr	5	8.96 ± 0.18	6.018 ± 0.436	8.30 ± 0.19
	Female		5	9.62 ± 0.32	7.723 ± 0.889	8.51 ± 0.19
	Male	22-May	5	10.16 ± 0.27	8.390 ± 0.919	7.86 ± 0.21
	Female		5	10.18 ± 0.40	8.693 ± 0.901	8.14 ± 0.16
	Male	26-Jun	5	9.40 ± 0.19	7.614 ± 0.305	9.14 ± 0.22
	Female		5	9.14 ± 0.22	5.689 ± 0.789	6.62 ± 0.36
	Male	31-Jul	5	10.44 ± 0.28	9.794 ± 0.633	8.57 ± 0.19
	Female		5	10.38 ± 0.44	9.724 ± 1.124	8.52 ± 0.41
	Male	23-Aug	5	10.50 ± 0.43	10.687 ± 1.212	9.10 ± 0.36
	Female		5	10.72 ± 0.36	10.979 ± 0.648	9.03 ± 0.81
	Male	11-Sep	5	11.04 ± 0.62	13.394 ± 1.648	10.00 ± 0.95
	Female		5	12.04 ± 0.19	16.948 ± 0.473	9.72 ± 0.25

B

Year	Sex	Date	*n*	FL (cm)	BW (g)	GSI (%)	Sperminatio or Ovulation
2006	Male	26-Jun	5	21.20 ± 1.46	89.52 ± 22.71	2.91 ± 1.37	No
	Female		5	22.28 ± 1.38	119.40 ± 30.51	3.21 ± 0.42	No
	Male	31-Jul	5	21.77 ± 1.42	106.42 ± 24.85	1.10 ± 1.05	No
	Female		5	21.90 ± 0.65	105.24 ± 7.91	4.83 ± 1.17	No
	Male	24-Aug	5	21.42 ± 0.46	112.22 ± 5.83	4.71 ± 0.30	No
	Female		5	24.96 ± 1.09	178.83 ± 29.01	10.64 ± 1.21	No
	Male	11-Sep	5	22.72 ± 0.53	129.56 ± 14.82	3.31 ± 0.27	No
	Female		5	23.14 ± 0.60	144.52 ± 13.84	12.99 ± 0.22	No
	Male	3-Oct	5	20.98 ± 0.34	98.22 ± 6.00	3.13 ± 0.28	Yes
	Female		5	22.18 ± 0.77	118.20 ± 14.37	12.92 ± 0.88	Yes

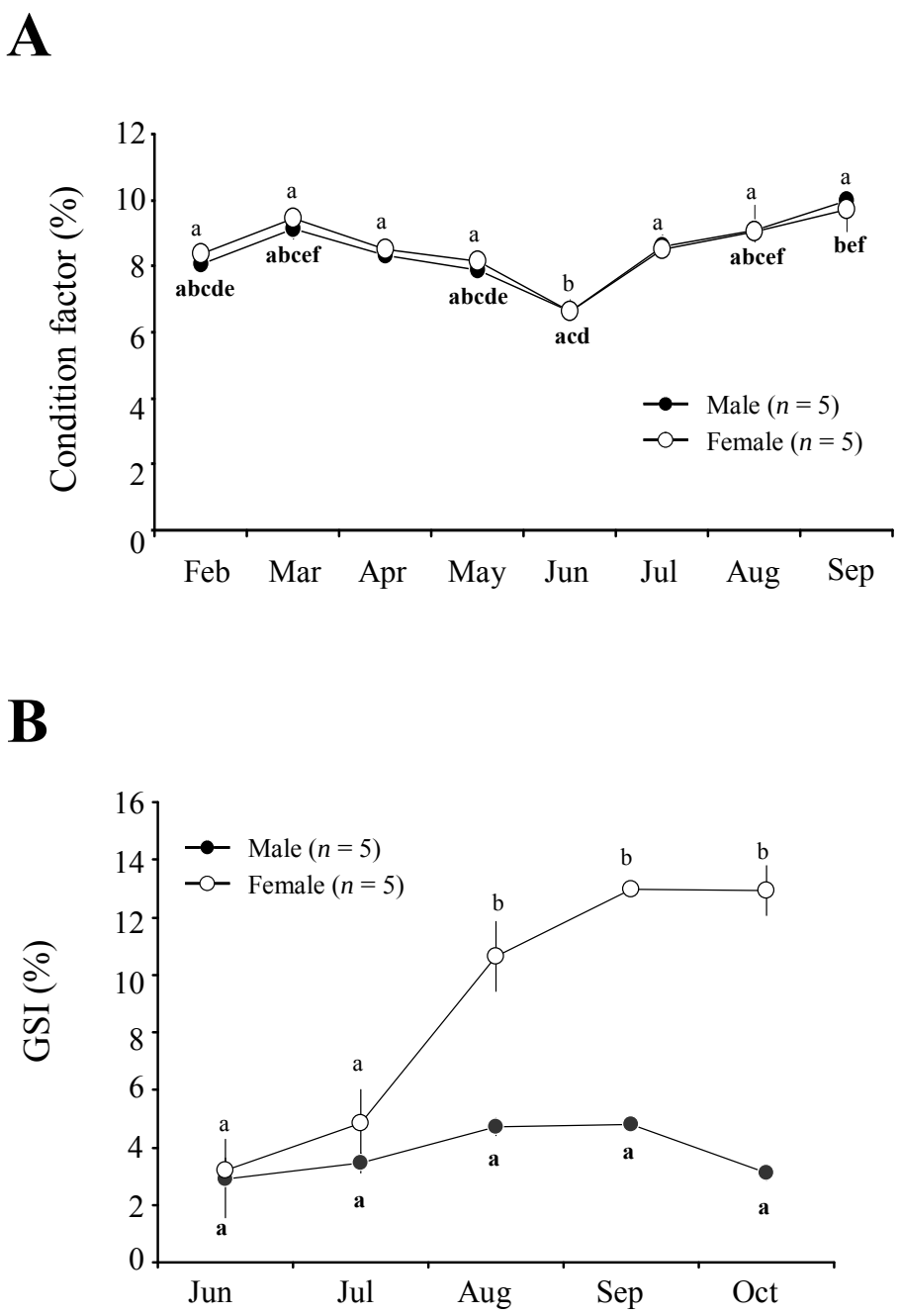


Fig. 4 Changes in condition factor (CF), and gonadosomatic index (GSI) of lacustrine sockeye salmon in 2006. (A) CF of one-year-old lacustrine sockeye salmon from February to September is shown. (B) GSI of three-year-old lacustrine sockeye salmon from June to October during sexual maturation is shown. The bold and normal letters are represented significant differences for males and females among the months, respectively. Values represent the means ± SEM. Significant differences among the months are indicated ($p < 0.05$ by one-way ANOVA following Tukey's test).

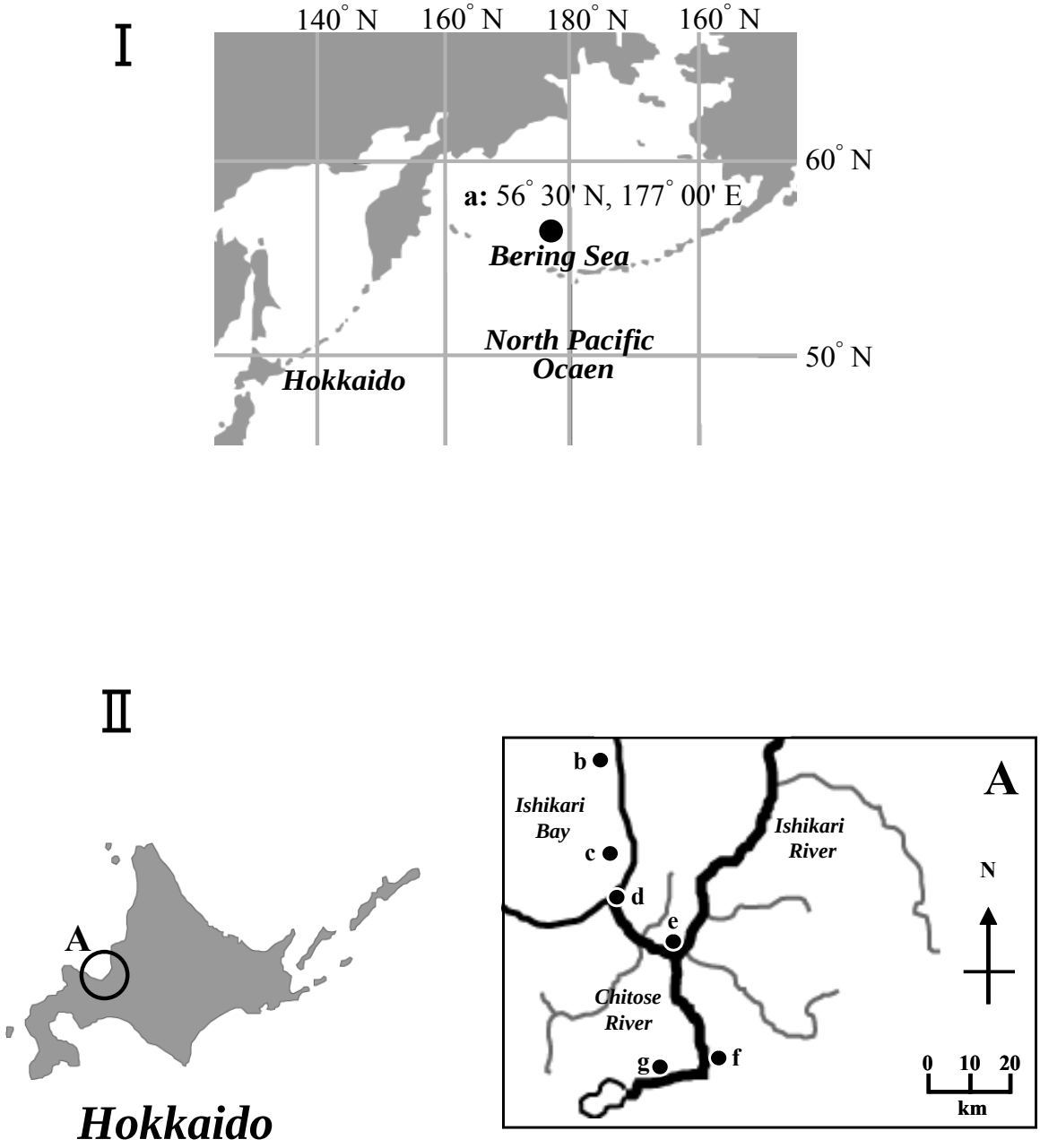


Fig. 5 Maps of North Pacific Ocean (I), from offshore to spawning ground (II) showing the sampling stations of chum salmon during homing migration. **a**: North Pacific Ocean (Bering Sea), **b**: offshore (Atsuta), **c**: coastal sea (Ishikari Bay), **d**: estuary (Ishikari), **e**: branch point (Ebetsu), **f**: pre-spawning ground (Hanazono), **g**: spawning ground (the Chitose Branch of National Salmon Resources Center).

Table 5. Fork length (FL), body weight (BW), and gonadosomatic index (GSI) of chum salmon. (A) Under yearling chum salmon from January to July in 2006. (B) Chum salmon during homing migration in 2003 and 2004. a: Bering Sea, b: offshore, c: coastal sea, d: estuary, e: branch point, f: pre-spawning ground, g: spawning ground.

A

Year	Sex	Date	*n*	FL (cm)	BW (g)
2006		23-Jan	5	4.15 ± 0.07	0.515 ± 0.030
		22-Feb	5	4.82 ± 0.06	0.961 ± 0.029
		3-Mar	5	6.06 ± 0.16	1.853 ± 0.120
	Male	18-Apr	5	6.98 ± 0.15	2.412 ± 0.085
	Female		5	7.12 ± 0.19	2.643 ± 0.215
	Male	22-May	5	7.18 ± 0.19	2.618 ± 0.244
	Female		5	7.26 ± 0.12	2.574 ± 0.155
	Male	28-Jul	5	7.68 ± 0.67	4.860 ± 1.623
	Female		5	7.32 ± 0.08	3.640 ± 0.154

B

Year	Sex	Point	Date	*n*	FL (cm)	BW (g)	GSI (%)	Spermiatio or Ovulation
2003	Male	a		5	42.9 ± 2.6	1010 ± 110	0.25 ± 0.04	No
		b	24-Sep	5	64.6 ± 1.8	3250 ± 310	2.74 ± 0.81	No
		c	29-Sep	5	61.9 ± 5.8	3360 ± 370	5.21 ± 0.64	No
		d	8-Oct	5	59.3 ± 3.2	3040 ± 570	6.35 ± 0.67	No
		e	2-Oct	5	61.6 ± 2.9	3060 ± 520	5.51 ± 0.15	No
		f	30-Sep	5	71.5 ± 2.9	4280 ± 580	3.75 ± 0.31	No
		g	1-Oct	5	68.0 ± 5.2	3790 ± 520	5.78 ± 0.19	Yes
	Female	a		5	44.9 ± 2.2	1050 ± 180	0.92 ± 0.11	No
		b	24-Sep	5	65.6 ± 1.9	3450 ± 330	15.62 ± 0.81	No
		c	29-Sep	4	63.0 ± 2.1	2950 ± 240	13.92 ± 3.36	No
		d	8-Oct	5	65.1 ± 1.0	3110 ± 90	15.09 ± 1.41	No
		e	2-Oct	3	67.5 ± 0.3	3710 ± 90	16.46 ± 0.67	No
		f	30-Sep	5	70.9 ± 1.2	4010 ± 330	21.30 ± 1.18	No
		g	1-Oct	5	67.6 ± 1.9	3940 ± 430	20.63 ± 2.66	Yes
2004	Male	a		5				
		b	30-Sep	5	67.0 ± 3.3	3390 ± 470	5.28 ± 0.36	No
		c	28-Sep	5	68.9 ± 2.2	3860 ± 400	5.70 ± 0.62	No
		d	1-Oct	5	67.7 ± 1.7	3680 ± 370	6.14 ± 0.14	No
		e	1-Oct	5	68.4 ± 1.5	3910 ± 340	4.54 ± 0.67	No
		f	29-Sep	5	69.4 ± 1.3	3590 ± 190	4.01 ± 0.29	No
		g	29-Sep	5	69.2 ± 1.8	3800 ± 400	4.70 ± 0.44	Yes
	Female	a		5				
		b	30-Sep	5	65.5 ± 0.5	3070 ± 120	14.80 ± 0.56	No
		c	28-Sep	5	66.9 ± 2.5	3660 ± 430	15.11 ± 0.92	No
		d	1-Oct	4	65.3 ± 1.2	3230 ± 170	16.63 ± 0.36	No
		e	1-Oct	5	65.5 ± 1.1	3340 ± 240	15.67 ± 1.25	No
		f	29-Sep	5	65.1 ± 0.7	2980 ± 140	22.73 ± 0.93	No
		g	29-Sep	5	66.5 ± 0.8	3330 ± 220	18.96 ± 1.03	Yes

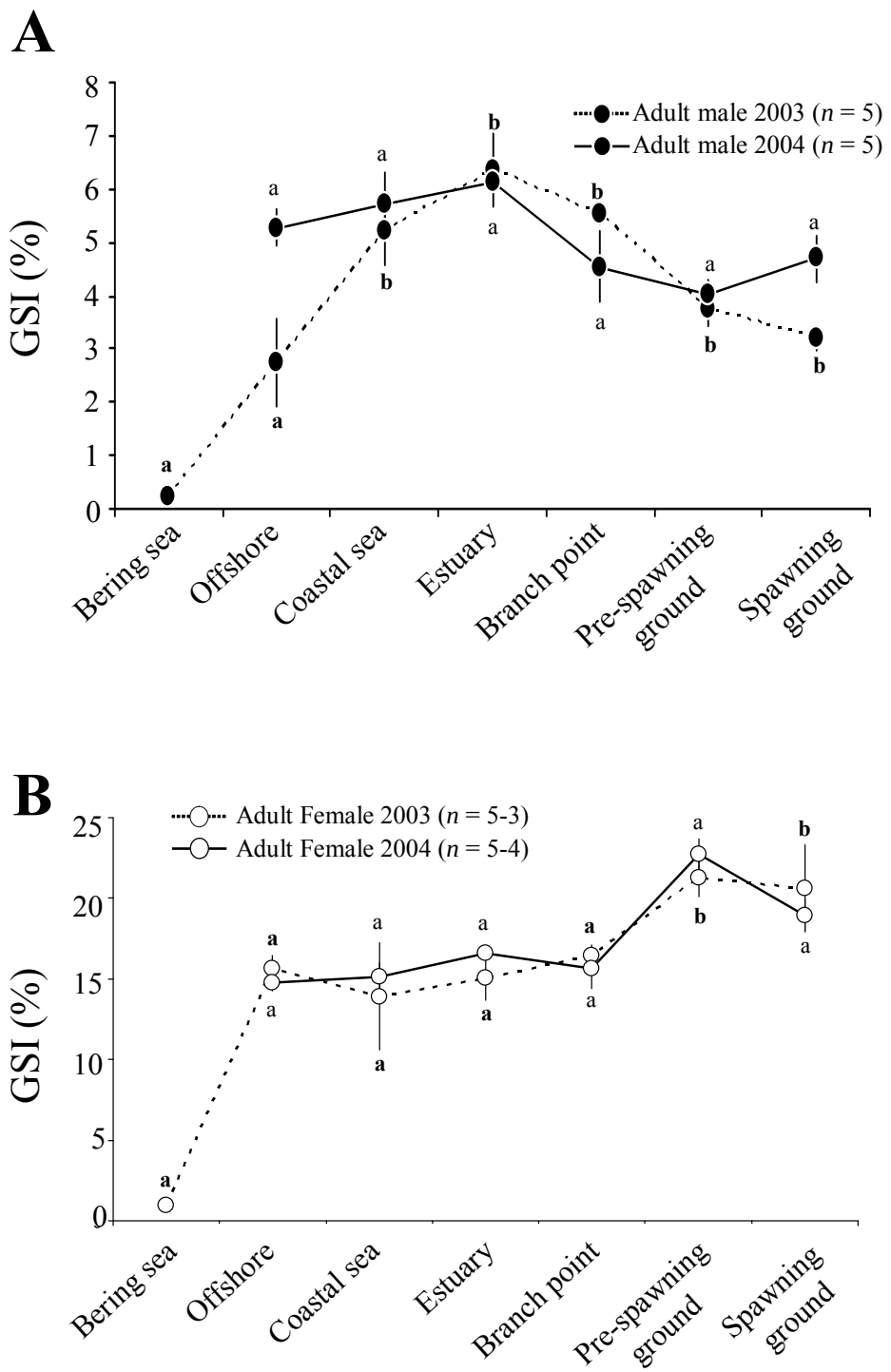


Fig. 6 Changes in gonadosomatic index (GSI) of chum salmon during homing migration. GSI of male (A) and female (B) fish in 2003 and 2004 are shown. The bold and normal letters are represented significant difference for 2003 and 2004 among the sampling points, respectively. Values represent the means ± SEM. Significant differences among the sampling points are indicated ($p < 0.05$ by one-way ANOVA following Tukey's test).

plasma samples, which were stored at -30˚C until assays of T_4. The olfactory epithelium was surgically isolated and then these tissues were stored at -80˚C until preparation of total RNA.

2) Northern blot analysis

Each Northern blot analysis was performed using pooled samples from 5 fish. Total RNA (10 μg) was electrophoresed on a 1% formaldehyde agarose gel and transferred to Hybond-N^+ membrane. The blotted membranes were air dried and baked at 60°C for 30 min. RNA probes were synthesized employing a non-radioactive method using a DIG RNA Labeling Kit (Roche). A partial fragment of clone *2* subtracted by previous experiment was subcloned into pBluescript II SK (+) vector, and then RNA probes were transcribed *in vitro* using T3 or T7 RNA polymerase. Membranes were prehybridized for 2 hr in a buffer of 50% formamide, 5×SSC, 0.1% N-lauroylsarcocine, 0.02% SDS and 2% blocking reagent (Roche) at 50˚C. Membranes were then hybridized in the same buffer at 50˚C for 16 hr with cRNA probes (100 ng/ml). Membranes were washed under the same conditions as used for differential screening. The signals were detected with a disodium-3-[4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro) tricycle [3.3.1.1.] 3,7 decan)-4-yl] phenylphosphate (CS PD; Roche) reaction.

3) *In situ* hybridization

The olfactory epithelia and olfactory bulbs of one-year-old lacustrine sockeye salmon were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2; PB), rinsed with 0.1 M PB, dehydrated in a graded series of ethanol, and embedded in

paraffin (Histosec; Merck, Darmstadt, Germany).

For *in situ* hybridization, digoxigenin (DIG)-labeled single stranded sense and anti-sense RNA probes were synthesized from the same template clones as for generation of Northern blot probes using T3 or T7 RNA polymerase. Serial sections were cut at approximately 5 μm thickness using a microtome, mounted on MAS-coated glass slides (Matsunami, Osaka, Japan), and deparaffinized with xylene.

Deparaffinized sections were treated with PBS buffer (100 mM NaCl, 10 mM, sodium phosphate, pH 7.4) containing proteinase K (10 μg/ml) at 37˚C for 15 min, then hybridization was carried out at 55˚C for 16 hr with hybridization buffer containing 500 ng/ml DIG-labeled RNA probe. The composition of hybridization buffer was 50% formamide, 17 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1×Denhard's solution (0.02% BSA, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll 400), 250 mM NaCl, 80 μg/ml yeast tRNA and 8% dextran sulfate. The sections were washed as follows: (1) 2×SSC/50% formamide at 50˚C for 30 min; (2) TNE buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA) at 37˚C for 10 min; (3) RNase A treatment (20 μg/ml) at 37˚C for 30 min; (4) TNE buffer at 37˚C for 10 min; (5) 2×SSC at 50˚C for 30 min; and (6) two washes in 0.2×SSC at 50˚C for 20 min. For signal detection, samples were incubated with anti-DIG antibody coupled to alkaline phosphatase (Roche) at a 1:1,000 dilution at 4˚C for 16 hr. The signals were detected with NBT and BCIP reaction. Sections adjacent to hybridization were stained with Carazzi's hematoxylin and eosin.

4) Construction of cDNA library

mRNA was purified from the total RNA extracted from the olfactory epithelium of one-year-old lacustrine sockeye salmon using an Oligotex™-dT30 (Super) mRNA

Purification kit (TaKaRa, Shiga, Japan) according to the manufacturer's instruction. Five μg poly $(A)^+$ RNA was used to synthesize cDNA primed with an oligo (dT) adaptor primer using a cDNA synthesis kit (Stratagene) according to the manufacture's instructions. The cDNA insert containing *Eco*RI and *Xho*I linkers were then ligated into the *Eco*RI/*Xho*I sites of Uni-ZAP XR vector (Stratagene). Approximately 1.2×10^6 primary clones were recovered with an average insert size of 2.0 kb.

5) Screening and cloning

Screening of the library was performed with DIG-labeled DNA probes. After plaques were transferred onto Hybond-N^+ membranes, the membranes were treated with denaturing solution, neutralizing solution and fixation solution. The membrane were prehybridized for 2 hr in a buffer of 50% formamide, 5×SSC, 0.1% N-lauroylsarcocine, 0.02% SDS and 1% blocking reagent (Roche) at 42˚C. Membranes were then hybridized in the same buffer at 42˚C for 16 hr with DIG-labeled probes (25 ng/ml). Membranes were washed under the same conditions as used for differential screening. Positive plaques were picked up to SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4 \cdot H_2O$, 0.01% gelatine] and *in vivo* excision was performed using ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. The obtained sequences were searched against 'Fasta Sequence Similarity search' (DDBJ), motif database (PROSITE: http://br.expasy.org/prosite/) and signal peptide analysis (SignalP 3.0: http://www.cbs.dtu.dk/services/SignalP/).

6) Accession numbers

The Gen Bank accession numbers for sequences cited in this study are as

follows: AAF71751, human uPAR; L03545, cattle uPAR; AF302074, monkey uPAR; P05533, mouse LY6 A; P09568, mouse LY6 C; and P13987, human CD59.

7) Cloning of cDNA fragments encoding SOIG from salmonids

To isolate cDNA fragments encoding SOIG from salmonids, oligonucleotide primers were designed based on SOIG nucleotide sequence (SOIG-F1, SOIG-R1 for masu salmon and chum salmon, SOIG-5'UTR, SOIG-3'UTR for pink salmon and rainbow trout; Table 6). Single-stranded cDNAs were synthesized from 2.5 μg of olfactory epithelium total RNA of these salmonids using a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The amplification profile comprised an initiating cycle of 2 min at 94˚C followed by 30 cycles as follows: 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min. The PCR-amplified DNA fragments were subcloned into pGEM T-Easy vector (Invitrogen) and sequenced. Sequence determination was performed as described in chapter II-1. Homology of the amplified cDNA fragments was confirmed by comparison with SOIG amino acid sequence in the ClustalW program (Thompson et al., 1994).

8) Amplification of 5' and 3' ends of cDNA fragments encoding SOIG of masu salmon and chum salmon

The 3' end of cDNA fragments encoding SOIG was amplified by the 3'-Full Race Core Set (TaKaRa) according to the manufacturer's instructions. Based on the nucleotide sequence of the partial cDNA encoding SOIG, sense primers were synthesized (SOIG-3'-F1; Table 6). PCR was initiated at 94°C for 4 min, followed by 30 cycles of amplification under conditions of 94°C for 1 min, 55°C for 1 min, and 72

Table 6. Primers and TaqMan probe used in this study.

Name	Description	Sequences
SOIG-F1	Forward primer for cloning	5’-GAGGACGCATCCATTTTAGC-3’
SOIG-R1	Reverse primer for cloning	5’-GTGGTGGTTGTTATCCCAAC-3’
SOIG-3’-F1	Reverse primer for 3’ RACE	5’-GGTTCTGGTTCTGACTCTGGTTCTG-3’
SOIG-5’-RT	Primer for reverse transcription for 5’ RACE	5’-CTGTCCAACACC-3’
SOIG-5’S1	Forward primer for 1st PCR of 5’ RACE	5’-CCTGCTGTGAGGGAAGTCTCTGCAA-3’
SOIG-5’AS1	Reverse primer for 1st PCR of 5’ RACE	5’-CTAGTGGGTCAGGGAGTGACAAGGT-3’
SOIG-5’S2	Forward primer for 2nd PCR of 5’ RACE	5’-GTGGGATCTGTTGCTTGTTGCTCATCAG-3’
SOIG-5’AS2	Reverse primer for 2nd PCR of 5’ RACE	5’-CAGGCTGAGCGGAATTCATAGCCCT-3’
SOIG-5’UTR	Forward primer for cloning	5’-GGTAATCATTGCTTCAGCTCAG-3’
SOIG-3’UTR	Reverse primer for cloning	5’-TCAAGAAACCCCCAATCAAGA-3’
SOIG-P	TaqMan probe for real time PCR	5’ ^{FAM}-TCAAGAAACCCCCAATCAAGA- ^{BHQ} 3’
SOIG-F2	Forward primer for real time PCR	5’-TTCACTTCTTCGTGCTGCAACT-3’
SOIG-R2	Reverse primer for real time PCR	5’-TGCAAGAAAAACACTCAAGTCCAT-3’

°C for 3 min. After the last cycle, elongation was extended to 10 min at 72°C.

5'-RACE was performed using a 5'-Full Race Core Set (TaKaRa) according to the manufacturer's instructions. Briefly, 500 ng of olfactory epithelium mRNA of masu salmon and chum salmon was reverse-transcribed using 5 units of AMV Reverse Transcriptase XL with specific primer (SOIG-5'-RT; Table 6). PCR was performed using first (SOIG-5'S1 and SOIG-5'AS1) and second specific primer sets (SOIG-5'S2 and SOIG-5'AS2; Table 6). The PCR-amplified DNA fragments were fractionated in a 2% agarose gel. DNA fragments obtained from the 3' and 5' RACE were subcloned into pGEM T-Easy vector (Invitrogen), and sequenced as just described.

9) Time-resolved fluoroimmunoassay for serum thyroid hormone

Serum levels of thyroid hormones (T_4 and T_3) were measured by time-resolved fluoroimmunoassay (TR-FIA) method using the protocols for T_4 and T_3 assays developed by Yamada et al. (1997). Antiserum for T_4 or T_3 was immobilized at the surface of the microtiter plates (Wallac Oy, Turku, Finland) by physical adsorption for 18 hr at 4˚C. After three washes with 0.9% saline, the plates were blocked with a blocking solution [50 mM Na_2HPO_4, 3% sucrose, 0.1% bovine serum albumin (BSA), 0.05% NaN_3] for 1 hr at room temperature. Then the plate was washed with 0.9% saline for immunoassay. In the assay, standards of T_4 or T_3 were applied in triplicate, and samples in duplicate. Assay buffer (50 mM Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN_3, 0.01% Tween 40, 20 μM diethylenetriamine-N,N,N',N",N"-pentaacetic acid, pH 7.75), standards of T_4 and T_3, serum samples, and europium (Eu) T_4 or T_3 BSA were added, then incubated at room temperature for 4 hr. After three washes with 0.9% saline, europium was dissociated from the antibody-antigen complex on the surface of

the wells with enhancement solution (Wallac Oy). Intensity of fluorescence from dissociated Eu was measured with a time resolved fluorometer (1234 DELFIA fluorometer, Wallac Oy) using DOS based multicalc software. Standard curves in each assay were plotted and values were calculated automatically by the time-resolved fluorometer. The intra-assay coefficient of variations (CV) ranged 7.9%, and inter-assay CV was 12.6%.

10) Real time PCR

Total RNA was extracted from whole bodies of embryos and alevins, and single olfactory epithelium, using ISOGEN (Nippongene) according to the manufacturer's instruction. The concentrations of total RNA were determined by measurement of optical density at 260/280 nm and its quantity and also integrity were verified by gel electrophoresis. RT reaction was performed with ExScript® RT reagent kit (TaKaRa). Total RNA (200 ng) was used for RT reaction in a mixture containing 1×ExScript® Buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.5 mM dNTP Mixture, 50 μM Random 6 mers, 25 U ExScript® RTase and 5 U RNase inhibitor. The reaction was performed at 42 ˚C for 15 min, and stopped at 92 ˚C for 2 min.

Real time PCR was carried out with Mx3000P QPCR System (Stratagene). Nucleotide sequences of primers and probes are shown in Table 6. The PCR reaction mixture contained 1×*Premix Ex Taq*™ (TaKaRa), 1×ROX Reference Dey II, 100 nM each forward and reverse primers and 130 nM of fluorogenic probe. Amplification profile was 40 cycles of 95˚C for 15 sec and 60˚C for 1 min (annealing and extension). To determine the amounts of SOIG mRNA, the full-length cDNA was used as a standard. The standard cDNA was serially diluted in the concentration of 1×10^4-1 ×

10^{10} copy (Fig. 7). In the assay, several doses of standard cDNA were applied in triplicate and sample cDNA prepared from total RNA were applied in duplicate. A standard sample was applied in triplicate to estimate coefficients of variation (CV) within and between runs. The range of intra-assay CV ranged 2.2-9.8% and inter-assay CV was 18.56%. The amounts of SOIG mRNA were expressed as copies per microgram total RNA.

11) Triiodothyronine (T_3) injection

As the treatment group fish were injected intraperitoneally with T_3 [0.1 and 1 μg/BW (g), 3,3',5-triiodo-$_L$-thyronine; Sigma, St. Louis, USA] dissolved in salmon Ringer solution (150 mM NaCl, 31 mM KCl, 3 mM $MgSO_4$, 10 mM $MgCl_2$, 34 mM $CaCl_2$, 40 mM HEPES, 0.1% glucose, pH 7.5, 0.01 N NaOH) and fish injected with salmon Ringer solution alone were served as the control group. The injected fish were returned to water tanks and five individuals were randomly sampled from each group 48 hours post-injection. Plasma samples were collected and stored at -30˚C until assays of T_3. Fish were anesthetized with 0.005% eugenol and then the olfactory epithelium was surgically isolated. These tissues were stored at -80˚C until preparation of total RNA.

12) Statistical analysis

All data are expressed as means ± SEM. Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered significant when $p<0.01$ and $p<0.05$.

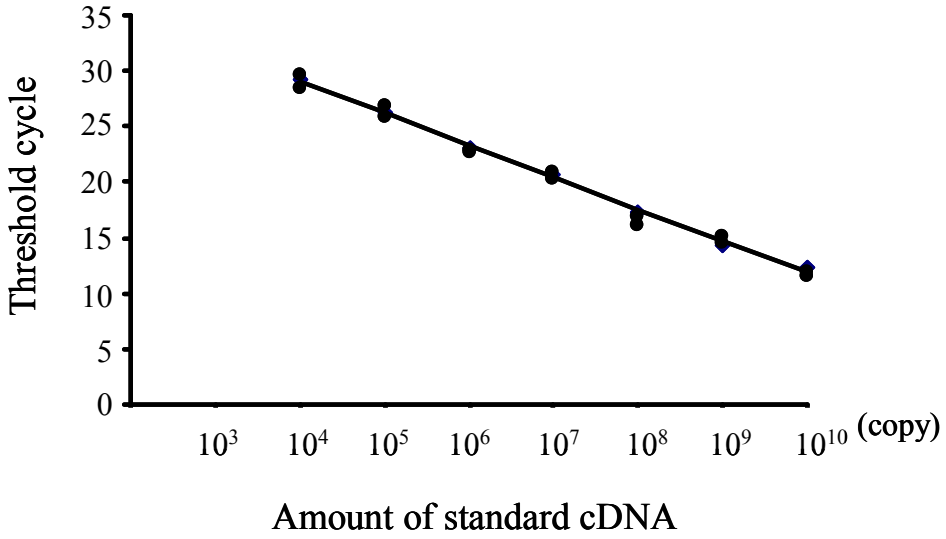


Fig. 7 Typical standard curve for quantitative real time PCR for SOIG mRNA.

Results

1) Northern blot analysis for SOIG mRNA expression in five salmonids

Expression of clone *2* mRNA in the brain (telencephalon, hypothalamus, optic tectum, cerebellum, and medulla oblongata) and body tissues (olfactory epithelium, gill, liver, heart, head kidney, spleen, muscle, intestine, and testis) from one-year-old lacustrine sockeye salmon, masu salmon, rainbow trout, under yearling chum salmon and under yearling pink salmon were analyzed by Northern blotting. Hybridized signal corresponding to 1.7 kb was detected in the olfactory epithelium of lacustrine sockeye salmon (Fig. 8). No signal was detected by Northern blot analysis in other tissues. I named this clone "Sockeye salmon Olfactory system Imprinting related Gene" (SOIG). Moreover, in four salmonid species, SOIG cRNA probe was hybridized only in the olfactory epithelium (Fig. 9).

2) Localization of SOIG mRNA in the olfactory system of lacustrine sockeye salmon

To determine the distribution of SOIG mRNA expression in the olfactory epithelium and olfactory bulb, *in situ* hybridization was performed using DIG-labeled sense and anti-sense RNA probes. The signals for SOIG were observed mainly in the olfactory receptor and basal cells in the olfactory epithelium of one-year-old lacustrine sockeye salmon (Fig. 10). The hybridization with labeled sense control probe showed no detectable hybridized signals in adjacent sections. On the other hand, no SOIG probe was detected in the olfactory bulb (data not shown).

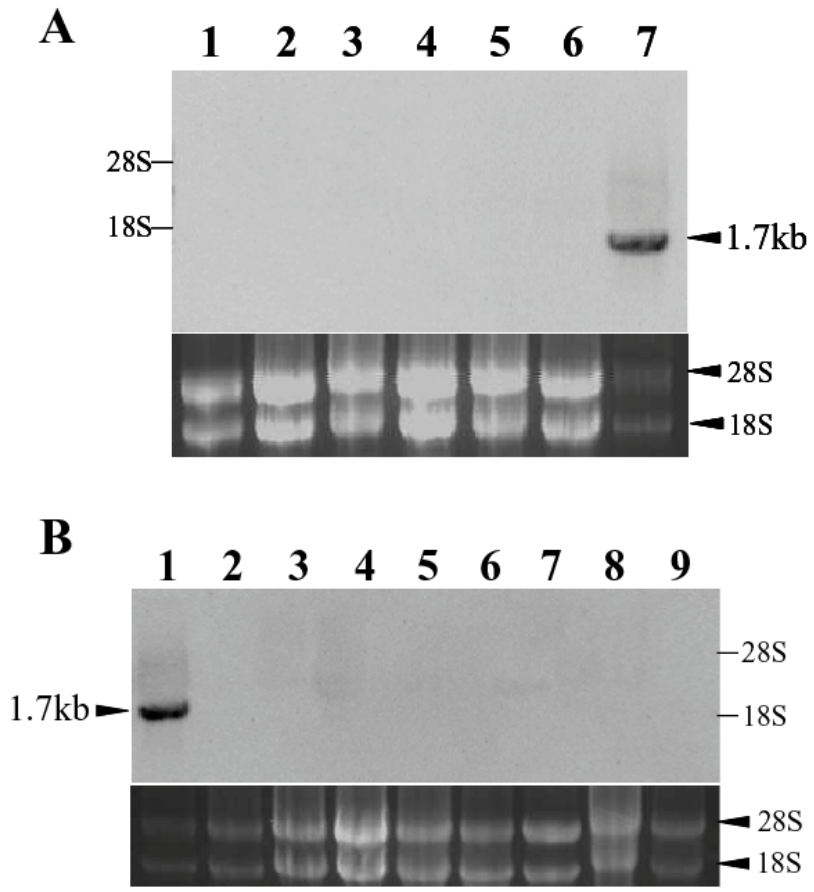


Fig. 8 Northern blot analysis of one-year-old lacustrine sockeye salmon SOIG mRNA. (A) Brain tissues (lane 1, olfactory bulb; lane 2, telencephalon; lane 3, hypothalamus; lane 4, optic tectum; lane 5, cerebellum; lane 6, medulla oblongata, and lane 7, olfactory epithelium). (B) Body tissues (lane 1, olfactory epithelium; lane 2, gill; lane 3, liver; lane 4, heart; lane 5, head kidney; lane 6, spleen; lane 7, muscle; lane 8, intestine, and lane 9, testis). Total RNA (10 μg) isolated from brain and body tissues were blotted to nylon membranes. The positions of SOIG transcripts are indicated by arrows. The relative positions of 28S and 18S ribosomal RNA are indicated. Northern blot analysis was performed using pooled samples from 5 fish.

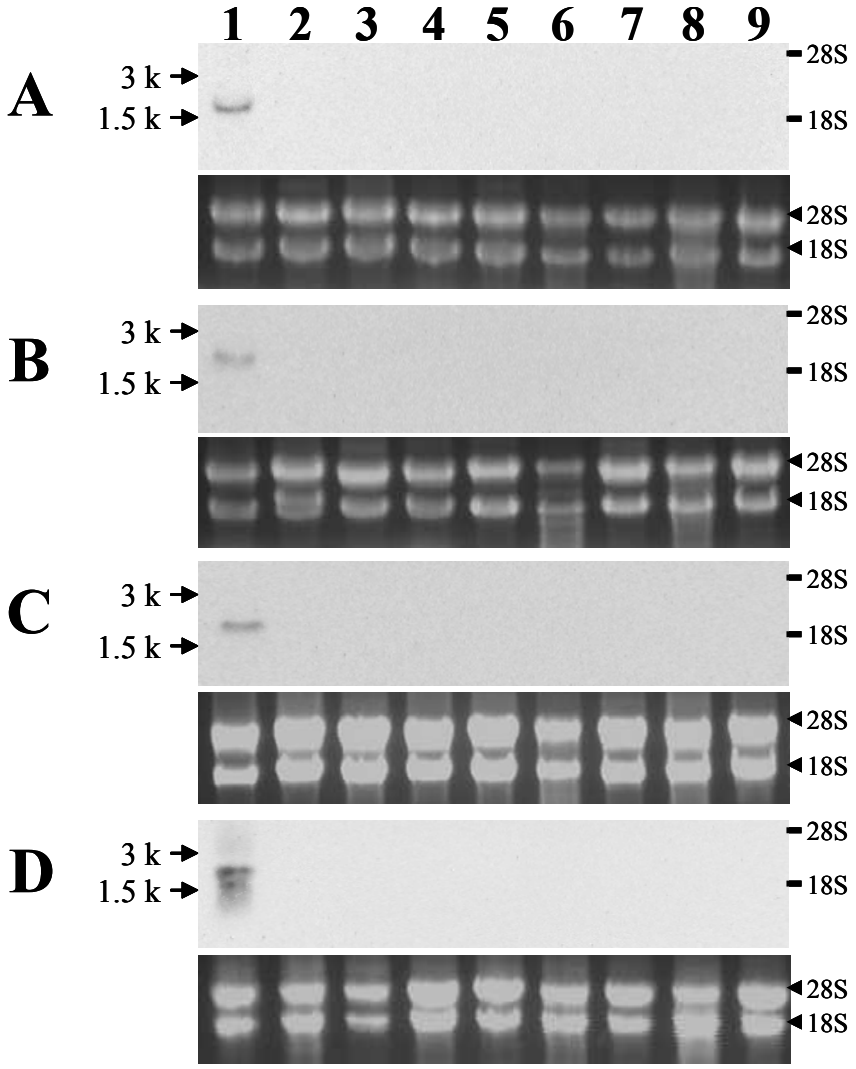


Fig. 9 Northern blot analysis of one-year-old masu salmon (A), under yearling chum salmon (B), under yearling pink salmon (C) and one-year-old rainbow trout (D) SOIG mRNA in the whole brain and body tissues. Lane 1, olfactory epithelium; lane 2, gill; lane 3, liver; lane 4, heart; lane 5, head kidney; lane 6, spleen; lane 7, muscle; lane 8, intestine; and lane 9, whole brain. Total RNA (10 μg) isolated from each tissues were blotted to nylon membranes. Maker is indicated by arrows. The relative positions of 28S and 18S ribosomal RNA are indicated. Northern blot analysis was performed using pooled samples from 5 fish.

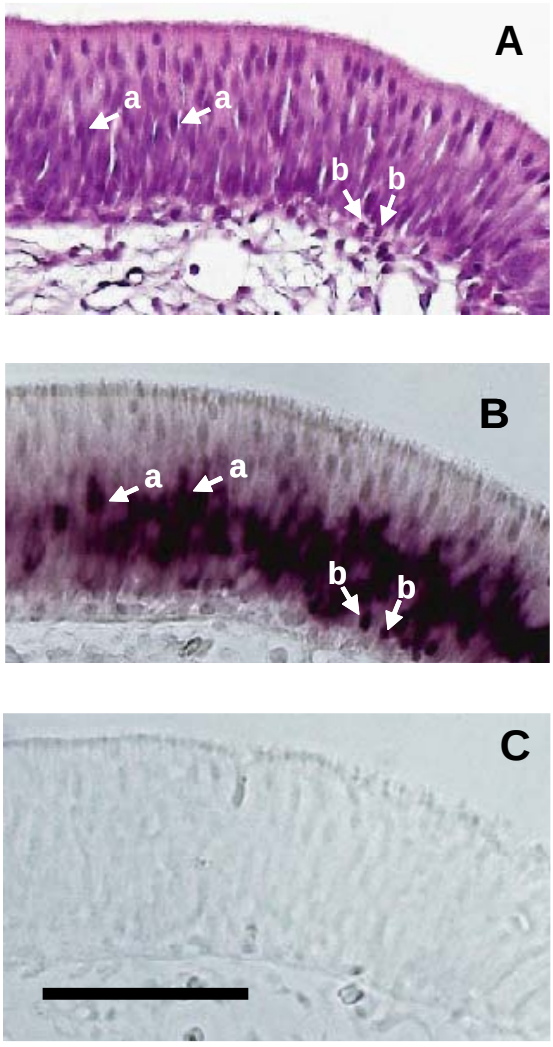


Fig. 10 Expression of SOIG mRNA demonstrated by *in situ* hybridization in the olfactory epithelium of lacustrine sockeye salmon. (A) Adjacent sections were stained with Carazzi's hematoxylin and eosin. (B) Section hybridized with antisense probes. (C) Section hybridized with sense probes as a negative control. a, olfactory receptor cell. b, basal cell. Scale bar: 100 μm.

3) Expression of SOIG mRNA in the olfactory bulb of one-year-old individual lacsutrine sockeye salmon

Semi-quantitative RT-PCR was performed to detect the expression levels of SOIG mRNA in the olfactory bulb of one-year-old individual male fish in May, 2003 (n=3). The saturation of β-actin in the olfactory bulb was detected in 30 cycles in all fish (data not shown). On the other hand, a weak band of SOIG mRNA in the olfactory bulb of fish 1, 2 and 3 were detected at 48-50 cycles (Fig. 11). I predicted that the characterization of SOIG mRNA in the olfactory bulb would be difficult, because the amount of SOIG mRNA in the olfactory bulb was very low. Hence, to obtain the characterization of SOIG, I carried out using the olfactory epithelium in the future analysis.

4) cDNA cloning and characterization of SOIG

To isolate a full-length SOIG cDNA, a cDNA library from the olfactory epithelium of one-year-old lacustrine sockeye salmon was constructed. The cDNA library was screened using the cDNA probe, which was synthesized from the same template as that used to generate Northern blot probes. The screening of approximately 1.2×10^6 plaques from the cDNA library yielded positive clones for SOIG, and these clones were subjected to sequence analysis.

The longest SOIG cDNA was 1,700 bp in length, having an open reading frame of 756 bp encoding 252 amino acids. A putative polyadenylation signal was located 19 and 29 nucleotides upstream of the poly (A) tail. The first ATG codon was located at nucleotides 7 to 9. The partial SOIG cDNA isolated from forward subtraction cDNA library was located at nucleotides 318 to 487 of the full-length cDNA (Fig. 12).

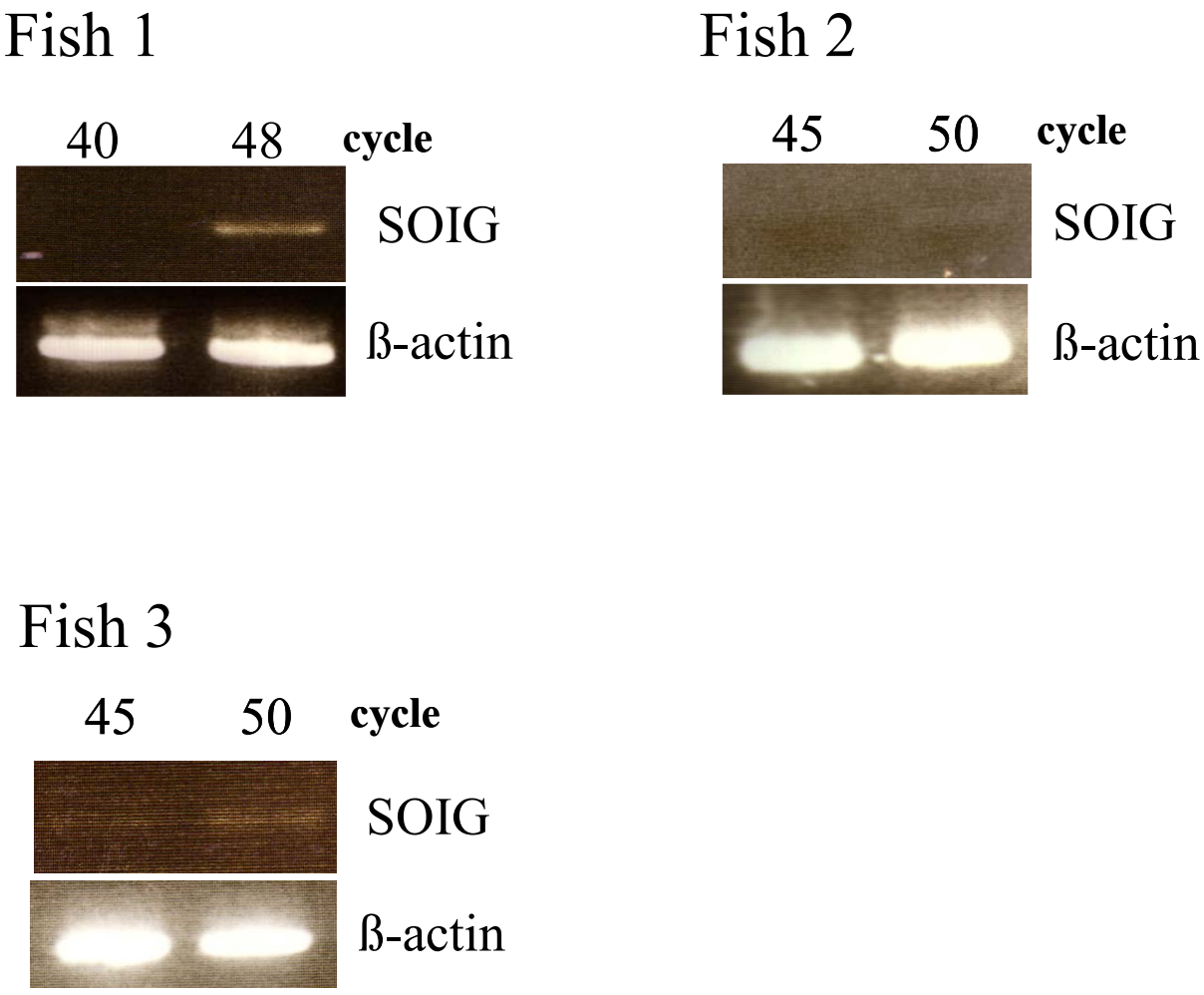


Fig. 11 Semi-quantitative RT-PCR amplification of SOIG mRNA in the olfactory bulb. RT-PCR was performed using the olfactory bulb of one-year-old individual fish. Results of 3% agarose gel electrophoresis of semi-quantitative RT-PCR products are shown. SOIG products and β-actin mRNAs were amplified from aliquots of the same mRNAs of the olfactory bulb of individual fish.

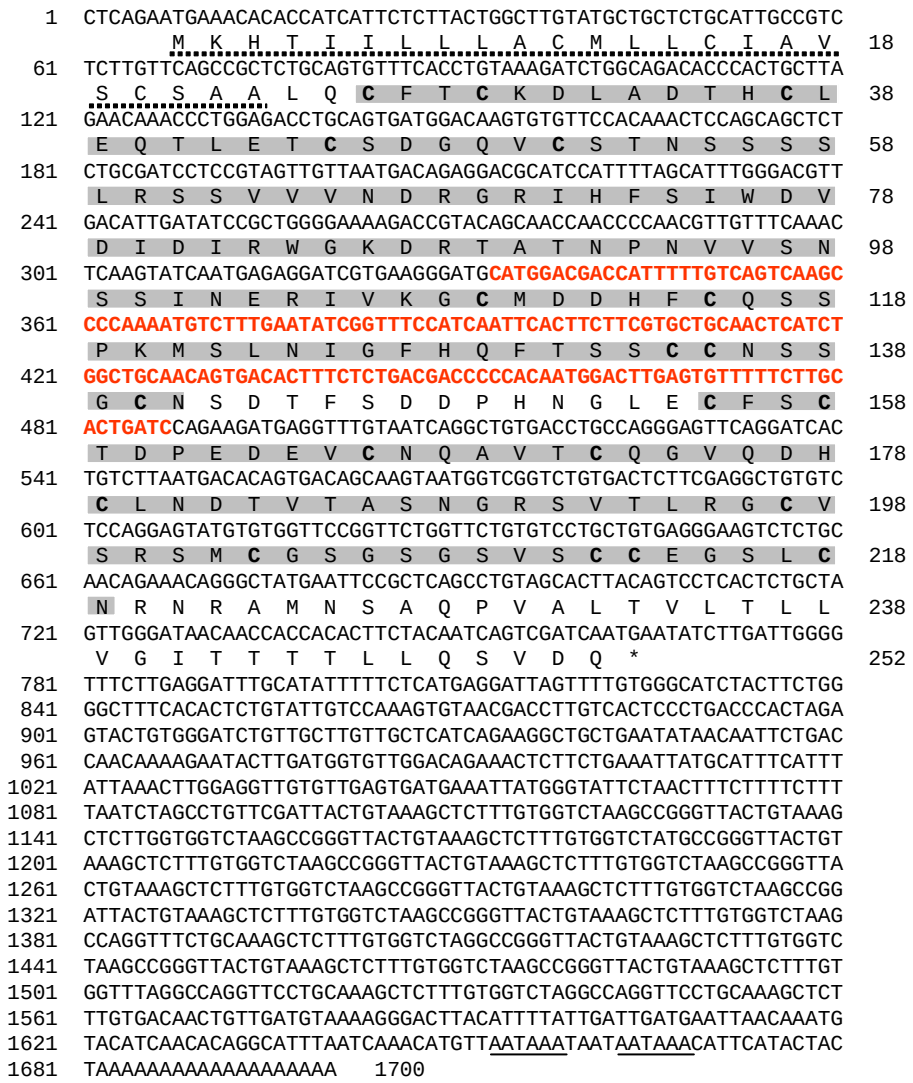


Fig. 12 Nucleotide sequence and deduced amino acid sequence of full-length SOIG cDNA. The stop codon (TGA) and polyadenylation signal (AATAAA) are indicated by an asterisk and thin underlines, respectively. The nucleotide sequence of partial SOIG cDNA isolated from forward subtractive cDNA library is colored in red. Conserved cysteine residues are in bold letters. Lymphocyte antigen-6 (Ly-6) domain is indicated by shade. The putative sequence of signal peptide is dotted underline.

The deduced amino acid sequence had a calculated molecular mass of 27.0 kDa, and signalP peptide analysis revealed that it contained a putative signal peptide of 23 amino acids. DDBJ search revealed SOIG identity 23, 21 and 21% similarity with the amino acid sequence of u-PAR from human (Zhu et al., 2000), cattle (Kratzschmar et al., 1993) and monkey (Engelholm and Behrendt, 2001), respectively (Fig. 13). The domain search revealed that SOIG amino acid contained two homologous cysteine-rich domains of lymphocyte antigen-6 (Ly-6; positions 26-141, 155-219; Figs. 12 and 14A). Ly-6 superfamily domain consists of 8 to 10 conserved cysteine residues with the characteristic spacing pattern. Comparison of each domains of SOIG with members of Ly-6 superfamily showed that SOIG contains six of these cysteines with the conserved spacing (Fig. 14B).

5) Isolation of cDNA encoding SOIG from four salmonid species

The cloned full-length cDNA encoding SOIG of masu salmon and chum salmon consists of 1,586 and 1,802 bp, respectively (Figs. 15 and 16). The open reading frame of masu salmon and chum salmon is composed of 251 and 256 amino acid residues, and the transcripts contain polyadenylation signal in 5' untranslated region (nucleotide 1,527-1,532, masu salmon; nucleotide 1,763-1,768 and 1,773-1,778, chum salmon). The deduced amino acid sequence had calculated molecular mass of 26.6 and 27.2 kDa. The putative open reading frame of cDNA of pink salmon and rainbow trout consists of 765 bp, encoding 255 amino acids (Fig. 17). The deduced amino acid sequence had a calculated molecular mass of 27.2 kDa. SignalP program predicted that the N-terminus would be cleaved at between Ala^{23} and Leu^{24} by translational processing in masu salmon, chum salmon, pink salmon and rainbow trout, and this

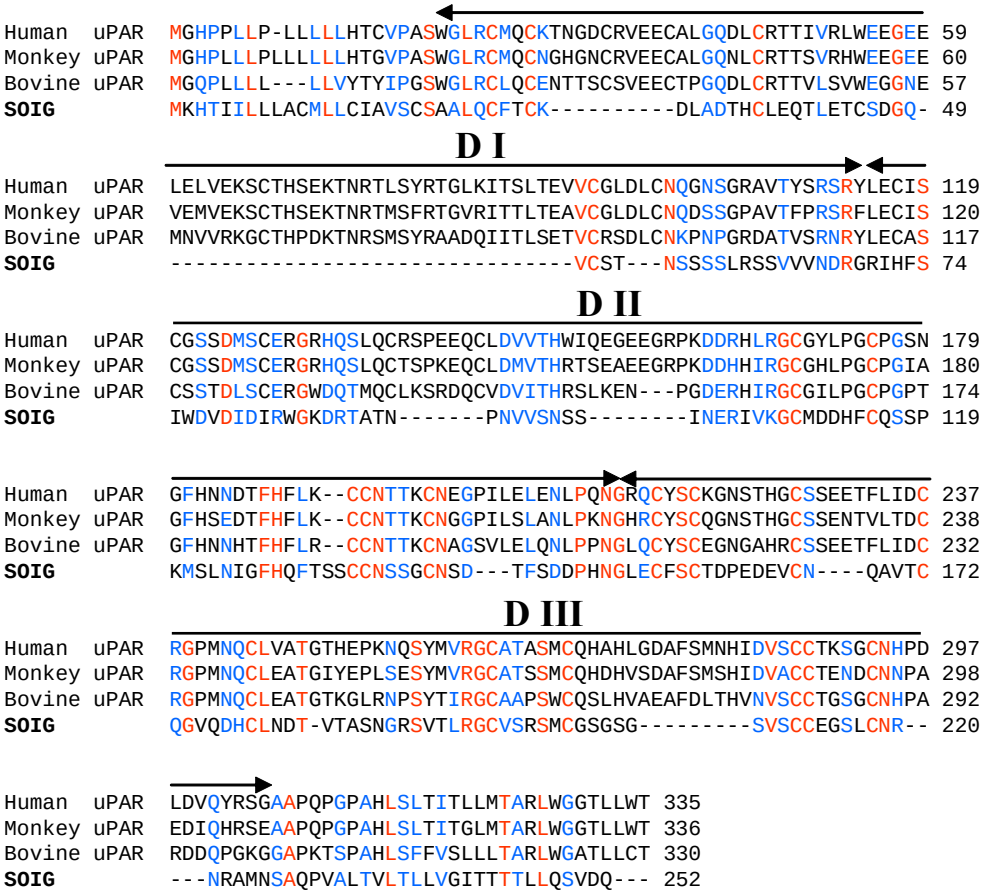


Fig. 13 Amino acid sequence comparison of SOIG, human uPAR, cattle uPAR and monkey uPAR. This alignment was performed using ClustalW (Thompson et al., 1994). Identical amino acids are indicated by red characters. Similar amino acids are depicted in blue characters. Dash represents a gap in the sequence introduced to maximize alignment. The three positions of LY-6 superfamily domains in human, cattle and monkey uPAR are indicated by lines (DI: positions 21-112, DII: 113-208, DIII: 209-300).

A

```
 26 CFTCKDLADTHCLEQTLETCSDGQVCSTNSSSSLRSSVVVNDRGRIHFSIWDVDIDIRWG 85
    CF*C*D  D        E*C*** *C*  ** *L***V**** G
155 CFSCTDPED--------EVCNQAVTCQGVQDHCLNDTVTASN-G---------------- 189

 86 KDRTATNPNVVSNSSINERIVKGCMDDHFCQSSPKMSLNIGFHQFTSSCCNSSGCN 141
      R**T               **GC**  *C S**      G    * SCC**S CN
190 --RSVT-------------LRGCVSRSMCGSGS------G----SVSCCEGSLCN 219
```

B

```
SOIG domain 2           153 --CFSCTDPEDEVCNQA----VTCQGVQDHCLNDTVT---ASN-GRSVTLRG-------- 196
Bovine uPAR domain 3    209 LQCYSCEGNGAHRCSSEETFLIDCRGPMNQCLEATGT---KGLRNPSYTIRG-------- 257
Mouse LY6 A domain       27 LECYQCYGVPFETSCPS----ITCPYPDGVCVTQEAA---VIVDSQTRKVKN-------- 71
Mouse LY6 C domain       27 LQCYECYGVPIETSCPA----VTCRASDGFCIAQNIE---LIEDSQRRKLKT-------- 71
SOIG domain 1            24 --CFTCKDLADTHCLEQT--LETCSDGQ-VCSTNSSS---SLRSSVVVNDRGRIHFSIWD 77
Human CD59 domain        26 LQCYNCPN-PTADCKTA----VNCSSDFDACLITKAG---LQVYNKCWKFEH-------- 69
Bovine uPAR domain 2    113 LECASCSS-TDLSCERGWDQTMQCLKSRDQCVDVITHRSLKENPGDERHIRG-------- 163

SOIG domain 2           197 -CVSRSMCGS---------GSGSVS------CCEGSLCN---------------------- 219
Bovine uPAR domain 3    258 -CAAPSWCQSLHVAEAFDLTHVNVS------CCTGSGCN--HPARDDQPGKG-------- 300
Mouse LY6 A domain       72 -NLCLPICPPNIESMEILGTKVNVKT----SCCQEDLCN--VAVPNGGSTWTMAG----- 119
Mouse LY6 C domain       72 -RQCLSFCPAGVP---IKDPNIRERT----SCCSEDLCN--AAVPTAGSTWTMAG----- 116
SOIG domain 1            78 VDIDIRWGKDRTATNPNVVSNSSINERIVKGCMDDHFCQS-SPKMSLNIGFHQFTSSCCN 136
Human CD59 domain        70 -CNFNDVTTR------LRENELTYY------CCKKDLCNFNEQLENGGTSLS-------- 108
Bovine uPAR domain 2    164 -CGILPGCPGPTG--FHNNHTFHFLR-----CCNTTKCNAGSVLELQNLPPNG------- 208

SOIG domain 2               -----
Bovine uPAR domain 3        -----
Mouse LY6 A domain          -----
Mouse LY6 C domain          -----
SOIG domain 1           137 SSGCN 142
Human CD59 domain           -----
Bovine uPAR domain 2        -----
```

Fig. 14 (A) The two homologous cysteine-rich domains of SOIG are shown aligned to each other. Upper and lower sequences are SOIG domain 1 and 2, respectively. (B) Amino acid sequence comparison of each domains of SOIG and Ly-6 superfamily. The protein sequences were retrieved from the Swissprot database (http://www.genome.jp) and aligned to ClustalW. Identical amino acids are shown in red. Conserved cysteine residues are enclosed in boxes. Dash represents a gap in the sequence introduced to maximize alignment. Similar amino acids are depicted in blue. Numbers preceding the sequences indicate the first amino acid of Ly-6 superfamily domain. Ly-6 motif (CCXXXXCN) is indicated by underlines.

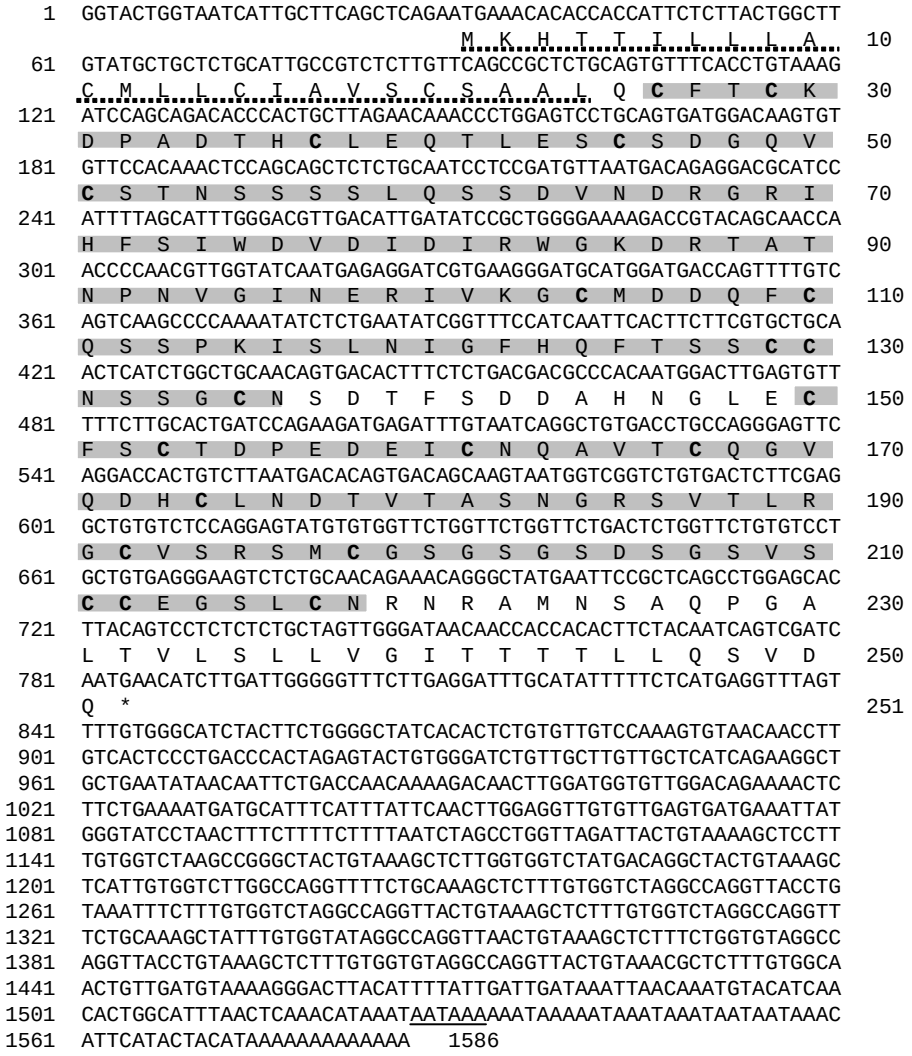


Fig. 15 Nucleotide sequence and deduced amino acid sequence of full-length cDNA encoding SOIG of masu salmon. The stop codon (TGA) and polyadenylation signal (AATAAA) are indicated by an asterisk and underline, respectively. Conserved cysteine residues are shown in bold letters. Ly-6 superfamily domain is indicated by shade. The putative sequence of signal peptide is dotted underline.

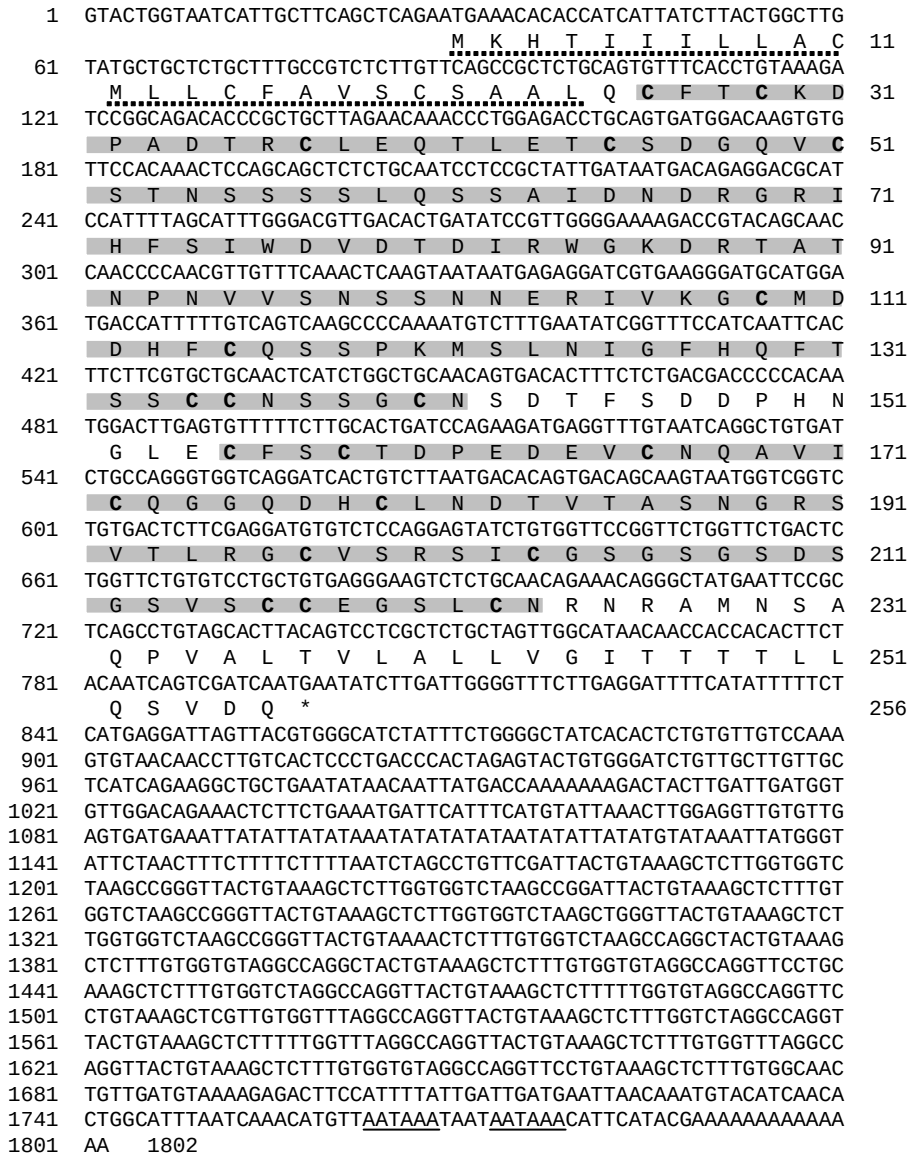


Fig. 16 Nucleotide sequence and deduced amino acid sequence of full-length cDNA encoding SOIG of chum salmon. The stop codon (TGA) and polyadenylation signal (AATAAA) are indicated by an asterisk and underline, respectively. Conserved cysteine residues are shown in bold letters. Ly-6 superfamily domain is indicated by shade. The putative sequence of signal peptide is dotted underline.

A

```
1   ATGAAACGCACCATCACTCTCTTACTGGCTTGTATGCTGCTCTGCATTGCCGTCTCTTGT
    M  K  R  T  I  T  L  L  L  A  C  M  L  L  C  I  A  V  S  C   20
61  TCAGCCGCTCTGCAGTGTTTCACCTGTAAAGATCCGGCAGACGCCCGCTGCTTAGAACAA
    S  A  A  L  Q  C  F  T  C  K  D  P  A  D  A  R  C  L  E  Q   40
121 ACCCTGGAGACCTGCAGTGATGGACAAGTGTGTTCCACAAACTCCAGCAGCTCTCTGCAA
    T  L  E  T  C  S  D  G  Q  V  C  S  T  N  S  S  S  S  L  Q   60
181 TCCTCCGTTGATAATGACAGAGGACGCATCCATTTTAGCATTTGGGACGTTGACATTGAT
    S  S  V  D  N  D  R  G  R  I  H  F  S  I  W  D  V  D  I  D   80
241 ATCCGCTGGGGAAAAGACCGTACAGCAACCAACCCCAACGTTGTTTCAAACTCAAGTATC
    I  R  W  G  K  D  R  T  A  T  N  P  N  V  V  S  N  S  S  I  100
301 AAAGAGAGGATCGTGAAGGGATGCATGGATGACCATTTTTGTCAGTCAAGCCCCAAAATG
    K  E  R  I  V  K  G  C  M  D  D  H  F  C  Q  S  S  P  K  M  120
361 TCTTTTAATATCGGTTTTCATCAATTCACTTCTTCGTGCTGCAACTCATCTGGCTGCAAC
    S  F  N  I  G  F  H  Q  F  T  S  S  C  C  N  S  S  G  C  N  140
421 AGTGACACTTTCTCTGACGACCCCCACAATGGACTTGAGTGTTTTTCTTGCACTGATCCA
    S  D  T  F  S  D  D  P  H  N  G  L  E  C  F  S  C  T  D  P  160
481 GAAGATGAGGTTTGTAATCAGGCTGTGATCTGCCAGAGTGTTCAGGATCACTGTCTTAAT
    E  D  E  V  C  N  Q  A  V  I  C  Q  S  V  Q  D  H  C  L  N  180
541 GACACAGTGACAGCAAGTAATGGTCGGTCTGTGACTCTTCGAGGCTGTGTCTCCAGGAGT
    D  T  V  T  A  S  N  G  R  S  V  T  L  R  G  C  V  S  R  S  200
601 ATGTGTGGTTCCGGTTCTGGTTCTGACTCTGGTTCTGTGTCCTGCTGTGAGGGAAGTCTC
    M  C  G  S  G  S  G  S  D  S  G  S  V  S  C  C  E  G  S  L  220
661 TGCAACAGAAACAGGGCTATGAATTCCGCTCAGCCTGTAGCACTTACAGTCCTCGCTCTG
    C  N  R  N  R  A  M  N  S  A  Q  P  V  A  L  T  V  L  A  L  240
721 CTAGTTGGCATAACAACCACCACACTTCTACAATCAGTCAATCAATGA   768
    L  V  G  I  T  T  T  T  L  L  Q  S  V  N  Q  *             255
```

B

```
1   ATGAAACGCACCATCACTCTCTTACTGGCTTGTATGCTGCTCTGCATTGCCGTCTCTTGT
    M  K  R  T  I  T  L  L  L  A  C  M  L  L  C  I  A  V  S  C   20
61  TCAGCCGCTCTGCAGTGTTTCACCTGTAAAGATCCGGCAGACGCCCGCTGCTTAGAACAA
    S  A  A  L  Q  C  F  T  C  K  D  P  A  D  A  R  C  L  E  Q   40
121 ACCCTGGAGACCTGCAGTGATGGACAAGTGTGTTCCACAAACTCCAGCAGCTCTCTGCAA
    T  L  E  T  C  S  D  G  Q  V  C  S  T  N  S  S  S  S  L  Q   60
181 TCCTCCGTTGATAATGACAGAGGACGCATCCATTTTAGCATTTGGGACGTTGACATTGAT
    S  S  V  D  N  D  R  G  R  I  H  F  S  I  W  D  V  D  I  D   80
241 ATCCGCTGGGGAAAAGACCGTACAGCAACCGACCCCAACGTTGTTTCAAACTCAAGTATC
    I  R  W  G  K  D  R  T  A  T  D  P  N  V  V  S  N  S  S  I  100
301 AAAGAGAGGATCGTGAAGGGATGCATGGATGACCATTTTTGTCAGTCAAGCCCCAAAATG
    K  E  R  I  V  K  G  C  M  D  D  H  F  C  Q  S  S  P  K  M  120
361 TCTTTTAATATCGGTTTCCATCAATTCACTTCTTCGTGCTGCAACTCATCTGGCTGCAAC
    S  F  N  I  G  F  H  Q  F  T  S  S  C  C  N  S  S  G  C  N  140
421 AGTGACACTTTCTCTGACGACCCCCACAATGGACTTGAGTGTTTTTCTTGCACTGATCCA
    S  D  T  F  S  D  D  P  H  N  G  L  E  C  F  S  C  T  D  P  160
481 GAAGATGAGGTTTGTAATCAGGCTGTGATCTGCCAGAGTGTTCAGGATCACTGTCTTAAT
    E  D  E  V  C  N  Q  A  V  I  C  Q  S  V  Q  D  H  C  L  N  180
541 GACACAGTGACAGCAAGTAATGGTCGGTCTGTGACTCTTCGAGGCTGTGTCTCCAGGAGT
    D  T  V  T  A  S  N  G  R  S  V  T  L  R  G  C  V  S  R  S  200
601 ATGTGTGGTTCCGGTTCTGGTTCTGACTCTGGTTCTGTGTCCTGCTGTGAGGGAAGTCTC
    M  C  G  S  G  S  G  S  D  S  G  S  V  S  C  C  E  G  S  L  220
661 TGCAACAGAAACAGGGCTATGAATTCCGCTCAGCCTGTAGCACTTACAGTCCTCGCTCTG
    C  N  R  N  R  A  M  N  S  A  Q  P  V  A  L  T  V  L  A  L  240
721 CTAGTTGGGATAACGACCACCACACTTCTACAATCAGTCGATCAATGA   768
    L  V  G  I  T  T  T  T  L  L  Q  S  V  D  Q  *             255
```

Fig. 17 Nucleotide sequences and deduced amino acid sequences of full-length cDNA encoding SOIG of pink salmon (A) and rainbow trout (B). The stop codon (TGA) is indicated by an asterisk. Conserved cysteine residues are showed in bold letters. Ly-6 superfamily domain is shaded. The putative sequence of signal peptide is dotted underline.

cleaved position corresponds to the cleaved position of SOIG. The amino acid sequence of SOIG was compared with SOIG homologs of four salmonid species (Fig. 18). SOIG showed high identity (93-94%) with SOIG homologs of masu salmon, chum salmon, pink salmon, and rainbow trout.

6) Changes in condition factor and serum T_4 concentrations of one-year-old lacustrine sockeye salmon

Condition factors (CF) in both sexes tended to decrease from March to June and then increased toward September (Fig. 4A). In the males, CF in June (6.62 ± 0.1%) tended to lower than in March (Fig. 4A). On the other hand, CF in June of the females (6.62 ± 0.36%) was significantly lower than those in other months.

Serum T_4 concentrations were measured in one-year-old lacustrine sockeye salmon from February to September 2006. Serum T_4 levels of the males peaked in May (3.06 ± 0.2 ng/ml) and decreased toward in June (Fig. 19). Especially, the serum T_4 concentrations in May to June were significantly higher than those in February and July. On the other hand, serum T_4 of the females also peaked in May (4.28 ± 0.8 ng/ml) and then sharply decreased in June. The T_4 serum concentrations in May were significantly higher than those in other months.

It is reported that CF decreases at the PST (Hoar, 1988), and plasma T_4 increased during the PST (Dickhoff et al., 1978; Yamauchi et al., 1984). Hence, samples using in this study correspond to pre-PST from February to March, PST from April to June, and post-PST from July to September.

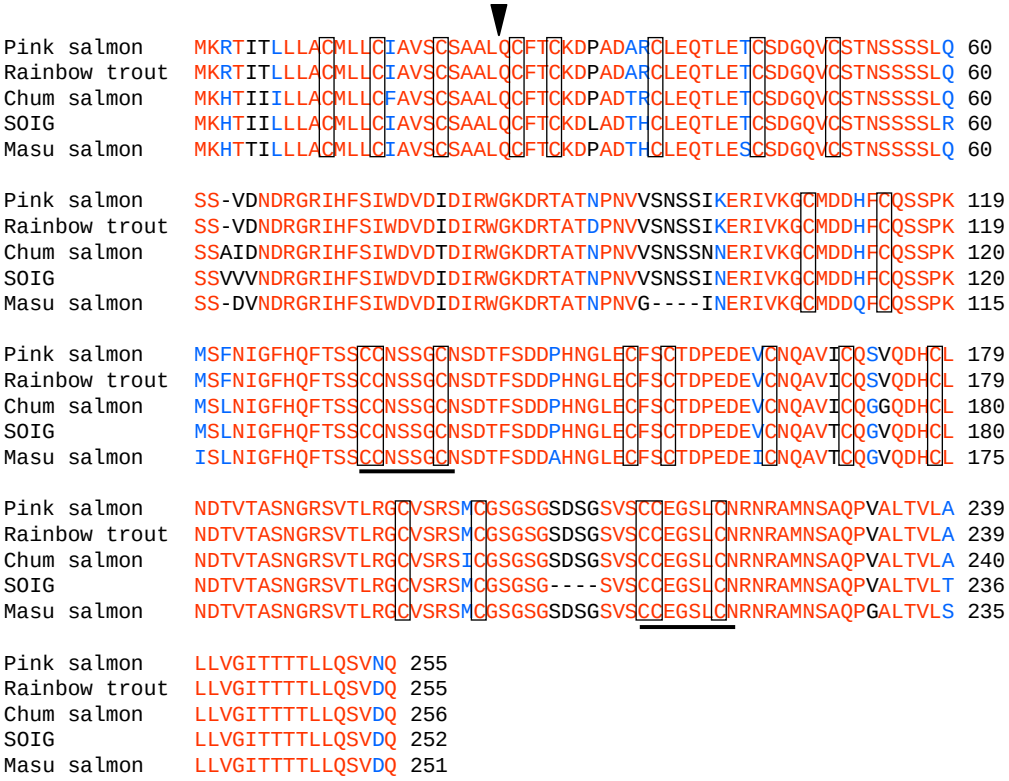


Fig. 18 The amino acid sequence alignment of SOIG and SOIG homologs of masu salmon, chum salmon, pink salmon, and rainbow trout. The multiple alignment was performed using ClustalW. Gaps are introduced to maximize homology. Identical amino acids are shown in red. Conserved cysteine residues are enclosed in boxes. Similar amino acids are depicted in blue. Ly-6 motif (CCXXXXCN) is indicated by underlines. The signal peptide cleavage site of SOIG and SOIG homologs of four salmonid species is indicated by arrowhead.

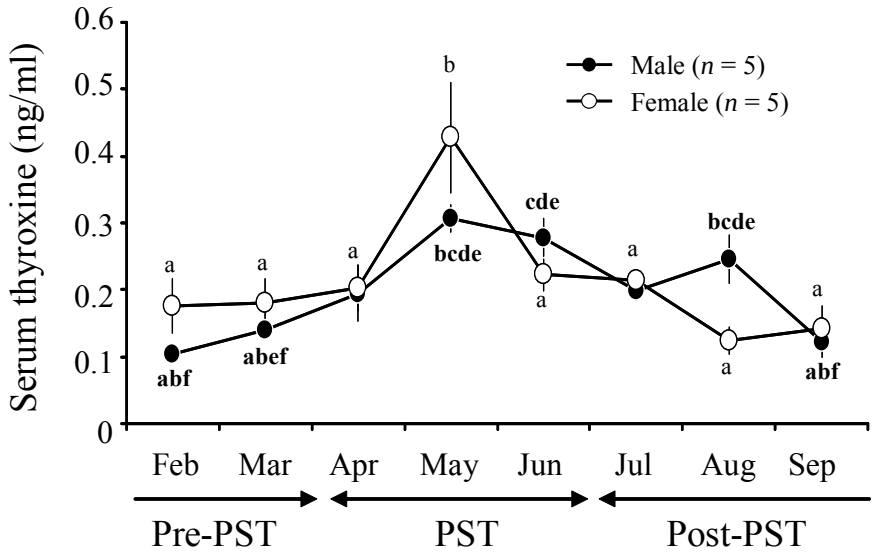


Fig. 19 Changes in serum thyroxine (T_4) concentrations of one-year-old lacustrine sockeye salmon in 2006. T_4 levels of one-year-old lacustrine sockeye salmon from February to September are shown. Values represent the means ± SEM. The bold and normal letters are represented significant differences for males and females among the months, respectively. Significant differences among the months are identified with different letters ($p < 0.05$ by one-way ANOVA following Tukey's test).

7) Sexual maturation of lacustrine sockeye salmon and chum salmon

In three-year-old fish of lacustrine sockeye salmon, GSI of female increased significantly in August (10.64 ± 1.21%) and reached maximum value in September (12.93 ± 1.98%; Fig. 4B). On the other hand, GSI of males peaked in August (4.71 ± 0.30%) and decreased toward September (Fig. 4B). Final maturations as ovulation and spermiation were confirmed all fish in September.

In male chum salmon, the GSI peaked in the estuary (6.35 ± 0.67%, 2003; 6.14 ± 0.14%, 2004) and then decreased toward the spawning ground, because of completion of spermatogenesis and release of milt into abdomen (Fig. 6A). In females, the GSI peaked in the pre-spawning ground (21.30 ± 1.18%, 2003; 22.73 ± 0.93%, 2004; Fig. 6B). In both sexes, ovulation and spermiation were confirmed for all fish in spawning ground.

8) Changes in SOIG mRNA levels during ontogeny of lacustrine sockeye salmon

Expression levels of SOIG mRNA of alevin were significantly higher than those of embryos 43 and 60 days after fertilization (Fig. 20). On the other hand, the SOIG mRNA levels at embryonic day 60 tended to be higher than those at day 43. However, the SOIG mRNA levels between embryonic day 43 and 60 were not statistically significant.

9) Changes in SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon from emergence to post-PST

In both sexes, the basal level of SOIG mRNA was 0.023-0.058 × 10^8 copies/μg total RNA as measured in fly, and constantly elevated till March at the pre-PST.

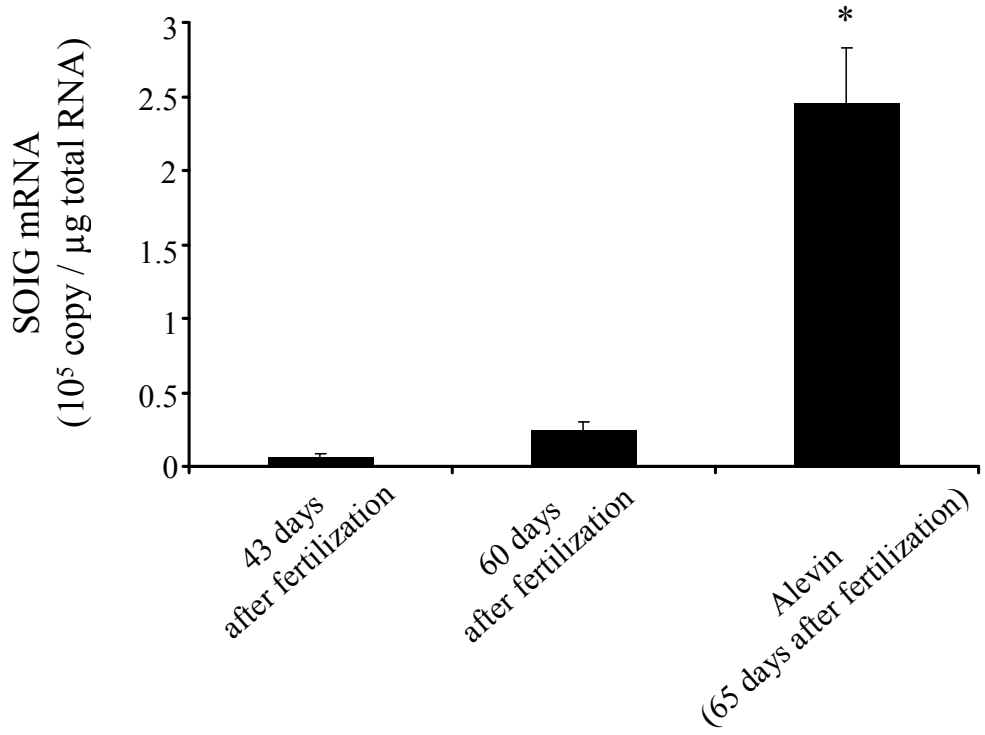


Fig. 20 Changes in SOIG mRNA levels during ontogeny of lacustrine sockeye salmon. Data are represented as means ± SEM. Significant differences among the sampling days after fertilization are indicated (*$p < 0.01$ by one-way ANOVA following Tukey's test).

During the PST, SOIG of male fish peaked in May and significantly increased when compared to SOIG mRNA levels from July to September in the post-PST (Fig. 21A). On the other hand, SOIG mRNA levels of female fish peaked in March, and then tended to decrease from March to May (Fig. 21A). Thereafter, SOIG mRNA levels of female fish tend to increase in June and toward the post-PST as in male fish. Especially, SOIG mRNA levels of females in June were significantly higher than in August at the post-PST.

10) Changes in SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon during sexual maturation

In the male fish, SOIG mRNA peaked in July and decreased gradually toward the spawning period (Fig. 21B). On the other hand, it increased gradually from June to August in the female fish. Thereafter, SOIG mRNA of the females decreased in September and peaked in October. In both sexes, these changes were not statistically significant.

11) Comparison of SOIG mRNA levels in one-year-old and three-year-old lacustrine sockeye salmon

In both sexes, SOIG mRNA levels of one-year-old fish in the PST were higher than three-year-old (Figs. 22A and B). In males, the SOIG mRNA levels showed twofold increase in April at the PST in comparison with June, August, September and October during sexual maturation. On the other hand, the mRNA levels in females in June at the PST were significantly higher than those in three-year-old fish in June.

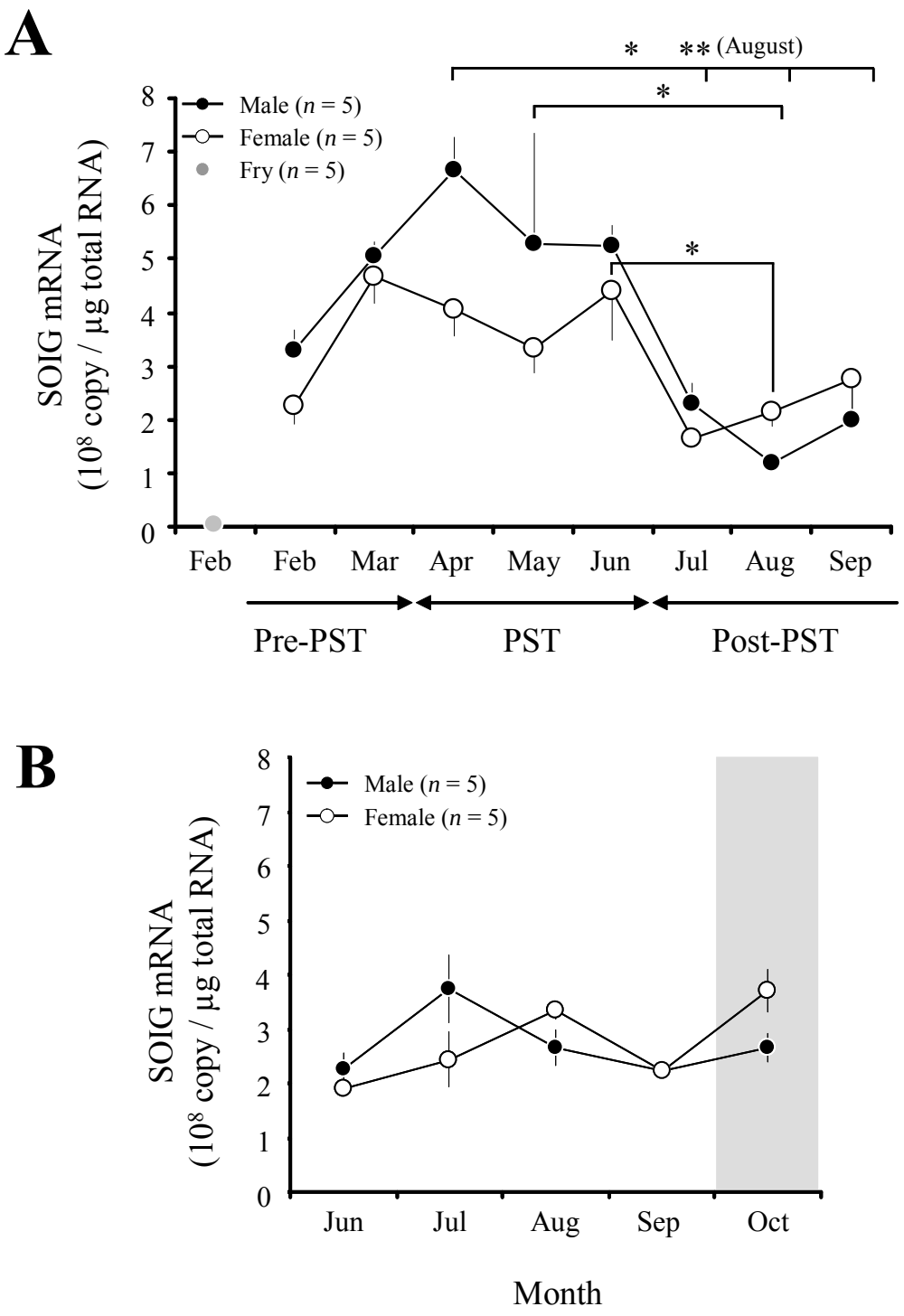


Fig. 21 Changes in SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon. (A) Changes in SOIG mRNA levels of lacustrine sockeye salmon from fry to post-PST in 2006. (B) Changes in SOIG mRNA levels of three-year-old lacustrine sockeye salmon during sexual maturation in 2006. Gray area indicates the spawning period. Data are represented as means ± SEM. Significant differences among the months are indicated (**$p < 0.01$, *$p < 0.05$ by one-way ANOVA following Tukey's test).

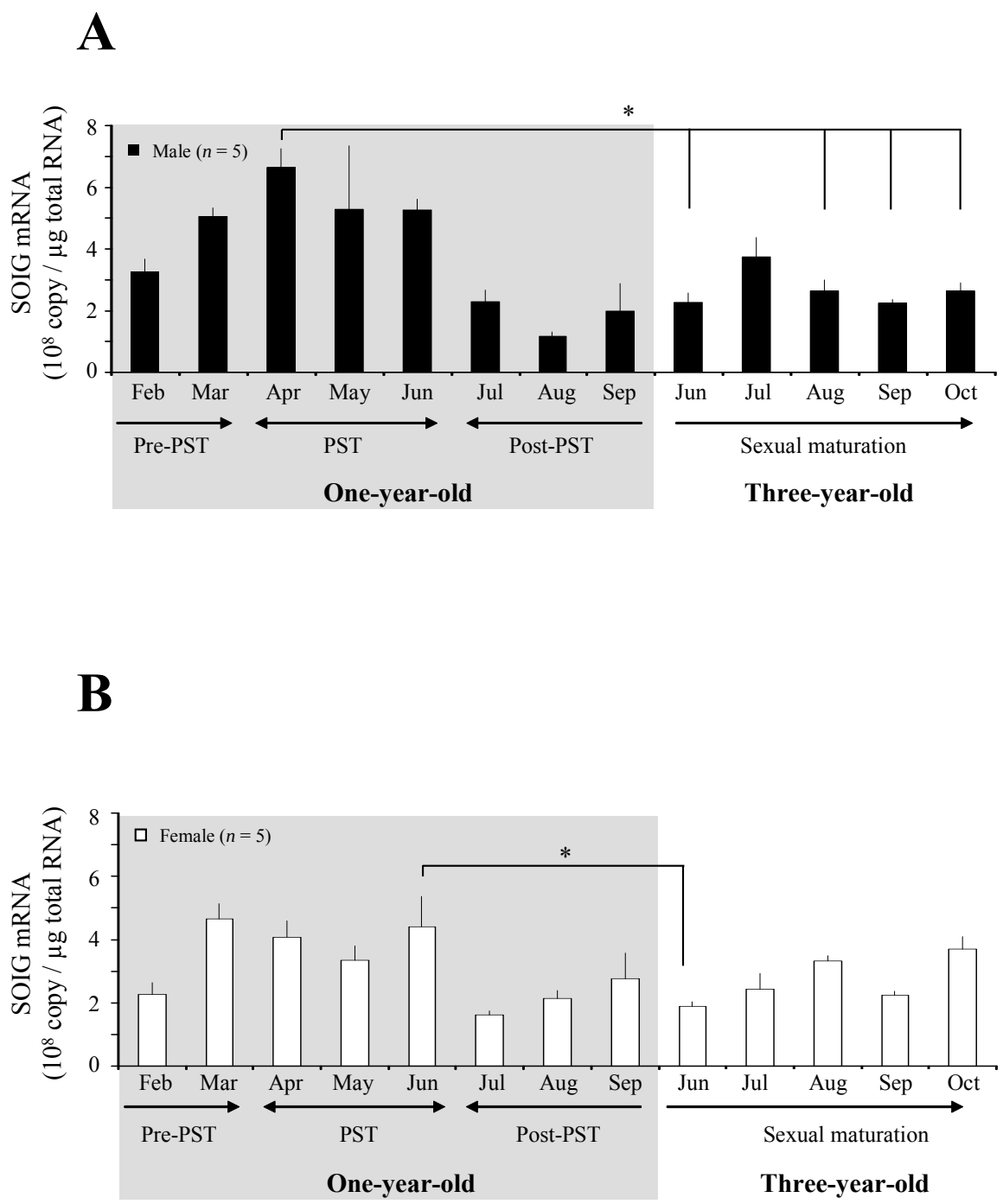


Fig. 22 Comparison of SOIG mRNA expression in the olfactory epithelium of males (A) and females (B) of one-year-old lacustrine sockeye salmon with those in three-year-old fish in 2006. *Significant differences among the months are indicated ($p < 0.05$ by one-way ANOVA following Tukey's test).

12) Changes in SOIG mRNA levels in the olfactory epithelium of chum salmon from early emergence to downstream migration

In under yearling chum salmon, SOIG mRNA indicated low levels from January to April, then increased dramatically in May, and decreased in July in both sexes (Fig. 23). In females, SOIG mRNA levels in May were significantly higher than in other months. In males, SOIG mRNA levels in May were significantly increased as compared to the rest of months except in April.

13) Changes in SOIG mRNA levels of chum salmon during homing migration

During homing migration from branch point to spawning ground, SOIG mRNA levels of males in 2003 increased at the pre-spawning ground, and then sharply decreased toward the spawning ground (Fig. 24A). These levels at the pre-spawning ground in 2003 were significantly higher than the rest of sampling points. On the other hand, these levels of males in 2004 increased at the pre-spawning ground, with a slight increase toward the spawning ground (Fig. 24A). In 2004, SOIG mRNA levels of males at the pre-spawning point and spawning point were significantly higher than those in the Bering Sea. In the females, the SOIG mRNA levels at the pre-spawning ground tended to be higher than those from the Bering Sea to coastal sea in both years (Fig. 24B). In 2004, SOIG mRNA levels of females at the pre-spawning ground significantly higher than those at the branch point and spawning ground. Although SOIG mRNA levels of females in 2003 also had the same tendency as in 2004 from branch point to spawning ground, these changes were not statistically significant. In both years, SOIG mRNA levels at the estuary tended to be higher than those at the coastal sea in both sexes, but these increases were not also statistically significant.

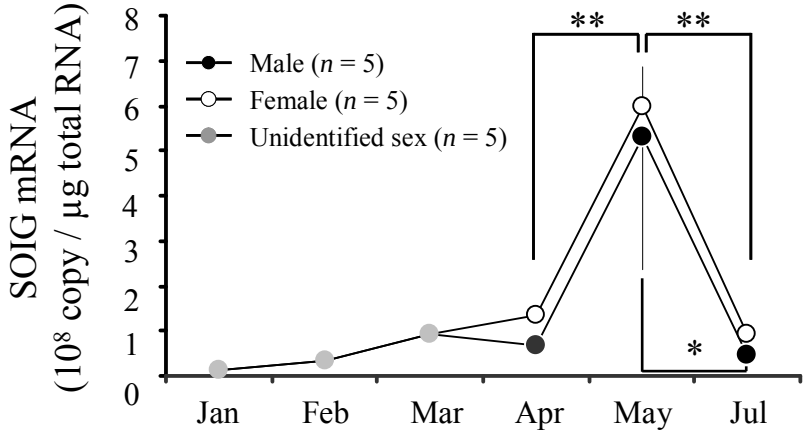
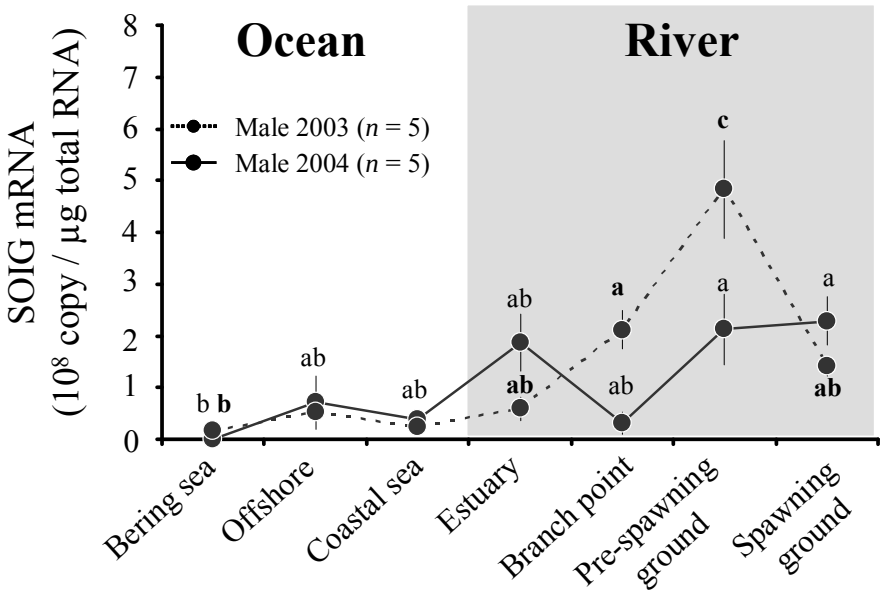


Fig. 23 Changes in SOIG mRNA levels in the olfactory epithelium of under yearling chum salmon from January to July in 2006. Data are represented as means ± SEM. Significant differences among the months are indicated (**p < 0.01, *p < 0.05 by one-way ANOVA following Tukey's test).

A



B

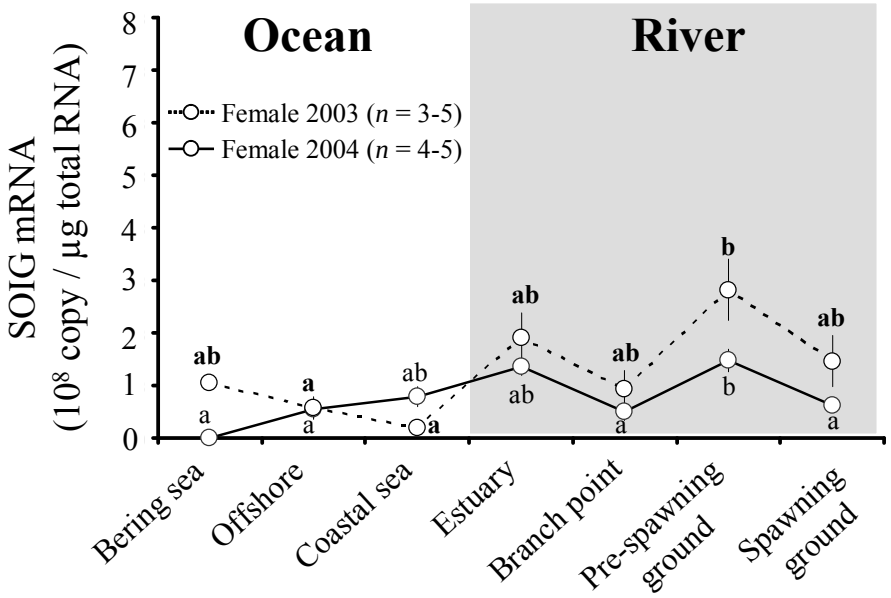


Fig. 24 Changes in SOIG mRNA levels in the olfactory epithelium of males (A) and females (B) during homing migration of chum salmon in 2003 and 2004. The bold and normal letters are represented significant difference for 2003 and 2004 among the sampling points, respectively. Significant differences among the sampling points are indicated ($p < 0.05$ by one-way ANOVA following Tukey's test).

14) Influence of T_3 treatment

I analyzed whether the expression levels of SOIG mRNA in the lacustrine sockeye salmon olfactory epithelium is influenced by the administration of T_3 by real time PCR analysis (Fig. 25). At 48 hours after injection of T_3, the serum concentration levels of T_3 in both experimental groups were significantly higher than control group. On the other hand, there were no significant changes in SOIG mRNA levels of the olfactory epithelium between the T_3-treatment groups and the control group.

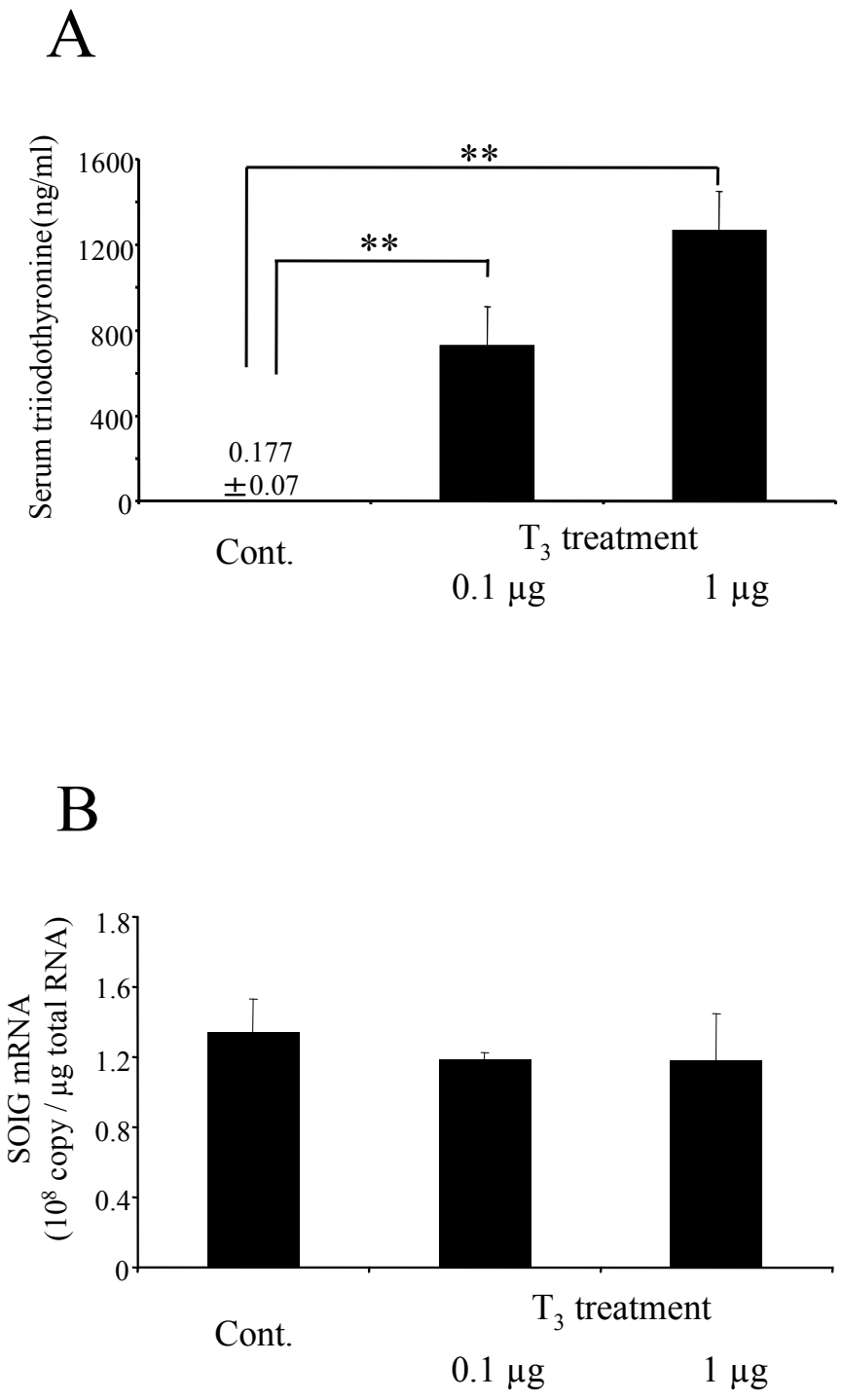


Fig. 25 Changes in serum concentrations of T_3 (A) and SOIG mRNA levels in the olfactory epithelium (B) by T_3 stimulation (0.1 and 1 μg/BW) in male lacustrine sockeye salmon. Each value represents the means ± SEM. Significant differences in T_3 dose are indicated (*$p < 0.01$ by one-way ANOVA following Tukey's test).

Discussion

In the present study, four partial cDNAs were isolated from a forward subtractive cDNA library, and it was found that the expression levels of clone *2* (SOIG) in the olfactory bulb of one-year-old lacustrine sockeye salmon was higher than that of three-year-old fish. The predicted open reading frame of SOIG encodes a protein of 252 amino acids and displayed low amino acid sequence similarity with u-PAR which belongs to Ly-6 superfamily. Overall amino acid identity between members of Ly-6 superfamily and SOIG is also low, ranging between 16% and 22%. However, this family is defined by the presence of cysteine-rich domains, which contains 8 to 10 conserved cysteine residues (Palfree, 1996). SOIG contains two cystein-rich domains that are homologous to each other, showing 35% identity, and conserved nine cysteine residues (Fig. 14A). In each SOIG domains, 6 cysteins are conserved in Ly-6 superfamily (Fig. 14B). However, all Ly-6 superfamily contain a "signature sequence", a conserved sequence found around last 2 C-terminal cysteine residues of the domain. This signature sequence consists of 8 residues with the sequence CCXXXXCN. This sequence is also found twice in SOIG (positions 134-141, 212-219; Fig. 14B).

The u-PAR, a member of Ly-6 superfamily, has an important role in proteolysis of extracellular matrix proteins (Tarui et al., 2001). The u-PAR is a heavily glycosylated signal-chain protein with a molecular weight of 50-60 kDa (Roldan et al., 1990), and organized into three Ly-6 superfamily domains, differently folded domains of approximately 90 amino acids, with four to five disulphide bonds (Ragno, 2006). In contrast, the calculated molecular weight of SOIG is 27.0 kDa, and SOIG contains only two Ly-6 superfamily domains. SOIG may be a novel member of the Ly-6 superfamily, or a distant relative of this suprefamily that shares similar structure.

Ly-6 superfamily members have been identified in several different species, including human and mouse (McKenzie et al., 1977; Bickmore et al., 1993; Suh et al., 1994), but the function of Ly-6 superfamily, except for uPAR, remains to be elucidated. However, Chou et al. (2001) reported that Ly-6-related protein, *odr-2*, which contains two Ly-6 superfamily domains has been isolated from *C. elegans*, and suggested that *odr-2* may regulate the olfactory neuron signaling within the neuronal network required for chemotaxis. SOIG may have an important role in neurotransduction related with olfaction in the olfactory epithelium of salmonids.

Northern blot analysis and *in situ* hybridization revealed that SOIG mRNA was expressed in the olfactory receptor cells and basal cells of the olfactory epithelium of lacustrine sockeye salmon (Figs. 8 and 10). The major role of the olfactory receptor cell may be the chemical stimulus detection, recognition of food and environmental conditions (Wysocki and Meredith, 1987; Zielinski and Hara, 1988, 1992). On the other hand, basal cells are the progenitor cells giving rise to all of the olfactory sensory neurons, and the development and turnover of the olfactory receptor cells in the olfactory epithelium occurs by proliferation of multipotent basal stem cells (Huard et al., 1998; Jang et al., 2003). In rodent, the differentiation period from basal cell to mature olfactory receptor cell was reported to be about one week using autoradiographic analysis (Graziadei and Monti-Graziadei, 1979). In teleost, Yanagi et al. (2004) reported that the differentiation period from basal cell to mature olfactory receptor cell of one-year-old lacustrine sockeye salmon was found to be 7 days by monitoring cell proliferation using 5-bromo-2'-deoxiyuridine (BrdU). Moreover, they demonstrated that differentiation period from basal cell to immature olfactory receptor cell was 3 days, and the present SOIG mRNA signal sites of the olfactory epithelium by *in situ*

hybridization seemed to be similar to the localization of the BrdU immunoreactive site in the immature olfactory receptor cells in the olfactory epithelium of lacustrine sockeye salmon. Moreover, the SOIG mRNA levels of olfactory epithelium tended to increase during homing migration of chum salmon (Fig. 24). Kudo (1994) reported that the number of ciliated and microvillous olfactory receptor cells of chum salmon tends to increase during homing migration by histological analysis. These reports suggest that SOIG may play an important role in the differentiation period from basal cell to olfactory receptor cell. In fact, Ly-6 superfamily related genes are expressed in the stem cell of hematopoietic cell or immature thymocyte cell, and these genes are used as a marker of precursor cells (van de Rijn et al., 1989; Classon and Coverdale, 1994; Capone et al., 1996). Taken together, it is likely that these molecules play roles in cell proliferation.

In this study, the amount of SOIG mRNA in the olfactory bulb of lacustrine sockeye salmon was very low. Wensely et al. (1995) observed the localization of olfactory maker protein (OMP) mRNA in the olfactory system of rat by *in situ* hybridization, and suggested that OMP mRNA was synthesized in receptor cell bodies in the olfactory epithelium and was transported into the axons and terminals in the olfactory bulb. So, there is possibility that SOIG mRNA which is expressed in the olfactory epithelium may be also transported in the olfactory bulb through the axons. That is why there was low amount of SOIG mRNA in the olfactory bulb and it may subject to change at the various periods of life history of salmon.

Yanagi et al. (2004) observed the development of the olfactory system during ontogeny of lacustrine sockeye salmon, and reported that the cilia of the ciliated receptor cells, and the olfactory nerve axons projecting from the olfactory placodes to

the olfactory bulbs were observed at embryonic day 43 after fertilization. They classified the component cells of the olfactory pit into olfactory receptor cells, basal cells, supporting cells, and goblet cells at embryonic day 70 (hatched). In the present study, the expression levels of SOIG mRNA of alevin was significantly higher than those of embryos 43 and 60 days after fertilization, and the SOIG mRNA levels at embryonic day 60 tended to be higher than embryonic day 43. These results raise a possibility that the expression of SOIG mRNA may depend on the development of olfactory system during ontogeny.

cDNAs encoding SOIG isolated from four salmonid species showed highly homologous to SOIG (amino acid level: 93-94%) and contained two Ly-6 superfamily domains and motifs (Figs. 15-18). The molecular weights of SOIG homologs were very closed to each other (27.0 kDa in sockeye salmon; 26.6 kDa in masu salmon; 27.2 kDa in chum salmon; 27.2 kDa in pink salmon; 27.2 kDa in rainbow trout). Moreover, SOIG RNA probe was hybridized in the olfactory epithelium of four salmonid species by Northern blot analysis (Fig. 9). These results indicate that SOIG was commonly expressed in the olfactory epithelium of four salmonid species. However, localization of SOIG expression in the olfactory epithelium of the four salmonid species remains to be established by means of *in situ* hybridization. Further histological study is required to investigate the localization of SOIG in four salmonids species.

The present study describes the changes in expression levels of SOIG mRNA in the olfactory epithelium during different stages of salmon life history by real time PCR. In the olfactory epithelium of lacustrine sockeye salmon, the SOIG mRNA levels in both sexes increased during the PST, and then the expression levels decreased at the post-PST (Fig. 21A). These changes in SOIG mRNA suggest that SOIG has a

particular role in the olfactory epithelium during the PST. Moreover, these changes in the olfactory sensitivity and cell proliferation during the PST are closely related with thyroid hormone. The olfactory sensitivity is enhanced during the PST and enhanced olfactory sensitivity coincided with increased thyroid activity and imprinting ability in Atlantic salmon (*Salmo salar*; Morin et al., 1989a, b, 1994; Morin and Døving, 1992). Moreover, Nevitt et al. (1994) showed that the peripheral olfactory system of coho salmon is sensitized to artificial odorant (PEA; phenyl ethyl alcohol) after exposure to that odorant during the PST. It has been reported in rodent that thyroid hormone stimulates proliferation of basal cells (Mackay-Sim and Beard, 1987; Paternostro and Meisami, 1989, 1994). In teleost, Lema and Nevitt (2004) reported that thyroid hormone induces the olfactory cell proliferation, and suggested that fluctuations in plasma thyroid hormone are reflected in changes in cell proliferation in the olfactory epithelium of coho salmon. Changes in expression levels of SOIG mRNA in the olfactory epithelium seem to be similar with changes in serum concentration levels of T_4 during the PST (Fig. 19). However, no significant correlation (Pearson's correlation test) was observed between SOIG mRNAs and serum levels of T_4 in the both sexes (data not shown). The influence of T_3 on the expression levels of SOIG mRNA in the olfactory epithelium during the PST was investigated by the administration of T_3. However, SOIG mRNA levels of the olfactory epithelium after 48 hours post T_3 injection revealed no changes (Fig. 25B). The previous study showed that thyroid hormone induces olfactory cellular proliferation in salmon by intraperitoneally implanted T_3 pellets for 16-20 days to mimic smolting using coho salmon parr (Lema and Nevitt, 2004). This report suggests that olfactory cellular proliferation may occur in fish before PST by long term administration of T_3. Changes in the expression levels

of SOIG mRNA in the olfactory epithelium may be induced by long term administration of T_3 in the early stage of the PST.

When compared SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon during the PST and sexual maturation showed that the SOIG mRNA levels during the PST were higher than those during sexual maturation (Fig. 22). Especially, the SOIG mRNA levels of male fish in April and female fish in Jun during the PST showed twofold increase in comparison with June during sexual maturation. On the other hand, SOIG mRNA levels in the olfactory epithelium during sexual maturation showed no significant changes. These results suggested that SOIG may act during the PST rather than sexual maturation in lacustrine sockeye salmon.

In the present study, the expression levels of SOIG mRNA in the olfactory epithelium of under yearling chum salmon increased dramatically in May during downstream migration and then sharply decreased in July (Fig. 23). The plasma T_4 concentration of under yearling chum salmon increased during downstream migration and decreased after downstream migration (Parhar and Iwata, 1996; Iwata et al., 2003). T_4 changes from these reports seem to be similar to the changes in SOIG mRNA levels in the olfactory epithelium of under yearling chum salmon. However, I did not analyze the plasma T_4 contents of fish used in this experiment during downstream migration. Moreover, under yearling fish in July used in this experiment were reared in freshwater. The analysis of under yearling chum salmon after river life is necessary to determine the changes in SOIG mRNA levels during downstream migration.

The expression levels of SOIG mRNA in the olfactory epithelium of chum salmon during homing migration were analyzed by real time PCR. The SOIG mRNA levels at the estuary tended to be higher than at the coastal sea (Fig. 24). The

concentration of Ca^{2+} (approximately 10 $mmoll^{-1}$) in seawater is quite higher than freshwater (Bentley, 1998; Withers, 1996). Recently, Shoji et al. (2000) showed that Ca^{2+} is not a major component of the home stream odorants by masu salmon, and suggested that amino acids are major component of the home stream odors by means of EEG. On the other hand, Bodznick (1978) proposed that Ca^{2+} in home stream water plays an important role in the olfactory discrimination of freshwater-reared sockeye salmon by recording extracellularly from the olfactory bulb (olfactory electro-encephalograph, EEG). In vertebrates, Ca^{2+} plays an important role in the olfactory reception. Odorants bind to G protein-coupled receptors to activate adenylate cyclase to generate transient increases in cAMP (Buck and Axel, 1991). cAMP in turn opens an olfactory cyclic nucleotide-gated channel, which allows influxes of Na^{+} and Ca^{2+} to enter the olfactory receptor cells (Nakamura and Gold, 1987). Changes in intracellular Ca^{2+} have effects of activation or inhibition on olfactory adenylate cyclase activity (Heldam and Lancet, 1986; Sklar et al., 1986). Hubbard et al. (2000) reported that the olfactory system of sea bream (*Sparus aurata*) is highly sensitive to reductions in environmental Ca^{2+} by EEG. The elevation of SOIG mRNA levels at the estuary may be caused by reduction of Ca^{2+} concentration due to change of water environment, and SOIG might involve in enhancing olfactory sensitivity for detecting the natal stream odors.

Sex pheromone plays an important role in finding for mating partners in vertebrates. The existence of sex pheromones that are released in water during pre- and post-ovulation has been shown in some teleost fish (Sorense, 1992). Moore and Scott (1991) reported that precocious Atlantic salmon parr was only sensitive to a putative pheromone, testosterone, during a brief period just prior to spawning.

Moreover, Yambe et al. (2006) showed that sex pheromone "$_{L}$-kynurenine" in the urine of ovulated female masu salmon detected only to precocious spermiating males by electrophysiological and behavioral analysis. On the other hand, Ito et al. (1992) showed that sex pheromones were released from males and affected females in the ayu (*Plecoglossus alltivelis*). The peak levels of SOIG mRNA levels in both sexes at the pre-spawning ground may be related to the detection of sex pheromones. It is known that pheromone is recognized in the olfactory receptor of VR type in mammals. In teleost, these receptors are expressed in the microvillous olfactory receptor cells. In this study, although SOIG mRNA was expressed in the olfactory receptor cells, it remains to be solved whether the SOIG mRNA is localized in the ciliated olfactory receptor cells and/or the microvillous olfactory receptor cells.

During homing migration, several hormones related to sexual maturation changes in anadromous salmonids. Fitzpatrick et al. (1986) reported that surges in plasma levels of the reproductive hormone gonadotropin II (luteinizing hormone: LH) and several steroid hormones occur just prior to spawning and coincide with enhanced olfactory sensitivity in coho salmon. Immature coho salmon injected with gonadotropins demonstrated increased olfactory sensitivity to their imprinted odor (Hasler and Scholz, 1983), and androgen treatment of a cyprinid fish (*Puntius schwanenfeldi*) enhanced peripheral olfactory sensitivity to a specific putative sex pheromone (Cardwell et al., 1995). The elevation of SOIG mRNA levels at the pre-spawning ground may be associated with reproductive hormone levels. In fact, the plasma testosterone levels at the pre-spawning ground in 2003 and 2004 were higher than at the spawning ground (Minywelet, unpublished).

In conclusion, SOIG mRNA was expressed in the olfactory epithelium in five

salmonid species, and the amount of SOIG mRNA increased during the PST in the olfactory epithelium of lacustrine sockeye salmon. On the other hand, the SOIG mRNA levels in the olfactory epithelium of chum salmon peaked in May during downstream migration and just prior to spawning during homing migration. These results suggest that SOIG may have an important role either as a neurotransduction in relation to the olfaction or as a promoter of cell proliferation in basal cells at the crucial period of olfactory imprinting and homing in salmonids.

III. Characterization of salmon glutamate carboxypeptidase (sGCP) in lacustrine sockeye salmon

Materials and methods

1) Fish

For semi-quantitative RT-PCR (5 male fish, FL 9.6-10.2 cm, BW 7.9-9.9 g) and construction of cDNA library (3 male fish, FL 9.8-10.8 cm, BW 7.2-11.4 g; 3 female fish, FL 9.8-11.8 cm, BW 7.5-14.0 g), one-year-old lacustrine sockeye salmon were obtained from the Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University in May, 2005.

For quantitative real time PCR of sGCP mRNA levels in the olfactory bulb, one-year-old lacustrine sockeye salmon reared at the Toya Lake Station were collected from February to September 2006. Five fish of both sex in each month, which are the same as the fish used for the study of SOIG were sampled (Table 4A).

Fish were anesthetized with 0.005% eugenol and then the olfactory epithelium, gill, liver, heart, head kidney, spleen, muscle, intestine, testis and brain were surgically isolated. For analysis of brain distributions, brains of lacustrine sockeye salmon were dissected into the olfactory bulb, telencephalon, hypothalamus, optic tectum, cerebellum, and medulla oblongata. Total RNA was isolated from each tissue using ISOGEN (Nippongene) according to the manufacturer's instruction.

2) Semi-quantitative RT-PCR

Single-stranded cDNAs were synthesized from 2.5 μg of the olfactory bulb, telencephalon, hypothalamus, optic tectum, olfactory epithelia, gill, liver, heart, head

kidney, spleen, muscle, intestine and testis from one-year-old fish using a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instruction. PCR amplification was performed with 0.5 μl of the first strand cDNA solution using primers for clone *3* (Table 2). The amplification profile comprised an initial cycle of 2 min at 94˚C followed by 30 cycles as follows: 94˚C for 1 min, 58˚C for 1 min, and 72˚C for 1 min. Amplification of ß-actin was also performed with the same first strand cDNA using primers ß-actin (Table 2). The amplification profile comprised an initiating cycle of 2 min at 94˚C followed by 30 cycles as follows: 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 30 sec. The amplified products were electrophoresed on a 3% agarose gel (Nippongene).

3) Construction of cDNA library and screening

The mRNA was purified from total RNA extracted from the olfactory bulb of one-year-old lacustrine sockeye salmon using an Oligotex™-dT30 (Super) mRNA Purification kit (TaKaRa) according to the manufacturer's instruction. Poly $(A)^+$ RNA (5 μg) was used to construct of cDNA library by the same method of chapter II-2. Approximately 1.3×10^6 primary clones were recovered with an average insert size of 2.5 kb.

Screening of the library was performed with DIG-labeled probes. Screening was carried out by the same method of chapter II-2. The obtained sequences were searched against 'Fasta Sequence Similarity search' (DDBJ), motif database (PROSITE: http://br.expasy.org/prosite/), signal peptide analysis (SignalP 3.0: http:// www. cbs. dtu. dk/services/SignalP/), and hydrophobicity analysis (SOSUI: http://sosui.proteome.bio. tuat.ac.jp/sosuiframe0.html).

4) Accession numbers

The Gen Bank accession numbers for sequences reported in this study are as follows: Q8AWF8, *Xenopus laevis* GCP; Q9P6I2, *Schizosaccharomyces pombe* GCP; Q4WWD5, *Aspergillus fumigatus* GCP; CAD10388, *Homo sapiens* hGCP-2; CAC69883, *Homo sapiens* hGCP; BAB22991, *Mus musculus* mGCP; AAM48397, *Drosophila melanogaster* dGCP; and Q04609, *Homo sapiens N*-acetylated-alinked acidic dipeptidase.

5) Real time PCR

Total RNA was extracted from the olfactory bulb using ISOGEN (Nippongene) according to the manufacturer's instruction. RT reaction was used ExScript® RT reagent kit (TaKaRa). Total RNA (250 ng) was used for RT reaction in a mixture containing 1×ExScript® Buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.5 mM dNTP Mixture, 50 μM Random 6 mers, 25 U ExScript® RTase, and 5 U RNase inhibitor. The reaction was performed at 42 ˚C for 15 min, and stopped at 92 ˚C for 2 min. Real time PCR was carried out with a Mx3000P QPCR System (Stratagene). The PCR reaction mixture contained 1×*Premix Ex Taq*™ (TaKaRa), 1×ROX Reference Dey II, 100 nM each forward and reverse primers (forward primer: 5'-GGCCACTGTGCCATCA-3', reverse primer: 5'-CATGGGAGGGAAGGTGGAA-3') and 150 nM of fluorogenic probe (5'- ^{FAM}GCTCTTGTACGCCGATGTCCACCA^{TAMRA}-3'). Amplification profile was 10 sec at 95 ˚C, followed by 40 cycles of 95 ˚C for 15 sec and 59 ˚C for 1 min (annealing and extension). To determine the amounts of sGCP mRNA, plasmid cDNA of full-length sGCP was used as the standard cDNA. The standard cDNA was serially diluted in the concentration of 1.41×10^3–1.41×10^9 copy (Fig. 26). In the assay,

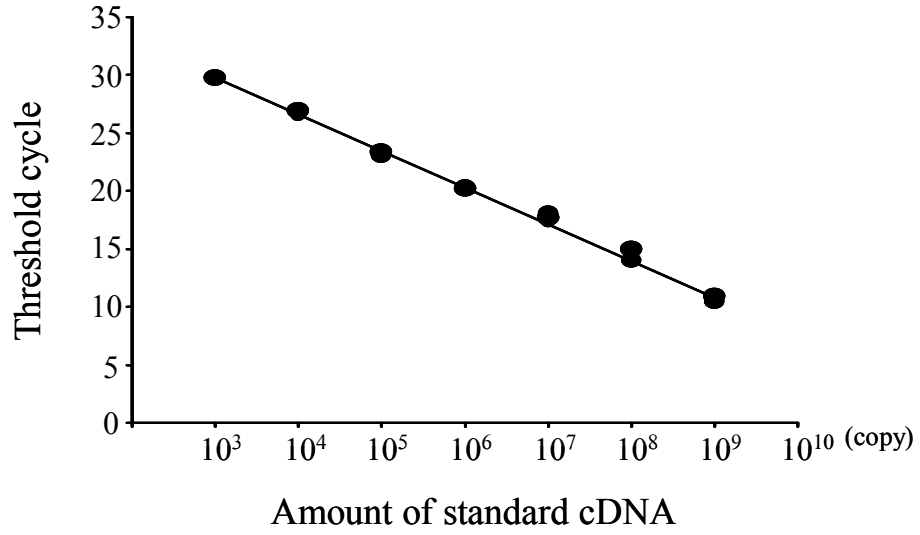


Fig. 26 Typical standard curve for quantitative real time PCR for sGCP mRNA.

several doses of standard cDNA were applied in triplicate and sample cDNA prepared from total RNA were applied in duplicate. In each assay, a standard sample was applied in triplicate to estimate coefficients of variation (CV) within and between runs. The range of intra-assay CV ranged 5.6-9.5% and inter-assay CV was 19.74%. The amounts of sGCP mRNA were expressed as copies per microgram total RNA.

6) Statistical analysis

All data are expressed as means ± SEM. Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered significant when $p<0.05$.

Results

1) Tissue distributions of sGCP mRNA in lacustrine sockeye salmon

Tissue specific distributions of sGCP mRNA in the brain (olfactory bulb, telencephalon, hypothalamus, optic tectum, cerebellum and medulla oblongata) and body tissues (olfactory epithelium, gill, liver, heart, head kidney, spleen, muscle, intestine and testis) from one-year-old lacustrine sockeye salmon were examined by semi-quantitative RT-PCR. The sGCP mRNA was widely expressed in the brain (Fig. 27A). Moreover, sGCP was expressed in the heart, intestine, head kidney, and testis (Fig. 27B).

2) cDNA cloning and characterization of sGCP

To isolate a full-length sGCP cDNA, a cDNA library from one-year-old lacustrine sockeye salmon whole brains was constructed. The cDNA library was screened using the sGCP cDNA probe which was synthesized from clone *3* (Table 3). The screening of approximately 1.2×10^6 plaques from the cDNA library yielded positive clones for sGCP, and these clones were subjected to sequence analysis.

The complete cDNA and deduced amino acid sequences for sGCP are shown in Figure 28. The full-length sGCP cDNA consists of 1,803 bp sequence which contains 1,221 bp open reading frame encoding a polypeptide of 407 amino acids. The 3' untranslated region contains a polyadenylation signal (AATAAA) 14 bp upstream from the poly (A) tail. The deduced amino acid sequence had calculated molecular mass of 45.1 kDa. Homology search revealed that sGCP has 53, 49 and 47% similarity with the amino acid sequence of glutamate carboxypeptidase of *Xenopus laevis*, *Schizosaccharomyces pombe* and *Aspergillus fumigatus*, respectively (Pera et al., 2003).

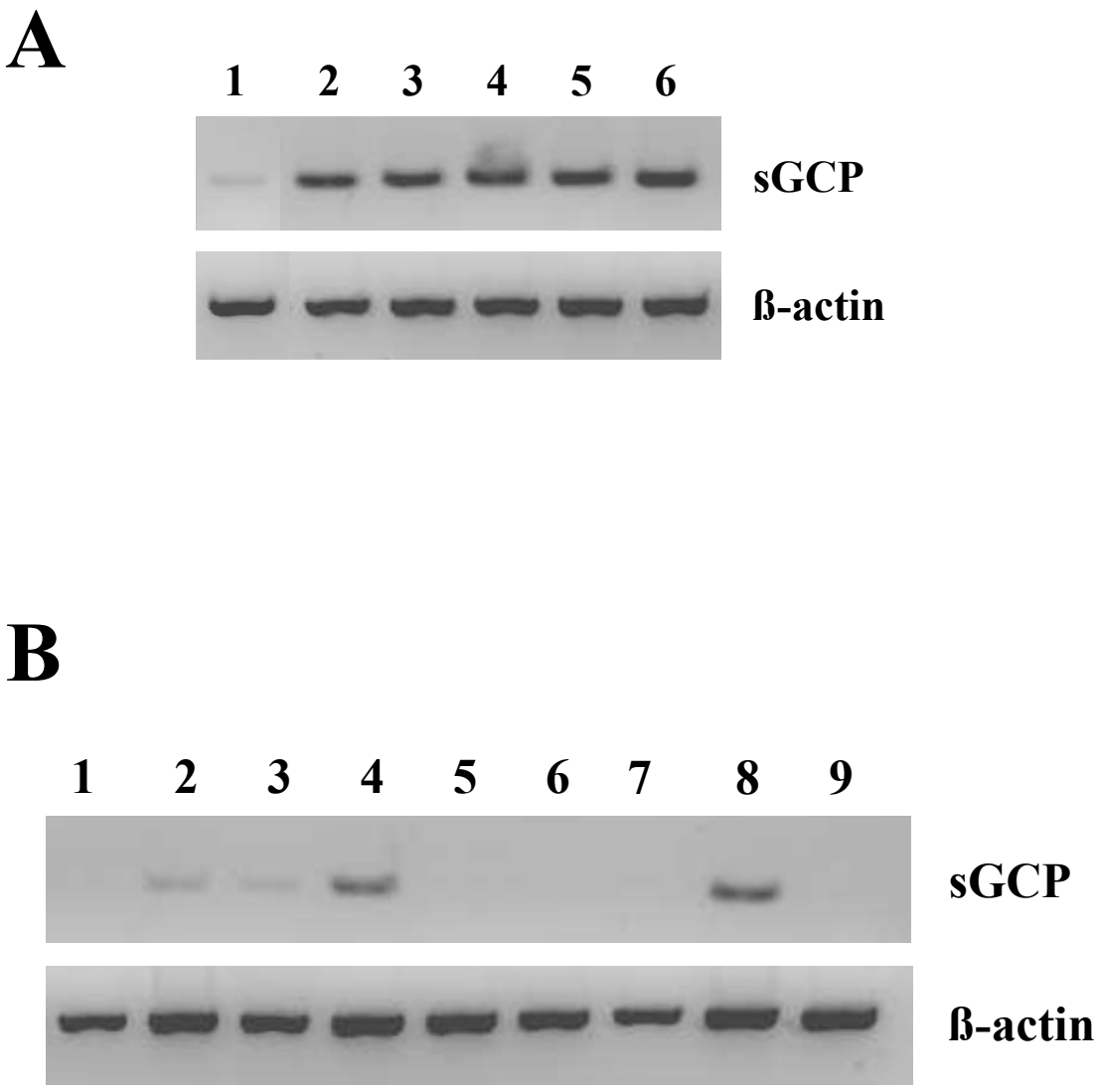


Fig. 27 Semi-quantitative RT-PCR analysis of one-year-old lacustrine sockeye salmon sGCP mRNA. (A) Brain tissues (lane 1, olfactory bulb; lane 2, telencephalon; lane 3, hypothalamus; lane 4, optic tectum; lane 5, cerebellum and lane 6, medulla oblongata). (B) Body tissues (lane 1, olfactory epithelium; lane 2, heart; lane 3, intestine; lane 4, head kidney; lane 5, spleen; lane 6, gill; lane 7, liver; lane 8, testis and lane 9, muscle). Results of 3% agarose gel electrophoresis of semi-quantitative RT-PCR products are shown.

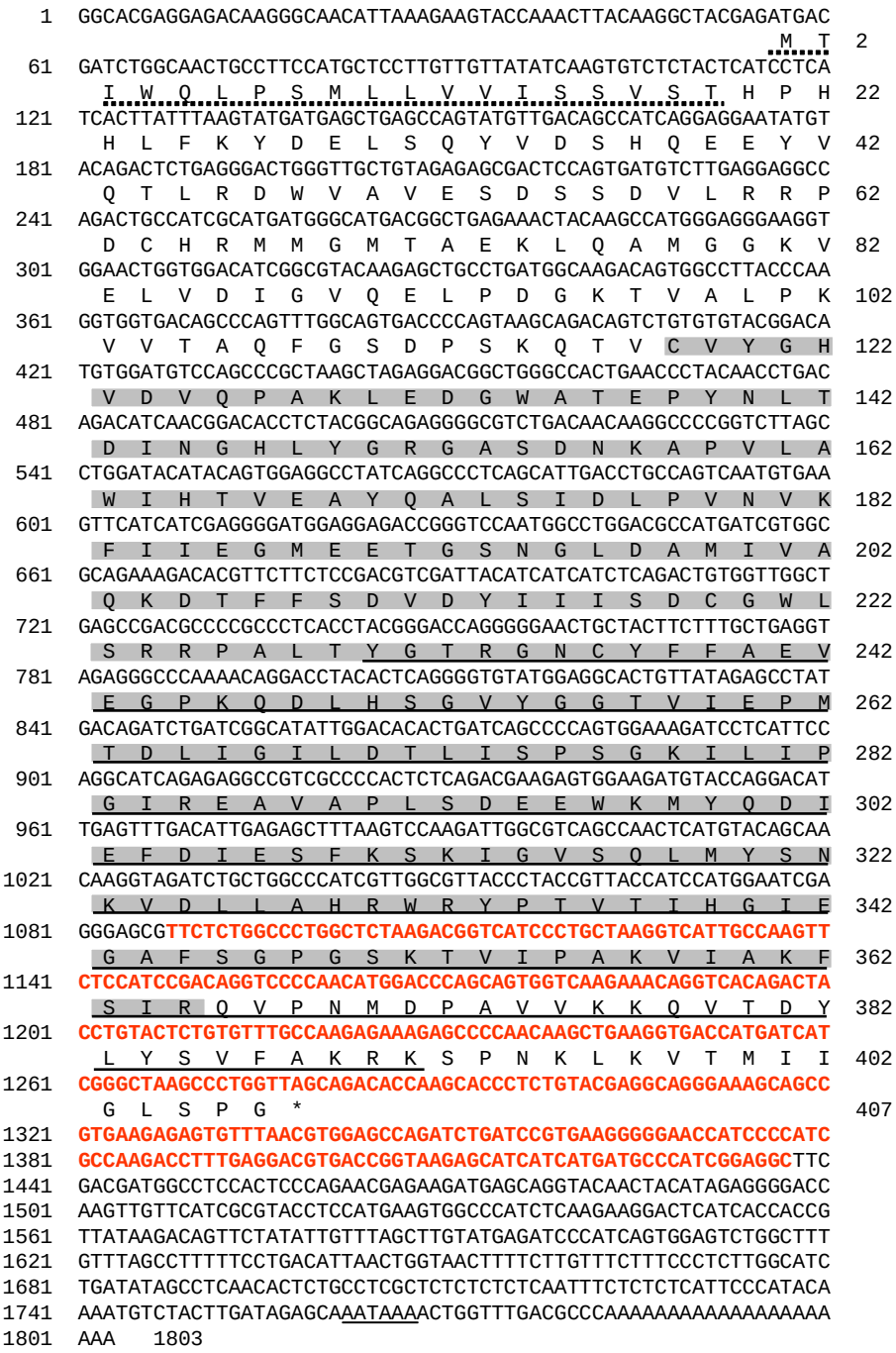


Fig. 28 Nucleotide sequence and deduced amino acid sequence of full-length sGCP cDNA. The stop codon (TAG) and polyadenylation signal (AATAAA) are indicated by an asterisk and thin underline, respectively. The nucleotide sequence of partial sGCP cDNA isolated from forward subtractive cDNA library are red characters. Peptidase family M20 domain and peptidase M20 dimerisation domain are indicated by shade and thick underlines, respectively. The putative sequence of signal peptide is dotted underline.

SignalP program predicted that the N-terminus would be cleaved out at between Thr^{19} and His^{20} by translational processing. Hydrophobicity analysis indicated that sGCP did not contain the transmembrane domain. The domain search revealed that sGCP amino acid contained peptidase family M20 domain (positions 118-365, Fig. 28) and peptidase M20 dimerisation domain (positions 230-391, Fig. 28). Peptidase family M20 domains are found in glutamate carboxypeptidases (Rawlings and Barrett, 1995; Sherwood and Melton, 1998), and these proteins belong to the group of zinc metallopeptidases that hydrolyze peptide bonds and depend on zinc for their activity. sGCP is most closely related to a African clawed frog glutamate carboxypeptidase-like protein (fGCP). The two proteins share 53% amino acid identity. Other proteins related to sGCP include human glutamate carboxypeptidase 2 (hGCP2), human glutamate carboxypeptidase (hGCP), homologous hypothetical proteins in mouse (mGCP) and *Drosophila melanogaster* glutamate carboxypeptidase (dGCP). The share of sGCP and these proteins was 51, 51, 51 and 48% amino acid identity, respectively (Figs. 29 and 30).

In order to determine whether the zinc-ligand residues are conserved in sGCP, I compared amino acid residues of *Pseudomonas* glutamate carboxypeptidase (pGCP) which was already identified as the zinc-ligand residues (His/Asp^{112}, Asp^{114}, Asp^{141}, Glu^{175}, Glu^{176}, Glu/Asp^{200}, His^{385}) with several species of glutamate carboxypeptidase including sGCP. As the result, six active sites were conserved in sGCP (His^{122}, Asp^{124}, Asp^{155}, Glu^{189}, Glu^{190}, Glu^{218}; Fig. 31).

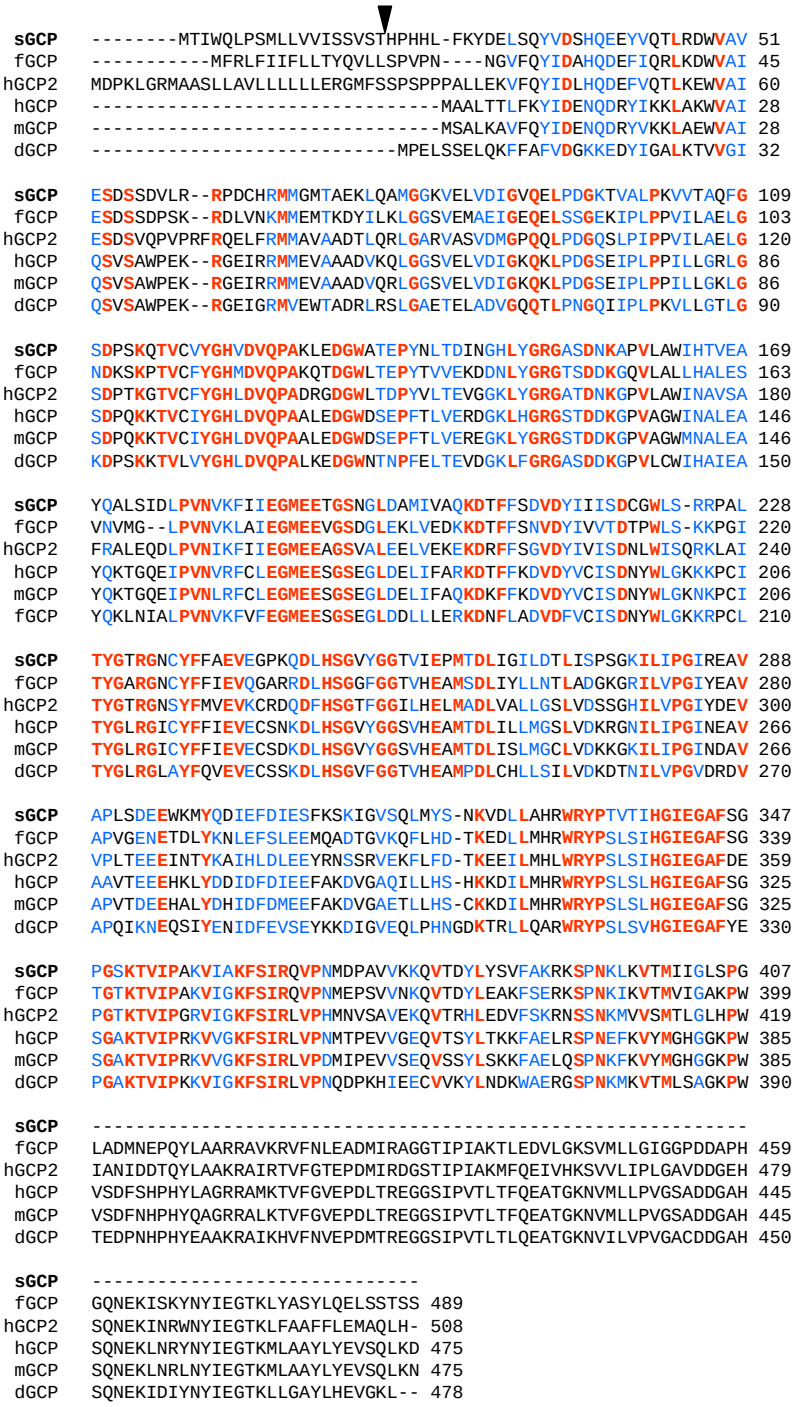


Fig. 29 Amino acid sequences alignment of sGCP, *Xenopus* glutamate carboxypeptidase (fGCP), human glutamate carboxypeptidase-like protein 2 (hGCP2), human glutamate carboxypeptidase (hGCP), mouse glutamate carboxypeptidase (mGCP) and *Drosophila melanogaster* glutamate carboxypeptidase (dGCP). The multiple alignment was performed using ClustalW. Gaps are introduced to maximize homology. Identical amino acids are shown in bold red. Similar amino acids are depicted in blue. The signal peptide cleavage site of sGCP, fGCP and hGCP2 is indicated by arrowhead.

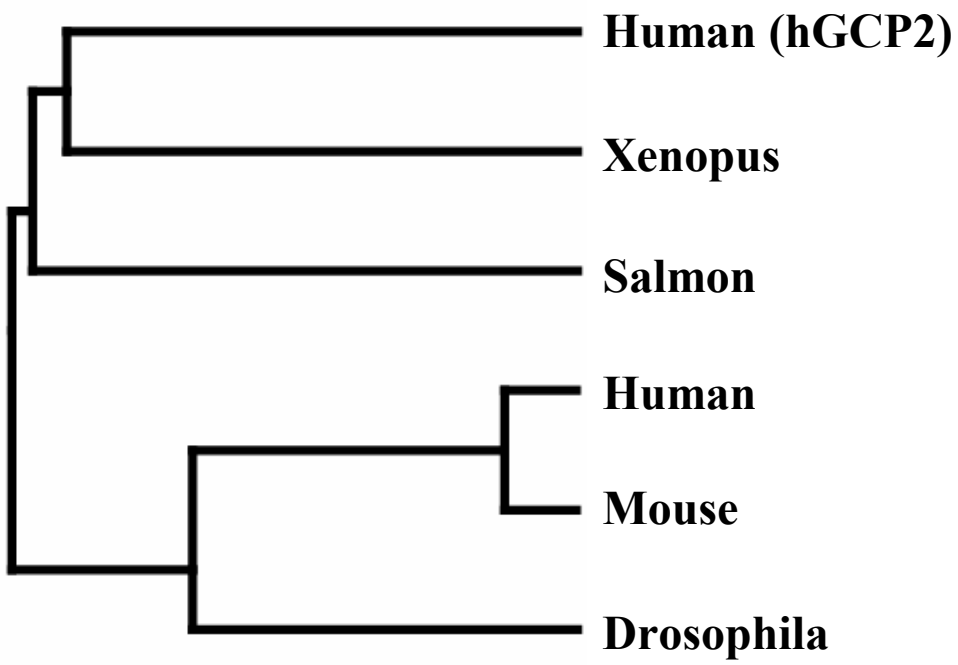


Fig. 30 Evolutionary relationships of sGCP and other members of the GCP family. Proteins displaying the greatest sequence similarity group together, and branch lengths are proportional to distance.

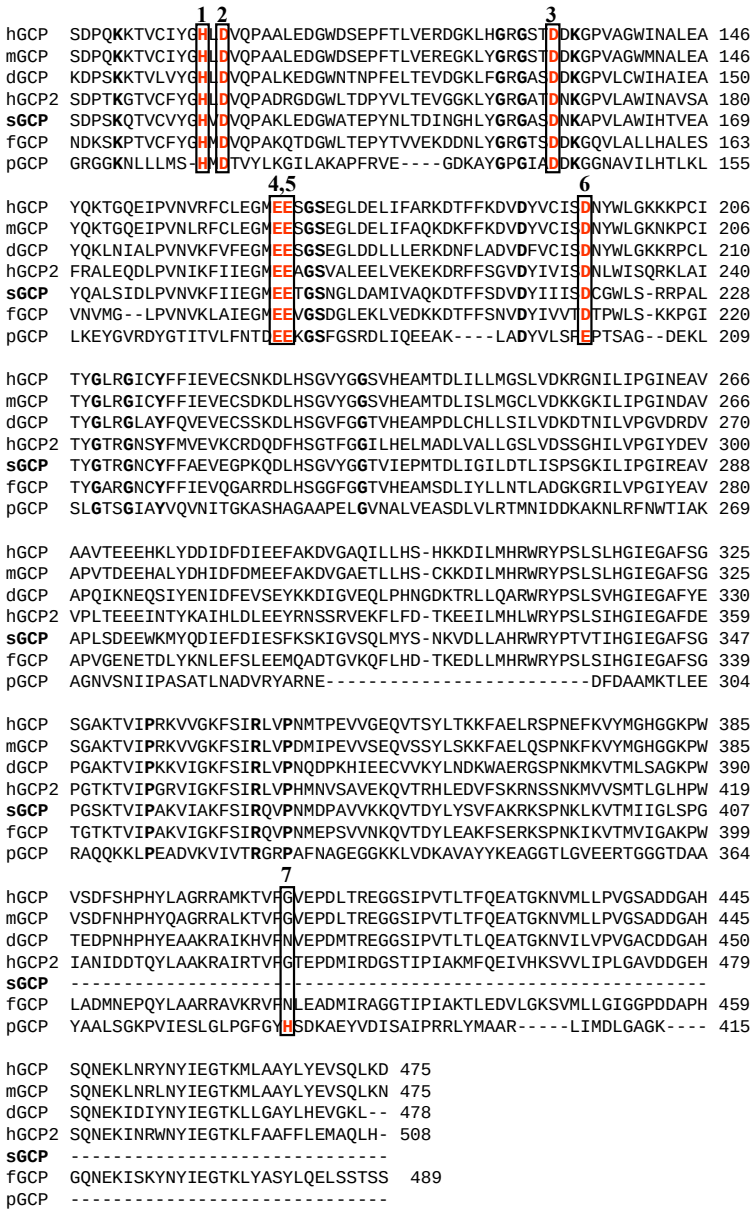


Fig. 31 Amino acid sequences alignment of sGCP, *Xenopus* glutamate carboxypeptidase (fGCP), human glutamate carboxypeptidase-like protein 2 (hGCP2), human glutamate carboxypeptidase (hGCP), mouse glutamate carboxypeptidase (mGCP), *Drosophila melanogaster* glutamate carboxypeptidase (dGCP) and *Pseudomonas* glutamate carboxypeptidase (pGCP). The multiple alignment was performed using ClustalW. Gaps are introduced to maximize homology. Predicted zinc-ligand residues and identical amino acid are shown in bold red and bold black characters, respectively. Numbers represent zinc-ligand residues.

3) Changes in the expression levels of sGCP mRNA in the olfactory bulb of lacustrine sockeye salmon from pre-PST to post-PST

During the PST, the expression levels of sGCP mRNA in the olfactory bulb of male lacustrine sockeye salmon peaked in May and decreased sharply in June (Fig. 32). Thereafter, sGCP mRNA levels of male fish increased from August to September at the post-PST. On the other hand, sGCP mRNA levels of female fish showed the low levels from February to August, and then tended to increase in September at the post-PST. However, in both sexes, these changes were not statistically significant.

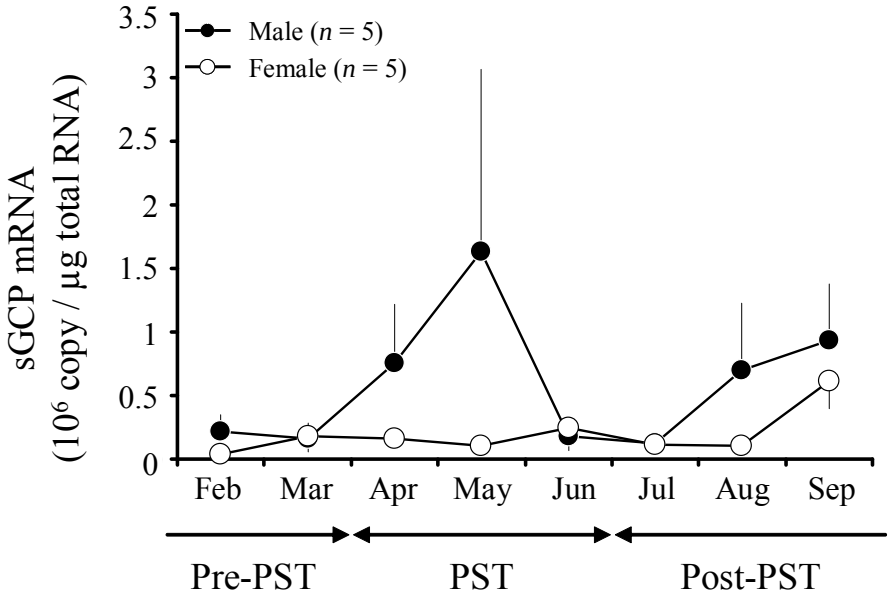


Fig. 32　Changes in sGCP mRNA levels in the olfactory bulb of lacustrine sockeye salmon from pre-PST to post-PST in 2006.　Data are represented as means ± SEM.

Discussion

In this chapter, the characterization of sGCP was carried out by molecular biological techniques. The full-length sGCP cDNA encoded a 407 amino acids protein containing a putative signal peptide at N-terminus, M20 peptidase family domain and M20 dimerisation domain (Fig. 28). M20 peptidase family domains are found in glutamate carboxypeptidases (Rawlings and Barrett, 1995; Sherwood and Melton, 1998). These proteins are predicted as the secreted protein and belongs to the group of zinc metallopeptidases that hydrolyze peptide bonds and depend on zinc for their activity. The sGCP showed 48-53 % similarity to glutamate carboxypeptidase of several species and was predicted in the same cleaved position of signal peptide with hGCP2 and fGCP (Fig. 29). On the other hand, sGCP is weakly homologous to human GCP II (*N*-acety-lated-alinked acidic dipeptidase; 9 % amino acid identity; Rawlings and Barrett, 1997). In contrast to M20 peptidase family, GCP II is a transmembrane protein and belongs to the M28 peptidase family (Rawlings and Barrett, 1997), and this family also has the function of catalytic zinc metallopeptidases. The function of GCP II plays an important role in neurotransduction with *N*-acetyl-aspartyl-glutamate (NAAG; Robinson et al., 1987; Coyle, 1997), and it is known that NAAG acts as a partial agonist/antagonist of a NMDA receptor in the hippocampus of rodents (Puttfarcken et al., 1993). Therefore, I conclude that sGCP is a novel member of M20 family of evolutionarily conserved glutamate carboxypeptidases, because the amino acid sequence of sGCP lacks a transmembrane sequence.

In M20 peptidases family, glutamate carboxypeptidase of *Pseudomonas* sp has active sites of seven zinc-ligand residues (His/Asp^{112}, Asp^{114}, Asp^{141}, Glu^{175}, Glu^{176}, Glu/Asp^{200}, His^{385}; Jozic et al., 2002). Amino acid residues of GCPs including sGCP

contained all active sites except for His^{385} of *Pseudomonas* sp (Fig. 31). These results suggest that these sGCP may contain other potential zinc-ligand residues replaced with His^{385} or receive zinc only by six zinc-ligand residues.

The sGCP mRNA was expressed in the brain, heart, intestine, head kidney and testis (Fig. 27). There has been little report about tissue distributions of M20 peptidase family. However, there are several reports about tissue distributions of GCP II. GCP II is mainly investigated in human, and it is expressed in several tissues as follows; kidney (Chang et al., 1999), testis (Pangalos et al., 1999), ovary (Sokoloff et al., 2000), brain (Luthi-Carter et al., 1998a, b, c; O'Keefe et al., 2004; Troyer et al., 1995), intestine (Troyer et al., 1995), liver (O'Keefe at al., 2004), spleen (O'Keefe et al., 2004; Pangalos et al., 1999), and skeletal muscle (Chang et al., 1999). Especially, GCP II was extensively expressed in the brain, and the localization of GCP II was observed in the cortical astrocytes in mouse (Cassidy and Neale, 1993). Astrocyte cells form an almost complete envelope around the olfactory glomerulus and send processes into the central neuron (Valverde and Lopez-Mascaraque, 1991; Bailey and Shipley, 1993). The expression of sGCP mRNA in tissues corresponded to these reports about the tissue distributions of GCP II in human, and the expression of sGCP mRNA in the brain tended to be higher than the body tissues of lacustrin sockeye salmon (Fig. 27). These findings suggest that sGCP may have similar function of GCP II which acts as a neurotransduction in the brain of salmon.

Metallopeptidases play an important role in tissue repair, neurological processes, protein maturation, hormone-level regulation, cell-cycle control and protein-degradation processes. Recently, Charli et al. (2006) reported that pyroglutamyl peptidase II (PP II)-related zinc metallopeptidase localized on the plasma membrane of TRH (thyro-

tropin-releasing hormone) target neurons and regulated the release of TRH in mouse brain. TRH is also expressed in the brain of teleost. Hamano et al. (1996) demonstrated that the concentration of TRH in the olfactory bulb increased significantly just before the upstream migration or entering the river and dropped after entering the natal stream in chum salmon, and suggested that TRH which distributed in the olfactory bulb may be related to the olfactory function of homing. However, the changes in the expression levels of sGCP mRNA in the olfactory bulb during the PST were not statistically significant (Fig. 32). sGCP may be related to olfaction during homing migration in salmon.

Zinc has crucial roles in enzyme function as an integral constituent of a large number of metalloenzymes. In rainbow trout, zinc deficiency causes growth depression, high mortality, lens cataracts, erosion of fins and skin, and short body dwarfism (Ogino and Yang, 1978; Satoh et al., 1983). However, there is little detailed report about the influence of zinc in the brain in teleost. The influence of zinc in the brain is mainly investigated in rodent. Zinc blocks the current of voltage-dependent calcium channel (Takahashi and Akaike, 1991). In contrast, Izumi et al. (2006) reported that low micromolar concentrations of zinc is dose not depress of LTP using slice of rat brain. Zinc also inhibits NMDA receptors (Traynelis et al., 1998) and may inhibit intracellular response induced by activation of metabotropic glutamate receptors (Xie et al., 1993). These studies suggest that the zinc-metallopeptidases, which shows activity depending on zinc, might be an important role in the formation of memory in rodent. The sGCP may function as a neuromodulator, the activation of which depends on the concentration of zinc in the olfactory bulb of lacustrine sockeye salmon.

In conclusion, sGCP contains zinc-ligand residues and peptidase family M20

domain. sGCP may be a novel member of peptidase family M20 which belongs to zinc-metallopeptidases. It remains for future studies to determine whether the activation of sGCP is caused by zinc or not.

IV. General discussion

The present study are attempted to isolate the olfactory imprinting related genes from the olfactory system by the cDNA-representational difference analysis (cDNA-RDA), aiming at elucidation of the mechanism of the olfactory imprinting in salmonids. I obtained two kinds of clones, SOIG (sockeye salmon olfactory imprinting related gene) and sGCP (salmon gulutamte carboxypeptidase), from subtraction cDNA library in chapter II-1. Several molecular biological techniques characterized SOIG and sGCP, and possible functions of these genes in the olfactory system of salmonids are discussed.

In chapter II-2, SOIG mRNA is found to be exclusively expressed in the olfactory epithelium of salmonids, and its levels increase both during the PST and just prior to spawning. It is suggested that SOIG may have relation to the olfaction or cell proliferation during the PST and the final stage of homing.

In chapter III, the characterization of sGCP mRNA reveals that sGCP contains peptidase family M20 domain which belongs to the group of zinc metallopeptidases with conserved zinc-ligand residues, and it is suggested that sGCP may be a novel member of this family.

The timing of olfactory imprinting of chum salmon differs from lacustrine sockeye salmon. Harden-Jones (1968) and Brannon (1982) proposed that juvenile chum and pink salmon learn a series of olfactory waypoints during their migration through freshwater, and later adult salmon retrace this odor sequence during homing migration. In this study, SOIG mRNA levels in the olfactory epithelium of chum salmon increased in May during the downstream migration and at the pre-spawning ground during homing migration. In chum salmon, SOIG may play important roles in

relation to the olfactory imprinting. However, there are few reports on the timing of the olfactory imprinting of chum salmon. Further study should elucidate the critical timing of the olfactory imprinting of chum salmon. On the other hand, many studies on the timing of the olfactory imprinting were carried out in sockeye salmon and it was suggested that specific odorant factor of the natal river was imprinted during the PST. The PST is a transitional period starting the downstream migration, where individuals undergo a serial morphological, physiological and behavioral changes (Dickhoff et al., 1982; Dickhoff and Sullivan, 1987; Yamauchi et al., 1984). During the PST, there are changes in cytosolic protein pattern of the olfactory epithelium of masu salmon by two-dimentional polyacrylamide gel electrophoresis (Shimizu et al., 1995). Morin and Døving. (1992) performed a stimulation experiment on the olfactory epithelium with L-alanine and recorded the electrophysiological response in the olfactory bulb of L-alanine-exposed and -nonexposed Atlantic salmon from parr to post-PST by means of EEG. They reported that the peaks of electrophysiological response to L-alanine occurred throughout the PST in nonexposed fish, whereas no peaks were observed in exposed fish. Nevitt et al. (1994) reported that the olfactory receptor neurons (ORNs) isolated from 6-9 months fish following phenyl ethyl alcohol (PEA) imprinted coho salmon during the PST was retained highly sensitivity to the imprinted PEA odor. This report suggested that the level of the peripheral olfactory system was linked to the olfactory imprinting and the recall of the imprinted odor. The present study showed that SOIG mRNA levels in the olfactory epithelium of lacustrine sockeye salmon drastically increased in the PST, suggesting that SOIG may be related in the olfactory imprinting. However, unlike wild chum salmon during homing migration, SOIG mRNA levels of lacustrine sockeye salmon during sexual maturation were not

significantly increased. Further study should analyze the expression levels of SOIG mRNA in the olfactory epithelium of wild sockeye salmon during sexual maturation.

In vertebrates, odor detection is normally initiated by the interaction of odorants with G protein-coupled receptors in the olfactory receptor cells. The activated receptor stimulates adenylate cyclase, causing a rapid increase in cAMP. Increased cAMP activates cyclic nucleotide-gated channels, resulting in membrane depolarization of action potentials. On the other hand, guanylate cyclase in the olfactory neurons also sutimulates cyclic nucleotide-gated channels. Normally, Cyclic 3',5'-guanosine monophosphate (cGMP) has a role in the photoreception in vertebrates, and the production of cGMP was controlled by soluble guanylate cyclase which was activated by nitric oxide (NO). NO is produced by the enzyme nitric oxide synthase (NOS). Chen et al. (2004) reported that NOS is transiently expressed in developing and regenerating OSNs in mice, suggesting that NO-mediated cGMP responses may play a role during the proliferation or maturation of developing and regenerating OSNs. Moreover, Kendrick et al. (1997) reported that the formation of olfactory memories mediated by NO in sheep. Nevitt and Moody. (1992) recorded the electrical properties of the ciliated olfactory receptor cells isolated from different stages such as the PST, sea-run and spawning of coho salmon using the whole-cell of patch-clamp technique, and reported that relaxation of the outward current was slowed by dialysis with cGMP only in the ciliated olfactory receptor cells at the PST and sea-run stage, but not in those of spawning stage. Dittman et al. (1997) reported that stimulation of guanylate cyclase activity by PEA was significantly greater in the olfactory cilia isolated from PEA-imprinted coho salmon compared with PEA-naive fish only at the time of the homing migration, 2 years after PEA exposure. Additionally, Harden et al. (2006)

measured the expression levels of transcription factor, *otx2*, which was isolated from the olfactory epithelium of PEA-exposed zebrafish, by *in situ* hybridization, and they demonstrated that *otx2* is up regulated in the olfactory epithelium of PEA-exposed fish comparison with non-PEA-exposed fish. These reports suggest that, unlike normally olfactory reception mediated cAMP, unique signaling pathway for the olfactory imprinting may exist in the olfactory epithelium of salmonids. The present study suggests that SOIG may have an important role in neurotransduction related to the olfaction during critical period such as the PST and homing of salmonids (Fig. 33-I). The function of SOIG may be able to be identified by the patch clamp methods if the expression of SOIG mRNA in the olfactory receptor cells which were isolated from artificial odorants-imprinted salmon may be silenced by RNA interference.

Several olfactory-specific proteins have been identified, including OMP (Margolis, 1972; Keller and Margolis, 1975, 1976) and salmonid olfactory system-specific protein (N24; Shimizu et al., 1993; Kudo et al., 1996, 1999). In the function of OMP, there is report that OMP contributes to olfactory sensitivity by electrophysio-logical technique using OMP-knockout mice (Buiakova et al., 1996; Ivic et al., 2000; Youngentob et al., 2001). On the other hand, N24, a glutathione *S*-transferase isoform, is suggested to play a role in the olfactory termination by xenobiotic function in the olfactory receptor cells and neuromodulation in the glomerulus of salmon (Kudo et al., 1996, 1999). Although both proteins are localized in the mature olfactory receptor cells, immunoreactive sites of these proteins seem to be different to the expression sites of SOIG mRNA. The SOIG mRNA was expressed also in the immature olfactory receptor cells which are the stem cell of the olfactory receptor cells. The present study described that SOIG was expressed in the basal cells, and suggested that SOIG may

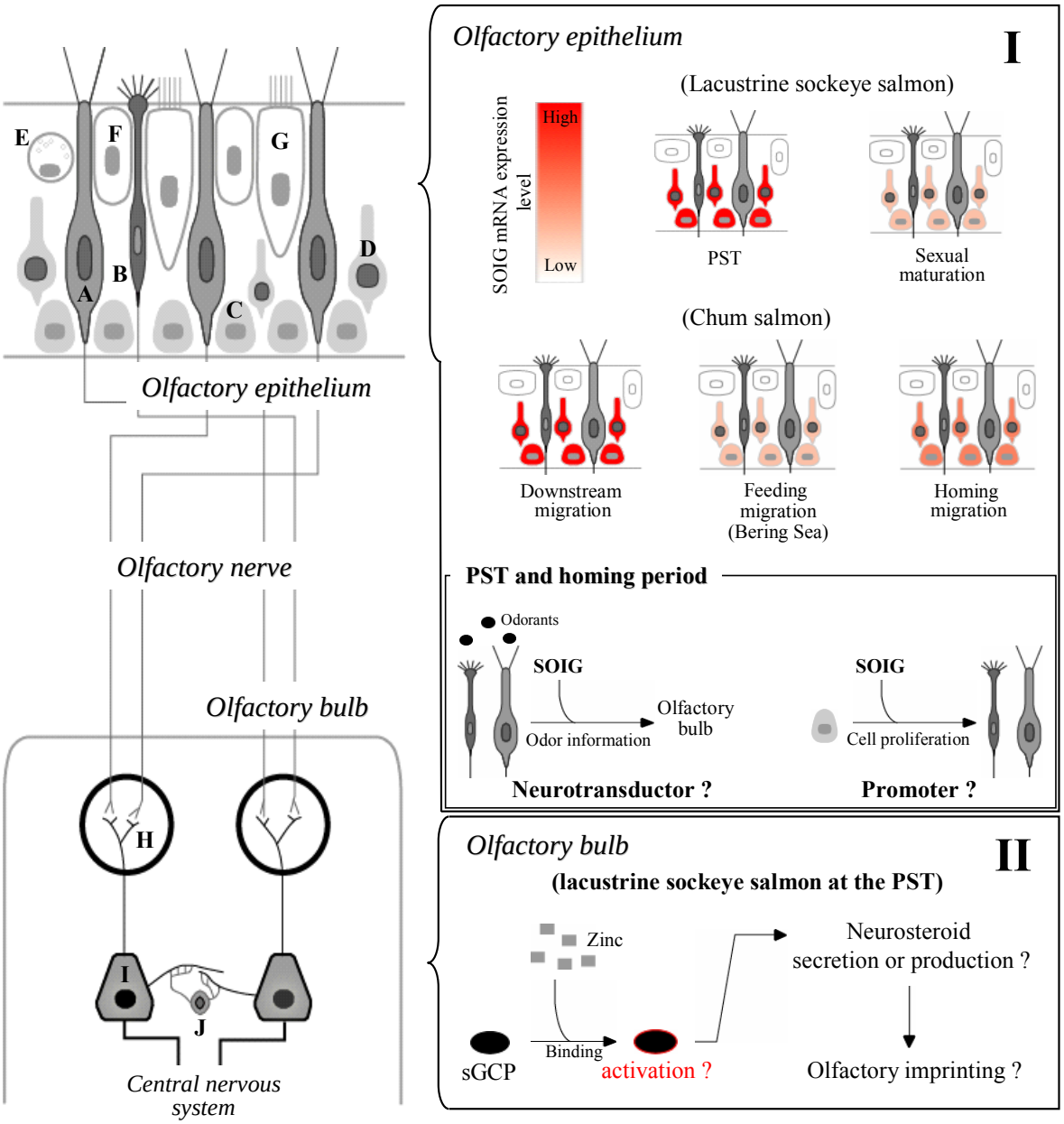


Fig. 33 Schematic illustration of the possible functions of SOIG (I) and sGCP (II) in the olfactory system of salmonid species. (I) SOIG mRNA expression level is indicated by vertical red bar. The intensity of red color (deep red, high intensity; light red, low intensity) shows the amount of SOIG mRNA expression in the immature olfactory receptor cells and basal cells at the several stages of life history in lacustrine sockeye salmon and chum salmon. A; ciliated olfactory receptor cell, B; microvillous olfactory receptor cell, C; basal cell, D; immature olfactory receptor cell, E; goblet cell, F; supporting cell, G; ciliated nonsensory, H; glomerular layer, I; mitral cell, J; granule cell.

promote the cell proliferation (Fig. 33-I). However, it has not been investigated yet whether SOIG is present as a protein in the olfactory system of salmon. Recently, the growth-associated protein B-50/GAP-43 (GAP-43) is used as an olfactory marker for recognizing immature olfactory receptor neurons in mouse olfactory epithelium (Holtmaat et al., 1999). Further work will be required to characterize SOIG protein by imunohistochemical and molecular biological study and to isolate GAP-43 mRNA from the olfactory epithelium of salmon.

In general, memory is thought to involve changes in neurons and neural connections in the brain, and many studies on the olfactory memory in mammals were focused on the main or accessory olfactory bulb. In mammals, zinc plays an important role in engaged memory and the activity of zinc-metallopeptidase might be involved in the formation of memory. The present study suggested that zinc-metallopeptidases (sGCP) belonging to peptidase family M20 were expressed in the olfactory bulb of lacustrine sockeye salmon (Fig. 33-II). Further study should elucidate the enzyme activity of sGCP with zinc using recombinant sGCP. Zinc causes several physiological changes in fish. Fish utilize zinc from water through the gills and the absorbed zinc is conveyed to the brain throughout the blood. In female channel catfish (*Ictalurus punctatus*), accumulation of zinc in the livers and ovaries increase during sexual maturation (Banks et al., 1999), thereby the relation of zinc and steroid hormones is suggested. Moreover, zinc deficiency causes reduction in testosterone production in male rats (Pekary et al., 1993). The distribution of sGCP mRNA in lacustrine sockeye salmon showed that sGCP was expressed in the testis and brain. In vertebrates, steroid hormone (neurosteroid) exists also in the brain, suggesting that steroid hormone functions as a neuromodulator. The action of neurosteroid is thought to be mediated

through GABA receptor and NMDA receptor (Kawato et al., 2002), and these receptors play important role in neuronal plasticity which are involved in memory and learning. GCP may play a role in relation to the secretion and production of steroid hormone (Fig. 33-II). However, the expression levels of sGCP mRNA in the olfactory bulb of salmonids during homing migration remains to be established by means of real time PCR. In addition, it has not been investigated yet whether the activation of sGCP is caused by zinc and which peptides are hydrolyzed by sGCP in salmon brain. Further research should explore the levels of sGCP mRNA and neurosteroid in the salmon brain during homing migration.

V. Summary

The present study was conducted to elucidate the mechanism of olfactory imprinting by means of molecular biological approaches using lacustrine sockeye salmon, masu salmon, chum salmon, pink salmon, and rainbow trout. In order to isolate the imprinting related genes from the olfactory system during the parr-smolt transformation (PST), subtractive hybridization technique of cDNA-representational difference analysis (cDNA-RDA) was carried out using the olfactory bulb of one-year-old lacustrine sockeye salmon at the PST as a tester and three-year-old fish at the feeding migration as a driver. SOIG (Sockeye salmon olfactory imprinting related gene) and sGCP (salmon glutamate carboxypeptidase), whose mRNA expression levels in the olfactory bulb of one-year-old fish were tended to be higher than the olfactory bulb of three-year-old, were obtained from subtractive cDNA library. Finally, these genes are characterized by molecular biological techniques.

1. By Northern blot analysis and *in situ* hybridization using several tissues of lacustrine sockeye salmon at the PST, the localization of SOIG mRNA was investigated. SOIG mRNA was expressed in the immature olfactory receptor cells and basal cells of the olfactory epithelium. On the other hand, the expression of SOIG mRNA in the olfactory bulb was not detected by Northern blotting.

2. The full-length of SOIG mRNA was isolated from a cDNA library constructed from the olfactory epithelium of lacustrine sockeye salmon at the PST. SOIG cDNA was 1,700 bp in length, having an open reading frame of 756 bp encoding 252 amino acids which two Ly-6 superfamily domains.

3. In the analysis of SOIG mRNA expression levels in the olfactory epithelium of lacustrine sockeye salmon from emergence to post-PST, the SOIG mRNA levels during the PST were higher than those at the post-PST in both sexes. Moreover, the SOIG mRNA levels during the PST tended to be higher than those in three-year-old fish during sexual maturation. During ontogeny, the expression levels of SOIG mRNA in alevin were significantly higher than those in embryos 43 and 60 days after fertilization.

4. Complementary DNA clones encoding SOIG homologs were isolated from the olfactory epithelium of masu salmon, chum salmon, pink salmon and rainbow trout. Predicted amino acid sequences of these cDNAs were very similar to SOIG (93-94% at the amino acid level) and contained two Ly-6 superfamily domains. Furthermore, these mRNAs were only observed in the olfactory epithelium by Northern blot analysis.

5. The changes in SOIG mRNA levels in the olfactory epithelium of chum salmon during downstream and homing migration were measured by real time PCR. SOIG mRNA levels sharply increased in May during downstream migration in both sexes. During homing migration of chum salmon, SOIG mRNA levels in the olfactory epithelium was elevated at the estuary and pre-spawning ground.

6. sGCP was expressed in the brain, heart, intestine, head kidney and testis of lacustrine sockeye salmon by semi-quantitative RT-PCR

7. sGCP has 1,221 bp open reading frame that encodes a polypeptide of 407 amino acids with peptidase family M20 domain and M20 dimerisation domain. Moreover,

six zinc-ligand residues were conserved in sGCP (His^{122}, Asp^{124}, Asp^{155}, Glu^{189}, Glu^{190}, Glu^{218}).

8. The changes in the expression levels of sGCP mRNA levels in the olfactory bulb of lacustrine sockeye salmon from pre-PST to post-PST were investigated by real time PCR. However, the changes in sGCP mRNA levels were not statistically significant.

VI. Acknowledgements

Sincere gratitude is due to Professor Hiroshi Ueda, Field Science Center for Northern Biosphere, Hokkaido University, for his invaluable guidance and encouragement throughout the present study. I would also like to express my gratitude to Professor Masakane Yamashita, Graduate School of Science, Hokkaido University, Professor Etsurou Yamaha, Field Science Center for Northern Biosphere, Hokkaido University and Associate Professor Hideaki Kudo, Graduate School of Fisheries Science, Hokkaido University, for their important suggestion and critical reading of the manuscript.

I would also like to take this opportunity to thank the following people: Associate Professor Takashi Todo, Graduate School of Fisheries Science, Hokkaido University, for providing a 3,3',5-triiodo-$_L$-thyronine. Dr. Toshiharu Iwai, Graduate School of Science, Hokkaido University for his advice and encouragement on molecular biological experiments; Dr. Masatoshi Ban, National Salmon Resources Center for providing chum salmon and advice; Mr. Motohiro Kikuchi, Chitose Salmon Aquarium for providing chum salmon and advice; Mr. Masaki Ichimura, Shibetsu Salmon Museum for providing pink salmon and advice; Mr. Hiroyuki Haruna, Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, for his help in maintenance of lacustrine sockeye salmon, chum salmon, masu salmon and rainbow trout used in the present study; Mr. Minwyelet Mingist Zerihun, a Ph. D. candidate, Graduate School of Fisheries Sciences, Hokkaido University for his help and useful comments; and all colleagues who have studied in Laboratory of Aquatic Ecosystem Conservation, Graduate School of Environmental Science, Hokkaido University, for their help.

VII. References

Bailey, M.S., Shipley, M.T., 1993. Astrocyte subtypes in the rat olfactory bulb: morphological heterogeneity and differential laminar distribution. J. Comp. Neurol. 328, 501-526.

Banks, S.D., Thomas, P., Baer, K.N., 1999. Seasonal variations in hepatic and ovarian zinc concentrations during the annual reproductive cycle in female channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 124, 65-72.

Barth, A.L., Dugas, J.C., Ngai, J., 1997. Noncoordinate expression of odorant receptor genes tightly linked in the zebrafish genome. Neuron. 19, 359-369.

Bentley, P.J., 1998. Hormones and calcium metabolism. In Comparative Vertebrate Endocrinology. Third edition. pp. 269-301. Cambridge: Cambridge University Press.

Bickmore, W.A., Longbottom, D., Oghene, K., Fletcher, J.M., van Heyningen, V., 1993. Colocalization of the human CD59 gene to 11p13 with the MIC11 cell surface antigen. Genomics. 17, 129-135.

Bodznick, D., 1978. Calcium ion: An odorant for natural water discriminations and the migratory behavior of sockeye salmon. J. Comp. Physiol. A. 127, 157-166.

Brannon, E.L., 1982. Orientation mechanisms of homing salmonids. In Salmon and Trout Migratory Behavior Symposium (ed. E. L. Brannon and E. O. Salo), pp. 219-227. Seattle: School of Fisheries, University of Washington.

Buck, L.B., 2000. The molecular architecture of odor and pheromone sensing in mammals. Cell. 100, 611-618.

Buck, L., Axel, R., 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 65, 175-187.

Buiakova, O.I., Baker, H., Scott, J.W., Farbman, A., Kream, R., Grillo, M., Franzen, L., Richman, M., Davis, L.M., Abbondanzo, S., Stewart, C.L., Margolis, F.L., 1996. Olfactory marker protein (OMP) gene deletion causes altered physiological activity of olfactory sensory neurons. Proc. Natl. Acad. Sci. USA. 93, 9858-9863.

Capone, M.C., Gorman, D.M., Ching, E.P., Zlotnik, A., 1996. Identification through bioinformatics of cDNAs encoding human thymic shared Ag-1/stem cell Ag-2. A new member of the human Ly-6 family. J. Immunol. 157, 969-973.

Cardwell, J.R., Stacey, N.E., Tan, E.S.P., McAdam, D.S.O., Lang, S.L.C., 1995. Androgen increases olfactory receptor response to a vertebrate sex pheromone. J. Comp. Physiol. A. 176, 55-61.

Carter, R.E., Coyle, J.T., 1998. In Handbook of Proteolytic Enzymes; Barrett, A.J., Rawlings, N.D., Woessner, J.F., (eds); Academic: New York, pp. 1434.

Cassidy, M., Neale, J.H., 1993. *N*-acetylaspartylglutamate carbolism is achieved by an enzyme on the cell surface of neurons and glia. Neuropeptides. 24, 271-278.

Chang, S.S., Reuter, V.E., Heston, W.D., 1999. Five different antiprostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. Cancer Res. 59, 3192-3198.

Charli, J.L., Chavez-Gutierrez, L., Matta, E., Cruz, R., Vargas, M.A., 2006. Structure and function of pyroglutamyl peptidase II, the TRH inactivating enzyme. Neuropeptides. 40, 1.

Chen, J., Tu, Y., Moon, C., Matarazzo, V., Palmer, A.M., Ronnett, G.V., 2004. The localization of neuronal nitric oxide synthase may influence its role in neuronal precursor proliferation and synaptic maintenance. Dev. Biol. 269, 165-182.

Chou, J.H., Bargmann, C.I., Sengupta, P., 2001. The *Caenorhabditis elegans odr-2* gene encodes a novel Ly-6-related protein required for olfaction. Genetics. 157, 211-224.

Classon, B.J., Coverdale, L., 1994. Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins. Proc. Natl. Acad. Sci. USA. 91, 5296-5300.

Cooper, J.C., Lee, G.F., Dizon, A.E., 1974. An elevation of the use of the EEG technique to determine chemical constituents in home stream water. Wisc. Acad. Sci. Arts Lett. 62, 165-172.

Coyle, J.T., 1997. The nagging question of the function of *N*-acetylaspartylglutamate. Neurobiol. Dis. 4, 231-238.

Curatolo, A., D'Arcangelo, P., Lino, A., Brancati, A., 1965. Brain concentration of glutamic, aspartic, *N*-acetylaspartic and *N*-acetylaspartyl-glutamic acids in rats chronically treated with aspartic and *N*-acetylaspartic acids. Boll. Soc. Ital. Biol. Sper. 41, 588-590.

Dickhoff, W.W., Darling, D.S., Gorbman, A., 1982. Thyroid function during smoltification of salmonid fish. Gunma Symp. Endocr. 19, 45-61.

Dickhoff, W.W., Folmar., L.C., Gorbman, A., 1978. Changes in plasma thyroxine during smoltification of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 36, 229-232.

Dickhoff, W.W., Sullivan, C., 1987. Involvement of the thyroid gland in smoltification, with special reference to metabolic and developmental processes. Am. Fish. Soc. Symp. 1, 197-210.

Dittman, A.H., Quinn, T.P., Nevitt, G.A., 1996. Timing of imprinting to natural and artificial odors by coho salmon, *Oncorhynchus kisutch*. Can. J. Fish. Aquat. Sci. 53, 434-442.

Dittman, A.H., Quinn, T.P., Nevitt, G.A., Hacker, B., Storm, D.R., 1997. Sensitization of olfactory guanylyl cyclase to a specific imprinted odorant in coho salmon. Neuron. 19, 381-389.

Dukes, J.P., Deaville, R., Bruford, M.W., Youngson, A.F., Jordan, W.C., 2004. Odorant receptor gene expression changes during the parr-smolt transformation in Atlantic salmon. Mol. Ecol. 9, 2851-2857.

Dukes, J.P., Deaville, R., Gottelli, D., Neigel, J.E., Bruford, M.W., Jordan, W.C., 2006. Isolation and characterisation of main olfactory and vomeronasal receptor gene families from the Atlantic salmon (*Salmo salar*). Gene. 371, 257-267.

Dulac, C., Axel, R., 1995. A novel family of genes encoding putative pheromone receptors in mammals. Cell. 83, 195-206.

Engelholm, L.H., Behrendt, N., 2001. Differential binding of urokinase and peptide antagonists to the urokinase receptor. Evidence from characterization of the receptor in four primate species. Biol.Chem. 382, 435-442.

Fitzpatrick, M.S., Van Der Kraak, G., Schreck, C.B., 1986. Plasma profiles of sex steroids and gonadotropin in coho salmon (*Oncorhnychus kisutch*) during final maturation. Gen. Comp. Endocrinol. 62, 437-451.

Fukaya, M., 1999. Molecular histochemical and pharmaco-behavioral studies on the glutamate receptor in the brain of salmonid fishes. Master thesis, Hokkaido University, Hakodate.

Graziadei, P.P.C., Monti-Graziadei, G.A., 1979. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J. Neurocytol. 8, 1-18.

Hamano, K., Yoshida, K., Suzuki, M., Ashida, K., 1996. Changes of thyrotropin-releasing hormone concentration in the brain and levels of prolactin and thyroxin in the serum during spawning migration of the chum salmon, *Oncorhynchus keta*. Gen. Comp. Endocrinol. 101, 275-281.

Harden-Jones, F.R., 1968. Fish Migration. Edward Arnold, London, pp. 325.

Harden, M.V., Newton, L.A., Lloyd, R.C., Whitlock, K.E., 2006. Olfactory imprinting is correlated with changes in gene expression in the olfactory epithelia of the zebrafish. J. Neurobiol. 66, 1452-1466.

Hasler, A.D., Scholz, A.T., 1983. Olfactory imprinting and homing in salmon. New York: Springer-Verlag.

Heldman, J., Lancet, D., 1986. Cyclic AMP-dependent protein phosphorylation in chemosensory neurons: identification of cyclic nucleotide regulated phospho-proteins in olfactory cilia. J. Neurochem. 47, 1527-1533.

Herrada, G., Dulac, C., 1997. A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. Cell. 90, 763-773.

Hoar, W.S., 1988. The physiology of smolting salmonids. In: Hoar, W.S. and Randall, D.J. (eds), Fish Physiology. vol. XIB, Academic Press, New York, pp 275-343.

Holtmaat, A.J., Huizinga, C.T., Margolis, F.L., Gispen, W.H., Verhaagen, J., 1999. Transgenic expression of B-50/GAP-43 in mature olfactory neurons triggers downregulation of native B-50/GAP-43 expression in immature olfactory neurons. Brain Res. Mol. Brain Res. 74, 197-207.

Huard, J.M.T., Youngentob, S.L., Goldstein, B.J., Luskin, M.B., Schwob, J.E., 1998. Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. J. Comp. Neurol. 400, 469-486.

Hubank, M., Schatz, D.G., 1994. Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. 22, 5640-5648.

Hubbard, C.P., Barata, N.E., Canario, M.V.A., 2000. Olfactory sensitivity to changes in enviromental [Ca^{2+}] in the marine teleost *Sparus aurata*. J. Exp. Biol. 203, 3821-3829.

Ito, F., Yamaguchi, M., Soyano, K., Yamauchi, K., 1992. Induction of ovulation by male sex pheromone in the ayu, *Plecoglossus altivelis*. In: Breton, B., Fostier, A., Kah, O., Le Bail, P.Y., Loir, M., Prunet, P., Weul, C. (eds), Abstracts of 2nd International Symposium on Fish Endocrinology, Saint-Malo, France, pp. 83.

Ivic, L., Pyrski, M.M., Margolis, J.W., Richards, L.J., Firestein, S., Margolis, F.L., 2000. Adenoviral vector-mediated rescue of the OMP-null phenotype *in vivo*. Nat. Neurosci. 3, 1113-1120.

Iwata, M., Tsuboi, H., Yamashita, T., Amemiya, A., Yamada, H., Chiba, H., 2003. Function and trigger of thyroxine surge in migrating chum salmon *Oncorhynchus keta* fry. Aquaculture. 222, 315-329.

Izumi, Y., Auberson, Y.P., Zorumski, C.F., 2006. Zinc modulates bidirectional hippocampal plasticity by effects on NMDA receptors. J. Neurosci. 26, 7181-7188.

Jang, W., Youngentob, S.L., Schwob, J.E., 2003. Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. J. Comp. Neurol. 460, 123-140.

Jozic, D., Bourenkow, G., Bartunik, H., Scholze, H., Dive, V., Henrich, B., Huber, R., Bode, W., Maskos, K., 2002. Crystal structure of the dinuclear zinc aminopeptidase PepV from *Lactobacillus delbrueckii* unravels its preference for dipeptides. Structure. 10, 1097-1106.

Kawato, S., Hojo, Y., Kimoto, T., 2002. Histological and metabolism analysis of P450 expression in the brain. Methods Enzymol. 357, 241-249.

Keller, A., Margolis, F.L., 1975. Immunological studies of the rat olfactory marker protein. J. Neurochem. 24, 1101-1106.

Keller, A., Margolis, F.L., 1976. Isolation and characterization or rat olfactory marker protein. J. Biol. Chem. 251, 6232-6237.

Kendrick, K.M., Guevara-Guzman, R., Zorrilla, J., Hinton, M.R., Broad, K.D., Mimmack, M., Ohkura, S., 1997. Formation of olfactory memories mediated by nitric oxide. Nature. 388, 670-674.

Kinoshita, M., Fukaya, M., Tojima T., Kojima, S., Ando, H., Watanabe, M., Urano, A., Ito, E., 2005. Retinotectal transmission in the optic tectum of rainbow trout. J. Comp. Neurol. 484, 249-259.

Kinoshita, M., Hosokawa, T., Urano, A., Ito, E., 2004. Long-term potentiation in the optic tectum of rainbow trout. Neurosci. Lett. 370, 146-150.

Kratzschmar, J., Haendler, B., Kojima, S., Rifkin, D.B., Schleuning, W.D., 1993. Bovine urokinase-type plasminogen activator and its receptor: cloning and induction by retinoic acid. Gene. 125, 177-183.

Kudo, H., 1994. Morphological study on the olfactory system of several salmonid species. Master thesis, Hokkaido University, Hakodate.

Kudo, H., Tsuneyoshi, Y., Nagae, M., Adachi, S., Yamauchi, K., Ueda, U., Kawamura, H., 1994. Detection of thyroid hormone receptors in the olfactory system and brain of wild masu salmon, *Oncorhynchus masou* (Brevoort), during smolting by *in vitro* autoradiography. Aquacult. Fish. Manag. 25, 171-182.

Kudo, H., Ueda, H., Mochida, K., Adachi, S., Hara, A., Nagasawa, H., Doi, Y., Fujimoto, S., Yamauchi., 1999. Salmonid olfactory system-specific protein (N24) exhibits glutathione *S*-transferase class pi-like structure. J. Neurochem. 72, 1344-1352.

Kudo, H., Ueda, H., Yamauchi, K., 1996. Immunocytochemical investigation of a salmonid olfactory system-specific protein in the kokanee salmon (*Oncorhynchus nerka*). Zool. Sci. 13, 647-653.

Lema, S.C., Nevitt, G.A., 2004. Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting. J. Exp. Biol. 207, 3317-3327.

Lisitsyn, N., Lisitsyn, N., Wigler, M., 1993. Cloning the differences between two complex genomes. Science. 259, 946-951.

Lowe, G., Nakamura, T., Gold, G.H., 1989. Adenylate cyclase mediates olfactory transduction for a wide variety of odorants. Proc. Natl. Acad. Sci. USA. 86, 5641-564 56.

Luthi-Carter, R., Barczak, A.K., Speno, H., Coyle, J.T., 1998 a. Molecular characterization of human brain *N*-acetylated a-linked acidic dipeptidase (NAALADase). J. Pharmacol. Exp. Ther. 286, 1020-1025.

Luthi-Carter, R., Barczak, A.K., Speno, H., Coyle, J.T., 1998 b. Hydrolysis of the neuropeptide *N*-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidase II. Brain Res. 795, 341-348.

Luthi-Carter, R., Berger, U.V., Barczak, A.K., Enna, M., Coyle, J.T., 1998 c. Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. Proc. Natl. Acad. Sci. USA. 95, 3215-3220.

Mackay-Sim, A., Beard, M.D., 1987. Hypothyroidism disrupts neural development in the olfactory epithelium of adult mice. Dev. Brain Res. 36, 190-198.

Margolis, F.L., 1972. A brain protein unique to the olfactory bulb. Proc. Natl. Acad. Sci. USA. 69, 1221-1224.

Matsunami, H., Buck, LB., 1997. A multigene family encoding a diverse array of putative pheromone receptors in mammals. Cell. 90, 775-784.

Matsuoka, I., Fuyuki, K., Shoji, T., Kurihara, K., 1998. Identification of *c-fos* related genes and their induction by neural activation in rainbow trout brain. Biochim. Biophys. Acta. 1395, 220-227.

McKenzie, I.F., Gardiner, J., Cherry, M., Snell, G.D., 1977. Lymphocyte antigens: Ly-4, Ly-6, and Ly-7. Transplant. Proc. 9, 667-669.

Mombaerts, P., 2004. Genes and ligands for odorant, vomeronasal and taste receptors. Nat. Rev. Neurosci. 5, 263-278.

Moore, A., Scott, A.P., 1991. Testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar L.*) parr. Phil. Trans. R. Soc. London [Biol.] 332, 241-244.

Morin, P.P., Andersen, O., Haug, E., Doving, K.B., 1994. Changes in serum free thyroxine, prolactin and olfactory activity during induced smoltification in Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 51, 1985-1992.

Morin, P.P., Dodson, J.J., Dore, F.Y., 1989 a. Cardiac responses to natural odorants as evidence of a sensitive period for olfactory imprinting in young Atlantic salmon, *Salmo salar*. Can. J. Fish. Aquat. Sci. 46, 122-130.

Morin, P.P., Dodson, J.J., Dore, F.Y., 1989 b. Thyroid activity concominant with olfactory learning and heart rate changes in Atlantic salmon, *Salmo salar*, during smoltification. Can. J. Fish. Aquat. Sci. 46, 131-136.

Morin, P.P., Døving, K.B., 1992. Changes in the olfactory function of Atlantic salmon, *Salmo salar*, in the course of smoltification. Can. J. Fish. Aquat. Sci. 49, 1704-1713.

Morin, P.P., Hara, T.J., Ealse, J.G., 1995. T_4 depresses olfactory responses to L-alanine and plasma T_3 and T_3 production in smoltifying Atlantic salmon. Am. J. Physiol. 269, 1434-1440.

Nakamura, T., Gold, G.H., 1987. A cyclic nucleotide-gated conductance in olfactory receptor neurons. Nature. 325, 442-444.

Nakamura, T., Tanaka, T., Nagano,T., Yoneda, T., Takagi, H., Sato, M., 1998. Distribution of mRNA encoding Tat-binding protein-1 (TBP-1), a component of 26S proteasome, in the rat brain. Mol. Brain Res. 53, 321-327.

Nam, R.H., Kim, W., Lee, C.J., 2004. NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish. Neurosci. Lett. 370, 248-251.

Nelbock, P., Dillon, P.J., Perkins, A., Rosen, C.A., 1990. A cDNA for a protein that interacts with the human immunodeficiency virus Tat transactivator. Science. 48, 1650-1653.

Nevitt, G.A., Dittman, A.H., Quinn, T.P., Moody, W.J., 1994. Evidence for a peripheral olfactory memory in imprinted salmon. Proc. Natn. Acad. Sci. USA. 91, 4288-4292.

Nevitt, G.A., Moody, W.J., 1992. An electrophysiological characterization of ciliated olfactory receptor cells of the coho salmon *Oncorhynchus kisutch*. J. Exp. Biol. 166, 1-17.

Ngai, J., Dowling, M.M., Buck, L., Axel, R., Chess, A., 1993. The family of genes encoding odorant receptors in the channel catfish. Cell. 72, 657-666.

Niwa, H., Harrison, C.L., DEAizpurua, J.H., Cram, D.S., 1997. Identification of Pancreatic β cell-related genes by representational difference analysis. Endocrinology. 138, 1419-1426.

Ogino, C., Yang, G.Y., 1978. Requirement of rainbow trout for dietary zinc. Bull. Jpn. Soc. Sci. Fish. 44, 1015-1018.

O'Keefe, D.S., Bacich, D.J., Heston, W.D., 2004. Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate-specific membrane antigen-like gene. Prostate. 58, 200-210.

O'Neill, M.J., Sinclair, A.H., 1997. Isolation of rare transcripts by representational difference analysis. Nucleic Acids Res. 25, 2681-2682.

Palfree, R.G., 1996. Ly-6 domain proteins-new insights and new members: a C-terminal Ly-6 domain in sperm acrosomal protein SP-10. Tissue Antigens. 48, 71-79.

Pangalos, M.N., Neefs, J.M., Somers, M., 1999. Isolation and expression of novel human glutamate carboxypeptidases with *N*-acetylated alpha-linked acidic dipeptidase and dipeptidyl peptidase IV activity. J. Biol. Chem. 274, 8470-8483.

Parhar, S.I., Iwata, M., 1996. Intracerebral expression of gonadotropin-releasing hormone and growth hormone-releasing hormone is delayed until smoltification in the salmon. Neurosci. Res. 26, 299-308.

Paternostro, M.A., Meisami, E., 1989. Selective effects of thyroid hormonal deprivation on growth and development of the olfactory receptor sheet during the early postnatal period: a morphometric and cell count study. Int. J. Dev. Neurosci. 7, 243-255.

Paternostro, M.A., Meisami, E., 1994. Quantitative [^{3}H]thymidine autoradiography of neurogenesis in the olfactory epithelium of developing normal, hypothyroid, and hypothyroid-rehabilitated rats. Dev. Brain Res. 83, 151-162.

Pekary, A.E., Lukaski, H.C., Mena, I., Smith, S.M., Bhasin, S., Hershman, J.M., 1993. Testosterone increases TRH biosynthesis in epididymis but not heart of zinc-deficient rats. Peptides. 14, 315-324.

Pera, E.M., Martinez, S.L., Flanagan, J.J., Brechner, M., Wessely, O., DeRobertis, E.M., 2003. Darmin is a novel secreted protein expressed during endoderm development in *Xenopus*. Gene Expr. Patterns. 2, 147-152.

Puttfarcken, P.S., Handen, J.S., Montgomery, D.T., Coyle, J.T., Werling, L.L., 1993. *N*-acetyl-aspartylglutamate modulation of *N*-methyl-$_D$-aspartate-stimulated [^{3}H] norepinephrine release from rat hippocampal slices. J. Pharmacol. Exp. Ther. 266, 796-803.

Ragno, P., 2006. The urokinase receptor: a ligand or a receptor ? Story of a sociable molecule. Cell. Mol. Life Sci. 63, 1028-1037.

Rawlings, N.D., Barrett, A.J., 1995. Evolutionary families of metallopeptidases. Methods Enzymol. 248, 183-228.

Rawlings, N.D., Barrett, A.J., 1997. Structure of membrane glutamate carboxypeptidase. Biochim. Biophys. Acta. 1339, 247-252.

Robinson, M.B., Blakely, R.D., Couto, R., Coyle, J.T., 1987. Hydrolysis of the brain dipeptide *N*-acetyl-$_L$-aspartyl-$_L$-glutamate. Identification and characterization of a novel *N*-acetylated a-linked acidic dipeptidase activity from rat brain. J. Biol. Chem. 262, 14498-14506.

Roldan, A.L., Cubellis, M.V., Masucci, M.T., Behrendt, N., Lund, L.R., Danø, K., Appella, E., Blasi, F., 1990. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. EMBO J. 9, 467-474.

Ryba, N.J., Tirindelli, R., 1997. A new multigene family of putative pheromone receptors. Neuron. 19, 371-379.

Satoh, S., Yamamoto, H., Takeuchi, H., Watanabe, T., 1983. Effects of growth and mineral composition of rainbow trout by deletion of trace elements or magnesium from fish meal diet. Bull. Jpn. Soc. Sci. Fish. 49, 425-429.

Satou, M., Hoshikawa, R., Sato, Y., Okawa, K., 2006. An *in vitro* study of long-term potentiation in the carp (*Cyprinus carpio L.*) olfactory bulb. J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol. 192, 135-150.

Satou, M., Sugiyama, S., Inadomi, T., Kitamura, S., 1996. Field-potential response and synaptic plasticity in the olfactory bulb of salmonid fish. Zool. Sci., Suppl. 13, 107.

Scholz, A.T., 1980. Hormonal regulation of smolt transformation and olfactory imprinting in coho salmon. PhD thesis, University of Wisconsin, Madison.

Sherwood, R.F., Melton, R.G., 1998. Glutamate carboxypeptidases. In: Barrett, A.J., Rowlings, N.D., Woessner, J.F., Editors, 1998. Handbook of Proteolytic Enzymes, Academic Press, London, pp. 1416-1420.

Shimizu, M., Kudo, H., Ueda, H., Hara, A., Shimazaki, K., Yamauchi, K., 1993. Identification and immunological properties of an olfactory system-specific protein in kokanee salmon (*Oncorhynchus nerka*). Zool. Sci. 10, 287-294.

Shimizu, M., Ueda, H., Kawamura, H., Shimazaki, K., Yamauchi, K., 1995. Electrophoretic changes in olfactory system proteins in masu salmon during parr-smolt transformation. J. Fish. Biol. 47, 1044-1054.

Shoji, T., Ueda, H., Ohgami, T., Sakamoto, T., Katsuragi, Y., Yamauchi, K., Kurihara, K. 2000. Amino acids dissolved in stream water as possible home stream odorants for masu salmon. Chem. Senses. 5, 533-540.

Sklar, P.B., Anholt, R.R.H., Snyder, S.H., 1986. The odorantsensitive adenylate cyclase of olfactory receptor cells. J. Biol. Chem. 261, 15538-15543.

Sokoloff, R.L., Norton, K.C., Gasior, C.L., 2000. A dual-monoclonal sandwich assay for prostate-specific membrane antigen: levels in tissues, seminal fluid and urine. Prostate. 43, 150-157.

Sorense, P.W., 1992. Hormones, pheromones and chemoreception. In: Hara, T.J. (ed), Fish Chemoreception. Chapman & Hall, London, pp. 199-228.

Suh, T.T., Nerlov, C., Dano, K., Degen, J.L., 1994. The murine urokinase-type plasminogen activator receptor gene. J. Biol. Chem. 269, 25992-25998.

Takahashi, K., Akaike, N., 1991. Calcium antagonist effects on low-threshold (T-type) calcium current in rat isolated hippocampal CA1 pryramidal neurons. J. Pharm. Exp. Ther. 256, 169-175.

Tarui, T., Mazar, A.P., Cines, D.B., Takada, Y., 2001. Urokinase-type plasminogen activator receptor (CD87) is a ligand for integrins and mediates cell-cell interaction. J. Biol. Chem. 276, 3983-3990.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence aligment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Traynelis, S.F., Burgess, M.F., Zheng, F., Lyuboslavsky, P., Powers, J.L., 1998. Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. J. Neurosci. 18, 6163-6175.

Troyer, J.K., Beckett, M.L., Wright, G.L.Jr., 1995. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. Int. J. Cancer. 62, 552-558.

Valverde, F., Lopez-Mascaraque, L., 1991. Neuroglial arrangements in the olfactory glomeruli of the hedgehog. J. Comp. Neurol. 307, 658-674.

van de Rijn, M., Heimfeld, S., Spangrude, G.J., Weissman, I.L., 1989. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci. USA. 86, 4634-4638.

Wensley, C.H., Stone, .DM., Baker, H., Kauer, J.S., Margolis, F.L., Chikaraishi, D.M., 1995. Olfactory marker protein mRNA is found in axons of olfactory receptor neurons. J. Neurosci. 15, 4827-4837.

Wickens, A., May, D., Rand-Weaver, M., 2001. Molecular characterisation of a putative Atlantic salmon (*Salmo salar*) odorant receptor. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 129, 653-660.

Wisby, W.J., Hasler, A.D., 1954. Effect of olfactory occlusion on migrating silver salmon *Oncorhynchus Kisutch*. J. Fish. Res. Bd. Can. 11, 472-478.

Withers, P.C., 1996. An exploration of salt and water balance in invertebrate and vertebrate animals. In The Comparative Endocrinology of Calcium Regulation (ed. C. Dacke, J. Danks, I. Caple, G. Flik), pp. 17-42. Bristol: J. Endocrinology Ltd.

Wysocki, C.J., Meredith, M., 1987. The vomeronasal system. In: Finger, T.E., Silver, W.L., (eds), Neurobiology of Taste and Smell. Wiley-Interscience, New York, pp. 125-150.

Xie, X., Gerber, U., Gahwiler, BH., Smart, T.G., 1993. Interaction of zinc with ionotropic and metabotropic glutamate receptors in rat hippocampal slices. Neurosci. Lett. 159, 46-50.

Yamada, H., Satoh, R., Yamashita, T., Kambegawa, A., Iwata, M., 1997. Development of a time-resolved fluoroimmunoassay (TR-FIA) for testosterone: measurement of serum testosterone concentrations after testosterone treatment in the rainbow trout (*Onchorhynchus mykiss*). Gen. Comp Endocrinol. 106, 181-188.

Yamauchi, K., Koide, N., Adachi, S., Nagahama, Y., 1984. Changes in seawater adaptability and blood thyroxine concentrations during smoltification of the masu salmon, *Oncorhynchus masou*, and the amago salmon, *Oncorhynchus rhodurus*. Aquaculture. 42, 247-256.

Yambe, H., Kitamura, S., Kamio, M., Yamada, M., Matsunaga, S., Fusetani, N., Yamazaki, F., 2006. L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. Proc. Natl. Acad. Sci. USA. 103, 15370-15374.

Yanagi, S., Kudo, H., Doi, Y., Yamauchi, K., Ueda, H., 2004. Immunohistochemical demonstration of salmon olfactory glutathione *S*-transferase class pi (N24) in the olfactory system of lacustrine sockeye salmon during ontogenesis and cell proliferation. Ant. Embryol. 208, 231-238.

Youngentob, S.L., Margolis, F.L., Youngentob, L.M., 2001. OMP gene deletion results in an alteration in odorant quality perception. Behav. Neurosci. 115, 626-631.

Zhang, X., Firestein, S., 2002. The olfactory receptor gene superfamily of the mouse. Nat. Neurosci. 5, 124-133.

Zhang, X., Rodriguez, I., Mombaerts, P., Firestein, S., 2004. Odorant and vomeronasal receptor genes in two mouse genome assemblies. Genomics. 83, 802-811.

Zhu, F.X., Jia, S.D., Gao, L.L., He,F.C., 2000. cDNA cloning and sequencing of human urokinase receptor. Sheng Wu Gong Cheng Xue Bao 16, 461-463.

Zielinski, B., Hara, T.J., 1988. Morphological and physiological development of olfactory receptor cells in rainbow trout (*Salmo gairdneri*) embryos. J. Comp. Neurol. 27, 300-311.

Zielinski, B., Hara, T.J., 1992. Ciliated and microvillar receptor cells degenerate and then differentiate in the olfactory epithelium of rainbow trout following olfactory nerve section. Micros. Res. Techn. 23, 22-27.