Physiological Mechanisms of Imprinting and Homing Migration of Pacific Salmon

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Abstract
Salmon are recognized for their amazing abilities to precisely migrate thousands of kilometers from their feeding habitat in the ocean to their natal stream for reproduction, but many mysteries are still unsolved in the mechanisms of imprinting and homing migration. Physiological mechanisms of imprinting and homing migration of Pacific salmon were investigated using three different research approaches. Homing behavior of adult chum salmon from the Bering Sea to Hokkaido as well as lacustrine sockeye salmon and masu salmon in Lake Toya (serves as a model ocean) were examined using physiological biotelemetry techniques, demonstrating that salmon can navigate in open water using different sensory systems. Hormone profiles in the brain-pituitary-thyroid and brain-pituitary-gonad axes were analyzed in chum salmon and sockeye salmon during their imprinting and homing migration, suggesting that thyrotropin-releasing hormone and salmon gonadotropin-releasing hormone in the brain are involved in imprinting and homing migration, respectively. The olfactory memory formation and retrieval of Pacific salmon were investigated using several neurophysiological techniques, suggesting that long-term stability of dissolved free amino acid compositions in natal streams are crucial for olfactory imprinting and homing, and *N*-methyl-D-aspartate receptor can be a useful molecular marker for olfactory memory formation and retrieval. These topics are discussed with physiological mechanisms of imprinting and homing migration of Pacific salmon.

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1. Introduction

Pacific salmon (genus *Oncorhynchus*) show dramatic and complex life cycles. In four Pacific salmon species in Japan, there are large differences in the timing of imprinting migration in juveniles and homing migration in adults (**Fig. 1**). In pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*), underyearling juveniles all carry out downstream migration within a few months after emergence, and adults do upstream migration within a few weeks of spermiation and ovulation. In contrast, in sockeye salmon (*O. nerka*) and masu salmon (*O. masou*), juveniles stay in freshwater for one and half year to grow into smolts that have seawater adaptability do downstream migration. Some river or lake residents also exist (lacustrine sockeye and masu salmon), and adults do their upstream migration at least several months prior to spermiation and ovulation (Groot and Margolis 1991).

Retropositional genome analyses have revealed that pink salmon are phylogenetically the most advanced salmon species, while masu salmon are considered to be the more primitive species (Murata *et al*. 1996). Pink salmon are also the most widely distributed species and have the largest population size, while masu salmon appear to have the most restricted distribution and the smallest population (Kaeriyama and Ueda 1998). Although the homing accuracy of these salmon has not been compared in detail, it is believed that masu salmon return to their natal stream with the highest precision, and that pink salmon are more likely to stray into a non-natal stream. If most salmon might show a highly accurate homing to the natal stream, there would be little chance to enhance their distribution area as well as to increase their population size. And, they might encounter the dangerous possibility to reduce their genetic diversity. The relationship between salmon evolution and homing accuracy is one of the most interesting questions from a viewpoint of biological evolution.

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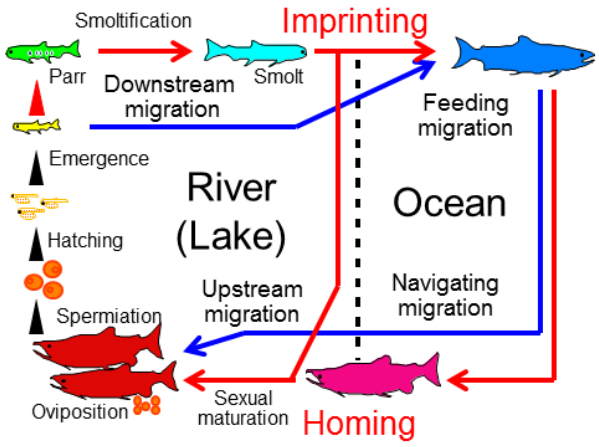

Fig. 1. Life history of two different types of Pacific salmon species in Japan. Blue line: chum and pink salmon; Red line: sockeye and masu salmon.

Salmon are recognized for their amazing abilities to precisely migrate thousands of kilometers from their feeding habitat in the ocean to their natal stream for reproduction. Since the olfactory imprinting hypotheses for salmon homing to their natal stream was proposed by Hasler's research group in the 1950's (Hasler and Wisby 1951; Wisby and Hasler 1954), physiological mechanisms of olfactory imprinting and homing abilities in salmon have been intensively studied by many researchers (Harden Jones 1968; Brannon 1982; Cooper and Hirsch 1982; Hasler and Scholz 1983; Smith 1985; Døving 1989; Stabell 1992; Hara 1994; Ueda and Yamauchi 1995; Dittman and Quinn 1996; Satou *et al*. 1996; Bertmar 1997; Nevitt and Dittman 1998; Quinn; 2005; Hamdani and Døving 2007; Ueda *et al*. 2007; Zielinski and Hara 2007; Hino *et al*. 2009; Ueda 2011, 2012, 2014). The pheromone hypothesis was proposed by Nordeng (1971, 1977) using Arctic char (*Salvelinus alpines*) and Atlantic salmon (*Salmo salar*) that juvenile salmon in a stream released population-specific odors that guided homing adults. Several studies have also suggested that juvenile salmonids produce population-specific odors or pheromones (Groot *et al*. 1986; Quinn and Tolson 1986; Courtenay *et al*. 1997). It has also been demonstrated that sex steroids and prostaglandins that have effects on the olfactory epithelium of salmonid fish may be acting as sexual pheromones (Moore and Scott 1992; Sveinsson and Hara 1995; Moore and Warning 1996; Sveinsson and Hara 2000). L-kynurenine, an amino acid was identified as a sex pheromone in the urine of ovulated female masu salmon (Yambe *et al*. 2006). Recently, the hierarchical navigation hypothesis was proposed by Bett and Hinch (2015) that migrating adult salmon relied on primary imprinting cues, secondary conspecific cues, and tertiary non-olfactory environmental cues. Large differences in imprinting and homing capability among salmonid fishes might be induced by their different life cycles, but all salmonid fishes have an amazing homing ability to leave offspring in streams.

There are still many mysteries regarding which sensory systems play important roles in open water orientation, which hormones control imprinting and homing migration, and how olfactory systems discriminate and retrieve stream odors to identify the natal stream. In this review, three different research approaches are used to clarify physiological mechanisms of imprinting and homing migration of Pacific salmon: physiological biotelemetry studies on salmon homing behavior, endocrinological studies on the brain-pituitary-thyroid (BPT) and brain-pituitary-gonad (BPG) axes, and neurophysiological studies on olfactory function. These topics are discussed with physiological mechanisms of imprinting and homing migration of Pacific salmon with special focus on navigation abilities in open water, hormonal control mechanisms during imprinting and homing migration, and olfactory memory formation and retrieval abilities of natal stream odors.

2. Physiological biotelemetry studies on homing migration

The recent rapid development of biotelemetry technologies, such as ultrasonic and radio telemetry, data logging, and pop-up satellite telemetry, makes it possible to continually observe the underwater behavior of salmon in open water (Cooke *et al*. 2004; Ueda 2004; Hussey *et al*. 2015). In particular, ultrasonic transmitters that emit pulsed signals have been useful for investigating the migratory behavior of salmon in coastal seas (Quinn and Groot 1984; Quinn *et al*. 1989) and the central Bering Sea (Ogura and Ishida 1994). Moreover, ultrasonic tracking has been used in conjunction with sensory ablation experiments, which block visual and olfactory cues or magnetic senses, permitting insight into the relative importance of individual senses to salmon homing (Døving *et al*. 1985; Yano and Nakamura 1992; Hansen *et al*. 1993; Yano *et al*. 1996).

2-1. Chum salmon homing behavior from the Bering Sea to Hokkaido, Japan

Tanaka *et al*. (2005) examined chum salmon homing behavior from the Bering Sea to Hokkaido, Japan. Japanese chum salmon were collected by longline in June, 2000 in the central Bering Sea (56°30′ N, 179°00′ E) in healthy condition. Individuals were determined to be of Japanese origin by scale analysis, as most of Japanese chum salmon were released from hatcheries and the width of their scale ring during fry stage was wider than wild salmon from other countries. A pro-

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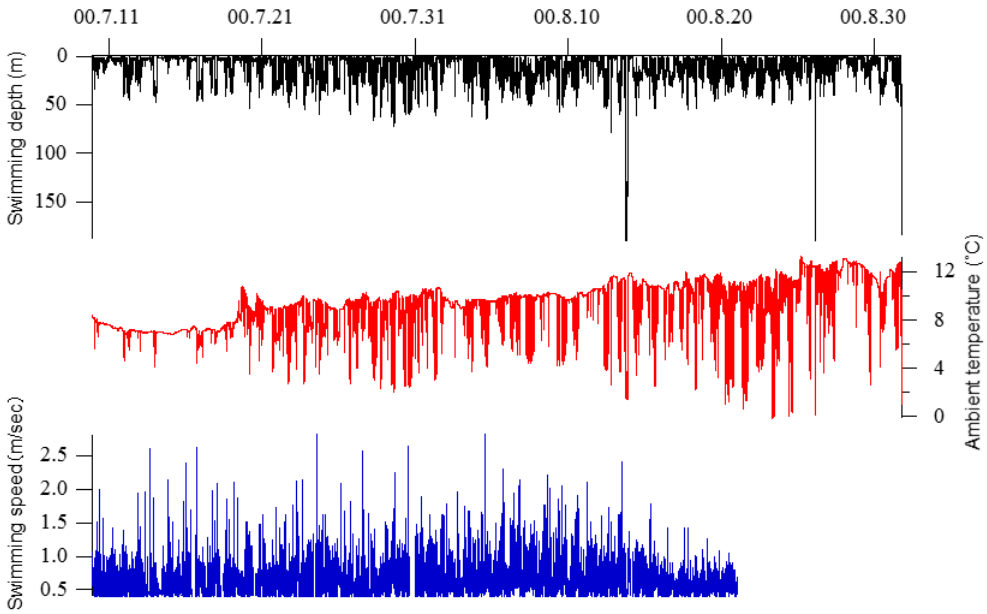

Fig. 2. Swimming depth, ambient temperature, and swimming speed of a chum salmon from the Bering Sea to Hokkaido, Japan recorded by a propeller data logger. Republished with permission of Elsevier, from *General and Comparative Endocrinology*, **170**, Ueda H., Physiological mechanism of homing migration in Pacific salmon from behavioral to molecular biological approaches, 222–232, Fig. 2, © 2011; permission conveyed through Copyright Clearance Center, Inc.

peller data logger, which recorded swimming speed (5 seconds sampling), depth (5 seconds sampling), and temperature (1 minute sampling), was attached externally through the dorsal musculature of the fish anterior to the dorsal fin. In total, 27 chum salmon were released with external data loggers, with one logger recovered from a set net on September on the east coast of Hokkaido, Japan (43°20′ N, 145°46′ E). The first recorded swimming profiles of homing chum salmon in the oceanic phase were collected over a period of 67 days covering a straight distance of 2,750 km. The salmon had travelled with an average swimming speed, depth, and temperature of 62 ± 12 (mean ± SD) cm/sec, 10.4 ± 14.7 m, and 9.2 ± 0.2°C, respectively (**Fig. 2**). Horizontal swimming speed was 36.4 ± 15.2 km/day, but the fish sometimes moved at speed less than 10 km/day during the initial 10 days after tagging. Excluding this less-active 10-day period from the analysis, mean horizontal speed was 42.3 ± 11.5 km/day. Both swimming speed and depth trimodally peaked around dawn and dusk, with an additional small peak around midnight. The fish showed sequential up-and-down movements near the thermocline during the twilight and during the daytime. These diurnal migratory patterns suggest that the homing chum salmon allocated a proportion of their time to foraging, and also that the foraging strategy was different in the daytime and the nighttime. Although Tanaka *et al*. (2005) could not calculate energy expenditures of chum salmon during the long distance migration, these foraging behaviors must be related to massive energy expenditures during migration. These results indicate that the homing chum salmon have navigation abilities in the homeward direction, and that salmon use ocean current transport to reduce energy expenditure during migration.

2-2. Lacustrine sockeye and masu salmon homing behaviors in Lake Toya, Hokkaido, Japan

For anadromous salmon research, it is difficult to carry out physiologically controlled and manipulated experiments as fish migrate from the sea in their pre-maturation phase to their natal stream where they become mature. In contrast, lacustrine salmon populations offer an excellent model system for studying homing behaviors from open water to natal areas for reproduction. Ueda *et al*. (1998) examined homing migrations of mature lacustrine sockeye salmon, whose sensory cues were impaired by ablations, tracking from the center of the Lake Toya, a large caldera lake (surface area 71 km^2, average and maximum depth 116 m and 179 m, respectively) to their natal area using ultrasonic tracking system. Both a mature male sockeye salmon (control fish) attached with a control brass ring on the head (**Fig. 3A-1**) and a similar mature male sockeye salmon whose magnetic cues were interfered with by attachment of a strong NdFe magnetic ring on the head (**Fig. 3A-2**) returned straight to the natal area after 1 h of random movement. A mature male sockeye salmon,

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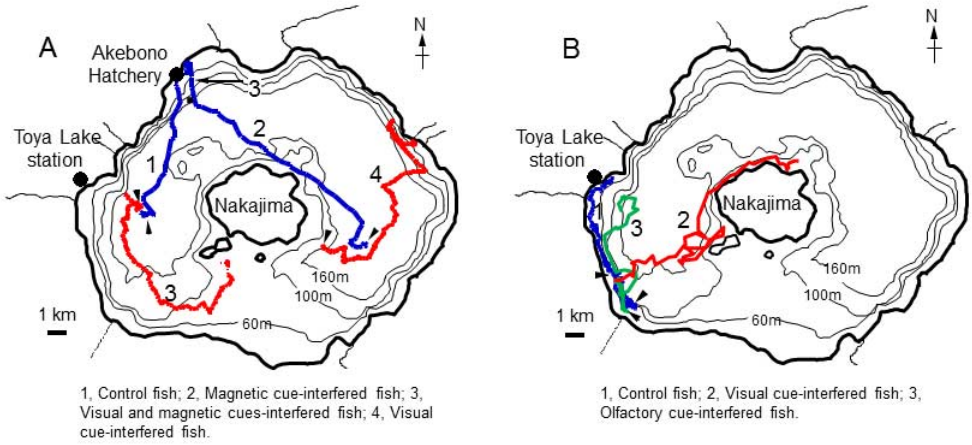

Fig. 3. Tracks of four mature male lacustrine sockeye salmon (A) and three mature lacustrine masu salmon (B) in Lake Toya during the spawning season using ultrasonic tracking system. Arrowhead indicates the releasing point of each fish.

whose visual cues were blocked by injection of carbon tonner and corn oil and whose magnetic cues were interfered with via a magnetic ring, moved in the direction opposite to the natal area, but was eventually rediscovered in the natal area on the following evening, suggesting the possible involvement of olfactory cues, in the absence of visual and magnetic senses, in finding the natal area (**Fig. 3A-3**). A blinded male sockeye salmon was also moved to the shore of Naka-Toya far from the natal area in the evening, where it stayed for a few days (**Fig. 3A-4**).

These data reveal that visual cues are critical to the straight homing of sockeye salmon, while magnetic cues do not appear to be necessary for successful return to the natal area. However, magnetoreceptor cells have been identified in the nasal cavity of rainbow trout (*O. mykiss*) (Walker *et al*. 1997) and empirical evidence for geomagnetic imprinting has recently been reported in Chinook salmon (*O. tshawytscha*) (Bracis and Anderson 2012), and pink and sockeye salmon (Putman *et al*. 2013, 2014). Recently, the Earth's magnetic forces during chum salmon homing migration in the Bering Sea was studied using magnetic tags that can record the magnetic force, and reported that the homing migration route was approximately along the isoline of magnetic intensity (Azumaya *et al*. personal communications).

Ueda *et al*. (2000) also tracked homing behaviors of mature lacustrine masu salmon in Lake Toya. A mature control male masu salmon moved constantly along the coast, and stopped his movement at the mouth of stream (**Fig. 3B-1**), while a blinded mature female masu salmon was released and moved randomly away from the coast (**Fig. 3B-2**), and a mature male masu salmon, whose olfactory cue was blocked by Vaseline, moved firstly along the coast, and eventually moved towards open water (**Fig. 3B-3**).

It is informative to compare the straight movements of sockeye salmon with the coastal movement behaviors of masu salmon (Ueda 2004). These two species show large differences in ocean distribution: sockeye salmon distribute widely in the North Pacific Ocean, while masu salmon are narrowly distributed in the west North Pacific Ocean (Kaeriyama and Ueda 1998). These data suggest some evolutionary aspects of successful homing migration of salmonids where the narrowly distributed masu salmon only require coastal recognition ability for successful migration, while the widely distributed sockeye salmon required the development of open water orientation ability in order to successfully return to their natal streams with high precision.

Spawning migration of lacustrine masu salmon attached with depth and temperature data loggers in Lake Toya (Matsushita and Ueda in preparation). A clear diurnal movement after they encountered the mouth of river for the first time at the beginning of spawning season was detected by the difference in ambient temperature between the mouth of river (14–20°C) and the surface of lake (20–22°C) in the surface water (**Fig. 4A**). After this encounter, fish swam vertically around thermocline depth in the daytime and stayed at the water surface at nighttime showing clear diurnal movement (**Fig. 4B**). This movement disappeared gradually toward the peak of the spawning season when they carried out upstream migration to the spawning ground (**Fig. 4C**). These behavioral changes in masu salmon during the spawning season show that masu salmon are able to calculate the day length with an internal biological clock, because the diurnal movement after encounter with the river mouth might be usable to calculate the timing of upstream migration to their spawning river.

To accurately home in open water, salmon must rec-

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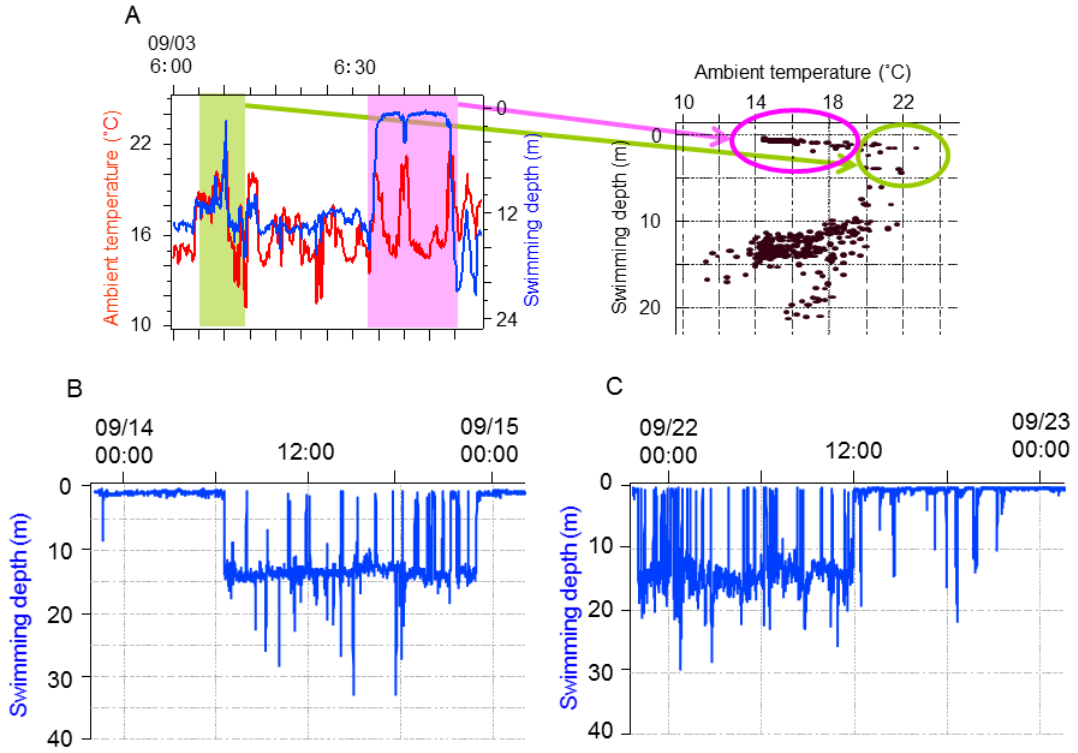

Fig. 4. Swimming depth and ambient temperature of a masu salmon in Lake Toya at the first swimming encountering the mouth of stream (A), after swimming in the stream water (B), and just prior to upstream migration (C) recorded by a depth and temperature data logger.

ognize exact locations (map) and compass direction (orientation) during migration, and must have an internal biological clock to calculate the duration from the feeding ground to the spawning ground. New research approaches to reveal how salmon can navigate in open water using compass, map, and biological clock are necessary to investigate how and which sensory systems involve in oceanic imprinting and homing migration of Pacific salmon.

3. Endocrinology studies on imprinting and homing migration

Salmon imprinting migration in juveniles and homing migration in adults are closely related to seawater adaptation and gonadal maturation, respectively. Juvenile imprinting downstream migration is associated with smoltification (parr-smolt transformation: PST), which is under the complex regulation of several endocrine hormones, such as growth hormone-releasing hormone (GHRH), corticotropin-releasing hormone (CRH), and thyrotropin-releasing hormone (TRH) in the brain; growth hormone (GH), adrenocorticotropin (ACTH), and thyrotropin (TSH) in the pituitary gland; and insulin-like growth factor I (IGF-I), cortisol, and thyroid hormones (thyroxine (T4) and triiodothyronine (T3)) in many endocrine organs (McCormick 2001; Björnsson *et al*. 2011, 2012). In particular, the BPT axis is thought to play critical roles during imprinting downstream migration (**Fig. 5A**). In contrast, adult homing migration is regulated mainly by the BPG axis (**Fig. 5B**). Two gonadotropin-releasing hormones (GnRH), salmon GnRH (sGnRH; commonly named GnRH3) and chicken GnRH-II (cGnRH-II; commonly named GnRH2), exist in various regions of the salmon brain (Amano *et al*. 1997). In particular, sGnRH neurons that have two different precursor genes sGnRH-I and sGnRH-II existed in the olfactory bulb (OB), terminal nerve (TN), and preoptic area (POA) are considered to play different roles in salmon homing migration, but their exact roles are not revealed yet. Mainly, sGnRH in the POA is believed to control synthesis and release of gonadotropin (GTH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GTHs induce steroidogenesis in the gonads, and steroid hormones stimulate gametogenesis and final gameto-maturation; estradiol-17β (E2) and testosterone (T) are active in vitellogenesis, T and 11-ketotestosterone (11KT) in spermatogenesis, and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in final gameto-maturation in both sexes (Nagahama 1999). It is very important to investigate hormone profiles in the BPT and BPG axes of salmon during juvenile imprinting and adult homing migration, in order to clarify

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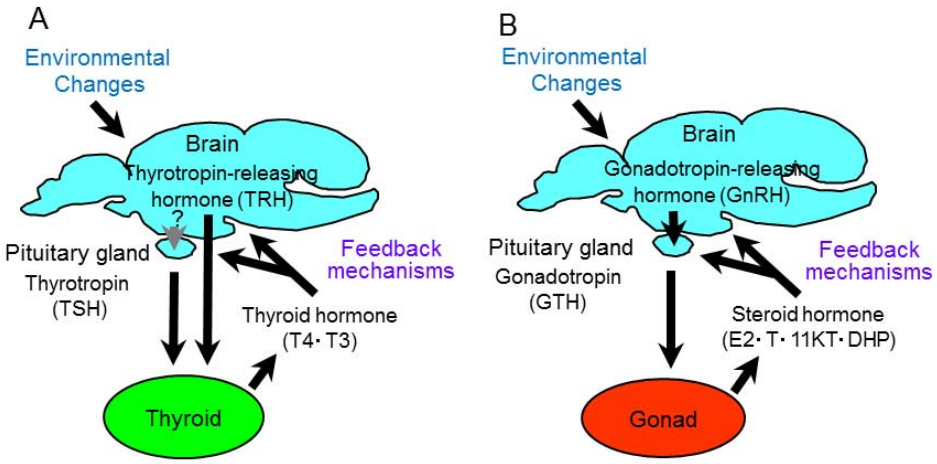

Fig. 5. Brain-Pituitary-Thyroid axis (A) and Brain-Pituitary-Gonad axis (B) in Pacific salmon.

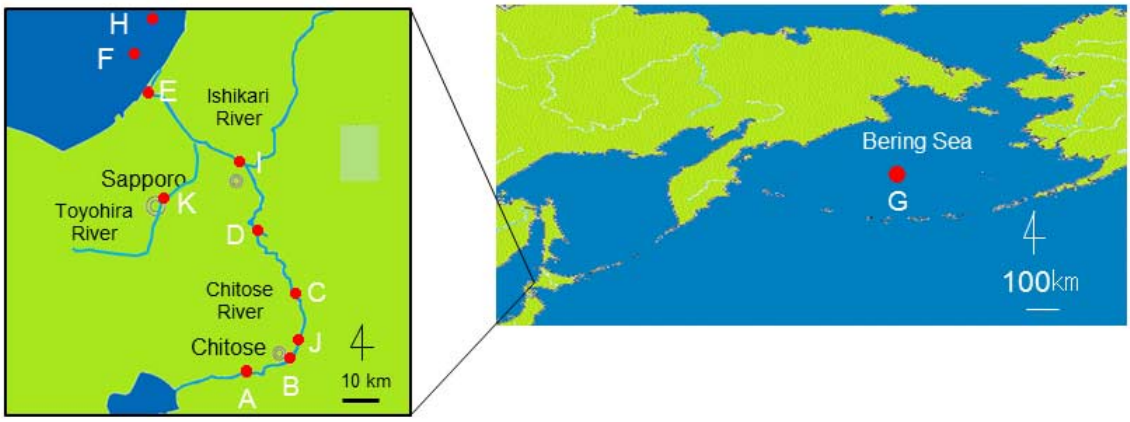

Fig. 6. Map showing the study area with the sampling sites of juvenile chum salmon during downstream migration, adult chum salmon during homing migration, and river water for electro-olfactogram experiments. A: Chitose Hatchery; B: second bridge of the Chitose; C: Chitose River in Kamaka Ward, Chitose; D: Confluence point of the Chitose and Yubari Rivers; E: Mouth of the main stem of the Ishikari River; F: Ishikari Bay; G: Bering Sea; H: Coastal area, Atsuta; I: Confluence point of the Ishikari and Chitose Rivers. J: Indian Waterwheel, Chitose River. River water sampling points were the second bridge of the Chitose River (B), the Toyohira River (K), and the mouth of the Ishikari River (E).

how these hormones control/mediate imprinting and homing migration, respectively. However, until now, it has been impossible to link hormonal control mechanisms to imprinting and homing because molecular markers that would permit the evaluation of olfactory memory formation and retrieval in the salmon brain were lacking.

Synaptic plasticity is thought to underlie the formation of memory in the brain of vertebrates. In the critical period, the formation of complex neural network by synaptic contact between axon and dendrite of neurons produce the synaptic plasticity that is the ability of chemical synapses to change synaptic strength. As memory is thought to be encoded by modification of synaptic strength, long-term potentiation (LTP), which is a persistent strengthening of synaptic activity produced a long-lasting increase in signal transmission between two neurons, is widely considered one of the major cellular mechanisms that underlies memory (Kotaleski and Blackwell 2010). LTP has been studied with a focus on *N*-methyl-D-aspartate receptor (NMDAR), which induces LTP (Martin *et al*. 2000). The NMDAR is a glutamate receptor channel subtype and mediates most of the fast excitatory synaptic transmission in the central nervous system. It plays important roles in memory formation and retrieval in higher vertebrates (Park *et al*. 2013) and in fish (Kinoshita *et al*. 2005; Nam *et al*. 2004; Cox *et al*. 2005; Gómez *et al*. 2006; Tzeng *et al*. 2007; Sison and Gerlai 2011). NMDAR is comprised of two subunits: the essential

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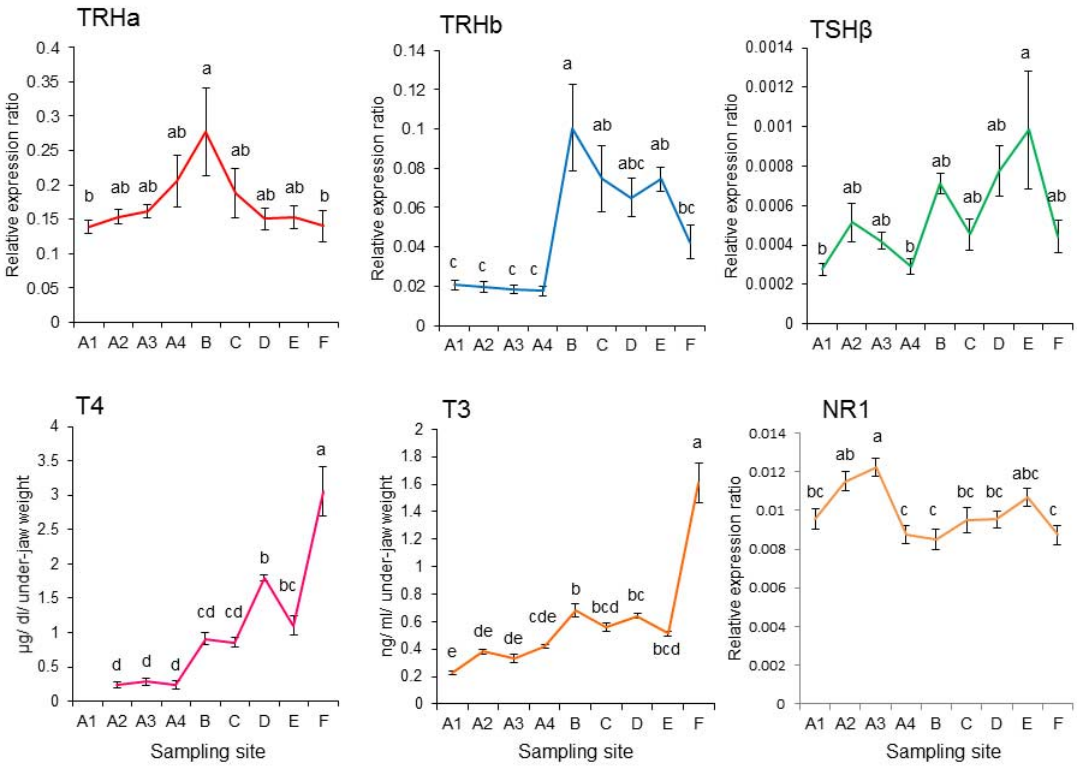

Fig. 7. Changes in the profiles of the brain-pituitary-thyroid hormones and the *N*-methyl-D-aspartate receptor essential subunit NR1 in juvenile chum salmon during downstream migration. Fish were sampled at the Chitose Hatchery (Site **A**) at four time points (January (A1), February (A2), March (A3) and April (A4)), and following release into the Chitose River from Site **B** to Site **F**. Data are presented as the means ± SEM (N = 10–15). Different letters represent significant differences at $p < 0.05$ level.

NR1 subunit and the differentially expressed NR2A-D subunit (Shipton and Paulsen 2013). The NR1 gene of chum salmon has recently been cloned and characterized (Yu *et al*. 2014), and the effects of changing salinity on NR1 gene expression have been reported (Kim *et al*. 2015). However, there have been no reports addressing how NR1 might be involved in olfactory memory formation and retrieval in salmon.

3-1. Hormone profiles of juvenile chum salmon during imprinting migration

Several studies have described the hormone profiles that occur during downstream migration and the artificial induction of downstream movement in juvenile Pacific salmon. A clear T4 surge was observed during downstream migration of chum salmon fry, suggesting that T4 plays an important role in the preparation for downstream migration; however, T4 is not the sole factor identified as responsible for the onset of downstream migration (Iwata *et al*. 2003; Ojima and Iwata 2007). Ojima and Iwata (2009) found that central administration of GHRH, CRH, melatonin, and *N*-acetyl serotonin, and serotonin stimulated downstream movements, whereas only GHRH also stimulated schooling behavior. In juvenile Chinook salmon, central administration of CRH was also found to induce downstream movement (Clements and Schreck 2004). The treatment with thyroid hormone has shown to prompt olfactory cellular proliferation in coho salmon (*O. kisutch*) (Lema and Nevitt 2004). In fall-smolting coho salmon, central administration of GHRH and CRH stimulated downstream moment and T4 secretion (Ojima and Iwata 2010). In the same species, the levels of TSHβ mRNA and TSH in the pituitary, as well as TSH, T4 and T3 in plasma, have been assessed and found to show correlations with the PST (Larsen *et al*. 2011). In the brain of sockeye salmon, the mRNA encoding the TRH precursor has been studied via *in situ* hybridization (Ando *et al*. 1998), and the expression of cDNA encoding TRH receptor subtypes has been examined (Saito *et al*. 2011). It was reported that T3 determines the onset of the sensitive period for imprinting and that it plays a critical role in later learning in chicks (*Gallus gallus domesticus*) (Yamaguchi *et al*. 2012). Although the onset of downstream migration, including smoltification, is under the complex regulation of several endocrine hormones, the BPT axis is considered to play critical roles in imprinting during downstream migration.

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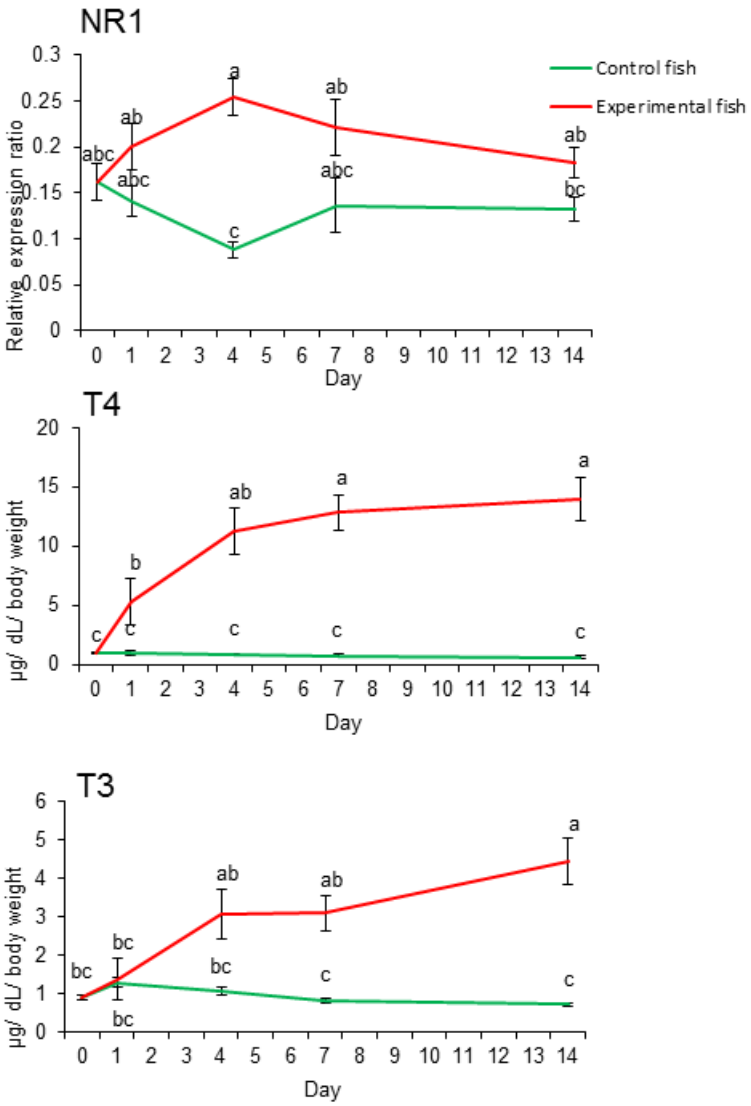

Fig. 8. Effects of oral thyroxine (T4) administration on the gene expression ratios of the *N*-methyl-D-aspartate receptor essential subunit NR1 in the whole brain and T4 and triiodothyronine (T3) levels in the whole body of juvenile chum salmon. Data are presented as the means ± SEM (N = 7–8). Different letters present significant differences at $p < 0.05$ level.

Underyearling juvenile chum salmon reared in the Chitose hatchery (Hokkaido, Japan) and then released into the Chitose River to allow migration to the sea (**Fig. 6**). The relationship between BPT hormones and natal stream odor imprinting was investigated based on whole-brain mRNA expression of TRHa/b and TSHβ in the upper head that contained the pituitary gland, levels of T4 and T3 isolated in the lower jaw that contained thyroid glands, and on whole-brain mRNA expression of NR1. Ueda *et al*. (2016) analyzed the gene expression profiles of TRHa/b and TSHβ in juvenile chum salmon, and revealed that environmental changes due to the release from the hatchery to the natal stream significantly enhanced the TRHa/b expression levels and then the TSHβ expression levels and T4/T3 levels increased significantly during downstream migration towards the sea (**Fig. 7**). Ueda *et al*. (2016) also demonstrated that NR1 expression levels showed a significant first peak correlated with larval brain development in the hatchery (Pouwels 1978), a gradual significant increase immediately after a significant TRHa/b expression surge at Site **C**, and a second significant peak at the mouth of their natal river at Site **E** (**Fig. 7**). The results of *in situ* hybridization confirmed that NR1 mRNA localization increased prominently in the ventral telencephalon (TE) at Site **C** compared to the hatchery immediately before release into the river. Moreover, in experiments with the oral administration of T4 (2 mg/g pellet) to juvenile salmon, Ueda *et al*. (2016) found increased T4 and T3 levels in the whole body and significant increases in NR1 gene expression in the whole brain for 4 days (**Fig. 8**). In-

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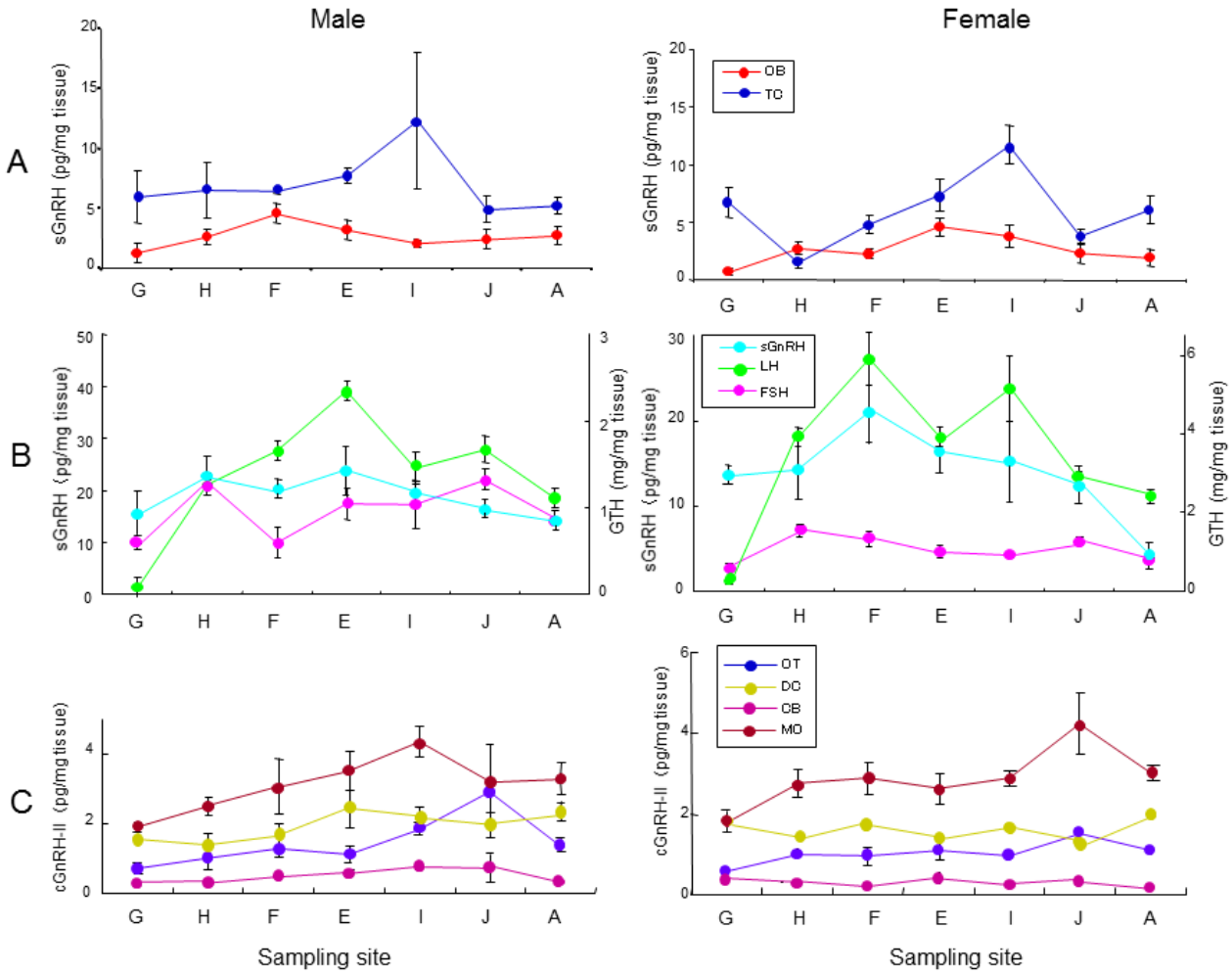

Fig. 9. Changes in salmon gonadotropin-releasing hormone (sGnRH) contents in the olfactory bulb (OB) and telencephalon (TE) (A), sGnRH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary gland (B), and chicken II GnRH (cGnRH-II) in the optic tectum (OT), the diencephalon (DC), the cerebellum (CB), and the medulla oblongata (MO) (C) of adult chum salmon during homing migration from Site **G** to Site **A**. Data are presented as the means ± SEM (N = 8–12).

terestingly, T4 and T3 levels increased for 7 and 14 days, but no significant difference was observed in NR1 gene expression, revealing that the critical period of imprinting is limited to less than 7 days (**Fig. 8**). These data clarify that during the initiation of the downstream migration of juvenile chum salmon, the environmental changes involved in the release into the river induce the expression of the BPT hormones, which then stimulate the upregulation of NR1, enhancing the olfactory memory formation capability.

3-2. Hormone profiles of adult chum salmon during homing migration

The hormone profiles in the BPG axis of adult chum salmon migrating from the Bering Sea to the natal hatchery in the Chitose River, were measured using specific time-resolved fluoroimmunoassay systems (Yamada *et al*. 2002). The level of sGnRH in the OB of both sexes peaked when the fish was located between Sites **E** and **F**; a location where the olfactory discriminating ability of the natal stream is expected to be important. Peak sGnRH in the TE was observed when the fish was at the branch point of the Chitose River from the Ishikari River (Site **I**), where the olfactory functions should be highly activated as individuals attempt to determine which branch to enter (**Fig. 9**). In the pituitary gland, sGnRH levels tended to increase in concert with LH levels around Sites **F** and **E**. In contrast, FSH levels did not show any clear correlations with sGnRH levels in the pituitary gland. Although the roles of cGnRH-II in these brain regions are yet unclear, levels increased in the medulla oblongata (MO) of both sexes at Sites **I** and **J**, and levels in the optic tectum (OT) increased in males at Site **J**. In the diencephalon (DC) and cerebellum (CB), cGnRH-

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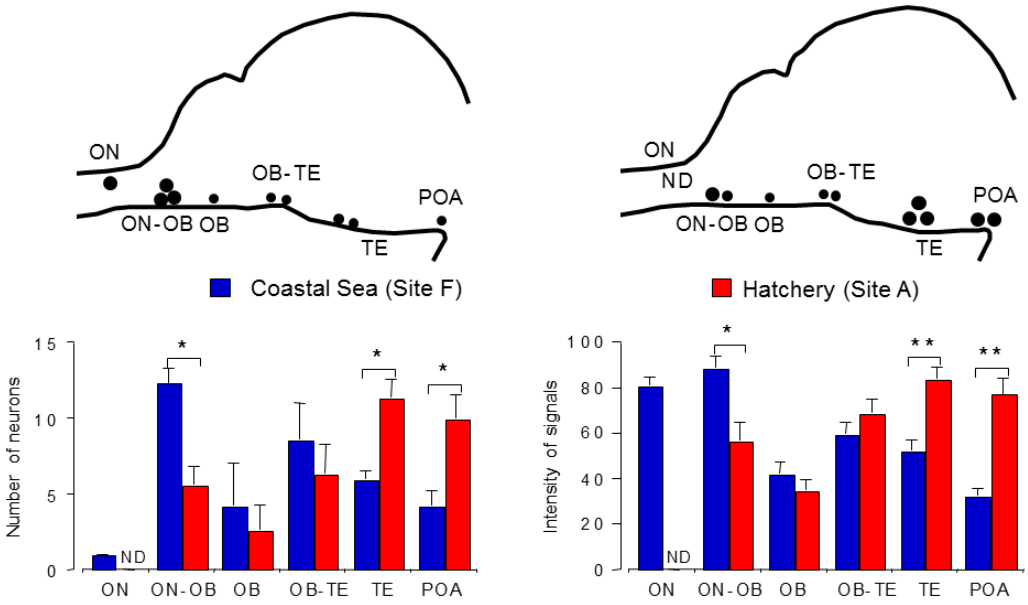

Fig. 10. Schematic illustrations of the distribution of sGnRH neurons and changes in the number of neurons showing signals for number of sGnRH neurons and intensity of pro-sGnRH signals in chum salmon at Sites **F** and **A**. Data are presented as the means ± SEM (N = 3–37). Significant differences are indicated at $*p < 0.05$ and $**p < 0.01$ levels. ND, not detected; OB, olfactory bulb; OB-TE, transitional area between olfactory bulb and telencephalon; ON, olfactory nerve; ON-OB, transitional area between olfactory nerve and olfactory bulb; POA, preoptic area; TE, telencephalon.

II levels showed no significant changes during homing migration (Ueda 2011; **Fig. 9**).

sGnRH neurons, which showed signals for pro-sGnRH mRNA, were observed in the dorsal olfactory nerve of chum salmon at Site **F**, but not in fish at Site **A** (Kudo *et al*. 1996; **Fig. 10**). Changes in the levels of GTH subunit mRNAs in the pituitary gland of pre-spawning chum salmon demonstrated that the levels of GTHα2 (there are two distinct α subunit proteins, and only α2 subunit is utilized as the composer of LHβ) and LHβ increase when an individual enters freshwater, but showed no changes in FSHβ level (Kitahashi *et al*. 1998a). It is interesting to note that both sGnRH levels in the TE and serum T levels in both sexes showed a coincident peak at the branch point of the Chitose River from the Ishikari River, Site **I**. Ueda *et al*. (2016) showed that the mRNA expression levels of sGnRH-I/II, and NR1 in the OB and TE appeared to increase, with some differences between males and females that might be related to sex-specific gonadal maturation (**Fig. 11**). The increase of NR1 gene expressions in the OB show that olfactory memory retrieval abilities increase during the upstream homing migration of adult chum salmon. Interestingly, sGnRH-II gene expression in the hypothalamus of males was also high in the Bering Sea (Ueda *et al*. 2016). These results reveal that sGnRH is involved in brain region-dependent roles on gonadal maturation and olfactory memory retrieval in adult chum salmon.

Serum steroid hormone levels showed similar profiles to those observed in our previous studies (Ueda *et al*. 1984; Ueda 1999): E2 in females and 11KT in males increased during vitellogenesis and spermatogenesis, respectively, during homing migration from the Bering Sea to the Chitose River (**Fig. 12**). Although E2 in males and 11KT in females showed interesting small peaks during upstream migration to their natal hatchery, the precious roles of these peaks were unknown. T, which is precursor of both E2 and 11KT, showed resemble peaks of E2 and 11KT. DHP increased dramatically at the time of final gonadal maturation in both sexes (**Fig. 12**). In addition, year-to-year differences in plasma levels of steroid hormones in pre-spawning chum salmon were also studied in comparison with sea surface temperature (SST) of coastal sea, and showed that there were year-to-year differences in plasma levels of steroid hormones and gonadal maturity, and some of them may be influenced by year-to-year variation of SST (Onuma *et al*. 2003).

3-3. Homing profiles of sockeye salmon in Lake Shikotsu, Hokkaido, Japan

Since it is difficult to carry out experimental treatments to manipulate endocrinological functions in anadromous salmon owing to the salinity differences between seawater and freshwater, lacustrine salmon populations offer a good model system for studying hormonal control mechanisms of salmon homing. In Lake Shikotsu (surface area 78 km^2, average and maxi-

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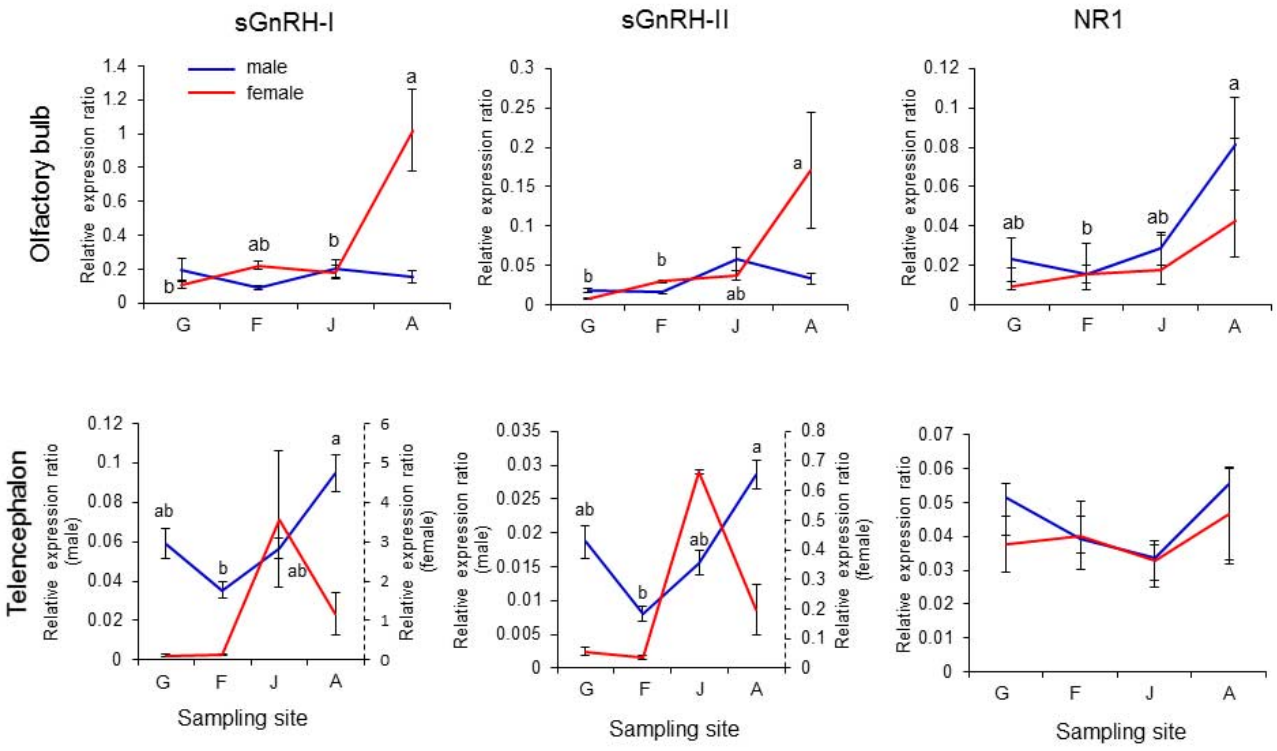

Fig. 11. Changes in the profiles of salmon gonadotropin-releasing hormone (sGnRH) and the *N*-methyl-D-aspartate receptor essential subunit NR1 in the olfactory bulb and telencephalon of adult chum salmon during homing migration. Adult fish were sampled from Site **G** to Site **A**. Data are presented as the means ± SEM (N = 10). Different letters represent significant differences at $p < 0.05$ level.

mum depth 265 m and 363 m, respectively), lacustrine sockeye salmon are artificially produced by hatchery propagation. Adult sockeye salmon were captured between September and November adjacent to their natal hatchery prior to spawning, and were sampled for serum steroid hormones, tagged, and released in the center of the lake. Fish were resampled at recapture to characterize changes in steroid hormone levels in individual migrants as well as to monitor homing duration and precision in each month (Sato *et al*. 1997). Homing duration was significantly truncated from September to October in males and from October to November in females (**Fig. 13A**). All males returned faster than females in September and October, although half of the males did not return to the natal site in November. In contrast, 78–90% of females returned over the entire three-month sampling period (**Fig. 13B**). It is interesting to note that the average homing percentage of both sexes for three months is 83%, with no sex difference in the total number of homing individuals. Although male salmon do not display territorial behavior, they are highly aggressive in order to compete for access to females, suggesting that early returning males accrue some benefits in securing females for breeding. It has been reported that increased T levels are correlated with compromised immune function (Harris and Bird 2000), suggesting a tradeoff whereby faster migration may be advantageous even with the risk of disease.

3-4. Homing profiles of sockeye salmon by hormone and antagonist treatments

GnRH analog (GnRHa) treatment has been reported to be highly effective in inducing GTH release, ovulation and spermiation in teleost fishes (Zohar 1996; Zohar and Mylonas 2001). In order to investigate GnRHa treatment on homing duration in lacustrine sockeye salmon in Lake Shikotsu, Sato *et al*. (1997) and Kitahashi *et al*. (1998c) investigated the effect of GnRHa implantation intramuscularly on both homing profiles and serum steroid hormone levels of fish in September. Although gonadectomy was not carried out in these experiments, the GnRHa implantation was highly efficient in shortening the homing duration, and caused dramatic increases in serum DHP levels in both sexes. An interesting discrepancy was observed between rapidly and slowly returning males: rapidly returning males had higher serum T levels and lower serum DHP levels than slowly returning males. To examine the direct action of T and DHP on homing duration, T and DHP were implanted in fish in September.

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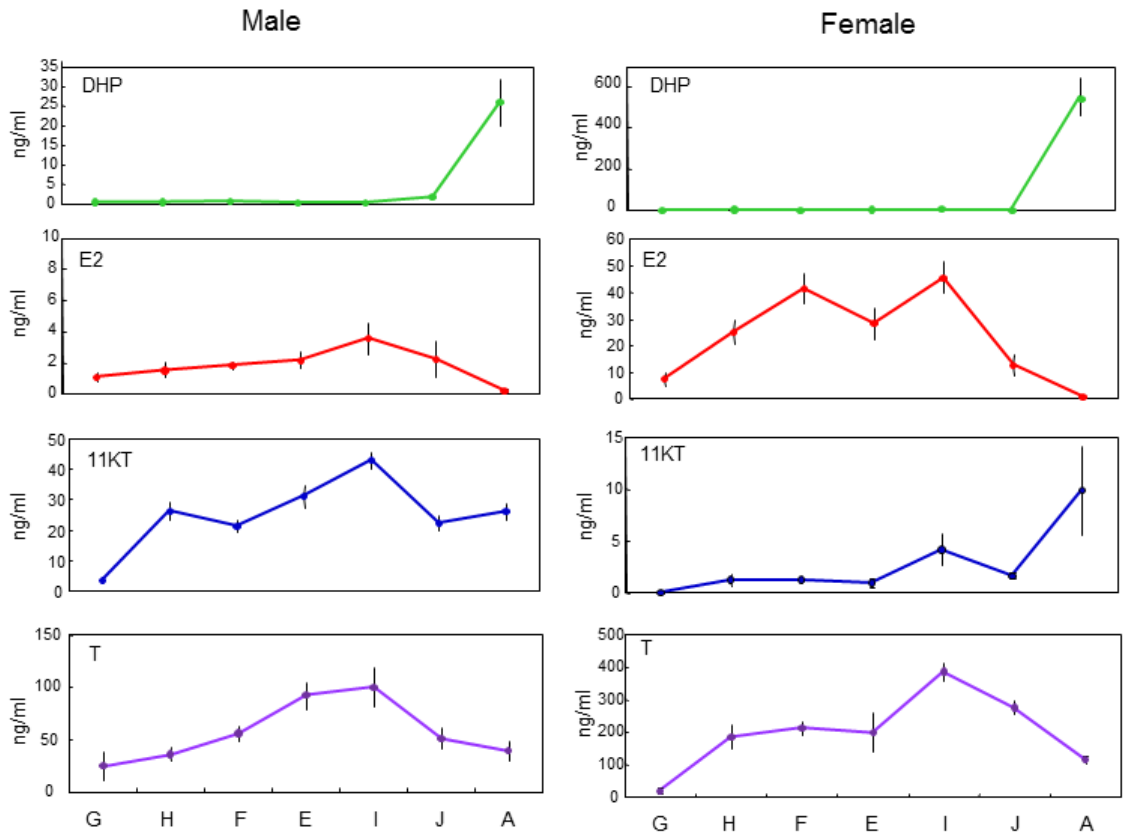

Fig. 12. Changes in serum steroid hormone levels of male and female chum salmon during homing migration from Site **G** to Site **A** (Fig. 6). Data are presented as the means ± SEM (N = 8–12). DHP, 17α,20β-dihydroxy-4-pregnen-3-one; E2, Estradiol-17β, 11KT, 11-ketotestosterone; T, testosterone.

T implantation tended to reduce homing duration in both males and females, but there was no statistical significance. DHP implantation significantly shortened homing duration in females, but it did not have any significant effect in males (**Fig. 13C**). It is interesting to note that the direct actions of T and DHP on homing migration are sex dependent. The peak of plasma T levels in lacustrine sockeye salmon of both sexes was observed at the time when they gathered at the mouth of their natal stream in Lake Chuzenji, Japan (Ikuta 1996). Androgens are known to be involved in stimulating aggressive behavior in teleost fishes (Villars 1983), and serum T and 11KT are the two major androgens that influence spawning behaviors, downstream and upstream migration, and the social dominance hierarchy (Kindler *et al*. 1989; Cardwell and Liley 1991; Pankhurst and Barnett 1993; Brantly *et al*. 1993; Cardwell *et al*. 1996; Munakata *et al*. 2001a, b). Munakata (2012) reviewed the correlation between migratory behaviors and steroid hormones in masu salmon. Although DHP is known to be a maturation-inducing steroid in salmonids (Nagahama and Adachi 1985), its function in the central nervous system has not yet been clarified. The functional roles of T and DHP on salmon homing migration should be further investigated with special attention to their action on the sensory and central nervous system.

In order to investigate involvement of NMDA receptor on homing duration in lacustrine sockeye salmon in Lake Shikotsu, the effect of NMDA receptor antagonists (APV and MK-801) on homing duration in late October was investigated (Fukaya and Ueda in preparation). Although there were no rescue experiments using agonists, homing duration of male lacustrine sockeye salmon was significantly prolonged by both blockers (**Fig. 13D**). These results suggest that NMDA receptors are deeply involved in retrieval of the imprinting memory in sockeye salmon.

GnRHa implantation was also highly effective in accelerating gonadal maturation in anadromous, maturing sockeye salmon of both sexes. Expression of GTH subunit genes in the pituitary gland was examined and revealed that the levels of GTHα and LHβ mRNAs in GnRHa-implanted fish were higher than those in control fish, but the levels of FSHβ mRNA showed no change (Kitahashi *et al*. 1998b). Implantation of GnRHa caused a significant elevation of serum DHP levels in both sexes, but had no effect on levels

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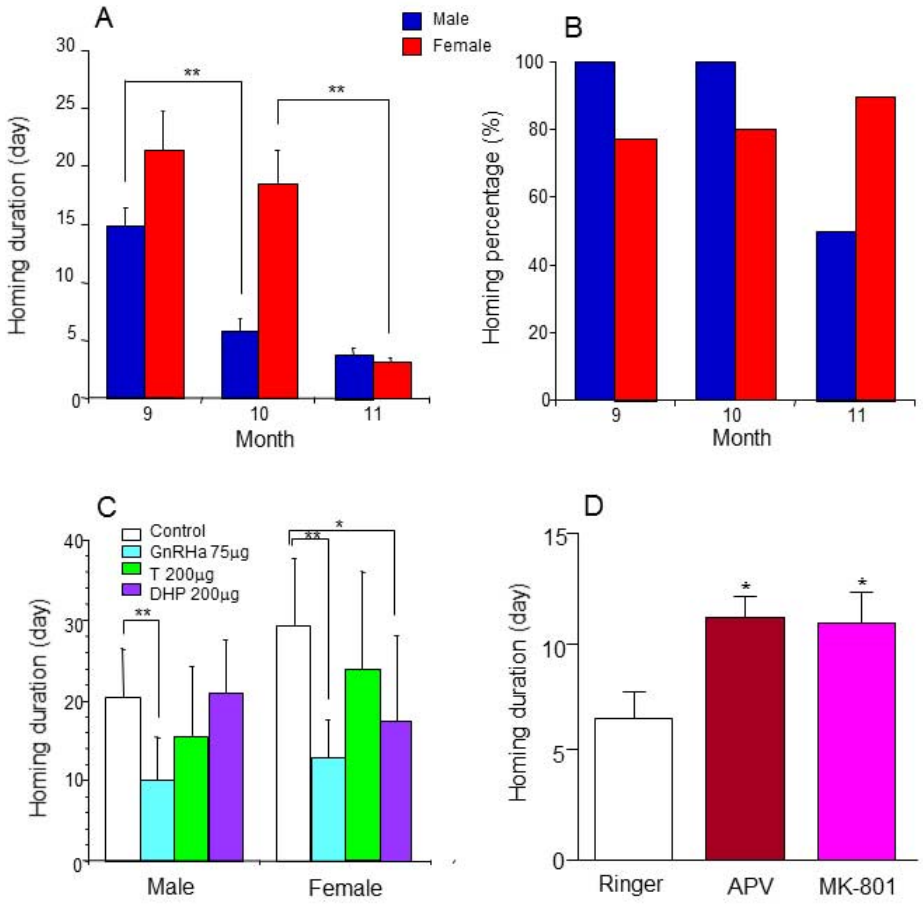

Fig. 13. Changes in homing duration (A) and percentage (B) of lacustrine sockeye salmon in Lake Shikotsu from September to November. Effects of GnRH analog (GnRHa), testosterone (T) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) implantation on homing duration of lacustrine sockeye salmon in Lake Shikotsu in September (C). (D) Effects of MNDA blockers (APV and MK-801) on homing duration of male lacustrine sockeye salmon in Lake Shikotsu in October (D). Data are presented as the means ± SEM (A and B, N = 9–12; C, N = 10; D, N = 10). Significant differences are indicated at *$p < 0.05$ and **$p < 0.01$ levels.

of T and 11KT in males or E2 and T in females (Fukaya *et al*. 1998). These data clarify that sGnRH in the brain stimulates LH release from the pituitary gland, and then LH enhances serum DHP levels in both sexes during the final phase of gamete maturation as well as the latter part of homing migration. GnRH is believed to play a prominent role in the homing migration of both sexes, but gonadal steroids, especially T and DHP, seem to differently affect the homing migration of males and females.

Since sGnRH neurons exist in the OB, TN, and POA of the brain, these sGnRH neurons are considered to play different roles during homing migration of Pacific salmon. However, it is difficult to examine the functional difference of these sGnRH neurons *in vivo*. Previous report demonstrated that NMDA treatment could stimulate GnRH release in the hypothalamic fragments in rats (Lopez *et al*. 1992). Effects of *in vitro* NMDA treatment on sGnRH release in brain slices of lacustrine sockeye salmon (3-year-old) in the non-breeding (June) and breeding periods (October) (Kozu and Ueda in preparation). Each slice was stimulated with 5 mM NMDA in Mg^{2+} free fish saline in both sexes in June and October. In comparison with fish between June and October, the NMDA treatment significantly enhanced the sGnRH release in the POA slices. In contrast, the NMDA treatment induced sGnRH release in the OB and TN slices, but no differences were observed between June and October (**Fig. 14**). These results demonstrate that sGnRH neurons in the POA must be involved in gonadal maturation, however, those in the OB and TN may have olfactory related functions in relation with olfactory-related homing migration. The sexually different roles of BPG hormones as well as the brain regional different roles of sGnRH on the sensory systems and the central nervous systems should be examined in future studies.

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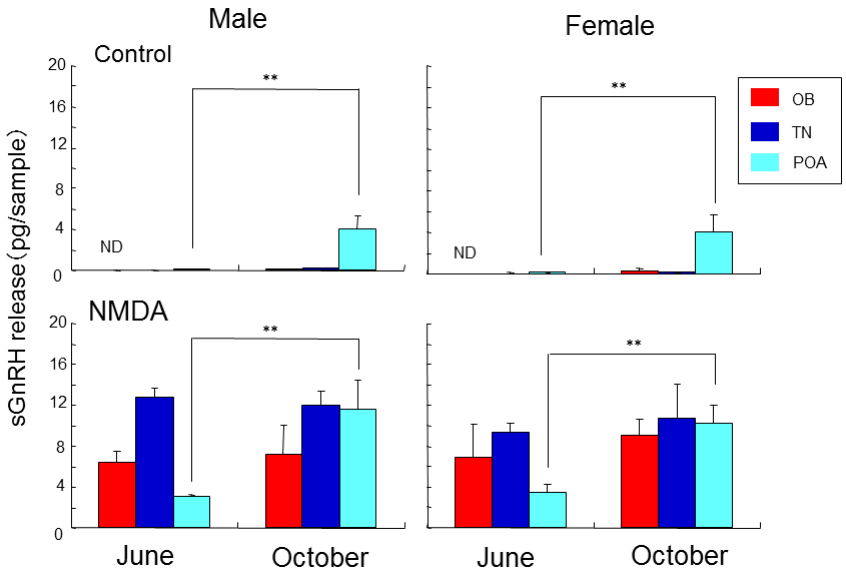

Fig. 14. Changes in the amount of sGnRH release from the olfactory bulb (OB), terminal nerve (TN), and preoptic area (POA) slices of the brain of lacustrine sockeye salmon in June and October. The release of sGnRH is significantly increased only in POA slices both in control and 5 mM MNDA in October. Data are presented as the means ± SEM (N = 10). Significant differences are indicated at $^{**}p < 0.01$ levels.

4. Neurophysiological studies on olfactory functions

Three different olfactory hypotheses have been proposed for salmon imprinting and homing. The first is the olfactory imprinting hypothesis developed by Wisby and Hasler (1954) using coho salmon that the specific odor of the natal stream is imprinted to the olfactory system before juvenile salmon migrate to the sea, and adult salmon retrieve this odor information as a cue for homing when they migrate through the natal stream network to their natal tributary. The second is the pheromone hypothesis developed by Nordeng (1977) using Arctic char and Atlantic salmon that juvenile salmon in a stream release population-specific odors that guide homing adults. And, the third is the hierarchical navigation hypothesis proposed by Bett and Hinch (2015), that migrating adult salmon relied on primary directional cue, is imprinted odors. Secondary directional cue is conspecific odors, and tertiary cue is non-olfactory environmental indicators.

The pheromone hypothesis is not applicable in Pacific salmon because that there are no juveniles of chum salmon or pink salmon present at the time that the adults return. The olfactory imprinting hypothesis, by which some specific odorant factors in the natal stream are imprinted on the olfactory system of juvenile salmon during downstream migration and adult salmon evoke these factors in the natal stream to attract adult salmon to carry out homing upstream migration, is the most reliable hypothesis in Pacific salmon. However, until now, it is uncertain to reveal properties of natal stream odors, and it is also impossible to link hormonal control mechanisms to olfactory imprinting and homing because we lacked molecular markers that would permit the evaluation of olfactory memory formation and retrieval in the salmon brain.

4-1. Properties of natal stream odors for Pacific salmon

Several attempts to identity the natal stream odors were made based on the olfactory bulbar response, suggesting that the natal stream odors were non-volatile (Fagerlund *et al*. 1963; Cooper *et al*. 1974; Bodznick 1978). Spectral analysis of the olfactory bulbar response suggested that the natal stream odor was absorbed on activated carbon and ion-exchange resin, insoluble in petroleum-ether, dialyzable, non-volatile, and heat-stable (Ueda 1985). Unlike olfactory organs of terrestrial animals, fish olfactory organs respond only to a limited suite of dissolved chemical species, such as amino acids, steroids, bile acids, and prostaglandins (Hara 1994).

Shoji *et al*. (2000) analyzed the compositions of dissolved free amino acid (DFAA), inorganic cations and bile acids in waters from three streams that flow into Lake Toya. Application of mixtures of inorganic cations or bile acids, combined based on their compositions in stream waters, to the olfactory epithelium induced only very small responses. On the other hand, application of mixtures of DFAA induced large re-

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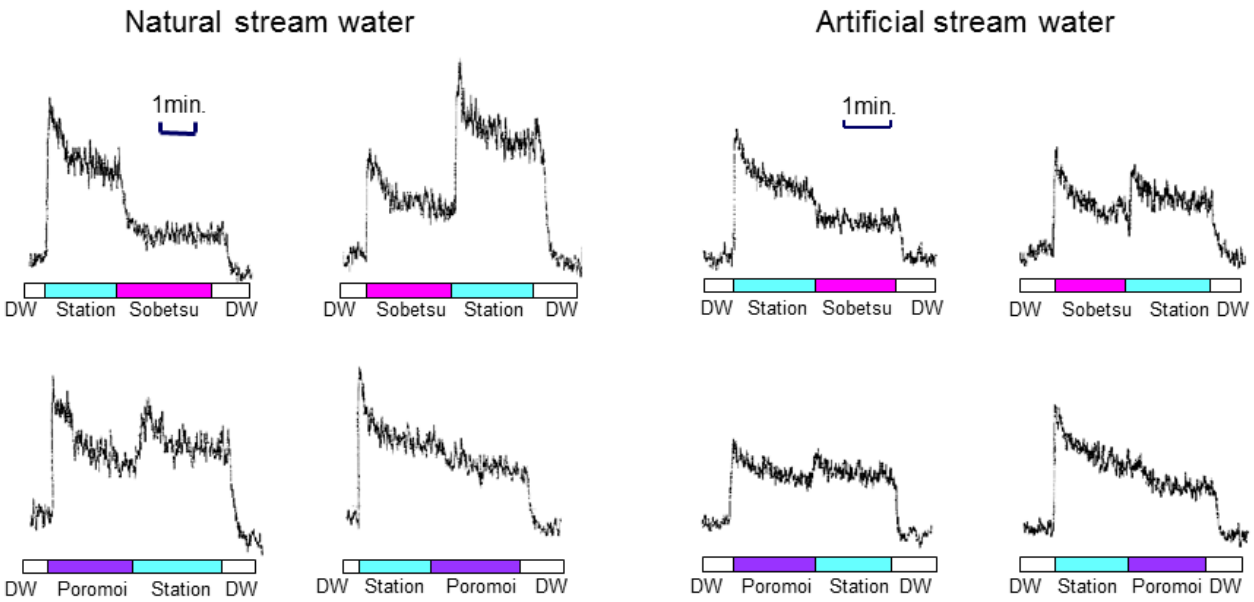

Fig. 15. Typical integrated olfactory nerve responses to natural stream waters and artificial stream waters reconstituted with amino acids and salts based on the compositions of natural stream waters after the olfactory epithelium was adapted to the lake water.

sponses. The response to artificial stream water based on the composition of DFAA and salts closely resembled the response to the corresponding natural stream water. Cross-adaptation experiments with three combinations of natural and artificial stream waters were carried out. The response pattern for each combination of artificial stream water closely resembled that of the corresponding combination of natural stream water (**Fig. 15**). Accordingly, DFAA compositions in the natal stream water are proposed to be natal stream odors, but these odors may change seasonally or annually.

Yamamoto *et al*. (2013) analyzed DFAA concentration and composition of water from the Teshio River in Hokkaido, Japan, where chum salmon returned for spawning, during juvenile downstream migration in spring and adult upstream migration in autumn with a 4-year difference. Among the 15 amino acids found in the Teshio River water, DFAA concentrations fluctuated greatly, but 5–7 stable DFAA compositions (mole %) were found between the spring and autumn samples within a 4-year span (**Fig. 16**). Two kinds of artificial stream water (ASW) were prepared using the same DFAA concentration in the Teshio River during the time of juvenile imprinting in spring (jASW) and adult homing in autumn (aASW), after a 4-year period. Behavioral experiments of upstream selective movement in a 2-choice test tank (Y-maze) consisting of two water inlet arms and one pool. Two different kinds of water were added to the water inlet of either left or right arms, and the fish movement monitored to determine the number of fish that moved into each arm. Four-year-old mature male chum salmon captured in the Teshio River showed significant preference for either jASW or aASW when compared to control water, with no preference for jASW or aASW (**Fig. 17**). Although in electro-olfactogram (EOG) experiments, adults were able to discriminate between jASW and aASW. These findings indicate that the long-term stability of the DFAA compositions in natal streams is crucial for olfactory homing in chum salmon.

Changes in the DFAA compositions in stream water are attributed mainly to complicated biological processes in the watershed ecosystem. There are many possible factors affecting the DFAA compositions both within and beyond the stream environment, such as soils, vegetation, litter, pollen, dew, and various microbial activities (Thomas 1997). Among these factors, the roles of complex microbial communities called biofilms have been intensively investigated (Costerton *et al*. 1994; Nosyk *et al*. 2008). A biofilm consists of various microorganisms, and is embedded into a matrix of extracellular polymeric substances. Ishizawa *et al*. (2010) investigated the origin of DFAA in stream water focusing on biofilms in the stream bed via incubation experiments in the laboratory. Stones were placed in the Toyohira River, for 3 months, allowing formation of biofilms, and then incubated for 24 hours in the laboratory at stream water temperature. After incubation, the composition and concentrations of DFAA in the incubation solution were measured by high-performance liquid chromatography. The DFAA

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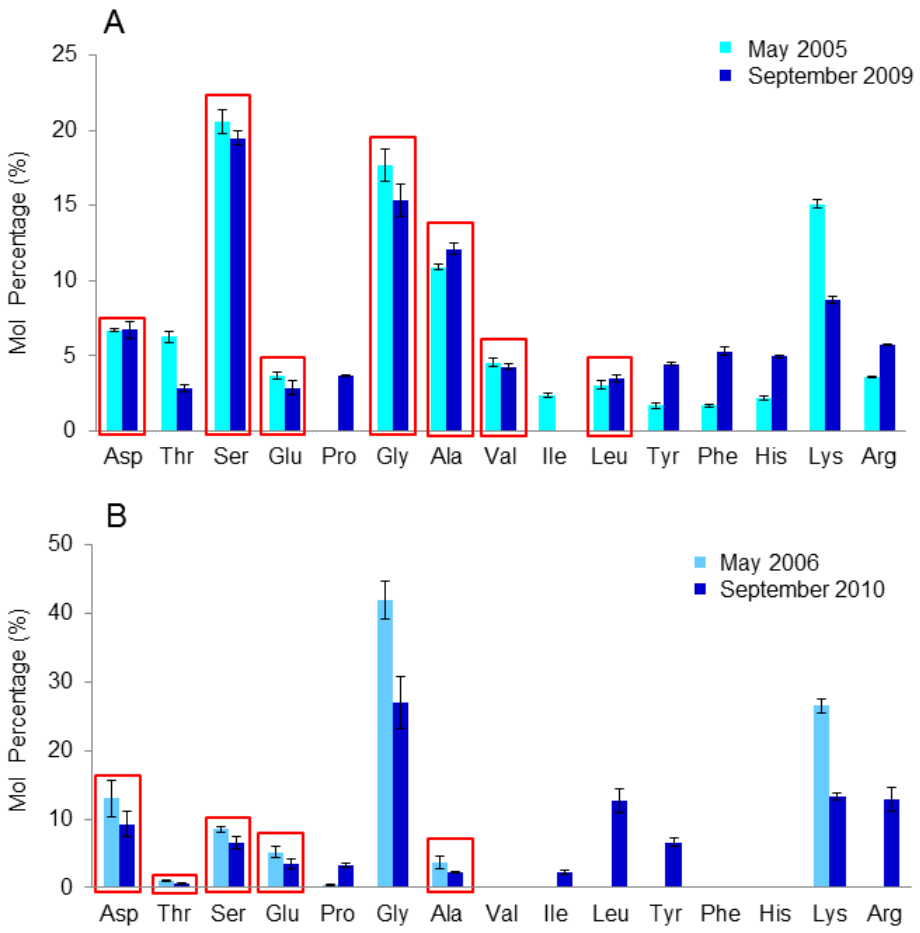

Fig. 16. Seasonal and yearly changes in the composition of 19 dissolved free amino acids in the Teshio River. Stable compositions of seven amino acids (Ala, Asp, Glu, Gly, Leu, Ser and Val) between May 2005 and September 2009 (A), and five amino acids (Ala, Asp, Glu, Ser and The) between May 2006 and September 2010 (B) are detected in red box.

concentration increased greatly in the biofilm incubation solution of the treatment group, but the DFAA composition (mole %) showed little change after 24-hour incubation, which was similar to stream water, suggesting that biofilms are a major source of DFAA in stream water.

4-2. Artificial imprinting studies using amino acid in sockeye salmon

Imprinting experiments have been used to demonstrate the reliance of salmon on olfactory cues. Coho salmon were experimentally imprinted with β-phenylethyl alcohol (PEA) or morpholine, during smoltification and lured into unfamiliar streams scented with these odors during homing migration a few years later (Cooper *et al*. 1976; Scholz *et al*. 1976). The olfactory receptor cells of coho salmon that had been imprinted with PEA had a higher sensitivity to PEA compared with non-imprinted fish (Nevitt *et al*. 1994), and only fish that were exposed to PEA or natural stream odors during smoltification formed an imprinted memory (Dittman *et al*. 1996). Using electrophysiological and behavioral experiments, Yamamoto *et al*. (2010) have revealed that 1-year-old lacustrine sockeye salmon can be imprinted at smoltification by a single amino acid, 1 μM L-proline (Pro) or L-glutamic acid (Glu). The EOG responses of test fish exposed to Pro in March (before smoltification) and April–June (during smoltification) for 2 weeks were significantly greater than those of non-exposed control fish, but not those of test fish exposed in July (after smoltification). When Pro and control water were added to the water inlets of the Y-maze during the spawning season 2 years after the test water exposure, 80% of maturing and mature test fish exposed before and during smoltification showed a preference for Pro, whereas those exposed after smoltification displayed no preference (**Fig. 18**). The EOG response of test fish exposed to Pro or Glu for 1 hour, 6 hours, 1 day, 7 days, or 14 days in May revealed that only the response after 14-days of expo-

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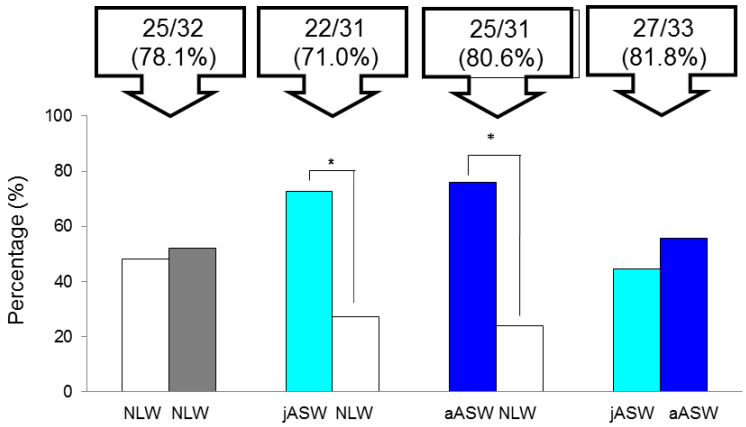

Fig. 17. Behavioral selectivity to artificial stream water by 4-year-old adult male chum salmon in the 2-choice test tank in 2009 and 2010. The numbers in boxes at the top indicate the number of fish that move upstream into either arm of the tank and the total number of fish that are experimentally tested. NLW, natural lake water; jASW, artificial stream water of juvenile downstream migration in May 2005 and 2006; aASW, artificial stream water of adult upstream migration in September 2009 and 2010. Data are presented as the means ± SEM (N = 31–33). Significant differences are indicated at $*p < 0.05$ and $**p < 0.01$ levels.

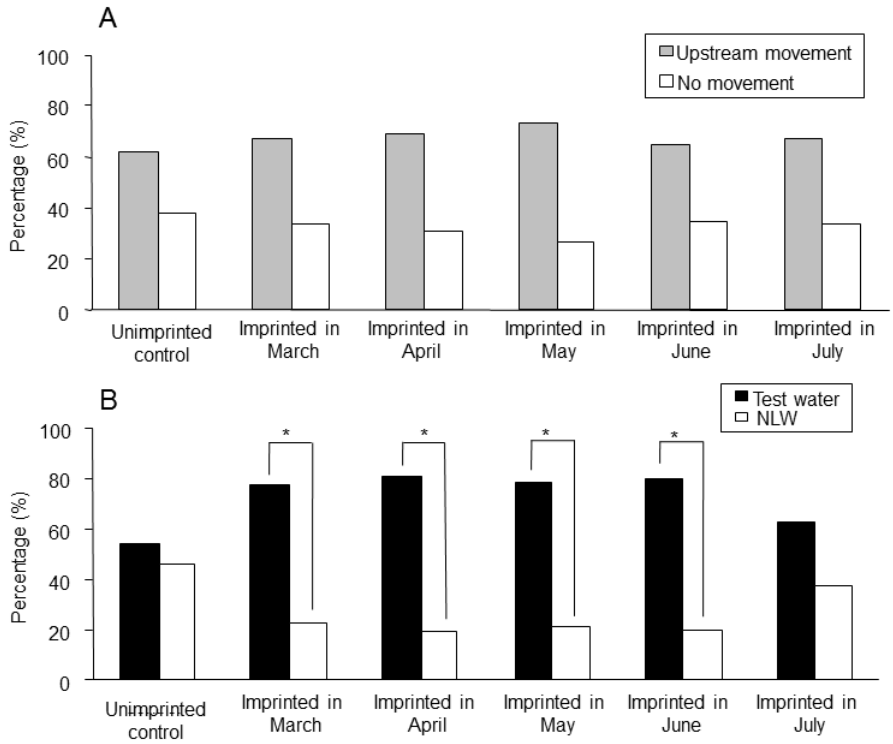

Fig. 18. Behavioral selectivity to the test water of mature sockeye salmon in a 2-choice test tank. Shown are the upstream movement (A) and selectivity (B) of mature sockeye salmon of experimental fish (exposed to 1 mM L-proline (Pro) from March to July in 2005) and unimprinted control fish in a two-choice tank containing either the test water (Pro) or natural lake water (NLW). Data are presented as the means ± SEM (N = 45–60). Significant differences are indicated at $*p < 0.05$ level.

sure was significantly greater than the control. Yamamoto *et al*. (2010) showed that 1-year-old sockeye salmon are able to imprint a single amino acid before and during smoltification, and that imprinting requires exposure for at least 14 days in the artificial rearing environment. In natural stream environment, however, smolts should be imprinted immediately by different odors when they encounter a branch stream that flows

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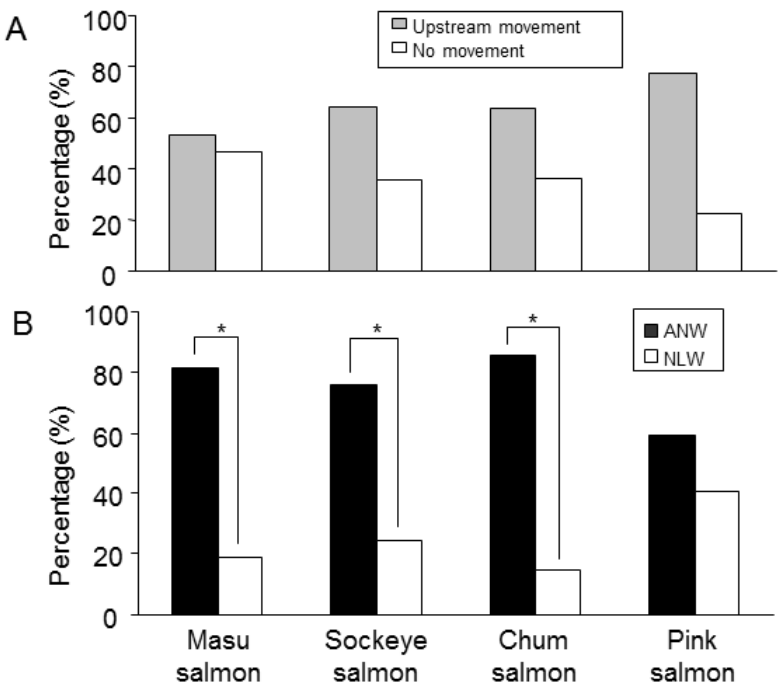

Fig. 19. Upstream movement (A) and selectivity (B) in each artificial natal stream water of mature male four Pacific salmon species in the two-choice test tank. Data are presented as the means ± SEM (N = 30–45). Significant differences are indicated at $*p < 0.05$ level.

into a main stream during downstream migration as in the sequential imprinting hypothesis proposed by Harden Johns (1968). Further experiments should be designed to clarify how smolts are imprinted when they encounter for a short time during downstream migration using artificial raceway tank.

4-3. Comparison of olfactory discriminating abilities among four Pacific salmon species

Behavior experiments were compared to test attractant effects on upstream selective movement among the four Pacific salmon species (pink, chum, sockeye, and masu salmon) using artificial natal stream water (ANW) prepared to the same composition and concentration of DFAA in their natural natal stream in the Y-maze (Yamamoto *et al*. 2008). In both ANW and natural lake water (NLW), pink salmon showed the highest percentage of upstream movement among the four Pacific salmon species, but showed the least selectivity (59.3%) in the arm running ANW, whereas the other three Pacific salmon species (chum, sockeye, and masu salmon) showed significant selectivity (85.7, 75.9, and 81.3%, respectively) in the arm running ANW (**Fig. 19**). It is quite interesting to note the significant selectivity to the test water of about 80% in the experimental conditions. The difference of olfactory discriminating ability merits further investigation to determine the source of error among the 20% of fish that select the wrong path.

If salmon always demonstrate accurate homing to their natal stream, there would be little chance of widening their distribution area or of increasing their population size; in addition, there is the dangerous possibility that this may reduce genetic diversity. Pink salmon, which have the widest distribution of the salmon species in Japan, may have evolved the capacity to adapt to non-natal stream odors, allowing this expanded distribution. The relationship between salmon evolution and homing accuracy should be investigated from an evolutionary perspective to determine the adaptive significance to homing inaccuracy.

4-4. Olfactory discriminating abilities of chum salmon during downstream and upstream migration

Ueda *et al*. (2016) examined olfactory discriminating ability of stream odors measured by EOG, showing significantly greater discrimination ability for Ishikari River (natal stream) water in juvenile chum salmon collected at Sites **B** and **D** compared to the fish at the hatchery (**Fig. 20A**). These data clarify that the capacity for forming olfactory memory of stream odors continues and increases during the downstream migration of juvenile chum salmon.

The EOG response of adult male chum salmon collected at Site J was significantly higher for both the

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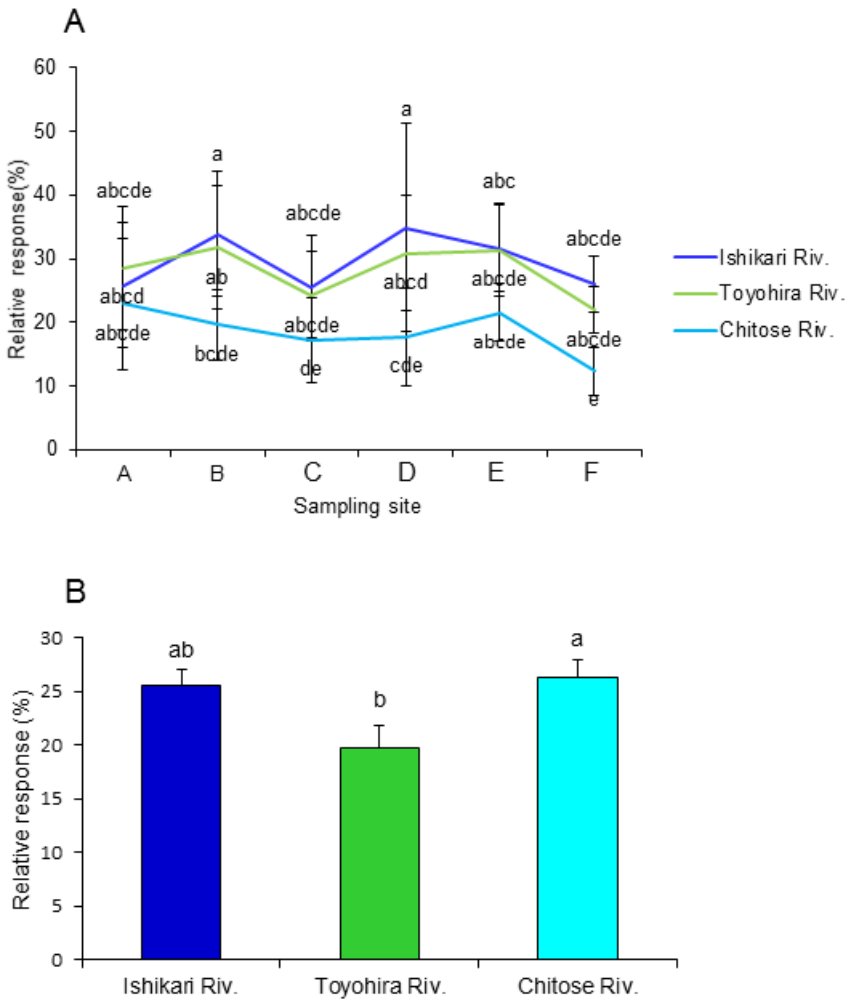

Fig. 20. EOG responses to water from three streams in juvenile chum salmon during downstream migration from Site **A** to Site **F** (A). EOG responses to waters from three streams in adult male chum salmon collected at Site **J** (B). Data are presented as the means ± SEM (A, $N = 3$; B, $N = 7$). Different letters represent significant differences at $p < 0.05$ level.

Ishikari River and Chitose River, their natal stream waters, than for the Toyohira River, a nearby tributary not on their migratory path (**Fig. 20B**). These data reveal that the olfactory memory retrieval abilities regarding natal stream odors increase during the upstream homing migration of adult chum salmon (Ueda *et al*. 2016).

The effects of implanting GnRHa on the olfactory discrimination and olfactory memory retrieval of natal stream odours were studied using adult male chum salmon caught in Ishikari Bay (Site **F**) prior to entering the Ishikari River. GnRHa implantation increased the serum levels of DHP after 48 h, and tended to enhance NR1 gene expression in the OB after 24 and 48 h (Ueda *et al*. 2016). GnRHa implantation for 48 h significantly increased the relative EOG response to the Ishikari River water in adult male chum salmon, including straying fish from another hatchery (**Fig. 21A**). The relative EOG response of adult male chum salmon originating from the Chitose Hatchery (confirmed by otolith thermal marks) showed significant discrimination of Chitose River water (**Fig. 21B**), revealing that GnRHa stimulated the ability to discriminate natal stream odor.

Since MK-801 is known to impair learning performance in zebrafish (*Danio rerio*) (Sison and Gerlai 2011), the effects of MK-801 injection (0.1 μg/g BW 4 times every other day for 2 weeks) on EOG responses in lacustrine sockeye salmon that were exposed to 1 μM Pro and L-glutamic acid Glu for 2 weeks in 1-year-old fish were examined 2 years later in the spawning period (3-year-old fish) (Nakamura and Ueda in preparation). The EOG response to Pro and Glu in the MK-801-injected fish was significantly lower compared to the control fish (**Fig. 22**). These results indicate that effects of NMDAR-dependent olfactory memory formation are blocked for two years.

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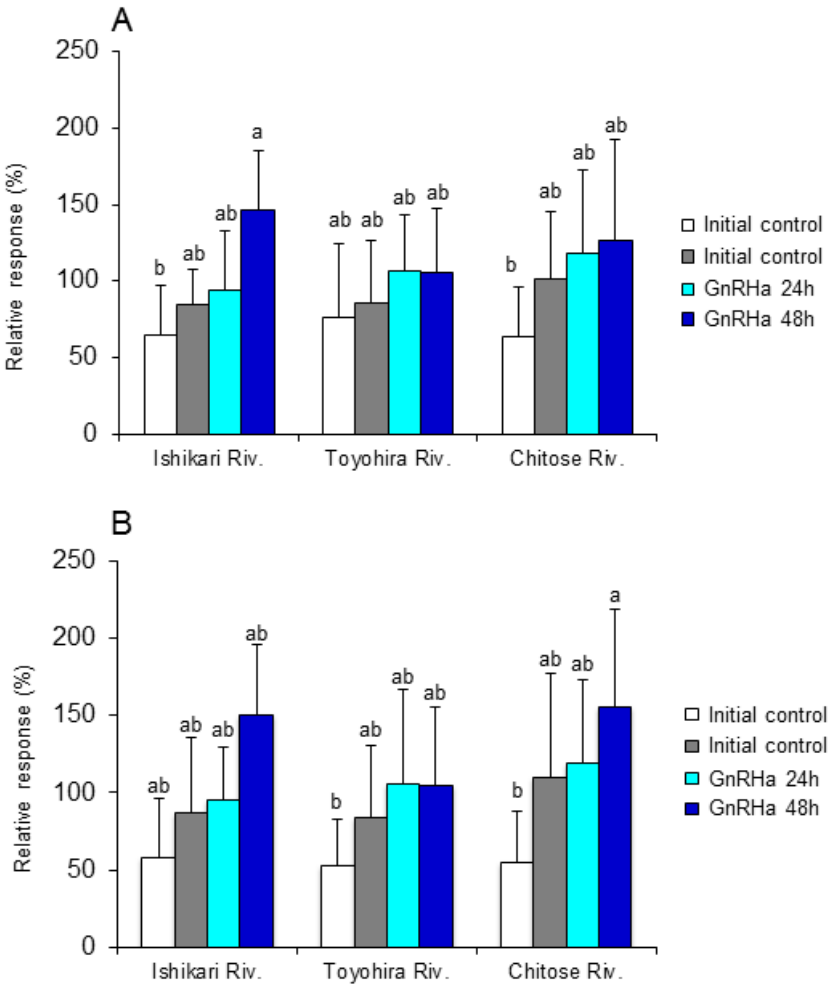

Fig. 21. Effects of GnRH analogue implantation on the EOG response to three stream waters in adult male chum salmon collected from Ishikari Bay. Adult chum salmon including strays from another hatchery (A) and adult chum salmon originating from the Chitose Hatchery (B) caught in Site **F**. Data are presented as the means ± SEM (N = 4–5). Different letters represent significant differences at $p < 0.05$ level.

4-5. Biochemical and molecular biological studies on Pacific salmon olfactory functions

An olfactory system-specific protein of 24 kDa (N24) was identified in lacustrine sockeye salmon by electrophoretic comparison of proteins restricted to the olfactory system with those found in other parts of the brain (Shimizu *et al*. 1993). In various species of teleosts, N24 immunoreactivity was found in the olfactory system of catadromous species, Japanese eel (*Anguilla japonica*), but not in non-migratory species, carp (*Cyprinus carpio*) (Ueda *et al*. 1994). Interestingly, N24 immunoreactivity was also observed in the testicular germ cells, spermatids and spermatozoa, suggesting its involvement in sperm chemotaxis (Ueda *et al*. 1993). cDNA encoding N24 was isolated and sequenced, and this cDNA contained a coding region encoding 216 amino acid residues. Protein and nucleotide sequencing demonstrated the existence of a remarkable homology between N24 and glutathione S-transferase class pi enzymes (Kudo *et al*. 1999). Northern analysis showed that N24 mRNA with a length of 950 bases was expressed in lacustrine sockeye salmon olfactory epithelium. The functional roles of N24 during salmon imprinting and homing migration should be clarified in future studies.

Hino *et al*. (2007) investigated that salmon olfactory imprinting-related gene (SOIG) from the olfactory system of lacustrine sockeye salmon has been identified by subtractive hybridization technique of cDNA-representational difference analysis using fish at smoltification as a tester and fish at the feeding migration term as a driver. SOIG mRNA was shown to be expressed in olfactory receptor cells and basal cells of the olfactory epithelium by *in situ* hybridization. The expression levels of SOIG mRNA in the olfactory epi-

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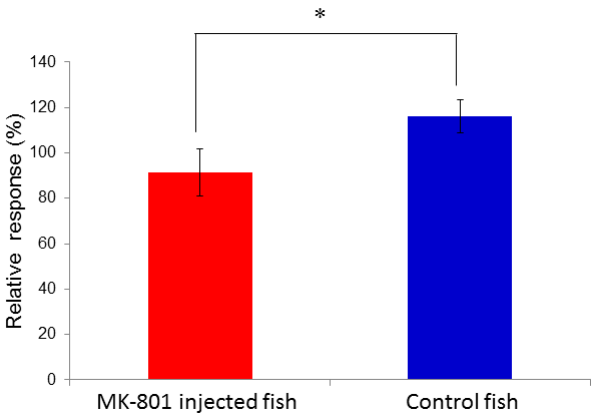

Fig. 22. EOG response to the test water (1 μM Pro and Glu) of MK-801 injected (0.1 μg/g BW 4 times every other day for 2 weeks in 1-year-old fish) and control lacustrine sockeye salmon (3-year-old). Data are presented as the means ± SEM (N = 3). Significant differences are indicated at $^*p < 0.05$ level.

thelium have been analyzed during smoltification and homing of sockeye salmon and chum salmon, respectively. During smoltification, SOIG mRNA levels increased before and during smoltification, and decreased after smoltification in sockeye salmon. These changes coincided with serum thyroxine changes during smoltification (Yamamoto *et al*. 2010). During homing migration, SOIG mRNA levels in the olfactory epithelium of chum salmon were elevated at Sites **F** and **J**. It is thought that SOIG might be related to olfaction or cell proliferation during both smoltification and the final stage of homing.

Olfactory chemoreception is accomplished via binding of the odorant substance to an olfactory receptor (OR), which is reportedly encoded by 100–200 genes (Alioto and Ngai 2005) in the olfactory epithelium with subsequent propagation of the information to the central nervous system. There are two types of OR genes, main olfactory receptors (MORs), which are expressed in ciliated olfactory receptor cells; and vomeronasal olfactory receptors (VORs), which are expressed in microvillous olfactory receptor cells. MOR genes have also been identified in a number of salmonids (Wickens *et al*. 2001; Dukes *et al*. 2004, 2006; Morinishi *et al*. 2007). The olfactory receptor gene expression was investigated in different life stages of Atlantic salmon, demonstrating that seven V2R-like (*OlfC*) genes were expressed at higher levels in juveniles (parr and smolts) than in adults (Johnstone *et al*. 2011, 2012). Although many MORs and VORs have been identified from several vertebrates owing to the progress of whole genome analysis, many ligands remain uncharacterized. Further intensive molecular biological studies must be performed to clarify the olfactory chemoreception during imprinting and homing migration in salmon.

Bandoh *et al*. (2011) have applied blood oxygenation level-dependent functional magnetic resonance imaging (fMRI) to investigate the odor information processing of natal stream in the brain of lacustrine sockeye salmon, and found that strong responses to odors of natal stream were mainly observed in the lateral area of dorsal TE, which is homologous to the medial pallium (hippocampus) in terrestrial vertebrates. Olfactory memory plays a key role in imprinting and retrieval natal stream odor information in salmon.

While measurement of NR1 transcription is a good first step, it is the protein that will be directly involved in olfactory memory formation. Further biochemical and molecular biological studies investigating the protein levels and the gene expression profiles of NR2A-D with treatments of NMDAR antagonist will be able to reveal olfactory LTP in salmon brain.

5. Conclusions

This review describes my career researches (for 40 years of PhD and for 25 years of faculty positions at Hokkaido University) on physiological mechanisms of imprinting and homing migration mainly using anadromous chum salmon from the Bering Sea to Japan as well as lacustrine sockeye and masu salmon in Lake Toya and Lake Shikotsu by means of three different approaches: physiological biotelemetry studies on salmon homing behavior, endocrinological studies on hormone profiles in the BPT and BPG axes, and neurophysiological studies on olfactory imprinting and discriminating abilities. Physiological biotelemetry studies show that salmon can navigate in open water using different sensory systems, but the sensory mechanisms of open water orientation remain unknown. The role of magnetic sensory systems should be further examined during oceanic imprinting and homing migration in salmon. Endocrinological studies suggest that TRH and sGnRH play leading roles in imprinting and homing migration, respectively. However, it is uncertain how the BPT and BPG hormones may control both the sensory systems and the central nervous systems during imprinting and homing migration, and the brain regional different roles of TRH and sGnRH on the sensory systems and the central nervous systems should be examined in future studies. Neurophysiological studies on olfactory function demonstrate that stable DFAA compositions in natal streams are crucial for olfactory imprinting and homing of Pacific salmon, and NMDAR is a useful molecular marker to clarify olfactory memory formation and retrieval. But the olfactory mechanisms of straying are still unsolved, and the relationship between salmon evolution and homing accuracy must be understood in order to explain straying mechanisms. Despite of the difficulties in a

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temporally limited spawning season, studies from molecular biology and behavioral biology using these model salmon species will provide new explanations for the precise imprinting and homing phenomenon among Pacific salmon.

Alphabetized list of abbreviated words

ACTH: adrenocorticotropin
Ala: L-alanine
ANW: artificial natal stream water
Arg: L-arginine
Asp: L-aspartic acid
aASW: artificial stream water at the time of adult homing in autumn
ASW: artificial stream water
BPG: brain-pituitary-gonad
BPT: brain-pituitary-thyroid
CB: cerebellum
cDNA: complementary deoxyribonucleic acid
cGnRH-II: chicken gonadotropin-releasing hormone-II
CRH: corticotropin-releasing hormone
DC: diencephalon
DFAA: dissolved free amino acid
DHP: 17α,20β-dihydroxy-4-pregnen-3-one
E2: estradiol-17β
EOG: electro-olfactogram
fMRI: functional magnetic resonance imaging
FSH: follicle-stimulating hormone
GH: growth hormone
GHRH: growth hormone-releasing hormone
Glu: L-glutamic acid
Gly: L-glycine
GnRH: gonadotropin-releasing hormone
GnRHa: gonadotropin-releasing hormone analog
GTH: gonadotropin
His: L-histidine
IGF-I: insulin-like growth factor I
Ile: L-isoleucine
11KT: 11-ketotostosterone
jASW: artificial stream water at the time of juvenile imprinting in spring
Leu: L-leucine
LH: luteinizing hormone
LPT: long-term potentiation
Lys: L-lysine
MO: medulla oblongata
MOR: main olfactory receptor
mRNA: messenger ribonucleic acid
N24: olfactory system-specific protein of 24 kilo Dalton
NLW: natural lake water
NMDAR: *N*-methyl-D-aspartate receptor
NR1: essential subunit of NMDAR
OB: olfactory bulb
ON: olfactory nerve
OP: optic tectum
OR: olfactory receptor
PEA: β-phenylethyl alcohol
POA: preoptic area
Phe: L-phenylalanine
Pro: L-proline
PST: parr-smolt transformation
Ser: L-seine
sGnRH: salmon gonadotropin-releasing hormone
SOIG: salmon olfactory imprinting-related gene
SST: sea surface temperature
T: testosterone
T3: triiodothyronine
T4: thyroxine
TE: telencephalon
Thr: L-threonine
TN: terminal nerve
TRH: thyrotropin-releasing hormone
TSH: thyrotropin
Tyr: L-tyrosine
Val: L-valine
VOR: vomeronasal olfactory receptor

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