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**Physiological mechanism of homing migration in Pacific salmon from behavioral
to molecular biological approaches**

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ABSTRACT

The amazing abilities of Pacific salmon to migrate long distances from the ocean to their natal streams for spawning have been investigated intensively since 1950's, but there are still many mysteries because of difficulties to follow their whole life cycle and to wait their sole reproductive timing for several years. In my laboratory, we have tried to clarify physiological mechanisms of homing migration in Pacific salmon, using four anadromous Pacific salmon (pink, *Oncorhynchus gorbuscha*; chum, *O. keta*; sockeye, *O. nerka*; masu, *O. masou*) in the north Pacific Ocean as well as two lacustrine salmon (sockeye and masu) in Lake Toya and Lake Shikotsu, Hokkaido, Japan, where the lakes serve as a model "ocean". Three different approaches from behavioral to molecular biological researches have been conducted using these model fish. First, the homing behaviors of adult chum salmon from the Bering Sea to Hokkaido as well as lacustrine sockeye and masu salmon in Lake Toya were examined by means of physiological biotelemetry techniques, and revealed that salmon can navigate in open water using different sensory systems. Second, the hormone profiles in the brain-pituitary-gonadal (BPG) axis were investigated in chum salmon and lacustrine sockeye salmon during their homing migration by means of hormone specific time-resolved fluoroimmunoassay (TR-FIA) systems, and clarified that salmon gonadotropin-releasing hormone (sGnRH)

plays leading roles on homing migration. Third, the olfactory functions of salmon were studied by means of electrophysiological, behavioral, and molecular biological techniques, and made clear that olfactory discriminating ability of natal stream odors. These results have discussed with the evolutionary aspects of four Pacific salmon, sexual differences in homing profiles, and the possibility of dissolved free amino acids (DFAA) as natal stream odors for salmon.

Key words: Homing; Imprinting; Olfaction; Vision; Biotelemetry; Neuroendocrinology; Electrophysiology; Molecular biology; Evolution; Pacific salmon; Lacustrine salmon

1. Introduction

Pacific salmon (genus *Oncorhynchus*) show dramatic and complex life cycles characterized by 4 different migrations: downstream migration, feeding migration, spawning migration and upstream migration. There are large differences in the timing of downstream migration in juveniles and the upstream migration in adults of four Pacific salmon in Japan. In pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*), all juveniles carry out downstream migration within a few months after emergence, and adults do upstream migration within a few weeks of final gonadal maturation. In contrast, in sockeye salmon (*O. nerka*) and masu salmon (*O. masou*), juveniles stay in fresh water for 16-18 months to grow into smolts that have the ability to tolerate sea water do downstream migration, some river or lake residents also exist (lacustrine sockeye and masu salmon), and adults salmon do their upstream migration 4-5 months prior to final gonadal maturation (Groot and Margolis, 1991). However, in either case, Pacific salmon have an amazing ability to migrate thousands kilometers from the open ocean to their natal stream for reproduction after several years of oceanic feeding migration. It is now widely accepted that some specific factors of the natal stream are imprinted to particular nervous systems of juvenile salmon during downstream migration, and that adult salmon evoke these factors to recognize the natal stream

during spawning and upstream migrations. The downstream migration must be the critical period for imprinting, and spawning and upstream migrations must be closely related to homing (Fig. 1).

Since the olfactory hypothesis for salmon homing was proposed by Wisby and Hasler (1954) in coho salmon (*O. kisutch*), the olfactory imprinting and homing mechanism has been studied in many behavioral, electrophysiological, biochemical, and neurobiological studies (see reviews; Cooper and Hirsch, 1982; Hasler and Scholz, 1983; Døving, 1989; Stabell, 1992; Ueda and Yamauchi, 1995; Satou et al., 1996; Bertmar, 1997; Nevitt and Dittman, 1998; Quinn, 2005; Ueda et al., 2007; Hino et al., 2009). The olfactory discriminating ability is believed to exert within a short distance from the coast of the natal stream, and it might be impossible for salmon to use only this ability for a long distance migration from the feeding area to the natal area. For open water orientation, the contributions of a map and compass system have been discussed (Quinn and Groot, 1984; Quinn et al., 1989; Hansen et al., 1993; Ogura and Ishida, 1995; Dittman and Quinn, 1996; Ueda et al., 2000). However, it is still unclear how the olfactory system discriminates various stream odors or which sensory systems play leading roles in open water orientation.

In this review, I focus on the physiological mechanisms of homing migration based

on our three different approaches using both anadromous and lacustrine salmon. First, the homing behaviors of adult chum salmon from the Bering Sea to Hokkaido as well as lacustrine sockeye and masu salmon in Lake Toya were examined by means of physiological biotelemetry techniques. Second, the hormone profiles in the brain-pituitary-gonadal (BPG) axis were investigated in chum salmon and lacustrine sockeye salmon during their homing migration by means of hormone specific time-resolved fluoroimmunoassay (TR-FIA) systems. Third, the olfactory functions of salmon were studied by means of electrophysiological, behavioral, and molecular biological techniques. These results have discussed with the evolutionary aspects of four Pacific salmon, sexual differences in homing profiles, and olfactory imprinting and discriminating abilities of natal stream odors.

2. Physiological biotelemetry of salmon homing behavior

Recent rapid advances in biotelemetry technologies make it possible to study underwater fish movement in great detail (Cooke et al., 2004; Ueda, 2004). In particular, ultrasonic transmitters that emit pulsed signals have been used to investigate the migratory behavior of salmon in the coastal sea (Quinn et al., 1989) and the central Bering Sea (Ogura and Ishida, 1994). Moreover, ultrasonic tracking in combination with

sensory ablation experiments, which blocked visual and olfactory cues or magnetic senses, have been performed several times with oceanic migratory salmonids (Døving et al., 1985; Yano and Nakamura, 1992; Yano et al., 1996).

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

Chum salmon caught by longline in June, 2000 in the central Bering Sea (56°30'N, 179°00'E) in a healthy condition were judged to be a Japanese origin by scale analysis. Since most of Japanese chum salmon were released from hatchery, the width of their scale ring during fry stage was wider than wild salmon from other countries. A propeller data logger, which recorded swimming speed (5 sec sampling), depth (5 sec sampling), and temperature (1 min sampling), was attached externally in the dorsal musculature of the fish anterior to the dorsal fin (Tanaka et al., 2005). We released 27 chum salmon with data loggers, and retrieved one data logger on September, 2000 from a set net on the east coast of Hokkaido, Japan (43°20'N, 145°46'E). The first record of swimming profiles of homing chum salmon in the oceanic phase for 67 days in the straight distance of 2,760 km revealed that average swimming speed, depth, and temperature were 62 ± 12 cm/sec, 10.4 ± 14.7 m, and 9.2 ± 0.2 °C, respectively (Fig. 2). Both swimming speed and depth had two peaks around the dawn and sunset with a small peak around the

midnight. The fish showed sequential up-and-down movements near the thermocline during the daytime and unfluctuating constant movements within one water column (at 10 °C around 10 m depth) during the nighttime. Since these diurnal patterns may be caused by the prey distributions that high-caloric fish during the day and gelatinous zooplankton during the night (Davis et al, 2000), it can be speculated that the homing chum salmon allocated its time to foraging and the foraging strategy differed between the daytime and nighttime. These results indicate that the homing chum salmon had navigation abilities in its homeward direction and that current transport may have assisted the successful migration. During the accurate homing migration in open water, salmon must recognize exact locations (map) and compass direction (orientation), and must have a biological clock (time).

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

It is difficult to carry out sensory manipulated experiments in sea-run anadromous populations because fish move from the sea in their pre-maturation phase to their natal stream where they become mature. In contrast, lacustrine salmon populations offer a good model system for studying homing behaviors from open water to the natal area for reproduction. Lake Toya (surface area 71 km², average and maximum depth 116 m and

179 m, respectively) is a large caldera lake in Hokkaido, Japan. The homing migrations of mature lacustrine sockeye salmon, whose sensory cues were impaired, were tracked from the center of the lake to the natal area using the ultrasonic tracking system (Ueda et al., 1998). Both a mature male sockeye salmon with attached control brass ring (Fig. 3A-1) and a mature male whose magnetic cues were interfered with magnetic ring (Fig. 3A-2) returned straight to the natal area after 1 h of random movement. A mature male sockeye salmon whose visual and magnetic cues were both blocked moved in a direction opposite to the natal area, and was rediscovered in the natal area on the following evening, suggesting the possibility of involvement of olfactory cues in finding the natal area (Fig. 3A-3). A blinded male was also moved to the shore of Naka-Toya far from the natal area (Fig. 3A-4).

The homing migrations of mature lacustrine masu salmon were also tracked in Lake Toya (Ueda et al. 2000). A mature control male masu salmon moved constantly along the coast, and stopped his movement at the mouth of river (Fig. 3B-1). A blinded mature female masu salmon was released and moved randomly away from the coast (Fig. 3B-2). A mature male masu salmon whose olfactory cue was blocked moved randomly along the coast, and then tended to move away from the coast (Fig. 3B-3).

The ultrasonic location transmitters were combined with sensory ablation to

153 evaluate homing capability, particularly orientation ability, of sockeye and masu salmon.

154 It is quite interesting to compare the straight movements of sockeye salmon with the

155 coastal movement behaviors of masu salmon. These two species show large differences

156 in ocean distribution. Sockeye salmon distribute widely in the North Pacific Ocean,

157 while masu salmon are narrowly distributed in the west North Pacific Ocean

158 (Kaeriyama and Ueda, 1998). These data suggest some ecological aspects of successful

159 homing migration of salmon where the narrowly distributed masu salmon only need

160 coastal recognition ability, but widely distributed sockeye salmon must obtain open

161 water cues for orientation. In the lake model, visual cues were critical to the straight

162 homing of sockeye salmon, while magnetic cues did not appear to be necessary for

163 straight return to the natal area. However, magneto-receptor cells have been identified in

164 the nose of rainbow trout (*O. mykiss*) (Walker et al., 1997). Further study should be

165 done to investigate the involvement of magnetic cues in salmon homing migration.

166

167 **3. Neuroendocrinological controlling mechanisms of salmon homing migration**

168 The salmon homing migration is closely related to gonadal maturation, which is

169 regulated mainly by the brain-pituitary-gonadal (BPG) axis. Two molecular types of

170 gonadotropin-releasing hormone (GnRH), salmon GnRH (sGnRH) and chicken II

GnRH (cII GnRH) exist in various brain regions (Amano et al. 1997). In particular, sGnRH in the olfactory system, the terminal nerve, and the preoptic area are considered to play important roles in salmon homing migration (Ueda and Yamauchi, 1995). Then, sGnRH in the preoptic area controls gonadotropin (GTH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and release from the pituitary gland. And then, GTHs induce steroidogenesis in the gonads, and steroid hormones stimulate gametogenesis and final gameto-maturation; estradiol-17 β (E₂) and testosterone (T) are active in vitellogenesis, T and 11-ketotestosterone (11KT) in spermatogenesis, and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) in final gameto-maturation in both sexes (Nagahama, 1999). It has been investigated hormone profiles in the BPG axis of salmon during homing migration as well as gonadal maturation (Ueda, 1999; Urano et al., 1999; Makino et al., 2007).

3-1. Hormone profiles of chum salmon during homing migration

The hormone profiles in the BPG axis of chum salmon migrating from the Bering Sea to the spawning ground in the Chitose River, Hokkaido, Japan, in 2001 were measured using specific time-resolved fluoroimmunoassay (TR-FIA) systems developed by Yamada et al. (2002) and Kitani (2006). The level of sGnRH in the

olfactory bulb (OB) of both sexes showed a peak during upstream migration from the coastal sea to the river mouth of the Ishikari River where the olfactory discriminating ability of the natal stream should be functioning, and also in the telencephalon (TC) where it increased at the branch point of the Chitose River from the Ishikari River where the olfactory functions should also be highly activated (Fig. 4A). In the pituitary gland, sGnRH levels tended to increase at the same time as elevation in LH levels from the coastal sea in females to the river mouth of the Ishikari River in males (Fig. 4B). In contrast, FSH levels did not show any clear correlations with sGnRH levels in the pituitary gland. Although the roles of cIIGnRH in these brain regions remains to be elucidated, the levels of cIIGnRH in the medulla oblongata (MO) increased in both sexes at the pre-spawning ground while that in the optic tectum (OT) also increased in males. In the diencephalon (DC) and cerebellum (CB), cIIGnRH levels showed no significant changes during homing migration (Fig. 4C).

Serum steroid hormone levels showed similar profiles as previous observations (Ueda et al., 1984; Ueda, 1999); E₂ in females and 11KT in males increased during vitellogenesis and spermatogenesis, respectively, and DHP increased dramatically at the time of final gonadal maturation in both sexes (Fig. 5). It is quite interesting to note that both sGnRH levels in the TC and serum T levels in both sexes showed a coincident

peak at the branch point of the Chitose River from the Ishikari River. These results confirm that sGnRH plays a role in GTH secretion in the pituitary of chum salmon, and sGnRH and cIIGnRH might be involved in brain region-dependent roles on gonadal maturation and homing migration in salmon. Moreover, 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 (Onuma et al., 2003). Although there were year-to-year differences in plasma levels of steroid hormones and gonadal maturity and some to them may be influenced by year-to-year variation of SST, the fundamental steroid hormone profiles of chum salmon during homing migration showed little differences.

3-2. Homing profiles and hormonal manipulation in lacustrine sockeye salmon

Since it is difficult to carry out experimental treatments to manipulate endocrinological functions in sea-run anadromous salmon because that the open sea is too large to allow the manipulation and tracking of salmon, lacustrine salmon populations also offer a good model system for studying hormonal controlling mechanisms of salmon homing. In Lake Shikotsu (surface area 78 km², average and maximum depth 265 m and 363 m, respectively), adult sockeye salmon were captured

225 from September to November adjacent to their natal hatchery prior to spawning. They
226 were sampled for serum steroid hormones, tagged, and released in the center of the lake.
227 Fish were sampled again at recapture to characterize changes in steroid hormone levels
228 in individual migrants as well as homing duration and percentage in each month (Sato et
229 al., 1997). Homing duration was significantly shortened from September to October in
230 males and from October to November in females (Fig. 6A). All males returned faster
231 than females early in September and October, although half of the males did not return
232 to the natal site in November. In contrast, 78-90% of females returned over the entire
233 three month sampling period. It is interesting to note that the average homing
234 percentage of both sexes for three months is 83%, indicating no differences in the total
235 number of homing individuals between male and female. Male salmon maintain high
236 levels of aggressive behavior to compete for access to females suggesting that early
237 returning males might accrue some benefits in securing females for breeding. The
238 drastic reduction of male homing percentage late in the season may be interpreted in
239 two ways; 1) some males prefer to go to other unsampled breeding sites to find females,
240 2) some males are prevented from returning to the natal sites by their early death. The
241 occurrence of relatively few non-homing females throughout the sampling period may
242 be related to the following two population-level hypotheses; 1) the conservative

protection of these individuals' strain from the disruption of being captured at their natal spawning site, 2) the enhancement of their strain arising from a wild spawning distribution within the lake. The sexual differences in homing behavior are thought to be reflected by the different steroid hormone profiles between males and females (Sato et al., 1997). In males, the shortening of homing duration coincided with an increase in serum T and 11KT levels. The reduction of homing percentage was associated with decreased serum T levels and increased serum DHP levels. In females, the shortening of homing duration corresponded to an elevation of serum T and DHP levels, and a drop in serum E₂ levels.

Since GnRH treatment has been reported to be highly effective in inducing GTH release, ovulation and spermiation in teleost fishes (Zohar, 1996), we investigated the effect of GnRH analog (GnRHa) implantation on both homing profiles and serum steroid hormone levels of fish in September (Sato et al., 1997; Kitahashi et al., 1998). 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 individual males: rapidly returning males showed higher serum T levels and lower serum DHP levels than slowly returning individual males. To examine the direct action of T and DHP on

homing duration, T and DHP were implanted in fish in September in comparison with GnRHa-implantation (Fig. 6B). GnRHa-implanted fish returned significantly earlier than the control fish regardless of sex. T implantation tended to reduce homing duration in both males and females, but there was no statistical significance. DHP implantation also significantly shortened homing duration in females, but it did not have any significant effect in males. It is quite interesting to note that the direct actions of T and DHP on homing migration are sex dependent. These data suggest GnRH in the brain stimulates LH release from the pituitary gland, and then LH enhances serum DHP levels in both sexes during the later part of the homing migration in salmonid fishes. GnRH is convinced to play a leading role in the homing migration of both sexes, but gonadal steroids, especially T and DHP, seem to have sexually different influences on homing migration. Further study using our model systems may reveal sexual differences in hormonal control of the homing migration in salmonid fishes with special reference to the early part of the homing migration.

4. Olfactory imprinting and discriminating abilities of salmon

Two different olfactory hypotheses have been proposed for salmon imprinting and homing. One is the imprinting hypothesis developed by Wisby and Hasler (1954) using

coho salmon. The other is the pheromone hypothesis developed by Nordeng (1971, 1977) using Arctic char *Salvelinus alpinus* and Atlantic salmon *Salmo salar*. The pheromone hypothesis assumes that juvenile salmon in a stream release population-specific odours that guide homing adults. However, there are no juveniles of chum salmon or pink salmon present at the time that the adults return. It is now widely accepted that some specific odorant factors in the natal stream are imprinted on the olfactory system of juvenile salmon during downstream migration, and that adult salmon evoke these factors to recognize their natal stream during homing migration (Dittman and Quinn, 1996; Quinn, 2005; Ueda et al., 2007; Hino et al., 2009). Harden Jones (1968) and Brannon (1982) proposed that juvenile Pacific salmon learn a series of olfactory waypoints during their migration through freshwater, and subsequently adult salmon retrace this odour sequence during homing migration.

4-1. Electrophysiological studies on olfactory discriminating ability

Since the olfactory transduction mechanism began to be examined by electrophysiological techniques, many electrophysiological studies have been carried out on the olfactory discriminating ability of salmon. The early studies reported that application of natal stream water to the olfactory epithelium of homing salmon induced

297 a large olfactory bulbar response (Hara et al., 1965; Ueda et al., 1967; Hara, 1970).
298 Later, it was shown that not only the natal stream water, but also waters from other
299 streams induced olfactory bulbar responses in salmon (Ohshima et al., 1969; Dizon et
300 al., 1973; Bodznick, 1975). Behavioral and electrophysiological studies using coho
301 salmon reported that imprinting with a synthetic odor such as phenylethyl alcohol
302 (PEA) was possible (Nevitt et al., 1994; Dittman et al., 1996).

303 We examined the olfactory discriminatory ability of lacustrine sockeye and masu
304 salmon, which were reared in the culture pond of Toya Lake Station, by recording the
305 integrated olfactory nerve response according to the technique of Sveinsson and Hara
306 (1990). The olfactory organs of both species elicited different response properties to
307 various freshwaters, regardless of sex or gonadal maturity (Sato et al., 2000). The
308 source and effluent water from the culture pond evoked the minimum and maximum
309 response magnitudes, respectively. These odors may modify the source water in such a
310 way as to make the culture pond water more detectable to the olfactory system. In
311 cross-adaptation experiments, the river waters abolished the secondary response to the
312 lake water, but the lake water did not abolish the secondary response to the river waters.
313 This phenomenon is quite reasonable because the salmon migrate from the lake to the
314 river. The minimum concentration (threshold) to induce the olfactory nerve response to

the culture pond water after adaptation to the lake water was between 0.1 and 1.0%.

This threshold level suggests that the olfactory discriminatory ability of salmonids during homing migration must function within a limited distance from the natal river.

Several studies have 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 salmonids may be acting as sexual pheromones (Moore and Scott, 1992; Moore and Warning, 1996). The mucus of fish body surface also released amino acids (Hara et al., 1984). Recently, L-kynurenine, an amino acid was identified as a sex pheromone in the urine of ovulated female masu salmon (Yambe et al., 2006).

4-2. Properties of natal stream odors

Several attempts to identify the natal stream odor were made based on the olfactory bulbar response, and suggested that the natal river 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 number of chemicals species dissolved in water. Chemicals that elicit the response from the olfactory organs of salmon are amino acids, steroids, bile acids, and prostaglandins (Hara, 1994). We analyzed the compositions of dissolved free amino acids (DFAA), inorganic cations and bile acids in waters from three streams which flow into Lake Toya (Shoji et al., 2000). 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 responses. 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 (Fig. 7). The response pattern for each combination of artificial stream water closely resembled that to the corresponding combination of natural stream water. According to these results, we concluded that amino acids dissolved in the natal stream water are likely natal stream odors.

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 inside and outside of 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. We investigated the origin of DFAA in stream water focusing on biofilms in the river bed by means of incubation experiments in the laboratory. Stones were placed in the Toyohira River, Hokkaido, Japan, 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 a high-performance liquid chromatography (HPLC). The DFAA concentration increased greatly in the biofilm incubation solution of the treatment group, but the DFAA composition (mole %) did not change relative to the inception of incubation, where it was similar to stream water. These results suggest that biofilms are a major source of DFAA in stream water (Ishizawa, 2008).

4-3. Behavioral studies on olfactory discriminating ability

Behavior experiments were compared to test attractive effects on upstream selective movement among four Pacific salmon (pink, chum, sockeye, and masu salmon) using artificial natal stream water (ANW) prepared by the same composition and concentration of DFAA in their natural natal stream in two-choice test tank (Y-maze) consisted of two water inlet arms and one pool. Either ANW or natural lake water (NLW) was added to the water inlet of left or right arms. The fish movement was monitored and the number of fish moved to each arm was counted. The two test pairs of natural and artificial waters were used: (1) both NLW, and (2) ANW and NLW. In pair (1), all species showed no selectivity for either arm. In pair (2), percentage of upstream movement of 4 Pacific salmon was 77.1, 63.4, 64.4, and 53.3 in pink, chum, sockeye, and masu salmon, respectively. In contrast, percentage of upstream selective movement in the arm running ANW was 59.3, 85.7, 75.9, and 81.3 in pink, chum, sockeye, and masu salmon, respectively (Fig. 8). These results indicated that ANW had different attractive effects on upstream selective movement among four Pacific salmon. Pink salmon showed the highest upstream movement and the lowest selectivity to the artificial natal stream water. It is interesting to note that the evolutionary relationship between the olfactory discriminating ability and the homing accuracy among four Pacific salmon. In a phylogenetic division of four Pacific salmon in Japan using

retropositional genome analyses, pink salmon is considered to be the most advanced species (Murata et al., 1993, 1996). An analysis of the relationship between the oceanic distribution and the population size of the four species revealed that pink salmon distributed the most widely and are most abundant (Kaeriyama and Ueda, 1998). If salmon conduct a highly accurate homing migration to their natal stream, there would be little chance to enhance their distribution area which would in turn affect population size and genetic diversity. Thus pink salmon may have evolved the ability to select natal streams with lower fidelity than other Pacific salmon, and therefore, pink salmon are the most widely and abundantly distributed among Pacific salmon. The relationship between Pacific salmon evolution and their homing accuracy should be investigated more in detail from a view point of evolution.

Further behavioral experiments of chum salmon captured in the Osaru River (OR), Hokkaido, were also conducted in Y-maze using various combinations of control water (NLW) and three artificial stream waters prepared by using the same composition and concentration of DFAA found in natural stream waters: 1. artificial OR water (AOR); 2. AOR without L-glutamic acid, the major amino acid in OR water (AOR-E); 3. Artificial water matching another stream (ALS) that had much higher amino acid concentrations than OR (Yamamoto and Ueda, 2009). In behavioral tests, the fish did not discriminate

between AOR and AOR-E, but displayed significant selection of AOR or AOR-E over NLW and AOR over ALS (Fig. 9). Electrophysiological cross-adaptation experiments indicated that mature male chum salmon have the olfactory capability to distinguish between AOR and AOR-E. These results suggest that migratory male chum salmon respond to DFAA mixtures in their natal stream water, and appear not to be affected by single amino acids.

4-4. Biochemical and molecular biological studies on olfactory functions

The other attempts to investigate olfactory homing mechanisms in salmon include biochemical and molecular biological analysis of the olfactory system. 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 species migrating between sea and river, such as Japanese eel (*Anguilla japonica*), but not in non-migratory species, such as 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).

Immunocytochemical and immunoelectronmicroscopic observations revealed that N24 positive immunoreactivity occurred in ciliated and microvillus olfactory receptor cells and the glomerular layer near the mitral cells in the olfactory bulb (Kudo *et al.*, 1996a; Yanagi *et al.*, 2004). Protein and nucleotide sequencing demonstrated the existence of a remarkable homology between N24 and glutathione S-transferase (GST; EC 2.5.1.18) class pi enzymes (Kudo *et al.*, 1999). Recently, salmon olfactory marker protein (OMP) has also been characterized in the olfactory epithelium of lacustrine sockeye salmon by molecular biological and histochemical techniques (Kudo *et al.*, 2009). N24 and OMP are useful molecular markers for studying olfactory functions during salmon homing migration.

Involvement of sGnRH in olfactory functions of masu and chum salmon was also examined by means of immunocytochemical technique with an antiserum to sGnRH and *in situ* hybridization techniques with an oligonucleotide encoding sGnRH precursor. Immunocytochemical analysis demonstrated that a sGnRH immunoreactive bipolar neuron, which might be related to the terminal nerve, was located in the dorsal portion of the olfactory nerve of both species. Immunoelectron microscopy revealed the presence of sGnRH immunoreactive electron-dense granule-like structure of 50 nm in the olfactory nerve of masu salmon (Kudo *et al.*, 1994). sGnRH immunoreactive

neurons, which also showed signals for pro-sGnRH mRNA, were observed in the dorsal portion of the olfactory nerve in chum salmon at the coastal sea, but not in fish at the spawning ground of the natal river (Kudo et al., 1996b). These findings suggest that sGnRH may participate in neurotransmission and/or neuromodulation in the olfactory system of salmonids.

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 (cDNA-RDA) using fish at the parr-smolt transformation (PST) as a tester and fish at the feeding migration term as a driver (Hino et al., 2007). SOIG mRNA was shown to be expressed in olfactory receptor cells and basal cells of the olfactory epithelium. The expression levels of SOIG mRNA in the olfactory epithelium have been analyzed during several lifecycle stages of lacustrine sockeye salmon and chum salmon, such as ontogeny, PST, and homing (Hino, 2007). During ontogeny, the expression levels of SOIG mRNA are significantly higher in alevin (juvenile fry) than in embryos at 43 and 60 days after fertilization, and then they surge at the PST in lacustrine sockeye salmon. On the other hand, SOIG mRNA levels in the olfactory epithelium of chum salmon during homing migration are elevated at the estuary and pre-spawning ground. It is thought that SOIG

might be related to olfaction or cell proliferation during both the PST and the final stage of homing.

The olfactory chemoreception is accomplished through binding of the odorant substance to an olfactory receptor (OR) in the olfactory epithelium with subsequent propagation of the information to the central nervous system. There are two types of OR genes namely, main olfactory receptors (MORs), which are expressed in ciliated olfactory receptor cells (cORCs); and vomeronasal olfactory receptors (VORs), which are expressed in microvillous olfactory receptor cells (mORCs). MOR genes have also been identified in a number of salmonids (Wickens *et al.*, 2001; Dukes *et al.*, 2004, 2006; Morinishi *et al.*, 2007). 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 researches need to be clarified the olfactory chemoreception during imprinting and homing migration in salmon.

5. Conclusions

This review describes our recent studies on the physiological mechanisms of homing migration in anadromous and lacustrine Pacific salmon. Using these model fish,

three different approaches in connection with homing behavior in the open water,
hormonal control mechanisms of homing migration, and olfactory discriminating ability
of natal stream odors provide several valuable findings on salmon homing migration.
However, many unknowns still remain such as the imprinting mechanisms during
downstream migration, the triggering mechanisms of the shift from feeding migration to
spawning migration, the sensory mechanisms of open water orientation, and the
hormonal control mechanisms for sensory systems. Despite the difficulties to follow
their whole life cycle and to wait their sole reproductive timing, comparative behavioral
to molecular biological studies using anadromous and lacustrine Pacific salmon will
provide a new concept for the physiological mechanisms of imprinting and homing
migration in salmon.

Ethical statement

This study involving experiments using anadromous and lacustrine Pacific salmon
has been carried out under the control of the committee along the “Guide for the Care
and Use of Laboratory Animals of Hokkaido University” and Japanese Governmental
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524

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779

FIGURE LEGENDS

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

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.

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. Arrowhead indicates the releasing point of each fish.

Fig. 4. Changes in salmon gonadotropin-releasing hormone (sGnRH) contents in the olfactory bulb (OB) and the telencephalon (TC) (A), sGnRH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary gland (B), and chicken II GnRH (cIIIGnRH) in the optic tectum (OT), the diencephalon (DC), the cerebellum (CB), and the medulla oblongata (MO) (C) of male and female chum salmon during homing migration from the Bering Sea to the spawning ground.

Fig. 5. Changes in serum steroid hormone levels of male and female chum salmon during homing migration from the Bering Sea to the spawning ground. DHP, $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; E2, Estradiol- 17β , 11KT,

798 11-ketotestosterone; T, testosterone.

799 Fig. 6. Changes in homing duration and percentage of lacustrine sockeye salmon in
800 Lake Shikotsu from September to November (A), and effects of GnRH analog
801 (GnRH α : 75 μ g/fish), testosterone(T: 200 μ g/fish) and
802 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP: 200 μ g/fish) implantation on
803 homing duration of lacustrine sockeye salmon in Lake Shikotsu in September..
804 Significant differences at 5% (*) and 1% (**) levels are indicated.

805 Fig. 7. Typical integrated olfactory nerve response in lacustrine masu salmon in the
806 cross-acclimation experiments to natural and artificial stream water: the Poromoi
807 river water (Poromoi), the Sobetsu river water (Sobetsu), and the Toya Lake
808 Station water (Station). DW: distilled water.

809 Fig. 8. Upstream movement (A) and selectivity (B) in each artificial natal stream water
810 of four mature male Pacific salmon in the two-choice test tank. Numbers in
811 parenthesis indicate the number of fish. Significant differences at 5% (*) levels
812 are indicated.

813 Fig. 9. Upstream movement (A) and selectivity (B) in each artificial stream water of
814 mature male chum salmon in the two-choice test tank. Numbers in parenthesis
815 indicate the number of fish. Significant differences at 5% (*) levels are

Fig. 1

Fig.2

1, control fish; 2, magnetic cue-interfered fish; 3, visual and magnetic cues-interfered fish; 4, visual cue-interfered fish.

1, control fish; 2, visual cue-interfered fish; 3, olfactory cue-interfered fish.

Fig. 3

Fig. 4.

Fig. 5

Fig. 6

Natural stream water

Artificial stream water

Fig. 7

Fig. 8

Fig. 9