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 evolutional 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

63 during spawning and upstream migrations. The downstream migration must be the
64 critical period for imprinting, and spawning and upstream migrations must be closely
65 related to homing (Fig. 1).
66 Since the olfactory hypothesis for salmon homing was proposed by Wisby and
67 Hasler (1954) in coho salmon (*O. kisutch*), the olfactory imprinting and homing
68 mechanism has been studied in many behavioral, electrophysiological, biochemical, and
69 neurobiological studies (see reviews; Cooper and Hirsch, 1982; Hasler and Scholz,
70 1983; Døving, 1989; Stabell, 1992; Ueda and Yamauchi, 1995; Satou et al., 1996;
71 Bertmar, 1997; Nevitt and Dittman, 1998; Quinn; 2005; Ueda et al., 2007; Hino et al.,
72 2009). The olfactory discriminating ability is believed to exert within a short distance
73 from the coast of the natal stream, and it might be impossible for salmon to use only this
74 ability for a long distance migration from the feeding area to the natal area. For open
75 water orientation, the contributions of a map and compass system have been discussed
76 (Quinn and Groot, 1984; Quinn et al., 1989; Hansen et al., 1993; Ogura and Ishida,
77 1995; Dittman and Quinn, 1996; Ueda et al., 2000). However, it is still either unclear
78 how the olfactory system discriminates various stream odors or which sensory systems
79 play leading roles in open water orientation.
80 In this review, I focus on the physiological mechanisms of homing migration based

81 on our three different approaches using both anadromous and lacustrine salmon. First,
82 the homing behaviors of adult chum salmon from the Bering Sea to Hokkaido as well as
83 lacustrine sockeye and masu salmon in Lake Toya were examined by means of
84 physiological biotelemetry techniques. Second, the hormone profiles in the
85 brain-pituitary-gonadal (BPG) axis were investigated in chum salmon and lacustrine
86 sockeye salmon during their homing migration by means of hormone specific
87 time-resolved fluoroimmunoassay (TR-FIA) systems. Third, the olfactory functions of
88 salmon were studied by means of electrophysiological, behavioral, and molecular
89 biological techniques. These results have discussed with the evolutional aspects of four
90 Pacific salmon, sexual differences in homing profiles, and olfactory imprinting and
91 discriminating abilities of natal stream odors.
92
93 **2. Physiological biotelemetry of salmon homing behavior**
94 Recent rapid advances in biotelemetry technologies make it possible to study
95 underwater fish movement in great detail (Cooke et al., 2004; Ueda, 2004). In particular,
96 ultrasonic transmitters that emit pulsed signals have been used to investigate the
97 migratory behavior of salmon in the coastal sea (Quinn et al., 1989) and the central
98 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°46E). 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 $\pm$ 12 cm/sec, 10.4 $\pm$ 14.7 m, and 9.2 $\pm$ 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

117 midnight. The fish showed sequential up-and-down movements near the thermocline
118 during the daytime and unfluctuating constant movements within one water column (at
119 10 ˚C around 10 m depth) during the nighttime. Since these diurnal patterns may be
120 caused by the prey distributions that high-caloric fish during the day and gelatinous
121 zooplankton during the night (Davis et al, 2000), it can be speculated that the homing
122 chum salmon allocated its time to foraging and the foraging strategy differed between
123 the daytime and nighttime. These results indicate that the homing chum salmon had
124 navigation abilities in its homeward direction and that current transport may have
125 assisted the successful migration. During the accurate homing migration in open water,
126 salmon must recognize exact locations (map) and compass direction (orientation), and
127 must have a biological clock (time).
128
129 *2-2. Lacustrine sockeye and masu salmon in Lake Toya, Hokkaido, Japan*
130 It is difficult to carry out sensory manipulated experiments in sea-run anadromous
131 populations because fish move from the sea in their pre-maturation phase to their natal
132 stream where they become mature. In contrast, lacustrine salmon populations offer a
133 good model system for studying homing behaviors from open water to the natal area for
134 reproduction. Lake Toya (surface area 71 km^2, 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 was 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

171 GnRH (cIIGnRH) exist in various brain regions (Amano et al. 1997). In particular,
172 sGnRH in the olfactory system, the terminal nerve, and the preoptic area are considered
173 to play important roles in salmon homing migration (Ueda and Yamauchi, 1995). Then,
174 sGnRH in the preoptic area controls gonadotropin (GTH), luteinizing hormone (LH)
175 and follicle-stimulating hormone (FSH) synthesis and release from the pituitary gland.
176 And then, GTHs induce steroidogenesis in the gonads, and steroid hormones stimulate
177 gametogenesis and final gameto-maturation; estradiol-17β (E_2) and testosterone (T) are
178 active in vitellogenesis, T and 11-ketotestosterone (11KT) in spermatogenesis, and
179 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in final gameto-maturation in both sexes
180 (Nagahama, 1999). It has been investigated hormone profiles in the BPG axis of salmon
181 during homing migration as well as gonadal maturation (Ueda, 1999; Urano et al., 1999;
182 Makino et al., 2007).
183
184 *3-1. Hormone profiles of chum salmon during homing migration*
185 The hormone profiles in the BPG axis of chum salmon migrating from the Bering
186 Sea to the spawning ground in the Chitose River, Hokkaido, Japan, in 2001 were
187 measured using specific time-resolved fluoroimmunoassay (TR-FIA) systems
188 developed by Yamada et al. (2002) and Kitani (2006). The level of sGnRH in the

189 olfactory bulb (OB) of both sexes showed a peak during upstream migration from the
190 coastal sea to the river mouth of the Ishikari River where the olfactory discriminating
191 ability of the natal stream should be functioning, and also in the telencephalon (TC)
192 where it increased at the branch point of the Chitose River from the Ishikari River
193 where the olfactory functions should also be highly activated (Fig. 4A). In the
194 pituitary gland, sGnRH levels tended to increase at the same time as elevation in LH
195 levels from the coastal sea in females to the river mouth of the Ishikari River in males
196 (Fig. 4B). In contrast, FSH levels did not show any clear correlations with sGnRH
197 levels in the pituitary gland. Although the roles of cIIGnRH in these brain regions
198 remains to be elucidated, the levels of cIIGnRH in the medulla oblongata (MO)
199 increased in both sexes at the pre-spawning ground while that in the optic tectum (OT)
200 also increased in males. In the diencephalon (DC) and cerebellum (CB), cIIGnRH levels
201 showed no significant changes during homing migration (Fig. 4C).
202 Serum steroid hormone levels showed similar profiles as previous observations
203 (Ueda et al., 1984; Ueda, 1999); E_2 in females and 11KT in males increased during
204 vitellogenesis and spermatogenesis, respectively, and DHP increased dramatically at the
205 time of final gonadal maturation in both sexes (Fig. 5). It is quite interesting to note that
206 both sGnRH levels in the TC and serum T levels in both sexes showed a coincident

207 peak at the branch point of the Chitose River from the Ishikari River. These results
208 confirm that sGnRH plays a role in GTH secretion in the pituitary of chum salmon, and
209 sGnRH and cIIGnRH might be involved in brain region-dependent roles on gonadal
210 maturation and homing migration in salmon. Moreover, year-to-year differences in
211 plasma levels of steroid hormones in pre-spawning chum salmon were also studied in
212 comparison with sea surface temperature (SST) of coastal sea (Onuma et al., 2003).
213 Although there were year-to-year differences in plasma levels of steroid hormones and
214 gonadal maturity and some to them may be influenced by year-to-year variation of SST,
215 the fundamental steroid hormone profiles of chum salmon during homing migration
216 showed little differences.
217
218 *3-2. Homing profiles and hormonal manipulation in lacustrine sockeye salmon*
219 Since it is difficult to carry out experimental treatments to manipulate
220 endocrinological functions in sea-run anadromous salmon because that the open sea is
221 too large to allow the manipulation and tracking of salmon, lacustrine salmon
222 populations also offer a good model system for studying hormonal controlling
223 mechanisms of salmon homing. In Lake Shikotsu (surface area 78 km^2, average and
224 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

243 protection of these individuals' strain from the disruption of being captured at their natal
244 spawning site, 2) the enhancement of their strain arising from a wild spawning
245 distribution within the lake. The sexual differences in homing behavior are thought to be
246 reflected by the different steroid hormone profiles between males and females (Sato et
247 al., 1997). In males, the shortening of homing duration coincided with an increase in
248 serum T and 11KT levels. The reduction of homing percentage was associated with
249 decreased serum T levels and increased serum DHP levels. In females, the shortening of
250 homing duration corresponded to an elevation of serum T and DHP levels, and a drop in
251 serum E_2 levels.

252 Since GnRH treatment has been reported to be highly effective in inducing GTH
253 release, ovulation and spermiation in teleost fishes (Zohar, 1996), we investigated the
254 effect of GnRH analog (GnRHa) implantation on both homing profiles and serum
255 steroid hormone levels of fish in September (Sato et al., 1997; Kitahashi et al., 1998).
256 The GnRHa implantation was highly efficient in shortening the homing duration, and
257 caused dramatic increases in serum DHP levels in both sexes. An interesting
258 discrepancy was observed between rapidly and slowly returning individual males:
259 rapidly returning males showed higher serum T levels and lower serum DHP levels than
260 slowly returning individual males. To examine the direct action of T and DHP on

261 homing duration, T and DHP were implanted in fish in September in comparison with
262 GnRHa-implantation (Fig. 6B). GnRHa-implanted fish returned significantly earlier
263 than the control fish regardless of sex. T implantation tended to reduce homing duration
264 in both males and females, but there was no statistical significance. DHP implantation
265 also significantly shortened homing duration in females, but it did not have any
266 significant effect in males. It is quite interesting to note that the direct actions of T and
267 DHP on homing migration are sex dependent. These data suggest GnRH in the brain
268 stimulates LH release from the pituitary gland, and then LH enhances serum DHP levels
269 in both sexes during the later part of the homing migration in salmonid fishes. GnRH is
270 convinced to play a leading role in the homing migration of both sexes, but gonadal
271 steroids, especially T and DHP, seem to have sexually different influences on homing
272 migration. Further study using our model systems may reveal sexual differences in
273 hormonal control of the homing migration in salmonid fishes with special reference to
274 the early part of the homing migration.
275
276 **4. Olfactory imprinting and discriminating abilities of salmon**
277 Two different olfactory hypotheses have been proposed for salmon imprinting and
278 homing. One is the imprinting hypothesis developed by Wisby and Hasler (1954) using

279 coho salmon. The other is the pheromone hypothesis developed by Nordeng (1971,
280 1977) using Arctic char *Salvelinus alpines* and Atlantic salmon *Salmo salar*. The
281 pheromone hypothesis assumes that juvenile salmon in a stream release
282 population-specific odours that guide homing adults. However, there are no juveniles of
283 chum salmon or pink salmon present at the time that the adults return. It is now widely
284 accepted that some specific odorant factors in the natal stream are imprinted on the
285 olfactory system of juvenile salmon during downstream migration, and that adult
286 salmon evoke these factors to recognize their natal stream during homing migration
287 (Dittman and Quinn, 1996; Quinn, 2005; Ueda et al., 2007; Hino et al., 2009). Harden
288 Jones (1968) and Brannon (1982) proposed that juvenile Pacific salmon learn a series of
289 olfactory waypoints during their migration through freshwater, and subsequently adult
290 salmon retrace this odour sequence during homing migration.
291
292 *4-1. Electrophysiological studies on olfactory discriminating ability*
293 Since the olfactory transduction mechanism began to be examined by
294 electrophysiological techniques, many electrophysiological studies have been carried
295 out on the olfactory discriminating ability of salmon. The early studies reported that
296 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

315 the culture pond water after adaptation to the lake water was between 0.1 and 1.0%.
316 This threshold level suggests that the olfactory discriminatory ability of salmonids
317 during homing migration must function within a limited distance from the natal river.
318 Several studies have suggested that juvenile salmonids produce population-specific
319 odors or pheromones, (Groot et al., 1986; Quinn and Tolson, 1986; Courtenay et al.,
320 1997). It has also been demonstrated that sex steroids and prostaglandins that have
321 effects on the olfactory epithelium of salmonids may be acting as sexual pheromones
322 (Moore and Scott, 1992; Moore and Warning, 1996). The mucus of fish body surface
323 also released amino acids (Hara et al., 1984). Recently, L-kynurenine, an amino acid
324 was identified as a sex pheromone in the urine of ovulated female masu salmon (Yambe
325 et al., 2006).
326
327 *4-2. Properties of natal stream odors*
328 Several attempts to identity the natal stream odor were made based on the olfactory
329 bulbar response, and suggested that the natal river odors were non-volatile (Fagerlund
330 et al., 1963; Cooper et al.., 1974; Bodznick, 1978). Spectral analysis of the olfactory
331 bulbar response suggested that the natal stream odor was absorbed on activated carbon
332 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

351 factors affecting the DFAA compositions both inside and outside of the stream
352 environment, such as soils, vegetation, litter, pollen, dew, and various microbial
353 activities (Thomas 1997). Among these factors, the roles of complex microbial
354 communities called biofilms have been intensively investigated (Costerton et al. 1994;
355 Nosyk et al. 2008). A biofilm consists of various microorganisms, and is embedded into
356 a matrix of extracellular polymeric substances. We investigated the origin of DFAA in
357 stream water focusing on biofilms in the river bed by means of incubation experiments
358 in the laboratory. Stones were placed in the Toyohira River, Hokkaido, Japan, for 3
359 months, allowing formation of biofilms, and then incubated for 24hours in the
360 laboratory at stream water temperature. After incubation, the composition and
361 concentrations of DFAA in the incubation solution were measured by a
362 high-performance liquid chromatography (HPLC). The DFAA concentration increased
363 greatly in the biofilm incubation solution of the treatment group, but the DFAA
364 composition (mole %) did not change relative to the inception of incubation, where it
365 was similar to stream water. These results suggest that biofilms are a major source of
366 DFAA in stream water (Ishizawa, 2008).
367
368 *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 selectively 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

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

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

405 between AOR and AOR-E, but displayed significant selection of AOR or AOR-E over
406 NLW and AOR over ALS (Fig. 9). Electrophysiological cross-adaptation experiments
407 indicated that mature male chum salmon have the olfactory capability to distinguish
408 between AOR and AOR-E. These results suggest that migratory male chum salmon
409 respond to DFAA mixtures in their natal stream water, and appear not to be affected by
410 single amino acids.
411
412 *4-4. Biochemical and molecular biological studies on olfactory functions*
413 The other attempts to investigate olfactory homing mechanisms in salmon include
414 biochemical and molecular biological analysis of the olfactory system. An olfactory
415 system-specific protein of 24 kDa (N24) was identified in lacustrine sockeye salmon by
416 electrophoretic comparison of proteins restricted to the olfactory system with those
417 found in other parts of the brain (Shimizu et al., 1993). In various species of teleosts,
418 N24 immunoreactivity was found in the olfactory system of species migrating between
419 sea and river, such as Japanese eel (*Anguilla japonica*), but not in non-migratory
420 species, such as carp (*Cyprinus carpio*) (Ueda et al., 1994). Interestingly, N24
421 immunoreactivity was also observed in the testicular germ cells, spermatids and
422 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 a*l*., 1994). sGnRH immunoreactive

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

446 Salmon olfactory imprinting-related gene (SOIG) from the olfactory system of
447 lacustrine sockeye salmon has been identified by subtractive hybridization technique
448 of cDNA-representational difference analysis (cDNA-RDA) using fish at the
449 parr-smolt transformation (PST) as a tester and fish at the feeding migration term as a
450 driver (Hino et al., 2007). SOIG mRNA was shown to be expressed in olfactory
451 receptor cells and basal cells of the olfactory epithelium. The expression levels of
452 SOIG mRNA in the olfactory epithelium have been analyzed during several lifecycle
453 stages of lacustrine sockeye salmon and chum salmon, such as ontogeny, PST, and
454 homing (Hino, 2007). During ontogeny, the expression levels of SOIG mRNA are
455 significantly higher in alevin (juvenile fry) than in embryos at 43 and 60 days after
456 fertilization, and then they surge at the PST in lacustrine sockeye salmon. On the other
457 hand, SOIG mRNA levels in the olfactory epithelium of chum salmon during homing
458 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 Law (No.105) and Notification (No.6).

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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 (cIIGnRH) 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, 17α,20β-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 (GnRHa: 75 μg/fish), testosterone(T: 200 μg/fish) and

802 17α,20β-dihydroxy-4-pregnen-3-one (DHP: 200 μg/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

indicated.

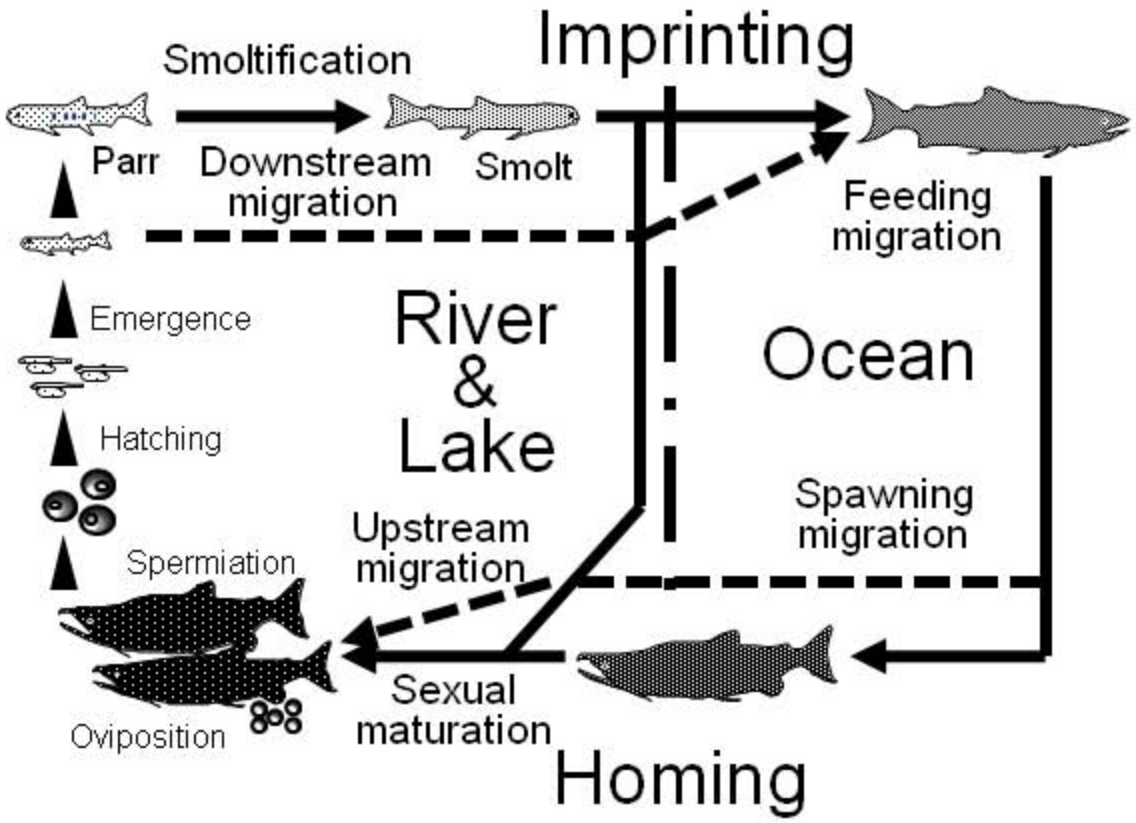

Imprinting
Smoltification
Parr
Downstream migration
Smolt
Feeding migration
Emergence
River & Lake
Ocean
Hatching
Spawning migration
Upstream migration
Spermiation
Sexual maturation
Oviposition
Homing

Fig. 1

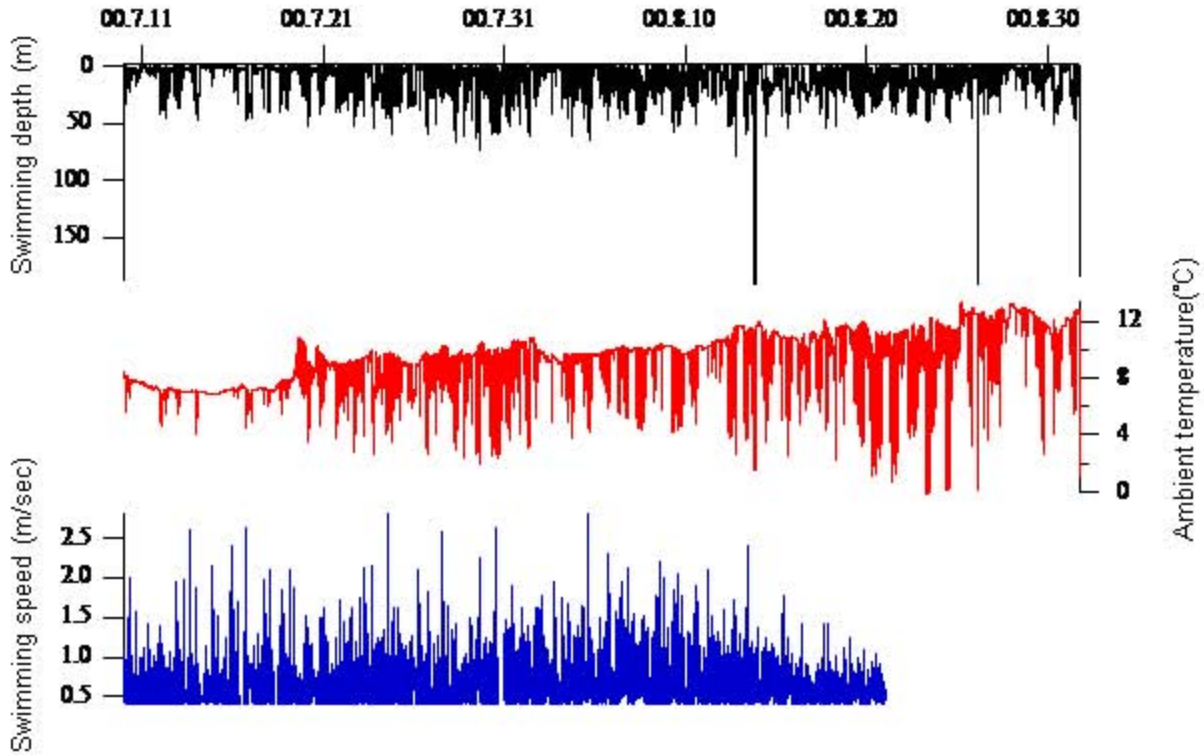
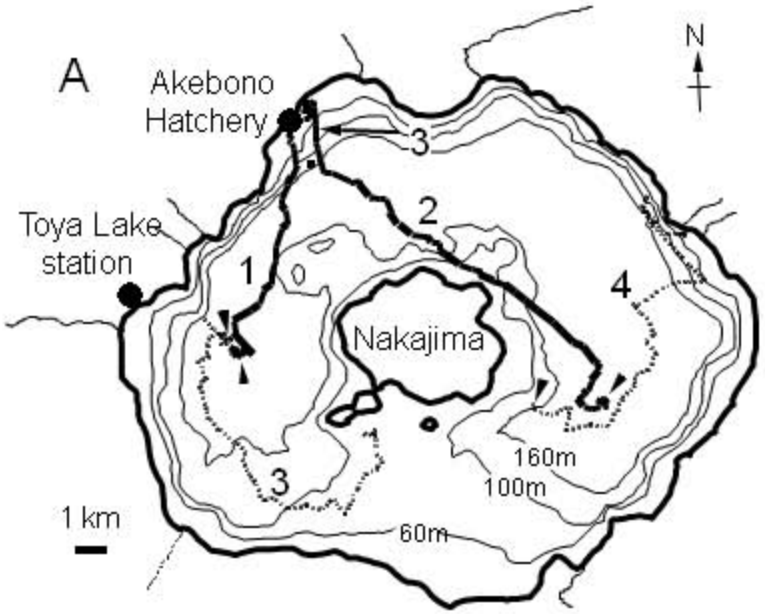
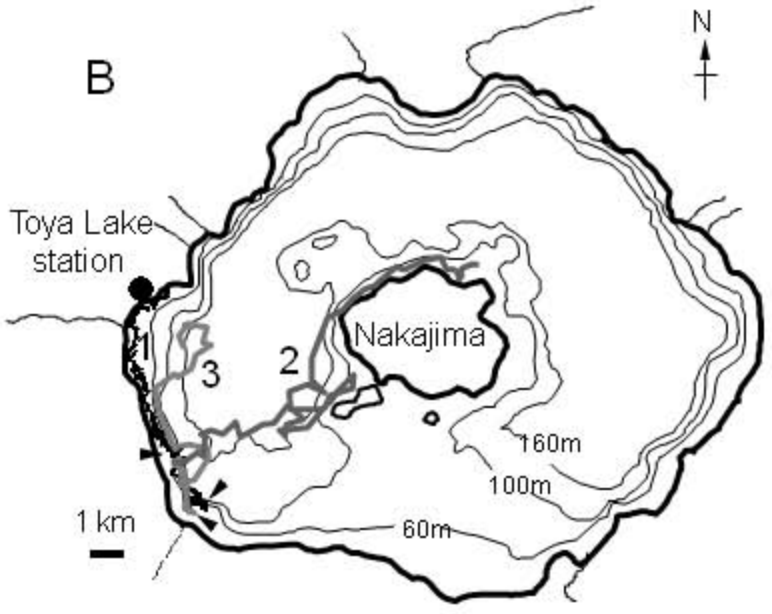

00.7.11
00.7.21
00.7.31
00.8.10
00.8.20
00.8.30
Swimming depth (m)
0
50
100
150
12
8
4
0
Ambient temperature(°C)
Swimming speed (m/sec)
2.5
2.0
1.5
1.0
0.5

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

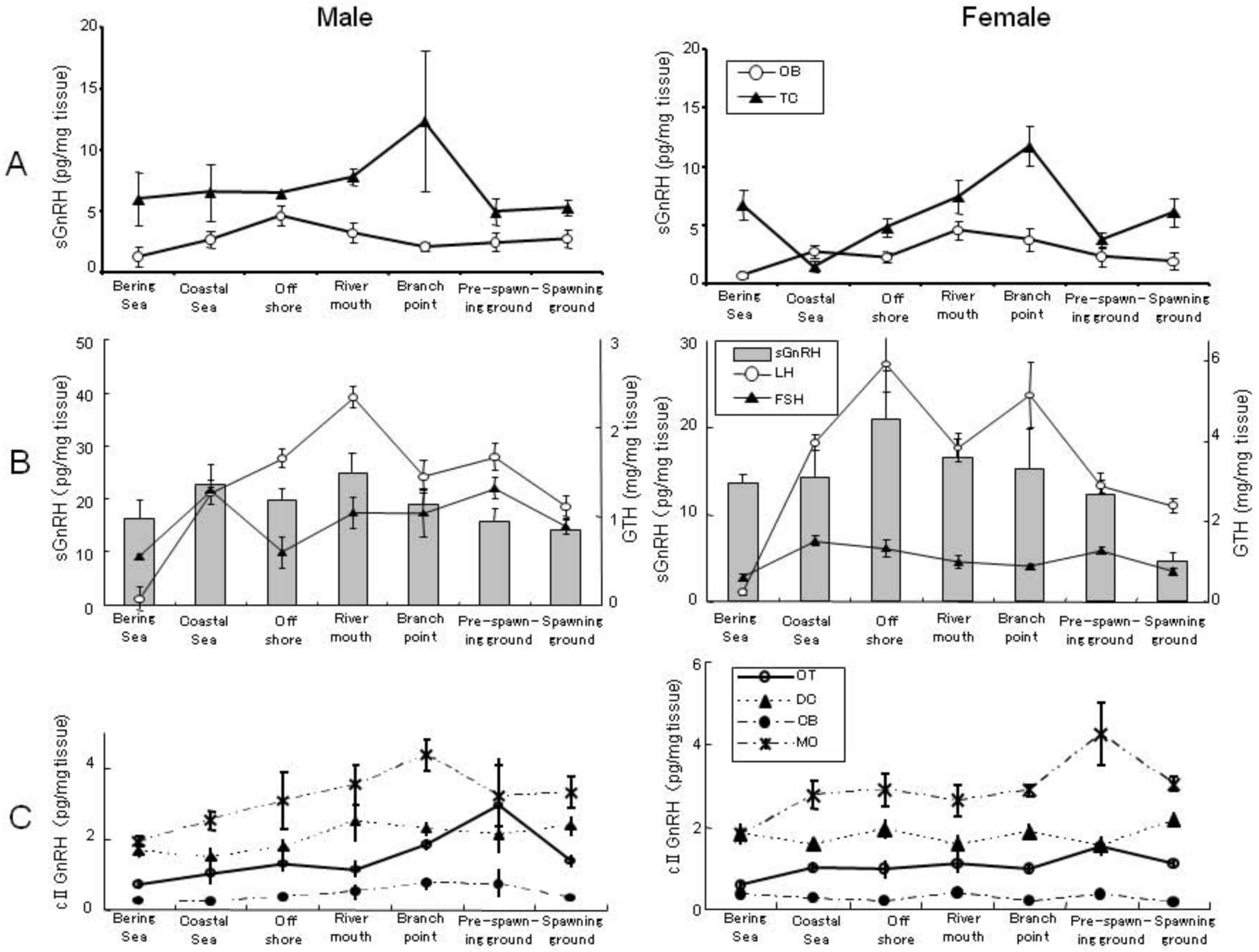

Male
Female
A
B
C
sGnRH (pg/mg tissue)
GTH (mg/mg tissue)
cII GnRH (pg/mg tissue)
OB
TC
sGnRH
LH
FSH
OT
DC
CB
MO
Bering Sea
Coastal Sea
Off shore
River mouth
Branch point
Pre-spawning ground
Spawning ground

Fig. 4.

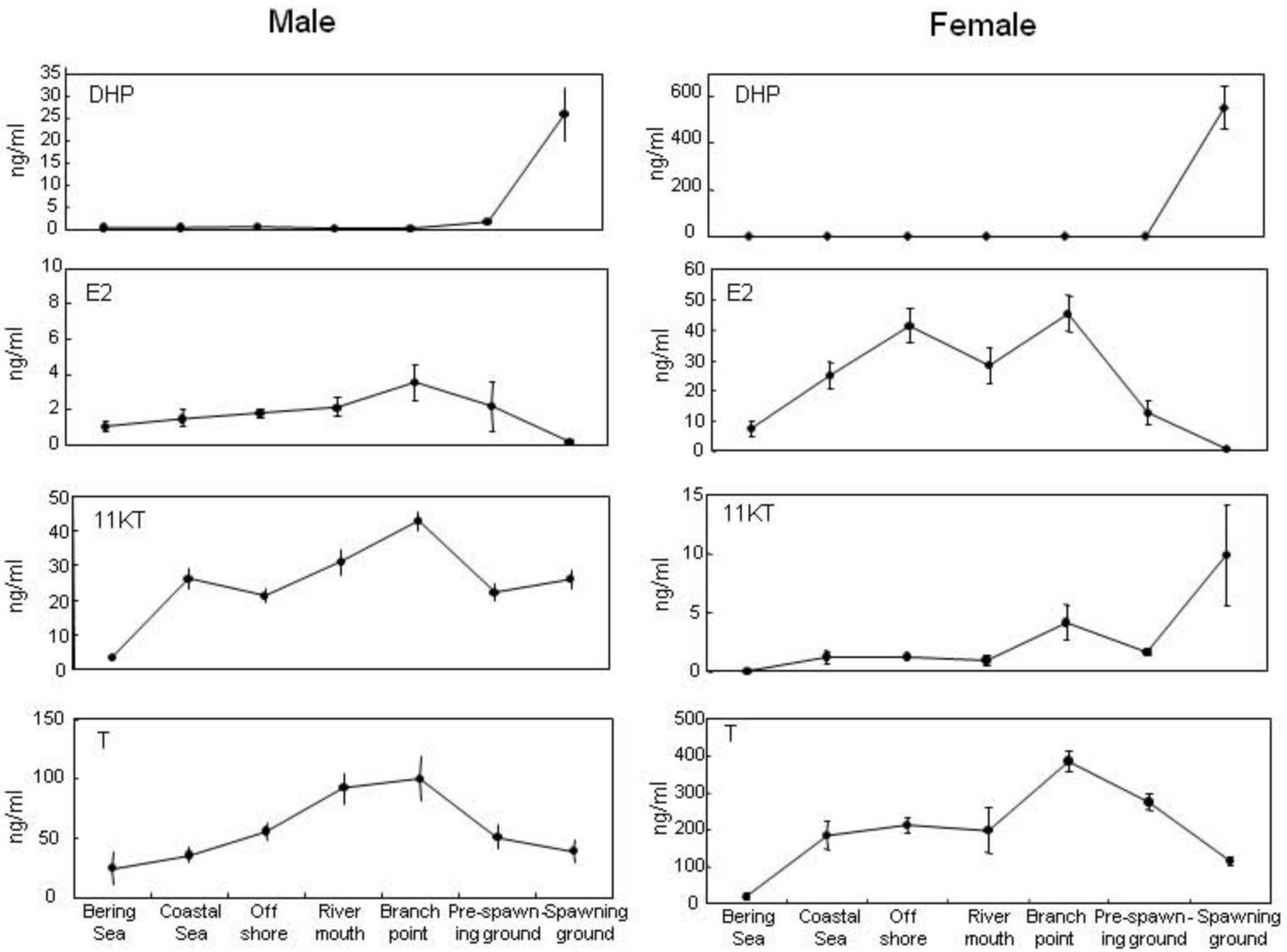

Male
Female
DHP
E2
11KT
T
ng/ml
Bering Sea
Coastal Sea
Off shore
River mouth
Branch point
Pre-spawning ground
Spawning ground

Fig. 5

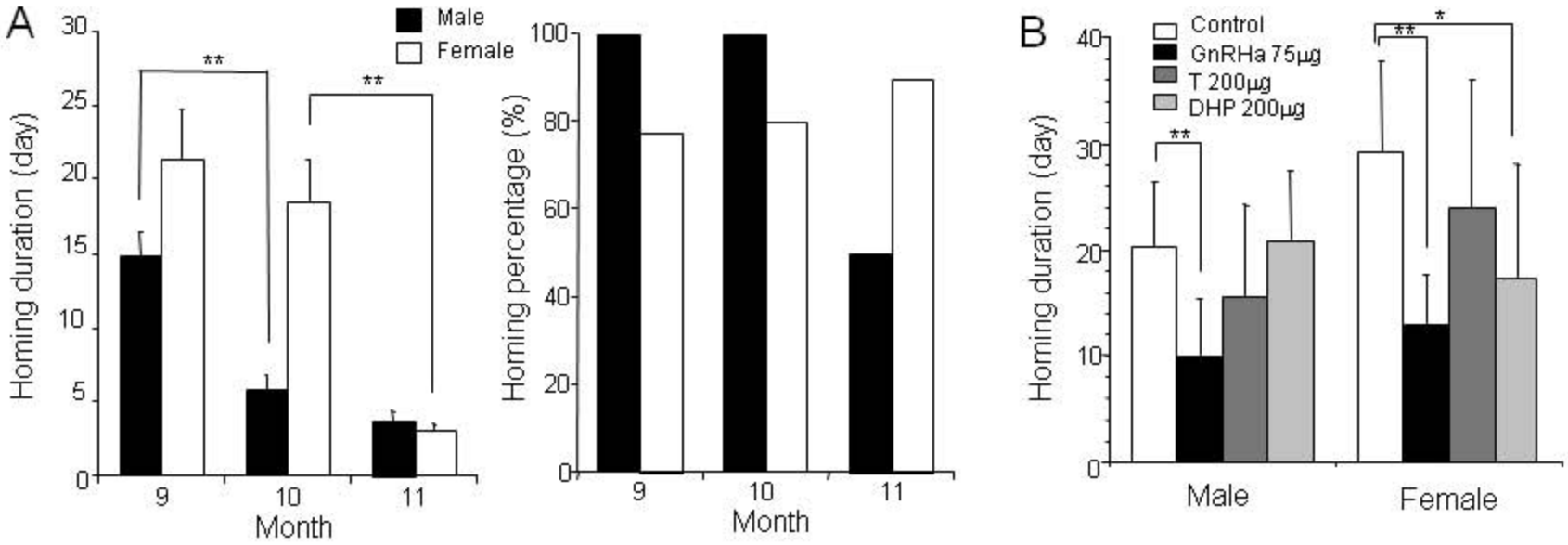

A
Male
Female
Homing duration (day)
30
25
20
15
10
5
0
**
**
9
10
11
Month
Homing percentage (%)
100
80
60
40
20
0
9
10
11
Month
B
Control
GnRHa 75μg
T 200μg
DHP 200μg
Homing duration (day)
40
30
20
10
0
**
**
*
Male
Female

Fig. 6

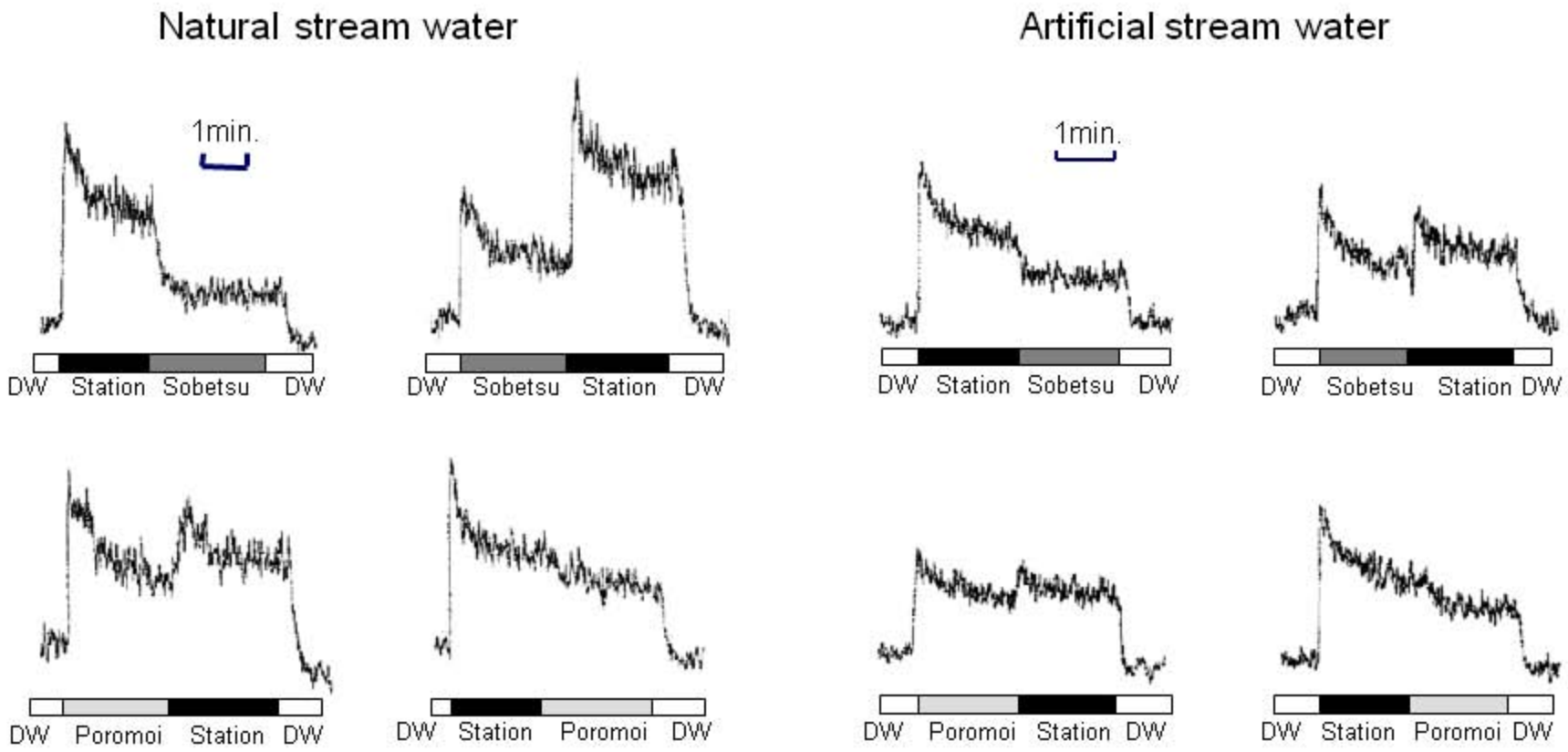

Natural stream water
Artificial stream water
1min.
1min.
DW Station Sobetsu DW
DW Sobetsu Station DW
DW Station Sobetsu DW
DW Sobetsu Station DW
DW Poromoi Station DW
DW Station Poromoi DW
DW Poromoi Station DW
DW Station Poromoi DW

Fig. 7

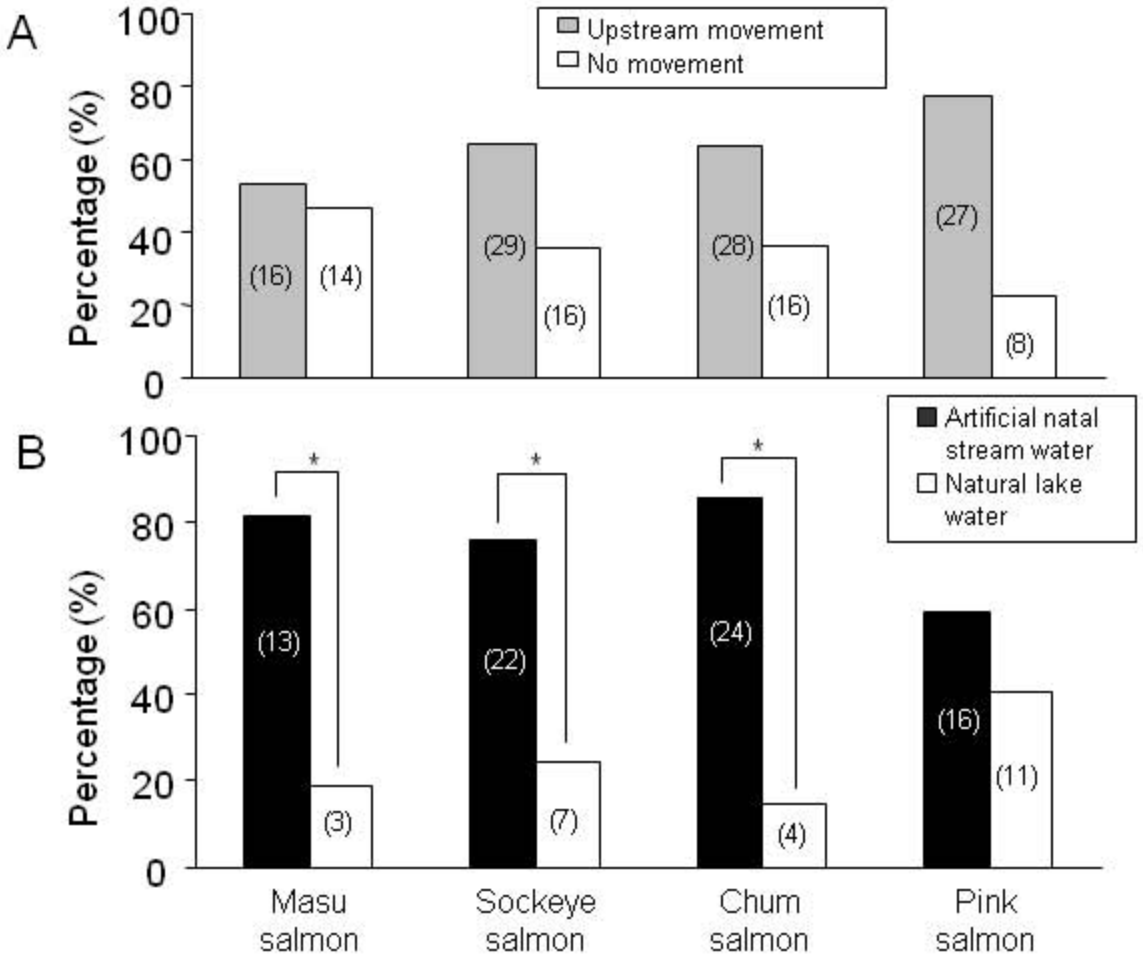

A
Upstream movement
No movement
Percentage (%)
100
80
60
40
20
0
(16)
(14)
(29)
(16)
(28)
(16)
(27)
(8)
B
Artificial natal stream water
Natural lake water
Percentage (%)
*
*
*
(13)
(3)
(22)
(7)
(24)
(4)
(16)
(11)
Masu salmon
Sockeye salmon
Chum salmon
Pink salmon

Fig. 8

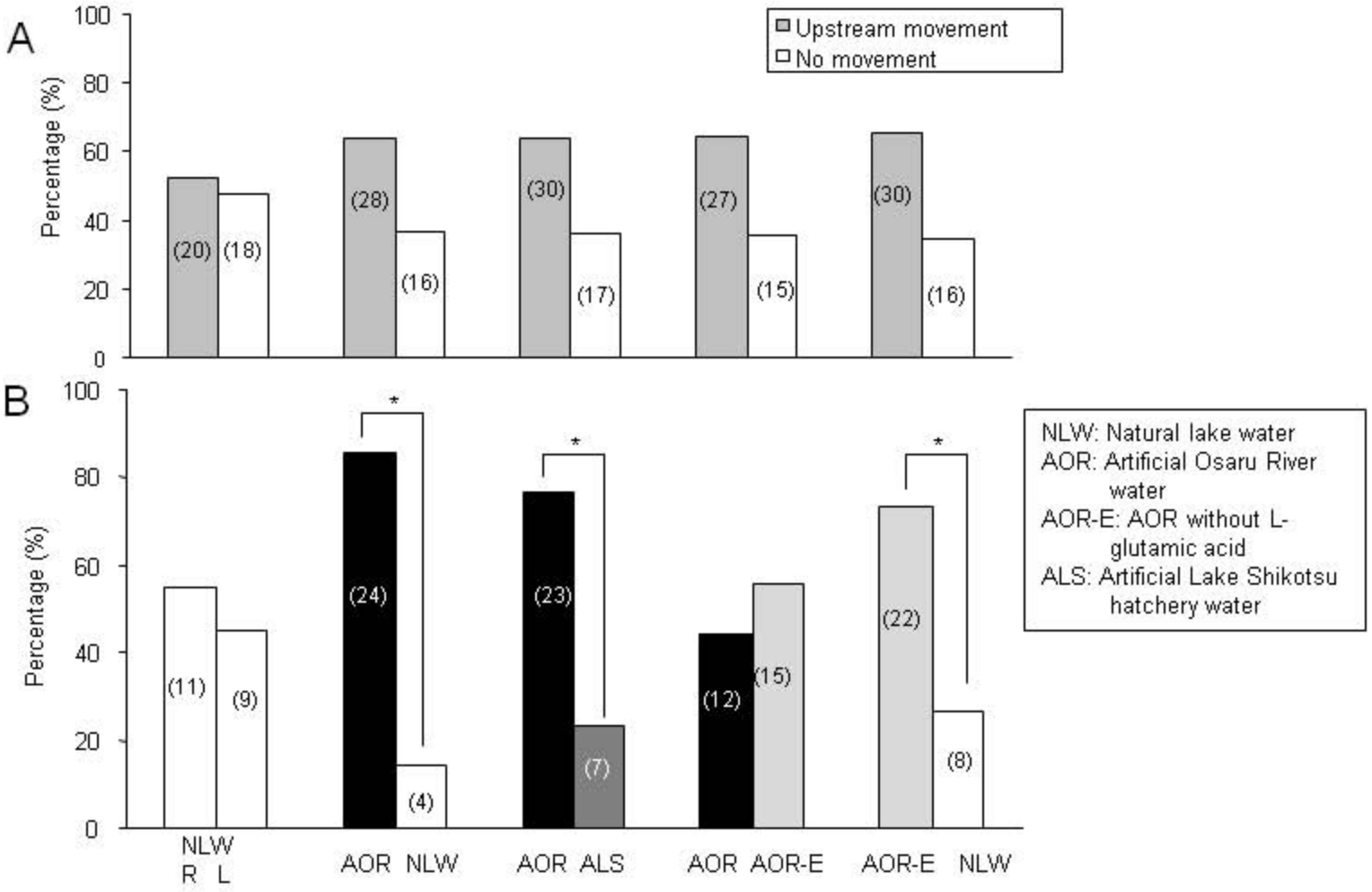

A
Upstream movement
No movement
Percentage (%)
100
80
60
40
20
0
(20)
(18)
(28)
(16)
(30)
(17)
(27)
(15)
(30)
(16)
B
Percentage (%)
100
80
60
40
20
0
(11)
(9)
(24)
(4)
(23)
(7)
(12)
(15)
(22)
(8)
*
*
*
NLW
R L
AOR NLW
AOR ALS
AOR AOR-E
AOR-E NLW
NLW: Natural lake water
AOR: Artificial Osaru River water
AOR-E: AOR without L-glutamic acid
ALS: Artificial Lake Shikotsu hatchery water

Fig. 9