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

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11    Running title: Mechanisms of salmon homing migration

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## ABSTRACT

16

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

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

40

41 Key words: Homing; Imprinting; Olfaction; Vision; Biotelemetry; Neuroendocrinology;

42 Electrophysiology; Molecular biology; Evolution; Pacific salmon; Lacustrine salmon

43

44

45 **1. Introduction**

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

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

102

### 103 *2-1. Chum salmon from the Bering Sea to Hokkaido, Japan*

104 Chum salmon caught by longline in June, 2000 in the central Bering Sea (56°30'N,  
105 179°00'E) in a healthy condition were judged to be a Japanese origin by scale analysis.  
106 Since most of Japanese chum salmon were released from hatchery, the width of their  
107 scale ring during fry stage was wider than wild salmon from other countries. A propeller  
108 data logger, which recorded swimming speed (5 sec sampling), depth (5 sec sampling),  
109 and temperature (1 min sampling), was attached externally in the dorsal musculature of  
110 the fish anterior to the dorsal fin (Tanaka et al., 2005). We released 27 chum salmon  
111 with data loggers, and retrieved one data logger on September, 2000 from a set net on  
112 the east coast of Hokkaido, Japan (43°20'N, 145°46'E). The first record of swimming  
113 profiles of homing chum salmon in the oceanic phase for 67 days in the straight distance  
114 of 2,760 km revealed that average swimming speed, depth, and temperature were  $62 \pm$   
115  $12$  cm/sec,  $10.4 \pm 14.7$  m, and  $9.2 \pm 0.2$  °C, respectively (Fig. 2). Both swimming speed  
116 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<sup>2</sup>, average and maximum depth 116 m and

135 179 m, respectively) is a large caldera lake in Hokkaido, Japan. The homing migrations  
136 of mature lacustrine sockeye salmon, whose sensory cues were impaired, were tracked  
137 from the center of the lake to the natal area using the ultrasonic tracking system (Ueda  
138 et al., 1998). Both a mature male sockeye salmon with attached control brass ring (Fig.  
139 3A-1) and a mature male whose magnetic cues was interfered with magnetic ring (Fig.  
140 3A-2) returned straight to the natal area after 1 h of random movement. A mature male  
141 sockeye salmon whose visual and magnetic cues were both blocked moved in a  
142 direction opposite to the natal area, and was rediscovered in the natal area on the  
143 following evening, suggesting the possibility of involvement of olfactory cues in  
144 finding the natal area (Fig. 3A-3). A blinded male was also moved to the shore of  
145 Naka-Toya far from the natal area (Fig. 3A-4).

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

152 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 (cIIIGnRH) 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-17 $\beta$  (E<sub>2</sub>) and testosterone (T) are  
178 active in vitellogenesis, T and 11-ketotestosterone (11KT) in spermatogenesis, and  
179 17 $\alpha$ ,20 $\beta$ -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<sub>2</sub> 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<sup>2</sup>, 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<sub>2</sub> 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 alpinus* 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 identify 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

333 heat-stable (Ueda, 1985). Unlike olfactory organs of terrestrial animals, fish olfactory  
334 organs respond only to a limited number of chemicals species dissolved in water.  
335 Chemicals that elicit the response from the olfactory organs of salmon are amino acids,  
336 steroids, bile acids, and prostaglandins (Hara, 1994). We analyzed the compositions of  
337 dissolved free amino acids (DFAA), inorganic cations and bile acids in waters from  
338 three streams which flow into Lake Toya (Shoji et al., 2000). Application of mixtures of  
339 inorganic cations or bile acids, combined based on their compositions in stream waters,  
340 to the olfactory epithelium induced only very small responses. On the other hand,  
341 application of mixtures of DFAA induced large responses. The response to artificial  
342 stream water based on the composition of DFAA and salts closely resembled the  
343 response to the corresponding natural stream water. Cross-adaptation experiments with  
344 three combinations of natural and artificial stream waters were carried out (Fig. 7). The  
345 response pattern for each combination of artificial stream water closely resembled that  
346 to the corresponding combination of natural stream water. According to these results,  
347 we concluded that amino acids dissolved in the natal stream water are likely natal  
348 stream odors.

349       Changes in the DFAA compositions in stream water are attributed mainly to  
350 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*

369 Behavior experiments were compared to test attractive effects on upstream  
370 selective movement among four Pacific salmon (pink, chum, sockeye, and masu  
371 salmon) using artificial natal stream water (ANW) prepared by the same composition  
372 and concentration of DFAA in their natural natal stream in two-choice test tank  
373 (Y-maze) consisted of two water inlet arms and one pool. Either ANW or natural lake  
374 water (NLW) was added to the water inlet of left or right arms. The fish movement was  
375 monitored and the number of fish moved to each arm was counted. The two test pairs of  
376 natural and artificial waters were used: (1) both NLW, and (2) ANW and NLW. In pair  
377 (1), all species showed no selectivity for either arm. In pair (2), percentage of upstream  
378 movement of 4 Pacific salmon was 77.1, 63.4, 64.4, and 53.3 in pink, chum, sockeye,  
379 and masu salmon, respectively. In contrast, percentage of upstream selective movement  
380 in the arm running ANW was 59.3, 85.7, 75.9, and 81.3 in pink, chum, sockeye, and  
381 masu salmon, respectively (Fig. 8). These results indicated that ANW had different  
382 attractive effects on upstream selective movement among four Pacific salmon. Pink  
383 salmon showed the highest upstream movement and the lowest selectivity to the  
384 artificial natal stream water. It is interesting to note that the evolutionary relationship  
385 between the olfactory discriminating ability and the homing accuracy among four  
386 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).

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

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

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

461 The olfactory chemoreception is accomplished through binding of the odorant  
462 substance to an olfactory receptor (OR) in the olfactory epithelium with subsequent  
463 propagation of the information to the central nervous system. There are two types of OR  
464 genes namely, main olfactory receptors (MORs), which are expressed in ciliated  
465 olfactory receptor cells (cORCs); and vomeronasal olfactory receptors (VORs), which  
466 are expressed in microvillous olfactory receptor cells (mORCs). MOR genes have also  
467 been identified in a number of salmonids (Wickens *et al.*, 2001; Dukes *et al.*, 2004,  
468 2006; Morinishi *et al.*, 2007). Although many MORs and VORs have been identified  
469 from several vertebrates owing to the progress of whole genome analysis, many ligands  
470 remain uncharacterized. Further intensive molecular biological researches need to be  
471 clarified the olfactory chemoreception during imprinting and homing migration in  
472 salmon.

473

## 474 **5. Conclusions**

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

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

488

#### 489 **Ethical statement**

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

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778

779

## FIGURE LEGENDS

780

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

783 Fig. 2. Swimming depth, ambient temperature, and swimming speed of a chum salmon  
784 from the Bering Sea to Hokkaido, Japan recorded by a propeller data logger.

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

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

795 Fig. 5. Changes in serum steroid hormone levels of male and female chum salmon  
796 during homing migration from the Bering Sea to the spawning ground. DHP,  
797  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; E2, Estradiol- $17\beta$ , 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<sub>a</sub>: 75 µg/fish), testosterone(T: 200 µg/fish) and  
802 17 $\alpha$ ,20 $\beta$ -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

816

indicated.



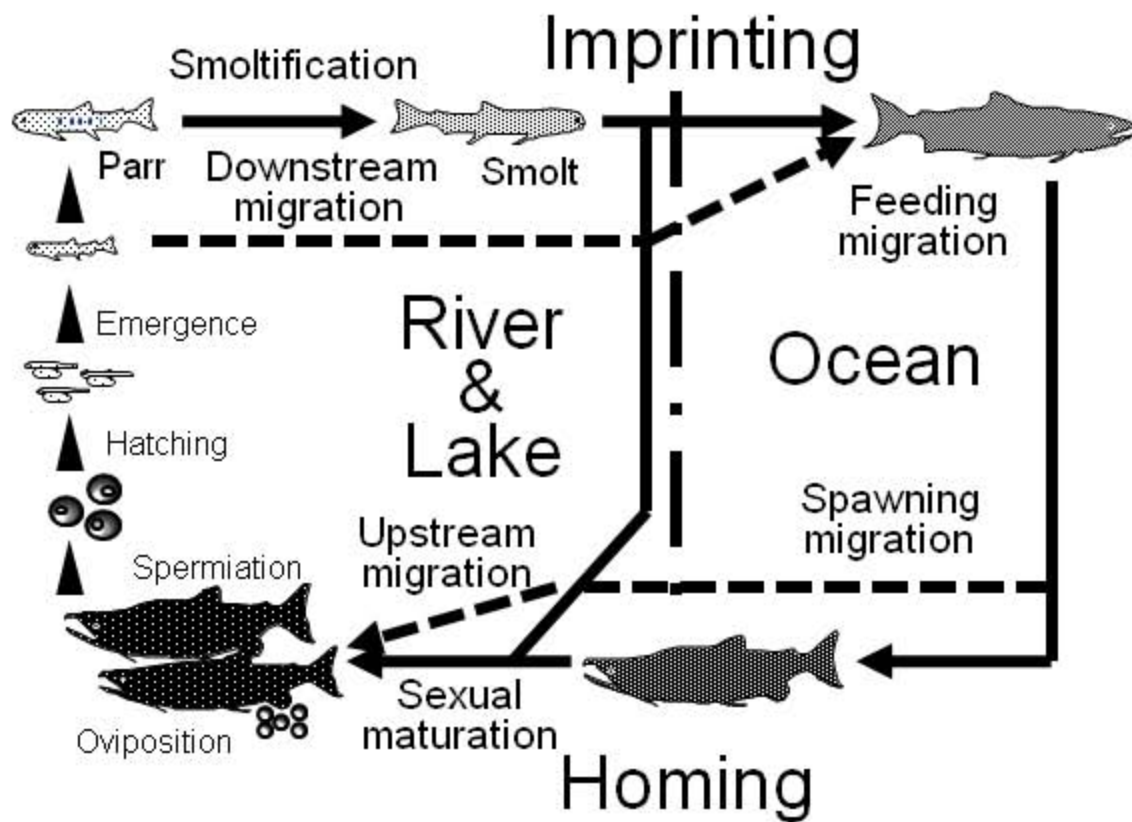


Fig. 1

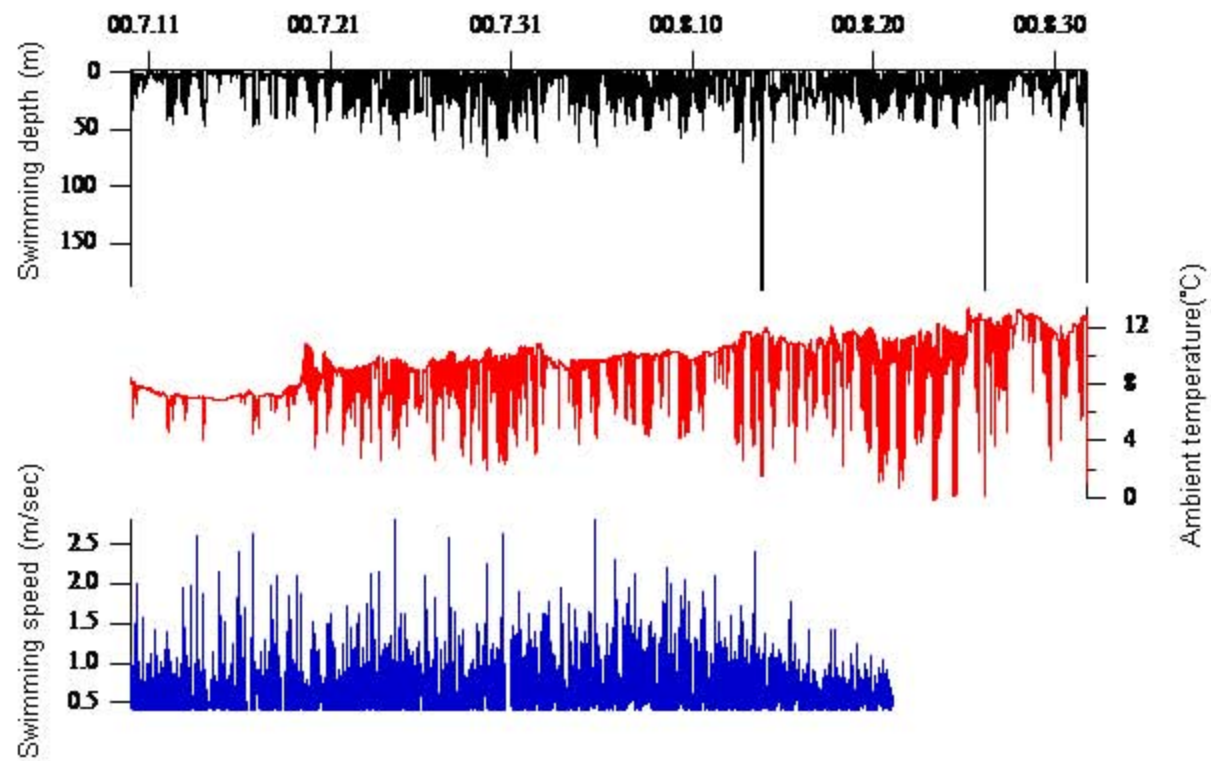
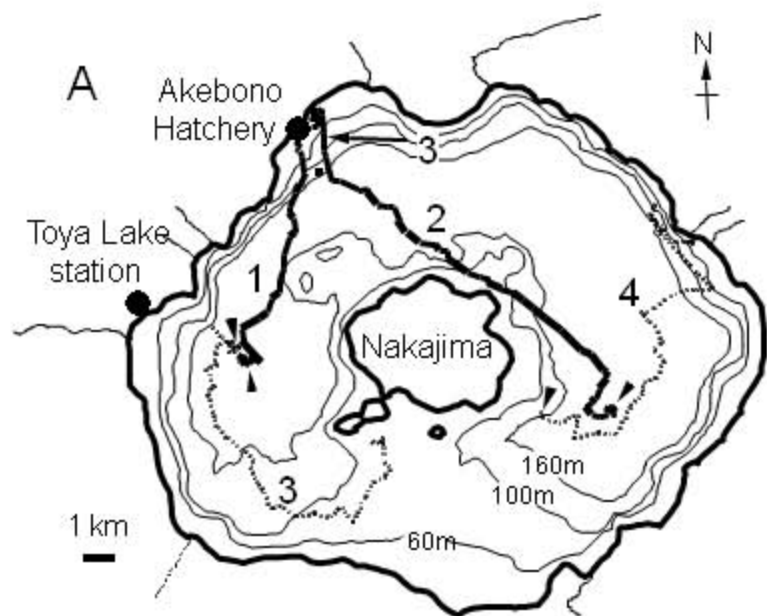
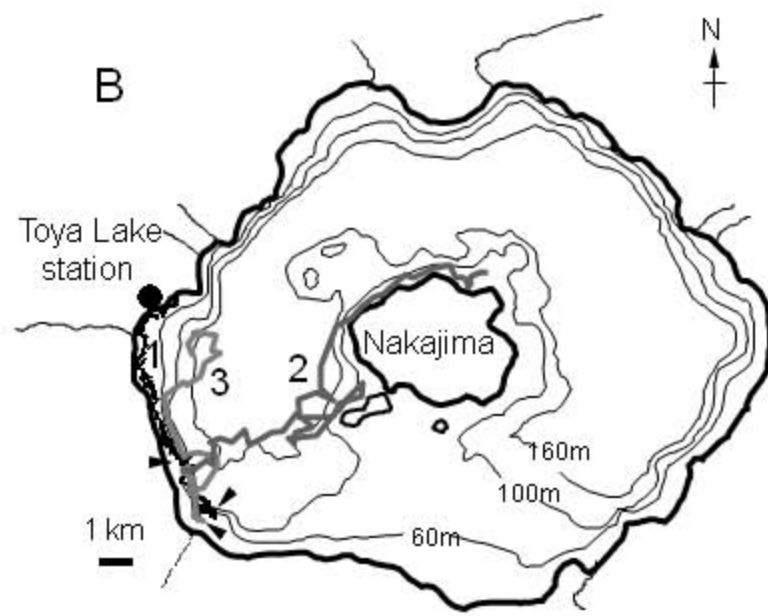


Fig.2



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

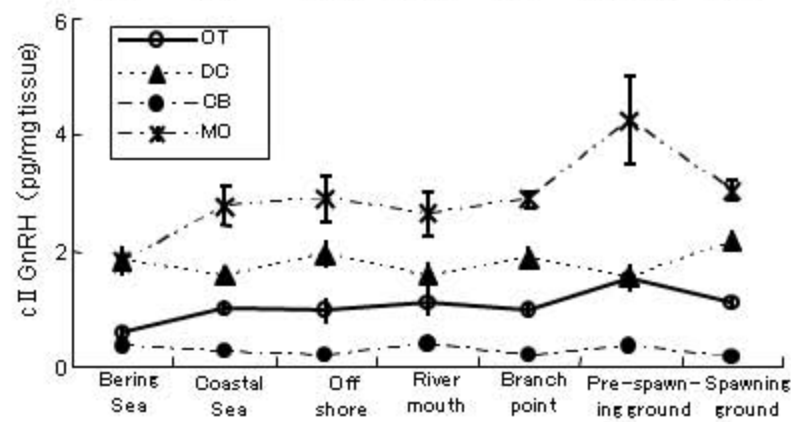
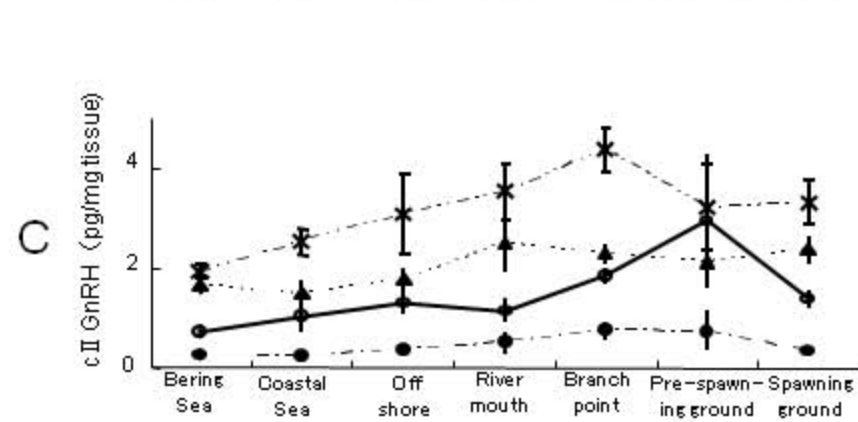
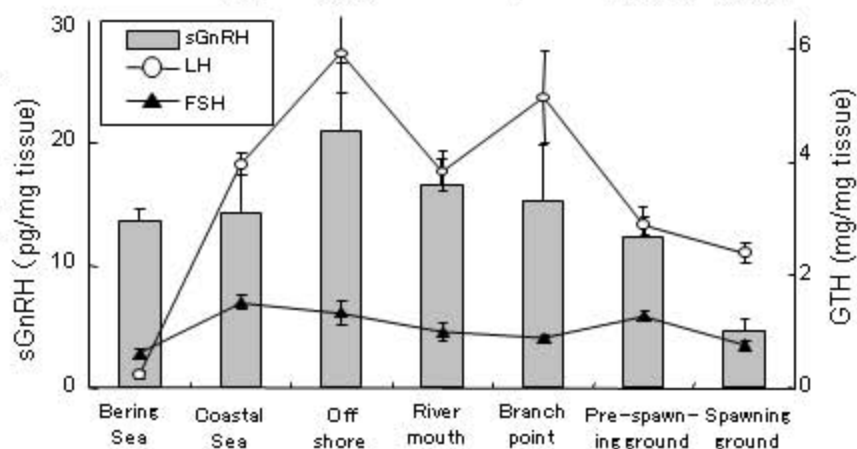
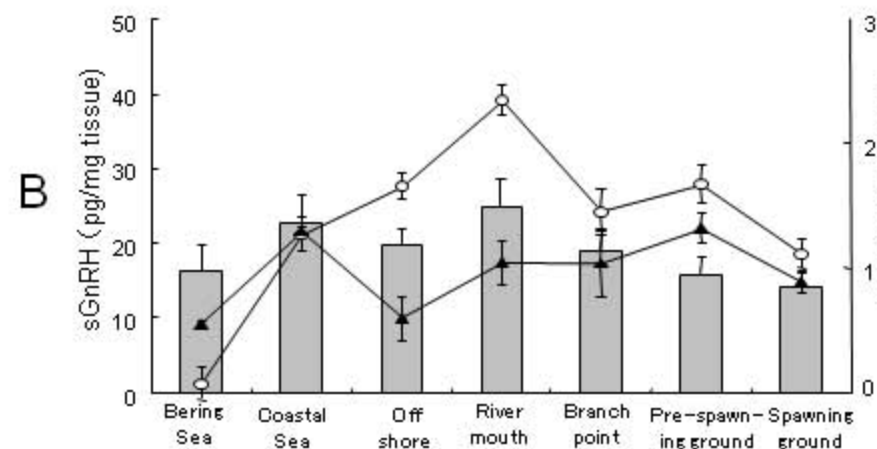
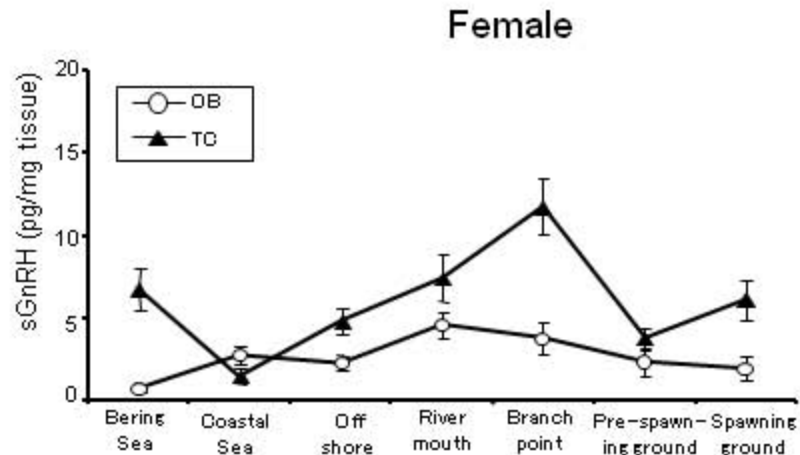
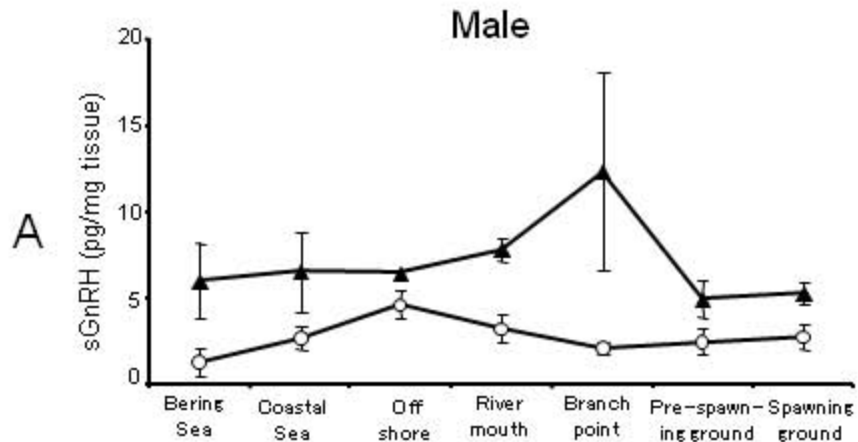


Fig. 4.

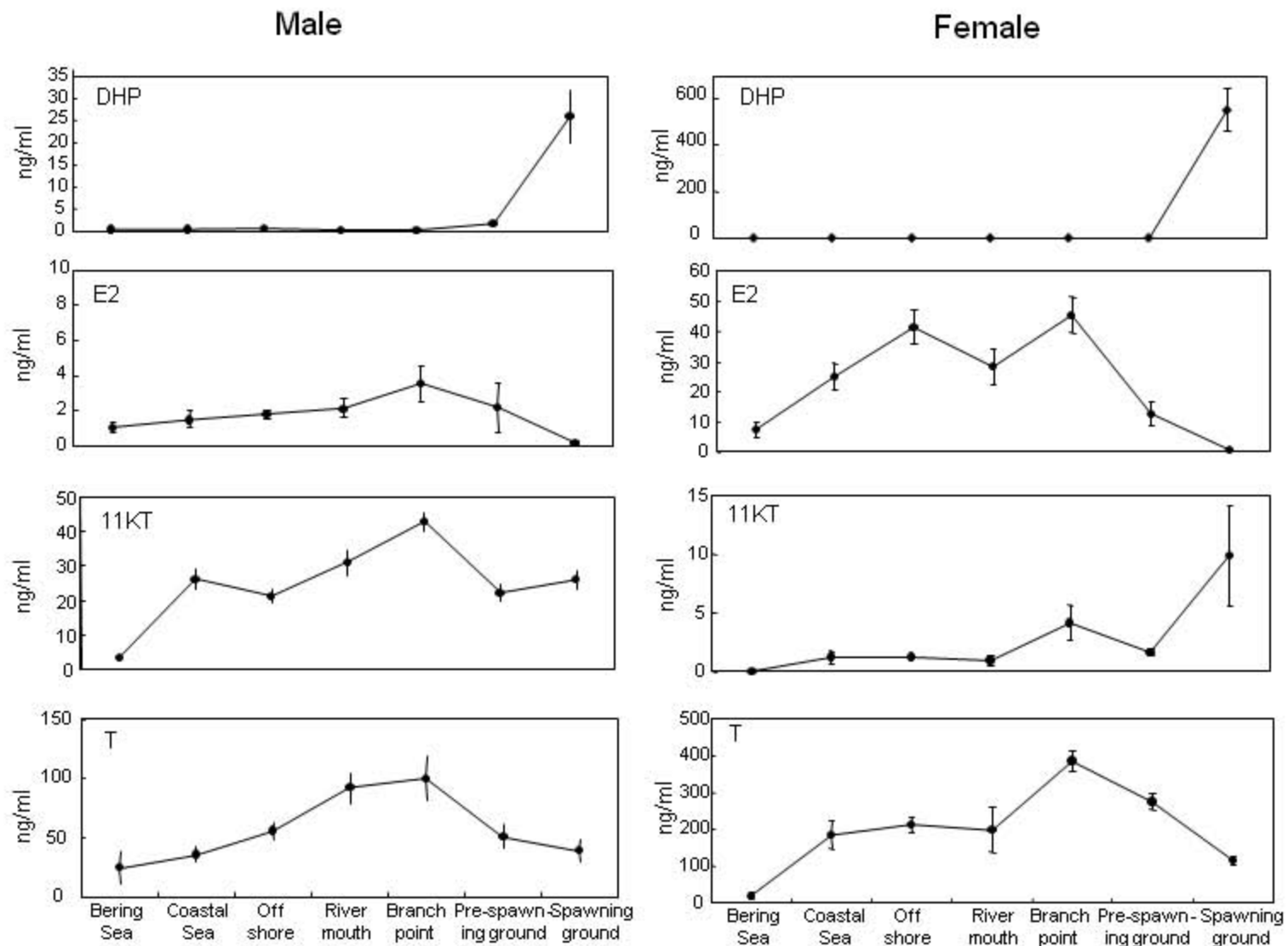


Fig. 5

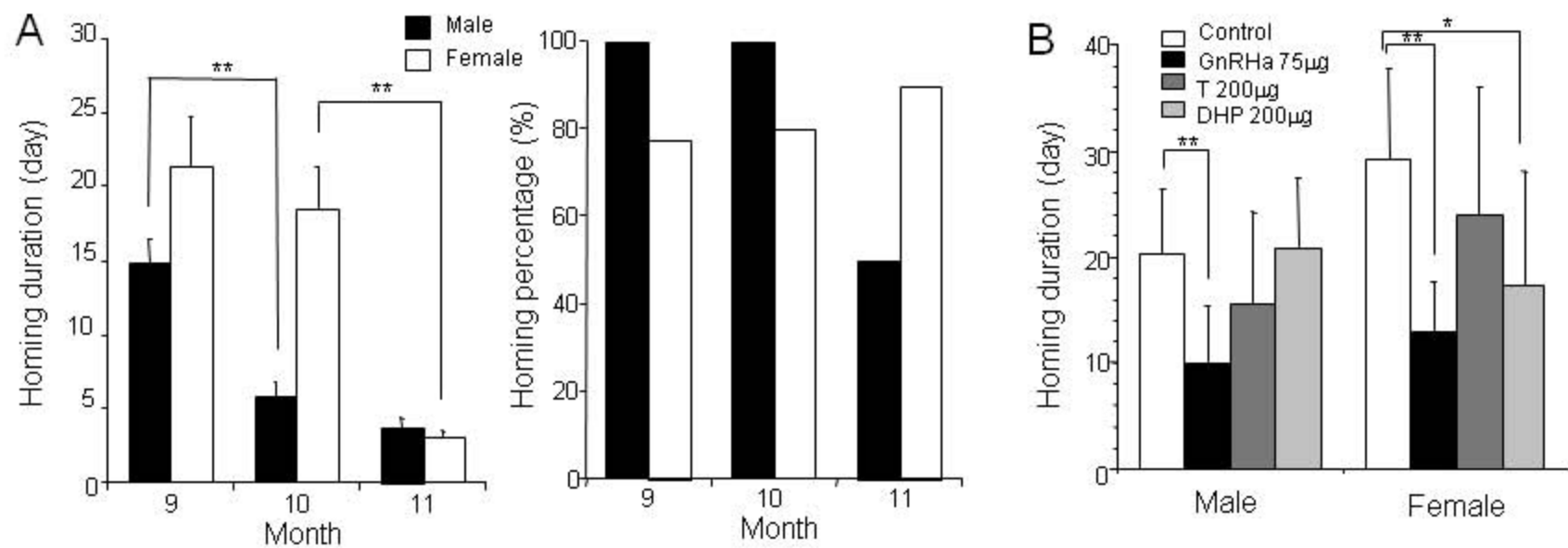


Fig. 6

### Natural stream water

### Artificial stream water

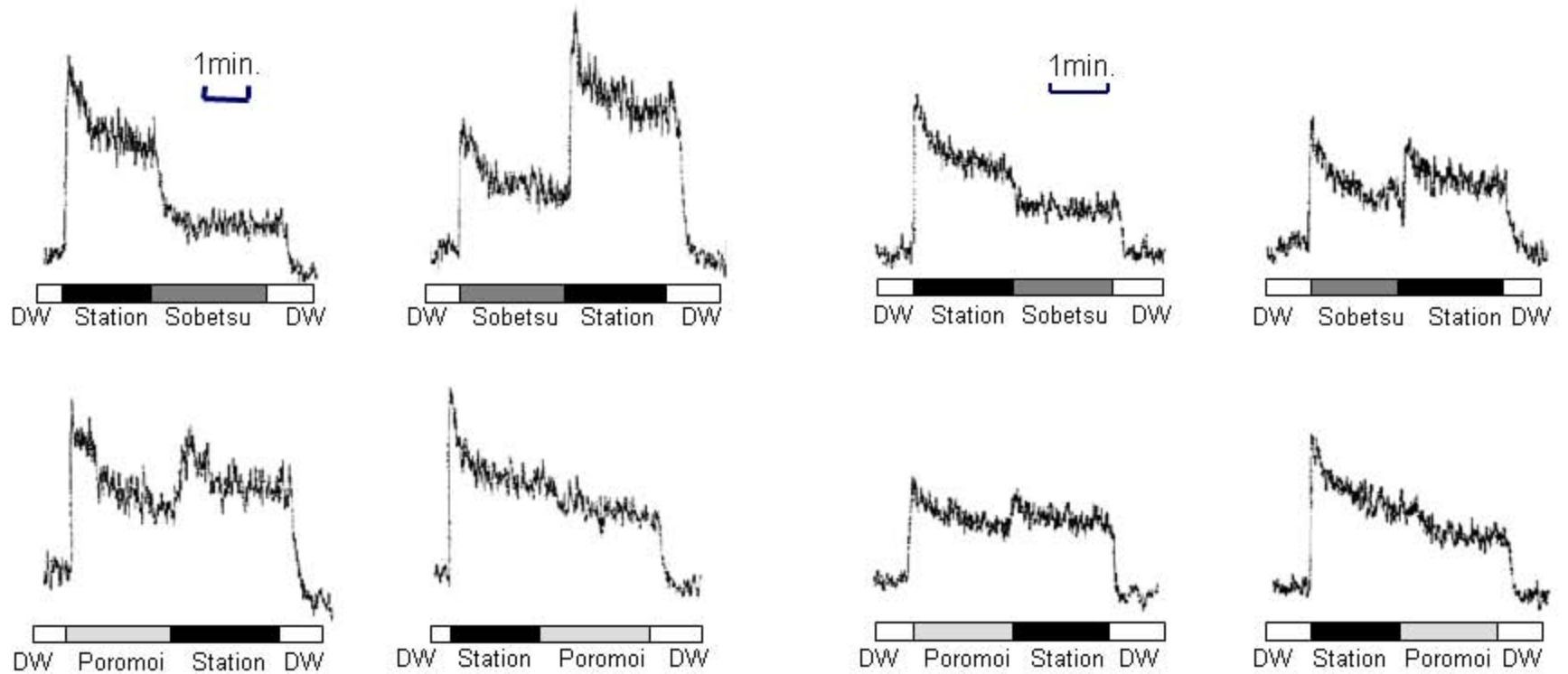


Fig. 7

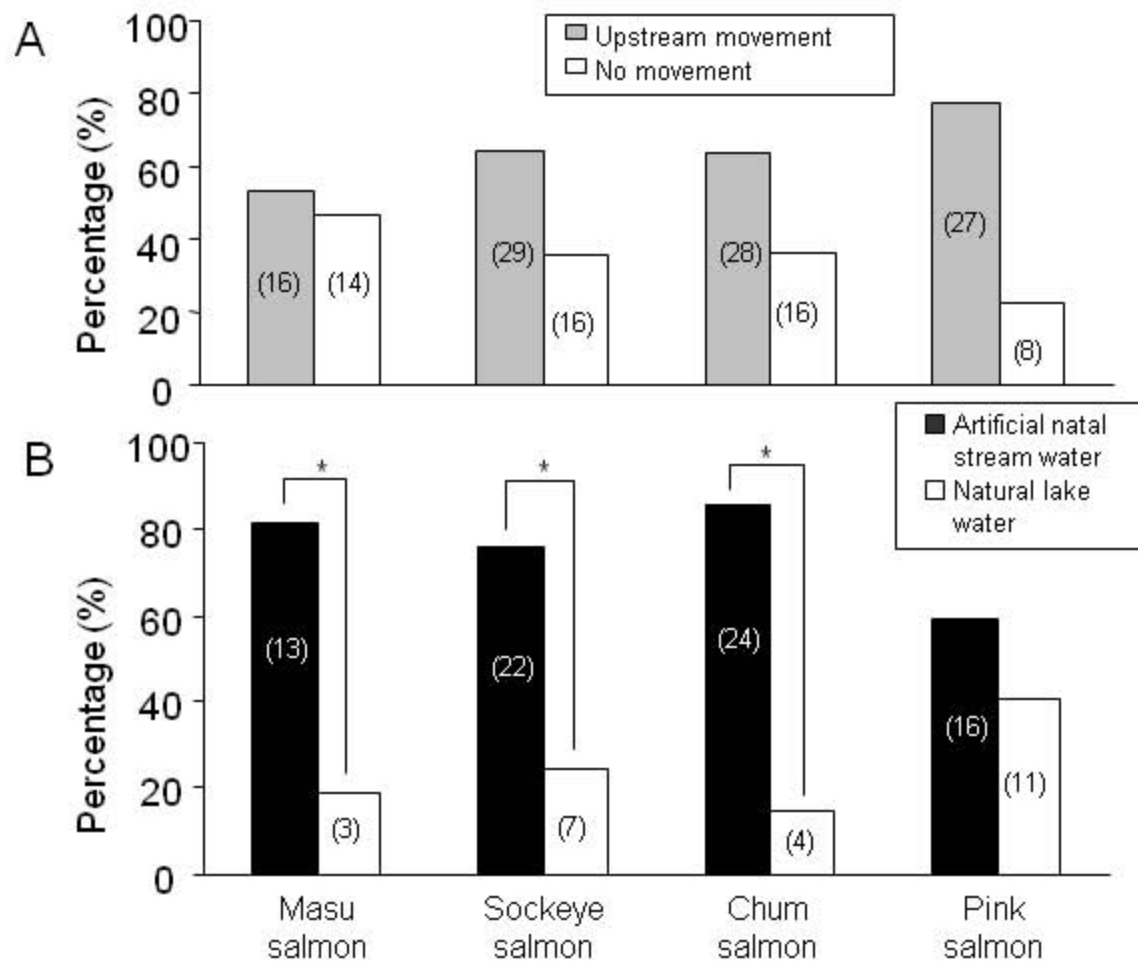


Fig. 8



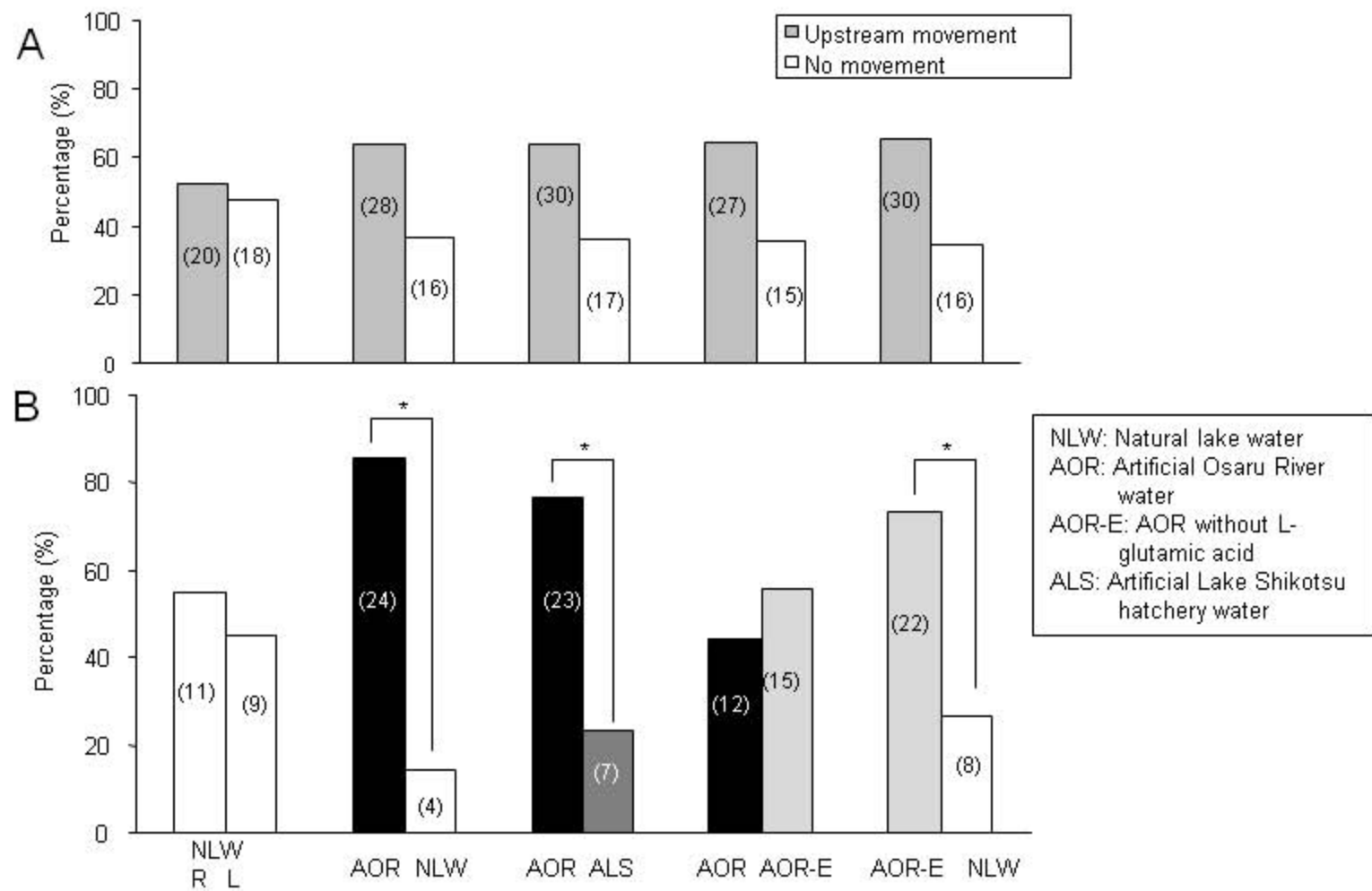


Fig. 9