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Authors: Yamamoto, Yuzo, Shibata, Hideaki, and Ueda, Hiroshi

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Olfactory Homing of Chum Salmon to Stable Compositions of Amino Acids in Natal Stream Water

Yuzo Yamamoto[1†], Hideaki Shibata[2,3], and Hiroshi Ueda[1,3*]

[1]*Aquatic Bioresources and Ecosystem Research Group, Field Science Center for the Northern Biosphere,*
Hokkaido University, North 9 West 9, Kita-ku, Sapporo, Hokkaido 060-0809, Japan
[2]*Watershed Function Research Group, Field Science Center for the Northern Biosphere,*
Hokkaido University, North 9 West 9, Kita-ku, Sapporo, Hokkaido 060-0809, Japan
[3]*Division of Biosphere Science, Graduate School of Environmental Science,*
Hokkaido University, North 9 West 9, Kita-ku, Sapporo,
Hokkaido 060-0809, Japan

Many attempts have been made to identify natal stream odors for salmon olfactory homing. It has recently been hypothesized that odors are dissolved free amino acids; however, it is unknown whether these odors change on a seasonal or annual basis. We analyzed dissolved free amino acid (DFAA) concentration and composition of water from the Teshio River in Hokkaido, Japan, where chum salmon (*Oncorhynchus keta*) returned for spawning, during juvenile downstream migration in spring and adult upstream migration in autumn with a 4-year difference. Among the 19 amino acids found in the Teshio River water, DFAA concentrations fluctuated largely, but 5–7 stable DFAA compositions (mole %) were found between the spring and autumn samples over a 4-year span. Two kinds of artificial stream water (ASW) were prepared using the same DFAA concentration in the Teshio River during the time of juvenile imprinting in spring (jASW) and adult homing in autumn (aASW), after a 4-year period. In behavioral experiments of upstream selective movement in a 2-choice test tank, 4-year-old mature male chum salmon captured in the Teshio River showed significant preference for either jASW or aASW when compared to control water, but did not show any preference with respect to jASW or aASW. In electro-olfactogram experiments, adults were able to discriminate between jASW and aASW. Our findings demonstrate that the long-term stability of the DFAA compositions in natal streams may be crucial for olfactory homing in chum salmon.

Key words: olfaction, imprinting, homing, behavior, salmon

INTRODUCTION

The olfactory hypothesis of salmon imprinting and homing to natal streams was first proposed by Hasler's research group in the 1950s (Wisby and Hasler, 1954; Hasler and Scholz, 1983). Since the publication of these studies, the mechanisms of olfactory imprinting and homing abilities in salmon have been intensively researched in behavioral, electrophysiological, biochemical, and neurobiological studies seeking to clarify the nature of natal river odors (Døving, 1989; Stabell, 1992; Dittman and Quinn, 1996; Quinn, 2005; Ueda, 2011). The pheromone hypothesis proposed by Nordeng (1971, 1977), which was based on findings in Arctic char (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*), postulated that juvenile salmon release population-specific odors that guide homing adults. However, for some Pacific salmon, such as chum salmon (*O. keta*) and pink salmon (*O. gorbuscha*), no juveniles are present when the adults return. It is now widely accepted that specific odors in the natal stream are the basis for olfactory imprinting and homing in salmon. Attempts to identify these natal river odors using an electrophysiological olfactory bulbar response have suggested that natal river odors can be adsorbed onto activated carbon and ion-exchange resins, and that these odors are insoluble in petroleum ether and are dialyzable, nonvolatile, and do not change in response to heat (Fagerlund et al., 1963; Ueda, 1985). Of the limited number of water-soluble chemicals tested, salmon olfactory organs respond to amino acids, steroids, bile acids, nucleotides, and prostaglandins (Hara, 1994).

In our previous study (Shoji et al., 2000), we analyzed compositions of dissolved free amino acids (DFAAs) in water from streams that flow into Lake Toya in Hokkaido, Japan. In that study, we revealed that when artificial stream waters (ASW) with the same DFAA compositions consistent with natural streams were applied to the olfactory epithelia of masu salmon (*O. masou*), electrophysiological olfactory nerve responses were induced that closely resembled the response to the corresponding natural river water. In contrast, the application of combinations of inorganic cations or

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* Corresponding author. Tel. : +81-11-706-2598;
Fax : +81-11-706-2598;
E-mail: hueda@fsc.hokudai.ac.jp

† Present address: Demonstration Laboratory, Marine Ecology Research Institute, 4-7-17 Arahama, Kashiwazaki, Niigata 945-0322 Japan

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bile acids that matched their compositions in river waters induced only very weak responses. We also conducted behavioral experiments in a 2-choice test tank (Y-maze) to examine the effects of an attractant on the upstream selective movement of chum salmon returning to the Osaru River in Hokkaido, Japan. This study revealed that adults prefer ASW with the Osaru River DFAA composition over both another ASW with amino acid concentrations four times higher than the Osaru River concentrations and another ASW with the most highly concentrated amino acid removed (Yamamoto and Ueda, 2009). In addition, we showed that 1-year-old lacustrine sockeye salmon (*O. nerka*) can be imprinted using a single amino acid for two weeks around the parr-smolt transformation (PST), and that maturing and mature fish exposed to these test waters before and during PST in the previous two years had the ability to select the test waters in the Y-maze experiment (Yamamoto et al., 2010). From these results, we hypothesized that the DFAA composition is the source of the natal river odors used for imprinting and homing by salmon. To exert this effect, the DFAA composition would need to be stable between juvenile imprinting in spring and adult homing several years later in autumn.

In the present study, the DFAA concentration and composition of water from the Teshio River in Hokkaido, Japan were analyzed during juvenile imprinting in spring and adult homing in autumn with a 4-year difference. Two types of artificial stream water (ASW) matching the DFAA concentration in the Teshio River during the time of juvenile imprinting in spring (jASW) and adult homing in autumn (aASW) after a 4-year period were prepared. Behavioral experiments using the Y-maze were investigated to compare selectivity of 4-year-old adult chum salmon returned to the Teshio River between jASW and aASW. Electro-olfactogram (EOG) experiments were conducted to determine whether adults can discriminate between jASW and aASW.

MATERIALS AND METHODS

Experimental animals

A total of 127 (average fork length 66.8 ± 4.3 cm and average body weight 3.32 ± 0.47 kg) and six (average fork length 65.7 ± 5.2 cm and average body weight 3.35 ± 0.32 kg) adult mature male chum salmon that were four years old as determined by counting of scale rings were used for behavioral experiments and EOG recording, respectively. The fish were captured with a fence net located about 1 km from the mouth of the Teshio River in September 2009 and 2010 (Fig. 1A), transferred to the Toya Lake Station, Hokkaido University (Fig. 1B), which was located approximately 400 km from the Teshio River, via a truck with a 2-ton live fish water tank, and reared for several days before experiments.

DFAA composition analyses and artificial river water preparation

Water samples (20 ml) were collected in May and September 2005–2010 from a single sampling point in the middle reach of the Teshio River (St. 5; Fig. 1A), frozen at –20°C, and transferred to the laboratory. A 20-µl aliquot of each sample was subjected to an ultra-high-speed liquid chromatography system (LaChrom Ultra, Hitachi High-Tech, Ibaraki, Japan) using a standard amino acid solution (P/N: 855-5953, Wako Pure Chemical Industries, Osaka, Japan). Measurements were performed in triplicate to reduce measurement error. The samples and standard solutions were incubated at 60°C for 1 min with the reaction solution set (P/N: 891-

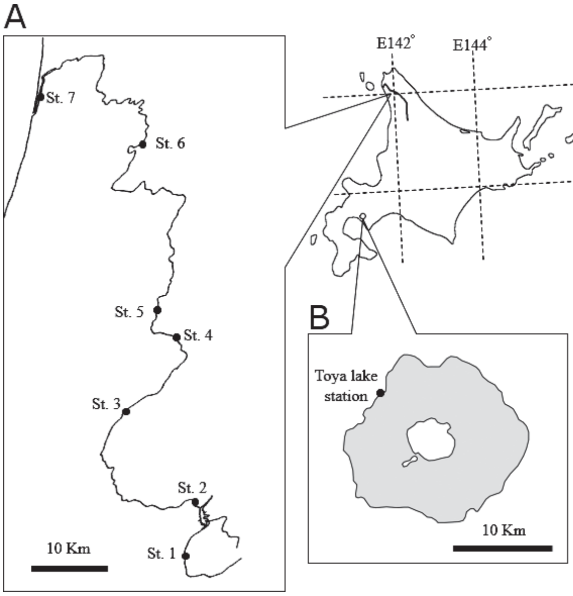

Fig. 1. Map of the Teshio River and Lake Toya in Hokkaido, Japan. **(A)** Water sampling points (Sts. 1–7) in the Teshio River. The main land uses at each station are as follows: St. 1, forest; Sts. 2–3, paddy field; Sts. 4–6, farmland; St. 7, river mouth. The water at St. 5 in the Teshio River is analyzed for seasonal and yearly changes. **(B)** Toya Lake Station, Hokkaido University, in Lake Toya.

Table 1. The concentration of dissolved free amino acids in the Teshio River waters at St. 5 in May 2005 and 2006, and September 2009 and 2010. Each sample was measured three times and presented as mean ± SEM.

	Amino acids	May-05	May-06	Sep-09	Sep-10
Concentration (nM)	Ala	121.63 ± 2.11	3.27 ± 1.14	143.66 ± 7.32	3.65 ± 0.48
	Arg	40.4 ± 0.75	0.00 ± 0.00	65.00 ± 1.25	20.65 ± 2.15
	Asn	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Asp	74.8 ± 1.22	12.69 ± 3.16	77.90 ± 4.36	14.96 ± 4.8
	Cys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Gln	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Glu	41.00 ± 3.00	4.82 ± 1.28	33.54 ± 4.57	5.43 ± 1.83
	Gly	197.00 ± 12.02	39.44 ± 2.34	176.48 ± 17.12	44.34 ± 2.31
	His	24.6 ± 1.44	0.00 ± 0.00	55.60 ± 2.21	0.00 ± 0.00
	Ile	26.2 ± 1.54	0.00 ± 0.00	0.00 ± 0.00	3.49 ± 0.75
	Leu	34.00 ± 3.61	0.00 ± 0.00	39.55 ± 2.16	20.33 ± 5.39
	Lys	168.6 ± 1.48	24.56 ± 2.36	98.77 ± 4.21	21.54 ± 3.82
	Met	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Phe	18.8 ± 0.53	0.00 ± 0.00	61.00 ± 1.49	0.00 ± 0.00
	Pro	0.00 ± 0.00	0.36 ± 0.18	41.66 ± 0.92	5.21 ± 0.95
	Ser	229.6 ± 8.76	7.94 ± 1.09	220.40 ± 8.96	10.73 ± 1.83
	Thr	69.80 ± 4.41	0.85 ± 0.19	32.94 ± 2.06	1.02 ± 0.27
	Tyr	19.00 ± 2.00	0.00 ± 0.00	50.00 ± 2.65	10.48 ± 1.7
	Val	51.00 ± 3.68	0.00 ± 0.00	47.66 ± 3.43	0.00 ± 0.00

5160, Wako) using NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole). Nineteen L-amino acids were analyzed (Table 1): alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val). Tryptophan was not detected, as it is non-fluorescent. On the basis of DFAA concentration determinations, we dissolved amino acids in distilled water to prepare four types of artificial river water during juvenile downstream migration in May 2005 and 2006 (jASW^{2005} and jASW^{2006}), and adult upstream migration in September 2009 and 2010 (aASW^{2009} and aASW^{2010}). Natural lake water (NLW; Table 2) from Lake Toya (Fig. 1B) was used as control water.

Upstream selective movement

Behavioral experiments were conducted in September and October of 2009 and 2010 using 32–35 randomly selected mature male 4-year-old chum salmon in four combinations of test water, as described previously (Yamamoto and Ueda, 2009). The Y-maze was constructed at the Toya Lake Station, Hokkaido University, and consisted of two upstream arms (12 m × 1.8 m, 0.6 m water depth) in which water flowed at a velocity of 50 L/min and a pool (3 m × 1.8 m, 0.6 m water depth) with a single outlet at the end. Before each experiment, three chum salmon were acclimated for three hours in the pool, which was covered with boards and fitted with a gate to prevent the fish from entering either arm. After the acclimatization period, one of the four combinations of test solutions was introduced into the left or right arm for a 9-hour period: (1) NLW in both arms, (2) jASW and NLW, (3) aASW and NLW, and (4) jASW and aASW. DFAAs in all test waters were 2000 times the concentration of their natural counterparts when added at the upper end of each arm and were diluted to the same concentration as the natural water when added to the Y-maze (confirmed by DFAA concentration analysis). The experiment was conducted between 19:00 h and 4:00 h to reduce the stress caused by intense light (Banks, 1969). The gate was opened 30 min before the introduction of the test and control waters, which were switched after each experiment. Fish were allowed to swim independently in and out of either of the two arms. The number of entries made into each arm was counted for nine hours after the initial opening of the gate. The preference for odors was compared with a chi-squared test, and the results of NLW in both arms were used as the expected value.

EOG recording

The EOG response was measured in September and October 2009 using six randomly selected fish from the Teshio River, and the magnitude of the response was expressed as a percentage of the response to 0.1 mM Ser dissolved in distilled water, as previously described (Yamamoto and Ueda, 2009). The EOG response was measured as described by Evans and Hara (1985). Fish were immobilized with an intramuscular injection of gallamine triethiodide (3 mg/kg). Gills were aerated through the mouth with an aerated solution of clove oil (0.005%), which was not allowed to contact the olfactory rosettes. The responsive properties of the olfactory receptor cells were recorded using a pair of glass microelectrodes filled with 2.5% agar-saline and bridged with silver wire. With the aid of a stereomicroscope and micromanipulators, an odorant perfusion tube was gently inserted into the in-current passage of the nose, and the recording microelectrode was inserted through the out-current passage and positioned above the midline of the rosette at the base of the posterior-most lamella. A reference microelectrode was placed on the head, and a separate ground electrode was clipped to the tail of the fish. The differential electrical signal was amplified 500-fold and filtered (100-Hz low-pass) by a direct-current amplifier (A-M System, Carlsberg, Washington, USA). The signals were digitized at 10 samples per second using PicoScope data-acquisition software (Pico Technology Ltd., St. Neots, UK), and signal amplitudes were measured in mV. After electrode placement, the olfactory rosettes were rinsed for 30 minutes with Toya Lake Station water at a steady rate of 1 ml/min. Each odor was then pulsed three times into the Toya Lake Station water for 10 seconds at 150-sec intervals. EOGs were recorded in response to 0.1 mM Ser dissolved

Table 2. The concentration of dissolved free amino acids in the Lake Toya water (NLW: natural lake water).

Amino acids	Concentration (nM)
Ala	3.5
Arg	2.44
Asn	0
Asp	2.44
Cys	0
Gln	0
Glu	2.72
Gly	7.53
His	1.25
Ile	9.4
Leu	1.23
Lys	9.88
Met	0
Phe	8.24
Pro	1.44
Ser	1.08
Thr	2.24
Tyr	8.56
Val	1.43

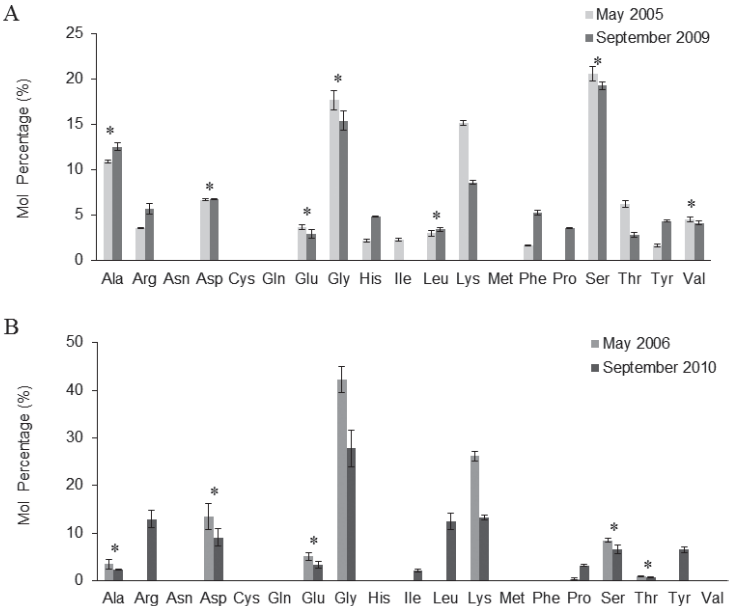

Fig. 2. Seasonal and yearly changes in the composition (mole %) of 19 dissolved free amino acids in the Teshio River. Stable compositions of seven amino acids (Ala, Asp. Glu, Gly, Leu, Ser and Val) between May 2005 and September 2009 **(A)**, and five amino acids (Ala, Asp, Glu, Ser and The) between May 2006 and September 2010 **(B)** are detected by one-way ANOVA followed by Turkey's test (*: $P > 0.05$). Error bars represent the SEM of three measurements.

in distilled water (standard), jASW, or aASW. The amplitude of the EOG response was quantified by the displacement of the odor-evoked peak relative to the pre-stimulus electrical baseline. The magnitude of the response was expressed as a mean percentage of the response to 0.1 mM Ser ± SEM. A cross-adaptation experiment was performed to evaluate the ability of chum salmon to discriminate between jASW and aASW. The olfactory epithelium was first exposed to distilled water for 3 min and then a test solution was applied until the response was unchangeable for 1 min. Then, the second test solution was applied until the response was unchangeable for 1 min. Finally, distilled water was applied again.

Statistical analyses

All data are expressed as the mean ± SEM. Significant differences were determined using a one-way analysis of variance (ANOVA) followed by Tukey's test for DFAA compositions. The behavioral selectivity of the test fish for each arm was analyzed using a chi-squared test with expected values representing a 50:50 distribution. The EOG recording analysis was assessed with Student's t-test. Data were considered significant when $P < 0.05$.

Ethics statement

This study (16-8) was carried out following the "Guide for the Care and Use of Laboratory Animals in Field Science Center for Northern Biosphere, Hokkaido University" and Japanese Government Law (No. 105) and Notification No. 6, and was approved by the Committee of Laboratory Animals, Field Science Center for Northern Biosphere, Hokkaido University.

RESULTS

Seasonal and Yearly Changes in DFAA composition

The DFAA concentration of water from the Teshio River showed large fluctuations in May 2005 and 2006 and September 2009 and 2010 (Table 1). We compared the seasonal and yearly changes in the DFAA composition (mole %) of water from the Teshio River between May 2005 and 2006 and September 2009 and 2010, and found some unchangeable DFAA composition between May and September over the 4-year span. No significant difference was detected with the ANOVA followed by Tukey's test ($P > 0.05$) either for seven amino acids (Ala, Asp, Glu, Gly, Leu, Ser, and Val) between May 2005 and September 2009 (Fig. 2A) or for five amino acids (Ala, Asp, Glu, Ser, and Thr) between May 2006 and September 2010 (Fig. 2B).

Upstream selective movement

To avoid variation caused by courtship behaviors, we only tested male chum salmon. We compared jASW^{2005}, aASW^{2009}, jASW^{2006}, aASW^{2010}, and NLW in 2009 and 2010. ASW or NLW was added to the water inlet of the left or right maze arm, and the number of fish that moved towards each arm was counted; these figures were summed to determine the total number of fish studied (Fig. 3). The four combinations of test and control waters compared were as follows: (1) both NLW, (2) jASWs vs. NLW, (3) aASWs vs. NLW, and (4) jASWs vs. aASWs. In each combination, 71–81% of the fish made upstream movements to one of the arms. Among fish that entered an arm when both arms contained (1) NLW, there was no difference between selected arms. However, when compared with NLW, 73% and 76% of fish showed a significant preference for (2) jASWs and (3) aASWs, respectively ($P < 0.05$ and $P < 0.01$, a chi-squared test). In contrast, there was no significant difference in selection between (4) jASWs and aASWs: 44% of fish selected jASWs, and 56% selected aASWs (Fig. 3).

Electrophysiological discriminating abilities

To evaluate the ability to discriminate between jASW and aASW, the relative EOG response magnitudes to jASW^{2005} and aASW^{2009} were compared in mature male chum salmon captured in the Teshio River in 2009. The EOG response of fish exposed to aASW^{2009} was significantly greater than that of fish exposed to jASW^{2005} ($P < 0.001$, Student's *t*-test; Fig. 4A). In cross-adaptation experiments, which are useful for demonstrating a response to

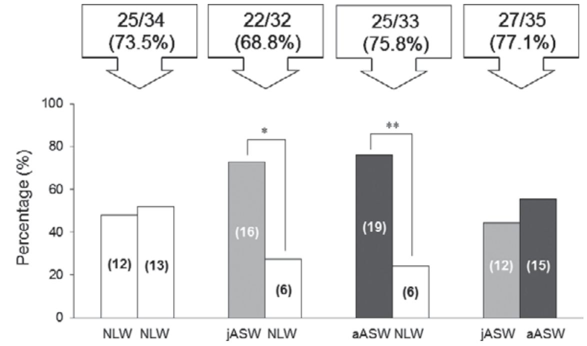

Fig. 3. Behavioral selectivity of artificial stream water by 4-year-old adult male chum salmon in the 2-choice test tank in 2009 and 2010. The numbers in boxes at the top indicate the number of fish that move upstream into either arm of the tank and the total number of fish that are experimentally tested. NLW, natural lake water; jASW, artificial stream water of juvenile downstream migration in May 2005 and 2006; aASW, artificial stream water of adult upstream migration in September 2009 and 2010. Statistical significance (*: $P < 0.05$; **: $P < 0.01$) is determined using a chi-square test.

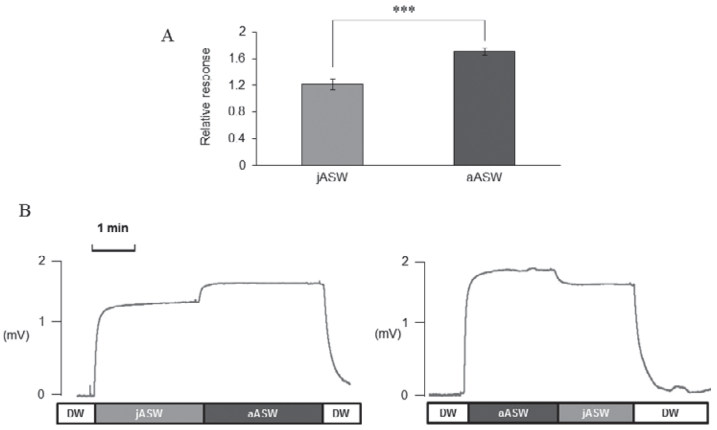

Fig. 4. Electro-olfactogram (EOG) response of adult chum salmon to artificial stream water. **(A)** The relative EOG responses to artificial stream water mimicking juvenile downstream migration in May 2005 (jASW) and adult upstream migration in September 2009 (aASW), expressed as a multiple of the mean response to 0.1 mM l-serine dissolved in distilled water (DW). **(B)** A typical EOG response in cross-adaptation experiments between jASW and aASW with DW used as adaptation water. Statistical significance (***: $P < 0.001$) was determined using Student's t-test. Error bars represent the SEM of six fish.

multiple odorants, jASW and aASW induced secondary responses when applied during adaptation to aASW and jASW, respectively (Fig. 4B).

DISCUSSION

In this study, we analyzed the DFAA concentration and composition of water from the Teshio River in Hokkaido, Japan during juvenile imprinting in spring and adult homing in autumn with a 4-year difference. Although the DFAA concentration fluctuated significantly among May 2005 and 2006 and September 2009 and 2010, there were a few stable DFAA compositions between the spring and autumn samples separated by four years. Two types of ASW matching the DFAA concentration of the natural stream water during juvenile imprinting in spring (jASW) and adult homing four years later in autumn (aASW) were prepared. These ASWs were compared to examine their attractive effects on upstream selective movement of adult chum salmon in behavioral experiments using the Y-maze. Adult male chum salmon captured in the Teshio River showed a significant preference for either jASW or aASW, but did not show any specific preference for jASW or aASW. EOG experiments showed that adults could discriminate between jASW and aASW. These findings indicated that some stable DFAA compositions in the natal stream over a 4-year period between juvenile downstream migration in spring and adult upstream migration in autumn might be deeply involved in olfactory homing in chum salmon.

The majority of adult chum salmon return after four years, but some adult chum salmon return after three to five years. We analyzed the seasonal and yearly changes in the DFAA composition in May from 2004 to 2007 and in September 2009 and 2010. A few stable DFAA compositions were detected between May and September after three to five years. No significant differences were detected for obe amino acid (Glu) over four years, three amino acids (Ala, Gly and Ser) over three years, and five amino acids (Asp, Ile, Leu, Thr and Val) over two years, on the basis of ANOVA followed by Tukey's test ($P > 0.05$; Supplementary Figure S1 online). Only a few 3-year-old adult chum salmon showed upstream movements in the Y-maze experiments, and only one exhibited upstream movement when comparing ASW with NLW. The solo 3-year-old adult chum salmon showed a preference for jASW^{2005} over NLW. However, there were no 5-year-old adult chum salmon that showed upstream movements in the Y-maze experiments. These stable DFAA compositions over several years in natal streams should be important for accurate homing of chum salmon with different ages.

Alterations in the DFAA composition of river water are attributable to complex biological processes in the watershed ecosystem, and it is thought that many factors may affect DFAA composition both inside and outside of the river environment, including soils, vegetation, litter, pollen, dew, and various types of microbial activity (Thomas, 1997). Of these, the roles of complex microbial communities known as biofilms have been thoroughly investigated (Romani et al., 2004). On the basis of incubation experiments in the laboratory, our previous research has reported that biofilms are one of the major sources of DFAAs in river water (Ishizawa et al., 2010). We analyzed the spatial changes in the DFAA compositions along seven different sampling stations from the headwaters to the mouth of the Teshio River in May 2007 (Supplementary Table S1 online). The ANOVA followed by Tukey's test revealed significant similarities in 11 amino acids (Ala, Asp, Glu, Gly, Ile, Leu, Pro, Ser, Thr, Tyr, and Val) at 2–7 stations (Supplementary Figure S2 online). As a result, we suggest that seasonal, yearly and spatial changes in the DFAA compositions of Teshio River water may reflect the activities of biofilms, and that unchangeable combinations of DFAAs showing little change contribute to the specific odor characteristics of each river.

Previous work has shown that coho salmon imprinted with the artificial odors β-phenylethyl alcohol (PEA) or morpholine during PST could be lured into unfamiliar streams scented with these odors at the time of their homing migration a few years later (Cooper et al., 1976; Scholz et al., 1976). Furthermore, the olfactory receptor cells of coho salmon that had been imprinted with PEA had a higher sensitivity to PEA than non-imprinted fish had (Nevitt et al., 1994), and only fish that were exposed to PEA or natural river odors during PST developed an imprinting memory (Dittman et al., 1996). Using electrophysiological, behavioral, and gene-expression (a salmon olfactory-imprinting-related gene; Hino et al., 2007) experiments, we have previously demonstrated that 1-year-old lacustrine sockeye salmon were able to be imprinted using a single amino acid (Pro or Glu) for two weeks around PST, and that maturing and mature fish exposed to these test waters before and during PST in the previous two years had the ability to select the test waters (Yamamoto et al., 2010).

Another unresolved mystery related to salmon homing concerns the sensory mechanisms that are involved in long-distance migration from open water to natal streams, as the ability to recognize natal river odors only emerges within a short distance from the mouth of the river. For example, it has been demonstrated that chum salmon are able to migrate 2760 km home from the Bering Sea to Hokkaido in 67 days (Tanaka et al., 2005). To home accurately over long distances in open water, salmon must possess a geomagnetic compass and be able to orient themselves based on their exact locations, as in sea turtles (Lohmann et al., 2008). Further physiological and behavioral studies are required to investigate whether chemical sensory mechanisms also apply to navigation and orientation in open water.

In conclusion, the present study demonstrates that adult chum salmon can use stable combinations of DFAA to home to their natal streams. We suggest that long-term stability of DFAA compositions in natal streams may be crucial for olfactory homing in chum salmon. The role of river chemistry should be considered in attempts to conserve salmon populations in their natal streams.

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