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Effects of aggressive interaction on downstream movement and olfactory function in masu salmon, *Oncorhynchus masou*

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ABSTRACT

In the present study, behavioral, electrophysiological, and molecular biological experiments were developed to examine the role of aggressive interaction ("fighting experience") on downstream movement and olfactory function in relation to smoltification in hatchery-reared 1-year-old masu salmon (*Oncorhynchus masou*). Individual salmon that lost the fighting contest in April and May moved downstream earlier than the winners. The olfactory response to salinity sensitivity of the losers, measured by electro-olfactogram to 10^{-1} M sodium chloride, was significantly higher than that of the winners during the period from April to June. In May, the olfactory response to imprinting activity of the losers, previously exposed for 1 week to 10^{-4} M L-glutamic acid was significantly high, and the salmon olfactory imprinting-related gene was also highly expressed. During the smoltification period, losing the aggressive interaction might exert strong influences on downstream movement and olfactory function in masu salmon. The present newly developed experiments provide new evidence for evaluating the relation between the aggressive interaction and the smoltification in masu salmon.

1. Introduction

Masu salmon (*Oncorhynchus masou*) in northern Japan spend 1.5 years in freshwater after hatching. Some individuals then migrate to the ocean in the late spring, becoming anadromous, while other individuals remain river-resident for their whole lives (Kiso, 1995). Prior to downstream migration, the anadromous form (smolt) acquires seawater adaptability, enabling it to survive in the ocean (Boeuf, 1993; McCormick et al., 1998). It is also imprinted by its natal stream odorant, enabling it to return as an adult for reproduction (Hasler and Scholz, 1983; Hino et al, 2009). Because anadromous and river-resident masu salmon exist in the same stream under the same photoperiod and water temperature conditions, environmental factors are not sufficient to explain why some masu salmon do not undergo smoltification.

Aggressive interactions have been speculated as a possible factor controlling smoltification because masu salmon show strong territorial behavior during the parr stage in reared condition (Kubo, 1974). It has also been shown that 1-year-old masu salmon that lost an aggressive interaction initiated downstream migratory behavior in an artificial raceway (Munakata et al., 2000). In addition, cortisol levels in individuals that recently lost a fighting contest were elevated in rainbow trout (*O. mykiss*; Øverli et al., 2004) and other species (Sakakura et al., 1998; Schuett and Grober, 2000), and testosterone levels in winners of aggressive interactions were generally lower than in losers (Hsu et al., 2006). Thyroid hormones have also been known to decrease the aggressive behavior of four salmonids during smoltification (Hutchison and Iwata, 1998). After cross-matching smoltification-related hormones and aggressive interaction-related hormones, fighting experience in the parr stage could be a factor that influences the initiation of smoltification. However, little is known about the relationship between aggressive interactions and smoltification in masu salmon.

The present study was conducted to examine the effect of aggressive interaction ("fighting experience") on downstream movement and olfactory function in 1-year-old masu salmon by means of newly developed behavioral, electrophysiological, and molecular biological experiments. After fighting contests during different months from April to August, masu salmon that either won or lost the fighting contest were tested for their downstream movement, olfactory sensitivity to NaCl, olfactory response to a 1-week exposure to L-glutamic acid (Glu), and expression levels of salmon imprinting-related gene (SOIG; Hino et al., 2007) in the olfactory epithelium (OE). Our results suggest that losing aggressive interactions has a strong influence on downstream movement and olfactory function in masu salmon from April to June, and especially in May.

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2. Material and methods

2.1. Animals

One-year-old masu salmon reared after hatching at the Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University were used in the present study. The experimental fish were reared in a 1400-L circular tank under a natural photoperiod with a continuous flow of spring water. The fish were fed on standard commercial pellets.

2.2. Experimental procedures

2.2.1. Fighting contests

The initial fighting contests were held between March and July of 2008. One month prior to each fighting contest from April to August, forty fish were selected randomly from the circular tank. All contests took place in a standard aquarium (28×28×28 cm) containing water 20 cm deep and gravel 2 cm deep. No geographic landmark except the sides of the aquarium was available for the fish to defend. Two individuals were placed in one standard aquarium with two compartments separated by an opaque partition and allowed to acclimate until the next morning. All contests were held between 0900 and 1300 and were videotaped from approximately 1 m directly in front of the aquarium. To reduce possible disturbances, the researcher left the room and closed the door after the partition was lifted. The researcher returned 1 hour later to separate the contestants by re-inserting the opaque partition into the aquarium. All contests resulted in clear winners and losers within 1 hour. Contest results were determined according to the methods described in a previous study of aggressive behavior study in the juvenile chum salmon, *O. keta* (Yamagishi et al., 1981). Winners occupied and swam freely in the center area of the aquarium, and losers were pushed against the corner of the aquarium. After the initial contest, each fish was transferred to an isolated tank (20×20×20 cm, containing water 10 cm deep) under a 12L/12D photoperiod with continuous flow and was fed on standard commercial pellets. To clear the effect of the initial fighting experience on the subsequent fighting contest, each fish was reared separately.

Each fish was paired with an opponent that had the same initial fighting contest result. For individual identification, the outer margins of the caudal fins of the fish were clipped 24 h prior to the fighting contests (one upper and one lower margin of the caudal fin, randomly assigned). The cut healed within 3–4 days and no sign of infection was ever observed. During marking, body length, fork length, and body weight were measured (Table 1). Because the body sizes of fish varied widely, two individuals with similar body sizes were used in each fighting contest. Although the large fish had a tendency to win the contest, there was no significant difference in body size between winners and losers from April to August.

After the fighting contests, fish were separated into groups for experiments on downstream movement, electrophysiological electro-olfactogram (EOG), and OE sampling.

2.2.2. Downstream movement

Two pairs of fish were used each month (from April to August) in the downstream experiment, except for June, when fish died by accident. Downstream movement was tested in a raceway tank made from the fiber reinforced plastics with no cover (150×33×35 cm, containing water 20 cm deep) under a 12/12D photoperiod with a continuous flow of spring water. The flow speed in the raceway tank was fixed at 30 cm/s. No food was given to the fish during the experiment. In the upstream part of the tank, a space (33×33×20 cm) separated by a removable net was created to allow the fish to acclimate. In the downstream part of the tank, a space (33×33×20 cm) separated by a movable wall was created to define the completion of downstream movement.

After the fighting contests, each fish was acclimatized in the upstream part of the raceway tank. The next morning, the net was removed, and each fish was allowed to move freely within the tank. The subsequent location of the fish in the tank at 9 am, noon, and 5 pm was recorded for 7 days. If the fish passed the movable wall and stayed in the downstream part of the tank for 3 days, the fish was classified as carrying out downstream movement.

Table 1
Body length (BL), fork length (FL) and body weight (BW) of 1-year-old masu salmon from April to August in 2008.

Year	Month	BL (cm)	FL (cm)	BW (g)
2008	April	7.0–12.9	7.7–14.3	3.7–23.2
	May	9.8–13.7	10.7–14.9	9.7–25.3
	June	9.6–13.7	10.5–15.3	9.8–25.3
	July	8.4–14.3	9.4–15.7	6.9–29.8
	August	9.2–14.2	10.1–15.3	8.7–29.0

2.2.3. Electrophysiological electro-olfactogram (EOG) experiments

EOG experiments were carried out according to the methods described by Scott and Scott-Johnson (2002). Fish were anaesthetized in 0.05 ml/L eugenol (4-allyl-2-methoxyphenol, Wako Pure Chemical Ins., Ltd., Osaka, Japan), immobilized by intramuscular injection of gallamine triethiodide (Sigma Chemical Co., St Louis, USA) at a concentration of 3 mg/kg body weight, and then placed in a holder on a vibration isolation table. The gills of the fish were irrigated through a mouthpiece with oxygenated spring water containing eugenol. The responsive properties of olfactory receptor neurons were recorded using a pair of glass microelectrodes, which were filled with 2.5% agar-salmon Ringer's solution (150 mM NaCl, 31 mM KCl, 3 mM $MgSO_4$, 10 mM $MgCl_2$, 34 mM $CaCl_2$, 40 mM HEPES, and 0.1% glucose, adjusted to pH 7.5 with 0.01 N NaOH) and bridged to a silver wire.

With the aid of stereomicroscope and micromanipulators, an in-current tube was inserted into the olfactory rosette, and the recording microelectrode was inserted and positioned above the olfactory epithelium. 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×) and filtered (100 Hz low-pass) using a direct current amplifier (A-M System, Carlsberg, WA, USA). The signals were digitized by PicoScope data acquisition software (Pico Technology Ltd., St. Neots, UK), and the signal amplitudes were measured in millivolts (mV).

After electrode settlement, the olfactory epithelium was rinsed for 15 min with spring water for acclimation. Stimulation solutions were then pulsed in three 10-s pulses, with each pulse 180 s apart. EOGs were recorded in response to each stimulator.

2.2.3.1. Salinity sensitivity. Eight pairs of fish were used each month for two salinity sensitivity experiments. After each fighting contest, fish were returned to their isolated rearing tanks. One week later, four losers and four winners were taken from the rearing tanks, and their olfactory response to 10^{-3} M and 10^{-1} M NaCl was measured. 10^{-1} M NaCl is 20% of the natural concentration of seawater, and 10^{-3} M of NaCl is the lowest concentration that produced an olfactory response in masu salmon.

2.2.3.2. Olfactory response to Glu. Three pairs of fish were used each month in this experiment. After the contests, six fish were transferred into a 20-L tank that was separated into six blocks by opaque partitions. The tank had a continuous flow of spring water, and a continuous drip of 10^{-4} M L-glutamic acid (Glu; Sigma) was used to keep the Glu concentration at 10^{-6} M. Fish were exposed to the Glu-containing water for 1 week and were then reared 3 weeks for recovery (without Glu). After 3 weeks of recovery, the olfactory response of the fish to 10^{-4} M Glu and 10^{-4} M L-serine (Ser; Sigma) was tested. If the relative olfactory response (Glu/Ser) of an individual

was higher than the overall average, it was determined that the individual was imprinted by Glu.

2.2.4. Real-time polymerase chain reaction (real-time PCR)

The olfactory epithelium was sampled 2 h and 1 week after the contest. Each month's data were derived from four pairs of fish 2 h after the contests and three pairs of fish 1 week after the contests.

Total RNA was extracted from single olfactory epithelia using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA concentrations were determined by optical density measurement at 260/280 nm, and RNA quantity and integrity were verified by gel electrophoresis. Reverse transcription polymerase chain reaction (RT-PCR) was performed with a TaqMan RT Kit (Applied Biosystems, Foster City, CA, USA). Each RT reaction contained total RNA (50 ng), 10× TaqMan RT Buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.5 mM dNTP mixture, 50 μM random primer 6-mers, 25 U TaqMan RTase, and 5 U RNase inhibitor. The reaction was incubated at 42 °C for 15 min and then terminated by incubation at 92 °C for 2 min.

Real-time PCR was carried out with an Mx3000P QPCR System (Stratagene, La Jolla, CA, USA). The PCR reaction mixture contained TaqMan Universal PCR Master Mix (Applied Biosystems), 100 nM each forward and reverse primer and 130 nM of fluorogenic probe. The amplification profile was 50 cycles of 95 °C for 15 s and 60 °C for 1 min (annealing and extension). To determine the amounts of SOIG mRNA, full-length cDNA was used as a standard. The standard cDNA was serially diluted to a concentration of 1×10^4–1×10^9 copies (Fig. 1). In this assay, several doses of standard cDNA were applied in triplicate, and sample cDNA prepared from total RNA was applied in duplicate. Amounts of SOIG mRNA were expressed as copies per microgram total RNA.

2.3. Statistical analysis

The Student's *t* test was used to analyze differences in salinity sensitivity, relative olfactory response to Glu, and SOIG expression in olfactory epithelium between winners and losers. Changes of salinity sensitivity and olfactory imprinting ability in different months were analyzed by Tukey's HSD test ($P<0.05$). JMP (v. 5.0.1 SAS Institute Inc., Cary, NC, USA), a commercial statistical package, was used for the statistical analysis in this study.

2.4. Ethics statement

This study (No. 16-22) was carried out under the control of a committee following the "Guide for the Care and Use of Laboratory Animals in Field Science Center for Northern Biosphere, Hokkaido University" and Japanese Governmental Law (No. 105) and Notification (No. 6).

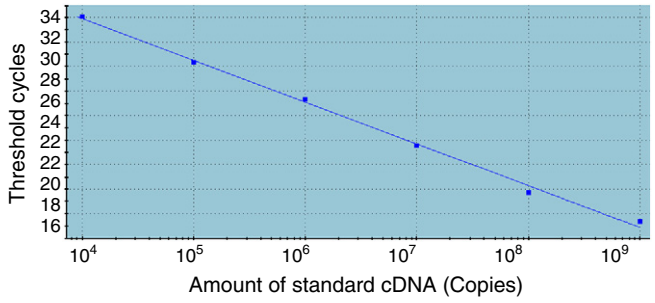

Fig. 1. Typical standard curve for quantitative real-time PCR of salmon imprinting-related gene (SOIG) mRNA.

3. Results

3.1. Downstream movement

In April and May, two individuals that lost the fighting contest moved downstream 3 to 4 days earlier than the ones that won the contest. In contrast, there was no downstream movement in individuals that either lost or won the contest in July and August (Table 2).

3.2. Salinity sensitivity

Winning or losing in the fighting contest had no significant difference on olfactory response to 10^{-3} M NaCl (data not shown). However, there were significant differences in olfactory response to 10^{-1} M NaCl between winners and losers from April to June (Fig. 2). Individuals that lost the contest from April to June demonstrated a significantly higher olfactory response to 10^{-1} M NaCl than the winners; this effect was especially prominent in June.

3.3. Olfactory response to Glu

Winners and losers from April to August differed in their relative olfactory responses to Glu (Fig. 3). Losers had significantly higher relative olfactory responses to Glu than winners from April to June, and especially in May. However, winners and losers showed almost similar olfactory response to Glu in July and August.

3.4. Expression of SOIG mRNA in the OE

Expression levels of SOIG mRNA in the OE did not significantly differ between winners and losers 2 h after the staging contests (data not shown). However, SOIG expression in losing individuals 1 week after the fighting contests peaked in May and was significantly higher than in winners (Fig. 4).

4. Discussion

In the present study, downstream movement, olfactory sensitivity to salinity, and olfactory imprinting activity were compared between winners and losers after aggressive interactions using newly developed experiments to understand the effects of fighting experience on the smoltification-related phenomena in 1-year-old masu salmon. Although only small-scale experiments in laboratory conditions were conducted, by measuring downstream movement, olfactory sensitivity to salinity, olfactory response to imprinted odor (Glu), and expression levels of SOIG mRNA in the OE, we found that losers from April to June became more like anadromous individuals (smolt) than winners. The present newly developed experiments provide new evidence for evaluating the relationship between aggressive interactions and smoltification in masu salmon.

In the downstream movement experiments, it was clear that the early moving individuals were those with losing interactions in April and May. A previous study of smoltification in salmon suggested that a loser moving from its territory to another area searching new territory or escaping from territory keepers (Hoar, 1988). Using 1-year-old masu salmon in an artificial raceway, Munakata et al. (2000) observed that relatively large fish, including precociously matured male,

Table 2
Downstream movement of winners (W) and losers (L) after fighting contests from April to August in 2008. (N: no downstream movement was detected in 7 days).

Month	April				May				July				August			
Experience	W	W	L	L	W	W	L	L	W	W	L	L	W	W	L	L
Days after contest	6	6	2	3	6	7	3	3	N	N	N	N	N	N	N	N

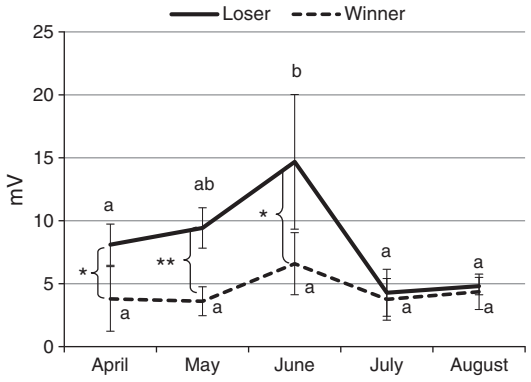

Fig. 2. Olfactory response of losers and winners (four fish each) to the 10^{-1} M sodium chloride after the fighting contests. Contests are conducted monthly from April to August. ab: Tukey's HSD test, $P<0.05$; Student's t test, *: $P<0.05$, **: $P<0.001$.

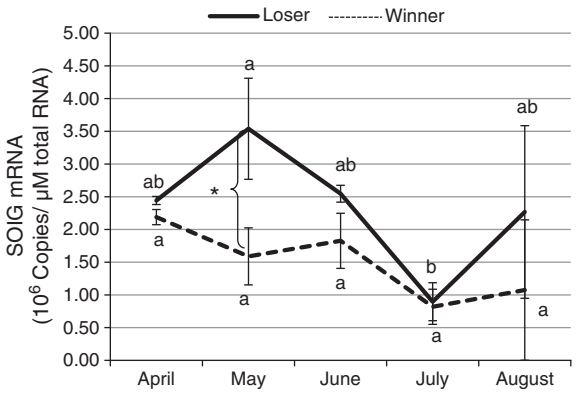

Fig. 4. Expression of salmon imprinting-related gene (SOIG) mRNA in the olfactory epithelium of losers and winners (three fish each) 1 week after the fighting contests. Contests are conducted monthly from April to August. ab: Tukey's HSD test, $P<0.05$; Student's t test, *: $P<0.05$.

exhibited territorial aggressiveness, and that some small smolts showed downstream migratory behavior. Although there was no significant difference in body size between winners and losers in the present study, losing had a strong influence on downstream movement. Because downstream migration occurred during the smoltification period, the observed downstream movement might be related to escaping or searching behavior after aggressive interactions.

The olfactory response of losers from April to June became more sensitive to 10^{-1} M sodium chloride (1/5 of seawater) than that of winners. While the reason for this increased sensitivity remains unclear, we would like to propose two possible explanations for why anadromous individuals could have a high sensitivity to salinity: first, migrating individuals might migrate to the ocean, where the salinity is high, and second, smolting individuals prepare for seawater adaptation by detecting higher concentrations of sodium chloride. Consistent with these explanations, a study of endocrine systems involved in seasonal development and seawater acclimation in juvenile Atlantic salmon (*Salmo salar*) revealed that the anadromous form displays a remarkable plasticity in adjusting ion homeostasis in response to changes in environmental salinity (Nilsen et al., 2008). The novel experimental methods to test olfactory sensitivity to salinity described in the present study could be used to examine seawater adaptability in salmon.

The olfactory response of losers that were imprinted by Glu for 1 week after the fighting contest was significantly higher than that of winners from April to June, and especially in May. Recently, Yamamoto et al. (2010) reported that 1-year-old sockeye salmon (*O. nerka*) can be imprinted by a single amino acid, either L-proline (Pro) or Glu, from March to June, and that this imprinting required a 2-week exposure to Pro or Glu at a concentration of 10^{-7} M. In addition, β-phenylethyl alcohol (PEA), an artificial odor, was imprinted at a concentration of 10^{-7} M for 10 days during smolt stages of coho salmon (*O. kisutch*; Dittman et al., 1996). Although the concentrations and exposure times of amino acids or PEA for sockeye and coho salmon were 1000 times lower and several days shorter, respectively, than those for masu salmon in the present study, all were in good accordance with imprinting profiles in these salmonid species.

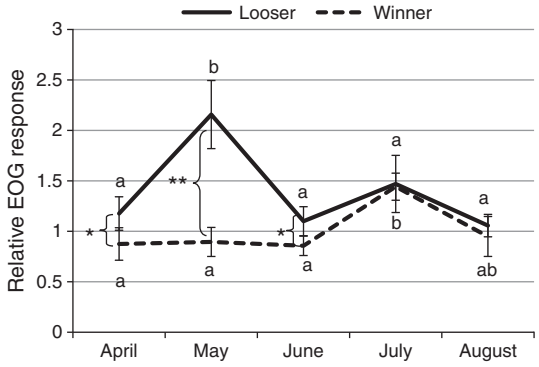

Fig. 3. Relative olfactory response of losers and winners (three fish each) to L-glutamic acid after the fighting contests. Contests are conducted monthly from April to August. ab: Tukey's HSD test, $P<0.05$; Student's t test, *: $P<0.05$, **: $P<0.001$.

SOIG mRNA expression in the OE of losers was significantly higher than in winners in May. Yamamoto et al. (2010) also observed that the expression of SOIG mRNA in 1-year-old sockeye salmon increased from March to June and decreased in July. The significantly different expression levels of SOIG mRNA in the OE between winners and losers were detected 1 week after the fighting contest, but not 2 h after the contest. Cortisol levels were similar between winners and losers just after the contest; however, differences in cortisol levels were detected between winners and losers 3 h after the contest (Øverli et al., 1999). Because changes in hormone levels between winners and losers might not happen immediately after the contests, the increase in SOIG mRNA expression in the OE might occur some time after the contest. These results regarding olfactory imprinting ability in masu and sockeye salmon could provide a starting point for future studies of smoltification-related olfactory function in salmon.

Both internal changes caused by aggressive interactions and external changes in environmental factors might modulate the initiation of smoltification in masu salmon. These modulations allow anadromous and river-resident masu salmon to coexist in the same stream. However, much remains unknown about the effects of aggressive interactions on smoltification-related hormone and the stress response after different fighting experiences. Using the newly developed experiments in the present study, further research should explore the relationship between the aggressive interaction and smoltification, with special attention paid to smoltification-related hormone and the stress response in masu salmon.

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