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Establishment and validation of an aquarium system to evaluate salinity preference in conscious rainbow trout

Yoshio Takei*, Hiroshi Miyanishi, Shigenori Nobata, Marty K. S. Wong, Taro Watanabe, Albert Ventura, Aya Shiozawa, Susumu Hyodo and Makoto Kusakabe

Department of Marine Bioscience, Atmosphere and Ocean Research Institute, The University of Tokyo, 5–1–5 Kashiwanoha, Kashiwa, Chiba 277–8564, Japan
**E-mail: takei@aori.u-tokyo.ac.jp*

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Abstract — Anadromous salmon migrate between fresh water (FW) and seawater (SW) during their life cycle, which most likely is driven by changes in salinity preference. There have been some studies examining salinity preference in juveniles during downstream migration, but no study has yet been reported in homing adults. In this study, we established an aquarium system to evaluate salinity preference that is usable not only for juveniles but for adult fish. The aquarium consists of three areas of different salinities, a FW area, a SW area and a brackish water (BW) area, among which fish can voluntarily move. By modifying the flow rate of FW/SW and other parameters, we could maintain the salinity of the FW area at <0.5 ppt (part per thousand) and that of the SW area at >20 ppt irrespective of the depth, but the salinity varies considerably by depth between the surface and bottom layer in the BW area. Two aquaria with the same system were prepared side by side, an acclimation aquarium that allowed fish to learn the system before the experiment and an experimental aquarium that was insulated from the environment and observed by a video system. Using this aquarium system, we found that cultured rainbow trout, *Oncorhynchus mykiss*, of ca. 200 g preferred areas in the order of BW≥FW>>SW. To further validate this system, we injected various hormones that have been implicated in osmoregulation/smoltification such as angiotensin II (Ang II), cortisol, thyroid hormone (T3), arginine vasotocin (AVT) and prolactin into the third brain ventricle of the trout and observed their preference behavior. There was a tendency toward high salinity preference after injection of hormones that promote SW acclimation and/or smoltification (Ang II, cortisol T3 and AVT), but fish tended to prefer a low salinity area after injection of prolactin that promotes FW adaptation. These results suggest that the newly established aquarium system can be used to evaluate salinity preference in salmonids and will contribute to future studies in identidying key factors that motivate downstream migration of juveniles and upstream migration of mature chum salmon, *O. keta*, homing to natal rivers in the Tohoku area of Japan.

Key words: Homing migration, salmonid fish, aquarium experiment, osmoregulatory hormones, motivation

Introduction

Salmonid fish migrate for long distances during their lifespan, which has attracted the attention of many investigators around the world. After hatching in the river, the juveniles experience a parr-smolt transformation (smoltification) to prepare for downstream migration into the sea (Folmer and Dickhoff 1980, Hoar 1988). After seaward migration, they spend several years circulating in the subarctic zone of the ocean for growth and maturation, and finally come back to their natal river for spawning (homing migration).

Smoltification is an essential process for downstream migration that enables adaptation to hyperosmotic seawater (SW). In fact, hyposmoregulatory ability and salinity preference increase in juvenile salmonids during the course of smoltification (Iwata 1995, McCormick 2013). Several hormones have been implicated in smoltification and subsequent downstream migration, of which thyroid hormones (tri- and tetra-iodothyronine, T3 and T4) and cortisol seem to have critical roles (Björnsson et al. 2011, Flores et al. 2012). A T4 surge occurs before the onset of downstream migration in the chum salmon (*Oncorhynchus keta*), which is the most common homing salmonid in Japan (Iwata et al. 2003). Growth hormone (GH) and cortisol, established hormones important for SW adaptation (McCormick 2001, Takei and Loretz 2005), also stimulate salt preference in juvenile coho salmon, *O. kisutch* (Iwata et al. 1990). With respect to the downstream migration, corticotropin-releasing hormone (CRH) injected centrally was inhibitory in juvenile chinook salmon, *O. tshawytscha* (Price and Schreck 2003, Clements and Schreck 2004), and growth hormone-releasing hormone (GHRH) was stimulatory in coho salmon juveniles (Ojima and Iwata 2010).

Although no study has thus far been reported on salinity

preference in homing salmon, changes in hormone gene expression have been studied extensively in the chum salmon (Makino et al. 2007). Gonadotropin-releasing hormone (GnRH) genes were upregulated during upstream migration from the coast (Onuma et al. 2010b). The expression of the GH gene was depressed but that of the prolactin gene was maintained at high levels in the pituitary of homing fish caught near the coast (Onuma et al. 2010a). As homing adults quickly lose hyposmoregulatory ability after arriving at the coastal area (Hirano et al. 1990), the loss of this ability may be due to the loss of SW-adapting hormone (GH) and high FW-adapting hormone (prolactin). There reported a behavioral study using a salinity logger in the homing chum salmon (Kitahashi et al. 2000), but nothing is known about the changes in salinity preference. This may be due in part to the difficulty to establish a large aquarium with stable salinity difference for adult salmonids. In juveniles, a small tank with a stable vertical salinity gradient or with two layers (upper FW and lower SW) has been used to test salinity preference (Iwata et al. 1986, Price and Schreck 2003, Maksimovich 2008).

We are interested in the factor(s) that trigger downstream migration in juveniles and upstream migration into the river in mature homing salmon. It is known that they stay for some time in the brackish waters (BW) of the estuary before moving into the sea or into the river. The final decision for movement most likely accompanies the change in salinity preference, as motivation is necessary for releasing the migratory behavior. We took advantage of large aquaria in the International Coastal Research Center of AORI at Otsuchi where chum salmon migrate back for spawning, and thus attempted to establish an aquarium system to examine salinity preferences. In this aquarium test system, fish can freely move among three areas of different salinities, FW, SW and BW areas. Although there are slight differences in the salinity between the surface layer and the bottom layer, the FW area was hypotonic and the SW area was hypertonic to trout plasma throughout each area. To test the validity and relevance of this preference test aquarium, we examined the effects of various osmoregulatory hormones on the salinity preference when injected into the third ventricle of rainbow trout, *O. mykiss*. The hormones used were angiotensin II (Ang II), cortisol, T3, arginine vasotocin (AVT) and prolactin, which are known to be involved in sodium appetite in mammals and/or osmoregulation/smoltification in salmonids (Folmer and Dickhoff 1980, Iwata 1995, Geerling and Loewy 2008).

Materials and Methods

Animals

Cultured, immature rainbow trout, *Oncorhynchus mykiss* (225.7±6.0 g, n=70) were provided from Iwate Prefectural Inland Fisheries Technology Center (Iwate, Japan) or purchased from a local dealer (Senjougataki Fish Culture Center, Iwate). They were maintained in a FW aquarium (see below) for several days without feeding before use. Natural well water (approximately 0.1 ppt) and seawater in the Otsuchi Bay (approximately 34 ppt) were used as FW and SW, respectively. All experiments were approved by the Animal Experiment Committee of the University of Tokyo and performed in accordance with the Manual for Animal Experiments.

Drugs

[Asn^{1}, Val^{5}]-angiotensin II (Ang II) and [Arg^{8}]-vasotocin (AVT) were purchased from the Peptide Institute (Osaka, Japan). Ang II and AVT were dissolved in distilled water (DW) at a concentration of 10^{-3} M. Triiodothyronine (T3) and cortisol were purchased from Sigma-Aldrich (St. Louis, MO, USA). It is known that T3 is an active thyroid hormone, which is converted from T4 for action (Bentley, 1998). One milligram of T3 was dissolved in 1 ml of 1 N NaOH, and DW was added to a concentration of 10^{-3} M. One gram of cortisol was dissolved in 1 ml of ethanol, and diluted to 10^{-3} M with DW. These stock solutions were kept at −20°C until use. All of these hormones are homologous to those in the rainbow trout. Chum salmon prolactin was generously provided by Prof. Akiyoshi Takahashi, School of Marine Biosciences, Kitasato University, which was isolated from the pituitary of homing fish (Kawauchi et al. 1983). All stock solutions were diluted with autoclaved 0.9% NaCl solution (saline) prior to administration.

Surgery and intra-cerebroventricular (i.c.v.) injection

Trout were anesthetized by immersion in ca. 0.05% (w/v) 2-phenoxyethanol (Wako Pure chemicals, Osaka, Japan) for 10 min. A hole (ca. 1 mm in diameter) was made in the skull by dental drill on the mid-sagittal line 5 mm posterior from the caudal margin of the eye. A stainless steel can-

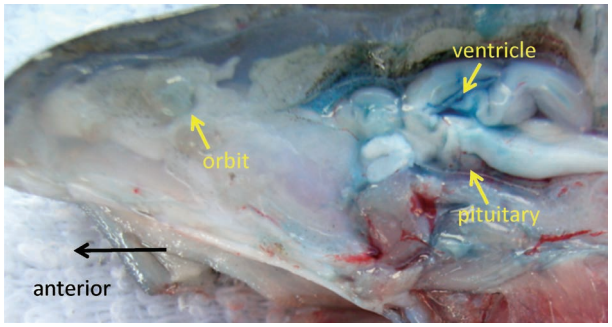

Fig. 1. A picture of a trout head cut sagittally at the midline after injection of a hormone into the third ventricle. Injected solution contained Chicago sky blue dye, showing that injection was successfully made into the ventricle of the trout.

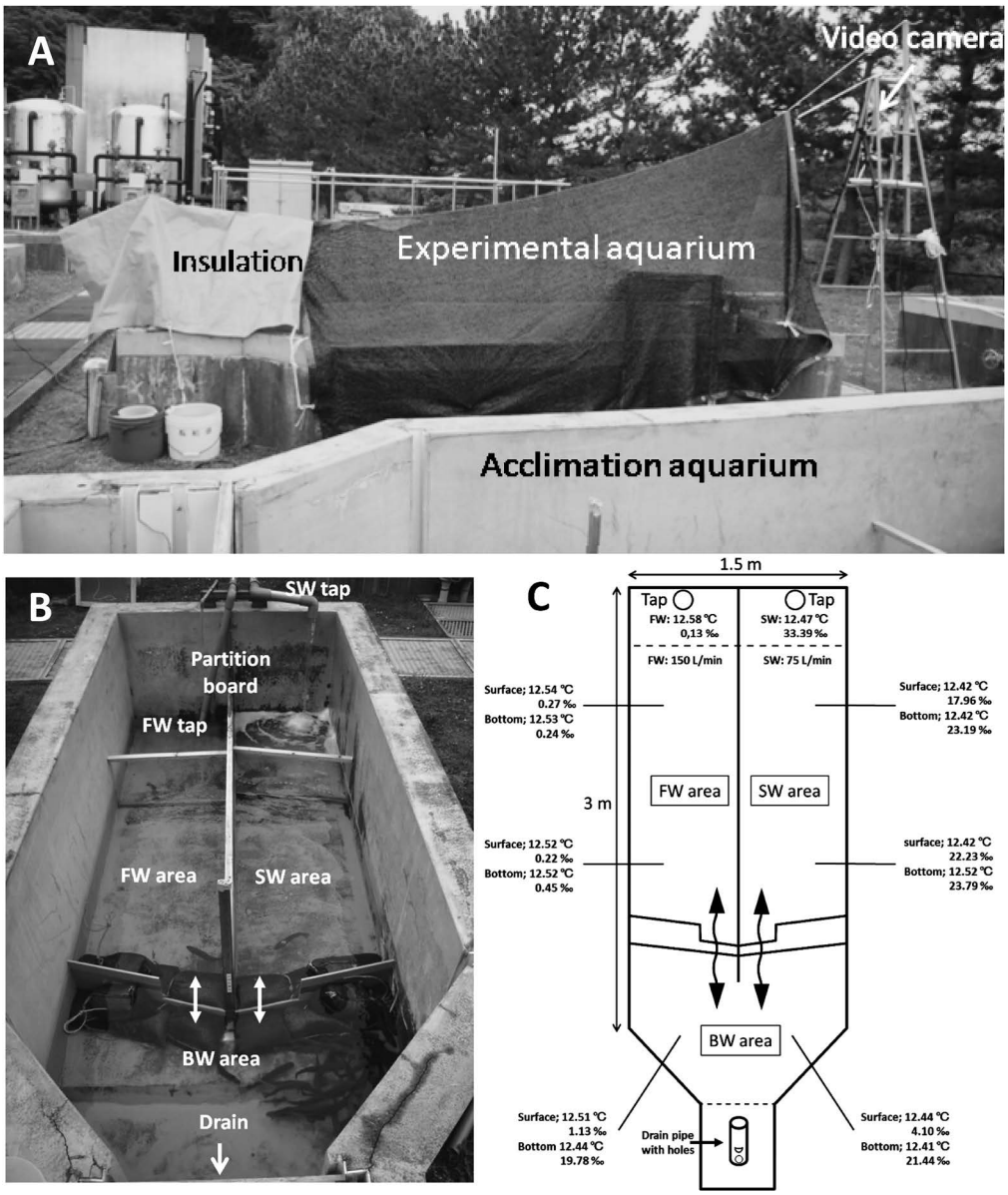

Fig. 2. (A) Outside view of the aquarium system. Experimental aquarium was insulated by shade curtains to decrease the influence from outside, and fish behavior is monitored by a video camera on the right. The acclimation aquarium is before the experimental aquarium. (B) The view of preference aquarium consisting of a freshwater (FW) area, seawater (SW) area and brackish water (BW) area. The tip of FW tap is placed in water and faced to the side wall to decrease the flow to the SW area. In this aquarium, most fish are in the BW area. (C) Schematic drawing of the preference aquarium. Salinity and temperature measured at the point in the surface and bottom layer are noted. Broken lines show the partition with a net. Height of the drainage pipe is 40 cm and holes are made as shown. FW is drained overflowing the pipe and high salinity waters through the two holes. For details, see text.

nula (o.d.: 0.3 mm) with a rubber stopper was attached to a 10 μl micro-syringe (Terumo, Tokyo Japan) by a polyethylene tube (o.d.: 0.6 mm) and inserted into the brain through the hole to a depth of 9.5–10.5 mm from the skull surface depending on the size of fish. Ang II, T3, AVT or cortisol was injected through the cannula into the third ventricle at the concentration of 10^{-4} M in saline containing 0.1% Chicago sky blue in a volume of 5 μl (5×10^{-10} moles/fish) except for prolactin which was injected at 2×10^{-4} M (10^{-9} moles/fish). To ensure the exact injection volume, the stainless steel cannula, polyethylene tube and micro-syringe were filled with liquid paraffin (Wako Pure Chemicals). Controls were injected with saline containing 0.1% Chicago sky blue. The injection into the third ventricle (i.c.v. injection) was confirmed by the presence of the dye in the ventricle after the experiment (Fig. 1). The hole in the skull was caulked by styptic gelatin sponge (Spongel: Astellas Pharma Inc., Tokyo, Japan) just after administration.

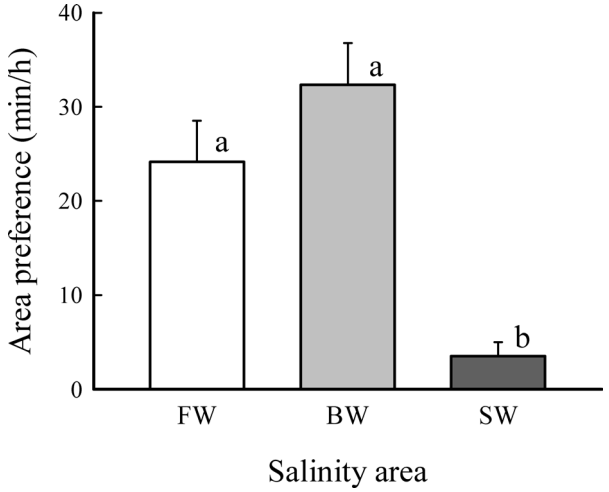

Fig. 3. Time in each salinity area (fresh water, brackish water or seawater) during 1 hour in control trout (n=27). The time in SW was significantly shorter than those in FW and BW.

Experimental protocol

Experimental and acclimation aquaria were prepared as shown in Fig. 2 at the International Coastal Research Center of AORI located at Otsuchi in the Tohoku area of Japan. In these aquaria, fish can move between the FW area and SW area through the BW area. Salinity and water temperature at the surface and bottom of each area were measured daily with logger version CTD profiler (RINKO-Profiler; JFE Advantech Co., Kobe, Japan) before and after the experiment (Fig. 2C). The experimental aquarium was covered with a shade curtain to minimize possible stress from environment (Fig. 2A). Before experimental testing, fish were kept in the acclimation aquarium of the same setup for more than 2 days to become familiarized with the aquarium organization consisting of different salinity areas (Fig. 2B). After i.c.v. injection, fish recovered during 5 min of gill perfusion and were then released in the BW area of the experimental aquarium for video recording. The behavior of the fish was recorded for 1 h with a digital camera (Sony DCR-TRV30) and the data were analyzed by a time-lapse digital video recorder system (DHV-470: Chiyoda Tokiwa Shoko Inc., Tokyo, Japan). The residence time in each of the FW, BW and SW areas during 1 h was cumulated by editing the video and the preference score was calculated by the sum of the time (min) in FW, BW and SW multiplied by 1, 3 and 5, respectively.

Statistical analysis

Results were expressed as means±SEM. Student's t-test was used for comparison between control and experimental groups. Significance was set at $P<0.05$. All statistical analyses were performed using Kyplot 5.0 software (Kyenslab, Inc., Tokyo, Japan).

Results

Establishment of the preference aquarium

Initially, salinity in the FW, BW and SW areas was adjusted by changing the flow rate from FW and SW tap, the height and position/size of the hole in the pipe used for drainage, and the size of the passageway between FW/SW area and BW area (Fig. 2C). FW overflowed through the drainage pipe and SW flowed out through the holes at the lower part of the pipe, but significant amount of FW and SW flowed into the SW and FW area, respectively, through the BW area. We adjusted these parameters and finally fixed them as follows; flow rate was 150 l/min from the FW tap and 75 l/min from the SW tap, the height of drainage pipe was 40 cm and the hole (5 cm in diameter) was at 7 cm above the bottom and a half hole 7 cm above it, and the passageway was 19 cm in width and 23 cm in height (see Fig. 2B, C). In this condition, the FW area was maintained at the salinity of <0.5 ppt in all regions and depth within the areas. As the trout used in this experiment were cultured in FW ponds, we thought it important to maintain the FW area at low salinity to offer a salinity option that was similar to the culture pond. Because of the double flow rate from the FW tap, significant FW entered into the SW area, resulting in dilution in the SW area (Fig. 2C). However, the salinity of the SW area was maintained ca. 20 ppt (60% SW), which is twice as high as that of body fluids of trout, and the salinity difference between the surface layer and the bottom layer was minimized by active bubbling. In the BW area, however, the salinity difference was fairly large according to depth. Because the temperature of well water (FW) and that of SW pumped from the Otsuchi Bay in May were similar (ca. 12.5°C), there was little difference in temperature among areas and depths.

In the acclimation aquarium, most intact fish spent time either in the FW area or BW area. Thus, we attempted to quantify the preference of controls injected with saline (n=27). We found that trout spent most of the time in BW or FW, and no difference was detected between the two areas (Fig. 3). It is not known whether fish in the BW area were in the surface or bottom in the current observation system. In contrast, the trout seldom entered the SW area with salinity of >20 ppt (only a few min in 1 h). Thus, we suspect that fish in the BW area may not be in deeper waters with a salinity of ca. 20 ppt.

Effects of i.c.v. injection of hormones on salinity preference

The i.c.v. injection of Ang II, AVT, T3, and cortisol tended to increase the salinity preference of the trout compared with controls, although the difference was not significant (Fig. 4). Detailed analyses in each area showed that Ang II tended to increase the time in the SW area, while AVT, T3

and cortisol tended to decrease the time in FW. In contrast, the i.c.v. injection of prolactin tended to decrease salinity preference in the trout, particularly decreased the time in the SW area (Fig. 5), suggesting an avoidance of high salinity. Although the effects of hormones were not significant compared with controls, there is a tendency towards high salinity preference by SW-adapting hormones and low salinity preference by a FW-adapting hormone. Hematocrit value was measured after experiment to confirm normal body fluid balance (34.2±0.4%, n=70).

Discussion

Migration, osmoregulation and hormonal regulation

Diadromous fish migrate between FW and SW twice during their life cycle. It is known that migrating fish, when going either upstream or downstream, stay in the brackish estuary for some time before entering into FW or SW. Therefore, there must be a trigger to motivate the final entry into the extremely different salinities. Two migratory species, anadromous salmonids (salmon and trout) and catadromous anguillids (eels), have been investigated with regard to the physiology of spawning migration (for review in salmonids, see Ueda 2011, McCormick 2013, in eels, see van der

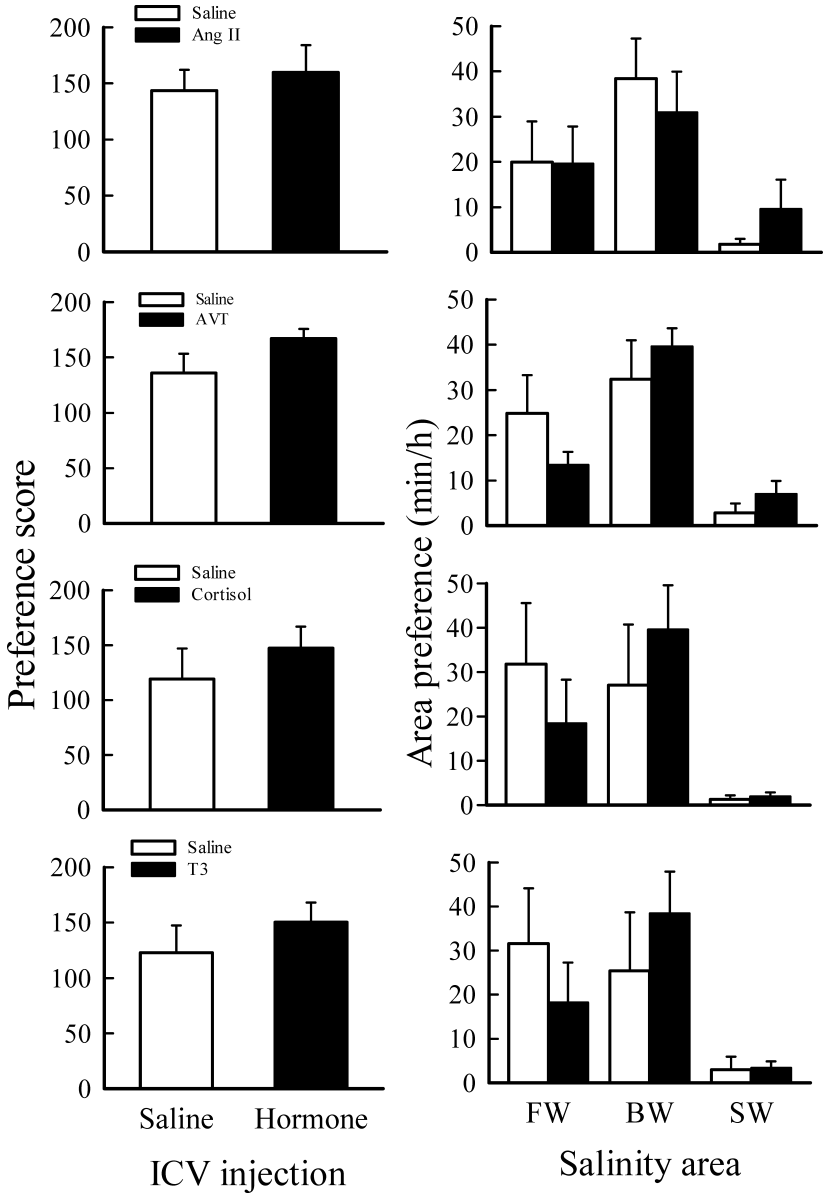

Fig. 4 Preference score and time in each salinity area (fresh water, brackish water and seawater) during 1 hour after injection of hormones (n=6 each) into the cerebral ventricle (ICV injection) in trout. Saline was injected in controls (n=4 each). For calculation of preference score, see Methods. Ang II, angiotensin II; AVT, arginine vasotocin; T3, triiodothyronine.

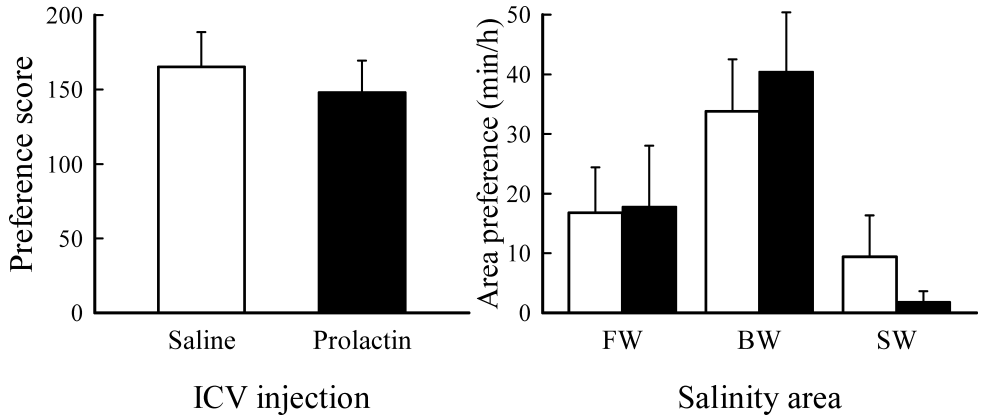

Fig. 5. Preference score and time in each salinity area (fresh water (FW), brackish water (BW) and seawater (SW)) during 1 hour after injection of prolactin (n=6) or saline (n=4) into the cerebral ventricle (ICV injection) in trout. For calculation of preference score, see Methods.

Thillart et al. 2009, Righton et al. 2012). The physiology underlying migration of juveniles to feeding grounds for growth has been investigated in salmon smolts (Iwata et al. 1986, 1990, Price and Schreck 2003) and in glass eels (Edeline et al. 2005). Migration between FW and SW requires regulation of water and ion fluxes in opposite directions, i.e., osmoregulation. In case of seaward migration, mild phenotypic changes (metamorphosis) occur to prepare for future hyposmoregulation, referred to as parr-smolt transformation (smoltification) in salmonids and silvering in eels, in which the body shape becomes streamline and the lower half of the body color changes to silver. Smolt physiology has been extensively studied in salmonids (Folmar and Dickhoff 1980, Hoar 1988, McCormick 2013).

Smoltification is a developmental event for future homeostatic adaptation to a high osmotic environment, in which hormones play critical roles. Several major hormones have been identified for regulation of smoltification in salmonids; thyroxine (T4), GH/IGF-I and cortisol for stimulation, and prolactin for inhibition (Björnsson et al. 2011). Cortisol and T4 also induce metamorphosis in fishes and other vertebrates (Tagawa et al. 1990, Kikuyama et al. 1993), and these hormones are also important for osmoregulation. GH and prolactin are pituitary hormones that are important for SW and FW adaptation, respectively, and cortisol plays permissive roles in both SW and FW adaptation in teleosts (Takei and McCormick 2013). GH and prolactin are protein hormones that induce the expression of various osmoregulatory genes and induce body changes for long-term adaptation to a new osmotic environment. Cortisol and T4 bind to their cytoplasmic receptors and function as long-acting hormones to induce gene expression of various transporter/channel genes. There are also small peptide hormones such as Ang II and AVT that are involved in SW adaptation (Takei and McCormick 2013). They are rapid-acting and change behavior and activity of existing transporters/channels to quickly cope with the environmental changes.

Important role of salinity preference in migration

Based on the data mentioned above, we started to seek the factors, particularly hormones, which play critical roles in the final stage of downstream and upstream migration. Salinity preference is thought to guide salmonids when they arrive at the river mouth where FW and SW are mixed (McInerney 1964). It is known that migrating salmonids stay in the BW of the river mouth for some time before migrating down into the ocean as in juvenile smolts or up into the natal river as in mature adults (Ueda 2011). In the river mouth, waters usually form two layers because SW has higher specific gravity and thus forms a layer below the FW flowing down the river, thus resembling the BW area of the current aquarium. Migratory behavior of wild Atlantic salmon smolts has recently been investigated along the coastal area using monitoring devices tagged to the fish (Hedger et al. 2008, Manel-La et al. 2009). These studies showed that the fish move horizontally and vertically in order to select preferred salinities and temperatures during seaward migration.

The salinity preference test has been performed on juvenile salmon and glass eels using tanks in which fishes can move between FW and SW. An example for salmonids is the tank used by Baggermann (1960), which is divided into two compartments (FW and SW) with an incomplete partition, and fish can move between the two compartments via the bridge of FW above the partition (see also McInerney 1964). A similar type of tank has also been devised with some modifications by Iwata et al. (1986). A shortcoming of this type of tank is that fish must cross the narrow and uppermost water bridge to enter water of a different salinity. Another type is a two-layered tank, which has a vertical salinity profile consisting of an upper FW layer and a lower SW layer (Price and Schreck 2003, Maksimovich 2008). The two layers are distinguishable by the visible halocline in the tank or by the use of slightly tinted SW. In these tanks, not only salinity but also hydrostatic pressure changes when fish choose a station in the vertical profile. Using these small tanks, pref-

erence studies have been performed on small fish such as fry and juvenile.

To our knowledge, however, such preference studies have not been done using mature fish homing back to their natal river. The lack of study may be due to the difficulty in preparing a large tank with a proper salinity gradient and in obtaining wild homing fish at similar stages of maturation and in sufficient numbers. In this experiment, therefore, the primary aim was to establish a large aquarium system to evaluate salinity preference in both small and large fish. Ideally, the aquarium consists of three horizontally separated areas of different salinities to mimic natural estuary conditions; a FW area (river), a SW area (bay) and a BW area (river mouth) where vertical salinity differences occur (low salinity on the surface and high salinity at the bottom). After their experience in the acclimation aquarium, fish can move among the areas according to their salinity preference when salinity preference changes. Since the trout used in this experiment were cultured in spring water (FW), and since the aim of the experiment is to detect preferred salinities, we ran FW to the FW area at a rate twice higher than that of SW to the SW area to maintain the salinity of the FW area lower than 0.5 ppt (Fig. 2C). Because of the difference in the flow rate, FW enters the SW area significantly and decrease the salinity of SW to ca. 20 ppt (original salinity of SW was 33.4 ppt). However, as the ion concentration of 20 ppt SW is twice as high as that of body fluids of trout, the fish voluntarily enter the SW area only when there is a strong salinity preference that motivates the movement into the hyperosmotic water. Salinity preference in fish may be similar to the salt appetite of terrestrial animals that motivates them to lick salts or drink aversive concentrations of NaCl solution. When rats were offered two bottles that contain tap water and 1.8% NaCl solution, rats drink only tap water (Fitzsimons 1998). However, injection of Ang II into the third ventricle induces significant intake of 1.8% NaCl solution, demonstrating the arousal of salt appetite. Similarly, entrance into the SW area indicates enhanced salinity preference.

Using this preference aquarium, we found that trout spent most of the time in BW and FW areas in the absence of administered hormones. In the BW area, the salinity differs considerably depending on the depth; <5 ppt on the surface and ca. 20 ppt at the bottom. It is possible that trout chose the depth in the BW area where the environmental water is isotonic to their body fluids, as isotonic 0.9% NaCl solution is palatable for animals and rats prefer to drink it than tap water when both are offered to them (Fitzsimons 1998) . It is also possible that fish stayed in the surface layer as we used the landlocked rainbow trout cultured in FW for this experiment, although vertical position in the water column could not be distinguished in our experimental system. Concerning the quantification of preference score, we gave the same score irrespective of the depth for fish that were in the BW area. However, preference may differ greatly between the surface and bottom layer of the BW area, but this could not be determined by video camera from above (Fig. 2A). In future experiments, therefore, it will be necessary to measure the environmental salinity using a tagged device such as salinity logger (Kitahashi et al. 2000) in order to quantify salinity preference more accurately. We are now repeating this experiment in the same preference aquarium using a new salinity logger with much smaller size and greater stability developed by Katsufumi Sato in the Biologging group of AORI.

It should also be noted that water flows rapidly at the boundary between the BW area and the FW/SW area; the flow rate from the FW area to the BW area was calculated to be 0.082 m/sec, and that form the SW area to the BW area was 0.041 m/sec, which may significantly affect the entry of fish from the BW area into the FW or SW area. It has been shown that water flow is sensed by the lateral line and regulates the rheotaxis of fish (Montgomery et al. 2013). In the current experimental setup, the flow rate against FW entry was twice that of SW entry but trout enter the FW area more frequently than the SW area. Thus, it seems that salinity preference overrides rheotaxic preference when trout chose the FW or SW area.

Using this aquarium system, we examined the effect of hormones on salinity preference when injected into the brain ventricle of trout. Ang II is an established hormone that induces drinking behavior in all vertebrate classes thus far examined except the cyclostomes (Kobayashi and Takei 1996). Ang II also induces strong salt appetite in rats and other herbivores and granivores (Geerling and Loewy 2008), but it is not known whether SW drinking induced by Ang II in marine fish is due to thirst or salt appetite. Another hormone that induces strong salt appetite is aldosterone (Geerling and Loewy 2008), which is a mineralocorticoid that has a potent sodium-retaining action on the kidney in mammals (Thomas and Harvey 2011). Therefore, Ang II and cortisol (functionally equivalent to mammalian aldosterone) are candidates that induce salinity preference in teleost fish. We also injected an important hormone for SW adaptation, AVT, and the most important hormone for smoltification, T3. We also injected prolactin, the most important hormone thus far known for FW adaptation. It is possible that SW entry is motivated by salt preference and FW entry is motivated by FW preference or inhibition of salt preference induced by these hormones.

Because of the large variation among individuals, we could not detect statistical significance in the hormone effects from controls injected with saline. The failure to detect the significance may be due in part to the depth-dependent difference in the salinity in the BW area as discussed above, and to the low sensitivity to the hormones in the rainbow

trout, a landlocked FW species. We used this species to test the validity of this aquarium system because of their availability throughout the year, but the sensitivity to salt preference may be suppressed compared with the migrating salmonid species. However, it seems that trout injected with Ang II, cortisol, AVT and T3 stay longer in the high salinity area, but prolactin extend the time in the low salinity area. These results are consistent with the role of these hormones in osmoregulation (Takei and McCormick 2013). Therefore, the preference aquarium established in the current study can be used to detect the changes in salinity preference, which regulates movement into waters with different salinities.

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