

Prolactin and Upstream Migration of the Amphidromous Teleost, Ayu *Plecoglossus altivelis*

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Changes in mRNA levels of prolactin (PRL) during the upstream migration were examined in fry of the amphidromous fish, ayu *Plecoglossus altivelis*. Quantification of mRNA has been done with real-time PCR and expressed as whole body or pituitary contents depending the body size of fry. PRL mRNA levels of ayu caught in seawater of the coastal area remained low during early spring. Prior to the start of the upstream migration, the fish caught in the coastal area in mid spring showed increased levels of PRL mRNA. There were further increases in PRL levels in the fish caught in the river. Analysis of proportions revealed that there were significant differences among PRL mRNA in the fish caught in different environmental salinities. Body weight showed a positive relation with PRL mRNA in ayu caught in seawater. A landlocked population of ayu, which migrates from lake to river, showed no significant change in PRL mRNA levels before and after upstream migration. Results in this study indicate the importance of up-regulation of PRL gene expression of ayu during the upstream migration from seawater to fresh water. There is a possible relationship between body size and PRL in the early developmental stage of ayu in seawater, but not in the fish in fresh water.

Key words: prolactin, osmoregulation, migration, fish, ayu *Plecoglossus altivelis*

INTRODUCTION

Prolactin (PRL) is a versatile hormone, which has been implicated in regulating a wide variety of functions, such as reproduction, growth, immunity, behavior, and osmoregulation (Drago, 1984; Hirano, 1986; Bentley, 1998; Bole-Feysot et al., 1998; Schradin and Anzenberger, 1999; Manzon, 2002; Yada and Nakanishi, 2002; Goffin et al., 2002; Angelier and Chastel, 2009). Importance of PRL in migratory behavior is well known in birds in relation to sexual maturation (Meier et al., 1965; Farner and Wingfield, 1980; Bentley, 1998; Holberton et al., 2008). Also in terrestrial amphibians, PRL plays an important role to stimulate "water drive" for spawning (Gona et al., 1973; Ishii et al., 1989; Rankin, 1991). In

fish, an increase in PRL gene expression has been found in chum salmon (*Oncorhynchus keta*) during the spawning migration (Makino et al., 2007; Onuma et al., 2010). However, few studies have examined the separate effect of prolactin on reproduction and migratory behavior using juvenile fish.

It is generally accepted that PRL is a hormone that promotes acclimation to fresh water (FW) in a large number of fish species, and an activation of PRL secretion has been observed in various studies after entry from seawater (SW) to FW (Hirano, 1986; McCormick, 2001; Manzon, 2002; Sakamoto and McCormick, 2006; Lee et al., 2006; Flores et al., 2012). In contrast, decreased secretion of PRL was found in SW-acclimated fish and after experimental transfer from FW to SW, since the sodium-retaining action of PRL is thought to be inhibitory for adaptation to hypertonic environments (Hirano, 1986; Hasegawa et al., 1986; Yada and Hirano, 1992; Yada et al., 1992; McCormick, 2001; Manzon, 2002; Sakamoto and McCormick, 2006). Although the importance of PRL on FW acclimation is well known, it is still unclear whether the increased expression of PRL gene is important for osmoregulation or behavioral regulation during spontaneous entry from SW to FW especially in juvenile stages without sexual maturation.

Some fish species migrate seaward just after hatching to grow in the marine habitat, and then migrate upstream to

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doi:10.2108/zs130181

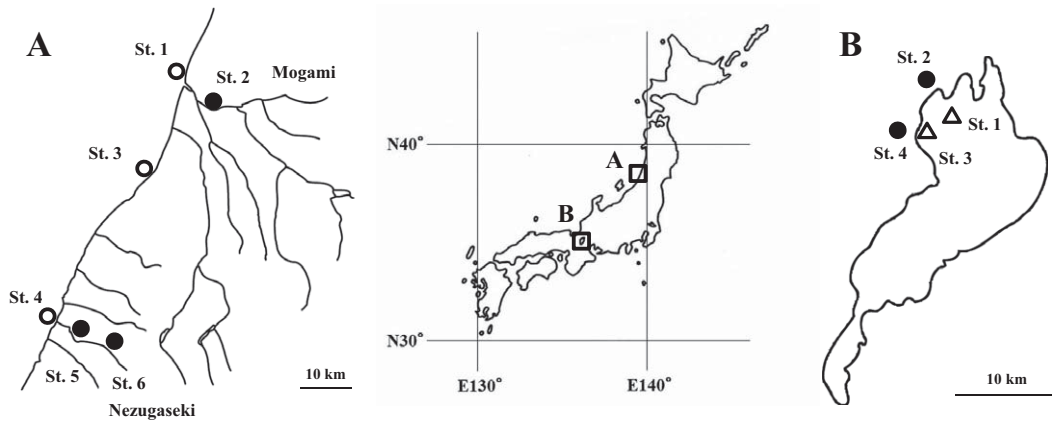


Fig. 1. Maps of the sampling sites in Yamagata (A) and Shiga (B) prefectures of Japan. Coastal (○), river (●), and lake (△) sampling sites are shown.

further grow and reproduce in FW; this type of diadromy is categorized as amphidromy (McDowall, 1992). Ayu (*Plecoglossus altivelis*) is an amphidromous salmonoid fish distributed in East Asia, and is semelparous with an annual life history (Iguchi and Tsukamoto, 2001). Immediately after hatching in autumn, larvae migrate from the bottom of rivers to drift with the current downstream and spend their early life in coastal areas over winter, and then ascend rivers in spring as juveniles (Senta and Kinoshita, 1985; Uchida et al., 1995; Takahashi and Niimi, 1998). Thus, conversion of osmoregulation from uptake to excretion of excess salts in hypertonic environments seems to be important for survival during the early stage of development in SW. Chronologically, immunoreactive-PRL cells appear in the pituitary of ayu one day before hatching (Saga et al., 1999). In a previous study by our group, whole body contents of PRL mRNA in the field samples taken during the downstream migration, in the newly hatched larvae experimentally transferred from FW to SW, and in the developing larvae reared in a vertical salinity gradient showed significant decreases after entry into SW (Yada et al., 2010).

In ayu, a landlocked population which shows migration within FW from lake to river, is available to distinguish between behavioral and osmoregulatory actions of PRL in comparison with the amphidromous population (Nishida, 1986; Takeshima et al., 2009). Ayu thus seems to be an appropriate fish species to examine a possible separate function of PRL in osmoregulation, migratory behavior, and sexual maturation. In contrast to PRL, growth hormone (GH) is known to stimulate osmoregulatory adaptation from FW to

Table 1. Position, year, date, water temperature and salinity of sampling sites, and fish body weight in Yamagata.

Site name	Latitude and longitude		Year	Date	Temperature (°C)	Salinity (ppt)	Body weight (g)
	°N	°E					
Site 1. Sakata North Bay	39.0	139.8	2006	March 23	9	28	0.17 ± 0.10
				April 27	13	28	0.71 ± 0.13
				May 30	16	27	2.37 ± 0.37
			2007	March 23	12	28	0.46 ± 0.02
				April 6	12	27	0.78 ± 0.12
				April 13	13	23	3.03 ± 0.39
2007	April 26	13	28	4.88 ± 0.62			
	May 10	16	27	2.06 ± 0.51			
2007	139.5	May 31	19	30	2.66 ± 0.30		
		May 10	14	0	1.76 ± 0.31		
Site 2. Mogami River	38.9	139.9	2007	May 10	14	0	1.76 ± 0.31
Site 3. Kamo Bay	38.8	139.7	2006	April 26	11	30	0.92 ± 0.11
				May 30	18	30	2.97 ± 0.26
Site 4. Nezugaseki Gulf	38.6	139.5	2007	May 10	16	24	0.96 ± 0.17
Site 5. Nezugaseki River, lower	38.5	139.5	2006	May 30	13	0	3.90 ± 0.33
				2007	May 10	15	0
			2006	May 30	18	0	4.38 ± 0.23
Site 6. Nezugaseki River, upper	38.5	139.6	2007	May 10	11	0	4.64 ± 0.65

Body weight is indicated as mean ± SE.

SW in several euryhaline fishes, irrespective with its structural similarity to PRL (Madsen and Bern, 1992; McCormick, 2001; Reinecke et al., 2005; Sakamoto and McCormick, 2006; Deane and Woo, 2009). In comparison with PRL, changes in mRNA levels of GH in ayu was also examined in this study.

MATERIALS AND METHODS

Animals

Wild ayu (*Plecoglossus altivelis*) were collected using a dip net or a casting net in coastal areas and rivers in Yamagata and Shiga prefectures (Fig. 1) and immediately immersed in RNA Later (Ambion, TX). Water temperature, body weight, and/or environmental salinity are shown in Tables 1 and 2. In some fish, the gonads were dissected and weighted; the gonadosomatic index (GSI, gonadal weight × 100/body weight) were less than 0.7% in all cases and identified as immature (Hirose et al., 1985). Samples were stored at -20°C until RNA extraction.

Table 2. Position, year, date, and water temperature of sampling sites, and fish body weight in Shiga.

Site name	Latitude and longitude		Year	Date	Temperature (°C)	Body weight (g)
	°N	°E				
Site 1 Momose Lake shore	35.4	136.1	2007	May 8	17	14.0 ± 1.1
Site 2 Shiotsu-Ohkawa River	35.5	136.2	2007	May 8	19	7.2 ± 0.6
Site 3 Imazugawa Lakeshore	35.4	136.0	2010	June 4	22	9.8 ± 1.3
Site 4 Imazugawa River	35.4	136.0	2010	June 4	22	12.9 ± 0.9

Body weight is indicated as mean ± SE.

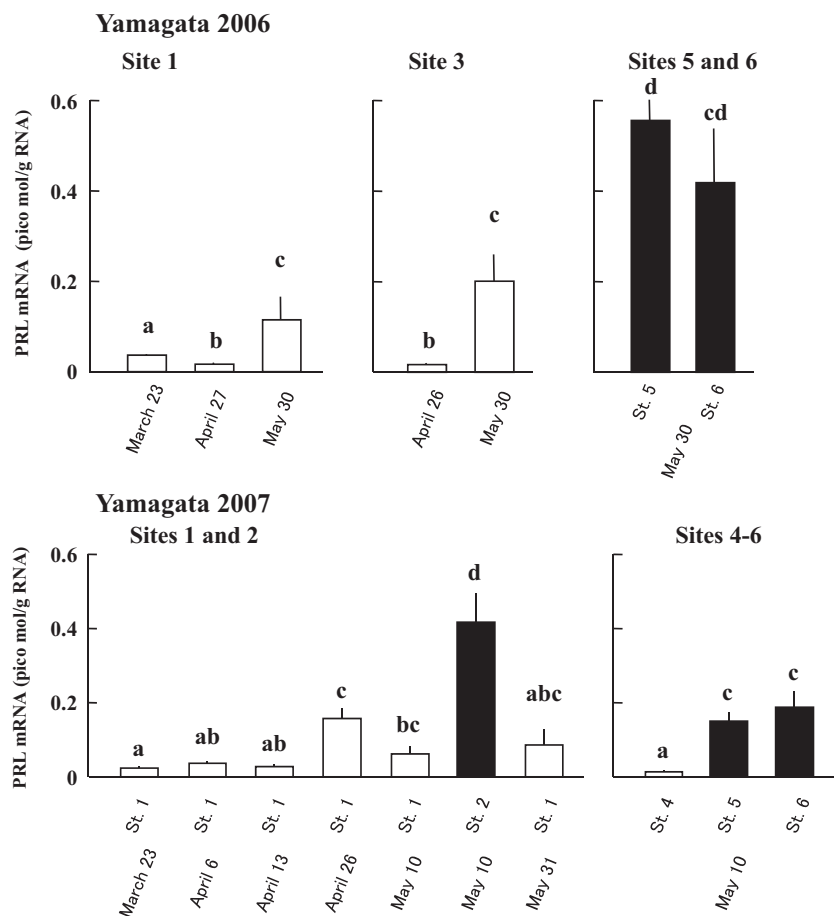


Fig. 2. Whole body contents of PRL mRNA in ayu caught in the coastal area (open column) and river (closed column) in Yamagata in 2006 and 2007. Data are expressed as means + SEM ($n = 3-10$). Different characters represent significance of difference at $P < 0.05$.

RNA extraction

Total RNA was extracted from each fry of ayu from 0.1 g body weight. When the fish were weighed more than about 1 g, RNA was prepared also in each pituitary isolated after storage in RNA Later. Whole ayu obtained from Yamagata in 2006 and 2007. They were minced using a razor blade, homogenized with a polytron homogenizer (Kinematica, Luzern) in ISOGEN (Nippon Gene, Tokyo), and total RNA was isolated following the manufacturer's manual. Pituitaries dissected from other ayu from Yamagata in 2007 and from Shiga in 2007 and 2010 were also applied for RNA extraction. Total RNA was treated with RNase-free DNase I (Takara, Shiga, Japan). After inactivation of DNase, reverse transcription was carried out using SuperScript II First-Strand Synthesis System (Invitrogen, CA).

Quantitative real-time PCR

Contents of PRL and GH mRNA of individual fish were estimated by real-time PCR (ABI Prism 7900HT) with TaqMan probes (Applied Biosystems) as described, previously (Yada et al., 2010). The partial cDNA of ayu PRL and GH, cloned and sequenced as described above, was used as the standard. The PCR mixture (10 μ L) contained 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM each of forward and reverse primers, 100 nM of fluorogenic probe, and either standard ($6 \times 10^2-6 \times 10^7$ copies/reaction) or product of reverse-transcribed RNA sample (2 ng/reaction). After denaturation at 95°C for 10 min, amplification was carried out by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Statistical analysis

After one way analysis of variance (ANOVA), significance of difference between two groups caught on different date or place was analyzed by Student's *t*-test for parametric or Mann-Whitney *U*-test for non-parametric groups. Analysis of proportions between mRNA level and external salinity was been done using the Kruskal-Wallis test. Spearman's rank correlation coefficient has been applied for analysis between mRNA level and fish body weight. Calculations were performed using a computer program, STATISTICA (Statsoft, OK).

RESULTS

Figure 2 shows the whole body contents of PRL mRNA in juvenile ayu captured in the coastal areas and rivers of Yamagata in 2006 and 2007. In 2006, ayu caught on 30 May in the coastal areas showed higher levels of PRL mRNA than before in both sites 1 and 3. On 30 May, fish caught in site 5 located about 500 m upward from the mouth of Nezugaseki River showed 3- to 4-times higher levels of PRL mRNA than levels of the fish in sites 1 and 3. Site 6 located 2 km upward from the river mouth also showed higher levels of PRL mRNA than the coastal areas on 30 May. However, there was no significant difference in PRL mRNA levels between the fish caught in site 6 and in the coastal areas on 30 May.

Whole body contents of PRL mRNA in ayu caught in the coast and river of Yamagata in 2007 showed essentially similar patterns to those in 2006. Samplings at site 1 in every two weeks indicated that PRL mRNA level on April 26 was significantly higher than before. That high level of PRL in the coast was transient and gradually decreased in May. Ayu caught in site 2 located about 1 km upward from the mouth of Mogami River showed higher PRL levels than all fish caught in site 1, significantly. Sites 5 and 6 in Nezugaseki River also showed higher levels of PRL mRNA than site 4 located in the mouth of same river. On the other hand, there was no significant difference in GH mRNA levels between sampling periods or sites in both 2006 and 2007 (Fig. 3).

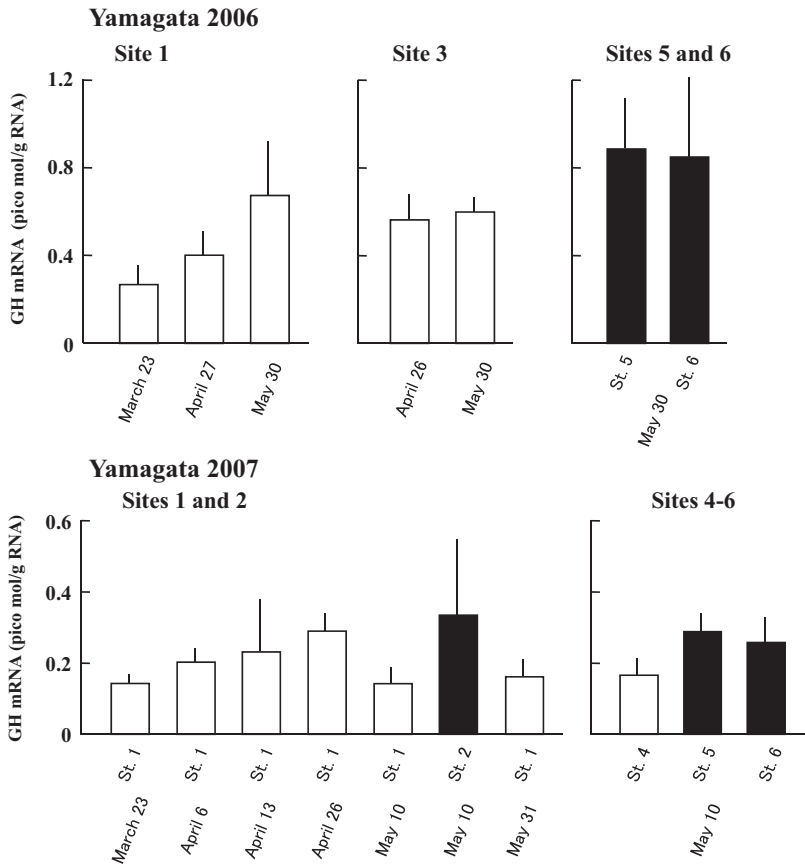


Fig. 3. Whole body contents of GH mRNA in ayu caught in the coastal area (open column) and river (closed column) in Yamagata in 2006 and 2007. Data are expressed as means + SEM ($n = 3-10$). Different characters represent significance of difference at $P < 0.05$.

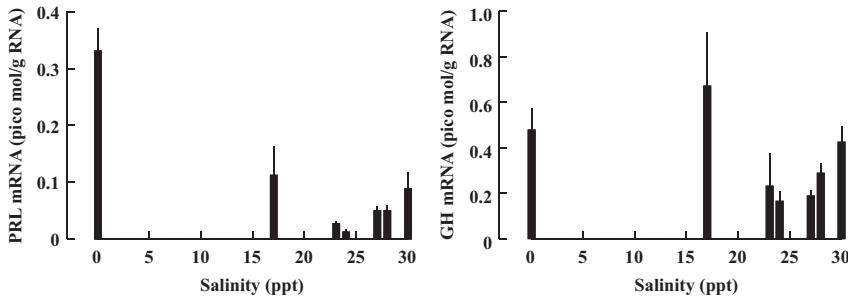


Fig. 4. Relations between salinity and whole body contents of PRL or GH mRNA in ayu caught in the coastal area in Yamagata in 2006 and 2007.

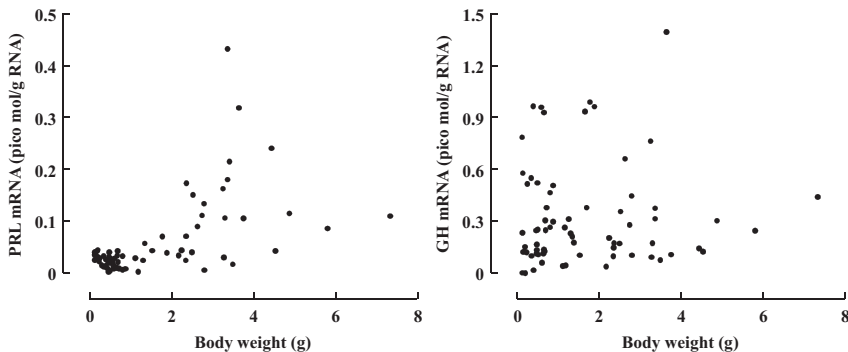


Fig. 5. Relations between body weight and whole body contents of PRL or GH mRNA in ayu caught in the coastal area in Yamagata in 2006 and 2007.

Figure 4 shows relations between environmental salinity of sampling sites and PRL or GH mRNA levels in ayu caught in Yamagata in 2006 and 2007. There were significant differences among PRL mRNA in the fish caught in different environmental salinities (Kruskal-Wallis test, $\chi^2 = 48.34$, $P < 0.01$). There was no significant relation between GH mRNA and salinity ($\chi^2 = 6.11$, $P > 0.05$). On the other hand, a positive correlation was found between body weight and PRL mRNA level in ayu caught in both river and coastal areas (Spearman's rank correlation coefficient, $r_s = 0.66$, $P < 0.05$). In Figure 5, when the fish were limited to the coastal area, there still was a positive relation between the two parameters ($r_s = 0.52$, $P < 0.05$). There was no significant relation between GH mRNA and body weight, again ($r_s = 0.10$, $P > 0.05$).

In 2007, PRL mRNA contents in each pituitary were examined in the juvenile ayu caught in the coastal areas and rivers in Yamagata for fish greater than 1 g in weight (Fig. 6). The values from dissected pituitary standardized with total RNA content were almost 10,000 \times higher than those from whole body-homogenate as described above. PRL mRNA on 26 April showed the highest levels among the fish caught in the coastal area in site 1, coinciding well with the changes observed in the whole body levels. Pituitary PRL contents of ayu in site 2 of Mogami River were significantly higher than those in the fish caught in site 1, except for 26 April. Similarly, in Nezugaseki River, an entry from SW in site 4 to FW in sites 5 and 6 resulted in a significant increase in the pituitary contents of PRL mRNA. There was no significant difference in GH mRNA levels in each pituitary between sampling periods or sites, as the results of whole body contents of mRNA (data not shown). Furthermore, there were significant differences among pituitary contents of PRL mRNA in the fish caught in different environmental salinities as shown among the whole body contents ($\chi^2 = 23.25$, $P < 0.01$). There was a positive relation between body weight and pituitary PRL, too (coast and river; $r_s = 0.79$, $P < 0.05$, coast only; $r_s = 0.84$, $P < 0.05$).

There was no significant difference in PRL mRNA contents in pituitary of land-locked ayu between the lake and river areas in Shiga in 2007 (Fig. 7). Again in 2010, PRL mRNA contents were compared between the lake and river at

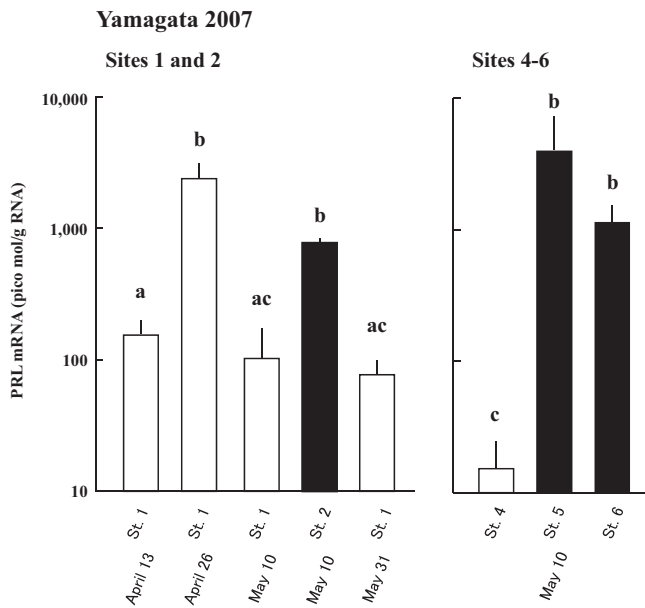


Fig. 6. Pituitary contents of PRL mRNA in ayu caught in the coastal area (open column) and river (closed column) in Yamagata in 2007. Data are expressed as means + SEM ($n = 4-6$). Different characters represent significance of difference at $P < 0.05$.

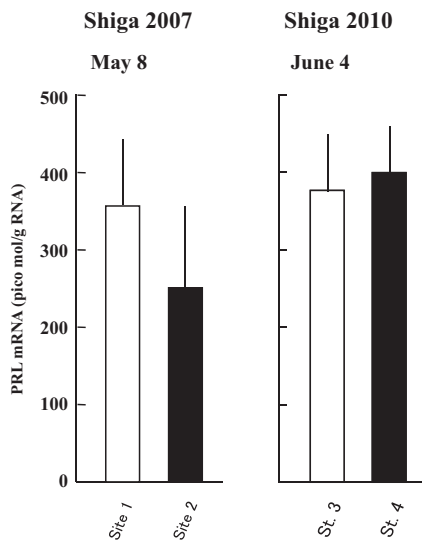


Fig. 7. Pituitary contents of PRL mRNA in ayu caught in the lake (open column) and river (closed column) in Shiga in 2007 and 2010. Data are expressed as means + SEM ($n = 6-10$).

Imazugawa; site 4 located about 100 m upward from site 3. Upward migration from the lake to river produced no significant change in PRL mRNA levels. There was no significant relation between PRL mRNA and body weight ($r_s = -0.003$).

DISCUSSION

Results in the present study showed increased levels of PRL mRNA in wild juvenile ayu after moving from the coastal area to the river. After forced experimental transfer from SW to FW, elevations of PRL production and release, which were deduced from increased plasma levels and pitu-

itary mRNA contents, have been reported in a large number of fish species (Hirano, 1986; McCormick, 2001; Manzon, 2002; Sakamoto and McCormick, 2006; Lee et al., 2006). On the other hand, few studies have examined the roles of PRL during spontaneous entry of wild fish from SW to FW in the field. Increased levels of circulating PRL and PRL-receptor mRNA in gills are observed in maturing wild sockeye salmon (*O. nerka*) during upstream migration (Flores et al., 2012). The spontaneous entry from the coastal area to the river is generally known in homing salmon, and their migratory behavior is thought to be closely linked to reproductive processes. In maturing chum salmon, influences of FW entry on plasma levels or pituitary mRNA contents of PRL are equivocal, some indicating an increase, and other reporting a decrease or no change (Hirano et al., 1985; Onuma et al., 2010). These contradictory results could be due to complex relationships between osmoregulation, behavior, and reproduction, or PRL and other sex steroids (Makino et al., 2007). Ayu is known to spawn normally from September to October, and plasma levels of sex steroids of immature ayu were much lower than the matured one (Hirose et al., 1985). The fish used in the present study from March to June were identified as immature based on their reproductive stages by the low GSI value (Yoshida et al., 2001). Juvenile ayu captured in the river showed higher whole body contents of PRL mRNA than those caught in the coastal area. In the pituitary contents, PRL mRNA levels in FW were significantly higher than those in SW. Furthermore, pituitary contents of PRL mRNA in the amphidromous ayu, which entered from SW into FW, were 4–10 times higher than those of the landlocked ayu dwelling only in FW. In wild juvenile ayu spontaneously selecting environmental salinity, higher PRL mRNA levels responding to lower salinity support the importance of up-regulation of PRL gene expression during upstream migration of amphidromous fish.

Migratory behavior of birds and amphibians is known to be driven by PRL in relation to reproduction (Meier et al., 1965; Gona et al., 1973; Ishii et al., 1989; Rankin, 1991; Bentley, 1998; Bole-Feysot et al., 1998; Holberton et al., 2008). Also in teleost species, PRL is thought to be an important regulator of behavior, especially in nesting and parental care (Ball, 1969; De Vlaming, 1979; Bentley 1998; Schradin and Anzenberger, 1999). In landlocked ayu, there was no significant difference in PRL levels between the before and after upstream migration from FW to FW. That negative result suggests that PRL is less important for migratory behavior or selection of habitat than adaptation for different salinities in this salmonoid fish species. In other words, PRL is necessary for the migration accompanying a change in environmental salinity.

The fish caught in the bay areas on 30 May, 2006, and 26 April, 2007, represented significantly higher whole body contents of PRL mRNA than before in the same sites. In 2007, serial samplings revealed that the high PRL mRNA was followed by a return to the low initial levels. That single elevation of PRL mRNA was more obvious when quantification has been done in each pituitary, suggesting an increased production of PRL prior to the entry to low salinity. In comparison of data from several years in Ishikari Bay in Japan, Makino et al. (2007) suggested that the amounts of PRL mRNA in homing chum salmon in SW were already

elevated before the entry to FW. The high level of PRL mRNA was also observed in maturing chum salmon caught in the Bering Sea in comparison with those in the Gulf of Alaska (Makino et al., 2007). Elevation of PRL mRNA levels in SW-dwelling fish seems to be paradoxical owing to the inhibitory action of PRL for adaptation to hypertonic environment (Hirano, 1986; McCormick, 2001; Manzon, 2002; Sakamoto and McCormick, 2006). On the other hand, with an opposite direction of salinity change to the present study, PRL mRNA content significantly decreased prior to a movement of larval ayu from FW to SW in a tank with vertical salinity layers (Yada et al., 2010). In the fish spontaneously migrating and selecting salinity, PRL production may change before the timing of entry to different salinities.

In contrast to the negative relation with salinity, PRL mRNA levels showed a significant positive relation to body weight in ayu caught in Yamagata. Even when the ayu was limited to the fish caught in SW, there still was a positive relation between body weight and PRL. Furthermore, that positive relation was also found for specific dates, 30 May in 2006 and 10 May in 2007 (data not shown). On the other hand, there was no significant relation between body weight and PRL levels in landlocked ayu in Shiga. These different results between the SW-dwelling and landlocked ayu suggest that the relation between body size and PRL was closely related to the environmental salinity. Larger ayu shows a tendency to migrate upstream from the coast to the river sooner than smaller fish (Tago, 2004). Similar to ayu, body size is known to relate to the timing of downstream migration in juvenile salmon; larger fish move sooner and acclimate easier to different salinities than smaller fish (Parry, 1958; Ewing et al., 1984; Bjerknes et al., 1992; Beckman et al., 1998). Water temperature is known to be an important environmental factor affecting the upstream migration of ayu in relation to the body size (Tago, 2004). However, in our previous study, there is no influence of water temperature on PRL mRNA levels in ayu larvae (Yada et al., 2010). Although there are significant interrelationships among body size, temperature, and upstream migration of ayu, the possible role of PRL in this process seems to be limited in the osmoregulatory adaptation.

As discussed above, PRL gene expression seems to be activated not only after, but also before the entry to FW in ayu. Larger ayu in SW may express PRL gene more than the smaller fish to prepare the upcoming moving to FW. A positive relation between body size and PRL mRNA levels in SW-dwelling ayu may be associated with a growth-promoting effect of PRL, which has been observed repeatedly among a wide variety to vertebrates (Ball, 1969; De Vlaming, 1979; Hirano, 1986; Bole-Feysot et al., 1998; Goffin et al., 2002). However, there is no significant relation between body size and PRL in landlocked ayu. We thus conclude that a positive relation between body size and PRL observed in ayu derives mainly from the osmoregulatory role of PRL but not from its growth-promoting action.

The variation of body size for the animals used in this study was large especially in SW-dwelling fish in Yamagata. Ayu is an annual fish and there must not be multiple year classes (Tsukamoto and Uchida, 1992). Then, one of the possible reason of variation of fish body size is the width of birth dates within a year. Ayu is known as a multiple spawn-

ing fish and the spawning period is varied for several months in autumn (Shimizu et al., 2005). And another important factor seems to be growth rate during ocean, which is varied by the opportunity to catch the food (Tsukamoto and Uchida, 1992).

One of the most important growth-promoting hormones, GH showed no significant difference in mRNA levels between sampling sites, periods, environmental salinities, or body sizes in this study. That lack of difference in GH coincided with our previous study with ayu larvae (Yada et al., 2010). In contrast to PRL, GH promotes acclimation from FW to SW in several euryhaline fishes (Madsen and Bern, 1992; McCormick, 2001; Reinecke et al., 2005; Sakamoto and McCormick, 2006; Deane and Woo, 2009). Increases in plasma levels and gene expression of GH have been observed repeatedly after entry from FW to SW (Yada et al., 1992, 1994; Sakamoto et al., 1993; Sakamoto and McCormick, 2006; Deane and Woo, 2009; McCormick et al., 2013). On the other hand, there was a significant decline in plasma levels of GH after SW entry of Atlantic salmon (*Salmo salar*), suggesting complicated changes in the kinetics of production, release, and clearance of GH (Arnesen et al., 2003). GH mRNA quantified in the present study may reflect gene transcription, but may not be a direct indicator of secretion, clearance, or reception of GH bringing about physiological functions, such as growth and SW acclimation.

Ayu during spontaneous upstream migration from SW to FW represents a necessity of PRL for adaptation to novel hypoosmotic environments. Comparisons between SW-dwelling ayu in Yamagata and landlocked ayu in Shiga imply that PRL have more important implications for osmoregulation than for behavior, growth, or reproduction in this salmonoid fish. In addition to PRL, several endocrine factors are thought to play important roles in the regulation of fish migration, such as thyroid hormone, gonadotropin-releasing hormone, corticotrophin-releasing hormone, GH, and insulin-like growth factor-I (Rankin, 1991; Iwata, 1995; Ueda and Yamauchi, 1995; Clements, 2002; Makino et al., 2007; Onuma et al., 2010; McCormick et al., 2013). The results of this study also indicate the importance of endocrine regulation during migration of amphidromous fish, and further studies are needed in a view of success of migration as survival and growth in the river.

ACKNOWLEDGMENTS

We are grateful to Professor Susumu Hyodo, Atmosphere and Ocean Research Institute, University of Tokyo, Dr. Stephen D. McCormick, Conte Anadromous Fish Research Center, United States Geological Survey, and Dr. Yasuhiko Tago, Toyama Prefectural Fisheries Research Institute, for valuable discussions and encouragements during the study. This study was supported in part by a grant-in-aid from Agriculture, Forestry and Fisheries Research Council, Japan.

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(Received December 10, 2013 / Accepted April 10, 2014)