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1	Assessment of the timing and degree of smolt development in southern populations
2	of masu salmon
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13	Running headline: PARTIAL SMOLTING OF MASU SALMON
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18	The present study assessed whether non-anadromous masu salmon Oncorhynchus
19	masou in Miyazaki, southern Japan, smoltify, and if so at what time of the year. Yearling
20	O. masou of Miyazaki and an anadromous population from Hokkaido, northern Japan,
21	were reared in hatcheries in their respective regions and sampled monthly from
22	February to June to examine the spring smoltification period. The Hokkaido population
23	showed a peak of gill Na ⁺ /K ⁺ -ATPase (NKA) activity in May, which was accompanied
24	with an increase in mRNA levels of the seawater (SW)-type NKA alpha subunit, nka
25	αlb . Increases in gill NKA activity and <i>nka alb</i> levels were not seen in Miyazaki
26	populations. Transferring yearling Miyazaki population to 70% SW (salinity of 23) in
27	mid-April resulted in an increased serum osmolality over four days. These results
28	suggest that they do not smoltify in their second spring. Next, profiles of gill NKA
29	activity and its subunit mRNA levels in under-yearling Miyazaki population in the
30	autumn were examined. Two phenotypes differing in body color during this period were
31	categorized as parr and smolt-like fish. Smolt-like fish had higher gill NKA activity
32	than parr in December while there was no significant difference in gill $nka \ \alpha lb$ levels.
33	Smolt-like fish acclimated to 70% SW better than parr as judged by lower serum

34	osmolality. However, serum osmolality in smolt-like fish did not return to the basal
35	level seven days after transfer to 70% SW, suggesting that their hypo-osmoregulatory
36	ability was not fully developed to a level comparable to anadromous populations of this
37	species. The present study suggests that, if O. masou in Miyazaki go though a
38	smoltification process, it occurs in its first autumn instead of the second spring and is
39	less pronounced compared to anadromous populations.

41 Key words: masu salmon; smoltification; Miyazaki Prefecture; Na⁺/K⁺-ATPase;
42 osmolality; autumn

INTRODUCTION

44	Masu salmon Oncorhynchus masou (Brevoot 1856) is one of the eight Pacific salmon
45	species distributed in the Asian side of Pacific Ocean. Their distribution is more
46	southerly than other Pacific salmon but spans most of Japan from Hokkaido to Kyushu
47	(Kato, 1991). The southern limit of their distribution in Japan is Miyazaki (32°N) but a
48	land-locked strain is found even further south in Taiwan (Kato, 1991; Kimura, 1989).
49	Anadromy of O. masou declines with latitude (Malyutina et al., 2009; Morita
50	& Nagasawa, 2010) as is the case for other salmonids (Dodson et al., 2013; Morita et al.,
51	2014). In Hokkaido (42°N), the latitudinal middle of their distribution, the majority of
52	females and minority of males are anadromous, going through parr-smolt
53	transformation (smoltification) in the second spring of their life (Kubo, 1980).
54	Smoltification is a series of pre-adaptive changes in morphology, behavior and
55	physiology by which river-dwelling parr become ocean-type smolt (Wedemeyer et al.,
56	1980; Hoar, 1988; Stefansson et al., 2008; Björnsson et al., 2011; McCormick, 2009,
57	2013). In one rearing experiment using O. masou of Hokkaido, 62% of females and
58	26% of males became yearling smolts when they were fed twice a ration at 1-2%/body

59	mass and reared between 5-12°C (Sano and Ozaki, 1969). In Miyazaki, on the other
60	hand, both females and males are believed to be non-anadromous (Kimura, 1989) due to
61	the warm Kuroshio Current running along the Miyazaki coast which reaches 30°C
62	during summer. This lethally high water temperature during the summer is a physical
63	barrier setting them in a unique situation like "seasonal landlocking".
64	During the last glacial period, however, O. masou in Miyazaki were
65	presumably anadromous since a landlocked strain of this species exists in the high
66	altitude of the far south Taiwan Island (24°N). Increases in water temperature along the
67	Miyazaki coast after the end of the last glacial period should have a negative impact on
68	seaward migration of O. masou for thousands of years and reduced selection on
69	anadromous lifestyle over many generations. However, it is not known whether they
70	abandoned the intrinsic rhythm of smoltification or if they smoltify in the spring or at
71	another time of the year.
72	O. masou may undergo smoltification in the autumn. In fact, a subspecies of
73	O. masou, amago salmon O. masou ishikawae (Jordan and McGregor 1925), smoltify
74	and migrate to the ocean in the autumn to avoid warm seawater influenced by the

75Kuroshio Current during the spring and summer (Kato, 1991). They spend a half-year in 76 coastal waters and return to the rivers around May before the seawater temperature rises. 77Thus, it is possible that O. masou in Miyazaki have shifted their timing of smoltification 78from the spring to the autumn. 79Acquisition of hypo-osmoregulatory ability is one of the most important 80 physiological changes during smoltification. The gills are a key organ responsible for 81 extruding sodium and chloride ions, using Na^+/K^+ -ATPase (NKA; the sodium pump) as 82 a driving force (Evans, 2008; Hiroi & McCormick, 2012; Hwang et al., 2011; Takei et 83 al., 2014). The activity of gill NKA is often used as an indicator of 84 hypo-osmoregulatory ability. NKA is composed of two essential subunits, α and β , and one regulatory 85 86 subunit, y (Blanco & Mercer, 1998; Mobasheri et al., 2000; Geering, 2006, 2008). 87 Multiple isoforms of NKA α and β subunits have been found in vertebrates (Blanco & 88 Mercer, 1998; Geering, 2008), and teleosts possess additional isoforms due to an extra 89 round of whole genome duplication (Rajarao et al., 2001; Serluca et al., 2001; Dalziel et 90 al., 2014). There are five isoforms of NKA α subunits in salmonids, of which an

91	isoform named α 1b is considered as seawater type while α 1a is a freshwater type based
92	on their responses to salinity increases (Richards et al., 2003; Bystriansky et al., 2006;
93	Madsen et al., 2009; McCormick et al., 2009). Gill NKA alb both at mRNA and
94	protein levels increase during smoltification of Atlantic salmon Salmo salar (L. 1758)
95	together with gill NKA activity (Nilsen et al., 2003, 2007; McCormick et al., 2013).
96	Much less is known about profiles of NKA β subunit isoforms during
97	smoltification. NKA β subunit localizes the catalytic α subunit on the cell membrane
98	and increases the translation efficiency of the α subunit (Blanco & Mercer, 1998;
99	Rajasekaran <i>et al.</i> , 2004; Geering, 2008). Four isoforms of NKA β subunit (β 1a, β 1b,
100	β 3a and β 3b) have been identified in rainbow trout <i>O. mykiss</i> (Walbaum 1792) and <i>S.</i>
101	salar (Gharbi et al., 2004, 2005). A few studies dealt with changes in gill $nka \beta l$ subunit
102	during smoltification of S. salar and reported it increased in parallel with $nka \alpha$ subunit
103	and increased NKA abundance during that period (Seidelin et al., 2001; Nilsen et al.,
104	2007). However, there are at present no studies measuring isoforms of $nka \beta l$ subunits
105	during smoltification.

Based on their osmoregulatory role during smoltification and seawater

107	acclimation, comparing profiles of gill NKA activity and its subunits between
108	anadromous and non-anadromous population is a useful tool to reveal the degree of
109	smoltification in O. masou in Miyazaki. The aims of the present study were to examine
110	whether these fish undergo smoltification in their second spring or the first fall and, if
111	so, evaluate the degree of smoltification in terms of their hypo-osmoregulatory ability.
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113	
114	MATERIALS AND METHODS
115	FISH REARING
116	Yearling O. masou in Hokkaido
117	O. masou of Hokkaido population were obtained from the hatchery of the South Branch
118	of the Salmon and Freshwater Fisheries Institute, Hokkaido Research Organization
119	(42°N; Futami-gun, Hokkaido, Japan). Eggs were collected from returning adults that
120	were released from the hatchery as smolts and returned from the ocean. Alevin were
121	maintained in indoor raceways under dark and fry were moved to outdoor ponds (24.6 x
122	3.5 m) run through river water in the spring. Fish were maintained in the same outdoor

123 ponds throughout the sampling period from February to June 2011 (water temperature

124 range: 4 - 14°C) and fed twice or three times a day on a commercial diet (Nippon

125 Formula Feed, Kanagawa, Japan) with standard rations at 0.4-1.9% per body weight.

126

127 Yearling O. masou in Miyazaki

128Yearling O. masou of Miyazaki populations were obtained from a local fish farm at 129 Gokase (33°N; Nishiusuki-gun, Miyazaki, Japan) from April to June 2014 (water 130 temperature range:9-13°C) and Kobayashi Branch, Miyazaki Prefectural Fisheries 131 Research Institute (32°N; Kobayashi, Miyazaki, Japan) from February to April 2015 132(water temperature range:8-15°C). Fish at the local farm were a captive broodstock and 133 were maintained in the river water in outdoor ponds (4 x 20 m) and fed five times a 134week on a commercial diet (Scientific Feed Laboratory, Tokyo, Japan) at 1.3%/body 135 weight. Fish at Kobayashi Branch were also a captive broodstock and reared in indoor 136 tanks with a standard ration (1.3%/body weight) until use. In February 2015, they were 137 moved to a 500-1 circular fibre-reinforced plastic (FRP) tank and reared until April 2015 138 in cooled water simulating the local river water temperatures (8-15°C).

140	Under-year	ling O. 1	masou in	Miyazaki
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141 Under-yearling *O. masou* were also reared at the local fish farm in Gokase, Miyazaki as
142 described above. Two phenotypes (parr and smolt-like fish) were sorted based on the
143 silvery color and visibility of parr marks on the body and sampled from September to
144 December 2014.

145

146 SEASONAL SAMPLING

147Experiments and samplings were carried out in accordance with the 148guidelines of Hokkaido University Animal Care and Use Committees (#17-0064). 149 Seven to eight fish were sampled from each month, region and phenotype. Fish were 150anesthetized by 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and measured 151for fork length (L_F) and body wet mass (M_W) . Condition factor (K) was calculated as follows: $M_W L_F^{-3}x$ 100. Gill arches were excised and a block of gill filaments was 152153immediately frozen on dry ice and stored at -80°C until analyzed for NKA activity. 154Another block of gill filaments was immersed in RNAlater (Ambion Inc., Austin, TX,

155 U.S.A.) and stored overnight at 4°C, then frozen and stored in RNAlater at -30°C until

156 laboratory processing for quantification of mRNA.

157

- 158 SEAWATER CHALLENGE TEST
- 159 Yearling O. masou in Miyazaki
- 160 On 14 April 2015, yearling *O. masou* of Miyazaki were transferred to 70%, recirculated

161 artificial seawater (70% SW; salinity of 23) (Napqo, Tokyo, Japan) in four 100-1 tanks

- 162 installed with a portable filter system (PowerBox 55, Kotobuki, Nara, Japan) in
- 163 Kobayashi Branch. Water temperature was maintained at 13.5°C by placing the
- 164 experimental tanks in a larger tank run through cooled water. Food was withheld during
- 165 the experimental period. Fish were sampled 0, 1, 2 and 4 days after transfer as described
- above. Blood was collected using syringe, transferred to a 1.5 ml centrifuge tube and
- 167 kept overnight at 4°C. After centrifuging at 8,000 g for 10 min at 4°C, serum was
- 168 collected and stored at -80°C until analysis for osmolality.

169

170 Under-yearling O. masou in Miyazaki

171	On 24 November 2016, parr and smolt-like fish of under-yearling O. masou reared at
172	the local fish farm at Gokase were transferred by a truck installed with a freshwater tank
173	with oxygenation to Nobeoka Marine Experimental Station (Nobeoka, Miyazaki, Japan)
174	and placed in 70% artificial, recirculated SW in 500-1 tanks with filter system. Water
175	temperature was maintained at 10°C by a water chiller. Fish were sampled 0, 1, 2, 4 and
176	7 days after transfer as described above. Fish were not fed throughout the experimental
177	period and sampled as described above.
178	
179	NA ⁺ /K ⁺ -ATPASE ACITIVITY ASSAY
180	Gill NKA activity was measured according to Quabius et al. (1997) with minor
181	modification (i.e. correction of a wrong concentration of sulfuric acid). Protein

- 182concentration was measured by using BCA (bicinchoninic acid) Protein Assay Kit
- (Thermo Scientific, IL, U.S.A.). The activity was expressed as Pi (μ mol) per protein 183

184(mg) per time (h).

185

186 RNA EXTRACTION AND CDNA SYNTHESIS

187	Total RNA was extracted from the gills using ISOGEN (Nippon gene; Tokyo, Japan)
188	according to the manufacturer's instruction. One and half micrograms of RNA was
189	reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad,
190	CA, U.S.A.) in a 10-µl reaction according to the manufacturer's instruction. cDNA was
191	stored at -30°C until use.

193 REAL-TIME QUANTITATIVE PCR (QPCR)

194 Sequences of primers for qPCR of *nka* αla , *nka* αlb and *ef-la* were the same as

- 195 described in Nakajima *et al.* (2014) (Table 1). Primers for qPCR of *nka* βla and *nka* βlb
- 196 were designed based on their sequences in O. mykiss (Genbank accession #CA374089
- and #CB492131; Gharbi et al., 2004) (Table I). Reverse transcribed-PCRs using these
- 198 primers were performed to prepare assay standards for O. masou. PCR products run on
- 199 1.5% agarose gel were excised and purified using QIAEX II Gel Extraction Kit (Qiagen,
- 200 Valencia, CA, U.S.A.). Copy numbers of the purified amplicon were calculated from the
- 201 molecular weight of the amplion and concentration. The standard cDNA were serially
- 202 diluted from 1×10^7 to 3×10^2 copies.

203	qPCR was set up using Power SYBR Green PCR Master Mix (Applied
204	Biosystems, Carlsbad, CA, U.S.A.) in a reaction volume of 20 μl with primer
205	concentration of 100 nM. qPCR was run on a 7300 Sequence Detector (Applied
206	Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min,
207	95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Measured
208	values were expressed as relative to those of <i>ef-1a</i> . Performance of qPCR was evaluated
209	by confirming a single peak of the dissociation curve in each assay and calculating the
210	amplification efficiencies of the standard curves, which were within the range of
211	97-100%. Coefficients of determination of the standard curves were also between
212	0.99-1.00.
213	
214	SERUM OSMOLALITY MEASUREMENT
215	Osmolality in serum was measured by using a vapor pressure osmometer (Wescor 5500;
216	Logan, UT, U.S.A.). Ten microliters of serum was used for the measurement.
217	

218 STATISTICAL ANALYSIS

219	Results on yearling O. masou in Hokkaido and Miyazaki were analyzed by one-way
220	ANOVA using the JMP program (SAS Institute Inc., Cary, NC, U.S.A.) followed by the
221	Fisher's protected least significant difference (PLSD) test. Results on under-yearling O.
222	masou in Miyazaki were first analyzed by two-way ANOVA (phenotype x time). When
223	significant effects were found, differences were further identified by one-way ANOVA
224	followed by the Fisher's PLSD test. Differences among groups were considered to be
225	significant at $P < 0.05$.
226	
227	
228	RESULTS
228 229	RESULTS Body size (L_F and M_w) of yearling <i>O. masou</i> in Miyazaki was larger than that of
228 229 230	RESULTS Body size (L_F and M_w) of yearling <i>O. masou</i> in Miyazaki was larger than that of Hokkaido salmon throughout the sampling period (from February to June) [L_F : two-way
228 229 230 231	RESULTS Body size (L_F and M_w) of yearling <i>O. masou</i> in Miyazaki was larger than that of Hokkaido salmon throughout the sampling period (from February to June) [L_F : two-way ANOVA, $F_{1,70} = 343.11$, <i>P</i> <0.001; M_w : two-way ANOVA, $F_{1,70} = 245.34$, <i>P</i> <0.001;
228 229 230 231 232	RESULTS Body size (L_F and M_w) of yearling <i>O. masou</i> in Miyazaki was larger than that of Hokkaido salmon throughout the sampling period (from February to June) [L_F : two-way ANOVA, $F_{1, 70} = 343.11$, $P < 0.001$; M_w : two-way ANOVA, $F_{1,70} = 245.34$, $P < 0.001$; Fig. 1]. In both groups, average <i>K</i> values were the lowest in May followed by
228 229 230 231 232 233	RESULTS Body size (L_F and M_w) of yearling <i>O. masou</i> in Miyazaki was larger than that of Hokkaido salmon throughout the sampling period (from February to June) [L_F : two-way ANOVA, $F_{1,70} = 343.11$, $P < 0.001$; M_w : two-way ANOVA, $F_{1,70} = 245.34$, $P < 0.001$; Fig. 1]. In both groups, average <i>K</i> values were the lowest in May followed by significant increases in June [Hokkaido: Fisher's PLSD ANOVA, $F_{4,30} = 12.10$,

235	Gill NKA activity in yearling O. masou in Hokkaido showed an increase from
236	March to April, peaked in May and decreased to near the basal level in June [Fisher's
237	PLSD ANOVA, $F_{4,30} = 8.93$, $P < 0.001$, Fig. 2(a)]. O. masou in Miyazaki also tended to
238	increase in gill NKA activity from March to May, but its highest values were only about
239	half of those in Hokkaido [Fig. 2(b)].
240	Gill <i>nka</i> αla mRNA levels were unchanged during spring both in yearling <i>O</i> .
241	masou in Hokkaido and Miyazaki and their levels were similar between the two groups
242	[Fig. 3(a), (b)]. On the other hand, yearling O. masou in Hokkaido showed a sharp peak
243	of gill <i>nka</i> αlb levels in May [Fisher's PLSD ANOVA, $F_{4,30} = 18.72$, $P < 0.001$; Fig.
244	3(c)] while that in Miyazaki salmon was remained at the basal level [Fig. 3(d)]. Both
245	gill <i>nka</i> βla and βlb levels in fish in Hokkaido also showed peaks in May [βla :Fisher's
246	PLSD ANOVA, $F_{4,29} = 10.00$, $P < 0.001$; βIb : Fisher's PLSD ANOVA, $F_{4,30} = 12.52$, P
247	<0.001; Fig. 4(a), (b)]. There was a significant increase in gill <i>nka</i> βla levels from
248	February to April in fish in Miyazaki [Fisher's PLSD ANOVA, $F_{2,18} = 6.03$, $P < 0.01$;
249	Fig. 4(c)] whereas gill <i>nka</i> βlb levels remained constant during that period [Fig. 4(d)].
250	Transferring yearling O. masou in Miyazaki from freshwater to 70% SW

252which it gradually decreased until day 4 after transfer but still significantly higher than 253those of the initial controls in freshwater [Fisher's PLSD ANOVA, $F_{3,25} = 11.87$, P 254<0.001; Fig. 5(a)]. Gill NKA activity tended to increase, but not significantly, until 4 255days after transfer [Fig. 5(b)]. 256Body size and K were compared between under-yearling parr and smolt-like 257fish in Miyazaki during fall [Fig. 6]. L_F was not significantly different between the two 258groups except in November, when smolt-like fish were larger than parr [two-way 259ANOVA, , $F_{1,55} = 8.14$, P < 0.001; Fig. 6(a)]. M_w was also similar between the two 260groups except in October, when parr was larger than smolt-like fish [two-way ANOVA, 261 $F_{1,55} = 4.66, P < 0.01$; Fig. 6(b)]. Overall, both phenotypes decreased K during the 262autumn and smolt-fish had lower K than parr [phenotype: two-way ANOVA, $F_{1,55}$ = 16.36, P < 0.001; month: two-way ANOVA, $F_{3,55} = 4.66$, P < 0.001; Fig. 6(c)]. 263264There was an overall effect of phenotype on gill NKA activity, which was 265higher in smolt-like fish (two-way ANOVA, $F_{1,56} = 6.48$, P < 0.05). The activity was

(salinity of 23) in mid-April resulted in an increase in serum osmolality on day 1, after

251

266 similar in parr and smolt-like fish during September and October, but smolt-like fish

267 exhibited significantly higher NKA activity than part in November and December 268 [Fisher's PLSD ANOVA, $F_{1,56} = 2.81$, P < 0.05; Fig. 7].

269	Overall, gill <i>nka</i> αla mRNA levels were higher in parr [two-way ANOVA,
270	$F_{1,55} = 4.16$, $P < 0.05$; Fig. 8(a)] but αlb mRNA levels were not significantly different
271	between phenotypes and remained relatively constant during September and December
272	[Fig. 8(b)]. Gill <i>nka</i> βla showed an increase in October in both phenotypes (two-way
273	ANOVA, $F_{1,55} = 13.66$, $P < 0.001$), but it remained constant thereafter and no significant
274	difference was seen between parr and smolt-like fish [Fig. 8(c)]. Gill nka βlb in both
275	phenotypes showed a gradual increase from September to December (two-way ANOVA,
276	$F_{3,55} = 6.74$, $P < 0.001$) and did not differ between parr and smolt-like fish [Fig. 8(c)].
277	When parr and smolt-like fish were transferred from freshwater to 70% SW,
278	there were overall effects of phenotype and time and their interaction on serum
279	osmolality (phenotype: two-way ANOVA, $F_{1,70} = 61.13$, $P < 0.001$; time: two-way
280	ANOVA, $F_{4,70} = 38.47$, $P < 0.001$; interaction: two-way ANOVA, $F_{1,70} = 10.11$, P
281	<0.001). The degree of increase in serum osmolality on day 1 after transfer was lower in
282	smolt-like fish than in parr [Fisher's PLSD ANOVA, $F_{9,70} = 26.74$, $P < 0.001$; Fig. 9(a)].

283	Serum osmolality in parr remained relatively high for 7 days. Serum osmolality in
284	smolt-like fish increased over 7 days as compared to the level in initial freshwater
285	controls. Gill NKA activity in smolt-like fish was higher than that in parr throughout the
286	experimental period [two-way ANOVA, $F_{1,70} = 68.30$, $P < 0.001$; Fig. 9(b)].
287	

- 288
- 289 DISCUSSION
- *O. masou* in Miyazaki may have been prevented from spring migration by warm
 seawater temperature, but they still may have opportunities to migrate to the ocean
 during the autumn and/or winter. Such a migratory strategy may have lead to increased
 ability of Miyazaki *O. masou* to tolerate seawater in autumn. In order to test this
 hypothesis, the present study evaluated their smoltification status in the spring and
 autumn.
- 2003; Björnsson *et al.*, 2012), one of the characteristic changes during smoltification is 2008 an increase in gill NKA activity. Since the activity is generally correlated with

299whole-body hypo-osmoregulatory ability, it is often used as an index of smoltification. 300 Moreover, recent findings showed that one of NKA α 1 isoforms, α 1b, is responsible for 301 branchial NKA function in salmon in seawater and also increases during smoltification. 302 Other parameters commonly used as indices of smoltifiation are silvering of body color 303 and a reduction of condition factor. 304 The results of the present study suggest that O. masou in Miyazaki do not 305 increase hypo-osmoregulatory ability in their second spring, which contrast with O. 306 masou in Hokkaido which show clear evidence of smolt development in spring (Kubo, 307 1980). Gill NKA activity in Hokkaido population showed a clear peak in May, 308 corresponding to their active migration period. This increase in gill NKA was 309 accompanied with increases in *nka* alb as reported in a previous study (Nakajima et al., 310 2014). In addition, *nka* βla and βlb subunits, which were measured separately for the 311 first time, also peaked in May. Parallel increases in αlb and βl subunits are in good 312agreement with the findings in S. salar (Nilsen et al., 2007). The present study suggests 313 that both NKA β 1 subunits play a similar role in enhancing the localization of NKA α 1b 314 subunit to the cell membrane and thus promoting the development of

315	hypo-osmoregulatory ability during smoltification. Although there was a peak in gill
316	NKA activity in Miyazaki population in May, its level was as low as that of parr in
317	Hokkaido and they had no increases in gill <i>nka</i> αlb , βla and βlb subunits. In addition,
318	their hypo-osmoregulatory ability was not high enough to restore serum osmolality
319	down to the basal level 4 days after transfer to 70% SW (salinity of 23). Although a
320	direct comparison cannot be made, smolts of Hokkaido population were capable of
321	restoring increased serum sodium ion levels within 24 h after full-strength SW (Ban et
322	al., 1987). Based on these findings, they unlikely go through a smoltification process in
323	their second spring.
324	Exact mechanisms for why O. masou in Miyazaki do not smoltify in the
325	spring are not known, but one reason may be due to a conflict between smolting and
326	maturation. Initiation of maturation in freshwater is known to inhibit smolting (Thorpe,
327	1986, 1994). High water temperature in Miyazaki accelerates growth, which in turn
328	promotes maturation in freshwater and inhibits smoltification in the second spring.
329	When body size was compared between Miyazaki and Hokkaiod populations, the

331	populations from March to May, some fish of both sexes had developing gonads (data
332	not shown), suggesting sexual maturation had already begun in this group. In support of
333	this suggestion, Morita & Nagasawa (2010) revealed by a combination of field survey
334	and modeling that warmer water temperature increased freshwater residency of O.
335	masou populations in northern Japan through improving early growth conditions.
336	Next, the possibility that they smoltify in their first autumn was assessed.
337	When comparing parr and smolt-like fish, smolt-like fish had higher gill NKA activity
338	than parr in December. However, the higher gill NKA activity was not accompanied
339	with higher gill <i>nka</i> αlb or βl subunits. These results suggest that the degree of the
340	acquisition of hypo-osmoregulatory ability in smolt-like fish in Miyazaki was not as
341	high as that of smolts in Hokkaido and that their preparatory changes for marine life
342	were not complete. This assumption was supported by the result of 70% SW transfer
343	experiment. Smolt-like fish had serum osmolality lower than that in parr throughout the
344	experimental period for 7 days. However, serum osmolality in smolt-like fish continued
345	to increase from 2 to 7 days after transfer to a diluted SW (70%).

It is of note that no/little increase in gill *nka* αlb and βl subunits in smolt-like

347	Miyazaki population may be characteristics of autumn smoltification. The patterns
348	observed in the present study were similar to those in O. masou ishikawae (Nakajima et
349	al., 2014), where increased NKA activity was not accompanied with $nka \ alb$ change.
350	Thus, decreasing water temperature or/and photoperiod during the autumn might cause
351	such discordant profiles of NKA activity and $nka \ alb$. Our unpublished data showed
352	that gill <i>nka</i> αlb levels in smolt-like fish in the autumn increased following seawater
353	transfer (Uchida et al., unpublished data), suggesting that this phenotype does not
354	increase gill αlb mRNA levels until they are actually exposed to seawater.
355	Smolting in the autumn has been reported in Chinook salmon O. tshawytscha
356	(Walbaum 1792) (Ewing et al., 1979; Youngson et al., 1983; Healey, 1991; Beckman &
357	Dickhoff, 1998; Schroeder et al., 2016). Autumn smolts in this species had increased
358	gill NKA activity and down migrated the rivers and entered the ocean (Beckman and
359	Dickhoff, 1998; Schroeder et al., 2016). In the case of S. salar in sourthern England,
360	about 25% of juveniles become autumn migrants but they were not sufficiently
361	physiologically adapted to permit permanent or early, entry into the marine environment
362	(Riley et al., 2008). Profiles of smolt-related characters of O. masou in Miyazaki may

363	be comparable to that of S. salar in sourthern England in terms of limited ability to
364	hypo-osmoregulate. Thus, it is not known if smolt-like fish in Miyazaki actually down
365	migrate the river and enter the ocean.
366	The present study was unable to examine an interaction between genetic and
367	environmental factors since O. masou populations with different genetic backgrounds
368	were reared at different environments (i.e. in Hokkaido and Miyazaki). O. masou in
369	Miyazaki were reared at higher water temperatures with enough feed throughout the
370	sampling period resulting in larger body size compared to the age-matched Hokkaido
371	population. Thus, the life-history patterns of Miyazaki population described in the
372	present study might be simply environmental responses without genetic difference. It is
373	possible that autumn smoltification might be driven by accelerated growth and
374	consequently spring smoltification might be blocked by the initiation of maturation in
375	freshwater. A common garden experiment using both O. masou populations from
376	Hokkaido and Miyazaki should disentangle environmental effect from genetic
377	influence.

The results of the present study are relevant to aquaculture in Miyazaki. There

379	is a growing interest in sea cage aquaculture for <i>O. masou</i> in this region as a local brand.
380	However, a challenge is that fish farmers need to transfer fish to seawater in the winter
381	due to lethally high warm seawater temperatures during the summer. A direct transfer of
382	under-yearling fish in the winter resulted in a high rate of mortality (approximately
383	70%) (Uchida et al., unpublished data). Thus, unraveling the degree of their
384	smoltification and adopting rearing strategy to stimulate the development of
385	hypo-osmoregulatory ability will be important for for the success of sea cage
386	aquaculture in this region. In O. tshawytscha, accelerated growth through the
387	summer-fall stimulated the development of smolt characters (Beckman et al., 2003).
388	In summary, the present study suggests that if O. masou in Miyazaki go
389	through the smoltification process, it is the first autumn instead of the second spring.
390	However, it is presumably an incomplete process compared to the robust development
391	of salinity tolerance that occurs in anadromous <i>O. masou.</i> The life-history patterns of
392	O. masou in Miyazaki provide a unique opportunity to understand how life-history
393	pathways are regulated and evolved.

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Target	Direction	Primer sequence (5'-3')	Product size (bp)
nka ala	Forward	CTTCGCTGCTGTTGTGATTGC	134
	Reverse	GAGCCAGGGCGGATTCTGA	
nka a1b	Forward	GGTACATTTCAACCAACAACATT	77
	Reverse	CCATCACAGTGTTCATTGGAT	
nka β1a	Forward	CTGGAGATGTACGATGAGGAGAGG	86
	Reverse	CCACGGTCCCTGTACGATT	
nka β1b	Forward	CTCCCCAACCATTTCTCAAAAGTAA	140
	Reverse	GATGAAGTGTCGTCCCGTATG	
ef-1α	Forward	GAATCGGCCATGCCCGGTGAC	142
	Reverse	GGATGATGACCTGAGCGGTG	

Table I. Primer sequences used for real-time PCR

Figure captions

2	Fig. 1. Mean fork length (L_F ; a,b), body mass (M_w ; c,d) and condition factor (K ; e,f) of
3	yearling Oncorhynchus masou in Hokkaido (a,c,e) and Miyazaki (b,d,f). O. masou in
4	Hokkaido were sampled from February to June in 2011 at Kumaishi (circle) and those
5	in Miyazaki were sampled from February to April in 2015 at Gokase (square) and from
6	April to June in 2014 at Kobayashi (triangle). Values are expressed as mean \pm S.E.
7	(n=7-8). Symbols sharing the same letters within a group are not significantly different
8	from each other (one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$). Note that
9	the results of statistical analyses of the data on O. masou of Miyazaki from different
10	locations/years are expressed in lower and upper letters.
11	
12	Fig. 2. Changes in gill NKA activity in yearling Oncorhynchus masou in Hokkaido (a)
13	and Miyazaki (b) during the spring. O. masou in Hokkaido were sampled from February
14	to June in 2011 at Kumaishi (circle) and those in Miyazaki were sampled from February
15	to April in 2015 at Gokase (square) and from April to June in 2014 at Kobayashi
16	(triangle). Values are expressed as mean \pm S.E. (n=7-8). Symbols sharing the same

17letters within a group are not significantly different from each other (one-way ANOVA 18 followed by Fisher's PLSD test, P < 0.05). Note that the results of statistical analyses of 19the data on O. masou of Miyazaki from different locations/years are expressed in lower 20and upper letters.

21

22Fig. 3. Changes in gill *nka* αla (a,b) and αlb (c,d) mRNA levels in yearling 23Oncorhynchus masou in Hokkaido (a,c) and Miyazaki (b,d) during the spring. O. masou 24in Hokkaido were sampled from February to June in 2011 at Kumaishi (circle) and 25those in Miyazaki were sampled from February to April in 2015 at Gokase (square) and 26from April to June in 2014 at Kobayashi (triangle). Values are expressed as mean \pm S.E. 27(n=7-8). Symbols sharing the same letters within a group are not significantly different 28from each other (one-way ANOVA followed by Fisher's PLSD test, P < 0.05). Note that the results of statistical analyses of the data on O. masou of Miyazaki from different 2930 locations/years are expressed in lower and upper letters.

31

32Fig. 4. Changes in gill *nka* βla (a,b) and βlb (c,d) mRNA levels in yearling

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33	Oncorhynchus masou in Hokkaido (a,c) and Miyazaki (b,d) during the spring. O. masou
34	in Hokkaido were sampled from February to June in 2011 at Kumaishi (circle) and
35	those in Miyazaki were sampled from February to April in 2015 at Gokase (square) and
36	from April to June in 2014 at Kobayashi (triangle). Values are expressed as mean \pm S.E.
37	(n=7-8). Symbols sharing the same letters are not significantly different from each other
38	(one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$).
39	
40	Fig. 5. Changes in serum osmolality (a) and gill NKA activity (b) in yearling
41	Oncorhynchus masou in Miyazaki after transfer to 70% seawater (70% SW; salinity of
42	23). Values are expressed as mean \pm S.E. (n=5-8). Symbols sharing the same letters are
43	not significantly different from each other (one-way ANOVA followed by Fisher's
44	PLSD test, <i>P</i> < 0.05).

45

Fig. 6. Changes in fork length (L_F ; a), body mass (M_w ; b) and condition factor (K; c) in 4647under-yearling Oncorhynchus masou in Miyazaki during the autumn. Fish were categorized as parr (open circule) or smolt-like fish (closed circule) based on the body 48

49	color. Values are expressed as mean \pm S.E. (n=8). Overall and interactive effects were
50	indicated by asterisks. Symbols sharing the same letters are not significantly different
51	from each other (one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$).
52	
53	Fig. 7. Changes in gill NKA activity in under-yearling Oncorhynchus masou in
54	Miyazaki during autumn. Fish were categorized as parr (open circule) or smolt-like
55	(closed circule) based on the body color. Values are expressed as mean \pm S.E. (n=8). An
56	interactive effects were indicated by an asterisk. (two-way ANOVA, $P < 0.05$).
57	
58	Fig. 8. Changes in gill <i>nka</i> αla , αlb , βla and βlb mRNA levels in under-yearling
59	Oncorhynchus masou in Miyazaki during the autumn. Fish were categorized as parr
60	(open circule) or smolt-like (closed circule) based on the body color. Values are
61	expressed as mean \pm S.E. (n=8). Symbols sharing the same letters are not significantly
62	different from each other (one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$).
63	

64 Fig. 9. Changes in serum osmolality (a) and gill NKA activity (b) in under-yearling

65 *Oncorhynchus masou* in Miyazaki after transfer to 70% seawater (70% SW; salinity of 66 23). Fish were categorized as parr (open circle) or smolt-like (closed circule) based on 67 the body color. Values are expressed as mean \pm S.E. (n=5-8). Symbols sharing the same 68 letters are not significantly different from each other (one-way ANOVA followed by 69 Fisher's PLSD test, *P* < 0.05).

















