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Assessment of the timing and degree of smolt development in southern populations of masu salmon

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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 *α1b*. Increases in gill NKA activity and *nka α1b* 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 α1b* 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.
40
41 Key words: masu salmon; smoltification; Miyazaki Prefecture; Na^+/K^+-ATPase;
42 osmolality; autumn

INTRODUCTION

Masu salmon *Oncorhynchus masou* (Brevoot 1856) is one of the eight Pacific salmon species distributed in the Asian side of Pacific Ocean. Their distribution is more southerly than other Pacific salmon but spans most of Japan from Hokkaido to Kyushu (Kato, 1991). The southern limit of their distribution in Japan is Miyazaki (32°N) but a land-locked strain is found even further south in Taiwan (Kato, 1991; Kimura, 1989).

Anadromy of *O. masou* declines with latitude (Malyutina *et al.*, 2009; Morita & Nagasawa, 2010) as is the case for other salmonids (Dodson et al., 2013; Morita et al., 2014). In Hokkaido (42°N), the latitudinal middle of their distribution, the majority of females and minority of males are anadromous, going through parr-smolt transformation (smoltification) in the second spring of their life (Kubo, 1980). Smoltification is a series of pre-adaptive changes in morphology, behavior and physiology by which river-dwelling parr become ocean-type smolt (Wedemeyer *et al.*, 1980; Hoar, 1988; Stefansson *et al.*, 2008; Björnsson *et al.*, 2011; McCormick, 2009, 2013). In one rearing experiment using *O. masou* of Hokkaido, 62% of females and 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

75 Kuroshio 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.
77 Thus, it is possible that *O. masou* in Miyazaki have shifted their timing of smoltification
78 from the spring to the autumn.

79 Acquisition 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.

85 NKA is composed of two essential subunits, α and β, and one regulatory
86 subunit, γ (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 α1b is considered as seawater type while α1a 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 α1b 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 *et al.*, 2004; Geering, 2008). Four isoforms of NKA β subunit (β1a, β1b,
100 β3a and β3b) have been identified in rainbow trout *O. mykiss* (Walbaum 1792) and *S.*
101 *salar* (Gharbi *et al.*, 2004, 2005). A few studies dealt with changes in gill *nka β1* subunit
102 during smoltification of *S. salar* and reported it increased in parallel with *nka α* 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 β1* subunits
105 during smoltification.
106 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.
112
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*
128 Yearling *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
134 week 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-l circular fibre-reinforced plastic (FRP) tank and reared until April 2015
138 in cooled water simulating the local river water temperatures (8-15°C).

139

140 *Under-yearling O. masou in Miyazaki*

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

147 Experiments and samplings were carried out in accordance with the

148 guidelines 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

150 anesthetized by 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and measured

151 for fork length (L_F) and body wet mass (M_W). Condition factor (K) was calculated as

152 follows: $M_W\ L_F^{-3}$x 100. Gill arches were excised and a block of gill filaments was

153 immediately frozen on dry ice and stored at -80°C until analyzed for NKA activity.

154 Another block of gill filaments was immersed in RNAlater (Ambion Inc., Austin, TX,

U.S.A.) and stored overnight at 4°C, then frozen and stored in RNAlater at -30°C until laboratory processing for quantification of mRNA.

SEAWATER CHALLENGE TEST

Yearling *O. masou* in Miyazaki

On 14 April 2015, yearling *O. masou* of Miyazaki were transferred to 70%, recirculated artificial seawater (70% SW; salinity of 23) (Napqo, Tokyo, Japan) in four 100-l tanks installed with a portable filter system (PowerBox 55, Kotobuki, Nara, Japan) in Kobayashi Branch. Water temperature was maintained at 13.5°C by placing the experimental tanks in a larger tank run through cooled water. Food was withheld during 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 kept overnight at 4°C. After centrifuging at 8,000 *g* for 10 min at 4°C, serum was collected and stored at -80°C until analysis for osmolality.

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-l 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
182 concentration was measured by using BCA (bicinchoninic acid) Protein Assay Kit
183 (Thermo Scientific, IL, U.S.A.). The activity was expressed as Pi (μmol) per protein
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.
192
193 REAL-TIME QUANTITATIVE PCR (QPCR)
194 Sequences of primers for qPCR of *nka α1a*, *nka α1b* and *ef-1α* were the same as
195 described in Nakajima *et al.* (2014) (Table 1). Primers for qPCR of *nka β1a* and *nka β1b*
196 were designed based on their sequences in *O. mykiss* (Genbank accession #CA374089
197 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.

qPCR was set up using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, U.S.A.) in a reaction volume of 20 µl with primer concentration of 100 nM. qPCR was run on a 7300 Sequence Detector (Applied Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Measured values were expressed as relative to those of *ef-1α*. Performance of qPCR was evaluated by confirming a single peak of the dissociation curve in each assay and calculating the amplification efficiencies of the standard curves, which were within the range of 97-100%. Coefficients of determination of the standard curves were also between 0.99-1.00.

SERUM OSMOLALITY MEASUREMENT

Osmolality in serum was measured by using a vapor pressure osmometer (Wescor 5500; Logan, UT, U.S.A.). Ten microliters of serum was used for the measurement.

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**
229 Body size (L_F and M_w) of yearling *O. masou* in Miyazaki was larger than that of
230 Hokkaido salmon throughout the sampling period (from February to June) [L_F: two-way
231 ANOVA, $F_{1,70} = 343.11$, $P < 0.001$; M_w: two-way ANOVA, $F_{1,70} = 245.34$, $P < 0.001$;
232 Fig. 1]. In both groups, average K values were the lowest in May followed by
233 significant increases in June [Hokkaido: Fisher's PLSD ANOVA, $F_{4,30} = 12.10$,
234 $P<0.001$; Miyazaki: Fisher's PLSD ANOVA, $F_{2,18} = 4.42$, $P < 0.05$; Fig. 1(e), (f)].

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 *nka α1a* mRNA levels were unchanged during spring both in yearling *O.*
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 *nka α1b* 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 *nka β1a* and *β1b* levels in fish in Hokkaido also showed peaks in May [*β1a*:Fisher's
246 PLSD ANOVA, $F_{4,29} = 10.00$, $P < 0.001$; *β1b*: Fisher's PLSD ANOVA, $F_{4,30} = 12.52$, P
247 < 0.001; Fig. 4(a), (b)]. There was a significant increase in gill *nka β1a* 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 *nka β1b* levels remained constant during that period [Fig. 4(d)].
250 Transferring yearling *O. masou* in Miyazaki from freshwater to 70% SW

(salinity of 23) in mid-April resulted in an increase in serum osmolality on day 1, after which it gradually decreased until day 4 after transfer but still significantly higher than those of the initial controls in freshwater [Fisher's PLSD ANOVA, $F_{3,25}$ = 11.87, P <0.001; Fig. 5(a)]. Gill NKA activity tended to increase, but not significantly, until 4 days after transfer [Fig. 5(b)].

Body size and K were compared between under-yearling parr and smolt-like fish in Miyazaki during fall [Fig. 6]. L_F was not significantly different between the two groups except in November, when smolt-like fish were larger than parr [two-way ANOVA, , $F_{1,55}$ = 8.14, P <0.001; Fig. 6(a)]. M_w was also similar between the two groups except in October, when parr was larger than smolt-like fish [two-way ANOVA , $F_{1,55}$ = 4.66, P <0.01; Fig. 6(b)]. Overall, both phenotypes decreased K during the autumn 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)] .

There was an overall effect of phenotype on gill NKA activity, which was higher in smolt-like fish (two-way ANOVA, $F_{1,56}$ = 6.48, P <0.05). The activity was similar in parr and smolt-like fish during September and October, but smolt-like fish

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

269 Overall, gill *nka α1a* mRNA levels were higher in parr [two-way ANOVA,
270 $F_{1,55} = 4.16$, $P < 0.05$; Fig. 8(a)] but *α1b* mRNA levels were not significantly different
271 between phenotypes and remained relatively constant during September and December
272 [Fig. 8(b)]. Gill *nka β1a* 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 β1b* 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**
290 *O. masou* in Miyazaki may have been prevented from spring migration by warm
291 seawater temperature, but they still may have opportunities to migrate to the ocean
292 during the autumn and/or winter. Such a migratory strategy may have lead to increased
293 ability of Miyazaki *O. masou* to tolerate seawater in autumn. In order to test this
294 hypothesis, the present study evaluated their smoltification status in the spring and
295 autumn.
296 Although defining and quantifying smoltification is difficult (Stefansson *et al.*,
297 2003; Björnsson *et al.*, 2012), one of the characteristic changes during smoltification is
298 an increase in gill NKA activity. Since the activity is generally correlated with

299 whole-body hypo-osmoregulatory ability, it is often used as an index of smoltification.
300 Moreover, recent findings showed that one of NKA α1 isoforms, α1b, 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 α1b* as reported in a previous study (Nakajima *et al.*,
310 2014). In addition, *nka β1a* and *β1b* subunits, which were measured separately for the
311 first time, also peaked in May. Parallel increases in *α1b* and *β1* subunits are in good
312 agreement with the findings in *S. salar* (Nilsen *et al.*, 2007). The present study suggests
313 that both NKA β1 subunits play a similar role in enhancing the localization of NKA α1b
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 *nka α1b*, *β1a* and *β1b* 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
330 former was much larger. Despite a reduction of condition factor in Miyazaki

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 *nka α1b* or *β1* 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%).

346 It is of note that no/little increase in gill *nka α1b* and *β1* 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 α1b* change.
350 Thus, decreasing water temperature or/and photoperiod during the autumn might cause
351 such discordant profiles of NKA activity and *nka α1b*. Our unpublished data showed
352 that gill *nka α1b* 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 *α1b* 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.

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

379 is a growing interest in sea cage aquaculture for *O. masou* 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 *O. masou*. 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.
394

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Table

Table I. Primer sequences used for real-time PCR

Target	Direction	Primer sequence (5'-3')	Product size (bp)
nka α1a	Forward	CTTCGCTGCTGTTGTGATTGC	134
	Reverse	GAGCCAGGGCGGATTCTGA	
nka α1b	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	

Figure captions

Fig. 1. Mean fork length (L_F; a,b), body mass (M_w; c,d) and condition factor (K; e,f) of yearling *Oncorhynchus masou* in Hokkaido (a,c,e) and Miyazaki (b,d,f). *O. masou* in Hokkaido were sampled from February to June in 2011 at Kumaishi (circle) and those in Miyazaki were sampled from February to April in 2015 at Gokase (square) and from April to June in 2014 at Kobayashi (triangle). Values are expressed as mean ± S.E. (n=7-8). Symbols sharing the same letters within a group are not significantly different from 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 locations/years are expressed in lower and upper letters.

Fig. 2. Changes in gill NKA activity in yearling *Oncorhynchus masou* in Hokkaido (a) and Miyazaki (b) during the spring. *O. masou* in Hokkaido were sampled from February to June in 2011 at Kumaishi (circle) and those in Miyazaki were sampled from February to April in 2015 at Gokase (square) and from April to June in 2014 at Kobayashi (triangle). Values are expressed as mean ± S.E. (n=7-8). Symbols sharing the same

17 letters 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
19 the data on *O. masou* of Miyazaki from different locations/years are expressed in lower
20 and upper letters.
21
22 Fig. 3. Changes in gill *nka* α*1a* (a,b) and α*1b* (c,d) mRNA levels in yearling
23 *Oncorhynchus masou* in Hokkaido (a,c) and Miyazaki (b,d) during the spring. *O. masou*
24 in Hokkaido were sampled from February to June in 2011 at Kumaishi (circle) and
25 those in Miyazaki were sampled from February to April in 2015 at Gokase (square) and
26 from April to June in 2014 at Kobayashi (triangle). Values are expressed as mean ± S.E.
27 (n=7-8). Symbols sharing the same letters within a group are not significantly different
28 from each other (one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$). Note that
29 the results of statistical analyses of the data on *O. masou* of Miyazaki from different
30 locations/years are expressed in lower and upper letters.
31
32 Fig. 4. Changes in gill *nka* β*1a* (a,b) and β*1b* (c,d) mRNA levels in yearling

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 ± 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 ± 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, $P < 0.05$).
45
46 Fig. 6. Changes in fork length (L_F; a), body mass (M_w; b) and condition factor (K; c) in
47 under-yearling *Oncorhynchus masou* in Miyazaki during the autumn. Fish were
48 categorized as parr (open circule) or smolt-like fish (closed circule) based on the body

color. Values are expressed as mean ± S.E. (n=8). Overall and interactive effects were indicated by asterisks. Symbols sharing the same letters are not significantly different from each other (one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$).

Fig. 7. Changes in gill NKA activity in under-yearling *Oncorhynchus masou* in Miyazaki during autumn. Fish were categorized as parr (open circule) or smolt-like (closed circule) based on the body color. Values are expressed as mean ± S.E. (n=8). An interactive effects were indicated by an asterisk. (two-way ANOVA, $P < 0.05$).

Fig. 8. Changes in gill *nka α1a*, *α1b*, *β1a* and *β1b* mRNA levels in under-yearling *Oncorhynchus masou* in Miyazaki during the autumn. Fish were categorized as parr (open circule) or smolt-like (closed circule) based on the body color. Values are expressed as mean ± S.E. (n=8). Symbols sharing the same letters are not significantly different from each other (one-way ANOVA followed by Fisher's PLSD test, $P < 0.05$).

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 ± 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, $P < 0.05$).

Figure 1

Inatani et al., Fig. 1

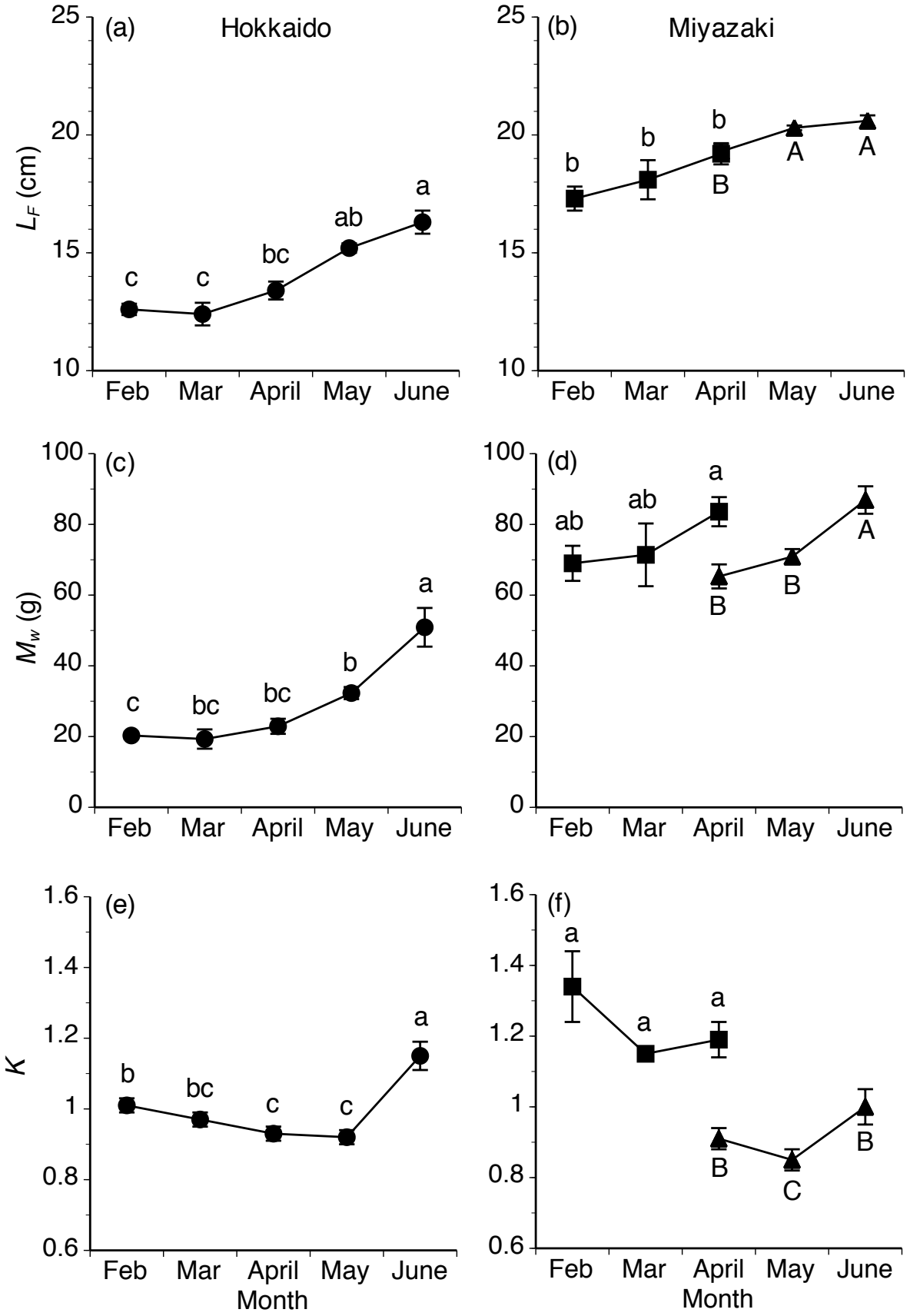


Figure 2

Inatani et al., Fig. 2

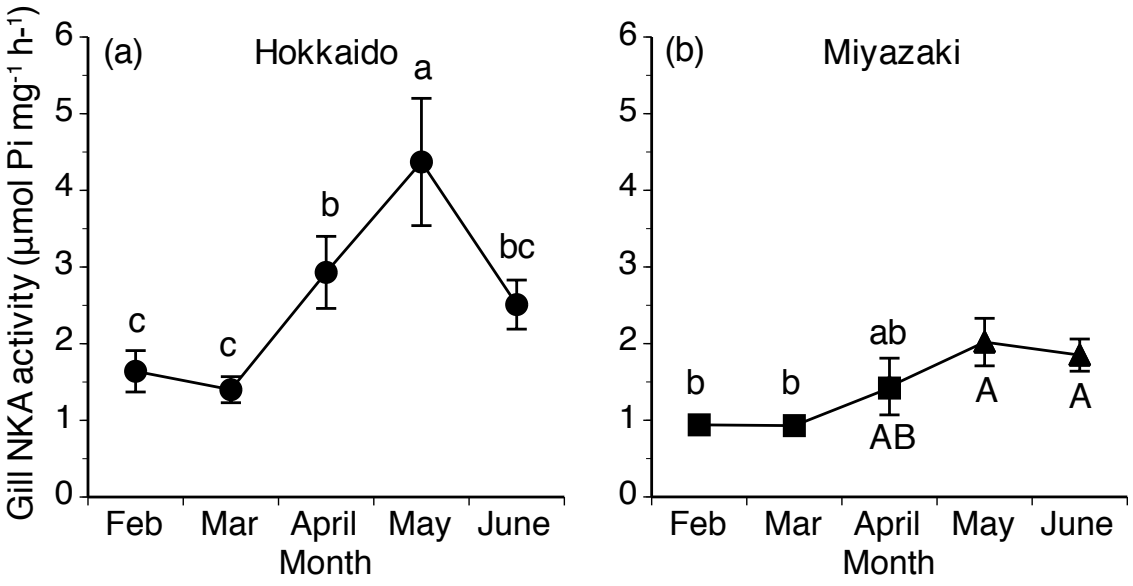


Figure 3

Inatani et al., Fig. 3

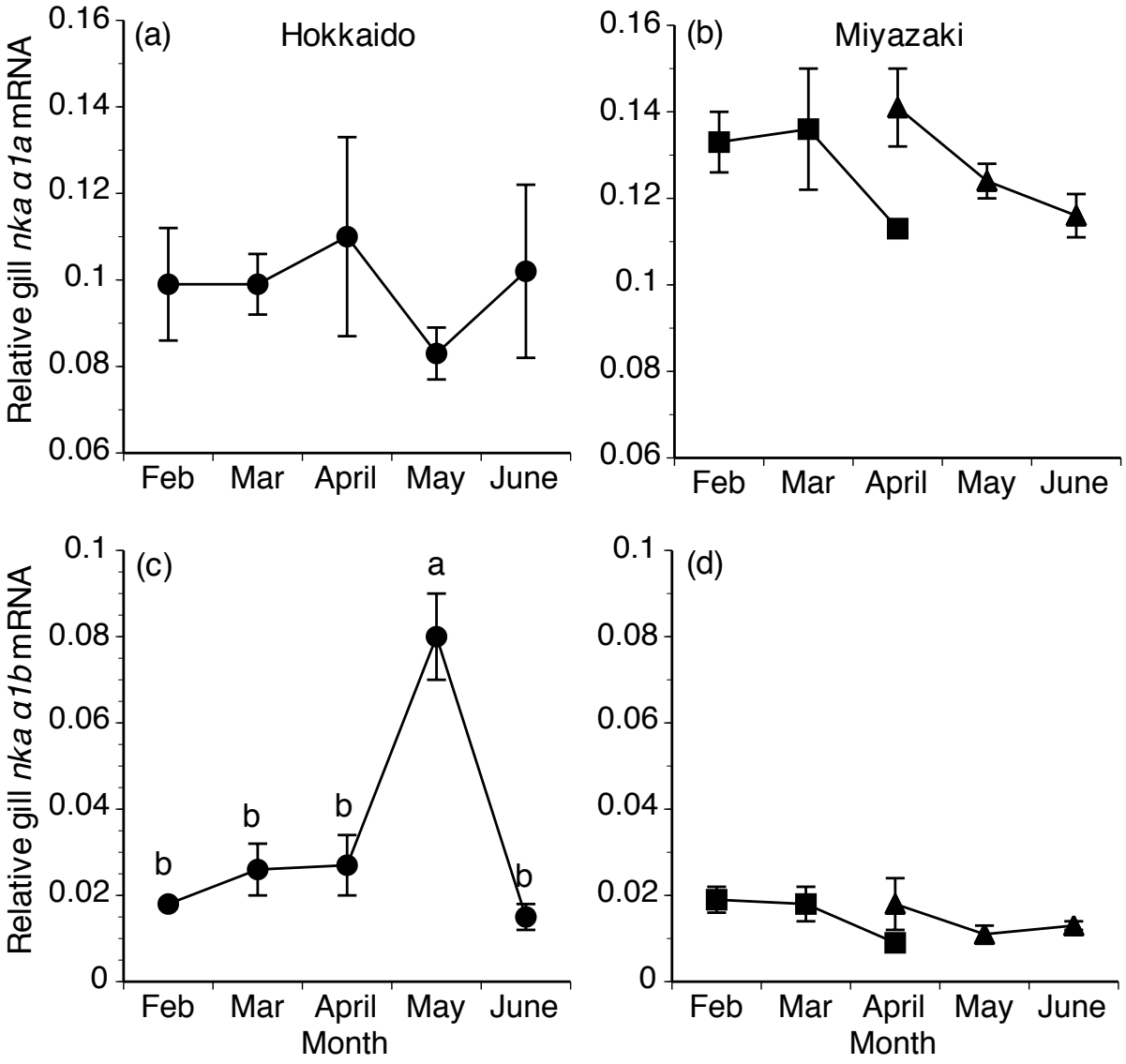


Figure 4

Inatani et al., Fig. 4

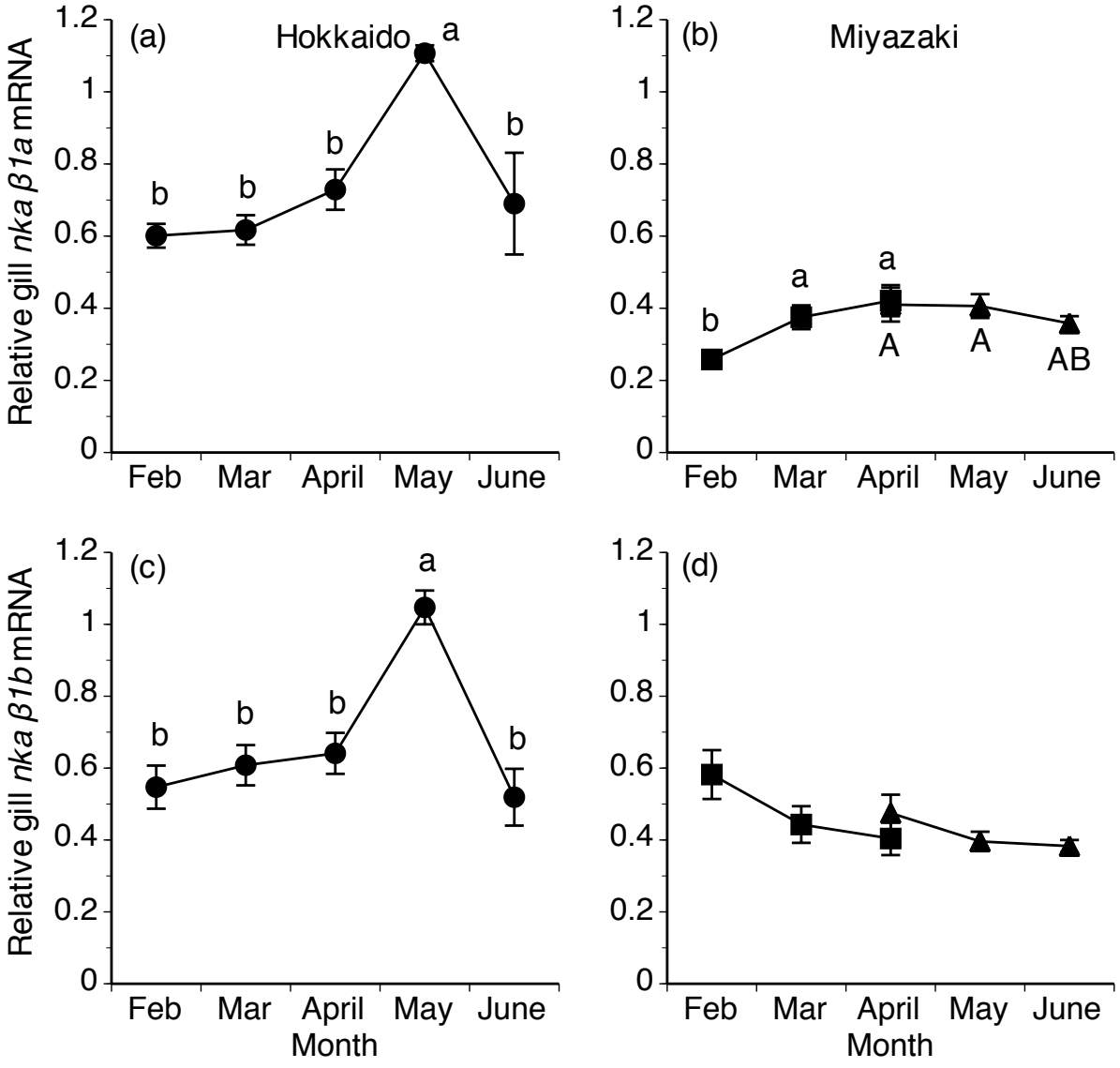


Figure 5

Inatani et al., Fig. 5

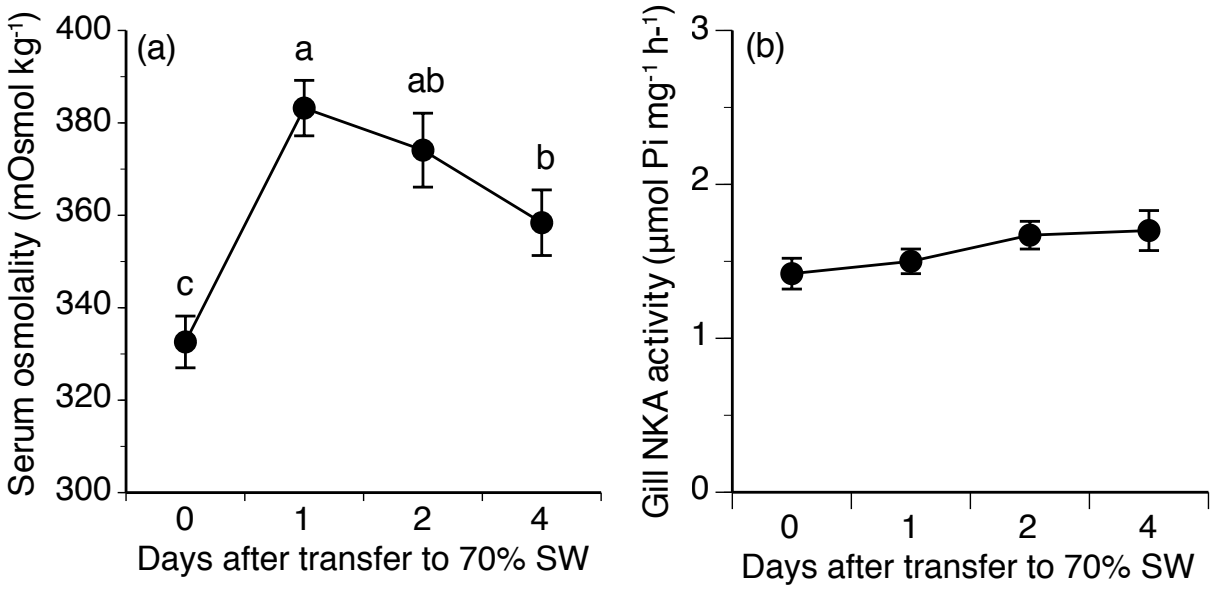


Figure 6

Inatani et al., Fig. 6

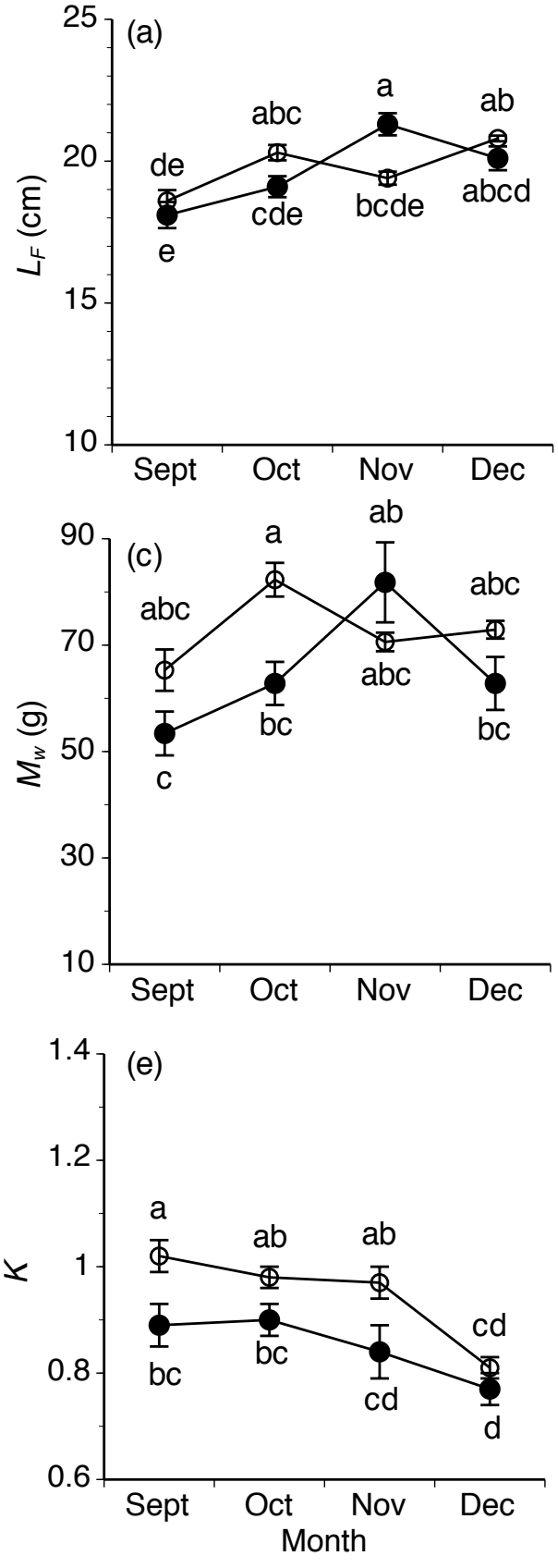


Figure 7

Inatani et al., Fig. 7

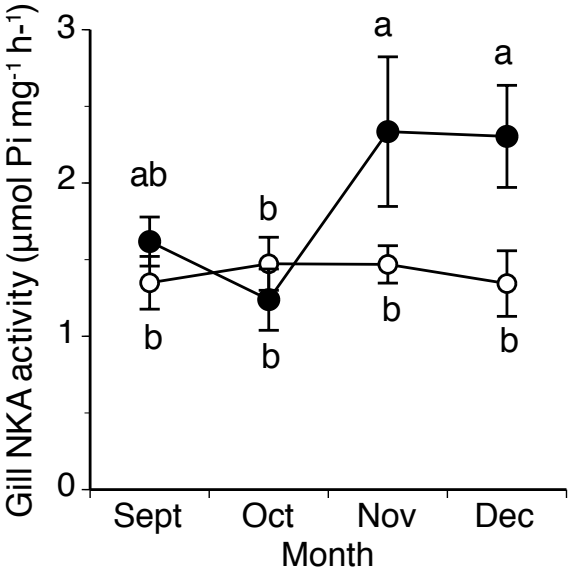


Figure 8

Inatani et al., Fig. 8

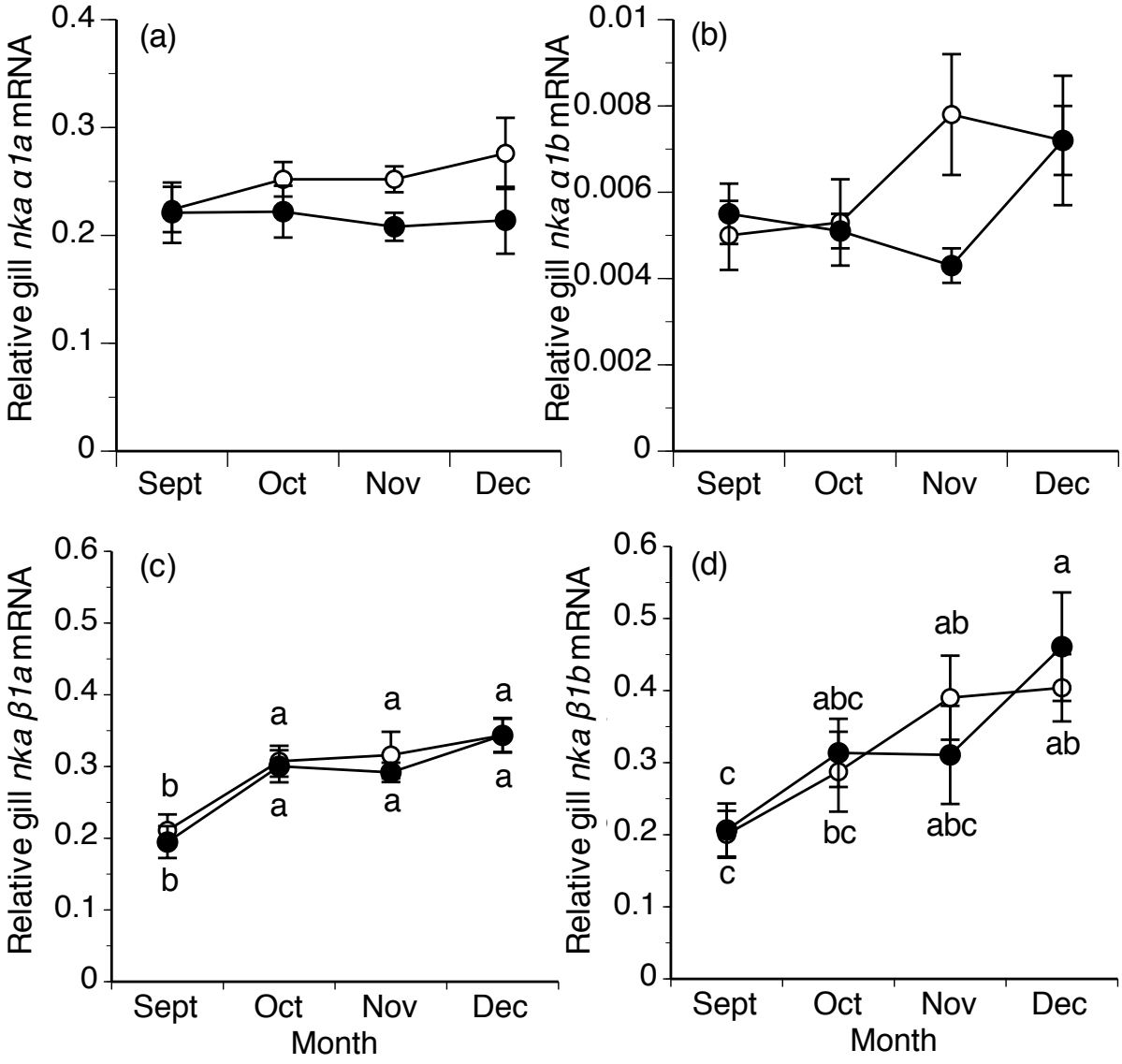
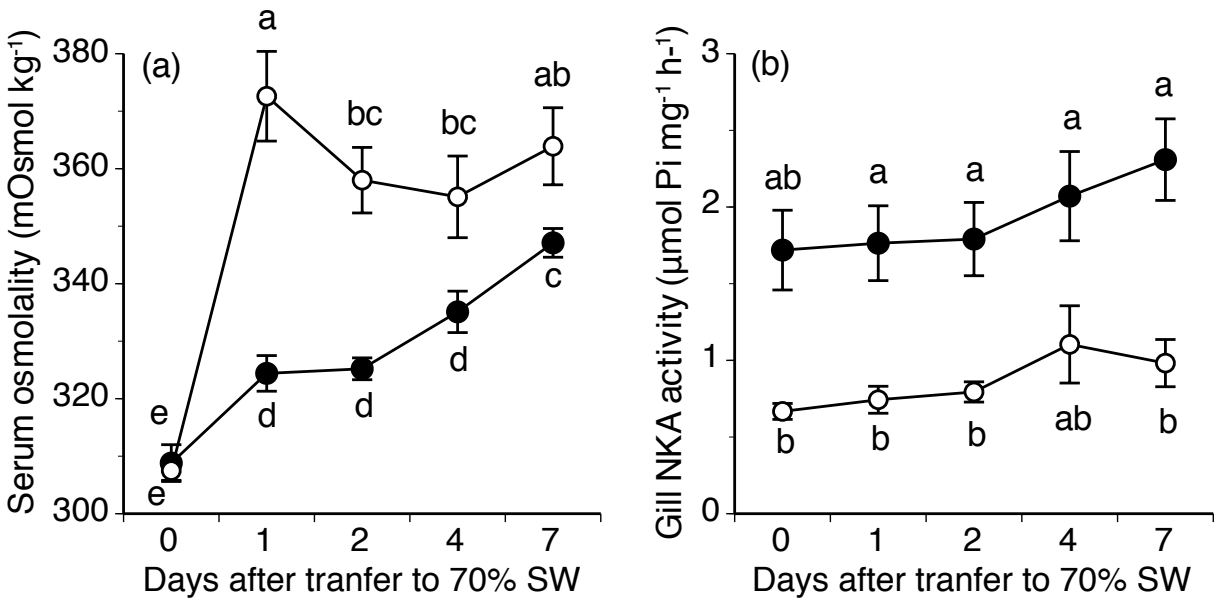


Figure 9

Inatani et al., Fig. 9