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Title

Circulating insulin-like growth factor-I in juvenile chum salmon: relationship with growth rate and changes during downstream and coastal migration in northeastern Hokkaido, Japan

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Abstract

Chum salmon (*Oncorhynchus keta*) migrate to the ocean in their first spring and growth during early marine life is critical for survival. We examined validity of circulating IGF-I and muscle RNA/DNA ratio as indices of growth rate using individually-tagged juvenile chum salmon fed or fasted for 10 days. Serum IGF-I level was highly, positively correlated with individual growth rate. Muscle RNA/DNA ratio also showed a positive correlation but its relation was not as high as that of IGF-I. We next measured these physiological parameters in chum salmon juveniles caught at river, estuary, port and nearshore of the northeastern Hokkaido, Japan, from May to June in 2013 and 2014, respectively. In both years, there was a trend that serum IGF-I levels were high in nearshore fish and low in river/estuarine fish in June. In contrast, muscle RNA/DNA ratio showed no clear temporal or spatial patterns. The present study shows that circulating IGF-I can be used as a growth index in juvenile chum salmon. Monitoring growth status using serum IGF-I suggests that growth of juvenile chum salmon in the survey area was activated when they left the coast.

Keywords

chum salmon, juvenile, migration, growth rate, RNA/DNA ratio, insulin-like growth factor-I

1. Introduction

Anadromous salmon hatch and reside in freshwater for a certain period, and migrate to the ocean as part of their life history. Salmon pass through the river and estuary during downstream migration and reach to the coast. They may stay in the coastal area until marine environments, such as water temperature, salinity and zooplankton abundance, become favorable (Wedemeyer et al. 1980; Fukuwaka and Suzuki 2002; Thorstad et al. 2012). The early marine stage including the life in the estuary, coast and offshore during the first year is known as the time of high mortality. A critical size, critical period hypothesis has been proposed to explain the high mortality (Beamish and Mahnken 2001). Based on this hypothesis and others, there are two critical periods; one occurs soon after sea entry mainly due to null adaptation to new environments and/or high predation pressure, while the other may be during fall to winter when shortage of energy reserve is lethal for young salmon (Healey 1982; Beamish and Mahnken 2001; Beamish et al. 2004; Farley et al. 2007; Kocik et al. 2009). Although the precise timing and mechanism are still need to be elucidated, the importance of fish size and energy reserve has been claimed and it is therefore of great value to measure growth of free-living fish for estimating their survival and stock recruitment. However, a direct measure of growth of the same individual requires marking and recapture, which is labor-tense, time-consuming and difficult to recover at sea. Instead, past growth can be estimated by back-calculation techniques using otolith and/or scale, which are widely used in population dynamics studies for fish resources. On the other hand, biochemical growth indices such as RNA/DNA ratio and hormone level may be more reflective of recent growth status since they are directly involved in the growth process (Buckley 1984; Chicharo and Chicharo 2008; Picha et al. 2008; Beckman 2011).

RNA/DNA ratio is most commonly used as a biochemical index of growth in marine organisms (Chicharo and Chicharo 2008). The rationale of using this parameter as a growth index is based on the facts that amount of ribosomal RNA in a cell reflects the capacity of protein synthesis whereas DNA is basically consistent, so that RNA/DNA ratio should be related to growth of whole animal. In a laboratory experiment using juvenile Atlantic salmon (*Salmo salar*), RNA/DNA ratio in

muscle was positively graded by feeding rations (Arndt et al. 1996). In addition, MacLean et al. (2008) found that muscle RNA/DNA ratio was strongly correlated with individual growth rate in weight ($r^2 = 0.79$) in Atlantic salmon smolts. In contrast, muscle or liver RNA/DNA ratio in post-smolt masu salmon (*Oncorhynchus masou*) reared in freshwater was not correlated with individual growth rate (Kawaguchi et al. 2013). These findings suggest that the relationship of RNA/DNA ratio with growth rate varies among species, life-history stages and rearing environments.

Growth hormone (GH) and insulin-like growth factor (IGF)-I play key roles in regulating animal growth. GH from the pituitary gland stimulates hepatic production of IGF-I (Daughaday and Rotwein 1989; Le Roith et al. 2001; Ohlsson et al. 2009). IGF-I is released into the bloodstream, delivered to target tissues and stimulates cell proliferation, differentiation and growth. Many studies using fish reported that IGF-I level responded to changes in nutritional status and circulating IGF-I has been shown to reflect recent growth rates of individuals, making it as a candidate of growth index (Picha et al. 2008; Beckman 2011; Kawaguchi et al. 2013). On one hand, the relationship between IGF-I level and growth rate was disrupted by an acute change in water temperature or altered seasonally in post-smolt coho salmon (*O. kisutch*) (Beckman et al. 2004b,c). Although care should be taken in certain situations, circulating IGF-I so far is a good candidate of growth index in fish.

Chum salmon (*O. keta*) is one of the eight Pacific salmon species widely distributed throughout the north Pacific Rim. Unlike other Pacific salmon except pink salmon (*O. gorbuscha*), chum salmon migrate to the ocean in their first spring. In Japan, this species is a target of intensive hatchery releases because of its importance in fishery catches along the north Japanese coasts (Miyakoshi et al. 2013). Several studies based on marking-recapture experiment have revealed that during the initial stage of their marine life (i.e. soon after sea entry) mortality is high losing at least 72% of released juveniles (Bax 1980; Fukuwaka and Suzuki 2002; Wertheimer and Thrower 2007). This high mortality is likely size-selective (Healey 1982). As most hatchery-reared chum salmon juveniles are released into the river, it is important to know how growth status is altered under changing food availability, salinity and water

temperature, and estimate their chance for survival during the critical periods at the estuary/coast and offshore. However, no study has linked the physiological parameters with individual growth rate in this species. The present study examined validity of RNA/DNA ratio and circulating IGF-I as indices of growth rate using individually-tagged juvenile chum salmon under captivity and measured these parameters during their downstream and coastal migration in northeastern Hokkaido, Japan.

2. Materials and methods

2.1. Rearing experiment

Chum salmon juveniles were transferred from a local hatchery (Kamisato Hatchery) in Abashiri area, northeastern Hokkaido, to the rearing facility at Faculty of Fisheries Sciences, Hokkaido University and reared in freshwater glass aquariums ($60 \times 29.5 \times 36 \text{ cm}^3$) in a temperature-controlled room (10°C). Each aquarium was closed-circulation system installed with a portable upper filter system. In order to maintain water quality, one third of water was replaced twice a week. Fish were fed daily on a commercial diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety until the beginning of the experiment. In May 2014, fish were acclimated to artificial seawater (Marine Salt Pro; Spectrum Brands Inc., Tokyo, Japan) by gradually increasing the salinity over one week. In June 2014, juveniles were lightly anesthetized using 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and individually marked with PIT-tags (size $\phi 1.4 \text{ mm} \times 8.4 \text{ mm}$, Biomark, Boise, ID, USA). They were randomly placed into two 90 L seawater tanks (25 fish per tank) and one group was fed twice daily on the commercial diet with a ration at 3.0% body weight/day for 10 days. Another group was fasted throughout the experimental period. Salinity was kept at full-strength seawater (31-34‰) and water temperature was maintained between $11.0\text{-}11.5^\circ\text{C}$ during the experiment. Water was not replaced during the experiment. The experiment was carried out in accordance with the guideline of the Hokkaido University Animal Care and Use Committee.

Fork length (FL) and body weight (BW) of all fish were measured at the beginning of the experiment and 10 days after treatment. Condition factor (K) was calculated as follows: $(\text{BW (g)}) \times$

1000/(FL (cm))³. Specific growth rate (SGR) was calculated as follows: $SGR (\%/day) = \ln (s_2 - s_1) \times (d_2 - d_1)^{-1} \times 100$, where s_2 is length or weight on day₁ and $d_2 - d_1$ is the number of days among measurements. At the initial sampling, eight fish were sampled for blood and muscle. On day 10, fish from each treatment (Fed: $n = 25$, Fasted $n = 24$) were sampled for blood and muscle. Blood was withdrawn using 10 or 20 μ L plain glass tubes (Microcap; Drummond Scientific Company, Broomall, PA, USA) from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C until use. A piece of muscle was excised from whole body, immediately frozen on dry ice and store at -80°C until use.

2.2. Field survey

Field survey was carried out around the Abashiri coast, which is the Sea of Okhotsk side of Hokkaido Island, four times from mid May to mid June in 2013 and 2014, respectively (Fig.1). Fish were caught every 10 days by castnet at the Abashiri River, dragnet at the estuary and two-boat trawling. Trawling was conducted using a net (8-m-wide x 5-m-deep mouth, 18-m-long with wing nets 7-m-long and a central bag with 5-mm mesh) towed through the 1-2 m surface layer for 1-2 km at 4-6 km/h in the morning (6:00-8:00). Total catch at each site, trawling time and boat speed were recorded to calculate catch per unit effort (CPUE). River water temperature or sea surface temperature (SST) was recorded at the beginning of catch at each site. Eight fish from each point were sampled for physiological analyses. After anesthetizing fish by 2-phenoxyethanol, FL and BW were measured. Blood was withdrawn using 10 or 20 μ L plain glass tubes from caudal vein, allowed to clot overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C. A piece of muscle was excised from whole body, which were mixture of white and red muscle but free of bone and other organs such as kidney, frozen on dry ice and store at -80°C until use.

2.3. Sample analyses

RNA/DNA ratio was measured by a spectrofluorimetric method modified from Grémare and Vétion (1994) as described in Kawaguchi et al. (2013). Briefly, amount of total nucleic acids (DNA + RNA) was measured using 4 µg/mL Thiazole orange (Sigma-Aldrich, St. Louis, MO, Japan) and that of DNA using 0.02 mg/mL Hoechst 33258 (Dojindo, Kumamoto, Japan). RNA/DNA ratio was calculated from these values.

For measuring IGF-I, serum was first extracted with an acid-ethanol as described in Shimizu et al. (2000). IGF-I was quantified by time-resolved fluoroimmunoassay (TR-FIA) based on the method described in Small and Peterson (2005) using recombinant salmon/trout IGF-I (GroPep Bioreagents Pty Ltd., Adelaide, SA, Australia) as a standard. Time-resolved fluorescence was measured using Wallac ARVO SX (PerkinElmer, Waltham, MA, USA).

2.4. Statistical analyses

Results of the tank experiments were first analyzed by two-way ANOVA (time x treatment) using the JMP program (SAS Institute Inc., Cary, NC, USA). Simple regression analysis was also conducted using JMP program and relations were considered to be significant at $P < 0.05$. Regression coefficients were compared after z-transformation. Results of field survey were also analyzed by two-way ANOVA (time x site). When significant effects were found, differences were further identified by one-way ANOVA followed by Tukey's HSD (honest significant difference) test. Differences among groups were considered to be significant at $P < 0.05$.

3. Results

In the fasting experiment, BW and K significantly decreased after 10 days of fasting (Table 1). Fasting for 10 days also caused little and negative SGR in length and weight, respectively (Fig. 2). Both muscle RNA/DNA ratio and circulating IGF-I levels in fasted fish were significantly lower than those in fed fish, although the magnitude of difference was greater for serum IGF-I (Fig. 3). Muscle RNA/DNA ratio was

positively correlated with SGRs in length and weight (Fig. 4a,c). Serum IGF-I showed stronger positive relations with SGRs than muscle RNA/DNA in both length and weight ($P < 0.05$; Fig. 4b,d). Muscle RNA/DNA ratio also had a significant correlation with K, but no relationships with fork length and body weight were seen (Table 2). In contrast, serum IGF-I level was significantly correlated with FL, BW and K (Table 2).

In the field survey, CPUE was highest at the estuary in May 2013, and the site of high CPUE shifted from the estuary to the port in June (Fig. 5). In May 2014, CPUE was high at the estuary as well as at the nearshore. The high CPUE at the nearshore was also observed in early June 2014. Water temperature in mid May 2014 was higher than that in 2013 (Fig. 5). FL and BW of chum salmon juveniles were similar among the sampling sites in May in both years except a significantly large size in fish caught at the nearshore in late May 2014 (Fig. 6 and Supplementary Fig. 1). In June, FL and BW tended to be small in fish at the estuary and large in fish at the nearshore in both years (Fig. 6 and Supplementary Fig. 1).

Muscle RNA/DNA ratio was generally similar among the sampling sites in both years (Fig. 7). When the overall average values were compared among the sampling dates in each year, it was high in mid June in 2013 and early June in 2014, respectively. Serum IGF-I levels were low in fish at the estuary when compared to those at the nearshore from early June to mid June in 2013 and in late May in 2014 (Fig. 8). Its levels gradually increased toward the nearshore during the same periods (Fig. 8).

4. Discussion

In the present study, we first tested if RNA/DNA ratio and/or circulating IGF-I could be used as indices of growth rate in juvenile chum salmon in seawater. Muscle may be the best part to measure RNA/DNA ratio since muscle constitutes majority of fish body's mass. MacLean et al. (2008) found that muscle RNA/DNA ratio was strongly correlated with individual growth rate in weight in Atlantic salmon smolts. On the other hand, Johnson et al. (2002) examined effect of variable rations on muscle RNA/DNA ratio in

juvenile red drum (*Sciaenops ocellatus*) and found that fasting was effective to see a significant reduction of RNA/DNA ratio but no significant differences were observed among different feeding rations. Thus, the sensitivity of muscle RNA/DNA ratio as a growth index needs to be evaluated in each case. In the present study, muscle RNA/DNA ratio significantly decreased in fasting group and had a weak positive correlation with individual growth rate both in length and weight. We did not examine varying feeding ratios in the present study but the correlation analysis suggests that muscle RNA/DNA ratio in juvenile chum salmon reflects nutritional status and recent body growth for some extent.

Circulating IGF-I in fish generally responds well to changing nutritional conditions; it is high when fish are fed and decreased when fish are restricted feeding or deprived of feed (Beckman et al. 2004b; Picha et al. 2008). In Chinook salmon (*O. tshawytscha*), circulating IGF-I levels started to decline in 4 days during fasting (Pierce et al. 2005). In the present study, serum IGF-I levels were significantly decreased by fasting for 10 days. There were positive correlations between serum IGF-I level and individual growth rate in length and weight, and the IGF-I-growth relationship was stronger than that of muscle RNA/DNA ratio. Although positive relationships between circulating IGF-I level and individual growth rate have been reported in Atlantic salmon (Dyer et al. 2004), coho salmon (Beckman et al. 2004a,b,c) and masu salmon (Kawaguchi et al. 2013), this study is the first to clarify the relationship in juvenile chum salmon. Our results suggest that IGF-I is a better growth index than muscle RNA/DNA ratio in chum salmon, which is consistent with the findings in masu salmon (Kawaguchi et al. 2013). On the other hand, time course of the IGF-I response may vary among experimental conditions such as temperature, season and fish status (Beckman et al. 1998, 2004c; Larsen et al. 2001; Beckman 2011). Seasonal changes in the basal IGF-I levels may also affect the relationship (Beckman et al. 2004b; Beaudreau et al. 2011). We also examined the IGF-I-growth relationship in August and found significant positive correlations in length and weight (data not shown). This result suggests that although the strength or slope of the relationship may vary, circulating IGF-I consistently reflects the growth rate at least during spring and summer in juvenile chum salmon.

We collected juvenile chum salmon at river, estuary, port, and nearshore by following their downstream and coastal migration route from May to June in Abashiri region, northeastern Hokkaido, Japan. Distribution of chum salmon juveniles along coastal waters in this region is largely influenced by SST (Nagata et al. 2007). When SST is low (i.e. $<8^{\circ}\text{C}$), their distribution is limited to the estuaries and littoral zones. SST above 8°C allows juveniles to disperse to nearshore area but high SST ($>14^{\circ}\text{C}$) drives them to leave for the offshore (Nagata et al. 2007). Thus SST ranging $8\text{--}13^{\circ}\text{C}$ is optimal for juvenile chum salmon to grow in this area. In the present study, relatively high CPUE was observed at the estuary in May 2013 where SST was $8\text{--}12^{\circ}\text{C}$. Fish moved to the port when SST at the estuary became $>14^{\circ}\text{C}$, which is in good agreement with the findings by Nagata et al. (2007). In 2014, high CPUEs were recorded at the estuary and nearshore in late May and their distribution shifted to the port and nearshore in June. The high CPUE at the nearshore in late May is somewhat peculiar since SST (7°C) was below the optimal range. Although the precise reason for this is not known, it may be related to the profile of SST at that area and fish conditions. SSTs in mid May 2014 at the port and nearshore were about 8°C which was favorable for juveniles. Fish caught at the nearshore were exceptionally large being 7-cm long and 3-g weight. These fish might be early migrants or fast-growing individuals that moved to the nearshore in late May just before SST slightly dropped.

Stefansson et al. (2012) monitored muscle RNA/DNA ratio in post-smolt Atlantic salmon migrating from the river through the fjord, coastal areas and the open ocean off the Norwegian coast. Muscle RNA/DNA ratio progressively increased as the fish migrated, suggesting activated muscle growth during this period. In the present study, muscle RNA/DNA ratio of juvenile chum salmon showed no such trend; it was relatively constant when different sampling sites were compared. However, when sampling dates were considered, the basal values were high in mid June 2013 and early June 2014. It might not be directly related to a change in growth status given the relatively weak relation with growth rate in the laboratory experiment. Water temperature or/and developmental stage should affect the basal levels, however, these need to be confirmed experimentally.

Although plasma/serum IGF-I levels in fish have been measured in many laboratory experiments, limited number of study have dealt with IGF-I in migrating salmon (Beckman et al. 2004a; Stefansson et al. 2012; McCormick et al. 2013; Ferris et al. 2014). Beckman and his colleagues validated IGF-I as an index of individual growth rate for immature coho salmon under laboratory conditions (Beckman et al. 2001, 2004b,c) and utilized it to evaluate growth status of post-smolt coho salmon caught at the Strait of Georgia, Canada, and Puget Sound, USA (Beckman et al. 2004a). This approach has been extended for sockeye, chum and Chinook salmon in British Columbian coastal waters (Ferriss et al. 2014). Variations in plasma IGF-I levels were found to reflect alterations of salmon growth status in response to local environments (Ferriss et al. 2014). In the present study, serum IGF-I levels in juvenile chum salmon also showed spatial and temporal variations. A general trend was that circulating IGF-I levels was low in fish at the estuary and high at the nearshore in June. The increased serum IGF-I levels well corresponded to the increased fish size, supporting the result of the laboratory experiment that IGF-I could be used as a growth index in this species. Based on these results, chum salmon juveniles activated growth when they left the nearshore, which would be important to reach a critical size by the end of the summer in the Sea of Okhotsuk. Our finding is also similar to that of Stefansson et al. (2012) who reported increased IGF-I levels during downstream and coastal migration in Atlantic salmon smolts in Norwegian coastal waters. In contrast, plasma IGF-I levels in Atlantic salmon smolts migrating in the Gulf of Maine, USA, were lower than those in the Penobscot River (McCormick et al. 2013). This discrepancy in IGF-I profile may be due to a temporal increase in IGF-I during smoltification or/and difference in water temperature between the river and the ocean (McCormick et al. 2009, 2013).

We found that small fish were consistently caught at the estuary throughout the sampling period and their serum IGF-I levels were low. These results suggest that fish in the estuary were under poor growth condition. Estuary is an important area for juvenile salmon especially for chum salmon (Salo 1991; Hillgruber and Zimmerman 2009) since it serves as a site for acclimation to seawater or a refuge from unfavorable temperature or feeding conditions. However, drawbacks are competition for food and

delay of migration timing. Indeed, fish in estuary were often small and found to be at high density (Magnusson and Hilborn 2003; Hillgruber and Zimmerman 2009; Kocik et al. 2009). We observed the distribution of juveniles was similar to that reported in the previous studies above; larger fish were caught in the nearshore and smaller fish were caught in the estuary in June in both years. Our results on IGF-I suggest that juveniles at the estuary had low growth rate and might suffer high mortality as indicated by marking-recapture experiments (Bax 1980; Fukuwaka and Suzuki 2002; Wertheimer and Thrower 2007). In order to test this hypothesis, more comprehensive sampling around the estuary and port is necessary in future survey.

In summary, the present study suggests that circulating IGF-I is a growth index in juvenile chum salmon in seawater. Monitoring growth status using IGF-I suggested that the growth of juvenile chum salmon in the Abashiri coastal waters was activated when they left the coast, while fish with low growth rate might stay in the estuary and experience size-selective mortality.

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379

380

Figure legends

Fig. 1. Locations of the survey area and sampling sites. Black circles indicate the collecting sites for chum salmon juveniles.

Fig. 2. Effect of fasting on specific growth rate (SGR) in length (A) and weight (B). Individually-tagged fish were fed or fasted for 10 days. Values are expressed as means $\pm$ SE (Fed: $n = 25$, Fasted: $n = 24$). Asterisks indicate significant differences between treatments.

Fig. 3. Effect of fasting on muscle RNA/DNA ratio (A) and serum IGF-I level (B). Values are expressed as means $\pm$ SE (Fed: $n = 25$, Fasted: $n = 24$). Asterisks indicate significant differences between treatments.

Fig. 4. Correlations between muscle RNA/DNA ratio and SGR in length (a) and weight (c), and between serum IGF-I level and SGR in length (b) and weight (d). Dots are data from both fed and fasted fish ($n = 46$).

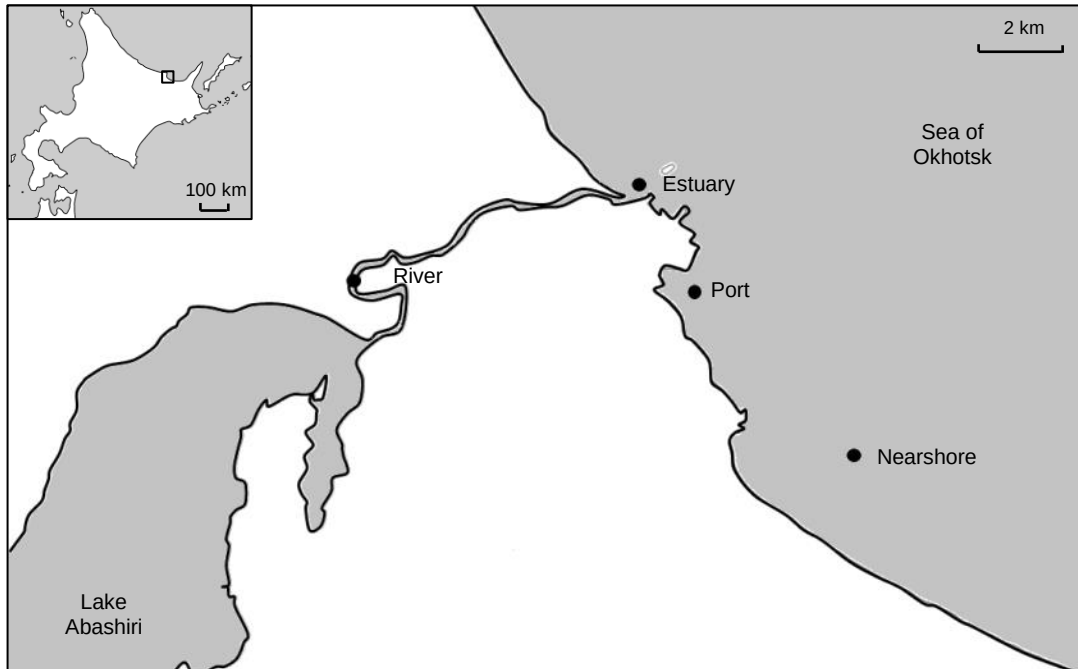
Fig. 5. Catch per unit effort (CPUE) for chum salmon juveniles and water temperature at the river, estuary, port and nearshore from mid May to mid June in 2013 (left) and 2014 (right). CPUE at the river is expressed as catch per casting and indicated by numbers, and that at estuary as catch per drag-netting. CPUEs at the port and offshore are expressed as catch per 1-km trawling. Shaded areas indicate temperature range 8-13°C which has been suggested to be optimal for juvenile chum salmon in that area (Nagata et al. 2007).

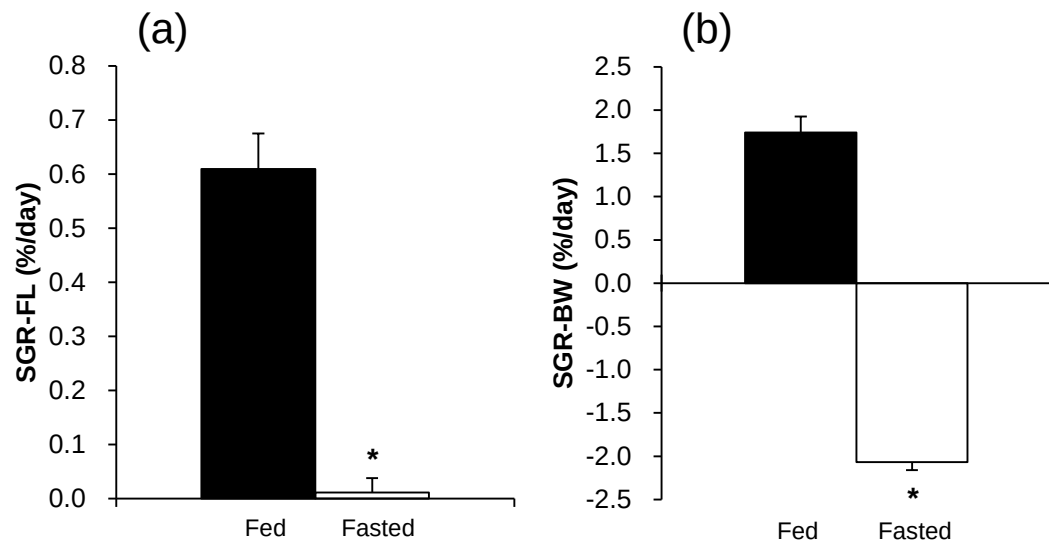
Fig. 6. Body weight (BW) of juvenile chum salmon at the river, estuary, port and nearshore from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means $\pm$ SE (n of each site is

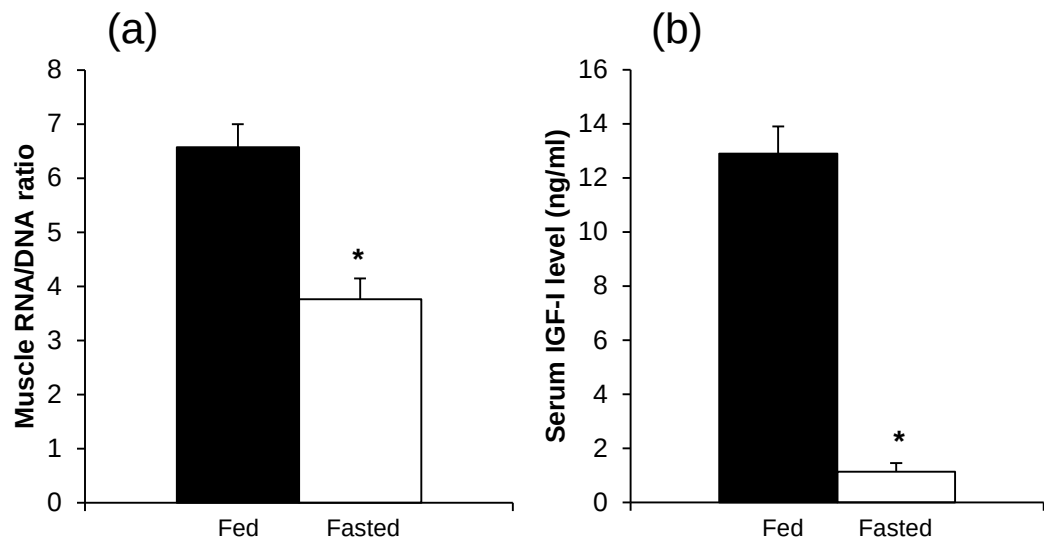
shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.

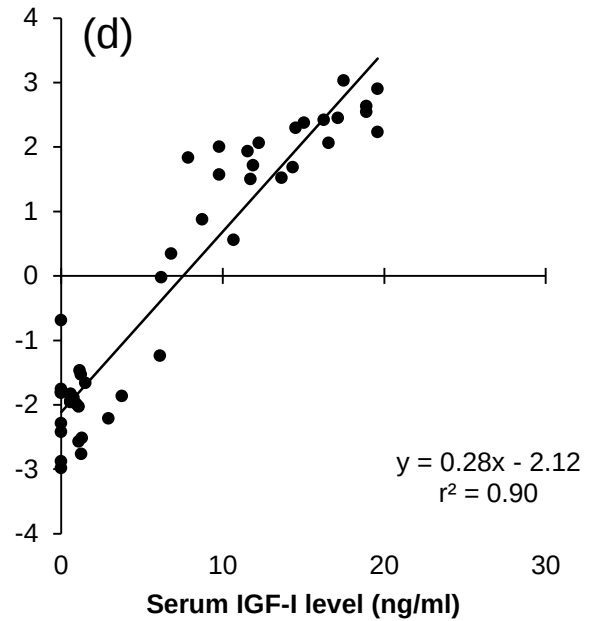
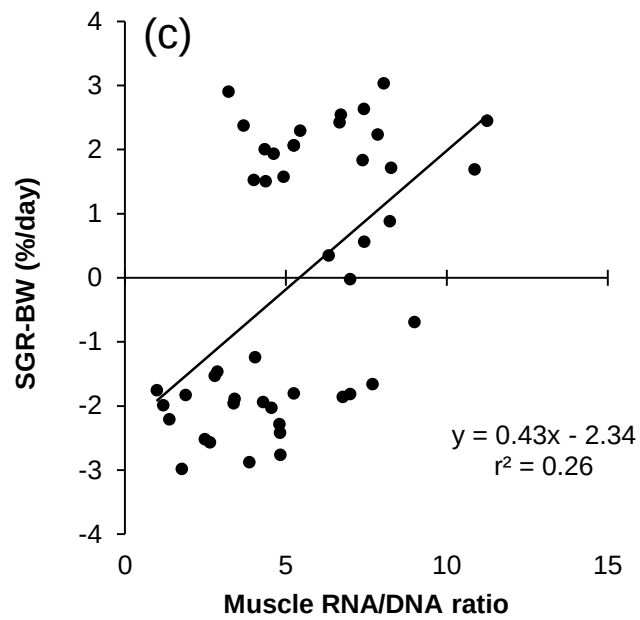
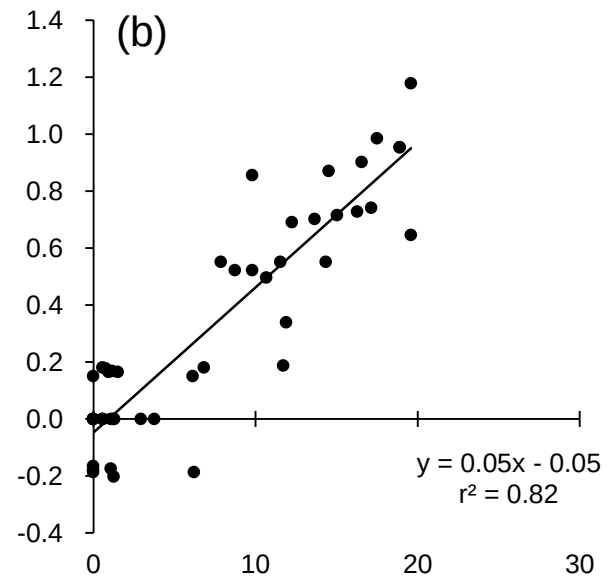
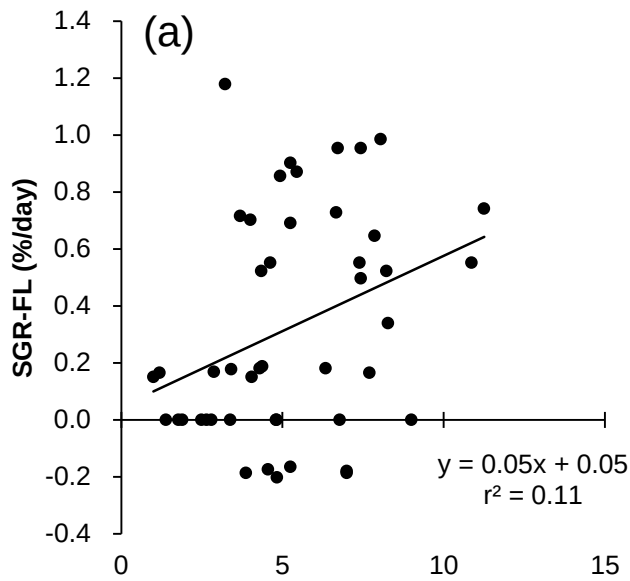
Fig. 7. Muscle RNA/DNA ratio of juvenile chum salmon at the river, estuary, port and nearshore from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means $\pm$ SE (*n* of each site is shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.

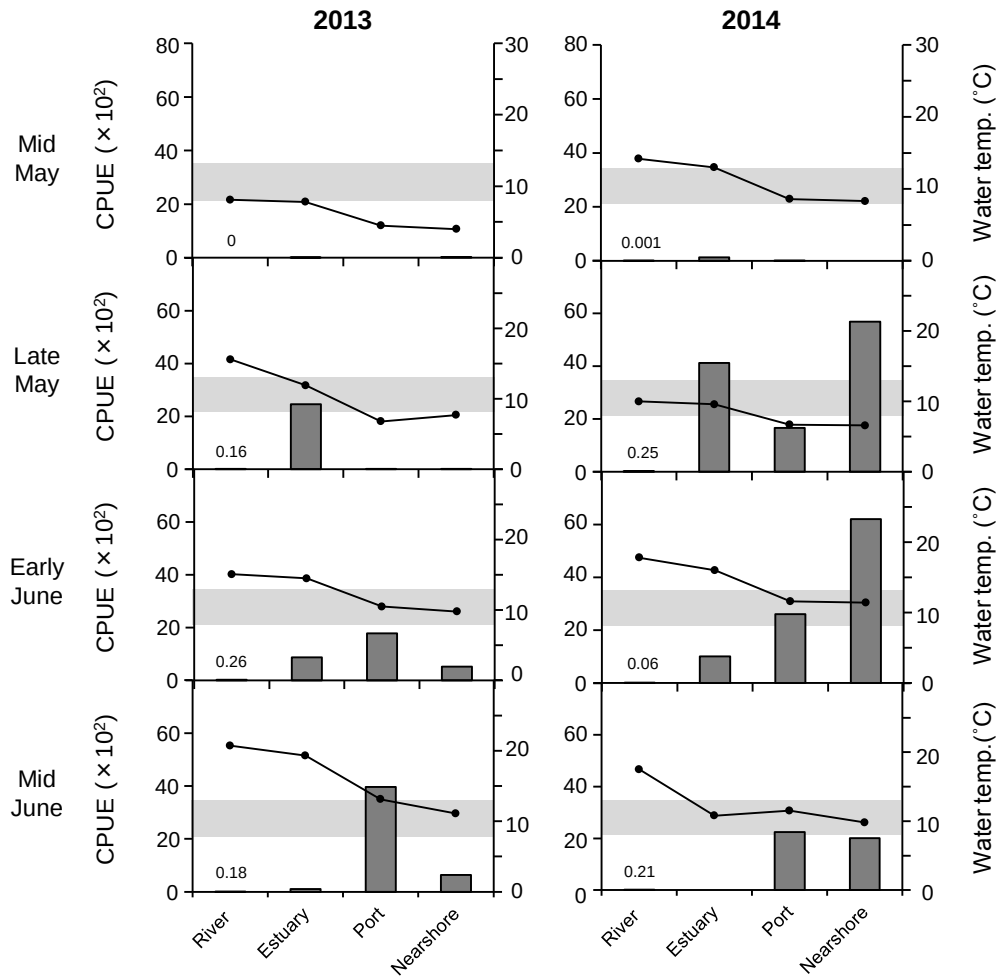
Fig. 8. Serum IGF-I level in juvenile chum salmon at the river, estuary, port and nearshore from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means $\pm$ SE (*n* of each site is shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.

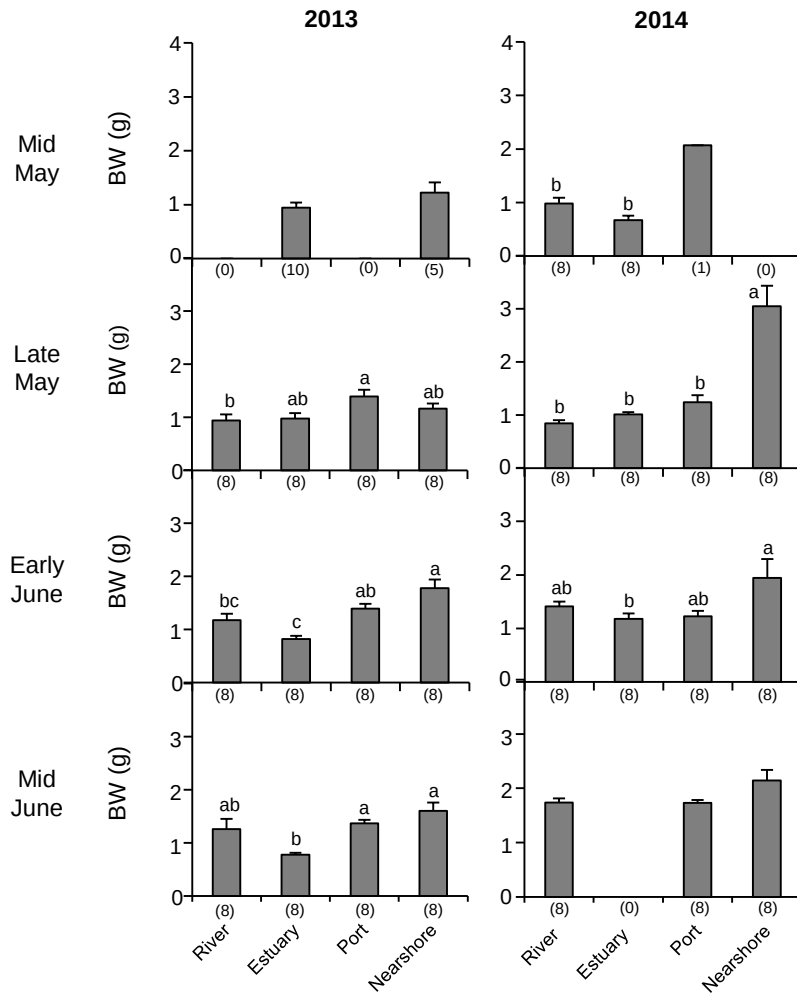


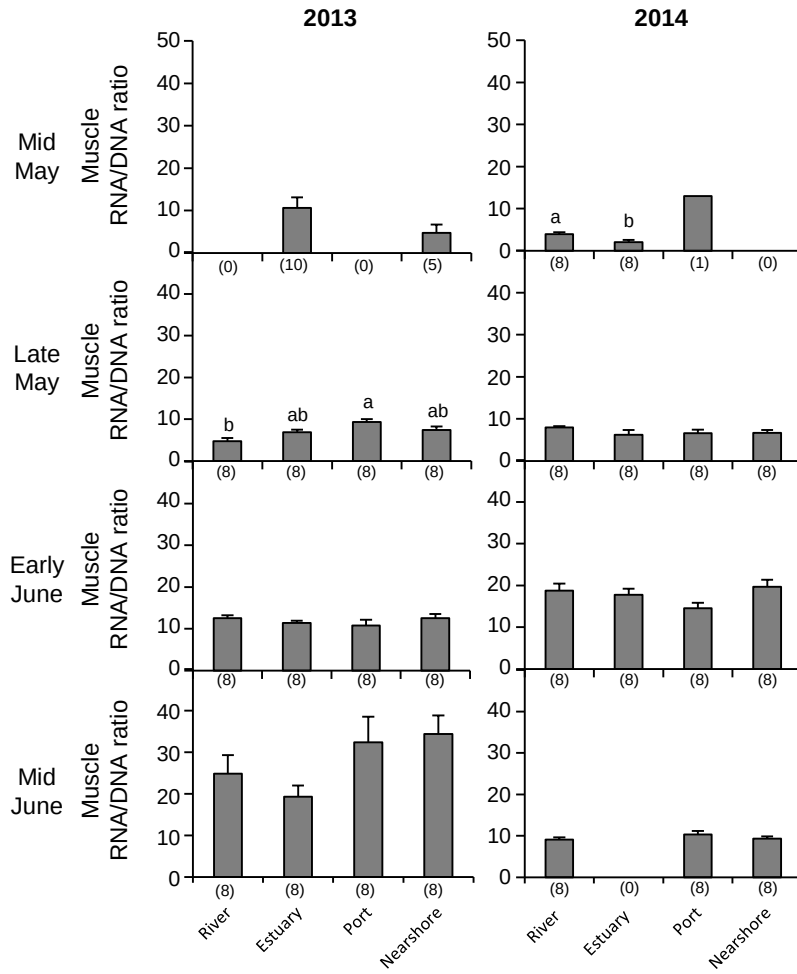












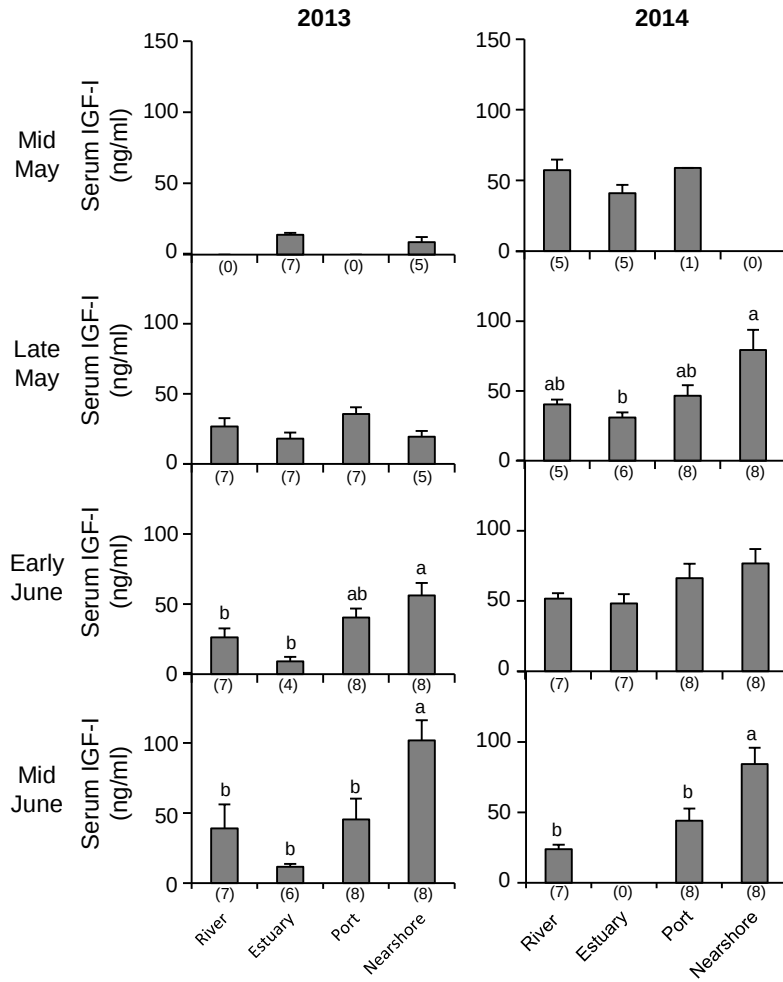


Table 1
Comparison of morphological parameters among treatments.

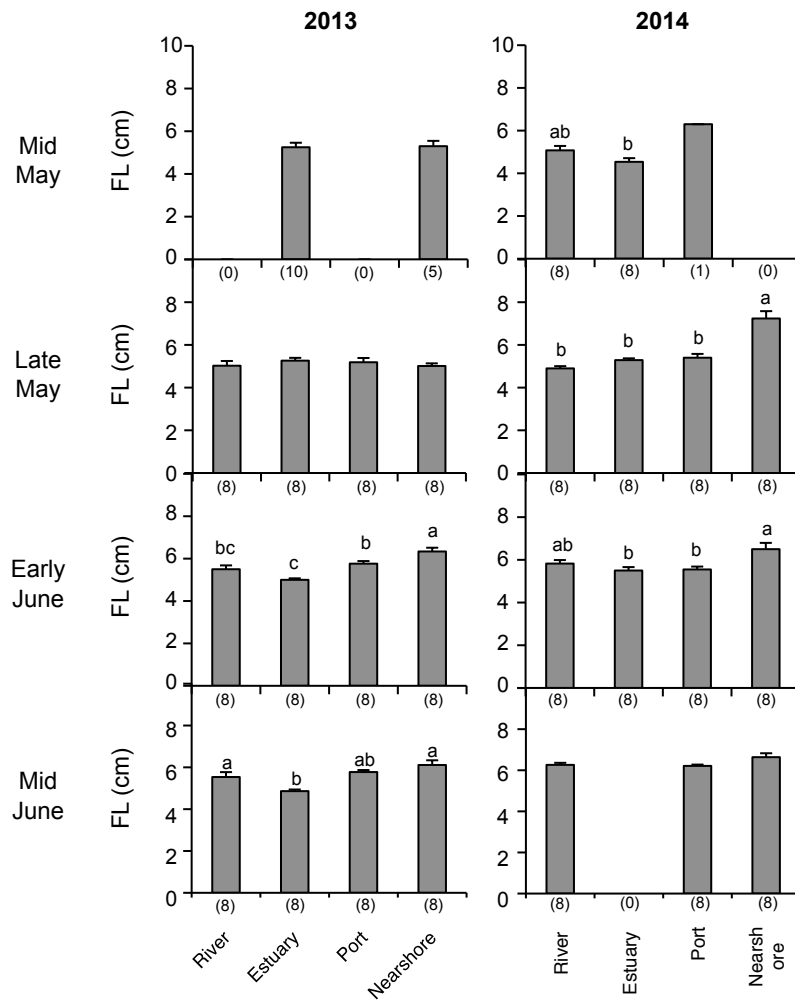
		Day 0	Day 10
FL	Fed	5.8 ± 0.17	5.9 ± 0.07
	Fasted		5.7 ± 0.11
BW	Fed	1.37 ± 0.12^{ab}	1.43 ± 0.06^a
	Fasted		1.13 ± 0.07^b
K	Fed	0.69 ± 0.02^a	0.69 ± 0.01^a
	Fasted		0.58 ± 0.01^b

Values are expressed as mean $\pm$ SE (Day 0: $n = 8$; Day 10: Fed $n = 25$, Fasted $n = 24$). Symbols sharing the same letters are not significantly different from each other.

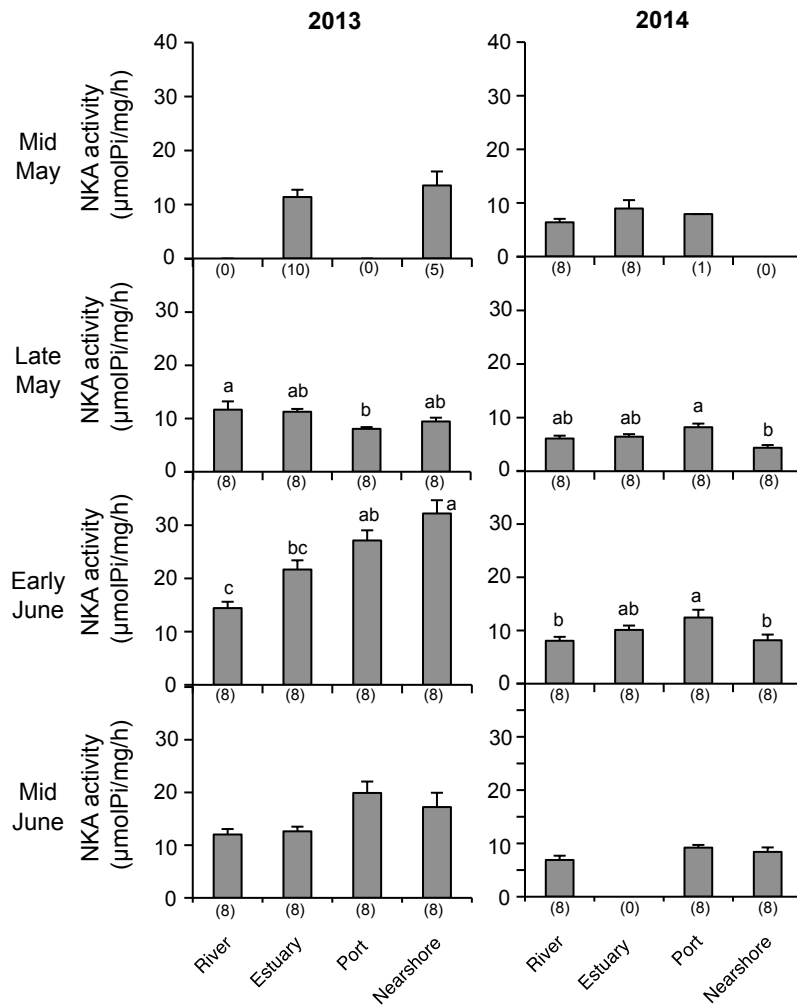
Table 2
Regression coefficients (r^2) among physiological and biochemical parameters.

	FL	BW	K	SGR-FL	SGR-BW	Muscle RNA/DNA
BW	0.87					
K	-	0.33				
SGR-FL	0.23	0.44	0.45			
SGR-BW	0.15	0.42	0.71	0.82		
Muscle RNA/DNA	-	-	0.21	0.11	0.25	
Serum IGF-I	0.17	0.41	0.58	0.82	0.90	0.23

(-): not significant.



Supplementary Fig. 1. Fork length (FL) of juvenile chum salmon at the river, estuary, port and nearshore from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as mean $\pm$ SE (n of each site is shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.



Supplementary Fig. 2. Gill $\text{Na}^+\text{K}^+\text{-ATPase}$ (NKA) of juvenile chum salmon at the river, estuary, port and nearshore from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as mean $\pm$ SE (n of each site is shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.