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Title

Response of the salmon somatotropic axis to growth hormone administration under two different salinities

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Key words

insulin-like growth factor-I, growth hormone, seawater, salmon

Abstract

We compared the response of plasma insulin-like growth factor-I (IGF-I) to growth hormone (GH) administration under two different salinities to test the hypothesis that environmental salinity alters the "activity" of the GH-IGF-I axis. In July, postsmolt coho salmon reared in fresh water (FW) were transferred to either FW or half seawater (1/2 SW) (15 ppt) tank. During the experiment, water temperature was maintained at 10°C for both salinities; photoperiod was adjusted to that of Seattle (48°N), and fish were not fed. Two days after transfer, fish were injected once with porcine GH (pGH) at a dose of 2 or 8 μg/g body weight. Liver and blood samples were collected 1, 2 and 3 days after injection. Liver GH receptor (GHR) mRNA expression was analyzed by quantitative real-time RT-PCR, and plasma IGF-I, 41-kDa IGF-binding protein (main carrier of IGF-I) and pGH were quantified by radioimmunoassays. Transfer to 1/2 SW resulted in transient increases in basal levels of liver GHR mRNA and 41 kDa IGF-binding protein (IGFBP) but not IGF-I. The GH-injection increased liver GHR mRNA, plasma IGF-I and 41-kDa IGFBP in fish in both FW and 1/2 SW. However, the time course and magnitude of the response differed between salinities. Fish in FW receiving 8 μg/g pGH had the highest IGF-I levels (63.7 ± 6.8 ng/ml) one day after injection, whereas fish in 1/2 SW showed a peak (88.8 ± 14.3 ng/ml) two days after injection of the same dose. It is speculated that the prolonged response to GH by fish in 1/2 SW may be due to slower disappearance of pGH from the circulation in fish in 1/2 SW. The transient increase in basal liver GHR mRNA may also contribute to a greater response for fish in 1/2 SW. These results suggest that salinity is capable of altering the "activity" of the GH-IGF-I axis in salmon.

Introduction

Smoltification in salmonids is a pre-adaptation to ocean life accompanied by a series of morphological, biochemical and behavioral changes (Hoar, 1988). One of the major achievements of smoltification is successful adaptation to seawater (SW). Salmon adapted to SW generally reach larger sizes than those in fresh water (FW). On the other hand, if juvenile salmon are transferred prematurely to SW, their growth is severely retarded and they become "stunts" (Folmar et al., 1982). These circumstances led scientists to hypothesize that salinity affects growth in salmonids, and successful SW adaptation results in an improved growth. However, the effect of salinity on growth in salmonids is inconsistent, probably due to varying experimental conditions such as water temperature, feeding ration, developmental stage, season and experimental period (Smith and Thorpe, 1976; McCormick et al., 1989; Morgan and Iwama, 1991; Usher et al., 1991; Duston, 1994; Handeland et al., 1998). On the other hand, the interaction between growth and salinity has been demonstrated in several fish species (for review, Bœuf and Payan, 2001). In tilapia (Oreochromis mossambicus), evidence indicates that SW rearing improves growth of this species (Kuwaye et al., 1993; Riley et al., 2002). In other marine fishes, optimal growth is sometimes seen at an intermediate salinity (Bœuf and Payan, 2001). Improved growth at intermediate salinity may be explained by a reduction of the metabolic cost for osmoregulation, whereas appetite and/or the endocrine system may also play a role (Bœuf and Payan, 2001).

Growth hormone (GH) and insulin-like growth factor-I (IGF-I) are key hormones regulating growth of animals. In the classical somatomedin hypothesis, GH from the pituitary gland stimulates hepatic production of IGF-I via the GH receptor, and IGF-I

from the liver mediates many of the actions of GH (Daughaday and Rotwein, 1989). Recent findings have pointed out that GH has direct actions independent of IGF-I, and virtually all tissues express IGF-I that acts through autocrine and paracrine manners (Butler and LeRoith, 2001). Regardless of the site of production, the activity of IGF-I is modulated by a family of six IGF binding proteins (IGFBPs). In the circulation, IGFBPs prolong the half-life of IGF-I and deliver IGF-I to certain tissues (Rajaram et al., 1997). Increasing evidence indicates that the fish GH-IGF-I axis also plays an important role in osmoregulation as well as growth regulation (Sakamoto et al., 1993; Dickhoff et al., 1997; McCormick, 2001). However, how the GH-IGF-I axis operates these two processes simultaneously is poorly understood and the endocrine mechanism, if any, by which salinity influences growth is not known. In a study by Riley et al. (2002) on tilapia, the GH-IGF-I axis was "activated" by treatment with 17α-methyltestostelone (MT) and SW rearing. The better growth of tilapia treated with MT and SW rearing was reflected by higher IGF-I levels (Riley et al., 2002). Circulating levels of IGF-I have been considered to be an index of growth in fish (Beckman et al., 1998; Uchida et al., 2003; Dyer et al., 2004). In coho salmon (Oncorhynchus kisutch), a series of experiment has shown that plasma IGF-I levels are positively correlated with growth rates of individuals in fresh water and seawater (Beckman et al., 2004a,b), implying that plasma IGF-I levels could be used to evaluate growth potential of salmon. In an attempt to understand how salinity might influence the "activity" of the GH-IGF-I axis and in turn growth in salmon, we examined response of the GH-IGF-I axis to GH administration under two different salinities.

Materials and Methods

Fish rearing conditions

One-year-old postsmolt coho salmon were reared in FW at the Northwest Fisheries Science Center in Seattle, WA, USA. They were maintained in recirculated FW (dechlorinated city water that is buffered with sodium bicarbonate) in circular fiberglass tanks under natural photoperiod adjusted to that of Seattle, WA (48°N); flow rate was 25 L/min; temperature ranged from 10.5°C to 13.0°C. Before fish were used for experiments, they were fed a ration of 1.25% body weight/day of a commercial diet (Biodiet Grower; Bioproducts Inc., Warrenton, OR, USA).

Treatment of fish

In early July, 2002, after 24 hr of fasting, 156 fish were transferred directly to one of eight tanks containing fresh water or half seawater (15 ppt; 1/2 SW) made from artificial sea salt (Aquarium Systems Inc., Mentor, OH, USA). The average body length and weight of fish were 15.6 ± 0.2 cm (mean ± SE) and 41.8 ± 2.2 g, respectively. Throughout the experiment, salinity was monitored daily; water temperature was kept at 10°C for both salinities, and fish were not fed. Blood and liver samples from untreated fish were collected 1, 2 and 5 days after transfer (day 1, 2 and 5, respectively) as described below. Other fish were injected intraperitoneally with porcine GH (Sigma, St. Louis, MO, USA) in saline at a dose of 2 or 8 μg/g body weight two days after transfer (day 4). Sham injected fish received saline only. Blood and liver samples were collected 1, 2 and 3 days after the single injection (day 3, 4 and 5, respectively).

115 Sample collection

116 Fish were anesthetized in 0.05% tricane methanesulfonate (MS-222; Argent Chemical
117 Laboratories, Redmond, WA, USA). Blood was withdrawn by cutting the caudal
118 peduncle and letting blood flow into a heparinized glass tube. Plasma was collected after
119 centrifugation at 700*g* for 15 min and stored at -80°C until use. Liver pieces were
120 excised, immediately frozen in liquid nitrogen and stored at -80°C until use.
121
122 Sample analysis
123 Expression of growth hormone receptor mRNA in the liver was measured by real-time
124 reverse transcript polymerase chain reaction (RT-PCR) as described in Fukada et al.
125 (2004). Expression levels were normalized with an acidic ribosomal phospoprotein P0
126 (ARP). ARP is superior to 18S ribosomal RNA as a reference gene and has been adopted
127 to RT-PCR for salmon IGF-I mRNA in the liver (Pierce et al., 2004). For measurement
128 of IGF-I, plasma IGF-I was extracted with an acid-ethanol followed by cryoprecipitation
129 as described in Breier et al. (1991) and quantified by RIA (Shimizu et al., 2000). Plasma
130 41-kDa IGFBP levels were measured by RIA as described in Shimizu et al. (2003b).
131 Porcine GH levels were measured by a commercial RIA kit (Linco Research Inc., St.
132 Charles, MO). This RIA showed no cross reactivity with sham-operated salmon plasma
133 (data not shown).
134
135 Statistical analysis
136 All measured values were not normally distributed and thus natural-log transformed
137 before analyses to obtain normal distribution (D'Agostino and Pearson omnibus

normality test). Data sets for each dependent variable (liver GHR mRNA expression, and plasma IGF-I, 41-kDa IGFBP and porcine GH levels) were first analyzed by two- or three-way analysis of variance (ANOVA) by including GH treatment, salinity and time as factors. When significant effects were found, the data were further analyzed by one- or two-way ANOVA for each time point. Differences between groups were identified by Fisher's protected least-significant difference (PLSD) test. Differences between groups were considered to be significant at $P < 0.05$.

Results

Changes in the basal levels of liver GHR mRNA, plasma IGF-I and 41-kDa IGFBP in fish transferred to FW or 1/2 SW were compared (Fig. 1). There were overall effects of salinity and time on the liver GHR mRNA and plasma 41-kDa IGFBP levels, but no interaction was found (two-way ANOVA). Salinity and time had no significant effect on plasma IGF-I. Hepatic GHR mRNA and circulating 41-kDa IGFBP were significantly higher in the 1/2 SW group one day after transfer (day 1) (Fig. 1a,c) and the difference became insignificant thereafter. A similar trend of higher levels in 1/2 SW was seen in plasma IGF-I, although this was not statistically significant ($P = 0.0551$).

There were overall main effects of GH treatment, salinity and time on the liver GHR mRNA expression (three-way ANOVA). An increase in liver GHR expression by the low dose of GH injection was evident in fish in both FW and 1/2 SW on day 3 (one day after injection) (Fig. 2a). On day 4 (two days after injection), the effect of GH was not seen in fish in FW but was seen in 1/2 SW (Fig. 2b). GHR mRNA levels became similar between the sham and treated groups on day 5 (three days after injection). When

fish received a high dose of GH, salinity enhanced the GH effect on the liver GHR expression one day after injection (Fig. 2b).

For plasma IGF-I, GH treatment, salinity and time had significant main effects, and an interactive effect was also seen (three-way ANOVA). Plasma IGF-I levels were increased by the low dose of GH treatment in both FW and 1/2 SW for two days (Fig. 3a). On day 3 (one day after injection), plasma IGF-I levels in the FW group with the low-dose GH injection were higher than those in 1/2 SW, and decreased gradually over time (Fig. 3a). When fish received the high dose of GH (8 μg/g), plasma IGF-I levels in the 1/2 SW group showed a peak on day 4 (two days after injection) and were higher than those in the FW group (Fig. 3b).

The response of 41-kDa IGFBP to GH treatment was essentially the same as that of IGF-I (Fig. 4). A positive effect of pGH injection lasted for two days. Salinity had a positive effect on the increase in 41-kDa IGFBP with the high-dose GH on day 4 (two days after transfer) (Fig. 4b).

The disappearance of exogenously injected pGH from the circulation was monitored by homologous RIA (Fig. 5). The pGH levels decreased rapidly after injection in both FW and 1/2 SW. However, the levels were always higher in fish in 1/2 SW than those in FW, showing that injected pGH was retained longer in the circulation in 1/2 SW.

Discussion

This study examined the effect of salinity on the basal levels of the GH-IGF-I axis components and their response to GH administration in postsmolt coho salmon. A relatively mild salinity change (FW to 1/2 SW) was chosen for the experiment as

transferring fish directly to full seawater (30-33 ppt) likely causes a stress response. A relatively short period of SW exposure (up to five days) was applied to the present study. Folmar and Dickhoff (1981) found that when yearling coho salmon were transferred to seawater, plasma ions (sodium, chloride and potassium) reached a steady state within a few days. On the other hand, Pierce et al. (2005) reported that fasting of coho salmon induced a decline of plasma IGF-I as early as day 4. For these reasons, fish were acclimated for two days prior to the GH administration in order to avoid the influence of the rapid physiological change during the initial phase of seawater adaptation and the negative effect of food deprivation on the GH-IGF-I axis. Under these experimental conditions, we evaluated the "activity" of the somatotropic axis by plasma IGF-I levels since several studies have revealed that circulating IGF-I levels are positively correlated with growth rates of fishes including salmon (Beckman et al., 1998, 2004a,b; Uchida et al., 2003; Dyer et al., 2004).

During seawater adaptation of salmonids, increases in secretion and metabolic clearance rate of GH, and occupancy and total number of liver GHR have been observed (Sakamoto et al.,1991; Sakamoto and Hirano, 1991, 1993). Plasma GH levels are also known to change during seawater adaptation of salmon (Björnsson et al., 1998). These changes in GH and its receptor support the concept that GH is a key hormone for seawater adaptation in salmonids. The action of GH in osmoregulation may be, at least partly, mediated by IGF-I. McCormick et al. (1991) demonstrated that IGF-I promotes osmoregulatory ability of salmon. Participation of the GH-IGF-I axis in osmoragulation has been also suggested in non-salmonid fishes (Mancera and McCormick, 1998). Despite the importance of the GH-IGF-I axis in osmoregulation, little is known about the

response of GHR mRNA, plasma IGF-I and its carrier protein (41-kDa IGFBP) levels during seawater adaptation in salmon. In the present study, liver GHR mRNA expression was transiently increased one day after 1/2 SW transfer. GHR expression tended to be higher in fish in 1/2 SW thereafter although it was not significantly different. This agrees with the finding by Sakamoto and Hirano (1991) that total GH binding sites in the liver of rainbow trout (Oncorhynchus mykiss) transferred to 80% SW tended to be high and became significantly higher two weeks after transfer. This suggests that GH binding capacity may be regulated at the transcriptional level. Average plasma IGF-I levels were similar between FW and 1/2 SW despite a tendency of higher IGF-I levels in 1/2 SW. There appears to be no negative impact of fasting for up to five days on the basal IGF-I levels because IGF-I levels were similar to those in fed fish (data not shown). Mixed results in the response of growth regulating hormones to salinity are seen in non-salmonid species. Transfer of tilapia from SW to FW for five months resulted in a decline in growth rate, increases in plasma GH and IGF-I, and decreases in pituitary GH and liver IGF-I mRNA levels (Riley et al., 2003). In black sea bream (Mylio macrocephalus) hepatic IGF-I increased in fish adapted to 1/3 SW and full SW after eight months (Deane et al., 2002). In the four spine-sculpin (Cottus kazika), adaptation to full SW for 44 days resulted in an elevation of hepatic IGF-I mRNA, but not pituitary GH mRNA (Inoue et al., 2003).

There is only one study examining the effect of salinity on circulating IGFBPs (Shepherd et al., 2005). In their experiment, juvenile rainbow trout were gradually acclimated to 66% SW over five days. As a result, the intensity of four IGFBP bands at 21, 32, 42 and 50 kDa on ligand blotting was higher in fish at higher salinity. In the

present study, plasma 41-kDa IGFBP levels increased one day after 1/2 SW transfer while its increase lasted for just one day. This conflicts with the finding by Shepherd et al. (2005). As discussed above, differences in species and experimental conditions may contribute to the discrepancy. Overall, direct transfer of postsmolt coho salmon to 1/2 SW had a transient, positive effect on liver GHR mRNA expression and 41 kDa IGFBP levels.

Many of the components in the GH-IGF-I axis are regulated by their own control system. For example, GH induces production of IGF-I and IGFBP-3 in the liver, and IGF-I is capable of inhibiting GH synthesis and secretion by the pituitary (Le Roith et al., 2001). This regulatory mechanism appears to be operative in teleosts (Duan, 1997; Björnsson et al., 2002). The present study examined the effect of salinity on the potency of GH to stimulate the GH-IGF-I components (i.e. GHR mRNA, plasma IGF-I and 41-kDa IGFBP) in salmon. Biological actions of GH are mediated by a transmembrane GH receptor. GHR is composed of two subunits and forms a dimer with another GHR upon binding GH (Argetsinger and Cater-Su, 1996). It is not clear, however, if GHR expression is under control by GH. In mouse hepatocyte culture, GH alone had no effect on GHR mRNA cellular concentrations, whereas a synergistic effect with estrogen was seen (Contreras and Talamantes, 1999). In fish, no study has examined the effect of GH on GHR mRNA expression. The present study showed that liver GHR mRNA was increased one day after GH injection. When fish were held in FW, the GH effect diminished in two days, whereas the effect was still evident in fish in 1/2 SW. This difference is presumably attributed to relatively high levels of GHR mRNA in the sham-

operated group in FW on day 4 (two days after injection). However, it is clear that GH induces its own receptor in the liver in salmon.

Induction of circulating IGF-I by GH administration is a well-known response in salmonids (Moriyama et al., 1994; Moriyama, 1995). A new finding of this study is that the time course and magnitude of the IGF-I response differed between two salinities. Fish in FW showed a maximum response on day 3 (one day after injection) and IGF-I levels gradually decreased thereafter. In contrast, plasma IGF-I levels in fish in 1/2 SW peaked on day 4 (two days after injection). Moreover, when fish were injected at the high dose (8 μg/g), IGF-I levels were higher in fish in 1/2 SW than those in FW on the same date. It is possible that IGF-I levels in fish in FW reached a peak in less than 24 hr, although Moriyama (1995) reported that in trout in FW plasma IGF-I levels followed by GH injection continued to increase until 24 hr.

The 41-kDa IGFBP is one of three major circulating IGFBPs in salmon (Shimizu et al., 2003a,b). Although its identity is still not clear due to the lack of complete amino acid sequence data, several lines of evidence from physiological and biochemical studies suggest that it is physiologic equivalent of mammalian IGFBP-3 (Shimizu et al., 2003a,b; Beckman et al., 2004a,b). IGFBP-3 prolongs the half-life of IGF-I and therefore forms a large pool of IGF-I in the circulation (Rajaram et al., 1997). A similar IGFBP with a molecular mass of 40-50 kDa has been detected in other fish species and shown to be induced by GH, as is mammalian IGFBP-3 (Siharath et al., 1995; Park et al., 2000). The result from the present study is in good agreement with the previous findings in fish. It is worth noting that the response of the 41-kDa IGFBP to GH is almost identical to that of IGF-I. In addition, simple regression analysis confirmed that their levels are positively,

highly correlated (data not shown). These results further support our assumption that the 41-kDa IGFBP is the main carrier of circulating IGF-I in salmon.

The present study showed that the response of circulating IGF-I to GH administration differed between two salinities. What caused the difference? An obvious possibility is a difference in the clearance of exogenous GH. Measurement of pGH by a specific RIA enabled us to monitor pGH levels after injection and revealed that pGH was retained longer in the circulation in fish in 1/2 SW. This may be due to a difference in the glomerular filtration rate at the kidney under different salinities. The kidney is one of the main sites of GH clearance in mammals, where 20-50% of circulating GH is cleared (Feld and Hirschberg, 1996). In salmonids, the glomerular filtration rate at the kidney is known to be reduced in a hyperosmotic environment (Brown et al., 1978). GH-binding protein (GHBP) might contribute to the slower disappearance of pGH from the circulation since GHBP has been shown to increase in SW in trout (Sohm et al., 1998). Another possible reason for the difference in the GH effect on IGF-I may be GHR. The transient increase in GHR mRNA levels in 1/2 SW might induce a greater response. The reason for the significantly higher IGF-I levels in fish that received 2 μg/g GH in FW is not clear. It might to due to an inhibition of GH effect by GHBP increased in 1/2 SW. In mammals, GHBP is capable of inhibiting GH interaction with GHR (Barnard and Waters, 1997). Levels of GHBP in fish in 1/2 SW might have been high enough to prevent the low dose of pGH, but not for the high dose of GH, from binding GHR. However, we have no data on GHBP levels.

The biological significance of the prolonged induction of IGF-I in SW observed in the present study is difficult to interpret; It is not known whether induced IGF-I would

298 be utilized for growth or osmoregulation. Collie et al. (1989) found that a two-day pre-
299 adaptation of trout in 1/3 SW prior to transfer to 80% SW enhanced the plasma ion
300 lowering effect of ovine GH. Although IGF-I levels were not measured in their study due
301 to the lack of the immunoassay at that time, IGF-I was most likely induced in the
302 circulation and might mediate the ion lowering action of GH. It is thus possible that in
303 the present study the induced IGF-I might have an osmoregulatory action rather than a
304 growth promoting action. To distinguish these two actions, a longer acclimation period
305 in SW (with feeding) should be helpful in future work.
306 In summary, the present study suggests that salinity is capable of altering the
307 "activity" of the GH-IGF-I axis. The prolonged response of plasma IGF-I in fish in 1/2
308 SW may be due to a slower clearance of pGH. The transient increase in the basal liver
309 GHR mRNA levels in fish in 1/2 SW may also contribute to the greater response.
310

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482

Figure legends

Fig. 1 Changes in liver GHR mRNA, plasma IGF-I and 41-kDa IGFBP levels after transfer to 1/2 SW.

Postsmolt coho salmon reared in FW were directly transferred to either FW or 1/2 SW . GHR mRNA was quantified by real-time RT-PCR using acidic ribosomal phospoprotein P0 (ARP) as an internal control. Plasma levels of IGF-I and 41-kDa IGFBP were measured by radioimmunoassays. Values are mean ± SEM (n = 6). For statistical analysis, values were natural-log transformed. Asterisks indicate significant difference between FW and 1/2 SW for a given time point (Fisher's PLSD, $P < 0.05$).

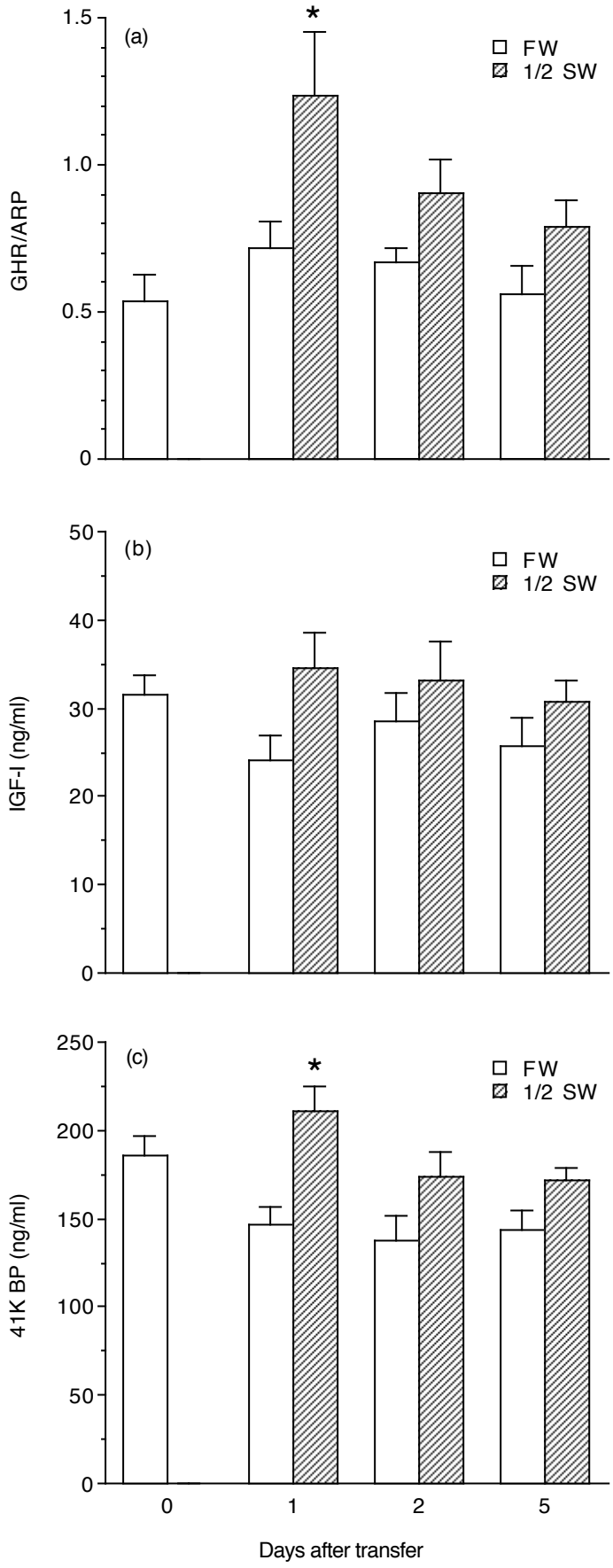
Fig. 2 Effect of GH administration on liver GHR mRNA levels in postsmolts in FW and 1/2 SW.

Fish were acclimated in FW or 1/2 SW for two days and injected once with 2 μg/g body weight porcine GH (a) or 8 μg/g (b). Sham fish received saline only. GHR mRNA was quantified by real-time RT-PCR using acidic ribosomal phospoprotein P0 (ARP) as an internal control. Values are mean ± SEM (n = 6). For statistical analysis, values were natural-log transformed. Symbols sharing the same letters are not significantly different from each other for a given time point (Fisher's PLSD, $P < 0.05$).

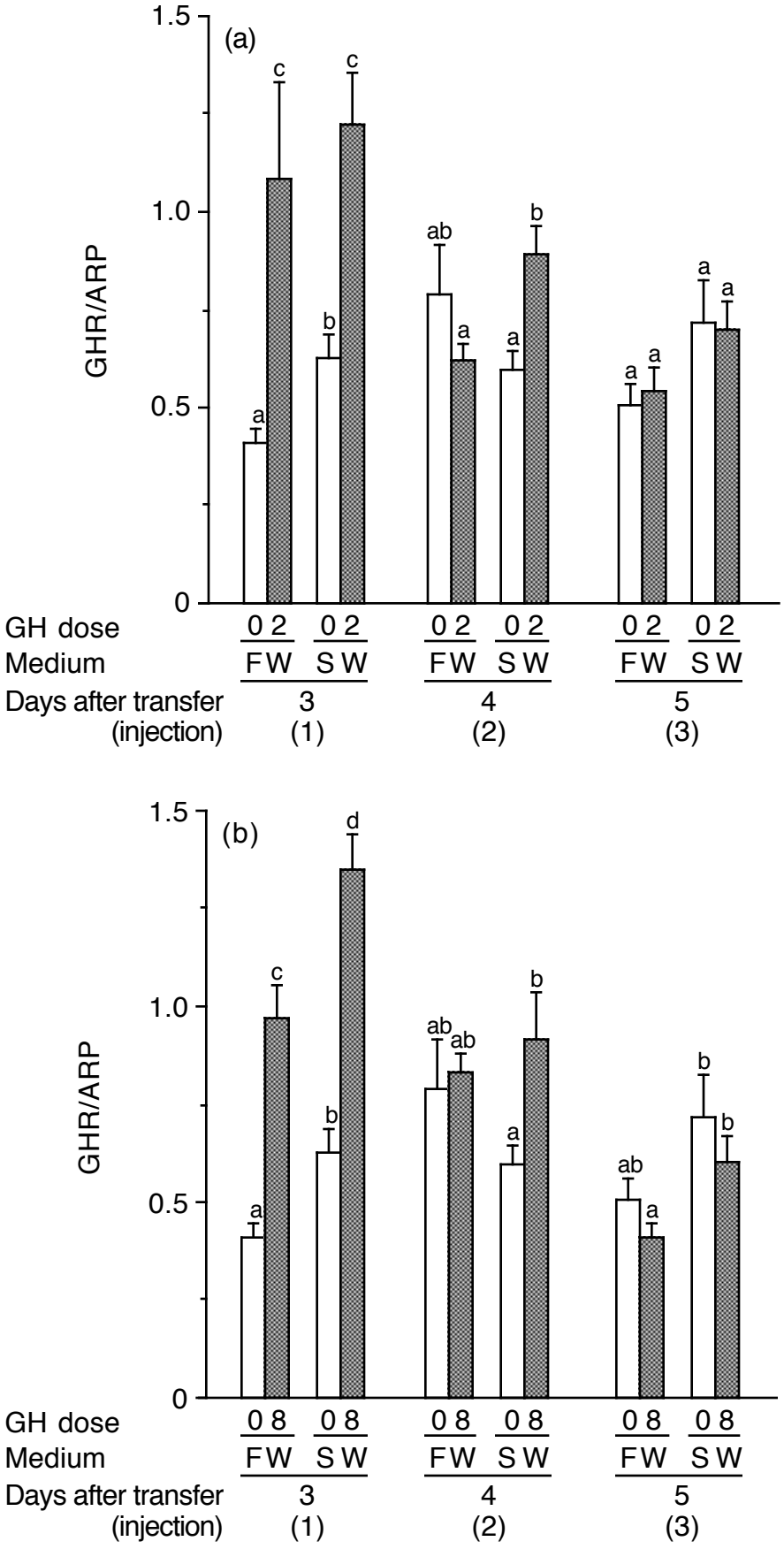
Fig. 3 Effect of GH administration on plasma IGF-I levels in postsmolts in FW and 1/2 SW.

Fish were acclimated in FW or 1/2 SW for two days and injected once with 2 μg/g body weight porcine GH (a) or 8 μg/g (b). Sham fish received saline only. Plasma levels of

505 IGF-I were measured by radioimmunoassay. Values are mean ± SEM (n = 6). For
506 statistical analysis, values were natural-log transformed. Symbols sharing the same
507 letters are not significantly different from each other for a given time point (Fisher's
508 PLSD, $P < 0.05$).
509
510 Fig. 4 Effect of GH administration on plasma 41-kDa IGFBP levels in postsmolts in FW
511 and 1/2 SW.
512 Fish were acclimated in FW or 1/2 SW for two days and injected once with 2 µg/g body
513 weight porcine GH (a) or 8 µg/g (b). Sham fish received saline only. Plasma levels of
514 41-kDa IGFBP were measured by radioimmunoassay. Values are mean ± SEM (n = 6).
515 For statistical analysis, values were natural-log transformed. Symbols sharing the same
516 letters are not significantly different from each other for a given time point (Fisher's
517 PLSD, $P < 0.05$).
518
519 Fig. 5 Disappearance of injected porcine GH from the circulation of postsmolts in FW
520 and 1/2 SW
521 Fish were acclimated in FW or 1/2 SW for two days and injected once with 2 µg/g body
522 weight porcine GH (a) or 8 µg/g (b). Plasma levels of porcine GH were measured by a
523 specific radioimmunoassay. Values are mean ± SEM (n = 6). For statistical analysis,
524 values were natural-log transformed. Symbols sharing the same letters are not
525 significantly different from each other for a given time point (Fisher's PLSD, $P < 0.05$).



Shimizu et al., Fig. 1



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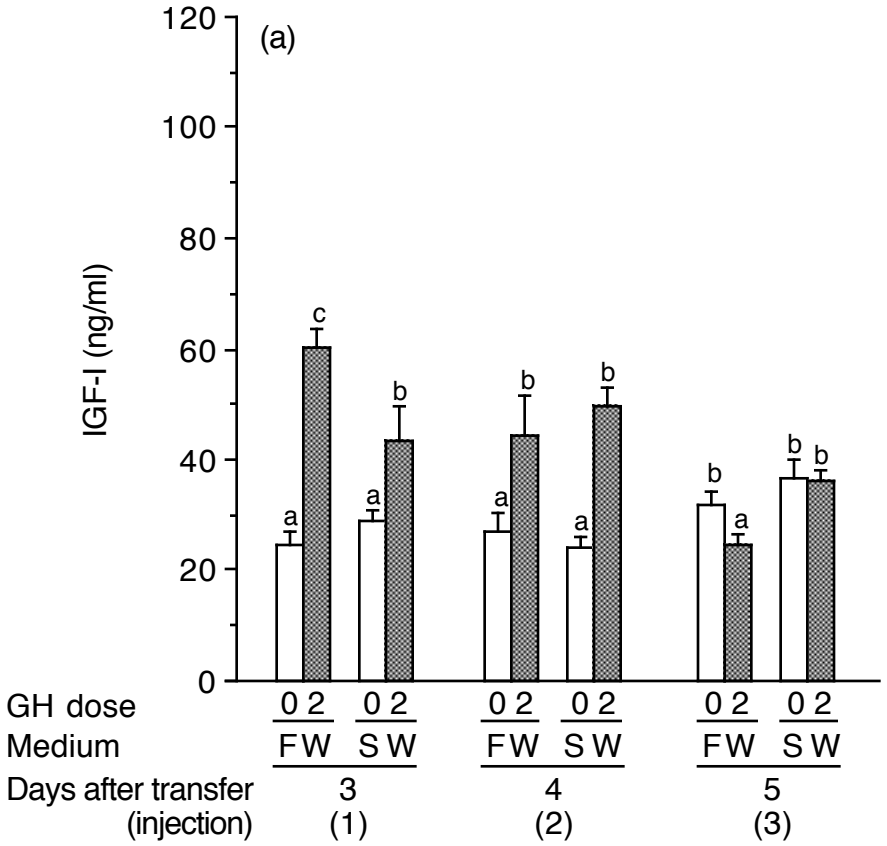

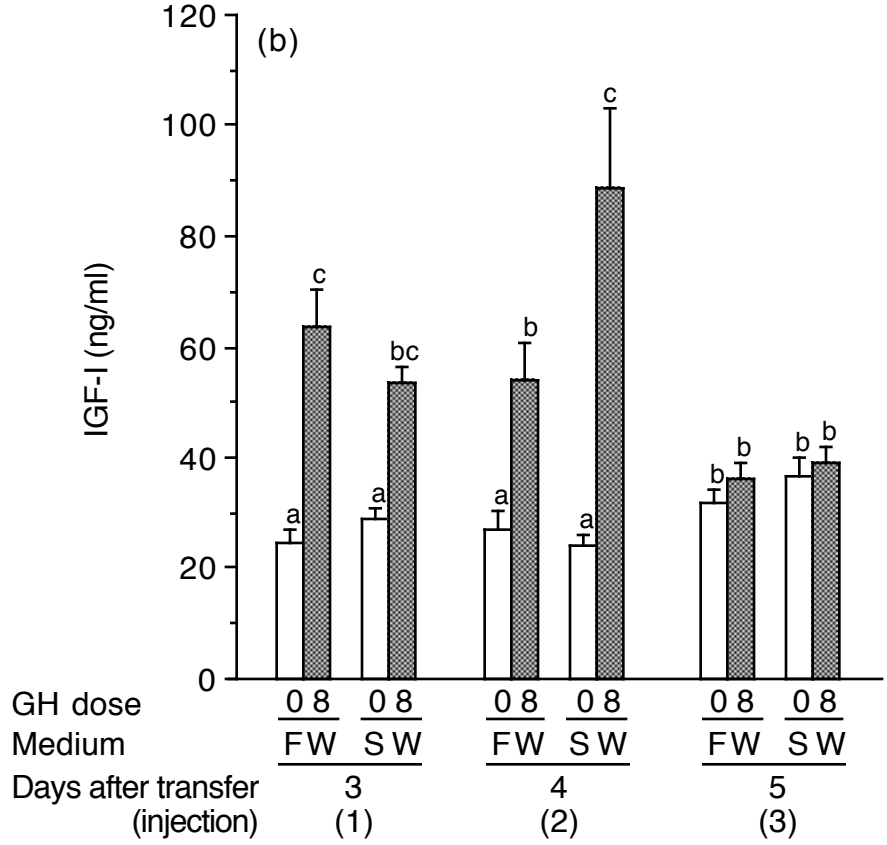

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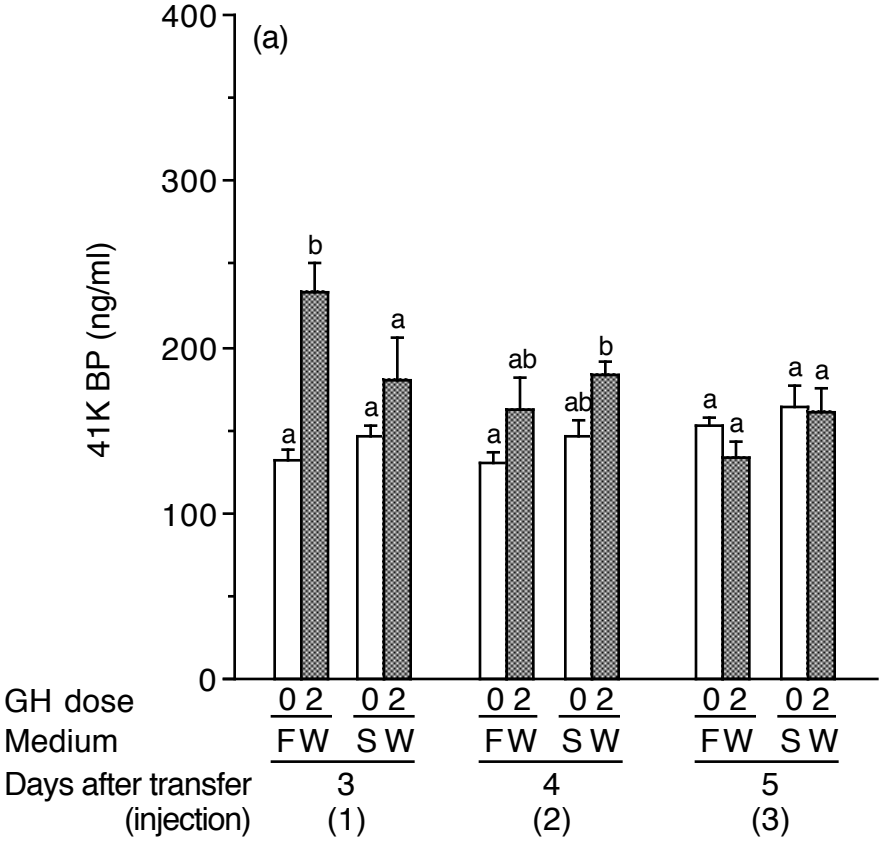
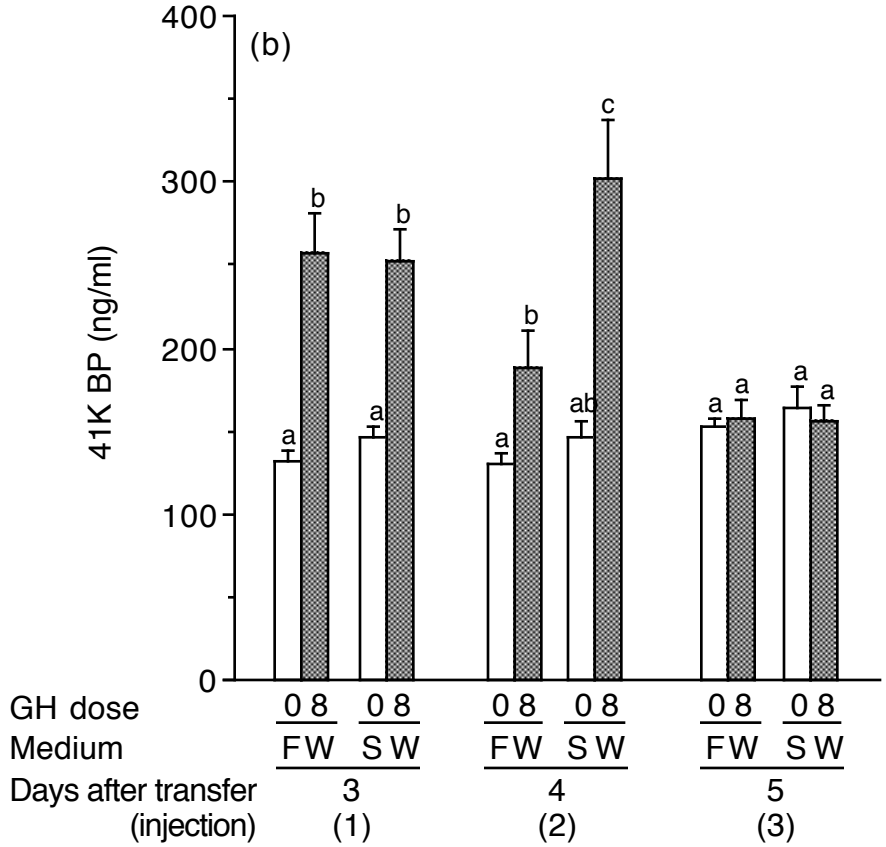
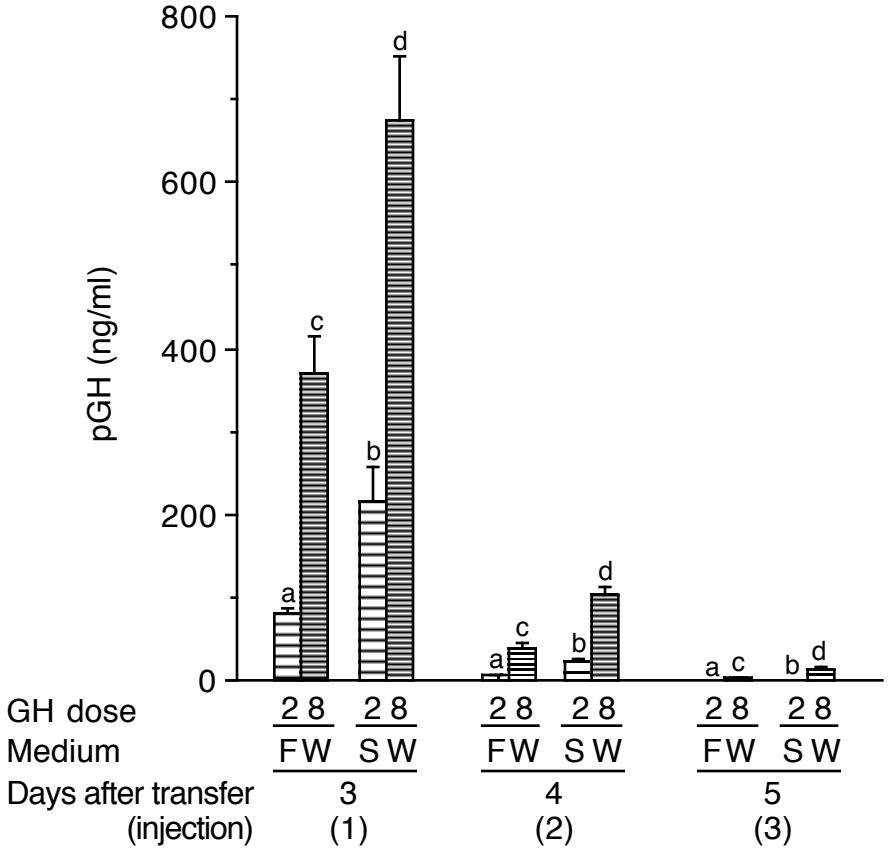




Shimizu et al., Fig. 4



Shimizu et al., Fig. 5