Insulin-like Growth Factor I (IGF-I) and Branchial IGF Receptor Expression and Localization During Salinity Acclimation in Striped Bass

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Running headline: Response of IGF-I and IGF receptor to salinity challenge

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Abstract

The initial response of the IGF-I system and the expression and cellular localization of IGF type I receptor (IGF-IR) were studied in the gill of a euryhaline teleost during salinity acclimation. Exposure of striped bass (*Morone saxatilis*) to hyperosmotic and hypoosmotic challenges induced small, transitory (< 24 hours) deflections in hydromineral balance. Transfer from fresh water (FW) to seawater (SW) induced an initial decrease in plasma IGF-I levels after 24 hours in both fed and fasted fish. There was an overall decrease in liver IGF-I mRNA levels after SW transfer suggesting that decreased plasma levels may be due to a decline in hepatic IGF-I synthesis. No changes were observed in gill IGF-I mRNA, but SW transfer induced an increase in gill IGF-IR mRNA after 24 hours. Transfer from SW to FW induced an increase in plasma IGF-I levels in fasted fish. In fed fish, no significant changes were observed in either plasma IGF-I, liver or gill IGF-I mRNA, or gill IGF-IR mRNA levels. In a separate experiment, FW acclimated fish were injected with saline or IGF-I prior to a 24 hours SW challenge. Rapid regain of osmotic balance following SW transfer was hindered by IGF-I. Immunohistochemistry revealed for the first time in teleosts that IGF-IR and Na⁺,K⁺-ATPase are localized in putative chloride cells at the base of the lamellae, identifying these cells in the gill as a target for IGF-I and IGF-II. Overall the data suggest a hyperosmoregulatory role of IGF-I in this species.

Key words: gene expression; osmoregulation; teleost; gill; liver.
**Introduction**

Most euryhaline teleosts are able to maintain their plasma osmolality at roughly the same level (~300-350 mOsm) following acclimation to fresh water (FW) and seawater (SW). The striped bass (*Morone saxatilis*) is an example of a euryhaline fish that regains ion-osmotic homeostasis on a very rapid time course (< 24 h) after salinity shifts between FW and full-strength SW (36). Hydromineral balance in euryhaline teleosts is regulated by a series of hormones. Generally, prolactin is FW-adaptive, growth hormone (GH) and insulin-like growth factor 1 (IGF-I) are SW-adaptive, whereas cortisol may play a dual role (20). The first findings of the influence of the GH/IGF-I on osmoregulation were derived from studies on salmonids, where IGF-I was shown to promote SW adaptation (18). In killifish, a single injection of recombinant bovine IGF-I (rbIGF-I) improves SW tolerance (15). By contrast, our preliminary investigations in the temperate striped bass indicate that IGF-I may antagonize SW osmoregulation. Thus, a single injection of rbIGF-I results in a greater hydromineral imbalance 24 hours after transfer to SW than that observed in saline-injected fish (14).

In the early development of gill filaments in Shi drum, IGF type I receptor (IGF-IR) immunoreactivity was found in gill epithelial cells, as well as chondroblasts of the gill arch (26). In 35-day old larvae of gilthead seabream, gill IGF-IR immunoreactivity was found to be strongest in epithelial cells localized at the base of the secondary lamellae (22). Collectively, these studies suggest an important role of IGF-I in the development of gill epithelia in teleosts. In osmoregulatory tissues the Na⁺,K⁺-ATPase is a major driving force for epithelial transport of NaCl. In the gills, this enzyme is primarily located in chloride cells and involved in active ion transport (39). Chloride cells are the site of active ion extrusion in SW and may also be involved in ion uptake in FW (7, 16) and therefore regulation of this epithelial cell type may be
particularly crucial to osmoregulation. Even though IGF-I stimulates gill Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in several teleosts (\textit{in vivo}: killifish, 15; Atlantic salmon, 19; brown trout, 30; \textit{in vitro}: coho salmon, 12) and chloride cell abundance in brown trout (30), direct evidence of chloride cells as target cells for IGF action is still lacking. Moreover, to our knowledge no studies have concurrently assessed changes in circulating IGF-I and branchial IGF-I and IGF-IR gene expression during the salinity acclimation process in teleost fishes.

The purpose of the present study was to examine the integrated response of IGF-I and its receptor during acclimation of striped bass to FW and SW. We examined, i) changes in systemic IGF-I, ii) its relationship to changes in hepatic IGF-I mRNA levels, iii) the expression of IGF-I and IGF-IR in the gill, and iv) branchial co-localization of IGF-IR with regard to key ion transporting proteins. Finally, we tested the short-term effect of exogenous IGF-I \textit{in vivo} on acclimation to SW.

**Materials and Methods**

**Animals**

Sexually immature, juvenile striped bass (\textit{M. saxatilis}; 30-60 g, mixed sex) were obtained from freshwater (FW) ponds at the Edenton National Fish Hatchery (Edenton, NC, USA) in December 2003 and 2004. All fish were transferred to tanks on the N.C. State Campus (Raleigh) supplied with de-chlorinated, recirculated FW (21-22°C, 12:12 hours light:dark artificial photoperiod). One group of fish was maintained in FW; another group was acclimated to full strength sea water (SW, 35 ppt). All fish were acclimated to the respective salinities in the salinity transfer tanks for a minimum of 3 weeks before start of experiments from January to
February each year. The fish were fed 3% body weight daily with a pelleted feed (Southern States, Richmond, VA, USA) until the day before sampling, unless otherwise indicated. All experimental procedures were performed in accordance with NIH *Guiding Principles for Care and Use of Laboratory Animals* and were approved by the North Carolina State University Animal Care and Use Committee.

**Salinity Challenges**

*FW-SW and SW-FW time course experiment 2004:* Striped bass were transferred directly from FW to SW (35 ppt) and from SW to FW and sampled at 0, 6, 24, and 96 hours after transfer. Fish sham-transferred to FW or SW were used as controls. In this initial experiment, the fish were not fed two days before or during the experiment. Since this experiment was also part of a drinking rate experiment using the $^{51}$Cr-EDTA method (6), the fish were transferred to a drinking tank 6 hours before sampling.

*FW-SW and SW-FW time course experiments, 2005:* Striped bass were transferred directly from FW to SW and from SW to FW and sampled at 0, 4, 24, 96 and 192 hours after transfer. Fish sham-transferred to FW or SW were used as controls. In these experiments, each tank of fish was fed 3% body weight daily until the day of sampling to eliminate any potential confounding factors associated with metabolic changes that might occur with feed deprivation in the first experiment. Feed consumption was similar among groups in this experiment.

**Effects of Exogenous IGF-I on SW Acclimation**

Fish acclimated to FW were mildly anaesthetized and given a single intraperitoneal injection of saline (0.9% NaCl, 0.05% NaOH, 0.4% bovine serum albumin) or barramundi IGF-I (100
ng/g body weight; GroPep, Adelaide, South Australia, Australia). The fish were allowed to recover from sedation and then transferred directly to FW or SW. All fish where sampled 24 hours after injection.

**Sampling**

Fish were anaesthetized in buffered MS-222, blood was drawn from the caudal blood vessels into a heparinized syringe, kept on ice, centrifuged at 5000 g for 3 min, and plasma was transferred to a separate tube and stored at –80°C until analyses. Before further sampling, the fish were killed by spinal section and pithing of the brain. For RNA analyses one first gill arch and a liver section was sampled from each fish and quickly snap frozen in liquid nitrogen and stored at –80°C. A piece (0.5 g) of muscle was sampled from the caudal region for determination of water content.

**Analyses**

*Plasma osmolality, plasma cortisol, plasma glucose and muscle water content:* Plasma osmolality (mOsm/kg) was measured by vapor pressure osmometry (Wescor, Logan, UT, USA). Plasma cortisol was measured by a Coat-A-Count radioimmunoassay according to the manufacturers instructions (Diagnostic Products, Los Angeles, CA). Plasma glucose was measured by a quantitative enzymatic assay for glucose according to the manufacturers instructions (Sigma, Saint Louis, MO). Muscle water content was measured gravimetrically after drying at 90 °C for 24 hours.
**IGF-I Radioimmunoassay (RIA):** Measurement of plasma IGF-I was carried out by using commercially available tuna IGF-I as standard and barramundi antisera previously validated for use as a universal IGF-I antibody in teleosts (GroPep). The hormone was iodinated and the procedure of the assay was according to Riley et al. (27) with slight modifications. In short, total IGF-I from each plasma sample was extracted by acidic ethanol, neutralized, and the extract was used in the RIA. Extracted plasma was assayed by incubating with the iodinated tracer and the primary antibody overnight (4 °C), followed by precipitation of bound antigen with secondary antibody and PEG for 2 hours (20 °C) and centrifugation at 3000 X g for 60 min (4°C). The validity of the IGF-I RIA for striped bass was confirmed by means of a concentration displacement curve of striped bass plasma which paralleled that of the standard.

**Extraction of RNA and cDNA synthesis:** Total RNA was extracted from gill and liver samples using Tri-Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA). An optional centrifugation step that aids in reducing genomic DNA contamination was included following tissue homogenization. For liver samples a high salt precipitation step was included, which reduces glycogen in extracted RNA. Following solubilization in DEPC-treated water, RNA for each sample was treated with DNA-free™ (Ambion, Austin, TX, USA) to remove any possible genomic DNA contamination. The volume of DNase-I enzyme was increased to 2 μl (4 units) per reaction and the incubation time was extended to 1 hour. RNA concentration was determined in duplicate for each sample using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purity (A260/A280) ranged from 1.8-2.0. Single-stranded cDNA was synthesized from 1 μg of total RNA using the Omniscript™ RT kit and oligo (dT) primers in 20 μl reactions according to the manufacturer’s protocol (Qiagen Inc., Valencia, CA, USA).
Quantification of IGF-I and IGF-IR mRNA: Gene-specific primers and dual-labeled FRET probes for qRT-PCR were designed within the partial coding sequences for striped bass IGF-I and IGF-IR (GenBank Accession Numbers AF402670 and AF402673, respectively) using Primer Express® 2.0 software (Applied Biosystems, Foster City, CA, USA). Primers and probes were purchased from Biosource (Camarillo, CA, USA). The IGF-I forward primer was 5´-TTGTGTGTTGGAGAGAGGCTTT-3´, the reverse primer 5´-TGACCGCCGTGCATTG-3´, and probe 5´-TTTCAGTAAACCTACAGGCTATGGCC-3´. The IGF-IR forward primer was 5´-CGAGGGCTTGGCAAAGG-3´, reverse primer 5´-CGACTCGTTGACCGGTCTTAATG-3´, and probe 5´-CAAAGATGAACCGGAGACCCGCG-3´. The reporter and quencher dyes for the FRET probes were FAM and TAMRA, respectively.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with a GeneAmp® 5700 Sequence Detection System (Applied Biosystems) using the standard cycling conditions recommended by the manufacturer: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Reactions for each sample were done in duplicate and reaction volumes were 25 µl. Each reaction consisted of cDNA template corresponding to 10 ng total RNA, 1X TaqMan® Universal PCR master mix (Applied Biosystems), 900 nM forward and reverse primers and 250 nM probe. Control reactions lacking cDNA template were included in each PCR plate to confirm that reagents were not contaminated with carryover PCR products. Control reactions containing RNA that was not reverse transcribed were also included to test for genomic DNA contamination in the RNA preparations. Serially diluted liver cDNA (for the IGF-I assay) and gill cDNA (for the IGF-IR assay) derived from RNA pooled from 4 fish was included in each plate to generate a standard curve. Dilutions ranged from 0.01-100 ng.
Relative abundance of IGF-I and/or IGF-IR transcripts in each experimental sample was extrapolated from the standard curves (for details see the Relative Standard Curve Method; ABI Prism 7700 Sequence Detection System, User Bulletin #2, P/N 4303859 Rev. A, Stock No. 777802-001 available from Applied Biosystems). Expression of IGF-I and IGF-IR mRNA was normalized to total RNA as recommended by Bustin (2). Similar to that previously reported and reviewed by Bustin (2), our initial validation studies show that normalization of target RNA to total RNA yields similar results as that for normalization to 18S RNA (23).

**Immunohistochemistry and antibodies:** Immunoreactive cells were visualized on 5 µm gill sagittal sections. Details of the immunoreactivity staining procedure are described in Seidelin et al. (30). To detect IGF-IR immunoreactive cells a polyclonal antibody raised against a highly conserved portion of the carboxy terminus of the β subunit of the human IGF-IR was used (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:110 dilution). We previously showed that this antibody binds Morone pituitary IGF-IR specifically (5). For the negative control a blocking peptide corresponding to the carboxy terminus of the IGF-IR β subunit was used in 5-fold surplus relative the primary antibody (Santa Cruz Biotechnology). The secondary antibody µ (Goat-anti-rabbit-HRP; Zymed, San Francisco, CA, USA) was diluted 1:400.

Detection of Na⁺,K⁺-ATPase and Na⁺,K⁺,2Cl⁻ cotransporter immunoreactive cells was done using a battery of monoclonal antibodies. The monoclonal α5 is directed against the chicken Na⁺,K⁺-ATPase α1 subunit, is specific for a cytosolic epitope, and reacts with all isoforms of distant species. It was developed by D.M. Fambrough (Johns Hopkins University, Baltimore, MD; see 34) and used in a 1:80 dilution. The monoclonal T4 antibody is specific for Na⁺,K⁺,2Cl⁻ cotransporter, and reacts with an epitope on the carboxy-terminus conserved between the
antigens two isoforms in distantly related species. It was developed by C. Lytle (University of California, Riverside, CA; see 11) and B. Forbush III (Yale University School of Medicine, New Haven, CT) and used in a 1:40 dilution. Both these primary antibodies were obtained from the Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Both α5 and T4 antibodies were used in combination with 1:200 dilution of the above secondary antibody from Zymed. After incubation with the secondary antibody, the peroxidase reaction was run for 10 min using 3,3*-diamino-benzidine as substrate, creating a brown precipitate. Nuclei were subsequently stained with Meyer’s haematoxylin.

**Statistics**

A two-factorial ANOVA was used to analyze overall effects of salinity exposure (i.e. FW or SW; factor 1) and sampling time (factor 2). The group of fish taken from the tank at time 0 was treated statistically as both a FW and a SW group. When there was a significant interaction between the two factors the two-way ANOVA was followed by Bonferroni-adjusted Fisher’s least significant difference test, comparing sham-control and challenged group at each time point. Data from the injection experiment were analyzed by one-way ANOVA and groups were subsequently compared with a Tukey multiple comparison test. All statistical analyses were performed using Statistica 7.0 (Tulsa, OK, USA) and significant differences were accepted when P < 0.05.
Results

In two initial experiments with fasted striped bass, the effects of salinity shifts on circulating IGF-I was investigated (2004 experiments). Transfer from FW to SW induced a transient decrease in plasma IGF-I after 24 hours, which returned to control levels by 4 days (Fig. 1A). By contrast, transfer of SW acclimated fish to FW induced a rapid increase in plasma IGF-I after 6 and 24 hours which returned to control levels by 4 days (Fig. 1B).

To further investigate the responses in the IGF-I system to salinity changes and to eliminate any potential metabolic changes associated with short-term feed deprivation in rapidly growing juvenile fish, these two experiments were replicated in 2005 to include hepatic and gill IGF-I and IGF-IR mRNA, and circulating cortisol and glucose analyses using fish fed daily. Transfer of FW acclimated fish to SW induced a rapid increase in plasma osmolality and a corresponding decrease in muscle water content (4 hours), both being corrected after 24 hours (Fig. 2A, 2B). The relative expression of hepatic IGF-I mRNA decreased in response to SW transfer (Fig. 2C). A corresponding decrease in circulating IGF-I was seen 24 hours after transfer and levels returned to control values at 4 days (Fig. 2D) as observed with the 2004 study. There was no statistically significant change in circulating cortisol and glucose in this experiment, albeit cortisol increased by 30% after 24 hours transfer to SW (Table 1).

Transfer of SW acclimated fish to FW induced a rapid decrease in plasma osmolality and a corresponding increase in muscle water content (4 hours), both being corrected after 24 hours (Fig. 3A, 3B). The relative expression of hepatic IGF-I mRNA did not change in response to FW transfer (Fig. 3C). In this experiment circulating IGF-I increased 24-h following FW transfer, although the effect was not statistically significant relative to sham-transferred animals (Fig. 3D). There was no significant change in circulating cortisol, although levels tended to decline over 24
hours of transfer to FW (34% reduction relative to sham transferred fish). Glucose levels did not differ significantly in this experiment (Table 2).

Gill IGF-I and IGF-IR mRNA expression were investigated during salinity transfer during experiments in 2005 to evaluate whether the receptor and paracrine sources of IGF-I might be involved in salinity adaptation at the level of the gill. Transfer to SW induced no significant change in gill IGF-I mRNA levels but an increase in the receptor expression was seen after 24 hours (Fig. 4). Transfer of SW acclimated fish to FW resulted in no change in the level of either IGF-I or IGF-IR transcripts (Fig. 5).

Since circulating IGF-I declines during the initial phase of SW transfer, an injection experiment was conducted to directly assess whether the growth factor might cause a further osmotic imbalance during the early phase of SW exposure. Freshwater fish were injected with saline (control fish) or IGF-I (100 ng/g body weight) and then transferred to SW or FW (sham-control) and sampled after 24 hours. Injection with IGF-I increased plasma osmolality and decreased muscle water content after SW transfer (Fig. 6A, 6B) compared to saline-injected controls. In addition, hormone injection counteracted the decrease in circulating IGF-I seen in SW-transferred control fish compared to FW-control (Fig. 6C).

To examine a possible cellular site of IGF-I regulation of gill function, localization of major ion transport proteins and the IGF-IR in gill was examined by immunostaining of serial sections. In initial studies, we found that gill cells at the base of the lamellae in the primary filament stained positively with the IGF-IR antibody. The immunostaining was specific, since exclusion of the primary antibody or pre-incubation with a blocking peptide both prevented localized staining (data not shown). Positive IGF-IR staining appeared to be localized to similar areas of the gill in both FW and SW acclimated fish (not shown). The Na\(^+\),K\(^+\)-ATPase and Na\(^+\),K\(^+\),2Cl\(^-\)
cotransporter antibodies stained large, putative chloride cells, at the base of the lamellae in the filament (Fig. 7A, 7B). The IGF-IR antibody stained similar, putative chloride cells, at the base of the lamellae (Fig. 7C).

**Discussion**

The relationship between systemic IGF-I and local expression of this growth factor and its receptor in the gill of fish during salinity adaptation is poorly understood. In the present investigations we assessed changes in plasma, liver and gill expression of IGF-I, as well as, branchial IGF-IR in the striped bass during acclimation to either FW or SW. We found that acclimation to FW induces a rapid increase in systemic IGF-I, that is more pronounced in fish deprived of feed versus those fed throughout the course of FW challenge. In contrast, SW acclimation is accompanied by a decrease in circulating IGF-I, as well as, liver IGF-I mRNA levels. Gill IGF-IR mRNA expression was stimulated during SW acclimation, and the receptor was localized in putative chloride cells.

In accordance with previous studies (13, 36), the striped bass acclimates rapidly following salinity changes (Fig. 2, 3), as judged by the restoration of plasma osmolality and muscle water content within 24 hours. This appears a very fast adjustment compared with most other euryhaline teleosts examined (e.g.: Mozambique tilapia, 3; Milkfish, 4; European sea bass, 9; brown trout, 31; Longjawed mudsucker, 40) where a longer period (≥ 3 days) of disturbance is generally observed.

Transfer of SW acclimated fish to FW induced a significant increase in plasma IGF-I in fasted fish (Fig. 1B) and an increase, albeit not statistically significant, in fed fish (Fig. 3D). Based on these results it appears that feeding status might interact with the response to FW
transfer, since the increase in IGF-I is more intensified in fasted striped bass. The underlying mechanism for this difference in responsiveness is uncertain. Fasting would be expected to decrease circulating insulin as seen in Chinook salmon (25), which in turn may decrease the sensitivity of the liver to GH and thereby IGF-I output (24). In contrast, in the present study a positive interaction of FW with fasting was observed with respect to plasma IGF-I indicating that this mechanism is not likely significant within the short time-course of the present study. On the other hand, short-term stress induces GH secretion in rainbow trout and the response is blocked by elevations in plasma glucose levels (glucose injection), independent of insulin (34). Therefore, potential reductions in plasma glucose levels during short-term fasting could intensify activation of GH, and hence the secretion of IGF-I, as observed in the present study. However, we are unable to confirm whether this is the case as glucose levels in short-term fasted fish of the 2004 trial could not be measured for lack of plasma to compare with fed fish (2005 study) whose glucose levels did not differ significantly in any of the salinity acclimation trials (Tables 1 and 2). Further studies are clearly warranted to directly assess in both fed and fasted fish any potential interactions of nutritional state with IGF-I responsiveness during the FW acclimation process.

In contrast to that observed with FW transfer, SW transfer of both fed (Fig. 2D and 6C) and fasted (Fig. 1A) fish induced a transient drop in circulating IGF-I after 24 hours, coinciding with the regain of osmotic balance. We found that IGF-I mRNA levels in liver declined in parallel with circulating IGF-I levels suggesting decreased hepatic synthesis of the hormone and thus secretion into the circulation (Fig. 2C and 2D). This supports the notion that hepatic IGF-I may be the primary source of circulating hormone as has been shown for mammals (32). One can not rule out the possibility that the decline in circulating IGF-I may reflect increased clearance of the
hormone, however.

Our preliminary results indicate that SW transfer produced an early decline in circulating GH levels 4 hours after transfer and then a subsequent increase above sham animals by 24 hours (4-hr sham control, 2.82 ± 0.51 and 4-hr SW transfer, 0.74 ± 0.18 ng/ml; 24-hr sham control, 2.16 ± 0.29 and 24-hr SW transfer, 5.07 ± 0.74 ng/ml [Mean ± SEM]; unpublished data). Thus, early declines in GH-stimulated production of hepatic IGF-I may be involved in a subsequent drop in circulating IGF-I and hepatic IGF-I mRNA levels reported here during the initial period following SW transfer. The lower IGF-I concentration at 24 hr post-SW transfer might, in turn, lead to a rebound in circulating GH levels due to the lower degree of negative feedback inhibition elicited by IGF-I on GH secretion and synthesis (5). In a previous study it was shown that SW transfer of adult striped bass induces an initial increase in circulating cortisol after 6 and 12 hours (13). In another euryhaline fish, the Mozambique tilapia, cortisol reduces hepatic IGF-I mRNA and circulating IGF-I despite exerting little effect on plasma or pituitary GH dynamics (10). In view of this, an alternative mechanism for the SW-induced drop in IGF-I may be that cortisol reduces IGF-I sensitivity to GH. Although, we found no statistically significant changes in circulating cortisol in these studies, levels gradually increased and peaked by 24 hrs after SW transfer with the opposite occurring during FW transfer (Tables 1 and 2). In light of previous findings in striped bass where cortisol levels increase following SW transfer (13), the lack of a more prominent change observed here may have been masked by the high basal levels of cortisol (100-200 ng/ml) that likely resulted from stress associated with netting and sedation during sampling. A preliminary follow up study using similar-sized fish and rearing conditions show that the netting/sedation (MS-222) and blood sampling process (15 min) produces elevated plasma cortisol levels of around 150-225 ng/ml that can be completely blocked with the addition
of metomidate (a blocker of glucocorticoid biosynthesis) to the sedation tank ([cortisol] = 15-25 ng/ml). Future studies, including those that utilize metomidate during sampling, are required to confirm whether cortisol or other regulators might contribute to reductions in IGF-I shown here during SW acclimation in both fed and fasted fish.

The up-regulation of circulating IGF-I during FW acclimation and down-regulation during SW acclimation, points to this growth factor as a possible hyperosmoregulatory hormone in striped bass. This hypothesis is further supported by direct evidence derived from injection experiments. Exogenous treatment of FW-fish with IGF-I induces physiological FW levels of the hormone after SW transfer and produced an anti-SW adaptive effect (Fig. 6). Twenty-four hours after SW transfer striped bass typically have regained osmoregulatory balance, however injection with IGF-I clearly prolonged the crisis seen initially after 4 hours (Fig. 2 and 6). The initial drop in IGF-I synthesis and circulating levels may be causally related to the ability to acclimate rapidly to SW in this species. Accordingly, opposing this decrease with a single IGF-I injection disturbs the rapid acclimation to SW.

Evidence for a possible function of IGF-I in salinity acclimation has been documented in other teleosts, mainly the salmonids, where it appears to function as a SW-adaptive hormone, which is opposite to that observed here for striped bass. The developmental process of smoltification in salmonids is a pre-adaptation to SW while the fish still resides in FW. This process is associated with increased hepatic expression of IGF-I mRNA (29) and circulating IGF-I (1), suggesting a hypo-osmoregulatory role of the hormone. On the other hand, direct transfer of rainbow trout to 80 % SW induced no change in liver IGF-1 mRNA levels (28) while acclimation to 66 % SW induced a gradual increase in circulating IGF-1 levels over 5 days (33). More conclusive evidence for IGF-I’s role as a SW-adaptive hormone in salmonids (rainbow
trout, 18; Atlantic salmon, 19; brown trout, 30) is offered by injection experiments, where IGF-I improves hypo-osmoregulatory ability.

It is possible that the opposing osmoregulatory functions of IGF-I seen in salmonids versus that of striped bass shown here are related to the relative preparedness these species have for coping with changes in salinity. Most salmonids require the developmental period of smoltification to acclimate gradually to SW, a process that requires increased expression of important gill ion transporters and proliferation and re-organization of chloride cells (21, 31, 37). By contrast, euryhaline fish like the striped bass maintain a high level of preparedness to cope with SW throughout their life (13, 36). Indeed, the striped bass already possesses high levels of expression and activity of gill Na⁺,K⁺-ATPase in FW environments, which may dramatically improve the ability of this species to adapt to SW. The enzyme also shows little change in response to either FW or SW challenge (13, 36). It appears therefore, that the striped bass has the pre-existing machinery needed to minimize osmotic perturbations that comes with SW entry. It is noticeable that all changes in circulating IGF-I observed in striped bass in response to salinity changes were transitory and not prolonged. This suggests that IGF-I may serve dual roles, both as a hyperosmoregulatory hormone during rapid salinity acclimation, and as a growth promoting and developmental hormone in both FW- and SW- acclimated fish. Interestingly, in several teleosts (striped bass and other Morone spp., 5; tilapia, 10) IGF-I has been reported to augment prolactin (PRL) release. Since PRL in general is thought of as a FW-adapting and SW-antagonistic hormone (20) the hyperosmoregulatory effect of IGF-I (or its opposing effect on hypo-osmoregulatory capacity) seen here with striped bass could be related, in part, to its stimulation of PRL secretion.

It has been suggested that locally produced IGF-I may mediate part of the osmoregulatory
function of the GH/IGF system in rainbow trout, since SW transfer induced increased IGF-I mRNA levels in gill and kidney, but not in liver (28). To examine a potential paracrine/autocrine role for IGF-I in salinity acclimation we measured gill IGF-I mRNA levels. Gill IGF-I mRNA levels were generally less than one order of magnitude lower than that measured in liver. The data showed considerable variation and no significant salinity effects on gill IGF-I mRNA levels were observed (Fig. 4, 5). Thus, it appears that local production of IGF-I may play a limited role on osmoregulatory function, at least at the level of the gill. Given the very heterogenous structure of the gill one cannot exclude that changes in individual cells may become masked by measuring responses in whole tissue. Nevertheless, we found that expression of gill IGF-IR transcripts were stimulated by SW challenge after 24 hours (Fig. 4). The elevated gill receptor mRNA and reduced circulating IGF-I levels seen with SW transfer could reflect alleviation of the negative feedback of receptor expression often observed in the presence of higher ambient ligand concentrations (38). The present results suggest that SW transfer may modulate the sensitivity of the gill to IGF-I. Future studies are required to assess whether protein levels of the receptor are similarly up-regulated during SW challenge.

Immunohistological evidence from teleost larvae indicates that early in development epithelia cells are target cells for IGFs (22, 26). In the present study on juvenile striped bass, an antibody raised against the carboxy terminus of the human IGF-IR, specifically stained large spherical cells at the base of the lamellae in the primary filament. The immunoreactive cells are most likely chloride cells, as their location and shape are similar to that of cells stained with Na\(^+\),K\(^+\)-ATPase and Na\(^+\),K\(^+\)-2Cl-cotransporter antibodies (Fig. 7 (13, 21, 31)).

In conclusion, this study demonstrates for the first time that SW challenge up-regulates gill IGF-IR transcript levels and that the receptor is specifically localized in putative chloride cells,
indicating that active ion transporting cells in the teleost gill are specific targets for IGF-I and/or IGF-II regulation. Successful and rapid SW acclimation in striped bass is blocked by exogenously administered IGF-I and appears to depend on a drop in circulating IGF-I, mediated, in part, through reductions in hepatic IGF synthesis. The mode of action and the targets for this IGF-I effect are not clear at present although interaction with gill chloride cells, as well as other osmoregulatory hormones (cortisol, PRL, and GH) may be involved.
Acknowledgements

The Edenton National Fish Hatchery (Edenton, NC, USA) is warmly thanked for supplying the experimental fish. The United States National Science Foundation supported this research by a grant to RJB (IBN-0215205). The Danish Natural Science Foundation supported this research by a grant to SSM (21-02-0520) and CKT (21-04-0035).
References


Legends

**Fig. 1.** Plasma IGF-I during salinity acclimation of striped bass fasted two days prior to and throughout the experiments in 2004. In A, FW-acclimated fish were transferred to SW (▲; SW challenged) or FW (■; sham control) at time 0. In B, SW-acclimated fish were transferred to FW (▲; FW challenged) or SW (■; sham control) at time 0. Asterisks indicate significant effects compared to sham-controls (*P < 0.05, ***P < 0.001). Values are means ± SEM (N = 8).

Overall, SW reduced (A; P < 0.05) and FW increased (B; P < 0.001) plasma IGF-I levels. There was also an overall effect of time of sampling and interaction between salinity and time in both experiments (two-way ANOVA).

**Fig. 2.** Effect of FW to SW transfer on plasma osmolality (A), muscle water content (B), liver IGF-I mRNA expression (C) and plasma IGF-I (D). Fresh water acclimated fish were transferred to SW (▲; SW challenged) or FW (■; sham control) at time 0. Asterisks indicate significant effects compared to sham-controls (*P < 0.05, **P < 0.01, ***P < 0.001). Values are means ± SEM (N = 10, except for liver IGF-I mRNA analysis where N = 5). Overall, SW reduced muscle water content (B; P < 0.001) and liver IGF-I mRNA levels (C; P < 0.05). There was an overall effect of time of sampling on plasma osmolality, muscle water content and plasma IGF-I (B, D; P < 0.001). There was also interaction between time and salinity for plasma osmolality (A; P < 0.05), muscle water content (B; P < 0.001) and plasma IGF-I (D; P < 0.05; two-way ANOVA).
**Fig. 3.** Effect of SW to FW transfer on plasma osmolality (A), muscle water content (B), liver IGF-I mRNA expression (C) and plasma IGF-I (D). Seawater acclimated fish were transferred to FW (▲; FW challenged) or SW (■; sham control) at time 0. Asterisks indicate significant effects compared to sham-controls (* P < 0.05, *** P < 0.001). Values are means ± SEM (N = 10, except for liver IGF-I mRNA analysis where N = 5). Overall, FW reduced plasma osmolality and increased muscle water content (A, B; P < 0.001). There was an overall effect of time of sampling for muscle water content (B; P < 0.01). There was also interaction between time and salinity for plasma osmolality (A; P < 0.05) and muscle water content (B; P < 0.001; two-way ANOVA).

**Fig. 4.** Effects of FW to SW transfer on gill IGF-I mRNA expression (A) and gill IGF-IR mRNA expression (B). Fresh water acclimated fish were transferred to SW (▲; SW challenged) or FW (■; sham control) at time 0. Asterisks indicate significant effects compared to sham-controls (*** P < 0.001). Values are means ± SEM (N = 5). Overall, SW increased gill IGF-IR mRNA expression (B; P < 0.01). There was an overall effect of time of sampling for receptor expression (B; P < 0.001). There was also interaction between the two factors for IGF-IR mRNA expression (B; P < 0.01; two-way ANOVA).

**Fig. 5.** Gill IGF-I mRNA expression (A) and gill IGF-IR mRNA expression (B) after SW to FW transfer. Seawater acclimated fish were transferred to FW (▲; FW challenged) or SW (■; sham control) at time 0. Values are means ± SEM (N = 5). There were no significant effects of salinity transfer on IGF-I or IGF-IR mRNA (A, B; P > 0.05; two-way ANOVA).
**Fig. 6.** Effect of IGF-I injection on plasma osmolality (A), muscle water content (B) and plasma IGF-I in fish transferred from FW to SW. Fresh water acclimated fish were injected with barramundi IGF-I (100 ng/g body weight) and transferred to SW. For comparison, FW fish were injected with saline and transferred to FW or SW. Different letters indicate significant differences (P < 0.05). Values are means ± SEM (N = 10).

**Fig. 7.** Neighboring 5 µm gill sections from SW-acclimated striped bass immunostained with the Na⁺,K⁺-ATPase α1 subunit antibody (A), the Na⁺,K⁺,2Cl⁻ cotransporter antibody (B), and the IGF-IR antibody (C). Sections were counterstained with haematoxylin. The arrow indicates an example of localization of antibody staining. Bar = 100 µm.
**Table 1.** Plasma Cortisol and Glucose after FW to SW transfer.

<table>
<thead>
<tr>
<th></th>
<th>Plasma Cortisol (ng/ml)</th>
<th>Plasma Glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours: FW</td>
<td>164 ± 38</td>
<td>4.64 ± 0.57</td>
</tr>
<tr>
<td>4 hours: FW control</td>
<td>185 ± 29</td>
<td>5.47 ± 0.61</td>
</tr>
<tr>
<td>SW</td>
<td>192 ± 35</td>
<td>4.11 ± 0.75</td>
</tr>
<tr>
<td>24 hours: FW control</td>
<td>173 ± 45</td>
<td>4.38 ± 0.61</td>
</tr>
<tr>
<td>SW</td>
<td>225 ± 20</td>
<td>5.50 ± 0.54</td>
</tr>
<tr>
<td>96 hours: FW control</td>
<td>184 ± 30</td>
<td>4.38 ± 0.59</td>
</tr>
<tr>
<td>SW</td>
<td>171 ± 23</td>
<td>4.70 ± 0.45</td>
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</tbody>
</table>

Values are Means ± SEM.
Table 2. Plasma cortisol and glucose after SW to FW transfer.

<table>
<thead>
<tr>
<th>Time</th>
<th>Condition</th>
<th>Plasma Cortisol (ng/ml)</th>
<th>Plasma Glucose (mmol/l)</th>
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</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>SW</td>
<td>197 ± 33</td>
<td>5.94 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>FW</td>
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<td></td>
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<tr>
<td>4 hours</td>
<td>SW control</td>
<td>127 ± 27</td>
<td>4.67 ± 0.35</td>
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<tr>
<td></td>
<td>FW</td>
<td>157 ± 27</td>
<td>5.25 ± 0.54</td>
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<tr>
<td>24 hours</td>
<td>SW control</td>
<td>202 ± 32</td>
<td>5.78 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>FW</td>
<td>134 ± 22</td>
<td>4.97 ± 0.58</td>
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<tr>
<td>96 hours</td>
<td>SW control</td>
<td>170 ± 30</td>
<td>4.32 ± 0.78</td>
</tr>
<tr>
<td></td>
<td>FW</td>
<td>183 ± 29</td>
<td>3.90 ± 0.65</td>
</tr>
</tbody>
</table>

Values are Means ± SEM.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A. Plasma Osmolarity (mOsm/kg)

<table>
<thead>
<tr>
<th>Condition</th>
<th>FW</th>
<th>SW for 24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline sw</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td></td>
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</tbody>
</table>

B. Muscle water (%)

<table>
<thead>
<tr>
<th>Condition</th>
<th>FW</th>
<th>SW for 24 hr</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Saline sw</td>
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<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td></td>
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</tbody>
</table>

C. Plasma IGF-I (ng/mL)

<table>
<thead>
<tr>
<th>Condition</th>
<th>FW</th>
<th>SW for 24 hr</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Saline sw</td>
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<tr>
<td>IGF-I</td>
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