Aquaculture 362–363 (2012) 177–183

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Effects of environmental salinity, biopsy, and GH and IGF-I administration on the expression of immune and osmoregulatory genes in the gills of Atlantic salmon (*Salmo salar*)

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ARTICLE INFO

Article history:
Received 22 October 2009
Received in revised form 1 November 2010
Accepted 23 December 2010
Available online 7 January 2011

Keywords:
Atlantic salmon
Gills
NKA
CFTR
GH
IGF-I

ABSTRACT

Immune-endocrine interactions are thought to be important for fish health, especially following injury of peripheral tissues. We investigated the influences of gill biopsy (surgical removal of several gill filaments with minimal bleeding) on mRNA levels of osmoregulatory and immune genes in peripheral tissues of Atlantic salmon (*Salmo salar*) smolts in freshwater (FW) and seawater (SW). The effects of growth hormone (GH) and insulin-like growth factor (IGF)-I, which are known as both osmoregulatory and immunomodulatory hormones, were also examined in FW- and SW-acclimated fish. Compared with the intact gill arch of the same fish, the residuary filaments after biopsy showed significant reduction in Na^+,K^+-ATPase (NKA) and cystic fibrosis transmembrane conductance regulator (CFTR) mRNA levels in FW fish. In contrast, mRNA levels of these genes in the filaments with biopsy were increased in SW fish. Increased caspase gene transcription was observed in the cut filaments of SW fish, but not in those of FW fish. Lysozyme gene transcription was stimulated in the residuary gill filaments after biopsy in both FW and SW. Administration of salmon GH or IGF-I increased mRNA levels of NKA, CFTR, caspase, and lysozyme in the gills after biopsy in FW fish. In SW-acclimated fish, there was no significant effect of GH or IGF-I on the transcription of osmoregulatory or immune genes. The results in this study showed tight relationships between immunity, osmoregulation, and endocrine system in fish gills, and that nonlethal gill biopsy can be used for investigations of fish health.

1. Introduction

In addition to their fundamental importance for respiration and ion transport, fish gills are also an important tissue in the immune system for trapping and processing of pathogens penetrating from environmental water (Zapata et al., 1996; Evelyn, 1996; Haugarvoll et al., 2008). Accordingly, injury of large amounts of gill tissues may cause lethal disruptions in respiration, osmoregulation, and defense mechanisms. On the other hand, nonlethal sampling of a small portion of gills is useful for monitoring of physiological status of living individual fish (McCormick, 1993). There was no detrimental influence of gill biopsy on subsequent survival, growth, and osmoregulatory ability in Atlantic salmon (*Salmo salar*) smolts (McCormick, 1993). Given the limited effect of the removal of small amounts of gill tissue on the physiological condition of whole individual, biopsy can be used as an experimental method to examine a possible interaction between osmoregulation and immune function at the limited site of tissue with physical damage.

Growth hormone (GH) and insulin-like growth factor (IGF)-I promote body growth among vertebrates, and in many teleost species the GH/IGF-I axis is also involved in osmoregulation during adaptation from FW to SW (Duan, 1998; Wood et al., 2005; Sakamoto and McCormick, 2006). Furthermore, it is becoming clear that GH and IGF-I are important modulators in the fish immune system and administration of those hormones results in enhancements of several immune functions, including lysozyme production (Yada, 2007, 2009). At the site of gill biopsy, it is likely that immunity, osmoregulation and repair of tissue with cell proliferation are simultaneously activated. Despite the importance of GH and IGF-I on the regulation of these physiological functions, few studies have examined effects of those hormones at the damaged tissues in fish.

The gill is the site for active ion uptake in freshwater (FW) and salt secretion in seawater (SW). Na^+,K^+-ATPase (NKA) is an ion-translocating enzyme and expressed in extremely high level in salt-transporting tissues such as gills. The functional NKA protein is composed of two essential subunits, α and β. The α-subunit contains the binding sites for ATP, Na^+, K^+, and ouabain, and is considered the catalytic unit (McCormick, 1995; Hirose et al., 2003; Richards et al., 2003; Evans et al., 2005; Bystriansky et al., 2006). In salmonid species, several isoforms of NKA α-subunit have been identified, and differential

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doi:10.1016/j.aquaculture.2010.12.029

expressions between those isoforms are observed during adaptation to different environmental salinities (Richards et al., 2003; Bystriansky et al., 2006); the abundance of gill NKA-α1a is greater in FW, whereas NKA-α1b is more abundant in SW (McCormick et al., 2009). Cystic fibrosis transmembrane conductance regulator (CFTR), which is an apical membrane cAMP-regulated chloride channel, also plays an important role in salt-transport in fish gills (Marshall and Singer, 2002; Hirose et al., 2003; McCormick et al., 2003; Evans et al., 2005). Two isoforms of CFTR gene have been cloned in Atlantic salmon (Chen et al., 2001). After exposure to SW of Atlantic salmon smolts, CFTR-I showed gradual and prolonged elevation of mRNA level expressed in gills, whereas increased CFTR-II was transient and returned to the initial level within several days (Singer et al., 2002). Those facts suggest the differential regulation between isoforms of those important components of salt-transport and osmoregulation in fish gills.

Lysozyme possesses a direct antibacterial effect by splitting peptidoglycan layers in the cell wall, and is found at many tissues where the risk of bacterial invasion is high, including gills of fish (Yano, 1996). Lysozyme activities in lymphoid tissues are well known to respond to pathogens (Yano, 1996). In Atlantic salmon, an increased enzymatic activity and mRNA levels of lysozyme responding to an intra-peritoneal injection of bacterial lipopolysaccharide are observed in the head kidney, which is thought to be equivalent to bone marrow in higher vertebrates as a hemopoietic and lymphoid tissue (Paulsen et al., 2003). Increased mRNA levels of lysozyme are also observed in gill epithelial cells of Atlantic cod (*Gadus morhua*) after *in vitro* administration of pathogens (Caipang et al., 2010). The possible influence of injury, salinity, or endocrine regulation of lysozyme expressed in gills has not yet been examined.

Cell proliferation should be enhanced during the process of recovery from tissue damage, accompanying activation of cyclin or proliferating cell nuclear antigen, which is an essential component of DNA replication (Kelman, 1997). Increased expression of cyclin is observed in the damaged gill filaments of Nile tilapia (*Oreochromis niloticus*) exposed to sublethal concentration of copper in water (Monteiro et al., 2009). Correspondingly, apoptosis is also observed in the damaged gills of several species of fish (Sollid and Nilsson, 2006). Morphologically, increased numbers of apoptotic cells have been detected not only in the damaged gills but also in the gills of fish exposed to SW (Lovy et al., 2007; Kammerer and Külts, 2009). Changes in expression of caspase gene required for the signal transduction of apoptosis have been observed in Atlantic salmon embryo after hyperthermic exposure (Takle et al., 2006). Increased activity of caspase is observed in the gills of sturgeon (*Acipenser medirostris*), responding to increase in environmental salinity (Sardella and Kültz, 2009). Although caspase seems to be a marker of apoptosis in the gills, little is known in the gene transcription of this important enzyme for tissue reconstruction during adaptation to different salinities.

To know the influence of physical damage and environmental salinity on the transcription of osmoregulatory and immune genes, mRNA levels of the two isoforms of NKA α-subunit, NKA-α1a and α1b, and those of CFTR, CFTR-I and II, and lysozyme were examined in Atlantic salmon after gill biopsy. Cell proliferation and apoptosis were also examined by quantification of β-actin, cyclin-B and caspase-3B mRNA levels. Furthermore, GH or IGF-I has been administered to the fish with gill biopsy to know the possible endocrine control on the immune-osmoregulatory interaction in the damaged gills.

2. Materials and methods

2.1. Fish

Atlantic salmon (*S. salar*) juveniles were obtained from the Kensington National Fish Hatchery, U.S. Fish and Wildlife Service (Kensington, CT, USA), and held at the Conte Anadromous Fish Research Center, U.S. Geological Survey (Turners Fall, MA, USA). Prior to the start of the study, the fish were held in fiberglass tanks receiving flow through Connecticut River freshwater (FW) maintained under seasonally changing light:dark cycle and ambient river temperatures (11–15 °C in September and October). They were transferred to four 1100-liter tanks containing recirculating FW or seawater (SW, 27 ppt) maintained at 15 °C. There were 50–55 fish in each tank, and they were fed to satiation twice daily with commercial feed (Zeigler Bros., Garners, PA, USA) for one month. Experiments were carried out in October, 2007 on parr weighing 15–25 g that had been acclimated to these conditions for at least 3 weeks. Food was withheld for 24 h prior to the start of the study and withheld for the duration of the experiment.

2.2. Sampling procedure

Distal half of 4 to 6 filaments of the first right gill arch were cut under anesthetization with MS-222 as described by McCormick (1993). After a short recovery, the fish were returned to their respective treatment tanks. Control fish were anesthetized, the operculum opened, and returned to the tanks without biopsy. Two days after biopsy, blood was collected from caudal vessels using a syringe treated with heparin ammonium in less than 6 min after the onset of anesthetization. As shown in Fig. 1, the residuary halves of filaments after biopsy were sampled and placed into RNA Later® (Ambion, Austin, TX), and stored at −80 °C for RNA extraction. The proximal halves of intact filaments from the first left gill arch were sampled as the control filaments. There were no significant differences in mRNA levels between the distal and proximal halves of the filaments in the same portion of gill arch (data not shown). The gill filaments of the other side from biopsy were also taken for the measurement of NKA activity, placed into 100 μl SEI (250 mM sucrose, 10 mM Na_2EDTA and 50 mM imidazole, pH 7.3) and stored at −80 °C for later analysis.

2.3. GH and IGF-I treatment

The fish received an intra-peritoneal injection of 0.9% NaCl (saline), salmon GH (0.2 μg g body weight^{-1}), or salmon IGF-I (0.1 μg g body weight^{-1}), immediately after removal of gill filaments. Salmon GH was prepared as described by Kawauchi et al. (1986), and the purity was confirmed by HPLC. Recombinant salmon IGF-I was purchased from Prospec-Tany TechnoGene (Rehovot, Israel). GH was dissolved first in 0.1 N NaOH, neutralized by addition of the same volume of 0.1 N HCl, and diluted by saline. IGF-I was dissolved in distilled water and diluted by saline. Two days after biopsy and injection, blood, and gills were collected as described above. Proximal halves of the filaments after biopsy were sampled from saline- or hormone-injected fish and placed into RNA Later®.

2.4. Biochemical assays

Gill NKA activity was measured as described by McCormick (1993) and calculated as the difference in the production of ADP in the absence and presence of 0.5 mM ouabain, and expressed as μmol ADP mg protein^{-1} h^{-1}. Plasma chloride level was measured by a chloridometer (Buchler-Cotlove, Fort Lee, NJ, USA) and expressed in milliequivalents (mEq l^{-1}). Plasma cortisol was measured by direct enzyme immunoassay (Carey and McCormick, 1998).

2.5. RNA extraction and real-time PCR

For the analysis of gene transcription, total RNA was extracted from gill samples (approximately 20 mg tissue) using FastPure RNA kit (Takara, Shiga, Japan) according to the manufacturer's instructions, and RNA concentrations were determined using a microplate spectrophotometer (SpectraMax 190, Nihon Molecular Devices, Kanda, Tokyo, Japan). Total RNA was then treated with RNase-free

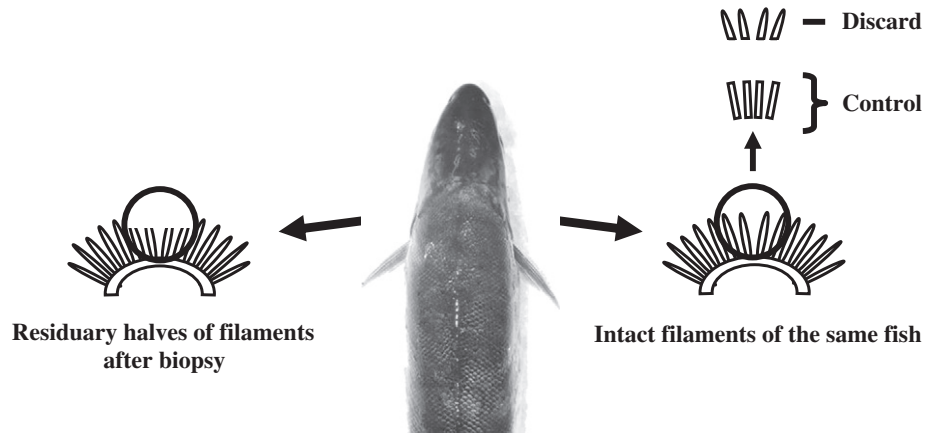

Fig. 1. Diagrams of sampling of gill filaments for mRNA quantification after biopsy in the first experiment.

DNase I (Takara). Reverse transcription was then carried out using PrimeScript 1st Strand cDNA Synthesis System (Takara). Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with TaqMan probe (Applied Biosystems). The quantifications of β-actin, caspase-3B, CFTR-I and II mRNA were carried out as described previously (Monette et al., 2010). The cDNA fragments as the standard for cyclin-B, lysozyme, NKA -α1a and α1b were amplified with the primers designed based on the GenBank accession numbers BG934275, AF179305, AY692142 and AY692143 (Table 1). After denaturation at 95 °C for 10 min, amplification was carried out by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The sequences of the primers and probes used in the assays are reported in Table 2. In each assay, standard cDNAs, positive control sample (cDNA from pooled gill tissue), and samples were run in triplicate. Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All data are expressed as pmol g^{-1} RNA in each sample.

2.6. Statistical analysis

In the first experiment, mRNA levels between filaments from both opercular sides of the same fish were compared using paired t-test or Wilcoxon test when the groups were not normally distributed or had unequal variance. For plasma chloride level, gill NKA activity, and mRNA levels in the second experiment, the significance of the differences between the two groups was determined by analysis of variance followed by Student's t-test or Mann–Whitney U-test when the groups were not normally distributed or had unequal variance. The calculations were performed using STATISTICA (Statsoft, Tulsa, OK).

3. Results

Fig. 2 shows gill NKA activity and plasma chloride level after biopsy in FW- or SW-acclimated Atlantic salmon smolts. Gill NKA activity significantly increased after SW acclimation, whereas there was no influence of gill biopsy itself in either FW- or SW-groups. There was no significant influence of gill biopsy on plasma chloride levels in FW or SW. There was a slight but significant increase in chloride level in biopsy fish in SW compared to FW. In FW fish, there was no significant difference in plasma cortisol levels between control (1.4 ± 0.7 ng ml^{-1}) and biopsy (1.6 ± 0.5 ng ml^{-1}) fish. SW-acclimation did not show significant effect on plasma cortisol levels either in control (2.9 ± 1.1 ng ml^{-1}) or biopsy (0.9 ± 0.4 ng ml^{-1}), and biopsy produced no significant effect in SW.

In FW-acclimated fish, gill biopsy resulted in significant increases in β-actin and lysozyme mRNA levels in the residuary filaments, when they were compared to those levels in the intact filaments from the other side of the same individual (Fig. 3). There was no influence of biopsy in cyclin-B or caspase-3B mRNA levels. NKA-α1a and CFTR-I showed lower mRNA levels in the residuary filaments after biopsy, whereas there was no significant change in NKA-α1b or CFTR-II. SW-acclimation enhanced transcription of cyclin-B, lysozyme, NKA-α1b and CFTR-I genes in the intact gill filaments, when compared to the levels in FW fish. NKA-α1a and CFTR-II showed significantly lower mRNA levels in SW than those in FW. As in FW fish, significant increases in β-actin and lysozyme mRNA levels were observed in the residuary filaments after biopsy of SW fish. In contrast to the results in FW fish, we found a significant increase in caspase-3B mRNA levels in the residuary gill filaments of SW fish. There was no effect of biopsy on mRNA levels of either isoforms of NKA-α, however, biopsy significantly enhanced the mRNA levels of both types of CFTR in the residuary filaments of SW fish.

Fig. 4 represents gill NKA activity and plasma chloride level in the GH- or IGF-I-injected fish with biopsy. Although SW-acclimation increased gill NKA activity, there was no significant effect of hormones on plasma chloride levels or gill NKA activity in FW or SW. On the other hand, as shown in Fig. 5, an intra-peritoneal injection with GH or IGF-I into FW fish resulted in significant increases in gene transcription of β-actin, cyclin-B, caspase-3B, lysozyme, NKA-α1a, and CFTR-II in the gill filaments with biopsy. However, there was no significant effect of GH or IGF-I on mRNA levels of those proliferative, apoptotic, immune, and osmoregulatory genes in the gill filaments of SW fish with biopsy.

4. Discussion

The results of this study indicate that the environmental salinity definitively influences transcription of lysozyme, cyclin and caspase

Table 1
Design of primers for cloning of standard cDNAs.

	Forward primer	Reverse primer
Cyclin-B	5′-ATGTTGGAGTCCCCAGACAGA-3′	5′-CGCATGTTGCCTGTAATTTCCT-3′
Lysozyme	5′-ATGGATGGCTACGCTGGAAAC-3′	5′-TGATTCTGACAGTGAAGGCGC-3′
NKA α1a	5′-GGCAGCTCTTTGGTGGGTT-3′	5′-TTCACCTCCACCAGATCTCCA-3′
NKA α1b	5′-CCCCCCAGCTCCAATGA-3′	5′-GTAGTACTTCACCTCCCCGATCTTAC-3′

Table 2
Design of primers and fluorogenic probes for real-time PCR.

	Forward primer	Reverse primer	Probe
Cyclin-B	5′-AAGGTCCCTGAGGTCCAACA-3′	5′-GGTAAAACCTGAACCTCCAGCTT-3′	5′-CCCCAAAAAGTTGCTATTGCCCCTGT-3′
Lysozyme	5′-CTACAATACCCAGGCCACCAA-3′	5′-GTCACACCAGTAGCGGCTGTT-3′	5′-CAACACCGACGGCTCCACCGA-3′
NKA α1a	5′-TGGTGCTATGCTCTGCTTCCT-3′	5′-TATCATTGGCCGGCTCATC-3′	5′-TACGGAATCCAGGCCGCCTCC-3′
NKA α1b	5′-GGGAGCGCAGACCAAGGT-3′	5′-GGCGTCCTCCTCTCTCTTGTT-3′	5′-CAGCCCAACGTCATGCCCATCTTT-3′

genes in the gills after surgical removal of filaments. Furthermore, despite the lack of change in gill NKA activity and plasma chloride level, transcriptions of the above mentioned genes in the gill filaments with biopsy were stimulated by GH and IGF-I administration in FW-acclimated fish, but not in SW. In addition to being the respiratory organ in fish, the gills are one of the most important sites of ion transport and barrier against external pathogens (Zapata et al., 1996; Evelyn, 1996; Haugarvoll et al., 2008). The results of this study show tight relationships among immunity, osmoregulation, and endocrine system in fish gills.

The removal of halves of gill filaments resulted in 2.5–6.8 times increase in mRNA level of lysozyme in the residuary parts both in FW and SW. Plasma lysozyme activity is thought to be produced mainly from monocytes and neutrophils in circulating blood (Yano, 1996). A significant amount of lysozyme is also detected in the mucus of Atlantic salmon, suggesting an important role of this humoral immune function in the prevention of infection through body surfaces (Yano, 1996; Fagan et al., 2003). Thus, the remarkable increase in lysozyme gene locally expressed in the gills after biopsy, when compared with the intact filaments of the same fish, supports the importance of this enzyme when there is local physical damage to gill tissue.

Lysozyme activity in the plasma of the brown trout (*Salmo trutta*) correlated positively with the plasma GH level (Marc et al., 1995). Administration of GH increased plasma lysozyme level in the rainbow trout (*Oncorhynchus mykiss*) acclimated to FW, and lysozyme secretion from cultured trout leucocytes is stimulated by GH added into the medium (Yada et al., 2001, 2004). IGF-I also stimulates fish immune functions, such as proliferation of lymphocytes, phagocytic activity and plasma lysozyme level (Calduch-Giner et al., 1995; Yada, 2007, 2009). Consistent with these results, in the present study we found a significant increase in lysozyme mRNA levels following GH treatment in FW. Although there was not a statistically significant effect of IGF-I, the mean values in this group were higher than the controls and similar to the GH-treated fish. In SW we were not able to detect a significant effect of either GH or IGF-I on lysozyme mRNA levels. This may in part be due to the fact that SW itself increased lysozyme mRNA levels, making it difficult to detect and/or bring about a further increase with GH or IGF-I. These results are similar to studies in rainbow trout in which there was no further stimulation of plasma lysozyme by GH in SW-acclimated trout, which has already shown significantly higher level of lysozyme compared to FW fish (Yada et al., 2001). After one-month acclimation in SW, the Atlantic salmon used in this study seem to reach a "steady state" in the endocrine regulation of hypoosmoregulation based on the low levels of plasma cortisol in both FW and SW groups. The absence of increased gill lysozyme transcription by GH and IGF-I injections in SW-acclimated fish may result from an attenuation of those hormonal effects by SW acclimation, which also stimulates endogenous GH/IGF-I axis in relation to hypoosmoregulation (Sakamoto and McCormick, 2006).

In several salmonid species, decreased mRNA levels of NKA-α1a but increased mRNA levels of NKA-α1b in gills have been observed after transfer from FW to SW (Richards et al., 2003; Bystriansky et al., 2006). Although NKA activity in fish gills is generally thought to be important mainly for salt excretion in SW, its necessity for ion uptake is also shown in the fish acclimated to FW (McCormick, 1995; Hirose et al., 2003). The differential regulations of the two NKA-α isoforms suggest difference in physiological roles during acclimation to salinities. Significant increase in NKA-α1b mRNA level in the gills after SW acclimation coincided well with the activation in NKA activity estimated as ouabain binding in SW fish, and suggests the importance of this isoform of NKA for ion balance in hypertonic environments. In contrast, NKA-α1a is more abundant in FW than NKA-α1b, and is likely to be involved in ion uptake in the gills of FW-acclimated salmonid fish (Bystriansky et al., 2006; McCormick et al., 2009). In this study, decreased NKA-α1a mRNA levels were observed in the residuary gill filaments after biopsy in FW, whereas no change was observed after biopsy in SW. The physiological significance of lower NKA-α1a transcription after biopsy is unclear, though it may reflect physical damage to the gill that reduces the number of

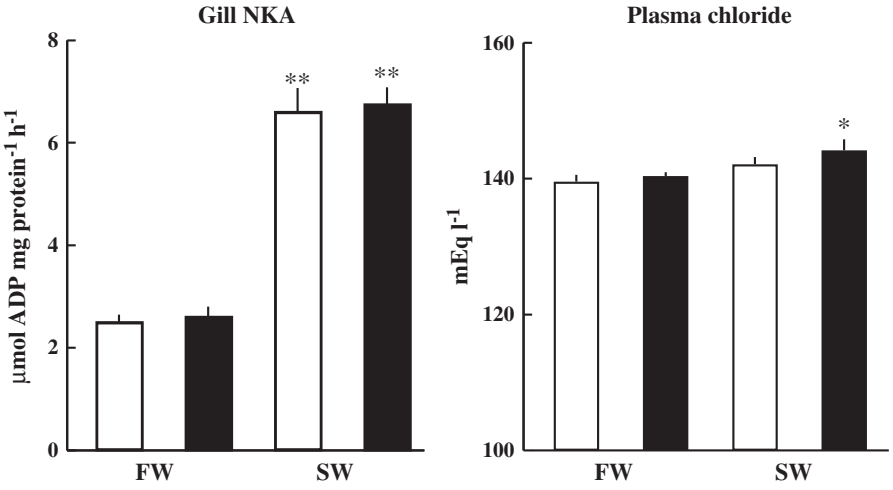

Fig. 2. Gill NKA activity and plasma chloride level in the control (open column) or gill biopsy fish (closed column) in Atlantic salmon parr acclimated to FW or SW. Data are expressed as means + SEM (n = 10). *, **Significantly different from FW-acclimated fish with the corresponding treatment at $P<0.05$ and 0.01, respectively.

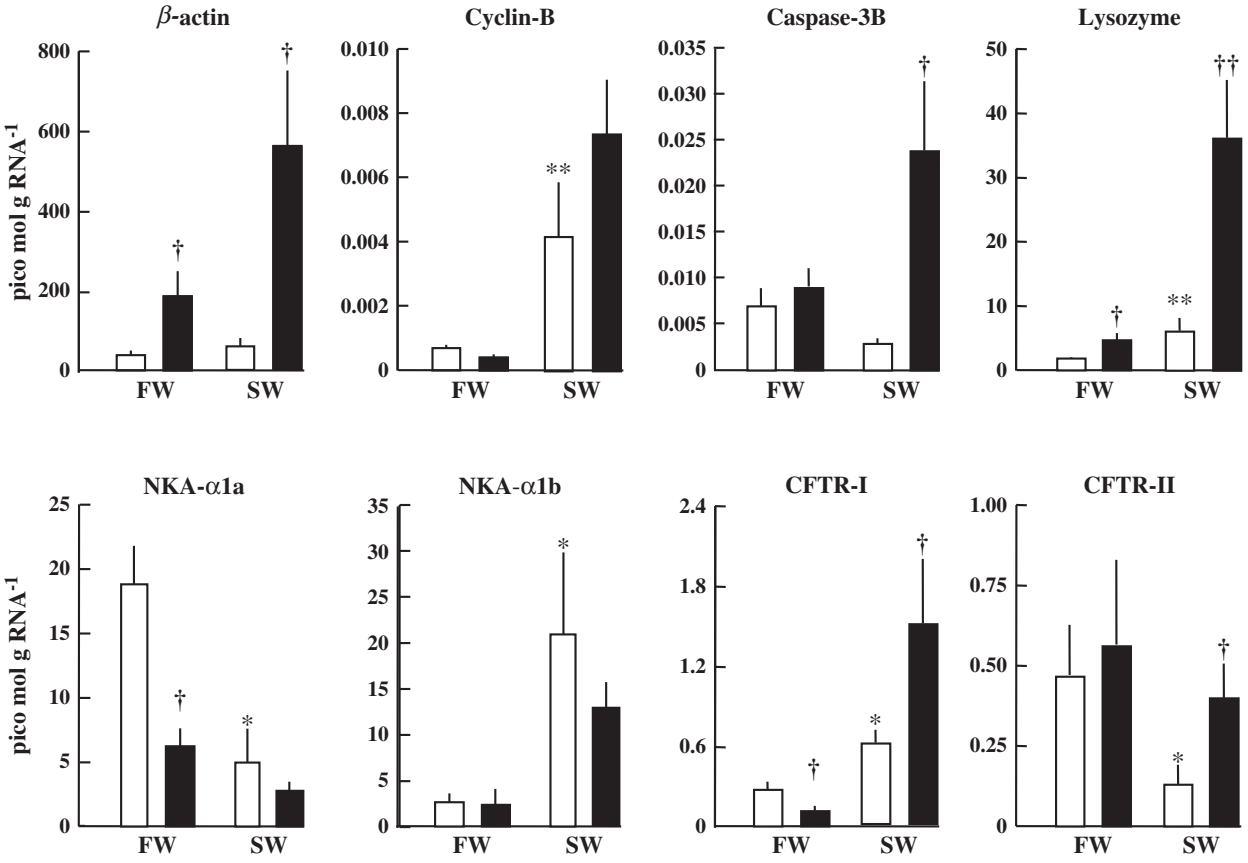

Fig. 3. Effects of biopsy (closed column) on β-actin, cyclin-B, caspase-3B, lysozyme, NKA -α1a and α1b, and CFTR-I and II mRNA levels in residuary filaments of FW- or SW-acclimated Atlantic salmon parr. Data are expressed as means + SEM (n = 10). *, **Significantly different from the control filaments of FW-acclimated fish at $P<0.05$ and 0.01, respectively. †, ††Significantly different from the control filaments of the same fish (open column) at $P<0.05$ and 0.01, respectively.

ionocytes in this region of the gill and its capacity for ion uptake. It is still unclear that the responsiveness of NKA-α1a to the physical damage of filaments reflected the function of this type of NKA in FW-acclimated fish. On the other hand, the lack of significant response in NKA-α1a to biopsy in SW may be related to the overall lowered levels observed in NKA-α1a in SW, consistent with the reduced importance of this NKA isoform in SW.

As in the differential response between the two isoforms of NKA to SW, mRNA levels of two CFTR expressed in the gills showed opposite changes after SW acclimation. Increased transcription of CFTR-I gene in the gills of SW-acclimated Atlantic salmon coincides well with the results of previous studies (Singer et al., 2002, 2003). CFTR-II shows a transient increase followed by a gradual decrease in mRNA levels in the gills after transfer from FW to SW (Singer et al., 2002). The fish examined in the present study have been reared in SW at least for a month, and decreased levels of CFTR-II are consistent with these previous observations. The two isoforms of CFTR show a high identity in amino acid sequence, and share essential structures for their

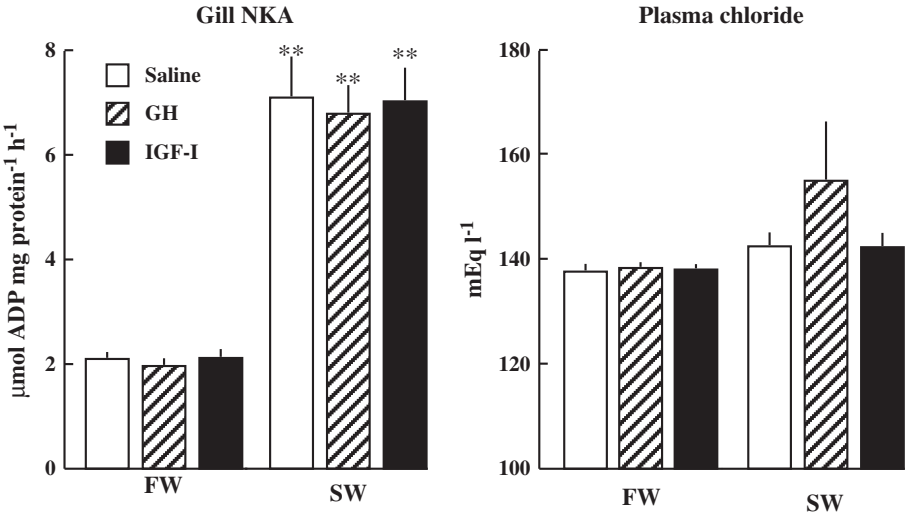

Fig. 4. Gill NKA activity and plasma chloride level after saline (open column), GH (shaded column), or IGF-I (closed column) injection in the FW- or SW-acclimated Atlantic salmon parr with biopsy. Data are expressed as means + SEM (n = 6). **Significantly different from the FW fish with the corresponding treatment at $P<0.01$.

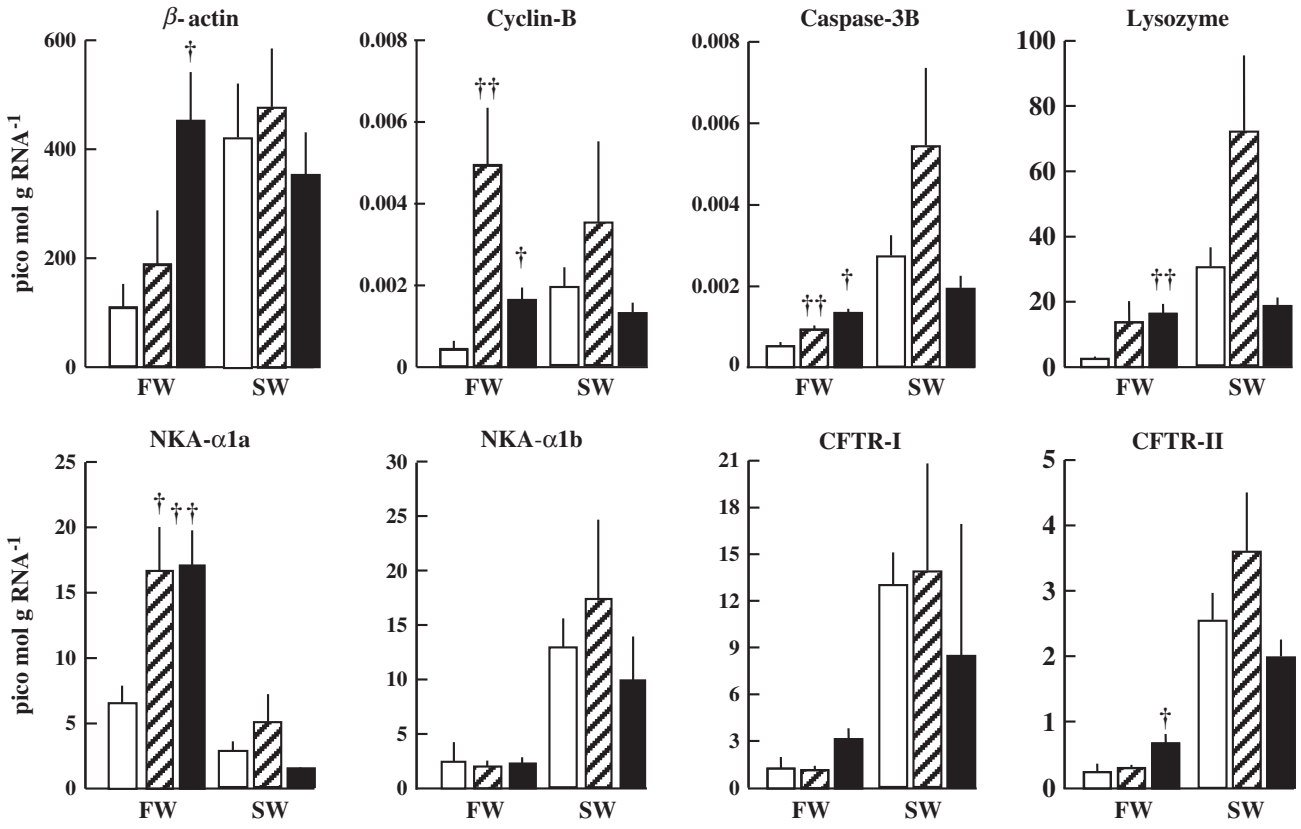

Fig. 5. Effects of saline (open column), GH (shaded column), or IGF-I (closed column) injection on β-actin, cyclin-B, caspase-3B, lysozyme, NKA -α1a and α1b, and CFTR-I and II mRNA levels in residuary filaments of FW- or SW-acclimated Atlantic salmon parr after biopsy. Data are expressed as means + SEM (n = 6). †, ††Significantly different from residuary filaments of the saline-injected fish (open column) at $P<0.05$ and 0.01, respectively.

function as chloride channel (Chen et al., 2001; Marshall and Singer, 2002).

There were differential responses in CFTR gene transcription to gill biopsy between FW and SW. For CFTR-I, gill biopsy resulted in a suppression in FW, but an enhancement in SW-fish. As described above, the role of CFTR for chloride excretion is generally accepted in the gills of SW-fish, but little is known of a possible role in ion uptake in FW (Singer et al., 2002, 2003; Marshall and Singer, 2002). Decreased mRNA levels of CFTR-I observed similarly to NKA-α1a may relate to the difference in FW-adaptive function to the other isoform. On the other hand, it seems paradoxical that gene transcription of both CFTR isoforms was stimulated in the damaged gill filaments after biopsy in SW. Between biopsy groups, there was a slight but significant increase in plasma chloride from 140 mEq l^{-1} in FW to 144 mEq l^{-1} in SW. Increased transcription of CFTR may be a compensative reaction to the physical damage caused by the biopsy that may increase the demand for salt secretion.

In euryhaline fishes, GH and IGF-I are hypoosmoregulatory hormones and stimulatory effects of exogenous administration of them on gill NKA activity are repeatedly observed especially in salmonid species (Sakamoto and McCormick, 2006). However, there was no significant stimulation of gill NKA activity in the fish treated with GH or IGF-I in SW, coinciding with the lack of change in mRNA levels of all examined genes in the gills of SW-acclimated fish in this study. The doses of hormones used in this study were effective on the transcription of several genes in the gills of FW fish. Higher doses may be needed to produce significant increases in NKA activity and gene transcription in SW.

There was no significant difference in mRNA levels of β-actin in gill filaments between FW- and SW-acclimated fish. However, in both FW and SW, there were 5–6 folds increases in β-actin in the residuary gill filaments after biopsy when compared to the proximal half of intact filaments of the other side of operculum. Although β-actin has been used as an internal standard for quantification of gene transcription, the function of this structural protein is primarily as the component of cell skeleton and could be influenced by physical damage and subsequent recovery processes. In contrast to β-actin, biopsy did not produce a significant change in mRNA levels of cyclin-B in the gill filaments of FW or SW fish. Cyclin is synthesized in the dividing cells especially during S-phage of the cell cycle (Kelman, 1997). The results in this study suggested that there was no obvious increase in cell division in the residual half of gill filaments 2 days after biopsy, but further time-course experiments are needed to know the details of changes in the cell cycle. On the other hand, acclimation to SW resulted in a 6-fold increase in cyclin mRNA in control filaments compared to those in FW fish. In the gills during adaptation from FW to SW, an increase in the number or transformation from inactive to active forms of chloride cells is observed in many species of fish especially in euryhaline teleosts (Hirose et al., 2003; Hiroi et al., 2005). Chloride cells, often referred to as mitochondrion-rich cells or ionocytes, in the epithelium of fish gills are well accepted to be the major sites of ion transport. Although chloride cells play important roles for ion uptake in FW, they show remarkable morphological activation and increase in number in the gill filaments of SW-acclimated fish (Hirose et al., 2003; Hiroi et al., 2005). An increased level of cyclin mRNA suggests an enhancement of cell division in the gills of SW fish, and that the effects of SW acclimation were greater than the effects of biopsy.

Caspase-3B mRNA level showed a significant increase in the residuary gill filaments after biopsy in SW-acclimated fish, but not in FW. However, environmental salinities did not produce significant changes in gene transcription of caspase in the control filaments without biopsy, coinciding with the observation in β-actin. In the gill chloride cells of tilapia (*Oreochromis mossambicus*) after exposure to

SW, an activation of caspase occurs coincident with apoptosis identified by DNA fragmentation (Kammerer and Külts, 2009). On the other hand, SW-acclimation does not produce significant changes in the number of apoptotic cells in the gill filaments of Atlantic salmon (Monette et al., 2010). There may be a species specific difference in the occurrence of apoptosis in gill chloride cells responding to environmental salinities. Collectively, those three proliferation and apoptosis genes examined in this study, β-actin, cyclin-B, and caspase-3B, showed different tendencies responding to salinities and gill biopsy. Reconstruction of cell skeleton deduced by gene transcription of β-actin was enhanced after biopsy, increased cell division accompanying with the activation of cyclin may reflect increased cell formation in SW fish, and significant apoptosis estimated by caspase mRNA level could be induced by the most severe condition under hypertonic environment and physical damage in gill filaments.

Similar to a previous study of biopsy effects on Atlantic salmon smolts (McCormick, 1993), there was a limited influence of biopsy on gill NKA activity and plasma ion level in FW- and SW-acclimated parr in the present study. There was also no significant elevation of plasma cortisol level after biopsy or SW-acclimation in this study. It is well known that cortisol secretion responds to stresses, and plasma level of cortisol is available to estimate stress status in fish (Mommsen et al., 1999). There were significant influences of biopsy in the peripheral expressions of several genes, however, they do not appear to be substantial physiological impacts on the fish 2 days after the disturbance by surgery. This indicates that the nonlethal gill biopsy examined in this study provides possibilities to estimate immune, osmoregulatory, proliferative and apoptotic status of individual fish from the small amounts of gill filaments with relatively little harm to fish. The fish gills are sites of the damage produced by many aspects of environmental conditions, such as pollutants and xenobiotics (Evans et al., 2005). We concluded that gill biopsy followed by the quantification of transcription of specific genes has applications for aquaculture and field studies in many fish species.

Acknowledgements

We thank the Kensington State Fish Hatchery (Kensington, CT) for providing us with juvenile Atlantic salmon. We are grateful to Michelle Monette, Darren Lerner, Mike O'Dea, and Amy Regish for help with sampling. This study was supported by FRA grants for international cooperation to TY and USGS base funds to SDM.

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