Available online at www.sciencedirect.com
SCIENCE DIRECT®
Aquaculture 222 (2003) 15–28
Aquaculture
www.elsevier.com/locate/aqua-online

Interactive effects of cortisol treatment and ambient seawater challenge on gill Na^+,K^+-ATPase and CFTR expression in two strains of Atlantic salmon smolts

Thomas D. Singer[a,*], Bengt Finstad[b], Stephen D. McCormick[c], Steve B. Wiseman[a], Patricia M. Schulte[a,1], R. Scott McKinley[a,2]

[a] *Department of Biology, Waterloo Biotelemetry Institute, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1*
[b] *Norwegian Institute for Nature Research, Tungasletta 2, N-7485 Trondheim, Norway*
[c] *USGS, Leetown Science Center, Conte Anadromous Fish Research Center, Turners Falls, MA, USA*

Received 29 October 2002; accepted 15 December 2002

Abstract

During peak smoltification, the interactive effects of cortisol and ambient seawater challenge were compared in two strains of Atlantic salmon (*Salmo salar*) smolts: a domesticated strain, AquaGen and a native River Imsa strain. Tissue and blood samples were taken from untreated fish on 20 May. Fish were then transferred to experimental tanks, allowed to recover for 24 h and cortisol (50 mg kg body mass^{-1}), dissolved in vegetable oil, or vegetable oil alone (sham) was implanted. Samples were taken 5 days post implantation. Fish were then exposed to 24 h ambient seawater challenge (FW–SW) or freshwater to freshwater replacement (FW–FW) and sampled as before. Sham implantation had no significant impact on any of the measured parameters. Cortisol implantation significantly elevated plasma cortisol in FW–FW Imsa smolts, while no effect was observed in FW–SW Imsa smolts. Cortisol implantation had no effect on the plasma cortisol levels of AquaGen smolts regardless of FW–SW challenge. Increased plasma cortisol corresponded with significantly higher plasma glucose levels in FW–FW Imsa smolts. Plasma Na^+ and Cl^- levels were not affected by cortisol implantation but were significantly increased in FW–SW smolts of both strains. Gill Na^+,K^+-ATPase activity

* Corresponding author. Tel.: +1-519-888-4567x3210; fax: +1-519-746-0614.
E-mail address: tsinger@sciborg.uwaterloo.ca (T.D. Singer).
[1] Present address: Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4.
[2] Present address: Faculty of Agricultural Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z4.

0044-8486/03/$ - see front matter
doi:10.1016/S0044-8486(03)00099-1

increased in response to cortisol implantation in only FW–FW smolts but not FW–SW smolts. Gill Na^+,K^+-ATPase α-subunit mRNA levels were not affected by strain, cortisol injection or transfer protocol, while both CFTR I and CFTR II mRNA levels were significantly higher in AquaGen versus Imsa smolts regardless of treatment. CFTR I mRNA was elevated following cortisol implantation in FW–FW smolts from both strains suggesting CFTR I expression is under the control of cortisol. These findings also suggest that plasma cortisol levels are regulated differently between strains and that cortisol implantation and ambient FW–SW challenge interact, interfering with the individual effects of each of these factors.

Keywords: Cortisol; Ambient seawater challenge; mRNA levels; Smoltification; Na^+,K^+-ATPase; CFTR

1. Introduction

The role of cortisol in seawater adaptation in teleost fish, in particular during smoltification of Atlantic salmon (*Salmo salar*), is well documented (Madsen, 1990; Bern and Madsen, 1992). Plasma cortisol levels rise in the spring prior to smoltification (Specker and Schreck, 1982; Barton et al., 1987) and in response to seawater exposure in Atlantic salmon smolts (Nichols and Weisbart, 1985). Although cortisol levels naturally increase during smoltification, little is known about the interactive effects of smoltification and chronic stress or cortisol implantation during this period. Aquaculture practices may induce chronic stress in juvenile salmonids, which could impact the ability of smolts to successfully adapt to seawater.

Increases in Na^+,K^+-ATPase activity during smolt development and following seawater exposure are characteristic of the smoltification process (for review see McCormick, 1995). The level of Na^+,K^+-ATPase α-subunit mRNA in the gill also shows similar increases during development and following seawater exposure (Madsen et al., 1995; D'Cotta et al., 2000). Less is known about the role of other important osmoregulatory genes in salmonid smoltification. It has recently been shown that the expression of an apically located chloride channel (CFTR) in the killifish (*Fundulus heteroclitus*) gill increases following seawater exposure, suggesting a role in teleost seawater adaptation (Singer et al., 1998). The cloning of two CFTR isoforms in Atlantic salmon has provided an opportunity to investigate their expression patterns (Chen et al., 2001).

The response of salmon to cortisol implantation during non-smolting stages has been shown to vary considerably both between studies and among species and developmental stages. For example, cortisol implantation increased seawater tolerance and gill Na^+,K^+-ATPase activity in juvenile Atlantic salmon during non-smolting periods in one study (Bisbal and Specker, 1991) while it failed to produce an effect on gill Na^+,K^+-ATPase activity in a second study of juvenile Atlantic salmon (Langdon et al., 1984) and induced an inhibition in coho salmon (*Oncorhynchus kisutch*) (Redding et al., 1984). McCormick et al. (1991) found that the in vitro responsiveness of gill Na^+,K^+-ATPase activity to cortisol was greatest just prior to completion of smolting. Increases in the level of Na^+,K^+-ATPase α-subunit mRNA have also been observed following cortisol implantation, suggesting cortisol regulation of this gene (Richman and Zaugg, 1987; Madsen et al., 1995; Seidelin

et al., 1999). The timing of these changes at both the protein and gene levels varies among studies. Similarly, a transient increase in plasma cortisol levels precedes the rise in CFTR mRNA levels in *F. heteroclitus*, suggesting cortisol involvement in CFTR expression (Marshall et al., 1999).

The objective of this study was to compare the interactive effects of cortisol implantation and ambient seawater challenge in two distinct strains of Atlantic salmon smolts during the peak period of natural smoltification. Ambient seawater challenge takes into account the temperature differences that are associated with direct transfer of cultured smolts from freshwater holding tanks to seawater culture cages. The use of an ambient seawater challenge betters approximates the conditions experienced in an aquaculture setting, in contrast to responses to the controlled temperatures associated with traditional seawater challenge. The 24-h time course was chosen to examine acute effects, which allowed for comparison of plasma cortisol levels associated with the cortisol implant to endogenous cortisol levels in response to ambient seawater transfer.

Atlantic salmon smolts used for this study were derived from two distinct Norwegian populations: (1) AquaGen, a strain which supplies most Norwegian commercial salmon farming operations and (2) Imsa, a strain native to the Imsa River. These strains show differences in behaviour and growth with the AquaGen strain having been shown to be competitively and reproductively inferior to the Imsa strain under natural conditions (Einum and Fleming, 1997; Fleming et al., 2000). Understanding the interactive response of different strains of smolts at the physiological and molecular level to both cortisol stimulation and ambient seawater exposure may lead to selection of appropriate strains and to improvements in aquaculture practices which are likely to reduce mortalities associated with seawater transfer.

2. Methods

2.1. Fish

Atlantic salmon smolts were derived from two distinct populations. The AquaGen strain originated in Norway's national breeding program at Sunndalsora (AquaGen) and were the offspring (Fall 1998) of parents taken as eyed eggs in 1996 to the Norwegian Institute for Nature Research (NINA) station at Ims, southwest Norway (Fleming and Einum, 1997). These fish constituted the eighth-generation of the first brood line (Gjedrem et al., 1991) that has undergone intentional selection such that it is now derived principally, if not solely from the river Namsen, central Norway (Gjedrem et al., 1991; Fleming and Einum, 1997). Selection was based upon survival and body weight under commercial aquaculture conditions (Gjedrem et al., 1991). Simultaneously, offspring were derived from spawning first-generation adult sea-ranched salmon collected from the River Imsa in the fall of 1998. The offspring of these salmon were considered native River Imsa stock. The eggs were hatched during January/February 1999 and subsequent juvenile fish from both populations were reared under similar hatchery conditions until smolting at age 1+. During this period, the fish were fed an ordinary commercial dry diet via automatic feeder, according to temperature and fish size. At the time of the experiments, the length and weight of the

strains were as follows (mean ± S.E.M.): AquaGen 21.8 ± 0.3 cm, 100.0 ± 4.3 g, $n = 56$; Imsa 20.7 ± 0.2 cm, 89.6 ± 3.5 g, $n = 56$.

2.2. Experimental design

All experiments were performed at the NINA Station at Ims during May 2000, coinciding with the annual natural smolt migration period in the River Imsa. Freshwater taken from the River Imsa had ambient temperatures that ranged between 14.5 and 16.0 °C during the course of the study. Seawater drawn from the neighbouring fjord was 7.9 °C at the time of ambient seawater challenge studies. At peak time of smoltification, six groups of eight fish (age 1+) from each strain were transferred from two 4000-l freshwater holding tanks to individual 100-l tanks (20 May). In addition, eight AquaGen and eight Imsa smolts were sampled directly from each of the holding tanks as undisturbed controls. The stocking densities within these holding tanks (approx. 15 kg m^{-3}) were similar to those used to hold smolts prior to being transferred to seawater cages. After each group of eight fish was held for 24 h in the smaller 100-l freshwater tanks, fish were lightly anaesthetized using MS222 (30 mg ml^{-1}) buffered with $NaHCO_3$ (60 mg ml^{-1}) given intraperitoneal implantations (1 ml kg body $mass^{-1}$) of either sunflower vegetable oil alone (sham) or vegetable oil containing cortisol (50 mg kg body $mass^{-1}$) and returned to their original tanks. This protocol has been shown to produce a slow release of cortisol into the circulation in teleosts (Vijayan et al., 1994). The fish were fed as described above and feeding was stopped 24 h prior to sampling to ensure the fish were in a post-absorptive state. Five days following the implantations, one group of cortisol- and sham-implanted AquaGen and Imsa fish were subjected to a 24 h ambient seawater challenge (FW–SW) which included a drop in water temperature of 7.1 °C (i.e., 15.0 °C freshwater to 7.9 °C seawater). Seawater replacement was accomplished by rapidly reducing freshwater volume from 100 to 10 l and then immediately replacing it with flow-through seawater. The salinity rose to full-strength seawater (32 ppt) within 60 min. To determine the impact of the water replacement protocol, all exposures were repeated identically using freshwater instead of seawater (FW–FW) using a separate group of sham- and cortisol-implanted AquaGen and Imsa fish. While such exposures do not account for the changes in temperature associated with ambient seawater challenge they do account for the acute handling stress. At the same time that the FW–SW and FW–FW protocols occurred (5-days post implantation), samples of eight fish for each strain and treatment group were taken to provide a further control group.

2.3. Sampling of fish

Fish were netted in pairs and exposed to a 20 l anaesthetic bath containing 100 mg l^{-1} MS-222 (Sigma) buffered with 200 mg l^{-1} $NaHCO_3$. Blood was obtained by caudal puncture using an NH_4-heparinized syringe and the sample was placed on ice. Immediately after drawing blood, the first pair of gill arches were removed and stored in RNA*later*™ (Ambion) overnight at 4 °C prior to long-term storage at −20 °C until future RNA extraction. The second pair of gill arches were removed and placed into ice cold SEI buffer (250 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3), frozen immediately in liquid nitrogen and stored at −80 °C for future analysis of Na^+,K^+-ATPase activity. After a total

of eight fish per treatment were sampled, blood was centrifuged for 5 min at 5000 × *g*, and plasma was separated and frozen at − 20 °C for future analysis.

2.4. Sample analysis

Plasma cortisol was quantified using commercial radioimmunoassay kit (ICN Biomedicals) from duplicate 20 μl samples. If needed, plasma was diluted as necessary to insure that levels fell within the range of the standard curve. In samples from certain cortisol implanted fish, where plasma cortisol levels were higher than expected, levels were confirmed using an ether extraction protocol (Schulz, 1985). Plasma glucose, Na^+ and Cl^- levels were determined using a Nova Biomedical Stat Profile Plus 9 blood gas analyzer. Gill Na^+,K^+-ATPase activity was measured according to McCormick (1993).

2.5. Semi-quantitative reverse transcriptase (RT)-PCR

To assess the gill Na^+,K^+-ATPase α-subunit, CFTR I and CFTR II mRNA levels in Atlantic salmon, we utilized a semi-quantitative RT-PCR approach (Harting and Wiesner, 1997), because northern blot analysis would be unlikely to distinguish between the two very similar Atlantic salmon CFTR isoforms. Gene-specific oligonucleotide primers were designed to amplify Atlantic salmon Na^+,K^+-ATPase α-subunit as well as CFTR I and CFTR II. Details of the primer design have been described in a companion study (Singer et al., 2002).

Total RNA was extracted from small amounts of gill tissue (100 mg or less) using an RNeasy® Mini kit (Qiagen). Samples were quantitated by spectrophotometry and qualitatively assessed by gel electrophoresis using 1% agarose formaldehyde gel. For each treatment group total RNA was extracted from each of the eight gill samples collected. First strand cDNA synthesis was performed using 1 μg of total RNA and oligo dT primer (0.5 mg ml^{-1}) (Promega) at a final volume of 12.5 μl. This mixture was heated at 70 °C for 5 min prior to adding first strand buffer (final concentration 50 mM Tris–HCl, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT), 20 U M-MuLV Reverse transcriptase (MBI Fermentas), 15 U RNasin ribonuclease inhibitor (Promega), 0.8 mM dNTPs in a final volume of 20 μl. The reaction was incubated at 37.5 °C for 1 h and stopped by heating at 70 °C for 10 min.

A sample of the resultant cDNA template (1 μl) was used for the PCR reaction (final concentrations: 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 pmol of each gene-specific oligonucleotide, 0.8 pmol of the β-actin control oligonucleotides (Ambion) and 2.5 U of Taq polymerase (MBI Fermentas) in a 25 μl total reaction volume). PCR reactions were carried out using two sets of primers within the same reaction (β-actin control oligonucleotide plus Atlantic salmon gene-specific Na^+,K^+-ATPase α-subunit CFTR I, or CFTR II oligonucleotide primers, respectively). Thermal cycling was performed according to the following protocol: 1 min denaturation at 94 °C, followed by 1 min annealing at 60 °C, followed by a 1 min extension step at 72 °C for 27 cycles. Before initiating experiments, the linear range of the PCR for each individual primer set alone and including β-actin primers was determined. These cycling parameters (27 cycles, 60 °C annealing) were chosen to insure that all sets of primers resulted in reactions that were within the exponential amplification phase of the PCR for all templates.

PCR products were separated according to size by gel electrophoresis in 1.5% agarose gels (1 × TAE containing 25 μg ethidium bromide 100 ml^{-1} gel). The PCR products were visualized by UV illumination and quantified by densitometry using a FluorChem gel documentation system (Alpha Innotech). For each treatment sample, the Integrated Density Value (IDV) obtained for the target gene-specific amplicon was divided by the signal obtained for the β-actin amplicon producing a relative mRNA abundance value. The β-actin amplicon IDV did not change significantly with treatment.

2.6. Data analysis

Plasma cortisol, glucose, Na^+ and Cl^- levels, gill Na^+,K^+-ATPase activity and gill Na^+,K^+-ATPase α-subunit, CFTR I and CFTR II mRNA levels were analyzed using three-way analysis of variance (ANOVA) with (1) strain (AquaGen or Imsa), (2) implantation protocol (cortisol or sham) and (3) water exposure protocol (FW–FW) or (FW–SW) as factors. A separate two-way ANOVA was used to compare resting and 5-day post implantation values with (1) strain and (2) implantation protocol as factors. Where necessary, dependant variable data were log-transformed to meet assumptions of homogeneity of variance within treatment group. Detailed analysis of the treatment means using Tukey's procedure was carried out only if the ANOVA revealed significant differences among main effects or interactions ($P < 0.05$). Statistical analysis was completed using SAS general linear model computer software. Data are presented as mean ± standard error calculated within each treatment group unless indicated otherwise. Fish length and weight were not significant covariants.

3. Results

3.1. Plasma cortisol and glucose

Resting plasma cortisol levels were low in both untreated AquaGen and Imsa smolts sampled directly from holding tanks (mean ± S.E.: 19.2 ± 4.9, 15.5 ± 3.2 ng ml^{-1}, respectively), and sham injection did not significantly increase cortisol levels (Table 1). In the fish sampled at 5 days post implantation (prior to water replacement), cortisol-implanted Imsa smolts had significantly elevated plasma cortisol levels versus the sham-implanted smolts ($P < 0.05$) (Table 1). In contrast, there was no significant difference in plasma cortisol level in the cortisol- and sham-implanted AquaGen smolts at this time ($P > 0.05$)(Table 1).

At 6 days post-implantation, following water replacement, Imsa smolts had significantly higher plasma cortisol levels compared with AquaGen smolts (irrespective of cortisol implantation or FW–SW challenge) ($P < 0.05$). Plasma cortisol levels were not affected by either cortisol implantation ($P = 0.78$) or FW–SW challenge ($P = 0.91$), but there were significant interaction effects between all factors, i.e., strain × cortisol implantation ($P = 0.01$), strain × FW–SW challenge ($P = 0.01$) and cortisol implantation × FW–SW challenge ($P = 0.03$). Post-hoc tests revealed significantly higher plasma cortisol levels in cortisol- versus sham-implanted FW–FW Imsa smolts ($P < 0.05$) but not in the FW–SW

Table 1
Plasma cortisol, glucose, Na^+, Cl^-, gill Na^+,K^+-ATPase specific activity, Na^+,K^+-ATPase α-subunit, CFTR I and CFTR II relative to β-actin mRNA levels in Atlantic salmon (*S. salar*) smolts given vegetable oil alone (sham) or vegetable oil containing cortisol at 50 mg kg^{-1} body mass (cortisol) and sampled 5 days later

	AquaGen		Imsa	
	Sham	Cortisol	Sham	Cortisol
Plasma cortisol (ng ml^{-1})	265.6 ± 66.3	542.8 ± 293.9	67.0 ± 28.8	2144.9 ± 448.4*
Plasma glucose (mmol l^{-1})	6.5 ± 0.2	6.8 ± 0.3	6.2 ± 0.2	7.7 ± 0.4*
Plasma Na^+ (mM)	139.0 ± 1.3	138.8 ± 2.8	133.8 ± 1.3	138.2 ± 2.4
Plasma Cl^- (mM)	131.6 ± 1.3	128.8 ± 2.7	123.7 ± 1.1	127.4 ± 2.1
Gill Na^+,K^+-ATPase activity (mmol Pi mg $prot^{-1}$ h^{-1})	11.2 ± 0.7	10.6 ± 0.3	13.8 ± 0.8	12.4 ± 1.1
Na^+,K^+-ATPase α-subunit: β-actin (gill relative mRNA abundance)	0.128 ± 0.024	0.153 ± 0.038	0.068 ± 0.005	0.135 ± 0.026
CFTR I: β-actin (gill relative mRNA abundance)	0.341 ± 0.066	0.343 ± 0.061	0.294 ± 0.047	0.278 ± 0.019
CFTR II: β-actin (gill relative mRNA abundance)	0.124 ± 0.022	0.140 ± 0.023	0.129 ± 0.039	0.133 ± 0.008

Values represent means ± S.E. ($n=8$); asterisk indicates significant difference between cortisol- and sham-implanted smolt within a single strain ($P<0.05$, Tukey's studentized range test, preceded by two-way ANOVA).

smolts (Fig. 1). In contrast, AquaGen smolts showed no increase in plasma cortisol levels 6 days following cortisol implantation, while FW–SW sham-implanted smolts had near significantly elevated plasma cortisol levels versus the FW–FW smolts ($P=0.10$).

Resting plasma glucose levels were low in untreated smolts of both strains sampled directly from holding tanks (mean ± S.E.: 6.7 ± 0.6, 6.9 ± 0.4 mmol l^{-1}, respectively), and did not change significantly with sham implantation. Five days following cortisol implantation, only Imsa smolts had significantly elevated plasma glucose levels versus sham-implanted smolts (Table 1). At 6 days post implantation, following water replacement, neither strain, implantation nor water replacement were significant factors affecting plasma glucose, but there was a significant interaction effect between cortisol implantation and FW–SW challenge ($P=0.01$) (Fig. 1). Cortisol-implanted Imsa smolts had significantly higher plasma glucose compared with sham-implanted smolts in the FW–FW replacement group ($P<0.001$).

3.2. Plasma ions

There were no significant differences between strains in resting or 5-day post implantation plasma Na^+ and Cl^- levels (Table 1). In contrast, both plasma Na^+ and Cl^- levels were significantly elevated in FW–SW challenged smolts compared with FW–FW replacement smolts at 6 days post implantation ($P<0.001$) (Fig. 1). There was no effect of strain or cortisol implantation on plasma Na^+ and Cl^- levels ($P>0.05$).

3.3. Gill Na^+,K^+-ATPase activity

There were significant differences in gill Na^+,K^+-ATPase activity between AquaGen and Imsa smolts at 5 days post implantation ($P<0.05$). Cortisol implantation had no significant

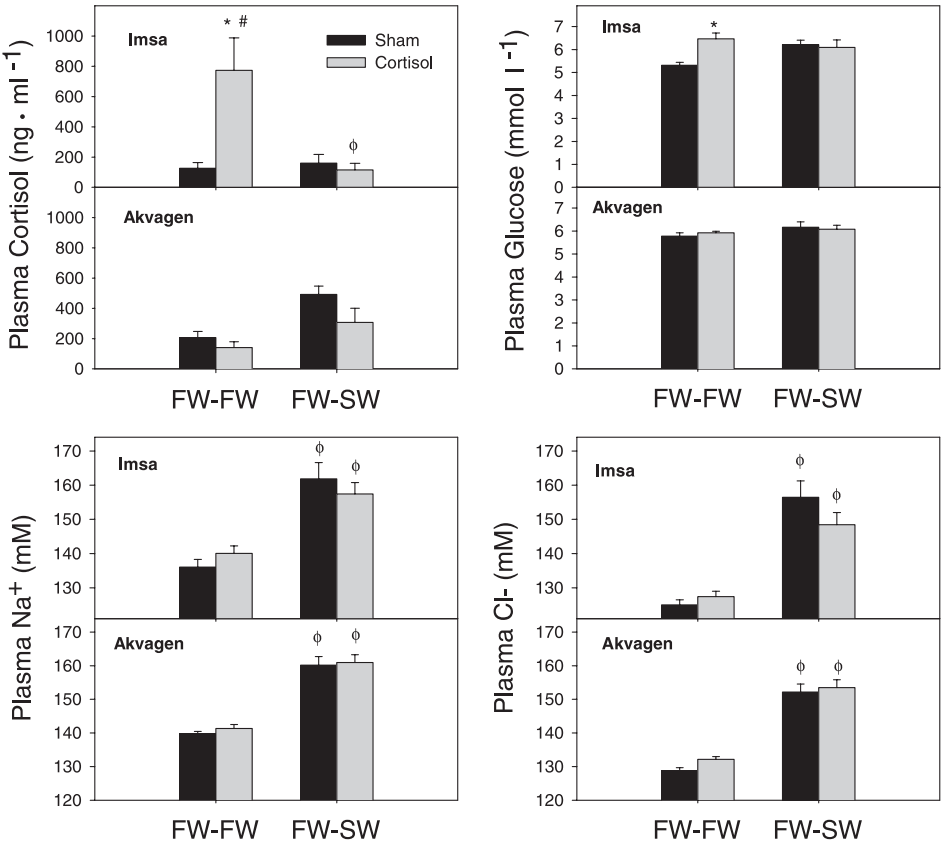

Fig. 1. Plasma cortisol, glucose, Na^+ and Cl^- levels (mean ± S.E.) of Atlantic salmon smolts exposed to ambient seawater challenge. Smolts ($n=8$) were implanted with vegetable oil alone (sham) or vegetable oil containing cortisol at 50 mg kg^{-1} body mass (cortisol) and sampled 6 days later following 24 h freshwater replacement (FW–FW) or 6 days later following acute 24 h seawater exposure (FW–SW). The following symbols denote significant differences between specific groups: (*) sham and cortisol implanted smolts from a specific strain/water replacement group; (#) Imsa and AquaGen smolts from a specific implantation/water replacement group; and (ϕ) FW–FW and FW–SW treated smolts for a specific strain/implantation group ($P<0.05$, Tukey's studentized range test, preceded by three-way ANOVA).

effect on Na^+,K^+-ATPase (Table 1). There was, however, a significant effect of strain ($P=0.0073$).

At 6 days post implantation, gill Na^+,K^+-ATPase activity was significantly affected by strain ($P=0.001$) and cortisol implantation ($P=0.004$), while FW–SW challenge had no effect, as determined by three-way ANOVA. There was a significant interaction effect between cortisol implantation and FW–SW challenge ($P=0.007$). Cortisol-implanted Imsa and AquaGen smolts had significantly elevated gill Na^+,K^+-ATPase activity compared with the sham-implanted smolts after undergoing FW–FW replacement protocol (Imsa: $P=0.01$; AquaGen: $P=0.002$) (Fig. 2) but not when exposed to a FW–SW challenge (Fig. 2).

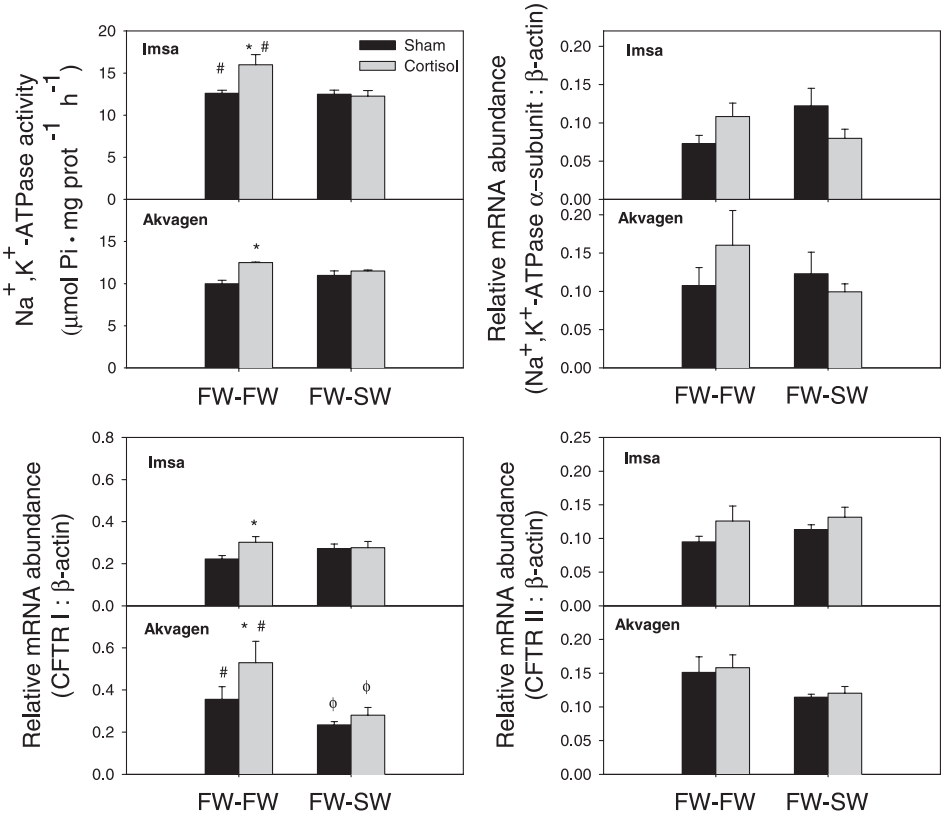

Fig. 2. Gill Na^+,K^+-ATPase specific activity, Na^+,K^+-ATPase α-subunit, CFTR I and CFTR II relative to β-actin mRNA levels of Atlantic salmon smolts (mean ± S.E.) exposed to ambient seawater challenge. See Fig. 1 text for details.

3.4. Gill mRNA abundance

In contrast to gill Na^+,K^+-ATPase activity, Na^+,K^+-ATPase α-subunit mRNA abundance was not affected by any of the factors examined at 6 days post implantation ($P>0.05$). Gill CFTR I was affected by strain ($P=0.01$), cortisol implantation ($P=0.01$) and FW–SW challenge ($P=0.01$), and there was a significant interaction effect between strain and FW–SW challenge ($P=0.007$). FW–FW replacement AquaGen smolts exhibited significantly higher CFTR I mRNA levels than any other group ($P=0.01$). Both AquaGen and Imsa smolts had significantly elevated CFTR I mRNA levels following cortisol implantation. CFTR II mRNA abundance was affected by strain ($P=0.03$), but neither cortisol implantation nor FW–SW challenge had any effect individually. There was a significant interaction effect between strain and FW–SW challenge ($P=0.01$) and, as was the case for CFTR I, FW–FW AquaGen smolts exhibited significantly higher CFTR II mRNA levels than any other group ($P=0.01$).

4. Discussion

4.1. Plasma cortisol and glucose

Plasma cortisol levels responded differently in AquaGen and Imsa smolts to both cortisol implantation and ambient seawater transfer. Imsa smolts responded to cortisol implantation with dramatically increased plasma cortisol levels in contrast to the AquaGen smolts, which showed no significant increases in plasma cortisol in response to administration of the same dose of exogenous cortisol in comparison with sham-implanted smolts. Cortisol levels in some injected Imsa smolts reached levels at 5 days post-injection that are beyond the normal physiological range for Atlantic Salmon (Table 1), which seldom exceed 250 ng ml^{-1} (Nichols and Weisbart, 1985; Carey and McCormick, 1998). The elevated glucose levels observed in cortisol-implanted Imsa smolts at 6 days post implantation correspond with the elevated plasma cortisol levels observed in these smolts. Such increases in plasma glucose may be due to increased cortisol-induced gluconeogenesis (Mommsen et al., 1999). Interestingly, 24 h FW–SW challenge ablated the increase in plasma cortisol in the cortisol-implanted Imsa smolts, while AquaGen smolts showed a near significant increase ($P=0.09$) in plasma cortisol as a result of FW–SW challenge, independent of cortisol implantation. Many studies have demonstrated that both smoltification and seawater exposure can elevate plasma cortisol levels in salmon (Specker and Schreck, 1982; Nichols and Weisbart, 1985; Barton et al., 1987). The changes in plasma cortisol levels have impacts on the numbers of corticosteroid receptors in the gill, which in turn is correlated to the development of seawater tolerance (Shrimpton and Randall, 1994). Cortisol implantation has also been shown to enhance seawater adaptation in pre-smolt stage Atlantic salmon (Bisbal and Specker, 1991; McCormick et al., 1991). Few studies, however, have examined the effects of cortisol implantation during smoltification. The findings here suggest that ambient seawater exposure has the ability to reduce the effects of exogenous cortisol on plasma cortisol levels in certain strains. These differences between strains may involve changes to total corticosteroid receptor numbers leading to variation in cortisol turnover rate. Further investigation will be required to test this hypothesis.

4.2. Plasma ions

The levels of plasma Na^+ and Cl^- were unaffected by cortisol implantation in both strains, but increased significantly following ambient FW–SW challenge. No significant differences between strains were observed for plasma Na^+ and Cl^- following FW–SW challenge. However, in a companion study, significant differences were detected between the same strains examined here 96 h after ambient seawater exposure, indicating that AquaGen smolts have a reduced ability to adapt to seawater between 24 h and 2 weeks (Singer et al., 2002).

4.3. Gill Na^+,K^+-ATPase activity

Neither strain showed an increase in gill Na^+,K^+-ATPase activity following 24 h FW–SW challenge. Salmon gill Na^+,K^+-ATPase activity has been shown to increase from

between 2 to11 days following seawater exposure and is species and developmental stage dependent (McCormick et al., 1989; Madsen et al., 1995; D'Cotta et al., 2000; Seidelin et al., 2000; Singer et al., 2002). In a companion study, we have shown that AquaGen and Imsa smolts exposed to a long-term ambient FW–SW challenge began to show significant increases in gill Na^+,K^+-ATPase activity at 96 h following seawater exposure (Singer et al., 2002). Gill Na^+,K^+-ATPase activities have also been shown to vary seasonally during the parr–smolt transformation with maximum activities coinciding with peak smoltification (McCormick et al., 1987; Nielsen et al., 1999; D'Cotta et al., 2000). Since we examined smolts at the peak of smoltification, gill Na^+,K^+-ATPase activity may have already been elevated and could not be stimulated to higher levels within 24 h of seawater challenge alone, although we have shown that longer periods of exposure result in increases in activity (Singer et al., 2002). Alternatively, the lower seawater temperatures associated with ambient seawater challenge may have reduced the increase in Na^+,K^+-ATPase activity. Handeland et al. (2000) reported significantly lower gill Na^+,K^+-ATPase activities 24 h after smolts were transferred from cold freshwater to cold seawater (both at 4.6 or 9.1 °C) compared with transfer from warm freshwater to warm seawater (both at 14.4 or 18.9 °C).

While seawater challenge alone did not increase gill Na^+,K^+-ATPase activity, cortisol implantation resulted in significantly elevated gill Na^+,K^+-ATPase activity in FW–FW replacement smolts from both strains. A similar response has been observed in both pre-smolt Atlantic salmon (McCormick et al., 1991; Bisbal and Specker, 1991) and brown trout (Madsen et al., 1995). In a separate study conducted during smoltification, Na^+,K^+-ATPase activity did not increase in smolts implanted with cortisol alone but did show increased activity when recombinant bovine insulin-like growth factor-I (rbIGF-1) was also administered (Seidelin et al., 1999). The responsiveness of gill Na^+,K^+-ATPase activity to cortisol has been shown to diminish during peak smoltification when seasonal Na^+,K^+-ATPase activity levels peak (McCormick et al., 1991). Despite being at peak of smoltification and having significant differences in plasma cortisol levels, the two strains examined here had the capacity to elevate gill Na^+,K^+-ATPase activity when implanted with cortisol. In contrast, the effect of cortisol implantation on gill Na^+,K^+-ATPase activity was absent in FW–SW smolts. While cortisol treatment prior to smoltification has been shown to increase the ability of smolts to survive seawater exposure (Bisbal and Specker, 1991), such effects were not evident in this study.

4.4. Gill mRNA abundance

Cortisol implantation had no effect on gill Na^+,K^+-ATPase α-subunit mRNA levels suggesting that expression is not induced by cortisol during peak smoltification. Gill Na^+,K^+-ATPase α-subunit mRNA levels have been shown to increase following a 6 day course of cortisol injections in Atlantic salmon and could be elevated further when rbIGF-I was co-injected (Seidelin et al., 1999). Similarly, we did not observe increases in gill Na^+,K^+-ATPase α-subunit mRNA levels following ambient FW–SW challenge in either strain. Increases in gill Na^+,K^+-ATPase α-subunit mRNA levels have been observed in Atlantic salmon smolts at 24 h post-seawater exposure (D'Cotta et al., 2000), and in juvenile pre-smolt Brown trout between 12 and 24 h post-seawater exposure (Madsen et al., 1995; Seidelin et al., 2000). All of these studies employed single temperature seawater

challenge, which could account for some of the differences between these and the present data.

In contrast to gill Na^+,K^+-ATPase α-subunit CFTR I mRNA levels were significantly affected by the three factors examined: strain, cortisol implantation and FW–SW challenge. Overall, the levels of gill CFTR I mRNA were significantly higher in AquaGen smolts compared with Imsa smolts. Comparison of treatment means revealed that both AquaGen and Imsa smolts elevated mRNA levels in response to cortisol implantation. Interestingly, the level of CFTR I mRNA following cortisol implantation was higher in the AquaGen smolts compared with Imsa smolts despite significantly lower plasma cortisol levels. These results suggest that CFTR I mRNA levels are under the control of cortisol and that this regulation varies between strains.

Gill CFTR II mRNA levels were significantly elevated in AquaGen versus Imsa smolts and there was an overall effect of FW–SW challenge. However, unlike CFTR I, there was no significant effect of cortisol implantation. Possession of two isoforms of CFTR is unique to Atlantic salmon (Chen et al., 2001), and these isoforms have been shown to have very different expression patterns following long-term seawater exposure (Singer et al., 2002). CFTR I mRNA levels increased steadily over 2-week time course while CFTR II mRNA levels transiently peaked at 24 h following seawater exposure. This study demonstrates that there are also differences in response to cortisol implantation between the two CFTR isoforms. The functional consequences of these differences between the two CFTR isoforms remain to be investigated.

4.5. Conclusions

Despite similar rearing conditions, the two strains of smolts examined here differ in their response to cortisol implantation as well as to ambient seawater challenge. Application of ambient seawater challenge has helped to identify differences in physiology between strains that are relevant to actual aquaculture practices. In a companion study, AquaGen smolts had inferior ability to adapt to seawater following a long-term ambient seawater challenge (Singer et al., 2002). The differences between strains in response to cortisol implantation may be a factor influencing the differences in physiology between the strains.

Clearly there are strain-specific differences in the response to cortisol implantation and ambient seawater challenge. These two factors appear to interact in complex ways that differ between strains. As a result, the effects (at a biochemical and molecular level) of either factor alone cannot be used to adequately predict their combined effects. This complex interaction may be particularly evident in fish that are at or near their peak of natural smoltification. Additional studies into the interaction of these factors in fish at a variety of developmental stages are clearly required.

Acknowledgements

We thank Sigurd Einum and the staff of the NINA Research Station, Ims, particularly Jon Backer and Knut Bergersen for their help with sampling and care of smolts. Wendy McCaul and Ngan Diep, Waterloo Biotelemetry Institute, for processing plasma and tissue

samples. We thank the University of Waterloo Statistical Consulting Service for advice on statistical analysis. Financial support was provided by grants from the European Commission (Contract FAIR CT 973498) to B. Finstad and R.S. McKinley, a Natural Sciences and Engineering Research Council of Canada (NSERC) operating grant to P.M. Schulte and an NSERC/Lotek Industrial Research Chair in Biotelemetry to R.S. McKinley.

References

Barton, B.A., Schreck, C.B., Barton, L.D., 1987. Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions and stress responses in juvenile rainbow trout. Dis. Aquat. Org. 2, 173–185.

Bern, H.A., Madsen, S.S., 1992. A selective survey of the endocrine system of the rainbow trout (*Oncorhynchus mykiss*) with emphasis of the hormonal regulation of ion balance. Aquaculture 100, 237–262.

Bisbal, G.A., Specker, J.L., 1991. Cortisol stimulates hypo-osmoregulatory ability in Atlantic salmon, *Salmo salar* L. J. Fish Biol. 39, 421–432.

Carey, J.B., McCormick, S.D., 1998. Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. Aquaculture 168, 237–253.

Chen, J.-M., Cutler, C., Jacques, C., Boeuf, G., Denamur, E., Lecointre, G., Mercier, B., Cramb, G., Ferec, C., 2001. A combined analysis of the cystic fibrosis transmembrane conductance regulator: implications for structure and disease models. Mol. Biol. Evol. 18, 1771–1778.

D'Cotta, H., Valotaire, C., Le Gac, F., Prunet, P., 2000. Synthesis of gill Na^+-K^+-ATPase in Atlantic salmon smolts: differences in α-mRNA and α-protein levels. Am. J. Physiol. 278, R101–R110.

Einum, S., Fleming, I.A., 1997. Genetic divergence and interactions in the wild among native, farmed and hybrid Atlantic salmon. J. Fish Biol. 50, 634–651.

Fleming, I.A., Einum, S., 1997. Experimental tests of genetic divergence of farmed from wild Atlantic salmon due to domestication. ICES J. Mar. Sci. 54, 1051–1063.

Fleming, I.A., Hindar, K., Mjolnerod, I.B., Jonsson, B., Balstad, T., Lamberg, A., 2000. Lifetime success and interactions of farm salmon invading a native population. Proc. R. Soc. Lond., B Biol. Sci. 267, 1517–1523.

Gjedrem, T., Gjøen, H.M., Gjerde, B., 1991. Genetic origin of Norwegian farmed Atlantic salmon. Aquaculture 98, 41–50.

Handeland, S.O., Berge, A., Bjornsson, B.Th., Lie, O., Stefansson, S.O., 2000. Seawater adaptation of out-of-season Atlantic salmon (*Salmo salar* L.) smolts at different temperatures. Aquaculture 181, 377–396.

Harting, I., Wiesner, R.J., 1997. Quantification of transcript-to-template ratios as a measure of gene expression using RT-PCR. BioTechniques 23, 450–455.

Langdon, J.S., Thorpe, J.E., Roberts, R.J., 1984. Effects of cortisol and ACTH on gill Na^+-K^+-ATPase, SDH and chloride cells in juvenile Atlantic salmon *Salmo salar* L. Comp. Biochem. Physiol. 77A, 9–12.

Madsen, S.S., 1990. The role of cortisol and growth hormone in seawater adaptation and development of hypo-osmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). Gen. Comp. Endocrinol. 79, 1–11.

Madsen, S.S., Jensen, M.K., Nøhr, J., Kristiansen, K., 1995. Expression of Na^+-K^+-ATPase in the brown trout, *Salmo trutta*: in vivo modulation by hormones and seawater. Am. J. Physiol. 269, R1339–R1345.

Marshall, W.S., Emberley, T.R., Singer, T.D., Bryson, S.E., McCormick, S.D., 1999. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. J. Exp. Biol. 202, 1535–1544.

McCormick, S.D., 1993. Methods for the nonlethal gill biopsy and measurements of Na^+,K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50, 656–658.

McCormick, S.D., 1995. Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation. Academic Press, New York, NY, pp. 285–315.

McCormick, S.D., Saunders, R.L., Henderson, E.B., Harmon, P.R., 1987. Photoperiod control of parr–smolt transformation in Atlantic salmon (*Salmo salar*): changes in salinity tolerance, gill Na^+,K^+-ATPase activity and plasma thyroid hormones. Can. J. Fish. Aquat. Sci. 45, 1462–1468.

McCormick, S.D., Saunders, R.L., MacIntyre, A.D., 1989. Mitochondrial enzyme activity and ion regulation during parr–smolt transformation of Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem. 6, 231–241.

McCormick, S.D., Dickhoff, W.W., Duston, J., Nishioka, R.S., Bern, H.A., 1991. Developmental differences in the responsiveness of gill Na^+-K^+-ATPase to cortisol in Salmonids. Comp. Endocrinol. 84, 308–317.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268.

Nichols, D.J., Weisbart, M., 1985. Cortisol dynamics during seawater adaptation of Atlantic salmon *Salmo salar*. Am. J. Physiol. 248, R651–R659.

Nielsen, C., Madsen, S.S., Bjornsson, B.T., 1999. Changes in branchial and intestinal osmoregulatory mechanisms and growth hormone levels during smolting in hatchery-reared and wild brown trout. J. Fish Biol. 54, 799–818.

Redding, J.M., Pàtino, R., Schreck, C.B., 1984. Clearance of corticosteroids in yearling coho salmon, *Oncorhynchus kisutch*, in fresh water and seawater after stress. Gen. Comp. Endocrinol. 54, 433–443.

Richman III, H.N., Zaugg, W.S., 1987. Effects of cortisol and growth hormone on osmoregulation pre- and desmoltified coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 65, 189–198.

Schulz, R., 1985. Measurement of five androgens in the blood of immature and maturing male rainbow trout, *Salmo gairdneri* (Richardson). Steroids 46, 717–726.

Seidelin, M., Madsen, S.S., Byrialsen, A., Kristiansen, K., 1999. Effects of insulin-like growth factor-I and cortisol on Na^+,K^+-ATPase expression in osmoregulatory tissues of brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. 113, 331–342.

Seidelin, M., Madsen, S.S., Blenstrup, H., Tipsmark, C.K., 2000. Time-course changes in the expression of Na^+,K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. Physiol. Biochem. Zool. 73, 446–453.

Shrimpton, J.M., Randall, D.J., 1994. Downregulation of corticosteroid receptors in gills of coho salmon due to stress and cortisol treatment. Am. J. Physiol. 267, R432–R438.

Singer, T.D., Tucker, S.J., Marshall, W.S., Higgins, C.F., 1998. A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. Am. J. Physiol. 274, C715–C723.

Singer, T.D., Clements, K.M., Semple, J.W., Schulte, P.M., Bystriansky, J.S., Finstad, B., Fleming, I.A., McKinley, R.S., 2002. Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. Can. J. Fish. Aquat. Sci. 59, 125–135.

Specker, J.L., Schreck, C.B., 1982. Changes in plasma corticosteroids during smoltification of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 46, 53–58.

Vijayan, M.M., Reddy, P.K., Leatherland, J.F., Moon, T.W., 1994. The effects of cortisol on hepatocyte metabolism in rainbow trout, a study using the steroid analogue RU486. Gen. Comp. Endocrinol. 96, 75–84.