General and Comparative Endocrinology 157 (2008) 35–40

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na^+,K^+-ATPase activity and isoform mRNA levels in Atlantic salmon

Stephen D. McCormick [a,*], Amy Regish [a], Michael F. O'Dea [a], J. Mark Shrimpton [b]

[a] USGS, Conte Anadromous Fish Research Center, One Migratory Way, PO Box 796, Turners Falls, MA 01376, USA
[b] Ecosystem Science & Management (Biology) Program, University of Northern British Columbia, Prince George, BC, Canada

ARTICLE INFO

Article history:
Received 29 February 2008
Revised 20 March 2008
Accepted 26 March 2008
Available online 31 March 2008

Keywords:
Cortisol
Osmoregulation
Gill Na,K-ATPase
Deoxycorticosterone
Aldosterone
Salinity tolerance
Ion regulation

ABSTRACT

It has long been held that cortisol, acting through a single receptor, carries out both glucocorticoid and mineralocorticoid actions in teleost fish. The recent finding that fish express a gene with high sequence similarity to the mammalian mineralocorticoid receptor (MR) suggests the possibility that a hormone other than cortisol carries out some mineralocorticoid functions in fish. To test for this possibility, we examined the effect of *in vivo* cortisol, 11-deoxycorticosterone (DOC) and aldosterone on salinity tolerance, gill Na^+,K^+-ATPase (NKA) activity and mRNA levels of NKA α1a and α1b in Atlantic salmon. Cortisol treatment for 6–14 days resulted in increased, physiological levels of cortisol, increased gill NKA activity and improved salinity tolerance (lower plasma chloride after a 24 h seawater challenge), whereas DOC and aldosterone had no effect on either NKA activity or salinity tolerance. NKA α1a and α1b mRNA levels, which increase in response to fresh water and seawater acclimation, respectively, were both upregulated by cortisol, whereas DOC and aldosterone were without effect. Cortisol, DOC and aldosterone had no effect on gill glucocorticoid receptor GR1, GR2 and MR mRNA levels, although there was some indication of possible upregulation of GR1 by cortisol ($p = 0.07$). The putative GR blocker RU486 inhibited cortisol-induced increases in salinity tolerance, NKA activity and NKA α1a and α1b transcription, whereas the putative MR blocker spironolactone had no effect. The results provide support that cortisol, and not DOC or aldosterone, is involved in regulating the mineralocorticoid functions of ion uptake and salt secretion in teleost fish.

Published by Elsevier Inc.

1. Introduction

Corticosteroids have two major functions in vertebrates: a glucocorticoid function affecting metabolism and growth, and a mineralocorticoid function, regulating the movement of ions and water. In many vertebrates these functions are served by two distinct hormones, cortisol (or in some species corticosterone) and aldosterone that each have a specific receptor, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), respectively. In teleost fish it has generally been thought that a single hormone, cortisol, carries out both glucocorticoid and mineralocorticoid actions (Wendelaar Bonga, 1997; Mommsen et al., 1999), and that this hormone works through a single class of receptors (Chakraborti et al., 1987).

Recent molecular data provide evidence that teleost fish express an MR-like gene that shares more sequence similarity with the tetrapod MR than with the fish GR (Colombe et al., 2000; Greenwood et al., 2003; Bridgham et al., 2006). Expression studies of the rainbow trout MR and GR in a mammalian cell line indicate that transactivation of the MR is more sensitive to aldosterone and deoxycorticosterone, whereas the GR is more sensitive to cortisol (Sturm et al., 2005). Similar studies with the cichlid MR and GR found no strong difference in transactivation affinity between aldosterone and cortisol (Greenwood et al., 2003). MR mRNA is found in a wide variety of tissues including those involved in ion regulation (Greenwood et al., 2003; Sturm et al., 2005). Use of the MR antagonist spironolactone has been shown to affect chloride cell density in rainbow trout following transfer to ion-deficient fresh water, implicating a function of the MR in ion uptake in fish (Sloman et al., 2001). While these studies suggest that a MR-like receptor is expressed in fish, its biochemical characterization, localization and function have yet to be established.

Aldosterone is present only in extremely low levels in teleost fish; lower than are likely to have actions on either the GR or MR (Prunet et al., 2006). However, deoxycorticosterone (DOC) is present in some teleosts in concentrations ranging from 0.5 to 10 nM. Although these levels are much lower than circulating levels of cortisol, they are near the range that could interact with a receptor that has affinities ascribed to the trout MR (Sturm et al., 2005).

* Corresponding author. Fax: +1 413 863 9810.
E-mail address: mccormick@umext.umass.edu (S.D. McCormick).

0016-6480/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ygcen.2008.03.024

Thus, Prunet et al. (2006) suggested that DOC may have biological actions in fish, possibly acting through the MR and resulting in physiologically relevant effects on osmoregulation.

The present studies were conducted to determine whether we were missing a mineralocorticosteroid in fish, and to determine the receptor pathway through which corticosteroids are acting to affect ion regulation. We used *in vivo* administration of cortisol, DOC and aldosterone to see if these steroids could carry out a well-known corticosteroid function in fish, the development of seawater tolerance. We examined whether these hormones could also regulate the levels of putative fresh water and seawater isoforms of the Na^+,K^+-ATPase (NKA), an ion transport enzyme involved in both ion uptake and salt secretion. Finally, we examined the impact of the GR inhibitor RU486 and MR inhibitor spironolactone on corticosteroid-induced regulation of these osmoregulatory parameters.

2. Materials and methods

Juvenile Atlantic salmon (*Salmo salar*) were obtained from the White River National Fish Hatchery (Bethel, VT, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in autumn. Fish were reared in 1.7 m diameter tanks supplied with ambient river water at a flow rate of 4 L min^{-1} and provided supplemental aeration. They were maintained under natural photoperiod conditions and fed to satiation (Zeigler Bros., Gardners, PA, USA) using automatic feeders.

In vivo treatment with corticosteroids and receptor blockers followed the methods outlined in McCormick (1996). Feed was withheld for 24 h prior to injection, and fish were anesthetized with 100 mg L^{-1} MS-222 neutralized to pH 7.0. Hormones, receptor blockers or both were suspended in 1:1 vegetable oil:shortening as outlined in Specker et al. (1994), warmed to 35 °C and injected intraperitoneally at 5 μl per g wet weight of fish. DOC has a molecular weight lower than cortsiol and aldosterone, so approximately 10% lower doses of DOC were used to achieve approximately equimolar treatments. Fish were placed in 1 m diameter tanks filled with dechlorinated tap water at 10–12 °C with particle and charcoal filtration and continuous aeration. Fifty percent water change occurred twice weekly. Seawater challenge tanks were identical to fresh water tanks but contained 25 ppt seawater (Instant Ocean). All experiments were conducted with 0+ parr (10–30 g) between October and January. Food was withheld during the entire treatment period. In experiments measuring physiological responses, fish were treated for 14 days, which included a gill biopsy at 6 and 12 days and transfer to 25 ppt seawater on day 13 followed by terminal sampling 24 h later. Aldosterone and DOC exposures were done in separate experiment, with separate vehicle controls in each. In experiments measuring changes in mRNA, treatments occurred for 6 days (terminal sampling) in order to best capture the changes in mRNA levels which are likely to precede biochemical and physiological responses and can become reduced over time.

For sampling, fish were anesthetized as above and fork length to the nearest mm and weight to the nearest 0.1 g were recorded. Gill biopsies consisted of 4–6 gill filaments that were severed above the septum (approximate half of total length), placed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C within 30 min. At terminal sampling, blood was drawn from the caudal vein into a 1 ml ammonium heparinized syringe and spun at 3200 g for 5 min at 4 °C. Plasma was aliquoted and stored at −80 °C. The second gill arch was removed, the gill filaments trimmed from ceratobranchials and placed in an autoclaved 1.5 ml microcentrifuge tube and frozen immediately at −80 °C for later extraction of total RNA.

Na^+,K^+-ATPase activity was determined with a kinetic assay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min as described in McCormick (1993). Gill tissue was homogenized in 125 μl of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000*g* for 30 s. Ten microliter samples were run in two sets of duplicates; one set containing assay mixture and the other assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity measurement is expressed as μmoles ADP/mg protein/h. Protein concentrations were determined using BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, Il, USA). Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA).

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as outlined in Carey and McCormick (1998). Sensitivity as defined by the dose–response curve was 1–400 ng ml^{-1}. The lower detection limit was 0.3 ng ml^{-1}. Using a pooled plasma sample, the average intra-assay variation was 5.5% (n = 10) and the average inter-assay variation was 8.8% (n = 10). Plasma aldosterone levels in aldosterone-treated fish were measured using a competitive enzyme immunoassay (Immuno-Biological Laboratories, Minneapolis, MN, USA) with plasma dilutions of 1:100. The lower detection limit at this dilution was 0.1 ng ml^{-1}.

Total RNA was purified by the TRIzol procedure (Invitrogen, Carlsbad, CA, USA) using 1 ml TRIzol/100 mg gill tissue according to manufacturer's recommendation. First strand cDNA was synthesized from 1 μg of total RNA using oligo(dT_{15}) primer. The expression of Na^+,K^+-ATPase α1a and α1b isoforms, GR1, GR2 and MR was estimated using quantitative real-time PCR (qRT-PCR; ABI Prism 7300 sequence analysis system; Applied Biosystems Inc., Foster City, CA, USA). PCRs contained 1 μL of cDNA, 4 pmoles of each gene specific primer and Universal SYBR green master mix (Applied Biosystems Inc., Streetville, ON, Canada) in a total volume of 20 μL. Primers designed for rainbow trout Na^+,K^+-ATPase α1a, Na^+,K^+-ATPase α1b and elongation factor 1α were used (Richards et al., 2003). Primers designed for rainbow trout GR1 were used (Vijayan et al., 2003). We designed primers for GR2 and MR using rainbow trout sequences (GenBank Accession Nos.: AY495372 and AY495584, respectively) to amplify ~100 bp product; GR2 forward primer 5′-CAT GGC AGA CCA GTG TGA AC-3′, GR2 reverse primer 5′-AGC AGC AGC AGA ACC TTC AT-3′, MR forward primer 5′- CTT CTT CCA GCT CAC CAA GC-3′ and MR reverse primer 5′-CCA CCT TCA GAG CCT GAG AC-3′. All qRT-PCRs were performed as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melt curve analysis was performed following each reaction to confirm the presence of only a single product of the reaction. Negative control reactions were performed for a selection of samples using RNA that had not been reverse transcribed to control for the possible presence of genomic contamination. Gene expression levels measured in qRT-PCR assays are often normalized to the mRNA level of an invariant reference gene. Normalization with a reference gene corrects for variation in reverse transcription (RT) efficiency and it is expected that variation in RT efficiency will affect target and reference genes equally. Richards et al. (2003) demonstrated that elongation factor 1α did not vary in response to salinity challenges in rainbow trout. Relative expression of the target genes was normalized to a reference gene by use of the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). mRNA amounts are expressed relative to the gill samples collected from vehicle-treated fish.

For each parameter a one-way analysis of variance (ANOVA) followed by Student–Neuman–Keuls test was used to test the significance of treatment with corticosteroids and/or receptor blockers (RU486 or spironolactone). All statistics were run using the Statistica (StatSoft Inc., Tulsa, OK) software package.

3. Results

Treatment with cortisol resulted in dose-dependent elevations of plasma cortisol (Table 1), while DOC and aldosterone had no effect (Tables 1 and 2). Cortisol treatment (50 μg/g) resulted in significant increases in gill Na^+,K^+-ATPase (NKA) activity after 6 and 12 days of treatment (Fig. 1), with the relative effect increasing over time (40% and 84% increases at 6 and 12 days, respectively). Although there were increases in gill NKA activity at 10 μg/g cortisol (10% and 18% after 6 and 12 days), these differences were not statistically significant (p = 0.46 and 0.31, respectively). Salinity tolerance as indicated by plasma chloride after 24 h in 25 ppt seawater was significantly increased by both 10 and 50 μg/g cortisol. Neither DOC at 9 and 45 μg/g (Fig. 1) nor aldosterone at 10 and 50 μg/g (Table 1) had a significant effect on gill Na^+,K^+-ATPase activity or salinity tolerance. Treatment of fish with aldosterone resulted in a dose-dependent increase in circulating aldosterone levels (Table 2).

Cortisol treatment for 6 days at 50 μg/g resulted in significant elevations in NKA α1a and α1b mRNA levels (90% and 2.8-fold, respectively; Fig. 2). DOC and aldosterone had no significant effect on NKA α1a and α1b mRNA levels. Cortisol, DOC and aldosterone did not significantly affect gill GR1, GR2 and MR mRNA levels (Table 3). However, gill GR1 mRNA was increased approximately 42% (p = 0.068) by cortisol treatment. Gill MR mRNA was also increased

Table 1
Plasma cortisol after treatment with corticosteroids for 13 days in fresh water followed by exposure to seawater (25 ppt) for 24 h

Treatments	Plasma cortisol (ng ml^{-1})
Vehicle	29.2 ± 14.6
Cortisol 10 μg/g	51.3 ± 16.5
Cortisol 50 μg/g	202.2* ± 58.9
DOC 9 μg/g	51.6 ± 25.6
DOC 45 μg/g	63.2 ± 36.7

Values are mean ± standard error (n = 10 fish per group). Asterisk indicates significant difference between the treated and vehicle group (p < 0.05, Student-Neuman-Keuls test).

Table 2
Effect of aldosterone on gill Na^+,K^+-ATPase activity and salinity tolerance (plasma chloride after 24 h seawater challenge) of Atlantic salmon parr

	Vehicle	Aldosterone 5 μg/g	Aldosterone 50 μg/g
Gill Na^+,K^+-ATPase activity (μmol ADP mg protein^{-1} h^{-1}) Day 6	1.5 ± 0.2	1.6 ± 0.2	1.4 ± 0.1
Day 12	1.9 ± 0.2	1.6 ± 0.1	1.5 ± 0.1
Plasma chloride (mM) Day 14 (SW challenge)	174.6 ± 2.4	169.4 ± 2.8	172.3 ± 2.2
Plasma cortisol (ng ml^{-1})	50.9 ± 10.4	14.7 ± 5.9	50.9 ± 19.3
Plasma aldosterone (ng ml^{-1})	nd (<0.1)	7.3 ± 2.8	18.2 ± 1.6

Values are mean ± standard error (n = 8–10 fish per group).

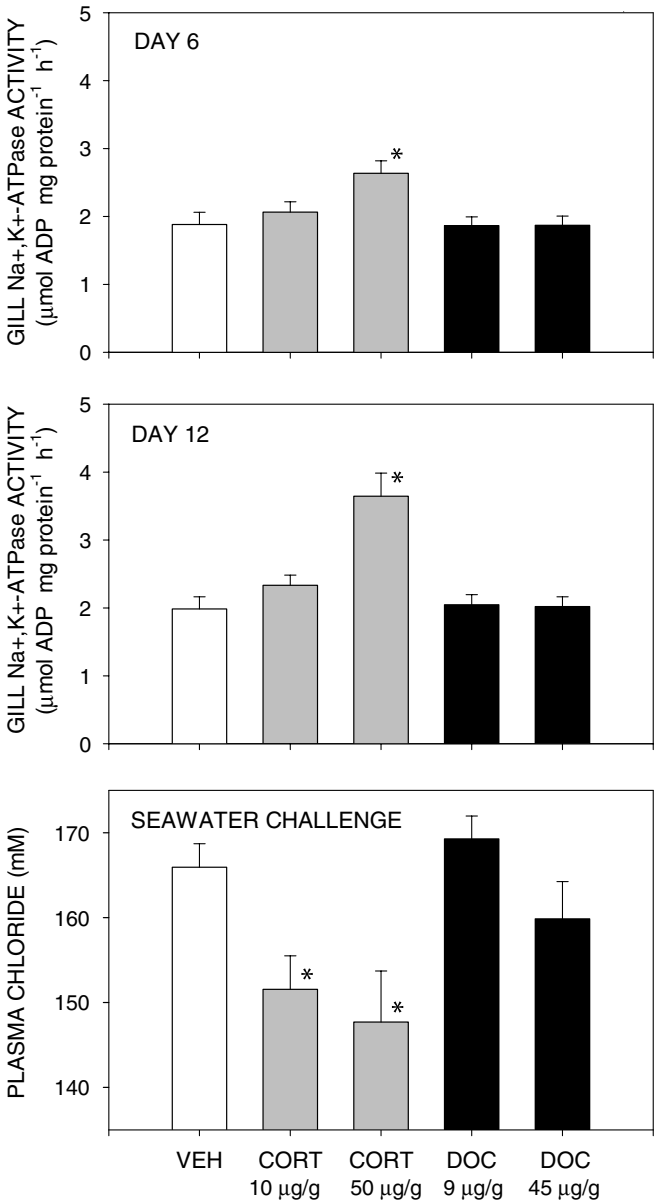

Fig. 1. Gill Na^+,K^+-ATPase activity and salinity tolerance (plasma chloride after 24 h seawater challenge) in Atlantic salmon parr treated with cortisol and 11-deoxycorticosterone (DOC). Values are mean ± standard error (n = 10 fish per group). Asterisk indicates significant difference between the treated and vehicle group ($p < 0.05$, Student-Neuman-Keuls test).

by approximately 67% ($p = 0.178$) by aldosterone treatment. The difference was largely attributed to one fish, however, with 3.6-fold greater number of MR mRNA copies.

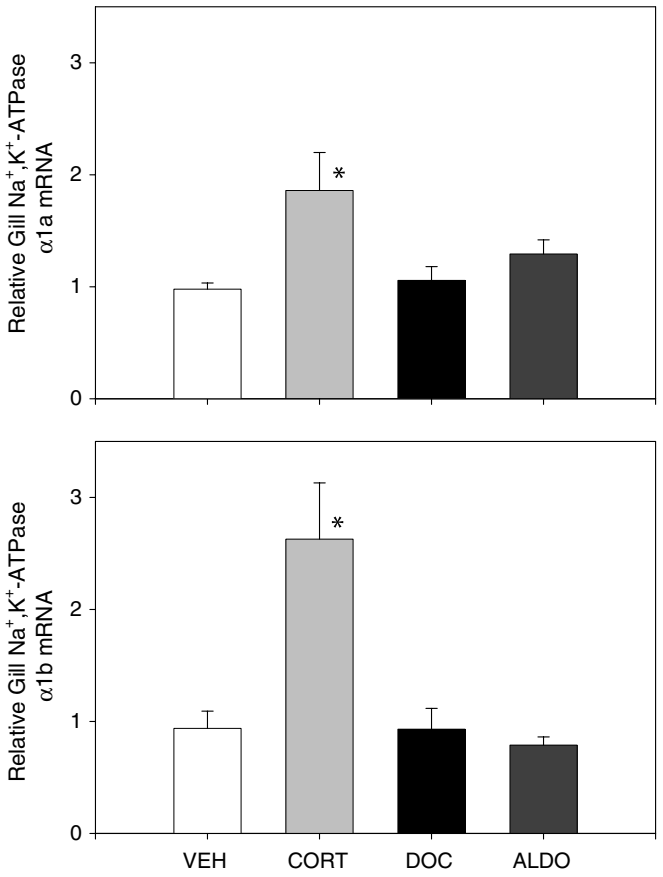

Fig. 2. Gill Na^+,K^+-ATPase α1a and α1b mRNA levels in Atlantic salmon parr treated with cortisol (50 μg/g), 11-deoxycorticosterone (DOC; 45 μg/g) and aldosterone (50 μg/g) for 6 days. Values expressed as mRNA per unit elongation factor 1α mRNA and are mean ± standard error (n = 6–8 fish per group). Asterisk indicates significant difference between the treated and vehicle group ($p < 0.05$, Student-Neuman-Keuls test).

RU486 and spironolactone had no effect on cortisol-mediated increases in gill NKA activity after 6 days (Fig. 3). After 12 days, however, RU486 partially decreased the effect of cortisol on gill NKA activity, whereas spironolactone had no effect. Cortisol-mediated increases in salinity tolerance were also partially blocked by RU486, with no impact of spironolactone. RU486 blocked the upregulation NKA α1a and α1b mRNA levels by cortisol (Fig. 4), whereas spironolactone had no detectable effect.

4. Discussion

The present results indicate that exogenous treatment with cortisol to achieve circulating physiological levels increases gill NKA activity and promotes salinity tolerance in juvenile Atlantic salmon. These results are consistent with a large number of *in vivo* and a smaller number of *in vitro* studies indicating an effect of cortisol on mechanisms of salt secretion (see reviews by McCormick, 2001; Evans et al., 2005). DOC and aldosterone, administered at two different doses and in the same fashion as cortisol, had no impact either on NKA activity or seawater tolerance. Few previous studies have examined the effect of DOC or aldosterone on the osmoregulatory physiology of teleosts fish. Umminger and Gist (1973) found that cortisol injections lowered serum sodium and chloride levels in fresh water goldfish (*Carassius auratus*), whereas aldosterone had no effect. Takahashi et al. (2006) found that treatment of eel with cortisol caused increased cell proliferation and apoptosis in the anterior intestine of seawater-acclimated mud-

Table 3
Gill corticosteroid receptor mRNA levels and gill Na^+,K^+-ATPase activity (μmol ADP mg protein^{-1} h^{-1}) in Atlantic salmon parr treated with cortisol, 11-deoxycorticosterone (DOC) and aldosterone for 6 days

	Vehicle	Cortisol 50 μg/g	DOC 45 μg/g	Aldosterone 50 μg/g
Gill glucocorticoid receptor 1	1.01 ± 0.06	1.43 ± 0.19	1.12 ± 0.13	1.11 ± 0.11
Gill glucocorticoid receptor 2	1.01 ± 0.07	0.99 ± 0.12	1.09 ± 0.07	0.96 ± 0.11
Gill mineralocorticoid receptor	1.03 ± 0.11	1.19 ± 0.22	1.21 ± 0.17	1.68 ± 0.36
Gill Na^+,K^+-ATPase activity	2.21 ± 0.44	3.13 ± 0.59	1.62 ± 0.13	2.15 ± 0.24

Values are expressed as mRNA per unit elongation factor 1α mRNA and are mean ± standard error (n = 6–8 fish per group). There was no significant difference among treatments ($p > 0.1$, one-way ANOVA), with the exception of cortisol treatment and glucorticoid receptor 1 ($p = 0.068$).

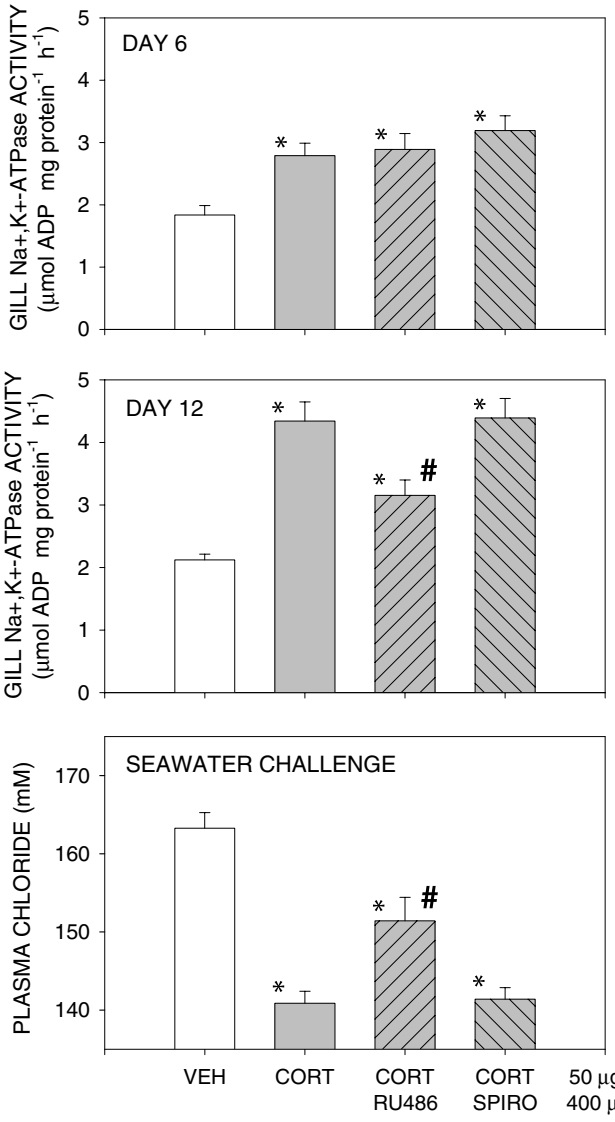

Fig. 3. Effect of RU 486 and spironolactone on the ability of cortisol to affect gill NKA activity and salinity tolerance (plasma chloride after 24 h seawater challenge) in Atlantic salmon parr. Values are mean ± standard error (n = 8–10 fish per group). * and # indicates significant difference between the vehicle and cortisol-treated groups, respectively ($p < 0.05$, Student-Neuman-Keuls test).

skipper (*Periophthalmus modestus*), whereas DOC had no effect. Though limited, these and the current results do not provide support for an osmoregulatory function of DOC or aldosterone in teleost fish.

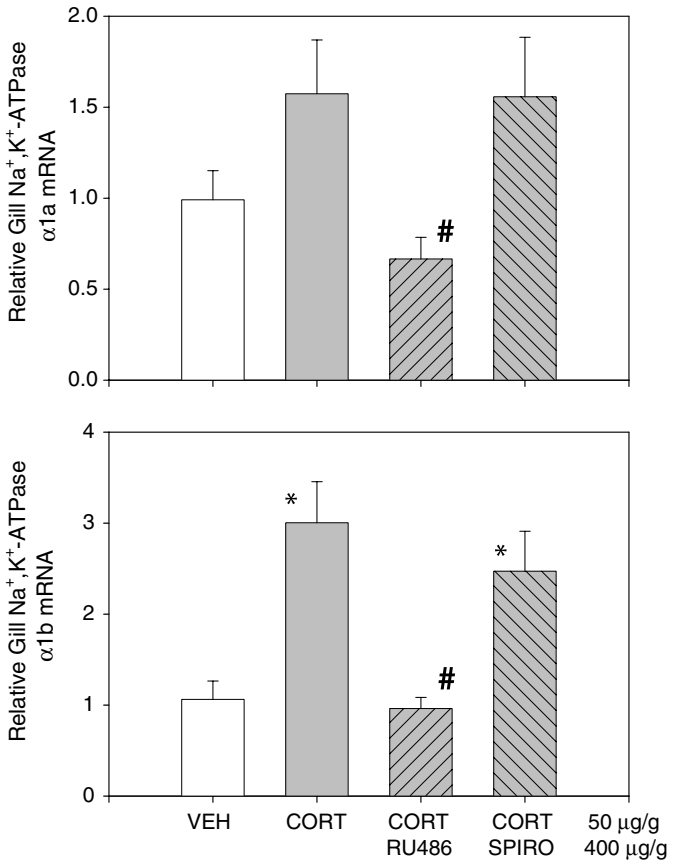

Fig. 4. Effect of RU 486 and spironolactone on the ability of cortisol to affect gill NKA α1a and α1b mRNA levels in Atlantic salmon parr. Values are mean ± standard error (n = 8–10 fish per group). * and # indicates significant difference between the vehicle and cortisol-treated groups, respectively ($p < 0.05$, Student-Neuman-Keuls test).

Na^+,K^+-ATPase is involved in both ion uptake and salt secretion by the gill of teleost fish (Evans et al., 2005). Recently, two isoforms of the alpha-subunit of NKA, α1a and α1b, were found to be differentially regulated in fresh water and seawater in the rainbow trout (*Onchorhynchus mykiss*) (Richards et al., 2003). Levels of NKA α1a mRNA increased following transfer from seawater to fresh water, whereas NKA α1b mRNA levels increased after exposure to seawater. This effect also occurs in Atlantic salmon, which also show increased NKA α1b mRNA during the parr–smolt transformation, consistent with the increased salinity tolerance that occursin this developmental stage (Nilsen et al., 2007). Shrimpton et al. (2005) found that NKA α1a subunit mRNA increased in adult sockeye salmon (*Oncorhynchus nerka*) migrating from the ocean to a fresh water environment. Although characterization, localization and function of the α1a and α1b proteins is still lacking, the differential mRNA levels found in these studies suggest that these isoforms have distinct functions, one active in fresh water and one active in seawater. In the present study both α1a and α1b mRNA levels were increased by cortisol treatment. These results are consistent with recent findings that *in vitro* cortisol increases mRNA levels of both of these isoforms (Kiilerich et al., 2007).

There is a large body of research in support of a physiological role for cortisol promoting mechanisms involved in salt secretion of teleost fish (Foskett et al., 1983). However, there is also substantial support for the action of cortisol on ion uptake in fresh water in phylogenetically diverse teleosts (McCormick, 2001). If we accept

the assumption that the α1a isoform is involved in ion uptake, then the upregulation of NKA α1a mRNA by cortisol found in the present study and by Kiilerich et al. (2007) provides further support for an ion uptake function of cortisol. We have found that cortisol can increase the capacity of Atlantic salmon to maintain plasma ions following transfer from seawater to ion-poor fresh water, whereas DOC is without effect (McCormick, unpublished results). Together, these results are consistent with cortisol acting as the major corticosteroid involved in both ion uptake and salt secretion in teleost fish.

Steroid treatment had little effect on gill GR1, GR2 and MR mRNA levels, despite the evidence that cortisol, DOC and aldosterone have high affinity for these receptors. Steroid binding *in vitro* to a translated protein from the rainbow trout GR1 gene indicates specificity and high affinity for cortisol, with much lower affinity for aldosterone (Ducouret et al., 1995). Bury et al. (2003) showed that dexamethasone had similar affinity for recombinant GR1 and GR2. Additionally these authors found that DOC could partially induce GR2 transactivation activity, but DOC was without effect on GR1. Binding studies have shown that cortisol has a higher affinity for MR than aldosterone and DOC (Colombe et al., 2000), but aldosterone and DOC are about 10 times more potent in stimulating transactivation of MR than cortisol (Sturm et al., 2005). These studies provide evidence that one or more of these hormones can bind and potentially transactivate the GRs or MR *in vivo*.

Ligand-receptor binding can result in significant changes in receptor mRNA levels. Cortisol treatment *in vivo* and *in vitro* results in significant decreases in hepatic GR protein levels but increases in hepatic GR mRNA levels, due to an autoregulation of hepatic GR mRNA by GR protein abundance (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). A negative feedback of cortisol on gill cortisol receptor levels has been shown in salmonids (Shrimpton and Randall, 1994), and consequently we expected to see significant changes in GR1 or GR2 (and possibly MR) mRNA levels in response to the presence of an agonist. Although, there was some indication of an increase in gill GR1 mRNA levels in cortisol-treated fish ($p = 0.07$), the differences were not significant. Our time course for the study and cortisol treatment were similar to those of Vijayan et al. (2003), so the lack of change in GR1 and GR2 receptor mRNA levels in response to potential agonists is puzzling but may be due to differences in liver and gill in the strength of the autoregulation of the receptor protein on gill GR mRNA levels.

In the present study we found that the effect of cortisol on salinity tolerance and gill NKA activity could be partially blocked by the GR receptor blocker RU486, whereas spironolactone had no effect. Treatment with RU486 inhibits the development of intestinal fluid absorption, important for normal seawater acclimation, during the parr–smolt transformation of Atlantic salmon (Veillette et al., 1995). In the killifish *Fundulus heteroclitus*, prior treatment with RU486 inhibits cAMP-stimulation of short-circuit current, a measure of chloride secretion, in the isolated opercular membrane (Marshall et al., 2005). More recently, Shaw et al. (2007) demonstrated that RU486 treatment resulted in high plasma ions and mortality after transfer of killifish from fresh water to seawater and reduced gill CFTR mRNA levels after seawater exposure. RU486 also reduces seawater tolerance in summer flounder (*Paralichthys dentatus*), a deficit that could be restored by treatment with cortisol (Veillette et al., 2007). Cortisol induction of NKA α1b and CFTR mRNA levels *in vitro* can be blocked by RU486 (Kiilerich et al., 2007). These results are consistent with cortisol acting through a GR to promote mechanisms of salt secretion in teleosts.

Spironolactone has only recently been used to examine possible MR function in fish, in part because of lack of information on its binding and specificity to teleost corticosteroid receptors. Spironolactone treatment caused a reduction in the number of gill chloride cells in rainbow trout following transfer to ion-deficient water (Sloman et al., 2001). In killifish, spironolactone reduced gill cell proliferation (BrdU incorporation) and NKA α1a mRNA levels following transfer from 10 ppt to fresh water (Scott et al., 2005). In Atlantic salmon, spironolactone *in vitro* blocks cortisol-induced upregulation of NKA α1a mRNA (though this effect is also blocked by RU486) (Kiilerich et al., 2007). These results are consistent with cortisol acting through an MR to promote mechanisms of ion uptake in teleosts. However, conclusions based on the use of RU486 and spironolactone as specific inhibitors of GR and MR in teleost fish should be made with some reservation. The relative specificity of spironolactone as an MR blocker in mammals has been questioned (Delyani, 2000). In a mammalian cell line expressing rainbow trout MR, RU486 was a slightly more potent competitive inhibitor of aldosterone-induced transactivation than spironolactone (Sturm et al., 2005). Furthermore, in the absence of aldosterone or other steroids, spironolactone was a partial agonist of the rainbow trout MR, whereas RU486 was not. These results bring into question the relative specificity of these compounds for blocking the teleosts MR and GR. In spite of these results, there is evidence that these inhibitors have some specificity in teleost fish. RU486 treatment of teleosts results in increased levels of circulating cortisol (Veillette et al., 1995; Marshall et al., 2005; Scott et al., 2005), whereas spironolactone has no effect (Scott et al., 2005, McCormick, unpublished results). The ability of RU486 to increase plasma cortisol can be attributed to blockage of the negative feedback in the pituitary, and suggests the RU486 is blocking a pituitary GR and that spironolactone is not. Differential effects of the two inhibitors within the same studies were found on gill cell proliferation in rainbow trout (Sloman et al., 2001), chloride cell proliferation and CFTR mRNA levels in killifish (Scott et al., 2005; Shaw et al., 2007), and on *in vitro* cortisol induction of NKA α1a and α1b mRNA levels in Atlantic salmon (Kiilerich et al., 2007). These differential effects of RU486 and spironolactone provide limited support that these receptor blockers are in fact specific, though it is clear that further work is necessary to fully establish these or other compounds as unreserved specific blockers of corticosteroid receptors in teleosts.

The cortisol treatments in the present study were successful in elevating circulating cortisol levels (Table 1). The levels of cortisol in the vehicle group may not represent 'basal' cortisol levels as these animals were exposed to seawater for 24 h which may have increased cortisol in the vehicle group. Nonetheless, the results indicate that dose-dependent and physiological levels were achieved in both low and high cortisol treatments. It is interesting to note that salinity tolerance was affected at both doses, whereas NKA only at the highest. This is perhaps due to an inherent variability in NKA activity, but could also suggest greater sensitivity or more rapid response of salinity tolerance to cortisol compared to that for NKA activity.

In summary, the absence of effects of DOC and aldosterone on NKA mRNA levels, NKA activity or salinity tolerance does not provide support for an osmoregulatory function of these corticosteroids. Our study, however, does not rule out the possibility that DOC is involved in some other aspect of ion regulation or physiology that we have not examined. The present results do provide support for cortisol acting through a glucocorticoid receptor as the main pathway for corticosteroid regulation of salt secretion. The capacity of cortisol to upregulate NKA α1a mRNA levels indicates that cortisol is also involved in regulating ion uptake.

Acknowledgments

We thank personnel of the White River National Fish Hatchery (US Fish and Wildlife Service), Kensington Fish Hatchery (Connecticut Department of Environmental Protection) and the Connecticut River Atlantic Salmon Commission for providing us with fish for

these studies. We also thank Amanda Keyes for technical assistance in various aspects of this study and Dr. P.M. Schulte (University of British Columbia) for allowing us access to her laboratory equipment.

References

Bridgham, J.T., Carroll, S.M., Thornton, J.W., 2006. Evolution of hormone-receptor complexity by molecular exploitation. Science 312, 97–101.

Bury, N.R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Robinson-Rechavi, M., Laudet, V., Rafestin-Oblin, M.E., Prunet, P., 2003. Evidence for two distinct functional glucocorticoid receptors in teleost fish. J. Mol. Endocrinol. 31, 141–156.

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.

Chakraborti, P.K., Weisbart, M., Chakraborti, A., 1987. The presence of corticosteroid receptor activity in the gills of the brook trout, *Salvelinus fontinalis*. Gen. Comp. Endocrinol. 66, 323–332.

Colombe, L., Fostier, A., Bury, N., Pakdel, F., Guiguen, Y., 2000. A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. Steroids 65, 319–328.

Delyani, J.A., 2000. Mineralocorticoid receptor antagonists: the evolution of utility and pharmacology. Kidney Int. 57, 1408–1411.

Ducouret, B., Tujague, M., Ashraf, J., Mouchel, N., Servel, N., Valotaire, Y., Thompson, E.B., 1995. Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. Endocrinology 136, 3774–3783.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

Foskett, J.K., Bern, H.A., Machen, T.E., Conner, M., 1983. Chloride cells and the hormonal control of teleost fish osmoregulation. J. Exp. Biol. 106, 255–281.

Greenwood, A.K., Butler, P.C., White, R.B., DeMarco, U., Pearce, D., Fernald, R.D., 2003. Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities. Endocrinology 144, 4226–4236.

Kiilerich, P., Kristiansen, K., Madsen, S.S., 2007. Cortisol regulation of ion transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway. J. Endocrinol. 194, 417–427.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25, 402–408.

Marshall, W.S., Cozzi, R.R.F., Pelis, R.M., McCormick, S.D., 2005. Cortisol receptor blockade and seawater adaptation in the euryhaline teleost Fundulus heteroclites. J. Exp. Zool. A Comp. Exp. Biol. 303A, 132–142.

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

McCormick, S.D., 1996. Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+,K^+-ATPase in Atlantic salmon (*Salmo salar*): interactions with cortisol. Gen. Comp. Endocrinol. 101, 3–11.

McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781–794.

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

Nilsen, T.O., Ebbesson, L.E., Stefansson, S.O., Madsen, S.S., McCormick, S.D., Björnsson, B.Th., Prunet, P., 2007. Differential expression of gill Na^+,K^+-ATPase α- and β-subunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. J. Exp. Biol. 210, 2885–2896.

Prunet, P., Sturm, A., Milla, S., 2006. Multiple corticosteroid receptors in fish: from old ideas to new concepts. Gen. Comp. Endocrinol. 147, 17–23.

Richards, J.G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na^+/K^+-ATPase a-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J. Exp. Biol. 206, 4475–4486.

Sathiyaa, R., Vijayan, M.M., 2003. Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Am. J. Physiol. Cell Physiol. 284, C1508–C1515.

Scott, G.R., Keir, K.R., Schulte, P.M., 2005. Effects of spironolactone and RU486 on gene expression and cell proliferation after freshwater transfer in the euryhaline killifish. J. Comp. Physiol. B175, 499–510.

Shaw, J.R., Gabor, K., Hand, E., Lankowski, A., Durant, L., Thibodeau, R., Stanton, C.R., Barnaby, R., Coutermarsh, B., Karlson, K.H., Sato, J.D., Hamilton, J.W., Stanton, B.A., 2007. Role of glucocorticoid receptor in acclimation of killifish (*Fundulus heteroclitus*) to seawater and effects of arsenic. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R1052–R1060.

Shrimpton, J.M., Patterson, D.A., Richards, J.G., Cooke, S.J., Schulte, P.M., Hinch, S.G., Farrell, A.P., 2005. Ionoregulatory changes in different populations of maturing sockeye salmon *Oncorhynchus nerka* during ocean and river migration. J. Exp. Biol. 208, 4069–4078.

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. Regul. Integr. Comp. Physiol. 267, R432–R438.

Sloman, K.A., Desforges, P.R., Gilmour, K.M., 2001. Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout. J. Exp. Biol. 204, 3953–3961.

Specker, J.L., Portesi, D.M., Cornell, S.C., Veillette, P.A., 1994. Methodology for implanting cortisol in Atlantic salmon and effects of chronically elevated cortisol on osmoregulatory physiology. Aquaculture 121, 181–193.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestion-Oblin, M.E., Prunet, P., 2005. 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Onchrhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146, 47–55.

Takahashi, H., Takahashi, A., Sakamoto, T., 2006. In vivo effects of thyroid hormone, corticosteroids and prolactin on cell proliferation and apoptosis in the anterior intestine of the euryhaline mudskipper (*Periophthalmus modestus*). Life Sci. 79, 1873–1880.

Umminger, B.L., Gist, D.H., 1973. Effects of thermal acclimation on physiological responses to handling stress, cortisol and aldosterone injections in the goldfish, *Carassius auratus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 44A, 967–977.

Veillette, P.A., Merino, M., Marcacclo, N.D., Garcia, M.M., Specker, J.L., 2007. Cortisol is necessary for seawater tolerance in larvae of a marine teleost the summer flounder. Gen. Comp. Endocrinol. 151, 116–121.

Veillette, P.A., Sundell, K., Specker, J.L., 1995. Cortisol mediates the increase in intestinal fluid absorption in Atlantic salmon during parr smolt transformation. Gen. Comp. Endocrinol. 97, 250–258.

Vijayan, M.M., Raptis, S., Sathiyaa, R., 2003. Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen. Comp. Endocrinol. 132, 256–263.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.