Implication of the mineralocorticoid axis in rainbow trout osmoregulation during salinity acclimation

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Abstract

Cortisol and glucocorticoid receptors (GRs) play an important role in fish osmoregulation, whereas the involvement of the mineralocorticoid receptor (MR) and its putative ligand 11-deoxycorticosterone (DOC) is poorly investigated. In this study, we assessed the implication of DOC and MR in rainbow trout (*Oncorhynchus mykiss*) osmoregulation during hypo- and hypersaline acclimation in parallel with the cortisol–GR system. A RIA for DOC was developed to measure plasma DOC levels, and a MR-specific antibody was developed to localize MR protein in the gill, intestine, and kidney. This is the first study to report DOC plasma levels during salinity change and MR localization in fish osmoregulatory tissue. Corticosteroid receptor mRNA abundance was investigated in osmoregulatory tissue during salinity acclimation, and the effect of cortisol and DOC on ionic transporters gene expression was assayed using an *in vitro* gill incubation method. Differential tissue-, salinity-, and time-dependent changes in *MR* mRNA levels during both hyper- and hyposaline acclimations and the ubiquitous localization of MR in osmoregulatory tissue suggest a role for the MR in osmoregulation. Presumably, DOC does not act as ligand for MR in osmoregulation because there were no changes in plasma DOC levels during either freshwater–seawater (FW–SW) or SW–FW acclimation or any effect of DOC on gill ionic transporter mRNA levels in the gill. Taken together, these results suggest a role for MR, but not for DOC, in osmoregulation and confirm the importance of cortisol as a major endocrine regulator of trout osmoregulation.

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Introduction

In mammals, the main mineralocorticoid hormone, aldosterone, plays a central role in the control of water and sodium homeostasis. Fish lack aldosterone because of their inability to synthesize this steroid (Jiang *et al.* 1998), but they have the ability to produce 11-deoxycorticosterone (DOC; Jiang *et al.* 1998) an intermediate of mammalian aldosterone synthesis. Recently, a mineralocorticoid receptor (MR) with high affinity for DOC has been characterized in rainbow trout (*Oncorhynchus mykiss*; Sturm *et al.* 2005) and in several other fish species (Greenwood *et al.* 2003, Kiilerich *et al.* 2007*a*, Stolte *et al.* 2008). The recent discovery of MR and its high affinity mineralocorticoid-like ligand (DOC) suggest the presence of a functional mineralocorticoid signaling axis in fish (Sturm *et al.* 2005). In this context, the corticosteroid signaling system in most fish consists of MR, DOC, and the glucocorticoid receptor isoforms, GR1 and GR2, along with their high-affinity ligand, cortisol. Moreover, cortisol activates not only the GRs but also the MR, thus cortisol has the potential to be a physiological ligand of both the glucocorticoid and the mineralocorticoid signaling systems.

As DOC is a specific ligand for MR and a tenfold more potent agonist compared with cortisol, this hormone could be the primary physiological agonist of the MR (Sturm *et al.* 2005) despite lower plasma levels of DOC compared with cortisol (Prunet *et al.* 2006). In fact, DOC has been detected in fish plasma at concentrations sufficient to activate the rtMR during specific physiological circumstances such as reproduction (Katz & Eckstein 1974, Campbell *et al.* 1976, 1980, Truscott *et al.* 1978), where an observed 10- to 50-fold increase in plasma DOC levels in mature trout males compared with immature fish suggests a physiological role of this hormone (Milla *et al.* 2008). Taken together, this

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suggests the presence of a functional mineralocorticoid signaling axis in fish; however it remains to be known whether cortisol, DOC, or both serve as the physiological relevant agonist(s).

The main organ involved in the maintenance of hydro-mineral homeostasis in fish is the gill, in association with the posterior kidney and the intestine. Ion transporters serve to regulate Na^+ and Cl^- flow over the gill epithelia. Na^+, K^+-ATPase α1b; Na^+, K^+, $2Cl^-$ co-transporter (NKCC); and cystic fibrosis transmembrane conductance regulator (CFTR) are involved in ion secretion to counterbalance passive ion uptake in hyperosmotic environment (Marshall & Grosell 2006), whereas V-type H^+-ATPase; epithelium Ca^{2+}-channel (ECaC); $Na^+ - HCO_3^-$ cotransporter (NBC); Na^+, K^+-ATPase α1a; and carbonic anhydrase (CA) govern ion uptake in hypoosmotic medium (Marshall & Grosell 2006, Parks *et al*. 2006, Perry & Gilmour 2006). Cortisol represents a major endocrine actor in the regulation of ionic homeostasis, particularly after environmental salinity change, by regulating some of these seawater-/freshwater- (FW) specific ion transporters (McCormick 2001, Kiilerich *et al*. 2007*b*). The GRs are most likely taking part in some of these osmoregulatory effects due to the positive correlation between cortisol receptor concentration and Na^+, K^+-ATPase activity responsiveness to cortisol in the gill (Shrimpton & McCormick 1999, McCormick 2001) and the inhibitory effects of the GR-specific antagonist, RU486 (Kiilerich *et al*. 2007*a*, McCormick *et al*. 2008). In contrast, information on the role of DOC and MR in fish osmoregulation is very limited. DOC administration promoted renal salt retention but branchial salt loss in rainbow trout (Holmes 1959), but McCormick *et al*. (2008) showed no effect of DOC on gill Na^+, K^+-ATPase activity; α1a, α1b mRNA expression; or overall salinity tolerance during Atlantic salmon (*Salmo salar*) seawater (SW) adaptation. Likewise, no effect of the specific MR antagonist, spironolactone, on cortisol stimulation of gill Na^+, K^+-ATPase activity or α1a, α1b expression was observed (McCormick *et al*. 2008). However, Kiilerich *et al*. (2011) have recently shown a seasonal dependent effect of DOC on ion transporter mRNAs in Atlantic salmon gill tissue explants. Furthermore, a role for MR in FW acclimation has been suggested on the basis of the inhibitory effects on some gill osmoregulatory parameters in rainbow trout and killifish (*Fundulus heteroclitus*; Sloman *et al*. 2001, Scott *et al*. 2005).

Thus, although cortisol–GRs are considered an important SW-/FW-adapting endocrine system, the exact role of DOC–MR in fish osmoregulation remains unclear. To assess this question, we compared the regulation of DOC–MR in parallel with that of cortisol–GRs in salinity adaptation of rainbow trout. Regulation of plasma cortisol and DOC levels during FW–SW and SW–FW adaptations and regulation of *MR*, *GR1*, *GR2* mRNA expression in osmoregulatory tissue (gill, posterior kidney, and intestine) were investigated. Furthermore localization of GR1 and MR in these tissues was investigated and co-localized with the Na^+, K^+-ATPase protein. Finally, an *in vitro* gill block incubation system was used to investigate cortisol and DOC regulation of important ion transporters in the gill.

Materials and Methods

Fish and salinity transfer

Investigations and animal care were conducted according to the guiding principles for the use and care of laboratory animals in compliance with French and European regulations on animal welfare. Immature rainbow trout (*O. mykiss*) were obtained from INRA/PEIMA fish farm (Sizun, France) and were maintained under natural photoperiod in a re-circulated water system at 12 °C at the INRA/SCRIBE experimental facilities (Rennes, France).

The immature rainbow trout (89 ± 18 g, mean $\pm$ S.D.) were randomly distributed in two 1 m^3 tanks (with 70 trout in each tank) containing either FW or synthetic 30% SW (Instant Ocean, Aquarium Systems, Mentor, OH, USA) 2 weeks before the start of the experiment. Before the start of the experiment, six fish from each salinity were sampled to constitute either the FW or the SW initial control group. FW-acclimated fish were transferred to two tanks containing either FW (FW–FW controls) or SW (FW–SW group), and vice versa for the SW-acclimated fish (SW–SW controls and SW–FW group). Such protocol allowed to leave each tank untouched during at least 24 h to avoid confounding effects due to sampling stress. No mortality was observed during the osmotic challenges. FW–SW-transferred fish were sampled at 12 h, and 1, 2, 5, 9, and 21 days post-transfer and SW–FW-transferred fish were sampled at 6 h, and 1, 2, 4, and 7 days post-transfer for plasma hormone measurements and the gill, intestine, and kidney RNA analysis ($n = 6$–8). Fish were killed with 2-phenoxyethanol; blood was withdrawn with a heparinized syringe, followed by cutting of the spine and pithing the brain. Blood was kept on ice until plasma was separated by centrifugation and frozen at −80 °C until hormone analysis. The gill, posterior kidney, and intestine were dissected out, snap-frozen in liquid N_2, and kept at −80 °C until RNA analysis.

Plasma cortisol measurements

Cortisol measurements were carried out using a RIA procedure described previously (Auperin *et al*. 1997).

Plasma DOC measurements

Fluoroimmunoassay A fluoroimmunoassay (FIA), adapted from a testosterone FIA (Fiet *et al*. 2004) with minor modifications, was developed for plasma DOC measurements: a rabbit anti-DOC antibody (Fiet 1979) was used, DOC was extracted from 1·5 ml plasma using cyclohexane/ethyl acetate (1/1, v/v), and DOC loss during extraction and HPLC was

monitored using 2000 d.p.m. tritiated DOC. The amounts of DOC added to the wells of the microtiter plates to establish the standard curve were in pg/well: 400, 200, 100, 50, 25, 12·5, 6·25, 3·12, 1·56, 0·78, and 0·39. This FIA assay was used to validate the specificity of the anti-DOC antibody and to validate the absolute levels of DOC measured in plasma samples by mass spectrometry (MS) (see below).

Validation of the anti-DOC antibody specificity by cross-reactivity test The specificity of the anti-DOC antibody was assessed by testing the cross-reactivity of the antibody with a panel of fish steroids as described in Fiet *et al.* (2004) complemented with cortisone and the piscine sex steroid 17,20β-dihydroxy-4-pregnen-3-one (17α,20β-dihydroxyprogesterone). No steroids had a cross-reactivity higher than 3·5%, and cortisol, the most abundant corticosteroid in fish, presented a cross-reactivity lower than 0·01%.

Validation of measurements of absolute DOC levels using MS Liquid chromatography (LC) coupled to MS was used to validate the measured levels by the FIA for DOC by determining the amount of DOC in 15 plasma samples. The LC separation was achieved on a Surveyor (Thermo Fisher, Les Ulis, France) HPLC system fitted with a C18 Purospher Star column (50×2 mm, 3 μm). The following gradient elution was used (flow rate, 0·2 ml/min): 0 min, 50% B, from 0 to 10 min, linear gradient from 50 to 100% B, then 100% B from 10 to 15 min, with A=H_2O/methanol/acetic acid (90/10/0·2, v/v/v) and B=H_2O/methanol/acetic acid (10/90/0·2, v/v/v). Mass spectrometric acquisition was carried out on a triple quadrupole mass spectrometer (TSQ Quantum Discovery, Thermo Fisher) fitted with an electrospray ionization source operated in the positive ionization mode. Typical ionization source operating conditions were as follows: electrospray needle, 5 kV; heated transfer capillary temperature, 350 °C; heated transfer capillary voltage, 35 V; and tube lens offset, 87 V. The detection and quantification of DOC was based on the monitoring of two MS/MS diagnostic transitions, i.e. m/z 331→109 and m/z 331→97, using a collision energy of 39 eV. The calibration curve was established from four standard DOC solutions at 1, 10, 50, and 100 pg/μl respectively (injected volume 10 μl). Linearity was checked between 1 and 100 pg/μl (R^2=0·9993). Detection limits and quantification limits were reached at 0·1 pg/μl (S/N=3) and 0·5 pg/μl (S/N=10) respectively. Repeatability was assessed by triplicate injections of the standard DOC solutions. The resulting residual standard deviations (RSDs) were in the range of 2–5%. Matrix effects were checked from spiked plasma extracts.

RIA To have an in-house DOC assay to measure plasma DOC levels routinely, the FIA, as described above, was modified to RIA using the same anti-DOC antibody. To estimate the DOC loss during extraction and HPLC before the immunoassay, 2000 d.p.m. of an ethanolic tritiated deoxycorticosterone solution (American Radiolabeled Chemicals, St Louis, MO, USA) were added to 1·5 ml of each plasma sample in 15 ml borosilicate glass tubes. After steroid extraction with 2 ml cyclohexane/ethyl acetate (1/1, v/v) two times, the organic phase was evaporated to dryness and steroids were re-dissolved in 65 μl ethanol. Before HPLC of plasma samples, 50 μl ethanolic tritiated DOC (2000 c.p.m.) were loaded on the column to determine the HPLC fraction containing DOC (normally between 2·5 and 3·5 min after injection), and samples were collected 20 s before and 40 s after this peak. Ethanolic plasma steroid extracts (50 μl) were then injected in a ZORBAX SB-C18 HPLC column (Agilent, Massy, France) and passed through a mobile phase containing acetonitrile/water (80/20, v/v) acidified with 0·01% sulfuric acid. The organic chromatographic elution fractions were evaporated to dryness and re-dissolved in 250 μl assay buffer (see Fiet *et al.* 2004). For RIA, 100 μl sample (measured in duplicates) or DOC standards (measured in triplicates) were incubated for 2·5 h at 4 °C with 100 μl rabbit anti-DOC antibody (Fiet 1979) followed by incubation for 3 h at 4 °C with 18 000 c.p.m. [^{3}H]-DOC tracer, 100 μl anti-rabbit scintillation proximity assay reagent (Bosworth & Towers 1989). Samples were then precipitated with 25% polyethylene glycol 6000 over night, after which the pellet was re-dissolved in 100 μl ethanol and mixed with 2·5 ml Insta-Fluor (Perkin-Elmer, Waltham, MA, USA) before counting on a Packard Tri-Carb 2100 TR β-ray counter. A DOC standard curve was routinely carried out with 7·8, 15·6, 31·2, 62·5, 125, 250, 500, 1000, 2000, 4000, and 8000 pg/ml.

The validity of the RIA was evaluated using a surcharge test, where six aliquots of 50 μl plasma were loaded with 50 μl of known quantities of DOC (final concentrations: 31, 62, 125, 250, and 500 pg/ml). Mean recoveries ranged from 83 to 123% dependent on the dose. A standard curve of DOC (1·9, 3·9, 7·8, 15·6, 31·2, 62·5, 125, 250, 500, 1000, 2000, 4000, and 8000 pg/ml) was included to assess the lower and upper detection limits of the assay. Finally, the inter-assay variability was assessed by quantifying DOC levels in triplicates in 14 different assays for three samples (7·8, 125, and 2000 pg/ml respectively) and the intra-assay variability was assessed by measuring the B_0 (containing gelatine buffer, anti-DOC antibody, and [^{3}H]-DOC tracer only) ten times in the same assay.

Immunohistochemistry

Antibodies GR1 localization studies were conducted using a GR1-specific rabbit polyclonal antibody developed against the N-terminal portion of rainbow trout GR1 (Tujague *et al.* 1998). Localization of mitochondria-rich cells by means of Na^+, K^+-ATPase was carried out with a monoclonal antibody raised against the α1 subunit of avian Na^+, K^+-ATPase (anti-Na^+, K^+-ATPase α5 antibody) developed by Douglas M Fambrough and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA

52242, USA). For localization of MR, a rabbit polyclonal antibody against the rainbow trout MR-AB domain was developed in the laboratory using a rainbow trout MR-AB domain-GST fusion protein (from now on called MR–GST) for immunization (Milla *et al*. 2008).

Validation of anti-MR antibody by immunoneutralization Recombinant MR–GST fusion protein used for immunization of rabbits and GST alone (to serve as control) was amplified in *Escherichia coli* in 2× YTA medium (per liter 2× YTA medium: 16 g tryptone, 10 g yeast extract, and 5 g NaCl) with 100 μg/ml ampicillin at 37 °C with shaking. Protein production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside when OD_{600} of the bacterial culture reached 0·6. Cells were harvested 2 h later, and sonicated, and the bacterial pellet was discarded before GST purification. MR–GST or GST alone was incubated with glutathione-sepharose 4B resin for 30 min at 4 °C, before washing two times with wash buffer 1 (PBS, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 2 mM NaCl) and one time with wash buffer 2 (PBS, 1 mM EDTA, and 1 mM DTT). MR–GST or GST was eluted for 30 min at 4 °C with 2 mM glutathione in 50 mM Tris–HCl. A denaturing SDS gel was run to verify the correct size of the purified MR–GST. Western blot was conducted in order to assess the specificity of the anti-rtMR antibody for MR, showing one single band for purified MR–GST and no bands for the GST protein alone. Immunoneutralization of anti-MR-AB domain antibody was carried out as described below (section Immunohistochemical localization of GR1 and MR in osmoregulatory tissue) except for 1 h incubation at room temperature of the antibody with graded doses of MR–GST or GST before incubation on the gill sections.

Immunohistochemical localization of GR1 and MR in osmoregulatory tissue One gill arch, a piece of the posterior kidney or 0·5 cm anterior and posterior intestine from three immature FW-adapted rainbow trout were excised and stored in 4% paraformaldehyde over night, dehydrated and embedded in paraffin. The tissues were sectioned (5 μm), mounted on polylysine slides, deparaffinated, and hydrated. Sections were de-masked for a total of 3×5 min in a microwave oven in 0·007 M citrate, pH=6, washed in 0·01 M PBS +1% Tween 20, and target protein was visualized using an Ultra Vision Anti-polyvalent, HRP/3,3′-diaminobenzidine (DAB) ready-to-use kit (Lab Vision Corporation, Thermo Fisher Scientific, Freemont, CA, USA) according to the manufacturer's recommendations. Briefly, sections were incubated for 10–15 min with hydrogen peroxide block, washed two times in PBS+Tween 20, incubated for 5–10 min in Ultra V block and washed in PBS before incubation at room temperature for 1 h with primary antibody (mouse anti-GR1 (dilution 1:1250), mouse anti-MR (dilution 1:1500), mouse anti-Na^+, K^+-ATPase (dilution 1:200), normal (pre-immune) rabbit serum (dilution 1:1500), or secondary antibody only (serving as control)). Thereafter the sections were washed four times in PBS+Tween 20, incubated 10 min with biotinylated goat anti-polyvalent rabbit antibody, washed 4 times, incubated for 10 min with streptavidin peroxidase, and washed four times. The chromogen DAB was used for visualization of antigens according to the manufacturers recommendations.

In vitro *gill block incubation*

All the gill arches were dissected from six FW-acclimated fish, and the cartilage was removed. Small pieces of gill (blocks of six filaments) were cut out, washed and incubated 1–2 h in pre-incubation medium (MEM (Sigma) with 25 mM HEPES, 650 mg/l bicarbonate, 200 mM glutamine, 5 mM pyruvate, 100 U/ml penicillin, 50 μg/ml streptomycin, and 2·5 μg/ml amphotericin, pH=7·55, 300 mOsm). Subsequently, the gill filament blocks were distributed in a repeated measures design in 12-well culture plates (~10–12 blocks of six filaments in each well) containing 2 ml incubation medium (MEM with 4% fetal bovine serum, 25 mM HEPES, 650 mg/l bicarbonate, 200 mM glutamine, 5 mM pyruvate, 50 U/ml penicillin, 25 μg/ml streptomycin, and 6·25 μg/ml amphotericin, pH=7·55, 300 mOsm) and were incubated with hormones for 24 h at 12 °C with gentle

Table 1 QPCR target genes, GenBank accession numbers and primer sequences

Target gene	GenBank no.	Forward primer	Reverse primer
rtGR1	Z54210	CCATCGTCAAGCGGGAAGAG	GGAACTCCACGCTAAGGGATTTATTC
rtGR2	AY495372	CTCCGCTTTCTCCAGCAGCTA	GTGAGCACCCCGTAGTGACAG
rtMR	AF209873	GAAACAGATGATCCGCGTGGT	TGGATCAGGGTGATTTGGTCCT
CFTR (*Salmo salar*)	AF161070	CAAGGCCCGCATACTGCT	GCAGTGGTTCCACTCTGTGTT
NKCC (*Salmo salar*)	NM_001123683	CGAGACCAAGGCATTCTACA	ATGTCTCCGTCCTTCCAGTC
rtNa$^+$, K$^+$-ATPase α1a	AY319391	GCAGACGCCTCTCGGAATT	CAATGAGAAAGATGATGGATG
rtNa$^+$, K$^+$-ATPase α1b	AY319390	GGAAGACGCCTATAGCCAAA	CGATGAGGAAGATGACAGCTT
rtNaHCO$_3$	NM_001124325	GGGGAACGTGGCGCAAGTATA	CCCATCCTCAAGTTCATCCCT
ECaC (*Danio rerio*)	AY383562	ACTTGGTCAACCGCAGAAAG	CAGATTCCACTTGAGCGTGA
rtH$^+$-ATPase (V-type)	AF140022	ATGTGCTGCCCTCTCTGTCT	CCAATGGCATAGCAGGCATA
rtCarbonic anhydrase	NM_001124220	CAGTCTCCCATTGACATCGTA	CGTTGTCGTCGGTGTAGGT
rt18S	AF309412	CGGAGGTTCGAAGACGATCA	TCGCTAGTTGGCATCGTTTATG

Table 2 Specificity of the rabbit anti-11-deoxycorticosterone (DOC) antibody used in RIA. Cross-reactivity of the anti-DOC antibody with a selection of fish steroid hormones is given as a percentage of the DOC signal

Steroid	Cross-reactivity (%)	Steroid	Cross-reactivity (%)
DOC	100	Aldosterone	<0·01
Progesterone	3·5	17-Hydroxypregnenolone	<0·01
Corticosterone	1·66	Δ4-Androsterone	<0·01
11-Deoxycortisol	1·25	Dehydroepiandrosterone	<0·01
Pregnenolone	0·28	Δ4-Androstenedione	<0·01
17-Hydroxypro-gesterone	0·05	Testosterone	<0·01
Cortisol	<0·01	Estradiol	<0·01

agitation. Hormone treatments (adjusted for total ethanol load) were as follows: control (2 μl ethanol), cortisol (F) alone (1 or 10 μg/ml) or together with the GR antagonist RU486 (10 μg/ml F+6 μg/ml RU486), RU486 alone (6 μg/ml), or DOC (10, 100 or 1000 ng/ml). Gill blocks were stored at −80 °C until RNA extraction. Cortisol and RU 10 and 6 μg/ml respectively were used to ensure equimolar concentrations (28 μM) of agonist (cortisol) and antagonist (RU). Cortisol in the doses used in this study has previously been demonstrated to be necessary to evoke an effect on Na^+, K^+-ATPase activity and α1-isoforms transcriptional levels in salmonids in the same type of experiment (McCormick *et al.* 1991, Kiilerich *et al.* 2007*a*, 2011). The actual tissue and cellular level of cortisol in this gill block incubation experiment may be much lower than 10 μg/ml, due to diffusion limitations caused by apical structure of the epithelial cells and mucus secretion by the gills during incubation.

Total RNA extraction, reverse transcription, and real-time PCR

RNA extraction from gill blocks, whole gill arches, posterior kidney and intestine was carried out using TRIzol (Invitrogen) as described previously (Milla *et al.* 2006). Likewise, reverse transcription and real-time PCR analyses were carried out on an iCycler IQ thermocycler (Bio-Rad Laboratories) as described previously (Milla *et al.* 2006). Relative expression levels of target gene mRNAs were calculated using the Pfaffl equation (Pfaffl 2001) and normalized to *18S* rRNA. Primers were designed using rainbow trout sequences when available (denoted rt) except ECaC (*Danio rerio*), NKCC, and CFTR (*S. salar*). Primer sequences and GenBank accession number are shown in Table 1. To compare the expression levels among the target genes, *rtGR1*, *rtGR2*, and *rtMR*, an absolute quantification was carried out. A dilution series of rainbow trout corticosteroid receptor containing plasmids (pCMV-rtGR1, -rtGR2, or -rtMR) from 0·01 attomole to 1 femtomole (corresponding to 6×10^6–6×10^{11} copies, considering the size of the receptor in question (8·6 kb for pCMV-rtGR1 and -rtMR and 7·5 kb for pCMV-rtGR2)) were subjected to

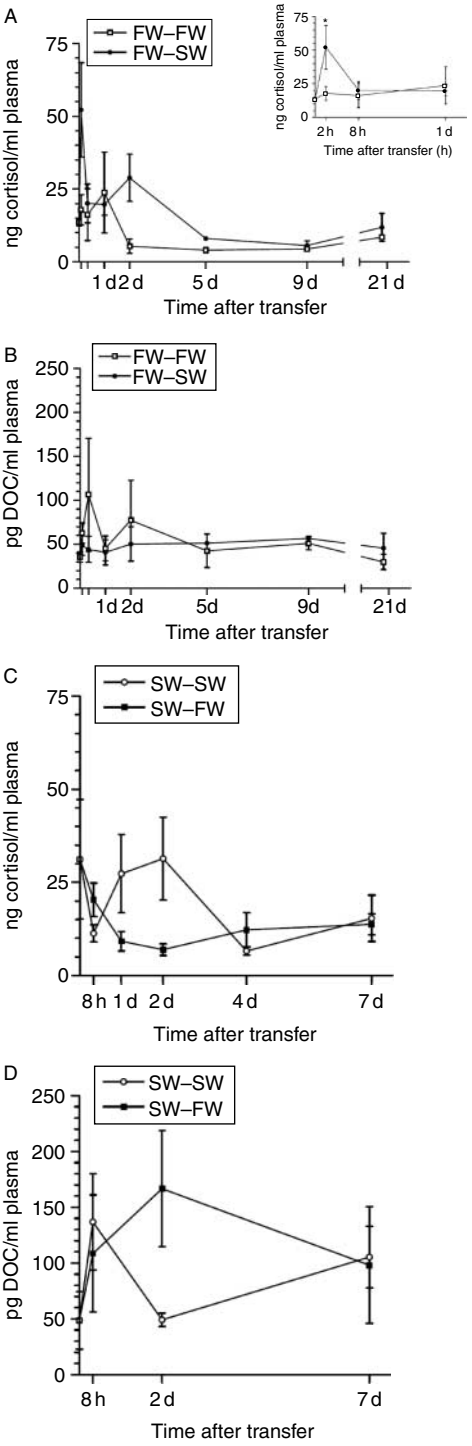

Figure 1 Variation in plasma cortisol (A and C) and DOC (B and D) during a FW–SW and a SW–FW acclimation time course. Open symbols denote control groups and black symbols denote transferred groups (n=6–8). No overall effects of time and salinity transfer were detected with a two-way ANOVA. Significant differences between control and transferred fish are marked with a star (P<0·05).

QPCR with specific primers. The resulting standard curves were fitted with regression lines and compared among primer sets to adjust for differences in QPCR efficiency due to differences in amplicon length and primer annealing and amplification efficiency. There were moderate differences in the absolute expression levels among the three primer sets (data not shown); thus, the expression levels of rtGR1, rtGR2, and rtMR in the gill, intestine, and kidney were adjusted accordingly to render a comparison of mRNA expression levels between receptors possible. Due to separate reverse transcription sessions for each tissue (gill, intestine, or kidney) at each transfer (FW–SW or SW–FW) – six in all – it is not possible to compare the expression level of corticosteroid receptors between tissues and salinity transfers.

Statistical analysis

All QPCR and plasma hormone data were analyzed using two-way ANOVA followed by Student's *t*-test. When necessary, data were transformed to obtain normality and homogeneity of variances. *In vitro* gill block incubation data were analyzed using a repeated-measures one-way ANOVA followed by multiple *t*-tests with Bonferroni's correction or, in case of non-normal distributed data sets, analyzed using a non-parametric Friedman test followed by Dunn's multiple comparison test (control column was compared with all treatment groups, and 10 F was compared with 1 F, 10 F+RU, and RU alone). In all cases, a significance level of $\alpha = 0{\cdot}05$ was used. All tests were performed using Prism3.03 (GraphPad Software Incorporated, La Jolla, CA, USA).

Results

Development and validation of a DOC RIA

To measure trout DOC plasma levels during salinity acclimation, a FIA was developed using a rabbit anti-DOC antibody. Absolute FIA measurements of DOC levels were validated using MS (LC–MS–MS) with no significant difference in absolute DOC levels measured with FIA and MS (results not shown). The specificity of the antibody was validated by assessing the cross-reactivity with a panel of fish

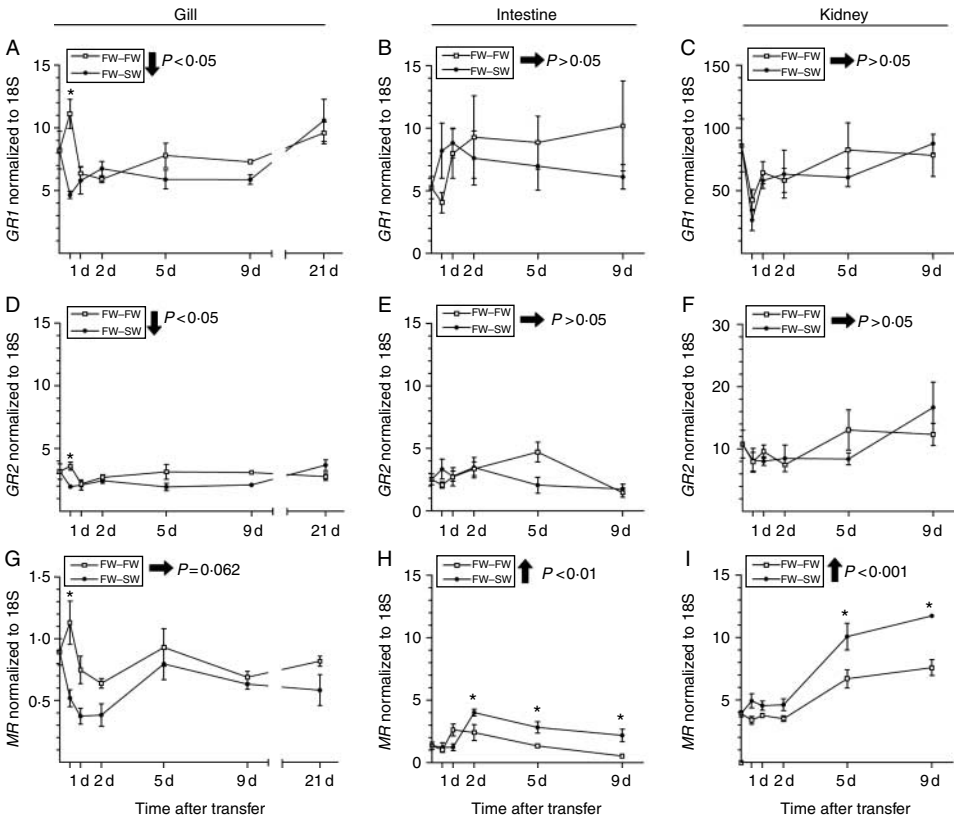

Figure 2 Variation in *GR1* (A–C), *GR2* (D–F), and *MR* (G–I) mRNA levels in gill (left column), intestine (middle column) and kidney (right column) during a FW–SW acclimation time course. Open and filled symbols denote control and transferred groups respectively ($n = 6$–8). Overall effects of time and salinity transfer were detected with a two-way ANOVA and significant differences between control and transferred fish are marked with an asterisk ($P < 0{\cdot}05$). Solid arrows accompanied by a *P* value indicate overall effect of salinity transfer and the direction of the movement in the transcript level. There was an overall effect of time in panels A, C, E, F, G, H, and I.

steroids given in Table 2. The antibody did not show any significant cross-reactivity with other common fish steroid hormones (Table 2). To have an in-house DOC assay, the FIA was modified to a RIA using the same antibody and only exchanging the biotin-labeled DOC with radioactive labeled ^{3}H-DOC. The repeatability of the RIA was assessed by calculation of the inter-assay variability coefficient (13·7, 22·5, and 12·9% for the doses 2000, 125, and 7·8 pg/ml respectively) and the intra-assay variability coefficient (6·2%) and sensitivity was assessed by determination of the minimum detectable limit of DOC (10 pg/ml) with an IC_{50} estimated to 21 pg/well (not shown).

Measurement of plasma cortisol and DOC levels following salinity changes

Apart from a significant increase in plasma cortisol levels 2 h after transfer from FW to SW (Fig. 1A, insert) neither salinity transfer gave rise to any overall changes in plasma cortisol (Fig. 1A ($P=0·118$), C ($P=0·2298$)). Cortisol concentration in plasma fluctuates around 15–25 ng/ml plasma – ~150–200 times higher than plasma DOC. There were no significant changes in DOC plasma levels after FW–SW ($P=0·4953$), SW–FW ($P=0·5619$), or control transfers, with DOC plasma levels fluctuating around 50–150 pg/ml regardless of acclimation salinity. Occasionally, DOC levels in plasma samples were lower than the detection limit in the RIA (10 pg/ml) resulting in the exclusion of two to three samples in some groups.

Regulation of corticosteroid receptors in osmoregulatory tissue during salinity acclimation

FW–SW transfer of rainbow trout resulted in an overall decrease in gill *GR1* ($P<0·05$) and *GR2* ($P<0·05$) mRNA levels, but not in *MR* ($P=0·062$) (Fig. 2A, D, and G). There is a significant difference between transferred and control fish 12 h post-transfer for all these transcripts, due to an increase in the control group concurrent with a decrease in the FW–SW-transferred group. Contrary to gill, MR is the only regulated

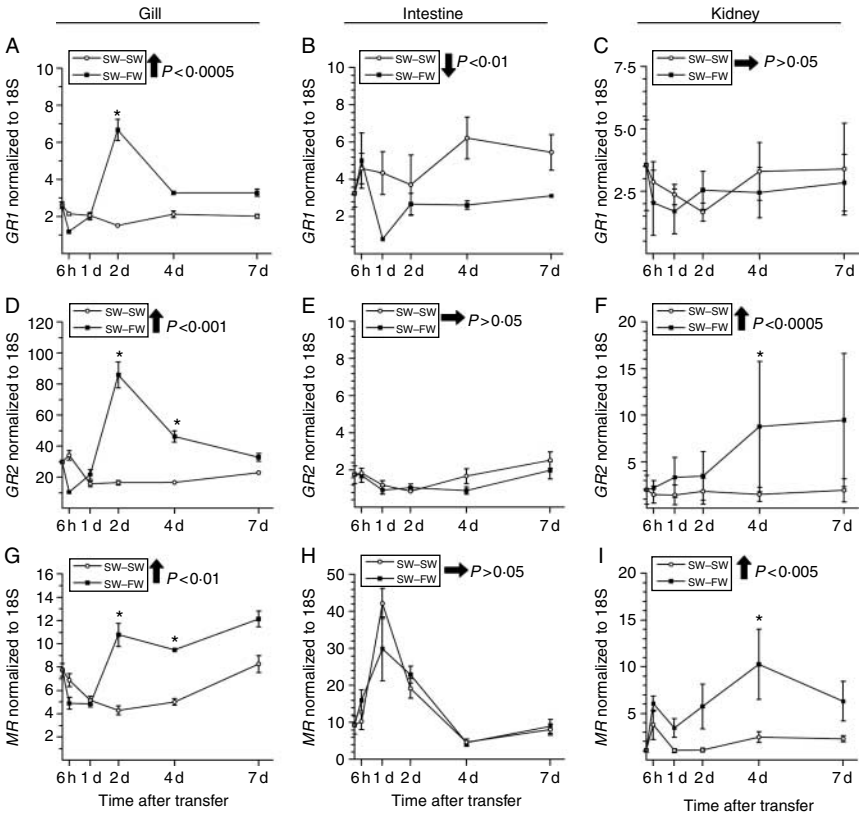

Figure 3 Variation in *GR1* (A–C), *GR2* (D–F), and *MR* (G–I) mRNA levels in gill (left column), intestine (middle column) and kidney (right column) during a SW–FW acclimation time course. Open and filled symbols denote control and transferred groups respectively ($n=6–8$). Overall effects of time and salinity transfer were detected with a two-way ANOVA, and significant differences between control and transferred fish are marked with an asterisk ($P<0·05$). Solid arrows accompanied by a *P* value indicate overall effect of salinity transfer and the direction of the movement in the transcript level. There was an overall effect of time in panels A, D, E, G, H, and I.

corticosteroid receptor during this FW–SW acclimation in the intestine and kidney. There is an overall increase in *MR* mRNA levels in the intestine ($P<0{\cdot}01$) and kidney ($P<0{\cdot}0001$) compared with the control transfer. Corticosteroid transcription levels are shown as relative expression levels normalized to 18S but are adjusted according to differences in the QPCR (due to differences in amplicon length, primer annealing, and amplification efficiency) among the target genes as determined by the absolute quantification and thus allow a comparison of expression levels among the target genes (but not among tissues). Accordingly, Fig. 2 shows that *GR1* is the highest expressed followed by *GR2* with *MR* as the lowest expressed transcript in the tissues investigated in this study.

SW–FW transfer resulted in an overall increase in gill GR1 ($P<0{\cdot}0005$), gill and kidney GR2 ($P<0{\cdot}001$ and $P<0{\cdot}0005$ respectively), and gill and kidney MR ($P<0{\cdot}01$ and $P<0{\cdot}005$ respectively). An overall decrease in GR1 in the intestine ($P<0{\cdot}01$; Fig. 3) was observed. Significant differences between SW–SW and SW–FW transferred fish were observed at days 2 and 4 for gill GR2 and MR, whereas differences are only significant at day 2 for GR1 in the gill and at day 4 for GR2 and MR in the kidney.

A summary of the overall effects of salinity transfer sorted by tissue is given in Fig. 4.

		FW–SW	SW–FW
Gill	*GR1*	↓	↑
	GR2	↓	↑
	MR	→	↑
Intestine	*GR1*	→	↓
	GR2	→	→
	MR	↑	→
Kidney	*GR1*	→	→
	GR2	→	↑
	MR	↑	↑

Figure 4 Summary of significant overall effects of salinity transfer on *GR1*, *GR2* and *MR* transcript levels in gill, intestine, and kidney. Upward and downward arrows indicate up- or down-regulation of the transcript level ($P<0{\cdot}05$), and horizontal arrows indicate no significant effect of salinity transfer ($P<0{\cdot}05$).

Validation of the specificity of the anti-rtMR antibody

To investigate the cellular distribution of MR in osmoregulatory tissue by immunohistochemistry (IHC), we developed an anti-rtMR antibody using an rtMR-AB domain-GST fusion protein (GST–MR) as antigen. Initially, the specificity of the anti-MR antibody for rtMR over GST was validated using western blot producing only one single band for the GST–MR fusion protein and no bands for the GST protein alone (not shown). Unfortunately, when used with tissue protein extracts (gill, intestine, kidney, brain, and heart), the anti-MR antibody did not prove applicable to detect MR. The specificity of the MR antibody in IHC was validated by immunoneutralization of the MR antibody with the immunogenic protein for 1 h before IHC. As shown in Fig. 5, the recombinant fusion protein (MR–GST) drastically reduces the immunoreactivity of the MR antibody (Fig. 5, compare panels A and B). Incubation of the gill tissue with MR–GST without MR antibody or pre-injection rabbit serum did not give rise to any artifacts (Fig. 5C and G respectively). The specificity of the rtGR1 antibody has previously been demonstrated in several studies (Teitsma *et al.* 1998, Tujague *et al.* 1998, Dang *et al.* 2000, 2001).

Co-localization of corticosteroid receptors in osmoregulatory tissue

In the gill, GR1 is expressed in chloride cells and pavement cells, with an occasional expression in deeper lying cells not in direct contact with water (Fig. 5D). The Na^+, K^+-ATPase antibody was used to localize gill chloride cells in the interlamellar epithelia in these FW-acclimated fish (Fig. 5E). Although GR1 is localized in most chloride cells in the interlamellar space (white arrows), GR1 is also located in other cell types on the lamellae and in the interlamellar space (black arrows). MR is ubiquitous and evenly distributed in all cell types in the gill epithelium on the lamellae and in the interlamellar space except pillar cells (Fig. 5F), thus an extensively co-localization of MR and GR1 can be observed in all the different gill cell types.

There is a high degree of co-localization of GR1 and MR throughout the columnar enterocytes in the anterior and posterior intestine (Fig. 6I, E, K, and G). Localization of the Na^+, K^+-ATPase protein indicates the basolateral membrane of the enterocytes in the intestine (Fig. 6F) as opposite to the apical brush border (Fig. 6J). Interestingly, MR staining is markedly more intense in the apical than in the basolateral part of the enterocytes.

Na^+, K^+-ATPase localization was employed to identify the different tubular cell types in the kidney showing mainly basolateral localization in proximal tubuli (marked with P), homogenous localization throughout the cells of the distal tubuli (marked with D), and for the collecting duct cells a widespread localization with a more prominent expression localized at the basolateral part (marked with C; Fig. 6B). The GR1 protein is mostly observed in the basolateral part of the cytoplasm and in the cell nuclei of renal cells showing a non-homogenous

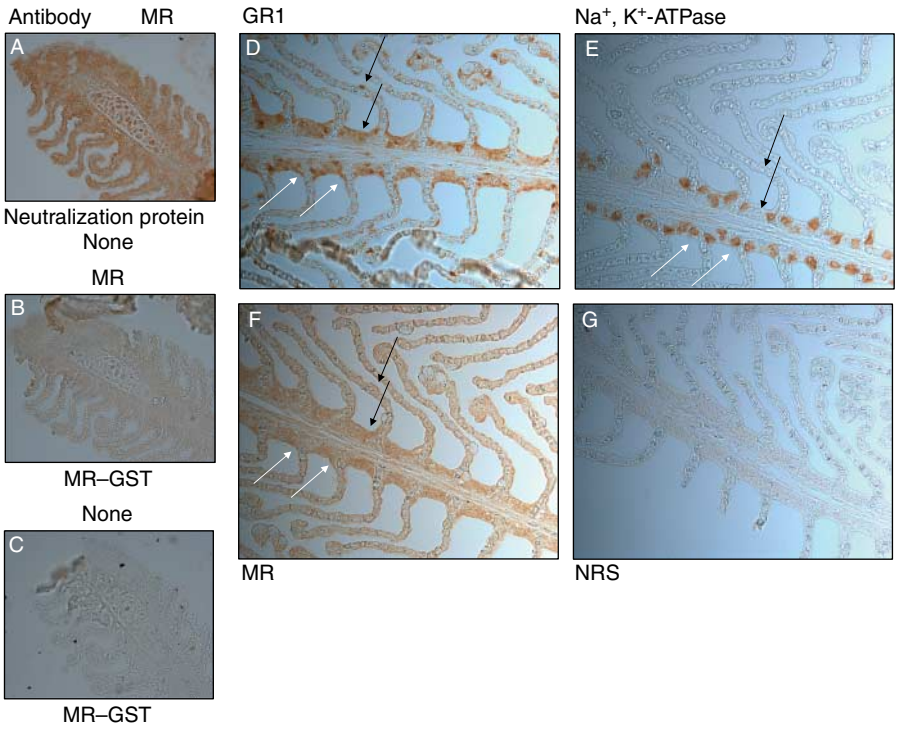

Figure 5 Validation of the anti-rtMR antibody and immunohistochemical localization of MR, GR1, and Na^+, K^+-ATPase in the gill. The specificity of the MR antibody was investigated using the MR-AB domain-GST fusion protein employed for immunization of rabbits. Coloration arising from MR localization (A) in the gill is completely competed away by the MR–GST fusion protein (B). The MR–GST fusion protein alone did not give rise to any coloration (C). Immunohistochemical localization of GR1 (D), Na^+, K^+-ATPase (E) and MR (F) in FW-adapted trout gill. Pre-immune rabbit serum was applied as control and did not result in any unspecific binding (G). Pavement and chloride cells in which GR1 and MR co-localize are shown with black and white arrows respectively. 40× magnification for panels A–C and 20× magnification for panels D–G.

expression among the different renal cell types (Fig. 6A). MR-specific staining in the kidney is very weak (Fig. 6C) with no apparent difference between the different tubular cell types.

In vitro *effects of cortisol and DOC in FW-acclimated fish gill*

To investigate the possible physiological functionality of the proposed DOC–MR axis in osmoregulation, an *in vitro* incubation of gill blocks was conducted to compare the effects of DOC and cortisol on mRNA expression of important proteins associated with ion transport in the SW- and FW-type chloride cells. To reflect the physiological situation in trout, plasma DOC was used at the same or lower concentration than cortisol. There were no effects of any dose of DOC (0·01, 0·1, or 1·0 μg/ml) or the lower dose of cortisol (1 μg/ml) on transcript level of the osmoregulatory target genes selected in this study (Fig. 7). However, cortisol treatment at 10 μg/ml resulted in significant increases in the SW-associated target genes *CFTR*, *NKCC*, and Na^+, K^+-ATPase α1b (Fig. 7A–C) and the FW-associated Na^+, K^+-ATPase α1a, *NBC*, and *ECaC* (Fig. 7D–F) transcript levels, but not in the FW-associated CA and H^+-ATPase (Fig. 7G and H).

Interestingly, when RU486, a validated antagonist for trout GR (Ducouret *et al.* 1995, Bury *et al.* 2003) was added to the 10 μg/ml cortisol treatment, we observed a significant inhibitory effect of the cortisol stimulation on *CFTR*, NKCC, Na^+-K^+-ATPase α1a, and *NBC* gene expression. These results confirm the involvement of a GR-mediated cortisol signaling.

Discussion

The aim of this study was to clarify the osmoregulatory role of the DOC–MR axis in rainbow trout during FW–SW and SW–FW acclimation. The structural relationship between the tetrapod and teleost mineralocorticoid signaling axis naturally suggests a role in osmoregulation in fish, but this involvement has only been investigated rather poorly and punctuated. Although the osmoregulatory effect of cortisol is well established (see reviews McCormick 2001, Evans *et al.* 2005), the regulation of cortisol receptors in osmoregulatory tissues requires re-investigation after the relatively recent discovery of two GRs and the possibility that cortisol may be

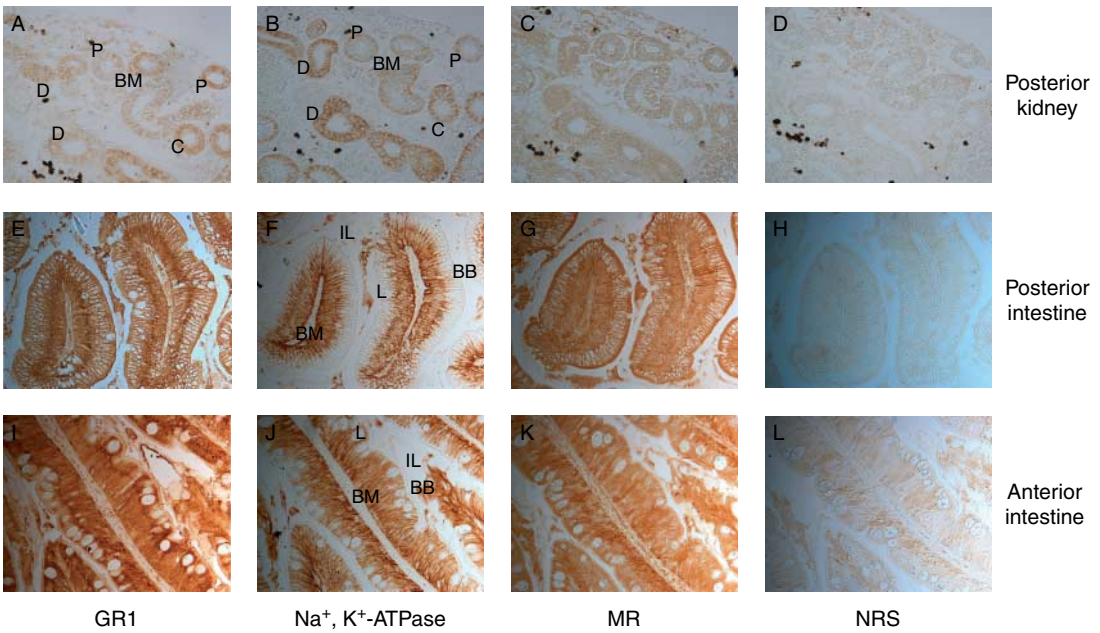

Figure 6 Immunohistochemical localization of GR1 (I, E, and A), Na^+, K^+-ATPase (B, F and J) and MR (C, G and K) in anterior intestine (leftmost column), posterior intestine (middle column), and posterior kidney (rightmost column) in FW-adapted trout. Pre-immune rabbit serum was applied as control and did not result in any notable coloration (D, H and L). Magnification is 20× for all panels. Brush border (BB), intestinal lumen (IL), lipid droplet (L), basolateral membrane (BM), collecting duct (C), proximal tubulus (P), and distal tubulus (D) are indicated on the Na^+, K^+-ATPase panels.

a physiological ligand for MR (Kiilerich *et al.* 2007*a*). In this context, we compare regulation of DOC and MR in osmoregulatory tissues (gill, intestine, and kidney) with that of cortisol and GR1/GR2, and compare cellular localization of MR and GR1 in these tissues. The data obtained in this study suggest involvement of MR but not of DOC in rainbow trout osmoregulation.

Measurement of plasma DOC levels: is DOC an osmoregulatory hormone?

In this study, measurement of plasma DOC levels by RIA has been carried out using an anti-DOC antibody after the specificity of the antibody was validated by analysis of cross-reactivity with other structurally related steroids. Moreover, levels of DOC in different plasma samples measured using this antibody were also confirmed by MS analysis. Our assay allows us to detect as low level as 10 pg DOC/ml, which should lead to detection of any physiologically meaningful changes in plasma DOC levels. Using the RIA for DOC, we followed plasma levels after transfer of fish from FW to SW or the converse using a time-course design including time points ranging from a few hours to several days or weeks. In both experiments, we did not observe any significant changes related to salinity transfer or time. These results suggest that DOC is not a major osmoregulatory hormone involved in salinity acclimation in rainbow trout. This is in agreement with the lack of DOC effects on SW tolerance *in vivo* judged by gill Na^+, K^+-ATPase activity and gill mRNA levels of Na^+, K^+-ATPase α1a and α1b in Atlantic salmon (McCormick *et al.* 2008) and no *in vitro* effect of DOC on gill ion transporter transcript levels (Fig. 6). However, a seasonal-dependent effect of DOC on Atlantic salmon gill Na^+, K^+-ATPase α1a and α1b mRNA levels *in vitro* (Kiilerich *et al.* 2011) suggests that further investigations of the osmoregulatory potential of DOC are still necessary.

The lack of results of DOC treatment in this study is not due to an overall lack of responsiveness of the gill tissue, as cortisol treatment results in up-regulation of mRNAs from both SW- and FW-adaptive ion transporters as expected (Kiilerich *et al.* 2007*a*).

Despite this probable absence of DOC effect on salinity acclimation in salmonids, one should not conclude that DOC has no physiological role in salmonids. For example, a highly significant change in plasma DOC was observed during male and female trout reproduction suggesting a function for DOC in reproduction (Milla *et al.* 2008). Clearly, further studies on regulation of DOC plasma levels in fish during different physiological conditions would be helpful for our understanding of the function of DOC.

Regulation of MR in gill, intestine, and kidney during salinity acclimation: does MR have an osmoregulatory function in trout?

In this study, *MR* mRNA levels in osmoregulatory tissues following salinity transfer (FW–SW and SW–FW) changed

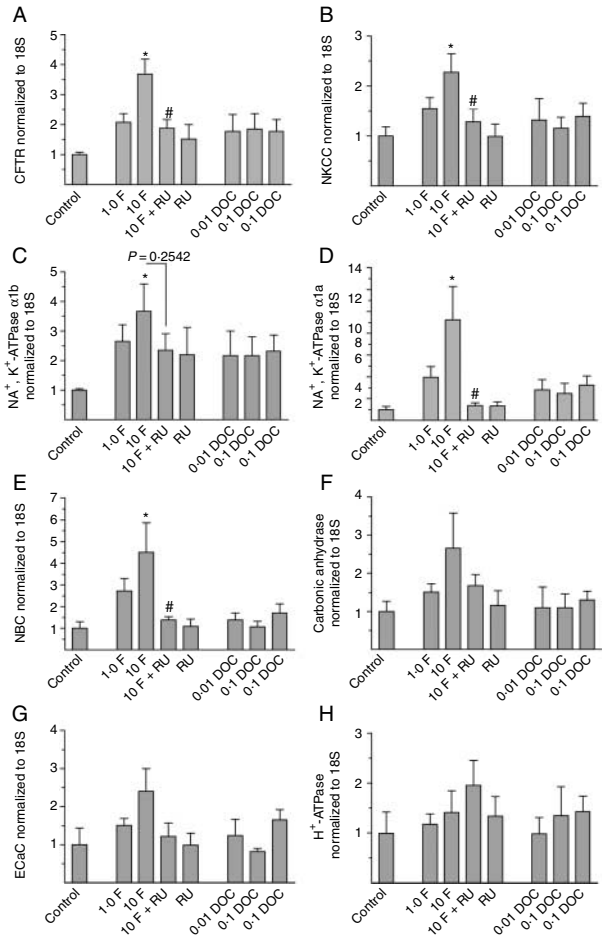

Figure 7 Effect of cortisol and DOC on selected ion transport protein mRNA levels in an *in vitro* gill block incubation. The three uppermost panels cystic fibrosis transmembrane conductance regulator (CFTR, A), Na^+, K^+, $2Cl^-$ co-transporter (NKCC, B), Na^+, K^+-ATPase α1b (C) represent SW-acclimatory genes. FW-acclimatory genes are represented by Na^+, K^+-ATPase α1a (D), $Na^+ - HCO_3^-$ co-transporter (NBC, E), epithelial Ca^{++} channel (ECaC, F), carbonic anhydrase (CA, G), and H^+-ATPase (H). Control levels are normalized to an arbitrary unit of 1 for each target gene ($n=6$). Gill blocks were incubated with 1 and 10 μg cortisol (1 F and 10 F respectively), 10 F+6 μg/ml RU486 (10 F+ RU), RU468 alone (RU), 0·01, 0·1 or 1 μg/ml DOC (0·01 D, 0·1 D and 1·0 D respectively). A repeated-measures one-way ANOVA followed by multiple *t*-tests with Bonferroni's correction (control column was compared to all treatment groups. 10 F was compared to 1 F, 10 F+ RU and RU alone) was conducted for each target gene. Star denotes significant difference from control and denotes significant difference from 10 μg/ml F.

significantly in a tissue- and/or salinity-dependent manner. Such an extensive analysis of MR expression through a wide time-course study has never been carried out, and the observed changes suggest an osmoregulatory role of MR in relation to salinity. The differential regulation of *MR* transcript levels shown in this study also suggests tissue-specific roles for MR in osmoregulation. Furthermore, the regulation might be of longer (intestine and kidney, FW–SW) or shorter (intestine, SW–FW) duration suggesting a dynamic role for MR.

During acclimation to hyperosmotic environment, this present study did not show any significant changes in *MR* transcript level in the gill and thus does not straightforwardly suggest a role for MR in this situation. This is along the suggestion from Kiilerich *et al.* (2007*b*, unpublished data), where a down-regulation of MR in Atlantic salmon gill during FW–SW acclimation was observed. However, up-regulation of MR during and after SW acclimation has also been reported (Nilsen *et al.* 2008, Yada *et al.* 2008), which suggests that the MR response may vary according to fish species and/or developmental status. Contrary to gill, an up-regulation of MR was observed in the kidney and intestine 2 days after transfer and onwards. Similar results have been reported by Yada *et al.* (2008) in the kidney of SW-adapted trout. This indicates an involvement of MR not only in the development of hypoosmoregulatory mechanisms in these tissues during acclimation to high salinity but also in the adapted fish, where MR might be a regulator of water/ion exchange mechanisms at the level of kidney and intestine.

Transfer of rainbow trout from SW to FW, which leads to development of hyperosmoregulatory mechanisms, is associated with significant increases in MR expression in the two main tissues involved in FW osmoregulation, gill and kidney (Fig. 3). This is in agreement with previous reports suggesting a role for MR in chloride cell proliferation and differentiation in FW-adapted rainbow trout and killifish (Sloman *et al.* 2001, Scott *et al.* 2005). Moreover, analysis of MR levels in the gill during Atlantic salmon smoltification lead Kiilerich *et al.* (2007*a*,*b*) to suggest that functionality of this receptor is coupled to hyper-osmoregulation.

Altogether, these data confirm the probable involvement of MR in osmoregulation. Further studies on MR in fish osmoregulatory tissue are now required to identify the molecular mechanisms concerning regulation of MR and MR signaling.

Which ligand could be the main activator of the MR signaling pathway?

Absence of changes in plasma DOC levels coincident with absence of DOC effects on gill gene expression *in vitro*, as shown in this study, raises the question regarding which corticosteroid hormone would activate the MR signaling pathway in response to salinity changes. In this study, measurement of plasma cortisol levels showed significant increase shortly after SW transfer which is in agreement with numerous previous similar studies carried out in salmonids (see review Mommsen *et al.* 1999). Furthermore, when compared with cortisol, plasma DOC levels are in general 150- to 200-fold lower. Although DOC activation of MR occurs at an only tenfold lower concentration than cortisol when tested in transfected COS cells (Sturm *et al.* 2005), the DOC plasma levels observed in this study do not favor a role

for DOC as the activating ligand in the MR signaling system compared with cortisol. However, local concentrations of cortisol might be different from those in plasma, especially due to the activity of the 11βHSD2 enzyme, which converts cortisol to receptor-inactive cortisone, and thus tentatively facilitates activation of MR signaling by DOC. This scheme is well known in mammals, where aldosterone is the activating ligand for the MR signaling system in distal nephrons of the kidney (Farman & Rafestin-Oblin 2001, Rashid & Lewis 2005). A functional 11βHSD2 enzyme was recently identified in rainbow trout (Jiang *et al.* 2003, Kusakabe *et al.* 2003) and changes in *11βHSD2* mRNA levels in gill during salmonid salinity acclimation have been reported (Kiilerich *et al.* 2007*b*, Nilsen *et al.* 2008, Kiilerich *et al.* unpublished data).

Taken together, cortisol seems to be the most probable activator of the MR signaling pathway in rainbow trout exposed to salinity changes.

Co-localization of MR and GR1 in the gill

The co-localization of GR1 and MR with Na^+, K^+-ATPase in gill chloride cells suggests a link between these corticoid receptors and osmoregulatory mechanisms. Interestingly, MR and GR are also localized in pavement cells, which also have been shown to have a function in osmoregulation (Marshall & Grosell 2006). As a result, both GR1 and MR possess the potential to regulate physiological processes related to ion/water exchanges in the two cell types associated with osmoregulation in gill. The present localization pattern of GR1 that shows higher expression in chloride cells than in pavement cells is in good agreement with previous IHC studies in other salmonids (Uchida *et al.* 1998, Dang *et al.* 2001, Kiilerich *et al.* unpublished data). Our development of a MR antibody functional in IHC made localization of MR in fish tissue possible, showing a similar localization of MR in trout (this study) and salmon gill (Kiilerich *et al.* unpublished data). The ubiquitous distribution of MR in many gill cell types might indicate non-specificity of the MR antibody on the gill tissue, but immunoneutralization of the MR antibody before IHC demonstrates the specificity of the antibody and confirms the distribution. This suggests that MR is not only involved in osmoregulation but may also be involved in other physiological processes in the gill, such as proliferation and differentiation of cells, acid–base regulation, and respiration.

Co-localization of GR1 and MR in intestine and kidney

This study is also the first to clearly demonstrate the localization of MR protein in the intestine and kidney showing a ubiquitous distribution among different cell types similar to the pattern observed in gill. The physiological or molecular significance of these data still needs further investigation but supports the probable involvement of this receptor in trout osmoregulation as suggested from *MR* mRNA regulation in the same osmoregulatory tissue as discussed earlier. The localization of GR1 in the intestine shown in this study is in good agreement with the localization shown in tilapia (*Oreochromis mossambicus*) posterior intestine (Takahashi *et al.* 2006*a*) and sea bass (*Dicentrarchus labrax*) intestine (Vazzana *et al.* 2008). In agreement with these data obtained in fish, co-localization of GR and MR has previously been shown in the mammalian kidney and intestine (Farman *et al.* 1991, Whorwood *et al.* 1993).

Differential functions of the GR isoforms in trout osmoregulation

Analysis of *GR1* and *GR2* mRNA levels in osmoregulatory tissues following salinity transfer overall confirms the involvement of these corticosteroid receptors in salinity acclimation and agrees with the major role of cortisol as a key hormone in activating these signaling pathways. Based on the difference in transactivation activity between GR1 and GR2 (Bury *et al.* 2003), it is tempting to speculate on differential roles of these two receptors in fish. In this study, both transcripts are overall similarly regulated during both FW–SW and SW–FW acclimation (except kidney and intestine, SW–FW), but differential roles for GR1 and GR2 still need to be considered. However, as the transcription levels given in this study are not supported by protein data, some caution has to be given to this interpretation.

Gill In this study there is an effect of FW–SW transfer on gill GR2 transcript levels in trout. Taken together with the lower EC_{50} value of GR2 compared with GR1, this indicates an osmoregulatory role of GR2. Nevertheless, it should be noted that Yada *et al.* (2008) reported an increase in GR2 transcript levels in SW-acclimated trout compared with FW-acclimated trout and Kiilerich *et al.* (unpublished data) did not observe any changes in gill GR2 levels during FW–SW acclimation in salmon. Taken together with an up-regulation of *GR2* mRNA level during SW–FW transfer in this study, it is impossible to reach any conclusion on GR2 function at this point. The downregulation of GR1 presented in this study is in contrast to the FW–SW acclimatory role of GR1 suggested by the up-regulation of GR1 in gill during FW–SW acclimation in Atlantic salmon (Kiilerich *et al.* 2007*b*, Nilsen *et al.* 2008, Kiilerich *et al.* unpublished data) and during the preparatory development of SW tolerance, smoltification (Mazurais *et al.* 1998, Mizuno *et al.* 2001, Kiilerich *et al.* 2007*b*). Interestingly, the up-regulation of *GR1* transcript level in the gill after SW–FW transfer presented in this study suggests a role for GR1 in FW-acclimation. The reason for this discrepancy is unknown and could be due to species differences between salmon and trout, but the need for more investigations on this matter is obvious.

Intestine Reports on regulation of GRs in the intestine are very few and scattered between different fish species. Furthermore, the lack of knowledge about the GR isoform

investigated further clouds up this discussion. Tomy *et al.* (2009) showed an early transient up-regulation of *GR* mRNA (probably GR2, when phylogenetically compared with other fish GR1 and GR2 sequences known, not shown) at day 1 but not at day 4 after FW–SW transfer of black porgy (*Acanthopagrus schlegeli*). Takahashi *et al.* (2006*a*) showed up-regulation of *GR* mRNA (probably GR2) at day 7 but not at day 1 after FW–SW transfer in the posterior intestine of tilapia. In this study we did not observe regulation of either GR1 or GR2 in the intestine when trout are transferred from FW to SW but a decrease in GR1 after SW–FW transfer. The reasons for these discrepancies are unknown and might stem from differences in acclimation strategy and physiology between the species investigated. A few indications suggest that the glucocorticoid axis is active in intestinal osmoregulation. Cortisol stimulated both apoptosis and cell proliferation in the anterior intestinal epithelium in FW–SW-transferred mudskipper – two opposite processes connected with intestinal fluid uptake in SW and FW adaptation respectively – whereas DOC had no effect (Takahashi *et al.* 2006*b*). Furthermore, cortisol treatment results in an increase in the intestinal fluid uptake in Atlantic salmon and brown trout (*Salmo trutta*; Veillette *et al.* 1995, Seidelin *et al.* 1999), while the intestinal fluid uptake decreased upon RU486 treatment (Veillette *et al.* 1995) – unfortunately, the effect of spironolactone has not been investigated.

Kidney As in the intestine, information on GR isoform regulation in kidney is more than scarce. The lack of regulation of GR1 and GR2 after FW–SW transfer is in disagreement with the up-regulation of both transcripts in SW-acclimated trout compared with FW-acclimated trout reported by Yada *et al.* (2008). However, FW–SW transfer of black porgy did not affect GR mRNA (probably GR2, Tomy *et al.* 2009). Although in this study we show an up-regulation of GR2 levels in kidney during SW–FW acclimation, Tomy *et al.* (2009) reported a down-regulation of *GR* mRNA in black porgy (again probably GR2). All in all, there is no good evidence pointing to differential function of the two GR isoforms in fish kidney at this moment.

Conclusion

Based on this extensive analysis of MR in osmoregulatory tissues during acclimation of trout to SW or FW, where we show localization of MR in the most important osmoregulatory tissues and cell types and salinity- and tissue-specific changes in MR expression in these osmoregulatory organs, we propose a role for MR in osmoregulation. This study suggests a regulatory role of MR in the kidney and intestine during FW–SW acclimation and in the kidney and gill during SW–FW acclimation. These conclusions are in agreement with regulation of MR in gill during salinity adaptation, which have already been observed by other researchers, although these regulations do not always move in the same direction perhaps due to seasonal, sampling or species-specific differences. On the other hand, the lack of regulation of DOC plasma levels during salinity acclimation shown in this study and the lack of effect on expression of classical osmoregulatory ion transporters in gill suggest that DOC is not a major regulator of the MR signaling system during rainbow trout salinity acclimation. On the other hand, this study confirms the major osmoregulatory role of cortisol in rainbow trout salinity acclimation through specific activation of the GR signaling system as shown by the *in vitro* effects of cortisol. Thus, the possibility that cortisol might be the physiological ligand for MR during salinity acclimation has to be considered.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research project.

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