

Differential effects of cortisol and 11-deoxycorticosterone on ion transport protein mRNA levels in gills of two euryhaline teleosts, Mozambique tilapia (*Oreochromis mossambicus*) and striped bass (*Morone saxatilis*)

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Abstract

The role of cortisol as the only corticosteroid in fish osmoregulation has recently been challenged with the discovery of a mineralocorticoid-like hormone, 11-deoxycorticosterone (DOC), and necessitates new studies of the endocrinology of osmoregulation in fish. Using an *in vitro* gill explant incubation approach, DOC-mediated regulation of selected osmoregulatory target genes in the gill was investigated and compared with that of cortisol in two euryhaline teleosts, Mozambique tilapia (*Oreochromis mossambicus*) and striped bass (*Morone saxatilis*). The effects were tested in gills from both fresh water (FW)- and seawater (SW)-acclimated fish. Both cortisol and DOC caused an up-regulation of the Na⁺,K⁺-ATPase α 1 subunit in SW-acclimated tilapia but had no effect in FW-acclimated fish. Cortisol conferred an increase in Na⁺,K⁺,2Cl⁻ cotransporter (NKCC) isoform 1a transcript levels in FW- and SW-acclimated tilapia, whereas

DOC had a stimulatory effect only in SW-acclimated fish. Cortisol had no effect on NKCC isoform 1b mRNA levels at both salinities, while DOC stimulated this isoform in SW-acclimated fish. In striped bass, cortisol conferred an up-regulation of Na⁺,K⁺-ATPase α 1 and NKCC transcript levels in FW- and SW-acclimated fish, whereas DOC resulted in down-regulation of these transcripts in FW-acclimated fish. It was also found that both corticosteroids may rapidly (30 min) alter the mitogen-activated protein kinase signalling pathway in gill, inducing phosphorylation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 in a salinity-dependent manner. The study shows a disparate organisation of corticosteroid signalling mechanisms involved in ion regulation in the two species and adds new evidence to a role of DOC as a mineralocorticoid hormone in teleosts.

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Introduction

It is generally accepted that the corticosteroid hormone, cortisol, plays a central osmoregulatory role during acclimation to both hyper- and hyposaline environments in teleosts (McCormick 2001, Sakamoto *et al.* 2001). However, the recent discovery of a potential mineralocorticoid receptor (MR) with high affinity for the mineralocorticoid hormone 11-deoxycorticosterone (DOC) and two glucocorticoid receptor (GR) isoforms has expanded the extent through which the corticosteroid family of hormones and receptors may work to regulate hydromineral balance in teleosts. Cortisol binds with high affinity to the three cytosolic corticosteroid receptors in fish, GR1, GR2 and MR, whereas DOC specifically binds to the MR (see Prunet *et al.* (2006)). The receptor type(s) through which cortisol exerts its osmoregulatory effects and the role of DOC signalling are

still not firmly established. Once associated with hormone, the classical cytosolic corticosteroid receptor translocates to the cell nucleus, where target gene transcription is modulated via a well-established slow-acting genomic pathway. However, rapid effects of corticosteroids have also been reported (Borski 2000, Haller *et al.* 2008) and may occur through additional membrane-bound receptors and/or interaction with cytosolic signalling cascade pathways.

Numerous studies in many fish species from a variety of genera have shown that cortisol may stimulate the level of important ion transport proteins (mRNA, protein and activity) in the gill. The Na⁺,K⁺-ATPase and Na⁺,K⁺,2Cl⁻ cotransporter (NKCC) are two examples of membrane proteins governing transport of Na⁺ and Cl⁻ ions across the gill epithelium against steep gradients between plasma and water. Accordingly, cortisol may regulate gill chloride cell differentiation and proliferation (reviewed by

McCormick (2001) and Sakamoto *et al.* (2001)), thereby improving overall ion-translocation capacity. Some effects of cortisol were shown to be mediated by the GR, although a few recent reports point to a role for the MR as well (Sloman *et al.* 2001, Scott *et al.* 2005, Killerich *et al.* 2007, 2010). There may, however, be species-specific differences in the role of corticosteroids for the development of ion transport mechanisms in relation to the degree of euryhalinity.

In this study, we used two Perciform teleosts, the Mozambique tilapia (*Oreochromis mossambicus*) and the American striped bass (*Morone saxatilis*), as model species to evaluate corticosteroid effects on gill Na^+ , K^+ -ATPase and NKCC expression. Both species are highly euryhaline; however, their acclimation 'strategy' in molecular terms seems different when exposed to increasing salinity. The Mozambique tilapia develops the appropriate hypo-osmoregulatory mechanisms gradually when stimulated by increased salinity (Morgan *et al.* 1997, Uchida *et al.* 2000). Thus, the energy to build the appropriate mechanisms is only spent when needed. On the other hand, studies in striped bass have shown that this species has important hypo-osmoregulatory mechanisms present (chloride cells and high levels of Na^+ , K^+ -ATPase) in the gill irrespective of salinity (Madsen *et al.* 1994, Tipsmark *et al.* 2004, 2007). In energetic terms, this must be viewed as a costly strategy. The different acclimation 'strategies' are also evident in the endocrine response when the two species are challenged with changing salinity. In tilapia, direct transfer from fresh water (FW)- to seawater (SW) results in drastically increased levels of plasma cortisol, and subsequently chloride cell number, NKCC1a and Na^+ , K^+ -ATPase mRNA, protein and activity levels in the gill (Assem & Hanke 1981, Morgan *et al.* 1997, Hwang *et al.* 1998, Hiroi *et al.* 2008, Inokuchi *et al.* 2008). Accordingly, cortisol seems to be an important osmoregulatory hormone in tilapia, where it stimulates Na^+ , K^+ -ATPase activity and chloride cell proliferation and differentiation (Dangé 1986, McCormick 1990). In striped bass, there is little or no deviation in the above parameters when challenged by increased salinity (Madsen *et al.* 1994, Tipsmark *et al.* 2004, 2007), and the available evidence suggests that cortisol is not involved in salinity acclimation. Even though FW striped bass transferred to SW showed increased plasma cortisol and gill Na^+ , K^+ -ATPase activity (Madsen *et al.* 1994), similar changes were observed in fish sham-transferred to FW, suggesting that these changes are related to the handling stress of the animals rather than salinity *per se* (Madsen *et al.* 1994). Accordingly, cortisol has either no effect *in vivo* on gill Na^+ , K^+ -ATPase activity or α -subunit mRNA or a slight stimulatory effect *in vitro* on gill α -subunit mRNA in FW striped bass (Madsen *et al.* 2007). We therefore hypothesise that the different salinity acclimation strategies and the role of cortisol herein may be reflected in differential responses of the gill to corticosteroids. In order to test this, we used an *in vitro* gill explant incubation system to investigate the potential for cortisol- and DOC-mediated regulation of transcript levels of two selected target genes (Na^+ , K^+ -ATPase and

NKCC) in relation to acclimation salinity of the two species. Transcriptional effects of steroids are classically considered as slow-acting effects. However, in order to test the possibility of rapid signalling effects, we also investigated the potential for regulatory effects of cortisol and DOC on the phosphorylation status (activation) of two members of the MAPK signalling cascade, extracellular signal-regulated kinase 1 (ERK1) and ERK2 in the two species. Activation of the MAPK pathway may have a central role in transducing osmosensory as well as endocrine signals in the gill (Madsen *et al.* 2007).

Materials and Methods

Fish: adult mixed sex tilapia (*O. mossambicus*) from Pamlico Aquaculture Field Laboratory (Aurora, NC, USA) and sexually immature, mixed sex, juvenile striped bass (*M. saxatilis*) obtained from FW ponds at the Edenton National Fish Hatchery (Edenton, NC, USA) were held at 12 h light:12 h darkness at 22 °C and fed with pelleted feed made available *ad libitum*. Fish were held in 500 l tanks and fully acclimated to either recirculated, filtered FW or full strength SW. When sampled, fish were stunned by a blow to the head and bled from the tail. Gills were removed and placed in ice-cold Ringer's solution (final concentration in mol/l: 0.14 NaCl, 0.015 NaHCO_3 , 0.0025 KCl, 0.0015 CaCl_2 , 0.001 KH_2PO_4 , 0.0008 MgSO_4 , 0.01 dextrose, 0.005 HEPES, pH 7.8; equilibrated with 99% O_2 /1% CO_2) with the addition of penicillin and streptomycin (400 000 IU/l) until further dissected.

In vitro gill incubation with corticosteroids and antagonists

Gill blocks of two to three filament pairs were carefully dissected from the cartilage arch and incubated for 24 h in Falcon multi-well six-well plates with gentle shaking at room temperature with 2 ml oxygenated (99% O_2 /1% CO_2) Ringer's solution containing hormones, antagonists or a combination (control: vehicle (0.003% (v/v) dimethyl sulfoxide (DMSO)); cortisol (F): cortisol-hemisuccinate (10 µg/ml); F+RU: F (10 µg/ml)+RU486 (20 µg/ml); F+Spi: F (10 µg/ml)+spironolactone (20 µg/ml); RU: RU486 (20 µg/ml); Spi: spironolactone (20 µg/ml); DOC (10 µg/ml); DOC+Spi: DOC (10 µg/ml)+spironolactone (20 µg/ml); 1.0 F: F (1 µg/ml); 0.1 F: F (0.1 µg/ml); 1.0 DOC: DOC (1 µg/ml); 0.1 DOC: DOC (0.1 µg/ml). Cortisol-hemisuccinate sodium salt (Sigma-Aldrich) was dissolved in Ringer's solution, whereas DOC, RU486 and spironolactone (Sigma-Aldrich) were dissolved in DMSO. All wells were adjusted to equal DMSO load. After incubation, gill blocks were transferred to RNAlater (Ambion, Austin, TX, USA) and frozen at -20 °C until RNA purification.

RNA purification, cDNA synthesis and qPCR

Total RNA was purified using GenElute Mammalian Total RNA kit (Sigma-Aldrich) according to the manufacturer's

recommendations. RNA concentration and purity were determined by measuring A260/A280 (values between 2.06 and 2.23). RNA (1 µg) was DNase treated, reverse transcribed and subjected to semi-quantitative real-time PCR as described previously (Küllerich *et al.* 2007). Reactions were carried out with 1 µl cDNA (corresponding to 20 ng RNA), 5 pmol forward and reverse primer (Invitrogen), 12.5 µl of Brilliant II SYBR green master mix (Stratagene, La Jolla, CA, USA) in a total volume of 25 µl. Cycling conditions were 95 °C for 30 s, 60 °C for 20 s and 72 °C for 10 s for 50 cycles. Melting curve analysis was routinely carried out for 30 s for each 1 °C interval from 60 to 95 °C. Calculation of relative copy number for target and normalisation genes was carried out using the Pfaffl equation (Pfaffl 2001), $(1 + E_a)^{-C_t}$, where E_a is the amplification efficiency, and C_t is the threshold cycle number. Normalised values were obtained by dividing the relative copy number of the target to that of the normalisation gene, 18S rRNA. The normalisation gene was not significantly affected by any treatment at any salinity, and the relative expression level did not differ between salinities in either tilapia or striped bass. Tilapia primer sequences and amplicon lengths are as follows (Genbank accession numbers in parenthesis): 18S rRNA (AF497908) forward 5'-TATTGTGCCGCTAGAGGTG-AA-3' and reverse 5'-CCTCCGACTTTCGTTCTTGA-3', 102 bp; Na⁺,K⁺-ATPase α 1 (U82549) forward 5'-GAAG-CCATTGTGGTGGTT-3' and reverse 5'-TGTGGCAGT-TGGCTCAGT-3', 136 bp; NKCC1a (AY513737) forward 5'-AAGACAAGAAGAGCCCCGACAG-3' and reverse 5'-AG-ACATCCACTGTGCCCTTCC-3', 105 bp; and NKCC1b (AY513738) forward 5'-CCGCGTTTGAATTAATG-TG-3' and reverse 5'-TCGGTGCATCACTGAGAGACA-3' mRNA sequence, 91 bp. Striped bass primer sequences and amplicon length are as follows: Na⁺,K⁺-ATPase based on the killifish Na⁺,K⁺-ATPase α 1 sequence (AY057072) forward 5'-GGGGATGGTGTAACGACTC-3' and reverse 5'-CAG-GGTGTAGGCAATGGATTTCTTCA-3', 194 bp; NKCC1 based on the *Dicentrarchus labrax* NKCC1 (AY532638 – this sequence was previously used to design a northern blot probe for striped bass in Tipsmark *et al.* (2004)) forward 5'-CGCCT-AAAGCCAAACACATT-3' and reverse 5'-CCTGGGAGGA-TAGCAAGTCA-3', 193 bp. Tilapia primers for 18S rRNA were also used for striped bass normalisation. The specificity of the primers was assessed with melting curve analysis, showing only one single product for each primer set, and confirmed by agarose gel verification of correct amplicon size. The amplification efficiencies were Na⁺,K⁺-ATPase α 1: 100.2%, NKCC1a: 107.2%, NKCC1b: 96.1%, 18S: 107.5% for tilapia; Na⁺,K⁺-ATPase: 98.5%, NKCC1: 86.4 and 18S: 71% for striped bass.

Cortisol and DOC effects on ERK-1 and -2 phosphorylation

Gill blocks of two to four filament pairs were incubated in Ringer's solution with or without 10 µg/ml cortisol or DOC as described earlier for 30 min. Initially, a time-course

experiment (10, 20 and 30 min) was conducted to determine the optimal incubation time for maximal ERK phosphorylation (activation), showing 30 min to be the optimal incubation time (data not shown). For western blotting, one gill block was sonicated in 100–120 µl NuPAGE LDS sample buffer (Invitrogen) with reducing agent for 15–20 s at 35% output (Fisher sonic dismembrator model 300, Fisher Scientific, Pittsburgh, PA, USA) and frozen at –20 °C until western blotting. Gel electrophoresis, blotting and immunological detection and quantification of dual phosphorylated (active) and total ERK1 and ERK2 by infrared imaging (Odyssey, LI-COR) were carried out as described previously (Madsen *et al.* 2007). In short, dual phosphorylated ERK 1/2 was detected using a monoclonal mouse phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr202/Tyr204) antibody from Cell Signaling (Beverly, MA, USA; dilution 1:2000). Total ERK 1/2 was detected using a polyclonal rabbit anti-MAPK (sc-94; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:1000). Following washing, membranes were incubated for 1 h with goat anti-mouse and/or anti-rabbit secondary antibodies conjugated to Alexa IRDye 680 or IRDye 800CW (LI-COR). Phosphorylated and total ERK 1/2 were detected on the same blot at 680 and 800 nm respectively. Active ERK1 and ERK2 were normalised to total ERK1 and ERK2 content.

Statistical analysis

Data in Figs 1–6 (mean \pm s.e.m., $n=6$) were analysed using a repeated-measures one-way ANOVA followed by a Student–Newman–Keuls test in an all pairwise fashion. In Fig. 7, a two-way ANOVA was followed by a Bonferroni's-adjusted Fisher's LSD test of selected pairs. When necessary, data were transformed to obtain normality and homogeneity of variances. In all cases, a significance level of $\alpha=0.05$ was used. All tests were performed using GraphPad Prism version 5.04 (San Diego, CA, USA).

Results

Effects of cortisol and DOC on Na⁺,K⁺-ATPase α 1 (NKA α 1) transcript level

In tilapia, no effect of hormones or their antagonists were observed on NKA α 1 in gills from FW-acclimated fish, whereas cortisol and DOC both increased this transcript in gills from SW-acclimated specimens (Fig. 1A and B). RU486 completely abolished the effect of cortisol. Spironolactone partly abolished the effect of cortisol but was unable to antagonise the effect of DOC on NKA α 1 (Fig. 1B).

In striped bass, cortisol induced an increase in the NKA α 1 mRNA level but significantly only in gills from SW-acclimated fish (Fig. 2A and B). RU486 and spironolactone both abolished the cortisol effect (Fig. 2B). DOC had an inhibitory effect on NKA α 1 mRNA in gills from FW- and

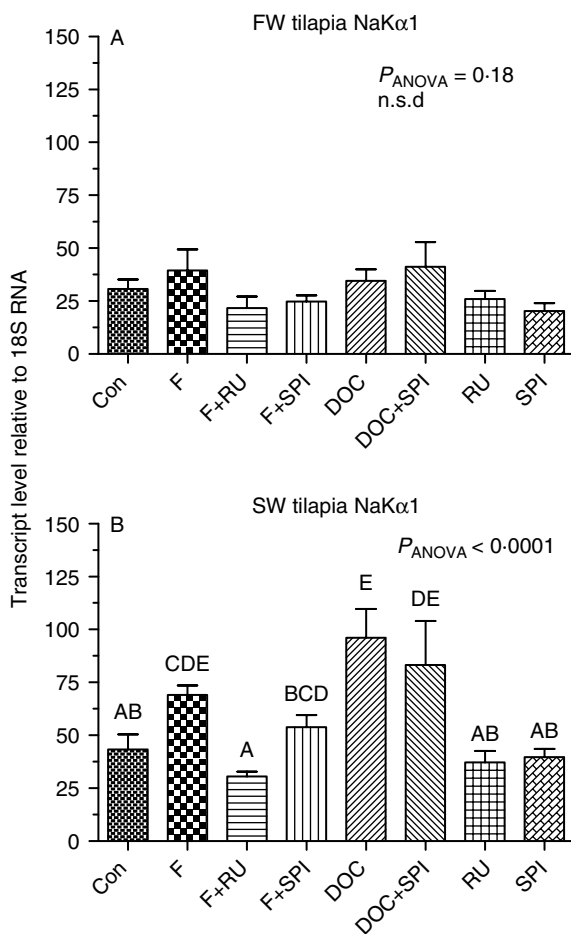


Figure 1 *In vitro* effect of 10 µg/ml cortisol (F) and 11-deoxycorticosterone (DOC) on Na⁺,K⁺-ATPase α1 (NaKα1) mRNA expression in FW- (A) and SW- (B) acclimated tilapia gill. The specificity of the corticosteroid signalling pathway was investigated using specific GR and MR antagonists (RU486 (RU) and spironolactone (Spi) respectively). Target gene mRNA levels are normalised to 18S rRNA levels and expressed as arbitrary units (mean ± S.E.M., n=6). One-way ANOVA *P* value is indicated on the figure. Letters above the bars indicate statistical groupings (Student–Newman–Keuls test). When letters are shared among groups, they are not statistically different (*P*<0.05). N.s.d: no significant difference.

SW-acclimated fish, but the effect was not antagonised by spironolactone (Fig. 2A).

Effect of cortisol and DOC on NKCC transcript levels

Cortisol treatment increased NKCC1a levels in gills from both FW- and SW-tilapia (Fig. 3A and B), and RU486 and spironolactone both abolished the effect. DOC stimulated NKCC1a levels in gills from SW-acclimated tilapia, and spironolactone reduced the effect of DOC (Fig. 3B). Cortisol had no effect on NKCC1b in both FW- and SW-acclimated tilapia (Fig. 4A and B), whereas DOC significantly increased

the NKCC1b levels in SW fish (Fig. 4B). This increase was completely abolished by spironolactone.

In striped bass, cortisol increased NKCC levels in gills from SW-acclimated fish but not from FW-acclimated fish (Fig. 5A and B). This effect was completely abolished by RU486 and to a smaller degree by spironolactone (Fig. 5B). DOC decreased the NKCC level in gills from FW-acclimated fish but had no effect in gills from SW-acclimated fish (Fig. 5A). The effect on gills from FW-acclimated fish was partly antagonised by spironolactone.

Sensitivity to hormones in relation to salinity

In total, three graded doses of cortisol and DOC were tested on tilapia and striped bass gill blocks from both FW and SW fish to compare the sensitivity to the hormones with respect to acclimation salinity. Cortisol doses <10 µg/ml (0.1 and 1.0 µg/ml) generally had no effect on any transcript level

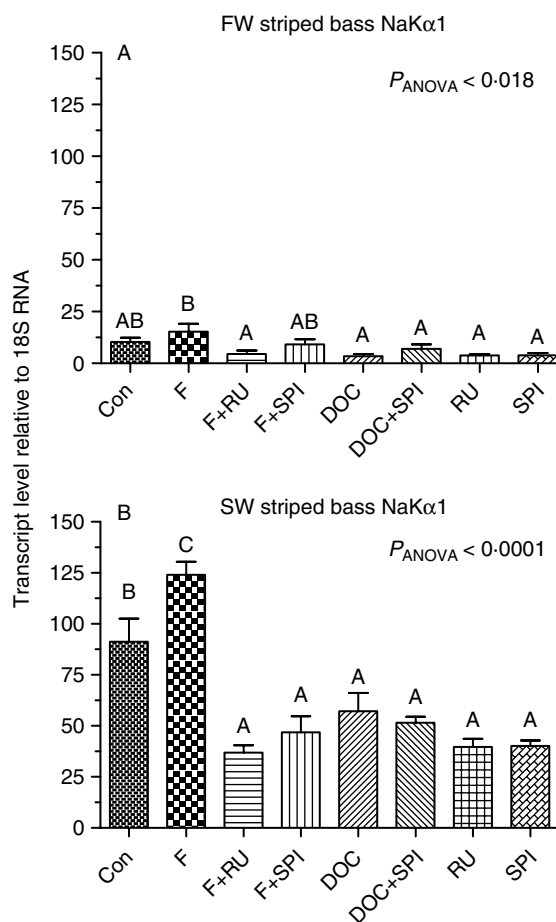


Figure 2 *In vitro* effect of cortisol and DOC on Na⁺,K⁺-ATPase α1 (NaKα1) mRNA expression in FW- (A) and SW- (B) acclimated striped bass gill. One-way ANOVA *P* value is indicated on the figure. Letters above the bars indicate statistical groupings (Student–Newman–Keuls test). When letters are shared among groups, they are not statistically different (*P*<0.05). For further details see legend to Fig. 1.

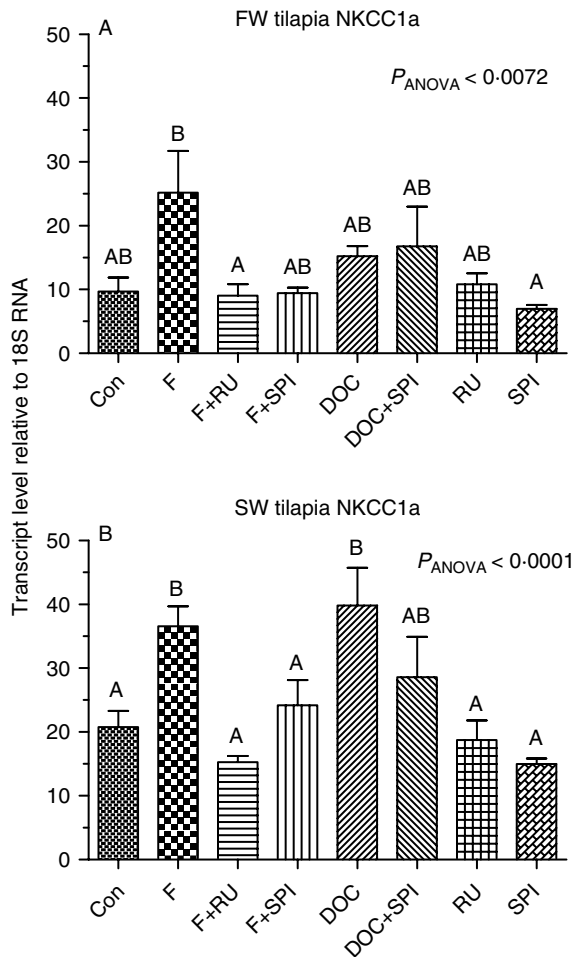


Figure 3 *In vitro* effect of cortisol and DOC on NKCC1a mRNA expression in FW- (A) and SW- (B) acclimated tilapia gill. One-way ANOVA P value is indicated on the figure. Letters above the bars indicate statistical groupings (Student–Newman–Keuls test). When letters are shared among groups, they are not statistically different ($P < 0.05$). For further details see legend to Fig. 1.

irrespective of salinity and species (data not shown). In FW striped bass gills, DOC decreased Na^+, K^+ -ATPase $\alpha 1$ subunit and NKCC transcript levels irrespective of dose (0.1, 1.0 and 10 $\mu\text{g}/\text{ml}$; Fig. 6), whereas in SW only the high dose (10 $\mu\text{g}/\text{ml}$) had an effect (not shown). There was no effect of DOC doses below 10 $\mu\text{g}/\text{ml}$ in FW- and SW-acclimated tilapia (not shown). A summary of the qualitative effects of cortisol and DOC in the two species is shown in Fig. 8.

Rapid effects of cortisol and DOC on ERK phosphorylation in tilapia and striped bass gills

Incubation of tilapia gill blocks with either cortisol or DOC for 30 min significantly increased the phosphorylation state of ERK1 and ERK2, thus activating the MAPK

phosphorylation cascade (Fig. 7). In FW-acclimated tilapia, ERK2 was activated by cortisol and DOC, whereas ERK1 was activated in gills from SW-acclimated fish by both hormones. In striped bass, cortisol and DOC activated ERK1 in gills from FW-acclimated fish, while they had no effect in gills from SW-acclimated fish.

Discussion

In fish, cortisol is well known for its ion-regulatory effects. The duality of effects on ion retention in FW and ion secretion in SW is in part a consequence of interaction with elements of the GH/PRL-IGF-1 axis (Tipmark & Madsen 2009), but the underlying molecular mechanisms and the signalling pathways remain largely unknown. We investigated the effect

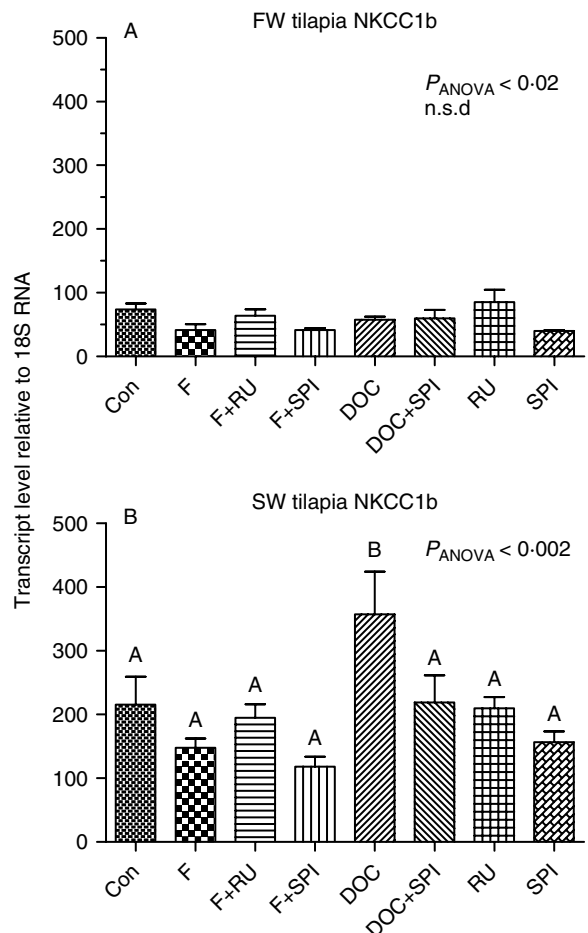


Figure 4 *In vitro* effect of cortisol and DOC on NKCC1b mRNA expression in FW- (A) and SW- (B) acclimated tilapia gill. One-way ANOVA P value is indicated on the figure. Letters above the bars indicate statistical groupings (Student–Newman–Keuls test). When letters are shared among groups, they are not statistically different ($P < 0.05$). N.s.d.: no significant difference. For further details see legend to Fig. 1.

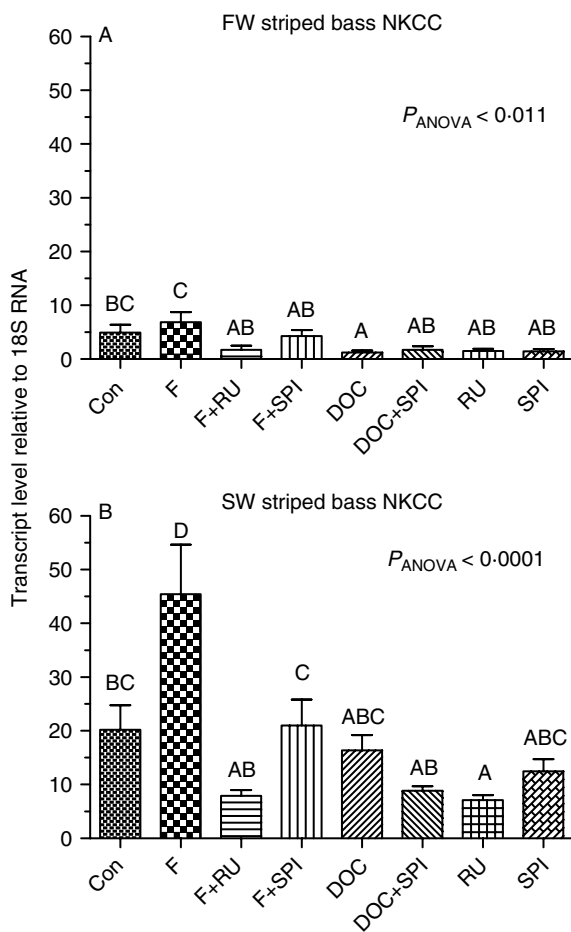


Figure 5 *In vitro* effect of cortisol and DOC on NKCC mRNA expression in FW- (A) and SW- (B) acclimated striped bass gill. One-way ANOVA *P* value is indicated on the figure. Letters above the bars indicate statistical groupings (Student–Newman–Keuls test). When letters are shared among groups, they are not statistically different ($P < 0.05$). For further details see legend to Fig. 1.

of cortisol and the recently discovered mineralocorticoid DOC in isolated gill tissue of FW- versus SW-acclimated fish to attain a better understanding of their function in salinity acclimation and the potential receptor-mediated process through which they act. We report that DOC has an ion-regulatory potential in the gill of euryhaline fish and demonstrate that cortisol's actions and signalling pathways in gill function may be species specific and related to the strategy for coping with fluctuations in salinity.

Doses of hormones

Only the highest dose of cortisol (10 $\mu\text{g/ml}$) had an effect on target gene mRNA levels in tilapia and striped bass gill in this study. This dose may be considered supraphysiological even though peak plasma levels during acute stress have been reported up to 0.3–0.4 and 0.8–2.0 $\mu\text{g/ml}$ in tilapia and

striped bass respectively (Mazik *et al.* 1991, Balm *et al.* 1994, Noga *et al.* 1994, Barcellos *et al.* 1999, Davis & McEntire 2009, Breves *et al.* 2010). For yet unknown reasons, relatively high levels of corticosteroids (1–10 $\mu\text{g/ml}$) are generally needed in order to evoke the effects on gill explants *in vitro* (e.g. McCormick & Bern 1989, McCormick *et al.* 1991, Killerich *et al.* 2010). The actual tissue and cellular level of cortisol in our study is unknown but may be much lower than 10 $\mu\text{g/ml}$ due to diffusion limitations caused by mucus secretion by the gills during incubation. The cortisol dose needed for a response may vary according to species and experimental conditions, since lower levels of cortisol can manipulate enzyme transcription and activity in other species such as rainbow trout (Na^+, K^+ -ATPase: Shrimpton & McCormick 1999, lactate dehydrogenase activity: Mazon *et al.* 2004) and sea bream (Na^+, K^+ -ATPase α subunit mRNA, but not enzyme activity: Deane & Woo 2005). In three-dimensional simpler models such as opercular membrane or cultured gill epithelia, cortisol also exerts effects at lower doses (0.1–1 $\mu\text{g/ml}$; e.g. McCormick 1990, Chasiotis *et al.* 2010).

With regard to DOC, information on plasma levels is indeed scarce. One study reported DOC plasma levels in tilapia around 0.0005–0.0006 $\mu\text{g/ml}$, with an increase up to 0.022 $\mu\text{g/ml}$ during spawning (Katz & Eckstein 1974). No data are available in striped bass. In order to compare the potency of DOC with that of cortisol, equal concentration of the two hormones was applied routinely. However, in a single experiment with FW striped bass, 10- to 100-fold lower doses of DOC induced the same qualitative effects as 10 $\mu\text{g/ml}$ (see later).

Na^+, K^+ -ATPase and NKCC isoforms and mRNA levels in gills from FW- and SW-acclimated fish

The transcript level of two response genes was investigated: the Na^+, K^+ -ATPase $\alpha 1$ subunit, being responsible for maintaining the electrochemical potential for ion transport

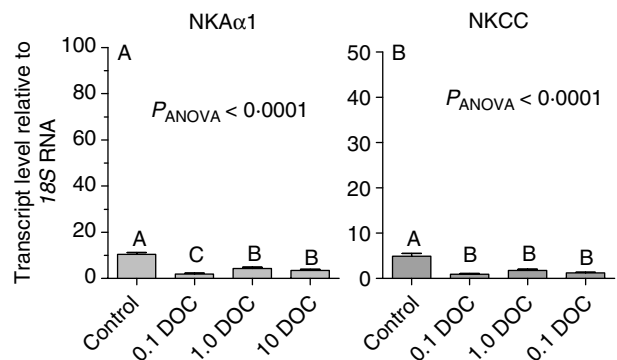


Figure 6 The effect of graded doses of DOC (10, 1 and 0.1 $\mu\text{g/ml}$) on Na^+, K^+ -ATPase $\alpha 1$ (NaK α 1; in A) and NKCC (B) transcript levels in FW-acclimated striped bass gills. One-way ANOVA *P* value is indicated on the figure. Letters above the bars indicate statistical groupings (Student–Newman–Keuls test). When letters are shared among groups, they are not statistically different ($P < 0.05$).

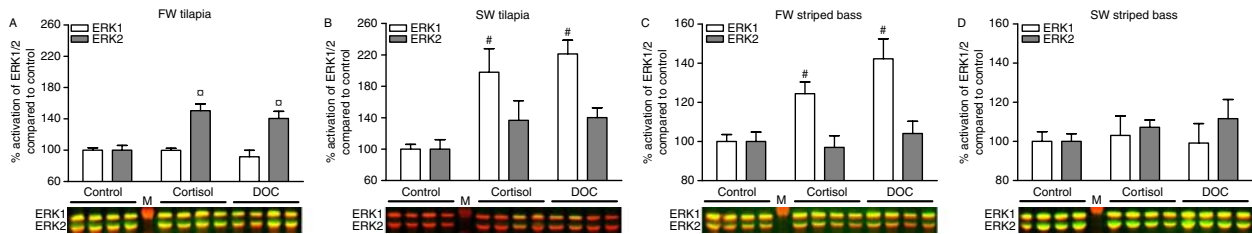
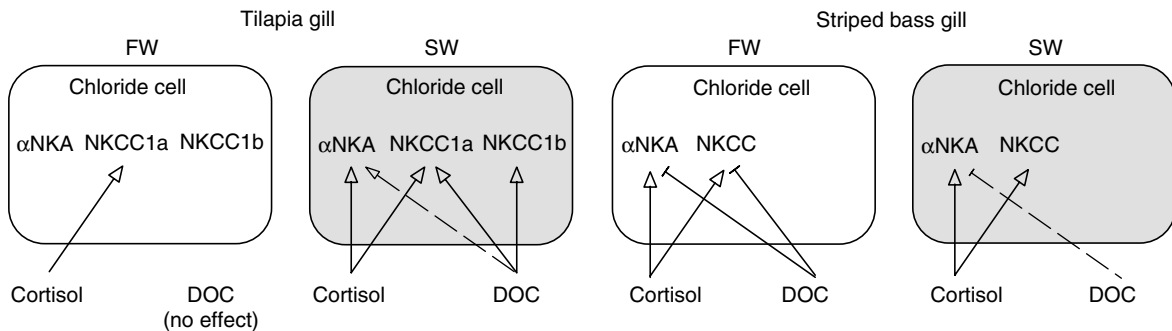


Figure 7 *In vitro* activation of ERK1 and ERK2 by cortisol and DOC in tilapia and striped bass gill is dependent on the acclimation salinity. Gill blocks from FW- (A and C) and SW- (B and D) acclimated fish were incubated with cortisol or DOC for 30 min at room temperature before homogenisation, gel electrophoresis and western blotting. The amount of activated (dual phosphorylated) ERK1/2 was calculated relative to the total amount of total ERK1/2 in each sample. Control wells are set to 100%, and the relative activation is given in percentage (values are mean \pm S.E.M., $n=4$). # and \square indicate significant difference from ERK1 and ERK2 control respectively. The western blot section below the graph illustrates the overlay of non-phosphorylated (red) and dual phosphorylated (green) ERK1 (upper band) and ERK2 (lower band). The protein marker band (M) is indicated at 50 kDa. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-10-0326>.

in both hypo- and hyperosmotic environment, and the NKCC, which facilitates ion excretion under hyperosmotic conditions (see Marshall (2002)). Until now, only one $\text{Na}^+, \text{K}^+ \text{-ATPase } \alpha 1$ isoform has been reported in the diploid tilapia (Hwang *et al.* 1998) and striped bass (Madsen *et al.* 2007) compared with the three $\alpha 1$ isoforms identified in the tetraploid salmon gill, $\alpha 1a$, $\alpha 1b$ and $\alpha 1c$ (Richards *et al.* 2003). Several studies in salmonids have demonstrated reciprocal changes of $\alpha 1a$ and $\alpha 1b$ isoforms when salinity changes, with $\alpha 1a$ as the predominant FW isoform and $\alpha 1b$ as the predominant SW isoform

(Richards *et al.* 2003, Madsen *et al.* 2009). The tilapia and striped bass sequences analysed in this study share highest homology with the salmonid $\alpha 1b$ (SW type) isoform (BLAST, $\alpha 1b > \alpha 1c > \alpha 1a$). Indeed, we found that SW striped bass had higher levels of $\text{Na}^+, \text{K}^+ \text{-ATPase } \alpha 1$ mRNA than FW fish (Fig. 2A compared with 2B, $P < 0.01$), whereas this was not significant in tilapia (Fig. 1A and B). An increase in $\text{Na}^+, \text{K}^+ \text{-ATPase } \alpha$ subunit mRNA may subsequently lead to a change in $\text{Na}^+, \text{K}^+ \text{-ATPase } \alpha$ subunit protein and thus enzymatic activity as described previously for FW-SW and FW-brackish water-transferred tilapia (Lin *et al.* 2004, Wang *et al.* 2009).



Species	Tilapia						Striped bass			
	FW			SW			FW		SW	
Salinity	NKA α 1	NKCC1a	NKCC1b	NKA α 1	NKCC1a	NKCC1b	NKA α 1	NKCC1a	NKA α 1	NKCC1a
F	0	+	0	+	+	0	(+)	(+)	+	+
F+RU	0	0	0	0	0	0	0	0	0	0
F+SPI	0	0	0	(-)	0	0	0	0	0	0
DOC	0	0	0	+	+	+	-	-	-	0
DOC+SPI	0	0	0	+	0	0	(0)	0	-	0

Figure 8 Schematic summary of the main findings of the study. The diagram illustrates target gene transcripts within gill chloride cells of FW- and SW-acclimated tilapia and striped bass. Observed effects of cortisol and DOC are shown by a line with arrowhead (stimulation) or bar (inhibition) near the target. α NKA, $\alpha 1$ -subunit of the $\text{Na}^+, \text{K}^+ \text{-ATPase}$; NKCC1(a/b), isoforms of the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter. Dashed line indicates that the effect is not verified by antagonist treatment. In the table given, '0' denotes no effect; '+' denotes stimulatory effect; '-' denotes inhibitory effect; () denotes tendency but not significant; light shading indicates 'blocking' with antagonist; dark shading indicates 'no blocking' with antagonist.

Our comparison was, however, based on control gill samples incubated in minimal medium for 24 h, during which time there may have been a degradation of mRNA due to natural turnover in the tissue (Madsen *et al.* 2007).

Two NKCC1 isoforms have been identified in tilapia, NKCC1a and NKCC1b (Hiroi *et al.* 2008). The NKCC1a was termed a secretory type based on its higher level in tilapia gill after SW transfer (Hiroi *et al.* 2008, Inokuchi *et al.* 2008). Only one NKCC1 (partial) sequence has been described in striped bass (Tipsmark *et al.* 2004), and a BLAST search suggests this to be the secretory NKCC1a isoform. Accordingly, the level of NKCC1a in striped bass and tilapia gills was higher in SW than in FW (Figs 3 and 5, $P < 0.01$ for both transcripts). This is consistent with other fish species (reviewed by Scott *et al.* (2004), Tipsmark *et al.* (2004), Evans *et al.* (2005), Lorin-Nebel *et al.* (2006), Nilsen *et al.* (2007) and Tang & Lee (2007)) and agrees with the ion-secretory roles assigned for this ion transporter (Evans *et al.* 2005).

We also found that the NKCC1b tilapia isoform was elevated in gills from SW versus FW-acclimated tilapia (Fig. 4, $P < 0.05$). Investigations of the role of NKCC1b in the gill are scarce, and we can only note that the higher level of NKCC1b in SW-acclimated tilapia compared with FW-acclimated tilapia in this study is inconsistent with the studies of Hiroi *et al.* (2008), where NKCC1b levels were the same or lower in SW compared with FW.

Na^+, K^+ -ATPase and cortisol

A stimulatory effect of cortisol on gill Na^+, K^+ -ATPase α subunit and isoform transcripts, protein and activity levels *in vivo* and *in vitro* is well established in salmonids (see Mommsen *et al.* (1999), McCormick *et al.* (2008) and Tipsmark & Madsen (2009)). In both tilapia and striped bass, cortisol also stimulated Na^+, K^+ -ATPase α subunit transcript levels significantly but only in gills from SW-acclimated fish and not in gills from FW-acclimated fish. For tilapia, this is at variance with Dang *et al.* (2000) who showed that cortisol stimulated Na^+, K^+ -ATPase protein density in chloride cells in FW-acclimated tilapia. On the other hand, Dangé (1986) showed that cortisol had a much stronger effect on gill Na^+, K^+ -ATPase activity when injected into SW-compared with FW-acclimated tilapia (Dangé 1986). In striped bass, only one previous study has analysed the effect of cortisol on gill Na^+, K^+ -ATPase. Madsen *et al.* (2007) found either no effect of cortisol on Na^+, K^+ -ATPase activity or α -subunit mRNA when injected into FW-acclimated fish *in vivo* or a very weak stimulatory effect when applied to gill tissue of FW-acclimated fish directly *in vitro* for 24 h. These authors did not, however, investigate SW-acclimated fish. Thus, the present experiments indicate that tilapia and striped bass gills respond similarly to cortisol *in vitro* – though only when acclimated to SW. The apparent salinity dependent effect is likely explained by differences in gill corticosteroid receptors (CR) and thus increased sensitivity after SW transfer (Dean *et al.* 2003).

The similar pattern of response to cortisol in the two Perciforms does not support our initial hypothesis that cortisol would be more important in tilapia than in striped bass during acclimation to increased salinity. However, when we analyse the cortisol signalling pathways involved in the response, the two species seem to differ. In SW-acclimated tilapia, the fully antagonistic effect of RU486, but not spironolactone, suggests that a higher proportion of cortisol signalling may occur through a GR type rather than a MR type receptor (Fig. 1B). In SW striped bass, both RU486 and spironolactone completely abolished cortisol's effect on Na^+, K^+ -ATPase $\alpha 1$ (Fig. 2B), suggesting a role for GR and MR alike. RU486 is considered to be an efficient and specific antagonist for the GR and has been used several times with success in fish (Bury *et al.* 2003, Scott *et al.* 2005, Kiilerich *et al.* 2007, Shaw *et al.* 2007). On the other hand, the efficiency of spironolactone as an antagonist in fish has been questioned by Sturm *et al.* (2005). While an agonistic effect of spironolactone was reported in mammalian COS cells using rainbow trout MR (Sturm *et al.* 2005), spironolactone has been used both *in vivo* and in *in vitro* organ culture without undesired agonistic effects in fish (Sloman *et al.* 2001, Scott *et al.* 2005, Kiilerich *et al.* 2007). Accordingly, there are no agonistic effects nor any consistent overall pharmacological effect of spironolactone observed in this study.

Collectively, gills from SW-acclimated tilapia and striped bass are more responsive to cortisol than gills from FW-acclimated fish in respect to regulation of Na^+, K^+ -ATPase $\alpha 1$ mRNA levels. This differs from the situation in Atlantic salmon, where both $\alpha 1a$ and $-1b$ subunits of the Na^+, K^+ -ATPase are stimulated by cortisol in gills from FW- as well as SW-acclimated fish (Kiilerich *et al.* 2007). Whether this difference between euryhaline salmoniform and Perciform teleosts reflects their evolutionary origin or their adopted life cycle is uncertain. Salmonids developmentally acquire salinity tolerance in a narrow time window during smoltification, whereas tilapia and striped bass may acclimate at all life stages. Further investigations are required to dissect the underlying mechanisms for the altered cortisol responsiveness among species with different acclimation strategies.

NKCC and cortisol

In striped bass, cortisol stimulated gill NKCC transcript levels only in SW-acclimated fish (Fig. 5). In FW, there was no effect of cortisol; however, RU486 significantly decreased the transcript level both alone and in combination with cortisol, suggesting that endogenous cortisol may have stimulated the GR in the cultured control gills. This resembles the regulation patterns observed with a similar experiment in Atlantic salmon (Kiilerich *et al.* 2007) and is in good agreement with the up-regulation of NKCC mRNA and protein levels after FW to SW transfer of striped bass (Tipsmark *et al.* 2004) and the SW adaptive role for cortisol and NKCC.

Tilapia differs from striped bass as cortisol caused an up-regulation of NKCC1a transcript levels in both FW and SW, which must be seen as a strong SW adaptive effect of cortisol in this species. Cortisol did not affect expression of the alternative isoform NKCC1b in tilapia gill, which supports different functions of the two isoforms as suggested by Hiroi *et al.* (2008). RU486 and spironolactone efficiently blocked the stimulatory effect of cortisol in both species suggesting that both GR and MR are involved in the signalling mechanism (Figs 3 and 5B). Together with the cortisol-mediated up-regulation of the secretory NKCC protein levels in the gill of salmonids (Pelis & McCormick 2001, Tipsmark *et al.* 2002), this suggests a general ability of cortisol to up-regulate the secretory NKCC isoform in the gill in these species, thereby promoting monovalent ion secretion.

Effect of DOC

Only very few studies have investigated the effects of DOC on osmoregulatory targets in fish. *In vivo* treatment with DOC does not affect gill Na⁺,K⁺-ATPase α subunit mRNA level or activity in Atlantic salmon, and blocking of the MR with spironolactone had also no effect (McCormick *et al.* 2008). However, Kiilerich *et al.* (2010) reported an ability of DOC to up-regulate FW Atlantic salmon gill Na⁺,K⁺-ATPase α 1a and α 1b mRNA *in vitro*, suggesting a potential osmoregulatory function of DOC. In this study, DOC conferred a significant up-regulation of Na⁺,K⁺-ATPase α 1, NKCC1a and NKCC1b transcripts levels in SW- but not FW-acclimated tilapia gill. In striped bass, DOC down-regulated Na⁺,K⁺-ATPase α 1 in gills from FW- and SW-acclimated fish, and NKCC in gills from FW-acclimated fish only. The inhibitory effect in gills from FW-acclimated fish was verified at doses ranging from 0.1 to 1 μ g/ml, whereas these doses had no effects on any other transcript at both salinities. Interestingly, while DOC and cortisol had similar effect on Na⁺,K⁺-ATPase α 1 and NKCC1a in SW tilapia, the two hormones had disparate effects on other targets (Fig. 7). For instance, in tilapia, DOC had no effect on NKCC1a in gills from FW-acclimated fish, while cortisol conferred an up-regulation. Further, DOC treatment induced an up-regulation of NKCC1b in gills from SW-acclimated fish, while cortisol had no effect. In striped bass, DOC induced down-regulation of Na⁺,K⁺-ATPase α 1 and NKCC in gills from FW-acclimated fish, while cortisol had a weak stimulatory effect. Finally, DOC had no effect on NKCC in gills from SW-acclimated fish, where cortisol induced an up-regulation. In some (SW tilapia NKCC1a, NKCC1b and FW striped bass NKA, NKCC), but not in all cases (SW tilapia NKA, SW striped bass NKA), the effect of DOC was effectively blocked by the mineralocorticoid antagonist spironolactone. Yet, since DOC is a specific agonist for MR only (Sturm *et al.* 2005), an inadequate concentration of spironolactone most likely explains the lack of inhibition of the DOC-MR signal.

Rapid effects of corticosteroids

In the classical view of steroid hormone action steroids mainly work through intracellular receptors to regulate transcriptional rates of target genes and the subsequent regulation of proteins and target cell activity. This signalling pathway is generally slow, typically with latency periods of hours to days. However, corticosteroids may also rapidly regulate target cell activity through non-genomic mechanisms of actions (Borski 2000). Corticosteroids may also exert rapid and/or non-genomic effects on ion transport (see Sakamoto *et al.* (2001) and McCormick & Bradshaw (2006)) as has been demonstrated for aldosterone on ion regulation in tetrapods (see Harvey *et al.* (2002)). In fish, rapid effects of glucocorticoids have been reported with regard to inhibition of pituitary prolactin release in tilapia (Borski *et al.* 2001, 2002, Hyde *et al.* 2004), lipogenic enzyme activity in tilapia liver cells (Sunny *et al.* 2002) and on Na⁺,K⁺-ATPase activity in the eel and tilapia gill (Marsigliante *et al.* 2000, Sunny & Oommen 2001). We demonstrate in this study that both cortisol and DOC may rapidly affect (within 30 min) the MAPK signalling cascade via an increase in the phosphorylation state of ERK1 and ERK2. We speculate that this may further affect ion channel and ion transport activity downstream as observed for MDCK cells (see Harvey *et al.* (2002)). Whether this effect is independent of gene transcription and is mediated through classical cytosolic or putative membrane-bound corticosteroid receptors remains to be determined. Interestingly, the observed effects were dependent on the acclimation salinity and fish species, which might reflect variation in corticosteroid receptor profiles in target cells.

Conclusion

In relation to our initial hypothesis, the corticosteroid sensitivity and response pattern on ion-transporting proteins in the gill indeed seems to differ in tilapia and striped bass (summarised in Fig. 7). Thus, the innate differences in salinity acclimation strategies among the two species are reflected by differences in corticosteroid regulation of ion transporter genes at the level of the gill. Especially when judged by the effect of DOC, which has an overall stimulatory effect on the three targets in the gill from SW-acclimated tilapia, an overall inhibitory effect on the two investigated targets in the gill from FW striped bass was observed. Based on our current understanding of the combined ion-regulatory role of Na⁺,K⁺-ATPase and NKCC in the gill, the effect of DOC seems in favour of hypo-osmoregulation in tilapia and hyperosmoregulation in the striped bass. With regard to cortisol's effects, there is evidence of both glucocorticoid and MRs being involved, the overall action of cortisol being in favour of hypo-osmoregulation in both species. DOC has distinct as well as overlapping actions on ion transporter

gene regulation, suggesting that the two corticosteroids may coordinate their actions, presumably through distinct GR and MR receptors to accurately regulate target gene transcription.

Declaration of interest

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

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