

Cortisol Receptor Blockade and Seawater Adaptation in the Euryhaline Teleost *Fundulus heteroclitus*

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ABSTRACT To examine the role of cortisol in seawater osmoregulation in a euryhaline teleost, adult killifish were acclimated to brackish water (10‰) and RU486 or vehicle was administered orally in peanut oil daily for five days at low (40 mg.kg⁻¹) or high dose (200 mg.kg⁻¹). Fish were transferred to 1.5 × seawater (45‰) or to brackish water (control) and sampled at 24 h and 48 h after transfer, when Cl⁻ secretion is upregulated. At 24 h, opercular membrane Cl⁻ secretion rate, as I_{sc}, was increased only in the high dose RU486 group. Stimulation of membranes by 3-isobutyl-1-methylxanthine and cAMP increased I_{sc} in vehicle treated controls but those from RU486-treated animals were unchanged and membranes from brackish water animals showed a decrease in I_{sc}. At 48 h, I_{sc} increased and transepithelial resistance decreased in vehicle and RU486 groups, compared to brackish water controls. Plasma cortisol increased in all groups transferred to high salinity, compared to brackish water controls. RU486 treated animals had higher cortisol levels compared to vehicle controls. Vehicle treated controls had lower cortisol levels than untreated or RU486 treated animals, higher stimulation of I_{sc}, and lower hematocrit at 24 h, beneficial effects attributed to increased caloric intake from the peanut oil vehicle. Chloride cell density was significantly increased in the high dose RU486 group at 48 hours, yet I_{sc} was unchanged, suggesting a decrease in Cl⁻ secretion per cell. Thus cortisol enhances NaCl secretion capacity in chloride cells, likely via glucocorticoid type receptors. *J. Exp. Zool.* 303A:132–142, 2005. © 2005 Wiley-Liss, Inc.

INTRODUCTION

Estuarine teleosts have the capacity to acclimate rapidly to changing salinity, and *Fundulus heteroclitus*, the mummichog or killifish, is able to adapt to large abrupt salinity increases (Wood and Marshall, '94). The hormonal control of adaptation to seawater involves the pituitary interrenal axis with cortisol as the final steroid product, because teleosts do not produce mineralocorticoids, such as aldosterone. The mineralocorticoid role has thus been considered to be filled by cortisol (review: McCormick, 2001). Growth hormone (GH) and insulin-like growth factor I (IGF-I) are also involved in seawater acclimation in salmonids (McCormick '96; Pelis and McCormick, 2001) and recent evidence suggests this may also occur in other euryhaline teleosts including killifish (Mancera and McCormick, '98). GH and cortisol appear to act synergistically in the sea-

water acclimation process to increase survival of brown trout (Madsen, '90) as well as gill Na⁺,K⁺-ATPase activity in brown trout (Madsen, '90) and Atlantic salmon (McCormick, '96). Also, GH and cortisol increase gill Na⁺,K⁺,2Cl⁻ cotransporter (NKCC) abundance in Atlantic salmon (Pelis and McCormick, 2001).

Seawater acclimation involves synthesis of new mitochondria-rich (MR) cells as well as modification of old MR cells. Hiroi et al. ('99) traced individual MR cells and found 70% of the initial MR cells to be still present and hypertrophied

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after 96 hours. In killifish, the anion channel Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is the apical membrane anion channel involved in Cl^- secretion, as the channel is present in MR cells and as determined by patch clamp is electrophysiologically similar to mammalian CFTR (Marshall et al., '95; Singer et al., '98). Also, an antibody to the carboxy terminus localizes CFTR protein to the apical membrane of MR cells from seawater acclimated killifish (Marshall et al., 2002). CFTR expression in the gill and opercular membrane is increased significantly at 8 h after transfer to seawater and is maximal at about 24 h after transfer (Marshall et al., '99). Immunocytochemical localization indicates that CFTR in freshwater MR cells is present in the basolateral membrane and, in this first 24 h in seawater, de novo synthesized CFTR is trafficked into the apical membrane, while basolateral CFTR diminishes and disappears by 72 h in seawater (Marshall et al., 2002). The appearance of CFTR in the apical membrane coincides with greatly increased transepithelial Cl^- secretion, as measured by short-circuit current (I_{sc}) in isolated opercular membranes (Marshall et al., '99, 2002).

Plasma cortisol levels rise rapidly after an increase in salinity, with a significant peak early (1 h) prior to CFTR enhancement (Marshall et al., '99), and the killifish and *Fugu* CFTR genes have a glucocorticoid responsive element in the promoter region upstream of the gene (Singer et al., 2000). Therefore, it is reasonable to assume that cortisol may be responsible, at least in part, for the increased CFTR expression leading to increased I_{sc} at 24–48 h. In support of the relationship between stress and glucocorticoid responsive elements in fish, Schulte et al. (2000) found that Northern strains of *Fundulus heteroclitus* that have a point mutation in the glucocorticoid responsive element of lactate dehydrogenase cannot express the gene (Schulte et al., 2000). While cortisol is clearly associated with seawater acclimation and upregulation of Na^+, K^+ -ATPase, NKCC and CFTR expression (McCormick, '96; Pelis and McCormick, 2001), it is not clear what other aspects of the acclimation response are induced by cortisol, nor is it known what receptors may mediate the response. Sloman et al. (2001) observed that MR cell hypertrophy in rainbow trout exposed to low calcium fresh water was blocked by spironolactone, a mineralocorticoid receptor antagonist, while the glucocorticoid receptor blocker RU486 was ineffective in preventing MR cell hypertrophy. A second class of cortisol

receptor was therefore suspected. Recently there have been two glucocorticoid receptor subtypes recognized in rainbow trout, a genetic duplication that may have arisen through a duplication of the entire genome early in actinopterygian history (Bury et al., 2003). The two receptors, termed rtGR1 and rtGR2 have similar affinities for dexamethasone, but rtGR2 is more sensitive to cortisol and less sensitive to the glucocorticoid antagonist RU486 (Bury et al., 2003). Meanwhile, a total of four corticosteroid receptors have been identified in the cichlid teleost *Haplochromis burtoni*, one similar to rtGR1 (HbGR1), two similar to rtGR2 (HbGR2a and 2b) and a fourth that resembles instead a mineralocorticoid receptor (HbMR) (Greenwood et al., 2003). A homolog of the mammalian mineralocorticoid receptor gene has been found in the testes of rainbow trout (Columbe et al., 2000). Thus teleosts may have multiple receptors for adrenal steroids. At least in mammals mineralocorticoid and glucocorticoid receptors can both operate through the same responsive element in the genome and no purely specific mineralocorticoid responsive genes have been identified (Fuller et al., 2000). Thus it is possible for cortisol bound to either receptor type to activate seawater adaptive genes.

To test whether a glucocorticoid receptor type is involved in the upregulation of ion transport by MR cells, killifish acclimated to brackish water were treated with RU486 (orally at a low and high dose) then challenged to adapt to $1.5 \times$ seawater, a hypersaline condition, expecting that a large salinity challenge should magnify detrimental effects of receptor blockade. It was reasoned that cortisol would have a high affinity for the glucocorticoid receptor and the complex in turn would have a high affinity for the glucocorticoid responsive element in the regulatory region upstream of CFTR. Effective blockade of cortisol action ought to result in diminished CFTR-dependent ion secretion capacity, perturbations of MR cell number and elevated plasma cortisol.

MATERIALS AND METHODS

Animals

Adult killifish (*Fundulus heteroclitus* L.) of both genders were captured in Antigonish estuary, transferred to indoor holding facilities, and adapted to brackish water (salinity 10‰) for at least 10 days at 20–25°C and ambient photoperiod under artificial light. Fish were then moved to

sectioned aquaria for a further 7 d (each section containing a pair of fish). During the acclimation period, fish were fed marine fish food blend (Nutrafin flakes; R. C. Hagen, Montreal, Canada) twice daily at a rate of $1.0 \text{ g } 100 \text{ g}^{-1}$ body mass day^{-1} .

Drug treatment

Groups of eight animals each were included: brackish water control group (not transferred), vehicle control group (transferred), low and high RU486 dose (transferred), and a hypersaline control group (not transferred). Following the acclimation period, a steroid hormone blocker, Mifepristone, also known as RU486 (Sigma, St-Louis, MO, USA) was dissolved in peanut oil and added to the fish flakes. The animals ($N=8$) were fed individually every morning for five days a low dose ($40 \text{ mg.kg}^{-1}.\text{d}^{-1}$) or a high dose ($200 \text{ mg.kg}^{-1}.\text{d}^{-1}$) of RU486 and regular flakes in the afternoon. Control animals received peanut oil vehicle on flakes ($5.0 \text{ } \mu\text{l/g}$ food quality, Sobey's best, Stellarton NS; polyunsaturates 33%, mono-unsaturates, 48.4%, saturates 19%), while untreated animals were fed regular flakes. On the sixth day salinity was changed in approximately four minutes by displacing the brackish water with hypersaline water ($1.5 \times \text{SW}$, salinity 45 ‰; seawater augmented with 13 ‰ artificial sea salt, Kent Marine Inc., Marietta, GA) without altering water temperature or water level. The fish were then kept at that salinity for 24 or 48 h. In addition, two untransferred control groups, one in brackish water and another in $1.5 \times$ seawater were maintained separately and fed regular flakes throughout the experiment.

Blood and tissue collection

After 24 or 48 h following transfer to $1.5 \times$ seawater, the killifish were anesthetized in buffered (pH 7.1) 1:5000 MS-222 in 150 mM NaCl and blood was collected from the severed caudal peduncle into heparinized hematocrit tubes. Fish were killed by decapitation and gill tissue was placed in ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen in liquid nitrogen. Blood was centrifuged (1000 g for 2 min), hematocrit taken, plasma was separated, and samples for determination of cortisol levels were heat-sealed in plastic ampoules. The balance of the plasma was diluted 1:9 with deionized water and stored in micro centrifuge tubes for analysis of ion levels and osmolality.

Plasma $[\text{Na}^+]$ ($\pm 1 \text{ mM}$) was measured by atomic absorption spectrophotometry (Varian AA375, Varian Techtron, Springvale, Australia). A 4–6 mm section of caudal peduncle was also taken for determination of muscle moisture (below); samples were frozen in liquid nitrogen. Both opercular epithelia were dissected, one was frozen and the other, for electrophysiology of ion transport (below), was placed in a modified Cortland's saline (composition in mmol.l^{-1} : NaCl 160, KCl 2.55, CaCl_2 1.56, MgSO_4 0.93, NaHCO_3 17.85, NaH_2PO_4 2.97, and glucose 5.55, pH 7.8, when equilibrated with a 99% $\text{O}_2/1\%$ CO_2 gas mixture.

Muscle moisture

The wet weight of each muscle sample was measured. The samples were then dehydrated to constant mass and the moisture content calculated as initial mass minus final mass divided by final mass and expressed as percent.

Plasma cortisol

Plasma cortisol levels were measured using a validated direct enzyme immunoassay as outlined in Carey and McCormick ('99). Sensitivity as defined by the dose-response curve was 1 – 400 ng.ml^{-1} . The lower detection limit was 0.3 ng.ml^{-1} . Using a pooled plasma sample, the average intra-assay variation was 5.5% ($N=10$) and the average inter-assay variation was 8.8% ($N=10$).

Na^+, K^+ -ATPase assay

Na^+, K^+ -ATPase activity was determined using a kinetic micro assay run in 96 well microplates at 25°C and read at a wavelength of 340 nm for 10 min as outlined in McCormick ('93). Gill tissue (approx. 10–15 mg, six gill filaments) was homogenized in 125 μl of SEID (150 mM sucrose, 10 mM EDTA, 50 mM imidazole at pH 7.3 with 0.1% v/v deoxycholic acid) and centrifuged at $5000 \times g$ for 30 s. Samples of the supernatant (10 μl) were run in two sets of duplicates; one set containing assay mixture only and the other containing assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity measurement is expressed in $\mu\text{moles ADP.mg}^{-1} \text{ protein.h}^{-1}$. Two control groups ($N=8$) were included, animals adapted to full strength seawater (32 ‰) and to dechlorinated Antigonish tap water (NaCl 0.15–0.30 mM, Ca^{2+} 0.25–0.30 mM, pH 5.5–6.5).

Electrophysiology

The opercular epithelium was removed and mounted in a modified Ussing chamber as described previously (Marshall et al., '98), except that the nerve supply was not dissected with the epithelium. The epithelium was supported by a nylon mesh and pinned out over the circular aperture (0.125 cm^2) with the rim area lightly greased and beveled to minimize edge damage. In the membrane chambers, the following epithelial electrophysiological variables were monitored: transepithelial potential V_t (mV), transepithelial resistance R_t (measured as the voltage transient divided by a fixed square current pulse of $10 \mu\text{amp}$, expressed as $\Omega \cdot \text{cm}^2$), transepithelial conductance (G_t , the inverse of R_t) and short-circuit current I_{sc} ($\mu\text{amp} \cdot \text{cm}^{-2}$). I_{sc} is expressed as positive for secretion of anions and is equivalent to the net secretion of Cl^- (Degnan et al., '77). Epithelia were left at open circuit and were clamped to 0 mV for short periods to allow recording of I_{sc} . A current-voltage clamp (D. LEE CO., Sunnyvale, CA), or DVC 1000 (WP Instruments, Longmont, OH) was used to measure the epithelial variables. A control period of 1h established the resting I_{sc} , after which 1.0 mM dibutyryl-cyclic AMP (db-cAMP) and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (both from Sigma, St-Louis, MO) were added to the serosal side of the epithelium. A test period of 1h followed the stimulation to determine the maximum stimulated I_{sc} .

MR cell size and density

After removal from the Ussing chambers, the opercular membranes were incubated for at least 5 min with $10 \mu\text{M}$ (2,4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide (DASPEI; ICN pharmaceuticals), rinsed three times with Cortland's saline and mounted on slides in mounting media (Geltol; Immunon Thermo Shandon, Pittsburgh, PA). Images were collected using a laser confocal microscope (Olympus FV300). A section for cell counts was selected randomly and XYZ-stack series were collected at $10\times$ and $40\times$, zoom 3.0, with optical sections of $1.0 \pm 0.1 \mu\text{m}$. Cell counts were performed later in single blind fashion by counting all DASPEI positive cells in a field of 0.1156 mm^2 per animal. MR cell size was estimated from the same confocal XYZ scans by taking images at the plane of the nuclei, extracting the cell surface area (microns squared; Image-Pro Plus software) for ten individual cells selected randomly per animal.

Statistical analyses

Data are expressed as the mean plus or minus one standard error. One way analysis of variance was followed by Bonferroni or Dunnett's post hoc tests with significance ascribed if $P < 0.05$, in a two-tailed test.

RESULTS

Plasma and blood

Transfer to hypersaline conditions from brackish water caused no morbidity or mortality among the killifish and the animals fed well at all stages of the experiment. The blood variables measured were hematocrit, plasma Na^+ concentration and cortisol content. Plasma Na^+ showed high individual variation. There were trends to higher Na^+ levels in the RU486 treated animals at 24 h and 48 h, compared to vehicle and untreated controls (Fig. 1). Pooling untreated and vehicle treated groups for 24 and 48 h and comparing this combined control group to the pooled 24 and 48 h RU486 treated animals (RL24, RL48) showed that the low dose of RU486 but not the high dose, had significantly higher plasma Na^+ ($P < 0.05$, Bonferroni post comparison following ANOVA). Surprisingly, plasma Na^+ showed a lower trend in the untreated salinity challenged groups (U24, U48) compared to brackish water controls.

Elevated hematocrit is an indicator of hemoconcentration and/or enlargement of erythrocytes, for instance by catecholamines (e.g., Ling and Wells, '85). The untreated transferred group, U24, had

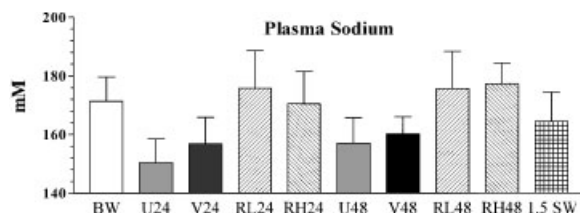


Fig. 1. Plasma sodium for killifish adapted to brackish water (BW, salinity 10 ‰) and transferred to $1.5\times$ seawater ($1.5\times$ SW, salinity 45 ‰) for 24 or 48 hours. Prior to the transfer, the fish were fed for five days a low dose ($40 \text{ mg} \cdot \text{kg}^{-1}$) (RL) or a high dose ($200 \text{ mg} \cdot \text{kg}^{-1}$) (RH) of RU486 dissolved in peanut oil and added onto the food flakes. Control animals received peanut oil vehicle on flakes (V). Untreated animals (U) were fed regular flakes. There were trends to higher plasma Na^+ levels in the RU486 treated groups that were significant for the low dose ($P < 0.05$, Bonferroni test after one way ANOVA) if 24 h and 48 h times were pooled. Values represent means \pm SEM ($N=8$ for all groups except for $1.5\times$ SW, where $N=11$).

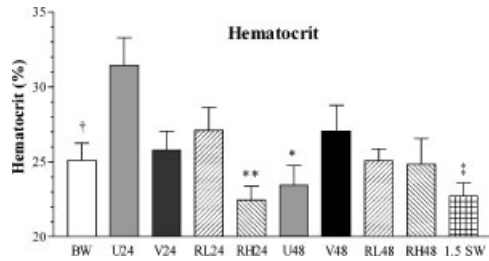


Fig. 2. Hematocrit levels of killifish transferred from brackish water (BW, salinity 10‰) to $1.5 \times$ SW for 24 or 48 hours. Treatment details as per Figure 1. U24 animals had a higher hematocrit than did the BW and $1.5 \times$ SW controls ($\dagger P < 0.05$ and $\dagger P < 0.001$). The effect was transient, as U24 hematocrit was significantly higher than the U48 animals ($* P < 0.01$) which were not different from either brackish or $1.5 \times$ SW controls. Further, the RH24 group had a significantly lower hematocrit than did the untreated animals at 24 h of transfer ($** P < 0.001$). Values represent means \pm SEM ($N=8$ for all groups except for $1.5 \times$ SW, where $N=11$). Significant differences were obtained using one-way ANOVA followed by Bonferroni's multiple comparison post test).

significantly higher hematocrit compared to BW ($P < 0.05$), RH24 ($P < 0.001$), U48 ($P < 0.001$) and $1.5 \times$ SW groups ($P < 0.001$) (Fig. 2). Vehicle controls (V24 and V48) had hematocrit approximately the same as control groups that were not transferred (BW and $1.5 \times$ SW). Hematocrit of blood from RU486 treated animals was not different from the vehicle controls at 24 or 48 h.

Plasma cortisol is elevated by salinity transfer and may be used as an indicator of efficacy of RU486 action (e.g., Veillette et al., '95), as receptor blockade should reduce negative feedback at the pituitary and hypothalamus. Indicative of cortisol responsiveness to salinity, salinity transfer in all groups caused elevated plasma cortisol levels compared to brackish water controls (Fig. 3). Consistent with blockade of cortisol receptors, there was significantly higher plasma cortisol in the low dose of RU486 at 24 h ($P < 0.05$) and the low and high doses of RU486 at 48 h ($P < 0.001$), compared to brackish water controls. There was also an increase in plasma cortisol in the untreated controls at 24 h after transfer to $1.5 \times$ SW that became significant ($P < 0.001$) at 48 h.

Opercular membranes

Chloride secretion was estimated from the short-circuit current (I_{sc}) (Fig. 4). The unstimulated I_{sc} was significantly elevated in the RH24 and V48 groups, compared to the vehicle treated control group at 24 h ($P < 0.01$). Further, the RH24, RL48, and RH48 had significantly higher

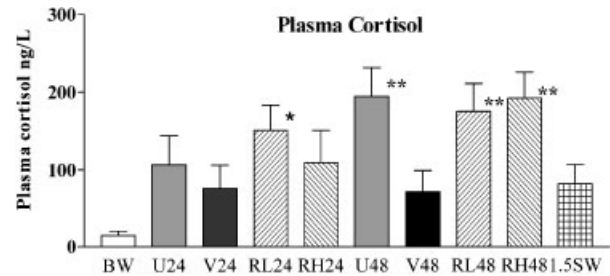


Fig. 3. Plasma cortisol levels were elevated in all the transferred groups compared to BW controls. Treatment details as per Figure 1. There was marginally significant elevation of plasma cortisol in the low dose of RU486 at 24 h ($* P < 0.05$) and more marked increases ($** P < 0.001$, Bonferroni post test after one way ANOVA) for the U48, RL48, and RH48 groups, compared to BW (untransferred) controls. The vehicle treated animals had a lower trend in cortisol levels compared to untreated animals, particularly at 48 h after transfer. $N=8$ for all groups except $1.5 \times$ SW, $N=11$.

I_{sc} , compared to brackish water controls ($P < 0.001$). At 48 hours after transfer, the untreated, vehicle and RU486 treated groups were all similar to each other.

Stimulation of the tissue with IBMX and db-cAMP here is taken as an indication of the total capacity of the tissue to excrete NaCl and typically this is about 1.5 times the resting Cl^- secretion rate (Marshall et al., '99). Treatment of tissues with IBMX and db-cAMP significantly increased I_{sc} in the untreated and vehicle treated controls at 24 h (U24+V24, Fig. 4, 5) compared to brackish water controls, while there was no change in I_{sc} in either of the RU486 treated groups ($P < 0.001$, Fig. 4, 5). At 48 h, the stimulation produced less of an increase in I_{sc} and the changes were similar between the vehicle and RU486 treated groups. Stimulation also did not increase the I_{sc} in the acclimated $1.5 \times$ SW group, while stimulation actually decreased I_{sc} in the acclimated brackish water controls (BW, Fig. 4, 5). The changes in transepithelial potential were overall similar to the pattern seen in I_{sc} , so they are not shown.

Epithelial conductance, an indicator of total tissue ionic permeability, was generally elevated in the groups transferred to high salinity (Fig. 6). The vehicle and low dose RU486 groups at 48 h had significantly higher conductance than brackish water controls ($P < 0.05$ and $P < 0.001$, respectively). Stimulation produced small increases in conductance in all groups except RU486 low dose at 24 h, where there was a trend to decreased conductance on stimulation (Fig. 6); the change made the RL48 group significantly higher

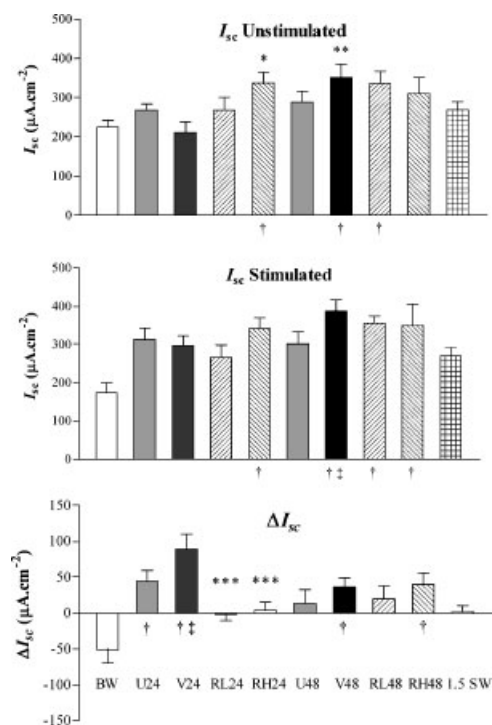


Fig. 4. Short circuit current (I_{sc}) of isolated opercular epithelia from brackish water (BW, salinity 10‰) killifish transferred to $1.5 \times$ SW for 24 or 48 hours. Treatment details as per Figure 1. Unstimulated opercular membranes (upper panel) from the high dose animals (RH24) showed a significant I_{sc} increase compared to the vehicle ($*P < 0.01$). There was also a significant increase in I_{sc} after transfer in the vehicle treated group (V48 vs. V24, $**P < 0.01$). Furthermore, the RH24, RL48, and RH48 groups had significantly higher I_{sc} than did the brackish water controls ($\dagger P < 0.001$). Following a stimulation with 1.0 mM db-cAMP+0.1 mM IBMX (middle panel), the vehicle controls transferred for 24 h were significantly greater than the $1.5 \times$ SW controls ($\dagger P < 0.05$). RU486 treated animals had less scope for I_{sc} increase (δI_{sc} , lower panel) on stimulation by db-cAMP+IBMX, compared to vehicle controls at 24 h ($***P < 0.001$). A negative δI_{sc} was noticed in brackish water animals. Values represent means \pm SEM ($N=8$ for all groups except for $1.5 \times$ SW, where $N=11$). Tests were ANOVA followed by Bonferroni's multiple comparison post test.

($P < 0.05$) than the $1.5 \times$ SW group as well as the brackish water group ($P < 0.05$).

Once the membranes were removed and stained with DASPEI, MR cell counts were performed and the density expressed as MR cells per mm^2 of membrane surface. All the groups had MR cell density in the range of 800–1300 cells. mm^{-2} , except the high dose of RU486 at 48 h that had a cell density greater than 1600 (Fig. 7). The RH48 group had higher MR cell density compared to BW, V24, U48, V48, RL48, and $1.5 \times$ SW ($P < 0.05$). Cell size was significantly higher in the vehicle treated animals at 48 hours and in the RU486 treated animals at 24 hours, compared to the long

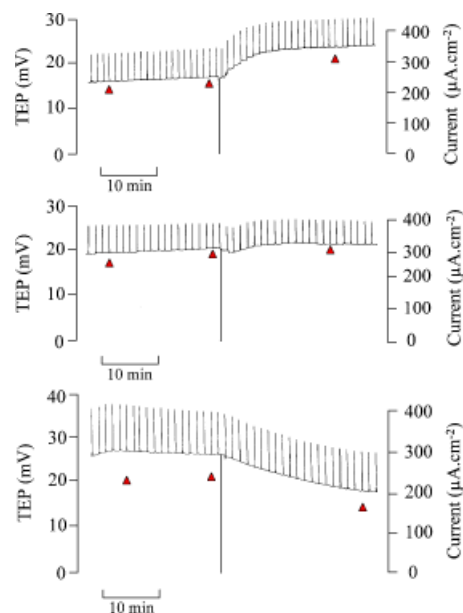


Fig. 5. Example tracings before and after addition of db-cAMP+IBMX for a V24 treated membrane (upper panel), RH24 treated animal (middle panel) and a brackish water adapted untransferred animal (lower panel). Normally at 24 h animals have large stimulation (here $\pm 30\%$) of I_{sc} , but RU486 reduces the scope for increase in I_{sc} , (here 0%) while brackish water adapted animals show a decrease in I_{sc} (here -27%) on stimulation. racing is the TEP, transients are in response to $80 \mu A \cdot cm^{-2}$ current pulses and the triangles are the I_{sc} .

term acclimated $1.5 \times$ SW animals ($P < 0.05$, Dunnett's test, two tailed, Figure 8) and there was a trend among most of the transferred animals to have enlarged MR cells. There was no consistent difference in cell size with RU486 treatment.

Gill Na^+, K^+ -ATPase activity

Gill Na^+, K^+ -ATPase activity is an indicator of the transport capacity of the gill and usually rises on adaptation to high salinity, but also in acclimation to very soft water. In addition to the groups included in the other measures, SW (32 ‰) and FW control groups ($N=8$ in each case) were added for comparison (Fig. 9). At 24 h there was no apparent increase in enzyme activity in control or treatment groups, although levels were all above 5 $\mu moles ADP \cdot mg^{-1} protein \cdot h^{-1}$, higher than many published activity levels for fresh water teleosts. At 48 h there was a trend to an increase in enzyme activity, especially in the RH48 group, but changes were not statistically significant. RU486 therefore produced a trend to an increase in Na^+, K^+ -ATPase activity after high salinity challenge. The $1.5 \times$ SW group stood out as having higher enzyme activity compared to BW, U24, V24, RL24,

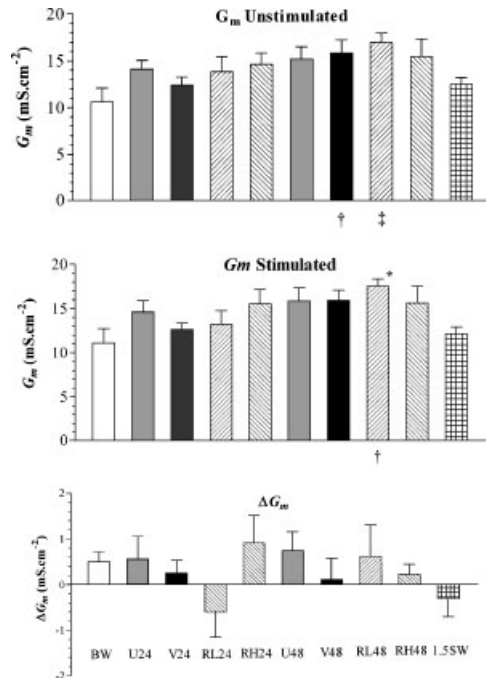


Fig. 6. Opercular membrane conductance (G_m) from brackish water (BW, salinity 10 ‰) killifish transferred to $1.5 \times$ SW for 24 or 48 hours. Treatment details as per Figure 1. Unstimulated opercular membranes from the vehicle and low dose animals (V48, RL48) had a significant G_m increase compared to the BW controls ($\dagger P < 0.05$ and $\ddagger P < 0.001$). Upon stimulation with 1.0 mM db-cAMP+0.1 mM IBMX, only the RH24 and the RL48 groups were significantly different from the BW controls ($\dagger P < 0.05$). The same groups had also a significantly higher G_m than did the $1.5 \times$ SW controls ($*P < 0.05$). The RH48 group had a larger increase in G_m (ΔG_m , lower panel) on stimulation by db-cAMP+IBMX, compared to $1.5 \times$ SW controls ($*P < 0.05$). Values represent means \pm SEM ($N=8$ for all groups except for $1.5 \times$ SW, where $N=11$; one-way ANOVA followed by Bonferroni's multiple comparison post test).

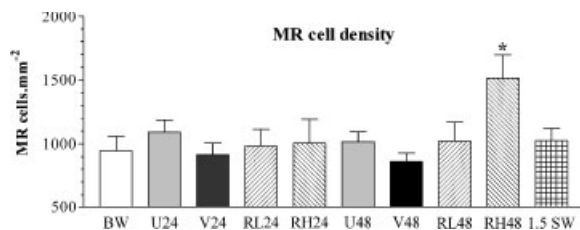


Fig. 7. Mitochondria rich (MR) cell counts from opercular membranes that were mounted in Ussing chambers for electrophysiology, then stained for mitochondria with DAS-PEI. Treatment details as per Figure 1. Density expressed as MR cells per mm^2 membrane. $N=8$ for all groups except $1.5 \times$ SW where $N=11$. *RH48 group has significantly high density of MR cells than V48 and V24 groups ($P < 0.05$, Bonferroni post test following one way ANOVA).

RH24, U48, V48, RL48, and SW groups ($P < 0.001$) and higher than RH48 ($P < 0.01$). Gill enzyme activity was also elevated by acclimation to the

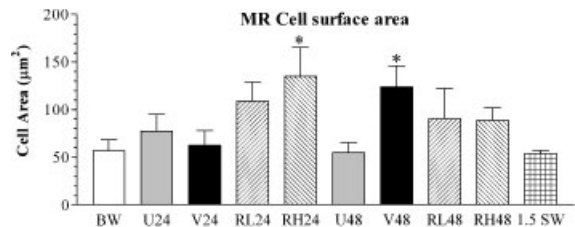


Fig. 8. Mitochondria rich (MR) cell surface area, taken by image analysis at the plane of the nucleus for 10 MR cells per animal. Treatment details as per Figure 1. Cell size was significantly elevated ($P < 0.05$, Dunnett's test) in the vehicle treated group at 48 hours (V48) and in the high dose of RU486 at 24 hours (RH24), compared to fully adapted $1.5 \times$ SW animals. There was also a trend toward larger cells in most groups in the transitional times versus the smaller cells of fully acclimated brackish water (BW) or $1.5 \times$ SW groups.

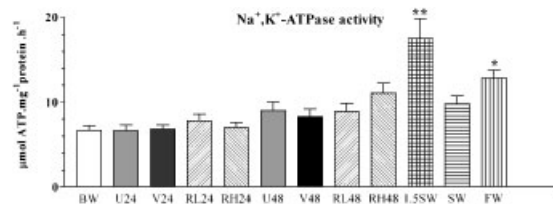


Fig. 9. Na^+, K^+ -ATPase assay of gill tissue. Included are the groups above plus a SW control group of eight; $N=8$ for all groups except $1.5 \times$ SW, $N=11$. Treatment details as per Fig. 1. The $1.5 \times$ SW group was significantly higher than BW, U24, V24, RL24, RH24, U48, V48, RL48, and SW $P < 0.001$ and higher than RH48 at $P < 0.05$. The FW control group was significantly higher than BW and U24 at $P < 0.05$ but not higher than SW or the test groups. There is a non-significant trend for the high dose of RU486 at 48 h to be higher than BW and U24. There is no apparent elevation of activity on transfer to $1.5 \times$ SW in the first 48 h, although at full acclimation activity is clearly augmented.

very soft Antigonish tap water and this group was significantly higher than BW and U24 groups ($P < 0.05$).

Muscle water content

Muscle water content was highest in the brackish water controls (76.9 ± 0.22 %, mean \pm SEM, $N=8$) and the $1.5 \times$ SW controls (76.6 ± 0.21 % mean \pm SEM, $N=11$). The U24, RH24, and RL48 groups were significantly lower in moisture than brackish water controls (75.4 ± 0.24 , 75.2 ± 0.23 , and 75.4 ± 0.25 , respectively, mean \pm SEM, $N=8$, $P < 0.05$), indicative of dehydration during acclimation to high salinity. The RH24 group was also significantly lower in muscle moisture than the $1.5 \times$ SW controls ($P < 0.05$), suggestive of a reduction of muscle water content by RU486.

DISCUSSION

Evaluation of oral RU486

Blockade of cortisol receptors by RU486 was apparently successful by oral administration of the drug, because cortisol levels were elevated by the treatment, compared to vehicle controls. The low levels of cortisol 15.5 ± 5.7 ng.ml⁻¹ ($N=8$) in the brackish water transfer group demonstrate the low level of disturbance for the animals. Previous values include 24 ± 2.2 for freshwater killifish (Marshall et al., '99), 19.1 ± 5.5 and 31.2 ± 2.5 also for freshwater killifish (Jacob and Taylor, '83), and 8.0 ± 2.5 for Atlantic salmon parr in fresh water (Veillette et al., '95). RU486 administered by implantation elevates plasma cortisol in smolting salmon to 60–300 ng.ml⁻¹, depending on the month around the smolting climax (Veillette et al., '95). These results are in the middle of this range at approximately 110–210 ng.ml⁻¹. RU486 blocks glucocorticoid receptors with a high affinity, and may therefore inhibit the normal negative feedback to the hypothalamus and pituitary, thus stimulating ACTH release and augmenting cortisol release by the interrenal and elevated cortisol in the plasma (review: Henderson, '97). Oral administration of the drug avoids repeated stress of injection and major stress of surgical implants. Oral administration probably also maintained drug levels, inasmuch as the animals maintained constant food intake throughout. The actual tissue levels of RU486 are unknown, but this is also true for animals treated by implants. The molecular action of RU486 and similar steroid inhibitors may be through competition for DNA binding sites and through recruitment of repressors so as to inhibit expression of normal steroid hormone stimulated gene expression (Leonardt and Edwards, 2002).

RU486 affects osmoregulatory ability

RU486 has been shown to affect hepatocyte metabolism of rainbow trout (Vijayan and Leatherland, '94) as well as intermediary and thyroid metabolism (Vijayan et al., '92), but clear osmoregulatory effects in teleosts have been difficult to demonstrate. Veillette et al. ('95) found that RU486 reduced intestinal fluid absorption rate in smolting salmon, but that the effect was only significant during a critical period. Sloman et al. (2001) found treatment with RU486 or spironolactone did not increase plasma cortisol levels nor alter plasma ions or gill Na⁺,K⁺-ATPase activity in freshwater rainbow trout. Spironolactone,

unlike RU486, blocks proliferation of MR cells normally seen in acclimation of trout to soft freshwater. It should be noted, however, that the specificity of these compounds for fish glucocorticoid and mineralocorticoid receptors, respectively, has yet to be demonstrated. All test animals survived the large salinity shock and adapted to the new environment, showing that RU486 clearly does not completely block the seawater acclimation process. However, RU486 did increase plasma sodium levels noticeably, and the change is significant compared to vehicle treated controls ($P < 0.05$) if 24 h and 48 h data are pooled. RU486 inhibits intestinal fluid reabsorption in salmon (Veillette et al., '95), an effect that would tend to force plasma volume down and plasma Na⁺ up. Previous work has shown that cortisol increases gill Na⁺,K⁺-ATPase activity and NKCC abundance in Atlantic salmon smolts (reviews McCormick, 2001; Pelis and McCormick, 2001). RU486 would tend to block this effect, and thus reduce NaCl secretion capacity, as was observed in the inability of opercular epithelia to respond normally to cAMP. Meanwhile, the observed variation in Na⁺,K⁺-ATPase activity fits a pattern for estuarine euryhaline teleosts, especially well shown in sea bream (*Sparus sarba*) (Deane and Woo, 2004), wherein very dilute freshwater as well as high salinity evoke increases in enzyme activity, with the lowest enzyme activities are seen in brackish water.

RU486 impairs NaCl secretion

RU486 treatment impaired the animals' ability to upregulate opercular membrane Cl⁻ secretion rate as measured by I_{sc}. In the RU486 treated groups at 24 h and 48 h in hypersaline conditions, opercular epithelium I_{sc} was not significantly increased by administration of IBMX and db-cAMP, while the control groups had large increases in I_{sc}. This indicates a lack of protein kinase A phosphorylation sites in the NaCl excretion system. Both NKCC and CFTR are phosphorylated and activated by PKA. Oocytes expressing killifish CFTR have cAMP stimulated anion current (Singer et al., '98) and membrane patches from apical membrane of MR cells have more anion channel openings if pretreated with cAMP (Marshall et al., '95). Shark rectal gland NKCC, the secretory isoform that is common to killifish gill and opercular membranes, is progressively phosphorylated in the presence of cAMP (Flemmer et al., 2002). The inability of the

opercular membranes to augment NaCl secretion rate implies that the animals are coping with hypersaline conditions but lack reserve transport capacity. Given that there is no change in the overall unstimulated I_{sc} in opercular epithelia with significantly more numerous chloride cells, this suggests a net decrease in transport of NaCl per cell, consistent with a lack of CFTR and NKCC. Killifish transferred to seawater show an early peak in cortisol levels, one hour after transfer (Jacob and Taylor, '83; Singer et al., '98) and increased CFTR expression in gill epithelium by Northern analysis, commencing at 8 hours and reaching highest levels at 24 hours after transfer (Singer et al., '98). Cortisol has also been shown to increase expression of CFTR mRNA in Atlantic salmon (Singer et al., 2003). These researchers hypothesize that CFTR and NKCC expression are upregulated by cortisol and it is the presence of these two transporters that allow upregulation of NaCl secretion. There was no significant change in gill Na^+, K^+ -ATPase activity in response to RU486 treatment, again pointing to the passive elements (CFTR and NKCC) as being affected by RU486. This result also suggests that Na^+, K^+ -ATPase is not rate limiting in killifish under the conditions imposed in this study.

RU486 treatment leads to chloride cell hypertrophy

The response in the high dose RU486 group is a significant increase in the density of MR cells at 48 h (Fig. 7). The lack of an increase at 24 h is understandable for a relatively protracted response involving cell division and differentiation. The cause of the increased density of MR cells may be a compensatory stimulation of growth hormone, insulin-like growth factor I, and/or other tissue-specific growth factors in fish that are induced when cortisol responsiveness is blocked. Growth hormone administration is known to increase chloride cell density in rainbow trout gills (Perry, '98) and in gills of Atlantic salmon parr (Pelis and McCormick, 2001). It is also possible that the elevated cortisol could operate through receptors not blocked by RU486, as cortisol administration is connected with chloride cell hypertrophy in freshwater rainbow trout gill (Perry, '98) in coho salmon presmolts (Richman and Zaugg, '87) and in Atlantic salmon parr (Pelis and McCormick, 2001). In either case, RU486 clearly does not block the ability of the animals to

augment MR cell number but it does block the ability of the cells to respond to cAMP.

RU486 had little effect on cell size, but there was a trend to increased cell size in the transferred animals, that was significant in the vehicle treated group at 48 hours and the RU486 group at 24 hours. This result is similar to the increased cell size seen in yolk sac membranes of tilapia embryos transferred to high salinity, where pre-existing cells enlarge during the 96 hours after transfer (Hiroi et al., '99).

Benefit of augmented caloric intake

An unintended result of this work is the apparent beneficial effects of peanut oil vehicle added to the diet. The vehicle treated transferred animals had lower cortisol levels than untreated transferred animals at 24 h and 48 h, a clear indication of a lower stress level. The vehicle treated animals also had a lower hematocrit (possibly indicative of reduced hemoconcentration) compared to the untreated controls and the highest level of cAMP stimulation of I_{sc} in spite of having the two lowest densities of MR cells in the opercular membranes. The extra transport capacity implies these animals were well on their way to adaptation to hypersaline conditions at an early stage and did so without hypertrophy of MR cells. The only difference between the groups was the daily supplement of peanut oil before transfer. It is possible that the added peanut oil was sufficient to bring about a positive nutritional impact on osmoregulatory capacity in killifish. The beneficial effects of augmented fat in the diet from the peanut oil may extend to reducing the stress effects of salinity transfer, thus the vehicle treated animals could have the observed lower than expected plasma cortisol level. Vijayan et al. ('96) found that fed tilapia had lower plasma ion levels, lower plasma cortisol, and lower plasma growth hormone three days after seawater transfer, compared to animals that were food deprived. Previous work has shown that the fatty acid components of gill membranes of eel change little with salinity (Crockett, '99) and that the gill tissue of several teleost species likely does not metabolize lipid efficiently (Crockett et al., '99). In contrast, Hansen and Grosell (2004) observed a transient shift in eel gill phospholipid profile from phosphatidyl ethanolamine to phosphatidyl choline with salinity challenge. Also, lipid augmentation of diet aids seawater adaptation in smolting salmon (Bell et al., '97). Presumably, the beneficial effect

is indirect, through stimulation of hepatic lipid metabolism.

Glucocorticoid and mineralocorticoid receptors

Rainbow trout have glucocorticoid receptors of at least two types (Bury et al., 2003; Greenwood et al., 2003) and a mineralocorticoid receptor homolog that binds cortisol when expressed in COS-1 cells (Colombe et al., 2000). In the cichlid teleost *Haplochromis burtoni*, one mineralocorticoid receptor and three glucocorticoid receptor isoforms exist and the former is more selective for aldosterone and cortisol than the latter, similar to the mammalian pattern (Greenwood et al., 2003). The role of mineralocorticoid type receptors in teleost osmoregulation is still unknown, but blockers that are designed for mammalian mineralocorticoid receptors (spironolactone) inhibit chloride cell proliferation that normally accompanies acclimation of rainbow trout to ion poor water, while RU486 is ineffective (Sloman et al., 2001). This suggests spironolactone has a high affinity for teleost steroid receptors, however, there is much overlap in ligand responsiveness, and at present it is unclear whether the putative mineralocorticoid and glucocorticoid receptor can be differentially affected by antagonists that are effective in mammals. Even in mammals, there appear to be no genes uniquely activated by aldosterone receptors, as glucocorticoid receptor activation also initiates transcription of those genes (Fuller et al., 2000). Finally, because aldosterone is not produced in substantial amounts by the teleost interrenal gland (Henderson and Kime, '87), presumably cortisol is the physiological agonist for the available teleost receptors.

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