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The involvement of cortisol and corticosteroid receptor types in mediating the effects of a high salt diet on rainbow trout (Oncorhynchus mykiss)

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The involvement of cortisol and corticosteroid receptor types in mediating the effects of a high salt diet on rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) are able to migrate between freshwater and seawater environments. These transitions induce remodeling of the gill, which aids in maintaining proper ionic balance in the face of changing salt and water requirements. Specific gill phenotypes for both freshwater and seawater rainbow trout have been observed, and are identifiable based on the locations and quantities of ion transporters and on the specific cell types present. Previous research has shown that internal salt loading is sufficient to induce a seawater gill phenotype in freshwater rainbow trout. The present study hypothesized that the developing phenotype was due to increases in circulating cortisol levels or corticosteroid receptor expression; this correlation was attempted by examining the time course of development of the seawater phenotype, and by examining concomitant changes in two other key osmoregulatory tissues, gut and kidney. A novel finding of the present study is that cortisol may play a role in promoting the development of a seawater phenotype in salt-fed freshwater fish, probably through regulation of corticosteroid receptor abundance rather than through modulation of cortisol levels.

Résumé

Oncorhynchus mykiss sont capable de migrer entre les environnements d'eau douce et eau de mer. Dans ces truites, on observe que les branchies sont remodeler, ce qui aide dans la propre maintenance d'équilibre ionique correct malgré les changements dans leur environnement. Il existe des phénotypes spécifiques de branchie, des truites qui vivent dans l'eau douce et l'eau de mer, qui sont identifiable a basé de l'emplacement et

les quantités des échangeurs d'ions et selon la présence des types de cellule spécifique. Dans cet étude, les truites d'eau douce sont nourrie un régime sel-enrichi (avec aucuns changements dans la salinité externe). Ceci et la recherche précédente faite dans notre laboratoire, a montré que ce changement de sel interne est suffisant de susciter un phénotype de branchie d'eau de mer dans ces poisson d'eau douce. Changements morphologiques et phénotypiques associé avec un régime sel-enrichi démontre le développement d'un phénotype d'eau de mer dans ces truites. On espérait que les expérimentes utilisant des inhibiteurs de récepteurs éluciderait les médiateurs du phénotype, les résultats suggère la possibilité d'une role pour les récepteurs cortocoïds.

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List of Abbreviations

Abbreviation	Description
11-DOC	11-deoxycorticosterone
11HSD2	11 β-hydroxysteroid dehydrogenase 2
ACTH	Adrenocorticotropic hormone
CFTR	Cystic fibrosis transmembrane conductance regulator
CRH	Corticotropin releasing hormone
DNA	Deoxyribonucleic acid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPI	Hypothalamic-pituitary-interrenal
MR	Mineralocorticoid receptor
MRC	Mitochondria rich cell
mRNA	Messenger ribonucleic acid
NKA	Na^+,K^+-ATPase
NKCC	Na^+, K^+, 2 Cl^- co-transporter
PCR	Polymerase chain reaction
PNA	Peanut lectin agglutinin
Spiron	Spironolactone

Acknowledgments

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I would also like to extend my thanks to many of the members of the two labs that I was a part of during the past two years. Without the help of many of them, I am sure that the two years would not have passed by as smoothly or as quickly.

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Introduction

The rainbow trout, *Oncorhynchus mykiss*, is a euryhaline teleost that is able to migrate between freshwater and seawater environments. Salt and water balance in environments of different salinity is achieved through many physiological and morphological adjustments (Evans, 1975; McCormick, 1995; Perry, 1997; Marshall, 2002; Hirose *et al.*, 2003; Evans *et al.*, 2005). In freshwater (Fig. 1A), ion loss and water gain occur primarily across the gills according to the prevailing trans-branchial ionic and osmotic gradients. Ion loss is countered by active uptake of salt across the gills, and to a variable extent, by salt obtained via the diet (for review, see (Perry, 1997)). The opposite situation is present in marine environments (Fig. 1B), where ionic and osmotic gradients favour ion entry and water loss (Evans, 1975; McCormick, 2001). The active excretion of salt across the gills coupled with water absorption through drinking allows marine teleosts to maintain salt and water balance (McCormick, 2001).

One of the major morphological changes observed during the transition of euryhaline fish from freshwater to seawater environments involves the branchial cells that are responsible for the active uptake or excretion of salt (Hirose *et al*, 2003) Current models suggest that the cells primarily responsible for active salt uptake or excretion are mitochondria-rich cells (MRCs), also referred to in the literature as chloride cells. Of the three main types of cells found within the gills of teleost fish, pavement cells, mucous cells and MRCs, MRCs are found sparsely distributed throughout the entire filamental surface and interlamellar regions of the gill (Laurent and Perry, 1991). Pavement cells are the most abundant cell type and cover much of the lamellar and filamental surfaces,

while mucous cells are the least abundant cell type (Laurent and Perry, 1991). Additionally, in seawater acclimated fish there is a fourth type of cell present, known as the accessory cell, that is involved in sodium excretion (Wilson and Laurent, 2002). Two populations of MRCs have been identified in the gill epithelium of freshwater rainbow trout based on their ability to bind peanut lectin agglutinin (PNA), PNA^+ and PNA^-. These two populations of cells appear to possess different ionoregulatory abilities (see below) and are present in a ratio of approximately 35:65 PNA^+:PNA^- (Goss *et al.*, 2001; Galvez *et al.*, 2002; Hawkings *et al.*, 2004). Upon seawater transfer, cell populations of the gill change; there is a significant reduction in the number of PNA^- cells following one day of seawater transfer and by three days the population has disappeared (Goss *et al.*, 2001; Galvez *et al.*, 2002; Hawkings *et al.*, 2004). This change is accompanied by a concurrent increase in the size of the remaining PNA^+ cells (Goss *et al.*, 2001; Galvez *et al.*, 2002; Hawkings *et al.*, 2004). Following seawater transfer the seawater type MRC (often called a chloride cell) remains in the gill. Each of these cell types is thought to have a unique complement of ion-transporting proteins that allows it to carry out a particular function (Goss *et al.*, 2001; Galvez *et al.*, 2002).

Ionic Regulation

In the rainbow trout there are three major osmoregulatory organs that are involved in ionic regulation; each has a specific role in regulating ion concentrations.

Gill

The principal tissue involved in osmoregulation in teleost fish is the gill (Smith *et al.*, 1995). In both types of freshwater MRCs, Na^+,K^+-ATPase (NKA) is a predominant ion exchanger (Galvez *et al.*, 2002) that probably functions to establish appropriate gradients to drive ion uptake. Research has suggested that the PNA^- cells are the main site of H^+ excretion via an apical transport protein such as the V-type H^+-ATPase that helps to generate an electrochemical gradient favourable for Na^+ uptake (Galvez *et al.*, 2002). The PNA^+ cells are thought to be the site of Cl^- uptake via a Cl^--HCO_3^- exchanger (Galvez *et al.*, 2002); Cl^- then exits the cells via a basolateral anion channel (Marshall, 2002). As in freshwater teleosts, MRCs in the gills of seawater trout are characterized by the expression of high levels of NKA on the basolateral surface (Marshall, 2002). NKA maintains Na^+ and K^+ gradients across the membrane and acts as the energy source for the active movement of ions out of the fish. Gradients established by the activity of NKA drive the Na^+,K^+,2 Cl^- cotransporter (NKCC) to permit Cl^- entry into the cell (Marshall, 2002; Bystriansky *et al.*, 2006). Cl^- exits the cells via the cystic fibrosis transmembrane conductance regulator (CFTR), an apical Cl^- channel, while Na^+ is secreted down its electrochemical gradient via a paracellular route that allows the Na^+ to pass through the extracellular space in the gill and out between the cells (Perry, 1997; Marshall, 2002; Hirose *et al.*, 2003). These pathways are summarized in Fig. 2, which presents a cartoon of the ion exchange mechanisms found in the MRCs of the gill.

Kidney

In freshwater fish, excess water gained by osmosis is lost through the production of a hypoosmotic urine with a low ionic content (Salman and Eddy, 1988). Clearly there is a need to keep urine as dilute as possible so as not to compound the problems of ion loss. Thus, the kidney needs to be very efficient at reabsorbing ions that appear in the filtrate (Salman and Eddy, 1988). By contrast, the main role of the kidney in seawater teleosts is the excretion of divalent ions (Salman and Eddy, 1988).

Intestine

In seawater acclimated teleosts, the intestine plays an important role in osmoregulation. Water lost down osmotic gradients must be continuously replaced; to achieve this replacement, seawater acclimated fish drink seawater (Grosell, 2006; Taylor and Grosell, 2006). The ingested water travels through the gastrointestinal tract where water absorption is driven by active NaCl uptake, which is accomplished in large part by NKA located on the basolateral membranes of the intestinal epithelial cells (Smith, 1930; Grosell, 2006; Vijayan and Leatherland, 1992; Veillette and Young, 2005). In euryhaline species, transfer from freshwater to seawater results in several changes that enable the intestine to contribute to osmotic regulation. These include increases in drinking rate, increased activity of NKA, and increased surface area of the pyloric caecae (Fuentes and Eddy, 1997a; Veillette *et al.*, 2005).

Freshwater to Seawater Transition

The rainbow trout is a teleost species that has the ability to shift between freshwater and seawater environments. Salt and water balance in environments of different salinity is maintained through many physiological and morphological adjustments. During the transition from freshwater to seawater, gills of euryhaline fish undergo a transition from an ion absorbing organ to an ion excreting organ (Tipsmark *et al.*, 2002; Wilson *et al.*, 2002; Marshall *et al.*, 2005a; Bystriansky *et al.*, 2006). This phenomenon is most often associated with an increase in gill NKA activity that can be seen in MRCs as well as the closely associated accessory cells of the gill epithelium (Mancera and McCormick, 2000; Marshall, 2002; Bystriansky *et al.*, 2006). Increases in NKA activity have been found to be a reliable indicator of seawater adaptability in teleost fish; moreover, as the salinity of the water increases, so does the NKA activity (Dang *et al.*, 2000; Martinez-Alvarez *et al.*, 2005). Increased NKA activity reflects at least in part increased gene expression, since increases in the expression of NKA, as well as NKCC and CFTR mRNA, have been observed in the gill epithelium during freshwater to seawater transfer (Scott *et al.*, 2004). There appears to be two different NKA isoforms (NKA α1a and α1b) whose expression patterns vary with salinity exposure (Bystriansky *et al.*, 2006). In Atlantic salmon, rainbow trout and Artic char upon seawater transfer it was observed that the α1a subunit decreased significantly; where as, the α1b subunit increased significantly (Richards *et al.*, 2003; Bystriansky *et al.*, 2006). This could suggest that the α1b subunit has particular importance in seawater acclimation and that the two subunits have different roles in the gills of freshwater and marine fish (Richards *et al.*, 2003; Bystriansky *et al.*, 2006). One of the major morphological changes observed

during the freshwater to seawater transition involves the branchial cells that are responsible for the active uptake or excretion of salt, the MRCs. Upon transfer to seawater there is an obvious increase in drinking rates in salmonid fish (from 0.1 mL kg^{-1} h^{-1} to values in the range of 2-6 mL^{-1} kg^{-1} h^{-1}) which is accompanied by increases in NKA activity in both gill and intestine (Usher *et al.*, 1988; Fuentes and Eddy, 1997a). In the kidney there is a decrease in urine output, and production of an iso-osmotic rather than a hypo-osmotic urine (Salman and Eddy, 1988). In the intestine, increases in ion and water permeability together with increased ion uptake result in increased water uptake across the intestinal epithelia (Takahashi *et al.*, 2005). The pyloric caecae of the intestine increase the absorptive surface area of the intestine thereby aiding in water uptake and overall osmoregulation (Veillette *et al.*, 2005). Thus, it has been shown that a good molecular marker that can be used during seawater transfer to indicate the development of a seawater phenotype is: an increase in NKA (Marshall *et al.*, 2005b; Singer *et al.*, 2007). Other indicators of the seawater phenotype are increased drinking rate, a reduced production of urine, and increased NKA activity in the gill and intestine.

Salt Fed Rainbow Trout

Although the physiological and gene expression changes that accompany the transition from freshwater to seawater are increasingly well documented and explained (Madsen, 1990; Daborn *et al.*, 2001; Dean *et al.*, 2003; Hawkings *et al.*, 2004; Marshall *et al.*, 2005a; Bystriansky *et al.*, 2006), the regulatory pathways governing this transition remain poorly understood. Rainbow trout fed a NaCl-enriched diet for ~1 month exhibit proliferation of branchial MRCs and increased branchial NKA activity (Salman and

Eddy, 1987), both changes suggestive of a transition from a freshwater to seawater phenotype. Recent work indicates that high salt feeding alone brings about a morphological transition of the gill epithelium in which seawater-type MRCs appear (Perry *et al.*, 2006). These cells exhibit characteristics typical of seawater fish, such as the appearance of accessory cells whose apical membranes share a common apical crypt with the MRCs (Perry *et al.*, 2006) (Fig. 3). Moreover, the morphological changes were accompanied by increased mRNA expression of three ion transporters, NKCC, NKA and CFTR; again, a response typically observed upon transfer of fish from freshwater to seawater (Perry *et al.*, 2006). Thus, salt feeding appears to induce a seawater phenotype at the gill. This suggests a model that is useful in investigating these pathways is dietary salt loading in freshwater fish (Salman and Eddy, 1987; Salman and Eddy, 1988; Perry *et al.*, 2006). One of the advantages of salt-feeding over seawater acclimation is that only the internal environment changes, not the external environment. Thus, the salt-feeding model may be a useful tool in determining what specific factors are involved in triggering the transition from the freshwater to seawater phenotype. Moreover, it is logistically a more convenient model to use in a lab that does not have access to seawater. This method of dietary salt loading is non-invasive, which is useful when studying variables such as stress hormone levels (see below) (Smith *et al.*, 1995). Although a few studies have characterized aspects of the salt-fed fish model, such as ion transport (both uptake and excretion) in the gill, morphology of the gill and some changes in ion transporter mRNA in the gill, little work has been carried out on the kidney or intestine, the two other major osmoregulatory organs in fish. The regulation of these changes, not only in the kidney and intestine but in the gill also, remains poorly

understood. However, there have been suggestions that the hormone cortisol may play a role in the development of the seawater phenotype (Madsen, 1990; McCormick, 2001; Marshall *et al.*, 2005b).

Cortisol

The mechanisms that regulate the morphological and physiological changes that accompany freshwater-to-seawater transitions (and vice versa) remain poorly understood. However, evidence suggests that important roles are played by several hormones, including thyroid hormones, prolactin and cortisol (reviewed by Bern & Madsen, 1992; McCormick, 2001). Cortisol, in particular, has been identified as a "seawater-adapting hormone", although recent work also suggests a role for cortisol in acclimation to dilute environments (McCormick, 2001; Sloman *et al.*, 2001). Cortisol has also been implicated in causing the increase in gill and intestinal epithelial NKA activity and the activity of the NKCC cotransporter seen in seawater-acclimated teleosts (Pelis and McCormick, 2001). While the receptors through which cortisol evokes its ionoregulatory and osmoregulatory actions remain unclear, many effects of cortisol have been identified. For example, cortisol treatment yields increases in branchial, renal and intestinal NKA activity (Laurent and Perry, 1990; Madsen, 1990; McCormick, 1995; Takahashi *et al.*, 2005; Veillette and Young, 2005). Cortisol has been shown to increase MRC numbers in various teleost fish, such as the American eel, rainbow trout, and Coho salmon (Madsen, 1990; McCormick, 1995). Cortisol probably functions as both a glucocorticoid and mineralocorticoid hormone in fish, but the receptor types responsible

for specific effects (McCormick, 2001; Takahashi *et al.,* 2005), have yet to be fully elucidated.

In rainbow trout, cortisol is released into the bloodstream via the hypothalamic-pituitary-interrenal (HPI) axis (Bonga, 1997). Nuclei located within the hypothalamus of the brain elicit the secretion of corticotropin releasing hormone (CRH) into the anterior pituitary, thereby stimulating the release of adrenocorticotropic hormone (ACTH) into the circulation. ACTH then acts on interrenal cells in the head kidney to elicit the synthesis of cortisol and its secretion into the bloodstream. This pathway is governed by negative control feedback mechanisms that regulate the concentration of cortisol in the blood plasma; cortisol itself feeds back negatively at both the level of the anterior pituitary to regulate ACTH release, and at the level of the hypothalamus to regulate CRH release (Balm *et al.,* 1994; Sloman *et al.*, 2002).

Cortisol Receptors

Cortisol, the main corticosteroid hormone in rainbow trout, is believed to act as both a glucocorticoid and mineralocorticoid hormone, being involved in the regulation of, respectively, metabolism and mineral homeostasis (Bonga, 1997). It is known to bind to and activate both types of corticosteroid receptors (GR and MR). Interestingly, the GR and MR in fish have been named according to the similarity of the sequences of the receptor types to corresponding sequences for mammals, where there is a two hormone, two receptor type system and where the two receptor types mediate the processes for which they are named (Sturm *et al.*, 2005). To date, in fish GRs are known to mediate

both metabolic and iono/osmoregulatory events (Bury *et al.*, 2003) but the function(s) of the MR remains unclear (Sturm *et al.*, 2005)

The GR was originally cloned from the rainbow trout liver and was shown to be similar in structure to the receptors of the steroid, thyroid, vitamin D and retinoic acid receptor superfamily (Ducouret *et al.*, 1995). The steroid receptor family consists of multiple receptor types: corticosteroid receptors (GR/MR); estrogen receptors (ERα/ERβ); progesterone receptor; and the androgen receptor (Bury *et al.*, 2003). Unusually, a nine amino acid insertion occurs in the trout GR between the two zinc fingers in the DNA-binding domain (Ducouret *et al.*, 1995). All of the receptors in this family have a DNA binding domain flanked by a variable amino terminal, and a carboxyl terminal which acts as the site for hormone binding (Cosfo *et al.*, 2005). Glucocorticoid hormones and the GR are found in all vertebrates that have been examined (Flik *et al.*, 2006). It is believed that there was an ancestral corticosteroid receptor that gave rise to the steroid receptor types, including the MR and GR's (Bury and Sturm, 2007). In many teleost species there are multiple glucocorticoid receptors which are thought to have arisen from a whole genome duplication event that occurred early in the teleostean lineage (Bury and Sturm, 2007).

Trout possess two forms of the GR, GR1 and GR2, as well as a recently-identified MR (Ducouret *et al* 1995; Colombe *et al.*, 2000; Bury *et al* 2003; Sturm *et al* 2005). The two isoforms of the GR are thought to be the result of a gene duplication that occurred relatively early in the teleost lineage, more specifically after the split of sturgeons and paddlefish (Acipenseriformes) from gar (Semionotiformes) and bowfin (Amiiformes), but before the divergence of the Osteoglossiformes; thus about 355 million years ago

(Fig. 5) (Bridgham *et al.*, 2006; Prunet *et al.*, 2006; Bury and Sturm 2007). Moreover, the GR1 undergoes alternative splicing in rainbow trout, yielding GR1a and GR1b forms that differ by only a 9 amino acid sequence (Flik *et al.*, 2006). Two alternative splicing forms of the MR also exist, termed A and B (Sturm *et al.*, 2005; Tallec and Lombres, 2005).

The corticosteroid receptors are arranged into four distinct functional domains (Bury and Sturm, 2007). The A/B domain is a variable domain located at the amino end of the receptor and is involved in the modulation of transcriptional activity, whereas, the C domain is a highly conserved DNA binding domain (Bury and Sturm, 2007) consisting of two zinc fingers separated by a short amino acid sequence. This amino acid sequence binds to glucocorticoid response elements (GRE) located in the promoter region of genes under the control of GR or MR genes so as to initiate their transcription or repression (Flik *et al., 2006*). Interestingly, both the MR and GR operate via the same hormone responsive elements (HRE) in the genome. The common HRE can be activated by either MR or GR homodimers or by heterodimers (Marshall *et al.*, 2005b). This arrangement makes it possible for cortisol to bind to either receptor type to induce transcription of, for example, seawater adaptive genes (Marshall *et al* 2005b). The D domain is involved in mediating conformational changes of the receptor, and finally, the E domain is located at the carboxy end of the receptor and is the domain responsible for ligand binding activity (Prunet *et al.*, 2006).

In mammals, activation of MRs by aldosterone plays a key role in regulating transepithelial sodium transport in the kidney (Bonvalet, 1998) Because the circulating levels of cortisol in mammals are 100 to 1000 fold greater than those of aldosterone, the

MR would be expected to be occupied by cortisol the majority of the time (Farman, 1999). However, in mammals the enzyme 11 β-hydroxysteroid dehydrogenase 2 (11HSD2) is co-localized with MR in aldosterone-activated tissues (e.g. kidney) (Kusakabe *et al.*, 2003). This enzyme catalyzes the conversion of cortisol to the inactive metabolite, cortisone, thus allowing opportunity for the mineralocorticoid aldosterone to bind to the MR (Edwards *et al.*, 1988). To date, research suggests that teleost fish lack aldosterone (Sturm *et al.*, 2005; Prunet *et al.*, 2006) and therefore may utilize cortisol for the corticosteroid-based regulation of salt and water balance, as well as the regulation of metabolism. However, a fish homologue of 11HSD2 was recently identified in the gills, intestine, heart and Leydig cells of rainbow trout and is able to convert cortisol into cortisone suggesting that an alternative mineralocorticoid hormone to cortisol may be present (Kusakabe *et al.*, 2003). This situation led (Sturm *et al.*, 2005) to propose 11-deoxycorticosterone (11-DOC) as an alternative mineralocorticoid.

The rainbow trout GR2 (EC_{50} = 404.8 ng mL^{-1}) is more sensitive to cortisol than the GR1 (EC_{50} = 57.6 ng mL^{-1}) (Bury *et al.*, 2003). In non stressful or mildly stressful conditions, the levels of plasma cortisol (<10 ng mL^{-1} resting; Gamperl *et al.*, 1999) are high enough to activate GR2 but not GR1, whereas, GR1 is activated during highly stressful conditions (Prunet *et al.*, 2006). This pattern suggests that there are distinct roles for the two receptors, with GR2 being the receptor involved in mediating the GR-activated effects of the stress response during periods of low stress, and the GR1 being more involved in mediating the GR-activated effects in more stressful conditions (Prunet *et al.*, 2006). On the other hand, the MR in teleost fish (as in tetrapod vertebrates) has a higher sensitivity to cortisol than either of the GRs; also that cortisol is about ten times

more active in stimulating the MR than aldosterone (Colombe *et al.*, 2000; Greenwood *et al.*, 2003; Prunet *et al.*, 2006). Immunocytochemical techniques have revealed that the glucocorticoid receptor (GR) mRNA is prominent in the epithelial cells of the posterior intestine of the teleost fish, Mozambique tilapia (*Oreochromis mossambicus*), and an increase in GR mRNA was observed in the intestinal epithelia of seawater acclimated tilapia (Takahashi *et al.*, 2005). Dean *et al.* (2003) reported an increase in GR mRNA in gill tissue of Mozambique tilapia during seawater acclimation (Dean *et al.* 2003; Scott *et al.*, 2004). Although cortisol is capable of activating the GR1, GR2 and MR receptors, the relative roles of the different receptors in osmoregulation have yet to be established.

Hypotheses and Predications

Given this background I hypothesized that the phenotype that develops when rainbow trout are fed a high salt diet is mediated by increases in the circulating levels of cortisol and/or by increases in the expression of the glucocorticoid and/or mineralocorticoid receptor. The two predictions derived from this hypothesis that were used for this study are the following:

1. An increase in circulating cortisol will be observed when fish consume a high salt diet.
2. An increase in the mRNA expression of the glucocorticoid and/or mineralocorticoid receptor will be observed when fish consume a high salt diet.

To better understand the phenotypic changes occurring as a result of dietary salt loading, I investigated the time course of these changes with the objective of relating the

phenotypic changes to modifications in either circulating cortisol levels or corticosteroid receptor expression. Although cortisol has been implicated in the increase in gill and intestinal epithelial NKA activity and the activity of the NKCC cotransporter in seawater-acclimated teleosts (Pelis and McCormick, 2001) it is as yet unclear whether cortisol is involved in the changes that occur in high salt fed rainbow trout and if so what receptors mediate the response. A combination of physiological, biochemical and molecular experimental approaches comparing the salt fed fish groups to matching control groups were adopted to achieve this goal.

Changes in NKA activity were used as a marker for high salt diet-induced changes in gill, intestine and kidney tissues. Light microscopy was used to identify and quantify MRC numbers within gill tissue of seawater-acclimated, high salt fed, and control fish (Garcia-Romeu and Masoni, 1970). Plasma cortisol concentrations and corticosteroid receptor mRNA expression were also assessed.

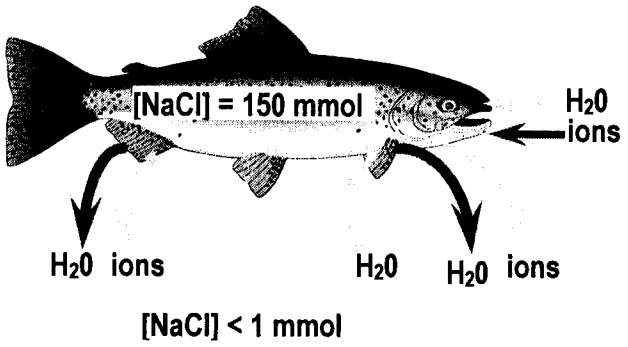
The data collected in the time course experiment were correlative in nature, and from there the goal was to establish causality. To this end, corticosteroid receptor blockade was utilized in an attempt to eliminate the phenotype evoked by the salt feeding protocol. GR and MR antagonists (RU486 and spironolactone, respectively) were mixed with the food and administered to the fish during high salt feeding.

Thus, there are two main points that I hope to establish: first, to shed light on the roles of GR and MRs in ionic and osmotic regulation. Second, that the salt feeding

model is useful because of the possibility that it allows one to distinguish between the roles of internal versus external receptor-mediated pathways in eliciting the morphological and physiological changes that accompany salinity transitions. Although many studies have been carried out on the changes that occur in teleost fish during seawater acclimation (Daborn *et al.*, 2001; Hawkings *et al.*, 2004; Bystriansky *et al.*, 2006) and the possibility of a linkage to cortisol (Madsen, 1990; Dean *et al.*, 2003; Marshall *et al.*, 2005a) only a few have studied the changes that occur during salt feeding, and little is known about the mechanisms through which these changes occur in salt fed fish.

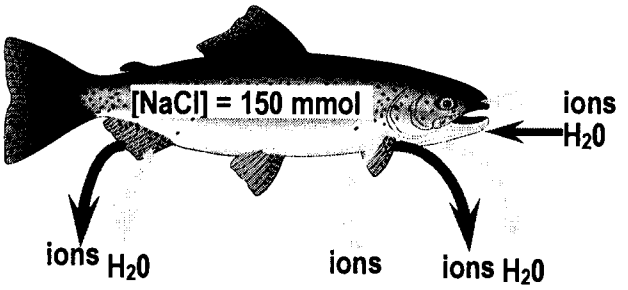
Figure 1. A schematic illustrating the major differences in the directions of water and ion flow in a teleost fish in environments of different salinity. In freshwater (Fig. 1A), ion loss and water gain occur primarily across the gills according to the prevailing trans-branchial ionic and osmotic gradients. The opposite situation is present in marine environments (Fig. 1B), where ionic and osmotic gradients favour ion entry and water loss. Solid red arrows represent active processes, dashed blue arrows represent passive processes.

A. Freshwater



[NaCl] < 1 mmol

B. Salt water



[NaCl] ~500 mmol

Figure 2. A schematic that highlights the differences in ionic regulation at the gill between freshwater and seawater teleost fish. MRC – mitochondria-rich cells; A – accessory cells; Na^+ - sodium ion; Cl^- - chloride ion; H^- - hydrogen ion; K^+ - potassium ion; HCO_3^- - bicarbonate ion; NKCC – $Na^+/K^+/2Cl^-$ cotransporter.

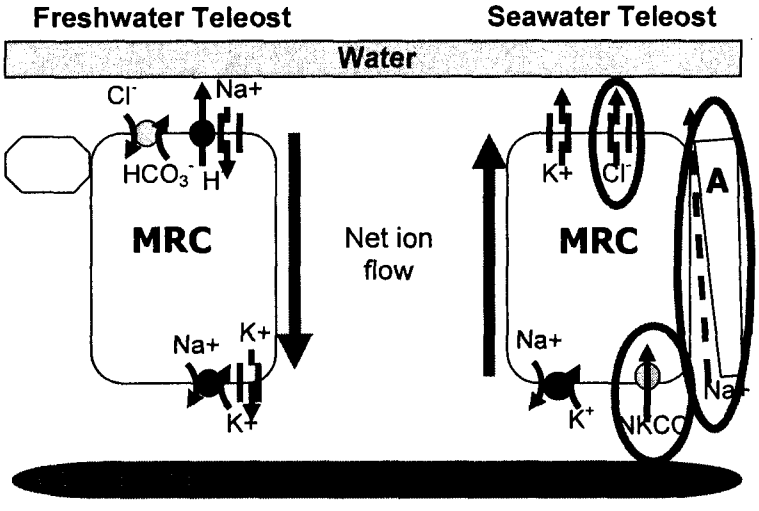

Freshwater Teleost
Seawater Teleost
Water
Cl-
Na+
HCO3-
H+
MRC
Net ion flow
Na+
K+
K+
K+
Cl-
MRC
A
Na+
K+
NKCC
Na+

Figure 3. Representative transmission electron microscope images of gill tissue from freshwater rainbow trout (*Oncorhynchus mykiss*) fed A) normal trout chow or B) NaCl-enriched trout chow. The arrow in panel B indicates where the apical membranes of the accessory cell and MRC are joined in a common apical crypt; whereas, the arrowhead in panel A indicates the flat apical membrane of the MRC. CC –mitochondrial rich cells; pvc – pavement cells; AC – accessory cells (Perry *et al.*, 2006). Scale bar represents 20 μm.

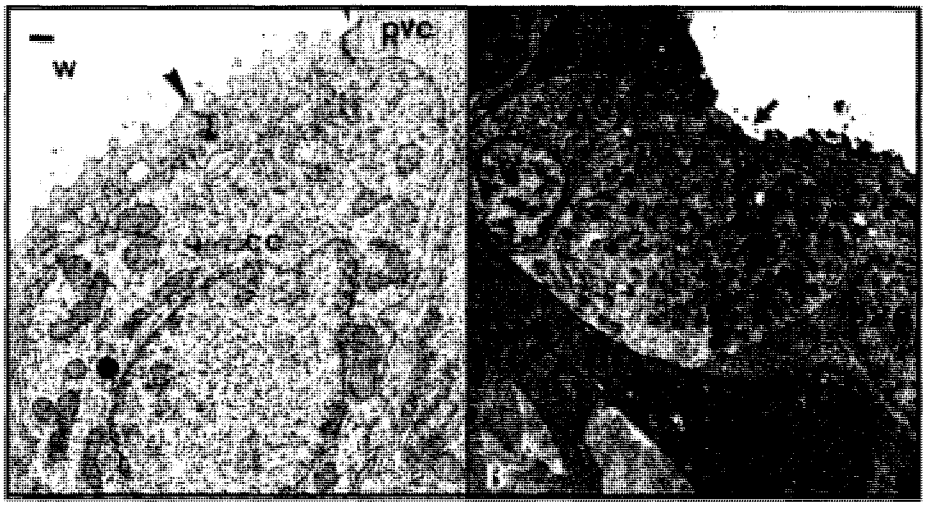

W
RVC
CC
b

Figure 4. A schematic of the pathway governing cortisol release in teleost fish.

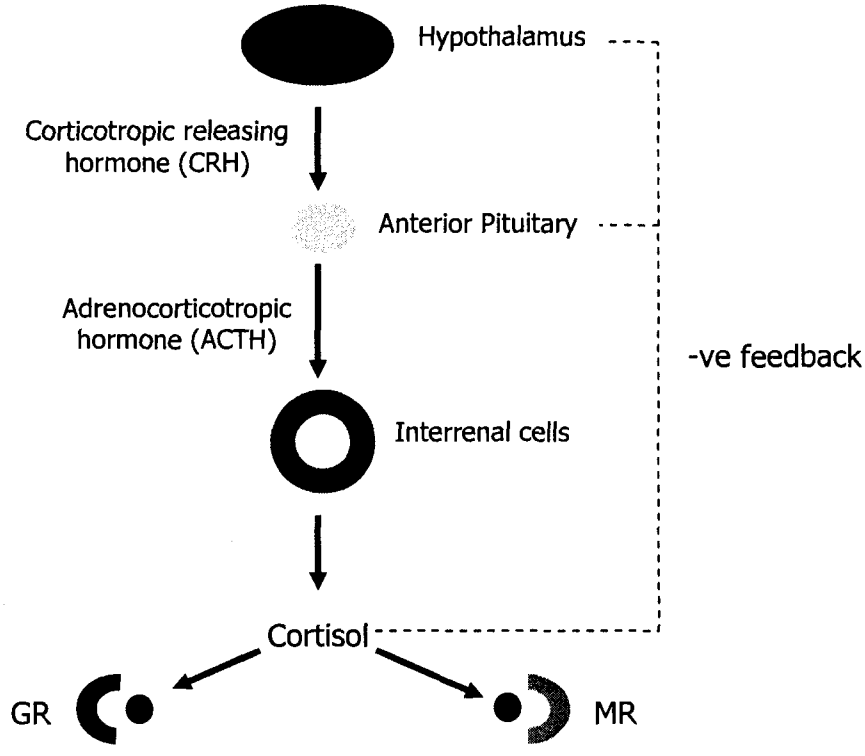

Hypothalamus
Corticotropic releasing hormone (CRH)
Anterior Pituitary
Adrenocorticotropic hormone (ACTH)
-ve feedback
Interrenal cells
Cortisol
GR
MR

Figure 5. A phylogenetic tree (Bury and Sturm 2007) showing the evolution of the GR and MR's. Multiple types of corticosteroid receptors appear to have evolved from an ancestral receptor.

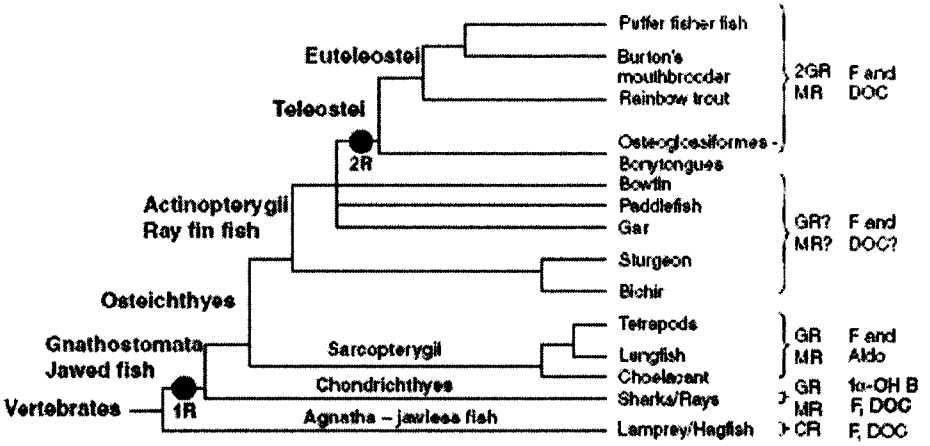
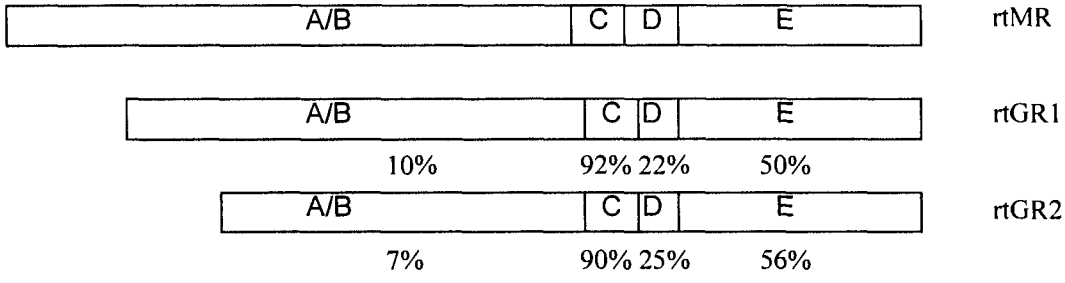

Euteleostei
Teleostei
2R
Actinopterygii
Ray fin fish
Osteichthyes
Gnathostomata
Jawed fish
Vertebrates
1R
Sarcopterygii
Chondrichthyes
Agnatha – jawless fish
Puffer fisher fish
Burton's mouthbrooder
Rainbow trout
Osteoglossiformes - Berytongues
Bowfin
Paddlefish
Gar
Sturgeon
Bichir
Tetrapods
Lungfish
Coelacanth
Sharks/Rays
Lamprey/Hagfish
2GR MR F and DOC
GR? MR? F and DOC?
GR MR F and Aldo
GR MR 1α-OH B F, DOC
CR F, DOC

Figure 6. Adapted from (Prunet *et al.*, 2006), this figure indicates the percent amino acid identity between the four domains of the corticosteroid receptors. As discussed in text.


A/B
C
D
E
rtMR
A/B
C
D
E
rtGR1
10%
92%
22%
50%
A/B
C
D
E
rtGR2
7%
90%
25%
56%

Materials and Methods

A. Experimental animals

Rainbow trout, *Oncorhynchus mykiss* were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and housed indoors at the University of Ottawa in circular fibreglass tanks supplied with flowing, aerated dechloraminated city of Ottawa tap water at 13°C. Fish were held in this facility for a minimum of three weeks before experimentation, were maintained on a 12h:12h light:dark photoperiod, and were fed daily at 12pm with commercial trout food. Fish were sampled between the hours of 12-2pm. Experimental protocols involving animals were approved by the University of Ottawa Animal Care Committee and carried out in accordance with the guidelines of the Canadian Council on Animal Care.

B. Experimental protocol

Series one: Salt feeding timeline

The goal of this series of experiments was to resolve the time course over 30 days of changes that occur as a result of salt feeding in freshwater rainbow trout. Fish were weighed and divided into four sets of two groups (6 fish per group for a total of 8 groups) housed in separate tanks. One group from each set was fed regular commercial trout food at 1% body mass per day while the second group from each set was fed a diet supplemented with 11% NaCl. To obtain 11% NaCl food, 1 kg of regular commercial trout food was ground up and mixed with 110 g of NaCl and 400 mL of water. Food was processed into small pellets and left to dry for 24 h before use. To ensure that both

groups of fish were receiving similar daily caloric intakes, salt-fed fish were fed 11% more food per day than the control group. All food was consumed within 20 minutes of feeding. After 3 (control: N=6; 69.1± 5.51 g; saltfed: N=6; 65.18 ± 8.24 g), 6 (control: N=6; 77.75± 6.39 g; saltfed: N=6; 80.15 ± 6.72 g), 14 (control: N=9; 128.14± 11.38 g; saltfed: N=9; 106.78 ± 12.27 g; this group as a whole was larger in mass than the others), or 30 (control: N=9; 96.87± 7.58 g; saltfed: N=9; 94.60± 10.36 g) days of control diet or salt feeding, fish were killed by anaesthetic overdose (0.1g L^{-1} benzocaine), a blood sample was withdrawn by caudal puncture, and tissues were sampled. Blood samples were centrifuged at 10,000 *g* for 1 min to separate plasma from red blood cells, and the plasma was flash frozen and stored at -80°C for later analysis of cortisol and ion concentrations. Gill tissue, kidney tissue, and intestinal epithelial cells were removed and either flash frozen in liquid N_2 then stored at -80°C for later analysis of NKA activity (in this case tissues were stored in SEI buffer composed of 250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, at a pH of 7.3) or corticosteroid receptor mRNA expression, or (in the case of the gill) fixed in a solution of ZnI/OsO_4^- (see protocol in later section) for later analysis of gill morphology. Intestinal epithelial cells were obtained by ligating and then removing the posterior portion of the gastointestinal tract, cutting the intestine open longitudinally and scraping the epithelial cells onto an ice cold glass microscope slide. Finally, separate groups of fish fed control or salt-enhanced diet for 30 days (control: N=6; 99.67± 4.30 g; saltfed: N=6; 92.00 ± 8.04 g) were exposed to [^{3}H] PEG-4000 (Perkin Elmer, Boston MA, USA) for the measurement of drinking rates. Note: experiments using 3, 6, and 30 day exposures were performed at the same time; the 14 day exposure was carried out approximately 6 months later.

Series two: Receptor antagonist treatment

Fish were divided into five groups: a regular diet control group (transferred to seawater after 30 days; N = 6; 135.43 ± 13.24 g), an RU486-treated regular diet group (transferred to seawater after 30 days; N = 6, 68.81 ± 3.17 g), a control group fed the salt-enriched diet sprayed with vehicle (not transferred to seawater; N = 18; 111.43 ± 7.35 g), an RU486-treated salt-fed group (not transferred; N = 18; 97.06 ± 5.40 g), and a spironolactone-treated salt-fed group (not transferred; N = 18, 76.52 ± 4.00 g). Following a one week acclimation period during which fish were fed a daily diet of regular trout chow at 1% of their body weight between the hours of 12 pm and 2 pm, the fish were fed different diets according to their experimental group. Fish treated with the corticosteroid receptor antagonists RU486 and spironolactone were fed a dose of either 40 mg kg^{-1} fish per day (RU486) or 0.1 mg kg^{-1} fish per day (spironolactone); in either case, the drug was dissolved in 95% ethanol (0.5 mL g^{-1} of food) and sprayed evenly onto either regular fish pellets or fish pellets prepared with 11% NaCl (DiBattista *et al.*, 2006; McDonald and Wood, 2004). To control for the ethanol vehicle, a control group was fed the high salt diet sprayed with 95% ethanol only. Fish were terminally sampled after 6, 14, or 30 days of feeding with treated food. Two groups were transferred into 50% seawater after 30 days of feeding; following 24 h in 50% seawater, these fish were transferred to 65% seawater and were terminally sampled after 3 days of exposure.

Blood samples were centrifuged at 10,000 *g* for 1 min to separate plasma from red blood cells, and the plasma was flash frozen and stored at -80°C for later analysis of cortisol and ion concentrations. Gill tissue, kidney tissue, and intestinal epithelial cells (obtained as described above) were removed and either flash frozen in liquid N_2 then

stored at -80°C for later analysis of NKA activity (in this case tissues were stored in SEI (sucrose, EDTA, imidazole) buffer as above) or corticosteroid receptor mRNA expression, or (in the case of the gill) fixed in a solution of ZnI/OsO_4 (see protocol in a later section) for later analysis of morphology.

C. Analytical techniques

Drinking Rates

Drinking rates were determined for control fish and fish fed the high salt diet for 30 days (control: N=6; 99.67± 4.30 g; saltfed: N=6; 92.00 ± 8.04 g). Drinking rates were measured according to the method of Wilson *et al.*, (1996). In brief, fish were transferred to individual 6 L opaque experimental chambers supplied with flowing, aerated and dechloraminated city of Ottawa tap water at 13°C, 24 h before experimentation. To begin the drinking rate measurement, water flow to the individual chambers was stopped and 10 MBq of [^{3}H] PEG-4000 (Perkin Elmer, Boston MA, USA) was added to each chamber to achieve a concentration of 185 MBq l^{-1}. Water samples (10 mL) were withdrawn after 15 min and 6 h; 1 mL of each water sample was mixed with 10 mL of scintillation fluid (Safety-Solve scintillation cocktail was obtained from Research Products International, Mount Prospect, IL, USA.) and counted for radioactivity using a Beckman-Coulter LS6500 scintillation counter. After 6 h, fish were killed by the addition of an anaesthetic overdose (0.1g L^{-1} benzocaine) to each individual experimental chamber, and the entire gastrointestinal tract (from the oesophagus to the rectum) was exposed by dissection, ligated at either end and removed. Gastrointestinal tracts were homogenized in five volumes of 8% $HClO_3$ and a 1 mL aliquot of this homogenate was mixed with 10 mL of

scintillation fluid (Safety-Solve scintillation cocktail was obtained from Research Products International, Mount Prospect, IL, USA.) and counted for radioactivity (Beckman-Coulter LS6500 scintillation counter).

Drinking rates were calculated using the following equation as described by (Pyle *et al.*, 2003):

$$D = C/MtW$$

where D represents the drinking rate in mL kg^{-1} h^{-1}, C is the activity of the gut homogenate (in cpm), M is the activity of the water (in cpm), t is time (in h), and W is the mass of the fish (in kg).

Plasma cortisol and ions

Plasma cortisol levels were measured using a commercial radioimmunoassay kit (MP Biomedical; Orangeburg, NY, USA). Plasma Na^+, K^+ and Ca^{2+} concentrations were determined by flame atomic absorption spectrophotometry (Varian). Plasma Cl^- concentration was determined using a colorimetric assay based on thiocyanate liberation from mercuric thiocyanate (Zall *et al.*, 1956). The original assay procedure was scaled for use with a Spectramax 340PC microplate reader (Molecular Devices).

Na^+, K^+-ATPase assay

A kinetic microplate assay was used to determine Na^+,K^+-ATPase activity (McCormick, 1993). Gill, kidney or intestine tissues stored at -80°C were homogenized,

using a hand held power homogenizor, in 250 μL of SEID (150 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, and 0.1% v/v deoxycholic acid at a pH of 7.3) and then centrifuged for 30 s at 5000 *g*. The resulting supernatant was assayed in duplicate. Supernatants were also assayed (in triplicate) for protein content using the bichinchonic acid method (BIORAD) as described in the manufacturer's manual. Ouabain-sensitive ATPase activity was measured and expressed in units of μmoles ADP mg^{-1} protein h^{-1} and compared to ATPase activity in the absence of ouabain.

Microscopy

To obtain gill tissues for morphometric analysis, the first gill arch was excised and rinsed in 0.9% saline. Small clusters of 4-5 filaments were then fixed and stained in an osmium tetroxide-zinc iodide preparation according to the method of (Garcia-Romeu and Masoni, 1970). The fixative was one part 2% OsO_4 to three parts ZnI_2, and after preparation was stored for 24 h at room temperature. This fixative yields a reduction of osmic acid to osmium, thereby staining black the thick phospholipid layers of the plasma membrane of branchial MRCs. Following fixation/staining, tissues were rinsed in 1X phosphate buffered saline containing Tween-20 (PBST) for 15 min. Gills were then cryoprotected by immersion in a 15% sucrose solution overnight, followed by immersion in 30% sucrose indefinitely. Thin (8 μm) sections were obtained using a cryostat (Leira CM 1850) at -30°C, allowed to dry overnight, and then mounted in 60% glycerol and cover-slipped for later analysis.

Morphometric analysis was carried out on gill tissue from four fish from each experimental group (3, 6, 14 and 30 day control and salt-fed fish). A light microscope (Zeiss Axiophot) fitted with a camera (Hamamatsu C5985 CCD) was used to capture images from areas selected randomly in the interior regions of gill filaments; all images were captured at 40x magnification (4 pictures per section, 2 sections per slide, 4 slides per fish; for a total of 32 pictures per fish). Digital images were analysed using Scion imaging software (Scion, Frederick, MD, USA) to estimate the fractional surface area of the gill occupied by MRCs.

Total RNA extraction and cDNA synthesis

RNA extraction and reverse transcription were carried out to obtain the cDNA necessary for real time RT-PCR analysis of corticosteroid receptor mRNA expression. Collected tissue samples were ground on dry ice, and RNA extraction was performed using the Trizol reagent extraction method as described by the manufacturer (Invitrogen Life Technologies; Burlington, Ontario, Canada). Trizol (1 mL per 100 mg of tissue) was added to each sample, and tissues were passed several times through a 22½ gauge needle using a 3 mL syringe before being centrifuged at 12,000 *g* for 10 min at 4°C. Following incubation at room temperature for 5 min, 0.2 mL of chloroform was added per mL of Trizol, and samples were centrifuged again at 12,000 *g* for 15 min at 4°C. The aqueous phase was transferred to a new tube and the RNA was precipitated overnight at -20°C by addition of isopropyl alcohol (0.5 mL per mL of Trizol). Samples were treated as per manufacturer's description and were then stored at -80°C for further analysis.

RNA was reverse transcribed into cDNA using the Revertaid H Minus M-MuLV reverse transcriptase enzyme (Fermentas; Burlington, Ontario, Canada). RNA samples were treated with DNAse (Invitrogen Life Technologies, Burlington, Ontario, Canada) before cDNA synthesis. Synthesis of cDNA was carried out following the Revertaid H manufacturer's method with one slight change: after the final incubation, samples were diluted 2x with deionized, distilled H_2O and stored at -20°C for further analysis.

Real time RT-PCR

Real time reverse transcriptase PCR (RT-PCR) was used to determine the relative abundance of GR1, GR2, and MR mRNA in gill, kidney and intestine. Primer pairs were designed against rainbow trout sequences for each of the three corticosteroid receptor genes: GR1 (GenBank accession number Z54210), GR2 (GenBank AY495372), and MR (GenBank AAS75842) and against the control gene 18S. Primers were designed using the DNAMAN computer package (Lynnon Corporation) and were specific to isoforms but not to splice variants. All primers were designed with melting temperatures between 58 and 62°C, and were tested by regular RT-PCR to ensure that they were specific to the gene for which they were designed, and that no primer dimers were present. Product sizes obtained using the primers were: 149 bp, 149 bp, and 250 bp, for respectively, the GR1, GR2 and MR, while for 18S the product was 230bp in size; all products were sequenced to ensure specificity). The primer sequences that were used can be found in table 1.

Real-time RT-PCR was carried out using an MX 3000 Multiplex Quantitative PCR System (Stratagene) and the Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) as per kit instructions, but with several modifications. Final reaction volume was reduced to 12.5 μL (from 25 μL); 1 μL of cDNA was added per reaction to 6.25 μL of master mix, 3 μL of DEPC H_2O, and 1 μL of 2 mmol primers. After the initial annealing step at 95°C for 10 min, 40 PCR cycles were performed with each cycle consisting of: a denaturation step at 95°C for 15 s; followed by an annealing step at 59°C for 30 s; and an extension step at 72°C for 30 s. Standard curves were generated for each primer set using serial dilutions of trout gill cDNA. A dissociation curve was created at the end of each run to determine the purity of amplification products from each reaction. Negative controls in which cDNA was omitted from the reaction were run alongside all reactions to check for genomic contamination. In addition, no reverse transcriptase controls were carried out.

D. Statistical analysis

All data are presented as mean values ± the standard error of the mean. All statistical analyses were carried out using Sigma Stat 3.0 (SPSS, Chicago, IL, USA). Two-way analysis of variance (ANOVA) was used to assess the statistical significance of all results except drinking rates, where an unpaired Student's *t*-test was used. In 2-way ANOVAs, the factors analysed were time and treatment, and this analysis allowed assessment of interactions between these two variables. One-way ANOVAs were used to analyze the results of the antagonist experiments. The fiducial limit of significance in all cases was set at 0.05.

Table 1. Primer sequences designed, tested and used for the glucocorticoid receptors (GR1 and GR2), the mineralocorticoid receptor, and for the control gene 18S.

Primer	**Direction**	**Sequence**
GR1	forward	5'-CCATCGTCAAGCGGGAAGAG-3'
GR1	reverse	5'-GGAACTCCACGCTAAGGGATTTATTC-3'
GR2	forward	5'-CTCCGCTTTCTCCAGCAGCTA-3'
GR2	reverse	5'-GTGAGCCACCCCGTAGTGACAG-3'
MR	forward	5'-CAACGTAGGCCTGGACCACATG-3'
MR	reverse	5'-GGACGGACCTGACTGGAAGAGAC-3'
18S	forward	5'-GGCGGCGTTATTCCCATGACC-3'
18S	reverse	5'-GGTGGTGGCCTTCCGTCAATTC-3'

Results

1. Series one: Salt feeding timeline

Trout fed a high salt diet for 30 days exhibited drinking rates that were approximately two fold higher (P = 0.038) than fish fed a normal diet (Fig. 7). In both cases, drinking rates were typical of those of freshwater rainbow trout (Pyle *et al.*, 2003).

Plasma cortisol concentrations were unaffected by treatment, time or the interaction between the two (2-way ANOVA, $P = 0.712$, 0.089 and 0.892) (Fig. 8). No significant differences with treatment or the interaction of time and treatment were observed for any of the four plasma ions assayed (Fig. 9 A-D). There were however significant effects of time on all plasma ion levels. For sodium, chloride, potassium and calcium, 2-way ANOVA analysis yielded the following results: (A) $P = 0.906$, 0.001 and 0.425 for, respectively, the effect of treatment, the effect of time and the interaction of these two factors; similarly, (B) $P = 0.405$, 0.006 and 0.552; (C) $P = 0.720$, 0.001 and 0.092; and (D) $P = 0.379$, 0.001 and 0.314. As a group the 3 day fish had higher sodium, chloride, and calcium than the rest of the time points (Fig. 9 A-D). There were no significant interactions therefore comparisons can only be made for time; control and salt-fed fish at a given time point will be grouped together. Thus, the observed effects are not significant for this study.

A high salt diet resulted in elevated gill and kidney NKA activity, but reduced intestinal NKA activity. More specifically, kidney NKA activity was significantly elevated at 14 days in salt-fed fish (2-way ANOVA, $P = 0.003$, 0.703 and 0.048 for, respectively, the effect of treatment, the effect of time and the interaction of these two

factors; Fig. 10B); whereas, there was no significant impact of time on NKA activity in any tissue in the control group. NKA activity was approximately three-fold higher in salt fed fish than in control fish in gill tissue after 30 days (2-way ANOVA, $P = 0.034$, 0.026 and 0.046 for, respectively, the effect of treatment, the effect of time and the interaction of these two factors; Fig. 10A). In addition, gill NKA activity tended to increase with time in the salt-fed fish but not the control group. No significant effects of time or the interaction of time and treatment were observed in intestinal NKA activity, but control fish (overall mean = 2.24 ± 0.74 μmoles ADP mg protein^{-1} h^{-1}) exhibited significantly higher intestinal NKA activity overall than did salt fed fish (overall mean = 1.58 ± 0.56 μmoles ADP mg protein^{-1} h^{-1}) (2-way ANOVA, $P = 0.018$, 0.582 and 0.795 for, respectively, the effect of treatment, the effect of time and the interaction of these two factors; Fig. 10 C).

The fractional surface area of MRCs increased significantly with salt feeding (Fig. 11), resulting in significantly greater MRC surface area in salt-fed over control fish at 6, 14 and 30 days. MRC surface area remained constant in control fish (Fig.11F).

Real-time PCR was used to determine the mRNA abundance of the GR1, GR2, and MR genes relative to the control or housekeeping gene, 18s. Variation in 18s mRNA expression within or between tissues (gill, kidney and intestine) was minimal (cycle threshold (CT) values varied from 14 to 17), making 18S an appropriate control gene for this experiment. In the gill, a difference in corticosteroid receptor relative mRNA expression between control and salt-fed trout was detected only for MR expression at 30 days, where MR relative mRNA expression in salt-fed fish was ~6-fold greater than that in control fish. Corticosteroid receptor relative mRNA expression was

significantly affected by time in the salt-fed trout but not in the control group for GR1 and MR and in both groups for GR2. In general, 30 days of salt feeding resulted in significantly higher corticosteroid receptor relative mRNA expression than 3, 6 or 14 days of the high salt diet (Fig. 12). In the kidney, neither GR2 nor MR relative mRNA expression was affected by time or treatment. By contrast, GR1 relative mRNA exhibited elevated expression in trout fed a high salt diet for 6 days. GR1 relative mRNA expression in trout fed a high salt diet for 6 days was significantly higher than that of control trout at 6 days, and also significantly higher than that of salt-fed trout at any other time point (Fig. 13). In the intestine, no significant effect of treatment on corticosteroid receptor relative mRNA expression was detected, nor was GR1 relative mRNA expression affected by time. However, both GR2 and MR relative mRNA expression were significantly higher at 30 days than at most or all earlier time points. Because the 2-way ANOVA did not indicate any significant interaction between the two factors of interest (time and treatment; $P = 0.994$ for GR2, and $P = 0.468$ for MR), it was not possible to examine the effect of time within separate treatment groups. However, trout fed the high salt diet exhibited a tendency towards higher MR relative mRNA expression at 30 days than did trout fed the control diet (Fig. 14).

2. Series two: Receptor antagonist treatment

This series of experiments was designed to establish whether cortisol was responsible for the changes observed with salt-feeding, and if so, whether the effects were mediated through a GR (it is not possible to distinguish pharmacologically between GR1 and GR2 at the present time) or the MR, or both receptor types. Antagonists were

administered to fish via food using the protocol developed by (Marshall *et al.*, 2005a). To establish the efficacy of the antagonist treatment, fish fed RU486-treated control diet (GR antagonist) for 30 days were transferred to seawater and gill NKA activity in these fish was compared to that in a control group (i.e. no RU486) also transferred to seawater after 30 days. Gill NKA activity was used because it is known to increase with seawater exposure and this increase is thought to be GR mediated (Hawkings *et al.*, 2004; Lin *et al.*, 2004; Veillette and Young, 2005). It was assumed that if RU486 were effective at 30 days, it would also be effective over shorter periods. Because at present there is no well-established MR-mediated effect to examine, it was also assumed that what was true for RU486 would also be true of spironolactone (MR antagonist).

To test the involvement of cortisol and corticosteroid receptor types in the phenotypic changes associated with salt feeding, variables in salt-fed fish were compared with those of salt-fed fish treated with GR or MR blockers (administered in the food). In this case, selected parameters were examined for effects, specifically, those parameters found in the time course experiment (experiment #1) to show treatment effects, i.e. gill NKA activity at 30 days, kidney NKA activity at 14 days, MRC surface areas (after 6, 14 and 30 days), and relative mRNA abundance of the gill MR at 30 days and the kidney GR1 at 6 days.

A. Fish sampled after seawater transfer

Unexpectedly, NKA activity in the gills of trout treated with the GR antagonist RU486 for 30 days was significantly higher upon transfer to seawater than that in a control group that was also subjected to seawater transfer (Fig. 15A). Plasma cortisol

concentrations exhibited the opposite trend, being significantly higher in the control group than in the fish treated with RU486 (Fig. 15B).

B. Antagonist effects at 6 days of high salt feeding

In experiment #1, trout fed a high-salt diet for 6 days exhibited elevated branchial MRC fractional surface area and elevated renal GR1 relative mRNA expression. Therefore, these variables were examined in trout treated with the GR antagonist RU486 or the MR antagonist spironolactone over a 6 day period of high salt feeding. Unfortunately, technical difficulties were experienced in fixing/staining gill tissue for morphometric analysis, and as a result, the tissue degraded making it impossible to quantify branchial MRC fractional surface area at 6 days of high salt feeding (or indeed, at 14 or 30 days). GR1 relative mRNA expression in the kidney of salt-fed fish was unaffected by antagonist treatment (Fig. 16A). Plasma cortisol concentrations were uniformly low (<10 ng mL^{-1}) across all groups, but were significantly higher in salt-fed fish treated with spironolactone than in control or RU486-treated salt-fed fish (Fig. 16B).

C. Antagonist effects at 14 days of high salt feeding

In experiment #1, trout fed a high-salt diet for 14 days exhibited elevated branchial MRC fractional surface area and elevated renal NKA activity. Therefore, these variables were examined in trout treated with the GR antagonist RU486 or the MR antagonist spironolactone during a 14 day period of high salt feeding. Unfortunately, no data were obtained for gill morphometry (see above). NKA activity was unaffected by antagonist treatment (Fig. 17A), and while plasma cortisol concentrations were again

uniformly low (<10 ng mL^{-1}), in this case they were significantly higher in salt-fed fish treated with RU486 than in control or spironolactone-treated salt-fed fish (Fig. 17B).

D. Antagonist effects at 30 days of high salt feeding

In experiment #1, trout fed a high-salt diet for 30 days exhibited elevated branchial MRC fractional surface area, elevated branchial NKA activity, and elevated branchial MR relative mRNA expression. Therefore, these variables were examined in trout treated with the GR antagonist RU486 or the MR antagonist spironolactone during a 30 day period of high salt feeding. In addition, intestinal NKA activity was uniformly lower in salt-fed fish than in control fish across all time points, and the 30 day time point was selected for investigation of the potential involvement of cortisol in this effect. Unfortunately, no data were obtained for gill morphometry (see above). At 30 days of high salt feeding, antagonist treatment was without effect on any measured variable, including branchial and intestinal NKA activities, branchial MR relative mRNA expression, or plasma cortisol concentration (Fig. 18).

Figure 7. Drinking rates in rainbow trout (*Oncorhynchus mykiss*) fed a control diet (control) and in those fed a high salt diet (salt fed) for 30 days. An asterisk denotes a significant difference between treatment groups (unpaired Student's *t*-test, P = 0.038).

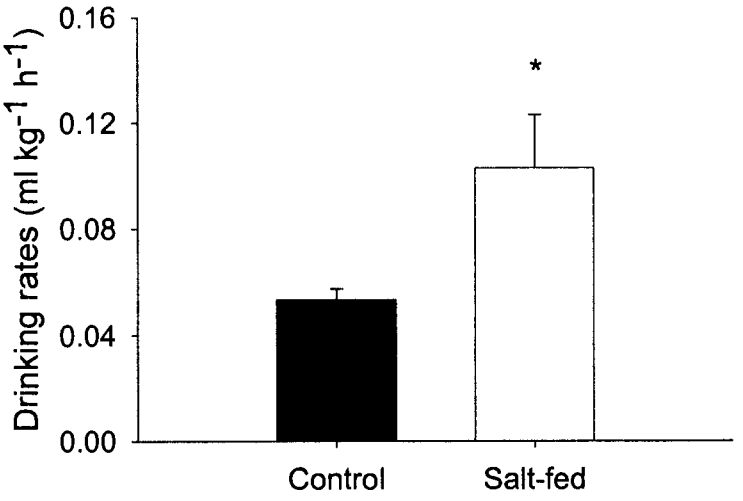

Drinking rates (ml kg-1 h-1)
0.16
0.12
0.08
0.04
0.00
*
Control
Salt-fed

Figure 8. Plasma cortisol concentrations in rainbow trout (*Oncorhynchus mykiss*) fed a control or high salt diet for 3, 6, 14, or 30 days. Values are means ± SEM, with $N = 6$ for each group. No significant effects of time or treatment were detected (2- way ANOVA, $P = 0.712$, 0.089 and 0.892 for, respectively, the effect of treatment, the effect of time and the interaction of these two factors).

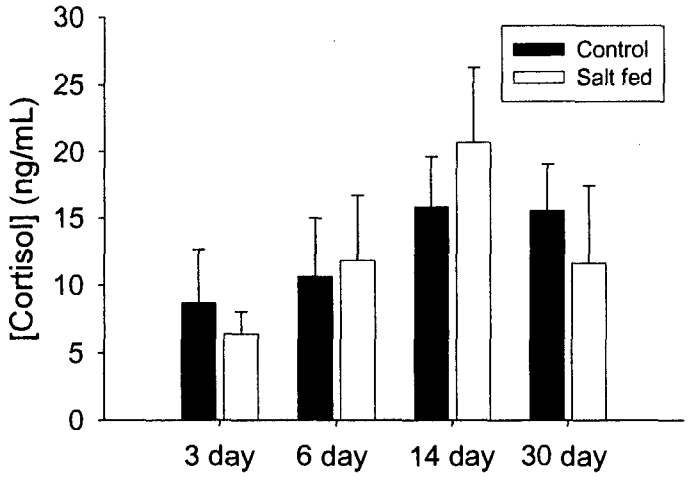
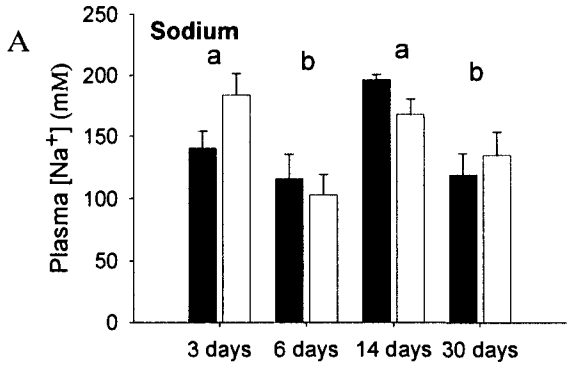
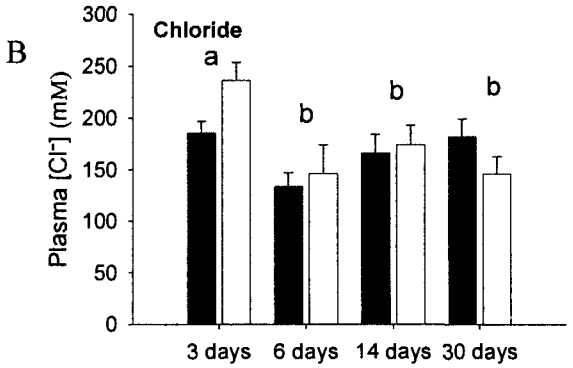
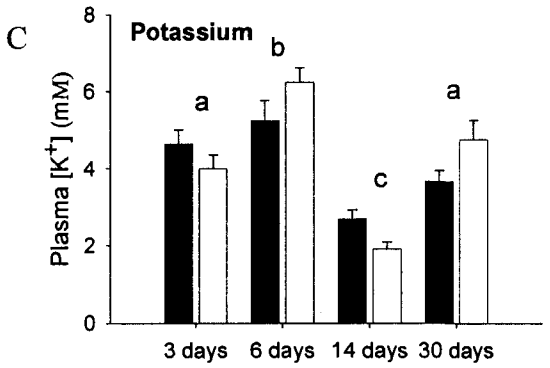

30
25
20
15
10
5
0
[Cortisol] (ng/mL)
Control
Salt fed
3 day
6 day
14 day
30 day

Figure 9. Plasma ion levels in rainbow trout (*Oncorhynchus mykiss*) fed a salt-enriched or control diet for 3, 6, 14 or 30 days. For each of the salt-fed and control groups, $N = 6$. No significant effects of time, treatment or the interaction of treatment and time were detected by 2-way ANOVA: (A) $P = 0.906$, 0.001 and 0.425 for, respectively, the effect of treatment, the effect of time and the interaction of these two factors; similarly, (B) $P = 0.405$, 0.006 and 0.552; (C) $P = 0.720$, 0.001 and 0.092; and (D) $P = 0.379$, 0.001 and 0.314.


A
Sodium
Plasma [Na+] (mM)
250
200
150
100
50
0
a
b
a
b
3 days
6 days
14 days
30 days


B
Chloride
Plasma [Cl-] (mM)
300
250
200
150
100
50
0
a
b
b
b
3 days
6 days
14 days
30 days


C
Potassium
Plasma [K+] (mM)
8
6
4
2
0
a
b
c
a
3 days
6 days
14 days
30 days

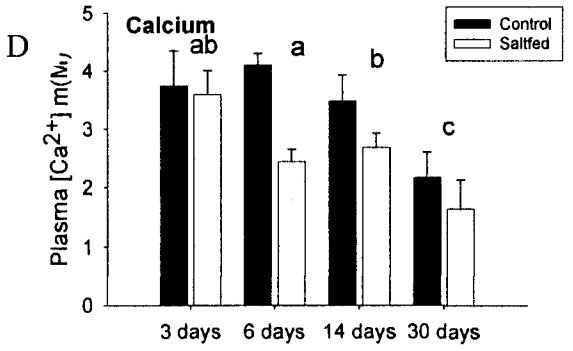
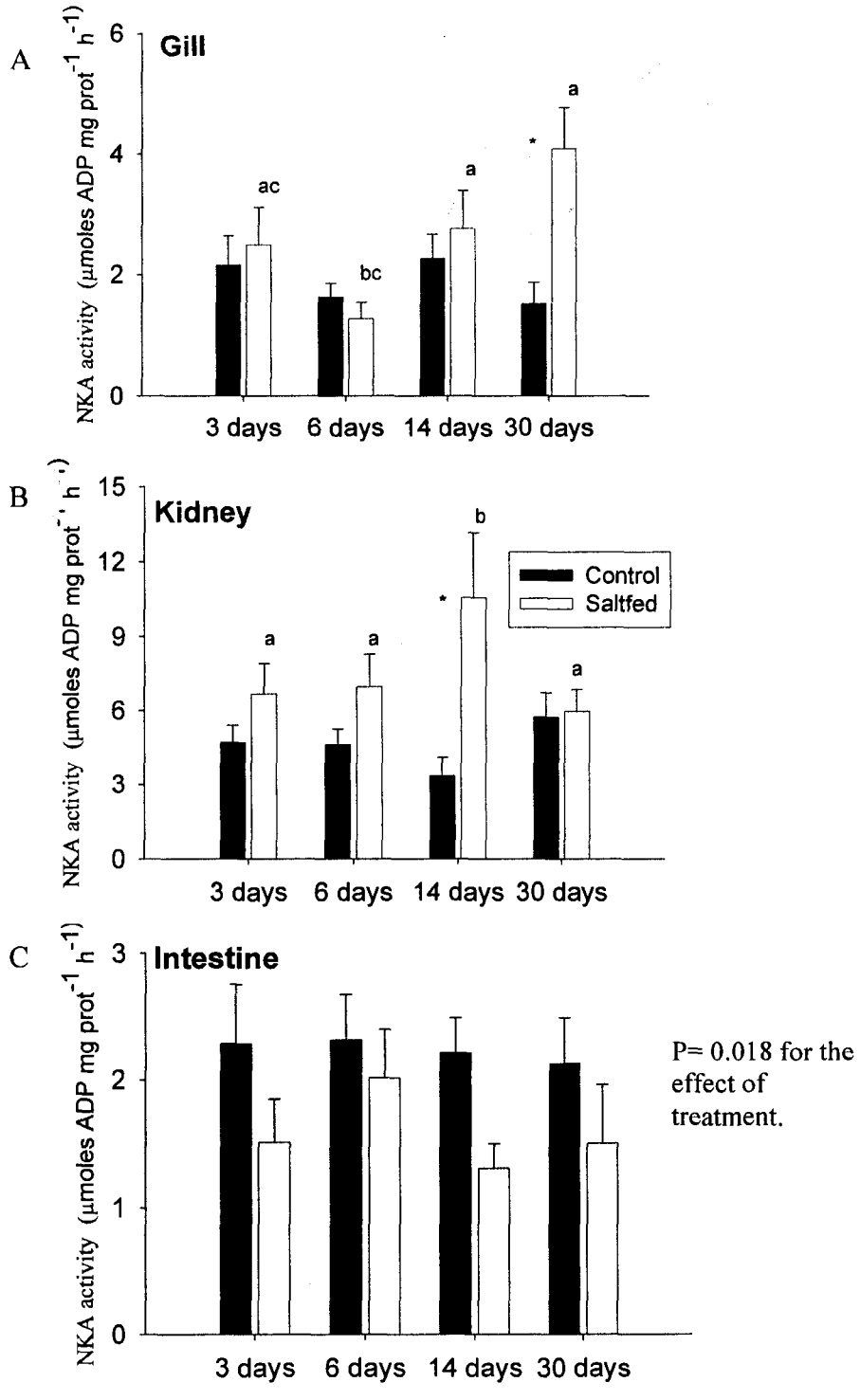
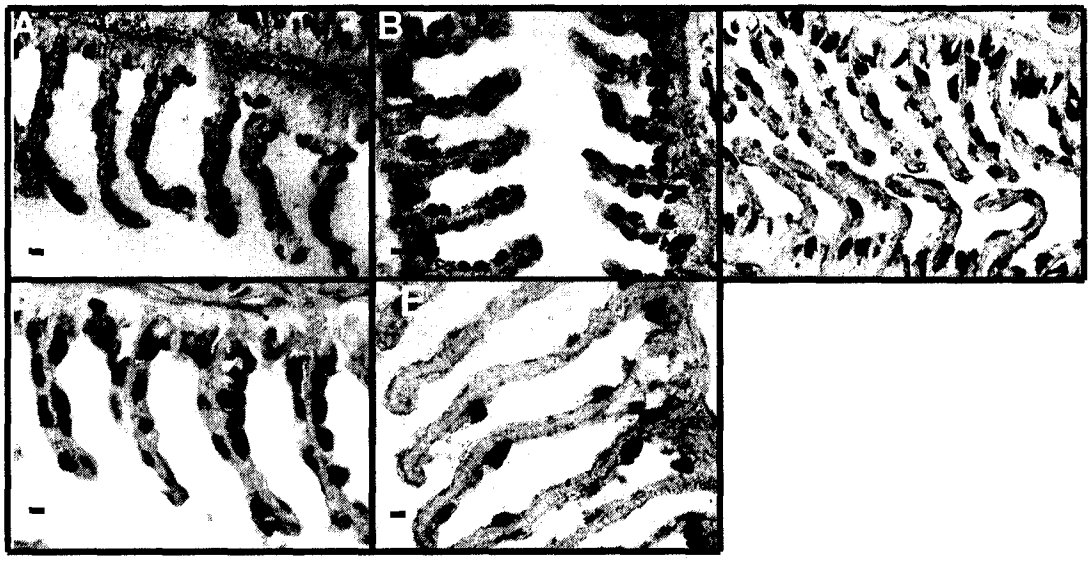

D
Calcium
Plasma [Ca2+] m(M,
5
4
3
2
1
0
ab
a
b
c
Control
Saltfed
3 days
6 days
14 days
30 days

Figure 10. The effect of salt-feeding on NKA activity in the (A) gill, (B) kidney and (C) intestine of rainbow trout (*Oncorhynchus mykiss*) fed normal trout chow or trout chow supplemented with 11% NaCl for 3, 6, 14 or 30 days. Values are presented as means ± SEM with $N = 6$ for each of the salt-fed and control groups. Within a treatment, groups that share a letter were not significantly different from one another, while an asterisk denotes a significant difference between treatment groups at a given time [2-way ANOVA, with P values for, respectively, the effect of treatment, the effect of time, and the interaction of these two factors of (A) 0.034, 0.026 and 0.046; (B) 0.003, 0.703 and 0.048; and (C) 0.018, 0.582 and 0.795].



P= 0.018 for the effect of treatment.

Figure 11. Representative light microscopy images of gill sections from rainbow trout (*Oncorhynchus mykiss*) fed a high salt diet for (A) 3 days, (B) 6 days, (C) 14 days, (D) 30 days, or (E) fed a control diet for 30 days. All images were taken at 40x magnification, and scale bars represent 20 μm. In panel (F), the results of morphometric analysis (N = 4 for each group) are presented as mean values ± SEM. Groups within a treatment sharing identical letters are not significantly different from one another, whereas an asterisk denotes a significant difference between control and salt-fed trout at a given time (2-way ANOVA; $P = 0.001$ for the effect of treatment, the effect of time, and the interaction of these two factors).


A
B

F

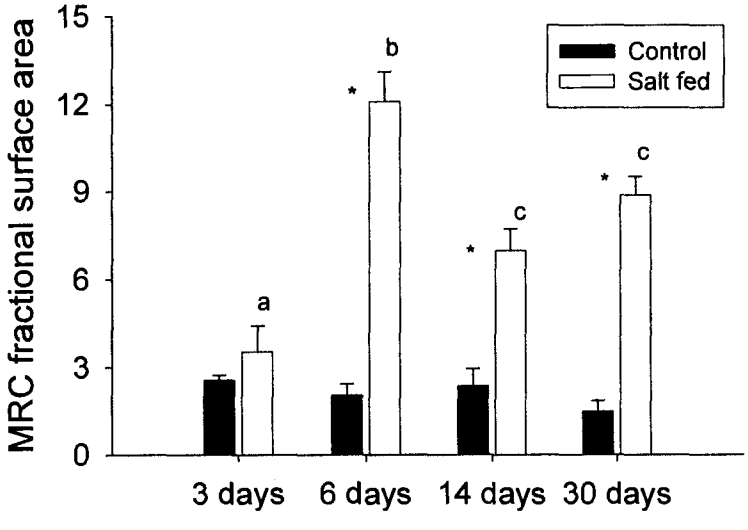
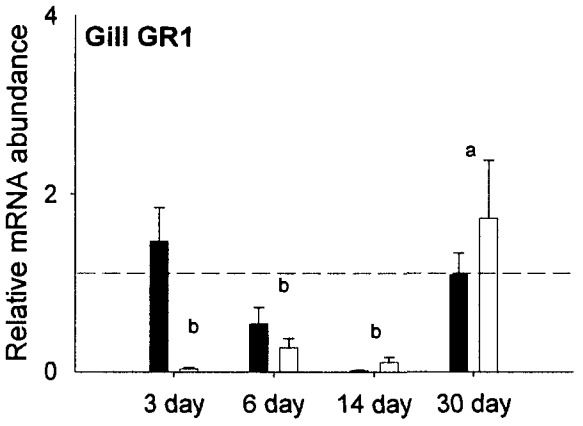
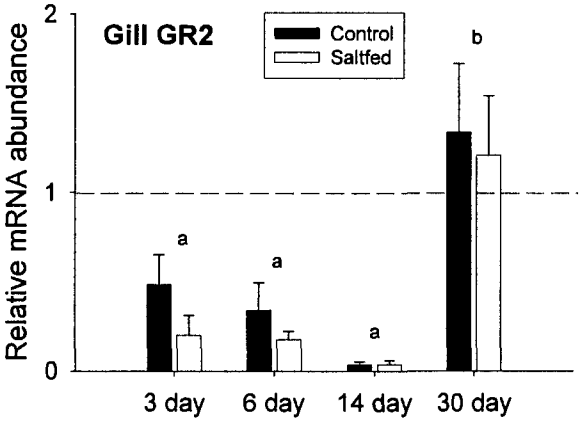

MRC fractional surface area
15
12
9
6
3
0
Control
Salt fed
a
b
c
c
*
*
*
3 days
6 days
14 days
30 days

Figure 12. Corticosteroid receptor relative mRNA expression in the gills of rainbow trout (*Oncorhynchus mykiss*) fed a control or high salt diet for 3, 6, 14 or 30 days. The mRNA abundance of (A) GR1, (B) GR2 and (C) MR was assessed by real-time RT-PCR using 18S as a standard by modification of the delta-delta Ct (threshold cycle) method (Pfaffl, 2001) in which expression in all groups was compared to that in a designated control group, namely trout fed a control diet for 30 days. Values represent means ± SEM (N = 6 for all groups in each of A, B and C). For statistical analysis, the mRNA expression of the designated control group was set to 1 and is indicated on the figure by a dashed line. Data were analyzed statistically by 2-way ANOVA with P values for the effect of treatment, the effect of time and the interaction of these factors, respectively, of (A) 0.172, 0.008, and 0.075, (B) 0.379, 0.001, and 0.944, and (C) 0.304, 0.001, and 0.002. An asterisk indicates a significant difference between control and salt-fed fish at a given time, while within a treatment, groups that share a letter are not significantly different from one another. Note that for GR2 (panel B), the interaction of time and treatment was not significant, and therefore statistical symbols indicating the effects of time apply to both treatments. Corticosteroid receptor relative mRNA expression in fish fed the control diet was unaffected by time for GR1 (panel A) and MR (panel C) and therefore symbols of statistical significance have been omitted for clarity.

A


Gill GR1
Relative mRNA abundance
4
2
0
a
b
b
b
3 day
6 day
14 day
30 day

B


Gill GR2
Control
Saltfed
Relative mRNA abundance
2
1
0
a
a
a
b
3 day
6 day
14 day
30 day

C

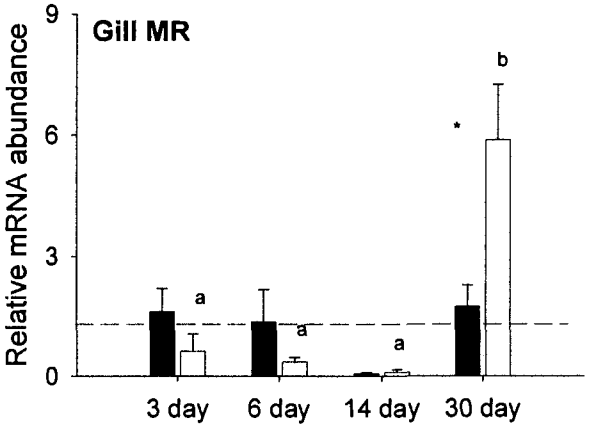
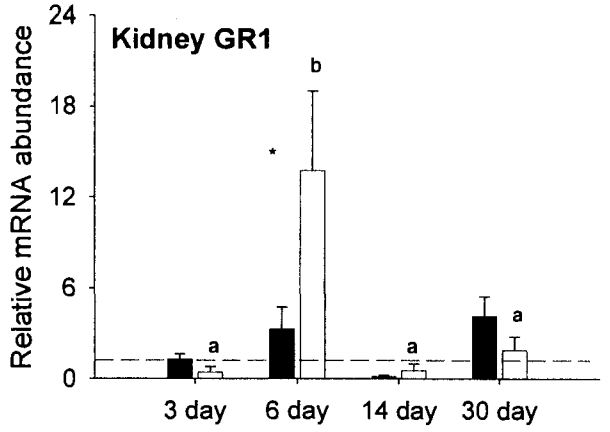
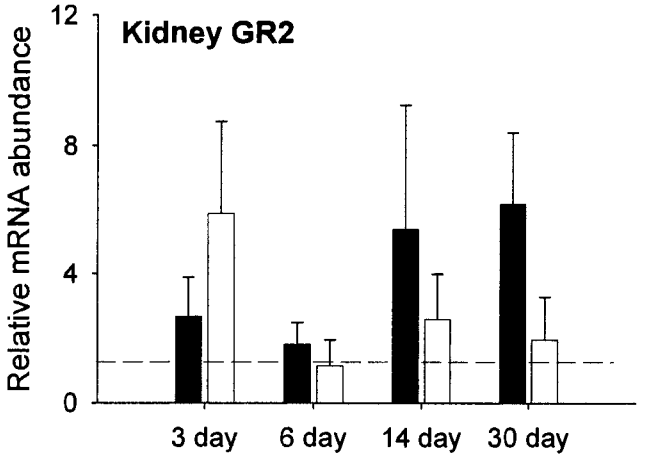

Gill MR
Relative mRNA abundance
9
6
3
0
a
a
a
*
b
3 day
6 day
14 day
30 day

Figure 13. Corticosteroid receptor relative mRNA expression in the kidney of rainbow trout (*Oncorhynchus mykiss*) fed a control or high salt diet for 3, 6, 14 or 30 days. The mRNA abundance of (A) GR1, (B) GR2 and (C) MR was assessed by real-time RT-PCR using 18S as a standard by modification of the delta-delta Ct (threshold cycle) method (Pfaffl, 2001) in which expression in all groups was compared to that in a designated control group, namely trout fed a control diet for 3 days. Values represent means ± SEM (N = 6 for all groups in each of A, B and C). For statistical analysis, the mRNA expression of the designated control group was set to 1 and is indicated on the figure by a dashed line. Data were analyzed statistically by 2-way ANOVA with P values for the effect of treatment, the effect of time and the interaction of these factors, respectively, of (A) 0.215, 0.003 and 0.024, (B) 0.430, 0.480 and 0.264, and (C) 0.511, 0.336, and 0.260. An asterisk indicates a significant difference between control and salt-fed fish at a given time, while within a treatment, groups that share a letter are not significantly different from one another. GR1 relative mRNA expression in fish fed the control diet was unaffected by time and therefore symbols of statistical significance have been omitted for clarity.

A


Kidney GR1
Relative mRNA abundance
24
18
12
6
0
a
b
*
a
a
3 day
6 day
14 day
30 day

B


Kidney GR2
Relative mRNA abundance
12
8
4
0
3 day
6 day
14 day
30 day

C

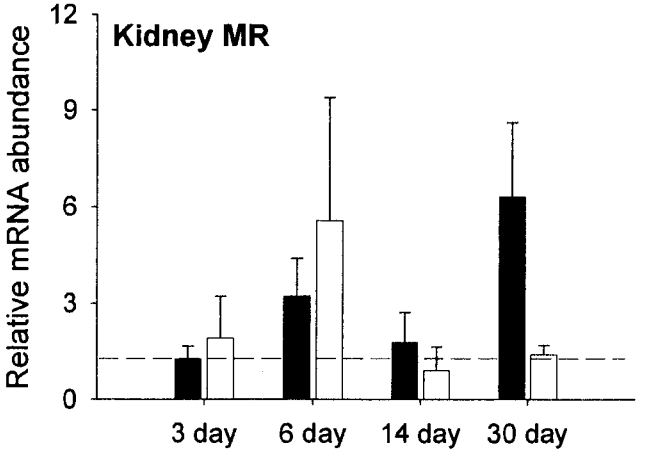
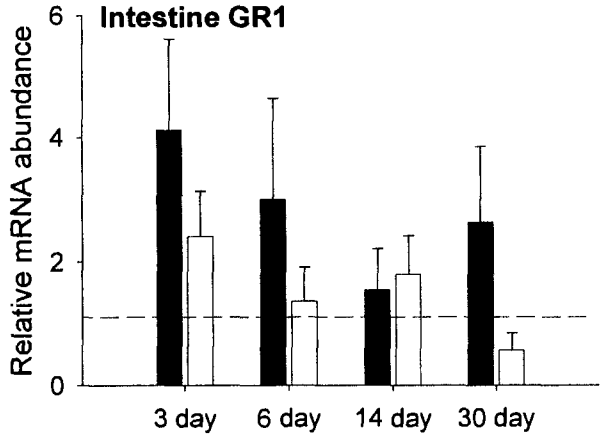
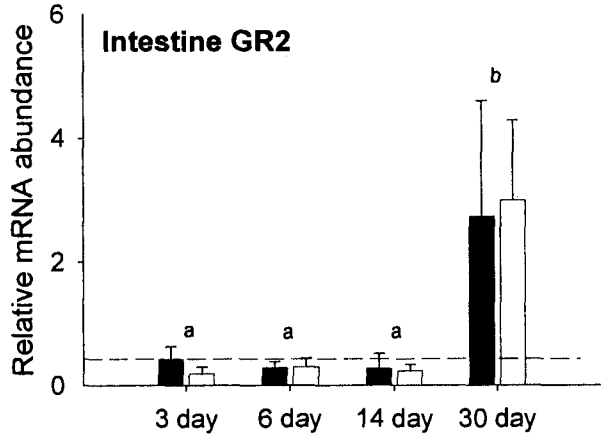

Kidney MR
Relative mRNA abundance
12
9
6
3
0
3 day
6 day
14 day
30 day

Figure 14. Corticosteroid receptor relative mRNA expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) fed a control or high salt diet for 3, 6, 14 or 30 days. The mRNA abundance of (A) GR1, (B) GR2 and (C) MR was assessed by real-time RT-PCR using 18S as a standard by modification of the delta-delta Ct (threshold cycle) method (Pfaffl, 2001) in which expression in all groups was compared to that in a designated control group, namely trout fed a control diet for 3 days. Values represent means ± SEM (N = 6 for all groups in each of A, B and C). For statistical analysis, the mRNA expression of the designated control group was set to 1 and is indicated on the figure by a dashed line. Data were analyzed statistically by 2-way ANOVA with P values for the effect of treatment, the effect of time and the interaction of these factors, respectively, of (A) 0.148, 0.646 and 0.644, (B) 0.997, 0.007 and 0.994, and (C) 0.058, 0.008 and 0.468. Groups that share a letter are not significantly different from one another [note that owing to the absence of significant interactions between time and treatment, the significant effects of time in (B) and (C) are indicated for the combined control and salt-fed groups at a given time].

A


Intestine GR1
Relative mRNA abundance
6
4
2
0
3 day
6 day
14 day
30 day

B


Intestine GR2
Relative mRNA abundance
6
4
2
0
a
a
a
b
3 day
6 day
14 day
30 day

C

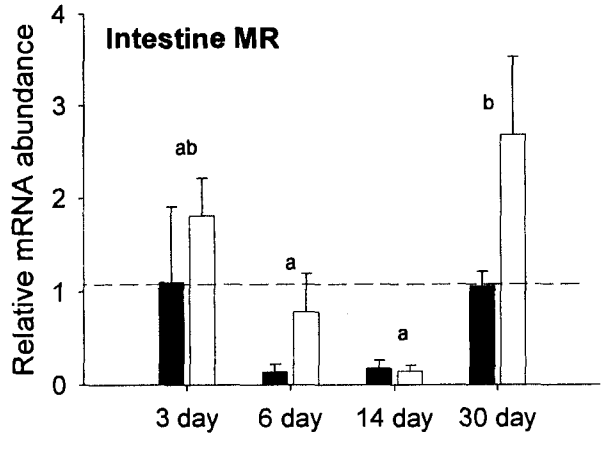
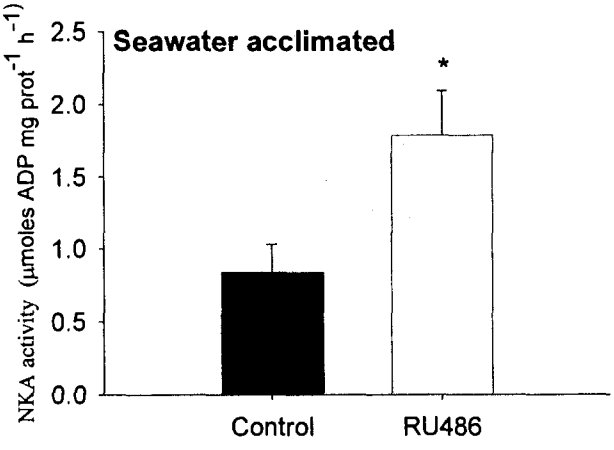

Intestine MR
Relative mRNA abundance
4
3
2
1
0
ab
a
a
b
3 day
6 day
14 day
30 day

Figure 15. The effect of acclimation to seawater for 96 h on (A) gill NKA activity and (B) plasma cortisol concentrations in rainbow trout (*Oncorhynchus mykiss*) fed regular trout chow with (N = 8) or without (N = 12) the GR antagonist RU486 (40 mg kg^{-1} fish per day). Values are means ± SEM. An asterisk denotes a significant difference between treatments [unpaired Student's *t*-test, P = 0.026 for (A) and P=0.002 for (B)].

A


Seawater acclimated
NKA activity (μmoles ADP mg prot-1 h-1)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
RU486

B

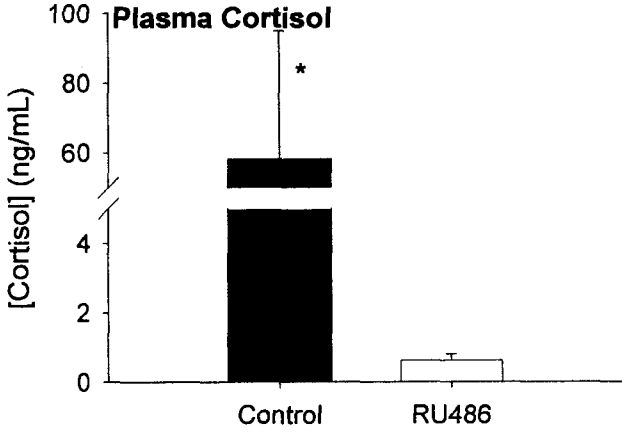
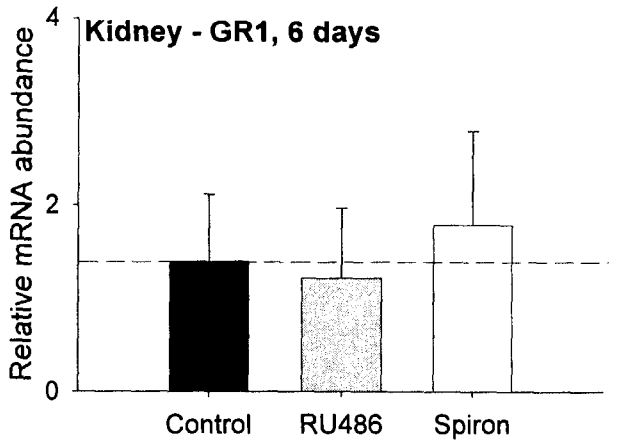

Plasma Cortisol
[Cortisol] (ng/mL)
100
80
60
4
2
0
*
Control
RU486

Figure 16. The effects of corticosteroid receptor antagonist treatment on (A) renal GR1 relative mRNA abundance and (B) plasma cortisol concentrations of rainbow trout (*Oncorhynchus mykiss*) fed a high-salt diet for 6 days. The GR antagonist RU486 (40 mg kg^{-1} fish per day; N = 6) or the MR antagonist spironolactone (spiron; 0.1mg kg^{-1} fish per day; N = 6) was administered via the food; food in the control group (N = 6) was sprayed with the ethanol vehicle (0.5 mL g^{-1} of food). GR1 relative mRNA abundance was assessed by real-time RT-PCR using 18S as a standard by modification of the delta-delta Ct method (Pfaffl, 2001) in which expression was compared to that in a designated control, namely the control group. For statistical analysis, the mRNA expression of the control group was set to 1 and is indicated on the figure by a dashed line. Values in both panels represent means ± SEM, and groups that share a letter are not significantly different from one another [one-way ANOVA; P = 0.296 for (A) and P = 0.001 for (B)].

A


Kidney - GR1, 6 days
Relative mRNA abundance
4
2
0
Control
RU486
Spiron

B

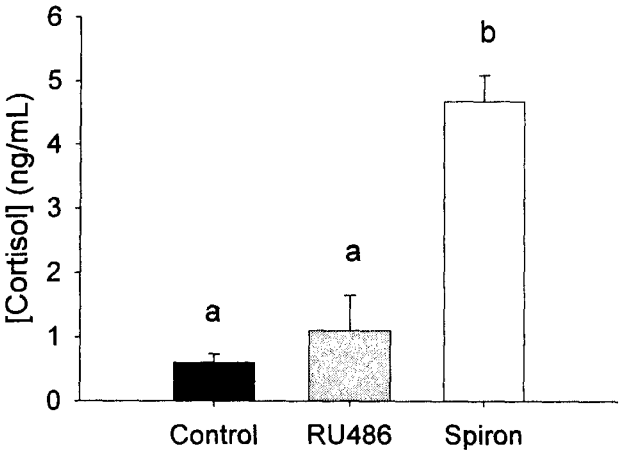
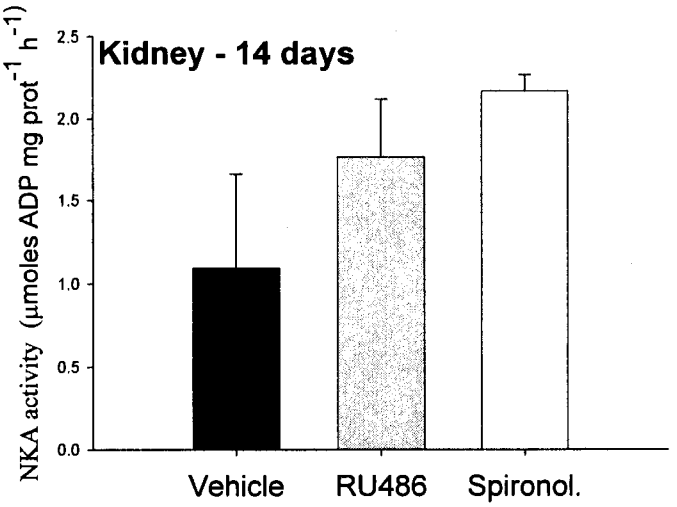

[Cortisol] (ng/mL)
6
5
4
3
2
1
0
a
a
b
Control
RU486
Spiron

Figure 17. The effects of corticosteroid receptor antagonist treatment on (A) renal NKA activity and (B) plasma cortisol concentrations of rainbow trout (*Oncorhynchus mykiss*) fed a high-salt diet for 14 days. The GR antagonist RU486 (40 mg kg^{-1} fish per day; N = 6) or the MR antagonist spironolactone (spiron; 0.1mg kg^{-1} fish per day, N = 6) was administered via the food; food in the control group (N = 6) was sprayed with the ethanol vehicle (0.5 mL g^{-1} of food). Values represent means ± SEM. Groups that share a letter are not significantly different from one another [one-way ANOVA; P = 0.217 for (A) and P =0.007 for (B)].

A


Kidney - 14 days
NKA activity (µmoles ADP mg prot-1 h-1)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
RU486
Spironol.

B

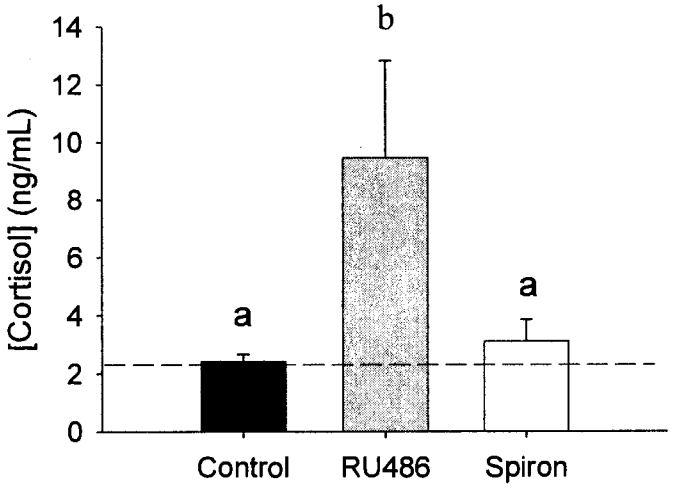
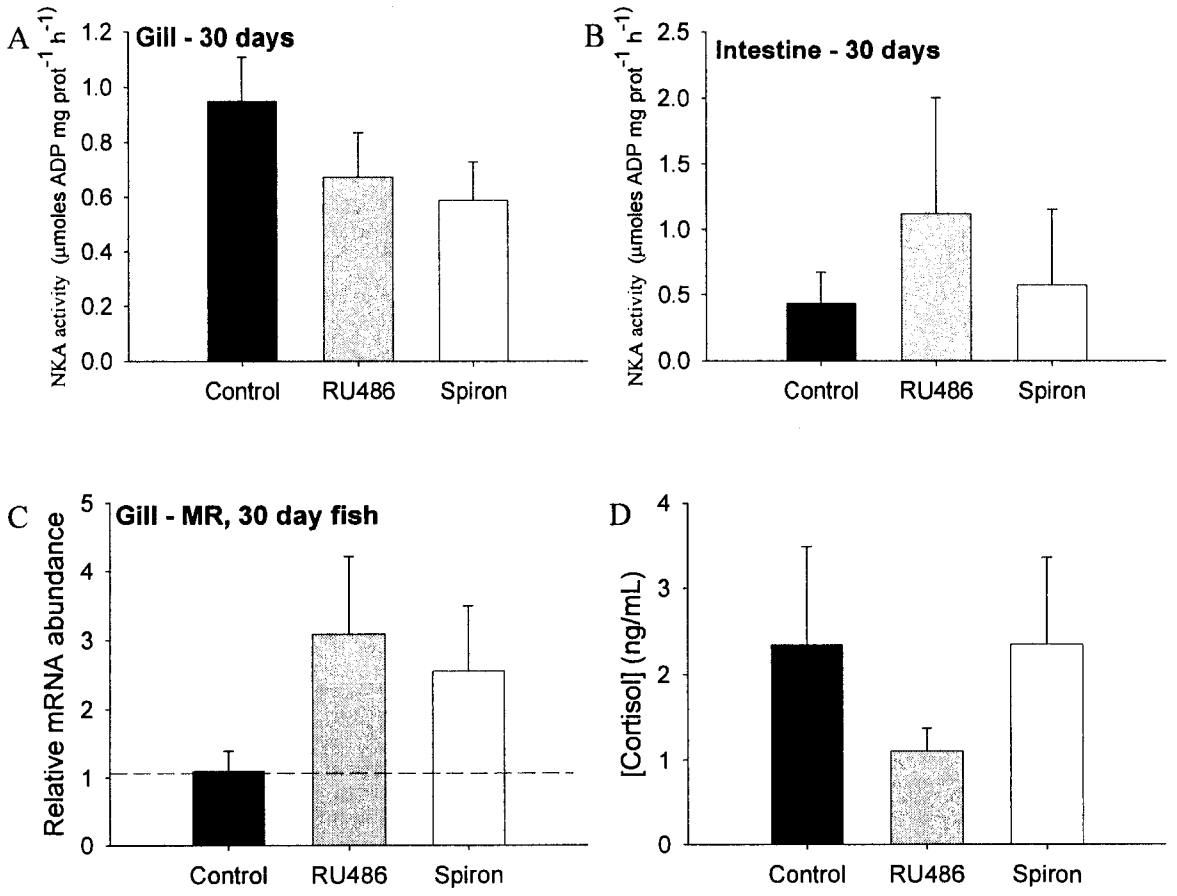

[Cortisol] (ng/mL)
14
12
10
8
6
4
2
0
a
b
a
Control
RU486
Spiron

Figure 18. The effects of corticosteroid receptor antagonist treatment on (A) branchial NKA activity, (B) intestinal NKA activity, (C) branchial MR relative mRNA expression and (D) plasma cortisol concentrations of rainbow trout (*Oncorhynchus mykiss*) fed a high-salt diet for 30 days. The GR antagonist RU486 (40 mg kg^{-1} fish per day; N = 6) or the MR antagonist spironolactone (spiron; 0.1mg kg^{-1} fish per day; N = 6) was administered via the food; food in the control group (N = 6) was sprayed with the ethanol vehicle (0.5 mL g^{-1} of food). MR relative mRNA abundance was assessed by real-time RT-PCR using 18S as a standard by modification of the delta-delta Ct method (Pfaffl, 2001) in which expression was compared to that in a designated control, namely the control group. For statistical analysis, the mRNA expression of the control group was set to 1 and is indicated on the figure by a dashed line. Values in all cases represent means ± SEM. No statistically significant differences were detected in any case [one-way ANOVA; P = 0.235 for (A), P = 0.718 for (B), P = 0.290 for (C), and P = 0.422 for (D)].


A
Gill - 30 days
NKA activity (µmoles ADP mg prot-1 h-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
RU486
Spiron
B
Intestine - 30 days
NKA activity (µmoles ADP mg prot-1 h-1)
2.5
2.0
1.5
1.0
0.5
0.0
Control
RU486
Spiron
C
Gill - MR, 30 day fish
Relative mRNA abundance
5
4
3
2
1
0
Control
RU486
Spiron
D
[Cortisol] (ng/mL)
4
3
2
1
0
Control
RU486
Spiron

Discussion

This study aimed to investigate the phenotypic changes observed in rainbow trout fed a high salt diet while at the same time attempting to correlate these changes to modifications in cortisol or corticosteroid receptors. The results of this research confirmed and extended the findings of Perry *et al.,* (2006) and Salman and Eddy, (1987); salt feeding appeared to induce at least a partial seawater phenotype, with increased branchial NKA activity and increased MRC fractional surface area. It further extended previous work by demonstrating that drinking rates increased in salt-fed fish, and by providing a finer resolution of the timing of these changes over a 30 day experimental period. In addition, the involvement of the kidney and the intestine, two tissues that play osmoregulatory roles complementary to that of the gills, in the documented changes was investigated. The final aim was to move beyond previous efforts (Salman and Eddy, 1987; Salman and Eddy, 1988; Perry *et al.,* 2006) by seeking to tie the documented changes in the phenotype of salt-fed fish to a hormonal trigger, specifically to cortisol via regulation of circulating cortisol concentrations and/or regulation of corticosteroid receptors (GR1, GR2 and/or MR).

Plasma ions were regulated to constant levels despite the greater salt intake. The phenotypic changes that accompany salt feeding are presumed to be responsible for the capacity of the fish to maintain constant ion levels. Transient fluctuations of plasma ion concentrations may occur immediately following salt consumption. For example, (Smith *et al.,* 1995) reported an increase in plasma Na^+ concentration in fish fed 12% NaCl when fish were sampled 7 h after feeding. Salt consumption likely causes an initial spike in

plasma NaCl levels as the salt load is absorbed from the gut. However, once the NaCl is in the circulation, blood ion levels are regulated effectively by excretion via the gills and kidney, accounting for the absence of plasma ion perturbations when measurements are made hours after salt consumption in fish fed a salt load for days, as in the present study. At the gill, increased NKA activity contributes to enhanced ion excretion (Pyle *et al.*, 2003), while at the kidney urinary ion excretion is increased through elevated urine flow (Salman and Eddy, 1988).

Drinking rates were measured after 30 days of salt feeding to expand the phenotypic picture of salt-fed fish – drinking is characteristic of marine teleost fish, where it serves to compensate for osmotic water loss across the body surfaces, but is avoided in freshwater teleosts, which face osmotic water influx (Shehadeh and Gordon, 1969). The 30 day time point was chosen based on previous work demonstrating NKA activity increases, increased mRNA expression of MRC ion transporters (NKA and NKCC), and the appearance of seawater type accessory cells within the gill at 30 days (Perry *et al.*, 2006). The significantly higher drinking rate measured in salt-fed fish (0.11 vs 0.055 mL kg^{-1} h^{-1}) was consistent with the idea of these fish developing a seawater phenotype, and was also in agreement with the results of (Pyle *et al.*, 2003), who demonstrated an increase in the drinking rate of freshwater fish fed an increased salt load in copper-loaded water (from 0.6 to 1.7 mL kg^{-1} h^{-1}). However, the absolute drinking rates remained typical of freshwater fish (Hickman, 1968; Pyle *et al.*, 2003; Scott *et al.*, 2006). Higher drinking rates in salt-fed fish may simply reflect the fact that the salt-fed fish received 11% more food and thus consumed more water during feeding, but the increase in drinking rate (approximately double) was higher than would be expected if

this were the sole explanation. Drinking behaviour may have been modified by the increased salt load through as yet unidentified mechanisms. For example, hormonal changes are thought to be involved in changes in water consumption during freshwater/seawater transitions (Fuentes and Eddy, 1997b). Angiotensin II has been found to increase drinking rates in marine fish (Beasley *et al.*, 1986), while atrial natriuretic peptide appears to inhibit drinking (Tsukada and Takei, 2006).

The time course of phenotypic changes

Examination of the time course of phenotypic changes with salt feeding suggested the existence of several different compensatory mechanisms, with different times of onset. For example, MRC fractional area increased very rapidly (by 6 days), with changes in branchial NKA activity lagging well behind (detectable only at 30 days). Increased MRC fractional area had been documented previously after 30 days of salt feeding (Salman and Eddy, 1987; Perry *et al.*, 2006), but the early onset (from 6 days) of elevated MRC fractional area was a new observation. Most MRCs were clustered along the lamellae and the outer edge of the filament (Fig.11). Because MRCs typically are thought to be NKA rich (McCormick, 1995; Goss *et al.*, 2001), this discrepancy in the timing of the two responses was unexpected, and raises questions about the types of MRCs in the gills at different time points. The two types of MRCs found in the freshwater fish gill, PNA^+ and PNA^-, exhibit somewhat different localization (Galvez *et al.*, 2002) PNA^+ cells tend to be found at the base of the lamellae and in the interlamellar regions of the filament, whereas PNA^- cells are localized to the base of the lamellae (Galvez *et al.*, 2002). Each type appears to have its own role in ionic and acid-base regulation. The PNA^+ cells are

believed to be base-secreting cells and possibly the main site of Cl^-/HCO_3^- exchange and NKA activity for Cl^- uptake, whereas the PNA^- cells are believed to be acid-secreting cells and possibly the main site of V-type H^+-ATPase for Na^+ uptake (Galvez *et al.*, 2002; Perry and Gilmour, 2006). The relative abundance of NKA activity in freshwater MRCs (PNA^+ or PNA^-) vs seawater MRCs (chloride cells) remains unknown. Thus, it is possible that the early stages of salt feeding are accompanied by a transition in freshwater MRC relative abundance, with the increase in branchial NKA activity occurring at a later time, when a transition to a marine MRC (chloride cell) is accomplished. Unfortunately, the method used to detect MRCs in the present study (ZnI_2/OsO_4 staining) did not allow different subtypes to be distinguished. Clearly, a more detailed examination of MRC subtype with salt feeding is warranted

A transient increase in kidney NKA activity was detected after 14 days of salt feeding; renal NKA activity was back to the control values by 30 days. The physiological significance of this response is unclear, because increased renal NKA activity would be expected to favour NaCl retention by enhancing NaCl reabsorption from filtrate in the nephron lumen (Nishimura and Imai, 1982), yet salt-fed fish must excrete a salt load to remain in ionic homeostasis (Salman and Eddy, 1988). Moreover, salt feeding is known to be without effect on blood acid-base status (L. Rivero-Lopez and SF Perry, unpublished results), and therefore the transient change in renal NKA activity probably does not reflect a compensatory response for the maintenance of acid-base status. Of importance to note in considering these data is the fact that the 14 day exposure was carried out approximately 6 months after the 3, 6, and 30 day exposures, on fish that were somewhat different in size and under conditions in the aquatic facility that

were less than optimal (high noise levels owing to an adjacent construction site). Thus, differences between the 14-day exposure fish and all other experimental groups should be interpreted with caution.

Unlike the changes in renal and branchial NKA activity, which were relatively slow in onset and towards higher activity in salt-fed fish, intestinal NKA activity was lower in salt fed fish. Moreover, the effect of salt-feeding on intestinal NKA activity was detectable by 3 days of dietary salt enrichment and was maintained across all time points. These observations suggest that lowering of gut NKA activity is a rapidly activated mechanism that is designed to limit salt uptake. Whether reductions in intestinal NKA activity impact upon nutrient absorption remains to be determined, as does the proximate mechanism responsible for triggering the reduction in intestinal NKA activity. Interestingly, the intestinal NKA activity response to salt feeding contrasts with that observed during seawater acclimation. Transfer from freshwater to seawater elicits increases in intestinal NKA mRNA expression and activity, responses that are presumed to support the intestinal water uptake required for the maintenance of osmotic homeostasis (Jensen *et al.*, 1998; Cutler *et al.*, 2000). Thus, while dietary salt loading promotes a branchial phenotype that is characteristic of a seawater-acclimated fish, this is not true of the intestine.

The involvement of cortisol and corticosteroid receptors

Owing to the extensive literature documenting a role for cortisol in ionic and osmotic regulation (McCormick, 1995; McCormick, 2001), cortisol was hypothesized to be involved in regulating the phenotypic alterations associated with dietary salt loading.

However, salt feeding did not appear to have any impact on plasma cortisol levels in the present study (Fig. 8). This was an unexpected result because previous studies have documented an increase in plasma cortisol concentration during transfer from freshwater to seawater (Mommsen *et al.*, 1999; McCormick, 2001). It is possible that a spike in plasma cortisol levels occurred but went undetected, if for example it occurred before the 3 day sampling time or perhaps between other time points. Another possibility is that plasma cortisol levels do not change, but rather that cortisol-mediated regulation is achieved through modulation of corticosteroid receptors, a possibility first suggested by (Sloman *et al.*, 2001) in response to the observation that MRs were involved, in the absence of detectable change in plasma cortisol concentrations, in the induction of MRC proliferation in response to softwater acclimation (Sloman *et al.*, 2001).

The data on corticosteroid receptor mRNA expression support the possibility of changes in corticosteroid receptors as a mechanism for the involvement of cortisol-mediated regulation. Specifically, gill MR mRNA abundance was increased in fish fed a high salt diet for 30 days (Fig.12), while kidney GR1 mRNA abundance was elevated in fish fed a high salt diet for 6 days (Fig.13). Although these findings are suggestive, they must be interpreted cautiously as changes in mRNA expression do not necessarily result in changes in protein expression. Unfortunately, the availability of antibodies for fish corticosteroid receptors is very limited. No commercially-available fish-specific antibodies are available, and antibodies against mammalian corticosteroid receptors are not specific (J. Lesnik, Santa Cruz Biotechnology, personal communication). The antibody against trout GRs used in some studies (Tujague *et al.*, 1998) does not distinguish between GR1 and GR2, and there is no MR antibody. Although antisera from

two rabbits immunized with trout-specific MR peptide antigen was obtained, I was unable to demonstrate any specificity against trout MR. In the absence of a means of assessing corticosteroid receptor protein, receptor mRNA expression was used as an indicator of corticosteroid receptor regulation. Coupled with the difficulty of assessing corticosteroid receptors directly, our knowledge of the physiological processes mediated specifically by GR1, GR2 and MR is at best incomplete (Prunet *et al.,* 2006; Bury and Sturm, 2007). Given these points, it is difficult to predict the physiological significance of the changes in corticosteroid receptor mRNA expression observed in the present study. However, the occurrence after 30 days of salt feeding of increases in both gill MR mRNA expression and NKA activity is in accordance with other work suggesting a link between MRC abundance and MRs. For example, (Sloman *et al.,* 2001) found that MR blockade prevented the proliferation of MRCs normally associated with softwater acclimation in freshwater rainbow trout, while increases in cell proliferation and NKA expression normally associated with transfer from brackish water to fresh water in killifish were inhibited by MR blockade (Scott *et al.*, 2005). It is tempting to speculate that the elevated GR1 mRNA expression in the kidneys of trout fed a high salt diet for 6 days is linked to the subsequent appearance of elevated renal NKA activity in salt-fed fish at 14 days. Finally, the rapidity of the changes in intestinal NKA activity (by 3 days) in the absence of any corresponding changes in gut corticosteroid receptor mRNA expression suggest that cortisol may not be involved in mediating the gut NKA activity response.

Given that the observed changes in corticosteroid receptor mRNA expression were suggestive of the involvement of corticosteroid receptors in mediating the changing phenotype in salt-fed fish, receptor blockade was utilized to establish a causal role of

cortisol in the changes observed with salt-feeding, and to determine the receptor type involved (GR vs MR). Although the data did not support a role for corticosteroid receptors in mediating the salt-induced phenotype, the results must be considered inconclusive because the experiments designed to confirm the efficacy of the antagonist protocol failed to demonstrate effective receptor blockade. Thus, although the protocol of Marshall *et al.*, (2005a) showed blockade was achieved, and this protocol was followed in the present study, the results of the seawater challenge validation experiment suggested that in this study, at least at 30 days, blockade was not effective. Fish treated with RU486 exhibited higher NKA activity than controls, an unexpected result in this case as seawater acclimation normally is associated with an increase in branchial NKA activity (Tipsmark *et al.*, 2002). Moreover, branchial NKA activity in seawater challenged fish was unusually low (Madsen ,1990; Hawkings *et al.*, 2004), again for reasons that were not clear. Blockers in the present study were administered via the food (as in Marshall *et al.*, 2005) and it is possible that differences in feeding of individual fish contributed to the apparent lack of blockade. Although other delivery methods [e.g. slow-release intraperitoneal implants (DiBattista *et al.*, 2005) or mini-osmotic pumps (Andersen *et al.*, 1991)] may more reliably elevate circulating blocker concentrations, they were not amenable to the length of the experimental period (30 days) of this study. If, indeed, blockade was achieved (despite the results of the validation experiment), then it was without effect on any measured variable, suggesting that cortisol is not involved in mediating these changes, or that blockade of both the GR and MR is required. Regardless, conclusions concerning the involvement of cortisol in mediating the

phenotypic changes associated with salt feeding must await the completion of an experiment in which corticosteroid receptor blockade is achieved.

This work has improved our understanding of the timing of changes involved in the development of the phenotype associated with salt feeding, has linked the development of the phenotype to a regulatory role for cortisol (although not as clearly as desirable), and further emphasizes the significance of the salt-feeding model both for studying the transition to seawater itself and for understanding the regulatory pathways involved in this transition.

The first series of experiments expanded upon the freshwater salt-fed fish model developed in previous studies. Phenotypic, biochemical, and molecular characteristics were investigated over a time course of salt feeding. Notable results included the demonstration after 30 days of salt feeding of increases in gill NKA activity, MRC total surface area, and MR mRNA abundance; of increased renal NKA activity after 14 days and GR1 mRNA abundance after 6 days of salt feeding; and of lowered intestinal NKA activity as early as after 3 days of salt feeding. The pattern suggests early roles for intestinal and renal modifications that are relinquished by 30 days of salt feeding owing to gill remodeling. Cortisol levels did not change during salt feeding, but changes in corticosteroid receptor mRNA abundance suggest a role for cortisol in the development of the salt-fed (seawater) phenotype via receptor regulation. Together, these results help to further develop the salt-fed fish model and support the idea that these fish develop a seawater phenotype, at least at the gill, despite their continued maintenance in fresh water (Perry *et al.*, 2006). An advantage of the salt-feeding model over seawater acclimation is

that the internal environment is manipulated in the absence of changes in the external environment. This feature makes the salt-feeding model a useful tool in determining what specific factors are involved in triggering the transition from the freshwater to seawater phenotype.

Future studies should include a repeat attempt at corticosteroid receptor blockade as this experiment is essential in establishing a causal role for cortisol in the development of the salt-fed phenotype. One endpoint that should be pursued is the morphometric analysis of the gill, if the fractional surface area could be shown to decrease with corticosteroid receptor blockade it would provide some of the data necessary to substantiate the involvement of the corticosteroid receptors in the development of the salt-fed seawater gill phenotype. In addition, a profile of protein expression for the three corticosteroid receptors is also necessary to gain an understanding of receptor expression and regulation; there are currently no fish specific antibodies available for the corticosteroid receptors and certainly none specific to the different GR isoforms. To this end there is a need for GR1, GR2 and MR-specific antibodies before useful protein expression profiles can be obtained. It would also be of interest to investigate other hormones that have been shown to activate the corticosteroid receptors. One would be 11-deoxycorticosterone (11-DOC), which has been observed to be as potent as aldosterone at activating the MR without activating the GR and that is present in the rainbow trout genome, unlike aldosterone (Sturm *et al.,* 2005; Prunet *et al.,* 2006; Bury and Sturm, 2007). However, circulating 11-DOC levels have not yet been shown to fluctuate with ionoregulatory challenges, and establishing such a response is a necessary first step to identifying 11-DOC as a mineralocorticoid hormone *in vivo.*

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