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Cellular Mechanisms of Ionoregulation in the Gill of Japanese Medaka and Rainbow Trout

Rebecca Jo Bollinger
University of Arkansas, Fayetteville

Cellular Mechanisms of Ionoregulation in the Gill of Japanese Medaka and Rainbow Trout

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Rebecca J. Bollinger
Southern Illinois University Edwardsville
Bachelor of Science in Biological Sciences, 2012

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University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Dr. Christian Tipsmark
Chair

_____
Dr. Mack Ivey
Committee Member

_____
Dr. Ralph Henry
Committee Member

_____
Dr. Ines Pinto
Committee Member

_____
Dr. Wayne Kuenzel
Committee Member

Abstract

Euryhaline fishes are capable of adapting to a wide range of salinities such as freshwater, brackish water or seawater. Through the combined effort of the gill, kidney and intestine, they are able to osmoregulate to maintain a constant internal hydromineral balance. As the gill is in direct contact with the external environment, it is continuously working to maintain ion and acid/base balance, gas exchange and eliminate nitrogenous waste. Fish in freshwater are subjected to osmotic water gain and diffusional ion loss across the gill and experience the opposite in seawater. Therefore, the gill exhibits extreme plasticity when experiencing a change in external salinity. Osmoregulation in fishes is controlled mainly by the endocrine system. Prolactin is a freshwater-adapting hormone as it decreases epithelial permeability and increases ion-retention in osmoregulatory tissues.

This dissertation examines the osmoregulatory function of the gill in two euryhaline teleosts, the Japanese medaka (*Oryzias latipes*) and rainbow trout (*Oncorhychus mykiss*). Gill of medaka exposed to seawater and freshwater were used to observe the effect of salinity on the expression of key ion transporters. Hormone *in vitro* studies were performed to understand the mechanism of prolactin-induced expression of the Na^+, Cl^- cotransporter in medaka gill. Finally, rainbow trout were subjected to ion poor water to expand our understanding of ion retention and ionocyte re-uptake function in a salmonid species.

Furthermore, several human diseases are related to (dys)function of osmoregulatory proteins including cancers, inflammatory bowel disease and chronic kidney disease. Because of the adaptability of the gill epithelia, euryhaline teleosts represent a unique model that may help us understand pathologies in human diseases related to epithelial dysfunction. The endocrine system of teleosts is also analogous to the human endocrine system and is therefore valuable to

better understand hormone-linked diseases in human such as breast cancer, diabetes and atherosclerosis. The experiments performed in this dissertation demonstrate the ability of euryhaline teleosts to provide an alternative model to study human diseases.

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List of Published Chapters

Chapter Two:

Bollinger, R.J., S.S. Madsen, M.C. Bossus, and C.K. Tipsmark. 2016. Does Japanese medaka (*Oryzias latipes*) exhibit a gill Na^+/K^+-ATPase isoform switch during salinity change? *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*. 186:485-501.

Chapter 1 Introduction

Stenohaline fishes are those that live in environments in which the salinity remains stable. Examples of such fishes would include the marine yellowfin tuna (*Thunnus albacares*) or the freshwater largemouth bass (*Micropterus salmoides*). Stenohaline fish have a low tolerance to salinity changes and spend their entire lives in one environment. Euryhaline fishes, on the other hand, are tolerant to changes in environmental salinity and can live in a wide range of salinities including both freshwater (FW) and seawater (SW) environments. For example, the anadromous rainbow trout (*Oncorhynchus mykiss*) is a species that spends its adult life at sea and travels up rivers and streams to spawn (Behnke, 2002). Other euryhaline species that are non-migratory and non-annual breeders, such as the Japanese medaka (*Oryzias latipes*), travel to FW environments where sperm are more motile and result in higher rates of hatching (Inoue and Takei, 2002).

As aquatic organisms, fish are under constant constraints to maintain a stable internal plasma osmolality independent of the osmolality of the external environment. Depending on their species, fish maintain an internal osmolality at about 300-350 mOsm kg^{-1} (Evans et al., 2005). With FW around 1 mOsm kg^{-1} and SW upwards of 1000 mOsm kg^{-1}, maintenance of an internal osmolality in those environments can be quite challenging (Edwards and Marshall, 2013). These osmoregulatory challenges are met by the concerted effort of the primary osmoregulatory organs (Fig. 1.1).

In FW, fish experience passive water gain and diffusive ion loss. The gill is responsible for active ion uptake, acid-base regulation, removal of nitrogenous waste, as well as a major site for gas exchange (Fig. 1.1; Evans et al., 2005). The kidney functions to reabsorb salt and thus produce copious amounts of dilute urine (Fig. 1.1; Loretz and Bern, 1980).

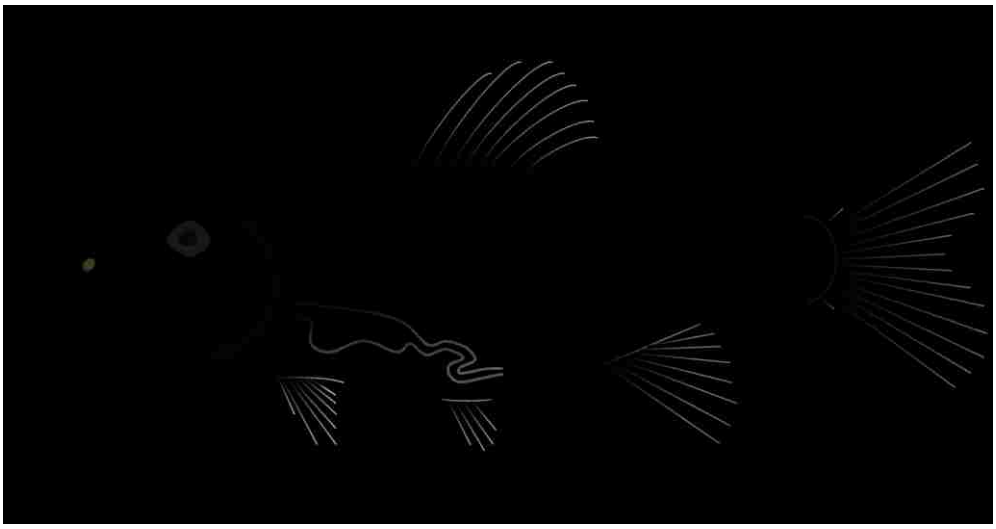

Figure 1.1 Diagram of water and salt exchange in freshwater fishes. Permeable tissues, including the gill, are subject to diffusional ion loss and osmotic water gain. Adapted from: Evans (2008)

In SW, fish experience osmotic water loss and diffusive ion gain. To compensate, SW is ingested and the intestine work to absorb the ions and water (Fig 1.2; Karasov and Hume, 1997). The kidney functions to excrete divalent ions and reabsorb water (Fig. 1.2; Beyenbach, 2004). And the gill actively excretes excess monovalent ions into the environment (Fig 1.2; Evans et al., 2005).

Figure 1.2 Diagram of water and salt exchange in seawater fishes. Permeable tissues, including the gill, are subject to diffusional ion gain and osmotic water loss. This results in ion and water uptake via the intestine and ion excretion through the gill and kidney. Adapted from: Evans (2008).

The gill can be argued as the most important of these organs as it is in direct contact with the environment. Additionally, the gill exhibits remarkable plasticity as its function can change from ion absorptive in FW to ion secretive in SW. Therefore, the gill will henceforth be the main focus of this review.

1.1 Gill function in FW osmoregulation: transcellular pathways

In FW, fish are in an environment with an osmolality much lower than their own and must therefore, hyperosmoregulate. In this case, the gill function is to uptake ions from the environment while also tightening intercellular junctions to prevent the loss of ions (Fig. 1.1). In the gill, the primary site for active ion regulation is the ionocyte, a cell known for being rich in mitochondria and possessing several ion transport proteins. The expression of these transporters in FW ionocytes seems to be quite diverse between species and therefore the primary function of each ionocyte has been shown to be highly variable between species as well.

1.1.2 Zebrafish (*Danio rerio*)

The zebrafish is a stenohaline teleost that naturally inhabit rivers in India (Briggs, 2002). Four ionocyte subtypes have been characterized in the zebrafish gill (Chang and Hwang, 2011; Hwang et al., 2011). H^+-ATPase- rich (HR) ionocytes function in acid-base regulation and Na^+ uptake with expression of a basolateral anion exchanger (AE), Na^+/K^+ -ATPase (NKA), and an Rh protein along with an apical H^+-ATPase (HA), Na^+/H^+ exchanger (NHE3) and Rhcg1 (Fig. 1.3). HR cells also contain two carbonic anhydrase isoforms (CA), one apically and another in the cytosol (Lin et al., 2008). Na^+/K^+-ATPase-rich (NaR) ionocytes work to uptake Ca^{2+} via an apical epithelial Ca^{2+} channel (ECaC) and a basolateral plasma membrane Ca^{2+}-ATPase (PMCA), Na^+/Ca^{2+} exchanger (NCX) and Nka (Fig. 1.3). Na^+, Cl^- cotransporter (NCC) ionocytes

take up Na^+ and Cl^- via an apical Ncc and a basolateral Na^+, HCO_3^- cotransporter (NBC) and Nka (Fig. 1.3). K^+ secreting (KS) ionocytes express an apical K^+ channel, a renal outer medullary K^+ channel (ROMK) and a basolateral Nka (Fig. 1.3).

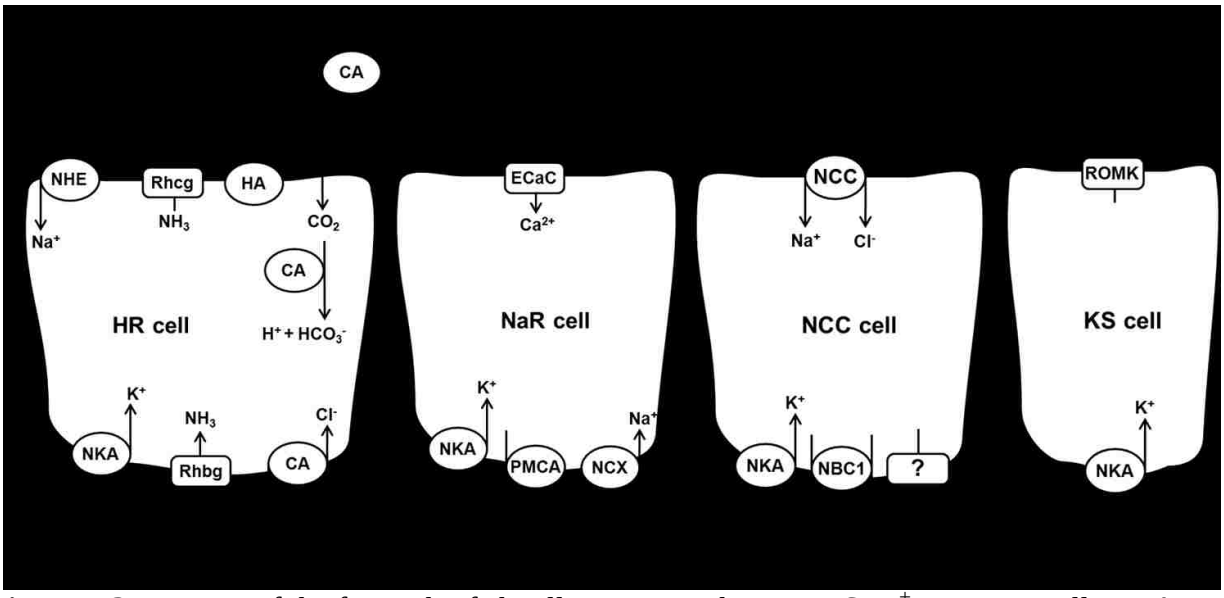

Figure 1.3 Diagram of the four zebrafish gill ionocyte subtypes. KS, K^+ secreting cell; NKA, Na^+/K^+-ATPase; ROMK, renal outer medullary K^+ channel; NCC, Na^+, Cl^- cotransporter; NBC, Na^+, HCO_3^- cotransporter; NaR, Na^+-rich; ECaC, epithelial Ca^{2+} channel; PMCA, plasma membrane Ca^{2+} channel; NCX, Na^+/Ca^{2+} exchanger; HR, H^+-ATPase-rich; CA, carbonic anhydrase; AE, anion exchanger; NHE3, Na^+/H^+ exchanger; Rhcg and Rhbg; Rh proteins. Adapted from: Dymowska (2012).

1.1.3 Tilapia (*Oreochromis*)

Tilapia are euryhaline teleosts that naturally inhabit FW environments but can tolerate full strength SW. Tilapia possess three FW ionocyte subtypes: Type I, Type II and Type III. The function of Type I ionocytes is currently unknown as it has only been found to express a basolateral NKA (Fig. 1.4; Hiroi et al., 2008). Type II ionocytes are believed to function in Na^+ and Cl^- uptake as it expresses an apical NCC, and basolateral NKA and NBC (Fig. 1.4; Hiroi et al., 2005a; Hiroi et al., 2008; Inokuchi et al., 2009; Inokuchi et al., 2008). The Type II ionocytes in the euryhaline tilapia appear to be analogous to the NCC cells on the stenohaline zebrafish in

terms of ion transporter expression and ion transport functions (Hwang, 2009; Hwang et al., 2011). Type III ionocytes are cells expressing apical NHE3, and basolateral NKCC (Na^+, K^+, $2Cl^-$ cotransporter) and NKA (Choi et al., 2010; Hiroi et al., 2005b; Inokuchi et al., 2009). Both NHE3 expression and Type III cell size and number increases rapidly after FW exposure suggesting that this ionocyte are involved in Na^+ uptake (Fig. 1.4; Hiroi et al., 2005a).

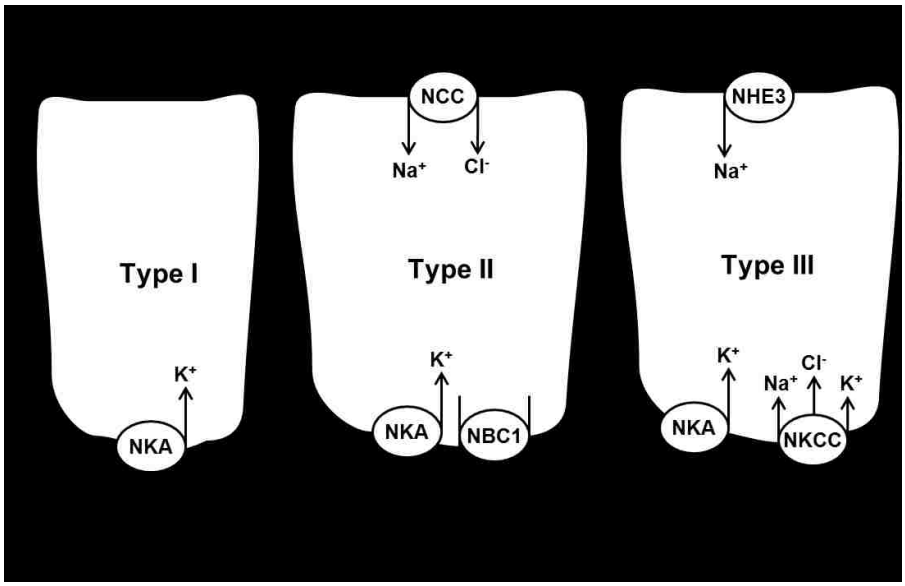

Figure 1.4 Diagram of the three FW ionocyte subtypes in tilapia gill. NKA, Na^+/K^+-ATPase; NCC, Na^+, Cl^- cotransporter; NBC, Na^+, HCO_3^- cotransporter; NKCC, Na^+, K^+, $2Cl^-$ cotransporter; NHE3, Na^+/H^+ exchanger. Adapted from: Dymowska (2012).

1.1.4 Killifish (*Fundulus heteroclitus*)

The killifish is a euryhaline species that naturally inhabits estuaries which naturally vary in salinity (Griffith, 1974). Distinct ionocyte subtypes do not seem to be present in the killifish gill. The only phenotypical evidence of a difference between a FW and SW ionocyte is that the FW ionocyte appears to be larger in size (Katoh et al., 2001; Katoh et al., 2003). While there is still much to be understood about the FW killifish gill ionocyte, there is evidence for basolateral

expression of HA and NKA (Katoh et al., 2003) and apical NCC and NHE (Fig. 1.5; Katoh et al., 2008).

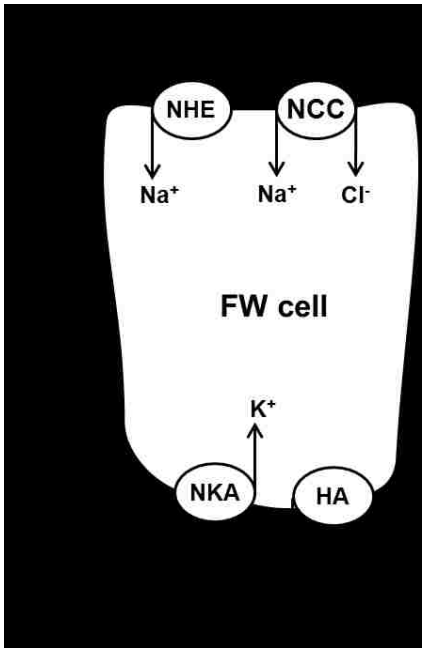

Figure 1.5 Diagram of the FW killifish gill ionocyte. NHE, Na^+/H^+ exchanger; NCC, Na^+, Cl^- cotransporter; HA, H^+-ATPase; NKA, Na^+/K^+-ATPase. Adapted from: Dymowska (2012).

1.1.5 Rainbow trout (*Oncorhynchus mykiss*)

Currently, studies have identified only two subtypes of ionocytes in the FW gill of rainbow trout. These ionocytes differed as to whether they expressed peanut agglutinin (PNA) or not (PNA+ or PNA-: Galvez et al., 2002). Due to variation in methods used to analyze expression and localization of transporters in these cell types, the model for ionocyte function in trout is still under debate. However, a model for both ionocytes can be constructed using the most current data. PNA+ ionocytes are proposed to be involved with Ca^{2+} and Cl^- uptake as well as the major site for Na^+/NH_4^+ exchange (Boyle et al., 2015; Dymowska et al., 2014; Galvez et al., 2008; Tresguerres et al., 2006; Zimmer et al., 2017). These ionocytes are suggested to express a basolateral NKA, NCX, PMCA, Rhbg and ClC (Cl^- channel), an apical CBE (Cl^-

/HCO_3^- exchanger), NHE2 and NHE3b, Rhcg1, HAT (H^+-ATPase) and ASIC (acid-sensing ion channel; Fig. 1.6). PNA- ionocytes are proposed to function in Na^+/H^+ exchange as well as Cl^- and Ca^{2+} uptake with apical expression of ECaC, HAT, and ASIC with basolateral NKA, Rhbg and ClC (Fig. 1.6; Dymowska et al., 2014; Galvez et al., 2008; Tresguerres et al., 2006; Zimmer et al., 2017). While both cell types seemingly serve the same functions, there is great debate as to which ionocytes are more active in extreme conditions such as high environmental ammonia, low ion conditions or acidic environments.

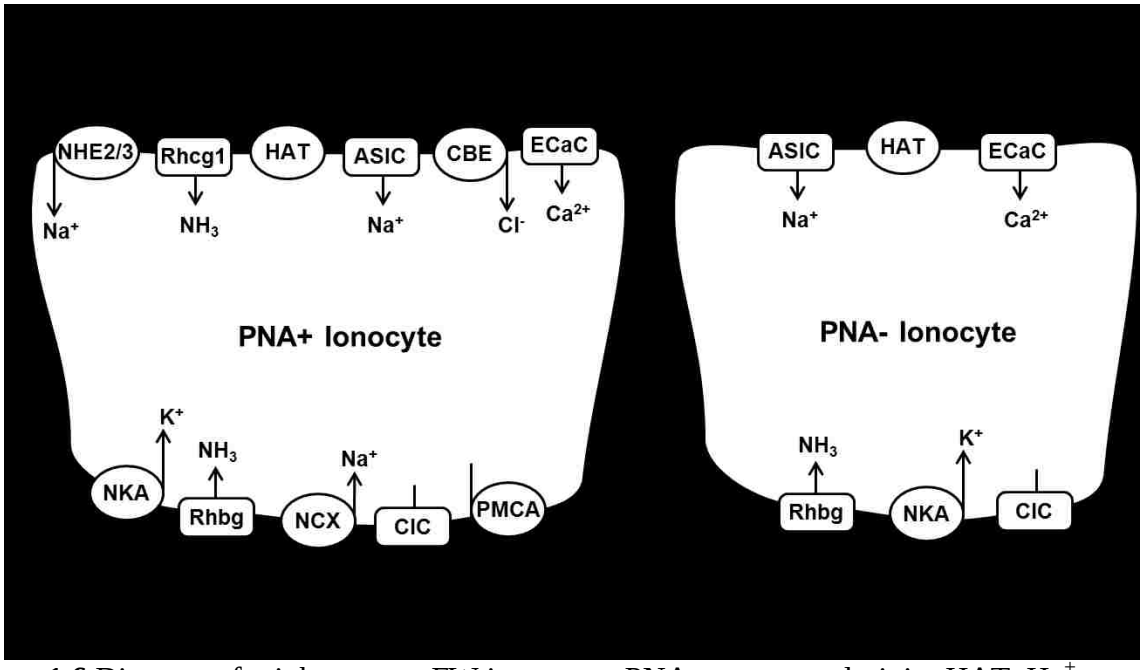

Figure 1.6 Diagram of rainbow trout FW ionocytes. PNA, peanut agglutinin; HAT, Ha^+-ATPase; Rhcg and Rhbg, Rh proteins; NHE, Na^+/H^+ exchanger; ASIC, acid sensing ion channel; CBE, Cl^-/HCO_3^- exchanger; ECaC, epithelial Ca^{2+} channel; ClC, Cl^- channel; NKA, Na^+/K^+-ATPase; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+} channel. Adapted from: Dymowska (2012) and Zimmer (2017).

1.1.6 Japanese medaka (*Oryzias latipes*)

The Japanese medaka is a euryhaline species that is capable of acclimating to a wide range of salinities as it is native to marshes and ponds in Japan, Korea and China (Takehana et al., 2003). Three FW ionocyte subtypes have been identified in the medaka gill (Hsu et al.,

2014). NHE cells express apical NHE3 and Rhcg1, along with basolateral NKCC, NKA, Rhbg and AE1. This cell type is proposed to function in Na^+ uptake, NH_4^+ excretion and acid secretion (Fig. 1.7). ECaC cells exhibit apical ECaC and basolateral NKA are thought to aid Ca^{2+} uptake (Fig. 1.7). NCC cells possess an apical NCC and basolateral HA and NKA and are either involved in NaCl uptake or acid-base regulation (Fig. 1.7).

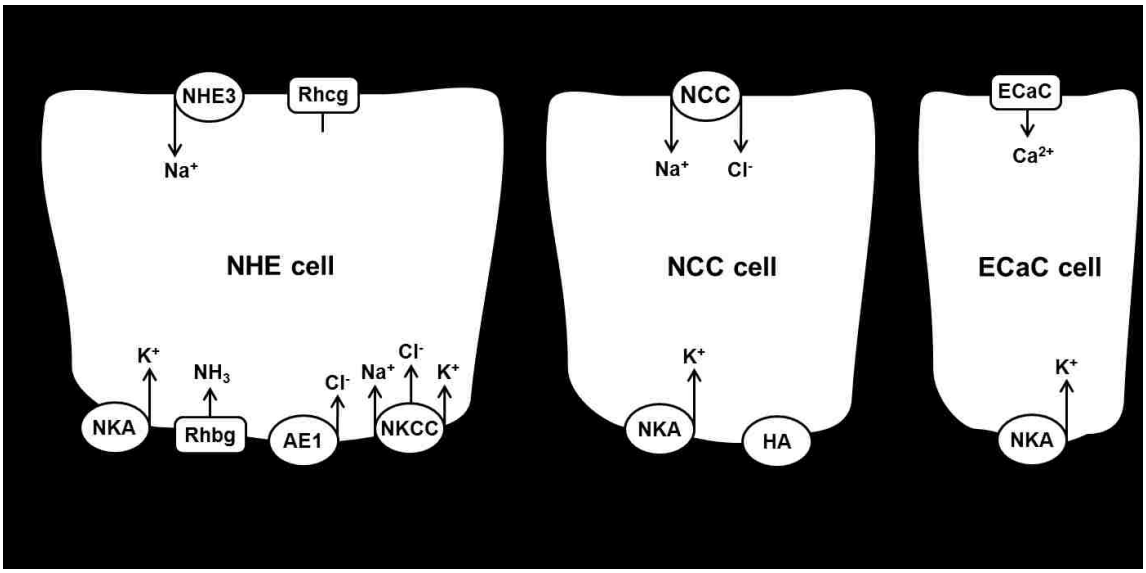

Figure 1.7 Diagram of the three FW ionocytes described in medaka gill. NCC, Na^+, Cl cotransporter; NKA, NA^+/K^+-ATPase; HA, H^+-ATPase; ECaC, epithelial Ca^{2+} channel; NHE, Na^+/H^+ exchanger; Rhcg and Rhbg, Rh proteins; NKCC, Na^+, K^+, $2Cl^-$ cotransporter; AE, anion exchanger. Adapted from: Hsu (2014).

1.2 Gill function in FW osmoregulation: paracellular pathways and tight junctions

While the membrane proteins in an ionocyte determine the movement of ions transcellularly, tight junctions are responsible for allowing the movement of ions paracellularly. The tight junction is the most apical cell-cell connection and is composed tricellulin, occludin and claudins (Gonzalez-Mariscal et al., 2008). In FW fishes, the gill epithelium is regarded as 'tight' and relatively impermeable to solute movement to prevent passive ion loss to the hypo-osmotic environment (Evans et al., 2005; Hwang and Lee, 2007; Marshall et al., 2002b). The

permeability of the tight junction is largely influenced by the proteins that bridge the space between epithelial cells, occludin and claudins (Gonzalez-Mariscal et al., 2003; Van Itallie and Anderson, 2006). Occludin has been shown to increase in expression in response to hypo-osmotic challenge (Chasiotis et al., 2012; Whitehead et al., 2011) and was accompanied by a reduction in Na^+ efflux across the gills (Scott et al., 2004). Occludin therefore functions to create a tighter gill epithelium.

1.2.1 Claudins

Claudin (Cldn) proteins have been shown to exhibit tissue-specific expression and it is proposed that the highly expressed claudins in these tissues are important regulators of epithelial permeability (Bagherie-Lachidan et al., 2008; Bagherie-Lachidan et al., 2009; Bossus et al., 2015; Chasiotis et al., 2012; Loh et al., 2004; Tipsmark et al., 2008a). While only 24 claudin genes have been identified in mammalian genome (Van Itallie and Anderson, 2006), over 50 claudin genes have been identified in teleosts (Baltzegar et al., 2013; Loh et al., 2004). However, only a few of these claudins exhibit barrier-forming expression patterns in the FW gill. In medaka, *cldn27a, cldn28a, cldn28b* and *cldn30c* were constitutively expressed in both FW and SW, with only *cldn28b* exhibiting an effect of FW (Bossus et al., 2015). In tilapia, transfer from FW to SW stimulated and increase in gill expression of *cldn28a* and *cldn30* (Tipsmark et al., 2008a). Acclimation of Atlantic salmon to SW resulted in a decrease of *cldn27a* and *cldn30* expression, while no effect of salinity was exhibited on *cldn28a* or *cldn28b* (Tipsmark et al., 2008b). These claudin isoforms (Cldn27a, 28a, 28b and 30c) appear to be particularly important in either increasing the gill epithelia or maintaining the integrity of the tight junction in FW and thus prevent ion loss.

The regulation of the expression of these tight-junction proteins as well as the transcellular osmoregulatory proteins is under the fine control of the endocrine system.

1.3 Hormonal control of FW osmoregulation

The endocrine system is an important factor in osmoregulation and is investigated in several ways to determine the role hormones play in these processes. One useful technique is the use of hormonal treatments of isolated tissues or cells (*in vitro*) or whole fish (*in vivo*) to examine the response of ionoregulatory and osmoregulatory proteins. Another common practice is the measurement of circulating hormones after transferring fish to a different salinity.

1.3.1 Prolactin

Prolactin is a pituitary hormone that has many functions related to osmoregulation in teleosts (Fig. 1.8; Bole-Feysot et al., 1998; Sakamoto et al., 2003). In particular, prolactin is considered a FW-adapting hormone as it has been shown to decrease epithelial permeability in osmoregulatory tissues and to increase ion-retention (Kelly and Wood, 2002b; Manzon, 2002). Several studies have shown increased prolactin expression and plasma levels in teleosts in response to a reduction in environmental salinity (Fuentes et al., 2010; Hoshijima and Hirose, 2007; Lee et al., 2006; Liu et al., 2006; Shepherd et al., 1999; Yada et al., 1994). Prolactin has proven to be critical to survival in FW as removal of the pituitary (hypophysectomy) was lethal to fish in FW (Ball and Ensor, 1965; Pickford and Phillips, 1959) but not to fish in SW (Breves et al., 2010). However, treatment with exogenous prolactin restored survival of the hypophysectomized FW fish.

Prolactin was shown to stimulate ion uptake in cultured branchial epithelia (Zhou et al., 2003), suggesting that prolactin regulates genes responsible for ionocyte function in FW.

Treatment with prolactin increased expression of *ncc* in both medaka and zebrafish gill (Bossus et al., 2017; Breves et al., 2013). Replacement therapy with ovine prolactin restored *ncc* expression and NCC-cell numbers in hypophysectomized tilapia (Breves et al., 2010). Prolactin replacement therapy stimulated the *nka-α1a* (FW isoform of the NKA alpha subunit) in hypophysectomized tilapia (Tipsmark et al., 2011) as well as increasing Nka activity in Atlantic salmon (Shrimpton and McCormick, 1998). Treatment with prolactin upregulated expression of *cldn28a* in Atlantic salmon gill and *cldn28b* in the gill of medaka (Bossus et al., 2017; Tipsmark et al., 2009). Furthermore, prolactin has also been shown to suppress function of SW-type ionocytes by downregulating expression of *nka-α1b* (SW isoform) in Atlantic salmon and *cftr* (a Cl^- channel, cystic fibrosis transmembrane conductance regulator) in medaka (Bossus et al., 2017; Tipsmark and Madsen, 2009).

These data demonstrate the importance of prolactin in FW teleost gill osmoregulation, as it plays a critical role ion retention by decreasing gill permeability and increasing ion absorption.

1.3.2 Cortisol

Cortisol is the major corticosteroid hormone produced by the interrenal tissue of the fish (Fig. 1.8; Patino et al., 1987). This hormone is important for osmoregulatory function and has been linked to maintenance of hydromineral balance in FW, SW and ion poor water (Laurent and Perry, 1990; McCormick and Bradshaw, 2006; Perry et al., 1992). Cortisol plasma levels increase in response to FW-acclimation (Kajimura et al., 2004; McCormick, 2001). Cortisol has been shown to enhance ion uptake, decrease transepithelial resistance, reduce paracellular permeability and ion efflux rates in FW fish gill epithelia (Chasiotis and Kelly, 2011; Kelly and Wood, 2001; Kelly and Wood, 2002b; Tipsmark et al., 2009; Wood et al., 2002).

Cortisol also works to ion absorption as it has been shown to stimulate Nka enzymatic activity in several salmonids in FW including coho salmon (McCormick and Bern, 1989), brown trout (Tipsmark et al., 2002) and rainbow trout (Shrimpton and McCormick, 1999). In Atlantic salmon, cortisol stimulated a parallel increase in Nka and Fxyd11 abundance (Tipsmark et al., 2010), suggesting the increase in Nka activity observed is possibly related to Fxyd11 modulation. Exogenous cortisol treatment promoted an increase in the density of Ncc-expressing cells which also resulted in an increase of *ncc* expression and whole-body Na^+ levels in zebrafish (Lin et al., 2016). Additionally, cortisol plays a role in promoting Ca^{2+} uptake by increasing ECaC mRNA expression in zebrafish and protein levels in rainbow trout gill (Lin et al., 2011; Shahsavarani and Perry, 2006). Cortisol increased HR cell proliferation and acid secretion via HR cells in zebrafish (Cruz et al., 2013; Lin et al., 2015). Exogenous cortisol also increased transepithelial resistance in FW trout gill as well and decreased paracellular permeability in pufferfish (Bui et al., 2010; Kelly and Wood, 2001; Kelly and Wood, 2002b). This tightening of the gill epithelium may be contributed to the increase in *cldn27a* and *cldn30c* expression that has been exhibited in medaka and *cldn27a* and *cldn30* in Atlantic salmon with cortisol treatment (Bossus et al., 2017; Tipsmark et al., 2009). Cortisol also stimulated expression of *cldn28a* and *cldn30* in tilapia gill (Tipsmark et al., 2016).

Taken together, these data provide evidence of cortisol's role as an osmoregulatory hormone by decreasing ion loss and increasing ion uptake in FW.

1.3.3 Prolactin and Cortisol

As a dual functioning hormone, cortisol's ability to aid in both FW and SW acclimation has been postulated to then work together with prolactin to work in synergy to promote FW acclimation (Fig. 1.8; Evans, 2002; Laurent and Perry, 1990; McCormick, 2001; Perry et al.,

1992). In primary cultures of gill cells of rainbow trout, prolactin and cortisol together promoted transepithelial resistance and potential (Zhou et al., 2003). They were also shown to directly decrease the permeability of rainbow trout branchial epithelia (Kelly and Chasiotis, 2011; Kelly and Wood, 2001; Kelly and Wood, 2002b). Prolactin and cortisol have also been shown to have an effect on gill ion regulation. Treatment with prolactin and cortisol exhibited an effect on Nka activity by increasing *fxyd11* expression (a Nka regulatory protein) in tilapia gill (Tipsmark et al., 2011). They have also increased *ncc* expression in medaka, possibly upregulating NaCl (Bossus et al., 2017). Together, prolactin and cortisol exhibit a greater effect on FW osmoregulation than either one alone.

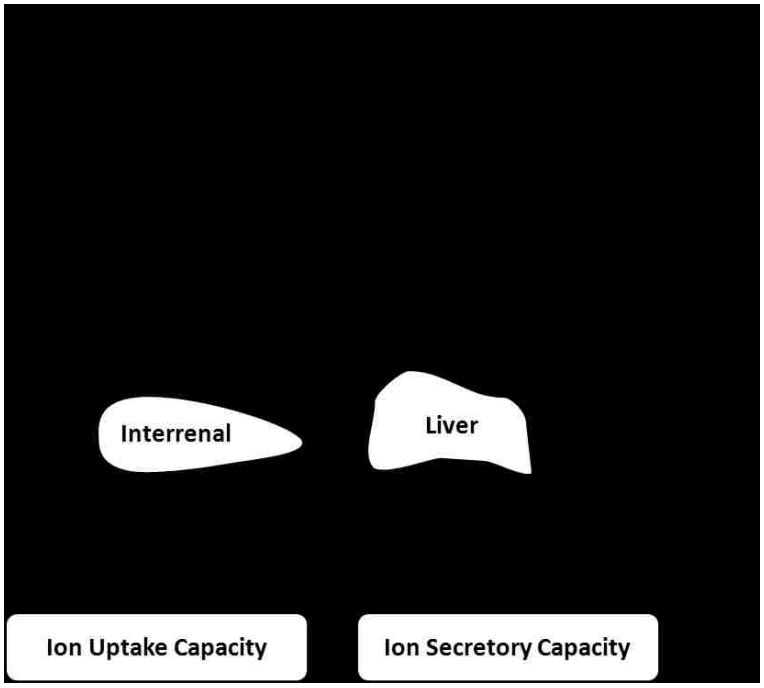

Figure 1.8 Schematic showing the effect of prolactin, cortisol, GH and IGF-1 on ion uptake and ion secretion in teleost gills. Prolactin and cortisol can either work alone or together to increase ion uptake in the gills. GH stimulates the expression of IGF-1, which can work in synergy with cortisol to increase ion secretion in the gills of SW fishes. Adapted from: Takei and McCormick (2013).

1.4 Gill function in SW osmoregulation: transcellular pathways

In SW, fish hypoosmoregulate, thus stabilizing their internal osmolality at a level much lower than that of the surrounding water. In this case, the gill functions to excrete excess monovalent ions from their plasma to the outer environment. The gill epithelium of a fish in SW is less tight to allow paracellular movement of ions. While the FW ionocyte is highly diverse, the mechanism of the SW ionocyte seems to be universal among teleosts.

1.4.1 SW ionocyte function

In the marine environment, fish are constantly losing water osmotically. In order to reverse the effect of this dehydration the fish must drink water and excrete excess NaCl. This is achieved by the active transport of Na^+ through basolateral NKA coupled to the secondary active transport of Cl^- via NKCC which then leaves the cell via an apical CFTR (Fig. 1.9; Evans et al., 2005). The NKA works to keep intracellular Na^+ levels low to create a gradient for Cl^- uptake via the NKCC (Fig. 1.9; Hirose et al., 2003). K^+ is in electrochemical equilibrium via a basolateral K^+ channel (Suzuki et al., 1999; Tse et al., 2006). Together, the NKA and the movement of Cl^- out of the cell generates a serosal positive transepithelial potential that allows for the paracellular extrusion of Na^+ via cation-selective tight junctions located between ionocytes and accessory cells. Furthermore, metabolic waste in the marine teleost involves apical NHE3, NHE2 and Rhcg1, and basolateral Rhbg (Fig. 1.9; Hsu et al., 2014). This model appears to be general among teleosts as it has been confirmed in tilapia (Hiroi et al., 2005b; Hiroi et al., 2008; Tipsmark et al., 2011), killifish (Marshall et al., 2002a), Japanese medaka (Hsu et al., 2014) and a number of salmonids (Bystriansky et al., 2006; McCormick et al., 2009; Nilsen et al., 2007; Tipsmark and Madsen, 2009).

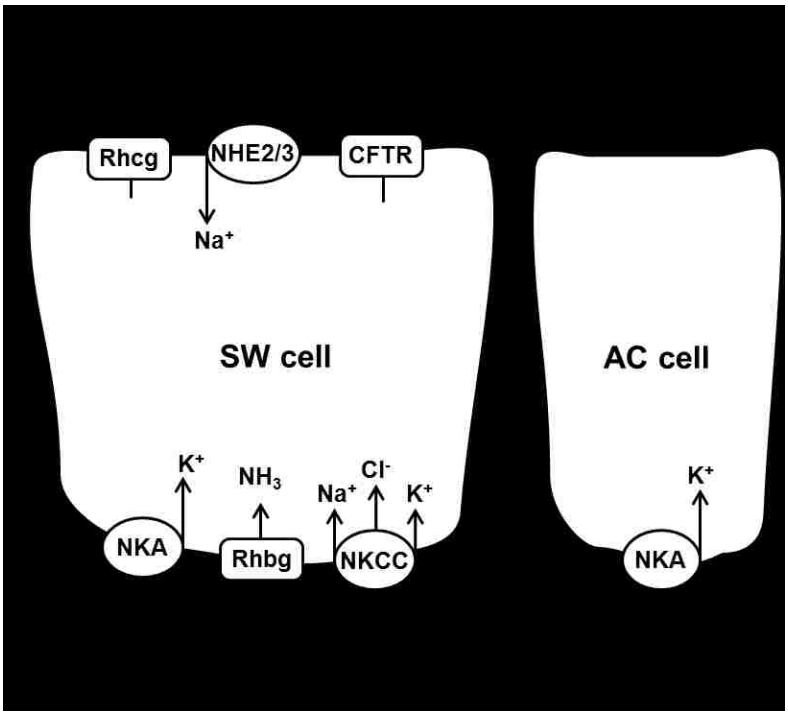

Figure 1.9 Diagram of the SW gill ionocyte. CFTR, cystic fibrosis transmembrane conductance regulator; NHE, Na^+/H^+ exchanger; Rhbg and Rhcg, Rh protein; NKCC, Na^+, K^+, $2Cl^-$ cotransporter; NKA, Na^+/K^+-ATPase; AC, accessory cell. From: Hsu (2014).

1.4.2 The Na^+/K^+-ATPase

The Nka is the primary driving force for ion secretion in the SW ionocyte (Foskett and Scheffey, 1982). The Nka uses one ATP to drive the exchange of two extracellular K^+ ions for three intracellular Na^+ ions, which maintains ion gradients favorable for vectorial transepithelial ion transport (Glynn, 1985; Glynn, 1993). Several euryhaline species exhibit an increase in gill Nka activity and abundance after transfer to SW (Johnston and Saunders, 1981; Madsen and Naamansen, 1989; McCormick et al., 1989). Furthermore, Nka α-subunit has been shown to be salinity dependent for several teleosts, involving a switch in the expressed isoform. Transfer from FW to SW leads to an increase in expression of *nka-α1b* and a decrease in *nka-α1a*, suggesting that Nka-α1b is the isoform responsible for driving Na^+ excretion in SW (Richards et al., 2003). This has been demonstrated in rainbow trout (Bystriansky et al., 2006; Richards et al.,

2003), Atlantic salmon (Bystriansky et al., 2006; Madsen et al., 2009; McCormick et al., 2009), arctic char (Bystriansky et al., 2006), tilapia (Feng et al., 2002; Lee et al., 1998; Tipsmark et al., 2011) climbing perch (Ip et al., 2012) and inanga (Urbina et al., 2013).

1.4.3 Na^+, K^+, $2Cl^-$ cotransporter

Nkcc1 is a member of the SLC12A family of proteins and is considered a secretory protein (Hwang 2011). Nkcc1 has been shown to co-localize with Nka in the basolateral membrane of the branchial epithelium in SW (Kang et al., 2010; Pelis et al., 2001). Two isoforms for Nkcc1 have been identified in teleost gills (1a and 1b), with Nkcc1a exhibiting higher expression and therefore presumed to be the more active isoform in NaCl secretion (Cutler and Cramb, 2002; Hiroi et al., 2008; Kang et al., 2010). Exposure to SW increased expression of Nkcc1a in several euryhaline teleosts including eel (Cutler and Cramb, 2002; Tse et al., 2006), tilapia (Hiroi et al., 2008; Wu et al., 2003), medaka (Kang et al., 2010), sea bass (Lorin-Nebel et al., 2006), killifish (Flemmer et al., 2010; Scott et al., 2004) and several salmonids (Hiroi and McCormick, 2007; Tipsmark et al., 2002). Thus, confirming the role of Nkcc1a in the Cl^- secretion mechanism in SW ionocytes.

1.4.4 Cystic Fibrosis Transmembrane Conductance Regulator

Cftr is a cAMP-activated Cl^- channel that primarily appears in SW branchial ionocytes (Bodinier et al., 2009; Hwang and Lee, 2007). Exposure to higher salinity increased expression of Cftr in sea bass (Bodinier et al., 2009), Atlantic salmon (Nilsen et al., 2007), killifish (Katoh et al., 2003; Marshall and Singer, 2002; Scott et al., 2004; Shaw et al., 2008), pufferfish (Tang and Lee, 2007), tilapia (Hiroi et al., 2005a) and eel (Tse et al., 2006). Furthermore, transfer of SW-acclimated fish to FW significantly decreased Cftr expression in tilapia (Scott et al., 2005),

sea bass (Bodinier et al., 2009) and medaka (Bollinger et al., 2016; Kang et al., 2012). Altogether, these results indicate a role for Cftr in Cl^- secretion.

1.5 Gill function in SW osmoregulation: paracellular pathways and tight junctions

The gill epithelium in SW-acclimated fishes is referred to as 'leaky' as the junctions are proposed to allow the passive movement of Na^+ ions from the interstitial fluid to the external environment. This is exhibited through increased permeability and expression of cation-selective claudins (Evans et al., 2005; Hwang and Lee, 2007). Claudin10 paralogs have shown higher expression in gills of SW-acclimated fish than in FW-acclimated fish and are suggested to aid in Na^+ extrusion. Cldn10d and 10e mRNA in pufferfish and Cldn10e in Atlantic salmon were expressed significantly higher in the gills of SW fish than FW fish (Bui et al., 2010; Tipsmark et al., 2008b). Furthermore, *cldn10c*, *cldn10d*, *cldn10e* and *cldn10f* all increased in expression following transfer of medaka to SW (Bossus et al., 2015), suggesting that claudin 10 paralogs are cation-pore forming claudins involved in forming a leaky epithelia necessary for Na^+ extrusion in SW. Other claudins do not exhibit an effect of salinity, suggesting their role in maintaining the integrity of the gill epithelium. Cldn27a, Cldn28a and Cldn30c mRNA expression in gill of medaka exposed to SW (Bossus et al., 2015). In Atlantic salmon, SW exposure resulted in a decrease in gill Cldn27a and Cldn30 mRNA expression and no overall effect on Cldn28a and Cldn28b (Tipsmark et al., 2008b).

1.6 Hormonal control of SW osmoregulation

Salinity changes induce endocrine signaling which mediate the acclamatory response needed to maintain homeostasis. While prolactin is integral to FW acclimation for several teleost species, its absence is also crucial when moving to SW. After transfer to SW, a decrease in

plasma prolactin levels has been observed in salmonids (Avella et al., 1990; Poppinga et al., 2007; Young et al., 1995) Furthermore, as an ion-retaining hormone, prolactin has been shown to oppose SW acclimation when injected into trout (Madsen and Bern, 1992; Seidelin and Madsen, 1997).

1.6.1 Cortisol

Cortisol plays a dual role in osmoregulation as it also aids in SW acclimation (Fig. 1.8). Treatment of FW fishes with cortisol improved survival after transfer to SW by increasing gill ionocyte size and abundance in rainbow trout, European eel, tilapia and catfish (Perry et al., 1992). Cortisol treatment led to a decrease in plasma ion levels and osmolality in SW acclimated teleosts and enhanced salinity tolerance (McCormick, 2001).Cortisol has been shown to increase expression of proteins integral to salt secretion in several teleost gill, including Nka-α1b, Nkcc1, Cftr and Fxyd11 (Bossus et al., 2017; Kiilerich et al., 2007; McCormick et al., 2008; Singer et al., 2003; Tipsmark and Madsen, 2009). Gill epithelium paracellular permeability decreases with exogenous cortisol treatment (Chasiotis et al., 2010; Kelly and Wood, 2002a) and is thought to be due to the regulation of tight junction protein expression. In medaka, cortisol treatment increased mRNA expression of the SW claudin10 isoforms; *cldn10d, cldn10e* and *cldn10f* (Bossus et al., 2017). Similar results were observed in tilapia (Tipsmark et al., 2016) Atlantis salmon (Tipsmark et al., 2009) and rainbow trout (Chasiotis and Kelly, 2011).

Together, these data provide evidence for the role of cortisol in promoting SW osmoregulation hormone by decreasing paracellular permeability and increasing ion secretion.

1.6.2 Growth hormone

Growth hormone (GH) is a pituitary hormone that promotes osmoregulatory action mainly through stimulation of insulin-like growth factor-1 (IGF-1) production (Fig. 1.8).

Transfer to SW results in increased gene expression, secretion, circulating levels and metabolic clearance of GH and IGF-1 (Link et al., 2010; Sakamoto and Hirano, 1993; Sakamoto et al., 1993; Sakamoto et al., 1990; Takahashi and Sakamoto, 2013). *In vivo* treatment with GH stimulated ionocyte cell size and number in sea trout (Madsen, 1990). Treatment with exogenous IGF-1 increases salinity tolerance of rainbow trout, Atlantic salmon and killifish (Mancera and McCormick, 1998). GH alone is incapable of increasing Nka activity in cultured gill tissues (Seidelin et al., 1999), however, IGF-1 can (Madsen and Bern, 1993). The GH-IGF-1 axis has also been shown to increase the expression of Nka-α1b, Fxyd11, Nkcc1 and Cftr; proteins linked with salt secretion (Pelis and McCormick, 2001; Tipsmark and Madsen, 2009; Tipsmark et al., 2010).

Gene expression of Nka-α1b, Nkcc1 and Cftr has also exhibited regulation by cortisol, which suggests an interaction between GH-IGF-1 and cortisol to regulate salt secretion in teleosts. GH and cortisol both increase gill ionocyte cell size and number (Pelis and McCormick, 2001; Perry et al., 1992). The combination of treatment with GH and cortisol on killifish gills resulted in Nka activity much greater than either hormone alone (Madsen, 1990; Mancera and McCormick, 1998; McCormick, 2001). Treatment of Atlantic salmon in FW caused an increase in both Nka-α1a and Nka-α1b, however, treatment with GH and cortisol led to an even greater expression of Nka-α1b and a decrease in Nka-α1a (Takei and McCormick, 2013).

Together these data support the synergy of the GH-IGF-1 axis and cortisol in the promotion of ion secretion in SW osmoregulation.

1.7 Synopsis

Euryhaline teleosts have proven to be beneficial models for understanding osmoregulation and the hormonal control over these osmoregulatory functions. The extreme

plasticity of the euryhaline gill provides a platform to observe the acclimatory response to change in salinity. While mammals have been the chosen laboratory model for most biomedical researchers, teleost fishes could be a valuable alternative. Teleosts are bilateral vertebrates that possess many of the same internal biological functions as humans, including a strikingly similar endocrine system. Furthermore, they express many of the same genes in their osmoregulatory tissues as humans do in their kidney and intestine. As a laboratory animal, fish are low cost to maintain, easy to keep in large numbers, can be bred easily in the lab, and are quick to develop transgenic models. Teleost fish are an overlooked model organisms that could be a valuable resource in many biomedical fields.

1.8 Dissertation outline

This dissertation presents unique research focusing on the cellular mechanism of ionoregulation of two euryhaline teleosts, the Japanese medaka (*Oryzias latipes*) and rainbow trout (*Oncorhychus mykiss*). Chapter 2 characterizes the expression of key osmoregulatory genes in response to salinity change in the Japanese medaka and tested the hypotheses that a switch between FW and SW is accompanied by a NKA α-isoform shift as observed in salmonid and tilapia species. Chapter 3 investigates the effect of prolactin on the expression of the Na^+, Cl^- cotransporter in Japanese medaka and the cellular signaling pathway involved. Chapter 4 aims to elucidate a model for ion retention and re-uptake in freshwater teleost ionocyte by exposing rainbow trout to ion poor water.

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Chapter 2 Does the Japanese medaka exhibit gill NKA isoform switch during salinity change?

Rebecca J. Bollinger[1], Steffen S. Madsen[1,2], Maryline C. Bossus[1,3], Christian K. Tipsmark[1]

[1] Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA

[2] Department of Biology, University of Southern Denmark, Odense M, Denmark

[3] Department of Biology, Lyon College, Batesville, AR 72501

2.1 Abstract

Some euryhaline teleosts exhibit a switch in gill Na^+/K^+-ATPase (Nka) α isoform when moving between freshwater (FW) and seawater (SW). The present study tested the hypothesis that a similar mechanism is present in Japanese medaka and whether salinity affects ouabain, Mg^{2+}, Na^+ and K^+ affinity of the gill enzyme. Phylogenetic analysis classified six separate medaka Nka α isoforms (α1a, α1b, α1c, α2, α3a and α3b). Medaka acclimated long-term (>30 days) to either FW or SW had similar gill expression of *α1c, α2, α3a* and *α3b,* while both *α1a* and *α1b* were elevated in SW. Since a potential isoform shift may rely on early changes in transcript abundance, we conducted two short-term (1-3 days) salinity transfer experiments. FW to SW acclimation induced an elevation of *α1b* and *α1a* after 1 and 3 days. SW to FW acclimation reduced *α1b* after 3 days with no other α isoforms affected. To verify that the responses were typical, additional transport proteins were examined. Gill *ncc* and *nhe3* expression were elevated in FW, while *cftr* and *nkcc1a* were up-regulated in SW. This is in accordance with putative roles in ion-uptake and secretion. SW-acclimated medaka had higher gill Nka V_{max} and lower apparent K_m for Na^+ compared to FW fish, while apparent affinities for K^+, Mg^{2+} and ouabain were unchanged. The present study showed that the Japanese medaka does not exhibit a salinity-induced α isoform switch and therefore suggests that Na^+ affinity changes involve altered posttranslational modification or intermolecular interactions.

2.2 Introduction

Teleost fishes maintain internal osmotic and ionic balance independent of external salinity and ion and water homeostasis are consequently under constant threat. In freshwater (FW), fish experience diffusive ion loss and osmotic water gain while seawater (SW) causes ion gain and dehydration (Marshall and Grosell, 2006). Ion homeostasis is dependent on key

osmoregulatory organs (gill, kidney and intestine) and in euryhaline fishes the phenotypic plasticity of the gill is especially remarkable. Here a complete reversal of active monovalent ion transport occurs, rendering the tissue from adsorptive in FW to secretory in SW (Evans et al., 2005).

Secretion of NaCl by SW ionocytes involves a basolaterally located Na^+/K^+-ATPase (Nka) and $Na^+,K^+,2Cl^-$ cotransporter (Nkcc) working in conjunction with an apical cystic fibrosis transmembrane conductance regulator chloride channel (Cftr: Hiroi and McCormick, 2012; Marshall and Singer, 2002; Silva et al., 1977) and a cation-selective paracellular exit path for sodium (Degnan and Zadunaisky, 1980). This model appears general and is largely confirmed in a series of teleosts species such as tilapia (*Oreochromis mossambicus*: Hiroi et al., 2005; Hiroi et al., 2008; Tipsmark et al., 2011), killifish (*Fundulus heteroclitus*: Marshall and Singer, 2002), Japanese medaka (*Oryzias latipes*: Hsu et al., 2014) and a number of salmonids (Bystriansky et al., 2006; McCormick et al., 2009; Nilsen et al., 2007; Tipsmark and Madsen, 2009). Ion absorption by branchial FW ionocytes is less well understood and has been a topic of controversy for quite some time, possibly due to species differences and diverse methods being applied to classify these cells (Dymowska et al., 2012; Evans et al., 2005; Hirose et al., 2003; Hwang et al., 2011; Marshall and Singer, 2002; Perry et al., 2003). In the gill of FW medaka, three distinct ionocytes were recently classified, all of which express a basolateral Nka (Hsu et al., 2014) but characterized by presence of other specific ion transport proteins. One cell type has high expression of apical Na^+,Cl^- cotransporter (Ncc) while another shows high apical Na^+/H^+ exchanger (Nhe3), both with a putative role in monovalent ion absorption. A third FW ionocyte with a putative role in calcium homeostasis has strong apical expression of the epithelial Ca^{2+} channel protein. Apical expression of Nhe3 has also been found in FW gill of rainbow trout

(*Oncorhynchus mykiss*: Hiroi and McCormick, 2012; Ivanis et al., 2008) and tilapia (O. mossambicus: Hiroi et al., 2008; Inokuchi et al., 2008). An apical vacuolar-type H^+-ATPase (Vata) was observed in the FW gill of rainbow trout (*O. mykiss*: Wilson et al., 2000) and was also expressed in FW Atlantic salmon gill (*Salmo salar*: Bystriansky and Schulte, 2011). Gill ionocytes with putative function in ion uptake and apical Ncc immunostaining have been reported in tilapia (*O. mossambicus*: Hiroi et al., 2008) and zebrafish (*Danio rerio*: Wang et al., 2009).

The Nka appears to be the primary driving force for ion uptake in FW and ion secretion in SW gills (Foskett and Scheffey, 1982). The Nka exchanges three intracellular Na^+ for two extracellular K^+ ions at the cost one ATP, maintaining ion gradients favorable for vectorial transepithelial ion transport (Glynn, 1985; Glynn, 1993). In most euryhaline teleosts, gill Nka activity and abundance increase after SW acclimation (Johnston and Saunders, 1981; Kelly and Woo, 1999; Madsen and Naamansen, 1989; McCormick et al., 1989). A switch in gill expression between specific α-subunit genes has been documented in several teleosts after transfer from hyposmotic FW to hyperosmotic SW or *vice versa*, and seems in these species to be instrumental for acclimation to the new environment (*O. myskiss*: Bystriansky et al., 2006; Richards et al., 2003; *S. salar*: Bystriansky et al., 2006; Madsen et al., 2009; McCormick et al., 2009; *Salvelinus alpinus*: Bystriansky et al., 2006; *O. mossambicus*: Tipsmark et al., 2011; *Anabus testudineus*: Ip et al., 2012; *Galaxias maculatus*: Urbina et al., 2013). Recent molecular analyses of these salinity responsive α1 isoforms suggests that they developed through parallel evolution in different fish species (Dalziel et al., 2014). The nomenclature in these fishes is based on similar regulation by salinity with Nka *α1b* elevated in SW and *α1a* in FW. Furthermore a conserved amino acid substitution (Lys-Arg) in Nka α1a transmembrane domain 5 (TM5) of salmonids and

tilapia could render it energetically suitable for Na^+ transport against steep electrochemical gradients as in FW (Dalziel et al., 2014; Jorgensen, 2008). This suggests that in these species, α1a provides the driving force for ion uptake in FW, while α1b appears critical to ion secretion in SW. In climbing perch the Lys-Arg substitution is present in both α1a and α1b but absent in α1c. However, in this species, expression of *α1a* is highest in FW while both *α1b* and *α1c* increase in SW suggesting an important role of the latter isoforms in the hyperosmotic environment (Ip et al., 2012).

Japanese medaka have been used for a while as a model for understanding ionoregulatory function in euryhaline fishes (Bossus et al., 2015; Hsu et al., 2014; Inoue and Takei, 2002; Inoue and Takei, 2003; Madsen et al., 2014; Sakamoto et al., 2001). This FW species is native to marshes, ponds and irrigation canals of rice fields in Japan, Korea and China (Takehana et al., 2003) and is capable of adapting to a wide range of salinities (Haruta et al., 1991; Inoue and Takei, 2002; Miyamoto et al., 1986; Shen et al., 2011). An essential step in developing this model is characterization of the molecular driving force for gill ion transport in FW and SW ionocytes. The hypothesis tested in the current study is that medaka exhibit α1 isoform shift in the gill when switching between hypo- and hyperosmoregulation. Given the scarcity of species on which studies have been published so far, this is by no means a trivial hypothesis as testing this occurrence is important for our understanding of euryhalinity. Therefore, the primary aim of the present study was to identify α isoforms expressed in Japanese medaka and examine if salinity-induced α isoform switch occurs in medaka gill during salinity acclimation. The expression of additional transport proteins (*ncc, nhe3, cftr, nkcc1a, vata, fxyd9, fxyd11*) were examined in parallel to confirm that medaka gill respond in accordance with studies in other teleosts (Dymowska et al., 2012; Hiroi and McCormick, 2012). A final goal addressed the

potential of functional differences in gill Nka activity in FW and SW fish by investigating the apparent enzyme affinity for Na^+, K^+, Mg^{2+} and ouabain.

2.3 Materials and Methods

2.3.1 Fish and maintenance

Adult Japanese medaka (*O. latipes*, Temmink & Schlegel; size range: 25-35 mm, weight range: 250-350 mg) were obtained from Aquatic Research Organisms (Hampton, NH, USA) and acclimated to recirculating de-chlorinated tap water, mechanically and biologically filtered (0.34 mM Na^+, 0.64 mM Ca^{2+}, 0.09 mM Mg^{2+}, 0.03 mM K^+). Fish were maintained at 20°C with a 14 hours light/10 hours dark photoperiod. They were fed daily with Tetramin tropical flakes (Tetra, United Pet Group, Blacksburg, VA) or frozen brine shrimp (San Fanscisco Bay Brand, Inc., Newark, CA, USA). Food was withheld during the short-term salinity transfer experiments from one day before and throughout the experiment. All handling and experimental procedures were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC 11005).

2.3.2 Experimental design and sampling

While Japanese medaka are capable of adapting to full strength SW, they first require transfer to a lower salinity in order to survive (Inoue and Takei 2002). Therefore a salinity (28-30 ppt) was chosen at which the medaka would be challenged and have a chance of surviving the transfer. For tissue distribution, 4 FW and 4 SW (Instant Ocean, Spectrum Brands, Blacksburg, VA) medaka were long-term acclimated to their respective salinities for a month prior to sampling. Food was withheld one day before sampling. Fish were anesthetized in 100 mg L^{-1} tricaine methanesulfonate (Western Chemical Inc., Ferndale, WA) and sacrificed by cervical

dislocation. The following tissues were dissected: gill, kidney, intestine, muscle and liver. Tissues were immediately placed on dry ice and stored at -80°C until further use. Gills from these fish were also used for comparison of specific targets between long-term acclimated FW and SW medaka. In the short-time course experiments (72 hours), medaka were acclimated to respective salinities for at least a month prior to experimentation. They were then transferred from FW to SW or from SW to FW. Sampling occurred 24 and 72 hours after transfer (n=6) and each experiment included a sham-transfer group to serve as control. Gill filaments were dissected and promptly frozen on dry ice.

2.3.3 RNA extraction, cDNA synthesis and real-time qPCR

Tissues were homogenized in TRI Reagent® (Sigma Aldrich, St. Louis, MO, USA) using a Power Max 200 rotating knife homogenizer (Advanced Homogenizing System; Manufactured by PRO Scientific for Henry Troemner LLC, Thorofare, NJ, USA). Total RNA was extracted following the manufacturer's protocol. RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water. NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to estimate quantity and purity (A_{260}/A_{280}) of each sample. cDNA was synthesized from 1 µg total RNA in a final volume of 20 µL using Applied Biosystems high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocol. mRNA sequences for Japanese medaka target transcripts were identified in the Ensembl genome browser (Flicek et al., 2014) and used to design specific primers (Table 2.1) to detect the following transcripts: *nka α1a, α1b, α1c, α2, α3a, α3b, nkcc1a, ncc, nhe3, vata, cftr, fxyd9 and fxyd11*. Elongation factor 1-alpha (*ef1a*), β-actin (*actb*) and ribosomal protein PO (*rplp0*) were used as normalization genes (Vandesompele et al., 2002). Primers were generated using Primer3 software (Koressaar and Remm, 2007; Untergasser et al.,

2012). Quantitative real-time qPCR was performed in a final volume of 15 µL using BioRad CFX96 platform (BioRad, Hercules, CA, USA) and SYBR® Green JumpStar™ (Sigma Aldrich). The following thermocycling protocol was used: 3 minutes initial denaturation/activation phase (94°C), 40 cycles of a 15 seconds denaturation step and an annealing/elongation steps for 60 seconds (60°C), followed by a melting curve analysis at an interval of 5 seconds per degree from 55-94°C. Amplification efficiency of each primer set was analyzed using the standard curve method and sequential dilutions from 2 to 16 times. Relative copy numbers of individual target genes was calculated using the primer set amplification efficiency. Relative copy numbers were calculated as $E_a^{\Delta Ct}$, where C_t is the threshold cycle number and E_a is the amplification efficiency (Pfaffl, 2001). GeNorm software (Biogazelle, Zwijnaarde, Belgium) was used to calculate a geometric mean of the three normalization genes. Normalized units were calculated by dividing the relative copy number of each target gene by the geometric mean of normalization genes.

Table 2.1 Primer sequences for quantitative PCR of medaka transcript targets

Target name	Forward primer	Reverse primer	GenBank Accession #
α1a	ATGACAAAGAACCGCATCCT	GTACATTCTGAGGGCGGTGT	XM_004084864
α1b	GAACCGTCACCATCCTCTGT	TGTCGCTCTCAGCTTCTTCA	XM_004066527
α1c	GATCGAGCACCATCCTCTGT	CCAAGGATGAGGGAGAGGAT	XM_004066525
α2	TTCAGTGGGCGGATCTTATC	CAGAGCCGTCTCAACAAACA	XM_004078573
α3a	CGTCATCATGGCTGAAAATG	ATTGCTGGCCATAGCTGTCT	XM_004074068
α3b	TTGCCCCCTTAATGTCACTC	GGGGCAGTTGTGATGAAAAT	XM_004078003
fxyd-9	GGTGTTTGCAGAAGAGACTTG	TGATGCCAATGAGACAAAGG	JX565422
fxyd-11	TGGAAACTGAAGCAAATCCA	TATCAACAGGCCGACAATGA	JX624726
cftr	GGGAAGAGGTGATGGAGACA	CACAATGGCGGAGAAGAAGT	XM_004086223
nhe-3	CATTTTTCGTGGTTGCCTTT	TTTTTGGTGCATCTGGTCAA	XM_004074310
ncc	GGGCCTATGTGCTCTGCTAC	TTGAGATCAAGGCTGCAATG	XM_004084987
nkcc-1a	CCCATCATCTCTGCTGGAAT	TTACACAGGGCCTGGAAAAC	XM_004084607
vat-a	GTGGCAACGAGATGTCTGAA	CAGGGCCGTTCTCTTCATAA	XM_004076355
s18	ACCCAGCTGCTGTCTCAGTT	TTGGACACCTCCTTCTGCTT	XM_004085258
rpl-7	CAGATGGAGAGGCGTGAGAT	CCTGATGACAAAGGCCAGTT	NM_001104870
actb	GAGAGGGAAATTGTCCGTGA	CTTCTCCAGGGAGGAAGAGG	NM_001104808
ef1a	ACGTGTCCGTCAAGGAAATC	TGATGACCTGAGCGTTGAAG	NM_001104662

2.3.4 Nka enzyme assay

Gill Nka activity was measured in gill homogenates using a NADH-coupled assay as described by McCormick (1993) with modifications for use with microplate spectrophotometer (SpectraMax® Plus384, Molecular Devices, Sunnyvale, CA, USA; (Tipsmark and Madsen, 2001). Whole gill apparatus was dissected and quickly frozen in SEI buffer (300 mM sucrose, 20 mM Na_2EDTA, 50 mM imidazole, pH 7.5). Prior to start the assay, gill apparatus was thawed on ice and homogenized in 0.5 mL ice-cold SEIDM buffer (SEI buffer with 0.1% Na-deoxycholate and 10 mM mercaptoethanol) with the Power Max homogenizer. Samples were then centrifuged at 8,000 rpm for 60 seconds. Supernatant was transferred to a new tube and diluted with 1.0 mL ice-cold SEIDM.

Maximal Nka activity (V_{max}) was measured by coupling ATP hydrolysis to the conversion of NADH to NAD^+ by pyruvate kinase (PK) and lactate dehydrogenase (LDH) and analyzed by kinetic readings at 340 nm for 10 min at 25°C. Each homogenate was assayed under the following conditions with or without the presence of ouabain (0.5 mM) in triplicate: (in mM) 49.1 NaCl, 10.4 KCl, 1.8 $MgCl_2$, 0.5 Na_3-phosphoenolpyruvate, 0.5 Na_2-ATP, 0.16 Na_2NADH, 0.4 KCN, 2.6 units mL^{-1} PK, 2.0 units mL^{-1} LDH, in 50 mM imidazole buffer, pH 7.5. Protein content of tissue homogenates was measured by the Bradford method modified for microplate reader (Bradford, 1976). Enzyme activity was normalized to protein content and expressed as µmoles ADP mg $protein^{-1}$ $hour^{-1}$ using the following equation: NKA Activity = $\frac{\frac{\Delta ABS_{340}}{min} x \frac{60\,min}{hr}}{\frac{\Delta OD_{340}}{nmol\ ADP}\ x\ (0.01\ mL\ x \frac{mg\ protein}{mL})}$. Where ΔABS_{340} is the difference in slopes between assays of the same sample with and without ouabain and ΔOD_{340}/nmol ADP is the calibration factor.

A series of assays were performed to examine the apparent affinity of Nka to the substrates Na^+ and K^+, the cofactor Mg^{2+} and inhibitor ouabain. In the salt substrate assays, the concentration of the cation examined was varied while the other two were held constant. Apparent K_m for Na^+ was measured (as described above) with varying concentrations of Na^+ (4.2, 9.2, 19.2, 34.2, 79.2, 104.2, 154.2 mM) while holding [K^+] (15 mM) and [Mg^{2+}] (2 mM) constant. Apparent K_m for K^+ was measured as described above holding [Na^+] (50 mM) and [Mg^{2+}] (2 mM) with varying concentrations of K^+ (0.4, 1.4, 3.4, 6.4, 10.4, 25.4, 50.4 mM). Additionally, apparent K_m for Mg^{2+} was measured as described above holding [Na^+] (50 mM) and [K^+] (15 mM) constant with varying concentrations of Mg^{2+} (0, 1.0, 1.5, 4.0, 8.0, 15.0 mM). Apparent Nka affinity to ouabain was evaluated by measuring Nka activity under conditions described above with varying concentrations of ouabain (0, $1.0x10^{-6}$, $3.0x10^{-6}$, $1.0x10^{-5}$, $3.0x10^{-5}$, $1.0x10^{-4}$, $3.0x10^{-4}$, $1.0x10^{-3}$, $3.0x10^{-3}$, 0.01, 0.03, 0.1, 0.3, 1.0 mM).

2.3.5 Phylogenetic analysis

Amino acid sequences of Nka from other species were obtained from Genbank using the following accession numbers: the climbing perch (*A. testudineus*) *α1a* (JN180940)*, α1b* (JN180941)*, α1c* (JN180942); rainbow trout (*O. mykiss*) *α1a* (NP001117933.1)*, α1b* (NP001117932.1)*, α1c* (NP001117931.1)*, α2* (NP001117930.1)*, α3* (NP001118102.1); inanga (*G. maculatus*) *α1a* (AFM73918.1), *α1b* (AFM73919.1), *α1c* (AFM73917.1), *α2* (AFM73922.1), *α3a* (AFM73920.1), *α3b* (AFM73921.1); tilapia (*O. niloticus*) *α1a* (XP_005452412.1), *α1b* (XP_003446597.1), *α1-3* (XP_005452414.1), *α1-4* (XP_003446598.1), *α1-5* (XP_003446653.1) *α2,* (XP_003447505.1), *α3-1* (XP_005459144.1), *α3-2*(XP_003450710.1); and the sea urchin (*Strongylocentrotus purpuratus*) *α* (NP_001116982.1). Based on the distant relationship of the sea urchin Nka, this protein was used as an outgroup in the analysis. Predicted sequences were aligned using ClustalW. The maximum likelihood consensus tree was generated using SEQBOOT, PROML and CONSENSE, all programs in the PHYLIP package (Felsenstein, 1989). Alignment was also used to compare Nka α1 isoform transmembrane domain amino acid sequences. As only a partial sequence for inanga α1b was available from Genbank, this sequence was excluded from the transmembrane domain comparison.

2.3.6 Alignment of select functional areas of medaka α-subunits

Amino acid sequences of Nka were obtained from Genbank using the following accession numbers: *O. latipes* α1a (XM004084864), α1b (XM004066527), α1c (XM004066525); *O. niloticus* α1a (XP005452412.1), α1b (XP003446597.1), α1-3 (XP005452414.1), α1-4 (XP003446598.1), α1-5 (XP003446653.1); *O. mykiss* α1a (AY319391), α1b (AY319390) and α1c (AY319389); *G. maculatus* α1a (AFM73918.1) and α1c

(AFM73917.1); *A. testudineus* α1a (JN180940), α1b (JN180941) and α1c (JN180942). Sequences were aligned using ClustalW.

2.3.7 Statistical analysis

Tissue expression data were analyzed by one-way ANOVA followed by Tukey's Honestly Significant Difference *post hoc* test. Time course experiments were analyzed by two-way ANOVA. When significant interaction between factors occurred this was followed by Bonferroni-adjusted Fisher's least significant difference test. When required, transformation of data was done to meet the ANOVA assumption of homogeneity of variances, as tested by Bartlett's test. Expression and Kinetics data of long-term acclimated FW and SW fish were compared using a student's t-test. A significance level of P-value<0.05 was used throughout. All tests were performed using GraphPad Prism 5.0 software (San Diego, CA, USA).

2.4 Results

2.4.1 Phylogenetic analysis

We identified three Nka α1 isoforms (a, b and c), one α2 isoform and two α3 isoforms (a and b) in the genome of the Japanese medaka. The phylogenetic relationship of medaka Nka α isoforms was examined by constructing a tree including sequences from trout, inanga, nile tilapia and climbing perch (Fig. 2.1). The α1c isoform was named based on its homology with tilapia α1-3, trout α1c and inanga α1c. The medaka α1a and b were clearly grouped together, separately from the other α1 isoforms and arbitrarily named a and b. Designation of medaka α2 was supported by the formation of a strong clade with inanga, trout and tilapia Nka α2. Medaka α3 isoforms were named based on their homology with the other teleost α3 isoforms.

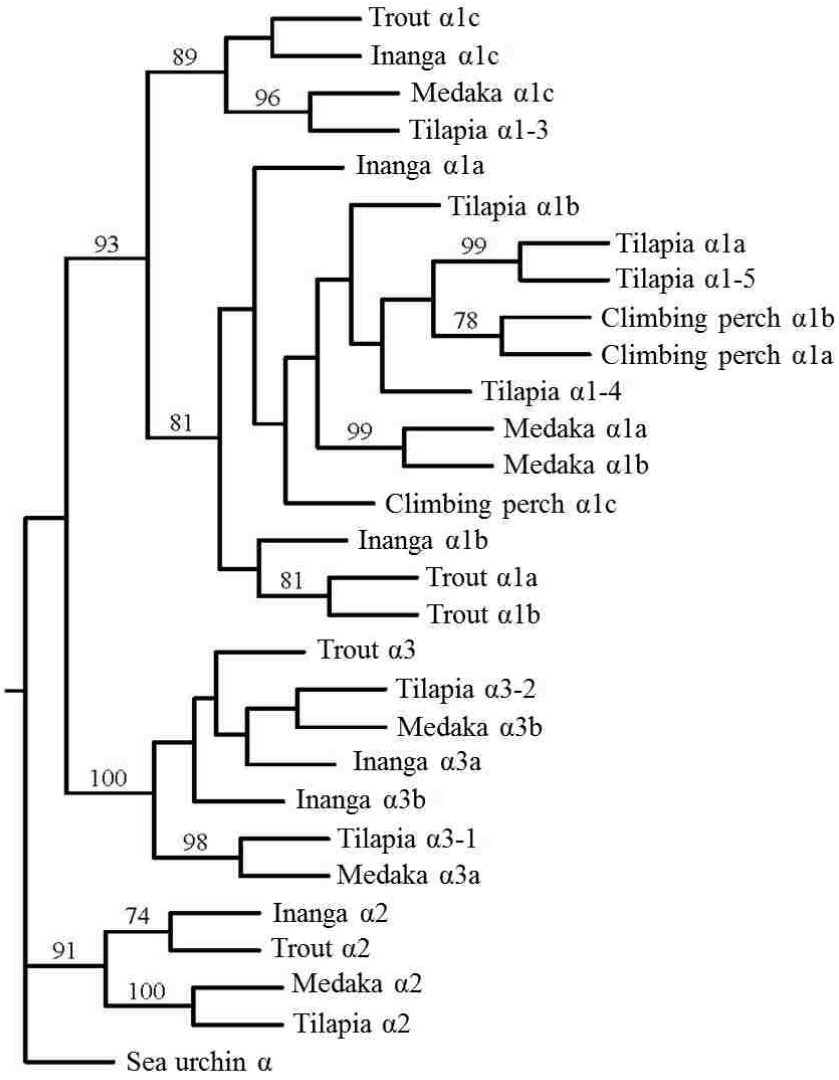

Figure 2.1 The consensus tree was assimilated based on homology of medaka (*Oryzias latipes*) with other teleosts using maximum likelihood; trout (*Oncorhynchus mykiss*), inanga (*Galaxius maculatus*) and climbing perch (*Anabus testudineus*). Numbers represent bootstrap values in percent of 1000 replicates. Sea urchin (*Strongylocentrotus purpuratus*) Nka α-subunit was used as outgroup.

2.4.2 Alignment of selected functional areas of medaka α-subunits

Amino acid sequences of Nka from medaka, rainbow trout, tilapia, inanga and climbing perch were aligned for comparison of the fifth (TM5), eight (TM8) and ninth (TM9) transmembrane domains (Fig. 2.2). Specifically, it shows that a lysine (Lys) substitution for asparagine 783 (Asn783) in TM5, a valine (Val) substitution for aspartate 933 (Asp933) in TM8

and a serine (Ser) substitution for glutamate 961 (Glu961) in TM9 are present in trout α1a compared with trout α1b and α1c. The Asn783-Lys substitution present in rainbow trout Nka α1a was also observed in tilapia α1a and α1.5 (latter not shown), inanga α1a (not shown) and climbing perch α1a and α1b. None of the medaka Nka α1 isoforms had this substitution. In TM8, the Asp933-Val substitution was only observed in rainbow trout α1a, while climbing perch α1a contained a threonine instead of Asp933. Finally, in TM9, only rainbow trout α1a exhibited a Glu961-Ser substitution while climbing perch α1b has a Val substitution at this position.

```
TM5    Ola.atp-α1a   IAYSLTSNIPELSPFLLFILASIP
       Ola.atp-α1b   IAYTLTSNIPEISPFLLFILASIP
       Ola.atp-α1c   IAYTLTSNIPEITPFLLFIIANIP
       Oni.atp-α1a   IAYTLTSKIPEMSPFLLFVIANIP
       Oni.atp-α1b   IAYTLTSNIPEISPFLLFIIANIP
       Omy.atp-α1a   ITYTLSSKIPEMTPFLFLLLANIP
       Omy.atp-α1b   IAYTLTSNIPEISPFLLFIIANIP
       Omy.atp-α1c   IAYTLTSNIPEITPFLFFIIANIP
       Ate.atp-α1a   IVYTLSSKIPEMSPFFFFAIANIP
       Ate.atp-α1b   IAYTLTSKIPEMSPFLFFVVASMP
       Ate.atp-α1c   IAYTLTSNIPEISPFLLFIIANIP

TM8    Ola.atp-α1a   CHTAFFISIVVVQWADLIICK
       Ola.atp-α1b   CHTAFFTSIVIVQWADLIICK
       Ola.atp-α1c   CHTAFFASIVIVQWADLIICK
       Oni.atp-α1a   CHTAFFSSIVIVQVADLLICK
       Oni.atp-α1b   CHTAFFASIVIVQWADLIICK
       Omy.atp-α1a   CHTAYFAAVVIAQWAVLIVCK
       Omy.atp-α1b   CHTAFFASIVVVQWADLIICK
       Omy.atp-α1c   CHTAFFASIVVVQWADLIICK
       Ate.atp-α1a   CHTAYFVNIVVIRWFTLIIAK
       Ate.atp-α1b   CHTAFFISIVIVQWTDLLICK
       Ate.atp-α1c   CHTAFFVSIVIVQWADLIICK

 TM9   Ola.atp-α1a   LIFGLFEETALAAFLSYCP
       Ola.atp-α1b   LIFGLIEETALAAFLSYCP
       Ola.atp-α1c   LIFGLFEETALAAFLSYCP
       Oni.atp-α1a   LIFGMFEELALAVFLSYCP
       Oni.atp-α1b   LIFGLFEETALAAFLSYCP
       Omy.atp-α1a   LIFGLCSESALALFLSYCP
       Omy.atp-α1b   LIFGLFEETALAVFLSYCP
       Omy.atp-α1c   LIFGLFEETALAAFLSYCP
       Ate.atp-α1a   LIFGLFEETALATFLSYCP
       Ate.atp-α1b   LIFGLFVETALAAFLSYCP
       Ate.atp-α1c   LIFGLFEETALAAFLSYCP
```

Figure 2.2 Sequence alignment of Nka TM5, TM8 and TM9 segments. Nka aligned protein sequences include: Japanese medaka α1a, α1b and α1c (*Ola.*); tilapia α1a and α1b (*Oni.*); rainbow trout α1a, α1b and α1c (*Omy.*); and climbing perch α1a, α1b and α1c (*Ate.*). *Bold letters* indicate the Asn783 → Lys in TM5, Asp933 → Val in TM8 and Glu961 → Ser or Val in TM9. Alignment numbers are set according to the rainbow trout sequence (see Jorgensen 2008).

2.4.3 Tissue distribution

The *α1a* and *α1b* isoforms had a mRNA expression more than 10-fold higher in kidney, intestine and gill than in muscle and liver (Fig. 2.3a, b), while *α1c* levels were significantly higher in kidney than in the other tissues examined (Fig. 2.3c). Nka *α2* was prominently expressed in muscle with a transcript level from 20- to 100-fold higher than in any other

examined tissues (Fig. 2.3d). Both *α3a* and *α3b* had highest expression in gill and kidney (Fig. 2.3e, f). Transcript levels of *fxyd9* were 2-fold higher in gill than in the other tissues (Fig. 2.4a). *fxyd11* showed more than 1000-fold higher expression in gill than in kidney and intestine and was not detected in muscle or liver (Fig. 2.4b). The *cftr* chloride channel was expressed in all five examined tissues with the highest levels in intestine>kidney>gill (Fig. 2.4c). *nhe3* had high transcript expression in gill and kidney with very low levels in the remaining tissues (Fig. 2.4e). Gill expression of *ncc* and *nkcc1a* were respectively 1000- and 10-fold higher than in the other examined tissues (Fig. 2.4d, f). Finally, the expression of *vata* was highest in gill and intestine (50-80% higher; Fig. 2.4g). Fig. 2.5 shows the relative abundance of the six Nka α isoforms in the gill of 12 FW and 12 SW medaka with *α1b* levels being 6-fold higher than *α1a* and 2-5 orders of magnitude higher than any other isoforms.

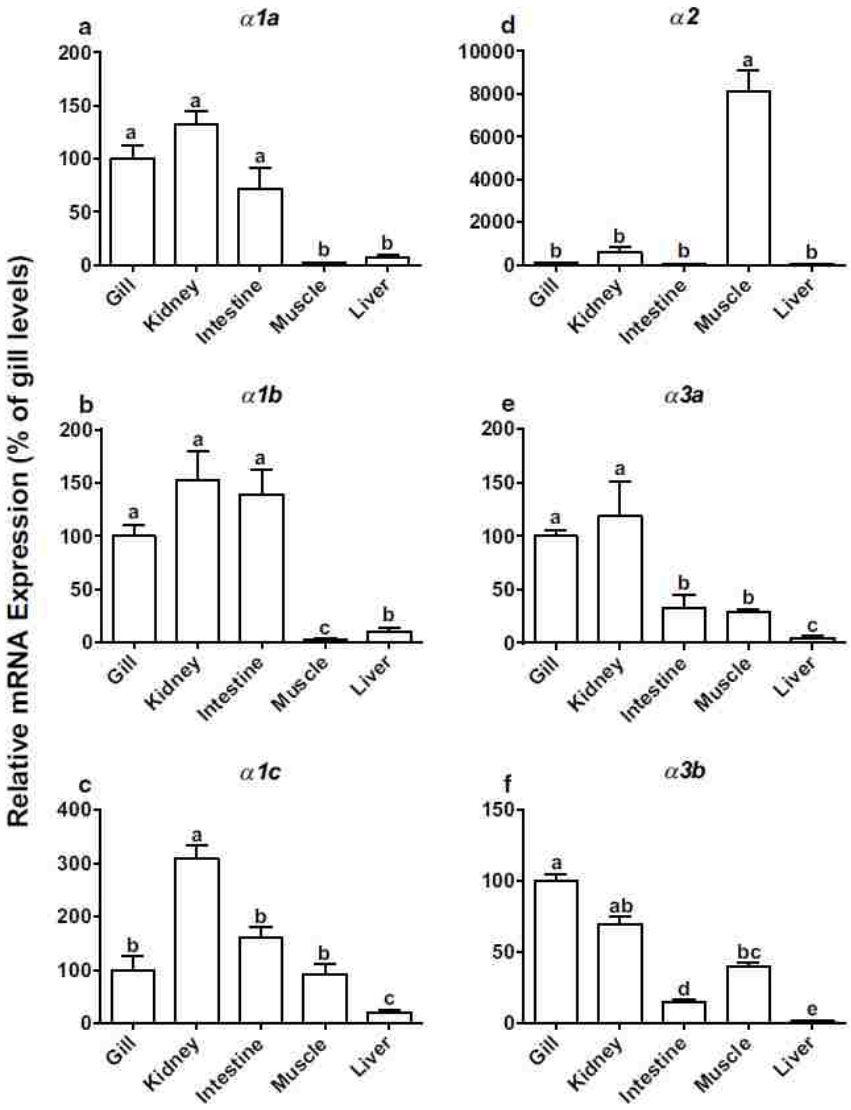

Figure 2.3 Transcript levels of Nka *α1a* (a), *α1b* (b), *α1c* (c), *α2* (d), *α3a* (e) and *α3b* (f) in various tissues from medaka. Expression levels represent the mean value ± SEM of both FW- and SW-acclimated fish (n = 8) in 100 % of the gill levels. Significant difference between means is indicated by *different letters* above bars: $P < 0.05$.

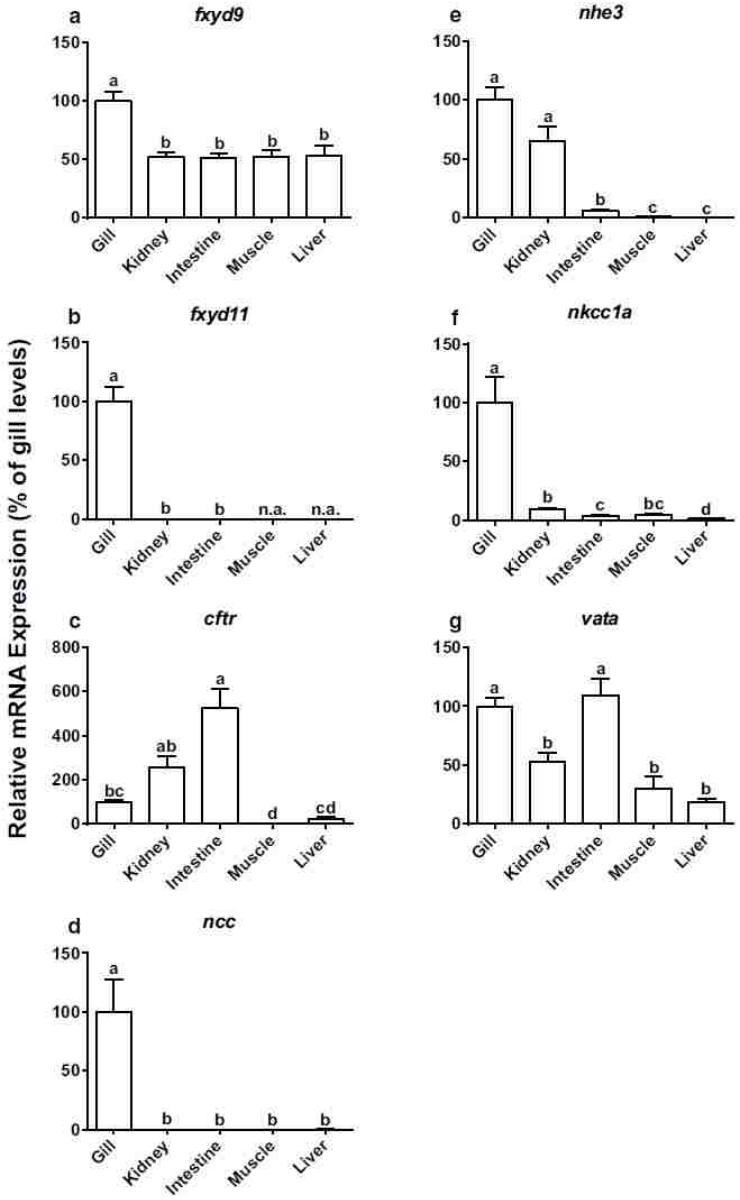

Figure 2.4 Transcript levels of *fxyd9* (a), *fxyd11* (b), *cftr* (c), *ncc* (d), *nhe3* (e), *nkcc1a* (f) and *vata* (g) in various tissues from medaka. Expression levels represent the mean value ± SEM of both FW- and SW-acclimated fish (n = 8) in 100 % of the gill levels. Significant difference between means is indicated by *different letters* above bars: $P < 0.05$.

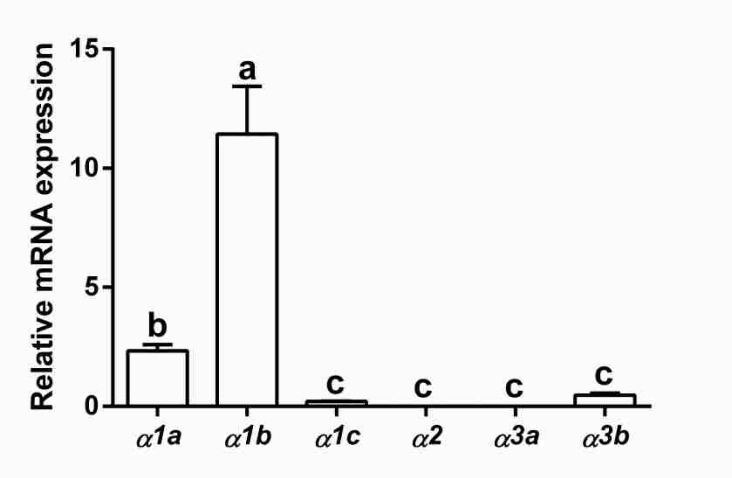

Figure 2.5 Relative mRNA expression of Nka *α1a, α1b, α1c, α2, α3a* and *α3b* in medaka gill. mRNA expression values are normalized to normalization genes and represent the mean of 12 FW and 12 SW acclimated fish. Expression levels represent the mean value ± SEM. Significant difference between means is indicated by *different letters* above bars: $P < 0.05$.

2.4.4 mRNA expression in FW and SW

In fish acclimated long-term to either FW or SW, expression of *α1a*, *α1b*, *fxyd11*, *cftr* and *nkcc1a* were elevated in SW gill (Fig. 2.6). FW-acclimated fish had a significantly higher gill transcript expression of *nhe3* and *ncc* when compared to the SW group (Fig. 2.6). No significant difference was exhibited in the transcript levels of *α1c*, *α2*, *α3a*, *α3b*, *fxyd9* or *vata* (Fig 2.6).

Transcript levels of those genes were also analyzed in the gill during the initial acclimation stages after FW to SW transfer (Fig. 2.7 and Fig. 2.8) or SW to FW transfer (Fig. 2.9 and Fig. 2.10). Transfer to SW induced a 1.5-fold increase in *α1a* gill expression after 72 hours (Fig. 2.7a) while *α1b* increased 2-3 orders of magnitude from FW controls (Fig. 2.7b). There was no effect of salinity on gill *α1c* in the short-term SW transfer experiment (Fig. 2.7c). Transfer to SW had no significant effect on *α2, α3a* or *α3b* expression (Fig. 2.7d, e, f). However, SW induced a significant increase in expression of *fxyd11*, *cftr* and *nkcc1a* (Fig. 2.8b, c, f). *ncc* and *nhe3* both exhibited a significant decrease in SW (Fig. 2.8d, e). Short-term transfer to FW did not induce any significant change in *α1a*, *α1c*, *α2*, *α3a* or *α3b* (Fig. 2.9a, c, d, e, f). Additionally,

there was no effect of FW on *fxyd9*, *fxyd11* or *vata* (Fig. 2.10a, b, g). After 72 hours in FW, a decrease in *α1b* was observed (Fig. 2.9b) along with *cftr* and *nkcc1a* (Fig. 2.10c, f). Both *ncc* and *nhe3* increased in the gill during the short-term FW transfer experiment (Fig. 2.10d, e).

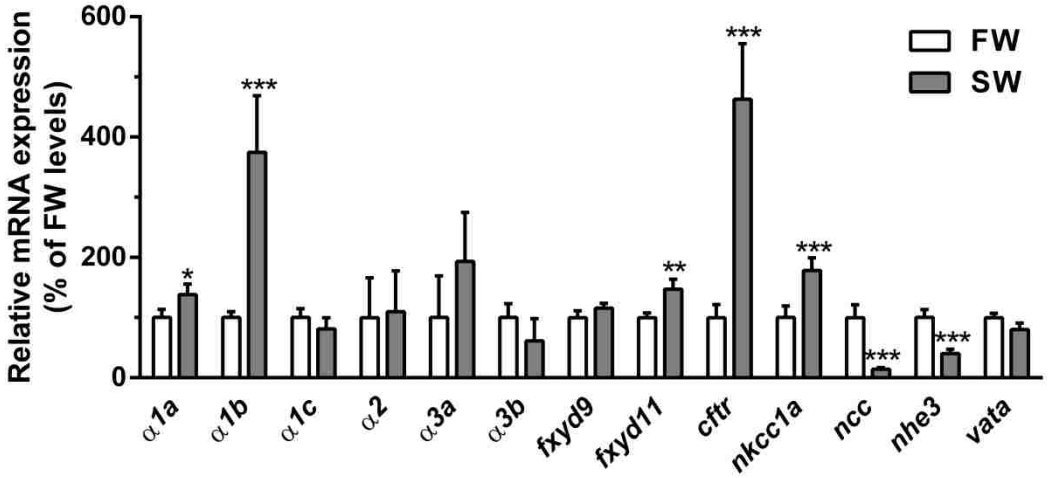

Figure 2.6 Transcript levels of Nka *α1a*, *α1b*, *α1c*, *α2*, *α3a*, *α3b*, *fxyd9*, *fxyd11*, *cftr*, *nkcc1a*, *ncc*, *nhe3*, *vata* in gill from medaka acclimated to freshwater (FW) or seawater (SW). Fish were acclimated to the respective salinities for at least one month prior to sampling (n = 12). Expression levels represent the mean value ± SEM relative to 100 % of FW levels. *Asterisks* indicate a significant difference from FW expression: *P < 0.05, **P < 0.01, ***P < 0.001.

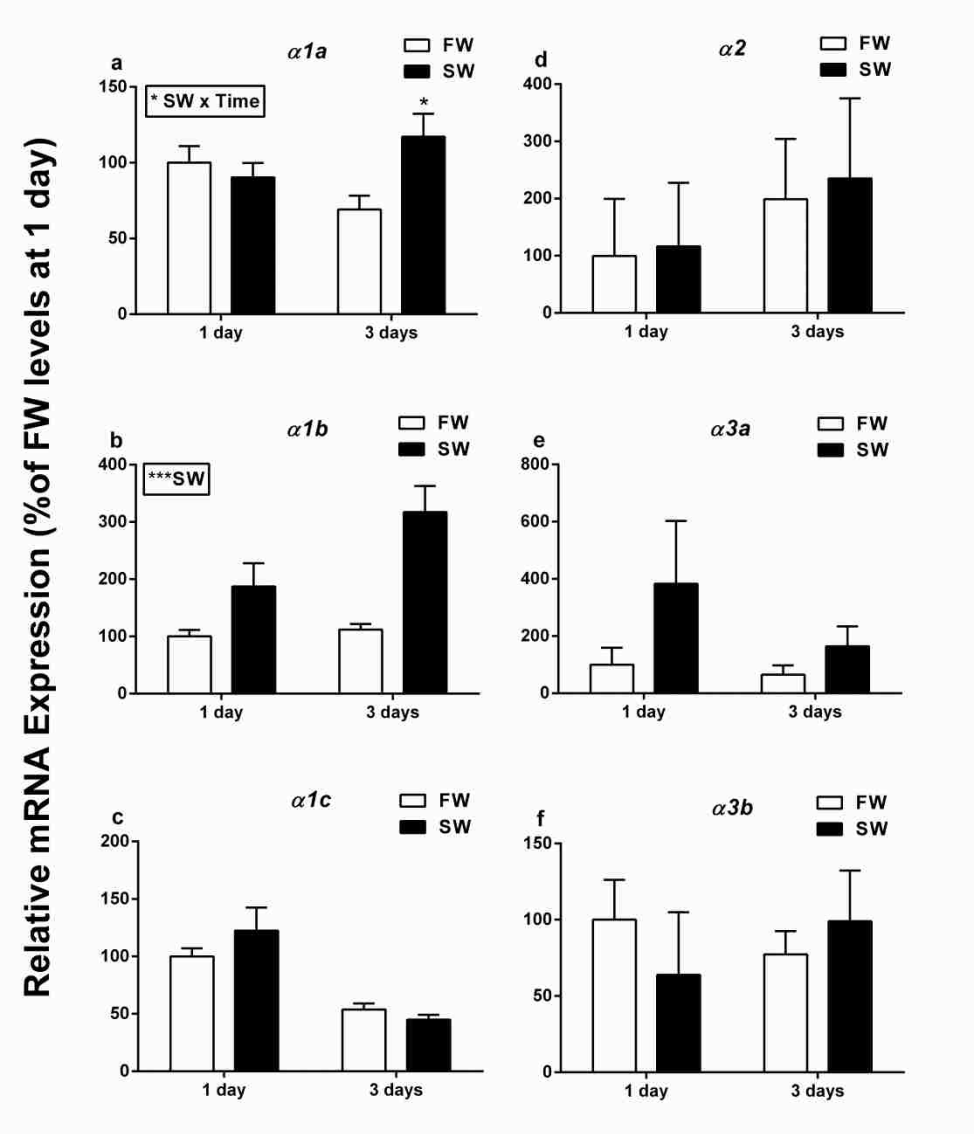

Figure 2.7 Effect of FW-to-SW transfer on gill transcript levels of Nka *α1a* (a), *α1b* (b), *α1c* (c), *α2* (d), *α3a* (e) and *α3b* (f). Fish were transferred from FW to SW or FW to FW as a control and sampled at 24 and 72 h (n = 6). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-FW group. "SW" and "SW × Time" refers to overall effects and statistical interaction between factors as indicated by *asterisks*: *P < 0.05, **P < 0.01, ***P < 0.001. When the interaction between factors are significant *asterisks* are placed above SW group at specific time-point: *P < 0.05.

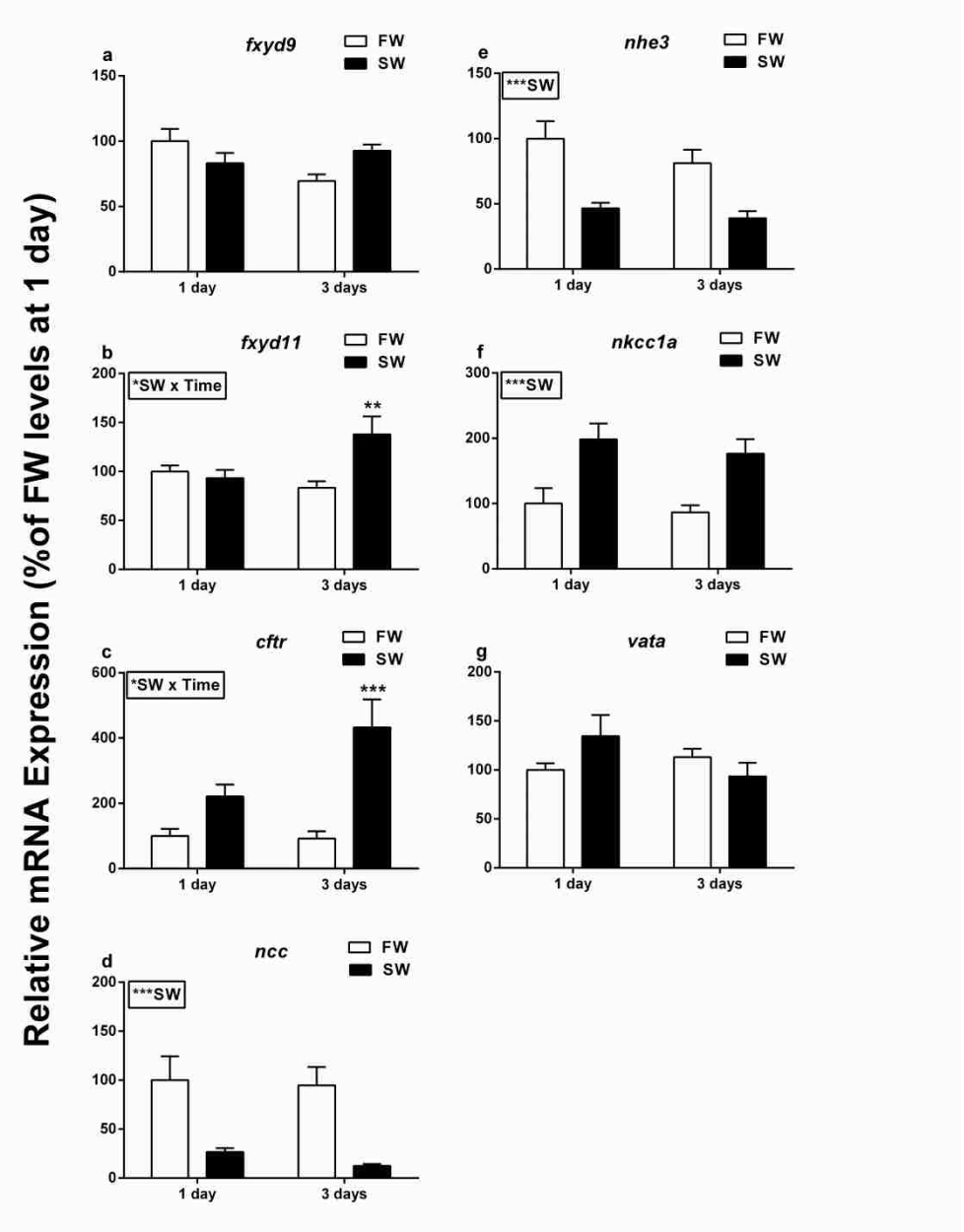

Figure 2.8 Effect of FW-to-SW transfer on gill transcript levels of fxyd9 (a), fxyd11 (b), cftr (c), ncc (d), nhe3 (e) nkcc1a (f) and vata (g). Fish were transferred from FW to SW or FW to FW as a control and sampled at 24 and 72 h (n = 6). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-FW group. "SW" and "SW × Time" refers to overall effects and statistical interaction between factors as indicated by *asterisks*: *P < 0.05, ***P < 0.001. When the interaction between factors are significant *asterisks* are placed above SW group at specific time-point: **P < 0.01, ***P < 0.001.

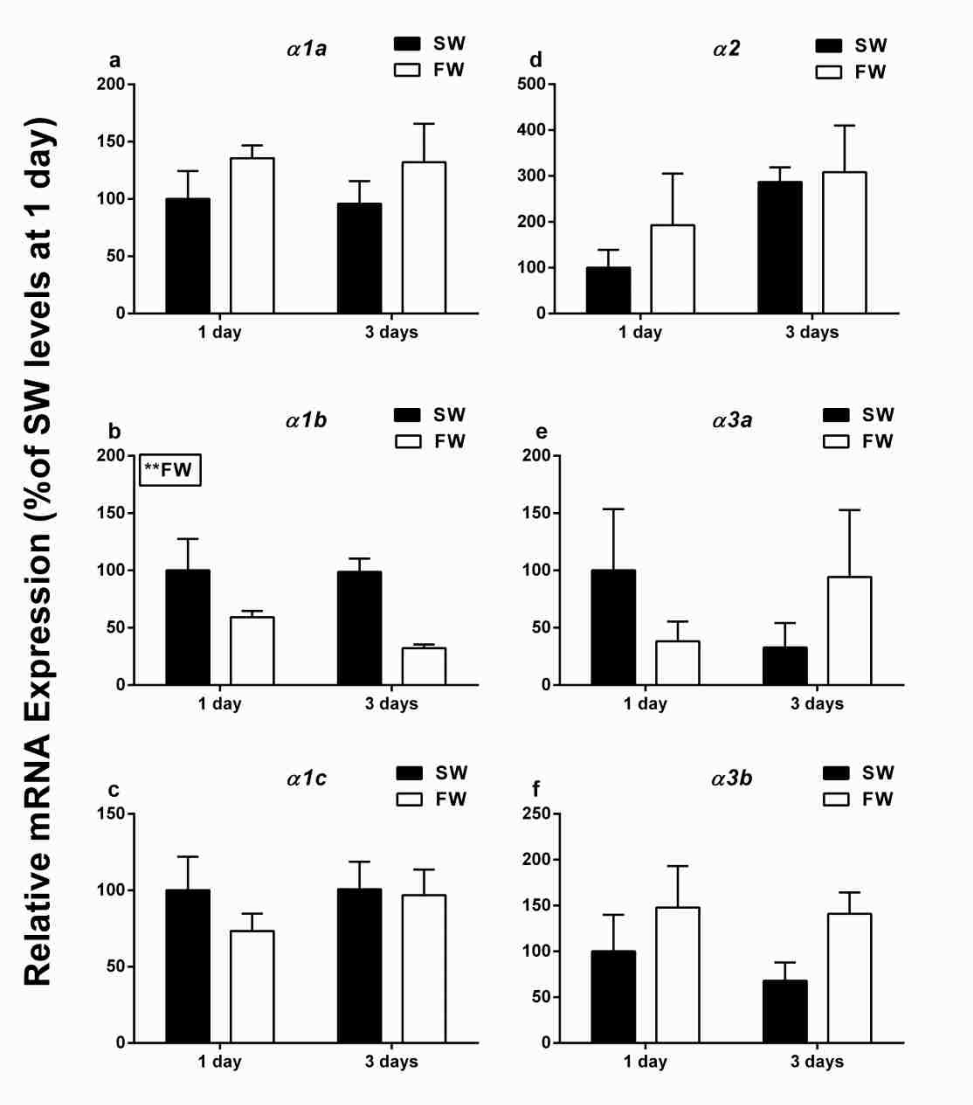

Figure 2.9 Effect of SW-to-FW transfer on gill transcript levels of Nka *α1a* (a), *α1b* (b), *α1c* (c), *α2* (d), *α3a* (e) and *α3b* (f). Fish were transferred from SW to FW or SW to SW as a control and sampled at 24 and 72 h (n = 6). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-SW group. "FW" refer to overall effects as indicated by *asterisks*: **P < 0.01.

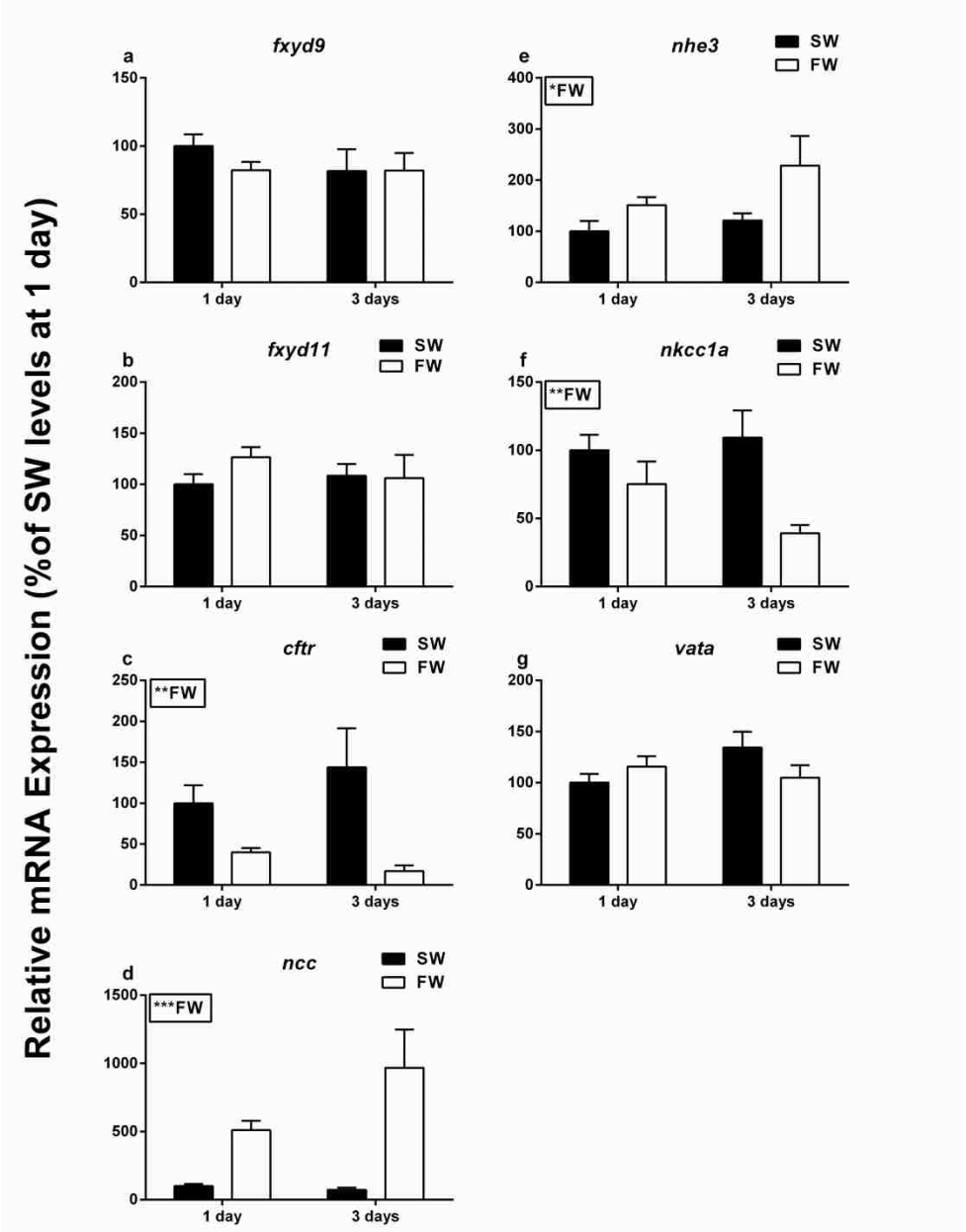

Figure 2.10 Effect of SW-to-FW transfer on gill transcript levels of *fxyd9* (a), *fxyd11* (b), *cftr* (c), *ncc* (d), *nhe3* (e) *nkcc1a* (f) and *vata* (g). Fish were transferred from SW to FW or SW to SW as a control and sampled at 24 and 72 h (n = 6). Expression levels represent the mean value ± SEM relative to 100% of the 24 h-FW group. "FW" refers to overall effects as indicated by *asterisks*: *P < 0.05, **P < 0.01, ***P < 0.001.

2.4.5 Gill Nka kinetic analysis

Maximal gill Nka activity (V_{max}) and apparent affinities for Na^+, K^+, Mg^{2+} and ouabain were analyzed in gill homogenates from long-term FW- and SW-acclimated fish (Table 2.2; Fig.

2.11). The apparent K_m for Na^+ was significantly higher in preparations from FW than SW gill. The other apparent affinities were not significantly different between the two salinities; however, V_{max} was highest in SW gill. Due to cofactor inhibition 8.0 and 15 mM (latter not shown) were excluded from K_m calculations for Mg^{2+} (Table 2.2; Fig. 2.11c) as well as 150 mM in Na^+ K_m for substrate inhibition (Table 2.2; Fig 2.11a).

Table 2.2 Nka apparent affinities (Na^+, K^+, Mg^{2+} and ouabain) and maximal activity in gill samples of freshwater (FW) and seawater (SW) acclimated medaka.

	FW	SW
K_m (mM)		
Na^+	8.97 ± 0.92	6.34 ± 0.67*
K^+	1.03 ± 0.25	0.86 ± 0.16
Mg^{2+}	1.03 ± 0.13	0.98 ± 0.10
V_{max} (µmol ADP mg^{-1} protein hr^{-1})	3.13 ± 0.42	4.54 ± 0.50*
IC_{50} (µM ouabain)	1.82 ± 0.74	1.09 ± 0.28

Values are expressed as mean ± SEM (*n*=12). Significantly difference is indicated by *asterisks*:*P<0.05.

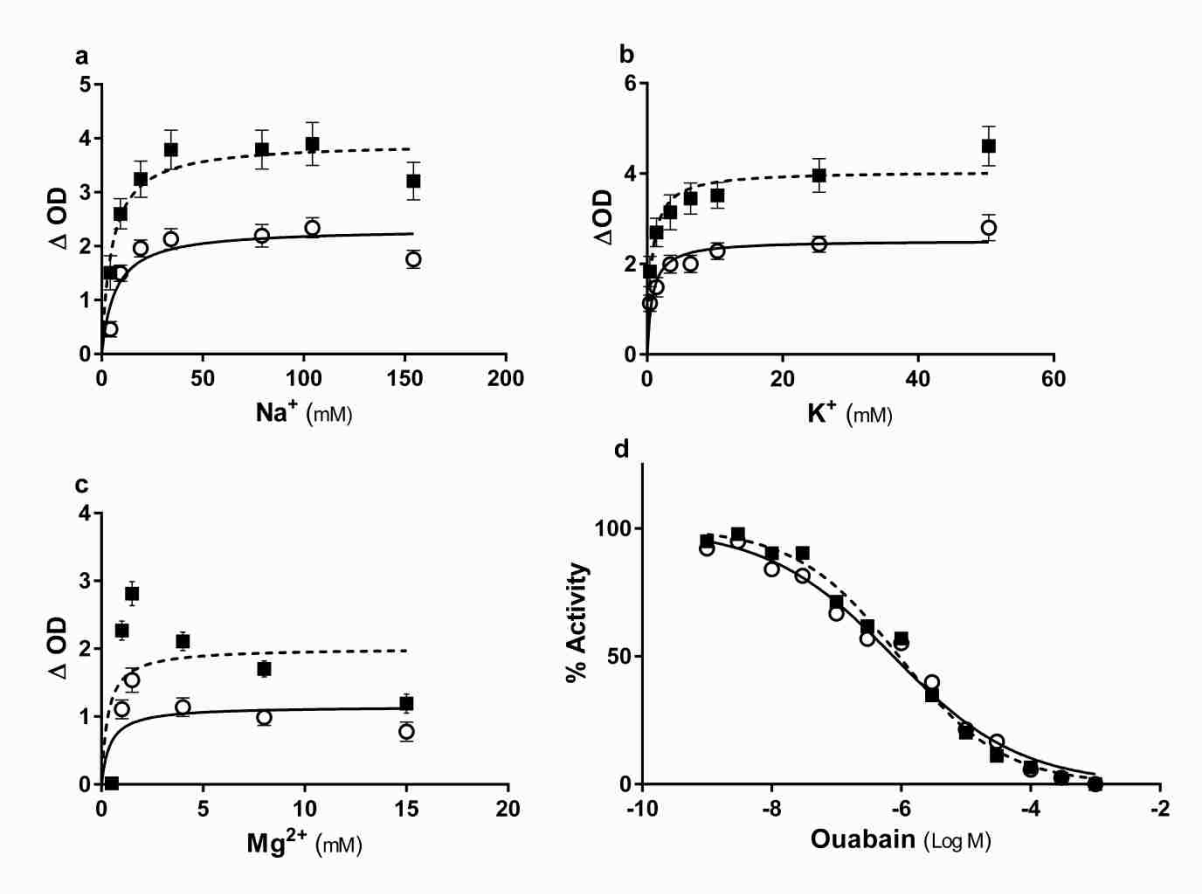

Figure 2.11 Kinetic analysis of apparent Nka affinity (Na^+, K^+, Mg^{2+} and ouabain) in gill samples from freshwater (FW) and seawater (SW) acclimated medaka. Effects of varying concentrations of K^+ (a), Na^+ (b), Mg^{2+} (c) and ouabain (d) on Nka activity in gill homogenates from FW- (*open circle*) and SW-acclimated (*filled square*) Japanese medaka. Values are means of 12 fish ± SEM K_m and IC_{50} values are listed in Table 2.2. Substrate and cofactor inhibition resulted in the exclusion of some data points in the curve fitting for Na^+ and Mg^{2+}, respectively.

2.5 Discussion

Over the last decade it has become clear that in some euryhaline teleosts, such as salmonids and tilapia, there is a salinity-specific effect on the expression of gill Nka α-subunit isoforms. In these species, the α1a isoform seems to be the prominent isoform in FW ionocytes, whereas the α1b isoform becomes predominant during SW acclimation (Dalziel et al., 2014). The present study demonstrated that among the six medaka Nka α-subunit isoforms (α1a, α1b, α1c, α2, α3a and α3b) only *α1b* and, to a lesser extent, *α1a* were regulated by salinity; however,

they were both stimulated by SW. When compared to relative transcript abundance *α1b* appears to be the most significant isoform in the gill, with *α1a* levels being somewhat lower and the other *α* isoforms detected at much lower levels. Sequence alignment of Nka α1 isoforms from medaka and other teleosts revealed that none of the medaka α isoforms have the amino acid substitution present in salmonids and tilapia α1a that is suggested to be critical to FW adaptation (Jorgensen, 2008; Tipsmark et al., 2011). While emphasizing the role of these α1 isoforms in the medaka gill, it also shows that Nka isoform shift is not part of the medaka acclimation strategy. As expected, medaka gill exhibited increased expression of marker genes for FW ionocytes (nhe3 and ncc: Hiroi et al., 2008; Hsu et al., 2014; Inokuchi et al., 2008) in FW and for SW ionocytes (cftr and nkcc1a: Hiroi and McCormick, 2012; Marshall and Singer, 2002) in SW. Enzyme analysis revealed that gills from SW-acclimated fish have higher maximal Nka activity and higher apparent affinity for Na^+ compared to FW-acclimated fish, while K^+, Mg^{2+} and ouabain affinity were unaffected by salinity.

2.5.1 In silico analysis of medaka Nka isoforms

The phylogenetic analysis grouped medaka α1c, α2, α3a and α3b with their corresponding α isoforms of the other species included. According to the present phylogenetic analysis and recently published molecular analysis performed by Dalziel et al. (2014), α1a and α1b isoforms may have developed separately by parallel evolution. In a functional study, Jorgensen (2008) examined amino acid substitutions in trout α1a and α1b by site-directed mutagenesis in critical ion-binding sites of the Nka α-subunit (TM5, TM8 and TM9). Using porcine α1-subunit as a template, the substitution of lysine (Lys) in trout α1a for asparagine (Asn) at site 783, a critical cation binding site in TM5, resulted in decreased binding affinity for Na^+ and K^+. In TM8, the Asp933-Val substitution, as observed in rainbow trout α1a, decreased

the binding affinity for K^+ (Jorgensen, 2008). The combination of these two substitutions are suggested to diminish the affinity for K^+, more than that for Na^+, thus allowing the Nka to preferentially pump Na^+. Additionally, this results in the insertion of the Lys ε-amino group of TM5 in the cation binding site, possibly reducing the Na^+/ATP ratio from $3Na^+$/ATP to $2Na^+$/ATP which could render sodium uptake from a dilute media more feasible for the FW-type Nka (α1a) in salmonids. Molecular reconstruction of Nka TM9 revealed that the Glu961 side chain points away from cation binding sites toward the regulatory Fxyd subunit. Therefore, the Glu961-Ser substitution in rainbow trout α1a may interfere with and/or change interactions with the regulatory subunit. The amino acid substitution in TM5 of Nka α1a is found in salmonids, climbing perch and tilapia, supporting the notion of an adaptive advantage of this substitution during FW acclimation. This thus lends insight as to why the α1 isoform switch is observed in some teleosts upon salinity challenge. In our study, the alignment of Nka α1 isoforms included the TM5, TM8 and TM9 domains of medaka, rainbow trout, tilapia, climbing perch and inanga (Ip et al., 2012; Urbina et al., 2013). The medaka Nka α1 isoforms did not exhibit any of the TM5, TM8 or TM9 substitutions observed in FW-type α1a in rainbow trout and other salmonids. If these amino acid substitutions in α1a are instrumental to ion absorption in trout ionocytes, the lack thereof in any medaka α isoforms may be important to our understanding of this euryhaline model. Thus, these solely *in silico* considerations do not support our initial hypothesis about salinity-induced Nka isoform shift in medaka.

2.5.2 Nka and Fxyd expression in the gills

The medaka Nka α isoforms identified were expressed in all tissues examined, however, with variable transcript levels. *α1a* and *α1b* were mostly expressed in osmoregulatory organs which is similar to *α1a* and *α1b* expression in rainbow trout (Richards et al., 2003), tilapia

(Tipsmark et al., 2011) and climbing perch (Ip et al., 2012). Additionally, *α1c* exhibited ubiquitous expression and was unchanged by salinity in the gill which is also similar to findings in salmonids (Richards et al., 2003). Based on its wide tissue distribution, we suggest that *α1c* may play a universal role as a general housekeeping gene. In addition, this isoform is possibly involved in driving transepithelial ion transport in the gut and kidney (Tipsmark et al., 2010b). The transcript of *α2* was mostly found in muscle, which parallels *α2* expression in rats (Mobasheri et al., 2000). *α3a* and *α3b* were expressed at approximately the same level in the tested tissues. This is in accordance with findings in rainbow trout (Richards et al., 2003) where ubiquitous expression of *α3* was reported.

In the few euryhaline fishes examined, gill α1 isoform expression is strongly influenced by salinity. The predominant FW and SW isoforms have been named α1a and α1b, respectively (Bystriansky et al., 2006; Ip et al., 2012; Madsen et al., 2009; Richards et al., 2003; Tipsmark et al., 2011; Urbina et al., 2013). Our initial long-term acclimation experiment suggested that an upregulation of *α1a* and *α1b* is associated with SW acclimation in medaka with no apparent isoform shift. To further validate this observation, two short-term transfer experiments were conducted (1-3 days; FW-to-SW and SW-to-FW). It was evident from these data that no switch in α1, α2 or α3 isoforms occurs as part of the osmoregulatory strategy in this species. Furthermore, α1a and α1b are the only salinity-sensitive isoforms suggesting that they are central to ionocyte function in the gill. In the present study, medaka *α1b,* and to a smaller extent *α1a,* showed elevated transcript expression in the SW gill. This is similar to expression of *α1b* exhibited in trout gill (Richards et al., 2003) with an increase in SW and decrease in FW. The present study showed that the medaka gill does not exhibit a salinity-dependent Nka α1 isoform switch. While this deviates from observations in some other euryhaline fishes examined to date,

it is similar to observations during salinity acclimation in the threespine sickleback (*Gastrerostereus aculeatus*) where there is no sign of isoform shift however there is an isoform-specific stimulation after SW entry (Judd, 2012 ; Madsen unpublished observations). In this species, mRNA levels of *atp1a1* isoform is highest in SW and lowest in FW as is the case for medaka *α1b* in the current study.

The Nka regulatory subunit is a single transmembrane protein that is often referred to as FXYD for its conserved extracellular motif: phenylalanine-X-tyrosine-aspartate (Sweadner and Rael, 2000). The family of FXYD proteins have been shown to interact with and modulate kinetic properties of Nka (Garty and Karlish, 2006). In Atlantic salmon eight FXYD isoforms were identified (Tipsmark, 2008) and of these, *fxyd11* was almost exclusively expressed in the gills. Elevated expression of *fxyd11* was also demonstrated in SW gill of two medaka species (O. dancena and O. latipes: Yang et al., 2013), in SW-acclimated Atlantic salmon (Tipsmark et al., 2010a) as well as in zebrafish exposed to ion-poor FW (Saito et al., 2010); all cases were correlated with elevated *nka* expression. Gill Fxyd11 has been shown to interact specifically with the Nka α-subunit in Atlantic salmon (Tipsmark et al., 2010a) and brackish medaka (Yang et al., 2013). In the present study, gill Nka *α1a* and *α1b* mRNA levels along with *fxyd11* were elevated during and after SW acclimation suggesting co-expression and co-localization as demonstrated in other species. It is possible that divergent interaction of Nka with Fxyd11 in FW and SW gill is responsible for the difference in apparent kinetic properties we observed at the two salinities.

2.5.3 Other ion transporters in the gill

The mechanism of ion absorption in the FW gill is still under debate; therefore, the current study measured several FW-type ion transporters. Gill expression of *nhe3* and *ncc* decreased rapidly upon exposure to SW and were significantly lower in SW long-term

acclimated medaka which is similar to the salinity effect on *nhe3* and *ncc* in tilapia (Hiroi et al., 2008; Inokuchi et al., 2008). Expression of *vata* seems to be unresponsive to salinity as there was no difference between FW and SW gills at the transcript level. In contrast, *vata* expression increased in gill of Atlantic salmon when transferred to FW (Bystriansky and Schulte, 2011). Our findings showing FW-induced *ncc* and *nhe3* for ion-absorption in gill is consistent with a recent study by Hsu and co-workers (2014), demonstrating apical localization of Ncc and Nhe3 in two separate populations of FW ionocytes in medaka. Furthermore, exposure of adult medaka to low Na^+ FW (0.03-0.05 mM) exhibited gill expression levels of *ncc-like2* comparable to those exposed to high Na^+ FW (9.2-10.5 mM; Hsu et al. 2014).Therefore, the inability of medaka to express a Nka α-subunit with a Lys substitution suggests that Nhe3 and Ncc may be more efficient in FW than previously expected.

In teleosts, the model for ion secretion in the branchial SW-ionocyte involves basolateral Nka and Nkcc1a, apical Cftr and a leaky paracellular pathway (Hiroi and McCormick, 2012). Accordingly, we showed that transfer of medaka to SW increased gill *cftr* and *nkcc1a* while expression was down-regulated within 72 hours in FW. Additionally, in long-term SW-acclimated medaka, gill Nka *α1a, α1b*, *cftr* and *nkcc1a* were significantly higher than in FW-acclimated medaka. This transcriptional data supports the idea that the SW medaka gill achieves ion secretion, at least in part, by the combined efforts of Nka α1b, Nkcc1a, Cftr and possibly Nka α1a.

2.5.4 Kinetic analysis

The present study revealed that SW-acclimated medaka display higher V_{max} than those acclimated to FW which is in accordance with previous findings in Japanese medaka (Kang et al., 2008). This is similar to findings in rainbow trout (Pagliarani et al., 1991), brown trout

(Tipsmark and Madsen, 2001), tilapia (Lin and Lee, 2005), sea bass (Jensen et al., 1998), climbing perch (Ip et al., 2012) and the giant mudskipper (Chew et al., 2014). Substrate affinity assays for Na^+, K^+ and Mg^{2+} revealed the apparent affinity for Na^+ to be salinity-dependent as SW gills exhibited a higher binding affinity for Na^+. There was no difference in apparent affinities for K^+, Mg^{2+} or ouabain. According to Jorgensen (2008), the combined effect of the Asn783-Lys and Asp933-Val substitutions in TM5 and TM8, respectively, decrease the binding affinity for K^+ thus allowing trout Nka α1a to preferentially bind Na^+. The Japanese medaka lacks these substitutions that may increase Nka affinity for Na^+ in other euryhaline teleosts while in FW. However, in the present study we observed a lower affinity for Na^+ in FW than in SW medaka which could possibly be due to interactions with Fxyd11. In contrast, we have previously found a higher affinity for Na^+ in gill of FW-acclimated brown trout and Atlantic salmon when compared to SW-acclimated fish (Madsen and Tipsmark, unpublished) similar to findings in rainbow trout (Pagliarani et al., 1991), which could be a result of TM5 substitutions. Furthermore, in European sea bass no salinity difference in apparent ion affinities was observed (Jensen et al., 1998).

2.6 Conclusions

The regulation of Nka α-subunits during salinity acclimation observed in Japanese medaka in the present study does not include a Nka isoform switch. While the current data are on the level of mRNA expression, the results from the long-term experiment are carefully verified by two separate short-term experiments that support the general conclusion. Furthermore, the mRNA levels of the dominant gill isoform, *α1b,* are stimulated during SW acclimation, suggesting a causal relationship to the elevated gill Nka activity observed. The regulatory pattern of Nka differs from the isoform shift observed in most euryhaline species examined so far

(salmonid species like *O. myskiss*: Bystriansky et al., 2006; *Anabus testudineus*: Ip et al., 2012; Richards et al., 2003; *O. mossambicus*: Tipsmark et al., 2011; *Galaxias maculatus*: Urbina et al., 2013). However, such different osmoregulatory patterns may not be altogether surprising especially given that euryhaline adaptation, including diadromous life cycles, is a reoccurring phenomenon in teleost evolution (Dalziel et al., 2014; Kultz, 2015). The regulatory pattern in Japanese medaka is similar to that of the diadromous stickleback (Judd, 2012) and emphasizes the importance of understanding Nka regulation at other levels, including interaction with other membrane components. The higher affinity for Na^+ observed in SW medaka may not be directly associated to changes in the primary structure of the catalytic α-subunit. The observed effects may instead relate to other mechanisms such as posttranslational modifications or intermolecular interactions with other membrane proteins or lipids (Cornelius and Mahmmoud, 2007). In this context, it will be important to understand the functional significance of Fxyd11-Nka interactions previously demonstrated in branchial ionocytes of various species (zebrafish: Saito et al., 2010; Atlantic salmon: Tipsmark et al., 2010a; brackish medaka: Yang et al., 2013) in chronic and rapid regulation of Nka kinetics.

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Chapter 3 Prolactin stimulates expression of Na^+, Cl^- cotransporter in the gill of Japanese medaka (*Oryzias latipes*) via the Stat5 pathway

Rebecca J. Bollinger[1], Laura V. Ellis[1], Maryline C. Bossus[1,2], Christian K. Tipsmark[1]

[1] Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA

[2] Department of Biology, Lyon College, Batesville, AR 72501

3.1 Abstract

Prolactin regulates gill Na^+,Cl^- cotransporter (Ncc2b) which is critical to ionic homeostasis in some freshwater fish, however the mechanism by which this hormone regulates extra-renal ion uptake is not understood. This study was carried out to examine the signaling pathways involved in prolactin-mediated salt retention using gill explants from Japanese medaka (*Oryzias latipes*). Ovine prolactin induced a concentration dependent stimulation of *ncc2b* with significant effects of 10, 100 and 1000 ng of hormone per ml media (2-6 fold). The effect was abolished by co-incubation with the Stat5 inhibitor, N-((4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide, but not PI3K/Akt and Erk1/2 pathway inhibitors. To understand the molecular mechanisms mediating prolactin control of gill function we analyzed early effects of prolactin on kinase signaling activation in a time course and concentration response experiment. Prolactin (1 μg mL^{-1}) induced a rapid phosphorylation (stimulation) of Stat5 (10 minutes) that reached a plateau after 30 minutes and was maintained for at least 120 minutes. The effect of prolactin on Stat5 phosphorylation was concentration dependent (4-12 fold). No activation of the PI3K/Akt or Erk1/2 pathways was observed in either experiment. The Stat5 activation was investigated in localization studies showing strong nuclear expression of phosphorylated Stat5 in prolactin treated gill ionocytes; identified as Na^+/K^+-ATPase positive cells. These findings shows that prolactin elicit its Ncc2b dependent ionoregulatory effect by downstream activation of Stat5 in branchial ionocytes.

3.2 Introduction

Prolactin (Prl) is a highly pleiotropic pituitary hormone and comparative studies have shown that its function in regulation of ion and water transport is highly conserved among

vertebrates (Bole-Feysot et al., 1998). In mammals, prolactin is involved in control of solute and water transport in a number of epithelia; including renal, intestinal, mammary and amniotic membranes. In teleost fishes, prolactin is an important freshwater (FW) hormone that adjusts gill and kidney function to the osmoregulatory challenges of dilute environments by promoting ion retention and water secretion (Hirano, 1986; Manzon, 2002). It has been known for a long time, that prolactin affects branchial function by decreasing permeability of the main surface epithelia and hence ion retention and water exclusion (Hirano, 1986). However, recent studies have made it clear that a critical part of prolactin's role is maintaining active re-uptake of NaCl by mitochondrion-rich cells (ionocytes) in the gill (Breves et al. 2010, Shu et al. 2016).

The significance of prolactin in osmoregulation was first demonstrated in a ground breaking study by Pickford and Phillips (1959) showing that replacement therapy with prolactin in hypophysectomized killifish (*Fundulus heteroclitus*) was critical to survival in FW; findings that were later confirmed in sailfin molly (*Poecilia latipinna*: Ball and Ensor, 1965) and Japanese medaka (*Oryzias latipes*: Utida et al., 1971). Consistent with these early studies, prolactin knock-out in zebrafish led to a phenotype that is only able to maintain larval ion content and survive until adulthood if kept in brackish water, where salt retention is less challenged (Shu et al., 2016). Reductions in environmental ion content induced elevated circulating prolactin levels and pituitary *prl* expression in several stenohaline and euryhaline teleost fishes including zebrafish (*Danio rerio*: Hoshijima and Hirose, 2007; Liu et al., 2006), tilapia (*Oreochromis mossambicus*: Shepherd et al., 1999; Yada et al., 1994), sea bream (*Sparus auratus:* Fuentes et al., 2010) and pufferfish (*Takifugu rubripes:* Lee et al., 2006).

The gill of FW fish contains several types of ionocytes expressing specific solute transporters responsible for transepithelial re-uptake of ions (Na^+, Cl^-, Ca^{2+}), and are to a large

degree similar to tubular cells of the mammalian nephron (Guh et al., 2015). Different and sometimes competing models for cellular mechanisms have been proposed and cell types appear to differ between species (see Takei et al., 2014). One type of gill ionocyte involved in re-uptake of Cl^-, and presumably Na^+, is characterized by apical Na^+, Cl^- cotransporter (Ncc) expression. This cell type has consistently been identified in tilapia (type II ionocyte: Hiroi et al., 2008), zebrafish (NCC cell: Guh et al., 2015; Wang et al., 2009) and medaka (NCC cell: Hsu et al., 2014), but appear to be absent in gill of salmonids or eel (Takei et al., 2014). Gill specific Ncc (slc12a10,) is a fish-specific paralog in the slc12a family and was recently re-named Ncc2 (formerly Ncc-like2: Guh et al., 2015; Takei et al., 2014). While Ncc2 is molecularly distinct from Ncc1 in the kidney (slc12a3), it is similar in its apical localization and NCC ionocytes furthermore functionally resembles salt retaining cells in the distal nephron.

In tilapia, apical Ncc2 expressing cells have been shown to actively absorb Cl^- (Hiroi et al., 2008; Horng et al., 2009). In both tilapia and zebrafish, exposure to low Cl^- conditions led to an increase in branchial Ncc gene and protein expression, thus indicating the importance of NCC cells in maintaining plasma Cl^- levels in FW (Hiroi et al., 2008; Inokuchi et al., 2008; Wang et al., 2009). The role of NCC cells in both Cl^- and Na^+ uptake is stressed by the fact that morpholino knock-down of Nhe3b in zebrafish led to the impairment of proton-coupled sodium uptake and resulted in an increase of NCC cell number with a concomitant recovery of Na^+ uptake function (Chang et al., 2013).

Prolactin is a potent stimulator of branchial Na^+,Cl^- cotransporter (Ncc2) expression in at least some teleost fishes; including stenohaline zebrafish (Breves et al., 2013) and euryhaline species like Mozambique tilapia (Breves et al., 2010) and Japanese medaka (Bossus et al., 2017). A study in zebrafish by Breves and co-workers (2013) demonstrated that ovine prolactin (oPrl)

stimulates zebrafish *ncc2b* in gill explant cultures in a concentration dependent manner and that an antagonist of the prolactin receptor blocked the effect completely. These *in vitro* findings showed that prolactin works by activating its cognate receptor on gill cells and not via alternate endocrine factors, which the intact animal approach could not exclude (Pickford and Phillips, 1959; Shu et al., 2016).

While endocrine regulation of NCC cells has been studied in some detail, the signaling pathway prolactin employs to stimulate *ncc2* gene expression is still unknown. Direct regulation of gene expression has been shown in prolactin-responsive cells where binding of prolactin to its receptor led to the activation of the Jak-Stat (Janus kinase – Signal Transducer and Activator of Transcription) pathway (Bole-Feysot et al., 1998). However, prolactin is known to activate other signaling pathways including MAPK (Mitogen-activated Protein Kinases) and PI3K (Phosphatidylinositol-3-kinase; Bole-Feysot et al., 1998; Freeman et al., 2000). For example, the MAPK pathway has been shown to be involved in cell volume responses in human leukemia cells (Pandey et al., 1999) and in rat hepatocytes (vom Dahl et al., 2001).

In this study, we used Japanese medaka to investigate the signaling pathways by which prolactin controls the expression of *ncc2b.* Since this is a NCC cell marker gene it may serve as a *proxy* for the capacity of active branchial salt retention by this ionocyte. Gill explant (*ex-vivo*) cultures make it possible to have defined media while maintaining the complexity of gill (Bossus et al., 2017; Breves et al., 2013). Specifically, we tested the hypothesis that Jak-Stat5, PI3K/Akt, and Erk1/2 signaling pathways mediate prolactin-stimulated *ncc2b* expression. This study examined the effect of specific signaling pathway inhibitors on prolactin-stimulated *ncc2b* expression while also evaluating kinase activation using dual labeling Western blots and cellular immunolocalizations.

3.3 Materials and Methods

3.3.1 Fish and maintenance

Adult Japanese medaka (*O. latipes*, Temmink & Schlegel; size range: 25-35 mm, weight range: 250-350 mg) were obtained from Aquatic Research Organisms, Inc. (Hampton, NH, USA). Fish were acclimated to recirculating de-chlorinated tap water, mechanically and biologically filtered (in mmol L^{-1}: 0.34 Na^{+}, 0.64 Ca^{2+}, 0.09 Mg^{2+}, 0.03 K^{+}). A 14 h light: 10 h dark photoperiod was maintained at a temperature of 20°C. Fish were fed daily with Tetramin tropical flakes (Tetra, United Pet Group, Blacksburg, VA) and food withheld 24 hours prior to sampling. Medaka were sacrificed by cervical dislocation and pithing of the brain and gill apparatus was removed and washed with PBS (Roche Diagnostics, Indianapolis, IN, USA). Gill arches were subsequently separated and immediately placed in Dulbecco's Modification of Eagle's Medium (DMEM; Cellgro by Corning, manufactured by Mediatech, Inc., Manassas, VA, USA). All handling and experimental procedures were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC 14042).

3.3.2 Experimental design

All in vitro studies used DMEM with 50 U mL^{-1} of penicillin and 50 µg mL^{-1} of streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Bossus et al., 2017). Purified ovine prolactin (oPrl) was obtained from the National Hormone and Peptide Program (Torrence, CA, USA).

3.3.2.1 Concentration-response experiment on oPrl effect on gill ncc2b expression

An initial *in vitro* experiment was performed to examine the effect of increasing concentrations of oPrl on *ncc2b* expression. In order to obtain more tissue for mRNA extraction,

one gill arch from two separate fish were pooled for each sample (n = 10). Gills were excised, rinsed with 1X PBS and immediately placed in DMEM. Samples were given a one hour pre-incubation. After pre-incubation, gills were transferred to media with 0, 0.01, 0.1 or 1.0 μg mL^{-1} oPrl for 16 hours. Experiments were terminated by transferring gill samples to TRI Reagent® (Sigma Aldrich) for mRNA isolation.

3.3.2.2 Effect of oPrl and kinase inhibitors

A two-factor designed *in vitro* experiment was used to examine interaction effects of kinase inhibitors and oPrl on gene expression. The first factor was oPrl and the second factor was kinase inhibitors. In order to obtain more tissue for mRNA extraction, one gill arch from two separate fish were pooled for each sample with 3-4 samples per treatment and pooled data from four separate experiments (n = 14-16). Samples were given a one hour pre-incubation. After pre-incubation, gill arches were transferred to either media alone (control) or media with an inhibitor for two hours. Gill arches were then transferred to one of the following media conditions for 16 hours; control, oPrl, an inhibitor or oPrl with an inhibitor. As the kinase inhibitors were reconstituted in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA), the same concentration of DMSO was added to control and oPrl solutions. Nico (STAT5 inhibitor; *N'*[(4-oxo-4H-chromen-3-yl)methylene]nicotinohydrazide) and Carb (Akt inhibitor; 1L6-hydroxymethyle-chiro-inositol-2-(R)-2-*O*-methyle-3-*O*-octadecyl-sn-glycerocarbonate) were obtained from EMD Millipore (Billerica, MA, USA). U0126 (inhibits MEK1/2 activation of ERK1/2) was obtained from Cell Signaling Technology (Beverly, MA). The following concentrations were used; oPrl [1.0 μg mL^{-1}], Nico [200 μmol L^{-1}], Carb [25 μmol L^{-1}] and U0216 [10 μmol L^{-1}]. Concentrations of inhibitors were based on those recommended to be maximally effective by the manufacturer. All incubations were performed at 20°C on a small

orbital rotator (Thermo Fisher). Experiments were terminated by placing gills in TRI Reagent® for mRNA isolation.

3.3.2.3 Effects of oPrl on Stat5, Akt and Erk1/2 phosphorylation

In an initial *in vitro* experiment, we examined the effect of oPrl on Stat5, Akt and Erk1/2 phosphorylation after 1 hour incubation with or without hormone. Whole gill apparatus was sampled from four fish and two gill arches from each fish was placed DMEM for a one hour pre-incubation (n = 4). Samples were then transferred to either DMEM (control) or 1.0 μg mL^{-1} oPrl. Termination occurred by placing gills in 1X LDS NuPAGE Sample Buffer (Thermo Fisher) with 50 mmol L^{-1} DTT (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) followed by Western blot analysis with dual detection of phosphorylated (active) and total kinase abundance. We ascertained that the detection was in a dynamic range by applying positive controls as supplied by the manufacturer.

3.3.2.4 Time dependence of oPrl on Stat5, Akt and Erk1/2 phosphorylation

In order to examine an early response was missed and to determine the optimal time for highest kinase phosphorylation, an *in vitro* experiment was performed to observe the time course of oPrl effects. In order to obtain more tissue for Western blot analysis, one gill arch from two separate fish were pooled for each sample (n = 4). Gill explants were immediately placed in DMEM for one hour for pre-incubation. Samples were then transferred to media with 1.0 μg mL^{-1} oPrl and sampled at 0, 10, 30, 60, 90 and 120 minutes. Control samples were transferred to fresh DMEM and terminated at 120 minutes. Experiment termination and kinase activation detection were performed as described previously.

3.3.2.5 Concentration dependence of oPrl on Stat5, Akt and Erk1/2 phosphorylation

In order to determine the optimal concentration for highest kinase phosphorylation, an *in vitro* experiment was performed to observe the concentration dependence of oPrl effects. In this experiment, the four largest gill arches were sampled from 6 fish and incubated with either DMEM (control) 0, 0.01, 0.1, 1.0 µg mL^{-1} oPrl for 60 minutes (n = 6). Experiment termination and kinase activation detection were performed as described previously.

3.3.3 Total RNA isolation, cDNA synthesis and quantitative real-time qPCR

Samples were homogenized with a Power Max 200 rotating knife homogenizer (Advanced Homogenizing System; Manufactured by PRO Scientific for Henry Troemner LLC, Thorofare, NJ, USA).Total RNA was extracted following the manufacturer's protocol. Nuclease-free water was used to dissolve RNA pellet. NanoDrop 2000 spectrophotometer (Thermo Fisher) was used to estimate concentration and purity (A_{260}/A_{280}) of each sample. Applied Biosystems high capacity cDNA reverse transcription kit (Thermo Fisher) was used for cDNA synthesis from 800 ng total RNA in a final volume of 20 µL following manufacturer's protocol. All primer sequences used (*ncc2b, ef1a, rplp0*) were previously described in Bollinger et al. (2016). Elongation factor 1-alpha (*ef1a*) and ribosomal protein PO (*rplp0*) were used as normalization genes (Vandesompele et al., 2002). Quantitative real-time qPCR was run on a Bio-Rad CFX96 platform (BioRad, Hercules, CA, USA) at a final volume of 15 µL using and SYBR® Green JumpStart™ (Sigma Aldrich). Cycling conditions were 3 minutes initial denaturation/activation phase at 94°C, a 15 seconds denaturation step and an annealing/elongation step for 60 seconds at 60°C for 40 cycles, and a melting curve analysis at an interval of 5 seconds per degree from 55-94°C. Relative copy numbers were calculated as $E_a^{\Delta Ct}$, where C_t is the threshold cycle number and E_a is the amplification efficiency (Pfaffl, 2001). Geometric mean of the two normalization

genes was calculated using GeNorm software (Biogazelle, Zwijnaarde, Belgium). Normalized values were calculated by dividing the relative copy number of each target gene by the geometric mean of normalization genes.

3.3.4 Western blot analysis

Gill tissues were sonicated in 1.5 mL sonication tubes in an ultrasonic bath (Ultrasonic Liquid Processor 3000, Farmingdale, NY, USA). Samples underwent four rounds of sonication with manual pestle homogenization in between sonication. Sonication procedure was as follows: 60 seconds on 20 seconds rest and 60 seconds on with a 1.0 output in ice water. All Western blotting solutions and materials were NuPAGE ^{TM} from Thermo Fisher unless otherwise stated. Following sonication, samples were transferred to microcentrifuge tubes and heated at 70°C for 10 minutes. Protein samples were run on a 4-12% Bis-Tris Gel with MES SDS Running Buffer with antioxidant, gels electrophoresed at 200 V for 34 minutes and subsequently blotted on a 0.2 µm nitrocellulose membrane for 1 hour at 30 V using transfer buffer with 10% methanol. Membranes were blocked with Li-Cor Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at room temperature and subsequently incubated with primary antibodies diluted in Li-Cor blocking buffer overnight at 4°C on a small orbital rotator. Membranes were washed four times with 1X TBST for 5 minutes each. Secondary antibodies were used according to manufacturer and incubated in the dark at room temperature for 1 hour in blocking buffer (IRDye® 800CW Goat anti-Rabbit IgG and IRDye® 680LT Goat anti-Mouse IgG, LI-COR). After washing, membranes were dried and imaged using an Odyssey infrared scanner (LI-COR). The following concentrations of antibodies were used and are according to manufacturer's protocol: Stat5 and pStat5 (1:1000), Akt and pAkt (1:1000), Erk1/2 (1:1000) and pErk1/2 (1:500), 800 CW and 680LT (1:10000). All primary antibodies for Western blot were obtained

from Cell Signaling Technologies except for Erk1/2 which was from Santa Cruz Biotechnology (Dallas, TX, USA).

3.3.5 Immunohistochemistry

Gills were incubated with or without 1.0 μg mL^{-1} oPrl for 1 hour before placing in 4% paraformaldehyde in 1X PBS (Mallinckrodt Chemicals, Phillipsburg, NJ, USA) overnight. Fixed samples were transferred to 100% methanol (EMD Millipore) for 30 minutes at -20°C. Gills were washed three times in 1X PBS for intervals of 30, 15 and 15 minutes. Post washing, samples were blocked for 1 hour with the following blocking buffer; 1X PBS, 5% normal goat serum (Sigma Aldrich) and 0.3% Triton™ X-100 (EMD Millipore). Blocked gills were rinsed to remove any remaining Triton and primary antibodies were subsequently added; pSTAT5 (1:1000) in conjunction with a5 (Na^+/K^+-ATPase alpha subunit; 0.7 μg mL^{-1}). pStat5 is a monoclonal antibody raised against human tyrosine 694 residue in rabbit (Cell Signaling) and was used to localize phosphorylated Stat5. The a5 antibody was obtained from The Developmental Studies Hybridoma Bank developed under auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA, USA). Antibodies were diluted in the following blocking buffer; 1X PBS, 1% bovine serum albumin (VWR International, Solon, OH, USA), and 0.3% Triton™ X-100. Gill tissues were incubated with primary antibodies overnight at 4°C on a small orbital rotator. Post primary antibody incubation, gills were rinsed with 1X PBS and incubated with secondary antibodies (Alexa Fluor® 488 (1:1000) and Alexa Fluor® 568 (1:1000); Molecular Probes, Burlington, ON, Canada) for 1 hour at room temperature on a small orbital rotator. Gill tissues were rinsed with 1X PBS before being mounted on slides with mounting media Prolong® Gold Antifade Reagent with DAPI and covered with a glass coverslip

(Molecular Probes). Slides were then placed in 4°C and stored flat until visualized on a Leica SP5 laser scanning confocal microscope (Buffalo Grove, IL) under 63x magnification and oil immersion. Images were collected using the Leica LAS AF software (Buffalo Grove, IL) and processed using Fiji/ Image J (Schindelin et al., 2012).

3.3.6 Statistical analysis

Concentration- and time-dependent data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. For the kinase inhibition studies, data from four separate experiments showed statistically similar results and were therefore combined (normalized relative to control incubation). Gill gene expression data from inhibitor incubations were analyzed by two-way ANOVA. With significant interaction between factors, data were further analyzed by Sidak's multiple comparisons test. Data from the 60 minutes incubation experiment with prolactin on Stat5, Akt and Erk1/2 phosphorylation were analyzed by two-sided t-test. A significance level of P-value < 0.05 was used throughout. All tests were performed using GraphPad Prism 6.0 software (San Diego, CA, USA).

3.4 Results

3.4.1 Prolactin stimulates expression of *ncc2b* in a dose-dependent manner

First, we examined the effects of ovine prolactin on expression of *ncc2b* in medaka gill. Gill filaments were incubated with increasing concentrations of prolactin (0, 0.01, 0.1, 1.0 µg mL^{-1}). Expression of *ncc2b* showed a concentration dependent increase as function of hormone dose; with a 2-fold elevation in the 0.01 µg mL^{-1} group up to a 6-fold stimulation in the 1.0 µg mL^{-1} group relative to control (Fig. 3.1).

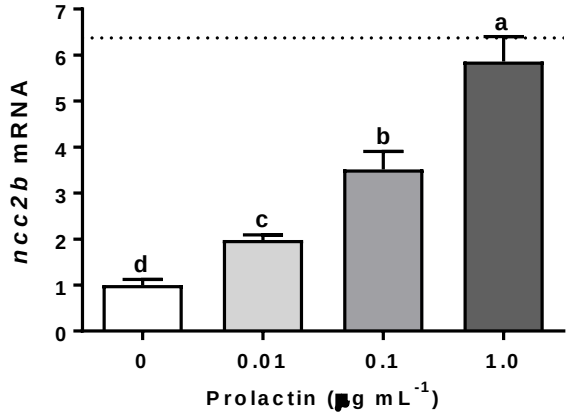

Figure 3.1 Concentration-dependence of prolactin effects on *ncc2b* expression in gill of Japanese medaka. Gill filaments where incubated with 0, 0.01, 0.1 and 1.0 µg ovine prolactin mL^{-1} for 16 hours. Expression levels where normalized to the geometric mean of three normalization genes. Values represent the mean value ± S.E.M. relative to control (*n*=10). *Dotted line* indicates pre-incubation expression levels. Groups not sharing letters are significantly different as determined by one-way ANOVA followed by Tukey's post-hoc test: $P<0.05$.

3.4.2 Prolactin's control of *ncc2b* expression is dependent on Stat5 activation

Next, we examined the effect of co-incubating prolactin with inhibitors of potential prolactin signaling pathways on *ncc2b* expression. In these experiments 1.0 µg mL^{-1} prolactin induced a 3-fold increase in *ncc2b*. Co-incubation with the Stat5 inhibitor (Nico) blocked prolactin-induced stimulation of gill *ncc2b* (Fig. 3.2). However, co-incubation with inhibitors of Akt-PI3K pathway (Carb) and Erk pathway (U0126) did not affect the stimulatory effect of prolactin (Fig. 3.2).

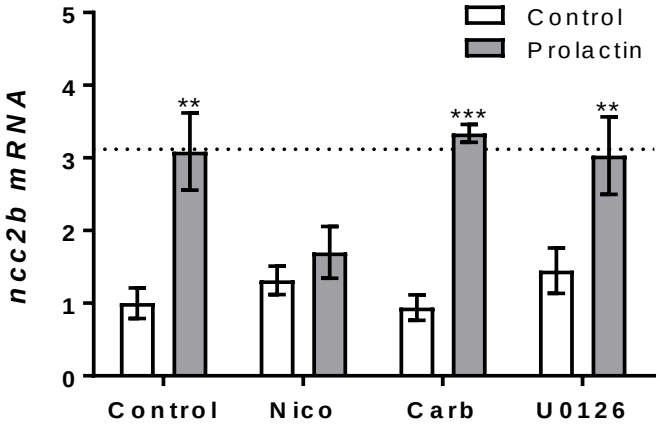

Figure 3.2 Effects of ovine prolactin (1.0 µg mL^{-1}) combined with control media, or specific inhibitors of Stat5 (Nico, 10 µmol L^{-1}), Akt (Carb, 10 µmol L^{-1}), Mek (U0126, 10 µmol L^{-1}) on *ncc2b* expression in gill from Japanese medaka. Gill filaments where incubated for 16 hours and expression levels normalized to the geometric mean of three normalization genes. There was a significant antagonistic effect of Nico (P<0.01) blocking the stimulatory effect of prolactin (two-way ANOVA). Each bar represents 3-4 samples per treatment from four separate experiments (i.e., a total of 14-16 samples per treatment). Asterisks indicate significant difference from respective control as determined by Bonferroni corrected Fisher's Least Significant Difference (LSD) post-hoc test: **P<0.01; ***P<0.001. Dotted line indicates pre-incubation expression levels. Values represent the mean value ± S.E.M. relative to control (n=14-16).

3.4.3 Prolactin activates the Stat5 but not the Erk1/2 or Akt signaling pathways in medaka gill

The activation of cell signaling pathways was studied using Western blots of lysates from medaka gill labelled with phospho-specific and general kinase antibodies. For all antibodies, only bands corresponding to the predicted size were observed with both general and phospho-specific antibodies (apparent M_r: 42-44 kDa for Erk1/2, 60 kDa for Akt, 90 kDa for Stat5), thereby validating the use of the antibodies in the medaka gill system. In an initial experiment, we examined the effect *in vitro* of a 60 minute incubation of gill filaments with 1.0 µg mL^{-1} prolactin. Gill filaments incubated with prolactin showed a 7-fold increase in pStat5 compared to total Stat5 (Fig. 3.3a). There was no significant change in phosphorylation of Akt (Fig. 3.3b).

Similar to Akt, incubation with prolactin did not cause any change in the phosphorylation of Erk1 or Erk2 (Fig.3.3c). Because Erk1 and Erk 2 reacted similar the sum of the two is used in all figures in the study. Phosphorylation of Akt and Erk1/2 in these Western blots was in the dynamic detection range, which was confirmed by commercial positive controls (data not shown). We went on to analyze the time- and concentration-dependence of prolactin activation of signaling kinases. Prolactin induced a marked 2-fold increase in phosphorylation of Stat5 after 10 minutes of exposure to prolactin which then reached a plateau after 30 minutes (4-fold increase; Fig. 3.4a), where it stayed for the duration of the experiment (120 minutes). Neither Akt (Fig. 3.4b) nor Erk1/2 (Fig 3.4c) were activated at the time-points of this experiment (10, 30, 60 90, 120 minutes). In another experiment we demonstrated the concentration dependence of the phosphorylation of Stat5 with increasing concentrations of prolactin (0.01 µg mL^{-1}: 4-fold increase; 0.1 µg mL^{-1}: 8-fold increase; 1 µg mL^{-1}: 12-fold increase; Fig. 3.5a). In this experiment none of the used concentrations affected the activation state of Akt (Fig. 3.5b), Erk1/2 (Fig. 3.5c).

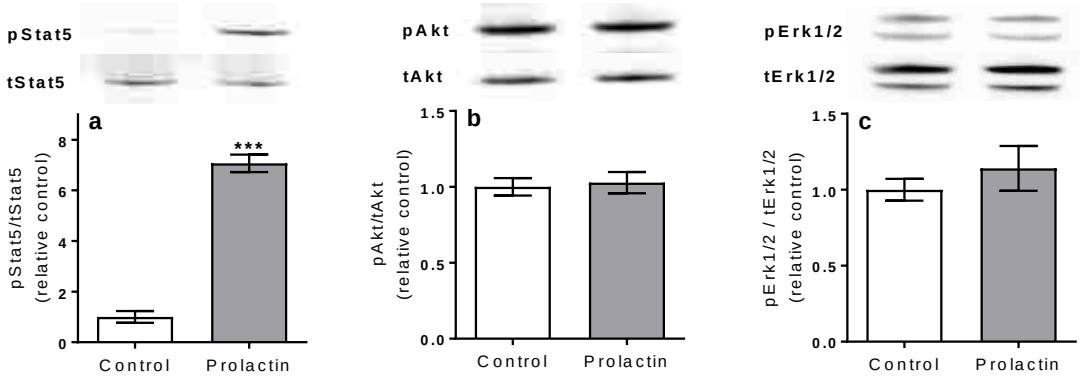

Figure 3.3 Effect of ovine prolactin (1.0 µg mL^{-1}) on phosphorylation (activation) of Stat5 (A), Akt (B) and Erk1/2 (C) in the gill. Gill filaments from Japanese medaka where incubated for 1 hour followed by Western blotting. Inserts show representative bands of phosphorylated (pStat5, pAkt, pErk1/2) and total (tStat5, tAkt, tErk1/2) signaling kinases. Phosphorylated signaling kinases were normalized to the total abundance of the specific kinase. Values are mean ± S.E.M. (*n*=4). *Asterisks* indicate significant difference from control as determined by two-tailed t-test: ****P*<0.001.

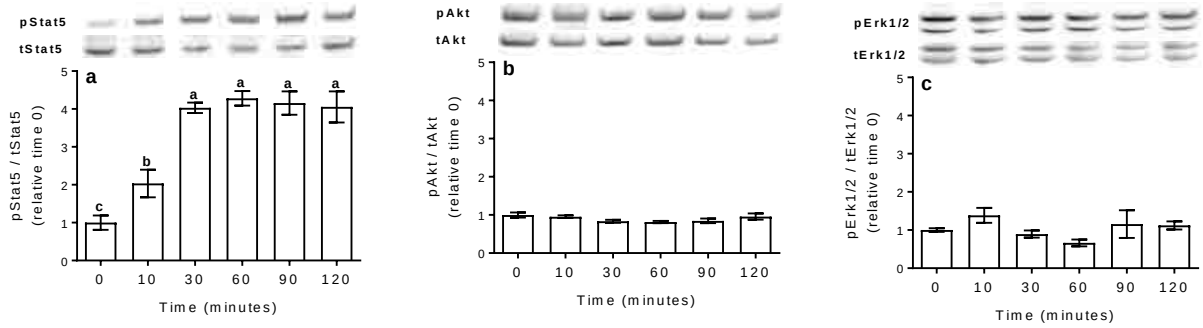

Figure 3.4 Time-dependent effect of ovine prolactin (1.0 µg mL^{-1}) on phosphorylation (activation) of Stat5 in gill filaments from Japanese medaka after 0, 10, 30, 60, 90, 120 minutes incubation. Phosphorylated (activated; pStat5, pAkt, pErk1/2) kinases were normalized to total (tStat5, tAkt, tErk1/2) kinase abundance as determined by Western blotting. Inserts show representative bands of phosphorylated (pStat5, pAkt, pErk1/2) and total (tStat5, tAkt, tErk1/2) signaling kinases. Values represent the mean value ± S.E.M. relative to control (*n*=4). Groups not sharing letters are significantly different as determined by one-way ANOVA followed by Tukey's post-hoc test: *P*<0.05.

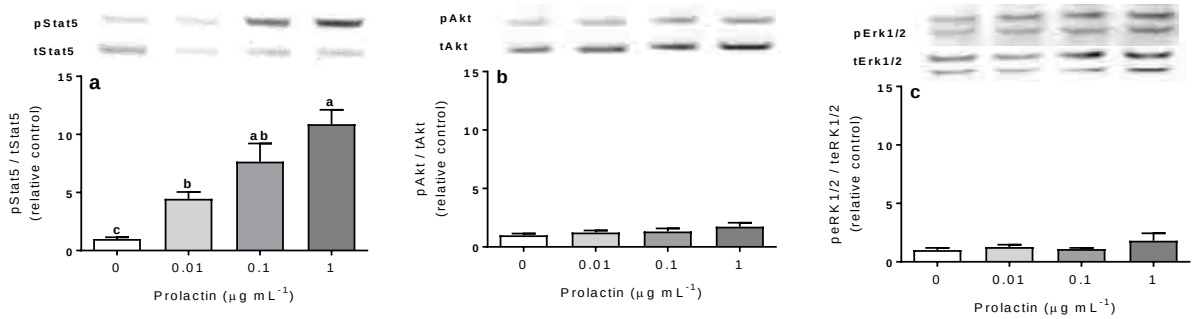

Figure 3.5 Concentration-dependence of prolactin effects on phosphorylation (activation) of Stat5 in gill filaments from Japanese medaka. Gill explants where incubated with 0, 0.01, 0.1 and 1.0 µg ovine prolactin mL^{-1} for 1 hour. Phosphorylated (activated; pStat5, pAkt, pErk1/2) kinase was normalized to total (tStat5, tAkt, tErk1/2) kinase abundance as determined by Western blotting. Inserts show representative bands of phosphorylated (pStat5, pAkt, pErk1/2) and total (tStat5, tAkt, tErk1/2) signaling kinases. Values represent the mean value ± S.E.M. relative to control (n = 6). Groups not sharing letters are significantly different as determined by one-way ANOVA followed by Tukey's post-hoc test: $P<0.05$.

3.4.4 Prolactin induce phospho-Stat5 immunoreactivity in nuclear region of ionocytes

To confirm the presence of pStat5 in the nucleus, we examined the subcellular localization of pStat5 in the afferent (trailing) edge of gill filaments following PRL stimulation. Gill filaments were excised from medaka post-mortem; and after 60 minute pre-incubation, treated with or without1 µg mL^{-1} prolactin for 60 minutes. Nuclear localization of pStat5 was detected by indirect immunofluorescence after staining with a monoclonal antibody to pStat5 and ionocytes identified as Na^+/K^+-ATPase positive cells using a pan alpha subunit antibody (a5). Ionocytes are characterized by strong Na^+/K^+-ATPase staining outside the nucleus, presumably in the vesiculotubular network (Fig. 3.6a, b, c). As shown in Fig. 3.6, pStat5 staining was weak and generally observed in the cytoplasm of unstimulated gill filament with weak nuclear staining detected in some ionocytes. In gill stimulated with prolactin, pStat5 was visible in the nucleus where staining was very intense; the staining appeared to be limited to ionocytes (Fig. 3.6). The

data support the nuclear localization of pStat5 in medaka gill ionocytes in response to prolactin stimulation.

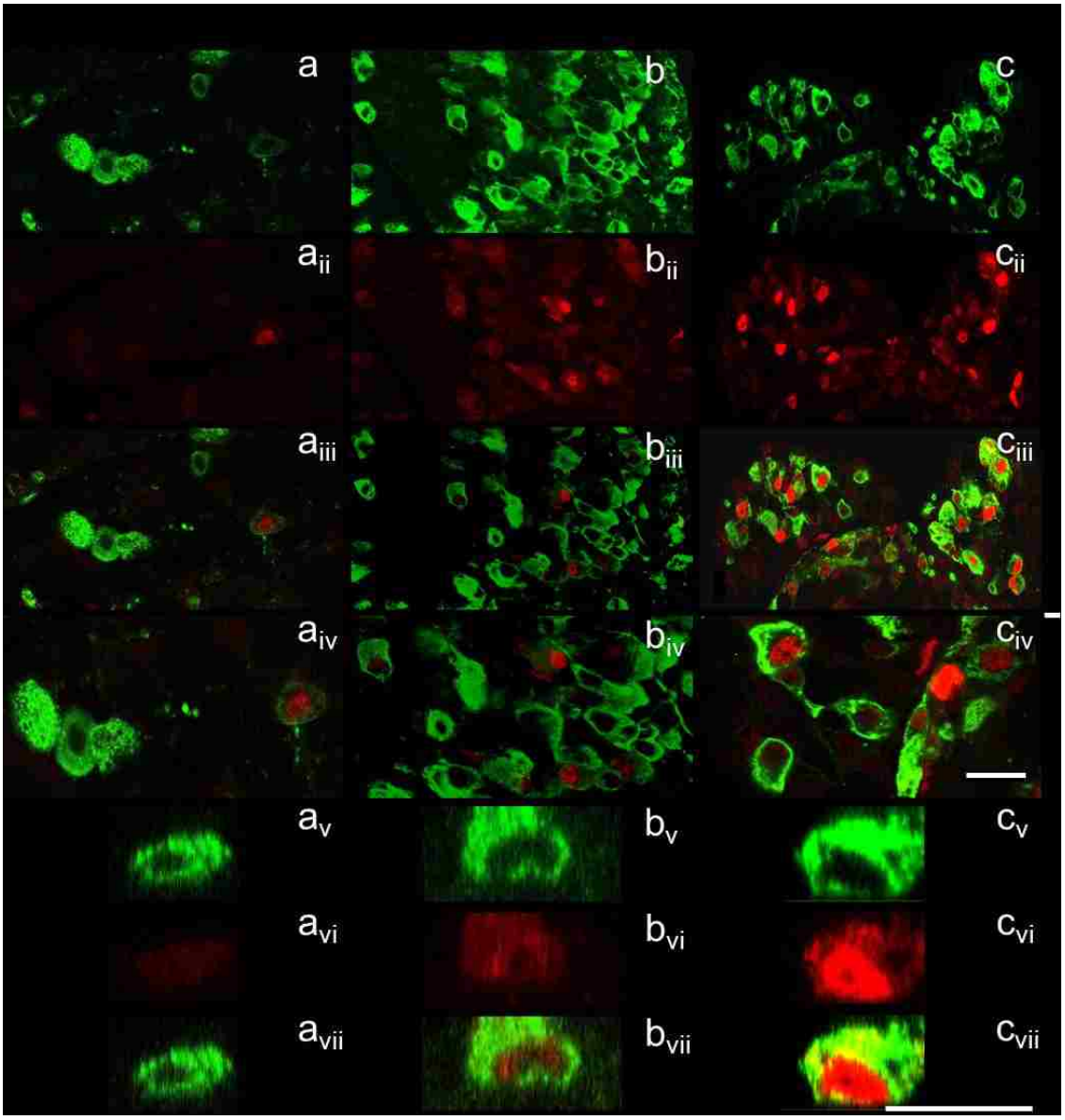

Figure 3.6 Double immunofluorescence whole-mount staining of trailing edge of gill filaments from Japanese medaka. The Na^+/K^+-ATPase (green) and pStat5 (red) in pre-incubation control (a), 4 hour control (b) and 4 hour 1 µg mL^{-1} ovine prolactin (c) in medaka gills. i-iv: *x*-*y* plane images at low magnification, scale bare = 10 µm. v-vii: *x*-*z* plane images at high magnification, scale bar 10 µm. iii-iv, vi-vii: merged images.

3.5 Discussion

Prolactin has a conserved function across vertebrates, regulating ion and water transport and, in teleosts, prolactin is a FW-adapting hormone as it promotes ion retention and water excretion (Bole-Feysot et al., 1998; Freeman et al., 2000; Hirano, 1986; Sakamoto and McCormick, 2006). In the gill prolactin upregulates expression of genes imperative to ion retention (reviewed by Breves et al., 2014); and in particular *ncc2* that plays an important role in the re-uptake of Na^+ and Cl^- across the gill (Horng et al., 2009; Hsu et al., 2014; Inokuchi et al., 2008). It has been shown that that prolactin stimulates expression of gill *ncc2* which in turn is instrumental in maintaining or increasing the abundance of NCC cells (Breves et al., 2010); and furthermore that the process is receptor mediated (Breves et al., 2013). This study is the first to examine the signaling pathways that mediate prolactin promotion of Ncc transcription. In the present work, ovine prolactin and kinase inhibitors that target putative prolactin signaling pathways (Freeman et al., 2000) were used in conjunction with kinase phosphorylation assays in a medaka branchial *in vitro* system to determine the mechanisms used by prolactin to stimulate the expression of Ncc. We thus demonstrated that prolactin utilizes the Jak-Stat5 pathway to stimulate *ncc2b* in the gills by a direct mechanism involving nuclear translocation of phospho-Stat5 in ionocytes. As *ncc2b* is commonly used as a proxy for NCC cell function this identifies one key mechanism by which prolactin mediates salt retention.

Treatment of cultured branchial rainbow trout epithelia with prolactin stimulated ion uptake (Zhou et al., 2003). In hypophysectomized killifish, prolactin replacement therapy promoted survival in FW fish (Pickford and Phillips, 1959). In tilapia, ncc gene expression and NCC cell numbers were restored in hypophysectomized fish following prolactin replacement treatments (Breves et al., 2010). Additionally, branchial expression of *ncc* in zebrafish was

upregulated with prolactin both *in vivo* and in culture (Breves et al., 2013). Based on these data it has been hypothesized that prolactin may be directly linked to Ncc-dependent ion uptake in both euryhaline and stenohaline teleosts. The present study sought to distinguish between the effects of ovine prolactin on three FW ionocyte subtypes. Our data show incubation with ovine prolactin stimulated an increase in *ncc2b* (Fig. 3.1) which is similar to that observed in Breves et al. (2013) and Bossus et al. (2017).

In teleosts, binding of prolactin to its receptor leads to dimerization and phosphorylation of Jak2-Stat5, activating the signaling cascade that leads to translocation of Stat5 into the nucleus and thus regulating transcription of genes responsive to prolactin (Bole-Feysot et al., 1998; Freeman et al., 2000; Han et al., 1997). However, in cells that respond to prolactin, there is still potential for activation of other signaling pathways including MAPK and PI3K (Bole-Feysot et al., 1998; Freeman et al., 2000). In order to identify the prolactin signaling cascade for *ncc2b* expression, we chose three inhibitors that act on the most downstream point of each pathway just prior to nuclear translocation. Nico, an inhibitor of the Jak-Stat pathway, selectively targets the SH2 (Src Homology) domain of Stat5 and prevents DNA binding. Carb is a competitive phosphatidylinositol ether analog that selectively inhibits Akt (also known as Protein Kinase B, a serine/threonine-specific protein kinase) thereby preventing the activation of PI3K. And U0126 is a highly selective inhibitor that binds Mek1 and Mek2 (MAPK/Erk kinases) thus preventing the activation of both Erk1 and Erk2 (extracellular signal-regulated kinases), blocking the MAPK signaling pathway.

In the present study, gills were first incubated with each inhibitor and subsequently treated with ovine prolactin. While prolactin induced expression of *ncc2b*, neither Carb nor U0126 affected the induced expression of *ncc2b* by prolactin (Fig. 3.2). The combination of Nico

and prolactin resulted in *ncc2b* levels comparable to control, thus indicating that inhibition of the Jak-Stat pathway stunted expression of *ncc2b*.

While qPCR data suggests that Akt and Erk1/2 are not involved in ovine prolactin signaling of *ncc2b* expression, Western blot analyses were performed to confirm there was no effect of ovine prolactin on the activation or deactivation of Akt or Erk1/2. Incubation with ovine prolactin led to increased levels of pStat5 while both pAkt and pErk1/2 where not affected (Fig 3.5a, b and c, respectively). Therefore, suggesting that prolactin regulates expression of *ncc2b* via the Jak-Stat pathway. Furthermore, using confocal microscopy, we showed an increase, not only in pStat5 abundance due to ovine prolactin, but also nuclear localization of pStat5 (Fig. 3.6). Thus further illustrating that ovine prolactin utilizes the Jak-Stat pathway in order to stimulate gene expression. We believe this study is the first to use the combination of inhibitors and ovine prolactin to determine the pathway ovine prolactin utilizes to stimulate gene expression. Future studies should aim to show co-localization of pStat5 in NCC cells specifically and a stimulation in apical Ncc expression with prolactin treatment.

3.6 References

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Chapter 4 Mechanisms of ion uptake and retention in the gill of rainbow trout (*Oncorhynchus mykiss)* exposed to ion poor water

Rebecca J. Bollinger[1], Maryline C. Bossus[1, 2], Joseph M. Gasser[1], Christian K. Tipsmark[1]

[1]Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA

[2] Department of Biology, Lyon College, Batesville, AR 72501

4.1 Abstract

Rainbow trout (*Oncorhynchus mykiss*) is a salmonid species able to live in very dilute freshwater habitats. This study aimed to advance our understanding of functional determinants of gill osmoregulatory mechanisms in a low ion environment. Expression of claudin (*cldn*) tight junction proteins, Na^+/K^+-ATPase (*nka*) subunits and ion transport proteins (*fxyd11, nhe3, rhcg1, hat, asic4*) potentially involved in ion uptake in the freshwater (FW) gill were examined during acclimation of FW trout to ion poor water (IPW, 1:4 diluted tap water) for 6, 24 and 168 hours. After 168 hours in IPW, *nka-a1a, nka-a1b, fxyd11, nhe3, asic4* and *rhcg1* were significantly elevated (2-8 fold) while no change in *hat* expression was observed during the time-course experiment. *cldn27a* mRNA levels were significantly elevated (2 fold) after 7 days in IPW while *cldn28a, cldn28b* and *cldn30c* remained stable. Western blot analysis demonstrated a significant increase of Nka-α1a protein abundance (6 fold) in fish acclimated to IPW for one week, while Nka-α1b and Fxyd11 abundances were unchanged. Enzyme analysis showed IPW-acclimated rainbow trout gills had a higher Nka enzymatic activity compared to FW gills. Taken together, the present study suggests that the mechanism for ion absorption in the rainbow trout gill exposed to IPW involves the basolateral Nka-α1a with an apical Nhe3-Rhcg1 metabolon loosely coupled to Hat and Asic4. The data suggests that Cldn28a, 28b and 30c maintain tight junction integrity while Cldn27a enforce barrier properties to prevent ion loss to the dilute IPW environment.

4.2 Introduction

Teleost fishes maintain an internal plasma osmolality around 300 mOsm kg^{-1} despite the salinity in their external surroundings. In both freshwater (FW: 1-10 mOsm kg^{-1}) and seawater

(SW: 1000 mOsm kg^{-1}) the osmoregulatory mechanisms of the fish are constantly working to maintain steep osmotic and ionic gradients (Evans et al., 2005). Hydromineral homeostasis is reliant on the concerted function of the gill, kidney and intestine. However, since the gill is directly exposed to the environment, its function is of the utmost importance as it maintains ion and acid/base balance, gas exchange and eliminates nitrogenous waste.

In SW, teleosts are subjected to ion gain and osmotic water loss and must therefore hypo-osmoregulate in order to maintain their plasma osmolality below that of the surrounding environment. The function of the SW gill in ionoregulation has been vastly studied and the proposed model for ionocyte function widely accepted. In this model, a basolateral Na^+/K^+-ATPase (Nka) and Na^+, K^+, $2Cl^-$ cotransporter (Nkcc) work in conjunction with an apical cystic fibrosis transmembrane conductance regulator chloride channel (Cftr) to excrete excess Cl^- while Na^+ exits paracellularly via cation-selective pores in the transmembrane tight junctions (Degnan and Zadunaisky, 1980; Hiroi and McCormick, 2012; Hwang and Hirano, 1985; Marshall and Singer, 2002; Silva et al., 1977). Fxyd proteins are modulatory proteins that interact with Nka to alter its kinetic properties (Garty and Karlish, 2006). The mechanisms for NaCl excretion in the SW ionocyte have been confirmed in numerous teleost species including tilapia (Hiroi et al., 2005; Hiroi et al., 2008; Tipsmark et al., 2011), killifish (Marshall and Singer, 2002), medaka (Bollinger et al., 2016; Hsu et al., 2014) and several salmonids (Bystriansky et al., 2006; McCormick et al., 2009; Nilsen et al., 2007; Tipsmark and Madsen, 2009).

In FW, the model for ion absorption is more complex and widely debated, possibly due to the diversity of species evolution and ecology. Currently there are three proposed mechanisms for Na^+ uptake in FW gills. The first model proposes an apical Na^+/H^+ exchanger (Nhe) while in the second model a vacuolar-type H^+-ATPase (HAT) assists in Na^+ uptake with an apical Na^+

channel, and the third employs an apical Na^+, Cl^- cotransporter (Ncc). Apical Nhe3 expression has been found in gill cells of rainbow trout (Hiroi and McCormick, 2012; Ivanis et al., 2008), tilapia (Hiroi et al., 2008; Inokuchi et al., 2008) and zebrafish (Esaki et al., 2007; Yan et al., 2007). Rhesus glycoproteins are ammonia transporters that are thought to form a functional metabolon with Nhe3 for simultaneous Na^+ uptake and nonionic ammonia excretion (Javelle et al., 2008; Khademi et al., 2004; Kumai and Perry, 2011). This model has been widely examined and has gained support with mounting evidence for the putative role of Rhesus (Rh) proteins in ammonia excretion. While no evidence of an Ncc has been found in rainbow trout, immunostaining of Ncc has been reported in tilapia (Hiroi et al., 2008) and zebrafish (Wang et al., 2009). Several studies have presented apical localization of Hat in rainbow trout (Ivanis et al., 2008; Reid et al., 2003; Sullivan et al., 1995; Wilson et al., 2000). Hat has also been proposed to drive electrogenic Na^+ uptake in rainbow trout through acid-sensing ion channels (Asic; Dymowska et al., 2014), voltage-insensitive Na^+ channels gated by extracellular H^+ (Waldmann and Lazdunski, 1998).

The mechanisms involved in determining paracellular permeability are also an important aspect of gill function in osmoregulation. Tight junctions make up the barrier between epithelial cells and function to regulate paracellular water and solute transport (Van Itallie and Anderson, 2006). Tight junctions are made up of the combination of several proteins including occludin, tricellulin, claudins and junctional adhesion molecules. Claudins are especially important as they determine the permeability and ion selectivity of the paracellular pathway (Colegio et al., 2001). Some claudins exhibit barrier-forming properties while others form cation-selective pores. For example, *cldn10d* and *cldn10e* expression increase in SW-acclimated trout (Tipsmark et al., 2008b) and medaka (Bossus et al., 2015) and are suggested to be associated with Na^+ excretion.

Cldn27a, 28a, 28b and *30c* exhibit increased expression in the gill of Atlantic salmon (Engelund et al., 2012; Tipsmark et al., 2008b), tilapia (Tipsmark et al., 2008a) and medaka (Bossus et al., 2015) when transferred from SW to FW, suggesting that these claudin isoforms work to create a tighter epithelia.

The steelhead trout and rainbow trout are two phenotypes of the same species (*Oncorhynchus mykiss*) which have distinctive life cycles. While rainbow trout go through its complete life cycle as a riverine while the migratory steelhead trout spawn in rivers but spend their adult life at sea. Moreover, *O. mykiss* are capable living in extremely dilute oligotrophic rivers and lakes of the Pacific Northwest, where they migrate to breed (Scott and Crossman, 1998). The FW rainbow trout, while landlocked, retain the ability to adapt to SW and have been widely used as a model species for ionoregulatory study (Flores and Shrimpton, 2012; Richards et al., 2003; Wood and Nawata, 2011). As an osmoregulatory organ, the rainbow trout gill has shown a remarkable plasticity in its ability to maintain hydromineral balance in SW, FW and in extremely dilute FW environments (10-50 µM; Bystriansky et al., 2007; Kirschner, 2004). In fact, Na^+ and Cl^- uptake has been measured in ion poor environments with Na^+ and Cl^- concentrations in the range of 0.5 mM (Kerstetter and Kirschner, 1972; Kerstetter et al., 1970).

In this study, we use rainbow trout, with its ability to osmoregulate in ion poor water (IPW) as a model to obtain a better understanding of the mechanism to Na^+ uptake and ion retention by the FW gill. FW rainbow trout were transferred to IPW (1:4 of tap water to deionized water) and sampled at 6, 24 and 168 hours (7 days). We examined the expression of key ion transporters; two alpha isoforms of Nka (*Nka-α1a*, *Nka-α1b*), *fxyd11*, *hat*, *nhe3*, a rhesus glycoprotein (*rhcg1*), (*asic4*) as well as putative barrier-forming claudins (*cldn27a, 28a, 28b* and *30c*).

4.3 Methods and Materials

4.3.1 Fish and maintenance

Juvenile rainbow trout (3.0 g ± 0.32, 70 mm ± 2.47) were obtained from the Norfork National Fish Hatchery (Mountain Home, AR, USA) and acclimated for one month to recirculating de-chlorinated municipal tap water, mechanically and biologically filtered (in mM: $[Na^+]$ 0.28, $[Cl^-]$ 0.21, $[Ca^{2+}]$ 0.62, $[Mg^{2+}]$ 0.08, $[K^+]$ 0.05 and pH 8.3). Fish were maintained at 20°C with a 14 hours light/10 hours dark photoperiod and fed daily with Purina® fish pellets (AquaMax® Grower 400; Gray Summit, MO, USA). Food was withheld from one day before the transfer experiments up to sampling. All handling and experimental procedures were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC 13052).

4.3.2 Experimental design and sampling

In order to achieve an environment similar to that observed in ion poor environments, a 1:4 ratio of tap water to deionized water was used. Water was mechanically and biologically filtered during transfer (in mM: $[Na^+]$ 0.08, $[Cl^-]$ 0.06 $[Ca^{2+}]$ 0.18, $[Mg^{2+}]$ 0.02, $[K^+]$ 0.02 and pH 7.9). All water analyses were performed by the Water Quality Lab at the University of Arkansas Water Resource center. Fish were anesthetized in 100 mg L^{-1} tricaine methanesulfonate (Western Chemical Inc., Ferndale, WA, USA) buffered with sodium bicarbonate and sacrificed by cervical dislocation. Blood samples were collected and spun at 2,000 x g for 15 minutes at 4°C for analysis of plasma osmolality. Plasma osmolality was measured by freezing point depression using a Fiske Micro-Osmometer (Advanced Instruments, Norwood, MA, USA). At the end of sampling, a piece of caudle musculature was excised and weighed for determination of muscle water content (MWC), Muscle samples were weighed after drying for 24 hours at 105°C and

MWC was calculated as percentage of wet weight. The gill apparatus was excised, rinsed with phosphate buffered saline (PBS), the cartilage was removed and the tissues were placed in either TRI Reagent® for RNA extraction, sucrose EDTA imidazole (SEI) buffer (300 mM sucrose, 20 mM Na_2EDTA, 50 mM imidazole, pH 7.5) for Western blot analyses and enzyme assay. Tissues were immediately placed on dry ice and stored at -80°C until further use.

4.3.3 RNA extraction, cDNA synthesis and real-time qPCR

Tissues were homogenized in TRI Reagent® (Sigma Aldrich, St. Louis, MO, USA) using a Power Max 200 rotating knife homogenizer (Advanced Homogenizing System; Manufactured by PRO Scientific for Henry Troemner LLC, Thorofare, NJ, USA). Total RNA was extracted following the manufacturer's protocol. RNA pellet was dissolved in molecular biology grade ultra-pure water (Quality Biological, Gaithersburg, MD, USA). NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to estimate quantity and purity (A_{260}/A_{280}) of each sample. All samples used had a ratio 2.0. cDNA was synthesized from 1 µg total RNA in a final volume of 20 µL using Applied Biosystems high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and following manufacturer's protocol. mRNA sequences for rainbow trout target transcripts were identified in the Ensembl genome browser (Flicek et al., 2014) and used to design specific primers (Table 4.1) to detect the following transcripts: *nka-α1a, -α1b, fxyd11, nhe3, rhcg1, asic4* and *hat.* Elongation factor 1-alpha (*ef1a*) and ribosomal protein (*rplp*) were used as normalization genes (Vandesompele et al., 2002). Primers were generated using Primer3 software (Koressaar & Remm, 2007; Untergasser et al., 2012) and synthesized by Sigma-Aldrich. Quantitative real-time qPCR was performed in a final volume of 15 µL using BioRad CFX96 platform (BioRad, Hercules, CA, USA) and SYBR® Green JumpStar™ (Sigma Aldrich). The following thermocycling protocol

was used: 3 minutes initial denaturation/activation phase (94°C), 40 cycles of a 15 seconds denaturation step and an annealing/elongation steps for 60 seconds (60°C), followed by a melting curve analysis at an interval of 5 seconds per degree from 55 to 94°C. Amplification efficiency of each primer set was analyzed using the standard curve method and sequential dilutions from 2 to 16 times. Relative copy numbers of individual target genes was calculated using the primer set amplification efficiency. Relative copy numbers were calculated as $E_a^{\Delta Ct}$, where C_t is the threshold cycle number and E_a is the amplification efficiency (Pfaffl, 2001). GeNorm software (Biogazelle, Zwijnaarde, Belgium) was used to calculate a geometric mean of the two normalization genes. Stability of normalization genes was verified using the GeNorm M value. Normalized units were calculated by dividing the relative copy number of each target gene by the geometric mean of normalization genes.

Table 4.1 Primer sequences for quantitative PCR of rainbow trout transcripts

Target	Forward Primer	Reverse Primer	NCBI acc. no.
ef1a	AGAACCATTGAGAAGTTGGAGAAG	GCACCCAGGCATACTTGAAAG	BT046846
rplp	GTTCCTGGAGGGTGTTCGTA	TCCGTTGATGATGGTGTGAG	BT073606
nka-α1a	CCCAGGATCACTCAATGTCAC	CCAAAGGCAAATGGGTTTAAT	AY319391
nka -α1b	CTGCTACATCTTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	AY319390
fxyd11	CTCTGTGCATTCTTTGTGGA	GGACAAACAATCCACCTGCT	BK006247
nhe3	GCCAAGAAGATCCAAACCAA	ATGGCTATGAGGTCGGACAC	EF446606
rhcg1	CATCCTCAGCCTCATACATGC	TGAATGACAGACGGAGCCAATC	DQ431244
hat	CAGGACAATGGACATCAACG	TCAGCCTTGGTTGTGAGATG	AF140022
asic4	TCAACCGCTTTCGTTTCTCT	GTCCAAATCAGTGGGCTTGT	KF964645
cldn27a	GACAGGTATCGTCGGCATCT	CCAGCCACAATACAGGCTCT	BK006400
cldn28a	TGACTGCTCAGGTCATCTGG	GGTAAGGCCAGAAGGGAGTC	BK006401
cldn28b	TTCTACCAGGGCTCCATCAG	ATGGGCAGAGCACAGATGAT	BK006405
cldn30	TGATCATTGGAGGAGGGTTC	AACATAGTCCCTGGGTGCTG	BK006405

4.3.4 Western blotting

Gill samples for Western blot were thawed on ice, transferred to a new tube and homogenized in 1 mL SEID buffer (SEI with 0.1% sodium deoxycholate) with a protease

inhibitor cocktail (P8340; Sigma Aldrich). Samples were centrifuged at 7,000 x g for 10 minutes at 4°C. The supernatant was transferred to a new tube and was subsequently centrifuged again for the membrane fraction (20,000 x g for 60 minutes at 4 °C). Protein content was measured using the Bradford assay (Bradford, 1976) adapted to a 96-well plate using a spectrophotometer (SpectraMax® Plus384, Molecular Devices, Sunnyvale, CA, USA). All Western blotting solutions and materials were NuPAGE ™ from Thermo Fisher unless otherwise stated. Western blot loading samples were prepared in 1X LDS NuPAGE Sample Buffer with 50 mM DTT (GE Healthcare Bio-Sciences, Pittsburgh, PA) and subsequently denatured for 10 min at 70°C. An equal quantity of gill protein (10 µg) was loaded into all lanes and run on a 4-12% Bis-Tris gel in MES buffer at 200 V for 34 minutes (Xcell II SureLock). A protein marker was included to estimate molecular size (Precision Plus Protein™ All Blue Prestained Standard, BioRad). Gels were subsequently blotted on a 0.2 µm nitrocellulose membrane for 1 hour at 30 V using transfer buffer with 10% MetOH. Membranes were blocked with Li-Cor Blocking Buffer (TBS; LI-COR Biosciences, Lincoln, NE, USA). Immunological detection was obtained by incubating with primary antibodies overnight at 4°C. Based on protein sequences of rainbow trout Nka we selected peptide sequences for homologous antibody production:
Nka-α1a: CLAATSEDDGKKKSE (NP001117933), Nka-α1b CRKSKKEVKKAREKK (NP001117932). The closest homology of the peptide sequences with other Nka α1 paralogs in Rainbow trout are for the respective peptides: α1a: Nka α1a (100%, 14/14), Nka α1b (64%, 9/14), Nka α1c (64%, 9/14); for α1b: Nka α1b (100%, 14/14), Nka α1a (64%, 9/14), Nka α1c (14%, 2/14). Affinity purified polyclonal antibodies were produced in rabbits by GenScript (Piscataway, NJ, USA). Immunoreaction specificity of Nka-α1a and Nka-α1b antibodies were validated by incubating with a peptide control antigen for each antibody and Western blotting.

The Fxyd11 antibody directed towards a c-terminal sequence in Atlantic salmon (see Tipsmark et al., 2010) is conserved in rainbow trout and detect one band with apparent molecular weight of 8 kDa in crude membrane preparations from trout gill. Primary antibodies were used at the following concentrations: Nka-α1a (0.5 μg mL^{-1}), Nka-α1b (1.0 μg mL^{-1}) and Fxyd11 (1.3 μg mL^{-1}) A mouse β-actin antibody was used as a control at a concentration of 0.2 μg mL^{-1} (Abcam, Cambridge, MA, USA). Secondary antibodies were used according to manufacturer's protocol and incubated in the dark at room temperature for 1 hour in blocking buffer (IRDye® 800CW Goat anti-Rabbit IgG and IRDye® 680LT Goat anti-Mouse IgG, LI-COR). After washing, membranes were dried on filter paper and imaged using an Odyssey infrared scanner (LI-COR).

4.3.5 Nka enzyme assay

Gill Nka activity was measured in gill homogenates using a NADH-coupled assay as described by Bollinger et al (2016) with modifications for use with microplate spectrophotometer. This method exploits the sensitivity of Nka to ouabain by coupling the production of ADP and NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of ouabain. Prior to start the assay, gill samples were thawed on ice. One half of a gill arch was homogenized in 0.2 mL ice-cold SEIDM buffer (SEI buffer with 0.1% Na-deoxycholate and 10 mM mercaptoethanol) with the Power Max homogenizer. Samples were then centrifuged at 8,500 x g for 60 seconds. The supernatant was transferred to a new tube and replaced on ice. The assay was initiated within 10 minutes of homogenization for each sample to ensure maximum activity. Optimal Nka enzyme activity was measured by coupling ATP hydrolysis to the conversion of NADH to NAD^+ by pyruvate kinase and lactate dehydrogenase and analyzed by kinetic readings at 340 nm for 10 min at 25°C. Each homogenate was assayed under the following conditions with or without the presence of ouabain (0.5 mM) in triplicate: (in

mM); [NaCl] 49.1, [KCl] 10.4, [$MgCl_2$] 1.8, [Na_3-phosphoenolpyruvate] 0.5, [Na_2-ATP] 0.5, [Na_2NADH] 0.16, [KCN] 0.4, [PK] 2.6 units mL^{-1}, [LDH] 2.0 units mL^{-1}, in 50 mM imidazole buffer, pH 7.5. The bradford assay was used to measure the protein content of tissue homogenates. Enzyme activity was normalized to protein content and expressed as µmoles ADP mg $protein^{-1}$ $hour^{-1}$ using the following equation: NKA Activity = $\frac{\frac{\Delta ABS_{340}}{min} x \frac{60\,min}{hr}}{\frac{\Delta OD_{340}}{nmol\ ADP}\ x\ (0.01\,mL\ x \frac{mg\ protein}{mL})}$

where ΔABS_{340} is the difference in slopes between assays of the same sample with and without ouabain and ΔOD_{340}/nmol ADP is the calibration factor for conversion of the measured decrease in OD340 to nmol ADP.

4.3.6 Statistical analysis

Time course data were analyzed by two-way ANOVA. If significant interaction between factors occurred data was further analyzed by Sidak's multiple comparisons test. Homogeneity of variances was analyzed by Bartlett's test. Statistical analysis of Western blot data was performed through one-way ANOVA followed by Dunnett's multiple comparisons post hoc test. A significance level of P-value<0.05 was used throughout. All tests were performed using GraphPad Prism 6.0 software (San Diego, CA, USA).

4.4 Results

4.4.1 Acclimation of rainbow trout to IPW

Transfer to IPW caused a slight decrease in plasma osmolality from 300 to 280 mOsm kg^{-1} after 6 hours (Fig. 4.1a). Osmolality remained in this range, increasing to only 290 mOsm kg^{-1} at 168 hours. MWC stayed steady during the experiment (Fig. 4.1b). Optimal enzymatic activity was not significantly different between FW and IPW gills at 6 or 24 hours (Fig. 4.1c). There was however a 1.4-fold higher activity in IPW gills after 7 days (Fig. 4.1c)

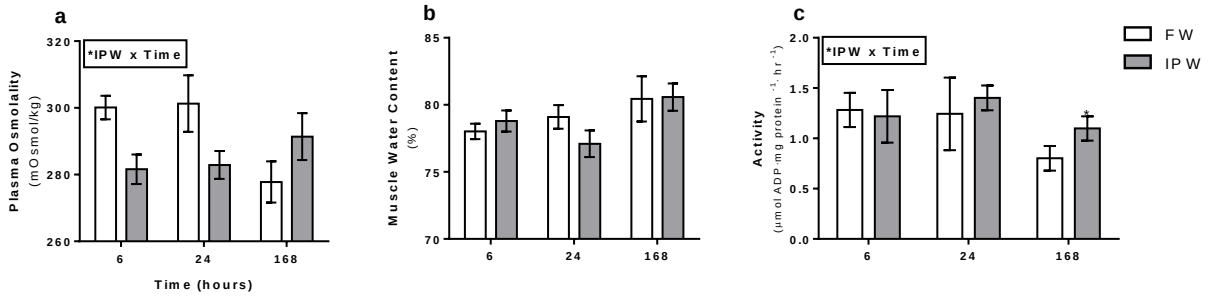

Figure 4.1 Plasma osmolality (a) muscle water content (b) and Nka enzyme activity (c) in rainbow trout following transfer to IPW. Fish were transferred from FW to IPW or FW to FW as a control and sampled at 6, 24 and 168 hours (n=10). Bars represent the mean values ± SEM. "IPW x Time" refers to statistical interaction between factors as indicated by *asterisks*: *$P <$ 0.05.

4.4.2 mRNA expression in FW and IPW

Transcript levels of genes expected to be critical to Na^+ uptake were analyzed in trout gills after 6, 24 and 168 hours (7days) after transfer to IPW (Fig. 4.2). Transfer to IPW induced a 1.7-fold increase in *nka-α1a* gill expression after 7 days (Fig. 4.2a). A similar effect was observed in *nka-α1b* expression, with an increase of 2 degrees of magnitude in 7 day IPW samples (Fig. 4.2b). *fxyd11* expression paralleled that of both *nka* isoforms with an increase 2.5-fold higher in IPW than FW at 7 days (Fig. 4.2c). Unlike the rest of the transcripts, *nhe3* exhibited a significant increase in expression after 24 hours (3-fold) in IPW which decreased after 24 hours and increased 2-fold in 7 day IPW gills (Fig. 4.2d). A drastic increase in *rhcg1* expression was observed in the 7 day IPW group with an 8x increase compared to FW (Fig. 4.2e). No effect of the IPW was exhibited by *hat* (Fig. 4.2f). And finally, no effect of IPW was exhibited in *asic4* after 6 or 24 hours, however, expression significantly increased at 7 days with IPW levels 2.7-fold greater than FW. c*ldn27a* expression exhibited an 2x increase in IPW gills after 7 days, while no effect of IPW was exhibited by *cldn28a, cldn28b or cldn30c* (Fig. 4.3).

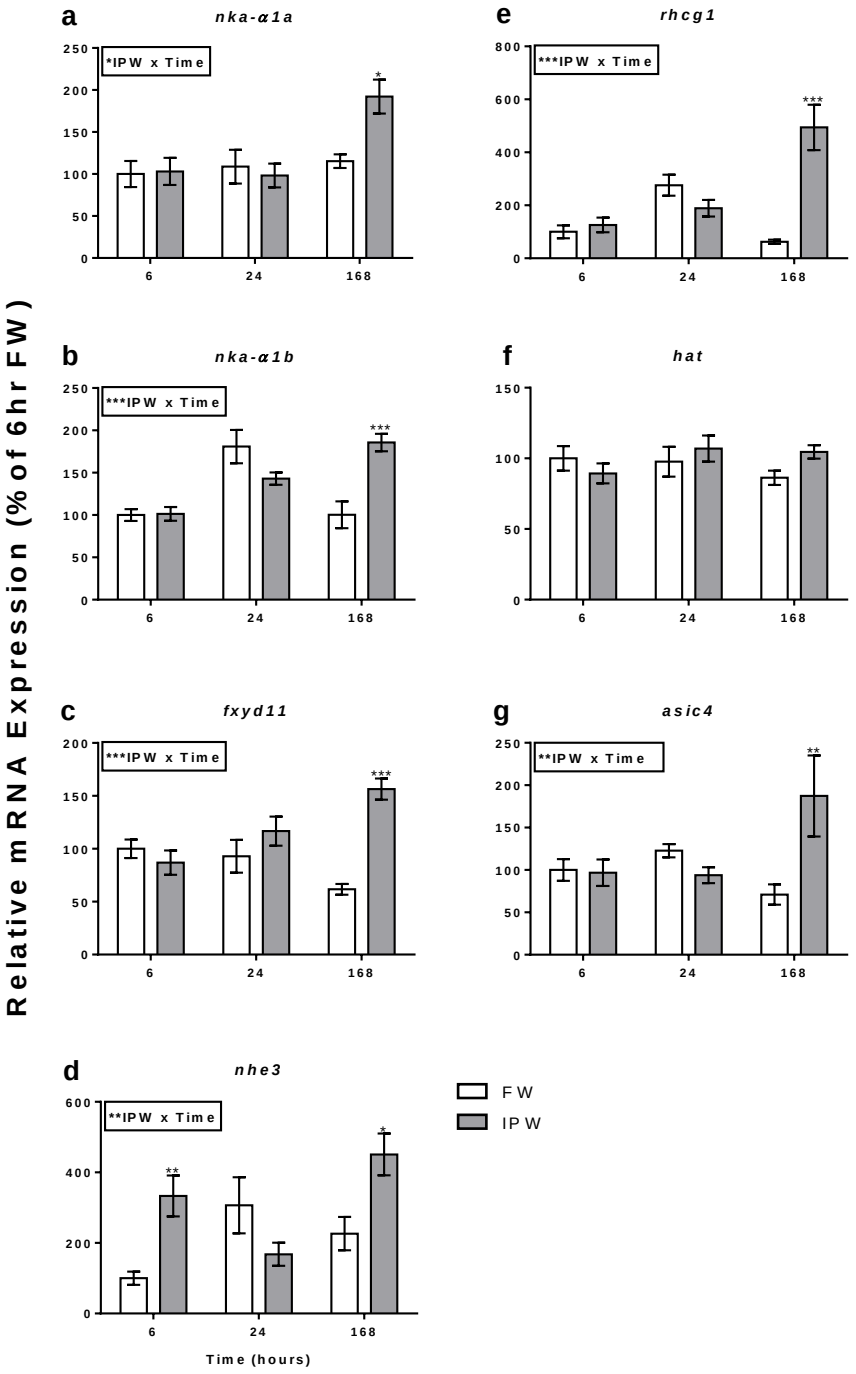

Figure 4.2 Effect of transfer from FW to IPW on gill transcript levels of *nka-α1a* (a), *nka-α1b* (b), *fxyd11* (c), *nhe3* (d), *rhcg1* (e), *hat* (f) and *asic4* (g). Fish were transferred from FW to IPW or FW to FW as a control and sampled at 6, 24 and 168 hours (n=10). Expression levels represent the mean value ± SEM relative to 100% of the 6 hour FW group. "IPW" and "IPW x Time" refers to overall effects and statistical interaction between factors as indicated by *asterisks*: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. When interaction between factors is significant, *asterisks* are placed above IPW group at specific time-point.

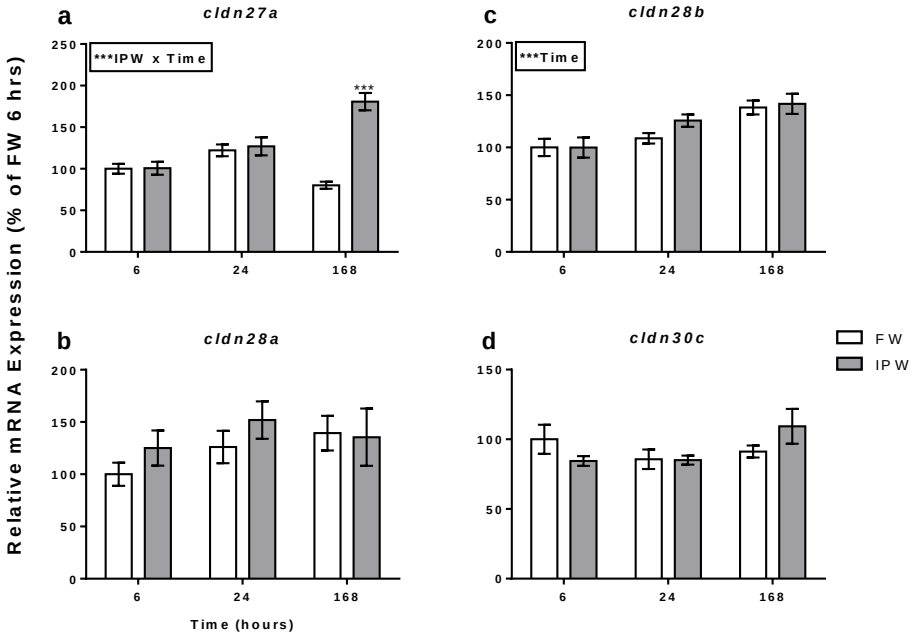

Figure 4.3 Effect of transfer from FW to IPW on gill transcript levels of *cldn27a* (a), *cldn28a* (b), *cldn28b* (c), *cld30c* (d). Fish were transferred from FW to IPW or FW to FW as a control and sampled at 6, 24 and 168 hours (n=10). Expression levels represent the mean value ± SEM relative to 100% of the 6 hour FW group. "Time" and "IPW x Time" refers to overall effects and statistical interaction between factors as indicated by *asterisks*: ****P* < 0.001. When interaction between factors is significant, *asterisks* are placed above IPW group at specific time-point.

4.4.2 Western blot analysis

Western blots of crude membrane fractions probed with Nka-α1a and Nka-α1b affinity-purified antibodies both identified one immunoreactive band with an apparent molecular weight around 100 kDa. This band was more intense in FW than SW samples for Nka-α1a (Fig. 4.4a) and more intense in SW than FW for Nka-α1b (Fig. 4.4b). For both antibodies, neutralization with 400-fold molar excess of the respective antigenic peptide blocked with immunoreactive band (Fig 4.4). Western blot analysis was used to observe the changes in protein expression of FW, IPW and SW gills after a 7 day acclimation. The SW transfer was performed in tandem to the IPW transfer with the sole purpose of obtaining 7 day samples for protein expression analyses. Nka-α1a protein levels were 4-fold higher in FW and SW while significantly greater abundances (6-fold) observed in IPW (Fig. 4.5a). Protein levels of Nka-α1b were significantly

higher in SW than FW, however, not significantly higher than IPW fish whose levels were comparable to FW fish (Fig. 4.5b). IPW fish had a tendency for higher expression of Fxyd11 than FW and SW fish (Fig. 4.5c).

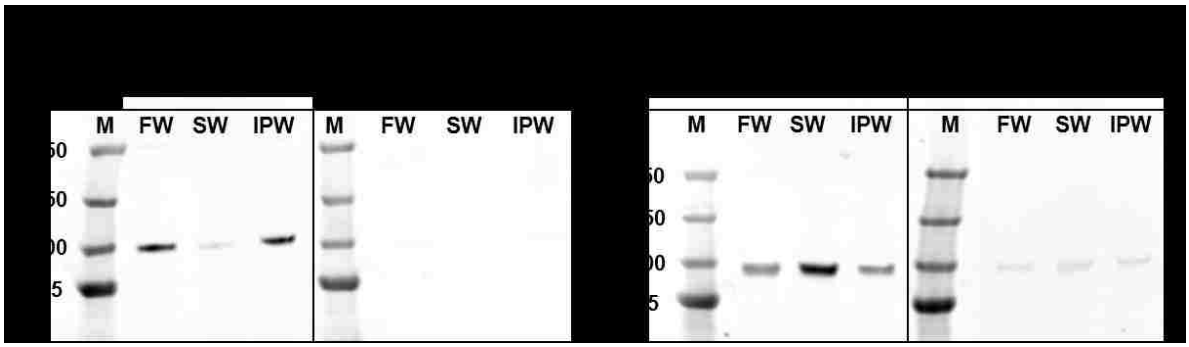

Figure 4.4 Western blots of four pooled membrane fractions of FW, SW and IPW rainbow trout gill samples probed with Nka-α1a and Nka-α1a with peptide (a) and Nka-α1b and Nka-α1b with peptide (b). Molecular weights in kDa are indicated on the left.

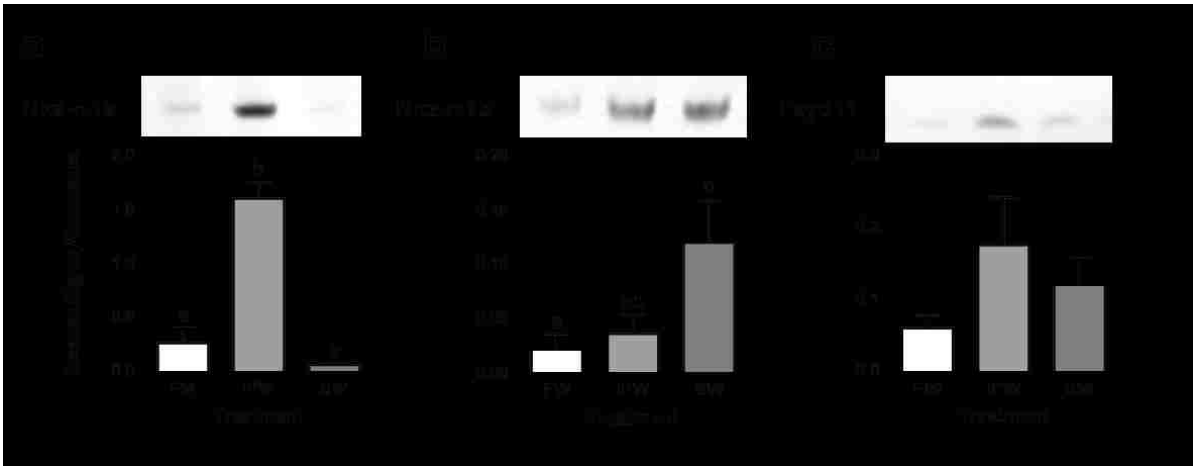

Figure 4.5 Western blot analysis on protein levels of Nka-α1a (a), Nka-α1b (b) and Fxyd11 (c) in gill of rainbow trout transferred from FW to IPW and SW. Signal abundance levels represent mean value ± SEM (N = 4). Significant difference between means is indicated by different letters above bars $P < 0.05$.

4.5 Discussion

4.5.1 Mechanisms of Na^+ uptake

For some time now the mechanisms involved in gill ionocyte Na^+ uptake in FW fishes have been widely debated (see reviews by Dymowska et al., 2012; Parks et al., 2008; Wright and Wood, 2009). The data in the current study supports the role of Nhe3 in Na^+ uptake in rainbow trout gill as our data shows an increase in *nhe3* expression upon exposure to IPW (Fig. 4.2d). Rainbow trout larvae exposed to soft water exhibited an increase in *nhe3* expression which also resulted in an increase in Na^+ uptake (Boyle et al., 2016). Similarly, our data supports this with *nhe3* expression increasing in juvenile trout exposed to ion poor water. Furthermore, using immunohistochemistry, Ivanis et al. (2008) showed localization of Nhe3 in branchial ionocytes of adult rainbow trout. A similar role for Nhe3 in Na^+ uptake in low [Na^+] environments has been observed in several teleost species including zebrafish (Craig, et al. 2007; Shih, et al. 2012; Yan, et al. 2007), medaka (Wu, et al. 2010), pupfish (Brix and Grosell 2012), tilapia (Inokuchi, et al. 2009) and Japanese eel (Seo, et al. 2013). Upon transfer to FW conditions, *nhe3* expression also increased in Atlantic stingray (Choe, et al. 2005) and killifish (Scott, et al. 2005). Localization of Nhe3 in ionocytes has been demonstrated in zebrafish (Esaki, et al. 2007; Yan et al. 2007), goldfish (Bradshaw, et al. 2012) and Atlantic stingray (Choe et al. 2005). Moreover, pharmacological inhibition of Nhe with EIPA (a selective Nhe inhibitor) significantly decreased Na^+ uptake in zebrafish (Esaki et al., 2007), goldfish (Preest et al., 2005) and stingray (Wood et al., 2002).However, because the function of Nhe relies solely on substrates (Na^+ and H^+), the ability of Nhe to function in dilute environments has been questioned (Avella and Bornancin, 1989; Parks et al., 2008). Therefore, it has been proposed by Wright and Wood (2009) that the function of Nhe be coupled to a proximal ammonia-conducting Rh protein, which excretes

ammonia working like a proton sink causing the external boundary layer of the cell to become alkalinized thus promoting Nhe mediated sodium uptake.

Several Rh protein isoforms (Rhag, Rhbg, Rhcg1 and Rhcg2) have been identified in the gills of rainbow trout (Nawata et al., 2007), puffer fish (Nakada et al., 2007b), killifish (Hung et al., 2007), medaka (Wu et al., 2010) and zebrafish (Nakada et al., 2007a). Rhcg1 was apically localized to rainbow trout gill ionocytes (Zimmer et al., 2017). Expression of *rhcg1* was higher in FW medaka gill ionocytes than SW (Liu et al., 2013) In the current study, expression of *rhcg1* increased in ion poor conditions (Fig. 4.2e). In zebrafish larvae, Rhcg1 was localized to apical membranes of H^+-ATPase rich cells (HR cells) and expression levels increased when exposed to dilute environments, suggesting that in addition to Na^+ uptake, HR cells are also involved in ammonia excretion (Nakada et al., 2007a). This was later supported by Shih et al. (2012) who reported increased expression of *rhcg1* in gill of adult zebrafish acclimated to low [Na^+]. Furthermore, Rhcg1 and Nhe3 co-localize in ionocytes of zebrafish (Nakada et al., 2007a), medaka (Wu et al., 2010) and rainbow trout (Zimmer et al., 2017). The separate knockdown of Rchg1 and Nhe3 in zebrafish larvae both led to a decrease in Na^+ uptake and NH_4^+ excretion (Shih et al., 2012), which together support the theory of a Rhcg1 and Nhe3 functional metabolon.

Several studies on Na^+ uptake in FW fishes have demonstrated the presence of a Nhe-Rh metabolon in the gills of various species including killifish (Hung et al., 2007), zebrafish (Nakada et al., 2007a), rainbow trout (Nawata et al., 2007) and medaka (Wu et al., 2010). While the proposed model of a Nhe-Rh metabolon alleviates the thermodynamic constraints associated with a low pH environment, it does not solve those imposed by a low Na^+ environment (Dymowska et al., 2012). It has therefore been proposed that this Nhe-Rh metabolon is not the sole mechanism for Na^+ uptake. Wright and Wood (2009) suggest these thermodynamic

constraints can be remedied with the coupling of Hat which can not only be loosely coupled to the Nhe-Rh metabolon, but also theoretically drive Na^+ uptake through its coupling with a Na^+ channel.

Hat was initially proposed as an alternative to Nhe, working to drive the uptake of Na^+ by a putative epithelial Na^+ channel (Avella and Bornancin, 1989). Hat was first apically localized to rainbow trout pavement cells (Sullivan et al., 1995) and was later located in ionocytes as well as pavement cells (Wilson et al., 2000). Several studies provide functional evidence supporting the role of Hat in Na^+ uptake. In rainbow trout gill, NEM-sensitive ATPase activity, that is presumably associated with Hat activity, decreased when transferred to higher salinities (Lin and Randall, 1991). An increase in *hat* gene expression has been observed repeatedly in the gill of rainbow trout exposed to high environmental ammonia (Nawata et al., 2007; Sinha et al., 2013; Tsui et al., 2009; Wood and Nawata, 2011; Zimmer et al., 2010). Additionally, morpholino knockdown of *hat* in HR cells of zebrafish led to a significant decrease in whole-body Na^+ (Horng et al., 2007). Strong inhibition of Hat has been shown to greatly decrease Na^+ uptake with the use of bafilomycin (a specific Hat inhibitor) in several species including tilapia and carp (Fenwick et al., 1999), zebrafish (Boisen et al., 2003; Esaki et al., 2007), goldfish (Preest et al., 2005) and rainbow trout (Bury and Wood, 1999; Goss et al., 2011; Reid et al., 2003). This suggests that Hat plays a critical role in Na^+ uptake in high environmental ammonia. It is therefore not surprising that our data shows no change of *hat* expression (Fig. 4.2f) as ammonia levels were consistently low throughout our experiment. We therefore propose that the Hat plays a generic role in Na^+ uptake and does not represent a bottleneck for the transport process.

Due to the lack of an epithelial Na^+ channel and any of the annotated fish genomes, researchers have gone in search for other potential Na^+ channels to complete the Hat-Na^+ channel

mechanism. Recently, six Asics have been described in zebrafish (Paukert et al., 2004). Of those six, one (Asic4.2) was localized in zebrafish gill and subsequently co-localized with Hat (Dymowska et al., 2015). Two Asics have been found in rainbow trout Asic1 and Asic4 were both found to be expressed in the gill of adult rainbow trout (Dymowska et al., 2014). To our knowledge, our data is the first to show the effect of external salinity on ASIC expression. In the current study, an increase in *asic4* expression was observed in rainbow trout gill exposed to IPW (Fig. 4.2g). Apical co-localization of Asic4 with NKA-rich ionocytes has also been observed in rainbow trout ionocytes and has lead researchers to believe that Asic4 is the putative Na^+ channel involved in Na^+ uptake (Dymowska et al., 2014). Pharmacological inhibition of Asic with DAPI (an Asic-selective inhibitor) resulted in a dose-dependent inhibition of Na^+ uptake in rainbow trout and zebrafish (Dymowska et al., 2015; Dymowska et al., 2014), thus further indicating the importance of Asic in Na^+ uptake.

In euryhaline teleosts, Nka appears as a primary driving force for ion absorption in FW and secretion in SW (Foskett and Scheffey, 1982). Nka is located in the basolateral membrane of ionocytes and exchanges three intracellular Na^+ for two extracellular K^+ ions, maintaining ion gradients favorable for vectorial transepithelial ion transport (Glynn, 1985). Several teleosts exhibit a change in Nka α-subunit expression in response to salinity change. The overall accepted model states that Nka-α1a is the FW "absorptive" isoform while Nka-α1b is the "secretive" isoform in SW gill. This has been observed in rainbow trout (Bystriansky et al., 2006; Richards et al., 2003), Atlantic salmon (Bystriansky et al., 2006; Madsen et al., 2009; McCormick et al., 2009), arctic char (Bystriansky et al., 2006), tilapia (Feng et al., 2002; Lee et al., 1998; Tipsmark et al., 2011), climbing perch (Ip et al., 2012) and inanga (Urbina et al., 2013). Additionally, Flores and Shrimpton (2012) showed an increase in *nka-α1a* expression in gill of rainbow trout

acclimated to IPW. Our mRNA results are consistent with these data (Fig. 4.2a) with a significant increase in *nka-α1a* in trout gills after 7 days in IPW. Furthermore, to our knowledge, this is the first study to show the significant increase in Nka-α1a at the protein level of gill exposed to IPW (Fig. 4.4a). While our mRNA data shows a significant increase in *nka-α1b* in IPW gill (Fig. 4.2b), Western blot data suggests that this is not associated with an elevated protein expression of this paralog (Fig. 4.4b). Taken together, it is likely that Nka-α1a is the Nka isoform responsible for driving ion absorption under ion poor conditions. Enzymatic activity of branchial Nka has been observed in several teleosts and has been shown to increase after acclimating to SW (Johnston and Saunders, 1981; Kelly and Woo, 1999; Madsen and Naamansen, 1989; McCormick et al., 1989). However, less is known about the enzymatic activity of the Nka in fish exposed to ion poor water. Flores and Shrimpton (2012) reported no difference in activity between trout after 7 days in FW or in IPW. In the present study there was a significant interaction for IPW and time ($P<0.05$) and the Nka activity increased in IPW at 168 hours (Fig. 4.1b). A possible explanation for these differences in results may be the ionic composition of the ion poor water used in each study. Our water analysis showed Na^+ (0.08 mM) and Ca^{2+} (0.18 mM) levels lower than that of Flores and Shrimpton with 0.14 mM Na^+ and 0.27 mM Ca^{2+} (2012). Our results suggest an increase in Nka activity is observed in trout exposed to ion poor water similar to that observed in trout exposed to SW (Madsen and Naamansen, 1989). FXYD proteins are single transmembrane proteins that interact with and modulate kinetic properties of the Nka (Garty and Karlish, 2006; Sweadner and Rael, 2000). Eight FXYD isoforms were identified in Atlantic salmon and of those, Fxyd11 was most highly expressed in the gill (Tipsmark, 2008). Most notably, elevated Nka activity is accompanied by elevated *fxyd11* expression in the SW gill of medaka (Bollinger et al., 2016; Yang et al., 2013) and

Atlantic salmon (Tipsmark et al., 2010). In the present study, Fxyd11 mRNA and protein expression increased after 7 days of IPW acclimation (Fig. 4.2c). These changes are similar to *fxyd11* expression levels observed in zebrafish exposed to IPW. The increase in Fxyd11 expression may contribute to the maintenance of Nka activity by association of the auxiliary subunit with Nka-α1a under the ion poor conditions.

4.5.2 Mechanisms for Na^+ retention

In FW, fish are challenged to maintain an internal plasma osmolality above that of their environment and therefore experience ion loss and osmotic water gain. The FW gill epithelium is described as "tight" with extensive tight junctions to prevent this loss of ions paracellularly. Claudins are transmembrane proteins involved in the formation of tight junctions and determine the permeability and ion selectivity of the tight junction (Furuse et al., 1998; Van Itallie and Anderson, 2006). Expression of these proteins has been shown in several teleost species to be tissue specific thus lending to osmoregulatory function of those organs (Bossus et al., 2015; Tipsmark et al., 2008b; Van Itallie and Anderson, 2006). The claudins examined in this study are based on isoforms found to be upregulated in the gill of Atlantic salmon transferred from SW to FW and therefore expected to be barrier-forming claudins (Tipsmark et al., 2008b).

We observed the expression of four claudin isoforms in the gill of rainbow trout exposed to ion poor water. Expression of *cldn28a, 28b* and *30c* did not exhibit a change in response to transfer to the dilute environment (Fig. 4.3b, c, d, respectively), while *cldn27a* expression increased after 7 days in IPW (Fig. 4.3a). Cldn27a expression levels increases in the gills of pufferfish acclimated to IPW (Duffy et al., 2011) and were decreased in the gill of Atlantic salmon in SW (Tipsmark et al., 2008b). Thus, the function for Cldn27a may be a barrier enforcing protein within the tight junction. However, no change in medaka gill *cldn27a* expression was observed

due to salinity in either long or short term acclimations to SW (Bossus et al., 2015). In Atlantic salmon, there seemed to be no effect of salinity on either Cldn28a or 28b (Tipsmark et al., 2008b). An increase in Cldn28b was exhibited in both medaka and tilapia transferred to FW (Bossus et al., 2015; Tipsmark et al., 2008a). Additionally, a decrease in *cldn30c* was observed in both tilapia and Atlantic salmon gill (Tipsmark et al., 2008a; Tipsmark et al., 2008b). With the exception of Cldn27a, our results are consistent with those from Bossus et al. (2015), who concluded that these claudins are cation barrier-forming isoforms in the gill of medaka. Cldn28a, 28b, 30c seem to be constitutively expressed in the FW gill and are proposed to maintain the integrity of the and tightness of the gill epithelium (Bossus et al., 2015). These claudin isoforms have been examined in several teleost species and it seems that the functions of these claudins may be species-specific and could possibly be attributed to differences in life history and ecology of the species (Chasiotis et al., 2012).

4.5.3 Conclusions

The aim of the present study was compose a more complete model for Na^+ uptake and retention in the rainbow trout FW ionocyte. Transfer of rainbow trout to IPW resulted in a slight decrease in plasma osmolality while MWC remained comparable to FW throughout the experiment. These data suggest that exposure to IPW was an osmoregulatory challenge the fish were able to overcome with time. Based on the current data, the ionocyte model for Na^+ uptake in trout gill exposed to IPW includes a functional Nhe-Rh metabolon loosely coupled to Hat, which also drives Asic. In this model Rhcg1 binds ammonium, removes a proton and transports ammonia across the membrane to the apical boundary layer (Fig. 4.6). This removed proton then drives the function of Nhe. Nhe then exchanges H^+ for Na^+ while Hat electrogenically drives Na^+ uptake via Asic4. Simultaneously both of these processes work to acidify the apical boundary

layer thus further driving Rhcg1 and the acid-trapping of ammonia. Finally, a basolateral Nka (α1a) pumps Na^+ out of the cell into the serosa creating a low intracellular Na^+ concentration favorable for driving Na^+ uptake from the environment. Cldn28a, 28b and 30c may work to maintain the integrity of the tight junctions gill epithelium while another putative cation barrier-forming claudin, Cldn27a, is upregulated to prevent loss of ions to the diluted environment.

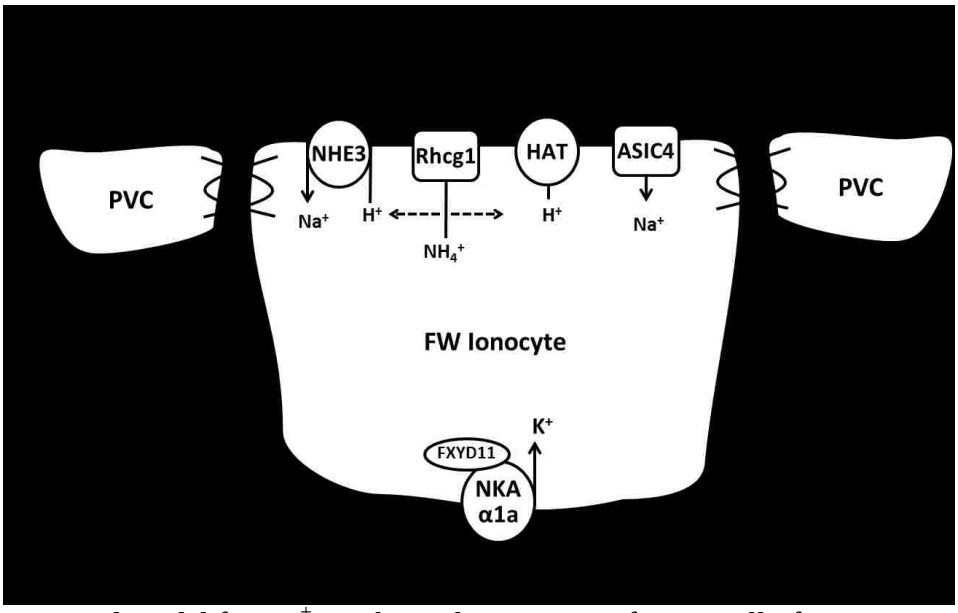

Figure 4.6 Proposed model for Na^+ uptake in the ionocyte of a FW gill of trout. NHE, Na^+/H^+ exchanger; Rhcg, Rhesus glycoprotein; HAT, H^+-ATPase; ASIC, acid-sensing ion channel; NKA, Na^+/K^+-ATPase; FXYD, NKA regulatory protein; PVC, pavement cell.

4.6 References

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Chapter 5 Conclusions

5.1 Teleost fishes as a model for human disease research

Biological model organisms have been employed in biomedical research for quite some time now and are used to provide insight into disease mechanisms, diagnostics and treatments. Researchers tend to employ models that most similarly resemble human physiology; therefore, it is understandable that mammals, rodents in particular, have been the model organism of choice. Despite this fact, fish have also been used for studying vertebrate gene function and lately are gaining traction as a model for studying human disease.

Fish diverged from humans over 400-million years ago, yet molecularly they are quite similar. In fact, 70% of human genes are found in the zebrafish genome (Howe et al., 2013). Moreover, one of the most frequently mutated genes in cancer, *HRAS*, exhibits 95% homology with the corresponding gene in medaka (Schartl, 2014). Interestingly, teleosts underwent a genome-wide duplication which means that fish in many cases have two copies of a gene while humans have only one (Amores et al., 1998; Braasch and Postkethwait, 2012; Meyer and Schartl, 1999; Postlethwait et al., 2000). This often results in modified gene expression patterns or protein function, such that paralogs could be expressed in different organs or exhibit different functions. Teleost fish models thus provide a unique advantage of studying genes related to human diseases.

Teleost fishes have been used as a model to study the function of prolactin and recently progress has been made in identifying the molecular mechanisms by which it regulates gene expression, cell proliferation and cell differentiation. Prolactin remains an area of interest in research as it has been shown to affect solute and water transport across intestinal, renal, mammary and amniotic epithelial membranes (Bole-Feysot et al., 1998; Freeman et al., 2000). Prolactin has been linked to breast cancer, diabetes, infertility, pregnancy-related hypertension

and atherosclerosis (Balbach et al., 2013; Bernichtein et al., 2010; Georgiopoulos et al., 2009; McHale et al., 2008; Neville et al., 2002; Rojas-Vega et al., 2015). Teleost fishes exhibit an endocrine system similar to that of mammals. In teleost fishes, prolactin acts on the gill, kidney, gut and urinary bladder to promote ion conservation and water secretion (Hirano, 1986). Therefore, teleost fishes, such as the Japanese medaka and the rainbow trout, may be a valuable alternative model organism to understand the involvement of prolactin in disorders that affect epithelial transport.

While the zebrafish is undoubtedly regarded as the most commonly used fish model for studying human disease, the medaka is quickly becoming a complementary alternative to the zebrafish. Medaka share many features with zebrafish including short generation time, ability to breed in large numbers in the laboratory and transparent eggs, making them ideal for studying embryonic development. Some of the many advantages of having medaka as a laboratory model include the ability to maintain large quantities easily, relatively cheap to acquire and maintain, and are generally easy to breed. The Japanese medaka has the added benefit of being euryhaline, which allows researchers to study proteins that zebrafish do not express. Compared to zebrafish, the medaka genome is considerably smaller which greatly reduces the palette of paralogues and isoforms. Furthermore, unlike mammal models, transgenic lines are quicker and more easily produced.

Rainbow trout, while less commonly used as a laboratory fish model, is one of the oldest models used for human cancer research (Schartl, 2014). Rainbow trout in particular have been utilized as a model organism for studying liver cancer as the histopathology is quite similar to humans (Jackson et al., 1968). Mutations in the *KRAS* oncogene are common in both human and trout liver cancer and both result in similar damages to the liver (Jackson et al., 1968). Rainbow

trout have since been employed as models for studying tumor-induction and tumor-inducing environmental chemicals.

The homology between the human and teleost genome would allow researchers to study diseases on a unique model organism. Several genes discussed in previous chapters are involved in human diseases. Alterations in the activity or expression of the Nka can affect Na^+ homeostasis and thus lead congestive heart failure, renal failure, cerebral stroke and myocardial infarction (McDonough et al., 1992). Gitleman's syndrome is characterized by a mutation in NCC of the distal convoluted tubule, which leads to loss of function (Valdez-Flores et al., 2016). Cystic fibrosis is caused by mutations in the CFTR gene that prevents successful directed trafficking into the apical membrane (Puchelle et al., 1992). Mutations in subunit B of the renal-specific H^+-ATPase leads to distal renal tubule acidosis (Karet et al., 1999). Gain-of-function mutations in the epithelial sodium channel causes hypertension, hypokalemia, low aldosterone levels and metabolic alkalosis (Bhalla and Hallows, 2008). Additionally, loss-of-function mutations lead to hypotension and hyperkalemia (Bhalla and Hallows, 2008). Several claudin genes have been identified in human diseases. Mutations in the claudin 1 gene have been identified in neonatal sclerosing cholangitis with ichthyosis (Hadj-Rabia et al., 2004). Claudin 2 is consistently upregulated in patients with inflammatory bowel disease (Weber et al., 2008; Zeissig et al., 2007). Claudin 12 has been linked to nonsyndromic deafness (Wilcox et al., 2001) and kidney stone disease (Thorleifsson et al., 2009). Epithelial cancers are associated with the upregulation of claudins 3 and 4 while claudins 1 and 7 can be either up- or downregulated (Turksen and Troy, 2011; Valle and Morin, 2010).

Teleost fishes offer an alternative to the more commonly used mammalian research model organism and could be a great benefit to biomedical research. The teleost genome

duplication offers the unique advantage of allowing researchers to study altered gene expression or protein function in fish. Euryhaline species, such as the Japanese medaka and rainbow trout, would be an alternative to the more popular zebrafish and allow an expanded view of the effect of salinity on protein function. Zebrafish and Japanese medaka can be an advantageous alternative to mammals, as they are easily maintained in large quantities, cheaper to acquire and maintain, easy to breed and allow quicker production of transgenic lines.

5.2 Summary of Results

The experiments discussed here were performed using an integrative approach examining the different aspects of fish osmoregulation from gene expression to protein expression and localization, and enzymatic assay. The following is a summary of the major findings of the experimental work presented in this dissertation.

5.2.1 Japanese medaka do not exhibit a salinity-dependent Na^+/K^+-ATPase isoform switch

Many salmonid and tilapia species have exhibited salinity-specific Nka α-subunit isoform expression. In these species, Nka-α1a is the more prominently expressed isoform in FW while Nka-α1b is more highly expressed in SW (Dalziel et al., 2014). In our studies (Chapter 2), we aimed to characterize the expression of Nka α-subunits in the Japanese medaka.

Japanese medaka do not follow the salinity specific isoform expression patterns observed in salmonids and tilapia. The salinity-specific isoform shift is proposed with be associated to three specific differences in the protein sequence of Nka-α1a and Nka-α1b. The first observed difference is in the fifth transmembrane domain of Nka-α1a, where a lysine-asparagine substitution at residue 783 is proposed to decrease the binding affinity for Na^+ and K^+ (Jorgensen, 2008). The second is an aspartate-valine substitution at site 933 in the eighth transmembrane domain of Nka-α1a, which decrease K^+ binding affinity (Jorgensen, 2008).

Together, these two substitutions are suggested to decrease the affinity for K^+ more than Na^+, thus Nka preferentially pumps Na^+. This also results in the insertion of an ε-amino group from lysine substitution into a cation binding site that could be reducing the Na^+/ATP ration from $3Na^+$/ATP to $2Na^+$/ATP, rendering sodium uptake more feasible for Nka-α1a. The third is a glutamate-serine substitution at residue 961 in the ninth transmembrane domain (Jorgensen, 2008) which imposes a side chain, pointing towards the regulatory Fxyd subunit, possibly interfering with or changing interactions with the regulatory subunit. The Japanese medaka Nka-α1a does not contain any of these amino acid substitutions described, instead it resembles that of Nka-α1b. Furthermore, there was no observed effect of salinity on Nka-α1a mRNA expression. Together these data suggest that medaka Nka-α1a may not be the favorable isoform for driving Na^+ uptake in FW and therefore may be the reason the Japanese medaka does not follow the pattern of expression for Nka-α1a observed in salmonids and tilapia. It is possible that medaka do not rely on a Nka α1 isoform shift as a part of its osmoregulatory strategy and may instead rely more on Nhe3 and Ncc for Na^+ uptake in FW.

5.2.2 Prolactin signals Na^+, Cl^- cotransporter expression via the Jak-Stat pathway

Prolactin is an essential regulator of salt and water transport in vertebrate osmoregulatory tissues. In mammals, prolactin acts on renal, intestinal, mammary and amniotic epithelial membranes. Similarly, in teleosts, prolactin influences osmoregulatory tissues by reducing ion and water permeability (Hirano, 1986). Prolactin stimulates expression of Ncc, co-transporter involved in ions absorption in FW, in the gills of tilapia (Breves et al., 2010), medaka (Bossus et al., 2017) and zebrafish (Breves et al., 2013). The present study (Chapter 3) utilized three kinase inhibitors to investigate the signaling pathway prolactin uses to stimulate expression of Ncc in the gill of medaka.

Co-incubation of gills with prolactin and kinase inhibitors of the Jak-Stat, Akt and MAPK pathways showed that prolactin stimulation of Ncc expression is dependent on the Jak-Stat pathway but not Akt or MAPK pathways. Gill explants incubated with prolactin led to a dose-dependent phosphorylation of Stat5 while no effect was observed in Akt or MAPK. Furthermore, gills incubated with prolactin exhibited an increase in nuclear localization of phosphorylated Stat5. Together, these data suggest that prolactin-stimulated expression of Ncc is regulated via the Jak-Stat pathway.

5.2.3 Mechanism of Na^+ uptake and retention in FW rainbow trout gill ionocytes

The mechanism for Na^+ uptake in the ionocytes of the freshwater (FW) gill has been a topic of debate for quite some time now. Our studies (Chapter 4) aimed to clarify these mechanisms by transferring fish from FW to an ion poor environment.

Several studies propose the use of Nhe3 (Na^+/H^+ exchanger) for Na^+ uptake in FW branchial ionocytes (Boyle et al., 2016; Bradshaw et al., 2012; Brix and Grosell, 2012; Choe et al., 2005; Craig et al., 2007; Esaki et al., 2007; Inokuchi et al., 2009; Ivanis et al., 2008; Preest et al., 2005; Scott et al., 2005; Seo et al., 2013; Shih et al., 2012; Wood et al., 2002; Wu et al., 2010; Yan et al., 2007). In the present study, rainbow trout exposed to ion poor water exhibited an increase in Nhe3 mRNA expression, an ion transporter known to exhibit apical localization in FW ionocytes of zebrafish (Esaki et al., 2007; Yan et al., 2007), goldfish (Bradshaw et al., 2012) and Atlantic stingray (Choe et al., 2005). However, the function of the Nhe3 relies on the availability of its substrates and based on thermodynamic considerations it is not likely to be functional in dilute environments.

It was therefore proposed that Nhe3 works in a metabolon with another transporter, Rhcg1 (rhesus glycoprotein), which has been apically localized in FW ionocytes of rainbow trout

gill (Zimmer et al., 2017). Rhcg1 functions to excrete ammonia and causes an alkalization of the external boundary layer and promotes Na^+ uptake by Nhe3 (Wright and Wood, 2009). Our data shows that Rhcg1 mRNA expression increases in rainbow trout exposed to ion poor water which parallels the observed increase in Nhe3. These results is similar to that observed by Shih et al. (2012) in zebrafish exposed to a low Na^+ environment and is strongly in favor of our hypothesis. The Nhe-Rhcg metabolon has already been supported before in rainbow trout that exhibited an increase in Nhe and Rhcg expression in response to high environmental ammonia (Nawata et al., 2007).

Theoretically, the coupling of Nhe and Rhcg to form a functional metabolon allows for Na^+ uptake in a low pH environment. However, this model is not thermodynamically ideal in a low Na^+ environment. It has therefore been proposed that the Nhe-Rhcg metabolon is loosely coupled to an H^+-ATPase (HAT), which also drives Na^+ uptake via an epithelial Na^+ channel, ASIC (acid-sensing ion channel; Avella and Bornancin, 1989; Dymowska et al., 2014). Apical localization has been observed in rainbow trout gill ionocytes (Wilson et al., 2000) and was shown to co-localize with ASIC4 (Dymowska et al., 2014). Our data show an increase in ASIC4 mRNA expression in the gill of rainbow trout exposed to ion poor water and to our knowledge is the first study to show the effect of salinity on ASIC expression in a teleost gill. While our data did not show a change in expression of HAT, several studies have shown an increase in HAT activity of rainbow trout exposed to high environmental ammonia (Nawata et al., 2007; Sinha et al., 2013; Tsui et al., 2009; Wood and Nawata, 2011; Zimmer et al., 2010), thus, suggesting a generic role in Na^+ uptake.

Teleost models propose the Nka as the primary driving force for Na^+ uptake as it maintains ion gradients favorable for vectorial transepithelial ion transport (Glynn, 1985).

Specifically, the Nka-α1a is known as the absorptive FW isoform in teleosts. The present study showed a significant increase in Nka-α1a mRNA and protein expression in ion poor water. The regulatory Fxyd11 protein has been proposed to modulate the kinetic properties of Nka (Garty and Karlish, 2006; Sweadner and Rael, 2000). Expression of Fxyd11 has been predominantly examined in response to SW with evidence of increased expression in the gill of medaka (Bollinger et al., 2016; Yang et al., 2013) and Atlantic salmon (Tipsmark et al., 2010) exposed to SW. Our data show an increase of Fxyd11 mRNA expression in rainbow trout exposed to ion poor water, which may contribute to the subsequently observed increase in Nka activity.

A small number of the claudin tight junction proteins exhibit salinity-dependent expression. Four putative barrier-forming claudins (Cldn27a, Cldn28a, Cldn28b and Cldn30c) exhibit high gill expression and species-specific expression in response to salinity. We hypothesize that these claudin paralog are involved in salt retention and blocking osmotic water gain across the gill when fish are in a dilute environment. In the present study, transfer to ion poor water did not affect mRNA expression of Cldn28a, Cldn28b or Cldn30c. Suggesting these claudin isoforms play a role in maintaining the general tightness of the FW gill epithelium. Cldn27a expression increased in the gill of pufferfish (Duffy et al., 2011) acclimated to ion poor water while exposure of Atlantic salmon to SW caused a decrease in Cldn27a (Tipsmark et al., 2008). Our data shows an increase in Cldn27a mRNA expression in rainbow trout gill exposed to ion poor water. Together, this data supports the putative cation barrier-forming role of Cldn27a in the FW gill epithelium of rainbow trout.

We propose the following model for Na^+ uptake and retention in the FW ionocyte of rainbow trout. An apical Nhe3-Rhcg1 metabolon loosely coupled to HAT, which also drives Na^+ uptake via the voltage-insensitive Na^+ channel, ASIC4, which is gated by extracellular H^+.

Rhcg1 binds ammonium, removes a proton and transports ammonia across the membrane to the apical boundary layer. The removed proton then drives the function of Nhe3. Nhe3 exchanges H^+ for Na^+ while HAT electrogenically drives Na^+ uptake via ASIC4. Simultaneously both of these processes work to acidify the apical boundary layer thus further driving NH_3 extrusion vie Rhcg1 and the acid-trapping of ammonia. A basolateral Nka-α1a pumps Na^+ into the serosa to create a low intracellular Na^+ concentration favorable for driving Na^+ uptake from the environment. Furthermore, the integrity of the tight junction is maintained in part by Cldn28a, 28b and 30c while Cldn27a is upregulated to prevent loss of cations to the ion poor environment.

5.3 Perspectives

The experiments discussed here demonstrate the benefits of using euryhaline teleosts as a model for understanding osmoregulation and the hormonal control of these functions. The Japanese medaka does not follow the salinity-dependent model for NKA α-subunit isoform expression observed in most euryhaline teleost species. This study provides insight to acclimation responses of a lesser studies euryhaline teleost and reminds researchers that euryhaline adaptation is a reoccurring phenomenon in teleost evolution. The Japanese medaka exhibits prolactin-induced expression of the Na^+, Cl^- cotransporter and appears to be mediated through the Jak-Stat pathway. This study provides insight to understanding the underlying mechanisms of hormones on cell function and gene expression. Future studies should aim to show both apical localization of Na^+, Cl^- cotransporter and nuclear localization of phosphorylated Stat5 in the ionocyte. Through the exposure of rainbow trout to ion poor water, expression of freshwater ionocyte transporters was observed in an extreme environment. This study allowed us to assemble a more complete model for Na^+ uptake and retention in the

freshwater ionocyte of rainbow trout gill. Future studies should work to validate this model through the expression and localization of these proteins.

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Chapter 6 Appendix

UNIVERSITY of ARKANSAS

Research Support and Sponsored Programs
Office of the Director
120 Ozark Hall
1 University of Arkansas
Fayetteville, Arkansas 72701-1201

(479) 575-3845
(479) 575-3846 (FAX)
E-mail: rsspinfo@uark.edu
http://www.uark.edu/admin/rsspinfo/

MEMORANDUM

TO: Christian K. Tipsmark

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: September 15, 2010

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date : September 30, 2013

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#11005- " THE PHYSIOLOGY AND ENDOCRINE CONTROL OF OSMOREGULATION AND GROWTH IN TELEOST FISH "**. You may begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the changes. If the study period is expected to extend beyond **09-30-2013,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

UNIVERSITY OF ARKANSAS

MEMORANDUM

TO: Dr. Christian Tipsmark

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee

DATE: June 26, 2014

SUBJECT: IACUC APPROVAL
Expiration date: June 25, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 14042: "Regulation of Epithelial Transport in Japanese Medaka".

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing(via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond June 25, 2017 you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Christian Tipsmark

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: May 9, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **May 8, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13052 - "Functional Regulation of Claudins in the Fish Gill Model"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **05-08-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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