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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CT</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate water</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>EDN</td>
<td>endothelin</td>
</tr>
<tr>
<td>EDNR</td>
<td>endothelin receptor</td>
</tr>
<tr>
<td>FW</td>
<td>fresh water</td>
</tr>
<tr>
<td>h</td>
<td>hour/hours</td>
</tr>
<tr>
<td>indel</td>
<td>insertion or deletion</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RPA16</td>
<td>RNA polymerase I 16kDa polypeptide</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>SPRY3</td>
<td>sprouty-3</td>
</tr>
<tr>
<td>SRX6c</td>
<td>sarafotoxin 6c</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride, sodium citrate solution</td>
</tr>
<tr>
<td>SW</td>
<td>seawater</td>
</tr>
<tr>
<td>SYBL1</td>
<td>synaptobrevin-like 1</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TMLHE</td>
<td>trimethyllysine hydroxylase, epsilon</td>
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<td>units</td>
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Researchers have been studying osmoregulation in fishes over the past 150 years. The one area that had received little attention is the role of paracrines/autocrines in the local regulation of ion transport, specifically in the teleost gill. The focus of my dissertation work was on the peptide, endothelin-1, and the components necessary for EDN1 signaling, including the enzyme that makes active EDN1, ECE1, and the sites of action, the EDN receptors (EDNRS). When I started this work, there were about a dozen papers on the effects of endogenous mammalian EDN1 on fish blood vessel tone or the effects of bolus injections of mammalian EDN1 on the cardiovascular system of fishes. The purposes of my dissertation work were to 1) localize the aforementioned components of EDN1 in the teleost gill; 2) determine if these components are regulated by environmental salinity, thus giving us some insight into whether or not EDN1 is involved in regulation of ion balance in fishes; 3) determine the evolutionary relationship among the protein sequences for the EDNs, ECEs, and EDNRs.

Through the use of molecular biology and immunohistochemistry, I determined that EDN1, ECE1 and the EDNRs are expressed in the euryhaline killifish (*Fundulus heteroclitus*) and longhorn sculpin (*Myoxocephalus octodecemspinosus*) gills. From my localization studies, I modeled the putative functions of EDN1, and proposed it acts as a paracrine and autocrine in the
fish gill. The localization of the EDNRs in the gill suggests that EDN1 signaling is involved in regulation of mitochondrion-rich cell functions, regulation of lamellar pillar cell tone (and ultimately perfusion of lamellae), and clearance of excess EDN1.

With the sequences obtained from the first part of my dissertation, I was able to determine the effects of environmental salinity on mRNA expression of each of these EDN1 signaling components using real-time quantitative PCR in both of these fishes. I was also able to measure protein level differences in these experiments. These mRNAs/proteins are regulated by hyperosmotic and hypo-osmotic stress, further suggesting that they are involved in not only ion balance, but also volume regulation. In addition, EDN1 signaling is postulated to be involved in cell survival during osmotic stress.

Finally, through the use of phylogenetics and bioinformatics, I determined that EDN1 and the EDNRs are vertebrate specific proteins, supporting the working hypothesis that EDN1 signaling was an important innovation leading to the development of the jaws and radiation of the vertebrates. Although ECE is found in Archaea, Bacteria and Eukarya, it is hypothesized to have shifted from being a monomer and general protease in all non-vertebrate organisms, to a dimer and a specific EDN1 protease in the vertebrates. Finally, the EDNRB2 was originally classified as an avian-specific EDNR; however, it is found in all non-therian gnathostomes, and I believe deleted from the therian genome 150 mya. From my Rate Shift Analysis of the EDNRA and EDNRB1, I hypothesize that therian EDNRA have different functions than the EDNRA in non-therian gnathostomes, but that the EDNRB1 is well conserved over gnathostome evolution.
CHAPTER 1
INTRODUCTION

Teleost fishes are the most specious group of vertebrates, and they have a diverse range of habitat use. Some are stenohaline freshwater or stenohaline marine, and these fishes can only maintain ion balance over a small range of environmental salinities. A third broad category is the euryhaline fishes, which can tolerate changes in environmental salinity. The osmoregulation of teleost fishes has been studied for over the past 150 years, and due to space limitations, I will briefly review teleost osmoregulation here (see these reviews Karnaky, 1998; Marshall and Farrell, 2006).

Depending on the salinity of their environment, fishes must overcome physiological challenges. For example, freshwater fishes are hyperosmotic to their environment, and consequently they tend to gain water and lose ions. To compensate for this water load, they tend to produce a dilute urine and uptake ions at the gill. In contrast, seawater fishes are hypo-osmotic to their environment, and they tend to lose water and gain excess salts. To combat this problem, marine fishes drink the seawater and transport salts across the gut epithelium, creating an osmotic gradient favorable for water to follow. These excess salts are transported in the blood to the gills where they are actively excreted. Euryhaline fishes like the killifish (*Fundulus heteroclitus*), experience large changes in environmental salinity over the course of a day (Marshall, 2003), and they must regulate ion uptake or excretion to maintain proper ion balance. The killifish does this by rapidly up- or down-regulating gill ion transporter density (Choe et al., 2006; Marshall et al., 1999; Scott et al., 2004; Scott and Schulte, 2005).

The fish gill is a multifunctional organ that is the main site of ion and acid/base regulation, gas exchange, and nitrogenous waste excretion. The gill epithelium is in direct contact with the environment, and it receives 100% of the cardiac output (Olson, 2002). In the gill, there are
specialized cells that are involved in ion transport termed the mitochondrion-rich cell (MRC, also called the chloride cell). These cells are characterized by many mitochondria and ion transporting proteins, and an extensive basolateral membrane. The MRCs have distinct freshwater- and seawater-type morphologies (Katoh and Kaneko, 2003; Perry, 1997). Freshwater MRCs have a flattened apical membrane with many microvilli. In contrast, the seawater MRC has an apical crypt, with no microvilli, and they are generally larger in size (Katoh and Kaneko, 2003; Perry, 1997). These cells also have different distributions of ion transporters. Freshwater MRCs function in ion uptake and this involves ion transporters such as, the apically expressed V-ATPase, HCO\(_3^-\)/Cl\(^-\), Na\(^+\)/H\(^+\) exchangers, and a hypothesized Na\(^+\) channel, and the basolaterally expressed Na\(^+\), K\(^+\)-ATPase (NKA) (Evans et al., 2005). The seawater MRC functions in ion excretion and apically expresses the cystic fibrosis transmembrane conductance regulator (a chloride channel), and basolaterally expresses the Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter, inward rectifier K\(^+\) channel (eKir), and NKA (Evans et al., 2005).

The hormonal regulation of osmoregulation has been extensively studied (see these reviews McCormick, 1995; McCormick and Bradshaw, 2006). Generally, prolactin is the hormone necessary for freshwater acclimation, and cortisol is the hormone necessary for seawater acclimation (McCormick and Bradshaw, 2006). In fishes acclimating to fresh water, prolactin stimulates an increase in the freshwater-type MRC morphology, and stimulates an increase in Na\(^+\), and Cl\(^-\) uptake (Evans, 2002; Lee et al., 2006; McCormick and Bradshaw, 2006). Although cortisol is classified as the “seawater-adapting hormone” (McCormick and Bradshaw, 2006), it is also is important in freshwater acclimation. Cortisol injections in freshwater-acclimated fishes leads to NA\(^+\) and Cl\(^-\) uptake (Perry, 1997), freshwater-type MRC morphology and an increase in Na\(^+\), K\(^+\)-ATPase activity (Dang et al., 2000). During seawater
acclimation, the cortisol and growth hormone/insulin-like growth factor axis is stimulated. In the killifish, one hour post a fresh water to seawater transfer, there is a sevenfold increase in plasma cortisol concentration (Marshall et al., 1999), and in the tilapia (*Oreochromis mossambicus*) it is released within minutes of a salinity transfer (Hegab and Hanke, 1984) (it maybe released within minutes in the killifish but this has not been tested). In addition, growth hormone is released and works synergistically with cortisol to increase gill Na\(^+\), K\(^+\), 2Cl\(^-\) density and increase gill Na\(^+\), K\(^+\)-ATPase activity (Pelis and McCormick, 2001). Also, these hormones stimulate development of the seawater-type MRC (Sakamoto and McCormick, 2006). Recently, an new “osmosensing” transcription factor, OSTF1, was cloned and characterized from the tilapia (Fiol and Kultz, 2005). Although there is little evidence that the OSTF1 actually senses changes in osmolality, it is rapidly upregulated within minutes of transferring the tilapia to seawater. Fiol and Kultz (2005) predict that the effects of cortisol are mediated via the OSTF1 and they are currently testing this hypothesis.

In addition to the few hormones that were described above, there are many other hormones that are believed to be involved in the osmoregulation of fishes. These include natriuretic peptides, urotensin II, thyroid hormones, arginine vasotocin, angiotensin II, insulin, vasoactive intestinal peptide (and likely others that are yet to be tested) (Evans, 2002; McCormick and Bradshaw, 2006).

The one area of teleost osmoregulation that has not been extensively studied is the role of locally produced signaling molecules in the gill. In addition to the aforementioned endocrine factors, there may also be paracrines and/or autocrines that are involved in the regulation of ion balance. A study from our lab suggests that the peptide endothelin-1 (EDN1), the gas nitric oxide (NO), and the arachidonic acid-derived prostaglandins (likely PGE\(_2\)) can regulate gill ion
transport (Evans et al., 2004). Evans et al. (2004) determined that EDN1 inhibits net chloride transport (as measured by the inhibition of short circuit current) by the killifish opercular epithelium. This single layer sheet of epithelium is a commonly used model tissue for the seawater teleost gill, because it contains many MRCs like the gill (Karnaky and Kinter, 1977; Karnaky et al., 1977). The inhibition of chloride transport was mediated through the EDN-1 stimulation of NO and PGE₂ (Evans et al., 2004). The mechanism of how the EDN1-NO-PGE₂ axis inhibits ion transport is unclear. For my dissertation, I focused on EDN1 in the fish gill. For more information on prostaglandins and NO in the killifish, see Choe et al. (2006) and Hyndman et al. (2006).

**Endothelins**

Endothelins (EDNs) are a family cardiovascular peptides with three isoforms, EDN1, EDN2 and EDN3 (Inoue et al., 1989; Yanagisawa et al., 1988c) and the genes encoding these proteins are located on different chromosomes (Masaki, 1993). They are regarded as the most potent vasoconstrictors yet identified. Endothelins are translated as ~200 amino acid (aa) preproendothelins (preproEDN) that are initially cleaved by a furin-like enzyme (Yanagisawa et al., 1988c) to form the relatively inactive 38 aa proendothelin (proEDN, also known as Big-EDN) (Kimura et al., 1989). Proendothelin is further cleaved to form the active 21 aa EDN by the endothelin converting enzyme (ECE-1 and/or ECE-2) (Shimada et al., 1994; Xu et al., 1994). Endothelin-1 release is stimulated by various factors including hypoxia, cortisol, growth factors, sheer stress, calcium ionophores, cytokines and endotoxins (Levin, 1995).

When I began this work, only a few endothelin-like peptide sequences were available from non-mammalian vertebrates. These included the rainbow trout (Wang et al., 1999), frog (*Rana ridibula*) (Wang et al., 2000) and the American alligator (*Alligator mississippiensis*) (Platzack et al., 2002). These were peptide sequences, as the coding sequences for these EDNs
had not been determined. In addition, there is a family of cardiotoxic peptides, the sarafotoxins, that shares 70% primary sequence identity and structural identity with mammalian EDN1 (Lee and Chiappinelli, 1989). The sarafotoxins are found in the venom of the Israeli burrowing asp (*Actractapis engaddensis*), and there is little evidence to suggest they are homologous to the EDNs. The most parsimonious hypothesis is that they occurred through convergent evolution (Froy and Gurevitz, 1998).

**Endothelin Receptors**

Endothelins bind to a specific group of seven transmembrane domain, G-protein-coupled receptors (GPRCs) that are part of the rhodopsin/β-adrenergic GPRC family. The classification of the EDN GPCRs has been quite confusing. For example, most researchers acknowledge that there are two EDN-specific receptors (EDNRs) termed, EDNRA (Arai et al., 1990), EDNRB (I will refer to this receptor as its official name, EDNRB1) (Sakurai et al., 1990); however, a third EDNR, an amphibian specific EDNRC, was cloned and characterized from melanophores (Karne et al., 1993). In 1998, a fourth GPCR was cloned and characterized from the chicken (*Gallus gallus*) and quail (*Coturnix japonica*), and termed the avian-specific EDNRB2. Traditionally, the EDNRs were classified based upon their pharmacological profiles (see Table 3-1). For example, mammalian EDNRA preferentially binds EDN1 and EDN2 over EDN3 (Arai et al., 1990). In contrast, mammalian EDNRB1 binds all three EDNs with equal affinity and binds the sarafotoxins, which are EDNRB1 specific agonists (Lecoin et al., 1998; Sakurai et al., 1990). In addition to these EDNRs, pharmacological and physiological studies in mammals suggest that there are multiple EDNRB-type receptors. For example, EDN1 binding to vascular smooth muscle EDNRB1 results in muscle contractions (Yanagisawa et al., 1988a; Yanagisawa et al., 1988b). On the contrary, EDN1 binding of endothelial EDNRB1 stimulates NO and prostacyclin...
production (De Nucci et al., 1988a; De Nucci et al., 1988b), and subsequent vasodilation of smooth muscle cells. Yet, there is no molecular evidence for two EDNRB-type genes in the mammals (Pollock and Highsmith, 1998)(Chapter 3). Most likely these differing responses are due to splice variants of the mammalian EDNRB1 (Elshourbagy et al., 1996). Unlike the mammals, the birds have a third receptor, the EDNRB2. This receptor binds all three EDNs with equal affinity (like the EDNRB1), but it has a very low affinity for the sarafotoxins (like the EDNRA) (Lecoin et al., 1998). In addition, the primary sequence of this receptor is more similar to mammalian EDNRB1 than EDNRA thus, Lecoin et al. (1998) termed it the EDNRB2. Finally, the amphibian-specific EDNRC binds preferentially binds EDN3 over EDN1 (Karne et al., 1993).

As with the EDNs, when I started my dissertation there was only pharmacological and physiological evidence for the EDNRs in the fishes. For example, physiological studies have suggested that the aortic vascular smooth muscle of the dogfish shark (Squalus acanthias) has EDNRB-like receptors (Evans et al., 1996), but that hagfish (Myxine glutinosa), sea lamprey (Petromyzon marinus), and eel (Anguilla rostrata) aortic vascular smooth muscles contain EDNRA-like receptors (Evans and Harrie, 2001). In addition, pharmacological studies using receptor binding assays demonstrated EDNRB-like receptors in the dogfish gill (Evans and Gunderson, 1999), but autoradiographic studies showed EDNRA-like receptors in the trout (Oncorhynchus mykiss) gill (Lodhi et al., 1995). The trout EDNRA-like receptors were specifically localized in the gill lamellae to a region termed the lamellar sinusoid by the authors (Lodhi et al., 1995). The characterization of the EDNR subtypes may be species and protocol specific. During my dissertation work, the EDNRB and EDNRA were immunolocalized in the gills of the cod (Gadus morhua) (using heterologous antibodies) (Stenslokkken et al., 2006), and
EDNRA in the gill of the tiger pufferfish (*Takifugu rubripes*) (using a homologous antibody) (Sultana et al., 2007). With the advancement of high throughput sequencing and genome sequencing, EDNR sequences can be found in all vertebrates and in Chapter 3 I explore the phylogenetic and evolutionary history of the EDNRs.

**Medical Importance of Endothelin-1**

A large portion of EDN research has gone into understanding the pathologies associated with errors in EDN signaling. These conditions include hypertension, atherosclerosis (Shreenivas and Oparil, 2007), congestive heart failure (Angerio, 2005), and glomerulonephritis (Richter, 2006). About 73 million Americans currently suffer from hypertension, and about 60% of them are under treatment for this disease (American Heart Association high blood pressure statistics), including EDNR antagonists (e.g. bosetan and ambrisentan). There are also developmental and genetic diseases associated with components of the EDN signaling cascades. This includes Hirschsprung disease, a developmental disorder that causes improper innervation of the gut resulting in a condition called anganglionic megacolon (Baynash et al., 1994; Puffenberger et al., 1994).

Initially, the EDN system was described as a regulator of vascular tone; however, it is quite evident that this system has many functions. A recent boom of research has been in understanding EDN signaling during development. Endothelin-1 and the EDNRs are necessary for craniofacial development, and gut innervation. This has been demonstrated in mammals and fishes (see Clouthier and Schilling, 2004) and Clouthier and Schilling (2004) have hypothesized that EDN1 signaling in jaw development was a gnathostome innovation.

**Cardiovascular Effects of Endothelin in Fishes**

Over the past 20 years, the mammalian EDN system has been extensively studied (see reviews Gandhi et al., 1994; La and Reid, 1995; Masaki, 1998; Duru et al., 2001) but
comparatively little is known about EDNs in non-mammalian vertebrates such as the fishes. Olson et al. (1991) evaluated the cardiovascular effects of ET in trout using cannulated dorsal aortas, *in situ* perfused hearts, isolated perfused gills, perfused trunks, and isolated systemic vascular rings. They determined that intra-arterial bolus injections of mammalian EDN1 (667 pmol·kg⁻¹) produced a triphasic (increase-decrease-increase) response in dorsal aorta pressure (P_DA), and continuous infusions produced an increased mean perfusion pressure in a dose-dependent manner. Hoagland et al. (2000) replicated this study using EDN1 isolated from the trout. They showed that intra-arterial bolus injections of trout EDN1 (667 pmol·kg⁻¹) produced a triphasic response in P_DA, and that continuous infusions produced a dose-dependent increase in P_DA, just as Olson et al. (1991) showed with mammalian EDN1. In a similar study, LeMevel et al. (1999) found that intracerebroventricular or intra-arterial injections of mammalian EDN1 (86 pmol·kg⁻¹) produced a transient increase in P_DA, but a triphasic response was not observed further suggesting dose-dependent effects of EDN1 on P_DA. Additionally, Olson et al. (1991) found that EDN1 increased mean perfusion pressure in isolated gills in a dose-dependent manner and that the half-maximal effective concentration (EC₀₅₀) was <10⁻⁸ M.

Other studies have examined constriction of isolated blood vessels in fishes (Poder et al., 1991; Sverdrup et al., 1994; Evans et al., 1996; Evans and Harrie, 2001). In the dogfish shark, mammalian EDN1 caused significant constriction of aortic vascular rings, with an EC₀₅₀ of 10⁻⁹ M (Evans et al., 1996). Evans and Harrie (2001) showed that ventral aortic rings, from hagfish, lamprey, and eels constricted in response to 0.1 μM mammalian EDN1.

Wang et al. (1999) compared the constrictive responses of isolated fish and rat vascular rings to trout EDN1 and mammalian EDN1. They found that trout anterior cardinal veins and branchial arteries were more sensitive to mammalian EDN1 than trout EDN1. The increased
sensitivity to mammalian EDN1 was hypothesized to be due to slower degradation of the mammalian EDN1 compared to the native trout EDN1 (Wang et al., 1999). Degradation of EDN1 has been proposed to be mediated through membrane bound metalloproteinases and internal lysosomes (Jackman et al., 1993). In rats and guinea pigs, the pulmonary circuit rapidly removes 60% of radioiodinated EDN1 and EDN3 in one minute (see La and Reid, 1995). In humans, 53% of EDN1 is removed by pulmonary clearance and this mediated through EDNRB1 (see La and Reid, 1995). In fishes, 55% of an EDN1 bolus was removed during a single pass through the gills (Olson, 1998). Presently, it is unknown which receptors mediate this clearance in fishes.

Endothelin induced vasoconstriction may have a large effect on gill haemodynamics. The gills are highly vascularized and are perfused by the entire cardiac output (Olson, 1998); thus, any change in blood flow or pressure would greatly influence their functions. The physiological function(s) of EDN1 in fish gills is unknown. It has been hypothesized that EDN1 redistributes lamellar blood flow, since a ventral aortic injection of EDN1 caused constriction of pillar cells in the gill lamellae of trout and cod (Nilsson and Sundin, 1998; Stenslokken et al., 1999). This contraction resulted in a shift of intralamellar blood flow to the outer marginal channels. Pillar cells contain contractile elements and although they do not appear to be innervated, hormones like EDN1 may signal pillar cells to contract (or dilate depending on the signal, Bettex-Galland and Hughes, 1973)(Mistry et al., 2004). Sundin and Nilsson (1998) found no evidence that the lamellar arterioles or filamental arteries (afferent or efferent) were constricted by mammalian EDN1. It appears the pillar cells may regulate microcirculation through the gill lamellae. From these studies, it appears that EDN1 may be one of the paracrine/autocrines controlling this system.
Endothelin Effects of Ion Transport

In addition to cardiovascular responses, EDN1 has been shown to have effects on renal transport in mammals (Garvin and Sanders, 1991; Plato et al., 2000; Zeidel, 1993). In the rat proximal tubule, EDN1 inhibited Na\(^+\), K\(^+\)-ATPase activity and bicarbonate transport (Garvin and Sanders, 1991). In the rabbit inner medullary collecting duct, EDN1 inhibited Na\(^+\)-K\(^+\)-ATPase activity and lead to sodium natriuresis (Zeidel et al., 1989). In the rat thick ascending limb of the loop of Henle, EDN1 inhibited net chloride flux and this was mediated by an EDNRB1 (Plato et al., 2000). Most recently, a series of papers has emerged from Donald Kohan’s lab at the University of Utah. They have developed collecting duct-specific EDN1, EDNRB1, or EDNRA knockout mice (Ahn et al., 2004; Ge et al., 2005a; Ge et al., 2006; Ge et al., 2005b). From their studies, they determined that during salt loading (through diet), EDN1 and EDNRB1 are necessary for sodium excretion. As a consequence, EDN1 and EDNRB1 collecting duct-knockout mice were severely hypertensive after salt loading (Ahn et al., 2004; Ge et al., 2006). The EDNRA collecting duct-knockouts were no different from control animals, suggesting that EDNRA in the mammalian collecting duct is not involved in blood pressure regulation (or sodium excretion) (Ge et al., 2005b).

Endothelin inhibition of solute transport also has been demonstrated in fishes. As mentioned above, mammalian EDN1 inhibits net chloride transport in the killifish opercular epithelium (Evans et al., 2004). In addition, the EDNRB1 agonist, sarafotoxin S6c (SRX S6c), also inhibited the net chloride transport in this preparation, suggesting that EDN1 is acting through an EDNRB1; however, there are no EDNRA agonists, thus it has not been determined if EDNRA also affect ion transport in the killifish.

Incubation of mammalian EDN1 with proximal tubules from the killifish kidney led to inhibition of transport by the multidrug resistance protein 2 (Mrp-2) in the tubules (Notenboom
et al., 2005; Notenboom et al., 2002). The Mrp-2 is found in the luminal membrane of the proximal tubules and transports a wide range of chemicals from lipophilic organic anions to polypeptides (Notenboom et al., 2002). Incubation of tubules with a mammalian EDNRB1 antagonist and EDN1 led to EDN1 mediated inhibition of Mrp-2 transport in the proximal tubules, thus suggesting that EDNRB1 are involved in the signaling cascade. Other than these few studies, the effects of EDN1 on transport in fishes are relatively unexplored.

**Overview of Dissertation Research**

When I started my dissertation I had three main objectives: 1) describe the endothelin system from the teleost gill; 2) determine the effects of changing environmental salinity on gill EDN1, ECE1, and EDNRs in two teleost with differing degrees of euryhalinity, with the aim to elucidate the function of EDN signaling in the gill; 3) determine the evolutionary and phylogenetic relationships among the EDNs, ECEs, and EDNRs, respectively.

The species used in my experiments were the killifish (*Fundulus heteroclitus*, Linnaeus) and the longhorn sculpin (*Myxocephalus octodecemspinosus*, Mitchill). The killifish is an excellent osmoregulator that tolerates direct transfers between fresh and seawater, giving researchers the opportunity to test the extreme effects of salinity acclimation. They are a commonly used model vertebrate to test a wide variety of physiological, ecological, and epidemiological questions (Burnett et al., 2007). The longhorn sculpin is classified as a marine teleost; however Claiborne et al. (1994) determined that these fish can tolerate direct transfer to 20% seawater indefinitely, and they can tolerate days in 8% and 4% seawater. This implies that they have some degree of euryhalinity. Interestingly, fishermen in the Gulf of Maine have described finding longhorn sculpin in estuaries during high tides, further suggesting some level of environmental salinity tolerance (Bigelow and Schroeder, 2002). These two fishes are found in the waters surrounding Mount Desert Island, ME, (where we have a summer lab), and afford
an interesting comparative system to test questions of the effect of changing environmental salinity on the gill EDN signaling axis.

In Chapter 2, I described EDN1 and ECE1 from the killifish. In addition, I explored the phylogenetic relationships among the EDNs and ECEs, respectively, and determined the effects of different environmental salinity on these genes/proteins. This work was recently published in the Journal of Experimental Biology, “Endothelin-1 and the endothelin converting enzyme in the fish gill: physiological and evolutionary perspectives” by Hyndman and Evans (2007).

In Chapter 3, I determined the functional and genomic relationships of the EDNRs. This chapter is completely different from all the other chapters, as it dives deep into the world of phylogenetics, bioinformatics, and genomics (three words I thought I would never say). In addition, I introduce the complete EDNR sequences from the killifish. This chapter will be refined for submission to PNAS over the next few months.

In Chapter 4, I described the EDNRs from the killifish and determined the effects of varying environmental salinity on these receptors. Using molecular biology and protein biochemistry, I determined that changing environmental salinity changes EDNR mRNA and protein concentrations in the gill. This work will be submitted to the Journal of Experimental Biology in the near future.

In Chapter 5, I determined the effects of low environmental salinity on the longhorn sculpin. Unlike the killifish, the effects of hypo-osmotic environments on plasma osmolality, ions, and gill ion transporter density has not been determined for the longhorn sculpin. This chapter was important for setting up the context of our EDN signaling effects in Chapter 5. This study is currently under review with the Journal of Experimental Biology
In Chapter 6, I described the effects of low environmental salinity on ECE1 and the EDNRs in the gills of the longhorn sculpin. Using molecular techniques, I sequenced portions of *ECE1* and the *EDNRs* and determined that this moderately euryhaline fish up-regulates *EDNRB2* and *EDNRB1* during acclimation to hypo-osmotic environments.

Finally, in Chapter 7 I summarize the findings from my dissertation work, and revisit my three main objectives, tying all the chapters together.
CHAPTER 2
ENDOTHELIN AND ENDOTHELIN CONVERTING ENZYME-1 IN THE FISH GILL:
EVOLUTIONARY AND PHYSIOLOGICAL PERSPECTIVES

Introduction

Endothelin (EDN) is a family of three autocrine/paracrine peptides (EDN1, EDN2 and EDN3) that function in a variety of physiological processes such as the regulation of vascular tone (Yanagisawa et al., 1988a) and natruresis in the kidney (Zeidel et al., 1989). Endothelins are translated as ~200 amino acid (aa) preproendothelins (preproEDN) that are initially cleaved by a furin-like enzyme (Yanagisawa et al., 1988a) to form the relatively inactive 38 aa proendothelin (proEDN, also known as Big-EDN) (Kimura et al., 1989). Proendothelin is further cleaved to form the active 21 aa EDN by the endothelin converting enzyme (ECE-1 and/or ECE-2) (Shimada et al., 1994; Xu et al., 1994). In mammals, EDNs’ actions are mediated via two G-protein coupled receptors: endothelin A receptor (EDNRA), which preferentially binds EDN1 (Arai et al., 1990), and endothelin B1 receptor (EDNRB1), which binds all three EDNs with equal affinity (Sakurai et al., 1990). In non-mammalian vertebrates, EDNs also equally bind to a third G-protein-coupled receptor, endothelin B2 receptor (EDNRB2) (Lecoin et al., 1998), and in amphibians EDN3 binds to an amphibian-specific receptor termed endothelin C receptor (EDNRC) (Karne et al., 1993).

Preproendothelin genes have been found in all major gnathostome clades (Fig. 2-1), and evidence for EDN1 regulation of vascular tone has been shown in fishes (Evans, 2001; Evans et al., 1996; Evans and Harrie, 2001; Olson et al., 1991; Wang et al., 2001). In addition, EDN1 inhibition of transport by the multidrug resistance-association protein was demonstrated in shark (Squalus acanthias) rectal tubules (Miller et al., 2002) and killifish (Fundulus heteroclitus) renal tubules (Masereeuw et al., 2000). Recently, Evans et al. (2004) determined that exogenous
(mammalian) EDN1 inhibited net chloride transport in the killifish opercular epithelium, a tissue used as a model for the SW teleost gill (Karnaky et al., 1977). In teleosts, the gill is the main site for ion balance, nitrogen excretion, acid/base regulation and gas exchange (Evans et al., 2005). Estuarine euryhaline fishes like the killifish (Fundulus heteroclitus) encounter varying environmental salinities throughout the day (Marshall, 2003), resulting in a net gain or loss of ions depending on the water salinity; thus the regulation of gill ion transport is an important mechanism to maintain ionic homeostasis. Evans et al. (2004) hypothesized that EDN1 signaling cascades in the gill may be a local regulator of ion balance in fishes. Thus, the purpose of this study was to determine if EDN1 and ECE1 are produced in the killifish, and secondarily determine if environmental salinity regulates gill EDN1 and/or ECE1 mRNA expression. We were also interested in determining the phylogenetic/evolutionary relationships of the EDNs and ECE family of proteins.

**Methods**

**Fish Maintenance**

Killifish, *Fundulus heteroclitus*, were trapped in North East Creek, Mount Desert Island, ME, and maintained in free flowing, 31 ppt seawater (SW) tanks at the Mount Desert Island Biological Laboratory, under a natural summer photoperiod, before being shipped to the University of Florida. There they were maintained in 32 ppt, 23°C SW, under a 12 light:12 dark photoperiod. Tank pH was maintained between 7.8 and 8.0, and ammonia, nitrate, and nitrite levels were below 1 ppm. The fish were fed commercial fish pellets to satiation every other day.

**EDN1 cDNA**

All protocols and procedures were approved by IACUC at the University of Florida. Molecular protocols of Hyndman et al. (2006) were used. Killifish were decapitated, and the gills of the right side were removed and snap frozen in liquid nitrogen. Total RNA was then
isolated with TRI-reagent (Sigma, St. Louis, MO), and 5’ and 3’ RACE cDNA was synthesized from 4 μg of total RNA with a GeneRacer™ Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. Published degenerate reverse EDN1 primers by Wang et al. (2006) were used in our initial 5’ Touchdown RACE PCR following Invitrogen’s protocols. The polymerase used was 0.625 U of Ex Taq, hot start, DNA polymerase (Takara Bio, Madison, WI) and the reactions were run in an Express thermocycler (ThermoHybaid, Franklin, MA). The PCR parameters were: 94°C for 2 min, 5 cycles of 94°C for 30 sec, 72°C for 30 sec, 5 cycles of 94°C for 30 sec, 70°C for 30 sec, 30 cycles of 94°C for 30 seconds, 45°C for 30 sec, 72°C 30 sec, and a final 72°C for 5 minutes. PCR products were visualized by ethidium bromide staining in 1.5% agarose gels, ligated into pCR® 4-TOPO vectors, and transformed into TOP10 chemically competent cells using a TOPO TA Cloning® Kit for sequencing (Invitrogen).

Plasmid DNA was then sequenced in both directions at the Marine DNA Sequencing Facility at the Mount Desert Island Biological Laboratory (Salisbury Cove, ME). Once we had the 5’ end, specific killifish EDN1, primers were designed (Table 2-1) and 3’ Touchdown RACE PCR was performed to complete the cDNA sequences. The PCR parameters were: 94°C for 2 min, 5 cycles of 94°C 30 sec, 72°C for 30 sec, 5 cycles of 94°C for 30 sec, 70°C for 1 min, 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final 72°C for 10 min. PCR products were cloned and sequenced like above.

**ECE cDNA**

To get ECE cDNA, 4 μg of total gill RNA (extracted as described above) was reverse transcribed using the First-strand cDNA Superscript™ III reverse transcriptase kit (Invitrogen) using oligo-dT primers. Initial degenerate primers used to get ECE were designed using CODEHOP (Rose et al., 2003) and are recorded in Table 2-1. PCRs were run on 0.5 μl of the
olgio-dT cDNA, with 0.625 U of Ex taq, hot start (Takara) and standard cycling parameters. PCR products were cloned and sequenced as described above.

**Sequence and Phylogenetic Analysis**

Sequence results for each transcript were assembled with GeneTools software (BioTools Inc., Edmonton, Alberta) and killifish EDNI and ECE1 nucleotide sequences were searched for open reading frames (ORFs). The resulting amino acid translations were analyzed with the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) website. The predicted amino acid sequences were aligned with other full-length vertebrate EDN or ECE proteins using Clustal X (Chenna et al., 2003). All sequences were taken from GenBank or the Genome projects in Ensembl (e:44 April 2007). Preproendothelin-1 sequences from each major vertebrate clade (mammals to teleosts) were separately aligned, and similarities among the sequences highlighted with GeneDoc (available at http://www.psc.edu/biomed/genedoc), including the expected cleavage sites for furin and ECE (Opogcnorth et al., 1992; Yanagisawa et al., 1988a). To determine the relationship among our sequences and those from other organisms, EDN and ECE alignments were exported to PHYML (Guindon et al., 2005) and a Fast Maximum-Likelihood test was performed, following the WAG model of amino acid substitutions and a gamma calculated of 1.023, and 1.03, respectively. Branches were then tested for statistical significance by bootstrapping with 500 replicates.

**Multiple Tissue Semi-Quantitative PCR**

To determine the distribution of EDN1A, EDN1B and ECE1 mRNA among tissues, relative duplexing semi-quantitative PCR was performed on total RNA from gill, opercular membrane, brain, heart, stomach, intestine, and kidney tissue as described previously (Choe et al., 2005; Choe et al., 2004). Briefly, cDNA was produced from the tissues of a SW killifish as described above, but random hexamer primers (not oligo-dT primers) were used so that
ribosomal and messenger RNA would be reverse transcribed. Non-degenerate primer pairs were
designed to amplify a product with high efficiency (e.g., high melting temperature) and to
minimize the chance of amplifying contaminating genomic DNA, the primer pair was designed
to include at least one intron-exon boundary when possible (Table 2-1). A QuantumRNA™ 18S
internal standard primer kit (Ambion, Woodward Austin, TX) was used to control for variability
in cDNA quality and quantity between the different tissues tested. Duplexing PCR with primers
for 18S and either EDN1A, EDN1B or ECE1 were then optimized to ensure that the reactions
were terminated during the exponential phase. Lastly, the products were visualized by ethidium
bromide staining in 1.5% agarose gels and digitized using the Biorad Gel Doc™ XR System.

Salinity Challenges

Killifish were acclimated to SW (approximate concentrations in mmol 1⁻¹: Na⁺ 517, Ca²⁺ 9,
K⁺ 12, Cl⁻ 486) (Choe and Evans, 2003) or fresh water (FW) (Gainesville dechlorinated tap
tap water approximate concentrations in mmol 1⁻¹: Na⁺ 4, Ca²⁺ 1, K⁺ 0.03, Cl⁻ 0.40) (Choe and
Evans, 2003) for 2 weeks, at which point the SW killifish were transferred into FW (SW to FW)
and the FW killifish were transferred into SW (FW to SW). An additional set of killifish were
removed and replaced into SW or FW as sham controls (SW to SW and FW to FW,
respectively). Immediately after transfer, 5 or 6 killifish from each treatment were sacrificed,
gills excised and snap frozen for RNA extraction and cDNA synthesis. Killifish (n=5 or
6/treatment) were further sacrificed at 3, 8 and 24 h post transfer (acute acclimations), as well as,
30 days post transfer (chronic acclimation). RNA was extracted from all of the samples and
oligo-dT cDNA synthesized as described above.

Quantitative Real-Time PCR

To determine the effects of environmental salinity on killifish gill EDN1A, EDN1B and
ECE1 mRNA levels, quantitative real-time PCR (qRT-PCR) was performed. Nondegenerate
primers were designed to amplify a product between 50-100 bp across a predicted intron-exon boundary (Table 2-1). L8 was used as an internal control gene as previously described (Choe et al., 2006; Choe et al., 2005). Each sample was run in triplicate using 2 μl of 1/10 diluted original cDNA, 7.4 pmol of primers and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μl. The cycling parameters used were: an initial denaturing step of 95°C for 10 min, 40 cycles of 95°C for 35 sec, 60°C for 30 sec and 72°C for 30 sec followed by a melting curve analysis to ensure only one product was amplified. Random samples were also sequenced following qRT-PCR confirming amplification of the target of interest. To determine the degree of possible genomic contamination, qRT-PCR was run using RNA samples that were not reverse transcribed, and we determined that there was no genomic contamination. All qRT-PCRs were run on a MyiQ quantitative thermocycler (Biorad, Hercules, CA).

Each primer pairs’ efficiency was determined by performing a 10 fold dilution curve using plasmid cDNA. Efficiency (E) for each primer pair was calculated using the equation:

\[ E = -1 + 10^{-\frac{1}{\text{slope}}} \]

where “slope” was the slope of the dilution curve. Each CT value was subtracted from a randomly chosen control sample resulting in a ΔCT, and were analyzed using the Pfaffl equation (Pfaffl, 2001):

\[ \text{ratio} = E^{\Delta CT \text{target}} / E^{\Delta CT \text{L8}} \]

Each Pfaffl ratio was then standardized to the average chronic seawater Pfaffl ratio.

Statistics

Data are expressed as mean +/- s.e.m. For the qRT-PCR data, a 2-Factor ANOVA was performed to determine whether effect of environmental salinity over time differed between SW to FW transfers and SW to SW shams or FW to SW transfers and FW to FW shams. If statistical significance was found a 1-Factor ANOVA was run to determine the effect of time over a
treatment group. Finally, all time points were compared to sham time points with unpaired T-tests to determine if salinity transfers altered mRNA expression. All values which did not meet homogeneity or equal variance tests were log transformed to meet the assumptions of the ANOVA. $\alpha=0.05$.

**Tissue Preparation for In Situ Hybridization and Immunohistochemistry**

Killifish gills were fixed in 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS) pH=7.3, for 24 h, dehydrated in an increasing concentration of ethanol, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin wax. The tissue blocks were cut at 7 microns, placed on Superfrost Plus slides (Fisher Scientific), and heated at 37°C overnight.

**In Situ Hybridization**

mRNA for *EDN1A*, *EDN1B*, and *sodium, potassium ATPase (NKA)* mRNA were visualized using *in situ* hybridization. An *ECE1* mRNA probe was not made because our partial sequence of that transcript was from the middle of the sequence, a region that in other fishes is >65% identical to *ECE2*, and we were afraid of the potential of cross-reactivity of this probe. Specific digoxigenin (DIG)-RNA probes (sense and antisense) were made against the 3’ end of the transcripts (including UTR for the *EDNs*, these regions were <60% identical). For *EDN1A* the probe was made from position 520 to the end of the transcript including poly A tail (420 bps long). The *EDN1B* probe was made from position 515 up to and including the poly A tail (419 bps). Both of these transcripts were cloned as described above. A killifish *NKA* mRNA probe was also made based upon the complete killifish NKA sequence (AY057072). The probe was made from bps 915 to 3115. This transcript was also cloned and sequenced to ensure it was indeed *NKA*. All of the transcripts were linearized by T3/T7 PCR amplification from the plasmids containing the sequences of interest. Dig-RNA probes were generated by incubating
100-200 ng of the linearized transcripts with the DIG RNA Labeling mix (Roche Applied Science, Indianapolis, IN) following manufacturer’s protocols, at 37°C for 16 h followed by treatment with DNase for 1 h at 37°C. The DIG-RNA probes were purified using mini Quick Spin RNA columns (Roche) following the manufacturer’s instructions, eluted in 80 μl of DEPC water and stored at -80°C until use.

To determine which gill cells expressed EDN1A or EDN1B mRNA, gill tissue slides were rehydrated in two changes of Citrisolv, followed by incubation in a series of decreasing concentration of ethanol washes. The slides were placed in sterile 10 mM PBS and post fixed in 4% PFA for 10 minutes at room temperature (25°C). Following this, the slides were rinsed in sterile 10 mM PBS and incubated in proteinase K (5 μg ml⁻¹) at room temperature for 5 minutes. Again, they were washed in 10 mM PBS and post fixed in 4% PFA for 10 minutes to inactivate the proteinase K. After a final PBS wash, the slides were incubated in prehybridization solution (50% formamide, 10% dextran sulphate, 2% Blocking reagent, 0.1% CHAPS, 1% Tween 20, 5 mM EDTA pH=8.0, 5X SSC, 50 μg ml⁻¹ heparin, 1 mg ml⁻¹ tRNA, in DEPC-water) for 2 h at room temperature. Next 200 to 500 ng of DIG-RNA probes were added to fresh prehybridization solution and the slides were left to incubate at 60°C for 18-24 h. Following this, the tissues were washed for 30 minutes in: 2X SSC at room temperature, 2X SSC at 60°C, two 0.2X SSC 60°C washes, one 0.2X SSC at room temperature and KTB (50 mM Tris pH=7.5, 100 mM NaCl, and 10 mM KCl) at room temperature. The tissues were then blocked in 20% normal goat serum diluted in KTB for 1 h at room temperature and incubated in 7.5 U ml⁻¹ of sheep anti-Digoxigenin-AP, Fab fragments (Roche, diluted in NGS) overnight at 4°C. The slides were then washed in 3 changes of KTB and incubated in alkaline phosphatase buffer (100 mM Tris pH = 9.5, 100 mM NaCl, 50 mM MgCl₂) for 30 minutes at room temperature. Visualization
of the probes was achieved by incubating the tissues in BCIP/NBT Substrate Kit, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Vector Labs, Burlingame, CA) with levamisole following manufacturer’s instructions, at room temperature until the signal developed (2-6 h). Images were captured using an Olympus BX60 light microscope with a Hitachi KP-D50 digital camera. Image contrast and brightness were adjusted with Photoshop CS (Adobe, San Jose, CA).

**Immunohistochemistry**

Slides were analyzed following the methods of Piermarini et al. (2002) and Hyndman et al.(2006). Slides with chronic SW and FW acclimated killifish gill tissue were incubated in primary antibodies: polyclonal, anti-human-proEDN1 (1/1000 diluted in NGS) (Phoenix Pharmaceutical, Burlingame, CA) made against the complete 38 aa of human proEDN1, which is 74% identical to both killifish EDN1s. Monoclonal, anti-NKA (α5, 1/1000) was developed by Dr. Douglas Fambrough, and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

**Results**

**Sequence Analyses**

From the killifish gill we have sequenced two EDN1 transcripts and have designated them EDN1A (accession EU009474) and EDN1B (accession EU009475). EDN1A is 917 bp with an ambiguous base at position 762 (C or T), and a predicted ORF of 483 bp that translates into a preproendothelin-1A (preproEDN1A) of 189 aa. EDN1B is 912 bp with a predicted ORF of 429 bp that translates into a preproendothelin-1B (preproEDN1B) of 143 aa. The predicted cleavage sites in the preproEDN1s for furin and ECE are depicted in Fig. 2-2A. As found in other
vertebrate preproEDN1 peptides, preproEDN1A and preproEDN1B contain the dibasic cleavage sites for furin and the conserved Trp^{21}-Val^{22} cleavage site for ECE (Fig. 2-2A). The two active (21 aa) killifish EDN1s are 80% identical to human EDN1 and are 100% identical to each other (Fig. 2-2B). As seen in Fig. 2-1, our killifish preproEDN1s group with the other fish preproEDN1 sequences and group outside of the fish preproEDN2 and preproEDN3 sequences. We were unable to find a preproEDN orthologue in the *Ciona intestinalis* or *Branchiostoma* genomes, suggesting that the EDNs are found only in vertebrates.

We have sequenced 1696 bps from the middle of the killifish ECE1 cDNA (accession EU009476). This translates into 565 aa from the killifish ECE1. Endothelin converting enzymes are found in all organism including Bacteria and Archaea (Fig. 2-3). As seen in Fig. 2-3, the non-vertebrate ECEs are not well resolved (i.e. lancelet ECE groups with locust and sea urchin, while sea quirt groups with hydra), and although there is no clear explanation for this, Fig. 2-3 shows three distinct ECE clades: ECE1, ECE2 and non-vertebrate ECE. Our partial sequence of ECE1 from the killifish groups with the other fish ECE1 sequences confirming it is ECE1.

**Tissue Distributions**

Using duplexing relative semi-quantitative PCR we found EDN1A mRNA in the gill, opercular epithelium, brain, heart, stomach, intestine and kidney of the killifish (Fig. 2-4). Relatively high expression was found in the gill, brain and kidney. EDN1B mRNA was not found in the opercular epithelium, and had very little expression in the gill, but was highly expressed in the brain, kidney and intestine. Finally, ECE1 mRNA was found in all of the tissues tested, with highest expression in the stomach, intestine, and gill (Fig. 2-4).

**In situ Hybridization**

EDN1A mRNA was localized to gill epithelial cells in the interlamellar region (Fig. 2-5A). These cells were large, round and were dispersed along the entire length of the filament (not
shown). \textit{NKA} mRNA was also found in epithelial cells in the interlamellar region (Fig. 2-5C) but were concentrated only near the afferent filamental artery (trailing edge of the filament, not shown). The morphology of these \textit{NKA} positive cells are consistent with mitochondrion-rich cells of the killifish gill (Choe et al., 2005; Hyndman et al., 2006; Katoh et al., 2001; Marshall, 2003). In contrast, \textit{EDN1B} mRNA expression was rare (as was seen in the \textit{EDN1B} tissue distribution described above); however it was found in some lamellar pillar cells (Fig. 2-5D, F) and in epithelial cells adjacent to the environment (Fig. 2-5D). Incubation of slides with sense probes produced a little non-specific staining (Fig. 5B, E).

\textbf{Immunohistochemistry}

Proendothelin-1, the precursor to active EDN1, was immunolocalized to epithelial cells in the interlamellar region of the killifish gill, and on the lamellar pillar cells (Fig. 2-6A, C). Proendothelin-1 immunoreactivity was seen in epithelial cells adjacent to cells immunoreactive for NKA (Fig. 2-6A, C), and these cells share similar morphology to NECs described in fishes (Goniakowska-Witalinska et al., 1995; Mauceri et al., 1999; Zaccone et al., 1992; Zaccone et al., 1996). This immunolocalization matches the killifish \textit{EDN1} mRNA localization shown in Fig. 2-5. Slides incubated in NGS and double labeled with NKA were done as negative controls, and showed no non-specific staining of the proEDN1 secondary antibody and chromagen (Fig. 2-6B, D).

\textbf{Salinity Acclimations}

When killifish were transferred from FW to SW there were no statistically significant changes in \textit{EDN1A} or \textit{EDN1B} mRNA levels over 24 h (acute acclimation) compared to sham (FW to FW) transfers (Fig. 2-7A, C). \textit{ECE1} mRNA increased four- and sixfold compared with sham \textit{ECE1} mRNA levels at 8 and 24 h post a FW to SW transfer (Fig. 2-7E). \textit{EDN1A} mRNA levels did not change significantly with acute acclimation from SW to FW compared to sham
(SW to SW) treatments (Fig. 2-7B), but 24 h $EDN1B$ mRNA levels were almost threefold higher compared to sham $EDN1B$ mRNA levels at 24 h (Fig. 2-7D). $ECE1$ mRNA levels were twofold higher after 3 and 24 h of acclimation to FW but did not differ from sham values at 8 h post transfer (Fig. 2-7F). With chronic acclimation (30 days), there were no statistical differences between the SW and FW acclimated killifish for $EDN1A$, $EDN1B$ or $ECE1$ mRNA levels (Fig. 2-8). In addition, preproEDN1 (protein) immunolocalization did not differ between fish chronically acclimated to FW (Fig. 2-6A, B) or SW (Fig. 2-6C, D).

**Discussion**

**Endothelin Sequences**

We have sequenced two cDNAs for $EDN1$ from the killifish gill. These two transcripts have identical 5 prime ends until position 510 where there is an AC insertion resulting in a frame shift mutation in $EDN1A$ that changes the stop codon TGA to ACT GAG. Conversely, $EDN1B$ may have lost an AC at position 510 resulting in a stop codon and a truncated preproEDN. Past this insertion/deletion (indel), the two transcripts differ along the rest of the sequence, but are only 5 bps different in total length. This suggests that the two transcripts are from duplicated $EDN1$ genes and are not transcript variants. We did not find duplicate EDN1 genes in the Takifugu, Tetraodon or Danio genomes, suggesting the $EDN1$ duplication was a *Fundulus* specific event (please see note at end of manuscript). As a consequence of the indel at position 510, the predicted preproEDN-1a is 46 aa longer than preproEDN-1b. Even though there are differences between the killifish preproEDN-1s, the proEDN-1s and EDN1s are 100% identical. Why would a tissue (cell) produce two different mRNAs if the end protein translated is 100% identical? We hypothesize that these two EDN1s have remained in the killifish because they have different regulatory pathways, and/or are stimulated by different signals. This hypothesis might suggest that the $EDN1$s would have different tissue/cellular distributions. Supporting this
hypothesis, from our tissue distribution analysis (Fig. 2-4), we found \textit{EDN1A} was ubiquitously expressed in all the tissues tested, with relatively high expression in the gill, brain, and kidney. Conversely, \textit{EDN1B} was found in very low levels in the gill and opercular epithelium, but relatively high in the brain. Nevertheless, until complete killifish \textit{EDN1A} and \textit{EDN1B} genomic sequences are determined, it is unclear how and what factors may regulate these genes, and thus suggest why these two have been retained in the killifish.

\textbf{Gill Expression of \textit{EDN1} mRNA and ProEDN1 Protein}

In the killifish gill we found \textit{EDN1A} mRNA expression in epithelial cells of the interlamellar region, and \textit{EDN1B} mRNA expression was found in pillar cells, and in cells adjacent to the environment, in the interlamellar region. Not only are these two transcripts expressed in different levels within a tissue (Fig. 4), they are also expressed in different cells within the gill. From our immunohistochemical experiments, we found proEDN1 immunoreactivity in epithelial cells adjacent to the NKA immunoreactive cells. NKA is commonly used as a marker for the ion transporting, mitochondrion-rich cell (MRC) of the fish gill (Evans et al., 2005; Katoh et al., 2001; Marshall, 2003). Proendothelin immunoreactivity was also found on pillar cells, which is in agreement with our \textit{in situ} hybridization findings. Zaccone et al. (1996) immunolocalized proEDN to gill neuroendocrine cells (NECs) of the eel (\textit{Conger congo}), catfish (\textit{Heteropneustes fossilis}) and dogfish (\textit{Scyliorhfnus canicula}), and the morphology of these cells matches that of the proEDN immunoreactive epithelial cells in the killifish gill.

Endothelin production in the pillar cells was suspected by Sundin and Nilsson (1998) and Stenslokken et al. (1999). They showed that infusion mammalian EDN1 into the lamellae of the rainbow trout resulted in a “constriction of the vascular sheet” (of the lamellae) and that this was likely due to constriction of the pillar cells. The authors hypothesized that hormonal control of
pillar cell tone may be one mechanism to match respiratory needs of a fish while minimizing ion fluxes. There is no evidence that pillar cells are innervated; thus endocrine/paracrine/autocrine signaling molecules may be the regulators of pillar cell tone. Pillar cells contain contracting filamentous material (Bettex-Galland and Hughes, 1973) and recently Mistry et al. (2004) described an actin-binding protein, FHL5, that is highly expressed in these cells, suggesting that they are capable of contraction. Stenslokken et al. (1999) demonstrated in vivo using video microscopy that pillar cells do contract with EDN1 infusion. Recently, EDN receptors, EDNRA and EDNRB, were immunolocalized in the fish gill. Stenslokken et al. (2006), found EDNRB throughout the gill vasculature, NECs, and pillar cells of the cod (Gadus morhua). EDNRA was described in nerve fibers running along the length of the filament and innervating the gill vasculature (Stenslokken et al., 2006). In the tiger pufferfish (Takifugu rubripes) EDNRA was found on the pillar cells and in erythrocytes (Sultana et al., 2007). Studies from our lab in the killifish have found EDNRBs throughout the gill vasculature and pillar cells, and EDNRAs on the mitochondrion-rich cell (Chapter 4). Evidently, there is species specific EDN receptor distribution in the gill of fishes.

**Acute and Chronic Salinity Acclimations**

Killifish usually live in estuaries where there are rapid changes in environmental conditions such as salinity and temperature (Marshall, 2003). We tested the effects of rapid changes of environmental salinity on mRNA expression of \textit{EDN1A}, \textit{EDN1B} and \textit{ECE1}. \textit{EDN1} transcript levels did not change with chronic acclimation to FW or SW (Fig. 2-8). In addition, proEDN1 immunoreactivity in the gill did not differ between SW and FW acclimated killifish (Fig. 2-6). However, \textit{EDN1B} and \textit{ECE1} mRNA levels increase with acute FW acclimation suggesting that more active EDN1 protein is produced. Evans et al.(2004), demonstrated that $10^{-8}$ M mammalian EDN1 can inhibit net chloride transport in the killifish opercular epithelium, and
this is predominately due to stimulation of cyclooxygenase (COX) and subsequent prostaglandin production. These findings suggest that during transfer to a hypo-osmotic environment, EDN1B and ECE1 protein levels increase resulting in an increase in active EDN1 that could potentially inhibit net chloride transport, helping the fish retain ions. However, we cannot rule out that EDN1 signaling in the gill is different from what was described in the killifish operculum by Evans et al. 2004 (see below). In addition, it is undetermined how volume stress, like that occurs during a rapid change to a hypo-osmotic environment, effects blood flow through the gill. EDN1B was found on gill pillar cells, and may play a role in regulating blood flow during blood volume increases however this is an unexplored area of fish gill physiology.

Although we found an increase in EDN1 during acclimation to FW, we unexpectedly found a sixfold increase in ECE1 mRNA levels with acute SW acclimation suggesting that there is an increase in ECE1 production during this period. This in turn would result in more EDN1 production because the proteolytic cleavage of proEDN1 to EDN1 by ECE1 is a rate limiting step (D'Orleans-Juste et al., 2003; Ikeda et al., 2002). Our attempts to measure EDN1 production in the fish gill by enzyme immunoassay, Tris-Tricine Western blotting, and MALDI-FTMS mass spectrophotometry, were unsuccessful, but measurements of EDN1 levels are necessary to fully understand the role of EDN1 cell signaling in the fish gill. Recently, Choe et al. (2006), showed a threefold increase in COX-2 mRNA levels in the killifish gill, 3 h post a FW to SW and a threefold increase 3 h post a SW to FW transfer, and hypothesized the increase in COX-2 is an important mechanism for gill cell survival during osmotic stress. A similar result has been demonstrated in mammalian medullary interstitial cells (that experience large changes in osmotic stress), which require functional COX-2 to survive (Hao et al., 1999; Hao et al., 2000). Medullary interstitial cells also contain EDNRs but do not produce EDN1 (Dean et al., 1996).
Endothelin has been shown to stimulate COX-2 in a variety of mammalian tissues (Chen et al., 2003; Hughes et al., 1995), and EDN1 signaling via endothelial ENDRB results in the production of prostacyclin (Hirata et al., 1993; Warner et al., 1989). Taken together, our findings suggest that during rapid changes in environmental salinity, gill cell survival during this osmotic stress may be accomplished by increased EDN1 production and subsequent stimulation of COX production of prostaglandins. To the best of our knowledge, it is unclear what aids cell survival during salt or water load in fishes, and it is plausible that since EDN1 and ECE1 are ubiquitously expressed that this may be a more global change in their signaling patterns, and that this is not a gill specific phenomenon; however this is yet to be determined and experiments testing these hypotheses are needed. In addition, studies blocking aspects of EDN1 signaling in the gill and subjecting these fish to salinity challenges are vital in understanding EDN1 function during osmotic stress. This technique has been successful in mice models, where kidney collecting duct EDN1 (or EDNRB1) knockout mice who are fed a high salt diet are unable to excrete the excess Na\(^+\) accumulated and are severely hypertensive (Ahn et al., 2004; Ge et al., 2006) suggesting that EDN1 is necessary for salt excretion in mammals. Applications of these types of techniques to fish models are necessary to full understand the in vivo role to EDN signaling in the fish gill.

**Evolution of EDNs and ECE**

In searching of the completed genome projects, we were unable to find EDN orthologues in any organism basal to the teleost fishes. From the maximum likelihood analyses presented in Fig. 1, there are three distinct groups representing: preproEDN1, preproEDN2 and preproEDN3 in vertebrates. Evans and Harrie (2001) showed that aortic vascular smooth muscle rings from the sea lamprey and Atlantic hagfish constrict in response to mammalian EDN1, suggesting that the receptors and EDN1 are expressed endogenously in these basal, Agnathan vertebrates.
Physiological responses to EDN1 have also been demonstrated in the spiny dogfish shark (Evans et al., 1996; Evans and Gunderson, 1999) again suggesting that EDN1 is produced endogenously. Currently, the exact evolutionary history of this family of peptides is not clear. EDN sequences from hagfish, lamprey, and sharks are necessary to determine when these peptides arose, and to determine if it was due to gene/genome duplications or other evolutionary events over vertebrate evolution. Interestingly, mutations in EDN1, endothelin receptors (EDNRs), or ECE result is severe craniofacial developmental abnormalities, and these phenotypes are often lethal (Brand et al., 1998; Clouthier and Schilling, 2004; Kurihara et al., 1994; Nair et al., 2007), suggesting the EDN signaling is necessary for development in vertebrates, and it may be a key innovation in the radiation of vertebrates.

Unlike EDNs which are only found in vertebrates, ECE is found in all organisms, including Bacteria and Archaea. In Fig. 2-3, our maximum likelihood analyses reveals three distinct groups of ECEs: prokaryote, fungal, and invertebrate ECE, vertebrate ECE1 and vertebrate ECE2. This suggests a gene duplication event sometime after the chordate-vertebrate split, but before the teleost radiation. Because there is no molecular evidence for EDNs or EDNRs in animals basal to the vertebrates why would they have an ECE? Endothelin converting enzymes are zinc-dependant metalloendoproteases and part of the Neprelysin and Kell family (Shimada et al., 1994; Xu et al., 1994). In vertebrates, ECE can function as a monomer or dimer; however for effective proteolytic cleavage of proEDN1 to EDN1, dimerization at Cys412 is preferential (Shimada et al., 1996). In contrast, hydra ECE (Zhang et al., 2001) and the other invertebrate, fungal and prokaryote ECEs are missing Cys412 and are believed to function as monomers (Zhang et al., 2001). Vertebrate ECE has been shown to cleave peptides other than proEDN including bradykinin, angiotensin I, and substance P (Hoang and Turner, 1997; Johnson
et al., 1999), suggesting that it may be a generalist protease. Although the native substrates cleaved by ECE in non-vertebrate organisms are undetermined, it is plausible that ECE originally cleaved substrates found in all organisms, and during vertebrate evolution started functioning as a dimer and preferentially cleaving proEDN.

**Tentative Model for EDN1 Signaling in the Killifish Gill**

To summarize our findings, we propose the following model (Fig. 2-9) of paracrine and autocrine EDN1 signaling in the fish gill. Diagrammed is a lamellar cross-section of the gill (same orientation as the gills in Figs 2-5 and 2-6), with pillar cells (PCs) highlighted in grey and adjacent pavement cells (PVCs) in white. In the intralemallar region there are two MRCs and an NEC above the gill vasculature. Cyclooxygenase-2 (COX-2) and neuronal nitric oxide synthase (nNOS) were previously immunolocalized in the killifish gill, to MRCs (Choe et al., 2006) and NECs, nerve fibers and lamellar arterioles (Hyndman et al., 2006), respectively. NKA was immunolocalized to the basolateral membrane of the MRC (Choe et al., 2006; Hyndman et al., 2006; see Katoh et al., 2001) and the chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), to the apical membrane of the MRC (Katoh et al., 2001). From our studies and others, EDNRB were found throughout the gill vasculature (Hyndman and Evans, unpublished; Stenslokken et al. 2006), and EDNRB and EDNRA were on the pillar cells depending on the species (Hyndman and Evans, unpublished Stenslokken et al., 2006; Sultana et al., 2007). EDNRA were found on MRCs in the killifish gill (Chapter 4). Here we present EDN1 expression in cells adjacent to the MRC (likely NECs) and pillar cells. This suggests a paracrine role of EDN1 signaling given that it is produced in the NEC and can bind to receptors on the adjacent MRCs (Fig. 2-9, pathway #1) where it potentially stimulates COX-2 activity resulting in cell survival during osmotic stress and/or alter ion transport by the MRC as previously hypothesized (Evans et al., 2004). EDN1 can also potentially act as a paracrine
binding to EDNRB on the gill vasculature and lamellar arterioles, suggesting it can regulate perfusion of the lamellae (Fig. 2-9, pathway #1). It also can act as an autocrine on the pillar cells, further supporting the role of regulation of local perfusion across a lamella to meet the respiratory needs of the fish (Fig. 2-9, pathway #2) (Stenslokken et al., 1999; Sundin and Nilsson, 1998). It may also help maintain lamella integrity during rapid increases in plasma volume during exposure to a hypoosmotic environment. This is the first model to depict EDN1 signaling in the fish gill, and in the future, studies determining the specific function of EDN1 in the gill and whole fish are necessary to understand its role in normal fish physiology.

**Note**

When this paper was under review a new version of ENSEMBL was released and in it, I found duplicate EDN1 genes in the fish genomes. Thus in contrast to what is stated above, the \textit{EDN1A} and \textit{EDN1B} is likely the result of the teleost-specific genome duplication and not just a killifish specific gene duplication.
Table 2-1. Primers used for cloning, tissue distributions (td) and quantitative real-time PCR (q)

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</tr>
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<td>sense</td>
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<td>sense</td>
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<td>sense</td>
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</tr>
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<td>antisense</td>
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<td>sense</td>
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<td>antisense</td>
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<td>sense</td>
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* Denotes degenerate primers
† Denotes primers used in RACE PCR.
Figure 2-1. Maximum likelihood analyses of the vertebrate preproendothelin amino acid sequences. Following the WAG model of amino acid substitutions (Whelan and Goldman, 2001) and a gamma=1.023, there are three distinct groups of preproendothelins, preproendothelin-1 (abbreviated as only EDN1 to save space), preproendothelin-2 (EDN2) and preproendothelin-3 (EDN3). Numbers at nodes represent the percent bootstrap (BS=500 replications). GenBank accession or Ensembl numbers: Chicken EDN1, XP_418943; chicken EDN2, XP_417707; chicken EDN3, XP_001231488; frog EDN1, AAS13535.1; frog EDN3, AAS13536.1; human EDN1, NP_001946; human EDN2, NP_001947; human EDN3, NP_000105; killifish EDN1A, EU009474; killifish EDN1B, EU009475; medaka EDN1, ENSORLP00000011633; medaka EDN2, ENSORLP00000010557; medaka EDN3, ENSORLP00000011814; mouse EDN1, NP_034234; mouse EDN2, P22389; mouse EDN3, NP_031929; rat EDN1, NP_036680; rat EDN2, NP_036681; rat EDN3, NP_001071118; salmon EDN1, BAF30875.1; Takifugu EDN2, NEWSINFRUP000000182956; Takifugu EDN3, NEWSINFRUP000000181774; Tetraodon EDN3, GSTENT00028275001; Tetraodon EDN2, GSTENT00026224001; Zebrafish EDN1, NP_571594; Zebrafish EDN2, NP_001038650. Scale bar represents the number of replacements per site.
Figure 2-2. An alignment of vertebrate preproendothelin-1 protein sequences. A) Similar amino acid residues (based on the BLOSUM 62 score table) are highlighted in gray, as compared to human preproendothelin-1. The cleavage sites for furin (▼) and ECE1 (†) are indicated. B) predicted active EDN1 structure from the killifish, modeled after Webb (1997). Amino acids different from human EDN1 are highlighted in gray. Two disulfide bonds are indicated at Cys1 to Cys15 and Cys3 to Cys10. Accession numbers are listed in Fig. 1.
Figure 2-3. Maximum likelihood analyses of the ECE family of proteins following the WAG model of amino acid substitutions (Whelan and Goldman, 2001), and a gamma distribution of 1.03. There are three distinct groups of ECE: ECE1, ECE2 and nonvertebrate ECE. Accession numbers: Archaea ECE, NP_616924; Bacteria ECE, NP_812722; chicken ECE1, NP_990048; chicken ECE2, ENSGALP00000010123; frog ECE1, AAH46653; frog ECE2, ENSXETP00000037627; fungus ECE, XP_754379; human ECE1, NP_001388; human ECE2, NP_055508; hydra ECE, AAD46624; killifish ECE1, EU009476; lancelet ECE, 86342 scaffold_150000101; locust ECE, AAN73018; medaka ECE2, ENSORLP00000025753; medaka ECE1, ENSORLP00000021332; mouse ECE1, NP_955011; mouse ECE2, NP_647454; opossum ECE1, ENSMODP00000019967; opossum ECE2, ENSMODP000000002887; platypus ECE1, ENSOANP00000023244; platypus ECE2, ENSOANP00000003016; rat ECE1, NP_446048; rat ECE2, NP_001002815; sea squirt ECE, ENSCSVP00000016300; sea urchin ECE, XP_798822; stickleback ECE1, ENSGACP0000006069; stickleback ECE2, ENSGACP0000005922; Takifugu ECE1, NEWSINFRUP000000136873; Takifugu ECE2, NEWSINFRUP000000151424; Tetraodon ECE1, GSTE0P00006535001; Tetraodon ECE2, CAG02177; zebrafish ECE1, XP_694687. Scale bar represents the number of replacements per site.
Figure 2-4. Tissue distribution of killifish *EDN1A*, *EDN1B*, and *ECE1* determined by duplexing semi-quantitative PCR with *18S* as a internal control.
Figure 2-5. Representative pictures of *in situ* hybridization of *EDN1A* and *EDN1B* mRNA in lamellar cross-sections of the seawater killifish gill. A) The *EDN1A* antisense probe was localized to epithelial cells in the interlamellar region of the gill. Little staining was seen in the gill when the *EDN1A* sense probe was used (B). C) The sodium, potassium ATPase (*NKA*) antisense probe was localized to mitochondrion-rich cells. D) *EDN1B* antisense probes bound to pillar cells and epithelial cells adjacent to the environment. Little staining was seen with the sense probe (E). F) A magnification of the pillar cell *EDN1B* staining. Scale bar = 50 μM.
Figure 2-6. Representative pictures of killifish lamellar cross-sections, labeled with anti-proendothelin-1 (brown) and anti-NKA (blue). A and B). Gill sections from a chronic (>30day) fresh water acclimated killifish, and C and D) a chronic SW acclimated killifish. B and D) were incubated in normal goat serum as a negative control for proendothelin immunoreactivity, and doubled labeled with anti-NKA (blue) to illustrate the position of the mitochondrion-rich cells (MRC) in the gill. Proendothelin immunoreactivity was found in a cell adjacent to the NKA immunoreactivity (MRC) and on gill pillar cells in both the FW and SW killifish. The immunoreactivity presented here matches the in situ hybridization of mRNA probes for EDN1s and NKA in Figures 5 and 6. Scale = 50 μM.
Figure 2-7. Acute changes in killifish gill EDN1A, EDN1B and ECE1 mRNA levels as determined by quantitative Real Time-PCR. FW to SW transfers are indicated by solid lines and circles (•) and FW to FW sham controls are represented by open circles (○) and dotted lines (A, C, E). SW to FW transfers are represented by solid lines and squares (■) and SW to SW sham controls by dotted lines and open boxes (□) (B, D, F). N=5 or 6 killifish per time and treatment and mean +/- s.e.m. are represent. Note the y axis is logarithmic. All values are normalized to L8 and standardized to chronic SW mRNA levels (Fig. 2-9). A and B) EDN1A mRNA levels, C and D) EDN1B mRNA levels and E, F) ECE1 mRNA levels. The asterisks (*) indicate statistical significance p<0.05 for that time point compared to sham value.
Figure 2-8. Chronic changes in gill \textit{EDN1A}, \textit{EDN1B} and \textit{ECE1} mRNA levels as measured by quantitative Real Time-PCR. Killifish (n = 6) were transferred from FW to SW (SW treatment, black bars) or SW to FW (FW treatment, gray bars) and sacrificed 30 days later. Mean +/- s.e.m. represented. Note the y axis is logarithmic. No significant changes in mRNA level were found between the SW and FW killifish for \textit{EDN1A}, \textit{EDN1B} or \textit{ECE1}. 
Figure 2-9. A model of paracrine and autocrine EDN1 signaling in the fish gill. Diagrammed is a lamellar cross-section (see Figs 5-7) with pillar cells (PCs) in gray, pavement cells (PVCs) and a lamellar arteriole (LA) adjacent to the interlamellar region of the gill containing mitochondrion rich-cells (MRCs), a neuroendocrine cell (NEC), and the gill vasculature. Cyclooxygenase-2 (COX-2) and neuronal nitric oxide were previously immunolocalized in the killifish gill (Choe et al., 2006; Hyndman et al., 2006). Sodium, potassium ATPase and the cystic fibrosis transmembrane conductance regulator (CFTR) are used as markers for the MRC (Katoh et al., 2001). See text for details.
CHAPTER 3
FUNCTIONAL AND GENOMIC STUDY OF THE ENDOTHELIN RECEPTORS

Introduction

Endothelins (EDNs) are small secreted peptides found in all gnathostomes (Hyndman and Evans, 2007; Yanagisawa et al., 1988a). They have many diverse physiological functions including: regulation of vascular tone (La and Reid, 1995; Yanagisawa et al., 1988a; Yanagisawa et al., 1988b); alteration of ion transport (Evans et al., 2004; Garvin and Sanders, 1991; Prasanna et al., 2001; Zeidel et al., 1989); and migration of neural crest cells (NCCs) during craniofacial development (Clouthier and Schilling, 2004; Kurihara et al., 1994). Traditionally researchers have acknowledged that EDN signals via two G protein coupled receptors (GPCRs), EDNRA and EDNRB1. These receptors are coded by separate genes, on different chromosomes (Arai et al., 1993; Hosoda et al., 1992) and have different pharmacological profiles (Table 3-1). In non-mammalian species, the classification of the EDNRs has been somewhat confusing. In the African clawed frog (Xenopus laevis) three EDNRs have been described: EDNRAx from the heart (Kumar et al., 1994); EDNRBx from the liver (Nambi et al., 1994); and EDNRC from melanophores (Karne et al., 1993). In addition, from the chicken (Gallus gallus) and quail (Coturnix japonica) an avian-specific receptor EDNRB2 was cloned and characterized (Lecoin et al., 1998). The classification of these GPCRs is generally based upon sequence homology and pharmacological profiles (Table 3-1) compared to the mammalian EDNRA and EDNRBs. For example, EDNRB2 was named such because the primary sequence was more closely related to human EDNRB1 than EDNRA or frog EDNRC; however it was pharmacologically different from human EDNRB1 (Lecoin et al., 1998). This classification system depicts a series of species-specific EDNRs in non-mammalian gnathostomes; however, our knowledge of non-mammalian EDNRs is quite limited. A thorough phylogeny of this gene family would help
elucidate the evolutionary relationships among these receptors, and it will help us better classify the receptors. For example, it will determine if frog EDNRAx is an orthologous or paralogous gene of the mammalian EDNRA.

The majority of EDN research has been in the biomedical field, because numerous pathologies have been linked to problems with EDN signaling. These conditions include hypertension, atherosclerosis (Shreenivas and Oparil, 2007), congestive heart failure (Angerio, 2005), and glomerulonephritis (Richter, 2006). The use of mammalian and non-mammalian model organisms has been imperative in understanding EDN signaling cascades. For example, experiments using chicks (Gallus gallus) (e.g. Kanzawa et al., 2002; e.g. Miller et al., 2003; Nagy and Goldstein, 2006) and zebrafish (Danio rerio) (e.g. Clouthier and Schilling, 2004; Kimmel et al., 2003; Miller et al., 2000; Miller et al., 2003; e.g. Nair et al., 2007; Walker et al., 2006) have highlighted the importance of EDN in embryonic development. Yet, given our knowledge of EDN signaling during development and mammalian physiology, we know comparatively little about the function of EDN in non-mammalian adult organisms.

We recently described the phylogenetic relationships among the EDNs (Hyndman and Evans, 2007), and explored the effects of environmental salinity on EDN gene expression from the euryhaline teleost, the common killifish (Fundulus heteroclitus). The killifish is a model organism used in a diverse range of studies including: ecological, epidemiological, evolutionary, physiological, and toxicological (Burnett et al., 2007). In addition, they have been valuable in understanding drug transport by multi-drug resistance proteins (Masereeuw et al., 2000; Notenboom et al., 2005; Notenboom et al., 2004; Terlouw et al., 2001). These fish are abundantly distributed in the estuaries along the eastern coast of the US (Bigelow and Schroeder,
2002; Marshall, 2003), and because they are small (~3 inches) and easily maintained in aquaria, they are an ideal species to use in laboratory experiments.

The purposes of this study were to: 1) organize the classification of the gnathostome EDNRs; 2) complete a thorough phylogenetic analysis of the EDNRs; and 3) determine if there are functional shifts between the therian mammal and non-therian EDNRS. Using the killifish as our model organism, we sequenced cDNA for three EDNRs and putatively called them EDNRA, EDNRB1, and EDNRB2. From our complete phylogenetic analysis, we determined there are three distinct groups of gnathostome EDNRs: EDNRA, EDNRB1 and EDNRB2. Interestingly, we did not find an EDNRB2 orthologue from any therian mammal, suggesting a loss of this receptor in this lineage. We used synteny to determine if the therian EDNRB2 was lost due to mutation or deletion. To understand better the potential consequence of not having EDNRB2 in the therians, we determined the replacement rate at each amino acid position, and determined if there were significant changes these rates between the therian and non-therian gnathostomes. We then mapped these sites to known regions of the EDNRA and EDNRB1 that are necessary for EDN binding, and tested the hypothesis that therian EDNRS have undergone rapid remodeling of functionally important sites as a consequence of losing EDNRB2. Our results suggest that therian EDNRA have been remodeled, but that EDNRB1 has remained conserved throughout gnathostome evolution.

Methods

Molecular Cloning, Sequencing, and Tissue Distribution

The RT-PCR, cloning, and sequencing protocols of Hyndman and Evans (2007) were used to sequence cDNA for the killifish EDNRs. Degenerate primers were designed against highly conserved regions for all EDNRs and are recorded in Table 3-2. Initial sequences were identify using homology and BLAST (Wheeler et al., 2007). To determine the tissue expression of the
EDNRs in an adult gnathostome, random hexamer cDNA was made from RNA extracted from the opercular epithelium, gill, brain, heart, stomach, intestine and kidney of an adult killifish as previously described (Hyndman and Evans, 2007). Gene specific primer pairs were designed to amplify a product with high efficiency (e.g., high melting temperature), and to minimize the chance of amplifying contaminating genomic DNA, the primer pair was designed to include at least one exon-exon boundary when possible (Table 3-3). Multi-tissue duplexing-PCR was run using gene-specific primers and QuantumRNA™ 18S internal standard primers (Ambion, Woodward Austin, TX) to control for variability in cDNA quality and quantity among the different tissues. The duplexing-PCRs were optimized to ensure they were terminated during the exponential phase. The products were visualized by ethidium bromide staining in 1.5% agarose gels and digitized using the Biorad Gel Doc™ XR System.

**Data Mining and Multiple Sequence Alignment**

We used sequence homology to find all of the EDNRs in GenBank (Wheeler et al., 2007) and the completed genomes in Ensembl (e! 44, April 2007) (Hubbard et al., 2007; Spudich et al., 2007). A number of BLAST searches were completed using the following query sequences: Killifish EDNRA (EU391601); Killifish EDNRB1 (EU391602); killifish EDNRB2(EU391603); Chicken EDNRB2 (NP_989451); Frog EDNRC (P32940); Human EDNRA (NP_001948; and Human EDNRB (NP_000106). This collection of sequences was refined to include only a single protein sequence for EDNR per species. If there were splice variants, only the longest one was included in the dataset. We preferentially selected RefSeq sequences over others because these sequences are curated. Finally, any therian species that did not have complete sequence data for EDNRA and EDNRB1 was not included in the analysis. With these criteria, we were left with a
phylogenetically enriched (species and protein) dataset, while limiting the size to run a complete and thorough phylogenetic analysis.

A recent phylogenetic analysis of a portion of human GPCR was completed, and the results suggest that the endothelin B receptor-like proteins (GPR37 and GPR37L) are the outgroup to the EDNRs (Fredriksson et al., 2003). Using the aforementioned protocol we compiled a list of sequences to include as the outgroup. All the sequences were aligned with CLUSTALX (Chenna et al., 2003; Larkin et al., 2007) and visually inspected to ensure no gaps were inserted into known structural regions (i.e. transmembrane domains) (Pollock and Highsmith, 1998).

**Phylogenetic Analyses**

The phylogenetic relationships among the EDNRs were determined by fast maximum likelihood analyses (ML) with PHYML (Guindon et al., 2005). The evolutionary model used was the Whelan and Goldman (WAG) model for amino acid replacements (Whelan and Goldman, 2001), with a free gamma distribution parameter optimized using eight rate categories to account for rate heterogeneity across positions. The proportion of invariable sites was calculated as zero during likelihood analyses, thus this parameter was excluded from the final analysis. The robustness of the ML tree was evaluate by non-parametric bootstrapping = 1000. This analysis was performed with and without the outgroup, and we determined that when the outgroup was included, there was a destabilization of the optimal tree. This was likely because of the great divergence time among the sequences and this issue was recently discussed by (Shavit et al., 2007). Thus, we ran the ingroup (study group) and outgroup ML separately and tried Lundberg rooting (Lundberg, 1972); a method of rooting the study group, after you run your analysis.
In addition to our ML analysis, we performed a Bayesian analysis (BA) with MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with our study group only. Using the same evolutionary model as in our ML analysis, we ran a Monte Carlo Markov Chain using 3 hot chains and 1 cold chain (Huelsenbeck et al., 2001), for 20 million generations, with a sample frequency of 500, and a burnin of 10%. The 50% majority consensus tree was compiled.

**Synteny**

Using NCBI’s Mapviewer (Wheeler et al., 2007) and Ensembl (Hubbard et al., 2007; Spudich et al., 2007), we determined the location of *EDNRB2* in the *Gallus gallus* (Chicken) genome. This gene is on chromosome 4, at bp 11255609-11264791. The genes surrounding *EDNRB2* are listed in figure 4. These include, upstream *trimethyllysine hydroxylase, epsilon* (*TMLHE*), and downstream *RNA polymerase I 16kDa polypeptide (RPA16)* and *synaptobrevin-like 1 (SYBL1)*. We then used these genes as markers for the position of *EDNRB2*, and searched for them in the Western clawed frog, platypus, opossum, mouse, rat, macaque, and human genomes. Only in the Western clawed frog, platypus, opossum, macaque, and human genomes were some of these genes mapped, so we restricted our searches to these organisms. Using this gene order, we calculated gene distances among our aforementioned markers. In the opossum, macaque, and human genomes we did not find *EDNRB2*. We preformed BLAST searches using any unidentified loci or pseudogenes from these genomes to determine if they had any significant homology to the *EDNRB2*.

**Rate Shift Analyses**

To test the significance of losing the *EDNRB2* to the therians, we tested the hypothesis that the remaining therian EDNRs have undergone changes at the sequence level related to functional shifts. This was determined using Knudsen and Miyamoto’s (2003) rate shift analysis (RSA). The RSA involved likelihood-ratio tests (LRT), on a site-by-site basis, to test for two types of
rate shifts in a gene family: Type I or Type II (Fig.3-1). As illustrated in Figure 3-1, a Type I sites is defined as a homologous position that is fixed for one subfamily, but variable in the second subfamily. A type II site is a homologous site that is fixed for different amino acids in both subfamilies (Fig. 3-1). This analysis also tests for slow evolving sites (conserved) between both subfamilies. We defined our two subfamilies based upon the split of the last gnathostome lineage where we found EDNRB2 and the lineage where it was putatively lost; 1) all the gnathostomes basal to the loss of EDNRB2 (thus the platypus to teleosts), and 2) all the therian mammals (opossum to human). Using the EDNRA sequences from our multi-sequence alignment, and the EDNRA grouping from the ML tree (Fig. 3-3), the RSA program developed by Knudsen et al. (2003), was used to determined the number of Type I and/or II (Type I/II) and conserved sites. This analysis was repeated using the EDNRB1 portion of the multi-sequence alignment, and EDNRB1 grouping from the ML tree (Fig. 3-3).

From the literature, we compiled a list of functionally important sites necessary for EDN1 binding (the endogenous ligand) for EDNRA (Adachi et al., 1993; Breu et al., 1995) and EDNRB1 (Wada et al., 1995). From those studies, we determined that EDNRA has a specific set of 26 amino acids (T50-I70, position based on human sequence ,Fig. 3-5A) in the N-terminal necessary for EDN1 binding (Adachi et al., 1993). In addition, site mutagenesis studies determined that G97, K140, K159, Q165, and F315 are necessary for EDN1 binding (Breu et al., 1995). EDNRB1s have a region of 60 amino acids, I138 to I197) that are necessary for EDN1 binding (Wada et al., 1995) (Fig. 3-5B).

Results

Killifish Endothelin Receptors

We initially sequenced three EDNR cDNAs from the killifish gill using degenerate primers made against conserved regions of the vertebrate EDNRs. These sequences were compared,
using sequence homology and BLAST, to the known gnathostome EDNRs, and determined that we had sequenced killifish: *EDNRA* (2543 bp), *EDNRB1* (1629 bp), and *EDNRB2* (1470 bp). These sequences were searched for open reading frames (ORF) using BioTools (Edmonton, Alberta) ORF tool and compared to other EDNRs. The putative killifish EDNRA has an ORF of 1278 bp, EDNRB1 1251 bp, and EDNRB2 1242 bp. From our phylogenetic analysis (see below), our putative killifish EDNRs (protein sequence) form groups with the other gnathostome EDNRA, EDNRB1 and EDNRB2, respectively (Fig. 3-3). Killifish EDNRA (EU391601) shares 55% amino acid identity to the killifish EDNRBs and is 63% identical to human EDNRA. Killifish EDNRB1 (EU391602) is 63% identical to killifish EDNRB2, and 70% identical to human EDNRB1. Killifish EDNRB2 (EU391603) is 63% identical to human EDNRB1 and 68% identical to quail EDNRB2.

The tissue distribution of the EDNRs in the adult killifish was determined, and *EDNRA* and *EDNRB2* were ubiquitously expressed in the opercular epithelium, gill, brain, heart, stomach, intestine, and kidney (Fig. 3-2). *EDNRB1* was found in all those tissues except the heart. *EDNRA* had relatively high expression in the heart and kidney, *EDNRB1* was highest in the gill, brain, and kidney, and *EDNRB2* was highest in the gill, brain, heart, and kidney (Fig. 3-2).

**Phylogeny**

Initially, we tried to root our phylogenetic tree using Lundberg rooting (Lundberg, 1972). We artificially placed the outgroup ML tree to each of the branches leading to the three EDNR groups (therefore, three artificial trees), and determined the ML score of each of these artificial trees (arrows in Fig. 3-3 depict the artificial roots). All three ML scores were very similar; however, the outgroup branching from the EDNRB2 grouping was slightly better, thus to test the statistical significance of this branch we used an approximate likelihood ratio test (PHYML-aRLT). The analysis determined that there was weak support for this branch (probability =
0.270); thus, we restricted our analyses to the study group, because of the inability to robustly root the outgroup.

By using homology and BLAST searches of NCBI’s Protein database, and the completed genome projects (in Ensembl e!44, April 2007), we did not find any EDNR in any animal basal to the teleost fishes. In addition, when EDNRB2 or EDNRC sequences were queried (see above) in BLAST, we found frog EDNRC, all the non-therian EDNRs, but the only therian sequences we found were EDNRA and EDNRB1. Thus, we conclude that if therian EDNRB2 or EDNRC were present, we would have found them. The results of our complete phylogenetic analyses of the gnathostome EDNRs are depicted in Figure 3-3. The log-likelihood of this tree was -18202, with a gamma distribution parameter calculated as 0.617. Our bayesian tree was very similar to this tree, and the percent posterior probabilities of each node are recorded in Figure 3-3. There were three, distinct groups found, representing the EDNRA, EDNRB1 and EDNRB2. The support for these groups was high (bootstrap> 99%, and posterior probability=100%). Within each group, the majority of the nodes followed the classic species relationships (i.e. human and chimp grouped together, outside of the macaque, Fig. 3-3).

The EDNRA group contains a set of teleost-specific EDNRA gene duplications, likely the result of a teleost specific genome duplication that occurred after their split from the tetrapods (Volff, 2005). We named these duplicates EDNRA2 (Fig. 3-3). The African clawed frog EDNRAx (Kumar et al., 1994) grouped with 60% support to the African clawed frog EDNRA. These also grouped with the Western clawed frog EDNRA, and this is outside of the rest of the tetrapod EDNRA, and teleost EDNRAs. We have termed these proteins EDNRA (AAH44316) and EDNRA2b (AAA19570.1), because they are of the A subtype, but the duplicate is independent of the duplicate EDNRA2s found in the teleost fishes.
The EDNRB1 group, also contains a group of teleost-specific EDNRB1 gene duplications, and we have named them EDNRB1b (Fig. 3-3). Interestingly, we found other duplicate EDNRB1s; the zebrafish has a duplicated EDNRB1b protein we have termed EDNRB1c, and the opossum has a duplicate EDNRB1 that groups with the opossum EDNRB1, outside of the teleost EDNRB1bs. We have termed this protein EDNRB1d, and it appears to be an opossum specific duplicate; no other therian mammal has a duplicate EDNRB1. The Western clawed frog EDNRB grouped with the tetrapod EDNRB1, suggesting it is an orthologous gene. We did not find an African clawed frog EDNRB1.

Unlike EDNRA and EDNRB1, we did not find a teleost-specific duplication of the EDNRB2; however, we did find a stickleback specific EDNRB2 duplicate (named EDNRB2b) (Fig. 3-3). To our surprise, we did not find a zebrafish EDNRB2, suggesting that they have lost this receptor. Interestingly, the African clawed frog EDNRC grouped with 100% support with the African clawed frog EDNRB2 (Fig. 3-3), suggesting it is a duplicate receptor. Again, we were unable to find a therian EDNRB2 orthologue.

**Synteny**

Using the gene order of *TMLHE, EDNRB2, RPA16,* and *SYBL1* as determined by the chicken genome (chromosome 4), we searched the other vertebrate genomes for this same gene order. Similar gene orders were found on the Western clawed frog (scaffold 258), platypus (Ultra519), opossum (chromosome X), macaque (chromosome X) and human (chromosome X). The total gene distances from the end of *TMLHE* to the beginning of *SYBL1* were calculated and the chicken genome has the smallest distance, followed by the opossum, Western clawed frog, macaque, human, and finally the platypus has the largest distance (Fig. 3-4). Other genes located between these markers in the therian genomes include some pseudogenes; however, none of
these regions of DNA showed homology to the *EDNRB2*. In addition, we identified some unnamed loci using homology and BLAST searches (Fig 3-4. asterisks).

**Rate Shift Analysis**

The results from the rate shift analysis (RSA) are depicted in Figures 5A and B. For EDNRA, between the therian and non-therian gnathostome subfamilies, there were 35 Type I/II and 119 conserved positions out of a total of 425 informative sites. Based upon published mutagenesis and bind domain experiments completed in therians, we determined that EDNRAs have 31 functionally important sites necessary for EDN1 binding (Adachi et al., 1993; Breu et al., 1995). As tabulated in Table 3-4A, 6/31 functionally important positions were type I/II sites, and 5/31 were conserved, functionally important positions (Table 3-4C). All of these 6 type I/II sites were located within one, consecutive region of 26 amino acids that is known to be necessary for EDN1 binding in therians (Adachi et al., 1993). We determined there was a greater than expected concentration of the 6 type I/II sites within this 26 amino acid binding region (the G-test was used because it is less sensitive than the Chi-squared to small column totals, $G= 4.54, p=0.022$) (Table 3-4B). In addition, there were significantly less conserved sites within this 26 amino acid region ($2/26$) than expected ($G=35.34, p<0.0001$) (Table 3-4C).

With the EDNRB1, between the therian and non-therian gnathostomes, there were 70 type I/II and 221 conserved positions out of a total of 567 informative sites. Wada et al. (1995) determined that a region of 60 amino acids was necessary for EDN1 binding, thus these sites were considered functionally important. Within this region of 60 positions, the RSA determined there was only 1 type I/II site, and 49 conserved sites (Fig. 3-5B, Table 3-4A, C). This was a significantly lower than expected number of type I/II sites in this functionally important region (Table 3-4A, $G=6.50, p=0.01$) and a significantly higher number of conserved sites (Table 3-4C, $G=35.34, p<0.0001$).
Discussion

Here we present three new EDNR sequences from the adult killifish, a commonly used model organism in a variety of physiological, ecological and epidemiological studies (Burnett et al., 2007). We are the first to determine the tissue distribution of all three EDNRs from an adult animal; all other studies have looked at the three EDNRs distribution during embryonic development (e.g. Lecoin et al., 1998; Nataf et al., 1996) or distribution of EDNRA and EDNRB1 in mammals (Molenaar et al., 1993; e.g. Ogawa et al., 1991). All three EDNR mRNAs are ubiquitously expressed in the adult killifish tissues tested, with the exception of EDNRB1 that was not in the killifish heart. These expression patterns are similar to those described in mammals, where the EDNRA is the predominant form in the heart (Molenaar et al., 1993), and the EDNRB1 is highly expressed in the brain, lung, and kidney (Ogawa et al., 1991). The fish gill is the main site of nitrogen excretion, and gas exchange (Evans et al., 2005), functioning like the mammalian kidney and lung, so it is not surprising that EDNRs are highly expressed in the gill.

This study is the first to complete a thorough phylogenetic analysis of the gnathostome EDNRs. There are three, distinct groups of EDNRs representing the EDNRA, EDNRB1 and EDNRB2/EDNRC (Fig. 3-3). In addition, there appears to be a teleost-specific duplication of the EDNRA and EDNRB1, that is likely a result of the teleost specific genome duplication after their split from the tetrapods (Volff, 2005). The EDNRs are gnathostome specific, with no orthologous genes in the early chordates (Ciona intestinalis, Branchiostoma floridae). These results are in agreement with our recent analysis of the evolution of the EDNs. We could not find a homologous EDN in any animal basal to the teleost fishes (Hyndman and Evans, 2007). Physiological studies strongly suggest that these EDN and the EDNRs are present in the early vertebrates: hagfish, lamprey, and cartilaginous fishes (Evans et al., 1996; Evans and Gunderson,
1999; Evans and Harrie, 2001); however until these genomes are sequenced and fully annotated, it is unclear if they have all three EDNRs. Preliminary searching of the ratfish and lamprey genomes, found small fragments of some highly homologous regions of the EDNRS, but these fragments are too small to determine if they are EDNRA, EDNRB1 or EDNRB2. When these genomes are completed, it will help to elucidate the question of the origin of the EDNRs.

Previously, a “novel” African clawed frog EDNRAx was characterized from the heart (Kumar et al., 1994). As seen in Figure 3-3, this receptor is a duplicate EDNRA, grouping with a second EDNRA from the African clawed frog. This also groups with the Western clawed frog, and the other tetrapod EDNRAs. Searching of the Western clawed frog genome revealed only one EDNRA gene, suggesting the duplicate EDNRAx may be specific to the African clawed frog and not the whole genus (*Xenopus*). We propose this receptor should be reclassified as EDNRA2b.

The “novel” African clawed frog EDNRBx, described by Nambi et al. (1994) was based upon pharmacology, and not sequence data. From our BLAST searches, we could not find a homologous African clawed frog EDNRB1; however, we did find an EDNRB1 from the Western clawed frog. Thus, it is possible that Nambi et al. (1994) were characterizing the African clawed frog EDNRB2 and not a novel EDNRBx. The EDNRB2 has been called “the avian-specific” EDNR (Lecoin et al., 1998), but this statement is clearly not true. The EDNRB2 is found in all non-therian gnathostomes (for which we have sequence data) except the zebrafish (Fig. 3-3). We also found a duplicate stickleback EDNRB2 (EDNRB2b), but unlike the EDNRA and EDNRB1 (Fig. 3-3), we did not find a teleost specific duplication of the EDNRB2. The African clawed frog EDNRC, first described by Karne et al. (1993), groups with high support to the African clawed frog and Western clawed frog EDNRB2 and non-therian EDNRB2s, suggesting it is a
duplicate of the EDNRB2 gene. The name EDNRB2 is a bit of a misnomer. Lecoine et al. 1998 originally termed it because the “novel” quail EDNR sequence was more similar on an amino acid level EDNRB1 than EDNRA or EDNR, but it had a distinct pharmacological profile (Table 3-1). The EDNRB2 is a distinct receptor, coded by its own gene, on a different chromosome from EDNRA and EDNRB1 (Wheeler et al., 2007) and groups with the frog EDNR. The term EDNR would be more appropriate for this gene, emphasizing the independence of this receptor from EDNRA and EDNRB1. The majority of these “novel” EDNR discoveries were before the advent of genome projects and high throughput sequencing, so it is not surprising that we are reclassifying some of these receptors based upon molecular and phylogenetic data. These early studies do highlight the important point that not all EDNRs have the same pharmacological profile; however, we argue that does not necessarily mean they are novel. A common assumption is that all gnathostome EDNRs will be pharmacologically identical to the mammalian EDNRA and EDNRB1. This is not the case (see Table 3-1) and caution should be used when describing the basal gnathostome receptors in this fashion.

One of our most interesting discoveries was the lack of EDNRB2 sequences from the therian mammals. We exhaustively searched GenBank and the therian genome projects, and could only find EDNRA and EDNRB1, suggesting that EDNRB2 was lost about 150 MYA. Interestingly, we did not find a zebrafish or opossum EDNRB2, but both of these animals have a duplicate EDNRB1 (opossum EDNRB1d and zebrafish EDNRB1c) (fig. 3), leading us to question whether the initial loss of the EDNRB2 lead to a duplication of the EDNRB1 in these organisms?

To determine if EDNRB2 was lost by mutation or deletion, we used the conserved gene order of genes that surround EDNRB2: TMLHE, RPA16, and SYBL1. This gene order was
similar in the Western clawed frog, chicken, and platypus genomes. A fourth gene, \textit{sprouty 3} (\textit{SPRY3}) was found after TMLHE in the chicken, but was not found in the frog or platypus contigs. In the opossum, macaque, and human genomes \textit{TMLHE}, \textit{SPRY3}, and \textit{SYBL1} were conserved. In addition, in the therians, other genes and pseudogenes were found between \textit{TMLHE} and \textit{SYBL1}. In the opossum, the genes at loc100024257 and loc100024284 were not homologous to any other vertebrate genes. The pseudogenes (loc100024309 and loc100024329) were similar to \textit{alpha-tubulin}, \textit{clef lip and palate associated transmembrane protein 1}, and \textit{coiled coil domain containing 127}. In the macaque and human, in the approximate region where \textit{EDNRB2} would be expected to be located, there is a gene, \textit{AMDP1}. None of these proteins from the therians share any common characteristics with the GPRC with seven transmembrane domains. Thus, this region appears to be one of active chromosomal change, resulting in the loss/gain of genes. We therefore hypothesize that \textit{EDNRB2} was lost in the therians and has not simply mutated to an unrecognizable state.

The loss of the \textit{EDNRB2} from the therian mammals is intriguing, and we hypothesized that perhaps the remaining therian EDNRs have undergone shifts in replacement rates of amino acids that are necessary for EDN binding. If true, this would suggest a functional shift in these receptors, perhaps resulting in a new range of functions for these receptors to compensate for the loss of \textit{EDNRB2}. Using an analysis of the site-by-site replacement rate, we determined that therian EDNRAs have more Type I/II sites in a region necessary for EDN1 binding (Fig.3-5A). On contrary, the therian EDNRB1 is highly conserved across the gnathostomes (Fig. 3-5B). These results suggest that therian EDNRA have been remodeled, while EDNRB1 have remained conserved. Thus, we hypothesize that EDNRA have different functions in therians compared to non-therian gnathostomes. Our results set up a framework for future comparative studies among
the therians and non-therians to help elucidate the potential functional differences of the EDNRs in these animals. One limitation to EDN research is that there is little information about the function of EDNRs in the non-therian gnathostomes. All EDNR antagonists and agonists are based upon mammalian studies, and as summarized in Table 1, not all EDNRs share a common binding profile, making this a complex system to understand. Studies determining the physiological role of the EDNRS in non-therian gnathostomes are greatly needed.

In conclusion, the majority of gnathostomes have three EDNRS: EDNRA, EDNRB1, and EDNRB2; however, the therian mammals have lost the EDNRB2. Also, teleost fishes have duplicate EDNRA and EDNRB1 genes that are co-orthologous to single copy tetrapod EDNRA and EDNRB1, and this may be a remnant of a teleost specific genome duplication after the split from the tetrapods (Volff, 2005). The EDNRB2 has been largely ignored in the literature. In order to understand vertebrate evolution, more EDN/EDNR research is necessary, because this signaling system is thought to be a key innovation in the radiation of vertebrates and the development of jaws (Clouthier and Schilling, 2004). In addition, in the future caution should be used in extrapolating findings in model, non-therian organisms, since there is clearly heterogeneity between the presence and expression of the EDNRs.
Table 3-1. A summary of the binding profiles, and embryonic and adult tissue distribution of the gnathostome EDNRs. EDN= endothelin, NCC= neural crest cell, SRXc= sarafotoxin 6c, ? = undetermined.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Binding Profile</th>
<th>Embryonic Tissue Distribution</th>
<th>Adult Tissue Distribution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td></td>
<td>EDN1=EDN2&gt;&gt;&gt;EDN3</td>
<td>?</td>
<td>Ubiquitous</td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td></td>
<td>EDN1&gt;END2&gt;&gt;&gt;EDN3&gt;&gt;SRXc</td>
<td>?</td>
<td>Ubiquitous</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td>EDN1=EDN2&gt;&gt;&gt;EDN3</td>
<td>NC-derived ectomesenchyme, branchial arches</td>
<td>melanocytes, lung*</td>
<td>Kempf et al. 1998, Scarparo et al. 2007, Gomez et al. 2007</td>
</tr>
<tr>
<td>Quail</td>
<td>Western</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clawed</td>
<td>EDN1&gt;&gt;&gt;EDN3&gt;&gt;&gt;SRXc</td>
<td>?</td>
<td>oocyte follicle, lung, heart*</td>
<td>Kumar et al. 1993, Kumar et al. 1994</td>
</tr>
<tr>
<td>EDNRAx</td>
<td>Zebrafish</td>
<td>?</td>
<td>migrating NCCs, ectomesenchymal cells of pharyngeal arches</td>
<td>?</td>
<td>Clouthier and Schilling 2004</td>
</tr>
<tr>
<td>EDNRB1</td>
<td>Human</td>
<td>EDN1=EDN2=EDN3 = SRXc</td>
<td>Ubiquitous</td>
<td>Ubiquitous</td>
<td>Brand et al. 1998, Ogawa et al. 1991, Sakamoto et al. 1991</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td>EDN1=EDN2=EDN3 = SRXc</td>
<td>?</td>
<td>Ubiquitous</td>
<td>Hori et al. 1992, Sakauri et al. 1990, Kozuka et al. 1991</td>
</tr>
<tr>
<td>Cow</td>
<td></td>
<td>EDN1=EDN3, EDN2? SRXc?</td>
<td>?</td>
<td>Lung*</td>
<td></td>
</tr>
<tr>
<td>Western</td>
<td>Clawed</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>frog</td>
<td>EDNRB1</td>
<td>Zebrafish</td>
<td>NCC (melanocytes), xanthophore and iridophore precursor cells, ?</td>
<td>Parichy et al. 2000</td>
</tr>
<tr>
<td>EDNRB2</td>
<td>Chicken</td>
<td>EDN1=EDN2=EDN3 &gt;&gt;&gt;SRXc</td>
<td>NCC migrating in dorsolateral pathway, kidney, skin, feather buds</td>
<td>?</td>
<td>Nataf et al. 1996, Lecoin et al. 1998</td>
</tr>
<tr>
<td>Quail</td>
<td></td>
<td>EDN1=EDN2=EDN3 &gt;&gt;&gt;SRXc</td>
<td>NCC migrating in dorsolateral pathway, kidney, skin, feather buds</td>
<td>?</td>
<td>Nataf et al. 1996, Lecoin et al. 1998</td>
</tr>
<tr>
<td>Protein</td>
<td>Species</td>
<td>Binding Profile</td>
<td>Embryonic Tissue Distribution</td>
<td>Adult Tissue Distribution</td>
<td>References</td>
</tr>
<tr>
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<td>--------------------------</td>
<td>----------------------</td>
<td>-------------------------------</td>
<td>---------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>EDNRBx</td>
<td>Western Clawed frog</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Nambi et al. 1994</td>
</tr>
<tr>
<td></td>
<td>Zebrafish</td>
<td>?</td>
<td>?</td>
<td>Liver*</td>
<td></td>
</tr>
<tr>
<td>EDNRC</td>
<td>Western Clawed frog</td>
<td>EDN3&gt;EDN1, EDN2?</td>
<td>?</td>
<td>Melanophores*</td>
<td>Karne et al. 1993</td>
</tr>
</tbody>
</table>

*these are the only tissues tested thus far
> represents one order of magnitude
Table 3-2. Degenerate and non-degenerate primers used to sequence the killifish endothelin receptors.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' to 3' Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDNRF1</td>
<td>AGC CGA CAG AGA TTA GAT ACT CCT TYA ART AYR</td>
</tr>
<tr>
<td>EDNRF2</td>
<td>CGC TAC TAC GAA TTA TTT ATC AGA ACA ART GYA TGM G</td>
</tr>
<tr>
<td>EDNRF3</td>
<td>GCA GTT CTA TCA GGA CGC GAA RGA YTG GTG G</td>
</tr>
<tr>
<td>EDNRR1</td>
<td>TTT GGA AAC CAA GTA TAG TTG CTA TAG GRT TDA TRA C</td>
</tr>
<tr>
<td>EDNRR2</td>
<td>TCT TCC ACT GGA TGG AGG TGM CRT TNA YNG G</td>
</tr>
<tr>
<td>EDNRB1R1</td>
<td>AGC TTC ATG CAG GAC TGC TTN TCR TCC AT</td>
</tr>
<tr>
<td>exEDNRAR1</td>
<td>GCA GAA GAC AGC TTT GGC AAC</td>
</tr>
<tr>
<td>exEDNRAR2</td>
<td>TTT CAG GTG TTC ACT GAG CGC</td>
</tr>
<tr>
<td>rEDNRAF1</td>
<td>TCC CTT TGC ACC TCA GCA GGA TCC</td>
</tr>
<tr>
<td>rEDNRAF2</td>
<td>ATT ACT TCG GCA TCA ACC TGG CGA C</td>
</tr>
<tr>
<td>rEDNRAF4</td>
<td>GGC ATC AAC CTG GCG ACA ATC AAC T</td>
</tr>
<tr>
<td>rEDNRAF2</td>
<td>ATT ACT TCG GCA TCA ACC TGG CGA C</td>
</tr>
<tr>
<td>rEDNRAR1</td>
<td>TTC CGG TGG TTC ATC TCA CA</td>
</tr>
<tr>
<td>rEDNRB1F1</td>
<td>TGG TGT CTC TGT GCT GGC TTC C</td>
</tr>
<tr>
<td>rEDNRB1F2</td>
<td>AAC CGC TGC GAG CTG CTC AGT TCC T</td>
</tr>
<tr>
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<td>ACG CCG ATG CCT TTG ATT CGA CTC C</td>
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<td>rEDNRB1R2</td>
<td>CCC CAC GGA CGC TTT CTG GAC AA</td>
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<tr>
<td>rEDNRB2F1</td>
<td>GCA TGC GGA TCG CAC TGA ACG ATC</td>
</tr>
<tr>
<td>rEDNRB2F2</td>
<td>CTC AGC CGC ATC CTG AAG AAA ACC TT</td>
</tr>
<tr>
<td>rEDNRB2R1</td>
<td>CCGTTCTCATGCACTTTGTCTGTAAG</td>
</tr>
<tr>
<td>rEDNRB2R2</td>
<td>CCACGAAGATCACAGGCAAGAAATGATGGA</td>
</tr>
<tr>
<td>Primer</td>
<td>5' to 3' Orientation</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>tdEDNRAF1</td>
<td>AAA AGC CCG GAA CCC AAC A</td>
</tr>
<tr>
<td>tdEDNRAR1</td>
<td>GGA CAT CAT TCT CCC TGA CAG C</td>
</tr>
<tr>
<td>tdEDNRB1F1</td>
<td>TTC TGG ACA AAC GCT CCG T</td>
</tr>
<tr>
<td>tdEDNRB1R1</td>
<td>TCC CGC CGC TGC TTA ATA T</td>
</tr>
<tr>
<td>tdEDNRB2F1</td>
<td>TGT GCC CTG AGC ATT GAC C</td>
</tr>
<tr>
<td>tdEDNRB2R1</td>
<td>AAC ACC GTC TTC GCC ACT TC</td>
</tr>
</tbody>
</table>
Table 3-4. Contingency tables summarizing the results of the rate shift analysis (Fig. 5A and B). Observed (expected) numbers recorded. Significance was determined using the G-test and Chi-squared distribution (see text).

<table>
<thead>
<tr>
<th></th>
<th>Type I/II at functionally important positions</th>
<th>Type I/II at other positions</th>
<th>Type I/II Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDNRA</td>
<td>6 (2.3)</td>
<td>29 (32.6)</td>
<td>35</td>
</tr>
<tr>
<td>EDNRB1</td>
<td>1 (4.7)</td>
<td>69 (65.3)</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>98</td>
<td>105</td>
</tr>
<tr>
<td>B</td>
<td>Type I/II sites in the EDNRA</td>
<td>Non type I/II sites in the EDNRA</td>
<td>Total</td>
</tr>
<tr>
<td>EDN1 Binding Domain</td>
<td>6 (2.1)</td>
<td>20 (23.8)</td>
<td>26</td>
</tr>
<tr>
<td>Other sites</td>
<td>29 (32.8)</td>
<td>370 (366.1)</td>
<td>399</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>390</td>
<td>425</td>
</tr>
<tr>
<td>C</td>
<td>Conserved sites at functionally important positions</td>
<td>Other sites at functionally important positions</td>
<td>Total</td>
</tr>
<tr>
<td>EDNRA</td>
<td>5 (18.6)</td>
<td>26 (12.7)</td>
<td>31</td>
</tr>
<tr>
<td>EDNRB1</td>
<td>49 (36)</td>
<td>11 (24.7)</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>37</td>
<td>90</td>
</tr>
</tbody>
</table>
Figure 3-1. An illustration of A) type I and B) type II sites determined by a rate shift analysis (Knudsen et al., 2003). Depicted is the phylogenetic relationship of a homologous site between two subfamilies: therian mammals and non-therian gnathostomes. Arrows indicate rerooting of the tree along the internode connecting therians to other non-therian gnathostomes.
Figure 3-2. mRNA tissue distribution of the killifish *EDNRA*, *EDNRB1*, and *EDNRB2* using duplexing, multi-tissue PCR. *18S* primers were used as an internal control gene and the *18S* amplicon displayed equal intensity for each tissue.
Figure 3-3. A maximum likelihood tree of the gnathostome EDNRs. Numbers at the arrows represent the maximum likelihood score for the attachment of the outgroup to the maximum likelihood study group tree (see text for explanation). The numbers at the nodes represent percent bootstrap/posterior probability from our bayesian analysis. Nodes that were not represented in our Bayesian analysis are represented by (-). Green represents EDNRA, light green EDRNA2, blue EDNRB1, light blue EDNRB1b and red EDNRB2. GB= GenBank and EN= Ensembl.
Figure 3-4. Genetic maps of the genes (defined by NCBI) surrounding the EDNRB2 in non-therian gnathostomes, and hypothesized location in the therians. * represent gene identities identified by our analyses. # represents pseudogenes as defined by NCBI, and ? represents sequences with no similarity to any other vertebrate gene (as determined by BLAST searches)
Figure 3-5. Illustration of the human A) EDNRA and B) EDNRB1. Residues necessary for endothelin-1 binding are highlighted in red. See text for explanation.
CHAPTER 4
EFFECTS OF ENVIRONMENTAL SALINITY ON GILL ENDOTHELIN RECEPTOR
EXPRESSION IN THE KILLIFISH FUNDULUS HETEROCLITUS

Introduction

The endothelin (EDN) family of paracrine peptides consists of three isoforms, EDN1, EDN2 and EDN3, (Inoue et al., 1989; Yanagisawa et al., 1988a), and is found only in vertebrates (Hyndman and Evans, 2007). These proteins are involved in diverse physiological functions including: regulation of vascular tone (La and Reid, 1995; Yanagisawa et al., 1988a; Yanagisawa et al., 1988b), alteration of ion transport (Ahn et al., 2004; Evans et al., 2004; Garvin and Sanders, 1991; Ge et al., 2006; Prasanna et al., 2001; Zeidel et al., 1989), and direction of migration of neural crest cell during craniofacial development (Clouthier and Schilling, 2004; Kurihara et al., 1994). Endothelins bind to three G-protein-coupled receptors: endothelin A receptor (EDNRA) (Arai et al., 1990), endothelin B1 receptor (EDNRB1) (Sakurai et al., 1990), and the endothelin B2 receptor (EDNRB2) (Lecoin et al., 1998). We recently determined that there are teleost specific duplications of the EDNRA (EDNRA2) and EDNRB1 (EDNRB1B), and multiple species have duplicate endothelin receptors (i.e. Western clawed frog has EDNRC that is a duplicate EDNRB2) (Chapter 3).

Endothelin receptors (EDNRs) have been characterized in some fishes, but the results are often species specific. For example, pharmacological studies have suggested that the aortic vascular smooth muscle of the dogfish shark (Squalus acanthias) has EDNRB-like receptors (Evans et al., 1996), but that eel, lamprey, and hagfish aortic vascular smooth muscle apparently contains EDNRA-like receptors (Evans and Harrie, 2001). In addition, pharmacological studies using receptor binding assays demonstrated EDNRB-like receptors in the dogfish gill (Evans and Gunderson, 1999), but autoradiographic studies showed EDNRA throughout the vasculature of the trout (Oncorynchus mykiss) gill (Lodhi et al., 1995). Recently, EDNRBs were
immunolocalized in filamental arteries, lamellar arterioles and pillar cells of the cod (*Gadus morhua*) gill and EDNRA were found on branchial nerves throughout the filaments (Stenslokken et al., 2006). Finally, EDNRA were immunolocalized to pillar cells in the *Takifugu rubripes* gill (Sultana et al., 2007). The characterization of the EDNRs may be species and protocol specific. We recently sequenced gill cDNA for EDNRA (accession EU391601), EDNRB1 (accession EU391602), and EDRNB2 (accession EU391603) (Chapter 3) from the euryhaline killifish, *Fundulus heteroclitus*, demonstrating that all three EDNRs are present in the fish gill; however, the specific localization of these receptors has not been determined in the killifish. Thus, by using molecular techniques, the question of EDNR expression and tissue distribution in these fishes can be resolved, and help one to understand the earlier pharmacological, autoradiographical and immunohistochemical experiments (Evans et al., 1996; Evans and Gunderson, 1999; Evans and Harrie, 2001; Lodhi et al., 1995). In addition to these studies, Evans et al. (2004) determined that EDN1 or the EDNRB1 agonist, sarafotoxin S6c (SRX S6c), inhibited net chloride transport as measured by a reduction of the short circuit current in the killifish opercular epithelium (a model for the SW teleost gill (Karnaky et al., 1977)). Thus in addition to cardiovascular functions, EDN1 and the EDNRs are involved in the regulation of gill ion transport in marine fishes.

Killifish are euryhaline fish, distributed in the coastal waters from Florida to Newfoundland (Bigelow and Schroeder, 2002). They live in a harsh environment, where there are daily changes in environmental salinity and temperature (Marshall, 2003). They are a commonly used model species test a variety of ecological, epidemiological, and physiological questions (Burnett et al., 2007). Killifish have been instrumental in understanding the effects of changing environmental salinity on fish physiology, because they can tolerate direct transfers
between fresh and seawater (Marshall, 2003). The purposes of this study were to
immunolocalize the EDNRs in the killifish gill and to determine the effects of environmental
salinity on gill EDNR expression, in an effort to begin to elucidate the putative role of EDN1
signaling in the fish gill.

Methods

Fish Maintenance

The University of Florida and Mount Desert Island Biological Laboratory (MDIBL)
Institutional Animal Care and Use Committee approved all protocols. Killifish were trapped in
the brackish waters of North East Creek, Mount Desert Island, ME, and maintained in free-
flowing, 15°C, 31 ppt seawater (SW) for three months before being transported to the University
of Florida. There they were maintain in 3 ft circular tanks, in 20°C, 32 ppt SW or dechlorinated
Gainesville Fl tap water for 30 days before experimentation. Fish were fed commercial pellets to
satiation every other day, and ammonia, nitrites, and nitrates were below 0.1 ppt, and pH was
maintained between 7.8-8.0.

Salinity Challenges

The killifish salinity challenge experimental design was described in our earlier study
(Hyndman and Evans (2007)). In brief, killifish were subjected to one of four treatments: 1) SW
to FW transfer; 2) SW sham (SW to SW); 3) FW to SW; or 4) FW sham (FW to FW). At 0, 3, 8,
24 h (acute acclimation) and 30 days (chronic acclimation) after transfer, 8 or 9 killifish were
sacrificed by decapitation, and the gills from the right side snap frozen for RNA analysis, and the
left side snap frozen for Western blotting. In addition, from 3 killifish, gill arch 2 from both
sides was fixed in 4% paraformaldehyde (in 10 mM phosphate buffered saline, PBS) for 24 h at
4°C for immunohistochemical analysis.
Quantitative Real-Time PCR

We previously sequenced killifish cDNA for EDNRA, EDNRB1, and EDNRB2 (Chapter 3). From these sequences, we designed non-degenerate primers for quantitative real-time PCR (qRT-PCR) (Table 4-1). These primers were designed to amplify a product of 50-100 bp, across a predicted exon-exon boundary to prevent amplification of genomic DNA. All reactions were run in duplicate, and all values were normalized to L8 mRNA values (Choe et al., 2005) and standardized to a cDNA standard curve as previously described by Hyndman and Evans (2007). With each reaction a melting curve analysis was completed to ensure only one product was amplified. In addition, we sequenced samples and confirmed that we had amplified the target of interest. Finally, qRT-PCR were run using RNA instead of cDNA as a negative control, which confirmed that there was no genomic contamination.

Immunohistochemistry and Immunoblotting

The immunohistochemical methods of Piermarini et al. (2002) and Hyndman and Evans (2007) were used. Five slides per animal (each slide was about 100 microns deeper into the filament) were analyzed. In addition, Western blots were made from gill samples using the protocols of Piermarini et al. (2002) and Hyndman et al. (2006). Finally, to accurately quantify protein level differences, we made immunoblots following the methods of Joyner-Matos et al. (2006). In all of these protocols, we used the following antibodies: anti-rat EDNRA (1/500, Alomone Laboratories, Jerusalem); anti-rat EDNRB (1/1000, Alomone Laboratories). Currently a commercial antibody that can discriminate between EDNRB1 and EDNRB2 is not available; thus we used a non-specific EDNRB antibody. Negative controls were run using peptide-absorbed antibodies (1 μg peptide/1 μg antibody, mixed overnight, shaking, 4°C, following the manufacturer’s protocol). All of the protocols were run using the preabsorbed antibody to determine any non-specific binding. To localize the EDNR-expressing cells relative to
mitochondrion-rich (ion transporting) cells (MRCs), slides were double-labeled with the EDNR antibodies and with anti-chicken Na\(^+\),K\(^+\)-ATPase as previously described (Hyndman et al., 2006). This anti-Na\(^+\),K\(^+\)-ATPase is a common marker for the MRC in the killifish gill (Choe et al., 2006; Hyndman et al., 2006; Hyndman and Evans, 2007; e.g. Katoh and Kaneko, 2003) Westerns and dot blots were digitized using a flat bed scanner, and analyzed using Biorad’s Quantity One software (Hercules, CA). The brightness and contrast of each slide image was adjusted with Photoshop CS3 (Adobe, San Jose, CA).

Statistics

All values were tested for normality and equal variance and if these were not met, the values were log-transformed for statistical analysis. Quantitative real-time PCR values were tested using two-factor ANOVA (treatment and time), and when significance was found specific differences between sham and treatment were determined using unpaired, two-tailed, T-test (\(\alpha=0.05\)). Protein level differences were analyzed with one-factor ANOVAs and Dunnett’s post hoc test. Chronic qRT-PCR and protein level differences were analyzed using unpaired, two-tailed, T-tests to test for differences between the SW and FW treatments.

Results

Endothelin Receptor mRNA Levels

In killifish transferred from FW to SW, there was a significant, threefold increase in gill \(EDNRA\) mRNA levels only at 24 h after transfer (\(p=0.03\)) (Fig. 4-1A). There was, however, a significant, twofold increase in gill \(EDNRB1\) mRNA compared to sham at 3 h (\(p<0.001\)), 8 h (\(p=0.003\)), and 24 h (\(p=0.016\)) after transfer (Fig. 4-1C). Gill \(EDNRB2\) mRNA levels increased 3 h after transfer (\(p<0.001\)), but the levels were not different from sham by 8 and 24 h (Fig. 4-1E). In killifish transferred from SW to FW, there were no significant changes in gill \(EDNRA\) or \(EDNRB2\) mRNA levels compared to sham over the 24 h acclimation period (Fig. 4-1B, F).
There was, however, a significant increase in gill *EDNRB1* mRNA 3 h after transfer (p<0.001), these levels were not different from sham at 8 or 24 h after transfer (Fig. 4-1D). After chronic acclimation (30 days) to SW or FW, there were no differences in *EDNRB1*, or *EDNRB2* mRNA levels; however, gill *EDNRA* mRNA were 55% lower in the FW killifish compared to SW killifish (p=0.022) (Fig. 4-2).

**Endothelin Receptor Protein Concentrations**

Using Western blots made from SW killifish gills, we found a single ~37-kDa band with the anti-EDNRA, and a single band of ~40-kDa with anti-EDNRB (Fig. 4-3). As we found for *EDNRA* mRNA, there was a threefold increase in EDNRA compared to control (time zero) only at 24 h after a FW to SW transfer (p=0.014) (Fig. 4-4A). Likewise, there was a twofold increase in EDNRB in the killifish gill compared to control at 24 h after transfer (p=0.002) (Fig. 4-4C). After a SW to FW transfer, there was a significant decrease in EDNRA protein level (3 and 8 h) (p<0.001); however, by 24 h EDNRA protein levels return to control values (Fig. 4-4B). There was no significant change in EDNRB protein level after a SW to FW transfer (Fig. 4-4D). After chronic acclimation to SW or FW, there was a significant 60% decrease in gill EDNRA protein levels in the FW killifish compared to the SW killifish (p=0.004) (Fig. 4-4E). There were no statistical differences between SW and FW chronic acclimated killifish gill EDNRB levels (Fig. 4-4E).

**Immunohistochemistry**

In the gill, epithelial cells in the interlamellar region were immunopositive for EDNRA (Fig. 4-5). In addition, on the afferent side of the filament where there are no lamellae, there were many, large ovoid, cells immunopositive for EDNRA (Fig. 4-5D, E, H, I). All of the EDNRA-immunoreactive cells (-IR) were also immunopositive for NKA (Fig. 4-5C, E, G, I), suggesting that EDNRA is expressed in the mitochondrion-rich cell (ion transporting) (MRC).
There were no immulocalization differences between SW control gills (Fig. 4-5A-C) and gills from killifish acclimated to SW for 24 h (Fig. 4-5D, E); however, 24 h after SW to FW transfer, the EDNRA immunoreactivity became punctate, and less diffuse throughout the cell compared to the other treatments (Fig. 4-5H, I). In addition, gills from killifish acclimated to FW for 30 days, had a shift in EDNRA distribution. Compared to SW gills where the EDNRA-IR took up the whole cell (Fig 4-5A, D), in FW gills there was a shift to only EDNRA along the basal aspect of the cell (Fig. 4-5F, G). Negative controls using a peptide-absorbed antibody were double labeled with anti-NKA, and showed no nonspecific binding of the EDNRA antibody (Fig. 4-5B).

Throughout the gill vasculature there was EDNRB-IR (Fig. 4-6A, C-H), including the prelamellar arterioles (Fig. 4-6E, F arrows). In addition, EDNRB-IR was found on lamellar pillar cells (Fig. 4-6D). Unlike EDNRA, the EDNRB-IR did not colocalize to the same cell as the NKA-IR (Fig. 4-6C, E, F, H). There were no obvious immunolocalization differences between the SW (Fig. 4-6A, B) and FW chronically acclimated gills (Fig. 4-6G, H). Also, there were no obvious localization difference 24 h after a SW or FW transfer (Fig. 4-6E, F). A negative control using peptide absorbed EDNRB antibody displayed no immunoreactivity (Fig. 4-6B).

**Discussion**

The killifish is an excellent osmoregulator, capable of tolerating direct transfers from FW to SW and SW to FW (Hyndman and Evans, 2007; Marshall, 2003). We have previously hypothesized that endothelin-1 (EDN1) signaling cascades may be a local regulator of gill ion transport, because EDN1 inhibits net chloride transport in the killifish opercular epithelium (Evans et al., 2004), and we could localize EDN1 (mRNA and protein) in the gill of this species (Hyndman and Evans, 2007). In the present work, we have found that 24 h after a FW to SW transfer, there was a significant threefold increase in gill EDNRA mRNA and protein, and a
significant twofold increase in gill EDNRB1 mRNA and protein. Our earlier study (Hyndman and Evans, 2007) determined that after a FW to SW transfer there was a significant, sixfold increase in killifish gill *endothelin converting enzyme 1 (ECE1)* mRNA (but no change in gill *EDN1* mRNA), suggesting that there will be an increase in ECE1 protein. Given that the cleavage of proendothelin to EDN1 by ECE1 is the rate limiting step in active EDN1 production (D'Orleans-Juste et al., 2003; Ikeda et al., 2002) one would predict that an increase in ECE1 would result in more active EDN1. Therefore, during short-term hyper-osmotic stress, EDN1 signaling is probably regulated through changes in the expression of both the receptors and the ligand.

Killifish that were acclimated to FW 24 h after transfer from SW had no significant changes in gill *EDNRA, EDNRB1* or *EDNRB2* mRNA (or EDNRA and EDNRB protein levels); however, we previously determined that there is a threefold increase in gill *EDN1B* mRNA and *ECE1* mRNA 24 h after a SW to FW transfer (Hyndman and Evans, 2007). This again suggests that more EDN1 protein will be produced, because there is more EDN1 and an increase in ECE1. Therefore, during short-term acclimation to hypo-osmotic environments this system is probably regulated by changes in the ligand only.

With chronic acclimation (30 days) to FW, we found a significant 55% decrease in killifish gill EDNRA mRNA and protein, but no significant changes in EDNRB1 or EDNRB2 mRNA or protein, compared to the chronically acclimated SW killifish. We previously determined that these chronic acclimations did not change *EDN1A, EDN1B* or *ECE1* mRNA levels (Hyndman and Evans, 2007). Therefore, during long-term acclimation it appears ligand levels are similar but that there are significantly fewer EDNRAs.
Given our findings of changes to the EDN1 system in the killifish gill during hyper-osmotic and hypo-osmotic stress, we can speculate on the function of EDN1 in the fish gill. First, our finding of EDNRA on the MRC, and short term and long term changes in EDNRA expression (mRNA, protein and localization), we hypothesize that EDN1 regulates MRC function via the EDNRA. This may include the regulation of ion transport by this cell. Our lab previously hypothesized that in the gill, EDN1 signaling stimulates cyclooxygenase-2 (COX-2) production of prostaglandins, and this subsequently leads to an inhibition of net chloride transport (Evans et al., 2004). We also previously reported that COX-2 is expressed in the killifish MRC, and that COX-2 mRNA levels are significantly lower in chronically acclimated, FW killifish (Choe et al., 2006). Collectively with our current findings, it appears that FW killifish have less EDNRAs and COX-2, suggesting that this EDN1 signaling axis may function in the long-term as a fine-tune control of ion balance, acting like a brake, during osmoregulation in hyper-osmotic salinities.

The EDNRBs were found throughout the gill vasculature, including prelamellar arterioles, and on lamellar pillar cells. Given the epithelial cell localization of EDN1 (Hyndman and Evans, 2007), it may function as a paracrine regulator of gill vascular tone, and perfusion of lamellae through control of the tone of the prelamellar arteriole; however, there is no evidence that EDN1 contracts filamental arteries or lamellar arterioles (Stenslokken et al., 1999; Stenslokken et al., 2006). An alternative hypothesis is that the EDNRBs found on the filamental arteries act as clearance receptors for EDN1. In mammals, EDNRB1 functions as a clearance receptor in the pulmonary circuit (see La and Reid, 1995). In fishes, 55% of an EDN1 bolus is removed during a single pass through the gills (Olson, 1998). Thus, we postulate that gill vascular EDNRB are the clearance receptors in the killifish.
Given that EDN1 (Hyndman and Evans, 2007) and EDNRB were both found on the lamellar pillar cells (Fig. 4-6D), it seems plausible that EDN1 is acting as an autocrine on these cells, potentially regulating pillar cell contractility. It was hypothesized that EDN1 can redistribute and regulate lamellar blood flow through the lamellae of the fish gill (Stenslokken et al., 1999; Sundin and Nilsson, 1998). In the trout (*Onycorhynchus mykiss*) and cod, injections of EDN1 into the ventral aorta resulted in constriction of pillar cells, resulting in a shift of intralamellar blood flow to the outer marginal channels (Stenslokken et al., 1999; Sundin and Nilsson, 1998). Recently EDNRB-like receptors were immunolocalized to the cod pillar cells (Stenslokken et al., 2006). Pillar cells contain contractile elements and are not innervated (Bettex-Galland and Hughes, 1973; Mistry et al., 2004); thus paracrine peptides, like EDN1, may cause pillar cells to contract, and our data support this hypothesis.

Recently, EDNRA were found on the pillar cells of the tiger pufferfish (*Takifugu rubripes*) using a homologous pufferfish EDNRA antibody, and these receptors were capable of increasing intracellular calcium *in vitro*, suggesting that they cause contraction of pillar cells (Sultana et al., 2007). Thus, perhaps the gill EDNR distribution is species-specific. Determining the cellular distribution of these receptors in more fishes will elucidate any such patterns.

An alternative hypothesis is that EDN1 signaling is involved in maintenance of cellular and tissue integrity during volume expansion. Recently, Mistry et al., (2004) sequenced and characterized an actin-binding protein, FHL5, from the pillar cells of the tiger pufferfish. They determined that EDN1 and volume expansion (from isotonic dextran-saline) both stimulate FHL5 expression in the lamellar pillar cells. In a comparative study between normal and hypertensive rats, volume expansion stimulated EDN1 production in both groups (Abdel-Sayed et al., 2003). In agreement with this finding, Wongmekiat and Johns (2003), determined during
volume expansion in normal, lean rats, that EDN1 signaling (likely acting via and EDNRA) leads to diuresis and natriuresis (but this was impaired in obese rats). Thus, collectively we hypothesize that during volume expansion (as occurs during rapid transfer from SW to FW) EDN1 signaling is involved in maintaining pillar cell integrity through the stimulation of an increase in the actin binding protein FHL5 and regulation of pillar cell tone. An alternative hypothesis is that initially a volume load leads to a stretch response by the pillar cell, and this increases FHL5 and EDN1 expression in this cell, and subsequent synergistic contraction of the pillar cell to maintain lamellar integrity. Further experiments used to determine the relationships between EDN1 and volume expansion are needed.

In summary, all the components necessary for EDN1 production and action (the receptors) are present in the killifish gill. We have recently determined that these same proteins are found in the sculpin gill (accessions EU440324-EU44032), and that EDNRs are present in the dogfish shark (accessions EU440328 and EU440329) and sea lamprey (accession EU440327) (Petromyzon marinus) gills, thus we conclude this signaling molecule is actively expressed in the early vertebrates (Appendix A, Hyndman and Evans, unpublished). We have previously shown that environmental salinity regulates the expression of EDN1, ECE1 (Hyndman and Evans, 2007), and present here that salinity also regulates the EDNRs in the killifish, but no clear picture has emerged of the role that EDN plays in the response to salinity changes. Studies determining the effects of other factors, like hypoxia, and functional studies (using morpholinos, silencing RNAs or knockout models) will be integral in our understanding of the regulation and potential functions of EDN1 in the gill.
Table 4-1. Non-degenerate primers used in the quantitative real-time PCR (q) experiments.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ to 3’ Orientation</th>
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<tr>
<td>EDNRAqF1</td>
<td>GCA TCA ACC TGG CGA CAA T</td>
</tr>
<tr>
<td>EDNRAqR1</td>
<td>CAG CAG CAC AAA CAC GAC TTG</td>
</tr>
<tr>
<td>EDNRB1qF1</td>
<td>CTG ATG ACC TGC GAG ATG CTA A</td>
</tr>
<tr>
<td>EDNRB1R1</td>
<td>TCC GCG CGC TGC TTA ATA T</td>
</tr>
<tr>
<td>EDNRB2qF1</td>
<td>CCT GCG AGA TGC TGA GTC G</td>
</tr>
</tbody>
</table>
Figure 4-1. Relative gill mRNA levels for endothelin receptors from killifish acclimating to seawater (SW) or fresh water (FW) over a 24 h period (n=5-6 fish/treatment). Dotted lines and closed symbols represent shams, and solid lines and open symbols represent treatments. A, C, E) fish transferred from FW to SW (●) or maintained in FW (sham, ○), and B, D, F) fish transferred from SW to FW (■), or maintained in SW (sham, □). A, B) EDNRA, C, D) EDNRB1, and E, F) EDNRB2. All values are normalized to L8 mRNA levels and are made relative to chronic SW levels (Fig. 4-2). Asterisks (*) represents statistically significant differences compared to sham determined by 2-Factor ANOVA and unpaired, two-tailed, T-tests (compared to sham). Mean ± s.e.m. Note that the scale is logarithmic.
Figure 4-2. Relative gill mRNA levels for the endothelin receptors from the killifish (n=5-6) acclimated for 30 days to either seawater (SW, black bars) or fresh water (FW, grey bars). All values relative to SW. Statistical significance was determined using unpaired, two-tailed, T-tests comparing SW to FW treatments (p values listed on figure). Mean ± s.e.m.
Figure 4-3. Western blots from seawater killifish gills. Lane 1 is a Coomassie blue stained ladder. Lane 2 is a representative blot stained with anti-EDNRA. Lane 3 is a representative blot stained with anti-EDNRB.
Figure 4-4. Killifish gill protein level differences of the endothelin receptors during acclimation to SW or FW (n=5-6/treatment). EDNRA levels during acclimation to SW (A, open circles) or FW (B, open squares). EDNRB levels during acclimation to SW (C, open circles) or FW (D, open squares). These means are relative to time zero. Statistical significance was determined using ANOVA and Dunnett’s post hoc test (p values listed on figure). E) Chronic (30 days) acclimations to either SW or FW, and all values are relative to SW values and statistical significance was determined using unpaired, two tailed, T-tests. Mean ± s.e.m.
Figure 4-5. Representative light micrographs of the immunolocalization of the endothelin A receptor (EDNRA) in the killifish gill. A-C) seawater (SW) chronically (30 days) acclimated fish. D, E) Gills from fish acclimated to SW for 24 h. F, G) Gills from fish chronically acclimated to FW. H, I) Gills from fish acclimated to FW for 24 h. A, D, F, H) EDNRA immunoreactivity (brown color). B) A section of gill 7 microns deeper into the gill from A and C, incubated in peptide-absorbed anti-EDNRA (no staining observed) and double-labeled with anti-Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (blue). C, E, G, I) Gill sections double labeled with anti-EDNRA (brown) and anti-Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (blue). The immunoreactivity is in the same cell giving a grey appearance to the cell. Scale bar = 50 μm
Figure 4-6. Representative light micrographs of the immunolocalization of the endothelin B receptors (EDNRB) in the killifish gill. A-D) Seawater (SW), chronically (30 days) acclimated fish. E) A gill filament from a fish acclimated to SW for 24 h. F) A gill filament from a fish acclimated to fresh water (FW) for 24 h. G, H) FW chronically acclimated fish. A, G) Immunoreactivity for EDNRB (brown). B) A section 7 microns away from A or C incubated in peptide-absorbed antibody as a negative control (no staining observed). C, E, F, H) Filaments immunoreactive for EDNRB (brown) and Na⁺,K⁺-ATPase (blue). D) Magnification of lamellar pillar cells immunopositive for EDNRB. Arrows are pointing to prelamellar arterioles immunopositive for EDNRB. Scale bar = 50 μm.
CHAPTER 5
SHORT TERM LOW-SALINITY TOLERANCE BY THE LONGHORN SCULPIN, *MYOXOCEPHALUS OCTODECEMSPINOSUS*

Introduction

Sculpin (Scorpaeniformes: Cottidae) are a specious (~400 species (Nelson, 2006)) group of teleost fishes that displays a great variety in habitat use including: rivers, demersal freshwater lakes, inshore coastal marine areas (including brackish waters), and demersal-marine areas. The genus *Myoxocephalus* consists of 17 species, of which nine are demersal-marine fishes, five enter brackish water, and three spend a portion of their life in fresh water (Froese and Pauly, 2000). The longhorn sculpin, *Myoxocephalus octodecemspinosus*, is distributed in coastal waters from Virginia to Newfoundland, Canada (Bigelow and Schroeder, 2002). Although they are primarily distributed in marine waters, they have been found entering estuaries during high tides, but never in fresh water (Bigelow and Schroeder, 2002), suggesting they have some level of low salinity tolerance. The only laboratory study to subject longhorn sculpin to low salinity challenges was conducted by Claiborne and colleagues (1994). They tested the effects of 4, 8, 20% seawater (SW) acclimations, and acid loads on longhorn sculpin, and determined that in 4 and 8% SW they lose Cl\^- to a lethal level by 48 and 60 h, respectively. In 20% SW, there is an initial decrease in plasma Cl\^-, but by 72 h this values has returned to control values; however, they did not determine what effect low environmental salinity had on gill ion transporter expression.

In teleost fishes, ion balance is regulated by specialized epithelial cells in the gill called mitochondrion-rich cells (MRCs, also termed chloride cells). As the name implies, they contain a high density of mitochondria, as well as ion transporters and channels necessary for ion movement. These include the basolateral membrane proteins: Na\(^+\), K\(^+\)-ATPase (NKA) and Na\(^+\)-K\(^+\)-2Cl\^- cotransporter (NKCC1); and the apical membrane chloride channel, the cystic fibrosis
transmembrane conductance regulator (CFTR) (recently reviewed by Evans et al., 2005).

Studies in euryhaline fishes, like the killifish (*Fundulus heteroclitus*), or anadromous fishes like the rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) or Atlantic salmon (*Salmo salar*), have explored the effects of changes in environmental salinity on gill ion transporter density (e.g. Hiroi and McCormick, 2007; e.g. Mancera and McCormick, 2000; Marshall et al., 2002; Pelis et al., 2001; Scott et al., 2004; Seidelin et al., 2000; Singer et al., 2002). In general, when these fishes move from fresh/brackish waters to marine waters, there is a rapid increase in plasma [Na⁺], [Cl⁻] and total osmolality (e.g. Scott et al., 2004; Seidelin et al., 2000), and to help maintain ion homeostasis, euryhaline fishes up-regulate gill NKA, NKCC1, CFTR, and other transporters and channels. The opposite occurs when going from marine waters to brackish/fresh; there is a loss of ions, and generally, these ion transporters are down regulated. In addition to turning off SW osmoregulatory proteins, FW ion transporters are upregulated during FW acclimation. These include Na⁺-H⁺ exchangers (NHEs), V⁺ -H⁺-ATPase, and Cl⁻/HCO₃⁻ exchangers, and these are also necessary for proper acid-base regulation (e.g. Evans et al., 2005). Recently, NKA, Na⁺-H⁺ exchanger-2, and V⁺ -H⁺-ATPase were immunolocalized to MRCs in the longhorn sculpin gill (Catches et al., 2006); however the effects of salinity on the expression on these ion transporters in the longhorn sculpin is yet to be elucidated.

The purpose of this study was to explore the effects of low environmental salinity on SW osmoregulatory ion transporter expression and distribution in the longhorn sculpin gill to further our understanding of why they are not found in FW. We hypothesize that longhorn sculpin can only tolerate low salinity environments for short periods (days) because they are unable to properly regulate these ion transporters necessary for ion homeostasis.
Methods

The following experiments were conducted in August of 2006 and 2007, and all methods were approved by Institutional Animal Care and Use Committee at the University of Florida and the Mount Desert Island Biological Laboratory (MDIBL, Salisbury Cove, ME). Longhorn sculpin, *Myoxocephalus octodecemspinosus* (Mitchill), were collected by local fishermen in Frenchman Bay, ME. Longhorn sculpin were transported to MDIBL and maintained in 6 ft circular tanks with free-flowing seawater (SW) from Frenchman Bay, under a natural summer photoperiod, and fed squid every other day. All animals were fasted 48 h (h) prior to and during experimentation.

**Low Salinity Acclimation**

In a preliminary experiment, we maintained two longhorn sculpin in 10% SW for 6 days before seeing visual signs of stress (sluggish behavior, color change, lack of righting) (data not shown). Thus, we terminated the full experiment after 3 days (72 h), when there were no obvious signs of stress. Longhorn sculpin were randomly assigned to one of four treatments: 24 h sham (100% SW); 24 h 10% SW; 72 h sham (100% SW); or 72 h 10% SW. Each sculpin was placed in five gallons of the appropriate, aerated solution in a 10-gallon bucket and air. The buckets were maintained in a trough with free flowing SW (15°C) for temperature control. For experiments that lasted longer than 24 h, 50% of the water was replaced in each bucket daily. Due to the confined space, only 8 fish were run in each experiment (3 shams, 5 treatments); thus the experiment was run four times (10% SW and 20% SW for 24 or 72 h). The buckets were kept in a trough with ~3 inches of flowing SW from Frenchman Bay, and this kept the bucket water temperature at 16 ± 1°C. 20% SW was made by mixing 2 parts SW with 8 parts of dechlorinated FW (by bubbling air into a bucket of FW for 24 h). The salinity was confirmed by measuring the osmolality with a Wescor Vapor Pressure osmometer 5520 (Logan, UT).
similar experiment was repeated using 20% SW and 100% SW (sham) as the treatments. After 24 or 72 h, fish were anesthetized in either 100% SW, 20% SW or 10% SW (depending on the assigned treatment) with 0.379 g l^{-1} benzocaine (initially dissolved in absolute ethanol, final concentration of ethanol was 0.1%) (Sigma, St. Louis, MO). Blood (0.5-1.0 ml) was taken from the bulbus arteriosus, and then the fish was pithed. Half of the filaments of second gill arch were cut off and snap frozen for RNA extraction and the other half was fixed in 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS, pH = 7.3) for immunohistochemical analyses. The rest of the filaments from the 7 gill arches were cut off the arch into a dish of 10 mM PBS, mixed, divided into two tubes, and snap frozen for protein analyses.

**Plasma Chemistry**

Blood samples were immediately spun at 1000 rpm for 5 min at 4°C, and the plasma was aliquoted and frozen (-20°C) until analyzed. Total plasma osmolality was measured using a Wescor Vapor Pressure Osmometer (Logan, UT). Plasma sodium and potassium were measured using an IL943 Automatic Flame Photometer (Instrumentation Laboratory, Lexington, MA) and chloride by the Labconco Digital Chloridometer (Kansas City, MO). All samples were measured in triplicate.

**Molecular Techniques**

RNA was extracted using TRI Reagent (Sigma) as previously described in Hyndman and Evans (2007). RNA pellets were reconstituted in 10 μl of diethyl pyrocarbonate (DEPC) treated water, and the concentration of RNA measured using a Nanodrop ND-1000 spectrophotometer (Fisher Scientific, Wilmington, DE). Total RNA (5 μg) was reverse transcribed using a First
Strand cDNA Synthesis kit with Superscript III (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions.

Degenerate primers were designed using CODEHOP (Rose et al., 2003) to amplify, NKA, NKCC1, and CFTR (Table 5-1). The polymerase used was 0.625 Units of Ex Taq, hot start, DNA polymerase (Takara Bio, Madison, WI) and the reactions were run in a Px2 thermocycler (Thermo Fisher Scientific, Waltham, MA). The PCR parameters were: 94°C for 2 min, 40 cycles of 94°C for 30 sec, 45-60°C (gradient) for 30 sec, 72°C 30 sec, and a final 72°C for 5 minutes. These products were then singly nested with a primer listed in Table 5-1, and the PCR was run using the PCR product from the first PCR. With all transcripts, there was a bright single band, and these transcripts were ligated into pCR®4-TOPO vectors, and transformed into TOP10 chemically competent cells using a TOPO TA Cloning® Kit for sequencing (Invitrogen). Cells were grown on agar plates with Kanamycin (500x) antibiotic and positive colonies were grown in LB broth (20 g l⁻¹) over night at 37°C, while shaking. Plasmids were extracted from the cells using a miniprep kit (Roche Applied Science, Indianapolis, IN), and sequenced at the Marine DNA Sequencing center at MDIBL. These partial sequences have been deposited into GenBank (accession numbers): NKA #EU391598; CFTR #EU391599; and NKCC1 #EU391600.

**Quantitative Real-Time PCR**

To determine the effects of dilute environments on longhorn sculpin gill CFTR, NKA, and NKCC1, mRNA levels, quantitative real-time PCR (qRT-PCR) was performed. Non-degenerate primers were designed to amplify a product between 50-100 bp (Table 5-1). L8 was used as an internal control gene as previously described (Choe et al., 2006; Choe et al., 2005). Each sample was run in duplicate using 2 μl of 1/10 diluted original oligo-dt cDNA, 7.4 pmol of primers and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 25 μl.
The cycling parameters used were: an initial denaturing step of 95°C for 10 minutes, 40 cycles of: 95°C for 35 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a melting curve analysis to ensure only one product was amplified. Random samples were also sequenced following qRT-PCR confirming amplification of the target of interest. To determine the degree of possible genomic contamination, qRT-PCR was run using RNA samples that were not reverse transcribed, and we determined that there was no genomic contamination. All qRT-PCRs were run on a MyiQ quantitative thermocycler (Biorad).

Each primer pairs’ efficiency was determined by performing a 10-fold dilution curve using plasmid cDNA. Efficiency (E) for each primer pair was calculated using the equation:

\[ E = 10^{\left(-\frac{1}{\text{slope}}\right)} \]

where “slope” was the slope of the dilution curve. Each cycle threshold (CT) value was subtracted from a randomly chosen control sample resulting in a \( \Delta \text{CT} \), and were analyzed using the Pfaffl equation (Pfaffl, 2001):

\[ \text{ratio} = \frac{E^{\Delta \text{CT target}}}{E^{\Delta \text{CT L8}}} \]

Each Pfaffl ratio was then standardized to the average sham Pfaffl ratio.

**Immunohistochemistry**

A portion of the second gill arch from the longhorn sculpin (see above) was fixed in 4% paraformaldehyde in 10 mM PBS, for 24 h, dehydrated in an increasing concentration of ethanol series, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin wax. The tissue blocks were cut at 7 microns, placed on Superfrost Plus slides (Fisher Scientific), and heated at 37°C for 30 min. Slides were analyzed following the methods of Piermarini et al. (2002) and Hyndman et al.(2006). In short, five slides/animal for each treatment were rehydrated, blocked with 3% \( \text{H}_2\text{O}_2 \) in water for 30 minutes, and washed in 10 mM PBS. Next, there was a 20 min protein block with Biogenex’s protein block (BPB; Biogenex, San Ramon, CA), followed by 10 mM PBS washes. Finally, slides were incubated in primary antibody (see below), overnight at 4°C. The primary antibody was washed off with 10 mM PBS, and the
immunoreactivity was visualized using Biogenex’s Super Sensitive™ Link-Label IHC Detection System. The chromagens used in this study were 3, 3′-diaminobenzidine tetrahydrochloride (DAB; brown color; Biogenex), Vector SG (blue color, Vector Laboratories, Burlingame, CA), and Vector VIP (purple color, Vector Laboratories). Following this, some sections were double-labeled with a second primary antibody, following the same procedures.

**Western and Dot Blotting**

Western blots were made following the methods of Hyndman and Evans (2007). Gills were homogenized in 2 ml of ice-cold homogenization buffer (250 mM sucrose, 30 mM Tris, 1 mM EDTA, 0.5% of Sigma’s protease inhibitor cocktail, and 100 μg ml⁻¹ phenylmethylsulfonyl fluoride; pH 7.8). The homogenates were centrifuged at 14 000 rpm for 10 min at 4°C and the supernatant decanted. Protein content of the supernatant was measured using Pierce’s BCA protein assay kit (Rockford, IL). A portion of the supernatant was diluted with an equal portion of Laemmli sample buffer with 0.01% bromophenol blue and 2% β-mercaptoethanol (Laemmli, 1970) and heated at 65°C for 10 min. Twenty-five micrograms of protein was separated using SDS-PAGE (10% Tris-HCl gels, Biorad, Hercules, CA) for 2 h at 100 V and then transferred to an Immuno-blot PVDF membrane according to the manufacturer's protocol (Biorad). Next the membrane was placed in blotto, 5% non-fat dry milk in 10 mM Tris buffered saline (TBS: 25 mmol l⁻¹ Tris, 150 mmol l⁻¹ NaCl; pH 7.4) for 1 h at room temperature (~25°C), shaking, and then placed in primary antibody and incubated at room temperature, overnight. Next the membrane was washed in thee changes of 10 mM TBS with 1% Tween 20 (TBST) and incubated in 1/3000 goat anti-mouse alkaline phosphotase secondary (Biorad) diluted in blotto for 1 h at room temperature while shaking. Again, the membrane was washed in thee changes of 10 mM TBST. The membrane was developed using a chemiluminescent signal (Bio-Rad,
Hercules, CA) following the manufacturer’s instructions, and developed on ECL Hyperfilm (Amersham, Piscataway, NJ). All films were digitized using a flat bed scanner.

Dot blots were used to accurately quantify ion transporter protein level differences among our treatments, and the methods of Joyner-Matos et al. (2006) were used. In short, gills were homogenized and centrifuged as described above. The supernatant was heated at 65°C for 15 min, and then diluted to 2.5 μg µl⁻¹ in 10 mM TBS, and continued heating at 65°C until blotted. Another randomly picked control sample was diluted (in 10 mM TBS) out in a series of 2-fold dilutions to make an 8-point dilution curve. Proteins were blotted in 1 μl dots (thus 2.5 μg of protein), in triplicate onto dry nitrocellulose membrane (Millipore, Billerica, MA) and left to air dry for 10-20 min. Next, the membrane was placed in blotto and followed the aforementioned western incubation protocol. The developed filmed was digitized using a flat bed scanner, and dot density determined using Biorad’s Quantity One software. All values were standardized to the dilution curve, and made relative to protein content (relative units mg protein⁻¹).

**Antibodies**

Monoclonal, anti-chicken NKA (α5, 1/1000) was developed by Dr. D. Fambrough, and monoclonal, anti-human NKCC1 (T4, 1/500) was developed by Drs. Lytle and B. Forbush III, and were obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Monoclonal anti-human CFTR (1/500 ) (R&D systems, Minneapolis, MN) was made against the c-terminal of human CFTR and is ~61% identical to teleost CFTR (Katoh and Kaneko, 2003; Singer et al., 1998). All antibodies were diluted in BPB.
Gill Na\textsuperscript{+}, K\textsuperscript{+}-ATPase Activity

Na\textsuperscript{+},K\textsuperscript{+}-ATPase (NKA) activity was measured using a NADH-linked, spectrophotometric microassay, similar to the one developed by McCormick (1993).Briefly, gills were homogenized on ice with a polytron in 125 \( \mu \)l of 5X SEID buffer (250 mM sucrose, 10 mM Na\textsubscript{2}EDTA, 50 mM imidazole, 0.05% deoxycholic acid, pH=7.3) and 1 ml 1X SEID (diluted in SEI: 250 mM sucrose, 10 mM Na\textsubscript{2}EDTA, 50 mM imidazole). Samples were centrifuged at 3000 g for 30 sec at 4 °C to remove any large particulates. The protein content of the supernatant was determined using Pierce’s BCA Protein Assay. In a 96-well microplate, 10 \( \mu \)l of the supernatants were added to 200\( \mu \)l of reaction mixture (80 mM NaCl, 20 mM KCl, 5 mM MgCl\textsubscript{2}, 50 mM imidazole, 3 mM ATP, 2 mM phosphoenol pyruvate, 0.2 mM NADH, 3.1 U/ml lactic dehydrogenase, 3.84 U/ml pyruvate kinase) either with or without 1 mM ouabain. All samples were run in triplicate. The plate was read every ten seconds, for a total of 20 minutes, at 25 °C on a Biorad Benchmark Plus microplate reader (340 nm). An ADP standard curve was also run for every lot of reaction mixture to determine the extinction coefficient for the ADP-dependent conversion of NADH to NAD\textsuperscript{+} (used in our final calculation of NKA activity).

The difference in the slopes (the rate of [NADH] reduction) between the non-ouabain and ouabain wells was calculated for each sample. These values were standardized to the ADP standard curve and normalized to total protein content per sample (\( \mu \)mol ADP mg protein\textsuperscript{-1}·h\textsuperscript{-1}) (McCormick, 1993).

Statistics

Plasma chemistry and gill NKA activity data are displayed as mean ± s.e.m. All other data were made relative to the mean sham value for each time, and are displayed as relative means ± s.e.m. With the plasma, qRT-PCR and dot blot data, statistical differences among the treatments
were assessed by 2-Factor ANOVAs (for salinity and time), followed by unpaired, two-tailed T-tests to determine differences compared to sham treatments (100% SW). The 10% and 20% SW experiments were not run concurrently (see above); however the shams within each time point (24 or 72 h) did not differ significantly, thus they were combined in the 100% sham mean that is reported (n = 6). Statistical significance was set at alpha = 0.05. All statistics were run using SPSS (v.15, Chicago, IL).

Results

Plasma Chemistry and Gill Na\(^+\), K\(^+\)-ATPase Activity

Total plasma osmolality, sodium, potassium, and chloride concentrations did not differ between the 24 h and 72 h 100% SW (sham) treatments (Table 5-2). These parameters were not different between the sham and 20% SW at 24 or 72 h; however plasma osmolality and sodium were 14% lower after 24 h acclimation to 10% SW (p < 0.001), and 22% lower after 72 h compared to sham (p < 0.001). Plasma potassium decreased 24% with 24 h acclimation to 10% SW (p = 0.009) and this decrease was maintained at 72 h. Chloride also decreased 20% and 27% with 24 and 72 h acclimation to 10% SW, respectively (p < 0.001). Longhorn sculpin acclimated to 10% or 20% SW for 24 or 72 h did not have a significant change in gill NKA activity compared to sham values (Table 5-2).

Immunolocalization of CFTR, NKA, and NKCC1

Longhorn sculpin gills from all of the treatments were immunopositive for CFTR, NKA, and NKCC1 (Fig. 5-1). Epithelial cells in the interlamellar region were immunopositive for all three transporters. CFTR was found on the apical membrane, as indicated by a small, brown dot near to the edge of the epithelial cells (Fig. 5-1A, D, G). NKA and NKCC1 were basolaterally immunolocalized (Fig. 5-1B, C, E, F, H). Longhorn sculpin that were acclimated to 20% (data not shown) or 10% SW for 24 h (Fig. 5-1D-F) had similar immunostaining patterns as the shams.
(Fig. 5-1A-C). These immunoreactive patterns were also found with longhorn sculpin acclimated to 20% (data not shown) or 10% SW for 72 h. Gills from longhorn sculpin that were acclimated to 10% SW for 72 h, were single-labeled for CFTR (Fig. 5-1G) and then the adjacent section (7 microns further) was double-labeled with CFTR and NKA (Fig. 5-1H). As shown in Figures 5-1G and 5-1H, CFTR and NKA were immunolocalized to the same epithelial cells, with CFTR staining the apical membrane, and NKA found on the basolateral membrane. Likewise, this double labeling was repeated using CFTR and NKCC1, and again CFTR and NKCC colocalized to the same epithelial cell (data not shown).

**Westerns and Dot Blots**

The CFTR, NKA and NKCC1 antibodies used in our Western blot experiments yielded bands of the expected molecular weights (Fig. 5-1I). As seen in Figure 5-1I, a single CFTR band of ~140 kDa, and a single NKA band of ~120 kDa were found in the sham sculpin gill. With the anti-NKCC1 antibody, we found two bands of ~200 and ~130 kDa in the longhorn sculpin gill. Because we found only single (CFTR, NKA) or double bands (NKCC1) with our western blots, and these findings mirror those from other teleosts (Hiroi and McCormick, 2007; Tipsmark et al., 2002), we quantified protein differences with dot blots (total protein, not proteins separate by molecular weight).

The sham treatments for 24 and 72 h were not statistically different from each other for CFTR, NKA, or NKCC protein levels. We did not find any significant changes in gill CFTR or NKA protein level with acclimation to 10% or 20% SW for 24 or 72 h compared to their respective sham treatments (Fig. 5-2A, C). There was a significant 2.5-fold increase in NKCC1 protein level with 24 h acclimation to 20% SW (p <0.001) (Fig. 5-2E); however there were no statistically significant changes in NKCC1 protein level within the 72 h treatment.
**Quantitative Real-Time PCR**

Sham treatments for 24 and 72 h were not significantly different for *CFTR*, *NKA*, or *NKCC1*. Gill *NKCC1* and *CFTR* mRNA levels did not significantly change with acclimation to 10% or 20% SW for 24 or 72 h (Fig. 5-2B, F). Longhorn sculpin gill *NKA* mRNA levels were not different from sham with 24 h acclimation to 20% SW (Fig. 5-2D); however they did increase 3.6-fold after 24 h acclimation to 10% SW (p = 0.001). After 72 h, gill *NKA* mRNA levels were 2.3-fold higher in the 10% SW treatment compared to sham (p = 0.004) (Fig. 5-2D).

**Discussion**

This study is the first to examine the effects of low-salinity seawater (SW) osmoregulatory ion transporters, from a marine teleost, in order to determine why they are incapable of inhabiting freshwater environments (FW). In the wild, longhorn sculpin have been found in estuaries during high tides suggesting they have some low-salinity tolerance (Bigelow and Schroeder, 2002). We determined that acclimation to 20% SW for 24 or 72 h did not elicit any significant changes in plasma osmolality or ion concentration (Table 5-2), but acclimation to 10% SW resulted in a significant loss of ions. This suggests that down to 20% SW, longhorn sculpin can regulate plasma ion concentrations. Claiborne et al. (1994)determined that longhorn sculpin could not survive past 60 h in 8% SW or 48 h in 4% SW, thus we conclude that longhorn sculpin can tolerate salinities down to 8-10% SW for days, but salinities below this level are lethal within a few days. We propose that longhorn sculpin are missing the mechanism that allows euryhaline (or anadromous/ catadromous) fishes to survive in fresh and marine environments--proper regulation of gill ion transporter densities. Longhorn sculpin do express *CFTR*, *NKA*, and *NKCC1* in epithelial cells that match the morphology of the mitochondrion-rich cells (MRCs) (e.g. Hiroi and McCormick, 2007; e.g. Katoh and Kaneko, 2003). Recently, Catches et al. (2006) immunolocalized NKA in the basolateral membrane of the MRC of the SW
longhorn sculpin gill. We determined that CFTR and NKCC1 are also expressed on the MRC as was determined for other teleosts (Hiroi and McCormick, 2007; Katoh and Kaneko, 2003; e.g. Pelis et al., 2001). Presented here, acclimation to 20% did not affect gill CFTR or NKA protein levels or immunolocalization of these proteins in the longhorn sculpin gill (Figs 5-1 and 5-2). There was a significant increase in NKCC1 protein level after 24 h acclimation to 20% SW, but the NKCC1 protein level was not significantly different from sham at 72 h. The increase in NKCC1 protein level while the longhorn sculpin were acclimating to a hypoosmotic environment is puzzling, because it is well documented that NKCC1 is stimulated by cell shrinking (as occurs during acclimation to marine environments), and is involved in volume regulation in teleosts (see a recent review, Hoffmann et al., 2007). Interestingly, we did not find a significant increase in NKCC1 mRNA at 24 h compared to sham. Unfortunately, the time lag between de novo mRNA production and de novo protein production is not known for these transporters, so it is plausible that there was an increase in NKCC1 mRNA hours before our 24 h sampling resulting in more NKCC1 protein at 24 h. It may also be that there are post-transcriptional modifications occurring, resulting in a change of protein without a change in mRNA for NKCC1. In any event, these longhorn sculpin were able to maintain a plasma osmolality of ~330 mmol kg\(^{-1}\) (this is within the normal range for euryhaline and stenohaline marine species, see Evans et al. (2005)) during these experiments, without any obvious changes in CFTR and NKA ion transporter density, NKA activity, or localization of all three proteins in the gill.

Unlike acclimation to 20% SW, longhorn sculpin acclimated to 10% SW suffered a significant loss of ions. Euryhaline, catadromous, or anadromous teleosts that experience changes in environmental salinity regulate gill ion transporters to maintain proper ion balance.
For example, the euryhaline killifish (*Fundulus heteroclitus*), down regulates CFTR and NKCC1 mRNA and protein levels within a day or two of entering fresh water (Choe et al., 2006; Katoh and Kaneko, 2003) resulting in a conservation of ions. Killifish that were transferred from low (0.1 ppt) to high salinity (35 ppt) increased NKA activity 3 h and 72 h post transfer (Mancera and McCormick, 2000) to help excrete excess ions. Unlike the killifish, the longhorn sculpin did not down regulate CFTR, NKCC1, or NKA during acclimation to 10% SW, and subsequently suffered a significant loss of ions at 24 h and 72 h (Table 5-2). *NKA* mRNA levels were higher than shams at 24 and 72 h in the 10% SW treatments, but there was no obvious change in NKA protein level or immunolocalization. Collectively, this could again suggest differences in time lag between *de novo* production of mRNA and protein. We did not find an increase in NKA activity level either (Table 5-2), so this observed increase in NKA mRNA is intriguing. An alternative hypothesis is that there is high NKA protein turnover, so to maintain constant NKA protein levels would require an increase in *NKA* mRNA. This hypothesis has been proposed to explain high increases in *carbamoyl phosphate synthetase III (CPSase III)* mRNA in the Gulf toadfish (*Opsanus beta*) to maintain a constant CPSase III activity level during ureagenesis in this fish (Kong et al., 2000). This may also be occurring with the NKA. Unlike euryhaline fishes like the killifish, the longhorn sculpin does not down-regulate gill SW osmoregulatory ion transporter densities in dilute environments. Recently a study determined that landlocked, freshwater populations of Atlantic salmon (*Salmo salar*) were not capable of up-regulating NKA, NKCC1 and CFTR during smoltification, as was observed in anadromous salmon (Nilsen et al., 2007). Likewise, landlocked Arctic char (*Salvelinus alpinus*) were incapable of maintaining ion homeostasis during hyperosmotic stress, and were incapable of up regulating NKA (specifically NKAα1b subunit). This is likely because these landlocked fishes have completed many
generations in only fresh water, and have adapted to a fresh water existence. To the best of our knowledge, we are the first to determine physiologically why a marine fish is incapable of surviving in low-salinity environments.

In the future, experiments should determine the effect of low-salinity on FW osmoregulatory proteins, such as the Cl⁻/HCO₃⁻, \( V^+ \)-ATPase or NHEs. Catches et al. (2006) determined that NHE2 is expressed in apical membrane of MRCs and \( V^+ \)-ATPase in the basolateral membrane of the SW longhorn sculpin gill. This suggests that machinery involved in ion transport uptake may be present in the sculpin; however, if it is expressed properly in the MRC to drive \( Na^+ \) absorption and/or upregulated during low-salinity exposure remains to be determined.

The exclusion of longhorn sculpin from fresh water is intriguing. There are records of them entering estuaries during high tide (Bigelow and Schroeder, 2002), and the salinity of this environment during this time is likely higher because of the tide. This is probably less of an osmotic challenge than entering during low tide or entering near the fresh water source, and we have determined that down to 20% SW there is no obvious detriment to the fish. Because longhorn sculpin are incapable of properly regulating ion transporter densities in the gill below 20% SW, they suffer a net loss of ions eventually to a level that is lethal for the fish. There are “freshwater” Myoxocephalus: \( M. \) polyacanthocephalus, \( M. \) sinensis, and \( M. \) thompsonii (Froese and Pauly, 2000). \( M. \) polyacanthocephalus is amphidromous, spending a portion of its lifecycle in fresh water and is distributed in the North Pacific; \( M. \) sinensis is a demersal-freshwater species found in China; and \( M. \) thompsonii is also a demersal, freshwater species distributed from the St. Lawrence River to the Arctic (Froese and Pauly, 2000). Within the sculpin, there are three other genera that have freshwater species: \( Cottus \), \( Trachidermus \), and \( Messocottus \). The genus \( Cottus \)
is a group of 57 species of freshwater sculpin (Froese and Pauly, 2000), that diverged from the marine sculpin approximately 2-5 mya (Yokoyama and Goto, 2005). It seems plausible that the marine ancestor(s) to the freshwater sculpin had the ability to regulate gill ion transporters density and or activity, and this lead to their invasion of freshwater habitats. To help elucidate this question, a complete sculpin phylogeny, mapping habitat use to the different species, would be helpful in understanding the evolution of this group of fishes. A portion of the Myoxocephalus phylogeny has been completed using 7/17 species, and it depicts two distinct groups of the Myoxocephalus: Arctic-Atlantic and Pacific groups (Kontula and Vainola, 2003). Both groups contain “freshwater” representatives, suggesting that there were independent invasions of freshwater by Myoxocephalus.

In conclusion, the Myoxocephalus is an interesting group to test mechanistic questions to help understand habitat invasion and use. The longhorn sculpin can tolerate short term exposure to low salinity water (<10% SW) for days but not much longer, because they can not regulate ion transporter density or activity, resulting in a significant loss of ions (eventually to a lethal level). The diverse habitat use of the species of Myoxocephalus, make it an excellent model to complete comparative studies to explore the relationship between environmental salinity and gill ion transporter density, furthering our fundamental knowledge of the mechanisms and evolution of salinity tolerance.
Table 5-1. Primers used in sequencing and quantitative real-time PCR of longhorn sculpin Na⁺, K⁺-ATPase (NKA), Na⁺, K⁺, 2Cl⁻ cotransporter (NKCC1), and the cystic fibrosis transmembrane conductance regulator (CFTR). The asterisks denote degenerate primers and q represents quantitative real-time PCR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' to 3' Orientation</th>
</tr>
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<tbody>
<tr>
<td>CFTRF1*</td>
<td>AAA TGT AAC TGC CTC CTG GGA YGA RGG</td>
</tr>
<tr>
<td>CFTRF2*</td>
<td>TCC CCT CAG ACC TCT TGG ATH ATG CC</td>
</tr>
<tr>
<td>CFTRR1*</td>
<td>CCT CGG CTT CCA GCT GTT TNA RYT GYT G</td>
</tr>
<tr>
<td>NKA F1*</td>
<td>GGA TGA ACT AAA GAA GGA AGT AGA TAT GGA YGA YCA YAA</td>
</tr>
<tr>
<td>NKA R1*</td>
<td>CCA GAC AAT TCT TTT TCG CCA TNC KYT T</td>
</tr>
<tr>
<td>NKA R2*</td>
<td>CAC GCC GGT GAT TAT GTG TAT RAA RTG NTC</td>
</tr>
<tr>
<td>NKCC1 F1*</td>
<td>CCC CCT CTC AGT CTC GGT TYC ARG TNG A</td>
</tr>
<tr>
<td>NKCC1 R1*</td>
<td>GGA TGT ACC CTC GTA GAG GCT CRT TRT TYT T</td>
</tr>
<tr>
<td>qCFTRF1</td>
<td>TTC GAC CTC ATT CAG CTC ACA</td>
</tr>
<tr>
<td>qCFTRR1</td>
<td>TGG CGG CGA TGA AGA TGT A</td>
</tr>
<tr>
<td>qNKA F1</td>
<td>ACG AAC CGG CCA ACG ATA A</td>
</tr>
<tr>
<td>qNKA R1</td>
<td>TTT GTA GTA GGA GAA GCA GCC A</td>
</tr>
<tr>
<td>qNKCC1 F1</td>
<td>GGA TTT GTA CGA GGA GGT GGA G</td>
</tr>
<tr>
<td>qNKCC1 R1</td>
<td>GCA AAG GCA AAG ATC AGA CCA A</td>
</tr>
</tbody>
</table>
Table 5-2. Plasma parameters and gill Na\(^+\), K\(^+\)-ATPase for longhorn sculpin acclimated to 100, 20 or 10% SW for 24 or 72 hours. Means (s.e.m.) are recorded. Asterisks represent significant differences from 100% SW within 24 or 72-hour treatments.

<table>
<thead>
<tr>
<th></th>
<th>24 hour</th>
<th>72 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% SW</td>
<td>20% SW</td>
</tr>
<tr>
<td>Plasma osmolality mOsm kg(^{-1})</td>
<td>340.0 (3.2)</td>
<td>331.6 (7.1)</td>
</tr>
<tr>
<td>Plasma [Na(^+)] mOsm L(^{-1})</td>
<td>178.2 (6.38)</td>
<td>162.7 (5.4)*</td>
</tr>
<tr>
<td>Plasma [K(^+)] mOsm L(^{-1})</td>
<td>4.2 (0.2)</td>
<td>3.6 (0.4)</td>
</tr>
<tr>
<td>Plasma [Cl(^-)] mEquiv L(^{-1})</td>
<td>166.1 (2.9)</td>
<td>158.0 (4.8)</td>
</tr>
<tr>
<td>Gill Na(^+), K(^+)-ATPase activity mmol ADP mg pr(^{-1}) hr(^{-1})</td>
<td>8.0 (0.66)</td>
<td>8.2 (0.7)</td>
</tr>
</tbody>
</table>
Figure 5-1. Representative light micrographs of the immunolocalization of the cystic fibrosis transmembrane conductance regulator (CFTR, 1A, D, G), Na+, K+-ATPase (NKA, 1B and E), and Na+, K+, 2Cl- cotransporter (NKCC1, 1C and F). The gills are from longhorn sculpin acclimated to: 100% SW for 72 h (A-C); 10% SW for 24 h (D-F); and 10% SW for 72 h (G and H). CFTR was immunolocalized to the apical membrane, and NKA and NKCC1 to the basolateral membrane of epithelial cells of the interlamellar region. H) Is the next serial section of gill (7 microns deeper) from G) and is double labeled with anti-CFTR, indicated by the arrows (brown, apical membrane), and anti-NKA (blue, basolateral membrane), and shows that CFTR and NKA are expressed in the same epithelial cells. I) Western blots of longhorn sculpin gills acclimated to 100% SW. The first lane is the molecular mass ladder (std), second lane is a blot incubated with anti-CFTR, third lane is a blot incubated in anti-NKA and fourth lane is a blot incubated with anti-NKCC. Scale bar = 50 μm.
Figure 5-2. Longhorn sculpin gill CFTR, NKA and NKCC1 expression levels following acclimation to 100, 20 or 10% seawater (SW) for 24 and 72 hours (h). A, C, E) are protein levels determined from immunoblots (dot blots). B, D, F) mRNA levels determined by quantitative Real-Time PCR. mRNA values are normalized to L8 protein levels determined from immunoblots (dot blots). B, D, F) mRNA levels are relative mRNA Level compared to the sham value at 24 or 72 h (p values are listed on the graph). All values are relative to the mean sham (100% SW) level at 24 or 72 h, and are mean ± s.e.m. Asterisks represent statistically significant differences compared to the sham value at 24 or 72 h (p values are listed on the graph).
CHAPTER 6
EFFECTS OF LOW ENVIRONMENTAL SALINITY ON ENDOTHELIN RECEPTOR AND ENDOTHELIN CONVERTING ENZYME-1 MRNA IN THE GILL OF THE LONGHORN SCULPIN, MYXOCEPHALUS OCTODECEMSPINOSUS

Introduction

The small secreted protein, endothelin-1 (EDN1), is found in all gnathostomes (Hyndman and Evans, 2007), and it acts to regulate vascular tone (La and Reid, 1995; Yanagisawa et al., 1988a; Yanagisawa et al., 1988b), modulate ion transport (Ahn et al., 2004; Evans et al., 2004; Garvin and Sanders, 1991; Ge et al., 2006; Prasanna et al., 2001; Zeidel et al., 1989), and direct the migration of neural crest cell during craniofacial development (Clouthier and Schilling, 2004; Kurihara et al., 1994). Endothelin-1’s actions are mediated via two G-protein-coupled receptors (GPCR) in mammals, EDNRA and EDNRB1. In non-therian tetrapods, a third GPRC, EDNRB2, is involved in EDN1 signaling, and the teleosts are unique because they have an additional EDNRA (termed EDNRA2) and EDNRB1 (termed EDNRB1b) (Chapter 3), likely a result of the teleost specific genome duplication (Volff, 2005). Endothelin-1 is produced through two cleavage events involving the enzyme furin (Yanagisawa et al., 1988c) and the endothelin converting enzyme-1 (ECE1) (Shimada et al., 1994; Xu et al., 1994). The active form of EDN1 is secreted from the cell and acts on neighboring cells as a paracrine or autocrine signaling molecule (Webb, 1997)(Chapters 1 and 4).

Evans et al. (2004) were the first to suggest that EDN1 may be a local regulator of ion transport in the fish gill. They determined that in vitro, EDN1 inhibits net chloride in the euryhaline killifish (Fundulus heteroclitus) opercular epithelium (Evans et al., 2004), a model tissue for the seawater (SW) teleost gill (Karnaky et al., 1977). Recently, we determined the effects of acclimation to SW or fresh water (FW) in vivo, on gill EDN1, ECE1 (Hyndman and Evans, 2007), and endothelin receptor (EDNR) (Chapter 4) mRNA levels in the killifish. We
determined that all these components of EDN1 signaling are affected by changes in environmental salinity; however, the physiological role of EDN1 in the fish gill is still unclear. One would hypothesize that if EDN1 inhibits ion transport, then during acclimation to SW where there is an increase in plasma osmolality (Marshall et al., 1999), that EDN1 levels would be low, allowing the fish to excrete excess ions and return to ion homeostasis. Conversely, during acclimation to FW, when ions tend to be lost to the environment (Scott et al., 2006), EDN1 levels would be high, resulting in an inhibition of ion transport out of the gill, thus retaining plasma ions. Our experiments in the killifish suggest that EDN1 levels are highest during acclimation to hyperosmotic environments, opposite to our predictions. Thus, it is still unclear what the physiological role of EDN1 is in the gill. A recent hypothesis by our lab, is that EDN1 stimulates cyclooxygenase-2 production of prostaglandins and that these paracrines aid in cell survival during osmotic stress (Choe et al., 2006). Similar results were found in mammalian medullary interstitial cells that experience rapid changes in extracellular osmolality (Hao et al., 1999; Hao et al., 2000).

In an attempt to understand further EDN1’s putative role in the gill, we determined the effects of low environmental salinity on the EDN1 system in the moderately euryhaline longhorn sculpin, *Myxocephalus octodecemspinus*. The longhorn sculpin, is a marine fish that is distributed from Virginia to the Arctic, and has been found in estuaries during high tides (Bigelow and Schroeder, 2002). We recently reported that the longhorn sculpin are capable of tolerating low environmental salinity (10-20% SW) for days (Chapter 5). Sculpin in 20% SW can maintain plasma ion homeostasis, while sculpin in 10% SW significantly lose ions over 72 h (Chapter 5). The purposes of this study were to: 1. sequence sculpin gill cDNA for the *EDNRs* and *ECE1*; 2. determine the tissue expression of *EDNR* mRNA; and 3. determine the effects of
low environmental salinity on EDNR and ECE1 mRNA levels in the gill. By examining the EDN system in a moderately euryhaline fish, we aim to better understand the role EDN may be playing in ion balance in fishes.

Methods

Longhorn sculpin (Myxocephalus octodecemspinosus Mitchell) were purchased from local anglers from Frenchman Bay, ME. The fish were maintained at the Mount Desert Island Biological Laboratory in 3 ft circular tanks supplied with free flowing SW (31 ppt) from Frenchman bay (15°C). The fish were fed squid to satiation every other day.

Molecular Sequencing and Tissue Distribution

We previously sequenced killifish (Fundulus heteroclitus Linnaeus) gill cDNA for EDNRA, EDNRB1, and EDNRB2 (Chapter 3) and ECE1 (Hyndman and Evans, 2007). Using the same degenerate primers used to initially sequence these killifish cDNAs (Table 3-2), we sequenced and cloned a portion of the coding sequence for each EDNR and ECE1 from the sculpin gill. These sequences were translated and compared to those in NCBI’s protein database using BLAST, and using sequence homology, we confirmed we had sequenced the longhorn sculpin orthologues of EDNRA (Accession EU440324), EDNRB1 (Accession EU440325), EDNRB2 (Accession EU44026), and ECE1. In addition, to determine which co-orthologous copy of the tetrapod EDNRA and EDNRB1 we had sequenced, and to determine the evolutionary relationship among these sculpin EDNRs, we performed a fast maximum likelihood analysis (Felsenstein, 2004). A portion of the sequences from in our complete phylogenetic analysis of the gnathostome EDNRs (Chapter 3) were used to constructed a multi-sequence alignment using ClustalX (Larkin et al., 2007). Only a portion of the sequences were used because we wanted to run this additional analysis in a timely manner, while including all sequences would have extended our analysis an additional 20-30 days. This alignment was
inspected to ensure no gaps were inserted into known structural regions (i.e. transmembrane domains). Using the WAG model of amino acid substitutions (Whelan and Goldman, 2001), a free gamma distribution parameter, and a fixed proportion of invariant sites (Pinv=0), the maximum likelihood (ML) tree was determined. The robustness of the ML tree was evaluate by non-parametric bootstrapping = 1000.

Using multi-tissue, duplexing PCR, we determined the relative tissue distribution of the sculpin EDNRs. Following the methods of Choe et al. (2006), the RNA from the sculpin gill, operculum, brain, heart, stomach, intestine, kidney and white muscle was extracted using TRIReagent (Sigma, St. Louis, MS) following manufacturer’s instructions. Total RNA (0.5 μg) was reverse transcribed into cDNA using random hexamer primers and Superscript III (Invitrogen, Carlsbad, CA). These cDNAs were diluted ¼ with diethyl pyrocarbonate water. Non-degenerate primer pairs (Table 6-1) were designed to amplify a product with high efficiency (e.g., high melting temperature) and to minimize the chance of amplifying contaminating genomic DNA; the primer pair was designed to include at least one exon-exon boundary when possible (Table 6-1). A QuantumRNA™ 18S internal standard primer kit (Ambion, Woodward Austin, TX) was used to control for variability in cDNA quality and the quantity between the different tissues tested. Duplexing PCR with primers for 18S and either EDNRA, EDNRB1 or EDNRB2 was optimized to ensure that the reactions were terminated during the exponential phase. Lastly, the products were visualized by ethidium bromide staining in 1.5% agarose gels and digitized using the Biorad Gel Doc™ XR System (Hercules, CA).

**Salinity Challenge**

The sculpin salinity challenge was previously described in Chapter 5, and was performed at MDIBL (summer 2006 and 2007). Sculpin (5 or 6) were subjected to one of three treatments:
1) 100% SW (sham) 2) 20% SW; or 3) 10% SW. Each sculpin was placed in five gallons of the appropriate, aerated solution in a 10-gallon bucket and air. The buckets were maintained in a trough with free flowing SW (15°C) for temperature control. For experiments that lasted longer than 24 h, 50% of the water was replaced in each bucket daily. At 24 or 72 h post transfer, killifish were anesthetized in benzocaine (0.375 g l⁻¹ dissolved initially in ethanol, then dissolved in treatment water, final ethanol concentration 0.1%) and the blood and gills removed (see Chapter 5). These time end points were based upon the time course determined by Claiborne and colleagues (1994). They determined significant decreases in plasma chloride at 24 h and a return to control values at 72 h, thus we used these end points for our experiment.

**Quantitative Real-Time PCR**

To determine the effects of dilute environments on longhorn sculpin gill EDNRA, EDNRB1, EDNRB2, and ECE1 mRNA levels, quantitative real-time PCR (qRT-PCR) was performed. Non-degenerate primers were designed to amplify a product between 50-100 bp (Table 6-1). L8 was used as an internal control gene as previously described (Choe et al., 2006; Choe et al., 2005). Each sample was run in duplicate using 2 µl of 1/10 diluted original oligo-dt cDNA, 7.4 pmol of primers and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 25 µl. The cycling parameters used were: an initial denaturing step of 95°C for 10 min, 40 cycles of: 95°C for 35 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a melting curve analysis to ensure only one product was amplified. Random samples were also sequenced following qRT-PCR, confirming amplification of the target of interest. To determine the degree of possible genomic contamination, qRT-PCR was run using RNA samples that were not reverse transcribed; no genomic contamination was found. All qRT-PCRs were run on a MyiQ quantitative thermocycler (Biorad).
Each primer pairs’ efficiency was determined by performing a 10-fold dilution curve using plasmid cDNA. Efficiency (E) for each primer pair was calculated using the equation: \[ E = -1 + 10^{-\frac{1}{\text{slope}}} \] where “slope” was the slope of the dilution curve. Each cycle threshold (CT) value was subtracted from a randomly chosen sham sample resulting in a \( \Delta \text{CT} \), and were analyzed using the Pfaffl equation (Pfaffl, 2001): \[ \text{ratio} = \frac{E^{\Delta \text{CT target}}}{E^{\Delta \text{CT L8}}} \] Each Pfaffl ratio was then standardized to the average sham Pfaffl ratio.

**Statistics**

Quantitative real-time PCR data was analyzed using 2-Factor ANOVAs (for salinity and time), followed by unpaired, two-tailed T-tests to determine differences compared to sham treatments (100% SW). Statistical significance was set at alpha = 0.05. All statistics were run using SPSS (v.15, Chicago, IL).

**Results**

**Phylogenetic Relationships**

Our predicted sculpin EDNRA, EDNRB1 and EDNRB2, were aligned with other gnathostome EDNRs sequences, and the phylogenetic relationships among these receptors determined. The maximum likelihood score calculated was -13083 and a gamma parameter of 0.612. The longhorn sculpin EDNRA grouped with the teleost EDNRAs and the tetrapod EDNRAs, but outside of the teleost specific duplicate of EDNRA2s (Fig. 6-1). Likewise, the EDNRB1 grouped with the teleost EDNRB1s and the tetrapod EDNRB1s, but outside the teleost specific duplicate of the EDNRB1s. Finally, the longhorn sculpin EDNRB2 grouped with the teleost EDNRB2 and the non-therian gnathostome EDNRB2 (Fig. 6-1); the EDNRB2 has been lost by the therians (Chapter 3).
**Longhorn Sculpin Tissue Distribution**

In the longhorn sculpin, *EDNRA, EDNRB1, EDNRB2* mRNA are ubiquitously expressed in the tissues tested (Fig. 6-2). There are relatively high levels of *EDNRA* in the gill, operculum, heart, and kidney. *EDNRB1* was relatively high in the gill, operculum, and heart, and *EDNRB2* was highest in the heart, kidney, and intestine (Fig. 6-2).

**Quantitative Real-Time PCR**

Longhorn sculpin gill *EDNRA* mRNA levels did not differ from 100% SW sham at 24 or 72 h (Fig. 6-3A). Longhorn sculpin acclimated to 20% SW had gill *EDNRB1* mRNA levels that were not different from sham at either time; however, there was a significant 2.5-fold increase in gill *EDNRB1* after 24 h and 72 h acclimation to 10% SW (Fig. 6-3B). Also, we found a significant increase in gill *EDNRB2* after 24 and 72 h acclimation to 20% SW, but 10% SW sculpin were not different from 100% SW shams (Fig. 6-3C). Finally, gill *ECE1* mRNA levels did not differ from shams with low-salinity acclimation after 24 or 72 h (Fig. 6-3D).

**Discussion**

Endothelin-1 signaling cascades are hypothesized to be local regulators of ion transport in the fish gill (Evans et al., 2004); however the physiological role of this system in the fish gill is unclear. From our phylogenetic analysis, it was determined that we sequenced a portion of the longhorn sculpin *EDNRA, EDNRB1, and EDNRB2*, but we did not sequence the teleost specific duplications, *EDNRA2* and *EDNRB1b* (Chapter 3). The sculpin *EDNRA, EDNRB1, and EDNRB2* are ubiquitously expressed throughout the fish, with relatively high levels in osmoregulatory tissues such as, the gill and kidney. We recently determined that in the euryhaline killifish *EDNRA, EDNRB1, and EDNRB2* are also ubiquitously expressed in these same tissues, suggesting a constitutive role of EDN1 signaling in teleost fishes. One unexplored area of EDN1 research is understanding the function of the teleost specific duplicates, *EDNRA2*
and EDNRB1b. Teleost fishes have at least five EDNRs, while mammals have two. What is the function of these duplicate receptors? Are they expressed in the gill? How does environmental salinity affect their expression or immunolocalization? Does the longhorn sculpin have a duplicate EDNRA2 and EDNRB1b? These are all important questions that have not been explored.

The longhorn sculpin can tolerate direct transfers from SW to 20% SW, but cannot survive for more than a few days in salinities less than 10% SW (Chapter 5, Claiborne et al. 1994), making it a moderate osmoregulator. We determined that ECE1 mRNA levels did not change with acclimation to low salinity water. The conversion of the EDN1 precursor, proendothelin-1, by ECE1 is a rate limiting step in the production of active EDN1 (D'Orleans-Juste et al., 2003; Ikeda et al., 2002). Here we postulate that because there was no observed change in ECE1 mRNA there is likely no change in ECE1 protein level. Thus, we hypothesize that in the longhorn sculpin, acclimation to 10% or 20% SW does not change active EDN1 levels. In contrast, there were increases in EDNRA1 and EDNRA2 mRNA in the sculpin during acclimation to 10% SW or 20% SW, respectively. It may be that there is an increase in EDN1 signaling during hypo-osmotic stress, because there are more receptors present. If the EDN1 signaling via an EDNRA1-type receptor results in the inhibition of ion transport as hypothesized by Evans et al. (2004), then in our 10% SW sculpin one would expect that the sculpin would stop losing ions. We previously determined that longhorn sculpin cannot survive in 10% SW more than 6 days, and 24 h after transfer to 10% SW they have a significant decrease in plasma osmolality and ions (Chapter 5). Therefore, we conclude that the EDNRA1 is not involved in the inhibition of ion transport as hypothesized by Evans et al. (2004).
Twenty percent SW is also hypo-osmotic to the sculpin, but in this salinity, the fish was capable of maintaining plasma osmolality over the 72 h test period (Chapter 5). There was a significant 1.75-fold increase in \textit{EDNRB2} mRNA level during this time. Perhaps the activation of the \textit{EDNRB2} can lead to local regulation of ion transport in the longhorn sculpin, but below 20% SW the sculpin is incapable of up-regulating \textit{EDNRB2} and subsequently loses plasma ions. In contrast, during FW acclimation by the killifish, there is no change in \textit{EDNRB2} mRNA (Chapter 4), and this fish is capable of returning to control plasma ion values during hypo-osmotic acclimation, thus the role of \textit{EDNRB2} in fish osmoregulation warrants more experimentation.

In the killifish, \textit{EDNRA} are localized on the MRC, and FW-acclimated killifish have significantly lower \textit{EDNRA} (mRNA and protein) compared to SW-acclimated killifish (Chapter 4). We attempted to immunolocalize the EDNRs in the longhorn sculpin gill (as we did in the killifish, Chapter 4); however, our controls (Western blots and peptide-absorbed antibodies) did not work, suggesting these antibodies were not functioning in the longhorn sculpin. At the mRNA level, though, acclimation to 20% and 10% SW did not elicit any changes in \textit{EDNRA} mRNA in the sculpin gill. There are two possible explanations for this difference: 1. the putative role of EDNRs in regulation of ion transport is species specific, with \textit{EDNRA} being the receptor responsible for these effects in the killifish, and potentially \textit{EDNRB2} fulfilling this role in the sculpin. 2. The sculpin cannot survive in <20% SW, and the regulation of gene expression in this fish is impaired. Perhaps the changes we see are due to a lethal stress and are do not reflect a normal physiological response. In the future, experiments to differentiate between these two hypotheses are needed.
In conclusion, the role of EDN1 in the fish gill is still not clear; however, it is evident that environmental salinity regulates gill EDNR. It is plausible that the EDN signaling axis is impaired during acclimation to low salinity water, and if this is upstream to the regulation of ion transporter density, it maybe one reason why they cannot maintain ion homeostasis. Future experiments will help elucidate this hypothesis.
<table>
<thead>
<tr>
<th>Primer</th>
<th>5' to 3' Orientation</th>
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<tbody>
<tr>
<td>tdEDNRAF1</td>
<td>TAT CTG GTT GCT GTC GCT CTT</td>
</tr>
<tr>
<td>tdEDNRAR1</td>
<td>CAA CAG GAA ATT GAG CAG CTC</td>
</tr>
<tr>
<td>tdEDNRB1F1</td>
<td>CAC TCG GAG ACC TGC TAC ACA T</td>
</tr>
<tr>
<td>tdEDNRB1R1</td>
<td>CCA ACG GCA GGC AGA AAT AT</td>
</tr>
<tr>
<td>tdEDNRB2F1</td>
<td>TAG ACT GCT GCC GTT CAT CCA</td>
</tr>
<tr>
<td>tdEDNRB2R1</td>
<td>TTC ATG TGG TCG TTG AGC G</td>
</tr>
<tr>
<td>qEDNRAF1</td>
<td>CTA TCT GGT TGC TGT CGC TCT T</td>
</tr>
<tr>
<td>qEDNRAR1</td>
<td>TGC GTA TGG TTT CGT TCC TGT</td>
</tr>
<tr>
<td>qEDNRB1F1</td>
<td>AAG AGA GGT GGC TAA GAC GGT G</td>
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<td>AGG ATA CGG CTG AGA TGG AGA G</td>
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</tr>
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<td>qECE1F1</td>
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</tr>
<tr>
<td>qECE1R1</td>
<td>AGA TCC TTG GCC TCC ATC TTG</td>
</tr>
</tbody>
</table>
Figure 6-1. Maximum likelihood analysis of the endothelin receptors including our new sculpin EDNRA, EDNRB1 and EDNRB2 protein sequences. Numbers at the nodes represent percent bootstrap. For accession numbers see Figure 3-3.
Figure 6-2. mRNA tissue distribution of the endothelin receptors from the longhorn sculpin determined using duplexing semi-quantitative PCR. 18S was used as an internal control.
Figure 6-3. Longhorn sculpin gill A) EDNRA, B) EDNRB1, C) EDNRB2, and D) ECE1 expression levels following acclimation to 100, 20 or 10% seawater (SW) for 24 and 72 hours (h). mRNA levels determined by quantitative Real-Time PCR. mRNA values are normalized to L8 mRNA levels, and all values are relative to the mean sham (100% SW) level at 24 or 72 h, and are mean ± s.e.m. Asterisks represent statistically significant differences compared to the sham value at 24 or 72 h (p values are listed on the graph).
CHAPTER 7  
CONCLUSIONS

This work is the first to explore fully the expression of EDN1, ECE1, and the EDNRs in the teleost gill, to test the effects of hyperosmotic and hypo-osmotic environments on gill EDN1 signaling, and completely analyze the evolution of EDNs, ECEs and EDNRs.

In the killifish and the longhorn sculpin gills, EDN1, ECE1, and the EDNRs are expressed, demonstrating for the first time that EDN1 signaling occurs in vivo in the fish gill. A summary of the localization of these proteins is found in Figure 2-9. Although, I could not immunolocalize these proteins in the sculpin (because Westerns did not work with sculpin gills), I was able to sequence ECE1 and EDNRs from the sculpin gill, and a recent papers on EDNRA and EDNRB immunolocalization in the cod and tiger pufferfish gills suggests that this system is active in all teleosts. The findings of EDN1 producing cells and EDNRs in the gill strongly suggest that EDN1 acts as a paracrine and autocrine in these fishes. There is mounting evidence that EDN1 acts as an autocrine to regulate pillar cell tone, and as a result regulates blood flow through the lamellae. The finding of EDNRA on the MRC of the killifish gill suggests a putative role in the regulation of ion transport by the MRC. Interestingly, EDNRA was immunolocalized (using heterologous and homologous antibodies) in two other teleosts, and each had a different EDNRA distribution through the gill. In the cod, EDNRA were found on hypothesized nerve fibers running along the length of the filament (Stenslokken et al., 2006). In the tiger pufferfish, EDNRA were found on pillar cells. Thus, the distribution of EDNRA in the teleost gill may be species-specific given these observed differences in localization. The expression of EDNRB throughout the gill suggests that these receptors may act as clearance receptors for the EDNs. More than half of a bolus inject of EDN1 is removed with one pass through the gills (Olson, 1998), and likewise in mammals a significant portion of EDN1 is removed through the lungs by
the EDNRB1 (La and Reid, 1995). Therefore, it seems plausible that either EDNRB1 or EDNRB2 is acting as a clearance receptor in the fish gill.

One limitation to my findings was that I was not able to differentiate on the protein level between EDNRB1 and EDNRB2. We did make killifish-specific EDNR antibodies; however, preliminary experiments were inconclusive. In the future, \textit{in situ} hybridization will be important in determining the mRNA localization of these receptors. Until more teleosts are tested, and perhaps even more homologous antibodies made (or more \textit{in situ} studies complete), the generality of my model (Fig. 2-9) is unknown. However, this is the first model to describe the potential functions of EDN1 in the fish gill using empirical data, and it is a reasonable hypothesis to test in other species.

The expression of EDN1, ECE1, and the EDNRs are affected by acclimation to seawater (hyperosmotic environment) and fresh water (hypo-osmotic environment). In the killifish, some changes were within 3 h of transfer, while others took at least 24 h to occur, suggesting that EDN1 may be involved in short-term regulation of ion balance as I originally hypothesized. When one compares the extremely euryhaline killifish with the moderately euryhaline sculpin during acclimation to hypo-osmotic environments, we find that the killifish after 24 h had small changes in EDNR expression (mRNA and protein), while the sculpin had a 2.5-fold increase in \textit{EDNRB1} mRNA levels. In 10% SW the sculpin loses ions, likely because it does not down-regulate ion transporters necessary for tolerance in hypo-osmotic environments (CFTR and NKCC1) (Chapter 5). In contrast, the killifish significantly decreases these same ion transporters, and their plasma ion levels start to return to control values (Choe et al., 2006; Scott et al., 2006). Thus, it is hard to differentiate between normal, physiological responses to changing environmental salinity, and the effects from the sculpin that are not osmoregulating.
properly, and ultimately lose ions to a lethal level (as the sculpin is doing in 10% SW). If we compared the sculpin in 20% SW and the killifish in fresh water for 24 h, we do see a similar response—no change in \( EDNRA \) or \( EDNRB1 \) mRNA levels; however, sculpin increased in gill \( EDNRB2 \) mRNA levels, and unlike the killifish, sculpin were not losing plasma ions during this acclimation period. There are no clear trends between these two fishes, but I think an important conclusion is that EDN signaling is present in the teleost gill, and there are some changes with acclimation to a hypo-osmotic environment.

During acclimation to hyperosmotic environments, killifish increase ECE1, EDNRA, and EDNRB, suggesting that there is more active EDN1 produced (because there is more ECE1) and more receptors to bind EDN1 (Chapters 2 and 4). In addition, chronically acclimated killifish had lower gill EDNRA levels (all other EDN components were unchanged), and the EDNRA was immunolocalized to the MRC (Chapter 4). Thus, I speculate that EDNRA is involved in regulation of ion transport in the killifish gill during hyperosmotic stress. As stressed throughout my dissertation, until functional studies are completed, through the use of tissue-specific knockouts, silencing RNAs, or morpholinos (and likely other techniques), all one can do is speculate on the function of EDN1 in fishes.

From my phylogenetic analyses, I determined the evolutionary relationships among the EDNs, ECEs and EDNRs, respectively. The first conclusion from these studies is that all three EDNs and EDNRs are found in teleosts, and they are not found in the early chordates (lancelets and sea squirts) or any invertebrates. The EDN signaling axis is only found in the vertebrates. I have sequenced portions of the EDNRs from the sea lamprey (EDNRA EU440327, Appendix A) and shark gill (EDNRA EU440328, and EDNRB1 EU440329), and physiological studies by Evans and Harrie (2001) in the Atlantic hagfish suggest that this system was present in the early
vertebrates. This signaling axis is necessary for proper craniofacial development and may have been a crucial step in the development of the vertebrate jaws (Clouthier and Schilling, 2004). My results support this hypothesis by determining that EDNs and EDNRs are vertebrate-specific. What was very interesting was to find ECEs in all organisms including prokaryotes. It seems likely that ECE was a general protease and as it evolved, it became specialized for cleaving preferentially the EDNs. ECEs in vertebrates function as dimers, while in all other organisms they function as monomers, so there has been a structural and functional shift of this enzyme in the vertebrates.

The second, and in some respects, the most interesting thing I discovered during the course of my dissertation, was that there are three groups of EDNRs. When I started (and even now) most researchers talked about the two EDNRs: EDNRA and EDNRB. What we all missed was in 1998 a third receptor was described (and mislabeled) as the avian-specific EDNRB2. Since then about four more “avian-specific” papers have been published on this receptor. I initially found out about this receptor because it was the first clone I sequenced from the killifish gill. This finding and discussions with Dr. M. Miyamoto prompted me to explore the field of bioinformatics and phylogenetics to further understand this family of G-protein-coupled receptors. As shown in Figure 3-3 there are EDNRB2 proteins in the teleosts, amphibians, birds, and platypus, and I determined that EDNRB2 was lost by the therian mammals about 150 mya. In addition, an interesting finding was that the EDNRA and EDNRB1 are duplicated in the teleost fishes, likely a result of the teleost-specific genome duplication after this split from the tetrapods. In the future, studies should look at these duplicates more closely and determine what function they may play in the teleosts.
With the loss of EDNRB2 in therians, I hypothesized that perhaps the remaining EDNRS had undergone modifications, and had different functions from the non-therian EDNRS to compensate for this missing protein/gene (Chapter 3). Using a Rate Shift Analysis developed by Knudsen et al. (2003) and the known functionally important sites in the mammalian EDNRA and EDNRB1, I determined that the therian EDNRA has 6/21 functionally important sites that are slow evolving Type I sites or Type II sites (see Fig. 3-1 for a definition) compared to the non-therian gnathostomes. In addition, the EDNRB1 is highly conserved among these gnathostomes, compared to the EDNRA. This suggests that EDNRA may have different functions in the therians than non-therian gnathostomes.

My dissertation work is the foundation for the future functional studies needed to determine the function of EDN1 in the fish gill. With these sequences from the killifish, morpholinos, knockouts and silencing RNA probes can be made and used to test the effects of inhibiting specific steps in EDN1 signaling cascades. When I started this work, the tiger pufferfish (*Takifugu rubripes*) genome was just released, and the zebrafish (*Danio rerio*) genome sequencing was underway. The tiger pufferfish does have some level of salinity tolerance; however, the zebrafish is strictly a freshwater species. Thus, the addition of the sequences from a fish with extreme salinity tolerance, like the killifish, are very useful for future functional studies. In addition, the model presented in Figure 2-9 summarizes the localization and signaling patterns of EDN1 in the gill, and is an excellent hypothesis for others to test in their species of interest.

Finally, my dissertation work points out some fundamental issues with the way EDN research has been progressing over the past 20 years. First, most people assumed that antagonists and agonists developed for mammals will behave the same in non-mammalian
vertebrates. We recently determined this was not true for a COX-2 antagonist (Choe et al., 2006), and as tabulate in Table 3-1, the EDNRs have different pharmacology profiles for many animals. Second, there is an assumption that these receptors have the same functions in all organisms, and given that many biomedical studies are conducted in non-mammalian model organisms (chicks, frogs and zebrafish), this is a faulty assumption. My functional analysis of the EDNRA and EDNRB1 suggests that there was a functional shift in the therian EDNRA, but that the EDNRB1 is well conserved across the gnathostomes. This needs to be tested using well-developed, functional studies in non-mammalian vertebrates. Thus, all one can do is hypothesize as to the function(s) of EDN in the lower vertebrates. Finally, the fact that a third group of EDNRs, the EDNRB2, is still largely ignored needs to be remedied. By exploring the biology of the EDNRB2, it will help researchers understand the evolution of EDNRA and EDNRB1 in the therians. Given the medical importance of these receptors, it seems important to understand how their functions have changed or remained constant over the past 500 mya. These types of comparative studies can lead to discoveries of new functions, give insight into drugs and treatments of EDN-associated pathologies, and further our basic knowledge of this system.
APPENDIX

TISSUE DISTRIBUTION OF THE LAMPREY ENDOTHELIN A RECEPTOR

Tissue distribution of the sea lamprey EDNRA mRNA (Accession EU440327). Like the killifish and longhorn sculpin, the lamprey EDNRAs are found in all tissues tested.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Kelly Anne Hyndman was born in Guelph, Ontario, Canada, in 1978. She received her Bachelor of Science Honours, with a specialization in marine and fresh water biology in 2001. As an undergraduate, Kelly was awarded an NSERC Undergraduate Research Experience grant and spent the summer of 1999 working as an aquaculture technician on an Arctic Char (Salvelinus alpinus) fish farm (Icy Waters, Limited, Whitehorse, Yukon). Following this, she worked as a research assistant to Dr. James Ballantyne (University of Guelph), examining the role of photoperiod on Na\(^+\),K\(^+\)-ATPase activity in the char and rainbow trout (Oncorhynchus mykiss). In August, 2001 she entered the graduate program in the Department of Zoology at the University of Florida in Gainesville. In May 2004, she bypassed the M.S. and entered the Ph.D. program in the department. She has spent many summers at the Mount Desert Island Biological Laboratory in Salisbury Cove, ME, where a significant portion of her dissertation was completed. In 2006, she attended the Workshop on Molecular Evolution at the Marine Biology Laboratory, Woods Hole, MA. In October 2008, she hopes to start a post doc position; however, the exact location of that has not been determined.