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Quantification of AQP11 Paralog Expression in the Osmoregulatory Organs of Anguilla rostrata

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QUANTIFICATION OF AQP11 PARALOG EXPRESSION IN THE OSMOREGULATORY ORGANS OF ANGUILLA ROSTRATA

by

KEITH C. KURT

(Under the Direction of Christopher P. Cutler)

ABSTRACT

Osmoregulation is facilitated by using an assortment of ion and water channels to assist acclimation to changing conditions and to maintain cellular homeostasis. Euryhaline fish can be found in both seawater and freshwater (SW and FW) environments. Expression of ion and water channels have been experimentally demonstrated to change as a fish acclimates to different environmental conditions. Relatively recently, a new group of water channels has been discovered that are primarily intracellular and includes aquaporin 11 (AQP11). Typically, AQPs are located on the cell plasma membrane to allow water to flow in and out of the cell by osmosis to assist in cellular homeostasis. Since AQP11 has been primarily located in the membrane of the endoplasmic reticulum, it was expected that the expression of AQP11 would not be affected by the acclimation of fish to SW. For this study, Anguilla rostrata (the American eel) was selected partly because it has two paralogs of AQP11 (AQP11a and AQP11b). Eels were subjected to a SW-acclimation experiment where they were acclimated to SW for predetermined times, while concurrent FW control samples were also kept. Initially, genomic sequences for AQP11a and AQP11b genes were available for the closely related Anguilla japonica (the Japanese eel), thus cloning and sequencing of the AQP11a and 11b paralog cDNA sequences from the American eel were performed. The obtained sequences were used to generate primers and antibodies for further studies. Quantification of mRNA/cDNA was performed using quantitative PCR (qPCR). The analysis of the qPCR data determined that both AQP11a and AQP11b had significant differences between FW and SW in the expression of mRNA/cDNA in multiple osmoregulatory tissues (i.e. kidney, gill, and gastrointestinal tract). Quantification of AQP11 proteins was facilitated using western blotting. The antibody generated for AQP11a was found to be non-specific and unusable for quantification potentially due to a possible
PDZ domain located at the C-terminal end where polypeptides for the antibody were made. The antibody made against AQP11b produced quantifiable western blots. Significant differences between AQP11b bands were found in all intestinal tissues. Additionally, localization of AQP11 paralogs was carried out using immunohistochemistry in multiple intestinal tissues as well as kidney and liver in both FW and SW-acclimated fish. In SW tissues AQP11a and AQP11b were seen to be present in smooth muscle in intestinal tissues as well as on the luminal side of epithelial cells and expression of AQP11a and AQP11b seems to be lower in corresponding FW tissues. In kidney tissue AQP11b was occasionally present in nephron tubules in SW tissue but not FW tissue, and AQP11b was also found in the liver surrounding the liver ducts. The within-group and between-group quantitative analysis of the FW and SW-acclimated AQP11 paralogs transcript and protein expression suggests that AQP11 paralogs are affected by SW-acclimation even though they are intracellular. These results may also point to the endoplasmic reticulum being affected by SW-acclimation due to subsequent related changes in AQP11 expression.

INDEX WORDS: *Anguilla rostrata*, AQP11, Aquaporin, DNA sequencing, CDNA quantification, Protein localization, Osmoregulation, Seawater-acclimation, Protein quantification.
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CHAPTER 1
GENERAL INTRODUCTION

**Teleost Fish and the Lifecycle of Anguilla rostrata**

There are over 26,000 species of teleosts that inhabit a wide range of environments of varying salinity. Some, less than 10% of fish species, are considered euryhaline (McDowall 1988). These fish are characterized by being able to survive in a range of salinities and migrate between ocean and river systems. Examples include the anadromous Atlantic salmon (*Salmo salar*) which migrates from the river to the ocean, or the catadromous American eel which migrates from the Sargasso Sea in the Atlantic to rivers along the East American coast. For this study, *Anguilla rostrata* (the American eel) was selected. Many studies (see later) have already been performed examining the expression and osmoregulatory functions of aquaporins (AQPs) and other ion transporters in the American eel or its cousins the European eel (*Anguilla anguilla*) and the Japanese eel (*Anguilla japonica*).

The American eel ranges between Southern Greenland to the North Eastern part of South America (Facey and Van den Avyle 1987). The eel’s lifecycle is believed to begin at the benthic zone of the Atlantic Ocean. Upon fertilization, eggs float to the surface and hatch into flat transparent larva, leptocephali, and are carried by the current from the Sargasso Sea to the Eastern American coast; as they approach they begin to grow fins and take on the shape of a typical eel, these juveniles are known as glass eels reflecting the fact that they are transparent (Kleckner and McCleave 1982, Kleckner and McCleave 1985). After arriving at estuaries along the coast, the eels begin to pigment to a dark green-brown or gray color. By this point, the eels have become juvenile elvers. These elvers either enter estuaries, making their way to rivers and lakes in the Eastern United States, or they may remain in the estuaries or in the ocean; in either case, they continue to grow for 3-5 years and eventually becoming the sexually immature, but adult, yellow eels (Haro and Krueger 1991). As a yellow eel, The American eel have the potential to grow for up to 30 years reaching lengths of well over a meter (Helfman *et al.* 1987), until they become sexually mature and experience other physiological changes resulting in a silver eel phase. Although they can live for many years, a study found that in the United States eels older than 7 years and longer than 65cm are
generally rare (Hansen and Eversole 1984). In the silver-phase of the lifecycle, the eels begin to prepare for the migration back to the ocean. Following significant physiological and morphological changes, silver eels are capable of making the long journey back to the Sargasso Sea to mate. After mating it is assumed that both the male and female eels die (Facey and Van den Avyle 1987). It should be noted, however, that there is no documentation of the American eel spawning in the wild and researchers have had difficulty in making eels spawn in captivity (ASMFC 2000).

**Acclimation to Environments and Osmoregulation**

Rarely do organisms live out their entire life in an unchanging, static, environment. Instead, environments are typically dynamic, with seasonal temperature, humidity, and other changes that necessitate organisms, plants and animals alike, to acclimate to changing conditions. Acclimations can be rapid, such as sweating when temperatures are high, or take place over a long period of time, such as the increase in red blood cells that occurs while acclimating to high altitudes (Zubieta-Calleja *et al.* 2007). Euryhaline fish are capable of migrating between ocean and freshwater systems. Being in constant contact with water presents an interesting challenge to euryhaline fish in that they must overcome the osmotic challenge of moving between freshwater (FW) and seawater (SW) systems. How euryhaline fish physiologically accomplish migration between environments of varying salinities is still not entirely known at a cellular and molecular level. It is understood that the ability to adjust osmoregulatory strategies plays a key role in a euryhaline teleost’s ability to tolerate environments with varying degrees of salinity (Evans and Claiborne 2009, Hwang *et al.* 2011, Marshall and Grosell 2006).

The main objective of osmoregulation in euryhaline fish is the maintenance of the ionic concentrations of sodium, chlorine, and other ions and regulation of osmotic pressure regardless of the surrounding environmental salinity (Evans and Claiborne 2009, Hwang *et al.* 2011, Marshall and Grosell 2006). Osmoregulation is facilitated by a multitude of ion transporters and water channels referred to as aquaporins (AQPs) (Madsen *et al.* 2015). Osmoregulation has been found to be carried out in multiple organs in teleost fish, of these, the most studied organs include the gills, kidneys, and gastro-intestinal tract (Madsen *et al.* 2015, Evans 2010).
**Osmoregulatory Organs and Their Roles in Euryhaline Fish**

The gills are the best studied osmoregulatory organ in teleost fish. In addition to gas exchange, acid-base regulation, and nitrogenous waste excretion, the gill plays a major role in regulating body fluid ion concentrations (Evans et al. 2005). In FW environments, a fish takes in significant quantities of water through the gills by osmosis, this excess water is removed through the urine due to the production of hypotonic urine (Hwang and Lin 2014). In SW the fish instead loses water by osmosis through the gills, this is compensated for by the fish ingesting large quantities of SW with subsequent secretion of excess ions across the gill (Hwang and Lin 2014). Branchial osmoregulatory mechanisms are achieved using various ion transporters and osmosis. In SW environments the gill is responsible for removing excess NaCl from the body, it accomplishes this utilizing multiple ion transporters, Na$^+$ K$^+$ ATPase (NKA), Na$^+$ K$^+$ 2Cl$^-$ cotransporter (NKCC1) and the cystic fibrosis transmembrane conductance regulator (CFTR; Madsen et al. 2007). NKA and NKCC1 in mitochondria-rich cells, such as chloride cells, work together to transport monovalent ions out of the gills against the prevailing electrochemical gradient (Marshall et al. 2002). Chlorine ions are transported out using the ATP-gated CFTR Cl$^-$ ion channel and sodium is passively secreted using a paracellular pathway (Marshall and Grosell 2006, Hirose et al. 2003). Abrupt changes in salinity have been shown in Atlantic killifish (*Fundulus heteroclitus*) to affect the expression of ion transporters such as NKCC1 (Scott et al. 2005). In addition to ion transporters the gill also has AQPs present; however, they are not believed to play a role in the water permeability of the gills. Instead, AQPs in the gill likely play a role in maintaining cell volume (Madsen et al. 2015).

The kidney plays an important role in producing urine to expel excess water in FW environments. In SW environments, however, due to the lack of a loop of Henle (as is found in mammalian kidneys), the teleost kidney cannot generate hyperosmotic urine. It has been suggested that AQPs play a major role in the reabsorption of water, however, the kidney is unable to produce anything more than isotonic urine even in environments with greater salinities than SW (Beyenbach 2004). An important function of the euryhaline kidney is how it can change from excreting excess water in FW environments to conserving it in SW environments (Brown et al. 1978). In addition to the role in water homeostasis, the kidney
functions to preserve monovalent ions in FW and SW conditions and secrete mainly divalent ions (mostly absorbed from imbibed SW in the intestine) such as Mg$^{2+}$ in SW conditions (McDonald 2007).

The nephron of the eel kidney begins with the glomerulus enclosed by the Bowman’s capsule, followed by proximal tubules I and II, distal tubule, and collecting tubule. The glomerulus filters water and excess ions out of the blood, with a rate 10x higher in FW than in SW reflecting the need to remove excess water in FW conditions and to conserve it in SW conditions (Brown et al. 1978, Elger et al. 1897, McDonald 2007); in fact, it has been seen in rainbow trout (*Oncorhynchus mykiss*) that up to 50% of nephron glomeruli are filtering when the fish is in FW and this sharply drops to 5% post-acclimation to SW (Brown et al. 1980). The main function of the proximal segments outside of nutrient reabsorption is to secrete Mg$^{2+}$ and other divalent ions in SW fish and they may also function to secrete excess water in FW fish (Beyenbach 2004). In the distal and collecting tubules of FW-acclimated fish, it has been noted that there is low water permeability whereas in SW-acclimated fish there is higher permeability, this allows the fish to create either hypotonic urine in FW or isotonic urine in SW, allowing for the conservation or excretion of water and ions respectively (McDonald 2007). It has been shown that NaCl absorption occurs in the collecting tubules to facilitate water being reabsorbed by osmosis from the urine leaving behind an isotonic solution containing Mg$^{2+}$ and other unwanted solutes (Beyenbach 2004). The expression of AQPs in the kidney has yet to be fully determined for all AQPs for *Anguilla* species and there seems to be no clear consensus across teleost fish species on whether expression of an AQP changes with SW-acclimation. For example, AQP10b mRNA expression has been found to decrease after SW-acclimation in the Japanese medaka (*Oryzias latipes*) and yellow eels of European eel whereas expression rose in Atlantic salmon and did not change in silver eels of European eel (Madsen et al. 2014, Martinez, et al. 2005, Tipsmark et al. 2010). Furthermore, there is evidence to suggest that the expression of some AQPs may be influenced by hormones. In European eel, mRNA of AQP1 paralogs in kidney were shown to be suppressed with the introduction of cortisol (a steroid hormone elevated during SW-acclimation). Suppression of AQPs mRNA by cortisol was prominent in yellow eels, suggesting that AQPs are possibly involved in water secretion, especially in the proximal tubule. In contrast, for silver-phase eels there were
no significant changes in AQP expression in the examined organs, this is presumably due to the eels having already undergone morphological changes necessary for the migration back to the ocean (Martinez et al. 2005).

When teleost fish are in SW the kidney is unable to produce hypertonic urine, that, coupled with a significant amount of water being lost through osmosis through the gills and skin, causes a need to rehydrate. Eels maintain an osmotic pressure of extracellular fluids of approximately 300-400 mOsm/l, compared to the approximate 1,000 mOsm/l of the SW, which results in the loss of water across permeable body surfaces such as the gills, leading to an increased concentration of body fluids, therefore requiring the replacement of water loses (Shehadeh et al. 1969). To replenish lost fluid volume, euryhaline teleost fish in SW typically drink 1-5 mL/Kg/h of seawater depending on the species, this drinking rate is approximately 10-fold to 15-fold higher than fish in FW; over 70% of this gets absorbed by the gastrointestinal tract with the rest released as rectal fluids (Carrick and Balment 1983, Marshall and Grosell 2006). Being able to remove ions from SW (i.e. against an osmotic gradient) is rare and only present in a handful of organisms outside of marine teleosts. In general, absorption of water by the gastrointestinal tract is facilitated by first desalinating ingested SW through the absorption of Na$^+$ and Cl$^-$ ions in the esophagus and the anterior intestines (Skadhauge 1969, Hirano et al. 1976, Grosell 2006). Water absorption mainly occurs via osmosis in the posterior intestines and rectum (Aoki et al. 2003, Kim et al. 2008). The Na$^+$ and Cl$^-$ ion uptake occurs in the esophagus (where the gastrointestinal tract has low permeability to water) by diffusion or secondary active transport (Hirano et al. 1976). Na$^+$ and Cl$^-$ uptake in the anterior intestine is achieved by multiple ion transporters including NKCC2 and, to a lesser extent, the related Na$^+$:Cl$^-$ cotransporter (NCC) (Cutler and Cramb 2008, Cutler et al. 2009, Moreno et al. 2016). The absorption of water in the posterior intestine and rectum is accomplished by a variety of AQPs. Numerous AQPs has been discovered to be present in the gastrointestinal organs of Anguilla species and it has been seen that the expression of some AQPs (specifically AQP1, AQP3, AQP8, AQP10) in the gastrointestinal organs of teleost fish generally increase in response to acclimation to SW (Martinez et al. 2005, MacIver et al. 2009, Kim et al. 2010).
Aquaporin Channels in Teleost Fish

AQPs were first identified in 1991 on the cell membrane of human red blood cells and were originally dubbed as a channel forming integral membrane protein of 28 kilodaltons or CHIP28, it was not until later they were discovered to channel water (Preston and Agre 1991, Preston et al. 1992). After the discovery of other water channels, the group was renamed “Aquaporins” (Agre, et al. 1993). For the discovery of AQPs the 2003 Nobel Prize in Chemistry was (jointly) awarded to Peter Agre.

Since their discovery there have been 3 distinct groups identified so far in mammals; group I AQPs (ex. AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8) that primarily transport water, group II AQPs (ex. AQP3, AQP7, AQP9, AQP10) that transport water, glycerol, and urea (sometimes referred to as aquaglyceroporins), and recently discovered, more divergent, group III AQPs (AQP11, AQP12) found primarily on the surface of organelle membranes. AQPs are usually found in multiple organs but may be limited to a few tissues depending on the organism (Li and Wang 2017). Each group has a distinct structure and therefore may play a unique role in osmoregulation or other processes (Agre, et al. 1993, Yang and Verkman 1997, Echevarria et al. 1996, Ishibashi et al. 1997, Ishibashi et al. 1998, Ishibashi et al. 2002, Ishibashi et al. 2006).

Group III AQPs have been found to have less than 20% homology to group I and II AQPs. One of the most notable distinctions that exist for group III AQPs is that their first asparagine-proline-alanine (NPA) box, which is normally conserved in other aquaporins and allows the formation of a water pore for the channel, varies significantly from the highly conserved group I and II AQP NPA boxes where they play a significant role in the molecular function of the channel pore (Ishibashi et al. 2006, Nozaki et al. 2008). NPA boxes were traditionally used to identify AQPs, and with the first NPA box not being conserved in group III AQPs, these were more difficult to discover, however these AQPs are now identified as having a highly conserved cystine downstream of the second NPA box (Ishibashi et al. 2006). Group III AQPs represented a new superfamily and thus were originally named as the “superaquaporin” subfamily but are now regularly referred to as unorthodox AQPs (Ishibashi et al. 2006, Ikeda 2011). Interestingly, not only do their NPA boxes differ, but also the physical location of group III
AQP proteins is unusual (compared to most Group I and II AQPs) in that they are primarily found intracellularly in organelle membranes, this is the case with AQP11 which is primarily located on the endoplasmic reticulum (ER) membrane (Ishibashi 2006, Ikeda et al. 2011). These AQPs have a charged amino acid clusters at the C-terminal end similar to a di-lysine motif which is understood to be an ER retention signal. (Cosson and Letourneur 1994, Ishibashi 2006).

**Expression of AQPs in Teleosts**

Teleost fish are known to have the highest quantity of Aquaporins (AQPs) of any animal, up to 42 paralogs in some species, due to whole genome duplications (WGD) believed to have occurred twice in early vertebrate history and once again as teleost fish developed (Brunet et al. 2006). Salmonids and some carp species are believed to have a further WGD resulting in them having even more AQP paralogs (Finn et al. 2014). These WGDs led to the divergence of distinct paralogs within the teleost group. Some species of teleost fish have lost some copies of these duplicate genes, for example, zebrafish (*Danio rerio*) has only one copy of AQP11, AQP11b, whereas stickleback (*Gasterosteus aculeatus*) has only AQP11a, furthermore, species such as Japanese medaka and the Japanese eel, a close relative of the American eel, have both AQP11a and AQP11b (Tingaud-Sequeira et al. 2010, Finn et al. 2014). And as demonstrated by PCR amplification, cloning and sequencing performed in Chapter 2, the American eel also has both AQP11a and AQP11b paralogs.

In general, AQPs play a role in preventing water loss in marine and euryhaline SW-acclimated fish. The expression of AQPs in osmoregulatory organs (gill, gastrointestinal tract, and kidneys) have been seen to change in response to SW suggesting they may play some role in SW-acclimation. Whether or not the expression of a specific AQP is affected by SW-acclimation has been found to vary between species, development stages, and organs (Cutler and Cramb 2002, Martinez, et al. 2005, Kim et al. 2010, Li and Wang 2017). A 2010 study found that the Japanese eel expressed AQP1 at a higher level in posterior intestine and rectum when acclimated to SW, AQP3 was expressed higher in SW-acclimated rectum, and AQP8 and AQP10 expression increased in the anterior intestine, posterior intestine, and rectum, suggesting that these tissues use those AQPs to absorb water in SW environmental conditions
(Kim et al. 2010). In contrast, Atlantic killifish and European eel branchial expression of AQP3 decreases with SW-acclimation (Cutler and Cramb. 2002, Jung et al. 2012). In some other studies, AQP10b mRNA/cDNA expression was found to decrease after SW-acclimation in Japanese medaka and sexually immature yellow eels of the European eel. In contrast, expression rose in Atlantic salmon and did not change in sexually mature silver eels of European eels (Madsen et al. 2014, Martinez, et al. 2005, Tipsmark et al. 2010).

**Expression and Function of AQP11 in Vertebrates**

For each AQP there exist varying degrees of information regarding their RNA expression; AQP11 had yet to be characterized in the American eel. Due to a genome-wide duplication, teleost fish would be expected to have duplicate paralogs of AQP11, however, it is common that teleost species have only one paralog, either AQP11a or AQP11b (presumably having lost the other paralog during evolution; Brunet et al. 2006). The American eel was selected as a study species partly because it was suspected that it would have both paralogs of AQP11 (AQP11a and AQP11b) allowing for the potential study of AQP11 paralogs and their neo- or sub-functionalization in various tissues.

AQPs are typically present on the cell plasma membrane and are rarely ever seen in an intracellular location outside of mitochondria where AQP8 possibly plays a role in the growth of mitochondria during cell division (Calamita et al. 2005). As previously stated AQP11 seems to be localized to the ER and also displays reduced water permeability compared to some AQPs (e.g. 8x lower than that of AQP1) possibly due to a tyrosine amino acid facing the channel pore (Yakata et al. 2011).

Although the role of AQP11 is largely unknown, some experiments have already been performed demonstrating the effects when lacking AQP11. In one study, AQP11 knockout (KO) mice developed polycystic kidneys and renal failure, eventually dying within a month (Morishita et al. 2005). The absence of AQP11 led to the generation of large intracellular vacuoles in the cells of the proximal tubule. Using an electron microscope, it was suggested that these vacuoles derived from the rough ER as it was discovered they had ribosomes attached to the cytoplasmic side of the vacuole membrane. This implies AQP11 may play an important role for letting water in and out of the ER, yet how exactly the disruption of AQP11
causes massive vacuole formation is still uncertain (Morishita et al. 2005). In another study, focusing on
the liver, it was discovered in liver-specific AQP11 KO mice that severe strain and degradation of
hepatocytes near the portal vein occurred due to similar vacuoles being produced from the ER, this
condition was further aggravated by fasting followed by feeding (Rojek et al. 2012). One study looking at
the expression of AQP11 in mouse brain, suggested that AQP11 may play a role in water transport in the
choroid plexus and in the blood-brain barrier (Koike et al. 2016). Additionally, zebrafish lacking
endogenous AQP11 have tail deformities during early development suggesting that AQP11 may exhibit a
variety of functions across different species (Ikeda et al. 2011).

**Localization of AQP11 Paralogs**

Publications dealing with the localization of the AQP11a and AQP11b proteins were far and few
between at the time of this study. Only a limited number of studies dealing with the
immunohistochemically localization of AQP11 paralogs in euryhaline teleost fish, let alone in their
osmoregulatory tissues, had been published. There have been multiple publications dealing with the
expression and localization of AQP11 proteins in mice. One paper localized AQP11 in proximal tubules
in the kidney, which is an osmoregulatory organ, and the choroid plexus and parenchyma of the mouse
brain (Koike et al. 2016). Another study localized AQP11 in mouse and human liver hepatocytes
(Gregoire et al. 2015). One study localizing various AQPs in the retinal tissue of multiple animals found
that AQP11 expression was present in the retina of many animals (including the euryhaline sturgeon
(Amann et al. 2016). This wasn’t the first paper to explore the expression of AQP11 proteins in the eye,
other studies localized AQP11 (and other AQPs) in Müller-glial endfeet in the retina of humans (Tran et
al. 2013,) and horses (Deeg et al. 2016). There have been a few publications dealing with the
mRNA/cDNA expression of AQP11 paralogs that might garner some insight to potential locations for
protein expression. For example, AQP11a mRNA/cDNA was expressed in multiple FW and SW-
acclimated tissues (brain, eye, fin, gill, intestine, muscle, heart, ovary, intestine, kidney, spleen and testes)
in medaka and there were some significant differences in expression in intestinal tissue (Kim et al. 2014).
Additionally, although not part of a SW-acclimation experiment, AQP11b mRNA/cDNA was found to be
expressed in multiple tissues in zebrafish as well (ovary, gill, anterior intestine, midgut, posterior intestine, and liver, Tingaud-Sequeira et al. 2010).

**Purpose of Study**

This study investigates whether either of the AQP11 paralogs, AQP11a or AQP11b, found in the American eel are affected by SW-acclimation. It also marks the first attempt to localize both AQP11 paralogs in the American eel. Chapter 2 describes the experiments used to obtain American eel tissue and total RNA samples and their use in the cloning and sequencing of the cDNA of AQP11a and AQP11b. Obtained mRNA samples were used to generate cDNAs of AQP11a and AQP11b which were cloned and sequenced. The obtained sequences were then used to generate primers for quantitative PCR (qPCR) and to derive the protein sequences of AQP11 paralogs which were used to enable antibody production used for western blotting and immunohistochemistry. The number of mRNA/cDNA molecules for osmoregulatory tissues obtained during the SW-acclimation experiment were quantified using qPCR. SW experimental groups at predetermined time points were compared to FW controls to determine if SW-acclimation had an effect on the expression of AQP11a or AQP11b mRNA/cDNA molecules in osmoregulatory tissues. In Chapter 3, AQP11a and AQP11b protein abundances were quantified using western blotting to examine changes in expression for osmoregulatory tissues. Immunohistochemistry was used to localize AQP11a and AQP11b proteins within the cells of osmoregulatory tissues.
CHAPTER 2
CLONING, SEQUENCING, AND MRNA/CDNA QUANTIFICATION OF AQP11 PARALOGS IN OSMOREGULATORY ORGANS OF ANGUILLA ROSTRATA

INTRODUCTION

There is relatively little information on the expression of AQP11 in osmoregulatory organs. AQP11a has been documented in the Japanese medaka to be expressed in the brain, eye, fin, gill, intestine, muscle, heart, ovary, intestine, kidney, spleen and testes and AQP11b has been documented in zebrafish and medaka to be expressed in ovary, anterior intestine midgut, posterior intestine, and liver (Kim et al. 2014. Tingaud-Sequeira et al. 2010). One of the questions concerning AQP11 is whether their expression in the American eel would be affected by SW-acclimation in euryhaline fish in the same manner that many other AQPs are. Prior to being examined, the determination of the DNA sequences for AQP11 paralogs was required.

In this chapter, a SW-acclimation experiment comparable to that performed for other AQPs (Cutler and Cramb 2002, and Jung et al. 2011) was carried out. Many AQPs have been documented to be affected by SW-acclimation in other euryhaline fish (Martinez, et al. 2005, Tipsmark et al. 2010, Madsen et al. 2014). For this study, the American eel was selected as tissue samples were readily available to perform quantification and localization of AQP11 paralogs mRNA and protein. It was suspected that the American eel, like its Japanese cousin, would have both paralogs of AQP11; this was verified by cloning and DNA sequencing. This chapter also investigated the expression of the mRNA/cDNA of AQP11 paralogs in various tissues. Quantitative PCR (qPCR also known as real-time PCR) was used to investigate the presence of AQP11a and AQP11b in various tissues and including osmoregulatory tissues (gill, kidney, esophagus, stomach, anterior intestine, posterior intestine, rectum). The levels of mRNA/cDNA for the AQP11a and AQP11b genes in a SW-acclimation experiment with SW experimental and FW control groups at comparable time-points were analyzed using qPCR. Because AQP11 paralogs are primarily intracellular and thus would not necessarily have a role in maintaining the
water homeostasis of the cell cytoplasm or cell volume, a change in AQP11 mRNA/cDNA expression following SW-acclimation was not anticipated.

MATERIALS AND METHODS

Anguilla rostrata Sampling and Processing for the Seawater-Acclimation Experiment

The American eel samples used for the SW-acclimation experiment were collected by fishermen in FW lakes and rivers surrounding Augusta, Maine, or from lakes and surrounding estuaries on Mount Desert Island, Maine. Eels were kept and processed at the Mount Desert Island Biological Laboratories (MDIBL). Eels were kept in FW tanks for at least one week before undergoing SW-acclimation. SW used in this experiment was obtained from Salisbury Cove off the coast of Mount Desert Island. Eels used for the SW-acclimation experiment were transferred into SW for various time-periods, these experimental groups were paired with FW controls taken at similar time points. Due to the time it took to process samples, FW and SW samples could not be processed at the same time; consequentially, FW control samples were taken at time=0, 1-day, 6-days, and 20-days, SW experimental samples were taken at 6-hours, 2-days, 7-days, and 21-days. Samples were kept and processed in compliance with IACUC. Eels were anesthetized with 500 or 1000 ppm MS-222 anesthetic prior to euthanasia. Animals were kept on ice during dissection of tissues. For certain organs (gastrointestinal tract tissues and gills) the epithelium was scraped off the underlying connective tissue (gastrointestinal tract tissues; using a glass slide) or cartilage (gill using a razor blade), these were then homogenized using a syringe and a 16-gauge needle. Other solid tissues (kidney, liver, etc.) were homogenized using a blender (Kinematica) or using a syringe and a 16-gauge needle (brain). The obtained total protein samples were measured using the test tube method of the Pierce BCA protein assay kit (Thermofisher).

Tissue samples of the American eel used for immunohistochemistry were collected in Maine at the Mount Desert Island Biological Laboratories (MDIBL). Osmoregulatory organs such as kidney, anterior intestine, and rectum were available, additionally, liver samples were available and used. These organs had been dissected from euthanized eels and fixed in 4% para-formaldehyde made up in PBS. Tissues were incubated in a series of increasing strength ethyl alcohol solutions up to 100% and then
twice in hydrophobic HistoChoice (Sigma-Aldrich) clearing agent and then put into three different 
beakers of molten paraffin wax and kept at 58° C before they were set and embedded in the paraffin wax 
blocks by rapid cooling on a cold plate.

In total, the SW-acclimation experiment used 6 eels taken at each of the SW experimental group 
and FW control group time-points (FW 0-days, SW 6-hours, FW 1-day, SW 2-day, FW 6-days, SW 7-
days, FW 20-days, SW 21-days). These samples were either used for total RNA extractions for qPCR in Chapter 2 or used for quantifying proteins in Chapter 3 western blotting experiments. Further tissue 
samples were dissected from another SW-acclimated eel (including liver, swim bladder, urinary bladder, 
spleen, skeletal muscle, heart, brain and eye), were homogenized and were stored in a -80°C freezer. 
These samples were used for total RNA extractions (for cloning, or for qPCR).

Total RNA Extraction for the Production of cDNA

RNA samples had been prepared by previous students at the beginning of these experiments but 
were prepared using the following technique: Tissue samples from osmoregulatory organs of the 
American eel were homogenized in 0.9 ml (10 ml/g of tissue) of a solution containing 4 M guanidinium 
isothiocyanate, 0.01 M 2-mercaptoethanol, 25mM tri-sodium citrate buffer, and 0.5% sarcosyl detergent. 
Then 108 µl of 1.66 M sodium acetate (pH 3.7) was added to the tissue. This was vortexed for a few 
seconds. Then 0.45 ml of water-saturated phenol was added and vortexed. 144 µl 1-Bromo, 2-Chloro 
Propane (BCP) was then added and vortexed until a white emulsion formed. The tube was spun in a cold 
(0°C) microfuge at 14,000 rpm for 15 minutes. This resulted in a two-phase mixture, an upper aqueous 
phase and a lower organic phase separated by a precipitate interface. The aqueous phase (containing the 
total RNA) was separated entirely and put into a clean tube and its volume was measured with a pipette. 
Then 0.1 volumes of BCP (relative to the amount of aqueous phase obtained) was added and vortexed. 
This was microcentrifuged at 0°C at 14,000 rpm for 15 minutes. As before, after centrifugation, an 
aqueous phase was formed at the top of the sample and this was transferred to a new tube and measured 
and 0.2 volumes (relative to this new aqueous phase) of isopropanol was added to the tube and vortexed.
Then 0.2 volumes of a high salt buffer ((1.2 M tri-sodium citrate and 0.8M NaCl) were added and vortexed. The tube was incubated at room temperature for 10 minutes to allow precipitation of the RNA. The RNA was then washed to remove precipitated salts. First, the tube was microcentrifuged at 14,000 rpm for 10 minutes to pellet the RNA. The supernatant was pipetted off leaving the pelleted RNA. 0.5 ml of 70% ethanol was added to the RNA pellet. The tube was microcentrifuged again for 5 minutes at room temperature after which the liquid was pipetted out. The ethanol wash step was repeated and then the tube was left to air dry. Finally, a volume of DI water was added proportionately to the size of the RNA pellet obtained (between 20 µl and 250 µl depending on the tissue) to re-suspend the RNA. Total RNA was then quantified using a UV-Vis spectrophotometer set to 260 nm wavelength. Agarose gel electrophoresis was performed to quantify and normalize the RNA samples from the SW-acclimation experiment using the 18S and 28S ribosomal subunit rRNAs. This was performed twice for each set of total RNA samples for each tissue from the 48 eels in the experiment. Total RNA samples from various eel tissues were also extracted previously and these RNA samples were extracted by an identical protocol to that above and were used for cloning and sequencing of the AQP11a and AQP11b cDNAs.

Production of cDNA from Total RNA for PCR

The protocol for preparing cDNA samples made with Superscript IV is as follows: The RNA samples were first defrosted and heated at 65°C to re-dissolve and denature them. Total RNA samples were kept at 0°C or on the ice at all other times. For each tissue sample, 2 µg of total RNA was pipetted into individual 200 µl PCR tubes. Added to each tube in order were 1 µl of Oligo (dT), 1 µl of 10 mM dNTPs and 0.5 µl of SUPERase in thermostable RNase inhibitor (20 U/µl; Ambion/Life Technologies), and the volume was made up to 6.5 µl with dH2O. The tubes were then heat denatured in a thermocycler at 65°C for 5 minutes and cooled on ice for 1 minute. Then 2 µl of 5x Superscript IV buffer and 1 µl of 0.1 M DTT were added, and samples were mixed by pipetting. Samples were then incubated in a thermocycler at 55°C. Individual tubes were removed from the thermocycler and 0.5 µl of Superscript IV reverse transcriptase (200 U/µl; Life Technologies) was added and each sample was again mixed by pipetting and returned to the thermocycler. Once all the tubes had had the enzyme added, the tubes were
incubated for a further 30 minutes at 55° C. After this time, the temperature was raised to 80° C for 10 minutes to heat-inactivate the reverse transcriptase. The cDNAs were then diluted by the addition of 190 µl of dH₂O to each tube. A further subset of working samples was produced (to allow more accurate pipetting of the cDNA samples), here, 50 µl of the original diluted cDNA was transferred to each new tube and 200 µl of dH₂O was added.

**Cloning of AQP11 Paralogs**

For preliminary experiments, to test if fragments of AQP11a or AQP11b would amplify, PCR was performed using cDNAs already available from a number of different tissues including those from liver and intestine (used for the AQP11a and the AQP11b cloning respectively). PCR to amplify AQP11 paralogs was performed using primers designed for AQP11a and AQP11b, based on Japanese eel (*Anguilla japonica*) genomic DNA sequences (Figure 2.1). AQP11a and AQP11b cDNA fragments were generated using Phusion DNA polymerase (New England Biolabs). Initial amplifications suggested the potential presence of an additional, smaller, form of AQP11b in gill and brain (Figure 2.2) reactions so further PCR amplification and cloning experiments were used in an attempt to isolate and sequence the unidentified band.

Fragments for cloning required adenine overhangs to be added and was done using 1.25 units of *Taq* polymerase with 1x *Taq* buffer and 200 µM dATP, incubated at 72° C for 10 minutes. Fragments were run on an agarose gel, cut out using a scalpel blade under a UV light, and purified using a Monarch DNA purification kit (New England Biolabs). The plasmid vector used for cloning was pCR4-TOPO (Figure 2.3) that has attached topoisomerase enzymes. The vector was mixed with either AQP11a, AQP11b, or RPLP0 (housekeeping control gene used for qPCR) cDNA fragments. The vector and the DNA were given 30 minutes to combine before being added to chemically competent *E. coli* bacteria (total volume of 12.5 µl). The tubes containing the bacteria and the plasmid were heat-shocked at 42° C in a dry block heater for exactly 30 seconds and returned to ice to allow the plasmid to be taken up by the bacteria. Each lot of bacteria had 62.5 µl of SOC medium added before being placed on a rotating wheel in an incubator set to 37° C for 1 hour to allow the bacteria to recover from the heat shock. After
recovery, 75 µl of the bacteria was pipetted onto an LB agar plate containing kanamycin and was spread out. Plates were incubated overnight in an incubator set to 37°C.

The next day, once the bacterial colonies had sufficiently grown, individual colonies were picked using sterilized toothpicks and these were used to inoculate individually numbered tubes with 1ml of terrific broth containing kanamycin at a concentration of 50 µg/ml. The tubes were put on a rotating wheel and left to grow overnight in an incubator set to 37°C.

The next day the colonies were retrieved and 50 µl of each culture was pipetted into a correspondingly labeled microfuge tube and then centrifuged for 30 seconds to pellet the bacteria. The medium was pipetted out of the tubes and discarded leaving only the pellet behind. 500 µl of distilled water was then added to each tube which was then vortexed to suspend and lyse the bacteria, releasing the plasmid. These crude lysates were then used as DNA templates for colony PCR.

Amplification and Sequencing of DNA from Colony PCR

To amplify the DNA inserted into the plasmid vector, 1 µl of M13 forward and 1 µl of M13 reverse bacteriophage primers at a concentration of 4 µM were added to multiple 200 µl PCR tubes each labeled with the corresponding number from the selected colonies. Then 0.5 µl of each colony lysate solution was added to its corresponding, numbered, tube. Next, 11 reactions worth of the master mix was prepared (22 µl 10x buffer, 4.4 µl of 10 mM dNTP, and 165 µl of distilled water) and thoroughly mixed by pipetting and 1.1 µl of Taq polymerase was added (5 units/µl) to the master mix tube. 17.5 µl of the master mix was added to each tube making sure to mix the DNA primers, polymerase, and the colony DNA. The tubes were placed into a programmed PCR thermocycler that was paused at 92°C. Once all the tubes were added the following PCR program was run: 96°C for 1 second, 60°C, for 15 seconds, and 72°C for 20-60 seconds for 25 cycles then heating once more at 72°C for 10 minutes. The PCR amplification products were then run on a 1.5% agarose gel and evaluated for the presence of inserts (bands of 180bp plus the size of the inserted DNA). Colonies that gave signals that looked clean and had no second bands and had approximately the correct length (as determined by primers used) were selected. The DNA from the (3) selected colonies were amplified and purified using a QuickStep 2 PCR Purification kit (EdgeBio)
according to manufacturer’s instructions. The samples were dried and sent to Clemson University Genomics Institute for Sanger DNA sequencing.

**Setup for Quantitative PCR**

Quantifying the transcription of genes is commonly performed using qPCR. The American eel samples (obtained in Chapter 1) used were from a SW-acclimation experiment. There were six eels in each SW experimental and FW control group. Sampling had been staggered between FW control and SW experimental groups due to time constraints. Samples used had been taken for FW 0-days, SW 6-hours, FW 1-day, SW 2-day, FW 6-days, SW 7-days, FW 20-days, and SW 21-days. To determine the expression of mRNA/cDNA for AQP11a and AQP11b primers were designed for qPCR using the sequencing information collected from the initial cloning and sequencing of DNA fragments made from PCR amplifications of the whole AQP11a and AQP11b coding regions. Primers were designed at expected intron-exon junctions where AQP11a and AQP11b differed the most so that they would be unlikely to amplify genomic DNA and would be unlikely to cross-react (this was tested using reverse transcriptase minus [ RT- i.e total RNA] and cross-reaction tests using cDNA standard fragments).

**Primer Design**

Oligonucleotide primers to be used for qPCR experiments were designed using the obtained cDNA sequence of AQP11a and AQP11b, and these were manufactured by Eurofins. The original sets of qPCR primers (QPCR1 and QPCR2) were found to be amplifying genomic DNA, this was tested using a reverse transcriptase negative (RT-) control using total RNA which contained some genomic DNA. The problem of genomic amplification was attempted to be solved by designing primers across intron-exon junctions (this primer was named QPCR3). Unfortunately, the primers were discovered, using RT-controls, to be generating fragments from genomic DNA as well (in AQP11a amplifications). The antisense QPCR3 primer was then redesigned producing QPCR4 primers. This still had not solved the problem for AQP11a as genomic amplification was still occurring, so multiple primers were designed simultaneously to be tested in various combinations (QPCR5-QPCR9). For AQP11b QPCR3 sense primers were redesigned producing QPCR5 primers. This shortened the extent of the primer that was in
the 2nd exon and lengthened the end in the 1st exon. A new antisense QPCR5 primer was also made so that both sense and antisense primers crossed intron-exon junctions. QPCR6 primers were unique for both AQP11a and AQP11b as neither primer was located at an intron-exon junction but crossed two introns attempting to make the genomic fragments sizes generated too large to amplify in the QPCR reactions.

After testing the redesigned AQP11a primers in various combinations, raising the annealing temperature, and testing the cross-reactivity of the AQP11a primers with the 1,000 molecule AQP11b standards (production of standards for qPCR see later) it was determined the sense QPCR8 and antisense QPCR7 primers made the best pairing as there was no visible genomic amplification and no cross-reactivity with AQP11b standards. The sense QPCR8 primer was made across the first intron-exon junction and the antisense QPCR7 primer was made across the second intron-exon junction (location shown in Figure 2.8).

QPCR6 primers, when tested, were found to be specific to AQP11b and as the generated fragment would cross both introns and, due to a short extension time, it was unlikely to amplify genomic DNA. However, by trying all combinations of sense and antisense primers from QPCR5 and 6, adjusting the annealing temperature up to 67°C, as well as performing cross reaction tests with using 1,000 molecule AQP11a standards it was determined that QPCR5 sense and antisense primers were the better, more efficient, option for AQP11b (location shown in Figure 2.8). All qPCR primers designed for AQP11a and AQP11b, their Tm’s, and their length are described in Figure 2.9.

**Production of cDNA from Total RNA**

Total RNAs extracted from organ tissues of the American eel during SW-acclimation experiments were available to make cDNA samples for qPCR experiments. Also, some cDNA samples from the SW-acclimation experiment had already been generated using RocketScript, (Bioneer) reverse transcriptase enzyme before this study began. Unfortunately, preliminary qPCR experiments using the RocketScript-generated cDNA suggested that the cDNA synthesis was not efficient enough to obtain adequate PCR amplification signals. Consequently, cDNA samples for qPCR were remade using Superscript IV reverse transcriptase (Life Technologies).
The protocol for preparing samples made with Superscript IV was as follows: First, total RNA samples from one of the osmoregulatory tissues (each with 6 biological replicates samples) were diluted to 222 ng/µl by adding a fixed 10 µl of total RNA to a tube and adding varying amounts of water to adjust the concentration to 222 ng/µl. This reduces the error produced by variably pipetting different volumes of RNA solutions. Diluted RNA samples were defrosted and heated at 65°C to re-dissolve and denature the RNA. Samples were kept at 0°C or on ice at all other times. A master mix of 50 µl of 100 µM Oligo(dT)37, 50 µl of 10 mM dNTPs and 25 µl of SUPERase in thermostable RNase inhibitor (20 U/µl; Life Technologies) was produced and 2.5 µl of this master mix was added to 48 individual 200 µl PCR tubes. 4.5 µl of each diluted total RNA sample was added to its respective tube of master mix. The RNA in the tubes was then denatured in a thermocycler heated to 65°C for 5 minutes and then cooled to 4°C. Meanwhile, a second master mix of 100 ul of 5x Superscript IV buffer, 25 µl of 100 mM DTT and 25 µl of Superscript IV reverse transcriptase (200 U/µl) was created. 3 µl of this master mix was then added to each tube in the thermocycler (final reaction volume per tube was 10µl). The tubes were heated to 45°C for 30 seconds to anneal the oligo-dT primer and then the temperature was raised to 55°C for 30 minutes for cDNA synthesis. After this time the temperature was finally raised to 80°C for 10 minutes to heat-inactivate the reverse transcriptase. The cDNAs were then diluted by the addition of 190 µl of water to each tube. A further subset of the samples was produced in additional tubes by further dilution (i.e. 50 µl of the original diluted cDNA was transferred to each new tube and 75 µl of water was then added to each new tube). These sub-stocks of diluted cDNA were then used (4.5 µl for AQP11a and AQP11b and GAPDH or 2.5 µl for RPLP0) in each qPCR reaction.

**Quantitative PCR using a Housekeeping Gene for Normalization**

To quantify the expression of AQP11a and AQP11b mRNA/cDNA production, qPCR was employed. Additionally, a housekeeping gene (RPLP0) was used for normalization of mRNA/cDNA to correct for any variation produced whilst preparing samples. This was in addition to the normalization of the rRNA concentrations that was previously carried out using the 18S and 28S rRNAs prior to the cDNA generation. As a housekeeping gene, RPLP0 should be expressed in equal amounts between individuals.
and in principle would not be affected by SW-acclimation. By comparing the expression of biological replicates of RPLP0 to the average RPLP0 expression of all samples, adjustments were made for any variation produced during the preparation of individual samples.

Initial tests for qPCR were performed using cDNA made from SW 21-day experimental groups and FW time=0 control groups for osmoregulatory tissues that would be later quantified, in addition to cDNA made using RNA from non-osmoregulatory tissues (eye, brain, heart, liver, swim bladder, urinary bladder, spleen, skeletal muscle). One individual fish from each group and tissue was tested with 3 technical replicates. This was performed to test the primers as well as to measure the expression levels in the various tissues once valid amplification parameters were established.

The tissues to be used for comparing the expression of AQP11a and AQP11b as well as RPLP0 (in FW versus SW) include esophagus, stomach, anterior intestine, posterior intestine, rectum, gill and kidney. Calculations for normalizing data were made using Microsoft Excel. Due to time constraints sampling of control and experimental group eels had to be somewhat staggered. Control group eels kept in FW were processed at 0-days (time=0), 1-day, 6-days and 20-days. The experimental group eels were kept in SW and processed after 6-hours, 2-days, 7-days, and 21-days. For each time point 6 biological replicates were performed, and for each biological replicate 3 technical replicates on the qPCR plate were performed.

Standards were made for absolute quantification of the number of molecules of AQP11a, AQP11b, and RPLP0 mRNA/cDNA, and were generated using PCR and subsequently quantified using Logic DNA marker (Lamda Biotech). The molecular weight of each fragment was calculated from its sequence and then Avogadro’s number was used to calculate the number of molecules/µl. The fragment was diluted to 1 billion molecules per 4.5 µl (2.5 µl for RPLP0) for the first standard, then serially diluted 1:10 down to 10 molecules per 4.5 µl for AQP11a and AQP11b and 1000 molecules per 2.5 µl for RPLP0 (a 1 molecule standard was also made for AQP11a, AQP11b, but this standard was not consistent enough to be used for quantification). RPLP0 standards were only made to 1,000 molecules because there was found to be consistent amplification of other products (as determined by the melt curve), but only in the
highly diluted samples, however, as the expression of RPLP0 was found to be approximately in the range of 1 million-100 thousand molecules this did not affect the final quantification results. Standards were replicated twice per plate.

The master mix selected for qPCR was Luna Universal qPCR Master Mix (New England Biolabs), it contains a hot-start taq polymerase and uses SYBR green-like fluorescent dye that intercalates into double-stranded DNA. A Thermo-Fischer/Applied Biosystems QuantStudio 6 Flex qPCR machine was used to perform the assays.

Statistically significant differences between corresponding FW and SW group mRNA/cDNA expression were evaluated post-hoc using ANOVA and Dunnett’s test. Significance within FW and SW groups were each evaluated post-hoc using ANOVA and Tukey’s test.

RESULTS

Cloning and Sequencing of AQP11 Paralogs

The investigation of a band that was a potential second form seen in AQP11b amplifications, was determined, after sequencing, to be identical to the main form of AQP11b. The cloning and sequencing were performed twice to confirm this.

AQP11a and AQP11b cDNA sequences were obtained (Figure 2.4). These nucleotide sequences were aligned with the Japanese eel sequences to compare homology and determine if they were successfully cloned (Figures 2.5). The nucleotide sequences for the American eel AQP11a and AQP11b were determined to be 98.3% and 98.7% homologous respectively to the equivalent coding sequences for the Japanese eel AQP11a and AQP11b genes. Anguilla rostrata AQP11a and AQP11b DNA sequences were then aligned and determined to be 68.5% homologous at the nucleotide level (Figure 2.6). The open reading frame of these cDNA sequences were then translated into amino acid sequences and compared to Japanese eel AQP11a and AQP11b sequences to determine the level of homology (Figure 2.7). The American eel AQP11a protein was estimated to be 273 amino acids long with a molecular weight of 29717.42 g/mol (29.72 kDa) and was 98.2% homologous to the AQP11a sequence derived from the. The American eel AQP11b protein was estimated to be 274 amino acids long with a molecular weight of
29984.06 g/mol (29.98 kDa) and was 98.5% homologous to the AQP11b sequence derived from the Japanese eel.

**Quantitative PCR of Various Tissues for AQP11 Paralogs:**

Samples that were normalized using their level of 28S and 18S rRNA for the adjustment and not additionally adjusted with a housekeeping gene, have been labeled “rRNA normalized” and samples that were additionally normalized with a housekeeping gene (RPLP0) have been labeled “RPLP0 Normalized”. Furthermore, stomach tissue samples were alternatively normalized using GAPDH gene expression levels due to some obvious anomalies within the RPLP0 data.

Initial tissue tests showed high levels of AQP11a and AQP11b in multiple osmoregulatory tissues. For AQP11a qPCR amplification found relatively high numbers of mRNA/cDNA molecules in the SW experimental and FW control samples of kidney (481 mRNA/cDNA molecules and 212 mRNA/cDNA molecules respectively), SW and FW samples of anterior intestine (555 mRNA/cDNA molecules and 99 mRNA/cDNA molecules respectively), SW and FW samples of posterior intestine (139 mRNA/cDNA molecules and 37 mRNA/cDNA molecules respectively), and SW rectum (57 mRNA/cDNA molecules) compared to the lower level of expression SW and FW gill (4 mRNA/cDNA molecules and 12 mRNA/cDNA molecules respectively), SW and FW esophagus (0 mRNA/cDNA molecules and 5 mRNA/cDNA molecules respectively), SW and FW stomach (2 mRNA/cDNA molecules and 1 mRNA/cDNA molecules respectively) and FW rectum (0 mRNA/cDNA molecules). Similar results were seen after normalization with RPLP0. Additionally, in anterior intestine, posterior intestine and rectum there were higher levels of mRNA/cDNA expression in SW compared to FW samples, although in kidney the difference became less marked after RPLP0 normalization (Figure 2.10).

For non-osmoregulatory tissues it was found that the liver expressed AQP11a at around 3800 mRNA/cDNA molecules per 9ng of total RNA (used to make cDNA in each reaction) and this increased to 4200 molecules after normalizing with RPLP0. Additionally, after RPLP0 normalization, brain (88 mRNA/cDNA molecules), and urinary bladder (56 mRNA/cDNA molecules) showed modest levels of expression, the other tissues, swim bladder (17 mRNA/cDNA molecules), skeletal muscle (16
mRNA/cDNA molecules), heart (10 mRNA/cDNA molecules), eye (9 mRNA/cDNA molecules), and
spleen (essentially 0 molecules of AQP11a mRNA/cDNA) expressing lower numbers of AQP11a
mRNA/cDNA molecules (Figure 2.11).

AQP11b mRNA/cDNA expression was more ubiquitous, with some level of AQP11b
mRNA/cDNA found in every tissue. In the rRNA normalized osmoregulatory tissues the SW
experimental group and FW control group samples of kidney (232 mRNA/cDNA molecules and 90
mRNA/cDNA molecules respectively), SW and FW samples of gill (211 mRNA/cDNA molecules and
136 mRNA/cDNA molecules respectively), SW and FW samples of esophagus (131 mRNA/cDNA
molecules and 136 mRNA/cDNA molecules respectively), and SW and FW samples of anterior intestine
(93 mRNA/cDNA molecules and 106 mRNA/cDNA molecules respectively) had somewhat elevated
levels of mRNA/cDNA expression. In comparison the SW and FW samples of stomach (78
mRNA/cDNA molecules and 48 mRNA/cDNA molecules respectively), SW and FW samples of
posterior intestine (35 mRNA/cDNA molecules and 48 mRNA/cDNA molecules respectively), and SW
and FW samples of rectum (29 mRNA/cDNA molecules and 63 mRNA/cDNA molecules respectively)
there were somewhat lower levels of expression (Figure 2.12). After RPLP0 normalization, however, the
mRNA/cDNA expression of AQP11b all decreased in samples of SW and FW anterior intestine (2-fold
and 1.5-fold respectively), SW stomach (nearly 2-fold) SW kidney (2-fold). In contrast, the expression of
AQP11b increased in SW and FW samples of gill (both by nearly 2-fold) and the SW and FW samples of
esophagus (56% and 62% respectively) after RLPL0 normalization.

In non-osmoregulatory tissues it was found that the brain expressed the highest levels (700
mRNA/cDNA molecules) of AQP11b, but it was expressed ubiquitously in other tissues, urinary bladder
(215 mRNA/cDNA molecules), eye (183 mRNA/cDNA molecules), spleen (85 mRNA/cDNA
molecules), heart (58 mRNA/cDNA molecules), liver (51 mRNA/cDNA molecules), swim bladder (44
mRNA/cDNA molecules), and skeletal muscle (11 mRNA/cDNA molecules); normalizing with RPLP0
yielded similar results (Figure 2.13).

**Absolute qPCR of AQP11 paralogs mRNA/cDNA in Osmoregulatory Tissues**
Regarding the osmoregulatory tissue qPCR results, first in the esophagus (Figure 2.14), this tissue only exhibited a significant decrease in the number of mRNA/cDNA molecules between rRNA normalized AQP11b gene SW 2-day and FW 1-day experimental groups (49.72% lower).

In the stomach, samples adjusted using RPLP0 gene mRNA/cDNA levels (Figure 2.15), exhibited significant lower number of molecules in RPLP0 normalized AQP11a, rRNA normalized AQP11b (78.6% lower), and RPLP0 normalized AQP11b genes between SW 2-day and FW 1-day mRNA/cDNA experimental groups (all significance is calculated with p<0.05). Since the FW 1-day group samples normalized with RPLP0 seemed to be an obvious outlier in the data set (being many hundreds of times higher than any other RPLP0 stomach time point group) it was decided to retry the samples using a different housekeeping gene, specifically GAPDH. When adjusted with GAPDH (Figure 2.16), the stomach SW experimental groups no longer showed any significant differences from the FW control groups. Within-group analysis found that the expression of rRNA normalized mRNA/cDNA of AQP11b in FW 20-day samples had significantly lower expression compared to FW 1-day samples, and SW 1-day samples were significantly lower than SW 7-day samples.

The anterior intestine (Figure 2.17) tissue samples exhibited a significantly lower number of mRNA/cDNA molecules for both AQP11a and AQP11b for the rRNA normalized data from the SW 2-day experimental groups compared to the FW 1-day control group (84.55% and 67.86% lower respectively). The RPLP0 normalized data showed similar results. Additionally, rRNA normalized AQP11a gene results showed significantly higher numbers of molecules of mRNA/cDNA between SW 7-day experimental group and FW 6-day control group (4.16-fold higher) and between the SW 21-day experimental group and the FW 20-day control group (3.89-fold higher). Similar significant increases were also seen in the RPLP0 normalized AQP11a data. Within-group analysis found that the expression of rRNA normalized mRNA/cDNA of AQP11a found that FW 6-day and 20-day samples had significantly decreased from FW 1-day samples, and SW 7-day samples had significantly increased from SW 2-day samples. After normalizing with RPLP0 the expression of AQP11a mRNA/cDNA in the FW samples no longer were significantly different, but the SW samples retained their significance. For within-
group analysis of AQP11b the FW 0-day group was significantly lower than the FW-1 day and FW 20-day samples, SW 7-day and 21-day samples had significantly increased compared to the SW 6-hour and SW 1-day groups. After RPLP0 normalization only SW groups retained their within-group significance.

The posterior intestine (Figure 2.18) tissue samples exhibited significantly higher numbers of mRNA/cDNA molecules for the rRNA normalized AQP11a gene between SW 21-day and FW 20-day samples (9.3-fold higher), between RPLP0 normalized SW 7-day and FW 6-day samples (5.37-fold higher), and between the SW 21-day experimental group and FW 20-day control group (6.22-fold higher). Additionally, both rRNA and RPLP0 normalized AQP11b gene mRNA/cDNA levels showed a significant decrease in number of molecules between the SW 2-day experimental group and the FW 1-day control group (56.31% and 53.58% lower respectively). Within-group analysis showed that RPLP0 normalized AQP11a SW 6-hour group had significantly higher expression compared to the other SW groups. For the within-group AQP11b rRNA normalized SW samples SW 6-hour and 1-day samples were significantly lower compared to the SW 7-day and 21-day groups. After normalization with RPLP0 only SW 7-day was significantly higher than SW 1-day.

The rectum (Figure 2.19) only exhibited a significant increase in the number of mRNA/cDNA molecules for the rRNA normalized AQP11b gene between the SW 21-day experimental group and the FW 20-day control group (58.76% higher). Within-group analysis found that SW 7-day and 21-day groups rRNA normalized mRNA/cDNA expression of AQP11b were significantly higher than SW 6-hours and the SW 7-day group had higher expression than the 1-day group. Normalizing with RPLP0 resulted in only the SW 7-day group having higher expression than the 1-day group.

The gill (Figure 2.20) exhibited a significant decrease in the number of mRNA/cDNA molecules for the rRNA normalized AQP11a gene between the FW 0-day and the SW 6-hours experimental groups (66.6% lower), and between SW 21-day experimental group and the FW 20-day control group (56.7% lower). Additionally, for AQP11b, there were significant increases in expression in the SW 7-day and 21-day SW groups in comparison to the FW 6-day and 20-day groups respectively (1.90-fold and 1.91-fold higher in rRNA normalized, and 2.18-fold and 2.9-fold higher in RPLP0 normalized groups respectively).
Within-group analysis found that the expression of SW-acclimated AQP11a rRNA normalized mRNA/cDNA was significantly higher in the SW 7-day group compared to the other SW groups. This was not retained after normalizing with RPLP0. For the within-group analysis of AQP11b mRNA/cDNA expression rRNA normalization resulted in SW 7-day samples being significantly higher than SW 6-hour samples. RPLP0 normalization negated differential SW expression and introduced a higher expression of FW 1-day samples compared to FW 7-day samples.

The kidney (Figure 2.21) exhibited significant decreases in expression for AQP11a in the SW 7-day experimental group compared to the FW 6-day control group, both in the rRNA and RPLP0 normalized data (74.19% and 79.18% lower respectively). In contrast, AQP11b showed a significantly higher level of expression in the SW 7-day group compared to the FW 6-day group in the rRNA normalized data (64.31% higher). Within-group analysis found that the AQP11a mRNA/cDNA expression in SW-21 was significantly higher than the other SW groups for both rRNA and RPLP0 normalization. For rRNA normalized mRNA/cDNA expression of AQP11b for both the FW 0-day and 1-day groups were significantly higher than the FW 6-day and 20-day groups. After normalizing with RPLP0 the FW 6-day group was significantly lower than the FW 0-day and 1-day groups and lower than the FW 20-day group. Additionally, the FW 20-day group had also decreased from the FW 0-day group.

DISCUSSION

Cloning and Sequencing

It is unclear as to why preliminary gel electrophoresis showed a second band for AQP11b in the brain samples but when this was sequenced it turned out to be the same DNA as the normal version of AQP11b. It could be that a portion of the DNA formed some alternative molecular substructure that caused it to move faster in the gel. In any case, the question of how or why there was a second band is beyond the scope of this study.

Due to the extremely high level of nucleotide and amino acid homology (Figures 2.5 and 2.7 respectively) of the American eel AQP11a and AQP11b sequences to those from the Japanese eel, it can be safely assumed that the obtained sequences were indeed the American eel AQP11a and AQP11b. The
high level of homology between the Japanese eel and the American eel sequences shows that despite the fact these species breed in separate parts of the world (Pacific and Atlantic oceans respectively), there has not been much genetic drift in the sequences since they diverged and became separate species. The fact the sequences are well conserved also implies that the proteins must be performing an important and conserved function or else the differences between species would likely be greater. Interestingly the C-terminal ends of AQP11a and AQP11b amino acid sequences are almost completely different to each other. This suggests that any functional differences between the two proteins would likely be related to this region of each protein. The amino acid sequence RGADIAGI found in the C-terminal end of the AQP11a protein sequence was later discovered to somewhat resemble a PDZ binding motif (Lee and Zheng, 2010), this is not present in the AQP11b sequence. This may contribute to any observed differences in function between AQP11a and AQP11b. Additionally the presence of a PDZ binding motif might also explain the poor results with the AQP11a antibody (see Chapter 3) as multiple other proteins might contain a similar PDZ motif and would therefore be able to bind the AQP11a antibody.

Quantitative PCR of Various Tissues for AQP11 Paralogs.

Alignment between AQP11a and AQP11b DNA sequences for Anguilla rostrata showed the sequences on either side of the intron regions were fairly conserved (Chapter 1). Intron-exon junctions were an ideal location to make primers that could not amplify genomic DNA during qPCR, so being fairly conserved in this area made designing primers that would not cross-react and amplify the other AQP11 paralog difficult. However, after multiple iterations and combinations followed by RT controls and cross-reactivity testing, suitable primers for qPCR were eventually generated (Figure 2.9).

AQP11a had the highest expression of mRNA/cDNA molecules in liver tissue by a wide margin (nearly 16-fold higher than the next highest tissues, kidney, anterior intestine; Figure 2.4) this would suggest that AQP11a may have an important role in the liver. Indeed, liver specific AQP11 knockout mice have displayed deformities in liver (Rojek et al. 2013), however, further studies are required to fully determine what, if any, role AQP11a has in the liver, or in the other tissues of these eels.
AQP11b was more ubiquitously expressed than AQP11a, as its expression occurred in a more widely distributed set of tissues where there was a reasonable level of AQP11b mRNA/cDNA expression. It was determined that AQP11b had the highest expression of mRNA/cDNA molecules in the brain, (2-fold higher than the next closest tissue, gill) (Figure 2.13). One study (Koike et al. 2016) found AQP11 to be present in the brain of mice. The increased presence of AQP11b molecules suggests that it may play a greater role in water transportation in the brain. However, to fully determine what, if any, role AQP11b has in the brain of these eels would require further studies. Several of the pairs of amplifications from FW and SW osmoregulatory tissue individual samples (kidney, gill and gastrointestinal tract) suggested there might be differences in the level of expression in some osmoregulatory tissues. That indeed turned out to be the case when the samples from the full SW-acclimation experiment were analyzed.

Quantification of mRNA Expression in Osmoregulatory Organs

As shown in the individual tissue sample experiments in esophagus it was found that there was almost no expression of the AQP11a gene (average of 15 mRNA molecules at most). Regarding the expression of the AQP11b gene, it was found that only the FW 1-day and the SW 2-day experimental groups had a significant difference (all significance is calculated with Dunnett’s test at p<0.05), with the FW 1-day group having a higher level of expression than any other group in the experiment for this tissue/gene, moreover, there was no significant difference between samples in the RPLP0 normalization data. These results suggest that it is debatable whether there was any real effect of SW-acclimation in the esophagus.

In the stomach rRNA normalized experimental groups exhibited significantly lower abundance of AQP11b mRNA/cDNA molecules in the SW 2-days experimental group compared to its FW 1-day control group, however, this difference appeared to be specific to elevated levels in the FW 1-day group which had much higher expression than other FW groups. When the data from the stomach experimental groups was normalized with the RPLP0 gene both the AQP11a and AQP11b exhibited extremely high values for FW 1-day experimental group (on average over 3-fold higher than any other experimental group for AQP11a, and over 10-fold higher than any other experimental group for AQP11b) due to
extremely low values for RPLP0 in that group, so, to attempt to alleviate this apparent anomaly, stomach experimental groups were additionally re-normalized using GAPDH gene expression levels. Stomach samples normalized with the GAPDH gene, showed much lower variability with more similar values found in the FW 1-day experimental group. Normalization using GAPDH resulted in no significant differences in expression between experimental and control groups for the AQP11a and AQP11b genes. Furthermore, the AQP11a gene had a low level in stomach tissue with a maximum average of 7 mRNA/cDNA molecules. Taken together, these results suggest that AQP11 paralog mRNA expression in the stomach was not affected by SW-acclimation.

RPLP0 normalized and rRNA normalized experimental groups in anterior intestine showed an initial decrease in AQP11a mRNA/cDNA gene expression for SW 2-day experimental groups compared to FW 1-day control group samples and an increase in the number of AQP11a and AQP11b mRNA/cDNA molecules in the FW 1-day samples compared to SW 2-day samples and the FW time=0 samples. The SW 7- and 21-days experimental groups, however, showed a significant increase in the number of AQP11a and AQP11b mRNA/cDNA molecules compared to the FW 6-day and 20-day experimental groups respectively. Additionally, AQP11b displayed a significant increase over time for the within-group analysis. Taken together, the expression of AQP11a and AQP11b mRNA in the anterior intestine seems to be affected by SW-acclimation.

The posterior intestine had fairly high variability in the data. As with the anterior intestine, there was a lower level of AQP11b expression in the 2-day SW group compared to the 1-day FW group for both of the rRNA and RPLP0 normalized AQP11b genes data sets. Otherwise, there were no other significant differences in the AQP11b gene expression data in the posterior intestine. The posterior intestine showed significantly higher number of AQP11a mRNA/cDNA molecules in the rRNA normalized 21-day SW group compared to the 20-day FW control group. This significant increase was also evident in the data after normalizing with the RPLP0 gene, and there was an additional significant increase in the SW 7-day compared to the FW 6-day group. Additionally, the rRNA normalized AQP11b
data showed a significant increase in expression over time. The data suggests that AQP11 paralogs are affected by SW acclimation.

The rectum had a significantly higher level of AQP11b mRNA/cDNA expression in the SW 21-day experimental group samples compared to those of the FW 20-day control group. After RPLP0 normalization there was no significant difference between samples. There was a relatively high level of variability in the rectum data (FW 1-day samples for AQP11b gene) that limits any statistical analysis, this was particularly true for the AQP11a gene, although this was caused partly by the low level of overall AQP11a expression (around a maximum of 30 mRNA/cDNA molecules per qPCR reaction). Finally, the expression of AQP11b in both SW 7-day and 21-days groups had significantly increased from the SW 6-hour group. Additionally, the rRNA normalized AQP11b data showed a significant increase in expression over time. Taken together, the data suggests that only the expression of the AQP11b gene in the rectum may be affected by SW-acclimation.

The gill data had a significant difference for the AQP11a gene between the SW 6-hour experimental group and the FW time=0 control group and between the SW 21-day experimental group and the FW 20-day control group, both having significantly lower amounts of AQP11a mRNA/cDNA molecules for SW groups, these differences in expression were not significant after RPLP0 normalization. However, considering how low expression of AQP11a was in general for gill it is unlikely it is affected by SW acclimation. For the AQP11b gene in the gill, rRNA normalized and RPLP0 normalized comparisons between the FW 6-day and the FW 20-day control groups and their respective SW 7-day and SW 21-day experimental groups showed a significantly higher number of mRNA/cDNA molecules in the SW samples. The data suggests that AQP11b gene expression increase during SW-acclimation.

The kidney had a significantly lower level of AQP11a mRNA/cDNA expression in the SW 7-day experimental group compared to the FW 6-day control group. This seemed to be due to an elevated level in the FW 6-day control group compared to the other FW groups. The relevance of this single significant difference (in both the rRNA and RPLP0 normalized data) in the middle of the experiment is hard to determine. The number of mRNA/cDNA molecules for SW rose considerably for the SW 21-day
experimental group but it was not significantly higher than the FW 20-day control group however the 21-day SW group was significantly higher than the other SW groups. The situation for the AQP11b gene was to some extent the opposite of that of AQP11a in that the mRNA/cDNA expression was significantly higher in the 7-day SW rRNA normalized experimental group (compared to the FW 6-day control group), although this was not preserved after RPLP0 normalization. Although the significant differences between the FW 6-day and SW 7-day time points in opposite directions (for AQP11a compared to AQP11b) suggest the possibility of some kind of real phenomenon is occurring at this time, it is hard to envision what might be the cause of these temporary changes in gene expression. Otherwise the data suggests that renal AQP11a and AQP11b gene expression was unaffected by SW-acclimation. However, it should be noted that FW samples had significantly decreased in the FW 7-day and 21-day groups compared to the FW 0-day groups. These changes in expression should be further explored before final conclusions are drawn.

Conclusions

In the qPCR data for the anterior intestine, posterior intestine, and gill (also for the rectum with the AQP11b rRNA normalized data) there was a significant difference between the FW 20-day control groups and SW 21-day experimental groups in the number of mRNA/cDNA molecules for either AQP11a or AQP11b for both rRNA and RPLP0 normalization. The within-group changes in the expression of AQP11 paralogs were seen in some organs (AQP11b in kidney and to a lesser extent in AQP11b rRNA normalized stomach and RPLP0 normalized gill data, as well as AQP11a rRNA normalized anterior intestine data) to have a significant reduction in expression for either the FW 6-day or 20-day groups compared to either the FW time=0 or 1-day groups (p < 0.05), a similar situation was also seen in the SW-acclimated RPLP0 normalized AQP11a posterior intestine groups where the 2-day, 7-day and 21-day SW groups were significantly lower than the 6-hour group. A potential explanation is that since the samples were wild-caught animals the eels used for the FW groups may have naturally had wide variability in the number of mRNA molecules of AQP11 paralogs that affected the results. Another potential contributing factor is that the temperature had changed considerably by the end of experiments;
due to space limitations, not all groups could be run at the same time such that by the end of the SW-acclimation experiment there had been unquantified warming of the environment which in turn warmed the water used for the experiments and potentially played a role in the overall decrease in AQP11 for later time point FW or SW groups in some tissues. Gene expression in European eel larvae has recently been documented to be affected by changes in temperature (Politis et al. 2017). Temperature changes however, do not explain how multiple tissues (AQP11a anterior intestine, AQP11a posterior intestine, and AQP11b gill, and for rRNA normalized data for AQP11b in rectum) had significantly higher expression of AQP11 paralog mRNA/cDNA molecules for the SW 21-day experimental group compared to the FW 20-day control group as the environmental temperature change was the same for both FW and SW groups. Since there was still a significant difference in AQP11a and AQP11b mRNA/cDNA expression in multiple SW and FW samples it would seem that AQP11 may be affected by SW-acclimation to some extent over the longer term. Furthermore, the AQP11a and the AQP11b gene expression in the anterior intestine and the gill respectively eventually increased after 21-days in SW beyond the level set after 6-hours or 2-days in SW and was significantly higher than their FW counterparts. In contrast, the RPLP0 normalized AQP11a gene expression in the posterior intestine had values that, overall, decreased from 6-hours to 21-days in SW; the decrease in SW was not as much as occurred in the FW fish over their respective time course. Another explanation is that since some of the eels came from estuaries it is conceivable that they may have already been somewhat acclimated to SW initially and so AQP11 amounts dropped during the course of the experiment. It should also be noted that eels had at least one week in FW tanks before the SW-acclimation experiments began. Gene expression is complex, and changes in gene expression cannot be completely attributed only to SW-acclimation as there are likely other contributing factors involved. However, the within-group and between-group data from the qPCR implies that SW-acclimation does affect AQP11a and AQP11b gene expression, to a degree, in select osmoregulatory tissues.
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Figure 2.1: Original eel (*Anguilla japonica*) AQP11a and AQP11b primers used to produce *Anguilla rostrata* cDNA clones/sequences.
Figure 2.2: Agarose gel electrophoresis showing 2-log ladder, and DNA samples from kidney, esophagus, stomach, anterior (Ant) intestine, posterior (Post) intestine, rectum, and brain. Possible genomic DNA products were present at around 2.7 kb, the cDNA (cloned) was present at a slightly larger than expected size of 1.1 kb, a second band with a size slightly closer to the expected size was seen directly below the cDNA band and this was thought to potentially be a second form of AQP11b, this is visible especially in kidney and brain samples, and a smaller band at around 500 bp was seen that was probably an unrelated amplification product.
Figure 2.3: Thermo Fisher pCR4-TOPO plasmid vector used for cloning and subsequent colony PCR of AQP11a and AQP11b.
### Anguilla rostrata AQP11a Sequence

| AGGAAAGGCC | CTAAAATAGA | GAGACGAGCT | CGAACAGAGA | AGTTTTAACCC
| ACTGCGCTGG | ATGGAATAT | CTAGCGATTTC | CTGTATTTATTC | GTTCGTCGTC
| ATTTGGCATT | TAAGCAGACC | AGCAAGGAGG | ACAACTTGTTTA | AATCTCGGCG
| CGGAAAGAT | TATGGCCCTTCT | ATCTGGTTTGTT | AATACTTTA | ACAATTCCGG
| TATGGGCTCT | CATACATGAA | CACAACTCCTC | TCGGGAATCTTT | GGGCCAAATTT
| GAGGCAAGAAG | TAGAGTTTGAC | TCTAAGCTGAC | ACAACTCGGG | TGGTCCACGC
| GCTAACATTT | CGTGGAGCTC | TTTTCAATCCAT | ATCCGGTGCCC | CTGCAAGACA
| TTATGCGCTTGG | AAACATCACC | GCCCAAGAGAGA | CTTTGGCAGC | GATCGTTGCC
| CAATTTATG | CGCAGCTGAC | TGCCGCTTGGT | GTGATATTAG | AAGTCTGGGC
| TCGAAGAGCTC | TCGACCTTC | ACCTCAAGCA | CAAATACGTTA | GGAATTTAAT
| GCACAAATGCC | ATTCAACTC | GTCTACCGAGA | AAGCACTGTC | TATCTTTGTGA
| GCTGGCGCTG | TCCAGTGCA | AAGAGCTGTC | ACTACCTTGAG | ACGACTGGGA
| CGAAAAATAC | AGGACCTCTTT | TCATTGCCGC | TGCTGGTCACCA | TCTTTGCTTT
| ATGGAAGG* | CAGCCTTAACT | GGAGCCTGAT | CTAAATCGTG | TCTGGCGTCTC
| TCCATCCTGTT | TCCCCCTCTG | GGAAAACACA | TTTCTTGAAGT | ATTCCTTCGTT
| GCCGCAGCTG | GGGGCCGTTT | TGTCATCCTCG | ATCTTTTTACCA | ATGCGACAGG
| GCTTAGGTCG | TCTGGTTT | *AGG CAGCTTACT | GGAGCAGCTG | TGACCTCCCGC
| GCCCGTGAGC | AGGCGAGTCT | ACAAACCGAT | TGCATCGGAT | TGCGTTAGTCT
| CTGCAGTTGAG | *AGG CACCCCTTCT | GGAGCAGTCG | TTTCCTTCT | GCCGACACCG
| CAAATCACTG | CTTCCTGCTT | AAACATCGTTA | GGGCGTTATC | TGGTGTTAGT
| ACACATCTGG | CCGCAGCTG | TGGCCACAGC | TGGCGGAATTT | ATCCCATTTC
| GACATTGTTCG | GCCATACGACG | GTTCAGGTGTT | ATTTTTTATT | ATGCAGAGG
| GCATGCGACG | AGCCTTCTCC | GCTATCGGAT | TGCCGACAGC | ATGCGCCAGC
| *GGTGGT | CTGCTGCTT | CGCATTTTAC | CGGCGGATAT | TGCCGCAAGC
| ATGGAATAT | CTAGCGATTTC | CTGTATTTATTC | GTTCGTCGTC | ATGCGCCAGC

**KEY**

**Start/Stop**

5’ and 3’ UTRs

Intron location (from Japanese eel sequences) =*

### Anguilla rostrata AQP11b Sequence

| AGTGGCCAC | ATCACAGTTT | CTCATTCCCT | GCTGGCACCCT | TTGTGCTTTA
| GTCATGGAAGT | TACAAGGAGG | ATGGCCACAG | AAGCTTTCCGC | AAAAAAGGAT
| TACGTTATTTC | ATGTGTGGAA | AACAACTCGG | ACCTTTAGGCG | TCTGTGCTCG
| CACCCCATGAG | CTGGAAATCC | TAGGGCGAGT | GGGCGAACTC | GAGCCCGAGA
| TCGAATTGAC | TTTCACTC | CTTGTGCTG | ATGGACGACT | TTTTACTCCG
| GCACGAGGCA | TGGTGAAATC | TCTGGTGAG | ATGATGGGCC | TCTAGGATTCC
| GCGCTTCACC | GGCAAGGTGT | TCTCTGACG | GATCGCCTG | CCTATTTGTTG
| GGCAGTGGC | TCTGCTGTTT | GGCCGCTAGT | ATGGTACCTT | AGCTATGGGC
| TCTGATTGTC | ACATGACGGA | CAAACTCTTC | GGAATTTAAT | GCATAAATCC
| AATTGACGCA | ACAGCTACCA | AAGGAGCGTC | AGTGGGCTTA | TTGGGCGCTTC
| TTGTAGTACA | AACCGGCATT | ACACATATGT | ACACATGATA | CGAAATAATAC
| CGTGCACATG | CCGTAGCGACG | AGTCACTACG | GCCCTCTGCCT | ATGCGAGG*

**KEY**

**Start/Stop**

5’ and 3’ UTRs

Intron location (from Japanese eel sequences) =*

**Figure 2.4: Anguilla rostrata** AQP11a and AQP11b sequences generated from colony PCR. Start and stop codons, untranslated regions (UTRs) and intron locations were identified using sequences of AQP11a and AQP11b from the Japanese eel (Anguilla japonica).
Figure 2.5: Homology of nucleotides between *Anguilla rostrata* and *Anguilla japonica* cDNA sequences for AQP11a (right) and AQP11b (left). Homology between nucleotides is denoted below the pair with an asterisk (*). Lack of homology is left blank.
<table>
<thead>
<tr>
<th>A_rostrata_AQP11a</th>
<th>MEYLALSVMILIVLSEARRTLKFARKDNYAVLLEISTFQLCATHELKLLGEV</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_japonica_AQP11a</td>
<td>MEYLISGVLLTVLSEARRTLKFARKDNYAVLLEISTFQLCATHELKLLGEV</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A_rostrata_AQP11a</td>
<td>GQIEPQIGLTLTYYTVSHALTFRGALCNPSGALHLIYRGNLTKRALAIRACQFIAAVV</td>
<td>120</td>
</tr>
<tr>
<td>A_japonica_AQP11a</td>
<td>GQIEPQIGLTLTYYTVSHALTFRGALCNPSGALHLIYRGNLTKRALAIRACQFIAAVV</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A_rostrata_AQP11a</td>
<td>ARLVILEWALELSDHKLHSSLGFKCTSPIHTVLFKAVELACFVQTAVERNLRD</td>
<td>180</td>
</tr>
<tr>
<td>A_japonica_AQP11a</td>
<td>ARLVILEWALELSDHKLHSSLGFKCTSPIHTVLFKAVELACFVQTAVERNLRD</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A_rostrata_AQP11a</td>
<td>EKYRALFIAAVTFLVYAGSGGFTGVNFVPLASIQPGCTNSFLEYESLYVGVCPVGLVA</td>
<td>240</td>
</tr>
<tr>
<td>A_japonica_AQP11a</td>
<td>EKYRALFIAAVTFLVYAGSGGFTGVNFVPLASIQPGCTNSFLEYESLYVGVCPVGLVA</td>
<td>240</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>A_rostrata_AQP11a</td>
<td>FSVLFDKIIPLSNGSTYERGLDIAGIKDNNA</td>
<td>273</td>
</tr>
<tr>
<td>A_japonica_AQP11a</td>
<td>FSVLFDKIIPLSNGSTYERGLDIAGIKDNNA</td>
<td>273</td>
</tr>
</tbody>
</table>

Figure 2.6: Homology of derived amino acids sequences between *Anguilla rostrata* and *Anguilla japonica* cDNA for AQP11a (top) and AQP11b (bottom) homology between amino acids is denoted below the pair with an asterisk. Amino acids of strongly similar properties are denoted below the pair with a colon. Amino acids of weakly similar properties are denoted below the pair with a period.
Figure 2.7: Alignment between *Anguilla rostrata* AQP11a and AQP11b cDNA showing homology (|) or lack of homology (•) between nucleotides. Primer design used this alignment to determine optimal locations where AQP11a and AQP11b differed the most. Gaps in the sequence (-) are inserted to maintain alignment. The intron location for AQP11a and AQP11b are also included.
Figure 2.8: Locations of the final primers selected for qPCR of AQP11a and AQP11b cDNA. For both AQP11a and AQP11b sense and antisense primers were made across intron-exon junctions to prevent the amplification of genomic DNA.
Figure 2.9: Primers designed to amplify fragments for AQP11a (left) and AQP11b (right) qPCR. QPCR8 sense and QPCR7 antisense was selected for AQP11a and was used for qPCR of AQP11a. QPCR5 sense and antisense were selected for real- time PCR of AQP11b.
Figure 2.10: Measuring the number of AQP11a mRNA/cDNA molecules in osmoregulatory tissues in SW experimental groups and FW control groups for rRNA normalized and RPLP0 normalized total RNA samples. Data is expressed as mean ± standard error of technical replicate means.
Figure 2.11: Measuring the number of AQP11a mRNA/CDNA molecules in SW-acclimated non-osmoregulatory tissues for rRNA normalized and RPLP0 normalized total RNA samples. Means are given for each tissue. Data is expressed as mean ± standard error of technical replicate means.
Figure 2.12: Measuring the number of AQP11b mRNA/cDNA molecules in osmoregulatory tissues in SW experimental groups and FW control groups for rRNA normalized and RPLP0 normalized total RNA samples. Data is expressed as mean ± standard error of technical replicate means.
Figure 2.13: Measuring the number of AQP11b mRNA/CDNA molecules in SW-acclimated non-osmoregulatory tissues for rRNA normalized and RPLP0 normalized total RNA samples. Means are given for each tissue. Data is expressed as mean ± standard error of technical replicate means.
Figure 2.14: Comparing the number of mRNA/cDNA molecules in esophagus tissue for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in esophagus total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
Figure 2.15: Comparing the number of mRNA/cDNA molecules in stomach tissue for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in stomach total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
Figure 2.16: Comparing the number of mRNA/cDNA molecules in stomach tissue for each SW experimental group and the corresponding FW control for rRNA normalized and GAPDH normalized AQP11a and AQP11b in stomach total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
Figure 2.17: Comparing the number of mRNA/cDNA molecules in anterior intestinal tissue for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in anterior intestine total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
Figure 2.18: Comparing the number of mRNA/cDNA molecules in posterior intestinal tissue for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in posterior intestine total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with upper and lowercase letters respectively.
Figure 2.19: Comparing the number of mRNA/cDNA molecules in rectal tissue for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in rectum total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
Figure 2.20: Comparing the number of mRNA/cDNA molecules in brachial tissue for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in gill total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
Figure 2.21: Comparing the number of mRNA/cDNA molecules in kidney for each SW experimental group and the corresponding FW control for rRNA normalized and RPLP0 normalized AQP11a and AQP11b in renal total RNA samples. Data is expressed as mean ± standard error of means. Statistical significance within SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) are denoted with uppercase and lowercase letters respectively.
CHAPTER 3
QUANTIFYING AND LOCALIZING THE PROTEIN EXPRESSION OF AQP11 PARALOGS
(AQP11A AND AQP11B) IN ANGUILLA ROSTRATA

INTRODUCTION

As previously stated, the function of AQP11 is not yet fully understood however AQP11 proteins have been localized primarily on intracellular organelles such as the ER membrane (Mortisha et al. 2005, Ishibashi 2006, Ikeda et al. 2011). In Chapter 2, the expected weight of the American eel AQP11a and AQP11b proteins were estimated to be 29.72 kDa and 29.98 kDa respectively by translating the obtained cDNA sequences into amino acids. The estimated weights for AQP11a and AQP11b are aligned with previous literature that has calculated the weight of the AQP11 protein to be around 30 kDa in size (Ikeda et al. 2011, Morishita et al. 2005). Western blotting in mouse and rat species and in killifish found that AQP11 bands typically show up at around 25-26 kDa (instead of the calculated 30 kDa) which the researchers suggested may be due to secondary, post-translational, modification of the protein; in addition bands at 50 kDa were also witnessed, which the researchers suggested are likely dimerized versions of AQP11 (Ikeda et al. 2011, Morishita et al. 2005).

Due to their relatively recent discovery, there was little information on changes in protein expression of group III AQPs. One of the questions concerning group III AQPs in teleost fish was whether their protein expression would be affected by SW-acclimation in the same manner that many other AQPs are. This chapter investigated whether there were any changes in protein expression of AQP11a and AQP11b that occurred in the American eel following SW-acclimation and subsequently localizing AQP11 paralogs proteins to cells within various tissues using immunohistochemistry. At the time of this study, few articles dealing with the immunohistochemical localization of AQP11 paralogs in euryhaline fish, however because both AQP11 paralogs were expressed, to some degree, in the qPCR, protein expression was expected in all tissues tested. AQP11 paralogs are, as far as is known, entirely intracellular and do not necessarily have a role in maintaining the homeostasis of a cell cytoplasm or cell volume, a change in AQP11 protein expression following SW-acclimation was therefore not anticipated.
MATERIALS AND METHODS

Antibody Production

Amino acid sequences for AQP11a and AQP11b were estimated using the nucleotide sequences which had been obtained from the sequencing performed in Chapter 1. These amino acid sequences were then aligned to determine homology and the best location to generate polypeptides for antibody production, these regions were also determined to be highly antigenic (Figure 3.1). Alignment of AQP11 paralogs indicated that the best location for the generation of polypeptides for antibody production was at the C-terminal end of the polypeptides. At this location, the two paralogs differed almost completely and the resulting antibodies for one paralog would be unlikely to bind to the other paralog’s protein.

Polyclonal antibodies for both AQP11a and AQP11b were made by GenScript using artificially generated polypeptide sequences from AQP11a and AQP11b respectively. These peptides were conjugated (by GenScript) with keyhole limpet hemocyanin protein and were injected multiple times with an adjuvant into New Zealand White rabbits. Antibodies were affinity-purified using the peptide antigens attached to a column and antisera for both AQP11a and AQP11b were obtained for western blotting and immunohistochemistry experiments. GenScript reported that affinity-purified antibodies made against AQP11a had a yield of 11.55 mg of protein at a concentration of 0.350 mg/ml, and antibodies made against AQP11b yielded 3.01 mg of protein at a concentration of 0.752 mg/ml (Figure 3.1).

Western Blotting Detection and Quantification of Proteins

Western blotting was used to investigate the presence of AQP11a or AQP11b proteins and quantify their amounts in gastrointestinal tissues. The homogenized tissue samples had been prepared using Protease Inhibitor Cocktail V (Research Products International).

Samples available for quantification using western blotting were as follows: Anterior intestine, posterior intestine, and rectum. There were 6 samples per FW and SW group for both anterior intestine and posterior intestine, however there were only 3 samples per group for rectum. Samples from other tissues came from a single fish and thus quantification was not necessary since it was not possible to do a
statistical analysis of the results; these tissues included stomach, esophagus, gill, kidney, brain, eye, heart, spleen, swim bladder, skeletal muscle, and liver.

The SDS-polyacrylamide gel electrophoresis followed the procedures of Laemmli (Laemmli 1970). Two gels were used for each experiment, each has an acrylamide resolving gel and stacking gel. A 40% stock of acrylamide with a ratio of 29:1 acrylamide: bis-acrylamide was used to make the gels. The resolving gel required 10% acrylamide and a lower percentage (5%) acrylamide was prepared for the stacking gel. 35 ml of resolving gel was prepared using: 16.70 ml H2O, 8.75 ml 40% acrylamide stock, 8.75 ml 1.5M tris pH 8.8, and 0.175 ml 20% SDS. These were mixed and then had 0.35 ml of freshly prepared 10% ammonium persulfate and 0.028 ml TEMED (setting agents) added. The liquid gel was added to the apparatus using a 50 ml syringe and left to set for 30 minutes. A small amount of iso-butanol was added to smooth out the top surface. After setting, the iso-butanol was removed, and the surface was washed with distilled water. The stacking gel was made right before the 30 minutes for the resolving gel setting expired. The stacking gel was made with 5.14 ml DI water, 0.875 ml 40% acrylamide stock, 0.875 ml 1M tris pH 6.8, 0.035 ml 20% SDS. These were mixed and then had 0.07 ml 10% ammonium persulfate, 0.007 ml TEMED polymerization agents added.

The gel was poured, and the comb was added. After setting for 30 minutes, the comb was removed, and the wells were rinsed using distilled water. The gel running buffer (25 mM tris, 250 mM glycine, and 0.1% SDS) was then added to the tank after the apparatus was prepared and the gel was attached to the electrophoresis apparatus.

The protein samples used were crude membrane extracts that had been made previously. The protein samples were made by homogenizing tissues in the presence of Protease Inhibitor Cocktail V (RPI). The crude membranes were then pelleted by centrifugation at 50,000 g. The supernatant was removed, and the pellet was re-suspended in buffer containing protease inhibitors and was stored frozen at -80°C.

The trial quantification western blots were performed using 50 µg of anterior intestinal tissue. However, after inconsistent sample loading, and considering that stronger bands may be achieved using a
higher amount of protein, future runs were more carefully performed using 100 µg of protein. As was the case with the qPCR (see Chapter 2), SW samples were compared to corresponding FW controls (FW 0-day with SW 6-hour, FW 1-day with SW 2-day, FW 6-day with SW 7-day, FW 20-day with SW 21-day). Anterior intestine and posterior intestine western blotting had to be performed using 2 separate gels simultaneously (FW 0-day to SW 2-day on one gel and FW 6-day to SW 21-day on a second gel) whereas rectum was able to be carried out using a single gel.

A 4x denaturing buffer was added to the samples, containing the following: 250 mM tris pH 6.8, 8% 2-mercaptoethanol, 8% SDS, 40% Glycerol, 0.02 Bromophenol Blue (dye). The samples were then heat-denatured in a PCR thermocycler to 99°C for three minutes. After the adding of the gel running buffer to the tank the samples were loaded into the gel wells. Additionally, kaleidoscope protein markers (Bio-Rad; 10 µl) were added to the first well of the gel. Two sets of 24 samples on two gels were run each time. The gels were run overnight for around 16 hours at around 45 volts. Subsequently, the gels were removed, and the gels proteins were electroblotted on to a polyvinylidene difluoride (PVDF) membrane filter (Sequiblot; Bio-Rad). For electroblotting, fiber pads and blotting paper were prepared. The blotting paper (48 mM tris and 39 mM Glycine) was wet in blotting buffer and a PVDF filter (used to bind the proteins) was wet using 100% methanol. The cassette was prepared by placing the fiber pad and then wet filter paper and the PDVF filter on the clear side of the cassette (positive electrode side). The gel was removed from the electrophoresis tank and the casting clamps were removed. The gel was placed on top of the PVDF filter and massaged to remove any air bubbles. Wet blotting paper was put on top of the gel and a fiber pad was added above that. The cassette was closed and inserted into the blotting tank. The tank was then filled with buffer and was left to blot overnight at 15 volts.

A large stock of 1X TNT buffer at pH 8 was prepared using 10 mM tris, 150 mM NaCl and 0.05% Tween 20. 5% nonfat dry milk powder (blocking agent) was added to 10 to 75 ml of 1x TNT buffer (depending on the size of the PVDF strip) in separate glass trays labeled for either AQP11a or AQP11b. The western blot strips were removed from the apparatus and placed into glass trays containing
the TNT buffer and the blocking agent. The dishes were put onto a rocking platform and incubated for 30 minutes.

The blots were washed three times using 10-75 ml of 1x TNT buffer. After the washing, 25-187.5 µl of anti-AQP11a or anti-AQP11b (at a 1/400 dilution) was added to the appropriate dish and was incubated on the platform for 1 hour.

The samples were then washed 4 times followed by the addition of 2.5 - 18.75 µl of the secondary donkey anti-rabbit antibody (Pierce/Thermofisher 1/4000 dilution) and this was allowed to incubate for 1 hour.

The blots were washed 4 times with the 1x TNT buffer and then 5-10 ml (depending on the size of the filter) of 1 step NBT/BCIP reagent (with 1 mM levamisole endogenous alkaline phosphatase suppressor; Pierce/Thermofisher) was added to the dish and 1-10 minutes of swirling was performed to allow the color to develop. Finally, the strips were washed with distilled water and dried and photographed.

The western blots were quantified using Syngene GeneTools. Statistical analysis was performed between SW groups and corresponding FW control groups (FW time=0 and SW 6-hours, FW 1-day and SW 2-day, FW 6-day and SW 7-day, FW 20-day and SW 21-day) using ANOVA with Dunnett’s test.

**Immunohistochemistry**

Since various tissues from eels that had been SW-acclimated and anti-AQP11a and anti-AQP11b antibodies that had been made for western blotting were readily available, localization of the aquaporin proteins was attempted in samples of liver, kidney, anterior intestine, and rectum. This is the first known effort to attempt to localize AQP11 paralog proteins in osmoregulatory organs of euryhaline fish.

Organ samples preserved in paraffin-embedded blocks had been prepared from previous experiments and were available to use for immunohistochemistry. Various SW eel samples of liver, kidney, anterior intestine, posterior intestine, rectum, were examined. FW eel samples were also available for kidney, anterior intestine, posterior intestine, and rectum. 5 µm thick tissue sections were cut using a microtome (Leica). Sections were transferred to a float bath of de-gassed distilled water heated to 35-37°
C to smooth out wrinkles in the sections using surface tension. After the wax and tissue had flattened, slides were brought up underneath the wax sections until the edge of the section attached to the slide surface. Slides were then slowly pulled out of the water to attach the whole section to the slide. Slides were place almost vertical to allow water to drain out from between the slide and the section. The slides were heated on a slide drier overnight at around 50-55° C to dry the sections and adhere the tissue to the slide surface.

When immunohistochemistry was to be performed on the slide, wax was removed using two amounts of HistoChoice clearing agent (Sigma-Aldrich) followed by incubation in a series of increasingly dilute ethanol solutions from 100% down to 50%, performing 5 minutes incubations per solution. Slides were then rinsed in phosphate buffered saline (PBS). The tissue sections on slides were then ringed with a hydrophobic barrier pen and the slides were placed horizontally in a slide box. Solutions containing tween-20 detergent were used to permeabilize the tissue, another containing ammonium chloride was used to block any remaining reactive paraformaldehyde fixative, and then sections were blocked first using a proprietary “Background Buster” blocking solution (Innovex) for 30 minutes and after washing the slides 3 times with PBS, slides were further blocked with a solution containing 1% BSA and 1% gelatin in PBS for 30 minutes. Slides were washed again 3 times with PBS. Slides were then incubated either rabbit anti-AQP11a or anti-AQP11b antibodies for 1 hour and then washed four times with PBS. In order to detect the primary antibodies, a secondary antibody, goat anti-rabbit IgG with a fluorescent tag attached (Alexa 488; Thermofisher) was used. Slides were washed 4 more times with PBS and mounted with Prolong Gold mounting medium (Thermofisher), which contained DAPI as a counter-stain (that stains cell nuclei). Some slides were further treated with “True Black auto-fluorescence quencher” (Biotium). All slides were visualized using a Zeiss LSM 710 laser-scanning confocal microscope.

RESULTS

*AQP11 Paralog Amino Acid Sequencing and Alignment*

Translation of the nucleotide sequences into amino acid allowed for the estimation of the size of the AQP11a and AQP11b proteins (29.72 kDa and 29.98 kDa respectively) which were used to identify
AQP11 protein bands during western blotting (Figures 3.2 and 3.3). Alignment of AQP11a and AQP11b amino acid sequences determined them to be 64% homologous, when accounting for amino acids of functional equivalence that increased to 77% (Figure 3.1).

**Western Blotting**

While performing the initial western blots using the antibodies on various tissue types AQP11a showed two unexpected bands at 40kDa and 50kDa (Figure 3.2). AQP11b, however, gave a single band at 24 kDa (Figure 3.3). Which, although smaller than the estimated 30 kDa, was similar in size to what has been documented previously (Ikeda et al. 2011 Morishita, et al. 2005). After a further round of tissue blots with further samples it was determined that the antibody for AQP11a was not specific enough to give acceptable results as there was excessive banding found throughout the entire blot (Figure 3.4). For AQP11b there were three bands that were now seen in some tissues, and these were estimated to be 60 kDa, 30 kDa, and at 24 kDa in size (Figure 3.5). Peptide-blocking was also performed as a control for both AQP11a and AQP11b (Figure 3.5).

For the western blotting of the SW-acclimation study samples, the first blots were performed on the anterior intestine samples. This was initially performed using 50 µg of protein per sample but loading of the samples was determined to be inconsistent and was more cautiously repeated using 100 µg of protein per sample (Figure 3.6). For the more accurately performed 100 µg anterior intestine blots there was a significant increase in AQP11b protein expression between the FW 20-day control groups and the SW 21-day experimental groups for both the 60 kDa and 24 kDa bands and there was a significant decrease in expression with the FW 6-day control group compared to the SW 7-day experimental group for the 30 kDa band. Within-group comparisons found that SW 21-day samples were significantly higher than the SW 2-day samples for the 60 kDa band, FW 6-day samples were significantly higher than the FW 20-day band, and that SW 21-day and FW 20-day groups were both significantly higher than any other within-group member.

Posterior intestine sample blots were carried out using 100 µg of protein (Figure 3.7). Significant increases were found for all 3 bands between the FW 20-day control groups and the SW 21-day
experimental groups samples. Within-group analysis determined that the FW 6-day and 20-day groups had significantly lower expression compared to the 0-day and 1-day groups, and that the SW 7-day group displayed significantly lower expression compared to the rest of the SW groups for the 60 kDa band. The 30 kDa band showed both the FW 6-day and 20-day having lower expression compared to the 1-day samples. The FW 20-day samples were also significantly lower than the 0-day samples. For the 24 kDa band the FW 20-day group was significantly higher than both the FW 0-day and FW 6-day groups, and SW-21 was significantly higher than the rest of the SW groups.

The rectum sample blots were also performed using 100 µg of protein (Figure 3.8). For the 60 kDa band significant increases were found only between the SW 21-day experimental groups samples and the FW 20-day control groups. For the 30 kDa band there was a significant decrease only between the SW 6-hour experimental groups sample and the FW time=0 control group. For the 24 kDa band there was a significant decrease between the SW 2-day experimental groups and the FW 1-day control groups and the and there was a significant increase in expression between the SW 21-day experimental group and the FW 20-day control group samples. Within-group analysis showed a significant decrease in protein expression for the FW 6-day and 20-day groups compared to the 1-day group, and the SW 21-day group was significantly higher compared to the 6-hour and 7-day groups for the 60 kDa band. For the 30 kDa band the FW 20-day group was significantly lower compared to the 0-day and 1-day groups, and SW 6-hour was significantly lower than the 2-day group. For the 24 kDa band the SW 21-day group was significantly higher than the rest of the SW groups.

The relative abundance of each band was combined to estimate the amount of total AQP11b protein for FW and SW samples in anterior intestine, posterior intestine and rectum (Figure 3.9). The SW 21-day group had the highest expression in all three tissues significantly higher than the FW 20-day control and other SW samples. Additionally, both posterior intestine FW 7-day and 20-day groups displayed lower expression compared to the FW 0-day and 1-day groups. Within-group analysis showed a significant increase in the SW 21-day group compared to the rest of the SW groups in the anterior intestine. In the posterior intestine the SW 7-day samples were significantly lower than the rest of the SW
groups, and the FW 6-day and 20-day groups were both significantly lower than the 0-day and 1-day groups. In the rectum there was a significant increase in the SW 21-day group compared to the rest of the SW groups, and FW 7-day and 20-day groups displayed lower expression compared to the FW 0-day and 1-day groups.

**Immunohistochemistry**

Peptide-blocking in various tissues (anterior intestine, rectum, kidney, liver) and negative controls (anterior intestine, liver) confirmed that the fluorescence the AQP11a and AQP11b antibodies displayed was at least specific to the antibody. However, it should be noted that there was a degree of auto-fluorescence in red blood cells in many tissues as seen in the negative controls and peptide-blocked slides in the liver and kidney tissues. AQP11a and AQP11b had some similar patterns of expression. Both AQP11a and AQP11b proteins were found to be in smooth muscle and the luminal side of epithelial cells in SW and FW anterior intestine tissue (Figures 3.10 and 3.11). This was also seen to a similar degree in SW posterior intestine tissue and SW rectum tissue, in FW rectum and posterior intestine tissue, however, AQP11b was not expressed on the luminal side of epithelial cells (Figures 3.13 and 3.14). In SW kidney tissue AQP11b proteins were occasionally localized to renal tubules and AQP11a was only present in tissue surrounding the tubules (Figure 3.15). FW kidney tissue displayed almost no AQP11a or AQP11b proteins (Figure 3.16). In SW liver tissue both AQP11a and AQP11b were present. AQP11a was seen in muscle surrounding the tissue (although this may have been intestinal in origin due to imprecise dissection) but not in the actual liver tissue and AQP11b proteins were localized around liver ducts and surrounding tissue (Figure 3.17). AQP11b seemed to be expressed in blood vessels in a couple tissues, namely posterior intestine and liver (Figures 3.13 and 3.17).

**DISCUSSION**

*Western Blotting to Quantify AQP11 Paralog Protein Expression*

As was mentioned previously, it was determined that the binding of the anti-AQP11a antibody to the protein for AQP11a was not specific enough (see Figure 3.4). This may be due to the amino acid sequence RGADIAGI found in the C-terminal end of the AQP11a protein sequence resembling a PDZ
binding motif (X-Φ-G-Φ where X is any amino acid and Φ is a hydrophobic amino acid, IAGI fits this motif) and thus the anti-AQP11a antibody that was created using the C-terminal end could bind a variety of other proteins (Lee and Zheng 2010). Since the antibody was not specific to AQP11a it was not worth carrying out the western blot analysis for AQP11a and therefore only blots with the AQP11b antibody were performed and analyzed.

During the western blotting of AQP11b three bands were documented at 60 kDa, 30 kDa, and 24 kDa. Since the calculated size for AQP11b was 29.98 kDa (Figure 3.1) it was assumed that the 30 kDa band was the unmodified protein. In regard to the 24 kDa band, previous studies (Ikeda et al. 2011, Morishita et al. 2005) documented similar bands of 25 kDa rather than at the expected 30 kDa. The presence of both 30 kDa and 24 kDa bands in these blots suggests there may be post-translational modification of the AQP11b protein, however, additional research would need to be done to verify this. The presence of a 60 kDa band is somewhat analogous to what was witnessed in killifish AQP11, with the presence of a 50 kDa band which the researchers concluded was a dimerized form of the post-translationally modified AQP11 protein (Ikeda et al. 2011). Other bands present in the blots have yet to be explained but could potentially be caused by minor levels of cross reactivity of the antibody to other proteins.

For some bands in some tissues of the western blotting of AQP11b, it was seen that AQP11b protein abundance had significantly decreased in the FW 20-day control groups compared to the other FW groups (posterior intestine and rectum 60 kDa and 30 kDa, anterior intestine and 30 kDa). A potential explanation is that since many of the eels originally came from estuaries it is conceivable that they may have had been somewhat acclimated to SW to start with before the experiment began and AQP11 amounts dropped during the course of the experiment. The temperature may have also played a role; due to space limitations, not all groups could be run at the same time such that by the end of the SW-acclimation experiment the weather had become warmer which may have played a role in the decrease in AQP11 protein for the FW 6-day group, SW 7-day group, FW 20-day group, and SW 21-day group eels. Rising temperatures, however, do not explain the sharp increase seen in the 24 kDa and 60 kDa band for
the SW 21-day groups in every tissue. It also does not explain how in each tissue the total protein abundance for the SW 21-day group was significantly higher than its FW 20-day counterpart and how the anterior intestine and the rectum total protein abundance was significantly higher in the SW 21-day group compared to the rest of the SW groups. Another potential explanation is that since some quantity of eels may have originally come from estuaries it is conceivable that they had been already somewhat acclimated to SW, although the fish had been in FW for at least a week before the start of the experiment they may have still been in the process to acclimating to FW.

**Immunohistochemistry**

Although quantification of immunohistochemistry was not viable due to a lack of available samples, there appeared to be a noticeable difference in the degree of which AQP11a and AQP11b fluoresced between SW and FW samples. However, as previously stated, the results of the western blotting casts doubt on the validity of the results of the AQP11a immunohistochemistry. In previous studies AQP11a was documented in the Japanese medaka to be expressed in the brain, eye, fin, gill, intestine, muscle, heart, ovary, intestine, kidney, spleen and testes and AQP11b has been documented in zebrafish to be expressed in ovary, anterior intestine midgut, posterior intestine, and liver (Kim et al. 2014, Tingaud-Sequeira et al. 2010), The qPCR results suggested that all the tested tissues would express AQP11a and AQP11b to some degree and that the liver would be a major site of expression for AQP11a. Indeed, AQP11b protein florescence was present to some degree in all tissues, but the immunohistochemistry showed little to no florescence for AQP11a proteins in the liver tissue (Figure 3.17). Even though mRNA expression does not always correlate directly to protein expression, these results are likely due to the AQP11a antibody not being particularly effective (this is corroborated by the AQP11a western blot results). AQP11b antibody florescence was present in SW intestinal tissue and to some degree the liver and kidney slides. This is in line with documented expression in intestinal tissue and liver during western blotting and qPCR of intestinal, liver, and kidney mRNA/cDNA, and by what has been documented by Tingaud-Sequeria et al. 2010. Although not quantified, there was a noticeable difference in AQP11b florescence between some FW and SW slides. In contrast to SW tissue slides,
AQP11b protein was not expressed on the luminal side of epithelial cells in FW tissue of posterior intestine and rectal tissue, suggesting that SW-acclimation may have some impact on the expression of the AQP11b protein in these tissues. Future studies should be performed to quantify and localize AQP11a proteins in osmoregulatory tissue.

**Conclusions**

Perhaps the most interesting result from the protein quantification and localization is what was documented with the 24 kDa band and the total relative protein abundances during the western blotting. The 24 kDa band had low expression for both FW and SW groups until it significantly increased in the SW 21-day samples compared to the FW 20-day group and the other SW samples. Additionally, when the relative abundances of the three quantified bands were combined into one value all of the tissues displayed a significantly higher expression of AQP11b in the SW 21-day group compared to the FW 20-day group. The significant differences documented between the FW 20-day controls and the SW 21-day groups seen in the 60 kDa band, in the 24 kDa band, and in the combined-bands protein abundances may be the most compelling evidence for AQP11b being affected by SW-acclimation.

When considering the results from both the qPCR along with the western blot data, although there are likely external factors contributing to changes in expression (which is true of expression of most genes and proteins in general), it seems that even though the AQP11 paralogs are intracellular, their expression like what has been documented for many other AQPs in teleosts, is affected by SW-acclimation in the American eel (Cutler and Cramb 2002, Martinez, et al. 2005, Tipsmark et al. 2010 Kim et al. 2010, Madsen et al. 2014, Li and Wang 2017). Because AQP11 paralogs are generally localized on the membrane of the ER (Mortisha et al. 2005, Ishibashi 2006, Ikeda et al. 2011), assuming that AQP11 paralogs do play a role in regulating the osmotic homeostasis of the organelle, these findings suggest that the ER of the cells in some osmoregulatory organs would be synchronously affected by SW-acclimation due to the significant changes witnessed in the expression of both AQP11 paralogs at the transcript or protein levels, however, more research and future studies will be required to confirm this.
FIGURES

**Anguilla Rostrata AQP11A Amino Acid Sequence**

MEYLAISVLLIIIVLLSEA ARRTTLKFFA RKDYAVYLLE IISTFQLCAG THEKLLGVE GQIEFPQGLT LTTTTVVAH LTFRGALCNP SGALEHTYRG NLITEGALAR IACQFIAAVV ALRVLLEVYN LSLSLHELK KSLGFKCSTP INTLVLPKVA VELACAFAVQ TAVTHLRLD EKRYALFIAAV VVTFLVYAGG SPTGAVNPV LAFSIQFPCG GNMTLESVY YGVGPVLGVA FSVLFLDKII PLLSGBKSTEY RGAADIGIRD KMA

Estimated weight: 29.72 kDa

AQPI11a Generated Polypeptide Antigen

CYERGADIAGIKDK

Affinity-purified Anti-AQPI11a antibody yield

11.55 mg at a concentration of 0.350 mg/ml

**Anguilla Rostrata AQP11B Amino Acid Sequence**

MADITVSLFL LAFLVISEV TRKIATKLLA KTGYYVYVE TISTFQLCAG THEKLKGEV GQIEFPQGLT LTTTTVVAH LTFRGALCNP SGALEHTYRG NLITEGALAR IACQFIAAVV ALRVLLEVYN LSLSLHELK KSLGFKCSTP INTLVLPKVA VELACAFAVQ TAVTHLRLD EKRYALFIAAV VVTFLVYAGG SPTGAVNPV LAFSIQFPCG GNMTLESVY YGVGPVLGVA FSVLFLDKII PLLSGBKSTEY RGAADIGIRD KMA

Estimated weight: 29.98 kDa

AQPI11b Generated Polypeptide Antigen

CYQNNLNPVFETIK

Affinity-purified Anti-AQPI11b antibody yield

3.01 mg at a concentration of 0.752 mg/ml

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Figure 3.1: Aligned AQPI11a and AQPI11b amino acid sequences (left). Homology (+), functionally identical amino acids (I), and lack of homology (-) are denoted. The sequences for AQPI11a and AQPI11b used for generation of polypeptides to make anti-AQPI11a and anti-AQPI11b antibodies are highlighted yellow. Individual sequences, molecular weights calculated from the sequences, the manufactured polypeptide antigen sequence and the yield for anti-AQPI11a and anti-AQPI11b antibodies are also shown (right)
Figure 3.2: Preliminary western blot for AQP11a testing various tissues liver, (Li) rectum (Re), esophagus (Es), posterior intestine (PI), anterior intestine (AI), stomach (St), kidney (Ki), gill (Gi), swim bladder (SB), brain (Br), eye (Ey), heart (He), skeletal muscle (SM) and spleen (Sp). Multiple bands are visible with two common bands located at 50kDa and 40kDa. Kaleidoscope marker (M) and its visible bands are labeled.

Figure 3.3: Preliminary western blot for AQP11b testing various tissues liver, (Li) rectum (Re), esophagus (Es), posterior intestine (PI), anterior intestine (AI), stomach (St), kidney (Ki), gill (Gi), swim bladder (SB), brain (Br), eye (Ey), heart (He), skeletal muscle (SM) and spleen (Sp). One main band is visible for some samples at 24 kDa. Kaleidoscope marker (M) and its visible bands are labeled.
Figure 3.4: Western blot of various tissues performed using AQP11a antibody showing excessive banding throughout the blot. Samples are as follows, marker (M), FW anterior intestine (FW AI), SW anterior intestine (SW AI), FW posterior intestine (FW PI), SW posterior intestine (SW PI) FW rectum (FW Re) SW rectum (SW Re), SW stomach (SW St), SW esophagus (SW Es), SW kidney (SW Ki), SW gill (SW Gi), SW brain (SW Br), SW liver (SW Li). Kaleidoscope marker (M) and its visible bands are labeled.

Figure 3.5: Western blot of various tissues performed using AQP11b antibody showing three prominent bands at 60 kDa, 30 kDa and 24 kDa. Samples are as follows FW anterior intestine (FW AI), SW anterior intestine (SW AI), FW posterior intestine (FW PI), SW posterior intestine (SW PI) FW rectum (FW Re) SW rectum (SW Re), SW stomach (SW St), SW esophagus (SW Es), SW kidney (SW Ki), SW gill (SW Gi), SW brain (SW Br), SW liver (SW Li). Also shown are peptide-blocked (PB) samples for AQP11a and AQP11b. Kaleidoscope marker (M) and its visible bands are labeled.
Figure 3.6: Western blots of anterior intestine using 100 µg of tissue (bottom) and graphs (top) comparing the relative protein abundance of the three main bands found using AQP11b antibodies and measured using kaleidoscope marker (M) at 60 kDa, 30 kDa, and 24 kDa. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) is denoted with uppercase and lowercase letters respectively.
Figure 3.7: Western blots of posterior intestine using 100 µg of tissue (bottom) and graphs (top) comparing the relative protein abundance of the three main bands found using AQP11b antibodies and measured using kaleidoscope marker (M) at 60 kDa, 30 kDa, and 24 kDa. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) is denoted with uppercase and lowercase letters respectively.
Figure 3.8: Western blot of rectum using 100 µg of tissue (bottom) and graphs (top) comparing the relative protein abundance of the three main bands found using AQP11b antibodies and measured using kaleidoscope marker (M) at 60 kDa, 30 kDa, and 24 kDa. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) is denoted with uppercase and lowercase letters respectively.
Figure 3.9: Relative abundance of the three protein bands (60 kDa, 30 kDa, and 24 kDa) combined for anterior intestine, posterior intestine, and rectum tissue. Data is expressed as mean ± standard error of means. Statistical significance between SW and FW time points (p<0.05) is denoted with asterisks (*). Significance within FW and SW groups (p<0.05) is denoted with uppercase and lowercase letters respectively.
Figure 3.10: Immunohistochemistry of SW anterior intestine using polyclonal rabbit anti-AQP11a antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. 

A. Negative control incubated only with the secondary antibody showing serosa (S), longitudinal smooth muscle (LSM), and circular smooth muscle (CSM). 

B. Peptide-blocking serosa, longitudinal smooth muscle, and circular smooth muscle. 

C. Peptide-blocked tissue showing columnar epithelium (CE), lamina propria (LP), and circular smooth muscle. 

D. Negative control showing columnar epithelium, lamina propria, and circular smooth muscle. 

E. AQP11a fluoresces on the luminal side of columnar epithelium cells and in circular muscle tissue. 

F. AQP11a antibody fluorescing around blood vessels (BV) in circular smooth muscle. 

G. AQP11a is present in circular smooth muscle and less so in the lamina propria. 

H. Longitudinal and circular smooth muscle both have abundant expression of AQP11a, the serosa also expresses AQP11a at a lower level.
Figure 3.11: Immunohistochemistry of SW anterior intestine using polyclonal rabbit anti-AQP11b antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. 

A. Peptide-blocking showing columnar epithelium (CE), lamina propria (LP), and circular smooth muscle (CSM). 

B. Control incubated with only the secondary antibody showing circular smooth muscle and longitudinal smooth muscle (LSM). 

C. AQP11b antibody fluoresces where the lamina propria meets circular smooth muscle. 

D. AQP11b antibody fluoresces in circular smooth muscle and in longitudinal smooth muscle.
Figure 3.12: Immunohistochemistry of FW anterior intestine using polyclonal rabbit anti-AQP11a or anti-AQP11b antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. A. AQP11a antibody fluorescing in columnar epithelial (CE) cells and circular smooth muscle tissue (CSM). B. AQP11a antibody fluorescing in columnar epithelial cells. C. AQP11a antibody fluorescing in longitudinal smooth muscle (LSM) and circular smooth muscle. D. AQP11a antibody fluorescing in cells in villus lamina propria (LP). E. AQP11a antibody lightly fluorescing in the luminal side of columnar epithelium cells and in circular muscle tissue. F. AQP11b antibody lightly fluorescing in the luminal side of columnar epithelium cells.
Figure 3.13: Immunohistochemistry of FW and SW posterior intestine using polyclonal rabbit anti-AQP11a and anti-AQP11b antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. A. AQP11a antibody fluorescing in SW serosa and in transverse cut smooth muscle (TSM). B. AQP11a antibody fluorescing faintly in SW tissue on the luminal side of columnar epithelial (CE) cells. C. AQP11b antibody fluorescing in SW tissue in circular smooth muscle (CSM) and in blood vessels (BV), and lightly on the luminal side of columnar epithelial cells. D. AQP11b antibody fluorescing in SW tissue in transversely cut smooth muscle (TSM). E. AQP11a antibody fluorescing in FW tissue lightly in the luminal side of columnar epithelial cells, and lightly in circular smooth muscle and serosa (S). F. AQP11b antibody fluorescing in FW tissue lightly in the lamina propria and smooth muscle.
Figure 3.14: Immunohistochemistry of SW and FW rectal tissue using either polyclonal rabbit anti-AQP11a or anti-AQP11b antibodies. Secondary anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) Stain AQP11. DAPI (blue) counterstains the nuclei of cells. A. Peptide-blocking of AQP11a in SW rectum showing columnar epithelium (CE), lamina propria (LP), and circular smooth muscle (CSM). B. AQP11a antibody lightly staining the luminal side of columnar epithelial cells, circular smooth muscle, and longitudinal smooth muscle (LSM) cells in FW rectum. C. AQP11a antibody heavily fluorescing in the luminal side of columnar epithelial cells in SW rectum. D. AQP11a antibody fluorescing in smooth muscle in SW rectum. E. Peptide-blocking of AQP11b in SW rectum. F. AQP11b antibody fluorescing in transverse smooth muscle (TSM) in SW rectum lightly in circular smooth muscle but not in serosa (S). G. AQP11b fluorescing in smooth muscle, lamina propria, and the luminal side of columnar epithelial cells in SW rectum. H. AQP11b fluorescing in smooth muscle in FW rectum.
Figure 3.15: Immunohistochemistry of SW Kidney using polyclonal rabbit anti-AQP11a or anti-AQP11b antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. A. AQP11a peptide-block incubated showing renal tubules (T) and glomeruli (G). B. AQP11b peptide-block control incubated with only the secondary antibody showing renal tubules. C. AQP11b antibody fluorescing in columnar epithelial cells of renal tubules. D. AQP11b antibody fluorescing in columnar epithelium cells of renal tubules. E. AQP11a antibody fluorescing in tissue between tubules and auto-fluorescence in red blood cells (RBC) inside a glomerulus.
Figure 3.16: Immunohistochemistry of FW Kidney using polyclonal rabbit anti-AQP11a (A) or anti-AQP11b (B) antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. A. AQP11a antibody fluorescence not present in FW renal tubules. B. AQP11b antibody fluorescence not present in FW renal tubules or in the glomerulus.
Figure 3.17: Immunohistochemistry of SW liver using polyclonal rabbit anti-AQP11a or anti-AQP11b antibodies and anti-rabbit IgG antibodies with Alexa 488 fluorescent tag (green) attached. DAPI (blue) counterstains the nuclei of cells. A. Negative control incubated with only the secondary antibody showing smooth muscle (SM) surrounding the liver. B. AQP11a peptide-block in SW liver showing red blood cell (RBC) auto-fluorescence in a portal vein (PV). C. AQP11b antibody fluorescence present in endothelium of liver arteries (AT) but not in the portal veins; red blood cells display auto-fluorescence. D. AQP11a staining smooth muscle surrounding the liver. E. AQP11b antibody fluorescence present around bile ducts (BD) and surrounding tissue. F. AQP11a antibody shows limited fluorescence in liver tissue auto-fluorescence of red blood cells in blood vessels (BV) is present.
REFERENCES


