Published in final edited form as:

Comp Biochem Physiol A Mol Integr Physiol. 2012 March ; 161(3): 320–326. doi:10.1016/j.cbpa.2011.11.014.

Expression of aquaporin 3 in gills of the Atlantic killifish (*Fundulus heteroclitus*): Effects of seawater acclimation

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Abstract

Estuarine fish, such as the Atlantic killifish (*Fundulus heteroclitus*), are constantly and rapidly exposed to changes in salinity. Although ion transport in killifish gills during acclimation to increased salinity has been studied extensively, no studies have examined the role of aquaglyceroporin 3 (AQP3), a water, glycerol, urea, and ammonia transporter, during acclimation to increased salinity in this sentinel environmental model organism. The goal of this study was to test the hypothesis that transfer from freshwater to seawater decreases AQP3 gene and protein expression in the gill of killifish. Transfer from freshwater to seawater decreased AQP3 mRNA in the gill after 1 day, but had no effect on total gill AQP3 protein abundance as determined by western blot. Quantitative confocal immunocytochemistry confirmed western blot studies that transfer from freshwater to seawater did not change total AQP3 abundance in the gill; however, immunocytochemistry revealed that the amount of AQP3 in pillar cells of secondary lamellae decreased in seawater fish, whereas the amount of AQP3 in mitochondrion rich cells (MRC) in primary filaments of the gill increased in seawater fish. This response of AQP3 expression is unique to killifish compared to other teleosts. Although the role of AQP3 in the gill of killifish has not been completely elucidated, these results suggest that AQP3 may play an important role in the ability of killifish to acclimate to increased salinity.

Keywords

Fundulus heteroclitus; aquaporin 3; osmoregulation; seawater acclimation; ammonia/ammonium

1. Introduction

The Atlantic killifish, or mummichog (*Fundulus heteroclitus*), is a euryhaline teleost that is distributed in estuaries throughout the eastern coast of the North American continent (Shute, 1980). Killifish are widely used as an environmental, physiological, and toxicological model organism (Burnett et al., 2007). As estuarine inhabitants, killifish are exposed to constant

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and rapid changes in the salinity of their external environment, and can tolerate a wide range of external salt concentrations. Therefore, killifish are a valuable model for elucidating the mechanisms of acclimation to osmotic stress, and the effects of environmental toxicants on acclimation (Burnett et al., 2007; Marshall et al., 2005; Scott et al., 2006; Shaw et al., 2010; 2007b; 2008; Stanton et al., 2006). Most of the studies examining acclimation to osmotic stress in killifish have focused on the regulation of ion transport, such as transport of Cl^- and Na^+ by the gill and opercular membrane (Flemmer et al., 2010; Marshall et al., 2005; Scott et al., 2008; Shaw et al., 2007a; 2008; Wood and Marshall 1994; Zadunaisky et al., 1995). These studies have demonstrated that in seawater, marine teleosts drink copious amounts of water, thus they must excrete the NaCl that is absorbed by the gastrointestinal tract to maintain plasma concentrations of Na^+ and Cl^- (i.e., plasma osmolality). This is achieved primarily by NaCl secretion by mitochondrion rich cells (MRC) located in the gill (Evans, 1987; Evans et al., 2005; Hwang et al., 2011; Marshall and Singer, 2002). Considerably less is known about the transport of water and non-ionic solutes by teleost gill.

Recent studies have demonstrated that aquaglyceroporin 3 (AQP3), a channel that is permeable to water, glycerol, urea and ammonia/ammonium, is expressed in gills of several teleosts (Deane and Woo, 2006; Giffard-Mena et al., 2007; Hamdi et al., 2009; Lignot et al., 2002; Martinez et al., 2005; Tipsmark et al., 2010; Watanabe et al., 2005). These studies have shown that transfer of fish from freshwater to seawater dramatically reduced (80~97% after 2 days) AQP3 mRNA levels in sea-bass (Giffard-Mena et al., 2007), European eel (Cutler and Cramb, 2002), Japanese eel (Tse et al., 2006), and Atlantic salmon (Tipsmark et al., 2010). Conversely, transfer of killifish from seawater to freshwater dramatically increased AQP3 mRNA levels (Whitehead et al., 2010). Congruently, AQP3 protein levels also decreased in most, but not all, fish moved from freshwater to seawater (Watanabe et al., 2005). However, the magnitude of the decrease was much lower than the decrease in AQP3 mRNAs (60~87%) (Deane and Woo, 2006; Lignot et al., 2002; Tse et al., 2006; Watanabe et al., 2005). However, the role of AQP3 during acclimation to increased salinity in killifish gill has not been reported. Thus, we examined AQP3 gene and protein expression in response to transfer from freshwater to seawater in the gill of killifish.

Aquaglyceroporins, including AQP3, 7, 9 and 10, are integral membrane proteins that passively transport water, small neutral solutes, including glycerol and urea, as well as ammonia (Ishibashi et al., 1997; Ishibashi et al., 1994; Kuriyama et al., 1997; Wu and Beitz, 2007) and metalloids including arsenic (Bhattacharjee et al., 2008; Hamdi et al., 2009; Rosen and Liu, 2009). Although the function of AQP3 in the gill of teleosts has not been established, several groups have suggested that AQP3 plays a role in water and ammonia transport across the gill (Cutler et al., 2007; Wilkie, 2002), and cell volume regulation by modulating intracellular levels of urea and glycerol (Lamitina et al., 2004). Because nothing is known about AQP3 in killifish the goal of this study was to test the hypothesis that transfer from freshwater to seawater decreases AQP3 gene and protein expression in the gill of killifish. We developed a polyclonal antibody to killifish AQP3 and tested this hypothesis by measuring mRNA and protein abundance. We also examined the cellular expression of AQP3 by quantitative confocal immunocytochemistry in gills of killifish transferred from freshwater to seawater.

2. Methods

2.1. Animals

Killifish were caught from Northeast Creek, Salisbury Cove, ME, USA and were kept in 15-17 °C water at Mount Desert Island Biological Laboratory (MDIBL). Some fish were moved to Dartmouth Medical School (DMS) for maintenance past the summer season. In both facilities, fish were kept in 100% seawater (natural seawater at MDIBL and artificial

seawater (Aquarium Systems Inc., Mentor, OH, USA) at DMS, 27‰) or kept in 10% seawater for at least two weeks (3‰) prior to transfer to freshwater (100 μM NaHCO_3 , 50 μM CaSO_4 , 50 μM MgSO_4 , 20 μM KCl (American Society for Testing and Materials, 1985)) for step-down acclimation. Fish were acclimated for at least two weeks in freshwater before experimental use, as described previously by several laboratories (Genz and Grosell, 2011; Marshall et al., 1999b; Scott et al., 2004; Wood and Grosell, 2009). Results were similar at both test sites, and results were similar in fish acclimated to freshwater for two weeks and one month before transfer to seawater. Fish were fed commercial flake food daily (Tetracichlid, Tetra, Blacksburg, VA, USA) and kept under natural light (MDIBL) or 15:9 (light: Dark) cycle (DMS). All fish care and experimental protocols were in accordance with the Institutional Animal Care and Use Guidelines of DMS (10-03-03) and MDIBL (10-01).

2.2. Quantitative real-time PCR

Fish acclimated to freshwater were moved to 100% seawater and sacrificed at 1 h, 1 day, 2 days, 7 days, or 14 days. A subset of freshwater fish were moved to freshwater and kept under the same condition throughout the experiment (freshwater control). Subsequently, gills were removed and stored in RNeasy Lysis Buffer (Qiagen, Austin, TX, USA) at -20°C . Total RNA was isolated with the Qiagen RNeasy Minikit (Valencia, CA, USA) and RNA concentration ($\text{ng}/\mu\text{L}$), and purity (260/280) were measured using the Nano Drop 1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA). Acceptable samples had RNA concentrations of $>105\text{ ng}/\mu\text{L}$, a 260/280 ratio of >2 and a 230/280 ratio of >0.75 . On the Bioanalyzer, each sample had to exhibit 18S and 28S peaks without significant degradation. cDNA was synthesized by Promega Reverse Transcription System (San Luis Obispo, CA, USA).

AQP3 gene expression was determined by quantitative-polymerase chain reaction (Q-PCR) in a Stratagene MX 4000 using SYBR Green (Stratagene, Cedar Creek, TX, USA) and killifish specific AQP3 primers (KF AQP3 F1: CTCCAAATCTCACCAGCC, KF AQP3 R1: CAGCAGTGAAGAATCCC, 458bp). The primers were designed from the kFAQP3 sequence obtained from the killifish contig dataset that was determined by 454 pyrosequencing of the killifish transcriptome (JS Shaw, JK Colbourne- Indiana University, TH Hampton, CY Chen, BA Stanton- Dartmouth Medical School). A serial dilution to develop a standard curve with kFAQP3 cDNA at 10 fold, 100 fold and 1000 fold was done using freshwater AQP3 cDNA on every Q-PCR plate. The cycling conditions were segment 1- 1 cycle for 15 min at 95°C , and segment 2- 40 cycles (30 s at 95°C and 1 min at 60°C). We evaluated the dissociation curve for AQP3 to confirm the product of a single amplicon and sequenced the amplicon to confirm the sequence. All samples were run in triplicate.

2.3. kFAQP3 antibody production and verification

In preliminary studies we tested commercially available AQP3 antibodies to determine if they detected kFAQP3 but found none that detected kFAQP3 as determined by Western blot of HEK293T cells expressing killifish AQP3 by transfection. Thus, we developed a custom antibody. During the antibody design process we compared the sequences of all known teleost aquaglyceroporins/aquaporins, as well as killifish aquaglyceroporins/aquaporins identified by 454 sequencing of the killifish transcriptome. Moreover, we also identified the N terminal sequence of kFAQP3 (MGRQKIYLDKLAR) as a unique sequence in the killifish transcriptome. As a result, an affinity purified, polyclonal antibody that recognizes the above-mentioned peptide was produced by New England Peptides (Gardner, MA, USA, IACUC registration 50-R-0013).

The specificity of the antibody was tested by transfecting HEK293T cells with kfAQP3, kfAQP7 or kfAQP9 followed by western blot analysis (See Results). Briefly, cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 µg/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine, and kept in a 5% CO₂-95% air incubator at 37 °C. Cells were transfected with an increasing amount of kfAQP3 cDNA using Effectene (Qiagen) according to the manufacturer's instructions. 48 h after transfection, cells were lysed with a lysis buffer containing 25mM Hepes, 1% v/v Triton X-100, 10% v/v glycerol, and Complete Protease Inhibitor Mixture (Roche Diagnostics, Indianapolis, IN, USA). After centrifugation for 15 min at 15,000 g at 4 °C, supernatant was mixed 1:1 with sample buffer (Bio-Rad, Hercules, CA, USA) containing 80 mM dithiothreitol (DTT) and heated for 5 min at 85 °C. 50 µg of protein was loaded onto 12% SDS-Page gels (Bio-Rad) and separated by electrophoresis. Proteins were transferred to PVDE membranes and probed with the kfAQP3 antibody (1:3000), then with a horseradish-conjugated goat anti-rabbit secondary antibody (Bio-Rad). The bands were visualized with WesternLightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Waltham, MA, USA) or Western Femto (Thermo Scientific, Rockford, IL, USA). The same blot was also probed with a β-actin antibody (1:1000, MP Biomedicals, Solon, OH, USA) for normalization, and normalized pixel counts relative to the plasmid-transfected group were plotted for quantification. We used antibody preincubated with 5 µg of antigenic peptide (2 h at 30 °C) to confirm the specificity of the antibody. Since we were concerned with possible contamination of the gill samples by red blood cells, we probed proteins from red blood cells with our antibody and did not observe any band at the predicted size.

Once we demonstrated the specificity of the kfAQP3 antibody, western blot analysis was performed to measure AQP3 levels in gill lysates from fish acclimated to freshwater and from fish exposed to seawater for 0 h, 1 h, 1 day, 2 days, 7 days, and 14 days. Fish gill tissue was homogenized in lysis buffer (described above) using a hand-held homogenizer. Lysed tissue was processed for western blot following the protocol described above.

2.4. Immunocytochemistry

To determine the cellular localization of kfAQP3, gills from four freshwater-acclimated fish and four seawater-acclimated fish were isolated and processed for immunocytochemical staining following a protocol modified from Loretz et al. (2009). Briefly, gills were incubated overnight at 4 °C in Bouin's solution. Tissues were rinsed with 70% ethanol for 48 h to clear fixatives, and then dehydrated with graded ethanol and xylene for paraffin embedding. Embedded tissue was cut into 5µm-thick slides. Slides were rehydrated by sequential transfer through xylene and graded ethanol. Rehydrated tissues were immersed in PBS, and blocked with a solution containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1.5 h.

Tissue sections were then incubated for 2 h at room temperature in kfAQP3 primary antibody-1:100 diluted in 0.5% BSA with 0.1% Triton X-100. Tissue sections were also incubated with a monoclonal antibody to Na⁺-K⁺ ATPase α5 (5mg/mL, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) to identify mitochondrion rich cells (MRCs) (Kato and Kaneko, 2003; Wilson and Laurent, 2002; Wilson et al., 2000). Additional tissue sections were incubated with antibody pre-incubated with immunizing peptide (control). After washing with PBS, slides were incubated in the dark for 1 h in 1:1000 Alexa-488 goat anti-rabbit secondary antibody and 1:1000 Alexa-568 goat anti-mouse secondary antibody (Molecular Probes, Carlsbad, CA, USA). After washing, slides were mounted in anti-bleaching medium and observed with a Nikon swept field confocal microscope (40X 0.75 NA objective). For each individual fish, five to six tissue sections were used for analysis. Images were obtained under the identical illumination condition for all slides. Pixel intensities were quantified using the NIS-Element software

(Nikon, Inc., ver. 3.1). All identifiable cells were outlined and the mean intensity for kfAQP3 and area for each cell were recorded.

2.5. Statistics

All statistics were performed using Prism 5 (GraphPad Software, San Diego, CA, USA). Q-PCR and protein expression were analyzed using one-way analysis of variance (ANOVA). When ANOVA yielded significance ($p < 0.05$), Tukey's multiple comparison test was used for *post hoc* pair-wise comparisons. Student's *t* test was conducted for analysis of immunocytochemistry data. Statistical significance was accepted at $p < 0.05$. Data are expressed as mean $\pm$ standard error of means.

3. Results

3.1. Expression of kfAQP3 mRNA decreases in response to an increase in salinity

To determine if the expression of kfAQP3 in the gill of killifish is influenced by changes in the salinity, fish acclimated to freshwater were moved to 100% seawater for 1 h, 1 day, 2 days, 7 days, and 14 days, and kfAQP3 mRNA levels were measured by Q-PCR (Fig 1). Levels of mRNA were unchanged one hour after fish were transferred to seawater; however, kfAQP3 mRNA levels decreased significantly one day after transfer (29.0 ± 7.4 % of freshwater-acclimated fish values) and remained decreased throughout the duration of the 14-day experiment. By day 14 after the transfer to seawater kfAQP3 mRNA decreased to 4.6 ± 1.6 % of the value measured in freshwater-acclimated fish.

3.2. kfAQP3 protein levels do not change in response to an increase in salinity

The next set of studies was conducted to determine if an increase in salinity also decreased kfAQP3 protein expression. First, we tested the specificity of the kfAQP antibody that we designed. HEK293T cells were transfected with a gradient of kfAQP3 cDNA and western blots of cell lysates were probed with the kfAQP3 antibody (Fig 2a and 2b). A protein of ~28 kDa increased as a function of the amount of kfAQP3 cDNA transfected into the HEK293T cells. This is slightly smaller than the size predicted from the amino acid sequence (33 kDa), but other teleost AQP3s run about 28 kDa in western blot studies (Lignot et al., 2002). Pre-incubation of the antibody with the peptide that the antibody was raised against eliminated the 28 kDa signal (data not shown). It is also notable that the intensity of the non-specific bands was similar in all samples (Fig 2a). In addition, to examine the specificity of the antibody HEK293T cells were transfected with kfAQP3, kfAQP7, or kfAQP9 and western blots were probed with the kfAQP3 antibody. The kfAQP3 antibody only recognized kfAQP3 (Fig 2c).

The kfAQP3 polyclonal antibody was used in the next set of studies to determine if an increase in salinity decreased kfAQP3 protein abundance. To this end kfAQP3 protein levels in killifish gills were measured by western blot after fish were transferred from freshwater to seawater (1 h, 1 day, 2 days, 7 days, and 14 days) (Fig 3). Although there was a small decrease in kfAQP3 protein levels at some time points after transfer to seawater, the decreases were not statistically significant.

3.3. kfAQP3 protein is differentially localized in the gills of killifish acclimated to seawater versus freshwater

Immunocytochemical studies were conducted to examine the cellular localization and abundance of kfAQP3 protein in gills of killifish acclimated to freshwater and seawater. As shown in figure 4a, the pattern of kfAQP3 immunolocalization in the gill was dramatically different between the two groups. In freshwater acclimated fish kfAQP3 was located both in the primary filament and the secondary lamellae of the gills. Within the primary filament,

kfAQP3 (green) co-localized with Na⁺-K⁺ ATPase (red) indicating that kfAQP3 localized to MRCs. In the secondary lamellae kfAQP3 was located in pillar cells (Evans et al., 2005; Laurent and Dunel, 1980; Marshall and Grosell, 2005). In contrast, in seawater acclimated fish kfAQP3 protein was localized primarily in MRCs of the primary filament, and was barely evident in pillar cells in secondary lamellae. Overall the mean immunofluorescence intensity of kfAQP3 was similar for both groups of fish (Fig 4b). Although there was no change in the overall fluorescence intensity of kfAQP3 in the gill, the fluorescence per cell type was different in freshwater and seawater fish. kfAQP3 fluorescence intensity was significantly increased in MRCs of the primary filament (Fig 4c), and significantly decreased in pillar cells of secondary lamellae in the seawater fish compared to freshwater fish (Fig 4d). Taken together, the western blot and immunocytochemical data demonstrate that kfAQP3 abundance in gill was similar in killifish acclimated to freshwater and seawater, but that transfer from freshwater to seawater resulted in a dramatic decrease in the abundance of kfAQP3 in pillar cells, and a dramatic increase in the abundance of kfAQP3 in MRC.

4. Discussion

Killifish, a sentinel environmental organism that is used extensively to study osmoregulation, demonstrated a unique response during acclimation to seawater. We observed that upon transfer from freshwater to seawater kfAQP3 mRNA levels in the gill fell dramatically, but the total gill kfAQP3 protein abundance did not change significantly. Moreover, immunolocalization revealed that acclimation to seawater was accompanied by a dramatic change in the localization of kfAQP3 in the gill. Whereas in freshwater kfAQP3 was expressed in pillar cells in the secondary lamellae and in the MRC in the primary filaments, in seawater fish kfAQP3 abundance decreased in pillar cells and increased in MRCs.

Our data on kfAQP3 mRNA agree with studies on eel, sea bass and Atlantic salmon. In all species examined to date, transfer from freshwater to seawater elicits a dramatic fall in AQP3 mRNA (Cutler and Cramb, 2002; Giffard-Mena et al., 2007; Tipsmark et al., 2010; Tse et al., 2006). However, our result with regard to kfAQP3 protein abundance is dramatically different from studies in other fish. kfAQP3 protein abundance in the gill of killifish, measured using a polyclonal antibody that was produced to specifically recognize kfAQP3, did not decrease when freshwater fish were transferred to seawater, whereas AQP3 protein abundance decreased in silver sea bream, and both European and Japanese eel gill when these fish were transferred to seawater (Deane and Woo, 2006; Lignot et al., 2002; Tse et al., 2006). Thus, there are significant species differences in the response of AQP3 when fish are transferred from freshwater to seawater.

The discrepancy between mRNA and protein levels that we see in this study has been reported previously for other genes (de Sousa Abreu et al., 2009; Maier et al., 2009). However, it should be noted that the decreases in AQP3 protein abundance in the gill of other teleosts, including silver sea bream, and the Atlantic and Japanese eel, is quantitatively less robust (60~87%) than the fall in mRNA (80~97%) (Deane and Woo, 2006; Lignot et al., 2002; Tse et al., 2006). The observation that kfAQP3 protein abundance did not change in the killifish gill even after 14 days in seawater, even though mRNA was reduced by ~95% during this time, is unlikely to result from changes in posttranslational mechanisms that increase the half-life of kfAQP3, since the half-life of proteins, including various aquaporins, is typically hours (~20 h), not weeks (Eden et al., 2011; Hendriks et al., 2004; Leitch et al., 2001; Wang et al., 2003; Zhou et al., 2007). Moreover, two observations suggest that it is unlikely that the lack of change in kfAQP3 protein abundance was an artifact related to the use of a non-specific antibody. First, we made a killifish-specific

AQP3 antibody and demonstrated that it recognizes killifish AQP3, but not other similar killifish aquaglyceroporins including AQP7, and AQP9 (Figure 2). Second, it is unlikely that our kfAQP3 antibody recognized other killifish proteins, since our pyrosequencing of the killifish transcriptome revealed that the immunizing peptide sequence was unique to kfAQP3 within the killifish transcriptome. The most parsimonious interpretation of our data is that upon transfer from freshwater to seawater kfAQP3 mRNA and protein abundance in pillar cells in the secondary lamellae decrease in parallel, but that in MRC both kfAQP3 mRNA and protein increase. This interpretation is supported by our immunolocalization studies, and makes the assumption that most of the AQP3 mRNA in the gill is derived from pillar cells. Additional studies, beyond the scope of the present study, which require measurements of kfAQP3 mRNA in pillar and MRC cells, are required to test this hypothesis.

The immunolocalization of kfAQP3 in pillar cells in the gill is a novel observation in fish. Previous studies in the European and Japanese eel have localized AQP3 in secondary lamellae to pavement cells by immunocytochemistry, but not to pillar cells (Brunelli et al., 2010; Lignot et al., 2002; Tse et al., 2006). The differences in the immunolocalization of AQP3 in the secondary lamellae of these fish are likely to be species dependent. Previous studies that examined the protein abundance and localization of AQP3 in teleosts used models that are not exposed to rapid fluctuations in the environmental salinity, but rather are either migratory (eels) or restricted to limited salinity ranges (tilapia, sea bream, rainbow wrasse). Therefore the unique localization of AQP3 in killifish may reflect adaptation that allows them to respond to relatively rapid changes in osmolality.

The role of AQP3 in pillar cells in secondary lamellae is unknown. Secondary lamellae play an essential role in gas exchange and are formed by two sheets of epithelial cells that are held apart by pillar cells, which also form small tunnels that serve as channels for blood to perfuse the lamellae (Evans, 1987; Wilson and Laurent, 2002). Pillar cells are thought to regulate gas exchange across the secondary lamellae surface by expanding or contracting, thereby increasing or decreasing, respectively, blood flow in the secondary lamellae (Wilson and Laurent, 2002). Increased blood flow enhances gas exchange. AQP3 transports water, urea, glycerol, ammonia and even arsenic across plasma membranes in a variety of cells (Cerdà and Finn, 2010; Verkman and Mitra, 2000). The observation in the present study that transfer from freshwater to seawater decreases the abundance of kfAQP3 in pillar cells suggests that the transport of water, urea, glycerol, ammonia and arsenic across plasma membrane of pillar cells may be reduced in seawater fish. Because plasma osmolality increases significantly when killifish are transferred from freshwater to seawater (Marshall et al., 1999a; Shaw et al., 2007a; Stanton et al., 2006), the decrease in kfAQP3 abundance in pillar cells may play an important role in pillar cell osmoregulation. When cells are exposed to a hyperosmotic solution they rapidly shrink because of osmotic water loss, and then cell volume slowly returns to normal as the cells accumulate organic osmolytes, including glycerol from intracellular metabolic reactions. Because AQP3 is a passive glycerol transporter, we speculate that a decrease in AQP3 abundance will slow the passive loss of glycerol from the cell. The decrease in AQP3 abundance would allow pillar cells to maintain an elevated intracellular glycerol and therefore maintain cell volume, and thus lamellae blood flow and gas exchange in seawater acclimated fish. Additional studies, however, are required to test this hypothesis and to elucidate the role of kfAQP3 in pillar cell function.

The role of AQP3 in MRC in the primary filaments is also not clear. MRCs play an important role in salt homeostasis in freshwater and seawater acclimated fish, absorbing NaCl and calcium in freshwater fish and secreting Cl⁻ via CFTR in seawater fish (Evans et al., 2005; Marshall and Grosell, 2005; Marshall, 2003). MRC also play an important role in maintaining acid-base balance, excreting ammonium and ammonia, and excreting urea to

maintain urea homeostasis (Marshall and Grosell, 2005; Wilkie, 2002). Studies have shown that AQP3 passively transports water, urea, ammonia and ammonium (Holm et al., 2005; Litman et al., 2009), thus, it is likely, but not proven, that killifish AQP3 plays a functional role in the transport of water, urea, ammonia and ammonium by MRC. The functional consequence of the increase in kfAQP3 protein abundance in MRC in seawater fish is not clear. It is unlikely that the increase in kfAQP3 enhances water transport across MRC cells, since, at least in the European eel osmotic water permeability in the gill decreases during seawater acclimation (Evans, 1969). However, because increased salinity enhances the ammonium and ammonia permeability of the gill it should be considered that the increase in kfAQP3 may mediate, at least in part, the increase in ammonia/ammonium permeability. This proposed role of AQP3 is consistent with the observation that copper, which inhibits the water permeability of eel AQP3 (MacIver et al., 2009), increases plasma urea and ammonia levels, and also reduces ammonia efflux across the gill (Blanchard and Grosell, 2006; Grosell et al., 2004). Clearly, additional studies are required to elucidate the role of kfAQP3, and perhaps other aquaporins, in MRC function and the role of increased kfAQP3 in MRC during seawater acclimation.

In conclusion, we have developed a polyclonal antibody to kfAQP3 and have demonstrated that upon transfer from freshwater to seawater AQP3 mRNA levels in the gills of the Atlantic killifish fall dramatically, but the total gill kfAQP3 protein abundance do not change significantly. Moreover, immunolocalization revealed that acclimation to seawater was accompanied by a dramatic and novel change in the localization of kfAQP3 in the gill. Whereas in freshwater fish kfAQP3 was expressed in pillar cells in the secondary lamellae and in the MRC in the primary filaments, in seawater fish kfAQP3 abundance decreased in pillar cells and increased in MRCs. These results suggest that kfAQP3 may play important roles in acclimation to seawater: in pillar cells, the reduction in kfAQP3 abundance may play an important role in cell volume regulation and thus gas exchange across the secondary lamellae, whereas in MRC cells the increase in kfAQP3 may play an important role in ammonium and ammonia excretion, and thus in regulating acid base balance. These observations set the stage for additional studies examining the role of kfAQP3 in acclimation to seawater.

Acknowledgments

We thank Dr. David Evans for valuable discussions and Marissa Dzioba, Ashley Dzioba, Chloe Taub and Sara Stanton for their laboratory support. We also thank Dr. Qianru Yu for technical support with imaging. This publication was made possible by NIH Grant Number P42 ES007373 from the National Institute of Environmental Health Sciences, NIH grant Number P20 RR-016463 from the National Center for Research Resources, and grant Number ER1503 from DOD-SERDP.

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Figure 1. kfAQP3 mRNA expression during acclimation to seawater

Expression of kfAQP3 mRNA was determined by Q-PCR. Freshwater acclimated fish (FW) were moved to seawater (SW) and kfAQP3 mRNA was measured in gills harvested at 1h, 1 day, 2 days, 7 days, and 14 days after transfer. Data expressed as mean $\pm$ standard error of means. * $p < 0.05$ compared to freshwater (FW). N= 5 or 6 per group.

Figure 2. Verification of the kfAQP3 antibody

A, B: To verify the specificity of the kfAQP3 antibody, HEK293T were transfected with kfAQP3a cDNA at the concentrations indicated and kfAQP3 abundance was determined by western blot analysis. (A) Representative experiment. (B) Summary of three experiments. None of the non-specific bands were different among the different samples. * $p < 0.01$ compared to control. N=3 per group. C: In addition, cells were transfected with 1.0 µg of either kfAQP3, kfAQP7, or kfAQP9 and processed for western blot analysis. A protein of the right size was only identified in cells transfected with kfAQP3.

Figure 3. kfAQP3 protein abundance during acclimation to seawater

Killifish acclimated to freshwater (FW) were moved to seawater (SW) and kfAQP3 protein abundance in the gill was measured by western blot at 1h, 1 day, 2 days, 7 days, and 14 days post-transfer (a). There was no significant difference among the different treatment groups ($p = 0.25$). $N = 8$ per time point. A representative blot of kfAQP3 and β -actin, as loading control, is shown in b.

Figure 4. kfAQP3 localization in gills of fish acclimated to freshwater or seawater

Gills from killifish acclimated to either freshwater (FW) or seawater (SW) were isolated and fixed and probed with an anti-kfAQP3 antibody (green) and subsequently viewed by immunofluorescence confocal microscopy. Tissue was also labeled with an anti- $\text{Na}^+\text{-K}^+$ ATPase antibody (red) to identify MRC. (A) Representative images. Background refers to tissue probed with antibody pre-incubated with immunizing peptide. Arrowheads indicate MRC in the primary filament and arrow indicates secondary lamellae. Scale bar is equal to 50 μm . (B) Quantification of the mean fluorescence intensity of the gill tissue in FW and SW fish. (C, D) Single cell quantification of kfAQP3 in primary filament (C) and secondary lamellae (D). Data are presented as mean $\pm$ SEM of the fluorescence intensity (arbitrary units). $N = 4$ per treatment. * $p < 0.001$