Published in final edited form as:

Comp Biochem Physiol C Toxicol Pharmacol. 2015 May ; 171: 1–7. doi:10.1016/j.cbpc.2015.03.001.

A novel variant of aquaporin 3 is expressed in killifish (*Fundulus heteroclitus*) intestine

Dawoon Jung^{a,b,1}, Meredith A. Adamo^b, Rebecca M. Lehman^b, Roxanna Barnaby^a, Craig E. Jackson^d, Brian P. Jackson^c, Joseph R. Shaw^{b,d,e}, and Bruce A. Stanton^{a,b}

^aDepartment of Microbiology and Immunology and of Physiology, The Geisel School of Medicine at Dartmouth, Hanover, New Hampshire 03755

^bMt. Desert Island Biological Laboratory, Salisbury Cove, Maine 04672

^cDepartment of Earth Sciences and Chemistry, Dartmouth College, Hanover, New Hampshire 03755

^dThe School of Public and Environmental Affairs, Indiana University, Bloomington, Indiana 47405

^eSchool of Biosciences, University of Birmingham, Birmingham, UK B15 2TT

Abstract

Killifish (*Fundulus heteroclitus*) are euryhaline teleosts that are widely used in environmental and toxicological studies, and they are tolerant to arsenic, in part due to very low assimilation of arsenic from the environment. The mechanism of arsenic uptake by the intestine, a major route of arsenic uptake in humans is unknown. Thus, the goal of this study was to determine if aquaglyceroporins (AQP), which transport water and other small molecules including arsenite across cell membranes, are expressed in the killifish intestine, and whether AQP expression is affected by osmotic stress. Through RT-PCR and sequence analysis of PCR amplicons, we demonstrated that the intestine expresses kfAQP3a and kfAQP3b, two previously identified variants, and also identified a novel variant of killifish AQP3 (kfAQP3c) in the intestine. The variants likely represent alternate splice forms. A BLAST search of the *F. heteroclitus* reference genome revealed that the AQP3 gene resides on a single locus, while an alignment of the AQP3 sequence among 384 individuals from eight population ranging from Rhode Island to North Carolina revealed that its coding sequence was remarkably conserved with no fixed polymorphism residing in the region that distinguishes these variants. We further demonstrate that the novel variant transports arsenite into HEK293T cells. Whereas kfAQP3a, which does not transport arsenite, was expressed in both freshwater (FW) and saltwater (SW) acclimated fish, kfAQP3b, an arsenic transporter, was expressed only in FW acclimated fish, and kfAQP3c was expressed only

2015 Published by Elsevier Inc.

Corresponding author: Dawoon Jung, Ph.D., Dawoon.Jung@Dartmouth.Edu, The Geisel School of Medicine at Dartmouth Medical School, HB 7701, Hanover, NH 03755, USA, Phone: 1-603-650-1534.

¹Current Address: School of Public Health, Seoul National University, Seoul 151-742, Republic of Korea

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

in SW acclimated fish. Thus, we have identified a novel, putative splice variant of kfAQP3, kfAQP3c, which transports arsenic and is expressed only in SW acclimated fish.

Keywords

Fundulus heteroclitus; aquaglyceroporin; arsenite uptake; intestine; salinity

1. Introduction

Arsenic is a naturally occurring metalloid that is the number one substance of concern in the priority list of hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR 2011). Globally, at least 500 million people are exposed to arsenic in their drinking water. In addition, recent studies have indicated that rice-based food products are a major source of arsenic exposure (Jackson et al., 2012). Chronic exposure to arsenic leads to a number of diseases including cancer of the skin, bladder, kidney and liver; diabetes; cardiovascular and lung diseases (Abernathy et al., 2003; Hughes et al., 2011). In teleosts, exposure to sublethal concentrations of arsenic has been linked to reduced growth, attenuated stress response, and increased oxidative stress (Bears et al., 2006; Eisler, 1988; Erickson et al., 2010; Ventura-Lima et al., 2009).

The Atlantic killifish (*Fundulus heteroclitus*) is an estuarine fish that is widely distributed throughout the coast of the eastern United States. Because killifish readily acclimate to sudden and extreme changes in salinity, this organism has been extensively used to study osmoregulation in teleosts (Burnett et al., 2007; Wood and Marshall, 1994). Interestingly, although high levels of arsenic interfere with osmoregulation in killifish (Shaw et al., 2007a), studies have shown that killifish are highly tolerant to waterborne arsenic exposure. Notably, killifish are able to survive exposure to very high concentrations of arsenic (Shaw et al., 2007b), and accumulation of arsenic from water is dramatically lower compared to accumulation of other metals (Dutton and Fisher, 2011b; Dutton and Fisher, 2014).

Although arsenic accumulation is greater in fish acclimated to seawater (SW) compared to accumulation by fish acclimated to freshwater (FW) (Dutton and Fisher, 2011b) the reason for this is unknown, as is the reason why overall accumulation of arsenic is low in killifish.

Arsenic uptake into cells is achieved by two major routes. Transport of organic and inorganic arsenate (As^{5+}) occurs by sodium/phosphate cotransporter (Ballatori, 2002; Huang and Lee, 1996). Transport of organic and inorganic arsenite (As^{3+}) into cells is primarily mediated by aquaglyceroporins (AQPs), which also transport water, urea and glycerol (Bhattacharjee et al., 2008; Lee et al., 2006). AQP3, AQP7, AQP9, and AQP10 have been identified in teleosts (Cerdà and Finn, 2010) and have been shown to play an important role in cellular arsenic uptake in zebrafish (Hamdi et al., 2009). The expression of kfAQP3 in the gill is salinity dependent reflecting its role in maintaining osmotic balance (Shaw et al., 2014; Whitehead et al., 2011). In a previous study we identified a novel AQP3 expressed in killifish gill (kfAQP3a), which is expressed in FW acclimated fish and does not transport arsenic (Jung et al., 2012a). In fact, kfAQP3a is the only known AQP3 that does not transport arsenic. Because kfAQP3a was the only aquaporin identified in killifish gill, this

observation supported the conclusion that the gill is not a route of arsenite assimilation in the killifish (Jung et al., 2012a).

In the present study we conducted experiments to identify AQP transporters in killifish intestine since it is well known that arsenic uptake by the intestine is a major pathway for arsenic assimilation in several species including humans (Calatayud et al., 2011; Dutton and Fisher, 2011a; Erickson et al., 2011; WHO, 2001). We hypothesized that arsenite uptake in killifish intestine is mediated in part by AQP3, and that transfer from FW to SW may affect AQP3 expression. To these ends we used quantitative real-time PCR (qRT-PCR) and Western blot to identify APQs in killifish intestine, and a functional assay to examine arsenite transport. We found two variants of killifish AQP3 that we previously identified (kfAQP3a, kfAQP3b) as well as a novel variant, kfAQP3c, which we demonstrate is likely a splice variant and an arsenic transporter. We further demonstrate that the expression of the kfAQP3 arsenic transporters 3b and 3c, is salinity dependent with kfAQP3b expressed only in FW fish and kfAQP3c only expressed in SW fish.

2. Materials and Methods

2.1. Fish care

Fish were caught in Northeast Creek, Salisbury Cove, ME and transferred to Mount Desert Island Biological Laboratory (MDIBL) or the Geisel School of Medicine at Dartmouth (GSM). Fish were either kept in 100% seawater (SW, natural seawater at MDIBL and artificial seawater (Aquarium Systems Inc., Mentor, OH) at GSM) or kept in 10% seawater for two weeks, then moved to freshwater (FW, 100 μ M NaHCO₃, 50 μ M CaSO₄, 50 μ M MgSO₄, 20 μ M KCl) (ASTM, 1985). Results from fish studied at MDIBL and GSM were identical, thus, the data were pooled. Fish were acclimated in each salinity condition for at least one month before experimental use. Fish were fed commercial flake food (Tetracichlid, Tetra, Blacksburg, VA) that had minimal arsenic concentration, as reported previously (Shaw et al., 2010). Fish were kept under natural light (MDIBL) or 15:9 (light: dark) cycle (GSM). All fish care and experimental protocols were in accordance with the Institutional Animal Care and Use Guidelines of GSM (stan.ba.1) and MDIBL (13-01).

2.2. kfAQP3 sequence verification of amplicons

Sequence analysis of the amplicons derived by RT-PCR of AQP3 identified a variant of kfAQP3 (kfAQP3a) in killifish gill that differed from our transcriptome database (kfAQP3b) in the three C-terminal amino acids (Jung et al., 2012a; Shaw et al., 2014). To examine the expression of these AQP3 variants in killifish intestine, intestines were isolated from nine fish acclimated to FW and nine fish acclimated to SW and stored in RNA later (Ambion, Austin, TX) at -20°C. Total RNA was isolated using Qiagen RNeasy Minikit (Valencia, CA) according to the manufacturer's protocol. The quality and quantity of the isolated RNA were measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Only samples that exhibited 18S and 28S peaks without significant degradation were further processed for cDNA synthesis. cDNA was synthesized using Ambion Reverse Transcription System (San Luis Obispo, CA) from 1 μ g RNA. PCR analysis was performed with primers that span nucleotides that code for the three C-terminal amino acids of kfAQP3 (5'-3':

GGTTTCCATGTGGAAGGAGA, 3'-5': TGCCCTCATGACTAGCCTTT). Specifically, the primers were designed to include a 150 base pair (bp) target sequence that spans the 38 terminal amino acids, the stop codon, and 33 bp 3' to the stop codon. The conditions for PCR reaction was as follows; segment 1- 1 cycle for 2 min at 95°C, segment 2- 35 cycles (45s at 95°C, 30s at 58°C, and 1 min at 72°C), segment 3- 10 min at 72°C. PCR products were run on 2.0% agarose gel to confirm the product of a single amplicon. Products were then cloned into PCR 4.0-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into DH5 α -T1 cells according to the manufacturer's protocol (Invitrogen). Transformation products were incubated overnight at 37°C on LB plates containing 50 μ g/mL ampicillin. At least twenty colonies from each plate (i.e. each individual fish from each condition) were picked, cultured overnight in LB medium containing 50 μ g/mL ampicillin. Plasmids were isolated with QIAprep Miniprep kit (Qiagen), and sequenced using ABI Big Dye technology with the ABI Model 3100 Genetic Analyzer at the GSM Molecular Biology Core using M13 reverse primer. Chromatograms of each sequencing result were visually inspected and checked with MacVector software with Phred for base calling (MacVector Inc., Cary, NC), and any sequence data with high background noise was excluded from further analysis.

2.3. Arsenic transport assay

Arsenic transport by kfAQP3 was measured in HEK293T cells transfected with kfAQP3a, kfAQP3b, or kfAQP3c. Vectors containing full-length kfAQP3a, 3b, and 3c were constructed as described previously. Briefly, full length AQP3a was cloned into TopoTA pCR2.1 vector (Invitrogen, from the gill of killifish acclimated to SW and sequence was verified by Sanger sequencing (Jung et al 2012a). Next, the 3'- terminus of the cloned kfAQP3a was mutated using the forward primer (5'- CTCCAAATCTCACCAGCC-3') and reverse primer (5'- CCTTTCTGCGCCTCTTTTTTAGCAGTTAGCCTCTTTGCCGTTGG-3') to match kfAQP3b, and forward primer (5'- CCAACGGCAAAGAGGGTAATTGCTAAAAAAGAGGCGCAGAA -3') and reverse primer (5'-TTCTGCGCCTCTTTTTTAGCAATTACCCTCTTTGCCGTTGG- 3') to match kfAQP3c, respectively. Mutated clones were sequenced for verification by Sanger sequencing. Once verified, full length cDNAs were cut from the vectors and inserted into pcDNA3.1(-) expression vectors (Invitrogen) by standard molecular cloning techniques.

HEK293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μ g/mL penicillin, 50 μ g/mL streptomycin, and 2 mM L-glutamine in a 5% CO₂-95% air incubator at 37°C, and transfected with 1.0 μ g of empty vector, kfAQP3a, or kfAQP3c in pcDNA3.1(-) using Effectene (Qiagen) as described previously (n=3 per treatment) (Jung et al., 2012a). Efficiency of transfection was validated by checking the expression of AQP3 mRNA with qRT-PCR using primers and conditions described in section 2.6. For each experimental treatment, 200,000 cells were plated in each well of a 6-well plate. At 48h after transfection, cells were cultured in serum-free media containing 2 μ g/L (2ppb) sodium arsenite. After 1h, cells were washed twice with PBS, detached from the plates, transferred into pre-weighed centrifuge tubes, and pelleted by centrifugation (600 $\times$ g for 10 min). Tubes were weighed again to calculate the mass of the cells. The concentration of arsenic and treatment time was chosen after preliminary

experiments identified the conditions where arsenic uptake was linear and was dependent on the expression of kfAQP3b or kfAQP3c. Cells were resuspended in 100 μ L of optima grade HNO_3 (Fisher Scientific, Pittsburg, PA) and placed in a laboratory oven at 70°C for 2h. The samples were then cooled and 50 μ L of H_2O_2 was added to each sample after which samples were exposed to another heating cycle. The digested samples were weighed and analyzed for total arsenic by inductively coupled plasma mass spectrometry (7700 $\times$, Agilent, Santa Clara, CA) using He as a collision gas (Shaw, et al., 2007b). Data are reported as μ g of total arsenic/kg of wet weight.

2.4. Arsenic uptake measurement

To measure the effect of salinity on arsenic uptake, fish acclimated to either FW or SW were moved to dosing tanks containing 10 L of aerated water and dosed with either 1000 μ g/L or 0 μ g/L sodium arsenite (n= 10 to 12 per treatment). After 48h, intestines were removed from the fish, washed with PBS, and kept at -20°C until further processing. Intestine samples were digested as wet weight samples using 250 μ L of HNO_3 at 90°C for 20 minutes followed by addition of 25 μ L H_2O_2 and additional heating at 90°C for 2 minutes. The average sample wet weight was 40 mg. Dorm3 was used as a reference material and digested by the same method using 20 mg. A fortified blank (n=3) at 4 μ g/L was also taken through the digestion process. All digested samples were brought up to 5 ml with deionized water and final digestion weight was recorded. All digestion measurements were recorded gravimetrically. Arsenic was analyzed by inductively coupled plasma mass spectrometry (ICP-MS, 7700 $\times$ Agilent, Santa Clara, CA) using He as a collision gas. Average recovery of DORM3 was 95.2 $\pm$ 8.0% (n=3) and average recovery of the laboratory fortified blank was 101 $\pm$ 10%. The average relative percent difference of five analysis duplicates was 6.2 $\pm$ 4.0% and average recovery of a 1.8 μ g/L sample spike was 95.0 $\pm$ 13.0 %.

2.5. Protein isolation and Western blot

Western blot was run on killifish acclimated to FW or SW to measure the amount of AQP3 protein expressed in the intestines. Intestines from fish acclimated to FW or SW (n=6 each) were isolated, washed in phosphate buffered saline (PBS) and lysed with a hand-held homogenizer in lysis buffer containing 25 mM Hepes, 1% v/v Triton X-100, 10% v/v glycerol, and Complete Protease Inhibitor Mixture (Roche Diagnostics, Indianapolis, IN). Samples were centrifuged for 15 min at 15,000 g at 4°C, then supernatant was pipetted to a new vial and an equal volume of sample buffer (Bio-Rad, Hercules, CA) containing 80 mM dithiothreitol (DTT) was added. Samples were heated for 5 min at 85°C before electrophoresis where 50 μ g of protein was loaded onto 4-20% SDS-Page gels (Bio-Rad) and separated. The separated proteins were transferred to PVDE membranes and probed with the kfAQP3 antibody (1:3000) (Jung et al., 2012b), then with horseradish-conjugated goat anti-rabbit secondary antibody (Bio-Rad). The bands were visualized with WesternLightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Waltham, MA) or Western Femto (Thermo Scientific, Rockford, IL). The same blot was also probed with a β -actin antibody (1:1000, MP Biomedicals, Solon, OH) followed by horseradish-conjugated goat anti-mouse secondary antibody (Bio-Rad) for normalization.

2.6. RNA isolation and quantitative real-time PCR

To measure the relative abundance of AQP mRNAs, we performed qRT-PCR on mRNA isolated from killifish intestine. Intestines from fish acclimated to FW (n=6) or SW (n=6) were isolated, washed in phosphate buffered saline (PBS) and stored in RNA later (Ambion, Austin, TX) at -20°C. Total RNA was isolated and cDNA was synthesized as described above. Synthesized cDNA was diluted to 100 ng/μL. 1 μL of the diluted cDNA was mixed with 50 nM primer, 12.5 μL SYBR Green Master Mix (Stratagene, Cedar Creek, TX), and nuclease-free water to make a 25 μL reaction mix. qRT-PCR was performed using ABI PRISM 7700 sequence detection system (Applied Biosystems, Carlsbad, CA) with primers described previously (Jung et al., 2012a). The cycling conditions for the qRT-PCR reaction were segment 1- 1 cycle for 15 min at 95°C, and segment 2- 40 cycles of 30 sec at 95°C and 1 min at 60°C, and a dissociation curve analysis for confirmation of a single amplicon production. All samples were run in triplicate. We used known amounts of AQPs cloned from killifish, subcloned into pcDNA3.1(-) and linearized, to quantify the amount expressed.

2.7. Statistics

Student's *t* test was used for analysis of the gene expression and protein expression data. One-way ANOVA and *post hoc* analysis with Tukey's multiple comparison test was used to analyze arsenic transport. Prism5 (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses. Significance was assessed using a cut-off of $p < 0.05$.

3. Results

3.1. Identification of a novel kfAQP3 variant in the intestine

Analysis of the killifish genome and transcriptome data available in www.fundulus.org, a publically available portal website, revealed that only AQP7 and AQP9 in addition to the previously identified AQP3 are found and expressed in killifish (Shaw et al., 2014). Therefore, qRT-PCR studies were conducted on SW and FW acclimated fish to determine if the mRNA for AQP3, AQP7, and AQP9, AQPs that are known to transport arsenite, and are expressed in the intestine. As shown in supplemental figure 1, AQP7 and AQP9 mRNA levels were not significantly expressed above baseline levels in either FW or SW acclimated fish, i.e. their levels were statistically not different from zero. In contrast, using PCR primers that identify all known variants of kfAQP3, kfAQP3 mRNA expression was significantly above background in both FW and SW fish.

Additional PCR studies were conducted to identify kfAQP3 variants. In a previous study that focused on killifish gill we identified two variants of kfAQP3, kfAQP3a, a variant identified in the gill and which is a unique AQP3 that does not transport arsenic, and kfAQP3b, sequence identified in our reference genome (www.fundulus.org), which is an arsenic transporter (Jung et al., 2012a). In order to identify the variants of kfAQP3 expressed in the intestine of SW and FW acclimated fish, we isolated intestines from nine fish from each condition (SW or FW), amplified and sequenced the nucleotides that span the C-terminal amino acids of kfAQP3, which is the variable region of killifish AQP3 that differs between kfAQP3a (glycine-lysine-serine (GKS)) and kfAQP3b (alanine-asparagine-cysteine (ANC)). Among the available sequences, kfAQP3a and kfAQP3b were 97.4 % similar,

whereas kfAQPb and kfAQP3c were 97.6 % similar. The mRNA for kfAQP3a was present in both FW and SW fish (Table 1). However, in SW fish we identified a new variant of kfAQP3, kfAQP3c (glycine-asparagine-cysteine (GNC)). This variant was not observed in any of the FW fish examined (Fig. 1, Table 1). Moreover, kfAQP3b was identified in only FW fish. Quantitative methods were utilized in which qRT-PCR primers were designed that distinguish the three terminal sequences to examine the effects of salinity on the expression of kfAQP3a, kfAQP3b and kfAQP3c. However, these were not able to reliably provide quantitative insight into the relative expression of kfAQP3a, kfAQP3b and kfAQP3c mRNA in the intestine of FW and SW acclimated killifish.

We investigated the nature of the variant (i.e., duplicate gene, allelic variation, alternate splice forms) using the killifish reference genome, and sequence data from a large population re-sequencing project (Whitehead, Reid and Shaw personal communication). A BLAST search revealed the AQP3 gene resides on a single locus within the *F. heteroclitus* reference genome. We also aligned the AQP3 gene from genome sequences obtained from 384 individuals, collected from eight populations ranging from Rhode Island to North Carolina, which were obtained from a separate population re-sequencing projects (Whitehead, Reid and Shaw personal communication). We observed that the coding was highly conserved and there were no fixed polymorphisms within the sixth exon that codes for the c-terminus, which distinguishes the three variants.

3.2. kfAQP3c is an arsenite transporter

Previously we demonstrated that kfAQP3a transports water, urea and glycerol, but does not transport arsenite (Jung et al., 2012a). By contrast, kfAQP3b transports water, urea, glycerol, and arsenite (Jung et al., 2012a). We investigated whether kfAQP3c transports arsenite by expressing the kfAQP3c in HEK293 cells, which do not express an endogenous arsenite transporter. As expected, the arsenic concentration measured in cells transfected with kfAQP3a and exposed to arsenite was not different from cells transfected with empty vector and exposed to arsenite (Fig. 2). In contrast, cells transfected with kfAQP3c and exposed to arsenite had a significantly higher concentration of arsenic compared to cells transfected with empty vector. The concentration of arsenite in cells expressing kfAQP3c was similar to the concentration of arsenic in cells transfected with kfAQP3b and exposed to arsenite, which supports our conclusion that kfAQP3c is a arsenite transporter (Jung et al., 2012a).

3.3. Salinity does not affect intestinal arsenic levels

To determine the potential role of kfAQP3b and kfAQP3c in arsenic uptake by the intestine, FW and SW acclimated fish were exposed to control or 1000 µg/L arsenic for 48h, and subsequently cellular arsenic levels in the intestine were measure by ICP-MS (Fig. 3). With arsenic exposure, intestinal arsenic concentration in FW fish increased significantly from 129.0 ± 15.9 µg/L to 207.3 ± 27.0 µg/L. Arsenic concentration in SW fish also increased significantly from 104.0 ± 14.1 µg/L to 275.5 ± 14.6 µg/L. Although the mean arsenic levels in the intestine of SW fish were higher than in FW fish, they were not statistically different (Fig. 3).

3.4. kfAQP3 mRNA and protein abundance in the intestine of SW and FW fish

When transferred to SW, killifish drink up to 10 times more water than FW fish (Potts and Evans, 1967), therefore, SW fish ingest up to 10-times more arsenic than FW fish. However, as noted above, the arsenic concentration in the intestine of SW fish was similar to FW fish, an observation suggesting that the expression of the kfAQP3 variants that transport arsenite were less in SW fish compared to FW fish. Thus, studies were conducted to determine if SW fish express less kfAQP3 than FW fish. We measured protein abundance of kfAQP3 in the intestine using a polyclonal antibody that recognizes all known variants of kfAQP3 (Jung et al., 2012b) (Fig. 4). Fish acclimated to SW expressed 33.0 ± 7.0 % less AQP3 than fish acclimated to FW. Although AQP3 protein abundance was less in SW fish compared to FW fish, total kfAQP3 mRNA was similar in FW and SW fish (Fig. 5), an observation suggesting that the reduction of kfAQP3 in SW fish compared to FW fish is a result of posttranslational modifications (see Discussion)

4. Discussion

The major new observation in this study is that we have identified a novel variant of kfAQP3, kfAQP3c, in the intestine of killifish and demonstrated that kfAQP3c is an arsenite transporter (Figs. 1 and 2). In addition, we have shown that intestinal arsenic levels are similar in FW and SW fish exposed to 1000 $\mu\text{g/L}$ arsenic, despite the fact that SW fish drink 10 times more arsenic containing water than FW fish (Fig. 3). Our results agree with Dutton and Fisher's previous work demonstrating that although whole-body arsenic accumulation is greater in SW acclimated fish, arsenic accumulation in the viscera is not influenced by salinity (Dutton and Fisher, 2011b; Dutton and Fisher, 2014). This lack of salinity dependent accumulation of arsenic in the intestine despite increased exposure to arsenic in SW is likely, at least in part, to be mediated by a 33% reduction in kfAQP3 protein abundance, which would be expected to decrease the rate of arsenite uptake into intestinal cells. Our kfAQP3 antibody detects all kfAQP3 variants, since it was made using a peptide sequence that is identical in all kfAQP3 variants, and we could not examine the relative abundance, in FW versus SW fish, of kfAQP3a, kfAQP3b, or kfAQP3c. These experiments will require the development and characterization of additional antibodies, which is beyond the scope of the present study.

Several aquaglyceroporins, including AQP3, 7, 9, and 10 have been identified in the digestive tract of other teleosts (Cerdà and Finn, 2010; Tingaud-Sequeira et al., 2010; Tipsmark et al., 2010). However, very little information exists regarding the function of these AQPs in the intestine. AQP3 is distributed throughout the digestive tract in teleosts (Lignot et al., 2002; Ramírez-Lorca et al., 1999). Previous studies have shown that salinity changes do not affect mRNA or protein levels of AQP3 in the European eel (Lignot et al., 2002). This observation has led researchers to speculate that AQP3 does not play a significant physiological role in intestinal water absorption, since intestinal water reabsorption in SW fish is higher than in FW acclimated fish (Potts and Evans, 1967). In the present study, SW fish had significantly lower kfAQP3 protein levels compared to FW fish, an observation that also questions the role of AQP3 in intestinal water reabsorption. If AQP3

played a key role in water absorption, we predict that AQP3 protein abundance should have increased rather than decreased in SW acclimated fish compared to FW acclimated fish.

The observations that total mRNA for kfAQP3 was similar in FW and SW acclimated fish, but that kfAQP3 protein was reduced in SW acclimated fish, are an interesting contrast to our previous study of kfAQP3 expression in gills where mRNA for total kfAQP3 was higher in FW fish compared to SW fish, but protein levels were similar (Jung et al., 2012b). Observations in which mRNA levels do not correlate with protein levels of kfAQP3 suggest that salinity regulated kfAQP3 protein abundance in the intestine is primarily regulated by post-translational mechanisms rather than by changes in mRNA levels. Indeed, previous studies have shown post-translational modification of various aquaporins; including glycosylation (Moeller et al., 2011), glutathionylation (Tamma et al., 2014), phosphorylation, deamidation, and extensive backbone cleavage (Ball et al., 2004; Shey et al 2000). In addition, it has been shown that aquaporins go through cellular trafficking process, such as endocytosis, recycling, as well as ubiquitination and degradation (Chaumont and Tyerman, 2014)-processes that may change cellular aquaporin protein levels independent of changes in mRNA levels. Moreover, a previous study has shown that AQP4 protein immunoreactivity increased in absence of accompanying increase of mRNA levels, which indicates either increase in protein levels without increase in mRNA levels, or increase in immunosignal due to post-translational modification at the epitope region (Vajda et al., 2000)

A novel finding in this study is the identification of salinity dependent expression of kfAQP3b mRNA, which is found only in FW acclimated fish, and expression of kfAQP3c mRNA, which is found only in SW acclimated fish. The three variants of kfAQP3 may have resulted from genome duplication events in teleosts (Jaillon et al., 2004) with these three polymorphisms preserved. However, AQP3 resides only on a single locus within the reference genome, suggesting these variants are not the result of multiple gene copies. Another potential explanation is that the AQP3 variants results from genetic variation between individuals, as speculated by Jung et al (2012a). However, the coding region of AQP3 gene is highly conserved between individuals with no fixed polymorphisms occurring in the region that distinguishes the three variants. These finding suggest that kfAQP3 a, b, and c most likely represent alternate splice forms.

Total body arsenic uptake in killifish has been reported to be salinity dependent. SW acclimated killifish accumulate more arsenic than FW acclimated fish (Dutton and Fisher, 2011b; Dutton and Fisher, 2014). One likely mechanism for increased accumulation in SW killifish is that SW fish drink more water than FW fish in order to regulate plasma osmolality. To compensate for the osmotic loss of water in SW and to maintain plasma osmolality in SW killifish drink more SW and the accompanying salts in the ingested SW are excreted by the kidneys and gill (Evans, 2008). By contrast, Dutton and Fisher (Dutton and Fisher, 2011b; Dutton and Fisher, 2014) and we have demonstrated that arsenic accumulation by the intestine is not salinity dependent.

Several mechanisms may explain why intestinal arsenic levels are similar in FW and SW fish, despite the fact that SW fish drink more arsenic containing water than FW fish. First, as

noted above, kfAQP3 protein abundance is 33% lower in the intestine of SW fish compared to FW fish. Second, the abundance (both absolute and relative) of the arsenic transporting variants of kfAQP3 may also be lower in SW fish compared to FW fish. However, as noted above, our kfAQP3 antibody does not discriminate between kfAQP3b and kfAQP3c, thus, investigation of this possibility requires the development and characterization of a novel antibody.

In addition, several other mechanisms may explain why intestinal arsenic levels are similar in FW and SW fish. These include the possibility that arsenic metabolism and excretion may be increased in SW fish compared to FW fish, and/or that other arsenic transporters such as the Na-phosphate transporter (Villa-Bellosta and Sorribas, 2008), may be down regulated in SW versus FW. Investigation of the role of altered metabolism and excretion of arsenic in FW and SW fish as well as examination of the role of other arsenic transporters in arsenic assimilation in FW and SW fish will require additional, extensive studies that are beyond the scope of the present study.

5. Conclusion

We have identified and characterized a novel variant of kfAQP3, kfAQP3c, which is an arsenic transporter, and have demonstrated that despite increased ingestion of arsenic in SW fish compared to FW fish, SW killifish have similar levels of arsenic in intestinal cells. Several mechanisms may explain why intestinal arsenic levels are similar in FW and SW fish despite the fact that SW fish drink more arsenic containing water than FW fish. First, kfAQP3 protein abundance is 33% lower in the intestine of SW fish compared to FW fish. Another mechanism is that the abundance (both absolute and relative) of the arsenic transporting variants of kfAQP3 may be lower in SW fish compared to FW fish. In addition, arsenic metabolism and excretion may be salinity dependent and/or that other arsenic transporters may be salinity dependent. Additional studies are required to determine the role of kfAQP3c, an ecoresponsive putative splice-variant of kfAQP3, and the contribution of other mechanism in arsenic accumulation in the intestine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Chris Smith at the Mt. Desert Island Biological Laboratory for assistance with the molecular biology studies. Funding was provided by NIH Grants P42 ES007373 and R01 ES019324 from the National Institute of Environmental Health Sciences, NIH Grant P20 RR-016463 from the National Center for Research Resources and grant Number ER1503 from the DOD-SERDP, and Grant DEB-1120512 from the National Science Foundation.

References

- Agency for Toxic Substances and Disease Registry (ATSDR). [Accessed December 9, 2011] The ATSDR 2011 Priority List of Hazardous Substances. 2011. Available at: <http://www.atsdr.cdc.gov/spl/>
- Abernathy CO, Thomas DJ, Calderon RL. Health effects and risk assessment of arsenic. J Nutr. 2003; 133:1536S–1538S. [PubMed: 12730460]

- ASTM. Water and Environmental Technology. Water (I). ASTM; Philadelphia, PA: 1985. Annual Book of ASTM Standards.
- Ball LE, Garland DL, Crouch RK, Schey KL. Post-translational Modifications of Aquaporin 0 (AQP0) in the Normal Human Lens: Spatial and Temporal Occurrence. *Biochemistry*. 2004; 43:9856–9865. [PubMed: 15274640]
- Ballatori N. Transport of toxic metals by molecular mimicry. *Environ Health Perspect*. 2002; 110:689–694. [PubMed: 12426113]
- Bears H, Richards JG, Schulte PM. Arsenic exposure alters hepatic arsenic species composition and stress-mediated gene expression in the common killifish (*Fundulus heteroclitus*). *Aquat Toxicol*. 2006; 77:257–266. [PubMed: 16445994]
- Bhattacharjee H, Mukhopadhyay R, Thiagarajan S, Rosen BP. Aquaglyceroporins: ancient channels for metalloids. *J Biol*. 2008; 7:33. [PubMed: 19014407]
- Burnett K, Bain L, Baldwin W, Callard G, Cohen S, Di Giulio R, Evans D, Gómez-Chiarri M, Hahn M, Hoover C, Karchner S, Katoh F, Maclatchy D, Marshall W, Meyer J, Nacci D, Oleksiak M, Rees B, Singer T, Stegeman J, Towle D, Van Veld P, Vogelbein W, Whitehead A, Winn R, Crawford D. *Fundulus* as the Premier Teleost Model in Environmental Biology: Opportunities for New Insights Using Genomics. *Comp Biochem Physiol Part D Genomics Proteomics*. 2007; 2:257–286. [PubMed: 18071578]
- Calatayud M, Devesa V, Montoro R, Vélez D. In vitro study of intestinal transport of arsenite, monomethylarsonous acid, and dimethylarsinous acid by Caco-2 cell line. *Toxicology Letters*. 2011; 204:127–133. [PubMed: 21554932]
- Cerdà J, Finn RN. Piscine aquaporins: an overview of recent advances. *J Exp Zool A Ecol Genet Physiol*. 2010; 313:623–650. [PubMed: 20717996]
- Chaumont F, Tyerman SD. Aquaporins: Highly Regulated Channels Controlling Plant Water Relations. *Plant Physiology*. 2014; 164:1600–1618. [PubMed: 24449709]
- Dutton J, Fisher NS. Bioaccumulation of As, Cd, Cr, Hg(II), and MeHg in killifish (*Fundulus heteroclitus*) from amphipod and worm prey. *Sci Total Environ*. 2011a; 409:3438–3447. [PubMed: 21658746]
- Dutton J, Fisher NS. Salinity effects on the bioavailability of aqueous metals for the estuarine killifish *Fundulus heteroclitus*. *Environ Toxicol Chem*. 2011b; 30:2107–2114. [PubMed: 21688308]
- Dutton J, Fisher NS. Modeling metal bioaccumulation and tissue distribution in killifish (*Fundulus heteroclitus*) in three contaminated estuaries. *Environmental Toxicology and Chemistry*. 2014; 33:89–101. [PubMed: 24105776]
- Eisler R. Arsenic hazards to fish, wildlife, and invertebrates: A synoptic review. 1988:1–65.
- Erickson RJ, Mount DR, Highland TL, Hockett JR, Leonard EN, Mattson VR, Dawson TD, Lott KG. Effects of copper, cadmium, lead, and arsenic in a live diet on juvenile fish growth. *Can J Fish Aquat Sci*. 2010; 67:1816–1826.
- Erickson RJ, Mount DR, Highland TL, Russell Hockett J, Jenson CT. The relative importance of waterborne and dietborne arsenic exposure on survival and growth of juvenile rainbow trout. *Aquatic toxicology (Amsterdam, Netherlands)*. 2011; 104:108–115.
- Evans D. Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. *American Journal of Physiology-Regulatory*. 2008
- Hamdi M, Sanchez M, Beene L, Liu Q, Landfear S, Rosen B, Liu Z. Arsenic transport by zebrafish aquaglyceroporins. *BMC Mol Biol*. 2009; 10:104. [PubMed: 19939263]
- Huang RN, Lee TC. Cellular Uptake of Trivalent Arsenite and Pentavalent Arsenate in KB Cells Cultured in Phosphate-Free Medium. *Toxicology and Applied Pharmacology*. 1996; 136:243–249. [PubMed: 8619232]
- M F, Beck BD, Chen Y, Lewis AS, Thomas DJ. Arsenic exposure and toxicology: a historical perspective. *Toxicological Sciences*. 2011; 123:305–332. [PubMed: 21750349]
- Jackson BP, Taylor VF, Karagas MR, Punshon T, Cottingham KL. Arsenic, organic foods, and brown rice syrup. *Environ Health Perspect*. 2012; 120:623–626. [PubMed: 22336149]
- Jaillon O, Aury J, Brunet F, Petit J, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard R, Jubin C, Castelli V, Katinka M,

- Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, de Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau J, Gouzy J, Parra G, Lardier G, Chapple C, McKernan K, McEwan P, Bosak S, Kellis M, Volff J, Guigo R, Zody M, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander E, Weissenbach J, Crollius H. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature*. 2004; 431:946–957. [PubMed: 15496914]
- Jung D, Maciver B, Jackson B, Barnaby R, Sato JD, Zeidel ML, Shaw JR, Stanton BA. A Novel Aquaporin 3 in Killifish (*Fundulus heteroclitus*) is Not an Arsenic Channel. *Toxicol Sci*. 2012a; 127:101–109. [PubMed: 22323512]
- Jung D, Sato JD, Shaw JR, Stanton BA. Expression of aquaporin 3 in gills of the Atlantic killifish (*Fundulus heteroclitus*): Effects of seawater acclimation. *Comp Biochem Physiol, Part A Mol Integr Physiol*. 2012b; 161:320–326.
- Lee TC, Ho IC, Lu WJ, Huang JD. Enhanced expression of multidrug resistance-associated protein 2 and reduced expression of aquaglyceroporin 3 in an arsenic-resistant human cell line. *J Biol Chem*. 2006; 281:18401–18407. [PubMed: 16672223]
- Lignot JH, Cutler CP, Hazon N, Cramb G. Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel *Anguilla anguilla* (L.). *J Exp Biol*. 2002; 205:2653–2663. [PubMed: 12151371]
- Moeller HB, Olesen ETB, Fenton RA. Regulation of the water channel aquaporin-2 by posttranslational modification. *American Journal of Physiology - Renal Physiology*. 2011; 300:F1062–F1073. [PubMed: 21307124]
- Potts WT, Evans DH. Sodium and chloride balance in the killifish *Fundulus heteroclitus*. *The Biological bulletin*. 1967; 133:411–425. [PubMed: 6062265]
- Ramírez-Lorca R, Vizuete ML, Venero JL, Revuelta M, Cano J, Ilundáin AA, Echevarría M. Localization of aquaporin-3 mRNA and protein along the gastrointestinal tract of Wistar rats. *Pflugers Arch*. 1999; 438:94–100. [PubMed: 10370092]
- Schey KL, Little M, Fowler JG, Crouch RK. Characterization of Human Lens Major Intrinsic Protein Structure. *Investigative Ophthalmology & Visual Science*. 2000; 41:175–182. [PubMed: 10634618]
- Shaw JR, Bomberger JM, VanderHeide J, LaCasse T, Stanton S, Coutermarsh B, Barnaby R, Stanton BA. Arsenic inhibits SGK1 activation of CFTR Clchannels in the gill of killifish, *Fundulus heteroclitus*. *Aquat Toxicol*. 2010; 98:157–164. [PubMed: 20207026]
- Shaw JR, Gabor K, Hand E, Lankowski A, Durant L, Thibodeau R, Stanton CR, Barnaby R, Coutermarsh B, Karlson KH, Sato JD, Hamilton JW, Stanton BA. Role of glucocorticoid receptor in acclimation of killifish (*Fundulus heteroclitus*) to seawater and effects of arsenic. *Am J Physiol Regul Integr Comp Physiol*. 2007a; 292:R1052–1060. [PubMed: 17038445]
- Shaw JR, Hampton TH, King BL, Whitehead A, Galvez F, Gross RH, Keith N, Notch E, Jung D, Glaholt SP, Chen CY, Colbourne JK, Stanton BA. Natural Selection Canalizes Expression Variation of Environmentally Induced Plasticity-Enabling Genes. *Molecular Biology and Evolution*. 2014; 31:3002–3015. [PubMed: 25158801]
- Shaw JR, Jackson B, Gabor K, Stanton S, Hamilton JW, Stanton BA. The influence of exposure history on arsenic accumulation and toxicity in the killifish, *Fundulus heteroclitus*. *Environ Toxicol Chem*. 2007b; 26:2704–2709. [PubMed: 18020683]
- Tamma G, Ranieri M, Di Mise A, Centrone M, Svelto M, Valenti G. Glutathionylation of the Aquaporin-2 water channel: a novel post-translational modification modulated by the oxidative stress. *Journal of Biological Chemistry*. 2014
- Tingaud-Sequeira A, Calusinska M, Finn RN, Chauvigné F, Lozano J, Cerdà J. The zebrafish genome encodes the largest vertebrate repertoire of functional aquaporins with dual paralogy and substrate specificities similar to mammals. *BMC Evol Biol*. 2010; 10:38. [PubMed: 20149227]
- Tipsmark CK, Sørensen KJ, Madsen SS. Aquaporin expression dynamics in osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation. *J Exp Biol*. 2010; 213:368–379. [PubMed: 20086120]

- Ventura-Lima J, de Castro MR, Acosta D, Fattorini D, Regoli F, de Carvalho LM, Bohrer D, Geracitano LA, Barros DM, Marins LFF, da Silva RS, Bonan CD, Bogo MR, Monserrat JM. Effects of arsenic (As) exposure on the antioxidant status of gills of the zebrafish *Danio rerio* (Cyprinidae). *Comparative Biochemistry and Physiology, Part C*. 2009; 149:538–543. [PubMed: 19138757]
- Vajda Z, Promeneur D, Dóczy T, Sulyok E, Frøkiær J, Ottersen OP, Nielsen S. Increased Aquaporin-4 Immunoreactivity in Rat Brain in Response to Systemic Hyponatremia. *Biochemical and Biophysical Research Communications*. 2000; 270:495–503. [PubMed: 10753653]
- Villa-Bellosta R, Sorribas V. Role of rat sodium/phosphate cotransporters in the cell membrane transport of arsenate. *Toxicology and Applied Pharmacology*. 2008; 232:125–134. [PubMed: 18586044]
- Whitehead, A.; Roach, J.L.; Zhang, S.; Galvez, F. Proceedings of the National Academy of Sciences. 2011. Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient.
- WHO. Environmental Health Criteria: Arsenic. 2001:1–114.
- Wood C, Marshall W. Ion Balance, Acid-Base Regulation, and Chloride Cell Function in the Common Killifish, *Fundulus heteroclitus*: A Euryhaline Estuarine Teleost. *Estuaries*. 1994; 17:34–52.

Abbreviations

AQP	aquaglyceroporin
FW	freshwater
SW	seawater
kf	killifish
As	arsenic
qRT-PCR	quantitative real time polymerase chain reaction

kfAQP3a	aagagaacgcagaagagagtctacaacttactatcaacccccg	878
kfAQP3b	aaaagaacacggaagagagtctacaacttaccatcaacccccg	878
kfAQP3c	aagagaacgcagaagagagtctacaacttactatcaacccccg	878
	** .***** .* .***** .*****	
kfAQP3a	actcaaagtctaactccaacggcaaagagggtaaatcctaa	909
kfAQP3b	actcaaaatctaacgccaacggcaaagaggctaactgctaa	909
kfAQP3c	actcaaagtctaactccaacggcaaagagggtaatgctaa	909
	***** .***** ***** ***** * *****	
	<u>STOP</u>	

Figure 1. Identification of kfAQP3 variants in killifish intestine

PCR analysis was performed with primers that span nucleotides that code for the three C-terminal amino acids of kfAQP3. We identified three distinct variants in the killifish intestine isolated from FW and SW fish that differ in their three terminal amino acids (indicated by overline on the nucleotide sequence). * indicates identical bases in all three variants and indicates differences in bases among the three variants.

Figure 2. kfAQP3c is an arsenic channel

Cellular arsenic levels were measured in HEK293T cells transfected with an empty vector, or cDNA coding for kfAQP3a, kfAQP3b or kfAQP3c, and exposed to 2 µg/L sodium arsenite for 1h. Cells expressing kfAQP3c took up significantly more arsenic (7.0 ± 0.8 µg/kg) than cells expressing empty vector (2.7 ± 0.2 µg/kg) or kfAQP3a (2.7 ± 0.1 µg/kg), but similar amount as kfAQP3b (5.8 ± 0.6 µg/kg). Data are expressed as mean $\pm$ standard error. N=3 per treatment group. Letters indicate significant differences ($p < 0.01$).

Figure 3. Arsenic levels in killifish intestine

Fish were acclimated to FW or SW and then exposed to vehicle (0 µg/L arsenic) or 1000 µg/L sodium arsenite for 48h. Subsequently, arsenic levels in the intestine were measured by ICP-MS. Data are expressed as mean ± standard error. Letters indicate significant differences ($p < 0.05$). N=10-12/group.

Figure 4. kFAQP3 protein abundance in killifish intestine

Total protein was isolated from intestines of killifish acclimated to either freshwater (FW) or seawater (SW) and Western blot was performed using a polyclonal antibody that identifies all variants of kFAQP3. (A): AQP3 levels were significantly higher in fish acclimated to FW compared to fish acclimated to SW (* indicates $p < 0.001$). (B): Representative blot showing relative abundance of kFAQP3 in intestines of fish acclimated to either FW or SW. Data are expressed as mean $\pm$ standard error. N=6 per treatment group. kFAQP3 and actin were probed on the same blot, which was cut for presentation.

Figure 5. Expression of kfAQP3 mRNA in the killifish intestine

Intestines of killifish acclimated to either freshwater (FW) or seawater (SW) were isolated and the abundance of kfAQP3 mRNA was measured using qRT-PCR. Known copy numbers of kfAQP3 was used as standards for measurement. No difference was observed between expression of kfAQP3 in FW fish and SW fish. Data are expressed as mean $\pm$ standard error. N=6 per treatment group.

Table 1
Number of killifish in each condition (FW or SW) that expresses each kfAQP3 variant

	FW	SW
AQP3a	7	4
AQP3b	-	-
AQP3c	-	4
AQP3a/b *	2	-
AQP3a/c **	-	1
Total	9	9

* Individuals that expressed both AQP3a and AQP3b

** Individuals that expressed both AQP3a and AQP3c

FW: individuals acclimated to freshwater

SW: individuals acclimated to seawater

At least 20 clones were analyzed in each individual fish (see text)