4-1-2016

# The potential role of polyamines in gill epithelial remodeling during extreme hypoosmotic challenges in the Gulf killifish, Fundulus grandis

Ying Guan
*Louisiana State University*

Guo xia Zhang
*Southern Medical University*

Shujun Zhang
*Louisiana State University*

Beau Domangue
*Louisiana State University*

Fernando Galvez
*Louisiana State University*



## Recommended Citation

Guan, Y., Zhang, G., Zhang, S., Domangue, B., & Galvez, F. (2016). The potential role of polyamines in gill epithelial remodeling during extreme hypoosmotic challenges in the Gulf killifish, Fundulus grandis. *Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology, 194-195*, 39-50. https://doi.org/10.1016/j.cbpb.2016.01.003

# The potential role of polyamines in gill epithelial remodeling during extreme hypoosmotic challenges in the Gulf killifish, *Fundulus grandis*

Ying Guan[a,b*], Guo-xia Zhang[c], Shujun Zhang[a,d], Beau Domangue[a], Fernando Galvez[a,*]

[a] Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

[b] Department of Biostatistics, School of Public Health and Tropical Medicine, Southern Medical University (Guangdong Provincial Key Laboratory of Tropical Disease Research), Guangzhou, Guangdong, 510515 China

[c] Department of Environmental Health, School of Public Health and Tropical Medicine, Southern Medical University (Guangdong Provincial Key Laboratory of Tropical Disease Research), Guangzhou, Guangdong, 510515 China

[d] Department of Spinal Surgery of the First Affiliated Hospital of University of South, Hengyang, Hunan, 421001 China

* Corresponding author at: Department of Biostatistics, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou, Guangdong, 510515 China

Phone:+(86)020-61360753,Fax:+(86)020-61640319

Email address: ynguan@echobelt.org

## ABSTRACT

Polyamines are a family of low molecular weight organic cations produced in part by the coordinated actions of arginase II (Arg II) and ornithine decarboxylase (Odc). Although gill polyamine homeostasis is affected by acute transfer to fresh water, little is known of its function in fish osmoregulation. The current study investigated the role of polyamines in the compensatory response of hypoosmotic challenge in the euryhaline fish, *Fundulus grandis*. Adult *F. grandis* were acclimated to 5 ppt water, transferred abruptly to 5, 2, 1, 0.5 and 0.1 ppt water, and assessed for osmoregulatory function, gill morphology, and polyamine homeostasis. The plasma osmolality, $Na^+$ concentration, and $Cl^-$ concentration were only significantly reduced during exposure to salinities at or below 0.5 ppt, although these effects were transient except in the 0.1 ppt treatment. The phenotype of mitochondrion-rich cells (MRCs) shifted from a seawater-type to a freshwater-type only at salinities that also produced a plasma osmotic disturbance. Hypoosmotic exposure increased the concentrations of putrescine, spermidine, and spermine in the gill over the entire 7 day period. Exposure to 0.1 ppt water also transiently increased gill caspase-3 activity and gill mRNA levels of the immediate-early response genes, *c-fos* and *c-myc*, thus tightly associating polyamines with gill remodeling during freshwater acclimation. Furthermore, arginase II and ornithine decarboxylase mRNA levels were most highly expressed in MRCs, and these levels were further increased only in the 0.1 ppt treatment. Reduction of gill polyamine levels following administration of the Odc inhibitor, alpha-DL-difluoromethylornithine (DFMO), inhibited gill caspase-3 activity, but surprisingly reduced the magnitude of the plasma osmotic imbalance elicited by exposure to 0.1 ppt water. We used isolated opercular epithelia mounted on Ussing chambers to assess the influence of polyamines on the attenuating response of hypotonic shock on active $Cl^-$ secretion. Spermidine partially reduced the decrease of short-circuit current ($I_{sc}$) and membrane conductance ($G_t$) produced by hypotonic exposure. These data suggest polyamines blunt the hypotonic inhibition of NaCl secretion and may lead to early apoptosis of seawater ionocytes and their replacement by FW-type ionocytes.



Key words: Cell volume regulation; Apoptosis; Cell signaling; Ionocytes; Polyamines


[1]Abbreviations

[1] Abbreviations: Arg II, arginase II; DFMO, alpha-DL-difluoromethylornithine; ESTs, expressed sequence tags; FW, freshwater; $G_t$, membrane conductance; Hypo, hypotonic; $I_{sc}$, short-circuit current; Iso, isotonic; MRCs, mitochondrion-rich cells; Odc, ornithine decarboxylase; PBS, phosphate-buffered saline; PUT, putrescine; PVCs, pavement cells; SEM, scanning electron microscopic; SP, spermine; SPD, spermidine; SW, seawater

## Introduction

The Gulf killifish, *Fundulus grandis*, and its sister taxa, *Fundulus heteroclitus*, reside in coastal marshes that are subject to frequent and episodic oscillations in salinity, dissolved oxygen, and temperature (Marshall, 2003; Wood and LeMoigne, 1991). The ability of these species to tolerate dynamic habitats has made them a widely accepted model for studying the physiological basis of tolerance to environmental stressors (Burnett et al., 2007). Although they prefer brackish to marine environments (Kaneko and Katoh, 2004; Wood and Grosell, 2008), euryhaline *Fundulus* species can cope with reduced environmental salinity and can take short forays into fresh water for feeding (Marshall, 2003). Killifish appear to tolerate these short bouts of fresh water by making rapid, yet easily reversible physiological adjustments (Kaneko and Katoh, 2004; Wood and Grosell, 2008). Only with prolonged exposure to fresh water do they make more substantial compensatory adjustments in the physiology of their ion-transporting epithelia (Scott et al., 2004; Whitehead et al., 2011a; Whitehead et al., 2011b; Whitehead et al., 2012; Wood and Grosell, 2008).

The fish gill, which consists of several cell types that mediate the active excretion of salts in marine fish and the active uptake of osmolytes in freshwater fish, is critical to whole animal osmoregulation (Evans et al., 2005). The gill epithelium has the ability to remodel itself in response to environmental and physiological perturbations (Evans et al., 2005); however, few studies have delineated the salinity at which the gill epithelium transitions from a seawater (SW) to a freshwater (FW) morphology (Katoh and Kaneko, 2003; Laurent et al., 2006). Most *Fundulus* species are marine teleosts that have the ability to retain their seawater physiology at salinities approaching fresh water (Copeland, 1950; Griffith, 1974). *Fundulus heteroclitus* make this physiological transition at a salinity between 2 ppt and 0.2 ppt based on a morphological assessment of the cell types on the fish gill surface in the days following hypoosmotic challenge (Copeland, 1950). Wood and Grosell (2009) report that the transepithelial potential, an important determinant of the electrochemical driving force of gill ion transport, reverses in polarity instantaneously following acute transfer of SW-acclimated *F. heteroclitus* to fresh water. Only with prolonged exposure to fresh water do the gills of *F. heteroclitus* functionally "switch" into one characteristic of a freshwater fish (Wood and Grosell, 2008, 2009). Recently, it has been shown that *F. heteroclitus* acclimated to sea water can be transferred abruptly to 0.4 ppt

with no significant effects to their plasma osmolality and osmolyte concentrations over the 14 d post-transfer (Whitehead et al., 2012). Although the ability of the gill to remodel with varying environmental salinities is well reported, few investigations have described the cellular mechanisms regulating the transient and long term responses of the fish gill to osmotic challenges.

Our group has recently shown that the up-regulation of gill mRNA transcripts associated with polyamine biosynthesis is amongst the most highly upregulated responses of *F. heteroclitus* to hypoosmotic exposure. Furthermore, these transcriptomic responses are most pronounced when the physiological compensation to the hypoosmotic exposure is initiated (Whitehead et al., 2011a). Polyamines are a family of organic cations in cells, and their synthesis is affected by environmental salinity in bacteria (Peter et al., 1978), plants (Zapata et al., 2004) and mammalian cells (Poulin et al., 1991). Polyamines, which include the putrescine, spermidine and spermine, are all increased in the killifish gill during extreme hypoosmotic challenge; a response including an increased transcription and enzyme activity of ornithine decarboxylase, the first and rate-limiting step converting ornithine to putrescine. Following 3 days of acute transfer from sea water to fresh water, only gill putrescine remained elevated in concentration in killifish either due to the increased synthesis from ornithine, reconversion of spermine/spermidine back into putrescine, or due to decreased catabolism. Regardless, these data indicate there may be a distinct role of this polyamine in freshwater acclimation and potentially in the maintenance of the freshwater gill phenotype. Despite this information, little is known about the physiological function of these molecules in fish.

The main goals of this study were to characterize the possible roles of ornithine decarboxylase and polyamines in whole-animal osmoregulation and gill remodeling in Gulf killifish during exposure to a highly-resolved range of low environmental salinities. A series of exposures to low environmental salinities were performed to assess the transition between the marine and freshwater gill phenotype as characterized by assessment of differences in the apical opening of mitochondrion-rich cells on the fish gill surface. These findings were also corroborated with plasma osmolality and markers of apoptosis during acclimation to hypoosmotic challenges. We then studied the influence of alpha-DL-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, on plasma chemistry, ornithine decarboxylase activity, polyamine concentrations, and the expression of various apoptotic

markers in fish during fresh water transfer. Finally, we studied the effects of polyamine supplementation on active ion transport and membrane conductance in the opercular epithelium under hypoosmotic conditions. The opercular epithelium has been used as a surrogate model of the marine fish gill (Marshall, 2011; Marshall et al., 2000), and has been used to study the mechanisms of cell volume regulation during osmotic challenges (Marshall et al., 2005). In this study, we will access the ability of polyamines to influence cell-swelling induced changes in active ion transport in this epithelium.

## 2. Materials and methods

### *2.1 Experimental animals*

Adult *F. grandis* were obtained from a local hatchery (Gulf Coast Minnows, Thibodeaux, LA) and kept in the Life Sciences Aquatic Facility at Louisiana State University (Baton Rouge, LA, USA). Fish were maintained in 5 ppt water for at least one month in a 570-liter glass aquarium with mechanical and biological filtration, and UV-sterilization. Water salinity was monitored using a water quality meter (YSI, Yellow Spring, OH, USA) and temperatures were maintained at 22-24 ˚C. Fish were kept on a light cycle of 12 h light and 12 h dark provided by fluorescent lighting set on an automatic timer. Fish were fed a commercial fish pellet (Cargill, Aquaxcell™ WW Fish Starter 4512, Franklin, LA, USA) twice daily at 2% body weight throughout experimentation, and partial water changes were performed at least twice per week to keep water quality constant.

### *2.2 Experimental protocols*

*Series* 1: Blood plasma chemistry and gill surface morphology were assessed during acute hypoosmotic challenges. One hundred and fifty adult fish (weight range 4.3-16.8 g) were acclimated to 5 ppt water for at least one month. Immediately prior to sampling, a subset of fish (n =6) were sampled as described below. The remaining fish were divided randomly into 5 groups, then transferred acutely to 5, 2, 1, 0.5, or 0.1 ppt water, which was made by diluting Instant Ocean® salt (Blacksburg, VA, USA) with reverse osmosis water. At 6 h, 1 d, 3 d, or 7 d

post-transfer (n=6 per salinity per time point), fish were net-captured and anesthetized with 0.5 g/L tricaine methanesulfonate (MS-222). The tricaine stock solution (Finquel vet., Western Chemicals Inc., Washington, USA) had a concentration of 4 mg/mL and was buffered to a pH between 7 and 7.5 with Tris buffer (Trizma base, Sigma-Aldrich). Whole gill baskets were dissected from fish and washed with Milli-Q-purified water for 10 s. The 3$^{rd}$ and 4$^{th}$ left gill arches were fixed in a solution of 2% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer for scanning electron microscopic (SEM) analysis of the gill surface, as described in Section 2.3. Blood was collected into micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) following caudal severance and centrifuged at 3,000 *g* for 3 min. Plasma was collected and stored at -20 ˚C while awaiting analysis of plasma osmolality, as described in Section 2.4.

*Series* 2: In these studies, the influence of inhibition of ornithine decarboxylase on plasma osmolality, plasma $Na^+$ and $Cl^-$ concentrations, polyamine concentration and caspase-3 activity were assessed during hypoosmotic exposure. Two hundred and forty adult *F. grandis* (weight range 4.9-12.3 g) were acclimated to 5 ppt water, and fish were weighed and injected intraperitoneally with 10 µL phosphate-buffered saline (PBS; sham control; 146 mM NaCl, 3 mM KCl, 15 mM $NaH_2PO_4$, 15 mM $Na_2HPO_4$, and 10 mM $NaHCO_3$ at pH 7.4) or DFMO (0.2 mg DFMO/µL PBS) per *g* fish for two days prior to hypoosmotic transfers. On day 3, half of the fish in the DFMO and PBS treatments were transferred to 0.1 ppt water and the other half of the remaining fish were transferred to 5 ppt water. Immediately prior to transfer, a subset of fish (n =6) were sampled in both DFMO and PBS treatments. The remaining fish were injected daily before morning feeding with either DFMO or PBS, as described above. PBS or DFMO was injected intraperitoneally using a Hamilton microsyringe (Hamilton, Reno, NV, USA). Plasma was collected from 120 fish using the same procedure, as described for Section 2.2 (Series 1), and stored at -20 $^{o}$C while awaiting analysis of plasma chemistry, as described in Section 2.4. All four gill arches from these fish were flash frozen in liquid nitrogen and stored at -80 ˚C for analysis of caspase-3 activity, as described in Section 2.6. Another 120 fish were net-captured and anesthetized in 0.5 g/L tricaine methanesulfonate. Whole gill baskets were dissected from fish and flash frozen in liquid nitrogen. The whole gill baskets were stored at -80 ˚C for analysis of polyamine concentrations, as described in Section 2.7.

153 *Series* 3: The effects of salinity challenges on the mRNA levels of arginase II (*arg II*) and
154 ornithine decarboxylase (*odc*) in isolated mitochondrion-rich cells (MRCs) and pavement cells
155 (PVCs) of the gill epithelium were investigated. Forty-eight fish (weight range 8.19-17.83 g)
156 were acclimated in 5 ppt water for at least one month. Half the fish were transferred to 5 ppt
157 water and the other 24 fish were transferred to 0.1 ppt water. After 1 d post-transfer, fish were
158 net-captured and anesthetized in 0.5 g/L MS-222. Whole gill baskets were dissected from fish
159 and washed with ultrapure water for 10 s. MRCs and PVCs were isolated from pooled fish gills
160 (i.e., 2 gills per isolation) using the procedure described in (Galvez et al., 2002), with
161 modifications described below. Briefly, *F. grandis* fish gills were washed three-times in ice-cold
162 phosphate-buffered saline (137 mM NaCl, 4.3 mM $Na_2HPO_4$, 2.7 mM KCl, and 1.4 mM
163 $NaH_2PO_4$ at pH 7. 8) for 10 min. Gill filaments were removed from their arches, cut into small
164 sections, and digested in 0.25% Trypsin-EDTA (Sigma, St. Louis, MO, USA) for 20 min at 300
165 RPM using a SK-300 shaker (Lab companion, Geumcheon-Gu, Seoul, Korea). Gill digests were
166 passed through a 96 μm mesh filter in 15 mL stop buffer (Kelly et al., 2000). Gills were trypsin-
167 digested 2 or 3 times to yield a mixed population of isolated gill cells, then centrifuged at 300 *g*
168 for 8 min at 4 ˚C. Gill cells were diluted in a 10-fold volume of PBS centrifuged at 500 *g* for 10
169 min at 4 ˚C. The pellet, which was resuspended in 2 mL PBS, was applied to the top of a
170 discontinuous Percoll gradient (Sigma, St. Louis, MO, USA) consisting of a 1.03 g/ml top layer,
171 a 1.06 g/ml middle layer, and a 1.09 g/ml bottom layer. The stacked cell suspension was
172 centrifuged (1,000 *g* for 35 min at 4 ℃) to isolate PVCs and MRCs at the 1.03-1.06 g/mL and
173 1.06-1.09 g/mL Percoll interfaces, respectively. Each isolated cell fraction was stored at -80 ℃
174 until RNA extraction, then analyzed by quantitative PCR for *arg II* and *odc* mRNA levels, as
175 described in Section 2.5.
176 *Series* 4: Killifish gill *c-fos* and *c-myc* mRNA transcript levels and caspase-3 activity were
177 assessed as putative measures of cell proliferation and cell apoptosis following acute freshwater
178 transfer. Sixty adult fish (weight range 3.83-12.61 g) were acclimated to 5 ppt water for at least
179 one month. Gills were sampled prior to transfer and at 6 h, 1 d, 3 d, and 7 d post-transfer to 5 ppt
180 or 0.1 ppt water (n=6 per salinity per time point). The first two left gill arches were immersed in
181 RNA*later* and stored at 4 ˚C overnight, then transferred to -20 ˚C while awaiting total RNA
182 extraction and qPCR analyses of *c-fos* and *c-myc* transcript abundance, as described in Section

2.5. All four gill arches from the right side were flash frozen in liquid nitrogen, stored at -80 ˚C, and used for analysis of caspase-3 activity, as described in Section 2.6.

*Series* 5: Tests were conducted to assess the putative role of polyamines in the compensatory response of fish to hypoosmotic challenge. The opercular epithelium was used as a surrogate of the fish gill to study the influence of polyamines on membrane conductance ($G_t$, mS $cm^{-2}$) and short-circuit current ($I_{sc,}$ µA $cm^{-2}$) (Marshall et al., 2000). Adult *F. grandis* were acclimated to 5 ppt water and anaesthetized in 0.2 g/L MS-222. Both opercular membranes of the fish were isolated as described by Marshall et al (2000). Briefly, each operculum was mounted between two plexiglass sliders (Physiological instruments, San Diego, CA, USA), exposing a 0.1 $cm^2$ diameter round aperture, and placed in individual Ussing chambers (Physiological Instruments, San Diego, CA, USA) (Marshall et al., 2000). The Ussing chambers contained isotonic saline (151 mM NaCl, 3 mM KCl, 0.88 mM $MgSO_4$, 4.6 mM $Na_2HPO_4$, 0.48 mM $K_2HPO_4$, 1 mM $CaCl_2$, 11 mM HEPES-free salt, and 4.5 mM urea at an osmolality of 334 mOsm/kg and pH 7.8) on each side of the membrane, as described previously (Daborn et al., 2001). The isotonic bathing solution was bubbled with 100% oxygen. The basolateral side of the membrane was bathed in 2 mL isotonic saline with 3 mM glucose added, whereas the apical side contained 2 mL isotonic saline. $I_{sc}$ and $G_t$ across the opercular epithelium were measured in real time with a data acquisition system (PowerLab and LabChart 7 software, ADInstrument Inc., CO, USA). A pulse of 0.002 V was given every min to calculate $G_t$ according to Ohm's law: $G_t = \Delta I_{sc}/\Delta V$. Once a baseline was achieved under symmetrical conditions (isotonic saline on both sides of the membrane), the isotonic solution was replaced with a hypotonic bathing solution (75% isotonic saline, 25% distilled water) under symmetrical conditions, resulting in an osmolality of 270 mOsm/kg. This hypotonic solution mimicked the plasma osmolality of killifish during the early stages of hypoosmotic shock, as described in Fig. 1.

$I_{sc}$ and $G_t$ were assessed in opercula immersed in isotonic (Iso) and hypotonic (Hypo) bathing solutions for 30-45 min each to allow steady-state values to be reached. In order to derive the relationship between changes in $I_{sc}$ versus polyamine concentrations for each of the three compounds, preliminary experiments were conducted. The results of preliminary experiments showed that there was no significant difference in change of $I_{sc}$ between low dose and high dose of individual polyamines. A polyamine mixture consisting of 0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine was used in subsequent experiments as described below. First,

214 the influence of the polyamine mixture on the change of $I_{sc}$ and $G_t$ was assessed. Briefly, a
215 steady state of $I_{sc}$ and $G_t$ was attained under the Iso condition, then a polyamine mixture (0.4 mM
216 putrescine, 0.3 mM spermidine, and 0.1 mM spermine, PA) was added to the basolateral side of
217 opercular epithelia, either: a) coincident with transfer from isotonic to hypotonic conditions (pre-
218 PA, n =7), or b) following initial hypotonic transfer and establishment of a steady-state $I_{sc}$ under
219 the hypotonic condition (post-PA, n =10). Second, the influence of individual polyamines on the
220 change in $I_{sc}$ and $G_t$ was assessed. Putrescine (PUT, 0.4 mM), spermidine (SPD, 0.3 mM), and
221 spermine (SP, 0.1 mM) were added individually to the basolateral side of the opercular
222 epithelium coincident with transfer from isotonic to hypotonic conditions (Hypo), respectively (n
223 =7 for Hypo + PUT, Hypo + SPD, and Hypo + SP). $I_{sc}$ and $G_t$ were recorded for 40 min until
224 reaching a steady-state and percent change of $I_{sc}$ and $G_t$ was calculated according to Equation 1,

225 $$Percent\ change = \frac{Value_{treat} - Value_{isotonic}}{Value_{isotonic}} \times 100\% \quad (1)$$

226 where Value $_{treat}$ represents the values of $I_{sc}$ or $G_t$ at pre-PA and post-PA in Iso conditions, Hypo
227 alone, Hypo + PUT, Hypo + SPD and Hypo + SP at different time points. Value $_{isotonic}$ indicates
228 a steady-state of $I_{sc}$ or $G_t$ when opercular epithelia were incubated in isotonic saline.
229
230 *2.3 Scanning electron microscopy (SEM) analyses*
231
232 Gills were fixed in 2% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer (pH
233 7.4-7.6) for 4 h, rinsed in 0.1 M cacodylate buffer containing 0.02 M glycine, post-fixed in 2%
234 osmium tetroxide for 1 h, and rinsed in water. Samples were dehydrated in an ethanol series (10
235 min at 50%, 10 min at 70%, 10 min at 80%, 2 rinses of 10 min at 95%, and 3 rinses of 15 min at
236 100% ethanol), dried with liquid $CO_2$ in a Denton critical point dryer (Cherry Hill, NJ, USA)
237 mounted on aluminum SEM stubs and coated with a gold:palladium solution (V:V of 60:40) in
238 an Edwards S150 sputter coater (Ashbord, Kent, UK). The afferent edge of each gill filament
239 was randomly imaged using a JSM-6610 high vacuum mode SEM (Tokyo, Japan).
240
241 *2.4 Plasma chemistry analyses*

Plasma osmolality was analyzed by freeze-point depression (Precision systems INC, Natick, MA, USA). Plasma $Na^+$ concentrations were measured using flame atomic absorption spectroscopy (Varian Australia Pty Ltd, Australia), and plasma $Cl^-$ concentrations were measured using a modified mercuric thiocyanate method (Zall et al., 1956).

*2.5 Relative arg II, odc, c-myc, and c-fos mRNA levels*

Commercial SYBR Green master mix (QIAGEN Inc. Valencia, CA, USA) was used to analyze the relative mRNA levels of *arg II*, *odc*, *c-myc*, *c-fos*, and the reference gene, 18S ribosomal RNA (*18s rRNA*). Expressed sequence tags (ESTs) for *arg II* (GenBank accession no. AB290198.1), *odc* (GenBank accession no. CN977303.1), *c-fos* (GenBank accession no. DN956552.1) and *c-myc* (GenBank accession no. CN985702.1) from *F. heteroclitus* were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Primers for quantitative PCR were developed for all genes using Integrated DNA Technologies Inc. software (Coralville, IA, USA) (Table 1). A 20 μL reaction mixture (0.1 μg cDNA, 10 nM forward and reverse primers, 5 μL SYBR Green reagents) was used for the ABI Prism 7000 SDS system (Carlsbad, CA, USA). The first strand of cDNA was reverse-transcribed from total mRNA of MRCs and PVCs isolated from the fish gill. Samples were cycled 40 times at 95 ˚C for 15 s and 60 ˚C for 1 min. Efficiency and relative changes in mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Fleige et al., 2006).

*2.6 Caspase-3 activity assay*

A caspase-3 assay kit (Sigma, St. Louis, MO, USA) was employed to measure caspase-3 activity, which is based on the hydrolysis of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin by caspase 3 that results in the release of the fluorescent 7-amino-4-methylcoumarin. Gill arches were homogenized with a 10-fold volume of 0.85% NaCl, and the original homogenate was used directly to measure caspase-3 activity. Gill homogenates were diluted 20-fold and measured for total protein (Sigma, St. Louis, MO, USA). Fluorescence intensity was measured with excitation at 355 nm and emission at 460 nm using a VICTOR™ *X*3 multilabel plate reader (Perkin Elmer, Santa Clara, CA, USA). A 1x assay buffer of 200 μl served as a blank to adjust the fluorimeter

to zero. Fold–change in gill caspase-3 activity was calculated by taking the ratio of the enzymatic activity of post-transfer to 5 ppt water and 0.1 ppt water over the enzymatic activity of pre-transfer at each time point.

*2. 7 Gill polyamine contents*

Gill baskets were thawed and homogenized with methanol (containing 0.1 mM HCl) (V:V for 1:3) in dry ice using a PRO200 Homogenizer (Lab depot Inc. Dawsonville, GA, USA). Samples were incubated in 0.6 M perchloric acid to extract polyamines and derivatized with o-toluoyl chloride using a slightly modified procedure from that described previously (Wongyai et al., 1988). Samples (20 µL) were then analyzed for polyamines by high performance liquid chromatography (HPLC) (Bio-Rad high-performance liquid chromatograph gradient module with a Model 996 photodiode array detector; Milford, MS, USA). HPLC separations were performed using an ACE C-18-PFP column (150 mm x 4.6 mm; 5 µm; Chadds Ford, PA, USA), a mobile phase consisting of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, with a flow rate of 1.0 mL/min, and UV detection at 254 nm. Each polyamine derivative was dissolved separately in water to yield stock solutions of 200 nmol /100 µL**.** A series of standard solution containing 53 to 1500, 144 to 2300, and 25 to 400 ng/100 µL of putrescine, spermidine and spermine, respectively, were prepared by serial dilution of the stock solutions with water. Each sample including standards was analyzed in triplicate, and an average peak area under the curve was obtained for each sample.

*2.8 Statistical analysis*

Results were presented as mean ± standard error of the mean (SEM). Statistical differences in plasma osmolality, mRNA transcript abundance, and $I_{sc}$ and $G_t$ between treatments were analyzed using a one-way ANOVA (v.9.3, SAS Institute Inc., Cary, NC, USA) followed by a LSD post hoc test. Statistical differences were considered significant at a *P*-value of 0.05 or less.

**3. Results**

304 *3.1 Plasma chemistry*

305

306 Initial experiments were performed to assess the influence of comparably small changes in
307 environmental salinity on plasma osmolality (Fig. 1) and gill surface morphology (Fig. 2). The
308 magnitude of the reduction of plasma osmolality disturbances depended on the severity of the
309 hypoosmotic transfer and on the time following post-transfer. Plasma osmolality decreased by
310 approximately 19.5% following transfer from 5 ppt water to either 0.1 ppt or 0.5 ppt water,
311 reaching their lowest values at 1 d and 6 h, respectively (Fig. 1). Plasma osmolality in the 0.5
312 ppt treatment recovered to its original level by 1 d, but it did not completely recover to the pre-
313 transfer value even after 7 d in the 0.1 ppt treatment ($P$=0.007). In comparison, plasma
314 osmolality in *F. grandis* was not affected by the abrupt transfer from 5 ppt to 1 ppt, 2 ppt, or 5
315 ppt water at any time during the 7 d post-transfer period.

316

317 *3.2 Gill morphology*

318

319 Scanning electron microscopy was used to assess the morphology of the apical opening of
320 MRCs on the gill surface of fish undergoing osmotic shock, as described in Section 2.3. The gill
321 surface of fish acclimated to salinities of 1 to 5 ppt water consisted of pavement cells (PVCs)
322 possessing concentric rings of microridges around the outer cell margins, and, on occasion,
323 microvilli projecting towards the center of cells (Fig. 2). MRCs in fish gills exposed to 5 and 2
324 ppt water were predominately SW-type, as noted by their small apical openings at the gill surface
325 localized at the afferent edge of the filamental epithelium. Exposure to 1 ppt water led to minor
326 changes in the surface appearance of the fish gill, with a slight enlargement of MRCs during the
327 initial 6 h to 1 d post-transfer. FW-type MRCs, characterized by their convex shape and well-
328 developed microvilli, began to emerge on the gill surface as salinity decreased to 0.5 ppt and
329 below. These cell types were only expressed transiently, being replaced by SW-type MRCs,
330 which reemerged by 3 d at 0.5 ppt or by 7 d at 0.1 ppt. Regardless, FW-type MRCs with smaller
331 apical openings were still present following 7 d exposure to 0.1 ppt and 0.5 ppt water (Fig. 2).

332

333 *3.3 argII and odc mRNA levels in pavement cells and mitochondrion-rich cells*

334

The mRNA levels of *argII and odc* in MRCs and PVCs isolated from the gills of 5 ppt and 0.1 ppt fish were assessed. Compared to the PVCs at 5 ppt, the levels of *arg II* and *odc* mRNA in MRCs did not change. During hypoosmotic exposure, *arg II* (Fig. 3 A) and *odc* (Fig. 3B) mRNA levels were 4.5-fold ($P$=0.022) and 2.6-fold ($P$=0.011) higher in MRCs relative to PVCs, respectively. In contrast, *arg II* and *odc* mRNA levels increased by 3.3-fold ($P$=0.005) and 5.8-fold ($P$=0.001) in MRCs after 0.1 ppt water compared to the 5 ppt control (Fig. 3).

*3.4 Plasma chemistry during Odc inhibition*

Administration of DFMO inhibited gill ODC activity and polyamine levels, and also reduced the magnitude of the plasma osmotic (Fig. 4A), hyponatremic (Fig. 4B), and hypochloremic (Fig. 4C) responses measured in fish following transfer to 0.1 ppt water. Transfer to 0.1 ppt water reduced plasma osmolality in both the sham and DFMO-treated fish, although the reduction was more pronounced in the sham treated fish ($P$=0.029). Plasma $Na^+$ concentrations were reduced by 14% at 6 h and 18% at 1 d in sham fish transferred to 0.1 ppt water compared to the pre-transfer levels of sham fish at 5 ppt. In fish administered DFMO, plasma $Na^+$ was reduced only by 7.7% ($P$=0.046) and 15.4% ($P$=0.021) at 6 h and 1 d post transfer to 0.1 ppt, respectively (Fig. 4B). Similarly, the magnitude of the hypochloremic effect of 0.1 ppt transfer was attenuated by DFMO administration compared to that seen in the sham controls at 6 h ($P$=0.013) and 1 d ($P$=0.016) transfer to 0.1 ppt (Fig. 4C). Both plasma $Na^+$ and $Cl^-$ returned to pre-transfer levels after 3 d of hypoosmotic exposure in sham controls and the DFMO-treated fish. In comparison to the differences seen in the 0.1 ppt exposed fish, plasma osmolality, plasma $Na^+$, and plasma $Cl^-$ were not significantly different between the DFMO-treated fish and PBS-treated fish maintained in 5 ppt water over time (Fig. 4). These data suggest that the reduction in the hypoosmotic effects of 0.1 ppt water by administration of DFMO was not simply due to the drug serving as an osmolyte.

*3.5 Polyamine concentration with or without Odc inhibition*

The sum total of putrescine, spermidine and spermine concentrations were measured in the gills of fish acclimated to 5 ppt and 0.1 ppt with and without the concomitant inhibition of Odc

activity with DFMO. When fish were acclimated to 5 ppt water, the polyamine levels in the PBS injection group (sham control) did not vary over time, whereas they increased by 23.8%, 62.8%, 46.8%, and 30.0% at 6 h, 1 d, 3 d, and 7 d, respectively, when transferred to 0.1 ppt. When fish were kept in 5 ppt water, inhibition of Odc activity using DFMO decreased the sum of polyamine concentrations by 31.5%, 39.7%, 38.4% and 72.5% comparing to the sham control at 6 h, 1 d, 3 d, and 7 d, respectively. In comparison, when Odc activity was inhibited in fish transferred to 0.1 ppt water, the gill polyamines concentrations decreased by 32.5%, 49.2%, 54.8% and 64.0% comparing to the sham control at 6 h, 1 d, 3 d, and 7 d, respectively (Table 2).

*3.6 Relative c-myc and c-fos mRNA levels and caspase-3 activity in gill*

The mRNA levels for *c-fos* and *c-myc* were measured in the gills following hypoosmotic exposure as a measure of cell proliferation in the tissue. Gill *c-fos* mRNA expression increased 14.1-fold at 6 h ($P$=0.001) and 3.5-fold at 1 d ($P$=0.027) following transfer to 0.1 ppt water (compared to the pre-transfer control levels), but returned to the pre-transfer level by 3 d (Fig. 5A). Compared to the 5 ppt control, the *c-fos* mRNA level increased 10.5-fold and 4.4-fold at 6 h and 1 d, respectively, following hypoosmotic exposure ($P$<0.020 for both time points) (Fig. 5A). Gill *c-myc* mRNA abundance was increased 6.1-fold after 6 h of hypoosmotic exposure, but returned to the pre-transfer level at 7 d (Fig. 5B). The level of *c-myc* mRNA increased up to 7.1-fold by 1 d post-transfer (compared to the pre-transfer level) ($P$<0.001) (Fig. 5B). The mRNA expression of *c-myc* increased at 6 h, 1 d, and 3 d in the gills of 0.1 ppt-acclimated fish ($P$=0.018, 0.025 and 0.011, respectively) compared to the 5 ppt control at the same time points (Fig. 5B).

The onset of apoptosis was assessed by analyzing caspase-3 activity in gill. In an initial experiment, gill caspase-3 activity increased approximately 3-fold following 3 d of hypoosmotic exposure ($P$=0.006) but returned to the control level at 7 d (Fig. 5C). In comparison, caspase-3 activity in the gill did not change significantly during exposure to 5 ppt water over 7 days (Fig. 5C). In a second experiment, caspase-3 activity and the influence of Odc inhibition with DFMO on caspase-3 activity during concomitant hypoosmotic challenge were assessed. Inhibition of Odc activity using DFMO was found to have no effect on gill caspase-3 activity in fish maintained at 5 ppt compared to the 5 ppt sham controls. The 0.1 ppt sham control continued to

show a significant increase in caspase-3 activity at 6 h and 1 d ($P$<0.001 for both time points) before returning to the control level (Fig. 5D). In comparison, inhibition of Odc activity with DFMO decreased the ability of the hypoosmotic treatment to induce gill caspase-3 activity. In the 0.1 ppt DFMO treatment, gill caspase-3 activity was not increased above the level of the 5 ppt sham control (Fig. 5D).

*3.7 The effect of polyamines on opercular $I_{sc}$ and $G_t$*

The influence of a polyamine mixture (0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine, PA) on the hypotonic-induced changes in $I_{sc}$ and $G_t$ in the opercular epithelium was investigated. After transfer to the hypotonic solution, $I_{sc}$ of the opercular epithelium decreased immediately, and reached a new steady state within 30-35 min (Fig. 6A). Hypotonic shock induced a 52% reduction in $I_{sc}$ compared to that measured in the isotonic medium (Fig. 6A). Compared to the hypotonic control, pre-PA significantly inhibited $I_{sc}$ reduction in the opercular epithelium after 20 min hypotonic exposure, reaching its maximum inhibition at 40 min ($P$=0.001) (Fig. 6A). However, post-PA did not affect $I_{sc}$ compared to the hypotonic control over time (Fig. 6A). Transfer of the opercular epithelium to the hypotonic solution immediately reduced its $G_t$ and it remained at this level for at least 40 min (Fig. 6B). Application of PA either before hypotonic transfer (pre-PA) or after immediately after hypotonic transfer (post-PA) did not significantly affect this hypotonically-induced inhibition of $G_t$ (Fig. 6B). Additional experiments were conducted to assess the contribution of individual polyamines to changes in $I_{sc}$ and $G_t$ during hypotonic shock. The $I_{sc}$ and $G_t$ of the opercular epithelium were decreased immediately after transfer to the hypotonic solution, reaching new steady states within 30-35 min (Fig. 7A). When individual polyamines were applied, only spermidine was shown to attenuate the hypotonically-induced reduction in $I_{sc}$ and $G_t$ over time (Fig. 7B). In comparison, application of putrescine or spermine did not affect the ability of hypotonicity to reduce the $I_{sc}$ and $G_t$ of the opercular epithelium over time (Fig. 7B).

## 4. DISCUSSION

*4.1 Plasma chemistry and gill epithelial morphology*

In the present study, *F. grandis* were acutely transferred to salinities ranging from 5 ppt to 0.1 ppt water following acclimation to 5 ppt water (~15 % sea water). Salinities at or above 1 ppt did not impair plasma osmotic balance nor produce any notable morphological alterations to the gill. In comparison, plasma osmolality was significantly reduced at 6 h post-transfer to 0.5 ppt water but recovered back to the 5 ppt control level within 1 d (Fig. 1). When fish were transferred to 0.1 ppt water, plasma osmolality was reduced to the same extent as measured in the 0.5 ppt treatment, although osmotic balance did not return to pre-transfer levels even after 14 days of exposure (Fig. 1). Water at 1 ppt water has 14 mM $Na^+$, which is about 11-fold lower than the extracellular fluid, whereas 0.5 ppt water has 7 mM $Na^+$, which is approximately 22-fold lower than that measured in the extracellular fluid. This represents a two-fold difference in the ionic gradient between the blood and the outside environment despite only a 0.5 ppt difference in salinity (0.5 ppt versus 1 ppt). One could also conclude that an approximate 20-fold dilution of the environment compared to the blood is enough to differentiate between a physiological benign and a physiological stressful salinity. Furthermore, the change in osmolality (about 73 mOsm/Kg) was larger than the sum of $Na^+$ and $Cl^-$ changes (25 and 30 mM), suggesting that the regulation of other plasma ions was adversely affected at these low salinities. Osmotic imbalance in dilute environments (0.5 ppt and below) may also be explained by the reduction in the concentration of other ions in salt water such as calcium. For instance, a 22-fold dilution of reconstituted sea water would lower the $Ca^{2+}$ concentration to approximately 500 μM, which represents a concentration only slightly higher than the Michaelis-Menten constant for the high affinity $Ca^{2+}$ transport in freshwater-acclimated *F. heteroclitus* (Patrick et al., 1997).

The multifunctionality of MRCs with environmental salinity has been demonstrated (Marshall and Bryson, 1998), and shown in euryhaline *Fundulus* species to transition from a seawater to a freshwater phenotype within a narrow range of salinity well below the isosmotic point of the extracellular fluid (Copeland, 1950; Whitehead et al., 2011b; Whitehead et al., 2012). In the current study, gill MRC cell types were identified using SEM (Goss et al., 1994; Perry et al., 1992). The apical surface of the gill MRCs of *F. grandis* retained an apical crypt characteristic of a seawater fish gill at a salinity of 1 ppt and above. Only when acutely exposed to a salinity at or below 0.5 ppt did the apical surface of MRCs in the fish gill attain a concave appearance more characteristic of a freshwater gill phenotype (Fig. 2). Although the

functionality of these putative MRC subtypes is not known, this phenotypic transition only occurred in treatments that also led to an impairment in plasma osmolality regulation (Fig. 1). At salinity challenges at which plasma osmolality was reduced, there was a tendency towards expression of MRCs on the gill surface that were of freshwater phenotype (Fig. 2). It has been demonstrated that MRCs alternate their morphology in response to abrupt environmental osmotic change (Copeland, 1950; Kaneko and Katoh, 2004; Katoh et al., 2001; Katoh et al., 2000; Laurent et al., 2006). Fish exposed to salinities of 0.1 ppt and 0.5 ppt exhibited few SW-type MRCs in their gills after 6 h. These cells were replaced by large MRCs that were predominantly triangular in shape and with apical surfaces that contained short microvilli flush with the surrounding PVCs (Fig. 2). The actual fate of these seawater MRCs during hypoosmotic transfer is unclear. Laurent and others (Laurent et al., 2006) suggested that seawater MRCs either undergo apoptosis, necrosis, exfoliation, or are simply covered by pavement cells, where they remain dormant in the underlying tissue during bouts of fresh water exposure. These FW-type MRCs were termed cuboidal cells, and were characterized by their wedge-like appearance between neighboring PVCs, analogous to the mitochondrion-rich PVCs described in rainbow trout (Galvez et al., 2002; Laurent et al., 2006). With continued extreme hypoosmotic exposure, the surface exposure of these cuboidal cells decreased over time and SW-type apical crypts emerged, even in fish acclimated to 0.1 ppt water (Fig. 2). This produced a mixed population of SW and FW MRCs, as previously described (Laurent et al., 2006; Scott et al., 2008; Scott et al., 2006; Whitehead et al., 2012). This ability to rapidly cover and uncover cell types is beneficial for euryhaline fish species such as *F. grandis*, which may need to under quick physiological transitions during fluctuations in environmental salinity but probably do so at a high energetic cost (Kidder et al., 2006). It is unclear why SW MRCs began to resurface in the 0.1 ppt- and 0.5 ppt-acclimated fish gill, although this capacity benefits *F. grandis* survival in unstable environments. Alternatively, the need for MRCs in dilute environments (i.e., 0.5 ppt and below) may relate to the active calcium uptake that occurs in parallel with monovalent ion transport in FW (and SW) ionocytes (Evans et al., 2005; Marshall and Bryson, 1998). Regardless, these data support a dynamic restructuring of the gill epithelium of *F. grandis* at low environmental salinity.

In individual multifunctional MRCs, many transport systems including ion pumps, cotransporters, channels and exchangers operate simultaneously to facilitate active ion secretion in marine water or active ion absorption in fresh water (Hiroi and McCormick, 2012; Marshall

and Grosell, 2005). The current model of active ion secretion in the marine fish gill involves a basolateral $Na^+$,$K^+$-ATPase, which provides the driving force for $Na^+$ and $Cl^-$ entry via a $Na^+$,$K^+$,2$Cl^-$ cotransporter (NKCC), and the accumulation of $Cl^-$ to facilitate its passive movement across the apical membrane into the water via the anion channel Cystic Fibrosis Transmembrane conductance Regulator (CFTR) (Marshall et al., 1999). Reduction of plasma osmolality during exposure to low environmental salinity causes immediate MRC swelling and inhibit active $Cl^-$ secretion (Marshall, 2011). With prolonged exposure to fresh water, morphological alterations to the gill would include the expression of MRC types that participate in active ion absorption rather than in active ion secretion. Although the exact model of active $Na^+$ transport varies between species, *Fundulus* lack an active $Cl^-$ absorption mechanism in the gill (Scott et al., 2004). As such, the regulation of paracellular $Cl^-$ loss is particularly relevant in killifish. The ability to mitigate the diffusive loss of $Cl^-$ upon acute transfer to fresh water is a feature defining freshwater-tolerant killifish species or populations of euryhaline species best adapted to tolerate hypoosmotic exposure (Brennan et al., 2015; Scott et al., 2004; Scott and Schulte, 2005; Scott et al., 2006; Whitehead et al., 2011a; Whitehead et al., 2011b; Whitehead et al., 2012). The appearance of cuboidal cells presumably would help to inhibit ion loss at the gill epithelium and maintain plasma ion concentrations in response to rapid fluctuations in environmental salinity.

*4.2 Possible roles of polyamines in cell apoptosis and cell proliferation*

In the present study, *arg II* and *odc* mRNA levels were significantly higher in MRCs than in PVCs regardless of salinity, and the concentrations of both transcripts were further increased in freshwater-type MRCs following transfer to 0.1 ppt water (Fig. 3). Arginase II is abundant in extrahepatic cells rich in mitochondria, where it may be involved in numerous physiological functions including glutamate, proline, and polyamine biosynthesis, or in the modulation of nitric oxide synthesis (Morris, 2004). The concurrence of elevated *arg II* and *Odc* mRNA levels in gill MRCs (Fig. 3) and gill MRC remodeling (Fig. 2) suggests that polyamines may be associated with the phenotypic plasticity of MRCs in the fish gill (Brennan et al., 2015; Whitehead et al., 2011b; Whitehead et al., 2012). A similar relationship between hypoosmotic challenge and Odc activity was described in the gills of *F. heteroclitus* (Whitehead et al., 2012) and brine shrimp

(Watts et al., 1996) and in the variant L1210 mouse leukemia cell line (Uthaiwan et al., 2001). As a key enzyme for polyamine biosynthesis, Odc upregulation suggests a link between polyamine biosynthesis and hypoosmotic exposure.

Polyamines have an important role in RNA transcription, cell growth, cell proliferation, and apoptosis (Pegg and McCann, 1982; Ray et al., 1999; Tabor and Tabor, 1984). Previous studies have shown that polyamines can positively regulate *c-fos, c-ras,* and *c-myc* transcription, and that inhibition of Odc activity using DFMO can inhibit *c-myc* and *c-fo*s mRNA expression in carcinoma cells in primary cultured rat kidney cells (Tabib and Bachrach, 1994). Evidence is provided here that ornithine decarboxylase, or putrescine and other polyamines produced irreversibly by Odc, may influence the compensatory response to hypoosmotic acclimation in fish. An increase in caspase-3 activity to environmental stress including osmotic challenges has been previously documented in fish (Krumschnabel and Podrabsky, 2009). An increase in caspase-3 activity has been shown in zebrafish (*Danio rerio*) during DNA damage (Lee et al., 2008), in Atlantic salmon (*Salmo salar)* during hyperthermia (Takle et al., 2006), in sea bass (*Dicentrarchus labrax L*.) during bacterial infection (Reis et al., 2007), in rainbow trout (*Oncorhynchus mykiss)* after hyperosmotic transfer (Rojo and Gonzalez, 1999), and in sturgeon (*Acipenser schrenckii*) during prolonged hypoxia (Lu et al., 2005). The role of caspase-3 in fish correlates with extensive apoptosis and morphological abnormalities in tissues (Yamashita et al., 2008). Inhibition of Odc activity with DFMO inhibited the ability of hypoosmotic exposure to stimulate caspase-3 activity in the gill (Fig. 5). These results are consistent with the inhibitory effects of DFMO on caspase-3 activity in intestine (Deng et al., 2005; Ray et al., 2000), bone marrow stromal cells (Muscari et al., 2005) and in cardiac myoblasts (Tantini et al., 2006). DFMO also reduced gill polyamine concentrations in fish acclimated to 5 ppt and in fish transferred acutely from 5 ppt to 0.1 ppt water. These data suggest that Odc activity is critical for the continual production of endogenous polyamines in the fish gill and that these polyamines can be reduced in concentration when Odc activity is inhibited. Furthermore, inhibition of Odc significantly affected the capacity of fish gills to stimulate polyamine synthesis during a hypoosmotic challenge.

Although the role of polyamines in gill remodeling remains unclear, marine *Fundulus* species that are not able to physiological transform their gills to a freshwater phenotype nor able to survive hypoosmotic transfer following acclimation to sea water, have a reduced capacity to

stimulate polyamine biosynthetic pathways compared to euryhaline, *F. heteroclitus* (Brennan et al., 2015). The fact that the increase in polyamine levels occurred concomitantly with an increase in gill caspase-3 activity following 0.1 ppt water exposure (Fig. 5C) demonstrated that polyamines may induce apoptosis in gills mediated through a caspase pathway. Although the link between polyamines and phenotypic plasticity to environmental stress such as that induced by a hypoosmotic challenge is unclear, several possible mechanisms are worth considering. First, polyamines may induce cell apoptosis by triggering the c-Myc or c-Fos pathway in gills (Fig. 5A and 5B). Increasing polyamine levels after 6 h and 1 d following FW-exposure (Table 2) were concomitant with an increase in *c-myc* and *c-fos* mRNA levels in killifish gills (Fig. 5A, 5B). The correlation between polyamine levels and the *c-myc* and *c-fos* mRNA levels was described in a previous study in rat kidney cells (Tabib and Bachrach, 1994). These studies suggest that polyamines have the ability to increase *c-fos* and *c-myc* mRNAs levels during environmental challenges. The relationship between the increase in polyamine levels and the increase in *c-fos* and *c-myc* mRNA levels during the first few days following hypoosmotic transfer (Fig. 5A, 5B) support a causal relationship between these variables. Second, polyamines may trigger the intrinsic cell apoptosis via the caspase pathway in the gills (Fig. 5C and 5D). The transient increase in gill polyamine levels and the simultaneous increase in the mRNA levels of pro-apoptotic factors and in the activity of markers of apoptosis, are intriguing and worth future study.

### *4.3 The influence of polyamines on the electrophysiological properties of the opercular epithelium*

Besides cell apoptosis and proliferation, polyamines have been implicated in the regulation of cell volume during hypotonic challenges (Watts et al., 1996). In order to test this hypothesis, the opercular epithelium of killifish was used as a surrogate epithelium to the fish gill due to its high abundance of MRCs, the presence of active $Cl^-$ secretion similar to that found in the marine fish gill, the existence of an electrical current under symmetrical conditions associated almost entirely with this active $Cl^-$ secretion, and the fact that this electrical current is almost entirely inhibited by exposure to hypotonic media due to epithelial cell swelling (Marshall, 2011; Marshall et al., 2000; Marshall et al., 2005). In this tissue, $I_{sc}$ in the opercular epithelium of

582 brackish and marine acclimated killifish is almost entirely due to $Cl^-$ secretion via the combined
583 actions of NKCC and CFTR (Karnaky et al., 1977).
584 The goal was to assess the influence of polyamines on the $Cl^-$-mediated short circuit current
585 ($I_{sc}$) before and after hypotonic exposure. The hypothesis was that polyamines would enhance the
586 ability of hypotonic exposure to reduce $I_{sc}$, which in a whole animal would attenuate the
587 diffusive loss of $Cl^-$ and presumably enhance freshwater tolerance. As observed in previous
588 studies (Marshall et al., 2000), the $I_{sc}$ of opercular epithelium (i.e., NaCl secretion) was reduced
589 by approximately 60%-70% within minutes of transfer from isotonic to hypotonic conditions
590 (Fig. 6). Despite the expectation that polyamines would actually stimulate a further reduction in
591 $I_{sc}$ with hypotonic exposure, polyamines inhibited the hypotonically-induced attenuation of $I_{sc}$
592 (Fig. 7A) and $G_t$ (Fig. 7 B). Surprisingly, these data suggest that polyamines function to inhibit
593 the ability of the MRCs in fish epithelia to reduce active $Cl^-$ secretion during hypotonic exposure.
594 Additional experiments suggested that this effect of polyamines was mediated almost exclusively
595 by micromolar concentrations of extracellular spermidine. These data suggest that polyamines
596 function to inhibit the ability of the MRCs in fish epithelia to reduce active $Cl^-$ secretion during
597 hypotonic exposure. This seemingly paradoxical response on isolated opercular epithelia was
598 independently validated in whole animal experiments in which endogenous polyamine
599 production during hypoosmotic exposure was blocked by administration of DFMO. It was found
600 that when polyamine concentrations were significantly reduced, even following freshwater
601 transfer, there was actually less of a reduction in the osmolality, and $Na^+$ and $Cl^-$ concentrations
602 of plasma of fish transferred to 0.1 ppt water. In other words, polyamines enhanced rather than
603 reduced the osmolyte imbalance during hypoosmotic shock.
604 Many different mechanisms could probably function to reduce the hypotonic response in the
605 opercular epithelium. Polyaminated polyamines such as spermine are strong agonists of the
606 calcium-sensing receptor (CaSR) and produce a transient peak and sustained rise in intracellular
607 $Ca^{2+}$ (Quinn et al., 1997). Previous studies have shown that the CaSR can regulate fluid
608 secretion in rat colonic crypts by increasing intracellular D-myo-inositol 1,4,5-trisphosphate
609 (IP3), which stimulates an increase in intracellular $Ca^{2+}$ (Cheng et al., 2004). Extracellular
610 spermine and external $Ca^{2+}$ appear to modulate the sensitivity of the colonic epithelial CaSR to
611 $Ca^{2+}$ stimulated NKCC and CFTR mediated $Cl^-$ secretion. CaSR is expressed in gill and other
612 fish epithelia, where it is thought to participate in systemic calcium homeostasis and

613 intracellular ion sensing (Loretz, 2008), and recently as a mechanism for protecting against $Ca^{2+}$-
614 induced cytotoxicity (Gu et al., 2014). In killifish, the hypotonic response in the gills and
615 opercular epithelium results in an increase in intracellular $Ca^{2+}$ mediated through an alpha-2
616 adrenergic pathway independent of adenylyl cyclase (e.g. (Marshall et al., 1993)). However,
617 unlike in mammalian systems, this hypotonically-induced rise in intracellular $Ca^{2+}$ inhibits rather
618 than increases NaCl secretion. Although a link between polyamines, CaSR, intracellular $Ca^{2+}$,
619 and suppression of active ion secretion in the gills and opercular epithelium of fish during
620 hypotonic stress in not known, it is possible that polyamines prolong the survival of SW type
621 ionocytes in very dilute environments. DMFO may reduce the immediate effects of polyamines,
622 thus blunting hypotonic inhibition of NaCl secretion and may lead to early apoptosis of seawater
623 ionocytes and their replacement by FW-type ionocytes. It would be worth considering whether
624 polyamines interfere with $Ca^{2+}$ responses either by blocking some downstream response of
625 intracellular signaling or by reducing the rise in intracellular $Ca^{2+}$ during hypotonic shock.
626 In the long term, induction of polyamine synthesis during hypotonic exposure may facilitate
627 apoptosis of SW type MRCs and result in their replacement by FW type MRCs. Polyamines may
628 stimulate cell apoptosis and cell proliferation with the goal of remodeling the gill, especially in
629 MRCs. However, in the short term, polyamines appear to inhibit the ability of hypotonic cell
630 swelling to attenuate NaCl secretion in the operculum. Further studies are needed to determine
631 the role of polyamines in cell volume regulation, gill remodeling, or in osmotic sensing.

**Table 1**

Sequences of forward and reverse primers used for amplification of *F. grandis, c-myc, c-fos, arg II, odc,* and *18s rRNA* amplicons for quantitative RT-PCR analysis.

| Name | Direction | Sequence 5'→3' |
|---|---|---|
| *c-myc* | Forward | TGCTTGTGCCTCTCACCAGTTCTA |
| *c-myc* | Reverse | AGCCTTCGAATCCTCCACGTTCTT |
| *c-fos* | Forward | ATCTGACAGCATCAAGTGCCTCCT |
| *c-fos* | Reverse | AGGTCTGGACGTTGCAGACTTCAT |
| *argII* | Forward | CAGCTGTGGTTACTGCATTGCGTT |
| *argII* | Reverse | ACAGGAAGCAACAAACCAGCACAG |
| *odc* | Forward | TTGCCACGCCAATGTGGTCTAA |
| *odc* | Reverse | ATGCTCCTGTCAAGTAACTGGCCT |
| *18s rRNA* | Forward | TTCCGATAACGAACGAGAC |
| *18s rRNA* | Reverse | GACATCTAAGGGCATCACAG |

637 Table 2 The total gill polyamine concentrations, which represent the sum concentrations of gill
638 putrescine, spermidine, and spermine, of DFMO-injected and PBS-injected fish relative to the
639 simultaneous controls at 6 h to 7 d post-transfer to 5 ppt or 0.1 ppt water (nmol/g gill).
640

| | 5ppt-PBS | 5ppt-DFMO | 0.1ppt-PBS | 0.1ppt-DFMO | *F* | *P* |
|---|---|---|---|---|---|---|
| 6 h | 76.14 ±2.08 | 52.13 ±1.99* | 94.25 ±10.89 | 63.54 ±2.41$^{\Delta\square}$ | 9.981 | 0.005 |
| 1 d | 73.13 ±2.56 | 44.13 ±1.70* | 119.05 ±3.07$^{\&}$ | 60.50 ±4.98$^{\Delta\square}$ | 94.815 | <0.001 |
| 3 d | 75.56 ±2.74 | 46.53 ±5.91* | 110.95 ±10.45$^{\&}$ | 50.18 ±4.08$^{\Delta\square}$ | 20.995 | 0.001 |
| 7 d | 75.32 ±0.88 | 20.73 ±1.17* | 97.89 ±11.71$^{\&}$ | 35.28 ±2.49$^{\Delta\square}$ | 3.421 | <0.001 |
| *F* | 3.339 | 4.275 | 3.837 | 20.811 | | |
| *P* | 0.846 | <0.001 | 0.038 | <0.001 | | |

641
642 *$P$<0.05 compared to the 5ppt-PBS injection (sham control) for that time point, $^{\Delta}$ $P$<0.05
643 compared to the 0.1ppt PBS injection group for that time point; $^{\&}$ $P$<0.05 compared to the 5ppt-
644 PBS injection group for that time point; $^{\square}P$<0.05 compared to the 5ppt-PBS injection group for
645 that time point

**Fig. 1.** Plasma osmolality (n = 6) in *F. grandis* acclimated to 5 ppt water then sampled at pre-transfer or at 6 h, 1 d, 3 d, and 7 d following acute transfer to 0.1, 0.5, 1, 2, and 5 ppt water. The vertical dashed line divides pre-transfer and post-transfer time points. Data represent the mean ± SEM, mOsM/kg Asterisks indicate a significant difference ($P \leq 0.05$) in comparison to the 5 ppt treatment for that time point; different letters indicate a significant difference ($P \leq 0.05$) in comparison to the pre-transfer for a given salinity.

**Fig. 2.** Representative scanning electron micrographs of the afferent filamental epithelium of *F. grandis* gills after acclimation to 5 ppt and acute transfer to 0.1, 0.5, 1, 2, or 5 ppt water at 6 h, 1 d, 3 d, and 7 d post-transfer. Asterisks indicate pavement cells, solid white arrows point to seawater-type mitochondrion-rich cells (MRCs), and dashed white arrows point to freshwater-type MRCs. Scale bar = 5 μm.

**Fig. 3.** Relative *arg II* and *odc* mRNA levels in mitochondrion-rich cells (MRCs) and pavement cells (PVCs) in gills of fish acclimated to 5 ppt, then acutely transferred to 5 ppt or 0.1 ppt water for 1 d. The mRNA levels were normalized to the mean value of expression in PVCs from 5 ppt-acclimated fish. Data represent the mean ± SEM (n =6). Different letters indicate a significant difference ($P \leq 0.05$) in comparison to the 5 ppt treatment for the same cell type; asterisks indicate a significant difference ($P \leq 0.05$) in comparison to PVCs for that salinity.

**Fig. 4.** Plasma osmolality (mOsM/kg) (A), plasma sodium concentration (mM) (B), and plasma chloride concentration (mM) (C) in *F. grandis* acclimated to 5 ppt and sampled at pre-transfer or at 6 h, 1 d, 3 d, and 7 d following acute transfer to 0.1 ppt or 5 ppt water. Fish received intraperitoneal injections of DFMO or PBS (sham control) (*see text for details*). The vertical dashed line divides pre-transfer and post-transfer. Data represent the mean ± SEM (n =6). Asterisks indicate a significant difference ($P \leq 0.05$) in comparison to the 5 ppt PBS treatment for that time point; letters indicate a significant difference ($P \leq 0.05$) in comparison to the pre-treatment for a given salinity.

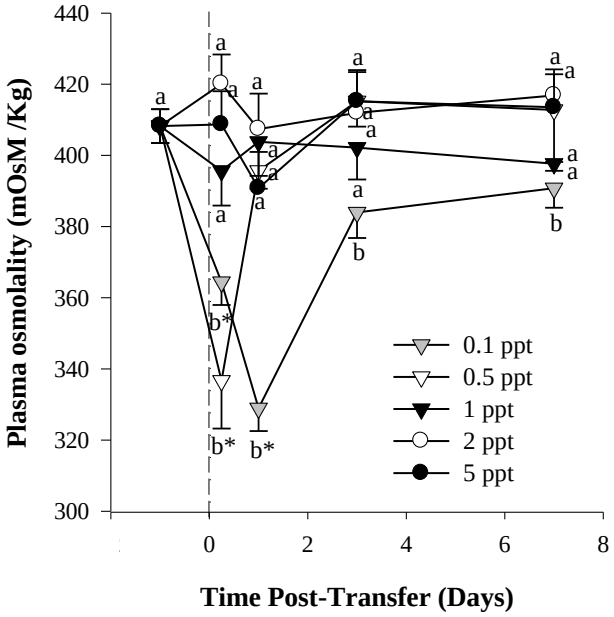
**Fig. 5.** Relative *c-fos* (A) and *c-myc* (B) mRNA levels (relative to the pre-transfer levels), and caspase-3 activity without DFMO application (C) and with DFMO application (D). The vertical

dashed line divides pre-transfer and post-transfer. Data represent the mean ± SEM (n =6). Asterisks indicate significant differences ($P \leq 0.05$) in comparison to the 5 ppt PBS treatment for that time point; letters indicate a significant difference ($P \leq 0.05$) in comparison to the pre-treatment for different time points.

**Fig. 6.** Percent change (relative to baseline isotonic conditions) in the short circuit current, $I_{sc}$ (A) and membrane conductance, $G_t$ (B) of opercular epithelia kept in symmetrical conditions to isotonic (Iso) (n =10) or hypotonic (Hypo) solutions (n =10). The vertical dashed line represents the transfer between these two media. Controls represent those epithelia receiving no administration of the polyamine mixture. Asterisks represent significant differences from hypotonic control exposures at the same time points ($P \leq 0.05$). A polyamine mixture (0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine) was added to the basolateral side of opercular epithelium, either a) coincident with transfer from isotonic to hypotonic conditions (pre-PA), or b) following initial hypotonic transfer and establishment of a steady-state $I_{sc}$ under hypotonic conditions (post-PA).

**Fig. 7.** Percent change (relative to baseline isotonic conditions) in short circuit current, $I_{sc}$ (A) and membrane conductance, $G_t$ (B) of the opercular epithelium in isotonic (Iso) or hypotonic (Hypo) solutions. The vertical dashed line represents the transfer between these two media. Controls represent those epithelia receiving no addition of polyamines. Data represent the mean ± SEM (n =6). Single asterisks represent significant differences from hypotonic challenges at the same time points ($P \leq 0.05$); double asterisks represent significant differences from hypotonic challenges at the same time points ($P \leq 0.01$).

701



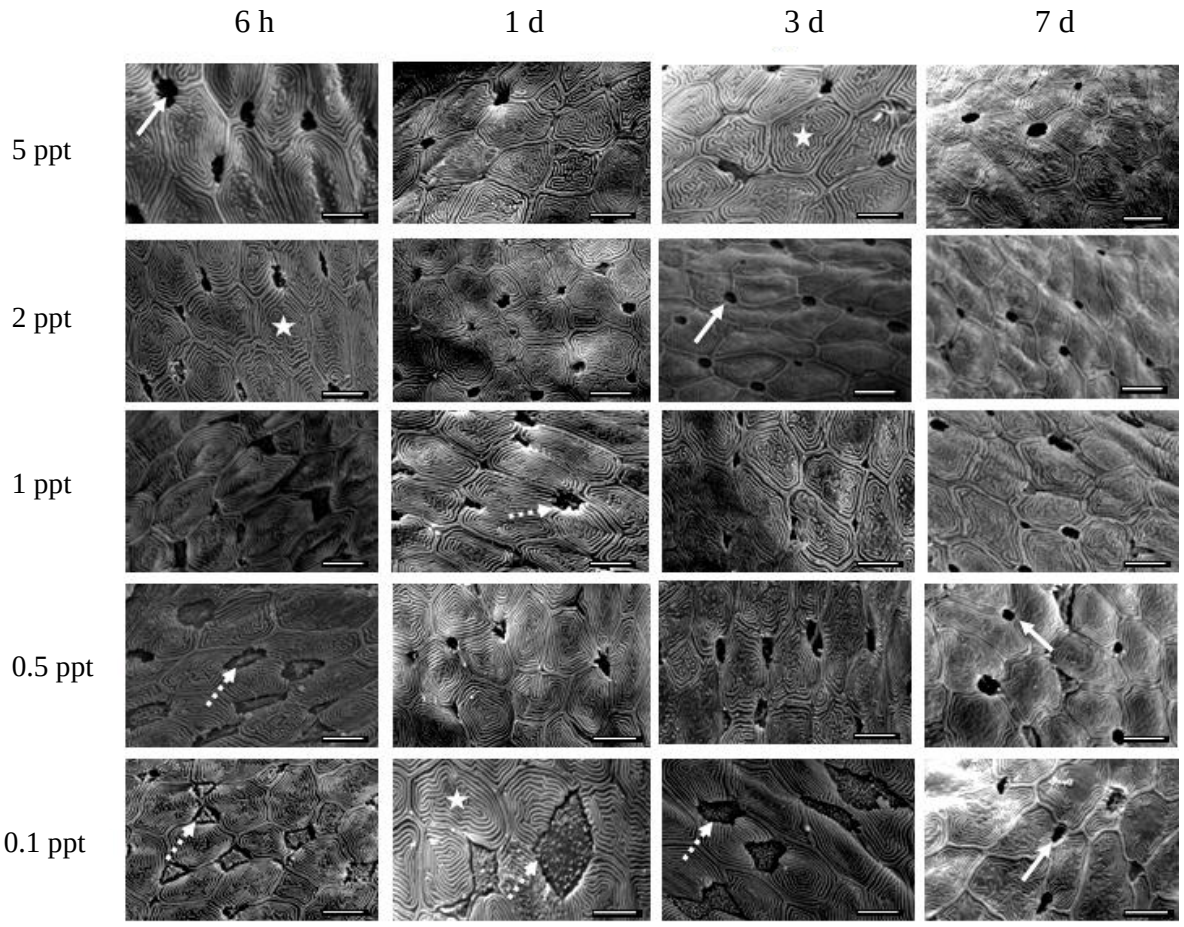

702

703

704 Fig.1

705

706

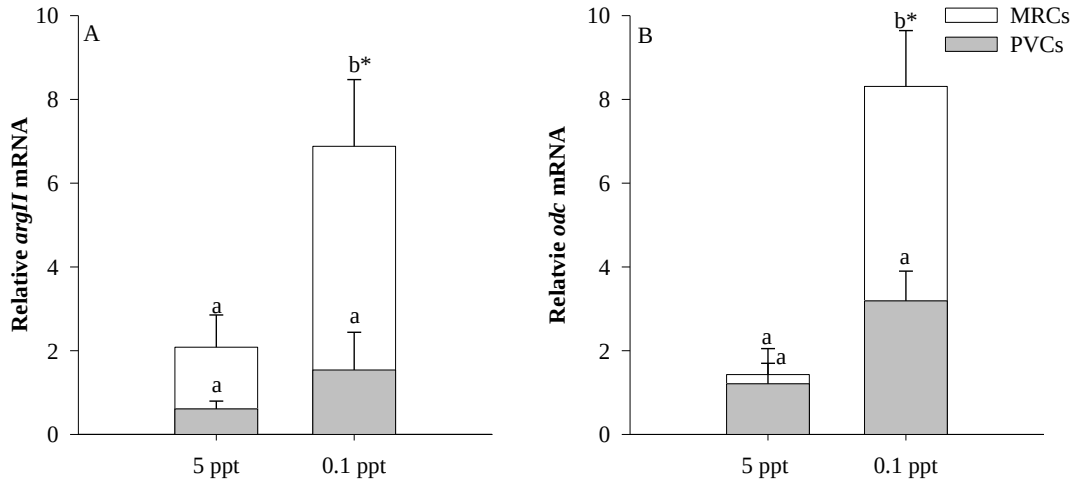



707

708 Fig.2

709

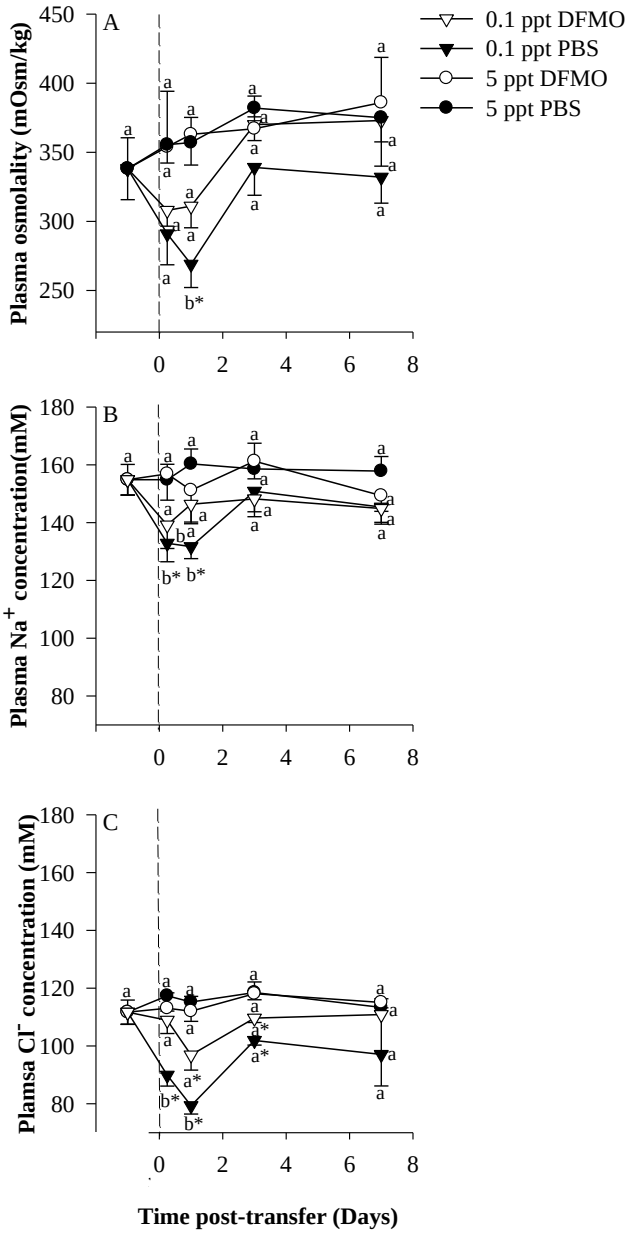
710




711

712 Fig.3

713




714

715

716 Fig.4

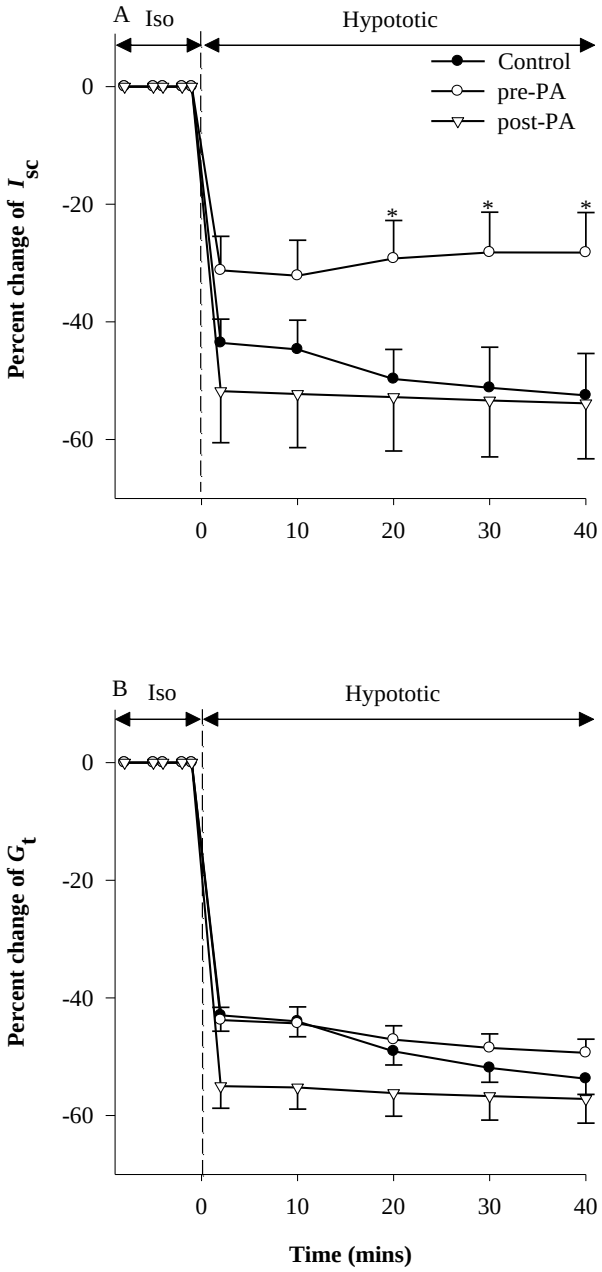
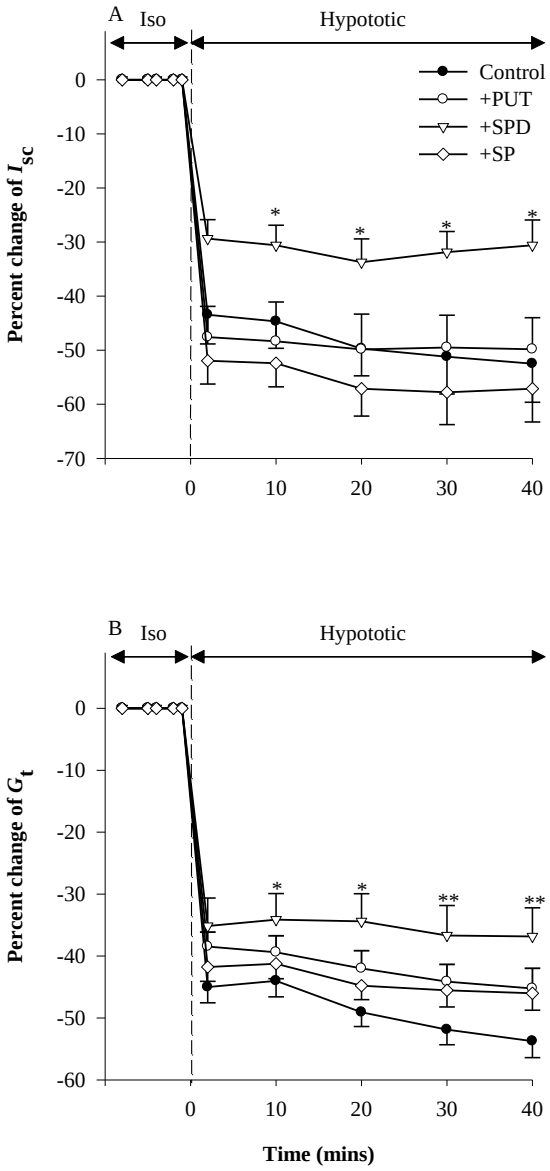
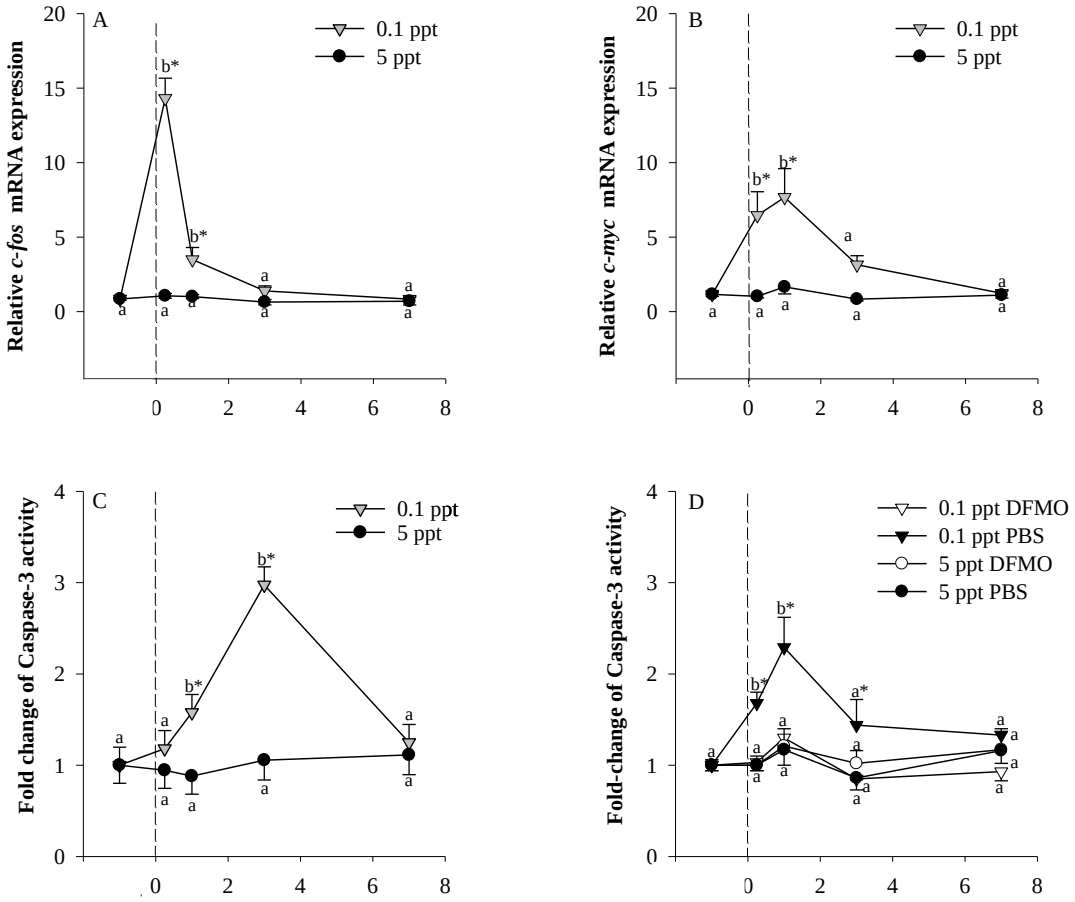

A
Iso
Hypototic
Control
pre-PA
post-PA
Percent change of $I_{sc}$
0
-20
-40
-60
*
*
*
0
10
20
30
40
B
Iso
Hypototic
Percent change of $G_t$
0
-20
-40
-60
0
10
20
30
40
Time (mins)

Fig.5


A
Iso
Hypotonic
Control
+PUT
+SPD
+SP
Percent change of $I_{sc}$
0
-10
-20
-30
-40
-50
-60
-70
0
10
20
30
40
*
B
Iso
Hypotonic
Percent change of $G_t$
0
-10
-20
-30
-40
-50
-60
*
**
Time (mins)

720

721 Fig.6


A
0.1 ppt
5 ppt
Relative c-fos mRNA expression
B
Relative c-myc mRNA expression
C
Fold change of Caspase-3 activity
D
0.1 ppt DFMO
0.1 ppt PBS
5 ppt DFMO
5 ppt PBS
Fold-change of Caspase-3 activity
Time Post-Transfer (Days)

722

723 Fig.7

## References:


Brennan, R.S., Galvez, F., Whitehead, A., 2015. Reciprocal osmotic challenges reveal mechanisms of divergence in phenotypic plasticity in the killifish *Fundulus heteroclitus*. J. Exp. Biol. 218, 1212-1222.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Gomez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., MacLatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn, R.N., Crawford, D.L., 2007. *Fundulus* as the premier teleost model in environmental biology: Opportunities for new insights using genomics. Comp. Biochem. Physiol. D 2, 257-286.

Cheng, S.X., Geibel, J.P., Hebert, S.C., 2004. Extracellular polyamines regulate fluid secretion in rat colonic crypts via the extracellular calcium-sensing receptor. Gastroenterology 126, 148-158.

Copeland, D.E., 1950. Adaptive behaviour of the chloride cell in the gill of *Fundulus heteroclitus*. J. Morphol. 87, 369-379.

Daborn, K., Cozzi, R.R.F., Marshall, W.S., 2001. Dynamics of pavement cell-chloride cell interactions during abrupt salinity change in *Fundulus heteroclitus*. J. Exp. Biol. 204, 1889-1899.

Deng, W., Viar, M.J., Johnson, L.R., 2005. Polyamine depletion inhibits irradiation-induced apoptosis in intestinal epithelia. Am. J. Physiol.- Gastro LIver Physiol. 289, G599-606.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97-177.

Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J., Pfaffl, M.W., 2006. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnol. Letters 28, 1601-1613.

Galvez, F., Reid, S.D., Hawkings, G., Goss, G.G., 2002. Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. Am. J. Physiol. Reg. I. 282, R658-668.

Goss, G.G., Laurent, P., Perry, S.F., 1994. Gill morphology during hypercapnia in brown bullhead (*Ictalurus nebulosus*)- role of chloride cells and pavement cells in acid base regulation. J. Fish Biol. 45, 705-718.

Griffith, R.W., 1974. Environment and salinity tolerance in the Genus *Fundulus*. Copeia 319-331.

Gu, J., Law, A.Y.S., Yeung, B.H.Y., Wong, C.K.C., 2014. Activation of gill $Ca^{2+}$-sensing receptor as a protective pathway to reduce $Ca^{2+}$-induced cytotoxicity. J. Mol. Endocrinol. 53, 155-164.

Hiroi, J., McCormick, S.D., 2012. New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. Respir. Physiol. Neurobiol. 184, 257-268.

Kaneko, T., Katoh, F., 2004. Functional morphology of chloride cells in killifish *Fundulus heteroclitus*, a euryhaline teleost with seawater preference. Fisheries Science 70, 723-733.

Karnaky, K.J., Degnan, K.J., Zadunaisky, J.A., 1977. Chloride transport across isolated opercular epithelium of killifish - membrane rich in chloride cells. Science 195, 203-205.

Katoh, F., Hasegawa, S., Kita, J., Takagi, Y., Kaneko, T., 2001. Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*. Can. J. Zool. 79, 822-829.

Katoh, F., Kaneko, T., 2003. Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique. J. Exp. Biol. 206, 4113-4123.

Katoh, F., Shimizu, A., Uchida, K., Kaneko, T., 2000. Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*. Zool. Sci. 17, 11-18.

Kelly, S.P., Fletcher, M., Part, P., Wood, C.M., 2000. Procedures for the preparation and culture of 'reconstructed' rainbow trout branchial epithelia. Methods Cell Sci. 22, 153-163.

Kidder, G.W., Petersen, C.W., Preston, R.L., 2006. Energetics of osmoregulation: II. Water flux and osmoregulatory work in the euryhaline fish, *Fundulus heteroclitus*. J. Exp. Zool. A Comp. Exp. Biol. 305, 318-327.

Krumschnabel, G., Podrabsky, J.E., 2009. Fish as model systems for the study of vertebrate apoptosis. Apoptosis 14, 1-21.

Laurent, P., Chevalier, C., Wood, C.M., 2006. Appearance of cuboidal cells in relation to salinity in gills of *Fundulus heteroclitus*, a species exhibiting branchial $Na^+$ but not $Cl^-$ uptake in freshwater. Cell Tissue Res. 325, 481-492.

Lee, K.C., Goh, W.L.P., Xu, M., Kua, N., Lunny, D., Wong, J.S., Coomber, D., Vojtesek, B., Lane, E.B., Lane, D.P., 2008. Detection of the p53 response in zebrafish embryos using new monoclonal antibodies. Oncogene 27, 629-640.

Loretz, C.A., 2008. Extracellular calcium-sensing receptors in fishes. Comp. Biochem. Physiol. A 149, 225-245.

Lu, G., Mak, Y.T., Wai, S.M., Kwong, W.H., Fang, M.R., James, A., Randall, D., Yew, D.T., 2005. Hypoxia-induced differential apoptosis in the central nervous system of the sturgeon (*Acipenser shrenckii*). Microsc. Res. Tech. 68, 258-263.

Marshall, W.S., 2003. Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. Biochimica Biophysica Acta- Biomembranes 1618, 95-105.

Marshall, W.S., 2011. Mechanosensitive signalling in fish gill and other ion transporting epithelia. Acta Physiol. 202, 487-499.

Marshall, W.S., Bryson, S.E., 1998. Transport mechanisms of seawater teleost chloride cells: An inclusive model of a multifunctional cell. Comp. Biochem. Physiol. A 119, 97-106.

Marshall, W.S., Bryson, S.E., Garg, D., 1993. Alpha-2-adrenergic inhibition of $Cl^-$ transport by opercular epithelium is mediated by intracellular $Ca^{2+}$. Proc. Natl. Acad. Sci. U. S. A. 90, 5504-5508.

Marshall, W.S., Bryson, S.E., Luby, T., 2000. Control of epithelial $Cl^-$ secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. J. Exp. Biol. 203, 1897-1905.

Marshall, W.S., Emberley, T.R., Singer, T.D., Bryson, S.E., McCormick, S.D., 1999. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. J. Exp. Biol. 202, 1535-1544.

Marshall, W.S., Grosell, M., 2005. Ion transport, osmoregulation, and acid-base balance, in: D.H. Evans, J.B. Claiborne (Eds.), Physiology of Fishes. CRC Press, Boca Raton, FL, 177-230.

Marshall, W.S., Ossum, C.G., Hoffmann, E.K., 2005. Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. J. Exp. Biol. 208, 1063-1077.

Morris, S.M., Jr, 2004. Enzymes of arginine metabolism. J. Nutr. 134, S2743-2747.

Muscari, C., Bonafe, F., Stanic, I., Flamigni, F., Stefanelli, C., Farruggia, G., Guarnieri, C., Caldarera, C.M., 2005. Polyamine depletion reduces TNFalpha/MG132-induced apoptosis in bone marrow stromal cells. Stem Cells 23, 983-991.

Patrick, M.L., Wood, C.M., Marshall, W.S., 1997. Calcium regulation in the freshwater-adapted *mummichog*. J. Fish Biol. 51, 135-145.

Pegg, A.E., McCann, P.P., 1982. Polyamine metabolism and function. Am. J. Physiol. Cell Ph. 243, C212-221.

Perry, S.F., Goss, G.G., Fenwick, J.C., 1992. Interrelationships between gill chloride cell morphology and calcium uptake in freshwater teleosts. Fish Physiol. Biochem. 10, 327-337.

Peter, H.W., Ahlers, J., Gunther, T., 1978. Dependence of polyamine and phospholipid contents in marine-bacteria on osmotic strength of medium. Int. J. Biochem. 9, 313-316.

Poulin, R., Wechter, R.S., Pegg, A.E., 1991. An early enlargement of the putrescine pool is required for growth in L1210 mouth leukemia cells under hypoosmotic stress. J. Biol. Chem. 266, 6142-6151.

Quinn, S.J., Ye, C.P., Diaz, R., Kifor, O., Bai, M., Vassilev, P., Brown, E., 1997. The $Ca^{2+}$-sensing receptor: a target for polyamines. Am. J. Physiol. Cell Physiol. 273, C1315-1323.

Ray, R.M., Viar, M.J., Yuan, Q., Johnson, L.R., 2000. Polyamine depletion delays apoptosis of rat intestinal epithelial cells. Am. J. Physiol. Cell Physiol. 278, 480-489.

824 Ray, R.M., Zimmerman, B.J., McCormack, S.A., Patel, T.B., Johnson, L.R., 1999. Polyamine depletion
825 arrests cell cycle and induces inhibitors p21(Waf1/Cip1), p27(Kip1), and p53 in IEC-6 cells. Am. J.
826 Physiol. Cell Physiol. 276, C684-691.
827 Reis, M.I.R., Nascimento, D.S., do Vale, A., Silva, M.T., dos Santos, N.M.S., 2007. Molecular cloning
828 and characterisation of sea bass (*Dicentrarchus labrax* L.) caspase-3 gene. Mol. Immunol. 44, 774-
829 783.
830 Rojo, C., Gonzalez, E., 1999. Ontogeny and apoptosis of chloride cells in the gill epithelium of newly
831 hatched rainbow trout. Acta Zoologica 80, 11-23.
832 Scott, G.R., Baker, D.W., Schulte, P.M., Wood, C.M., 2008. Physiological and molecular mechanisms of
833 osmoregulatory plasticity in killifish after seawater transfer. J. Exp. Biol. 211, 2450-2459.
834 Scott, G.R., Rogers, J.T., Richards, J.G., Wood, C.A., Schulte, P.M., 2004. Intraspecific divergence of
835 ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of
836 freshwater adaptation. J. Exp. Biol. 207, 3399-3410.
837 Scott, G.R., Schulte, P.M., 2005. Intraspecific variation in gene expression after seawater transfer in gills
838 of the euryhaline killifish *Fundulus heteroclitus*. Comp. Biochem. Physiol. A 141, 176-182.
839 Scott, G.R., Schulte, P.M., Wood, C.M., 2006. Plasticity of osmoregulatory function in the killifish
840 intestine: drinking rates, salt and water transport, and gene expression after freshwater transfer. J. Exp.
841 Biol. 209, 4040-4050.
842 Tabib, A., Bachrach, U., 1994. Activation of the protooncogene c-myc and c-fos by c-ras involvement of
843 polyamines. Biochem. Biophys. Res. Commun. 202, 720-727.
844 Tabor, C.W., Tabor, H., 1984. Polyamines. Annu. Rev. Biochem. 53, 749-790.
845 Takle, H., McLeod, A., Andersen, O., 2006. Cloning and characterization of the executioner caspases 3, 6,
846 7 and Hsp70 in hyperthermic Atlantic salmon (*Salmo salar*) embryos. Comp. Biochem. Physiol. B
847 144, 188-198.
848 Tantini, B., Fiumana, E., Cetrullo, S., Pignatti, C., Bonavita, F., Shantz, L.M., Giordano, E., Muscari, C.,
849 Flamigni, F., Guarnieri, C., Stefanelli, C., Caldarera, C.M., 2006. Involvement of polyamines in
850 apoptosis of cardiac myoblasts in a model of simulated ischemia. J. Mol. Cell. Cardiol. 40, 775-782.
851 Uthaiwan, K., Chatchavalvanich, K., Noparatnaraporn, N., Machado, J., 2001. Scanning electron
852 microscopy of glochidia and juveniles of the freshwater mussel, *Hyriopsis myersiana*. Invertebr.
853 Reprod. Dev. 40, 143-151.
854 Watts, S.A., Yeh, E.W., Henry, R.P., 1996. Hypoosmotic stimulation of ornithine decarboxylase activity
855 in the brine shrimp *Artemia franciscana*. J. Exp. Zool. 274, 15-22.
856 Whitehead, A., Galvez, F., Zhang, S.J., Williams, L.M., Oleksiak, M.F., 2011a. Functional genomics of
857 physiological plasticity and local adaptation in killifish. J. Hered. 102, 499-511.

Whitehead, A., Roach, J.L., Zhang, S., Galvez, F., 2011b. Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. Proc. Natl. Acad. Sci. U. S. A. 108, 6193-6198.

Whitehead, A., Roach, J.L., Zhang, S.J., Galvez, F., 2012. Salinity- and population-dependent genome regulatory response during osmotic acclimation in the killifish (*Fundulus heteroclitus*) gill. J. Exp. Biol. 215, 1293-1305.

Wongyai, S., Oefner, P., Bonn, G., 1988. HPLC analysis of polyamines and their acetylated derivatives in the picomole range using benzoyl chloride and 3,5-dinitrobenzoyl chloride as derivatizing agent. Biomed. Chromatogr. 2, 254-257.

Wood, C.M., Grosell, M., 2008. A critical analysis of transepithelial potential in intact killifish (*Fundulus heteroclitus*) subjected to acute and chronic changes in salinity. J. Comp. Physiol. B 178, 713-727.

Wood, C.M., Grosell, M., 2009. TEP on the tide in killifish (*Fundulus heteroclitus*): effects of progressively changing salinity and prior acclimation to intermediate or cycling salinity. J. Comp. Physiol. B 179, 459-467.

Wood, C.M., LeMoigne, J., 1991. Intracellular acid-base responses to environmental hyperoxia and normoxic recovery in rainbow trout. Resp. Physiol. 86, 91-113.

Yamashita, M., Mizusawa, N., Hojo, M., Yabu, T., 2008. Extensive apoptosis and abnormal morphogenesis in pro-caspase-3 transgenic zebrafish during development. J. Exp. Biol. 211, 1874-1881.

Zall, D.M., Fisher, D., Garner, M.Q., 1956. Photometric determination of chlorides in water. Anal. Chem. 28, 1665-1668.

Zapata, P.J., Serrano, M., Pretel, A.T., Amoros, A., Botella, M.A., 2004. Polyamines and ethylene changes during germination of different plant species under salinity. Plant Science 167, 781-788.