

RESEARCH ARTICLE

Tilapia (*Oreochromis mossambicus*) brain cells respond to hyperosmotic challenge by inducing *myo*-inositol biosynthesis

Alison M. Gardell^{1,2,*}, Jun Yang^{2,3}, Romina Sacchi¹, Nann A. Fangué⁴, Bruce D. Hammock^{2,3} and Dietmar Kültz^{1,2}

¹Department of Animal Science, ²NIEHS Superfund Research Program, ³Department of Entomology and UCDMC Comprehensive Cancer Center and ⁴Wildlife, Fish, and Conservation Biology, University of California Davis, One Shields Avenue, Davis, CA 95616, USA

*Author for correspondence (amgardell@ucdavis.edu)

SUMMARY

This study aimed to determine the regulation of the *de novo myo*-inositol biosynthetic (MIB) pathway in Mozambique tilapia (*Oreochromis mossambicus*) brain following acute (25 ppt) and chronic (30, 60 and 90 ppt) salinity acclimations. The MIB pathway plays an important role in accumulating the compatible osmolyte, *myo*-inositol, in cells in response to hyperosmotic challenge and consists of two enzymes, *myo*-inositol phosphate synthase and inositol monophosphatase. In tilapia brain, MIB enzyme transcriptional regulation was found to robustly increase in a time (acute acclimation) or dose (chronic acclimation) dependent manner. Blood plasma osmolality and Na⁺ and Cl⁻ concentrations were also measured and significantly increased in response to both acute and chronic salinity challenges. Interestingly, highly significant positive correlations were found between MIB enzyme mRNA and blood plasma osmolality in both acute and chronic salinity acclimations. Additionally, a mass spectrometry assay was established and used to quantify total *myo*-inositol concentration in tilapia brain, which closely mirrored the hyperosmotic MIB pathway induction. Thus, *myo*-inositol is a major compatible osmolyte that is accumulated in brain cells when exposed to acute and chronic hyperosmotic challenge. These data show that the MIB pathway is highly induced in response to environmental salinity challenge in tilapia brain and that this induction is likely prompted by increases in blood plasma osmolality. Because the MIB pathway uses glucose-6-phosphate as a substrate and large amounts of *myo*-inositol are being synthesized, our data also illustrate that the MIB pathway likely contributes to the high energetic demand posed by salinity challenge.

Key words: osmoregulation, compatible osmolyte, *myo*-inositol, brain, tilapia.

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INTRODUCTION

The accumulation of osmolytes, specifically ‘compatible’ osmolytes, is a key regulatory mechanism utilized by cells to compensate for hyperosmolality (Yancey et al., 1982). Compatible osmolytes are small, organic molecules that are concentrated inside of cells in order to restore osmotic equilibrium (Burg et al., 2007; Hoffmann et al., 2009). *myo*-Inositol, a polyol, is one example of a compatible osmolyte (Parthasarathy et al., 2006; Yancey et al., 1982). The use of *myo*-inositol in countering the negative effects of hyperosmolality is ubiquitous across phyla ranging from plants to animals (Burg and Ferraris, 2008). Various molecular pathways relating to osmolyte accumulation, including cotransporter activity and biosynthesis enzymes, have been shown to be induced in order to accumulate *myo*-inositol within a number of different cell types of various tissue origins (Fiess et al., 2007; Hasegawa and Eisenberg, 1981; Law, 1994; Strange et al., 1991).

In fishes, the *myo*-inositol biosynthesis pathway (MIB) has been demonstrated to play an important role in *myo*-inositol accumulation in osmoregulatory tissues in response to hyperosmotic challenge. The MIB pathway utilizes two enzymes, *myo*-inositol phosphate synthase (MIPS) and inositol monophosphatase (IMPA), to generate *myo*-inositol endogenously from glucose-6-phosphate (G-6-P) in a two-step reaction (Geiger and Jin, 2006). The study by Fiol et al. (Fiol et al., 2006b) was the first to identify upregulation of MIPS

mRNA in response to acute hyperosmotic challenge in tilapia gill, and since then, several studies have confirmed induction of the MIB pathway in other euryhaline species exposed to salinity challenge (Evans and Somero, 2008; Kalujnaia et al., 2009; Kalujnaia et al., 2010; Kalujnaia et al., 2013). In mammals, IMPA has at least two isoforms, originating from different genes (Ohnishi et al., 2007; Shamir et al., 2001), while MIPS can have alternative splice variants (Seelan et al., 2009) derived from the same gene. Interestingly, differential expression of isoforms following gene duplication events may have evolved in animals that are frequently exposed to variable environments (Schulte, 2004) as an adaptive mechanism for responding to environmental challenges, as seen in fishes. For example, Richards et al. (Richards et al., 2003) found that sodium potassium ATPase isoforms are differentially regulated in rainbow trout gill following acclimation to various salinities. It has not yet been investigated whether IMPA isoforms or MIPS splice variants are differentially regulated by environmental salinity in fishes.

The fish brain, particularly the hypothalamus, and the neighboring pituitary gland serve important roles in maintaining osmotic homeostasis in the whole organism (Bernier et al., 2009). The specific regulatory mechanisms and signaling events involved in osmoreception and osmoregulation of the fish brain in response to hypo-osmotic stress have been extensively studied (Seale et al.,

2002; Seale et al., 2005; Seale et al., 2012b). For example, in euryhaline teleosts, prolactin has been identified as primary hormone utilized in freshwater acclimation in order to decrease water uptake and allow for the retention of ions (Bernier et al., 2009; Manzon, 2002). In contrast, the molecular mechanisms employed by fish brain cells in response to hyperosmotic challenge still require additional investigation. Although brain cells (primarily neurons and glia) are not directly exposed to changes in environmental salinity, these cells still encounter and respond to hyperosmotic challenge. Mammalian brain cells have evolved to be very sensitive to small changes in extracellular osmolality, which are thought to be perceived through changes in blood plasma osmolality (Davson and Segal, 1996). Interestingly, the organum vasculosum of the lamina terminalis in the mammalian brain is believed to serve as the primary 'osmostat' and provides feedback regulation for the osmoregulatory response (Bourque et al., 2007). The acute osmosensitivity of the mammalian brain is critical because increases in plasma osmolality outside of the normal range (~280–310 mOsm kg⁻¹) can have damaging consequences for brain function, including interference with the maintenance of cell volume, which is crucial for proper functioning of the central nervous system (Bourque et al., 2007; Strange, 1992).

Teleost fishes have evolved to regulate their blood plasma osmolality around the set point of 300 mOsm kg⁻¹; however, rapid changes (+100 mOsm kg⁻¹) in plasma osmolality are known to occur after salinity acclimation (Baldisserotto et al., 2007). Interestingly, the baseline plasma osmolality of saltwater-acclimated fish (~350 mOsm kg⁻¹) is higher than that of freshwater-acclimated fish (~310 mOsm kg⁻¹) (Evans and Claiborne, 2006; Seale et al., 2003). Although the brain is additionally protected by the blood–brain barrier (BBB), deviations in plasma osmolality are reflected rapidly through changes in volume of the extracellular fluid surrounding brain cells due to water movement across the BBB (Abbott et al., 2010). This increase in extracellular osmolality induces water flux out of brain cells to the surrounding fluid through the semi-permeable cell membrane. To avoid prolonged cell shrinkage, brain cells employ regulatory volume increase by temporarily accumulating inorganic ions (e.g. Na⁺, Cl⁻) within the cell *via* various membrane transport systems and ion channels (Lang et al., 1998; Wehner et al., 2003). These damaging ions are rapidly (hours to days) replaced by compatible osmolytes, which do not disrupt macromolecular structure and function when accumulated at high concentrations (Burg et al., 2007; Hochachka and Somero, 2002). Volume regulation has been documented as a response of fish cells to osmotic challenge, with more studies focusing on regulatory volume decrease in response to hypo-osmotic challenge (Chara et al., 2011).

Mozambique tilapia [*Oreochromis mossambicus* (Peters 1852)], a euryhaline teleost, is a commonly used species for studying elements of the osmotic stress response. *Oreochromis mossambicus* can tolerate a variety of salinities ranging from 0 to 120 ppt (Stickney, 1986). Many novel physiological and molecular mechanisms have evolved in tilapia in order to promote a high tolerance to saline and hyper-saline conditions, including the rapid induction of osmotic stress transcription factor and ubiquitin ligase (Fiol et al., 2006a; Fiol et al., 2006b; Fiol et al., 2011; Kültz et al., 2007). A previous study by Fiess et al. (Fiess et al., 2007) measured compatible osmolyte accumulation in multiple tissues of tilapia using high-performance liquid chromatography after acclimation to increased environmental salinity and temperature. The authors identified *myo*-inositol as a major osmolyte in tilapia brain and suggested that it may play a central role in responding to these environmental stressors (Fiess et al., 2007). However, the specific

biochemical pathway utilized for the observed *myo*-inositol accumulation in tilapia brain in response to hyper-salinity has not yet been investigated. Interestingly, *myo*-inositol has also been demonstrated to be important for dietary health in cultured juvenile tilapia, suggesting that the implications of this compatible osmolyte go beyond osmotic regulation (Shiau and Su, 2005). For example, inositol derivatives are important for the structure of many second messengers and are also involved with multiple cell signaling events (Downes and Macphee, 1990; Michell, 2008).

To gain mechanistic insight into how tilapia brain cells respond to increases in environmental salinity, this study investigated the osmotic regulation of the MIB pathway in brain and its relationship to brain *myo*-inositol levels and key blood chemistry parameters, including plasma osmolality.

MATERIALS AND METHODS

Animals

Sub-adult *O. mossambicus* weighing (57.7±3.4 g) were maintained in de-chlorinated freshwater at 25–27°C in 208 l recirculating tanks at the UC Davis Cole B Animal Facility. The room was kept on a 12 h:12 h light:dark photoperiod and fish were fed daily with a commercial trout pellet diet (Nelson's Sterling Silver Cup Fish Feeds, Murray, UT, USA) at ~1% of fish body mass. All animal procedures used in this study were approved by the UC Davis Institutional Animal Care and Use Committee (protocol nos 16604 and 16609).

Salinity acclimations

Acute

Oreochromis mossambicus were acclimated to 25 ppt with a single increase in salinity from freshwater conditions using Instant Ocean salt mix (Spectrum Brands, Blacksburg, VA, USA). This increment is approximately the maximum salinity tolerated during acute acclimation without showing signs of behavioral stress or mortality (Kammerer et al., 2010; Stickney, 1986). Three 76 l tanks were used to repeat the 25 ppt salinity treatment and two fish from each tank replicate (*N*=6 fish total) were randomly collected after 2, 4, 8, 16 and 24 h. The whole brain was quickly harvested from each fish and snap-frozen in liquid nitrogen.

Chronic

Oreochromis mossambicus were acclimated to 7.5 ppt day⁻¹ increases in salinity using Instant Ocean salt mix. This increment of salinity increase was selected because behavioral stress and/or mortality was generally not observed (A.M.G., unpublished observations). Daily salinity changes were made by quickly (~10 min) replacing 20% of the water with a hyperosmotic stock solution, and the final salinity was validated using a calibrated YSI Model 85 handheld dissolved oxygen, conductivity, salinity and temperature system (YSI, Yellow Springs, OH, USA). Three 76 l replicate tanks were used to repeat salinity increases, and fish (*N*=6) were sampled randomly after being held for 24 h at each of the three salinity end points (30, 60 and 90 ppt) following a 4, 8 and 12 day step-wise increment in salinity. The whole brain was quickly harvested from each fish and snap-frozen in liquid nitrogen.

Control

Oreochromis mossambicus for each of the three salinity end points were held at constant salinity (0.3 ppt) in three 76 l freshwater tank replicates. Control fish underwent identical water change procedures as described above in order to account for handling stress imposed during the acute and chronic salinity increases. All samples collected from fish (*N*=6 per time point) were kept at -80°C until analysis.

Blood chemistry analyses

Fish ($N=6$ per time point and salinity treatment) were anesthetized using a moderate blow to the head and then blood was collected immediately *via* caudal severance using heparinized capillary tubes. Capillary tubes were kept at 4°C and then centrifuged in a microhematocrit centrifuge (International Equipment Co., Needham, MA, USA) at 5000 *g* for 3 min. Percent hematocrit was measured using a hematocrit reader and plasma was then separated from red blood cells. Plasma was stored at -80°C until analyses.

Plasma osmolality (mOsm kg⁻¹) was measured on a vapor pressure osmometer (Wescor Vapro 5520, Logan, UT, USA). Sodium and potassium ion concentrations (mmol l⁻¹) were measured on a flame photometer using lithium as the internal standard (Instrumentation Laboratory 343, Bedford, MA, USA). Chloride concentration (mmol l⁻¹) was measured using a chloride titrator (Radiometer CMT10, Copenhagen, Denmark). Subsamples of blood plasma were run in technical duplicates and an average of the two values was reported.

mRNA expression assay

Reaction conditions

Quantitative real-time PCR (qRT-PCR) was performed in order to measure mRNA levels of MIPS alternative transcripts (MIPS-160, MIPS-250) and IMPA isoforms (IMPA1, IMPA2) as described previously by our laboratory (Fiol et al., 2006b; Sacchi et al., 2013). Briefly, total RNA was extracted from tilapia brain samples from both acute and chronic acclimations using the RNeasy Kit (Qiagen, Hilden, Germany). RNA was then treated with Turbo DNA-free (Life Technologies, Grand Island, NY, USA) in order to remove any residual DNA contamination. Samples were incubated with an inactivation reagent according to the manufacturer's (Life Technologies) instructions in order to inactivate the DNase enzyme. Complementary DNA (cDNA) was then synthesized by reverse transcribing 2 µg total RNA using Superscript III (Life Technologies) using a 1:1 mix of random hexamers and oligo(dT) as primers. Semi-quantitative PCR reactions were set up with tilapia-specific primers using synthesized cDNA as template. PCR amplicons were run on a 2% agarose gel stained with GelRed (Biotium, Hayward, CA, USA) to confirm expected PCR product size for a given gene. In order to optimize qRT-PCR reaction conditions, a standard curve was run for each gene primer pair (Table 1) to determine proper dilutions of cDNA. For IMPA1, different dilutions of control (freshwater) and treated (salinity-exposed) cDNA were run in order to obtain cycle threshold (C_t) values within an acceptable working range (C_t=18–32). Samples were run in duplicate on a 96-well plate using Sybr Green (Life Technologies) as the method of detection. β-actin and 18S rRNA, which do not significantly change in response to hyperosmotic challenge in tilapia brain, were used as reference genes.

qRT-PCR data analysis

Target C_t values for MIB enzymes were first normalized against the reference genes (β-actin and 18S rRNA) after correcting by efficiency using LinRegPCR software (Ruijter et al., 2009). C_t values were then converted to a fold-change value using the Pfaffl method (Pfaffl, 2001). A dilution factor was applied to calculated fold changes in IMPA1 mRNA in order to account for differences in cDNA dilutions used for control and treated samples. Data presented are normalized against β-actin; however, 18S rRNA produced similar results (data not shown).

***myo*-Inositol quantitative assay**

This method was established using Kindt et al. (Kindt et al., 2004) as a reference for optimization of extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) procedures.

Tissue extraction

First, frozen whole brain was quickly weighed and then placed in a 15 ml glass mortar and pestle (Corning, Tewksbury, MA, USA). Tissue mass (~20-fold dilution, w/v) was homogenized in LC-MS/MS-grade water (Thermo Fisher Scientific, Rockford, IL, USA) spiked with 9.3 ng ml⁻¹ d₆-*myo*-inositol at room temperature. The homogenate was then centrifuged at 10,000 *g* at 4°C for 5 min and the liquid interface (excluding cell pellet and lipids) was removed. The interface was centrifuged again at 10,000 *g* for 5 min and homogenate was stored at -80°C until analyses.

To prepare samples for downstream processing, tissue homogenates were first thawed slowly on ice and then samples were centrifuged at 10,000 *g*. The supernatant was transferred to an Ultrafree Durapore PVDF 0.1 µm centrifugal filter (EMD Millipore, Billerica, MA, USA) and centrifuged at 14,167 *g* at 4°C for 15 min. Flow through was then transferred to sterile LC-MS vials (Waters, Milford, MA, USA) that contained 150 µl inserts (Waters).

LC-MS/MS method conditions

The concentrations of *myo*-inositol were measured using an Agilent 1200 SL ultra-high performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) coupled with an AB Sciex 4000 QTRAP quadrupole/linear ion trap tandem mass spectrometer (Foster City, CA, USA) equipped with an electrospray source (Turbo V, AB SCIEX, Concord, ON, Canada). The stationary phase was a Luna 5 µm NH₂ 150×2.0 mm column (Phenomenex, Torrance, CA, USA) and the mobile phase was 5 mmol l⁻¹ ammonia acetate in 50% MeOH/water solution. An isocratic method was used with a 0.2 ml min⁻¹ flow rate with an injection volume of 10 µl. The column oven was set at 40°C and the autosampler was kept at 4°C. In order to establish the LC-MS/MS method, the standards of *myo*-inositol and d₆-*myo*-inositol were first infused into the mass spectrometer and MRM transitions and source parameters were optimized for each compound. Source parameters were then re-

Table 1. Primers and cDNA dilutions of control and treated samples used for qRT-PCR

Gene	Forward (5' to 3')	Reverse (5' to 3')	Product size (bp)	Accession number	cDNA dilution (control; treated)
MIPS-160	CAGAGTCGCGCAGACAATGT	CGTTGACCCCTGGGATGATA	164 and 251	DQ465381	1:9
MIPS-250	GTGCATGATCTTCCAGATGGAGCG	AGAAGCGCTCGGTGTTGGCG	110	Sacchi et al., 2013	1:9
IMPA1	CGAAACTCTCCTAAGCAAGCCCC	CCAGCTTTCCTAATTTCCGCGCCA	114	JQ943581	1:1; 1:9
IMPA2	TACCAGAATCCTCTTCTTGGCCACACC	ACCAGGACACTGATGCACAGCTA	121	XM_003439148	1:1
β-actin	CCACAGCCGAGAGGGAAAT	CCCATCTCCTGCTCGAAGT	104	AB037865	1:9
18S rRNA	CGATGCTCTTAGCTGAGTGT	ACGACGGTATCTGATCGTCT	260	AF497908	1:729

Table 2. Mass spectrometer parameter settings for the analyte of interest, *myo*-inositol, and the internal standard (d_6 -*myo*-inositol) used in the LC-MS/MS quantitative assay

	Q1	Q3	Collision energy (eV)
<i>myo</i> -inositol	178.5	86.7	-24
d_6 - <i>myo</i> -inositol	185.3	88.9	-24

Q1 and Q3 represent the first and third quadrupole, respectively.

optimized under flow injection acquisition mode (infusion of analytes into the column eluent flow). The instrument was operated in negative MRM mode for analyzing samples and the final optimized mass spectrometric parameters are given in Table 2.

LC-MS/MS data analysis

The concentrations were quantified according to the calibration solutions of *myo*-inositol (Sigma-Aldrich, St Louis, MO, USA, >99% purity), ranging from 0.1 to 1000 ng ml⁻¹, using the Analyst 1.5.2 Software (AB Sciex). The total concentration of *myo*-inositol in each tissue sample was calculated based on the standard curve and converted to mg ml⁻¹. A standard curve of d_6 -*myo*-inositol (98% purity; Cambridge Isotope Laboratories, Tewksbury, MA, USA) at concentrations ranging from 0.745 to 93 ng ml⁻¹ was run in parallel with samples in order to determine percent recovery of the target analyte.

Statistical analysis

ANOVA was performed separately on data obtained from the acute and chronic experiments using SPSS (v. 21, IBM, Armonk, NY, USA). Normality and homoscedasticity were tested prior to ANOVA using the Shapiro–Wilk test and Levene’s test, respectively. In some cases, data were logarithmically transformed, or occasionally, statistically significant outliers were removed in order to meet the assumption of normality. In order to determine whether there was an effect of sampling time on control fish, a one-way ANOVA with time as a factor was performed for all freshwater samples (‘handling

controls’) for every time point or salinity end point. If non-significance was determined (data not shown), all freshwater fish were grouped together in a zero time point. As heteroscedasticity was generally observed in data sets, weighted least squares (WLS) analysis was conducted by grouping samples by the factors of salinity and time (Draper and Smith, 1998; Hartmann et al., 1998; Sadray et al., 2003). A weight for each sample grouping was calculated by taking the inverse of the unstandardized residual variance per assigned group. Subsequently, a one-way WLS ANOVA was performed to compare salinity-treated groups with the combined freshwater control group (0 h), with time or salinity as a factor. *Post hoc* analyses were then conducted using Tukey’s multiple comparison test to determine differences between time points (acute acclimation) and salinity end points (chronic acclimation). Correlation analyses were also performed on MIB enzyme mRNA data and plasma osmolality for both chronic and acute data sets. In acute data sets, MIB enzyme mRNA data and plasma osmolality were log-transformed prior to performing the correlation analysis. Statistically significant correlations were identified using Pearson’s correlation coefficient; however, because of heteroscedasticity in these data, a non-parametric Spearman correlation analysis was also performed. Slope differences between correlation lines were evaluated using a repeated-measures analysis of covariance, identifying plasma osmolality as the covariate. The significance threshold of $P < 0.05$ was set for all statistical tests.

RESULTS

Hematocrit, plasma osmolality, Na⁺, Cl⁻ and K⁺

Percent hematocrit was significantly higher in treatment fish compared with freshwater controls (0 h) at 2 and 24 h. At 16 h, hematocrit levels in treated fish were lower than those in treated fish at 2 and 24 h (Fig. 1D). Percent hematocrit did not change significantly for the chronic salinity acclimation. Total blood plasma osmolality, Na⁺, and Cl⁻ all showed similar patterns of regulation under acute and chronic acclimations. In the acute acclimation, salinity-treated fish had significantly higher blood plasma osmolality, Na⁺ and Cl⁻ at 2, 4, 8, 16 and 24 h compared with freshwater controls (0 h). These parameters

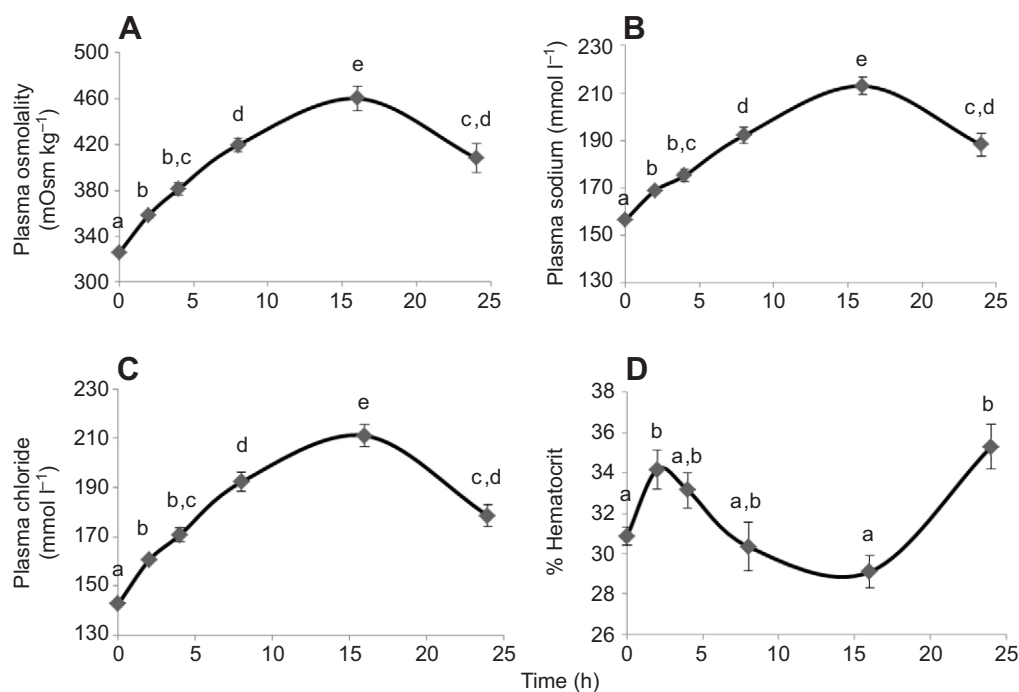


Fig. 1. Blood plasma osmolality (A), sodium concentration (B), chloride concentration (C) and percent hematocrit (D) after acute acclimation of *Oreochromis mossambicus* to 25 ppt salinity. Data are means \pm s.e.m. ($N=5-6$). Different letters indicate significant differences between groups evaluated by *post hoc* Tukey’s honestly significant difference (HSD) test ($P < 0.05$) following a weighted least squares (WLS) ANOVA.

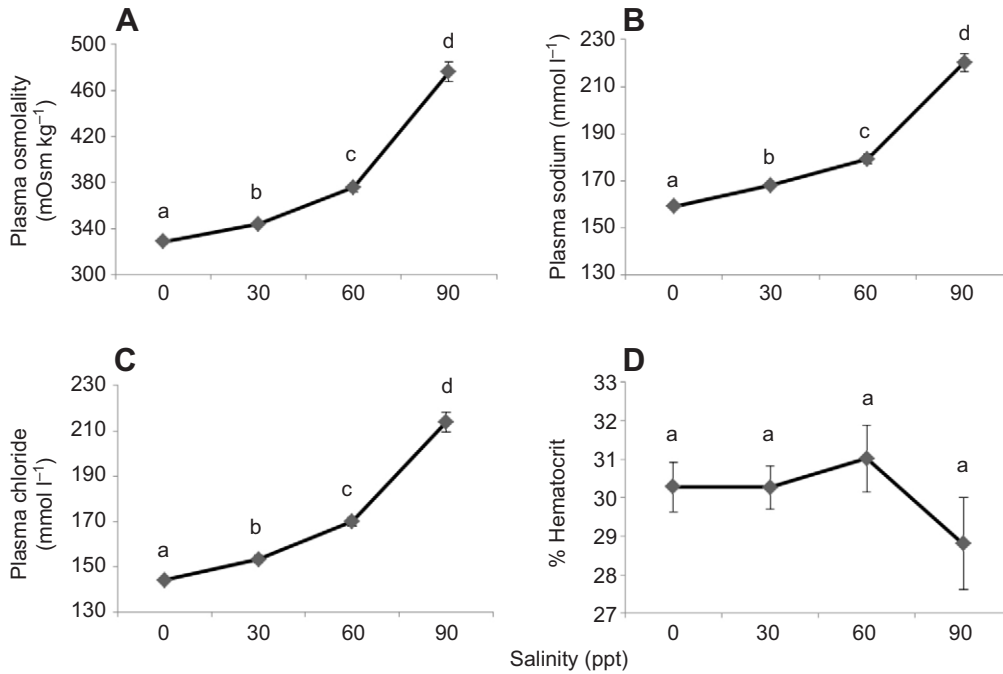


Fig. 2. Blood plasma osmolality (A) sodium concentration (B), chloride concentration (C) and percent hematocrit (D) following chronic acclimation of *O. mossambicus* to 30, 60 and 90 ppt treatments. Data are means \pm s.e.m. ($N=5-6$). Different letters indicate significant differences between groups evaluated by *post hoc* Tukey's HSD test ($P<0.05$) following a WLS ANOVA.

all reached peak values (~ 460 mOsm kg^{-1} , ~ 215 mmol l^{-1} Na^+ and Cl^-) at 16 h post acclimation and gradually tapered off to levels similar to those at 4–8 h by 24 h (Fig. 1A–C). In the chronic acclimation, a typical dose response was observed with regard to total osmolality and the plasma ions. The highest plasma osmolality and Na^+ and Cl^- concentrations (~ 475 mOsm kg^{-1} , ~ 215 mmol l^{-1} Na^+ and Cl^-) were observed at 90 ppt (Fig. 2A–C). Plasma K^+ concentration was not found to be significantly different from that of controls in acute and chronic acclimations (data not shown).

MIB pathway mRNA expression

MIPS-160, MIPS-250 and IMPA1 mRNA abundances were significantly higher in acute hyperosmotic treatment groups *versus* the freshwater control at every time point tested (Fig. 3A–C). The

MIPS-160 alternative splice variant was generally expressed at a higher level (approximately two times higher) than the MIPS-250 splice variant in the later time points in the acute acclimation (16, 24 h) or higher salinity end points for the chronic acclimation (Fig. 3A,B, Fig. 4A,B). IMPA1 mRNA abundance was much higher than that of IMPA2 in brain under both acute and chronic salinity acclimations (Fig. 3C, Fig. 4C). Under acute acclimation, IMPA1 mRNA peaked (~ 1500 -fold relative to controls, $P<0.001$) at 16 h, which is the same time that MIPS-160 (~ 100 -fold relative to controls, $P<0.001$) showed the highest expression. IMPA1 was expressed at the highest level (~ 780 -fold relative to controls, $P<0.001$) at the 90 ppt salinity end point for the chronic experiment. The IMPA2 isoform remained relatively stable across treatments, but was significantly higher than controls at 8, 16 and 24 h (acute, Fig. 3D) and 90 ppt (chronic, Fig. 4D).

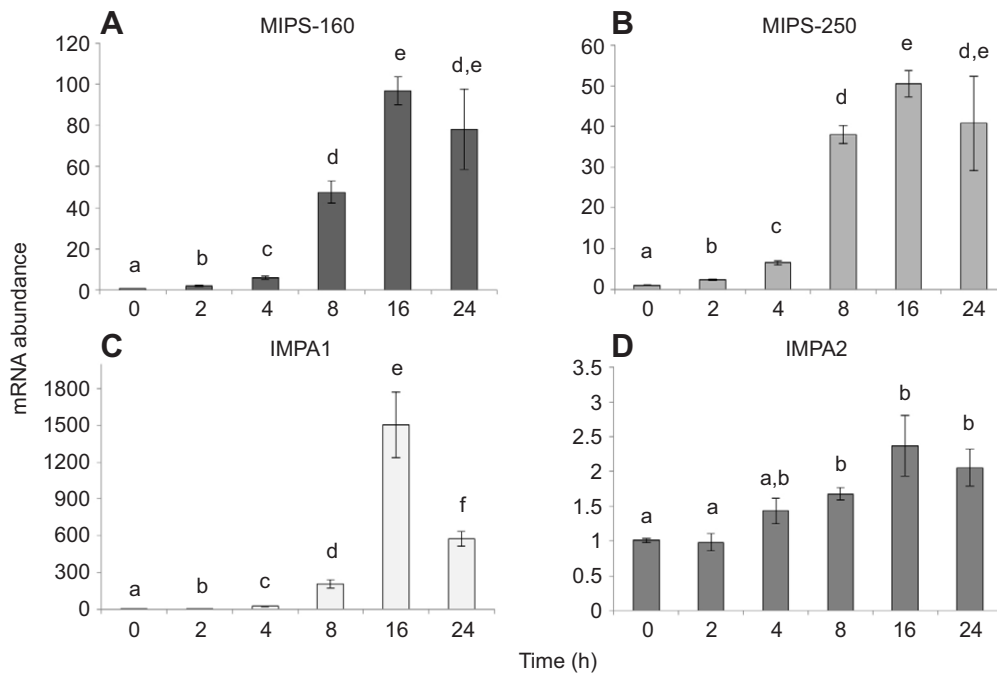


Fig. 3. MIPS-160 (A), MIPS-250 (B), IMPA1 (C) and IMPA2 (D) mRNA abundances following acute acclimation of *O. mossambicus* to 25 ppt at 0, 2, 4, 8, 16 and 24 h. Data are means \pm s.e.m. ($N=5-6$). Different letters indicate significant differences between groups evaluated by *post hoc* Tukey's HSD test ($P<0.05$) following a WLS ANOVA.

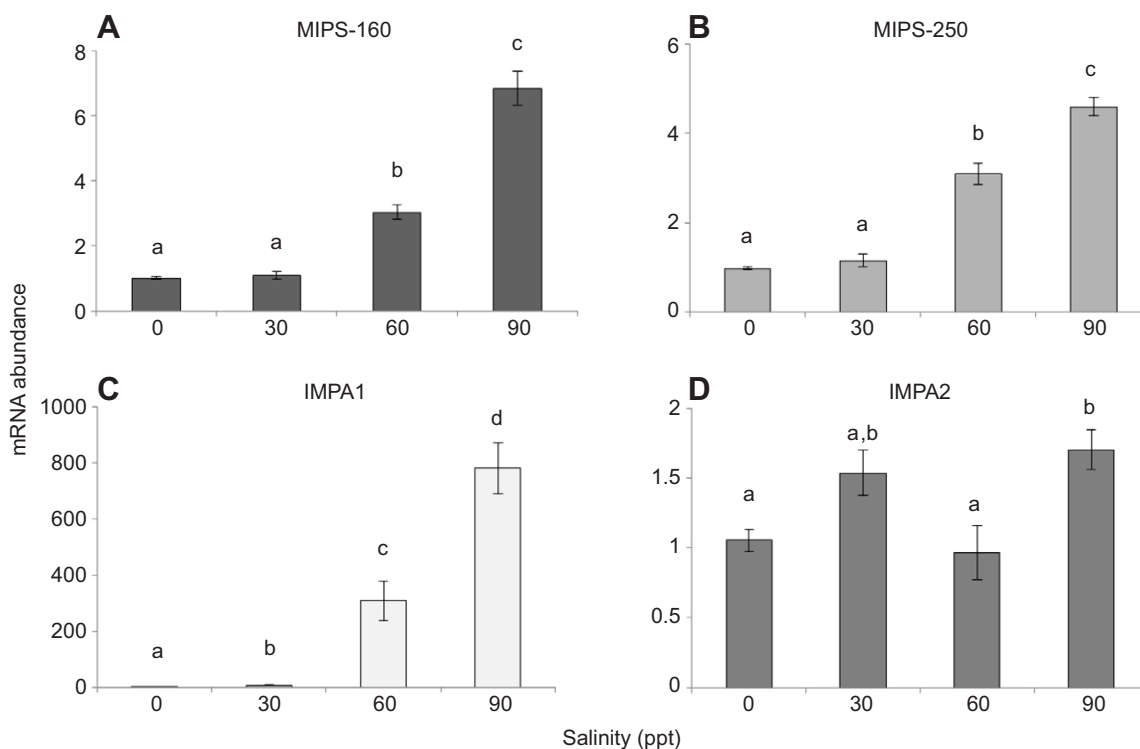


Fig. 4. MIPS-160 (A), MIPS-250 (B), IMPA1 (C) and IMPA2 (D) mRNA abundances following chronic acclimation of *O. mossambicus* to 30, 60 and 90 ppt. Data are means \pm s.e.m. ($N=5-6$). Different letters indicate significant differences between groups evaluated by *post hoc* Tukey's HSD test ($P<0.05$) following a WLS ANOVA.

myo-Inositol levels

Sample recovery was determined to be an average of $\sim 50\%$ across samples as determined with the internal standard d_6 -myo-inositol. myo-Inositol concentrations were the most significantly increased in later time points (24 h, $P<0.001$) following acute hyperosmotic challenge and trailing the mRNA induction of MIB enzymes (Fig. 5A). myo-Inositol concentration was also significantly higher ($P<0.001$) at all salinity end points in the chronic acclimation compared with the freshwater control (Fig. 5B), and was more robustly regulated under chronic acclimation (especially 90 ppt) compared with acute acclimation.

Correlation and slope analyses

MIPS-160, MIPS-250 and IMPA1 mRNA relative abundances (salinity treated/handling controls) were positively correlated ($P<0.01$, Pearson and Spearman) to blood plasma osmolality in both acute and chronic acclimation data sets (Fig. 6). Generally, IMPA mRNA abundance changes showed a stronger correlation to blood plasma osmolality than MIPS splice variant mRNA abundance changes. Significant differences between the slopes of MIPS-160 and MIPS-250 correlation lines were observed under acute ($P<0.044$) and chronic ($P<0.017$) acclimations.

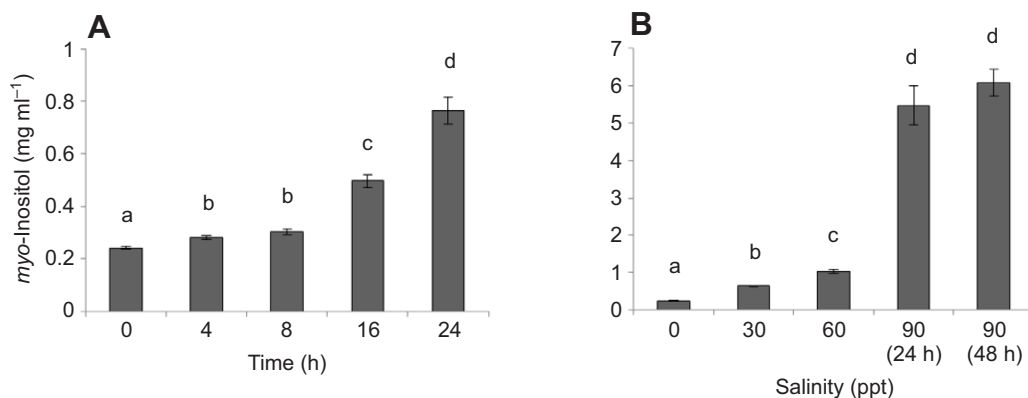


Fig. 5. myo-Inositol concentration (mg ml⁻¹) in *O. mossambicus* whole-brain homogenate after acute (A) and chronic (B) salinity acclimation detected using LC-MS/MS. Acute acclimation of fish involved a one-step increase to 25 ppt salinity and collection of brain after 4, 8, 16 and 24 h. Fish in the chronic exposure received 7.5 ppt increases in salinity per day, and brain was collected at the final salinities of 30 and 60 ppt following 24 h and at 90 ppt salinity following 24 and 48 h. Data are means \pm s.e.m. ($N=6-7$). Different letters indicate significant differences between groups evaluated by *post hoc* Tukey's HSD test ($P<0.05$) following a WLS ANOVA.

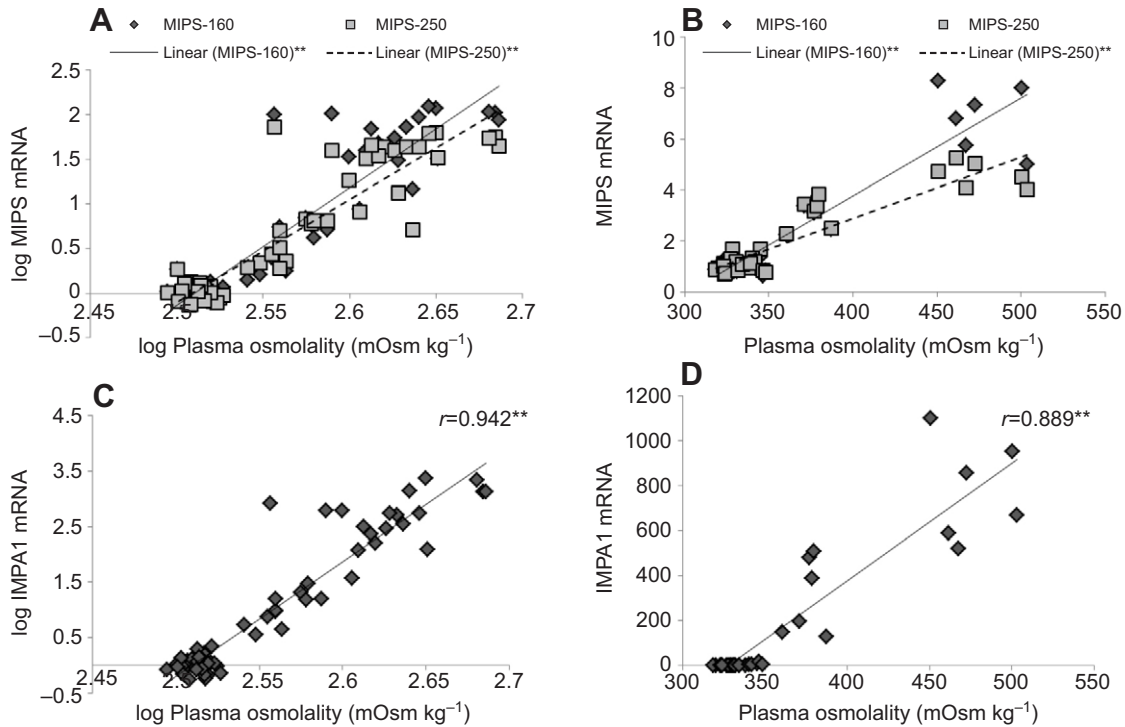


Fig. 6. Correlation analysis of MIPS-160 and MIPS-250 (A,B) or IMPA1 (C,D) mRNA abundance increase versus blood plasma osmolality increase performed separately for acute (A,C) and chronic (B,D) salinity acclimations in *O. mossambicus*. Pearson's correlation coefficients are reported (** $P < 0.01$).

DISCUSSION

The objective of this study was to gain deeper insight into how tilapia brain cells respond to acute and chronic hyperosmotic challenge. Specifically, we were interested in determining firstly whether the MIB pathway is an important mechanism used by tilapia brain cells to accumulate the compatible osmolyte, *myo*-inositol, and secondly, whether blood plasma osmolality changes precede and brain *myo*-inositol levels follow salinity-induced changes in in MIB enzyme mRNA expression.

Acute and chronic salinity acclimation alters blood plasma chemistry

Salinity treatment had a clear effect on blood plasma chemistry. Plasma osmolality was found to be significantly increased under both acute and chronic salinity acclimations. Na^+ and Cl^- displayed the same overall pattern as plasma osmolality, suggesting that these two ions are key contributors to increased plasma osmolality. Under acute acclimation, ion concentrations and plasma osmolality were both highest at 16 h. Similarly, Hwang et al. (Hwang et al., 1989) also detected peak values in inorganic ions and plasma osmolality before 24 h after acute hyper-salinity acclimation of tilapia. Under chronic acclimation, plasma osmolality, Na^+ and Cl^- were highest at the 90 ppt salinity end point. Surprisingly, we found that plasma osmolality in chronic acclimation was maintained at elevated levels ($\sim 475 \text{ mOsm kg}^{-1}$) for 24 h without mortality. Sardella et al. (Sardella et al., 2004) reported that Mozambique ‘hybrid’ tilapia kept at 95 ppt salinity for 5 days following transfer maintained an elevated plasma osmolality of $\geq 450 \text{ mOsm kg}^{-1}$, which is indicative of osmoregulatory failure. In contrast, tilapia transferred to 60 ppt salinity were able to return to lower plasma osmolality values following 24 h exposure (Sardella et al., 2004). The present study and others clearly demonstrate that fish are able to tolerate much larger deviations from the plasma osmolality set point than their

mammal counterparts (Evans and Claiborne, 2006). In mammalian systems, a blood plasma osmolality of only 15% above the baseline level will not be tolerated without significant damage to the central nervous system (Bourque and Oliet, 1997; Hall and Guyton, 2011; Seale et al., 2012b). In particular, elevated sodium concentration in the plasma above 160 mmol l^{-1} will cause hypernatremia in humans (Hall and Guyton, 2011).

Blood hematocrit was found to be significantly higher in salinity-treated fish compared with freshwater control fish during acute acclimation at 2 and 24 h time points. This increase has not been previously documented in other work performed in tilapia (Kammerer et al., 2010; Sardella et al., 2004). However, Kammerer et al. (Kammerer et al., 2010) observed significantly increased opercular ventilation at 3 h and a significant increase in respiration at 24 h in tilapia that were acutely exposed to 25 ppt salinity challenge. Increases in respiration rate may signal for increased production of red blood cells for delivery of oxygen to the tissues and could be related to the observed increase in hematocrit in this study. Our acute hematocrit data may also indicate that tilapia could have experienced dehydration in response to the acute salinity challenge, which may have also resulted in decreased plasma volume. Under chronic acclimation, hematocrit is likely to be more stable because fish have had time to adjust to the stressor and have overcome the physiological crisis stage that occurs around 6–12 h in tilapia (Hwang et al., 1989).

MIB enzymes in tilapia brain robustly respond to plasma osmolality

This study has clearly demonstrated that the MIB pathway is a major component of the biochemical response to hyperosmotic challenge in tilapia brain cells. We have shown that the two MIB enzymes, MIPS (MIPS-160 and MIPS-250 alternative splice variants) and IMPA (IMPA1 isoform), are robustly upregulated in tilapia brain

at the mRNA level. The mRNA abundance increase of IMPA1 was generally much higher than that of MIPS alternative transcripts. This is surprising given the fact that the synthesis of *myo*-inositol phosphate from G-6-P is the more rate-limiting step of the MIB pathway (Majumder et al., 1997), but does agree with a previous study that compared the regulation of the two enzymes (Sacchi et al., 2013). Significant upregulation or downregulation of IMPA1 mRNA under hyper- and hypo-osmotic challenge, respectively, has been reported in other teleost fishes (Evans and Somero, 2008; Kalujnaia et al., 2010; Whitehead et al., 2012). In leopard sharks, an osmoconforming species, inositol-related proteins have also been reported to be regulated in rectal gland and gill tissues in response to hypo-osmotic challenge (Dowd et al., 2010). Under acute acclimation, MIPS splice variants and IMPA1 displayed peak induction around 16 h, which closely corresponds with the highest inorganic ion concentration (Na^+ and Cl^-) and plasma osmolality. Similarly, in the chronic state, peak induction of MIB enzymes is at the highest salinity (90 ppt), when inorganic ion (Na^+ and Cl^-) concentrations and plasma osmolality were most significantly elevated. This agreement between a key physiological stimulus (plasma osmolality) and MIB mRNA levels suggests that hyperosmotic blood plasma triggers transcriptional induction of MIB enzymes to alleviate the damaging effects of hypertonicity.

The brain is an important tissue for systemic osmoregulation (Bourque, 2008; Seale et al., 2012b; Sinke and Deen, 2011). The significant positive correlations between blood plasma osmolality and MIB mRNA abundance observed in this study strongly suggest a role for hyperosmotic blood plasma in prompting induction of the MIB pathway in tilapia brain. Although ions are tightly regulated across the BBB, water can move more freely, which results in rapid changes in intracranial extracellular osmolality. In fish, deviations in blood plasma osmolality from the $\sim 310\text{--}350\text{ mOsm kg}^{-1}$ set point are probably detected by osmosensitive neurons and supporting glial cells in the extracellular fluid (Sinke and Deen, 2011). Some other aspects of the neuroendocrine response to osmotic challenge in tilapia, such as prolactin release, have also been reported to be stimulated under changing plasma osmolality (Seale et al., 2006). Interestingly, prolactin cells of freshwater-acclimated tilapia are more sensitive to changes in osmolality than prolactin cells of saltwater-acclimated fish (Seale et al., 2012b; Watanabe et al., 2012). The osmotic sensitivity of tilapia brain cells (neurons and glia) has not yet been investigated in response to changes in extracellular osmolality. It is also still unclear whether plasma osmolality or changes in particular ions trigger induction of MIB enzymes in brain. Some studies in mammals have suggested that osmoreceptor cells in the brain primarily respond to plasma tonicity rather than to total plasma osmolality (Bitoun and Tappaz, 2000; Verbalis and Gullans, 1991; Weber et al., 2004). The reported increase in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ would lead to hypertonicity and, therefore, support such a scenario.

It is possible that tilapia brain cells indirectly respond to salinity challenge by inducing the MIB pathway in response to a second messenger (e.g. neurotransmitter), which binds to a brain cell membrane receptor. Osmotic homeostasis in fish is maintained by multiple osmosensors that activate neural and hormonal signals that are involved in regulating ion and water transport across osmoregulatory tissues (Kültz, 2012). These signaling events, which precede the induction of the MIB pathway in tilapia brain cells, are yet to be investigated in fish. In mammals, stretch-activated proteins are known to prompt vasopressin release, which then acts on the kidney, driving increased thirst (Bourque et al., 2007). Tonicity response element binding protein has been shown to be responsive

to hyperosmotic challenge (Woo et al., 2002) and may also serve a function in osmosensing signaling cascades in fish (Fiol and Kültz, 2007).

Differential regulation of MIPS splice variants by environmental salinity

In this study we found evidence for differential regulation of MIPS splice variants by environmental salinity. The MIPS-160 variant was more responsive to changes in plasma osmolality than the MIPS-250 variant in brain tissue under both acute and chronic salinity challenges. Similarly, prolactin (PRL) isoforms of tilapia, PRL177 and PRL188, have been found to be differentially responsive to extracellular osmolality (Borski et al., 1992). Additionally, Seale et al. (Seale et al., 2012a) found that transient receptor potential vanilloid 4, a member of the TRP channel family, is differentially regulated by plasma osmolality. These studies clearly indicate that environmental selection pressure has allowed for diversification in the function and use of genetic elements used in osmoregulation and osmoreception in euryhaline tilapia.

Positive selection for various isoforms of IMPA and MIPS may have occurred differentially in various tissues of tilapia. Kalujnaia et al. (Kalujnaia et al., 2013) studied the transcriptional and protein regulation of four IMPA isoforms in response to environmental salinity and found evidence for tissue-specific profiles in two euryhaline species, the European eel and Nile tilapia. In particular, the osmoregulatory tissues (gill, kidney and intestine) showed the most robust regulation of IMPA isoforms (Kalujnaia et al., 2013). It is also possible that differential regulation of isoforms can occur during various stages of fish life history (Ayson et al., 1994). This may be particularly true for anadromous and catadromous fishes that live in very different osmotic environments during juvenile and adult stages, like salmon and eel.

myo-Inositol stably accumulates in tilapia brain cells to offset hyperosmotic challenge

We have demonstrated that *myo*-inositol content in tilapia brain cells is significantly upregulated under both acute and chronic salinity acclimations as quantified by LC-MS/MS. This salinity-induced effect is confirmed by other hyperosmotic challenge studies on mammalian systems (Heilig et al., 1989; Lien et al., 1990; Lohr et al., 1988) and in fish (Fiess et al., 2007). On a broader level, our *myo*-inositol data agree with the proposed role of osmolytes as molecules that are used for compensating increased extracellular osmolality. Increasing or stable levels of *myo*-inositol in both acute and chronic experiments were observed, suggesting that this metabolite is accumulated and is not biochemically metabolized to produce cellular energy equivalents. A similar observation was made in rat brain exposed to acute and chronic hypernatremia (Lohr et al., 1988). The high stability of *myo*-inositol is quite surprising given that its structure is almost identical to that of glucose, and further research is needed to explain its relatively inert nature.

The teleost fish brain is separated from the systemic circulation by a BBB (Soengas and Aldegunde, 2002). Most of the energy consumed in the brain is used for maintaining ionic gradients that are essential for neural processing (Soengas and Aldegunde, 2002). Hyperosmotic challenge puts severe energetic demands on the organism and glucose is limited as it is needed to sustain essential cellular functions. It has been well documented that blood plasma and tissue (gill and fin) glucose levels become elevated in tilapia following hyperosmotic challenge and that this is likely due to mobilization of glycogen reserves, primarily from the liver (Fiess et al., 2007; Kalujnaia et al., 2013). Chang et al. (Chang et al., 2007)

found that glycogen content in tilapia gill and liver significantly decreased after acute transfer to saltwater (25 ppt). As mentioned earlier, the MIB pathway uses G-6-P as a substrate for the generation of *myo*-inositol. G-6-P normally enters the glycolytic pathway for aerobic respiration, which is necessary during times of stress. Because cells are heavily relying on intrinsic glucose reserves for supplying the MIB pathway with substrate, glucose must be taken up across the BBB. Under hypoglycemic conditions, glucose transporters are known to increase in the brain (Duelli et al., 1999; Kumagai et al., 1995; McCall et al., 1986). Additionally, glucose transporter activity has also been demonstrated to significantly increase in fish brain following salinity acclimation (Balmaceda-Aguilera et al., 2012). Sangiao-Alvarellos et al. (Sangiao-Alvarellos et al., 2003) found an increased use of exogenous glucose in brain after acclimation of gilthead sea bream to hyper-saline conditions. Our findings strongly suggest that the high demand for *myo*-inositol production in brain cells exposed to hyperosmotic challenge increases the requirements for glucose uptake across the BBB; however, specific glucose analyses would need to be conducted in order to support this hypothesis.

Selective permeability of teleost BBB favors MIB pathway

The BBB serves to provide a microenvironment for the brain while maintaining brain homeostasis and protection from toxic agents (Cserr and Bundgaard, 1984). The endothelial cells of the BBB form tight junctions that act as a seal to protect the brain (Abbott et al., 2010). The accumulation of *myo*-inositol in brain cells can occur through multiple mechanisms including both endogenous and exogenous biochemical pathways. Brain cells can endogenously produce *myo*-inositol via the MIB pathway or membrane phospholipid (phosphatidylinositol) recycling in the cell, or alternatively, exogenous *myo*-inositol can be brought into the cell through various plasma membrane cotransporters, such as sodium/*myo*-inositol cotransporter (SMIT) and hydrogen/*myo*-inositol transporter (HMIT) (Bitoun and Tappaz, 2000). Even though *myo*-inositol levels are much lower in the blood than in brain cells (Davson and Segal, 1996), mammalian cells have the ability to increase intracellular concentrations of *myo*-inositol from exogenous sources at high enough levels to counter damage from hyperosmotic stress (Handler and Kwon, 1993).

Our current data favor the MIB pathway over other *myo*-inositol accumulation pathways in teleost fish brain in response to hyperosmotic challenge. Previous studies have indicated that the MIB pathway is particularly important for those tissues that possess a blood barrier, like the testis and brain (Stein and Geiger, 2002). Moreover, no known reported literature has cited exogenous pathways as highly inducible under salinity challenge in the brain of fishes. In contrast, several studies on mammalian systems highlight the exogenous cotransporter pathways as a primary mechanism for intracellular *myo*-inositol accumulation (Fenili et al., 2011; Ibsen and Strange, 1996; Inoue et al., 1996) in the brain. It is possible that mammalian systems have diverged in their preferred mechanism for *myo*-inositol accumulation in the cell. Future studies should quantify the relative contribution of MIB pathways *versus* cotransporter activity to overall *myo*-inositol accumulation in tilapia brain in order to determine the relative contribution of each pathway. These studies could be performed by systematic inhibition of the various MIB enzymes or cotransporters using pharmacological agents. In mammalian systems, SMIT and other pathways have been localized in the brain of mammalian systems, demonstrating specialization in various areas of the brain (Ibsen and Strange, 1996; Inoue et al., 1996), and may also reflect differences in permeability

of the capillaries. This may also be the case for MIB enzyme activity, and future studies should localize the specific region(s) of tilapia brain that contribute to the observed high MIB induction.

The permeability of molecules across the BBB in fish has yet to be investigated. It is possible that the MIB pathway may be favored over the use of cotransporters in the brain because of lower BBB permeability towards *myo*-inositol relative to glucose (Pasquali et al., 2010). Interestingly, in mammals, an increase in general permeability of the BBB can be induced by hyperosmotic challenge (Lu et al., 2004; Mackie et al., 1986; Wilhelm et al., 2008). The likely mechanism for this effect is temporary cell shrinkage, which creates openings in the tight junctions between brain capillary endothelial cells (Davson and Segal, 1996). This effect has been applied in human patients with central nervous system disorders by simultaneous administration of hyperosmotic challenge (e.g. mannitol, urea) with drugs to increase BBB permeability and improve drug delivery to the brain (Dorovini-Zis et al., 1987). Increased uptake of glucose across the BBB to supply the MIB pathway and meet energetic demands is likely mediated by activation of specific glucose transporters *versus* a result of non-specific increases in BBB permeability. In future studies, sampling of cerebrospinal fluid for osmolality and ion measurements as well as determination of glucose transporter activity in the BBB could serve to elucidate the roles of BBB permeability changes and glucose transport for supplying the large amount of substrate required by the MIB pathway during hyperosmotic challenge in euryhaline fish.

In summary, this study has clearly demonstrated that both enzymes of the MIB pathway are highly induced, which leads to extensive accumulation of *myo*-inositol in tilapia brain in response to acute and chronic salinity challenge. Based on its rapid and high level of accumulation, *myo*-inositol appears to be a major compatible osmolyte that protects tilapia brain cells from hyperosmotic challenge. MIB pathway induction and *myo*-inositol accumulation are both preceded and presumably triggered by an increase in plasma osmolality and plasma inorganic ion concentration. In particular, the role of plasma Na⁺ and Cl⁻ concentrations for regulating compatible osmolyte levels in brain cells of euryhaline fish merits further attention and represents a promising avenue for future research.

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AUTHOR CONTRIBUTIONS

A.M.G. designed and performed the experiments, analyzed and interpreted the data, and wrote the article. J.Y. and R.S. participated in research activities (LC-MS/MS and qRT-PCR, respectively), data interpretation and manuscript editing. N.A.F., B.D.H. and D.K. were involved in experimental design, data interpretation and manuscript editing.

COMPETING INTERESTS

No competing interests declared.

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