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Increased polyamine levels and maintenance of γ-aminobutyric acid (Gaba) homeostasis in the gills is indicative of osmotic plasticity in killifish

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ABSTRACT

The *Fundulus* genus of killifish includes species that inhabit marshes along the U.S. Atlantic coast and the Gulf of Mexico, but differ in their ability to adjust rapidly to fluctuations in salinity. Previous work suggests that euryhaline killifish stimulate polyamine biosynthesis and accumulate putrescine in the gills during acute hypoosmotic challenge. Despite evidence that polyamines have an osmoregulatory role in euryhaline killifish species, their function in marine species is unknown. Furthermore, the consequences of hypoosmotic-induced changes in polyamine synthesis on downstream pathways, such as γ-aminobutyric acid (Gaba) production, have yet to be explored. Here, we examined the effects of acute hypoosmotic exposure on polyamine, glutamate, and Gaba levels in the gills of a marine (*F. majalis*) and two euryhaline killifish species (*F. heteroclitus* and *F. grandis*). Fish acclimated to 32 ppt or 12 ppt water were transferred to fresh water, and concentrations of glutamate (Glu), Gaba, and the polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) were measured in the gills using high-performance liquid chromatography. *F. heteroclitus* and *F. grandis* exhibited an increase in gill Put concentration, but showed no change in Glu or Gaba levels following freshwater transfer. *F. heteroclitus* also accumulated Spd in the gills, whereas *F. grandis* showed transient increases in Spd and Spm levels. In contrast, gill Put, Spm, Glu, and Gaba levels decreased in *F. majalis* following freshwater transfer. Together, these findings suggest that increasing polyamine levels and maintaining Glu and Gaba levels in the gills may enable euryhaline teleosts to acclimate to shifts in environmental salinity.

1. Introduction

Killifish of the *Fundulus* genus consist of species that occupy a wide range of osmotic niches and, thus, differ in their capacities to tolerate and acclimate to salinity challenges (Burnett et al., 2007; Crego and Peterson, 1997). Previous work suggests that polyamine synthesis plays a role in enabling osmotic plasticity in *Fundulus* species during acute salinity challenge (Guan et al., 2016; Whitehead et al., 2012). Polyamines are a class of low molecular weight, aliphatic nitrogenous cations that are synthesized endogenously in all living organisms. The diamine putrescine (Put) is produced from the amino acids ornithine or agmatine by the enzymes ornithine decarboxylase 1 (Odc1) or agmatinase, respectively (Benítez et al., 2018; Cohen, 1998). Polyamines have been implicated in numerous physiological processes, including the regulation of gene expression (Childs et al., 2003; Xiao and Wang, 2011), cell membrane stabilization (Minocha et al., 2014; Schuber, 1989), ion channel modulation (Forsythe, 1995; Williams, 1997), cell-cell communication (Desforges et al., 2013; Shore et al., 2001), and cell proliferation and apoptosis (Moschou and Roubelakis-Angelakis, 2014; Thomas and Thomas, 2001).

In addition, prior studies in brine shrimp (Watts et al., 1994; Watts et al., 1996), blue mussels (Lockwood and Somero, 2011), crabs (Lovett and Watts, 1995; Silva et al., 2008), and killifish (Brennan et al., 2015; Guan et al., 2016; Whitehead et al., 2012; Whitehead et al., 2013) suggest that polyamine biosynthesis may have a role in the compensatory response of aquatic organisms to changes in environmental salinity. We have demonstrated that killifish show divergent transcriptomic responses, including differences in the expression of genes involved in

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polyamine synthesis, in the gills following acute salinity challenge, both between species that differ in their osmotic physiologies and within populations that differ by native salinity (Brennan et al., 2015; Whitehead et al., 2011; Whitehead et al., 2012; Whitehead et al., 2013). In freshwater (FW)-native populations of *F. heteroclitus*, a euryhaline species, the mRNA expression of arginase 2 (*arg2*), an enzyme that catalyzes the conversion of arginine to ornithine; and *odc1*, an enzyme that catalyzes the conversion of ornithine to Put (Rhee et al., 2007; Wallace et al., 2003); are significantly upregulated, and the expression of spermidine/spermine binding protein (*sbp*), a protein that binds free polyamines (Goueli et al., 1985; Mills et al., 1987); and transglutaminase 1 (*tgm1*), a protein that facilitates protein-polyamine conjugation (Greenberg et al., 1991); are significantly downregulated in the gills during acute hypoosmotic exposure (Brennan et al., 2015; Whitehead et al., 2011; Whitehead et al., 2012; Whitehead et al., 2013). In contrast, these changes in *arg2*, *odc1*, *sbp,* and *tgm1* mRNA expression are less pronounced in the gills of *F. majalis*, a marine species (Whitehead et al., 2013), and in *F. heteroclitus* populations that are native to brackish or marine habitats (Brennan et al., 2015; Whitehead et al., 2011; Whitehead et al., 2012; Whitehead et al., 2013), suggesting that polyamine synthesis may be associated with osmotic plasticity in killifish. Moreover, we have shown that *F. grandis*, a sister species of *F. heteroclitus*, exhibits increases in Put levels, *odc1* mRNA expression, and the activity of Odc1 and caspase-3, an enzyme associated with apoptosis, in the gills following FW transfer (Guan et al., 2016). This increase in caspase-3 activity, however, is attenuated in *F. grandis* administered the irreversible Odc inhibitor alpha-DL-difluoromethylornithine (DFMO; Guan et al., 2016), suggesting that polyamines may mediate apoptosis, a process that is necessary for gill epithelial remodeling, during acute hypoosmotic exposure. While our prior work suggests that euryhaline and marine killifish show distinct transcriptomic responses in the gills during acute hypoosmotic challenge, it is unclear whether these responses produce biochemical effects that are consistent with upregulation of polyamine biosynthesis. Furthermore, the effects of hypoosmotic-induced changes in gill polyamine production on downstream metabolic pathways are not yet investigated.

Polyamines serve as a precursor of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the vertebrate central nervous system that has also been implicated in environmental stress tolerance and osmoregulation, both in the brain and in peripheral tissues (Mohler and Enna, 2007; Shelp et al., 2012). The synthesis of Gaba (referred to here and throughout the text as a protein and not as a neurotransmitter and, thus, abbreviated with only the first letter capitalized based on the general format for acronym use in fish; http://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx) from Put occurs during the early stages of central nervous system development in birds (De Mello et al., 1976; Hokoc et al., 1990); during times of hyper-excitability in mice (Halonen et al., 1993; Shimosato et al., 1995), rats (Camon et al., 2001; Hayashi et al., 1993) and *Xenopus* tadpoles (Bell et al., 2011), when glutamate (Glu) is produced in excess and overstimulates certain neural circuits; and in some mature tissues, such as the adrenal glands (Caron et al., 1988) and postnatal subventricular zone in rats (Sequerra et al., 2007). Moreover, there is evidence that Put is a major source of Gaba during anoxic and hypoxic conditions in fish (reviewed in Nilsson and Lutz, 2004; Nilsson and Renshaw, 2004). Gaba has also been implicated in osmotic regulation in neurons and non-neuronal cells via the $GABA_A$ receptor ($GABA_AR$), a ligand-gated Cl^- channel (reviewed in Cesetti et al., 2011; reviewed in Kahle et al., 2008). Because *Fundulus* species lack an active Cl^- uptake mechanism in the gills (Patrick et al., 1997; Patrick and Wood, 1999), extraneural $GABA_AR$ may be especially important in the recovery of Cl^- homeostasis following acute hypoosmotic challenge. Despite this information, it is unknown whether Gaba signaling via $GABA_AR$ is an important osmoregulatory mechanism in teleosts.

The goal of this study was to assess how gill polyamine, Glu, and Gaba levels differ between species of killifish during acute salinity challenge. Specifically, we measured the concentrations of key metabolites involved in polyamine and Gaba synthesis [Glu, Gaba, Put, and the polyamines spermidine (Spd) and spermine (Spm)] in the gills of three killifish species with different tolerances to FW: *F. majalis*, a marine species that has a limited physiological ability to tolerate FW exposure; and *F. heteroclitus* and *F. grandis*, two euryhaline species that exhibit physiological plasticity in response to FW exposure (Griffith, 1974). *F. majalis* and *F. heteroclitus* were transferred from 32 ppt (control) to FW, and *F. grandis* were transferred from 12 ppt (control) to FW. In each species, we quantified levels of polyamines and related amino acids (i.e., Glu and Gaba) in the gills at 0 h (pre-transfer), 6 h, 1 d, and 3 d post-transfer using high-performance liquid chromatography. We predicted that the two euryhaline killifish species in this study, *F. heteroclitus* and *F. grandis,* would show elevated polyamine and Gaba levels in the gills in response to acute hypoosmotic exposure. In contrast, we predicted that the marine species *F. majalis* would exhibit no change or a decrease in gill polyamine and Gaba levels following FW transfer.

2. Materials and methods

2.1. Experimental animals

Adult *Fundulus heteroclitus* were collected from Chincoteague Island, Virginia (GPS coordinates 37°52′41.24″N, 75°21′9.69″W), adult *Fundulus majalis* were collected from Jekyll Island, Georgia (31°7′3.83”N, 81°25′0.59”W), and adult *Fundulus grandis* were collected from the Florida State University Coastal and Marine Laboratory in St. Teresa, Florida (29°54′56.9”N, 84°30′44.1”W). Each of these sites are along the coast, where salinity was near full-strength sea water at the time of collection. Fish were housed in the Department of Biological Sciences at Louisiana State University (Baton Rouge, LA, USA). All fish care and experimental protocols were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Prior to experimentation, fish were maintained in 570-L recirculating aquaria with mechanical, biological, and UV filtration. *F. heteroclitus* and *F. majalis* were acclimated to 32 ppt water (full-strength sea water) for at least 6 months, and *F. grandis* were acclimated to 12 ppt water for at least 2 months. Each of these species maintain their marine gill physiology at these salinities; thus, any results obtained from exposing 32 ppt-acclimated fish or 12 ppt-acclimated fish to FW should be analogous. Salinity was maintained using reverse osmosis water mixed with artificial sea salt (Instant Ocean®, Blacksburg, VA, USA), and water temperature was ambient (21–24 °C). Photoperiod was maintained on a 12 h light: 12 h dark cycle using an automated timer to control fluorescent lighting.

During the acclimation period, water salinity, temperature, pH, dissolved oxygen, and ammonia, nitrate, and nitrite levels were monitored weekly using either a water quality meter (YSI, Yellow Spring, OH, USA) or a commercially-available kit (Aquarium Pharmaceuticals Inc., Chalfront, PA, USA). System water was partially replaced at least once weekly to maintain ammonia, nitrate, and nitrite below 1 ppm, 20 ppm, and 1 ppm, respectively. During experimentation, water quality was checked daily, and water was replaced as necessary. Fish were fed a commercial pellet (Cargill, Franklin, LA, USA) at 2% body weight daily, except for 24 h prior to salinity transfer and 24 h prior to tissue and blood collection.

2.2. Experimental design

Gill polyamine, Glu, and Gaba concentrations were measured in killifish acclimated to 32 ppt water (*F. majalis* and *F. heteroclitus*) or 12 ppt water (*F. grandis*) and following acute transfer from 32 ppt or 12 ppt water to 0.1 ppt water (FW). Gill samples for *F. majalis* and *F. heteroclitus* were derived from previously published studies (Whitehead et al., 2011; Whitehead et al., 2013), allowing us to assess whether the transcriptomic responses observed in the gills of these fish, which support a

putative role of polyamine biosynthesis during acute hypoosmotic exposure, yield biochemical changes that are consistent with upregulation of this pathway. *F. grandis* were utilized from a concurrent study (Munley, Liu, and Galvez, unpublished results), in which fish were weighed and injected intraperitoneally daily with 5 μL phosphate-buffered saline (PBS; 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) using a Hamilton microsyringe (Hamilton, Reno, NV, USA) starting 2 d before salinity transfer. Previous work by Guan et al., 2016 suggests that ion homeostasis, an indicator of stress in fish, is not adversely affected in PBS-injected controls. Immediately prior to FW transfer, a subset of *F. majalis* ($n = 6$) and *F. grandis* ($n = 6$) was sampled for blood and gills, and a subset of *F. heteroclitus* ($n = 6$) was sampled for blood, as described below. Half of the fish of each species were netted and transferred to control water [transfer controls; 32 ppt water for *F. majalis* ($n = 18$) and *F. heteroclitus* ($n = 18$) and 12 ppt water for *F. grandis* ($n = 20$)], and the remaining fish were transferred to FW (*F. majalis*: $n = 18$, *F. grandis*: $n = 20$, *F. heteroclitus*: $n = 18$). Post-transfer exposures were performed in 120-L glass aquaria connected to a recirculating system. Fish were randomly sampled from each salinity treatment at 6 h, 24 h, and 72 h post-transfer ($n = 4$–7 per species, salinity, and time point) for blood and gills. Fish were net-captured, anesthetized using 0.15 g L^{-1} MS-222, and sacrificed by spinal cord severance. Blood was immediately collected in micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) following euthanasia, and plasma was isolated via centrifugation at 3000 x*g* for 3 min. Plasma was collected, and samples were flash frozen in liquid nitrogen and stored at −80 °C for analysis of plasma osmolality and Na^+ and Cl^- concentrations (see Section 2.3). Gill baskets were excised from fish, washed briefly in exposure water, blotted dry, and flash frozen in liquid nitrogen. Gill samples were stored at −80 °C for analysis of Glu, Gaba, and polyamine concentrations (see Section 2.4).

2.3. Plasma chemistry

Plasma chemistry data for *F. majalis* and *F. heteroclitus* were curated from previously published studies (Whitehead et al., 2011; Whitehead et al., 2013). For all species, plasma Na^+ concentrations were determined using flame atomic absorption spectroscopy (Varian Australia Pty. Ltd., Melbourne, Australia), and plasma Cl^- concentrations were measured using the mercuric thiocyanate method with minor modifications (Zall et al., 1956). Plasma osmolality was analyzed via freeze-point depression (Precision Systems Inc., Natlick, MA, USA).

2.4. Glutamate, Gaba, and polyamine contents in the gills

Frozen gill baskets were ground into a fine powder using a mortar and pestle chilled with liquid nitrogen, weighed, and transferred to sterile 2.0 mL microcentrifuge tubes. Samples were thawed and homogenized on ice in methanol supplemented with 0.1 M HCl (1:3 V:V) using a PRO200 homogenizer (Lab Depot Inc., Dawsonville, GA, USA). Glu, Gaba, Put, Spd, and Spm were extracted by adding 50 μL 0.6 M $HClO_4$, and samples were centrifuged at 15,000 x*g* for 15 min at 4 °C. The centrifugation process was repeated after adding an additional 20 μL of 0.6 M $HClO_4$. Supernatants were collected, neutralized using 7 M KOH, and stored at −20 °C for high-performance liquid chromatography (HPLC) analysis.

Prior to HPLC, samples were thawed, freeze-dried, and mixed with 100 μL phenylisothiocyanate (PITC) labeling reagent (7:1:1:1 ratio of ethanol:water:triethylamine:phenylisothiocyanate, respectively) for 30 min at room temperature. Samples were then dried using a lyophilizer and dissolved in 1 mL of diluent (5% acetonitrile in water) at room temperature. Following filtration through a 0.2 μm pore-size syringe filter, 20 μL of sample was injected into a HPLC system, which included a Waters 616 pump coupled to a Waters 2707 Autosampler and 996 Photodiode Assay Detector that was controlled by Waters Empower 2 software (Waters Corporation, Milford, MA, USA). Metabolite separation was performed on a 5 μm ACE C18-PFP column (4.6 × 150 mm; MAC-MOD Analytical, Chadds Ford, PA, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (eluent A) and 0.1% TFA in acetonitrile (eluent B), and a flow rate of 1 mL min^{-1} was used during the analysis. The following linear gradients were used over a period of 33 min: 75% to 35% eluent A from 0 to 24 min, 35% to 25% eluent A from 24 to 28 min, and 25% to 5% eluent A from 28 to 33 min. PITC-labelled amino acids that eluted from the column were detected at 254 nm and recorded. Metabolites of interest were characterized by the following elution times: Glu, 4.94 min; Gaba, 5.64 min; Put, 14.78 min; Spd, 20.12 min; and Spm, 23.48 min (Fig. 1A). Following each sample injection, the column was regenerated and equilibrated with 75% eluent A and 25% eluent B for 10 min.

Standards for L-glutamic acid monosodium salt monohydrate (≥98%), Gaba (≥99%), Put (98%), Spd trihydrochloride (≥98%), and Spm tetrahydrochloride (95–98%; all purchased from Sigma Aldrich, St. Louis, MO, USA) were dissolved separately in deionized water to yield stock solutions of 1.00 mg mL^{-1} (Glu: 5.34 μmol mL^{-1}, Gaba: 9.70 μmol mL^{-1}, Put: 11.34 μmol mL^{-1}, Spd: 3.93 μmol mL^{-1}, Spm: 2.87 μmol mL^{-1}). Standard curves were generated for each metabolite by serially diluting each 1.00 mg mL^{-1} stock solution with deionized water (Fig. 1B-F). Sample metabolite concentrations (in nmol g^{-1} gill) were determined by calculating the peak area of each metabolite and interpolating these values using known concentrations of metabolite generated via standard curves. Each standard and sample was analyzed in triplicate, and an average peak area was obtained for each sample. The Gaba concentration of 12 samples from *F. majalis* (32 ppt: $n = 3$, 0.1 ppt: $n = 9$) was below the lowest standard on the standard curve. These values were considered non-detectable, and the Gaba content of these samples was set to the limit of detection for the assay (0.039 μg, or 0.378 nmol) for the purpose of statistical analysis.

2.5. Statistical analyses

All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using R version 4.0.2 (R Core Team, 2020), and differences among means were considered statistically significant at an α level of 0.05 after controlling for false discovery rate (Verhoeven et al., 2005). Normality was assessed with Shapiro-Wilk tests using the *shapiro.test* function of the *stats* package in R (R Core Team, 2020), and homogeneity of variances was assessed with Levene's tests using the *leveneTest* function of the *car* package (Fox and Weisberg, 2019). In some cases, data were log transformed to attain a normal distribution. Data that exhibited a non-normal distribution were visualized with Cullen and Frey plots using the *descdist* function of the *fitdistrplus* package, and distribution fit was assessed using the *fitdist* function of the *fitdistrplus* package (Delignette-Muller and Dutang, 2015). Generalized linear models (GLMs) with Gaussian or gamma distributions and survival models with Weibull distributions were used to assess the effects of salinity (32 ppt and 0.1 ppt for *F. majalis* and *F. heteroclitus*, 12 ppt and 0.1 ppt for *F. grandis*), time [0 h (pre-transfer), 6 h, 1 d, and 3 d], and the interaction of salinity and time on plasma chemistry and gill metabolite concentrations for each species. If a statistical test reported a significant effect of salinity, time, or the interaction of salinity and time, Tukey's Honestly Significant Difference (HSD) post-hoc tests (for data exhibiting normal distribution) or Dunn's post-hoc tests for multiple comparisons (for data exhibiting non-normal distribution) were conducted to examine pairwise comparisons. Data were analyzed using the *glm* function of the *stats* package (GLMs; R Core Team, 2020), the *survreg* function of the *survival* package (survival models; Therneau, 2020), the *glht* function of the *multcomp* package (Tukey's HSD post-hoc tests; Hothorn et al., 2008), and the *dunn.test* function of the *dunn.test* package (Dunn's post-hoc tests; Dinno, 2017). Summaries of the GLMs and survival models for each species are available in the Supplementary Material (Tables S1-S3). For each model, χ^2 values were used to assess goodness of fit, and Nagelkerke's pseudo-

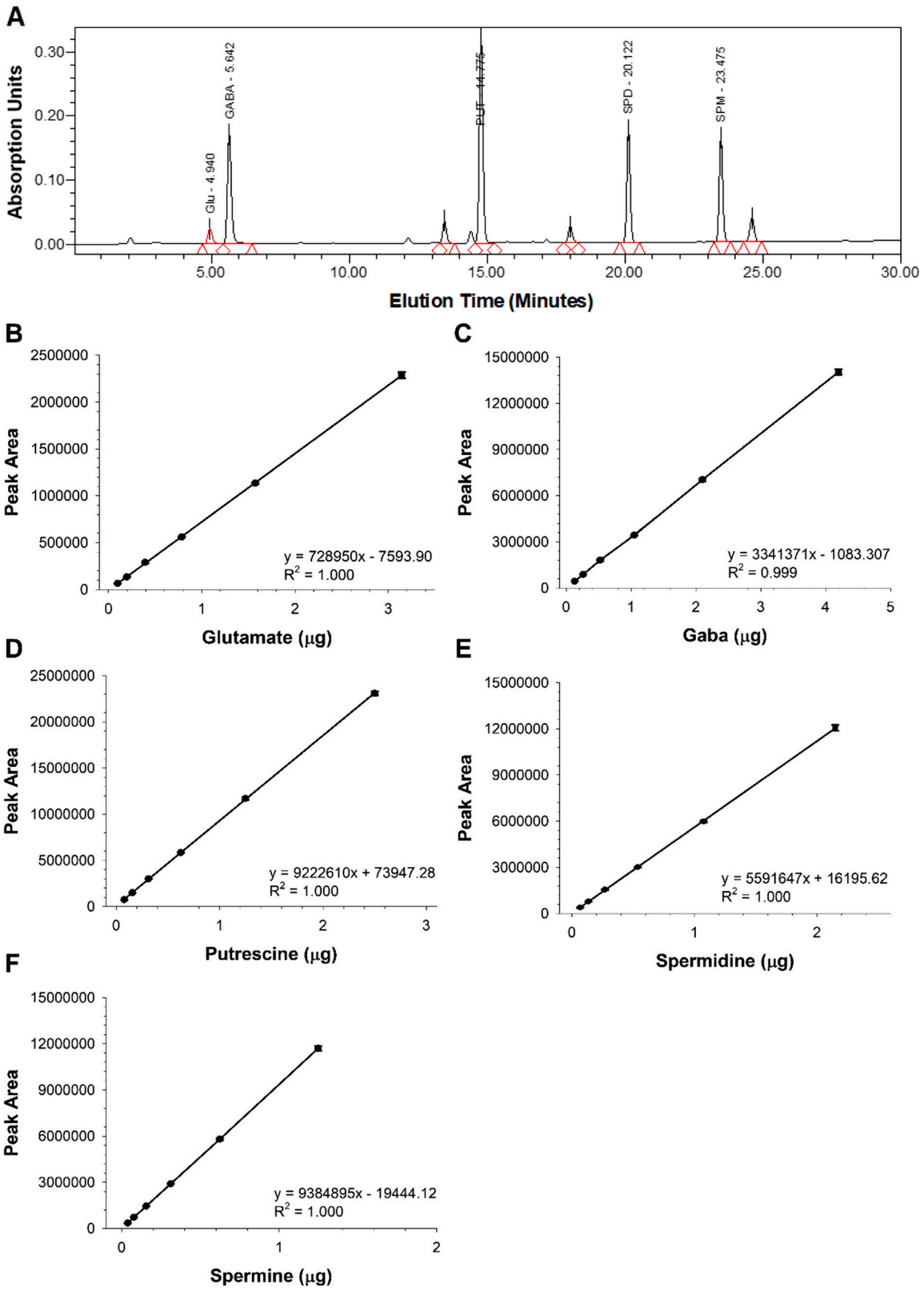

Fig. 1. Representative chromatograph generated from a mixture of 1.00 mg mL^{-1} solutions of glutamate (Glu), γ-aminobutyric acid (Gaba), putrescine (Put), spermidine (Spd), and spermine (Spm) (A). Metabolites were characterized by the following retention times: Glu, 4.94 min; Gaba, 5.64 min; Put, 14.78 min; Spd, 20.12 min; and Spm, 23.48 min. Representative standard curves for glutamate (B), Gaba (C), putrescine (D), spermidine (E), and spermine (F) generated via high-performance liquid chromatography (HPLC)Masses (µg) represent the amount of metabolite present in 20 µL of solution (sample injection volume). Standard curve ranges: 0.09–3.14 µg (0.50–16.80 nmol mL^{-1}) for glutamate, 0.13–4.20 µg (1.27–40.73 nmol mL^{-1}) for Gaba, 0.08–2.50 µg (0.89–28.36 nmol mL^{-1}) for putrescine, 0.07–2.15 µg (0.26–8.44 nmol mL^{-1}) for spermidine, and 0.04–1.25 µg (0.11–3.58 nmol mL^{-1}) for spermine.

R^2 values, which are based on the adjusted likelihood ratio, were used to estimate effect size (Magee, 1990; Nagelkerke, 1991). χ^2 and Nagelkerke's pseudo-R^2 values were calculated using the *summ* function of the *jtools* package (for GLMs; Long, 2020) and the *pam.survreg* function of the *PAmeasures* package (for survival models; Wang and Li, 2018).

3. Results

3.1. Plasma chemistry

Following FW transfer, the two euryhaline species, *F. heteroclitus* and *F. grandis*, and the marine killifish species, *F. majalis*, exhibited a significant reduction in plasma osmolality relative to pre-transfer levels; however, this reduction persisted longer and was more pronounced in *F. majalis* and *F. grandis* than in *F. heteroclitus* (Figs. 2A-C; Supplementary Material, Table S1). The mean plasma osmolality was reduced by as much as 41.9% in *F. majalis* at 3 d post-transfer to FW ($P < 0.05$) and was significantly lower than that of pre-transfer control fish throughout the exposure period ($P < 0.05$, Fig. 2A). In *F. grandis*, there was a significant effect of salinity, but there was no effect of time nor an interaction between salinity and time on plasma osmolality (GLM, Gaussian distribution: $\chi^2(3) = 36{,}621$, $P < 0.01$, $R^2 = 0.39$; salinity: $P = 0.002$; time: $P = 0.931$; interaction: $P = 0.710$). The mean plasma osmolality decreased by as much as 28.3% in *F. grandis* at 6 h post-transfer to FW ($P < 0.001$), and plasma osmolality in these fish remained significantly lower than the pre-transfer value after 3 d post transfer ($P = 0.006$; Fig. 2B). In *F. heteroclitus*, there was a significant effect of salinity, but there was no effect of time nor an interaction between salinity and time on plasma osmolality (GLM, Gaussian distribution: $\chi^2(3) = 3807$, $P = 0.29$, $R^2 = 0.08$; salinity: $P = 0.023$; time: $P = 0.424$; interaction: $P = 0.404$). On average, this species exhibited up to an 18.3% reduction in plasma osmolality at 1 d post-transfer to FW ($P < 0.001$); however, plasma osmolality recovered to pre-transfer levels in these fish at 3 d post-transfer ($P = 0.974$, Fig. 2C).

Similarly, while each species showed significant decreases in plasma Na^+ and Cl^- concentrations following FW transfer, the magnitude of plasma Na^+ and Cl^- reduction was more pronounced in *F. majalis* and *F. grandis* than in *F. heteroclitus* (Figs. 2D-I; Supplementary Material, Table S1). In *F. majalis*, there was a significant effect of salinity, time, and a significant interaction between salinity and time on plasma Na^+ (survival model, Weibull distribution: $\chi^2(3) = 43.32$, $P < 0.001$, $R^2 = 0.03$; salinity: $P < 0.001$; time: $P = 0.023$; interaction: $P = 0.026$) and plasma Cl^- concentrations (survival model, Weibull distribution: $\chi^2(3) = 45.93$, $P < 0.001$, $R^2 = 0.03$; salinity: $P = 0.003$; time: $P < 0.001$; interaction: $P = 0.001$). Plasma Na^+ and Cl^- significantly decreased in *F. majalis* following FW transfer, and the mean plasma Na^+ and Cl^-

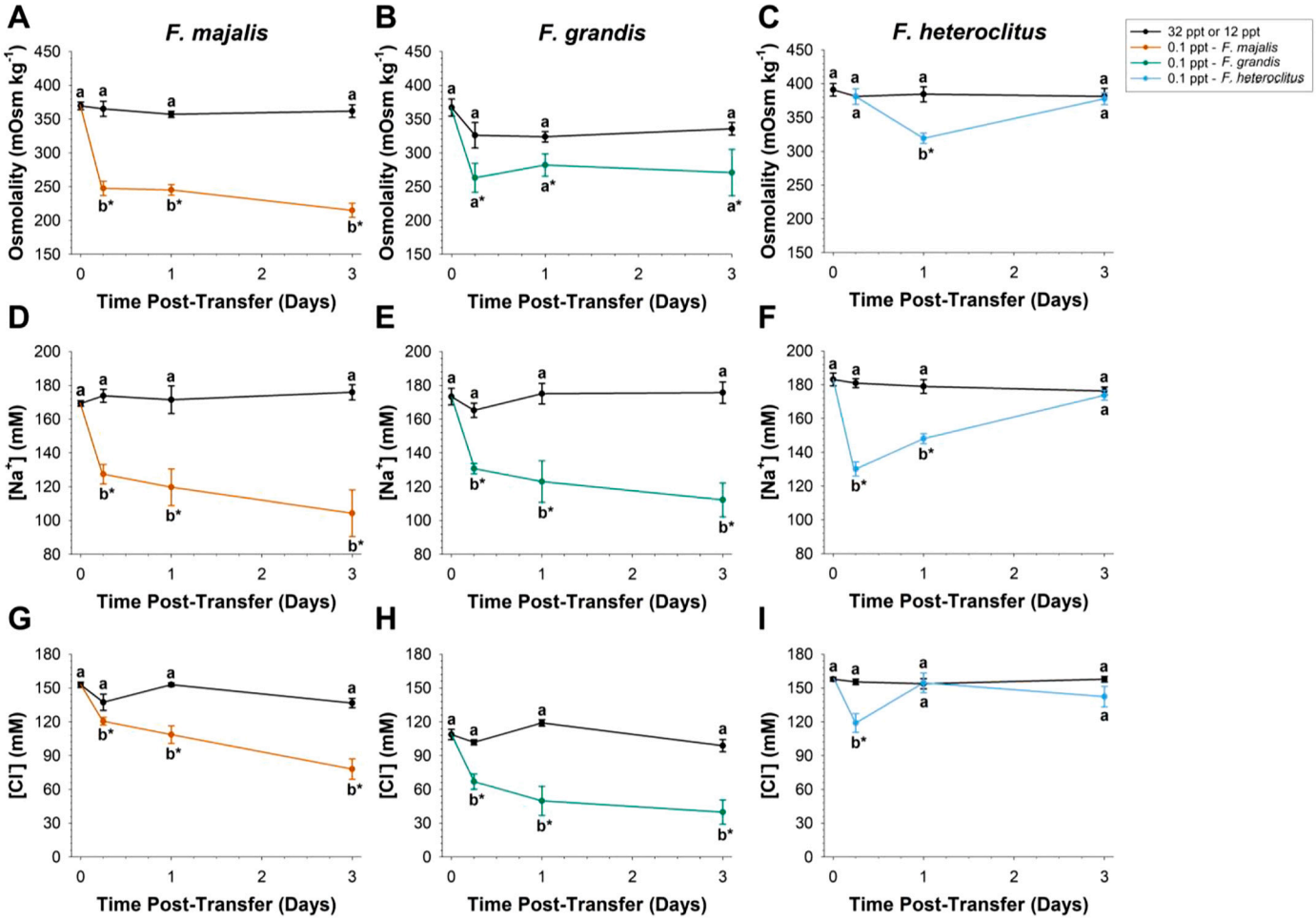

Fig. 2. Plasma osmolality (A-C), plasma sodium (D-F), and plasma chloride (G-I) concentrations in *F. majalis* (A, D, G), *F. grandis* (B, E, H), and *F. heteroclitus* (C, F, I) following acclimation to 32 ppt water (*F. majalis* and *F. heteroclitus*; 0 h, pre-transfer) or 12 ppt water (*F. grandis*; 0 h, pre-transfer) and at 6 h, 1 d, and 3 d following transfer to 32 ppt (black line; *F. majalis* and *F. heteroclitus*), 12 ppt (black line; *F. grandis*), or 0.1 ppt water (FW, colored line; *F. majalis*: orange, *F. grandis*: green, *F. heteroclitus:* blue). Data are presented as mean ± SEM (*F. majalis*: $n = 5$–6, *F. grandis*: $n = 4$–7, *F. heteroclitus*: $n = 4$–6). "*" indicates a significant difference compared to the pre-transfer time point for a given salinity, whereas different letters indicate a significant difference from the control group (32 ppt for *F. majalis* and *F. heteroclitus*, 12 ppt for *F. grandis*) at a given time point ($P < 0.05$, generalized linear models and survival models with Tukey's HSD or Dunn's post-hoc tests). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

levels remained 38.4% and 48.9% lower than pre-transfer levels at 3 d post-transfer, respectively (plasma Na^+: $P = 0.002$; plasma Cl^-: $P = 0.011$; Figs. 2D, G). In *F. grandis*, there was a significant effect of salinity and trends toward an effect of time and an interaction between salinity and time on plasma Na^+ levels (GLM, Gaussian distribution: $\chi^2(3) = 30{,}053$, $P < 0.01$, $R^2 = 0.66$; salinity: $P < 0.001$; time: $P = 0.091$; interaction: $P = 0.099$). There was also a significant effect of salinity and time, but not an interaction between salinity and time on plasma Cl^- concentration in this species (GLM, Gaussian distribution: $\chi^2(3) = 33{,}619$, $P < 0.01$, $R^2 = 0.69$; salinity: $P < 0.001$; time: $P = 0.038$; interaction: $P = 0.245$). On average, plasma Na^+ and Cl^- were reduced by as much as 35.3% and 63.4% in *F. grandis* at 3 d post-transfer, respectively, and neither plasma Na^+ nor plasma Cl^- returned to pre-transfer levels by the end of the study (plasma Na^+: $P < 0.001$; plasma Cl^-: $P < 0.001$; Figs. 2E, H). In *F. heteroclitus*, there was a significant effect of salinity, time, and a significant interaction between salinity and time on plasma Na^+ concentration (GLM, Gaussian distribution: $\chi^2(3) = 14{,}563$, $P < 0.01$, $R^2 = 0.85$; salinity: $P < 0.001$; time: $P < 0.001$; interaction: $P < 0.001$), whereas there was a trend toward an effect of salinity, but there was not an effect of time nor an interaction between salinity and time on plasma Cl^- concentration (survival model, Weibull distribution: $\chi^2(3) = 3.700$, $P = 0.30$, $R^2 = 0.03$; salinity: $P = 0.076$; time: $P = 0.100$; interaction: $P = 0.208$). The mean plasma Na^+ and Cl^- levels decreased by as much as 29.0% and 24.6% at 6 h post-transfer to FW in this species, respectively (plasma Na^+: $P < 0.001$; plasma Cl^-: $P = 0.001$), but both plasma Na^+ and Cl^- returned to pre-transfer levels by the end of the 3 d exposure period (plasma Na^+: $P = 0.429$; plasma Cl^-: $P = 1.000$; Figs. 2F, I).

3.2. Polyamine concentrations

The concentrations of several polyamines (Put, Spd, and Spm) were measured in the gills of a marine killifish species, *F. majalis,* and two euryhaline killifish species, *F. grandis* and *F. heteroclitus,* maintained in 32 ppt (*F. majalis* and *F. heteroclitus*) or 12 ppt water (*F. grandis*) and following acute transfer to FW. Overall, gill polyamine levels tended to decrease in *F. majalis* and increase in *F. heteroclitus* and *F. grandis* following FW transfer (Fig. 3; Supplementary Material, Table S2). While *F. majalis* had basal gill Put levels that were 5 times higher than those of *F. grandis* and 15 times higher than those of *F. heteroclitus*, gill Put concentration in *F. majalis* was significantly lower in FW fish than in 32 ppt fish at 3 d post-transfer (GLM, Gaussian distribution: $\chi^2(3) = 465{,}357$, $P = 0.27$, $R^2 = 0.11$; 3 d post-transfer: $P = 0.030$; Fig. 3A). In *F. grandis,* there was a significant effect of salinity and a significant

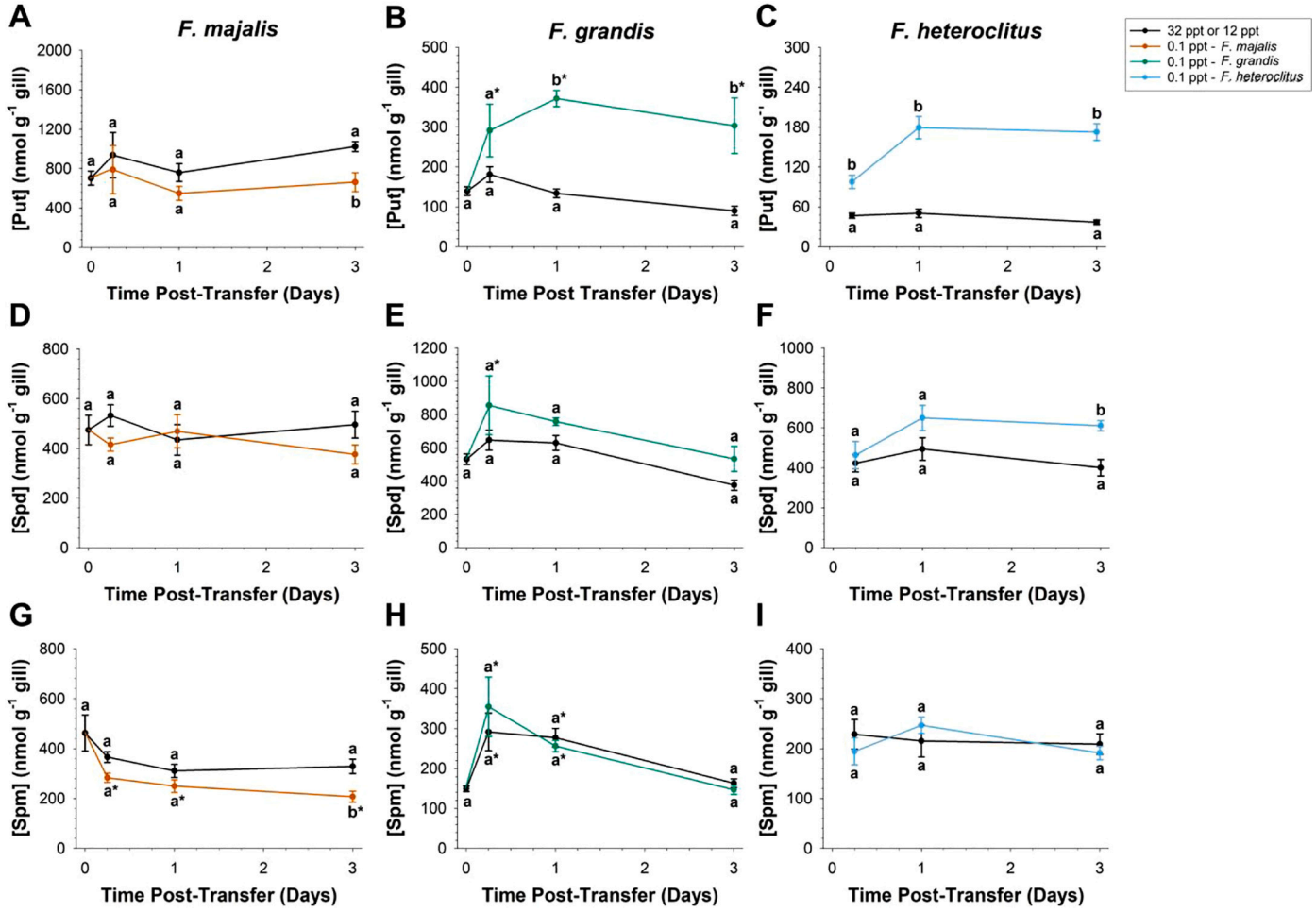

Fig. 3. Concentrations of putrescine (A-C), spermidine (D-F), and spermine (G-I) in the gills of *F. majalis* (A, D, G), *F. grandis* (B, E, H), and *F. heteroclitus* (C, F, I) following acclimation to 32 ppt water (*F. majalis* and *F. heteroclitus*; 0 h, pre-transfer) or 12 ppt water (*F. grandis*; 0 h, pre-transfer) and at 6 h, 1 d, and 3 d following transfer to 32 ppt (black line; *F. majalis* and *F. heteroclitus*), 12 ppt (black line; *F. grandis*), or 0.1 ppt water (FW, colored line; *F. majalis*: orange, *F. grandis*: green, *F. heteroclitus:* blue). *Note: the pre-transfer time point for F. heteroclitus was not available for analysis*. Data are presented as mean ± SEM (*F. majalis*: $n = 4$–6, *F. grandis*: $n = 6$–7, *F. heteroclitus*: $n = 5$–6). "*" indicates a significant difference compared to the pre-transfer time point for a given salinity, whereas different letters indicate a significant difference from the control group (32 ppt for *F. majalis* and *F. heteroclitus*, 12 ppt for *F. grandis*) at a given time point ($P < 0.05$, generalized linear models with Tukey's HSD or Dunn's post-hoc tests). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction between salinity and time, but there was no effect of time on gill Put levels (GLM, gamma distribution: $\chi^2(3) = 10.10$, $P < 0.01$, $R^2 = 0.59$; salinity: $P < 0.001$; time: $P = 0.866$; interaction: $P = 0.013$), whereas there was a significant effect of salinity, time, and a significant interaction between salinity and time on gill Put concentration in *F. heteroclitus* (GLM, Gaussian distribution: $\chi^2(3) = 13.65$, $P < 0.01$, $R^2 = 0.98$; salinity: $P < 0.001$; time: $P = 0.011$; interaction: $P = 0.003$). Both *F. grandis* and *F. heteroclitus* exhibited significant increases in gill Put levels following FW transfer, and these species showed similar magnitudes of Put elevation in response to FW exposure ($P < 0.001$; Fig. 3B-C).

Although the three species had similar basal concentrations of Spd in the gills, they differed in their relative change in gill Spd levels following FW transfer (Figs. 3D-F; Supplementary Material, Table S2). In *F. majalis*, there was not an effect of salinity, time, nor an interaction between salinity and time on gill Spd levels (GLM, Gaussian distribution: $\chi^2(3) = 42{,}054$, $P = 0.37$, $R^2 = 0.09$; salinity: $P = 0.445$; time: $P = 0.499$; interaction: $P = 0.636$; Fig. 3D). Conversely, there was a significant effect of salinity and time, but not an interaction between salinity and time on gill Spd concentration in *F. grandis* (GLM, Gaussian Distribution: $\chi^2(3) = 876{,}245$, $P < 0.01$, $R^2 = 0.36$; salinity: $P = 0.006$; time: $P = 0.004$; interaction: $P = 0.400$). On average, gill Spd levels increased by as much as 37.9% in *F. grandis* at 6 h post-transfer to FW ($P = 0.044$), but returned to pre-transfer levels by the end of the 3 d exposure period ($P = 1.000$; Fig. 3E). In *F. heteroclitus*, gill Spd concentration was significantly higher in 0.1 ppt fish than in 32 ppt fish at 3 d post-transfer (GLM, Gaussian distribution: $\chi^2(3) = 211{,}129$, $P = 0.01$, $R^2 = 0.28$; 3 d post-transfer: $P = 0.035$; Fig. 3F).

As observed for Spd, the three species had similar basal levels of Spm in the gills, but exhibited distinct changes in gill Spm levels following FW transfer (Figs. 3G-I; Supplementary Material, Table S2). In *F. majalis*, there was a significant effect of salinity and time, but not an interaction between salinity and time on gill Spm concentration (GLM, Gaussian distribution: $\chi^2(3) = 1.570$, $P < 0.01$, $R^2 = 1.29$; salinity: $P = 0.011$; time: $P = 0.047$, interaction: $P = 0.591$). Gill Spm levels significantly decreased in this species following FW transfer and, on average, remained 55.2% lower than pre-transfer levels at 3 d post-transfer ($P < 0.001$; Fig. 3G). In *F. grandis,* there was a significant effect of salinity, time, and a significant interaction between salinity and time on gill Spm levels (GLM, Gaussian Distribution: $\chi^2(3) = 2.570$, $P < 0.01$, $R^2 = 0.52$; salinity: $P = 0.008$; time: $P < 0.001$; interaction: $P = 0.016$). On average, gill Spm concentration increased by as much as 57.9% at 6 h post-transfer to FW ($P < 0.001$), but returned to the pre-transfer value at 3 d post-transfer ($P = 0.999$; Fig. 3H). In contrast, there was not an effect of salinity, time, nor an interaction between salinity and time on gill Spm concentration in *F. heteroclitus* (GLM, Gaussian Distribution: $\chi^2(3) = 2708$, $P = 0.86$, $R^2 = 0.02$; salinity: $P = 0.925$; time: $P = 0.528$; interaction: $P = 0.928$; Fig. 3I).

3.3. Glutamate and Gaba concentrations

To assess the effect of acute hypoosmotic exposure on Gaba production, concentrations of Glu and Gaba were quantified in the gills of a marine killifish species, *F. majalis*, and two euryhaline killifish species, *F. grandis* and *F. heteroclitus,* following FW transfer. In general, *F. majalis* exhibited reductions in gill Glu and Gaba levels, whereas *F. grandis* and *F. heteroclitus* exhibited few changes in gill Glu and Gaba concentrations in response to FW transfer (Fig. 4; Supplementary Material, Table S3). In *F. majalis*, there was a significant effect of salinity, but there was not an effect of time nor an interaction between salinity and time on gill Glu concentration (GLM, Gaussian Distribution: $\chi^2(3) = 6.630$, $P < 0.01$, $R^2 = 0.45$; salinity: $P < 0.001$; time: $P = 0.424$; interaction: $P = 0.315$). Although *F. majalis* had basal gill Glu levels that were two times higher than those of *F. grandis* and three times higher than those of *F. heteroclitus*, gill Glu concentration in this species significantly decreased following FW transfer and, on average, remained 56.7% lower than the pre-transfer value at 3 d post-transfer ($P = 0.023$, Fig. 4A). Conversely, there was a significant effect of time, but not an effect of

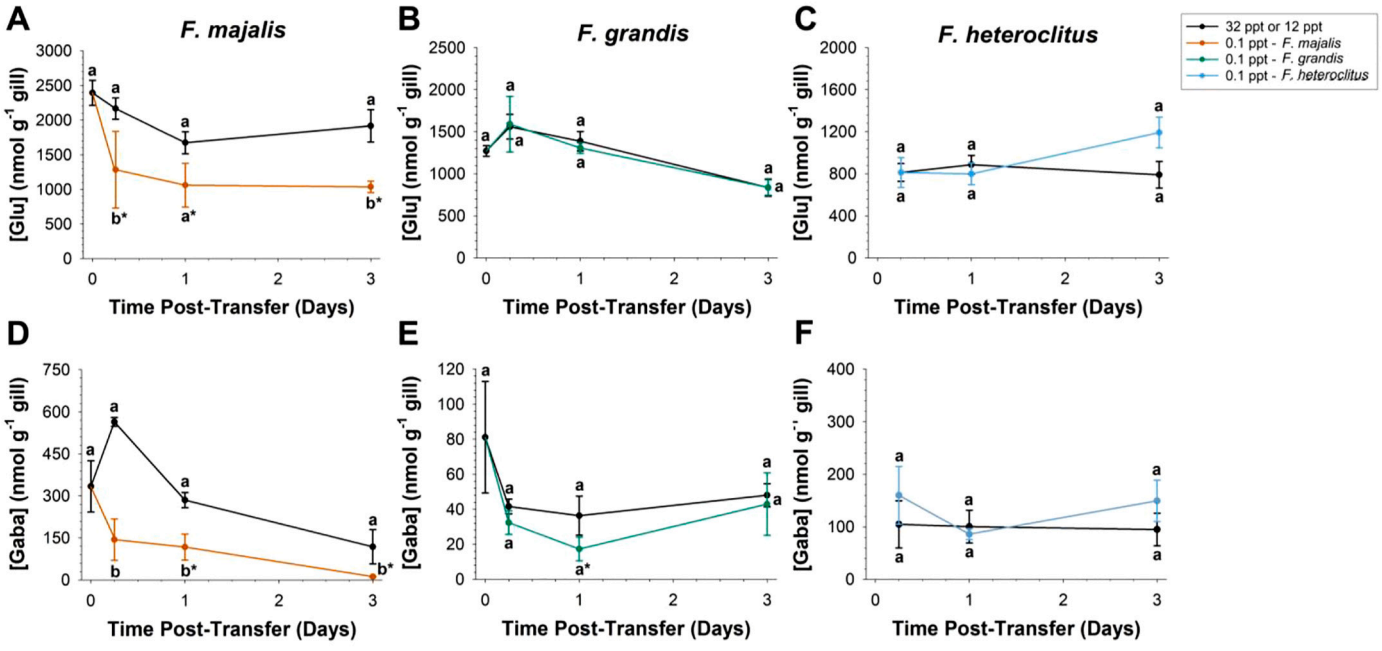

Fig. 4. Concentrations of glutamate (A-C) and γ-aminobutyric acid (Gaba) (D-F) in the gills of *F. majalis* (A, D), *F. grandis* (B, E), and *F. heteroclitus* (C, F) following acclimation to 32 ppt water (*F. majalis* and *F. heteroclitus*; 0 h, pre-transfer) or 12 ppt water (*F. grandis*; 0 h, pre-transfer) and at 6 h, 1 d, and 3 d following transfer to 32 ppt (black line; *F. majalis* and *F. heteroclitus*), 12 ppt (black line; *F. grandis*), or 0.1 ppt water (FW, colored line; *F. majalis*: orange, *F. grandis*: green, *F. heteroclitus:* blue). *Note: the pre-transfer time point for F. heteroclitus was not available for analysis*. Data are presented as mean ± SEM (*F. majalis*: $n = 4$–6, *F. grandis*: $n = 6$–7, *F. heteroclitus*: $n = 4$–6). "*" indicates a significant difference compared to the pre-transfer time point for a given salinity, whereas different letters indicate a significant difference from the control group (32 ppt for *F. majalis* and *F. heteroclitus*, 12 ppt for *F. grandis*) at a given time point ($P < 0.05$, generalized linear models with Tukey's HSD post-hoc tests). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

salinity nor an interaction between salinity and time on gill Glu levels in *F. grandis* (GLM, Gaussian Distribution: $\chi^2(3) = 2.640$, $P < 0.01$, $R^2 = 0.78$; salinity: $P = 0.665$; time: $P < 0.001$; interaction: $P = 0.686$) and *F. heteroclitus* (GLM, Gaussian Distribution: $\chi^2(3) = 615{,}046$, $P = 0.03$, $R^2 = 0.26$; salinity: $P = 0.420$; time: $P = 0.012$; interaction: $P = 0.075$). Neither *F. grandis* nor *F. heteroclitus* exhibited a change in gill Glu concentration following FW transfer, and there were no significant differences in gill Glu concentration between 0.1 ppt fish and control fish at any point during the 3 d exposure period (*F. grandis*: $P \geq 0.999$; *F. heteroclitus*: $P \geq 0.271$; Figs. 4B-C).

As observed for Glu, *F. majalis*, *F. grandis*, and *F. heteroclitus* showed differences in both basal Gaba levels and in the relative change of Gaba levels in the gills following FW transfer (Fig. 4D-F; Supplementary Material, Table S3). In *F. majalis*, there was a significant effect of salinity and time, but there was not an interaction between salinity and time on gill Gaba concentration (GLM, Gaussian Distribution: $\chi^2(3) = 47.18$, $P < 0.01$, $R^2 = 0.64$; salinity: $P = 0.003$; time: $P = 0.006$; interaction: $P = 0.868$). While *F. majalis* had basal Gaba levels that were approximately 5 times higher than those of *F. grandis* and *F. heteroclitus*, this species exhibited a significant decrease in gill Gaba concentration following FW transfer (Fig. 4D). In *F. grandis*, there was a significant effect of salinity, but there was not an effect of time nor an interaction between salinity and time on gill Gaba levels (GLM, Gaussian Distribution: $\chi^2(3) = 4.990$, $P = 0.04$, $R^2 = 0.18$; salinity: $P = 0.024$; time: $P = 0.504$; interaction: $P = 0.520$). On average, gill Gaba levels were reduced by as much as 65.1% in *F. grandis* at 1 d post-transfer to FW ($P < 0.001$), but recovered to pre-transfer levels at the end of the 3 d exposure period ($P = 0.413$; Fig. 4E). In *F. heteroclitus*, there was not an effect of salinity, time, nor an interaction between salinity and time on gill Gaba concentration (GLM, Gaussian Distribution: $\chi^2(3) = 0.830$, $P = 0.65$, $R^2 = 0.06$; salinity: $P = 0.490$; time: $P = 0.853$; interaction: $P = 0.890$; Fig. 4F).

4. Discussion

In the present study, we used a comparative approach to assess the effects of acute hypoosmotic exposure on the concentrations of polyamines, Glu, and Gaba in the gills of three closely-related killifish species with differing osmotic tolerances to FW. We found that the two euryhaline species examined in our study, *F. heteroclitus* and *F. grandis*, exhibited an increase in polyamine levels, but maintained Glu and Gaba levels in the gills following FW transfer. Conversely, *F. majalis*, a marine species, showed decreases in gill concentrations of polyamines, Glu, and Gaba. Collectively, our findings suggest that euryhaline killifish stimulate polyamine production and better maintain the concentrations of other metabolites that are peripherally associated with polyamine biosynthesis, including Glu and Gaba, in the gills relative to marine killifish. To our knowledge, our study is among the first to characterize the concentrations of polyamines, Glu, and Gaba in the fish gill during acute osmotic challenge and to suggest a role for these metabolites in osmoregulation in teleost fishes.

4.1. Euryhaline and marine killifish exhibit distinct changes in gill polyamine production during acute FW exposure

F. heteroclitus, a euryhaline killifish species, exhibited an attenuated impairment of osmotic imbalance relative to *F. majalis*, a marine species, and was able to recover plasma ion homeostasis following FW transfer. Unexpectedly, the euryhaline species *F. grandis* exhibited more pronounced reductions in plasma osmolality and plasma Na^+ and Cl^- levels than *F. heteroclitus* and did not reestablish plasma ion homeostasis following FW transfer (Fig. 2). Because we have previously shown that *F. grandis* exhibits a recovery of plasma osmolality and plasma Na^+ and Cl^- concentrations 3 days following the start of hypoosmotic exposure (Guan et al., 2016), differences in plasma osmolyte recovery between studies may have been due to the stress induced by handling during PBS administration. Indeed, other studies have shown that stress affects osmoregulatory processes in teleosts (Almeida et al., 2013; Dang et al., 2000). The changes in gill metabolite concentrations in *F. heteroclitus* and *F. grandis* following FW transfer (Fig. 3) were consistent with other studies. We have shown that arg*2* and *odc1* mRNA levels in the gills are increased and *sbp and tgm1* mRNA levels are reduced in the gills of FW-native populations of *F. heteroclitus* during acute hypoosmotic exposure (Brennan et al., 2015; Whitehead et al., 2011; Whitehead et al., 2012; Whitehead et al., 2013; see also Introduction section). More recently, we also demonstrated that polyamine metabolites and *odc1* mRNA expression increase in *F. grandis* during acute hypoosmotic exposure, but that DFMO administration prevents the accumulation of polyamines in the gills (Guan et al., 2016). Collectively, these results suggest that polyamine synthesis is elevated in the gills of euryhaline killifish during acute hypoosmotic challenge, a response that likely mediates physiological processes that aid in osmotic stress tolerance.

In this study, *F. majalis* failed to recover plasma ion homeostasis and decreased gill Put and Spm levels following FW transfer (Figs. 2-3). These results suggest that marine killifish exhibit a diminished capacity to increase polyamine biosynthesis during acute hypoosmotic challenge relative to euryhaline species. These findings are in agreement with our previous work, in which we showed that *F. majalis* exhibits less pronounced changes in gill arg*2*, *odc1*, *sbp*, and *tgm1* mRNA levels than *F. heteroclitus* during acute hypoosmotic exposure (Whitehead et al., 2013). Perhaps paradoxically, we also found that *F. majalis* also had higher basal concentrations of Put, Spm, Glu, and Gaba in the gills compared to *F. heteroclitus* and *F. grandis*; yet, these metabolites decreased in concentration during FW exposure (Figs. 3-4). High basal levels of polyamines and related amino acids in the gills could suggest that the synthesis of these metabolites is upregulated in marine killifish species, such as *F. majalis,* in sea water (SW), a mechanism that may enable the stable acclimation of these fish to their native habitat. Consequently, *F. majalis* may have a limited ability to upregulate genes that are related to polyamine and Gaba production in the gills in response to FW transfer, which could contribute to this species' diminished capacity to acclimate to acute salinity challenge. While the potential consequences of endogenous amino acid levels on osmotic stress tolerance are largely unexplored in vertebrates, studies in plants suggest that high endogenous polyamine levels can prevent some of the beneficial effects of these molecules during abiotic stress. In maize plants, high endogenous Put levels are associated with growth inhibition, increased oxidative stress, and the accumulation of salicylic acid, a plant hormone involved in abiotic stress signaling (Szalai et al., 2017). Thus, although abiotic stress tolerance in plants is associated with an ability to elevate polyamine synthesis in response to stress exposure (reviewed in Liu et al., 2015), these studies suggest that higher endogenous polyamine levels may have adverse effects under environmentally-stressful conditions. It would be worthwhile to explore the potential relationships between high endogenous amino acid levels, decreased polyamine production in response to environmental stress, and reduced stress tolerance in vertebrates, as little is known about these associations.

Prior studies have demonstrated that Odc1, which catalyzes the rate limiting reaction in polyamine biosynthesis, is upregulated during hypoosmotic exposure in almost every metazoan cell type that has been examined (Lockwood and Somero, 2011). As observed in the current study, however, the pattern of change in polyamine metabolites during acute salinity challenge may be species-specific. Lockwood and Somero (2011) found that, although *odc1* mRNA levels in the gills of the invasive marine mussel *Mytilus galloprovincialis*, were upregulated during acute hypoosmotic challenge, *odc1* mRNA was reduced in the gills of the native mussel species *M. trossulus*. Similarly, whereas the brine shrimp (*Artemia franciscana*) had elevated Odc activity in the gills following transfer to less salty waters (Watts et al., 1994; Watts et al., 1996), other crustaceans showed either increased Odc activity (*Callinectes sapidus*; Lovett and Watts, 1995) or higher polyamine levels in the gills during hyperosmotic exposure (*Callinectes danae*; Silva et al., 2008).

While the role of polyamines in osmotic stress tolerance is still being

investigated, previous work suggests that polyamine synthesis is upregulated in teleost fishes in response to environmental stress. Specifically, studies have shown that liver *odc1* mRNA expression is elevated in populations of the silverside *Basilichthys microlepidotus* that are native to polluted habitats (Vega-Retter et al., 2018; Véliz et al., 2020) and that gill *odc1* mRNA expression is upregulated in the euryhaline killifish species *F. heteroclitus* and *F. grandis* during acute hypoosmotic exposure (Guan et al., 2016; Whitehead et al., 2011; Whitehead et al., 2012). Polyamines have also been implicated in cell volume regulation in plants (Kotakis et al., 2014; reviewed in Groppa and Benavides, 2008) and brine shrimp (Watts et al., 1996) during acute salinity challenge. Furthermore, some studies suggest that polyamines may be important in gill epithelial remodeling during acute hypoosmotic challenge. The relative mRNA expression of *arg2, odc1*, and caspase-3 significantly increase in gill ionocytes of *F. grandis* during acute hypoosmotic exposure. However, gill caspase-3 activity is inhibited in *F. grandis* administered DFMO, suggesting that stimulating polyamine synthesis following FW transfer may support gill epithelial remodeling (Guan et al., 2016). During acute hypoosmotic challenge, the ability to transition from SW- to FW-type ionocytes is critically important in reestablishing plasma ion homeostasis. Prior studies have shown that euryhaline killifish species, such as *F. grandis* and *F. heteroclitus*, exhibit phenotypic plasticity of gill ionocytes; whereas marine species, such as *F. majalis,* exhibit a reduced capability to transition from a SW-type to a FW-type gill morphology following FW transfer (Guan et al., 2016; Whitehead et al., 2013). Because the *F. heteroclitus* and *F. majalis* gills that were analyzed in the present study are from the same fish that were used in Whitehead et al., 2013, our findings suggest that increases in gill polyamine levels are associated with an ability to undergo gill epithelial remodeling in response to acute hypoosmotic exposure. Collectively, induction of polyamine biosynthesis during acute hypoosmotic challenge may mediate morphological and physiological changes in the gills that are required for surviving large fluctuations in environmental salinity. It is worth examining whether polyamines facilitate apoptosis of SW-type ionocytes and their replacement by FW-type ionocytes following FW transfer.

4.2. Species differences in gill glutamate and Gaba production following FW transfer

Here, we also demonstrated that euryhaline and marine killifish show distinct changes in gill Glu and Gaba levels during acute hypoosmotic challenge. In the euryhaline species *F. heteroclitus* and *F. grandis*, Glu and Gaba concentrations in the gills remained constant following FW transfer, whereas gill Glu and Gaba levels were reduced in the marine species *F. majalis* following FW transfer (Fig. 4). For euryhaline teleosts, Glu could serve as an important energy source for metabolically-costly processes associated with acclimating to changes in environmental salinity, such as gill epithelial remodeling (Nilsson, 2007; Suresh et al., 1983). A recent study showed that the concentrations of Glu and three related amino acids (leucine, glycine, and valine) increase in the gills of the euryhaline tongue sole (*Cynoglossus semilaevis*) during acute hyperosmotic exposure (Jiang et al., 2019). Moreover, the euryhaline Japanese medaka (*Oryzias latipes*) exhibits increases in levels of Glu, glutamine, and proline and the relative mRNA expression of glutamate/glutamine transporters and synthesis enzymes in the gills during acute hyperosmotic challenge (Huang et al., 2020), suggesting that Glu may be important in mediating processes such as protein synthesis, energy metabolism, and cell volume regulation during acute salinity challenge (reviewed in Tseng and Hwang, 2008). Gaba signaling via the ligand-gated $GABA_AR$ has also been implicated in cell volume regulation in the central nervous system and non-neuronal cells of vertebrates (reviewed in Cesetti et al., 2011; reviewed in Kahle et al., 2008). Thus, maintaining the concentrations of Glu and Gaba in the gills, as observed in *F. grandis* and *F. heteroclitus*, may allow euryhaline fishes to sustain ion and water homeostasis in gill cells during acute salinity challenge. Conversely, an inability to maintain Glu and Gaba in the gills, as exhibited by *F. majalis*, may prevent these animals from carrying out the physiological mechanisms necessary to recover osmotic balance in response to changing salinity.

Interestingly, we found that *F. heteroclitus* and *F. grandis* exhibited an increase in Put levels, but no change in Glu or Gaba levels in the gills following FW transfer, whereas *F. majalis* showed concomitant decreases in gill Put, Glu, and Gaba concentrations. Importantly, these decreases in gill Glu and Gaba levels in *F. majalis* were larger than could be explained by the decrease in plasma osmolality resulting from FW transfer, suggesting that the synthesis of these amino acids is downregulated in the gills during acute hypoosmotic exposure (Figs. 3-4). In vertebrates, Gaba can be synthesized from one of two precursors: (1) Glu, via the enzyme glutamate decarboxylase 1 (Gad1; EC 4.1.1.15); and (2) Put, via the enzyme aldehyde dehydrogenase 9 family, member A1 (Aldh9A1; EC 1.2.1.3). While Gaba is typically synthesized by Glu, recent work suggests that there are conditions under which Put serves as an important source of Gaba, including during anoxic and hypoxic conditions in fishes and turtles. Anoxia-tolerant vertebrates, such as the crucian carp (*Carassius carassius*) and some species of FW turtles (e.g., *Trachemys scripta*, *Chrysemys picta*), are capable of surviving anoxia for prolonged periods of time due to their ability to suppress brain activity, a response that involves elevating Gaba production while reducing levels of Glu (reviewed in Nilsson and Lutz, 2004; Nilsson and Renshaw, 2004). Thus, this physiological response requires an increase in neural Put production, which allows these organisms to elevate Gaba in the brain despite low Glu levels. To distinguish the roles of Glu and Put as precursors of Gaba following FW transfer, future work should compare the effects of administering a Gad1 inhibitor, such as 3-mercaptopropionic acid, with those of administering an Odc inhibitor, such as DFMO, on gill polyamine, Glu, and Gaba levels during acute hypoosmotic exposure. This approach would allow researchers to assess how blocking Gad1 or Odc1 activity in the gills alters Gaba homeostasis during acute salinity challenge and to determine whether these effects differ between marine and euryhaline fishes.

4.3. Potential functions of Gaba signaling in the gills during acute hypoosmotic challenge

Although the role of Gaba signaling in the gills during acute salinity challenge has yet to be investigated, there are several potential mechanisms by which Gaba could aid in osmotic stress tolerance. In teleost fishes, exposure to low environmental salinity causes a reduction in plasma osmolality, which results in gill ionocyte swelling and inhibits active Cl^- secretion (Marshall, 2003; Marshall, 2011). Killifish lack an active Cl^- absorption mechanism in the gills (Scott et al., 2004), which makes paracellular Cl^- loss particularly relevant in *Fundulus* species and potentially problematic during acute FW transfer. The mitigation of diffusive loss of Cl^- following acute FW transfer is a feature that characterizes FW-tolerant killifish species and populations of euryhaline species that are best adapted to tolerate hypoosmotic exposure (Scott et al., 2004; Whitehead et al., 2011). In FW and euryhaline killifish, polyamines may serve a protective role against hyperexcitability during acute hypoosmotic exposure, a problem that could be exacerbated by a loss of extracellular Cl^- balance and may be sufficient to change the reversal potential of $GABA_AR$. Once activated, $GABA_AR$ allows Cl^- to move down its electrochemical gradient, which predominately results in hyperpolarization of cell membranes (MacDonald and Botzolakis, 2009; Magnaghi et al., 2006). However, when the electrochemical gradient of Cl^- is directed outwards, $GABA_AR$ can depolarize cell membranes, which has been observed in the mammalian airway epithelium (Xiang et al., 2007), dorsal root ganglia (Rohrbough and Spitzer, 1996; Sung et al., 2000), and the hippocampus (Huberfeld et al., 2007; Palma et al., 2006). Thus, an increase in intracellular Cl^- or a loss of extracellular Cl^-, as observed during the initial stages following FW transfer, may be enough to affect the reversal potential of $GABA_AR$, allowing FW-tolerant

killifish species to reestablish Cl^- balance in the gills. Interestingly, there is also evidence that $GABA_AR$ works in accordance with two co-transporters, NKCC1 and KCC2, to prevent excessive cell swelling or shrinking in neurons and non-neuronal cells (reviewed in Cesetti et al., 2011; reviewed in Kahle et al., 2008). In *F. heteroclitus*, acute salinity stress has been shown to activate signal transduction pathways, including mitogen-activated protein kinase (MAPK) and the dephosphorylation of focal adhesion kinase (FAK), via osmosensing cells in the opercular epithelium, a mechanism that regulates the activity of NKCC1 and cystic fibrosis transmembrane conductance regulator (CTFR) transport proteins (Marshall et al., 2005; Marshall et al., 2008; Marshall et al., 2009; reviewed by Fiol and Kultz, 2007; reviewed by Kültz, 2015). Thus, it is possible that $GABA_AR$ interacts with these transporters to prevent cell swelling and shrinkage in the gills of euryhaline killifish following changes in environmental salinity, specifically by maintaining intracellular Cl^- balance. Future work should measure the activity of $GABA_AR$ (accession number: NM_001077326) in the gills to assess whether euryhaline teleosts utilize this mechanism to tolerate osmotic imbalance following acute hypoosmotic exposure.

5. Conclusions

Our results suggest that increasing polyamine levels in the gills is associated with the compensatory response of euryhaline killifish, such as *F. heteroclitus* and *F. grandis,* during acute hypoosmotic exposure. Specifically, polyamines may stimulate gill apoptosis and remodeling in euryhaline teleosts following acute salinity challenge. Furthermore, our findings suggest that maintaining Glu and Gaba levels in the gills may be important in osmotic stress tolerance in euryhaline fishes. Future studies are needed to examine the osmoregulatory roles of polyamines, Glu, and Gaba in the gills during acute salinity challenge and to assess how these mechanisms may differ between euryhaline and marine teleost fishes. Collectively, this study highlights how euryhaline and marine teleosts differ in their capabilities to make compensatory changes in their physiology when exposed to changing environmental salinity.

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Declaration of Competing Interest

The authors declare no competing interests, financial or otherwise.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.110969.

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