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The Effects of Salinity on Gene Expression of Ion Transporters in Gulf Killifish *(*Fundulus *grandis)* Embryos

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Abstract. The Gulf killifish (*Fundulus grandis*) is a euryhaline species inhabiting the coastal marshes of the Gulf of Mexico. Although there is considerable information on the mechanisms of salinity tolerance and osmoregulation in adult fish, little is known of the ontogeny of osmoregulatory systems in developing embryos or of the physiological consequences of exposure to varying environmental salinities to developing embryos. This study investigated the ontogeny of gene expression for vacuolar H^+-ATPase (VHA), Na^+/H^+ exchanger 3 (NHE3), Na^+/HCO_3^- co-transporter 1 (NBC1), $Na^+/K^+/2Cl^-$ co-transporter 1 (NKCC1), and carbonic anhydrase II (CAII). These transport proteins were chosen based on their responsiveness to environmental salinity in adult killifish, and because they are important in maintenance of osmoregulation and acid-base balance. Embryos were exposed to 0.4, 7, 15, or 30 ppt from 1 hour post-fertilization to hatch, and sampled at three time points including the initiation of the embryonic shield (stage 15), onset of heart beat (stage 25), and the extension of the head (stage 35). Embryo mortality was highest during exposure to 0.4 ppt, but was not significantly different between the 7 ppt, 15 ppt and 30 ppt treatments. There was no detectable NHE3 at any time throughout development, whereas VHA, NBC1, and NKCC1were expressed as early as stage 15, albeit at varying levels. In contrast, CAII was only expressed at stage 25 and beyond. This study suggests that transcripts for important osmoregulatory proteins are expressed early during embryonic development, and that their mRNA levels are influenced moderately by environmental salinity.

Keywords: *Fundulus*, embryogenesis, salinity, ion transporter.

1. Introduction

Adult Gulf Killifish Fundulus grandis can tolerate a wide range of environmental salinities, which is consistent with its distribution in coastal waters of the Gulf of Mexico; an environment also subject to frequent and episodic salinity changes (Hubbs et al., 2008). Fluctuations in environmental salinity are often large during the peak of F. grandis reproduction in the spring and summer months, when large fresh water inputs from the Mississippi River or from inclement weather often occur. These variations in environmental salinity are likely to influence factors such as hatching rate, developmental time, and the emergence of physiological systems, and these alterations in embryonic ontogenesis are likely to affect an animal's fitness or physiological resilience. Adult females tend to spawn with salinity between 5 ppt to 39 ppt from May to September in the marsh grass (Nordilie, 2006), where they are prone to fresh water from rainfall during summer. Although Fundulus eggs are able to tolerate fluctuations in environmental salinity, they are susceptible to mortality at high osmolalities (Rao, 1974). Therefore, development of osmoregulatory mechanisms is critical during early development of the Gulf Killifish, Fundulus grandis. Ion-regulated proteins may be involved in osmoregulation of early teleost ontogenesis. Vacuolar H+-ATPase (VHA), Na+/H+ exchanger 3 (NHE3), Na+/HCO3- co-transporter-1 (NBC1), and Na+/K+/2Cl- co-transporter 1(NKCC1) are all expressed in ion-transporting cells, where they help mediate the transepithelial movement of ions for the purpose of osmoregulation in F. heteroclitus (Scott et al., 2008). Carbonic anhydrase II **(**CAII**)** is another enzyme that is involved in ion transport, but also has important implications in acid-base balance. VHA provides an electrochemical gradient for ion transport proteins such as Cl-/HCO3– co-transport (Harvey et al., 1999), however, there is a need to clarify the presence of Cl- /HCO3– co-transports in F. grandis because of an apparent lack of active Cl- uptake in this species (Patrick et al., 1997). Moreover,

research has shown that NKCC1, NHE3 and CAII mRNA expression were increased after seawater transfer in F. heteroclitus (Scott et al. 2008), but it is unclear if F. grandis, especially as embryos, shows a similar response to environmental salinity challenges. Therefore, our objective was to observe how environmental salinity affects both mortality and the mRNA levels of key ion-transport proteins during embryonic development of Gulf killifish.

2. Material and Methods

2.1. Animal Care and Experimental Design

Adult *F. grandis* were obtained from Grand Isle, Louisiana and transferred to the Louisiana State University AgCenter's Aquaculture Research Station (Baton Rouge, LA, USA) where they were held for at least one month prior to their use as a brood stock. Fish were maintained at a water temperature of 28 ± 1˚C and salinity of 7 ppt, and at a natural light cycle for the months of May to September. Fish were fed a commercial fish pellet twice a day at 2% body weight throughout experimentation.

2.2. Effect of Environmental Salinity on Embryos Mortality

We had a separate batch of animals that were not sampled but instead were placed in water at the four different salinities to measure time to hatch (data not shown) and mortality. A group of embryos were exposed to 0.4 ppt, 7 ppt, 15 ppt, or 30 ppt to assess the influence of salinity on embryo mortality and time-to-hatch (data not shown). Embryos were obtained using a manual fertilization procedure from a brood stock of 40 females and 5 males (Brown et al., 2011). Fertilized eggs were randomly assigned to 12 baskets (n=39 per basket), and three baskets were placed into one of four salinity solutions (0.4 ppt, 7 ppt, 15 ppt and 30 ppt). Each salinity was maintained within a separate circulating system, and all treatments were kept at a 12 h light: 12 h dark cycle. Eggs were monitored at days 0, 2, 4, 6, and 11 days post- fertilization for mortality, and the time at hatch for surviving animals recorded. Embryo mortality was assessed by calculation of dead embryos divided by total embryos.

2.3. Water Chemistry

Each salinity was made by mixing Instant Ocean® Salt and reverse-osmosis system water. Water salinity was monitored daily using a hand-held salinity meter, and water samples taken for analyses of total Na^+, K^+, Ca^{2+}, and Mg^{2+} by flame atomic absorption spectrophometer (Varian AAS240) and total Cl^- using a modified mercuric thiocyanate method (Zall et al., 1956) (Fig. 1).

2.4. Staging Embryos and RT-PCR

Two-hundred and eight fertilized eggs were randomly assigned to three replicates of each of four salinities (0.4 ppt, 7 ppt, 15 ppt and 30 ppt). Embryos were staged according to the descriptions provided by Armstrong and Child (1965). Based on physiological characteristics, three developmental points were chosen in order to ensure sampling at a consistent developmental stage. Embryos at stage 15 showed a germ ring around the margin of the blastoderm, which was a rudimentary embryonic shield. Stage 25 was determined by the onset of a functional cardiovascular system as determined by a pumping heart monitored under a stereomicroscope. Embryos at stage 35 had an obvious head extension and dorsal fin formation. After staging the embryos, three embryos were collected randomly from each stage per replicate. Total mRNA was isolated from embryos using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and mRNA were converted into cDNA following the instruction of Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). cDNA was amplified and primers were designed according to Scott **(**2008**).**

2.5. Statistical Analyses

ANOVA was used for statistical analysis using SAS (V. 9.3, SAS Institute Inc., Cary, NC, USA). Statistical differences were considered significant at *P*-values of 0.05 or less.

3. Results

Exposure **o**f F. grandis embryos to low salinity approaching that of fresh water (0.4 ppt) enhanced mortality (Fig. 2), and mortality increased as development progressed. There was no significant difference in

embryo mortality between embryos exposed to7 ppt and 15 ppt or 30 ppt (Fig. 2). Total mRNA was extracted from the embryo and was reverse-transcribed into cDNA. cDNA from embryos at different stages was used for PCR amplification and the products' lengths were between 53 to 77 base pairs. NHE3 was not expressed at any time between stages 15 and 35, and CAII was only expressed in embryos at stages 25 and 35. However, other genes of ion transporters such as NBC1, NKCC1, and VHA were expressed in embryos between stages 15 to 35 (Fig. 3).

4. Discussion

This study described the effects of salinity on embryo mortality and the ontogeny mRNA expression of critical ion transporters in embryos developing at salinities ranging from fresh water to sea water. Mortality was used as an indicator to assess embryo tolerance to salinities commonly found by this species in the environment during development. Our experiment demonstrated that water salinity as low as 0.4 ppt can kill about 90 % F.grandis embryos (Fig. 2), which corroborates the conclusion of Brown and workers (Brown et al., 2011). Embryos were not significantly more sensitive to rearing to salinities ranging between 7 ppt and 30 ppt, which is consistent with previous findings (Nordlie 2006). Embryos were characterized as gastrulae (stage 15-18) at 11 days post-fertilization according to physiological characteristics (Armstrong and Child, 1965). High pre-gastrulation mortality of embryos incubated in 0.4 ppt suggested that this salinity was below the lower hypo-osmotic limit for embryonic survival in F. grandis embryos.

Acclimation of fertilized eggs to a different salinity may produce an osmotic stress which may induce changes in the ontogeny of transcriptional responses in embryos. In order to compare transcriptional responses at similar stages of development, embryos were sampled at three easily identifiable developmental time points. This sampling regime was necessary considering the significant effect that salinity had in developmental rate, which may have confounded direct comparison in mRNA levels between treatments. We assessed the mRNA levels of VHA, NHE3, NBC1, NKCC1 and CAII since their gene products are transport proteins with important roles in osmoregulation and acid-base balance in adult killifish (Scott et al., 2008). We found regardless of salinity, VHA, NBC1 and NKCC1 mRNA were expressed as early as stage 15 of F. grandis. This observation suggested that these proteins are critical to early-life survival of fish embryos, and that their transcriptional response is relatively refractory to environmental salinity (Fig. 3). In comparison, CAII mRNA was only expressed at stages 25 and 35 during embryonic development. The exact reason is unclear, but it may be related to the tissue development in the embryos. As a critical protein in acid-base regulation, CA is also coupled to ion regulation and its function is based on tissue distribution. CA expressed in red blood cells can excrete CO2 from the body, however, gill CA catalyzes the hydration/dehydration reactions of CO2 within the branchial epithelium to provide counter ions for ion exchange processes that regulate acid-base balance and ionic homeostasis (Henry and Swenson, 2000). We also found no detectable NHE3 mRNA expression at any time throughout development. This observation was surprising considering that NHE2 and NHE3 mRNA are found in high abundance in the gills of in adult killifish, where they mediate transepithelial Na+/H+ transport (Edward et al., 2005). Overall, this study suggests that transcripts for important osmoregulatory proteins are expressed during embryonic development, and that their mRNA levels are influenced moderately by environmental salinity.

5. References

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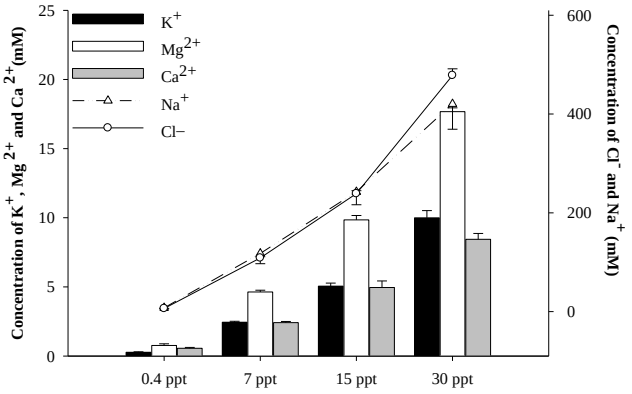

Fig. 1: Total measured concentrations of Na+, K+,Mg2+, Ca2+ and Cl -. Data represent mean ±SE (n=17 days).

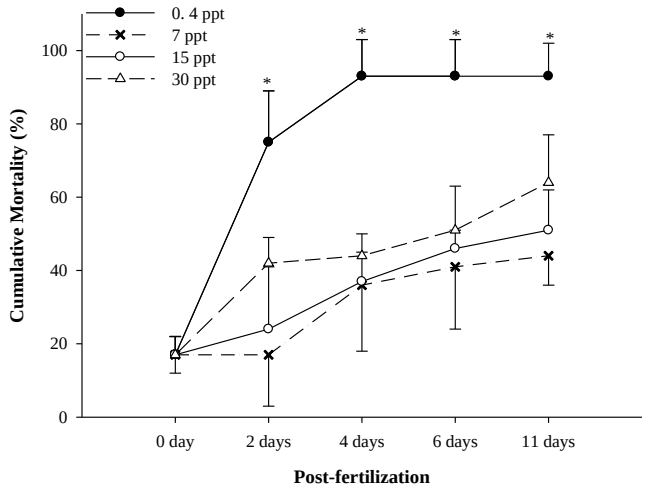

Fig. 2: Cumulative percent mortality of eggs in relation to time in various incubation salinities. Data represent mean ±SE (n=39). * means significant difference compared with mortality at day 0.

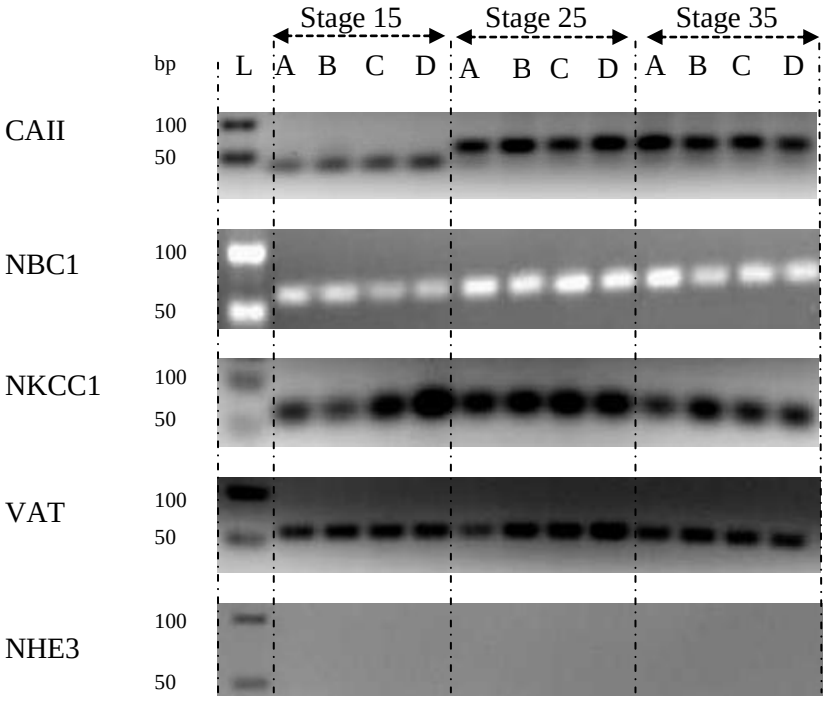

Fig. 3: Ion-regulated gene expression in different salinity water of different embryo stages. ABCD stands for 0.4 ppt, 7 ppt, 15 ppt and 30 ppt. L means 50 base pairs ladder, and bp means base pairs.