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Effects of potassium ion supplementation on survival and ion regulation in Gulf killifish *Fundulus grandis* larvae reared in ion deficient saline waters

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ABSTRACT

Teleost fish often live in an environment in which osmoregulatory mechanisms are critical for survival and largely unknown in larval fish. The effects of a single important marine ion (K^+) on survival and ion regulation of larval Gulf killifish, an estuarine, euryhaline teleost, were determined. A four-week study was completed in four separate recirculating systems with newly hatched larvae. Salinity in all four systems was maintained between 9.5 and 10‰. Two systems were maintained using crystal salt (99.6% NaCl) with K^+ supplementation (1.31 ± 0.04 mmol/L and 2.06 ± 0.04 mmol/L K^+; mean ± SEM), one was maintained with crystal salt and no K^+ supplementation (0.33 ± 0.05 mmol/L K^+), the fourth system was maintained using a standard marine mix salt (2.96 ± 0.04 mmol/L K^+), the salt mix also included standard ranges of other ions such as calcium and magnesium. Larvae were sampled throughout the experiment for dry mass, Na^+/K^+-ATPase (NKA) activity, whole body ion composition, relative gene expression (NKA, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR)), and immunocytochemistry staining for NKA, NKCC, and CFTR. Larvae stocked into water with no K^+ supplementation resulted in 100% mortality within 24 h. Mortality and dry mass were significantly influenced by K^+ concentration ($P \leq 0.05$). No differences were observed among treatment groups for NKA activity. At 1 dph NKA mRNA expression was higher in the 0.3 mmol [K^+] group than in other treatment groups and at 7 dph differences in intestinal NKA and CFTR staining were observed. These data indicate that the rearing of larval Gulf killifish may be possible in ion deficient water utilizing specific ion supplementation.

1. Introduction

Many teleost fishes live in an environment with an external osmolality differing from their own internal conditions, where efficient osmoregulatory mechanisms are crucial for survival and maintaining homeostasis. It is well documented that adult teleost homeostasis is maintained at several sites, primarily the gills, intestine, and kidneys. Less information is available for early developmental stages during which the gills, intestine, and kidneys are not yet fully developed (Katoh et al., 2000; Varsamos et al., 2005; Bodinier et al., 2010). Ion regulation is controlled by ionocytes where there are several proteins involved in ion and water exchange (Evans et al., 2005; Lorin-Nebel et al., 2006). Previous investigations have focused on Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and the cystic fibrosis transmembrane conductance regulator (CFTR) as the three primary proteins involved in ionoregulatory processes (Hirose et al., 2003). In gill ionocytes, ion transport is activated by the basolaterally located NKA which generates an electrochemical gradient by coupling two extracellular K^+ with three intracellular Na^+, driving the ions according to expression, location, and abundance of other proteins such as NKCC and CFTR (Kang et al., 2008; Bodinier et al., 2009a). Two NKCC isoforms have been identified (NKCC1 and NKCC2). NKCC1, the secretory isoform is expressed basolaterally in the gill ionocytes, while NKCC2 is identified as an absorptive isoform and is expressed apically along the intestinal and urinary bladder epithelium of saltwater and euryhaline teleosts (Lorin-Nebel et al., 2006; Kang et al., 2010). In gill ionocytes, NKA is responsible for lowering intracellular Na^+ allowing the basolateral NKCC1 to import Na^+, K^+, and two Cl^- ions. Excess intracellular Cl^- is then secreted through the CFTR chloride channel (Christensen et al., 2012). Additional ion regulation and water exchange in marine and euryhaline teleosts occurs intestinally to absorb Cl^- and water (Marshall and Grosell, 2005; Bodinier et al., 2009b). The exact mechanisms by which intestinal ion and water regulation take place are still unclear. The apically located NKCC2 most likely plays an active role in the reabsorption of ions (Lorin-Nebel et al., 2006). By exposing

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euryhaline teleosts to varying salinities or individual ion concentrations, it may be possible to immunolocalize specific osmoregulatory proteins in the gill filaments and intestinal epithelium to better understand the physiological adaptations these species undergo to cope with environmental challenges.

There are five major ions found in marine water; chloride (Cl^-), sodium (Na^+), magnesium (Mg^{2+}), sulfate (SO_4^-), and potassium (K^+), with numerous other minor ions present in lower concentrations. Potassium, sodium, and calcium serve as critical ions in electrolyte and acid–base homeostasis (Fielder et al., 2001). Potassium and sodium are also involved in ion regulation of intracellular fluids, as previously described. The high cost of synthetic marine salt mixes has facilitated interest in alternative low-cost salt sources and supplementing physiologically important ions. Potassium can be added directly to the water in the form of KCl or supplemented in the diet to maintain health. Juvenile channel catfish (*Ictalurus punctatus*) had no need for dietary supplementation of K^+ when it was supplied in the water at concentrations ≥0.1 mmol/L (Wilson and El naggar, 1992). Dietary K^+ supplementation for juvenile hybrid tilapia (*Oreochromis niloticus*×*Oreochromis aureus*) was found to significantly improve growth performance as compared to tilapia only supplemented with K^+ in the water (Shiau and Hsieh, 2001). Several studies have demonstrated the possibility to culture marine species using inland saline groundwater or water from brackish aquifers with K^+ supplementation (Fielder et al., 2001; Doroudi et al., 2006; Fotedar et al., 2008). Australian snapper (*Pagrus auratus*) maintained in saline groundwater low in K^+ began to lose equilibrium and feed response within two days and after four days all individuals had either lost equilibrium or died (Fielder et al., 2001). When the saline groundwater was supplemented with K^+, the Australian snapper demonstrated significantly increased growth, survival, and feed conversion ratio as compared to those with little or no K^+ supplementation. These studies are evidence that culture of marine species is possible through utilization of ion deficient waters by employing either dietary or environmental ion supplementation. Although many studies have explored the importance of salinity in euryhaline species for juveniles and adults (Doroudi et al., 2006; Roy et al., 2007; Fotedar et al., 2008; Coulon et al., 2012; Patterson et al., 2012), few have attempted to explore the importance of specific ion concentrations such as K^+, Ca^{2+}, and Mg^{2+} in larval fish.

Fundulus sp. have been recommended as a powerful model concerning environmental challenges such as extreme salinity, oxygen concentrations, and temperatures. The combination of attributes such as ease of breeding, euryhalinity, resilience to toxicity, and wide distribution is shared by few other species making *Fundulus* sp. unique as a model regarding biological responses to environmental changes (Burnett et al., 2007). Reviews of teleost ion and acid–base regulation through the gill epithelium have relied heavily on previous research conducted with *Fundulus heteroclitus* as a model (Evans et al., 2005). The Gulf killifish (*Fundulus grandis*) is a hardy species able to survive in salinities ranging from freshwater to an upper limit of almost 80‰ (Perschbacher et al., 1990). The ability of the Gulf killifish to withstand a wide variety of temperatures and salinities, accompanied by growing popularity among anglers gives them high potential as a cultured marine baitfish within the Louisiana and northern Gulf of Mexico (Oesterling et al., 2004). The unique osmoregulatory capabilities of Gulf killifish may also present an opportunity for rearing individuals in ion deficient waters with specific ion supplementation. The objective of this study was to examine the effects of K^+ supplementation on a biochemical, molecular, and whole organism level in Gulf killifish larvae reared in ion deficient water. Specifically we investigated whole body ion concentrations, Na^+/K^+-ATPase activity, and expression/localization of osmoregulatory proteins (NKA, NKCC, and CFTR) at selected biological membranes in newly hatched Gulf killifish.

2. Materials and methods

2.1. Animals

Newly hatched (<4 h old) Gulf killifish larvae were stocked, in triplicate, at a density of 7 larvae per L into 50-L aquaria maintained on a common recirculating system utilizing biological and ultra violet filters. Embryos were terrestrially incubated to ensure all larvae were at similar developmental stages upon hatch (Coulon et al., 2012). Once hatched, a Jensorter, LLC model FCM fry counter (Sweet Home, OR, USA) was used to count the larvae. Larvae were fed a diet of *Artemia* sp. nauplii (30 *Artemia* $fish^{-1}$ d^{-1}) for the first 7 days (d) at three times per day followed by a 50% protein, 14% lipid powdered diet fed to apparent satiation 4 times per day for the remainder of the study. After 4 weeks, survival was calculated by draining all aquaria and counting each individual.

2.2. Experimental design and water quality

Treatment groups with increasing concentrations of potassium were established by adding crystal salt with K^+ supplementation or a marine salt mixture to a potable municipal water supply with an initial salinity of 0.1 ppt and a K^+ concentration of 0.33±0.05. The experiment consisted of four treatments, in triplicate, each with a fourth tank reserved for biological sampling. All treatments were maintained at a salinity of 9.4–10‰ (Table 1). Salinity was maintained using crystal salt (Diamond Crystal Solar Salt, 99.6% NaCl) in the three treatments while the salinity of a fourth reference group was maintained using Crystal Sea marine mix salt (Marine Enterprises International, Inc.; Baltimore, MD, USA), which contained standard concentrations of all essential ions (Table 1). Potassium ion concentration was left at municipal concentration for treatment 1 and supplemented in treatments 2–3 in the form of KCl (Sigma Aldrich, St. Louis, MO, USA), to give concentrations of 1.31±0.04 and 2.06±0.04, respectively. Salinity was monitored daily and adjusted as needed. Total ammonia nitrogen (TAN) and nitrite nitrogen were measured once a week using the salicylate and diazotization method, respectively. Alkalinity and hardness were measured every two weeks using titration kits and reported as $CaCO_3$. An ion meter was used to measure pH every week. Dissolved oxygen (DO) was checked regularly to ensure concentration greater than 6.0 mg L^{-1}, but not recorded. Each recirculating system remained at ambient temperature and was monitored using

Table 1
Mean salinity and ion concentrations (±SEM) given for each K^+ concentration treatment group. Superscript letters denote statistically significant differences in specific ion concentrations among treatment groups (P≤0.05).

Parameter	Treatment group			
	1	2	3	4**
K^+ (mmol)	0.33 ± 0.05^{A}	1.31 ± 0.04^{B}	2.06 ± 0.04^{C}	2.96 ± 0.04^{D}
Na^+ (mmol)	170.19 ± 3.40^{A}	157.52 ± 6.08^{AB}	151.56 ± 7.03^{AB}	138.01 ± 0.83^{B}
Mg^{2+} (mmol)	0.04 ± 0.00^{A}	0.12 ± 0.01^{A}	0.11 ± 0.01^{A}	11.55 ± 0.10^{B}
Ca^{2+} (mmol)	0.31 ± 0.00^{A}	0.43 ± 0.05^{A}	0.30 ± 0.03^{A}	2.25 ± 0.02^{B}
Salinity (‰)	9.8±0.3	9.4±0.4	9.4±0.4	9.6±0.1
pH	8.56±0.05	8.27±0.10	8.24±0.09	8.07±0.11
TAN (mg/L)	0.03±0.01	0.02±0.00	0.01±0.00	0.04±0.03
NO_2 (mg/L)	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.00
Hardness (mg/L)*	37.25±1.55	39.17±2.06	33.83±2.20	1442.67±11.04
Alkalinity (mg/L)*	184.00±25.16	186.00±6.55	182.50±6.98	209.83±3.06

* Reported as $CaCO_3$.
** Represents reference salt group.