水研センター研報，第40号，97－107，平成27年
Bull. Fish. Res. Agen. No. 40, 97－107, 2015

Application of Physiological Tests to Determine Specific Monovalent and Divalent Ion Supplementation for Culture of Marine Species

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Abstract: The culture of marine and euryhaline fishes in low salinity or ion deficient waters has been an area of interest for many aquaculturists due to the expense of marine salt mixes. The Gulf killifish (*Fundulus grandis*) is a euryhaline teleost found abundantly in coastal marshes along the Gulf of Mexico. The ability to investigate the molecular underpinnings of specific ions in this model species could have a number of implications for other commercially important marine finfish species. Although studies have examined the influence of salinity on adults and juveniles, few have investigated the role of salinity or specific ion concentrations in larvae. These investigations utilize a model teleost to determine the role of specific monovalent and divalent ions at biochemical and molecular levels.

Separate four week trials were conducted exposing newly hatched Gulf killifish to concentration gradients of potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}). The K^+ supplementation consisted of 0.3, 1.3, and 2.9 mM. Treatment groups for Ca^{2+} consisted of 0.2, 1.1, 1.5, and 2.1 mM Ca^{2+}, while trials using Mg^{2+} consisted of 0.1, 2.7, 5.1, and 10.4 mM Mg^{2+}. All treatments were maintained at a salinity of 9.5–10‰ using crystal salt (99.6% NaCl). Each investigation consisted of four 50-L aquariums stocked at 7 larvae per liter for each concentration. Fish were sampled at 0, 1, 3, 7, 10, 14, and 28 d post hatch (DPH). Upon each sampling the standpipe was adjusted to maintain a constant density for each treatment. Collected samples were analyzed for whole body ion concentrations (K^+, Na^+, Mg^{2+}, Cl^-), Na^+/K^+-ATPase (NKA) activity, dry weight, and expression/localization of ion transport proteins (NKA, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR)).

Mortality and growth was significantly influenced by K^+ concentration ($P < 0.05$). No differences were observed among treatment groups for NKA enzyme activity, however at 28-d post hatch (dph) there were significant differences in dry weight among K^+ treatment. At seven dph differences in intestinal NKA and CFTR staining were observed, and NKA mRNA expression was also found to be higher in the 0.3 mM [K^+] group than in other treatment groups. Survival was significantly influenced by both Mg^{2+} and Ca^{2+} concentration ($P \leq 0.05$). Highest survival (71.1%) in the Ca^{2+} trial was noted in the 0.2 mM [Ca^{2+}] treatment. In the Mg^{2+} trial, highest survival was noted in the 2.7 mM [Mg^{2+}] treatment (82.9%). NKA enzyme activity was reduced and delayed peaks were observed in whole body ion composition in the Mg^{2+} treatments. In addition, decreased CFTR intensity was observed at the gill and intestine epithelium for the 0.05 mM [Mg^{2+}] treatment at 1 dph, indicating that Mg^{2+} deficiency has a possible effect on larval osmoregulatory capability. The role of intestinal epithelium in ion uptake not only allows the potential for uptake and maintenance of ion balance from dietary sources, but also demonstrates the potential to modulate concentrations of specific ions in prepared waters for euryhaline and marine teleosts.

Key words: Gulf killifish, osmoregulation, potassium, magnesium, euryhaline

2015年１月30日受理（Received on January 30, 2015）
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The major ions in seawater are; chloride (Cl^-), sodium (Na^+), magnesium (Mg^{2+}), Calcium (Ca^{2+}), sulfate ($SO4^-$), and potassium (K^+), with a variety of other ions present in low concentrations. The three ions Ca^{2+}, Mg^{2+}, and K^+ are necessary for maintaining electrolyte and acid-base homeostasis, and the regulation of osmolality and intracellular fluids (Fielder *et al*., 2001). The high cost of synthetic marine salt mixes has facilitated interest in alternative low-cost salt sources and supplementing physiologically important ions. Potassium is involved in ion regulation of intracellular fluids and can be added directly to the water, usually in the form of KCl, or supplemented in the diet (Wilson and El Naggar, 1992).Calcium and magnesium both influence the permeability of osmoregulatory membranes making them critical ions in teleost ionic regulation (Silva *et al*., 2005). Magnesium is responsible for the activation of numerous enzymes making it a necessary cofactor to the transfer of phosphate groups and the activation of ATP-dependent ion pumps such as NKA (Bijvelds *et al*., 1998). In freshwater fish, Mg^{2+} deficiency is associated with increased Na^+ and Ca^{2+} levels as well as low K^+ levels (Bijvelds *et al*., 1997).

Ion regulation is controlled by ionocytes in which 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). The ionoregulatory process is initiated by a basolaterally located NKA which couples two extracellular K^+ with three intracellular Na^+ generating an electrochemical gradient that drives the ions according to expression, location, and abundance of NKCC and CFTR (Bodinier *et al*., 2009; Kang *et al*., 2008). NKA is responsible for the lowering of intracellular Na^+ concentrations allowing the basolateral NKCC to import Na^+, K^+, and Cl^-, the excess Cl^- is then secreted through the chloride channel CFTR. Additional water exchange and Cl^- ion regulation occurs intestinally (Christensen *et al*., 2012) and it has also been suggested that substantial Mg^{2+} uptake occurs intestinally (Marshall and Grosell, 2005). The apically located NKCC2 most likely plays an active role in the reabsorption of ions (Lorin-Nebel *et al*., 2006). The exact mechanism by which Mg^{2+} uptake occurs has yet to be described in detail. It is suggested that significant differences in Mg^{2+} regulation exist among species, knowledge of Mg^{2+} transport across epithelia and cell membranes is still limited (Bijvelds *et al*., 1998). By exposing 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.

The Gulf killifish, *F. grandis*, inhabits the estuarine waters of the Gulf of Mexico and Atlantic coast of Florida where it is commonly used as a baitfish by anglers for speckled trout, *Cynoscion nebulosus*, flounder, *Paralichthys lethostigma*, and red drum, *Sciaenops ocellatus*. From Texas to Florida it is referred to by many regional names including but not limited to: mudminnow, mudfish, cocahoe minnow, and bull minnow. *F. grandis* are closely related to the mummichog, *F. heteroclitus*, which inhabits the Atlantic coast and is similarly utilized by anglers as a live bait. Both species are characterized as a "hardy" bait tolerant of wide swings in salinity, temperature, and other conditions encountered by live marine bait. *F. grandis* occupy coastal marshes where salinity can change dramatically over a short time period. These fish have the ability to live in salinities that range from freshwater (0 ‰) to near double the concentration of seawater (up to 70 ppt) for several days. Exploitation of this salinity tolerance in *F. grandis* allows investigators the ability to illicit physiological responses across a wide range of ion concentrations.

Several studies have investigated the use of low salinity inland seawater or water from brackish aquifers as a source for marine aquaculture. Much of these inland saline waters were found to have ion deficiencies, where specific manipulation of the major ions in saltwater have significantly increased growth and survival (Doroudi *et al*., 2006; Fielder *et al*., 2001; Fotedar *et al*., 2008). Many studies have investigated the effects of low salinity waters on juvenile and

adult euryhaline species (Coulon *et al*., 2012; Doroudi *et al*., 2006; Fielder *et al*., 2001; Fotedar *et al*., 2008; Patterson *et al*., 2012; Roy *et al*., 2007). Few studies have attempted to explore the importance of specific ion supplementation in euryhaline teleost larviculture. The objectives of this work were to demonstrate the use of physiological examinations at biochemical and molecular levels to determine specific supplementation of monovalent (K^+) and divalent ions (Ca^{2+} and Mg^{2+}) as replacements for saltwater in the culture of *F. grandis* larvae.

Materials and Methods

Three separate four-week experiments were conducted to investigate the impact of external K^+, Ca^{2+} and Mg^{2+} concentrations on larval *F. grandis*. Newly hatched (< 4 h old) *F. grandis* 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. Larvae were fed a diet of *Artemia sp.* nauplii (30 *Artemia* $fish^{-1}$ d^{-1}) for the first 7 d at a rate of three times per day followed by Otohime^{TM} B1 (Reed Mariculture Inc., Campbell, CA, USA) 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.

Treatments were established by adding crystal salt (Diamond Crystal Solar Salt, 99.6% NaCl) to 10‰ with ion supplementation.Potassium supplementation consisted of 0.3, 1.3, 2.0and 2.9 mM (Table 1). This investigation represents [K^+] from values similar to

Table 1. Mean salinity and ion concentrations (± SEM) given for each K^+ ,Ca^{2+}, and Mg^{2+} Concentration treatment group. Superscript letters denote statistically significant differences in specific ion concentrations among treatment groups ($P < 0.05$)

	K^+ Treatment group			
Parameter	1	2	3	4**
K^+ (mM)	0.33 ± 0.05^A	1.31 ± 0.04^B	2.06 ± 0.04^C	2.96 ± 0.04^D
Na^+ (mM)	170.19 ± 3.40^A	157.52 ± 6.08^{AB}	151.56 ± 7.03^{AB}	138.01 ± 0.83^B
Mg^{2+} (mM)	0.04 ± 0.00^A	$0.12 \pm .01^A$	0.11 ± 0.01^A	11.55 ± 0.10^B
Ca^{2+} (mM)	0.31 ± 0.00^A	0.43 ± 0.05^A	0.30 ± 0.03^A	2.25 ± 0.02^B
Salinity(‰)	9.8 ± 0.28	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

	Ca^2 Treatment groups			
Parameter	1	2	3	4
K^+ (mM)	2.37 ± 0.03	2.32 ± 0.02	2.33 ± 0.02	2.31 ± 0.05
Na^+ (mM)	165.31 ± 3.07	166.72 ± 4.83	166.72 ± 4.83	165.84 ± 2.97
Mg^{2+} (mM)	0.11 ± 0.01	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.01
Ca^{2+} (mM)	0.20 ± 0.02^A	1.08 ± 0.03^B	1.50 ± 0.04^C	2.07 ± 0.05^D
Salinity(‰)	10.3 ± 0.03	10.1 ± 0.07	10.1 ± 0.06	10.0 ± 0.09
TAN (mg/L)	0.03 ± 0.02	0.03 ± 0.02	0.01 ± 0.01	0.02 ± 0.02
NO_2 (mg/L)	0.05 ± 0.02	0.03 ± 0.02	0.02 ± 0.01	0.01 ± 0.00
Hardness (mg/L)*	193 ± 32.76^A	1090 ± 15.81^B	1433 ± 32.24^C	1740 ± 31.52^D
Alkalinity (mg/L)*	178 ± 17.97	263 ± 57.64	140 ± 4.08	170 ± 7.07

*Reported as $CaCO_3$

	Mg^{2+} Treatment groups			
Parameter	1	2	3	4
K^+ (mM)	2.37 ± 0.03	2.40 ± 0.04	2.29 ± 0.05	2.41 ± 0.02
Na^+ (mM)	168.48 ± 3.64	169.96 ± 4.11	162. 64 ± 4.83	170.55 ± 4.97
Mg^{2+} (mM)	0.05 ± 0.01^A	2.88 ± 0.08^B	5.76 ± 0.16^C	11.52 ± 0.19^D
Ca^{2+} (mM)	0.19 ± 0.02	0.24 ± 0.02	0.15 ± 0.01	0.26 ± 0.02
Salinity(‰)	9.9 ± 0.04	10.2 ± 0.03	10.1 ± 0.03	10.0 ± 0.05
TAN (mg/L)	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.02	0.03 ± 0.02
NO_2 (mg/L)	0.01 ± 0.03	0.03 ± 0.02	0.00 ± 0.00	0.02 ± 0.01
Hardness (mg/L)*	102 ± 25.82^A	320 ± 44.82^B	530 ± 29.35^C	1040 ± 36.21^D
Alkalinity (mg/L)*	165 ± 19.53	160 ± 4.71	150 ± 10. 62	185 ± 21.83

*Reported as $CaCO_3$

freshwater sources (0.33 mM) to concentrations of 2.96 mM, found saline waters at 10 ‰. Potassium concentration in [Ca^{2+}] and [Mg^{2+}] experiments were maintained at 2.35 mM, as determined by a previous study (Fisher *et al.*, 2013). Calcium was supplemented to give final concentrations of 0.20, 1.08, 1.50, and 2.07 mM (Table 1), while Mg^{2+} was supplemented to give concentrations of 0.05, 2.88, 5.76, and 11.52 mM. The [Mg^{2+}] experiment also received Ca^{2+} supplementation to the water in the form of $CaCO_2$, as determined in the previous experiment (Table 1). Weekly water samples were collected to quantify ion concentrations via ICP analysis by the LSU Agriculture Center; Agriculture Chemistry Department (Baton Rouge, LA, USA).

Fish were sampled at 0, 1, 3, 7, 10, 14, and 28 d post hatch (DPH). Upon each sampling the standpipe was adjusted to maintain a constant density for each treatment. Three replicates of 5 larvae were used to establish an average desiccated dry mass due to the inability to accurately measure individual dry mass of larvae less than 7 dph. Whole body ion concentration (Na^+, K^+ and Mg^{2+}) was analyzed using the methods described by (Van Genderen, 2003; Fisher *et al.*, 2013). Three replicates of 5 larvae were collected at each time point to determine Na^+/K^+-ATPase activityusing the protocol described in McCormick (1993) and expressed as μmol ADP mg protein^{-1} h^{-1}.

Six larvae were collected per treatment group at 1, 3, and 7 dph and preserved in Z-fix (Anatech, LTD, Battle Creek, MI, USA). Immunocytochemistry reactions were performed according to methods described in Bodinier *et al.* (2010) and Fisher *et al.* (2013) for NKA, CFTR, and NKCC1. Ionocyte length measurements were taken using Image Tool® version 3.0 (University of Texas Health Science Center, San Antonio, TX, USA). A minimum of 50 cells were measured per sampling period for each treatment.

Results

Growth and survival: The 0.3 mM [K^+] treatment resulted in 100% mortality within 24 h of hatch. No significant differences were seen in survival between the 1.3 and 2.0 mM [K^+] groups (survival ≤ 5%); however the 2.9 mM [K^+] treatment resulted in significantly greater survival after 4 weeks (survival ~60%). Final dry mass of the 2.9 mM [K^+] reference group was significantly higher than both the 1.3 and 2.0 mM [K^+] treatments ($P \leq 0.05$). The 2.9 mM [K^+] reference group had a final dry mass of 4.93 ± 0.54 mg while the 1.3 and 2.0 mM [K^+] treatments had dry mass of 2.39 ± 0.19 mg and 2.21 ± 1.55 mg, respectively.

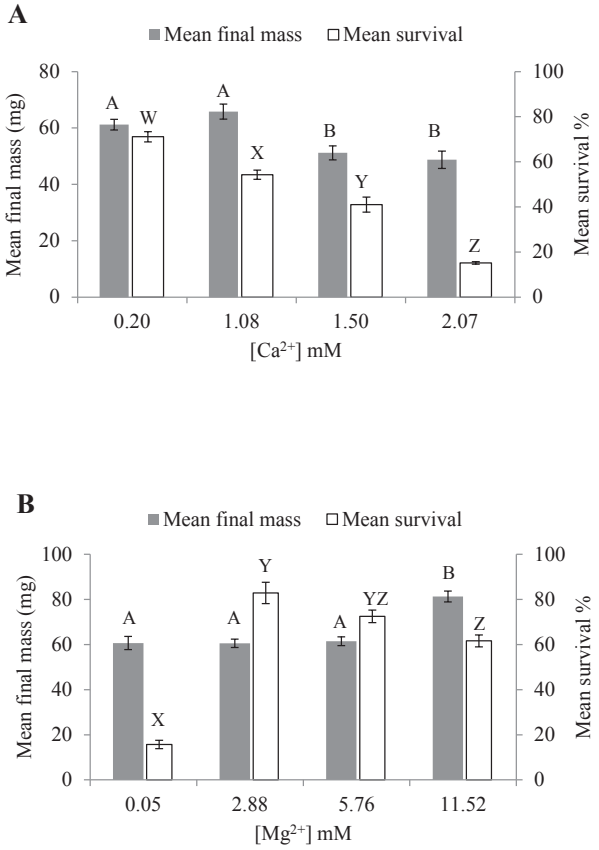

Fig. 1. Final mass and survival of Gulf killifish larvae after 4 weeks in (A) [Ca^{2+}] treatments or (B) [Mg^{2+}] treatments. Letters represent significant differences among treatment groups. Letters denote significant differences among treatments.

Survival and dry mass were significantly influenced by both [Ca^{2+}] and [Mg^{2+}] experiments. Supplementation of Ca^{2+} showed a decrease in mean final mass and mean survival with increasing concentrations of Ca^{2+} in the absence of Mg^{2+} (Fig. 1A). Supplementation of Mg^{2+} resulted in approximately a 5-fold increase in survival with Mg^{2+} supplementation as compared to no supplementation, no differences were observed among supplemented treatment groups. Final mass was not significantly

influenced until a concentration of 11.52 mM Mg^{2+} was reached (Fig. 1B).

Whole-body ion concentrations & Na^+/K^+-ATPase activity: No differences were found in Na^+/K^+-ATPase activity among [K^+] treatment groups or among time ($P \geq 0.05$). Whole body Mg^{2+} concentration showed no difference for treatment or treatment × dph interaction; however significant difference was determined for dph ($P \leq 0.05$). There was a significant increase in [Mg^{2+}] within the first week followed by a rapid decrease for all three[K^+] treatment groups. Whole body K^+ concentration had no significant difference in treatment × dph interaction, although significant differences were observed among treatments and for dph ($P \leq 0.05$).

No differences were observed among treatments for whole body ion composition in the [Ca^{2+}] study. The [Mg^{2+}] experiment showed delayed peaks in whole body [Na^+], [Ca^{2+}], and [K^+] for the 0.05 mM [Mg^{2+}] treatment group, with ion concentrations reaching their highest point 7 days later than other treatments. As expected, the Mg^{2+} deficient treatment groups (0.05 and 2.70 mM [Mg^{2+}]) also showed a significant decrease in whole body [Mg^{2+}].

Na^+/K^+ -ATPase activity in the [Ca^{2+}] study was significantly lower at 0 and 1 dph followed by a significant increase in activity throughout the experiment, however no treatment specific differences in activity were determined at any specific sample dates. The [Mg^{2+}] study demonstrated significant differences in Na^+/K^+-ATPase activity between Mg^{2+} deficient treatments and those with sufficient supply at 0 dph followed by minor, but not significant, increases throughout the experiment (Fig. 2).

Immunocytochemistry & cell volume: All negative slides, with no primary antibody, showed no immunostaining as expected (not illustrated). In [K^+] treatments at 7 dph, no significant differences were determined among treatments for gill ionocyte length. Gill ionocytes from the 1.3 mM [K^+] treatment were found to have a significantly larger area than those in the 2.0 and 2.9 mM [K^+] treatments. Intestinal CFTR and NKA were present at 7 dph in all surviving [K^+] treatment groups. No changes were observed in NKA or CFTR in the gill ionocytes. Intestinal NKA and CFTR staining appeared to show a decrease in intensity as K^+ concentration increased (Fig. 3). Very little staining was observed for NKCC both intestinally and at the gill epithelium.

No differences among treatments were observed

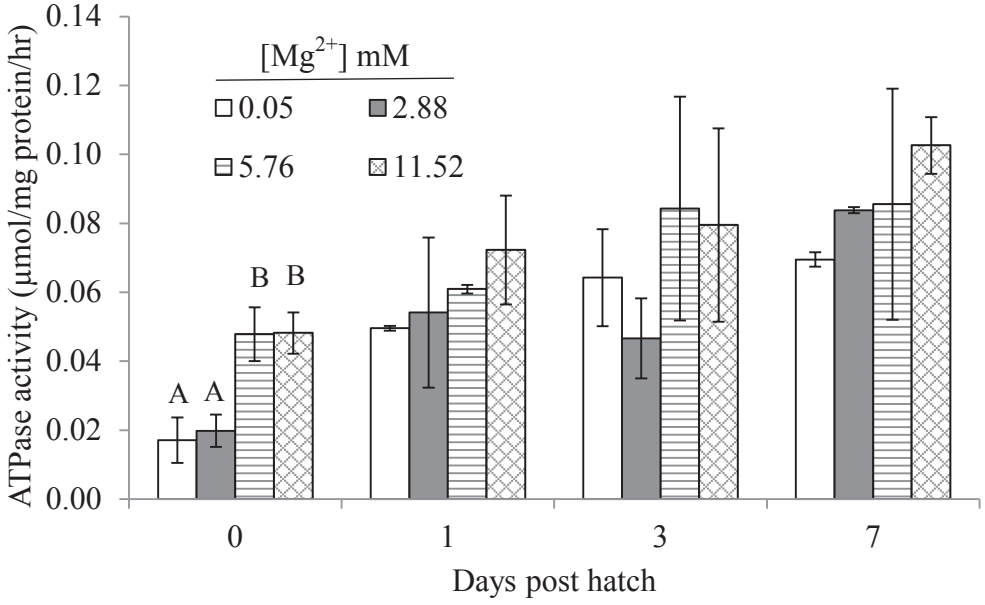

Fig. 2. Whole body NKA activity for larvae stocked in [Mg^{2+}] treatments after 0, 1, 3, and 7 dph. Letters represent significant differences among treatment groups at that day.

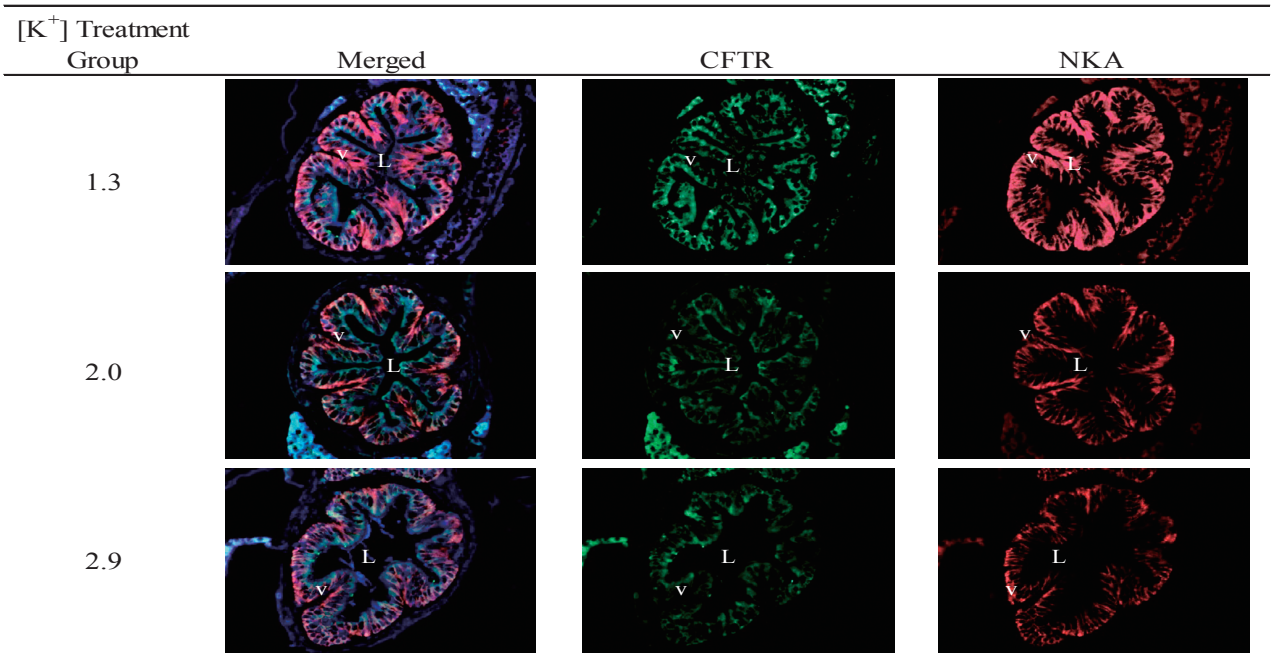

Fig. 3. Representative histological sections from larval intestines after one week in [K^+] treatment groups cut transversely at 5 μm intervals. Immunofluorescence of NKA (red) and CFTR (green). The 2.9 mmol K^+ group represents a reference salt used andcontains other ions which may be essential for the function of osmoregulatory proteins. L: lumen; v: villi

for NKA or NKCC staining intensity in the gills or intestine, nor were there any significant differences among treatments for gill ionocyte length or quantity at 1, 3, and 7 dph for both the [Ca^{2+}] and [Mg^{2+}] experiments. In the [Mg^{2+}] experiment, a decrease in CFTR staining intensity was observed at 1 dph in both the intestine (Fig. 4) and gill in the 0.05 mM [Mg^{2+}] treatment, no other changes in CFTR staining intensity were observed.

Discussion

The chemical gradient generated by the activation of NKA is one of the primary driving forces behind ion regulation, thus the ability to measure whole body NKA activity may allow for the evaluation of larval fish osmoregulation capacity in hyper and hypo-osmotic environments. It is possible that K^+ is a limiting factor in killifish larval osmoregulation and a deficiency can restrict the ability of NKA to function. The current model for chloride secretion as described by McCormick *et al.* (2003) suggests a basolateral NKA and NKCC coupled with an apically located CFTR-like protein. Because K^+ is also used by the chloride cotransporter, NKCC, to assist in the transport of chloride ions, an extracellular K^+ shortage could reduce a larval fish's ability to regulate sodium and chloride ion concentrations at critical biological membranes. Despite the fact that there were no statistical differences in NKA activity between any treatment groups, it is still conceivable that sufficient K^+ was not available for the function of NKCC. If so then the inability of the larvae to regulate either Na^+ or Cl^- ions may possibly have been a factor in the low survival of the 0.3, 1.3 and 2.0 mM [K^+] groups. The reference group (marine mix salt) was the only group that did not display poor survival and it is possible that the presence of other essential ions such as Mg^{2+} and Ca^{2+} played a significant role in the survival of this treatment group.

Potassium supplementation across the gradient examined in the current study at a constant salinity not only altered survival, but affected differences among molecular components of intestinal epithelium. Although potassium treatments in the current study did not appear to alter whole body Na/K ATPase activity, immunofluorescence

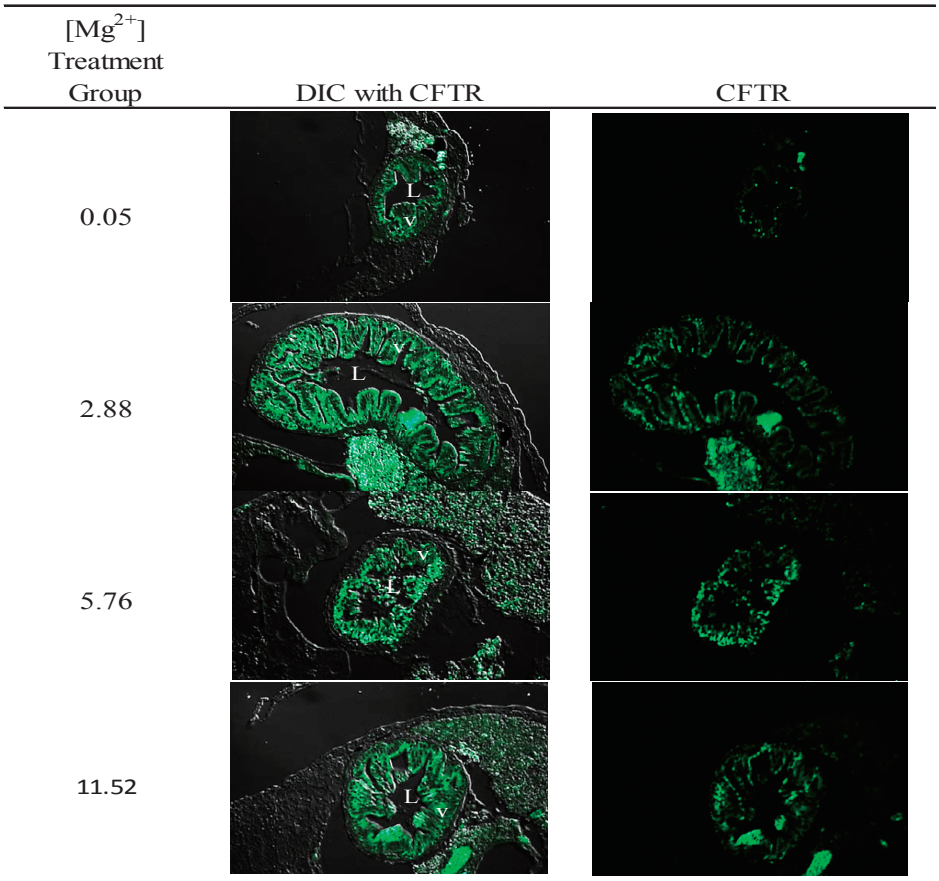

Fig. 4. Representative histological sections of the intestine from 1 day post hatch (dph) Gulf killifish larvae in [Mg^{2+}] treatment groups, cut transversely at 5 μm intervals. Differentialinterference contrast (DIC) images with immunofluorescence of CFTRL: lumen; v: vill

showed increased density of NKA and CFTR proteins as K^+ concentration decreased. Whole body K^+ concentration was greater in the reference as compared to treatments with 1.3 and 2.0 mM of K^+ both of which remained lower throughout the study as expected due to the inability to selectively retain K^+ ions. It is important to note that the reference group containing the highest concentration of K^+ was also comprised of greater concentrations of Mg^{2+} and Ca^{2+}, which could have modulated Na^+ and Cl^- absorption in the intestine relative to lower K^+ treatments in the current study that were relatively low in these divalent ions (Marshall and Singer, 2002; O'Grady, 1989). Growth and survival metrics for the reference treatment may reflect combined effects of a full to partial complement of other ions in addition to the K^+ gradient examined. The role of intestinal epithelium in ion uptake not only allows the potential for uptake and maintenance of ion balance from dietary sources and thus important in aquaculture, but also demonstrates the potential to modulate concentrations of specific ions in prepared waters for euryhaline and marine teleosts.

Calcium and Magnesium are critical in teleost ion regulation, varying concentrations of Ca^{2+} and Mg^{2+} can impact the permeability of osmoregulatory epithelia to both ions and water (Silva *et al.*, 2005). In the sea water adapted eel, removal of Ca^{2+} from the external medium diminished the active excretion of sodium by half, excretion returned to normal level after the reintroduction of Ca^{2+} to the medium (Isaia and Masoni, 1976). Proper development of many euryhaline teleosts is related to environmental Ca^{2+} concentration, for example red drum (*Sciaenops ocellatus*) fry displayed a drop in blood osmolarity when transferred from salt to freshwater, this

decline in osmolarity was reduced by the addition of Ca^{2+}(Wurts and Stickney, 1989). Previous studies have determined that Mg^{2+} is critical in the activation of ATP-dependent ion pumps (Bijvelds *et al*., 1998) making it critical for the activation of NKA. In this study, both the 0.05 and 2.88 mM [Mg^{2+}] treatment groups displayed significantly reduced NKA activity at hatch.

In the current study both Ca^{2+} and Mg^{2+} had effects on survival, growth, and molecular components at both the gill filaments and intestinal epithelium. In the absence of Mg^{2+} supplementation larval *F. grandis* had reduced survival, decreased Na^{+}/K^{+} -ATPase activity, and a delayed peak in whole body compositions for several ions essential for larval development. A decrease in CFTR intensity was also observed in the gills and intestine of Mg^{2+} deficient treatment as seen by immunocytochemistry staining at 1 dph. This is an indication that Mg^{2+} is a necessity for larval *F. grandis* growth, survival, and ion regulation. However, the Mg^{2+} of 10‰ seawater is approximately 11.52 mM and the greatest growth and survival were achieved in this study at a concentration of 2.88 mM Mg^{2+}. These results demonstrate that the potential for euryhaline teleost culture in prepared water may only require a fraction of the ion concentrations found in 10‰ seawater. Also the role of intestinal epithelium in ion uptake allows for the potential for ion maintenance through dietary sources.

Acknowledgements

This work was supported, in part, through grants from the Southern Regional Aquaculture Center and Louisiana SeaGrant. The authors would like to acknowledge the faculty, students and support staff that assisted with these investigations: Paige O'Malley, Emily McArdle, Chris Mariani, Mike Coulon, Josh Patterson, Charlie Brown.

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Annotated Bibliography

Fisher C., Bodinier C., Kuhl A., and Green C., 2013: Effects of potassium ion supplementation on survival and ion regulation in Gulf killifish *Fundulus grandis* larvae reared in ion deficient saline waters. *Comp. Biochem. Physiol., Part A.*, **164**, 572–578.

This study encompasses results pertaining to the K^+ ion manipulation portions of the abstract presented above for the UJNR Scientific Symposium. This investigation increased [K^+] from values similar to freshwater sources (0.33 mM) to concentrations of 2.96 mM, found in saline waters at 10‰. A number of biochemical and molecular techniques

were performed to examine the effect of this K^+ ion gradient, which included: whole body ion composition, Na^+/K^+ -ATPase (NKA) activity, gill ionocyte morphometrics, relative gene expression (NKA, NKCC, and CFTR), and corresponding immunocytochemistry at the gill and intestinal epithelium. Results more tangible to aquaculturist, such as growth and survival, indicated the presence of a threshold within the gradient examined whereby survival was not different between 1.3 to 2.9 mM [K^+]. Utilizing immunocytochemistry, the differences between these seemingly similar treatment groups indicated that the treatments groups between 1.3 to 2.9 mM [K^+] was different in terms of gill ionocyte area, NKA and CTFR localization.

Ostrowski A. D., Watanabe W. O., Montgomery F. P., Rezek T. C., Shafer T. H., Morris Jr J. A., 2011: Effects of salinity and temperature on the growth, survival, whole body osmolality, and expression of Na+/K+ ATPase mRNA in red porgy (*Pagrus pagrus*) larvae. *Aquacult.*, **314**, 193–201.

These authors investigated a number of physiological parameters pertaining to salinity and temperature in embryos and larvae of red porgy (*Pagrus pagrus*), which is viewed as a high-market value marine species with good potential as an aquaculture species. Embryos and resulting larvae were reared at four temperatures (17, 19, 21, 23℃) and two salinities (24 and 34‰). Larvae (16 dph) were transferred from their respective salinities to 44‰ to represent a sublethal hyperosmotic challenge. These authors demonstrated significant increases in NKA mRNA expression in individuals acclimated to 24‰ at 24 h after transferred to increased salinity, while individuals from 34‰ exhibited no significant changes in NKA expression. Temperature was not observed to influence expression of NKA, while metabolic parameters related to growth were influenced by the temperature gradient in their study. The authors utilized traditional growth metrics including molecular tools to anticipate optimum salinity (24‰) and temperature (23℃) conditions that are optimum for larval rearing of this species.

Bodinier C., Sucré E., Lecurieux-Belfond L., Blondeau-Bidet E., Charmantier G., 2010: Ontogeny of osmoregulation and salinity tolerance in the gilthead sea bream Sparus aurata. Comp. Biochem. Physiol. – Part A: Mol. Integrative Physiol., **157**, 220–228.

The gilthead sea bream (*Sparus aurata*) is a commercially important aquaculture species, which spawns in the ocean and whose resulting larvae and juveniles migrate to lower salinity estuaries and lagoons. These authors investigated the development of salinity tolerance in gilthead sea bream from 3, 30, 75, 96, and 300 d post hatch (dph) by challenging them with 9 salinities ranging between freshwater and 45.1‰. Utilizing immunohistochemistry, these authors localized the NKA throughout these challenges to document location of ion regulation within respect to this ionoregulatory protein. Initially, immunopositive NKA ionocytes were laocted in the integument along the yolk sac and integumentary folds representing the branchial slits. A functional shift from integument to gills was demonstrated 30 and 70 dph, when both the integument and gills were observed to locally express NKA, whereby from 70 to 300 dph the gills remain the main site of osmoregulation. Increases in osmoregulatory capacity for this species at the intervals examined within this study related to the shifts and patterns observed through immunohistochemistry.

Christensen A. K., Hiroi J., Schultz E. T., McCormick S. D., 2012: Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater. *J. Exp. Biol.*, **215**, 642–652.

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. Christensen *et al.* investigated changes in alewife physiology and branchial epithelium as individuals were acclimated to freshwater or saltwater and represents the first study of its kind to characterize multiple ion-transport proteins in a non-salmonid anadromous fish. Corresponding

increases in NKA, NKCC1, and CFTR abundance at the gill epithelium with increasing salinity was used to establish a gill model for hypo-osmoregulation. 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. 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. The authors determined the key differences between freshwater and seawater acclimated alewives in the context of ion transporters at the gill epithelium. The implications of these investigation has assisted in increasing the information multicellular complexes of mature ionocytes and the role of salinity and specific ion in the maintenance of homeostasis.