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# Low Salinity Tolerance in Gulf Killifish Fundulus Grandis with Relevance to Aquaculture

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# Low salinity tolerance in Gulf killifish *Fundulus grandis* with relevance to aquaculture

By

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The Gulf killifish *Fundulus grandis* is a euryhaline baitfish species with commercial aquaculture potential. The low salinity and freshwater tolerance of early life-stages of *F. grandis* were evaluated to determine the potential for inland culture and guide production protocols. Egg fertilization and embryo survival decreased in fresh water, but freshwater incubation improved larval freshwater tolerance. In juveniles, survival and growth were reduced in fresh water from 2-6 weeks post-hatch but not from 7-11 or 12-16 weeks post-hatch. $Na^+/K^+$-ATPase activity and whole body $Na^+$ content were elevated in low salinity treatments of the 7-11 weeks post-hatch age group, indicating substantial osmoregulatory ability at that age. RNA expression data from this age group indicated that freshwater acclimation is accompanied by an initial up-regulation of carbonic anhydrase followed by prolonged up-regulation of $Na^+/K^+$-ATPase. By 12-16 weeks post-hatch, *F. grandis* osmoregulatory ability is well developed with minimal differences among osmoregulatory data.

DEDICATION

I would like to dedicate this research to my parents, Tom and Ann Ramee, for their continual support in all my endeavors.

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# CHAPTER I

# INTRODUCTION

## Background

The Gulf killifish *Fundulus grandis* is a euryhaline species native to the coastal salt marshes and estuaries along the Gulf of Mexico from northern Mexico to the northern Atlantic coast of Florida (Tatum 1982; Pattillo et al. 1997; Williams et al. 2008). The species has several regional common names including bull minnows, mud minnows, cocahoe minnows, mudfish, and chub. Gulf killifish are very popular baitfish for inshore and near-shore sport fish species along the Gulf of Mexico coast such as red drum *Sciaenops ocellatus*, speckled seatrout *Cynoscion nebulosus*, and flounder *Paralichthys lethostigma* (Green et al. 2010). They are able to tolerate broad ranges of temperature (2-35°C), salinity (0 - >40 ppt), and temporarily low oxygen levels ($P_wO_2$=14 torr) (Umminger 1971; Perschbacher et al. 1990; Nordlie and Haney 1998; Virani and Rees 2000; Nordlie 2006). Though this species has been sampled in the wild at extremely low salinities (<1 ppt) (Griffith 1974; Hillis et al. 1980; Pattillo et al. 1997), it is still considered to have a marine physiology and is rarely found in inland habitats (Whitehead 2010). Gulf killifish resiliency to a broad range of salinities and dissolved oxygen levels make them prized by fisherman due to their ability to survive in bait buckets (Wallace and Waters 2004).

Though culture methods have been laid out over the past few decades (Trimble et al. 1981; Tatum 1982; Waas et al. 1983; Strawn et al. 1986; Wallace and Waters 2004; Anderson and Green 2012), commercial bait dealers are still reliant on wild caught stocks, leading to inconsistent availability and sizes of fish (Green et al. 2010). Surveys of marinas and bait shops in coastal Louisiana (Anderson and Green 2012) showed that the majority of dealers are not able to meet the year round demand for the baitfish. Most dealers estimate selling more than 1000 Gulf killifish per week and over 25% estimate selling over 5000 per week (Anderson and Green 2012). Louisiana is only one example of a Gulf coast state where demand is exceeding supply.

Saltwater fishing in the Gulf of Mexico is a massive economic industry. The National Oceanic and Atmospheric Administration (NOAA) reported $10 billion dollars spent on recreational fishing trip-related expenses in the Gulf of Mexico in 2012 by a total of 3.1 million recreational anglers and $1.4 billion was spent directly on bait and tackle (NOAA 2012). The live bait sector is a small but important part of the larger recreational fishing industry and economic analyses have concluded that commercial production of Gulf killifish can be profitable (Waas et al. 1983; Adams and Lazur 2008; Anderson and Green 2012). Thus, with the popularity of saltwater sport fishing, there is considerable opportunity for farming Gulf killifish to supplement the wild caught supply.

## Biology

Gulf killifish are fractional spawners with breeding occurring during the spring, summer, and fall, and with peaks in spawning activity near the beginning and end of this period (Gothreaux and Green 2012). Spawning starts once water temperature reaches 20°C (Tatum 1982). Gulf killifish reproductive output is relatively low compared to other

baitfish species. An individual can produce 0.72-0.90 eggs/g of female (Green et al. 2010). Eggs are usually deposited in several small groups of 10-20 eggs (Wallace and Waters 2004). In the wild, fish deposit their eggs on marsh grass during high tides and especially at night during spring tides (Greeley Jr. and MacGregor III 1983; Taylor 1999; Green et al. 2010). Their spawning pattern shows biweekly peaks, which correlate with the full and new moon phases and is also observed in captivity (Green et al. 2010). The eggs are able to withstand air exposure in humid environments and hatch about two weeks later (9-28 d) depending on the temperature (Wallace and Waters 2004).

Gulf killifish are opportunistic omnivores and easily adapt to commercial diets. In the wild, they feed on crustaceans including grass shrimp (*Palaemonetes spp.*), small crabs, amphipods, polychaete and annelid worms, fish, insects, vegetation, and detritus (Tatum 1982). In outdoor pond systems, fish have gained weight using a variety of feeding and fertilization regimes (Tatum 1982; Perschbacher and Strawn 1985). Supplemental feeding is recommended in ponds even though it is unclear whether the food is directly eaten or just provides further nutrients for fertilization (Anderson and Green 2012). In indoor culture systems, a nutritionally complete commercial feed must be provided (Anderson and Green 2012; Patterson 2014).

In the wild, 1 year-old fish have a length of 18-30 mm, and 2 year-old fish average 68 mm (Wallace and Waters 2004). Fish reach sexually maturity between 40-50 mm and have a maximum length of 140 mm (Wallace and Waters 2004). After 6 to 10 months, fish reach market size at 8.5-9 cm and 5-7 g (Anderson and Green 2012).

## Culture Techniques

Over the past few decades, there has been interest in developing techniques to commercially culture Gulf killifish. The most recommended method of culture is the three-phase system, which utilizes separate ponds for spawning, hatching, and grow-out (Tatum 1982; Waas et al. 1983; Wallace and Waters 2004; Anderson and Green 2012). Culture can be conducted in ponds, above ground pools, or recirculating systems with the same basic principles (Green 2013; Ofori-Mensah et al. 2013). Gulf killifish can also be cultured in a two-phase system, with a spawning pond and one pond for both hatching and grow-out (Tatum 1982; Strawn et al. 1986). A two-phase system is not as productive as a three-phase system but requires less land. The use of terrestrial incubation is a newer technique that could help coordinate hatching of larger cohorts and reduce cannibalism (Coulon et al. 2012).

Spawning ponds (phase 1) are recommended to be free of other fish and all aquatic vegetation before stocking. Removing vegetation limits the natural substrate available for egg deposition. Spawning ponds are stocked with a 2:1 female to male ratio at 24,710 fish/ha (10,000 fish/acre) (Tatum 1982; Wallace and Waters 2004). Green et al. (2010) found the optimal stocking density to be 30 fish/$m^3$ in static outdoor spawning pools. Water quality must be monitored and maintained. Ideal salinity is between 3 and 20 ppt (Strawn et al. 1986) or 5 to 15 ppt (Anderson and Green 2012). Broodstock are fed 3% body weight per day with food that contains 28-40% protein (Tatum 1982).

When water temperature reaches 20°C, spawning mats are placed along the edges of the pond (Tatum 1982; Wallace and Waters 2004). Spawning mats have been made out of cured Spanish moss, but commercially available Spawntex (Blocksom & Co.,

Michigan City, Indiana) spawning mats are more productive and easier to use (Green et al. 2010). Spawning mats are cut to a convenient size (i.e. 35 x 70 cm) and designed to suspend horizontally about 8-20 cm below the surface and not in contact with the bottom (Wallace and Waters 2004; Green et al. 2010). Spawning mats are left in the pond for 3-7 d depending on the rate of egg deposition and temperature (Strawn et al. 1986; Wallace and Waters 2004; Green et al. 2010; Anderson and Green 2012). It is important not to leave mats in too long in order to keep the cohort as evenly sized as possible and thus prevent cannibalism (Tatum 1982; Wallace and Waters 2004; Anderson and Green 2012). Harvested mats are then immediately replaced by clean mats.

Harvested spawning mats are moved to a hatching pond (phase 2). Hatching ponds are free of other fish and treated for aquatic insects before use in order to prevent competition and predation (Tatum 1982; Wallace and Waters 2004; Anderson and Green 2012). The ponds are then fertilized two weeks prior to stocking with inorganic and organic fertilizer. Fertilization is maintained so that there is a water transparency of approximately 30 cm using a Secchi disk (Strawn et al. 1986). This level of fertilization promotes algae growth, which is an important first feed for the freshly hatched larvae (Anderson and Green 2012). Killifish can grow equally as fast receiving only fertilizer as being fed 3% body weight per day (Strawn et al. 1986). Salinity of 4-40 ppt is recommended for hatching and survival of fry (Strawn et al. 1986). Eggs are stocked at a density of approximately 3.75 million eggs/ha (1.5 million eggs/acre) with an expected 65-80% survival to the fry stage (Tatum 1982). Hatching generally takes 10-21 d, with faster times occurring as a result of increased temperature and decreased salinity (Strawn et al. 1986). Hatching success may range from 50-80% depending on egg density, silt

levels, and dissolved oxygen (DO) levels (Strawn et al. 1986). Once fish hatch, they are fed finely ground minnow meal at 5.6 kg/ha (5 lb/ acre), though it is unclear whether this functions more as fertilizer or actual feed.

Fish are moved to grow-out ponds (phase 3) when they weigh between 0.3-0.5 g (Tatum 1982) or 60-80 d after hatch (Anderson and Green 2012). Grow-out ponds are typically the same salinity as the hatching and broodstock ponds because most production facilities have access to a saline water source. However, grow-out ponds may not have as strict of a minimum salinity requirement, but further research is needed. The fry are graded (usually with a 6.3 mm minnow grader), and different size classes are stocked in separate ponds (Wallace and Waters 2004). Growth rate in the grow-out ponds is largely determined by stocking density. Grow-out of market-sized 64 cm minnows takes 5 weeks at a stocking density of 123,500 fish/ha (50,000 fish/acre), 6-7 weeks at 247,100 fish/ha (100,000 fish/acre), and little growth occurs at 494,200 fish/ha (200,000 fish/acre) (Tatum 1982; Wallace and Waters 2004). Therefore density can be manipulated to help producers plan harvesting around times of peak demand. In grow-out ponds, fish are fed 10% of body weight for the first 10 d (Tatum 1982). A subset of fish are then sampled and weighed, and the feeding rate is reduced to 3-5% body weight. Thereafter, every 2 weeks a subsample is removed from the pond and weighed, and feeding rate is adjusted (Tatum 1982).

Recent research has developed terrestrial incubation methods (Perschbacher et al. 1995; Coulon et al. 2012). Terrestrial incubation can be used to synchronize egg hatching and reduce cannibalism (Coulon et al. 2012). Terrestrial incubation temperatures can also be manipulated so that 2 cohorts of eggs can be hatched at the same time. Prior to

terrestrial incubation, eggs are removed from spawning mats by shaking or striking the mats on a rigid screen above a plastic container of water (Anderson and Green 2012). The eggs are then poured onto a window screen sieve and sorted. Eggs are then placed between two pieces of moist (5-15 ppt) foam inside a plastic incubation chamber (Anderson and Green 2012). An incubator can be made out of a refrigerator, a thermostat, and at least one small (i.e. computer) fan (Anderson and Green 2012). Incubation temperature maintained between 20 and 24°C allows for hatching to be initiated by embryo submersion after 9 to 23 d (Anderson and Green 2012). Time to embryonic maturity is affected by incubation temperature, with incubation at 30°C resulting in embryos reaching maturity 6 d faster than incubation at 20°C (Brown and Green 2014). Foam and eggs are sprayed with saline (5-15 ppt) water every 2-3 d in order to maintain moisture and control water mold (Anderson and Green 2012). Perschbacher et al. (1995) reported that eggs terrestrially incubated for 6 and 12 d at 26°C had 95% and 92% survival, respectively. Coulon et al. (2012) recorded their highest egg survival using water at a salinity of 7.5 ppt and had the most success using foam substrate as opposed to burlap or bamboo matting. Terrestrially-incubated embryos were also larger than water-incubated embryos when incubated at 22°C for 19 d (Coulon et al. 2012). In the future, it may be effective to use spawning mats as the substrate for terrestrial incubation. This would save time and labor but has not been tested yet. Terrestrial incubation could make two-phase production a much more viable option, though grow-out density would still need to be controlled.

## Previous Salinity Research

Several studies have provided insight into the salinity requirements of Gulf killifish. Many of these studies have focused on differences in egg survival and hatching success at different salinities. Perschbacher et al. (1990) tested hatching success and time to hatch of wet incubated eggs between 0 and 80 ppt. They found no difference in percent hatch between 0 and 35 ppt (77-93%), except for a low value at 10 ppt (69%). They measured an 86% hatch at 0 ppt and found a correlation between lower salinities and reduced time to first hatch and median hatch. Brown et al. (2011) found embryos incubated in water at 10 ppt were less influenced by different incubation temperatures (20, 21.6, 23, and 29°C) than eggs incubated at 20 ppt. Other studies have shown hatching success and larval survival to be reduced by salinities <1 ppt(Brown et al. 2012). Brown et al. (2012) recorded their highest hatching success of 80.0±2.6% at 7 ppt, followed by 39.1±4.3%, 45.4±4.5%, and 36.3±12.0% success for 0.4, 15, and 30 ppt, respectively. They found that as salinity increased, more waste was produced as ammonia rather than urea. Length at hatch was not affected by salinity. The 30 ppt was the only treatment to differ in time to hatch (greater), body depth at hatch (greater), and body cavity area at hatch (smaller) (Brown et al. 2012). Coulon et al. (2012) observed their highest percent hatch of terrestrially incubated eggs at 7.5 ppt when all substrates were included. The most successful substrate was foam with a 69.9±3.6% hatch rate at 7.5 ppt, and a 51.6±7.6% hatch at 3.5 ppt, though there was no difference of hatching success on foam across the salinities tested (3.5, 7.5, 14, 20, and 27 ppt).

Larvae have been known to survive well from 5-40 ppt (Wallace and Waters 2004). Perschbacher et al. (1990) found mean 2.5-week larval survival to be lower at 0

and 80 ppt (60.0% for each) than at 5-60 ppt (95-100%). Mean length was highest at 20 ppt and lowest at 60 and 80 ppt, with no difference among the other salinities. Mean weight was greater at 5, 20, and 35 ppt than at 0, 60, and 80 ppt. They concluded that 5-40 ppt is the ideal range of salinities for larval survival and growth. Fisher et al. (2013) approached larval ionic requirements by testing the varying concentrations of potassium ($K^+$) in a 9.5-10 ppt environment on larval development and osmoregulation from 0 to 14 days post hatch (dph). They found that $K^+$ supplemented treatments had higher survival rates (≤5%) than the non-supplemented (NaCl) treatment (0% survival), but not as high as the sea salt treatment (~60% survival), which included greater diversity and higher concentrations of secondary ions.

Very little research has been done with salinity tolerance of juveniles, which is a very important life stage in commercial production. In general, their preferred salinity range is believed to be between 5-18 ppt (Strawn et al. 1986), though high survival has been seen in ponds below 1 ppt with almost no dissolved oxygen (Wallace and Waters 2004). Patterson et al. (2012) studied the effects of osmoregulation on growth and survival of juvenile Gulf killifish at 0.5, 5, 8, and 12 ppt over 12 weeks. Weight gain, specific growth rate (SGR), and final condition factor were lowest at 0.5 ppt and highest at 12 ppt. Survival was also lowest at 0.5 ppt (59.3%), although it was not different between the higher salinity treatments. This suggests that a critical salinity threshold exists somewhere between 0.5 and 5 ppt.

Kolok and Sharkey (1997) studied the metabolic cost of adult Gulf killifish acclimation to freshwater by measuring the critical swimming speed ($U_{crit}$) of fish acclimated to 0 ppt and 10 ppt. $U_{crit}$ in the freshwater acclimated fish was lower. The

freshwater fish also experienced 40% mortality within 7 d of the swimming challenge, although sample size was relatively small (N=10). This suggests that killifish divert a portion of energy to osmotic regulation in freshwater, which could be used for other purposes such as activity. These results also suggest that Gulf killifish may not be able to handle stressful situations as well in freshwater.

Phelps et al. (2010) approached the expansion of killifish aquaculture differently by using naturally occurring saline groundwater in Alabama's Black Belt region. They compared spawning success between water from four different sites ranging in salinity from 4.2 to 11.1 ppt with 5.0 ppt artificial seawater (as a control). They found no difference in spawning production. They also tested 104 d growth of Gulf killifish (initial weight = 8.6±1.4 mg/fish) in two different water sources, one of which was previously fertilized. Site A (4.01±2.09 ppt) came from a fertilized shrimp production pond and had over twice the chlorophyll-*a*, a much lower Secchi depth, and higher concentrations and diversity of secondary ions than site B (3.18±0.19 ppt). Site A had greater fry growth than Site B at each of the stocking densities tested (25, 50, and 100 fry/$m^2$). This reinforces the importance of fertilization and primary production described previously, but also suggests that the presence of secondary ions may help relieve osmotic stress. Site A contained higher concentrations of magnesium (A: 26.1±3.82 mg/L, B: 5.6±2.89 mg/L), potassium (A: 60.0±6.79 mg/L, B: 11.1±9.60 mg/L), and total alkalinity (A: 126.4±14.44 mg/L, B: 48.0±2.83 mg/L) and killifish fry thrived under these conditions (Phelps et al. 2010).

## Objectives

Previous research on commercial Gulf killifish culture using brackish water has yielded promising results. The three-phase pond or pool production method has worked well, and the incorporation of terrestrial incubation improves culture productivity. Current knowledge and techniques provide a basis to develop inland-specific culture techniques. To this point, all production systems have relied on a saline water source. Salt costs are likely to be prohibitive for inland culture if saline conditions are required during grow-out. However, specific minimum salinity requirements for each culture phase have not been determined. This information is needed in order to determine the feasibility of inland Gulf killifish culture.

For the phase-one spawning pond, it has been suggested that spawning is inhibited in freshwater (Strawn et al. 1986) but freshwater reproductive success has not been quantified. Pilot studies at Mississippi State University have produced viable embryos from freshwater reared fish (Ramee and Allen, in review). Submerged and terrestrial incubation at a salinity range from 5-10 ppt appears to be optimal (Brown et al. 2011; Coulon et al. 2012). This would be an inexpensive stage for a farmer to use artificial seawater since only low water volumes are required, especially for terrestrial methods. In addition, previous research has not examined the effect of salinity on survival during incubation nor the effect of incubation salinity on subsequent hatching and larval survival.

For the phase-two hatching/larval pond or tank, previous research on salinity tolerance indicates that Gulf killifish do not grow or survive as well at 0 ppt as they do between 5 and 30 ppt. Studies focusing on salinity effects on hatch rate and larval growth

and survival suggest the overall optimum salinity for these early life stages is between 5 and 15 ppt (Perschbacher et al. 1990). This is important since larvae are likely the most salinity-sensitive life stage (Varsamos et al. 2005). Yet, there is still a gap in knowledge between 0 and 5 ppt. This is a production stage where artificial saline water could be used, because less water is needed compared to later grow-out in ponds. It is unknown at which life history stage that hypo-osmotic tolerance begins to increases in Gulf killifish. Transfer experiments comparing low salinity tolerance at increasing juvenile ages will help guide the best time to transfer fish into low salinity or freshwater grow-out ponds.

Salinity studies applicable to stage-three grow out ponds are very limited. Only one study (Patterson et al. 2012) has directly tested the effect of osmotic stress on older juvenile Gulf killifish. This study also found that Gulf killifish grow and survive better above 5 ppt, yet there was 59.3% survival at 0.5 ppt. Fish also did well (72.4% survival at 50 fry/$m^2$) in fertilized water at 4 ppt with unusual ionic ratios (Phelps et al. 2010). Clearly, more research on outdoor grow-out needs to be conducted, especially between 0 and 5 ppt. There are several factors besides salinity that may contribute to Gulf killifish survival and growth in fresh water. Anecdotally, adult Gulf killifish appear healthy in freshwater ponds on Mississippi State's South Farm Aquaculture Facility (Allen, unpublished data), but further research needs to be done to determine the extent of survival and growth in these conditions and the water quality factors that are involved.

Gaps in knowledge of the effects of low salinities on growth and survival of Gulf killifish need to be addressed, especially between 0 and 5 ppt, the salinities that most inland production facilities would utilize. To address these knowledge gaps, it is necessary to determine the actual minimum salinity required for all three culture phases.

Growth and survival rates need to be determined at salinities that could be utilized for inland culture in systems that mimic potential culture conditions. Salinity transfer experiments also need to be conducted in order to better understand the relationship between age and hypo-osmotic tolerance. These transfer experiments should cover the ages and salinity ranges that would be used in three-phase culture methods. Finally, a better understanding of how water quality in earthen ponds affects Gulf killifish production is needed. Establishing the extent of Gulf killifish low salinity tolerance is the first step in determining practical, feasible inland culture protocols. To address these information gaps, the following objectives will be evaluated: **1)** Determine the effects of low salinities (0 and 7 ppt) and salinity transfer on Gulf killifish embryo incubation, hatching, and larval survival, **2)** Determine how the effects of low salinities (0, 2.5, 5, and 7.5 ppt) on juvenile Gulf killifish growth, survival, and osmoregulation change with age/size, and **3)** Determine the effects of low salinities (0, 2.5, 5, and 7.5 ppt) on the regulation of important osmoregulatory genes.

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# CHAPTER II

# FRESHWATER HATCHING SUCCESS AND LARVAL SURVIVAL OF GULF KILLIFISH *FUNDULUS GRANDIS*


## Abstract

Gulf killifish *Fundulus grandis* are euryhaline baitfish with promising potential for commercial culture. Currently, the market is almost entirely supplied by wild-caught fish. This experiment evaluated the freshwater tolerance of Gulf killifish embryos and larvae in order to investigate the potential for inland production. Gulf killifish eggs were collected from spawning mats in freshwater ponds. Eggs were separated volumetrically into 1 ml samples (mean±SE of 113.5±1.2 embryos) and terrestrially incubated at salinities of either 0 ppt or 7 ppt. After 10 d of incubation at 24°C (±1°C), samples from both salinity treatments were hatched at either 0 ppt or 7 ppt, for a total of four treatments. Larval survival was determined after 15 d. Embryo survival was greater at 7 ppt than 0 ppt. There were no differences in relative hatching success between these salinities. Further, there were no differences in post-incubation survival between treatments incubated at 0 ppt, but larvae incubated at 7 ppt and hatched at 0 ppt had a lower post-incubation survival than the treatment continually held at 7 ppt. Thus, embryo survival is greater in saline water, and larval freshwater tolerance may be dependent on incubation salinity, although further studies of larval and juvenile survival in low salinities are merited.

## Introduction

Gulf killifish *Fundulus grandis* (Baird & Girard) are a euryhaline species native to the coastal salt marshes and estuaries along the Gulf of Mexico from northern Mexico to the northern Atlantic coast of Florida (Pattillo et al. 1997). They are able to tolerate broad ranges of temperature (2-35°C), salinity (0 - >40 ppt), and temporarily low oxygen levels ($P_wO_2$=14 torr) (Umminger 1971; Perschbacher et al. 1990; Nordlie and Haney 1998; Virani and Rees 2000; Nordlie 2006).

Gulf killifish are a very popular baitfish for inshore and near-shore sport fish species along the Gulf of Mexico coast (Green et al. 2010). The resilience of Gulf killifish to a broad range of environmental conditions makes them particularly apt to survive in fishermen's bait buckets (Wallace and Waters 2004). Commercial bait dealers are currently reliant on wild caught stocks, which leads to inconsistent availability and sizes of fish (Green et al. 2010).

Culture techniques for Gulf killifish have been developed over the past several decades (Tatum 1982; Strawn et al. 1986; Wallace and Waters 2004; Anderson and Green 2012). The preferred culture method consists of 3 phases: spawning pond, hatching pond, and grow-out pond. Ideal stocking density, feeding rate, and timing of each stage have been described by Tatum (1982), Strawn et al. (1986), Wallace and Waters (2004), and Anderson and Green (2012). Economic analyses by Anderson and Green (2012) and Waas et al. (1983) conclude that Gulf killifish production should be profitable given a large enough (≥4 ha) facility. Notably, these production studies all relied on a saline water supply with salinities between 5-18 ppt. Gulf killifish have also been produced in above-ground, static, fiberglass pools between 5-15 ppt (Anderson and Green 2012).

Despite this research, Gulf killifish production is very limited, largely due to lack of access to saline grow-out conditions. If inland low-salinity or freshwater production methods can be developed, it would make commercial culture far more feasible. However, there have been few studies conducted on low salinity requirements of Gulf killifish.

Several studies have provided insight into the salinity requirements of Gulf killifish through evaluation of hatching success. Perschbacher et al. (1990) tested hatching success of eggs incubated in water at salinities ranging from 0 ppt to 80 ppt. They found no difference in hatching success between 0 ppt and 35 ppt (77-93%), except at 10 ppt (69%), which may have been affected by water mold. Brown et al. (2012) also found salinity affected hatching success, with the highest hatching success at 7 ppt (80.0±2.6%), followed by 15 (45.4±4.5%), 0.4 (39.1±4.3%) and 30 ppt (36.3±12.0%). Length at hatch was also measured and was not affected by salinity (Brown et al. 2012).

In more recent studies, terrestrial incubation methods have been developed (Perschbacher et al. 1995; Coulon et al. 2012). Terrestrial incubation has the benefit of reducing cannibalism through synchronous hatching, and producing larger embryos than water incubation (Coulon et al. 2012). Further, terrestrial incubation has high hatching success, which may approach 95% (Perschbacher et al. 1995). Coulon et al. (2012) recorded their highest embryo survival using water at a salinity of 7.5 ppt across all substrates. Hatching success was higher on the foam substrate than the bamboo or burlap across all salinities, although there was no difference of hatching success on foam among the salinities tested (3.5, 7.5, 14, 20, and 27 ppt). They observed a trend of improved hatching success at 7.5 ppt (69.9±3.6%) compared to 3.5 ppt (51.6±7.6%) (Coulon et al.

2012). Thus, for the terrestrial incubation and wet incubation methods, optimal salinities for embryo survival and hatching appear to be near 7.5 ppt, and hatching success of freshwater terrestrial incubation has not been tested.

Current research suggests the minimum acceptable salinity for larval growth and survival is 5 ppt (Perschbacher et al. 1990). Perschbacher et al. (1990) found 2.5-week post-hatch larval survival to be lower at 0 and 80 ppt (60.0% for each) than at 5-60 ppt (95-100%). Perschbacher et al. (1990) found mean 2.5-week post-hatch length to be highest at 20 ppt and lowest at 60 ppt and 80 ppt, with no difference among the other salinities (0, 5, 10, 15, 25, 30, 35, and 40 ppt). They found mean weight to be higher at 5, 20, and 35 ppt than at 0, 60, and 80 ppt. In juvenile fish, Patterson et al. (2012) found 12 week survival was lowest at 0.5 ppt (59.3%), and was not different among the 5, 8, and 12 ppt treatments (86.5, 96.3, and 89.7%, respectively). Patterson et al. (2012) found weight gain, specific growth rate, and final condition factor were lowest at 0.5 ppt and highest at 12 ppt.

Despite this initial research on the effects of salinity on hatching success and larval growth, relatively little is known in regard to low salinities (0-5 ppt). Fecundity is known to be lower in freshwater (Tatum 1982), although egg viability has not been investigated in broodstock from freshwater. Also, no culture studies have been conducted in which fish were transferred between salinities. It is very plausible that some life stages are more tolerant to lower salinity conditions. Determining the minimum salinity requirements for each life stage and the corresponding production phase is critical for developing low salinity production methods.

In order to investigate low salinity tolerance on early life stages, this study compared the effects of salinities of 0 and 7 ppt on embryo survival during terrestrial incubation, hatching success, and larval survival. It was hypothesized that embryo survival during incubation, hatching success and larval survival would be greater at 7 ppt. Identification of life stages where freshwater treatments were as successful as the saline treatments would be beneficial for guiding the development of freshwater culture methods.

## Methods

### Egg Collection

Gulf killifish eggs were collected from three freshwater ponds (0.4 ha) from August 4-24, 2012 following Anderson and Green (2012). Ponds were supplied with well water and aeration, and had been stocked over a year prior with adult fish collected from the northern Gulf of Mexico.

Two different methods were used to suspend spawning mats (Spawntex, Blocksom & Co., Michigan City, IN, USA) 20 cm below the surface. The first utilized a rigid 1.3 cm diameter polyvinyl chloride (PVC) frame (61 cm X 61 cm) with foam flotation pads (pool noodles) added for buoyancy. In order to ensure collection of a sufficient quantity of eggs, three more mats were made using a simpler design. A smaller rigid wire frame (51 cm X 30 cm) was suspended with twine attached to two sealed 5.1 cm pieces of PVC as floats. One mat of each style was placed in each pond and left for three nights before collection. The eggs were vigorously shaken off the spawning mats against a rigid screen, over a tub of water, similar to Anderson and Green (2012). The water and eggs were then poured over a sieve (500 micron nylon screening stretched

across a PVC frame), where they were consolidated and rinsed. The spawning mats were then returned to the ponds and egg collection was repeated two more times for a total of three batches. For each batch, the fertilized eggs were separated out by hand using a scoopula. Fertilized eggs were identified by their brown-flecked pigmentation (compared to the clear unfertilized eggs) (Anderson and Green 2012). Total fertilized and unfertilized eggs were measured volumetrically from each batch of eggs collected in order to calculate percent viability.

Viable embryos were measured into eight 1 mL samples per batch. Every sample was counted as they were placed in their individual incubation chambers in order to ensure consistency (mean±SE=113.9±1.2 eggs/mL) and later calculate incubation survival.

**Incubation**

Samples were placed into plastic incubation chambers (15 cm X 10 cm X 3 cm) containing two pieces of foam (Polly-Fil True-foam, Fairfield, Danbury, CT) (2.5 cm thick) (Anderson and Green 2012). For the two different incubation treatments, the foam was moistened with either fresh water (0 ppt) or brackish water (7 ppt), which was derived from non-chlorinated well-water and diluted artificial sea salt (Instant Ocean, Mentor, OH, USA). There were 4 replicate salinity treatments per batch and 3 batches, for a total of 12 replicate 1 mL hatching samples per treatment. For this experiment, 7 ppt was chosen because it is well within this preferred range while remaining at the lower end of it. Coulon et al. (2012) and Brown et al. (2012) also recorded peak hatching success at 7-7.5 ppt.

Eggs were incubated at 24.7 ± 0.02°C in a small, thermostatted incubator following Anderson and Green (2012). Temperature was recorded every 15 minutes using a data logger (HOBO, Onset, Cape Cod, MA, USA), and every three d the incubation chambers were removed from the incubator and moistened by spraying with their respective treatment water. At this time, mortalities (predominately due to water mold, presumably *Saprolegnia spp.*) were counted and removed. After 10 d, viable eggs were counted and then transferred to hatching tanks.

**Hatching and Survival**

All viable eggs from each treatment were submerged in 3-L static tanks in either their original salinity treatment or the opposite treatment, making a total of four treatments (SS: incubated at 7 ppt, hatched at 7 ppt; SF: incubated at 7 ppt, hatched at 0 ppt; FS: incubated at 0 ppt, hatched at 7 ppt; FF: incubated at 0 ppt, hatched at 0 ppt), with two replicates per batch. Three batches were used for analysis, for a total of 6 replicate hatching containers per treatment. Hatching success was determined by counts from three replicate digital images that were taken approximately one hour after eggs were submerged and before food was added to the tanks. Digital images were sufficient to compare relative hatch success between treatments because 90% of hatch can be determined within two hours (Perschbacher et al. 1995).

Larvae were held in 3-L static tanks, at their specified treatment salinity (0 or 7 ppt), each aerated with separate air stones, and maintained at the same temperature by a common water bath. Larvae were fed *ad libitum* once a d with a mix of *Artemia sp.* and formulated diet (larval AP100 powdered feed, >100 μm, Zeigler, Gardners, PA, USA). Tanks were siphoned and water was exchanged (3 L/d) one hour after feeding. Tanks had

overflow holes covered with a fine screen (750 micron nylon mesh) to prevent escape of larvae and allow for easy exchange of water. This design was determined during a pilot study in order to prevent larvae from escaping the chambers during water exchanges.

Water quality parameters (temperature, salinity, pH, dissolved oxygen, ammonia, and nitrite) were measured from each tank twice weekly in the afternoon, at least 2 hours after feeding and cleaning. After 15 d, larvae were removed from the tanks and manually counted to determine survival. A subsample of 10 larvae was taken from each sample tank and measured using calipers for total length (to the nearest 0.1 mm) and weighed (fish were blotted dry using paper towels and weighed to the nearest 0.1 mg).

**Statistical Analysis**

Unless otherwise noted, all means are reported with the standard error (SE). All analyses were conducted using program R (R Foundation for Statistical Computing, Vienna, Austria) and significance was determined at $\alpha = 0.05$. Normality of data was tested using the Shapiro-Wilk normality test and homogeneity of variance was tested using the Bartlett test. Differences in incubation survival were tested using a two-sample t-test. Differences in incubation survival resulted in different stocking densities between the FF/FS and SS/SF treatments. For this reason, post-incubation comparisons were only made between treatments that experienced a common incubation salinity. A t-test was used to confirm that the initial embryo stocking densities were not different in treatments incubated at the same salinity. The effect of pre-hatch salinity transfer on relative hatching rate and post-incubation survival was then determined with a t-test between each pair of treatments. A Dixon's Q-test was used to test for outliers (Dean and Dixon 1951),

and it was determined with 99% confidence that there was one outlier in the FF treatment in the post-incubation survival data, which was removed from the analysis.

## Results

### Water Quality

Salinity was maintained at designated levels for all hatching tanks, resulting in a difference between saline and fresh water hatching treatments (Table 2.1). There were no other differences in measured water quality parameters between the hatching tanks of each treatment (Table 2.1). Nitrite levels were similar among treatments and below harmful and toxic levels for warm water species (Colt et al. 1981; Lewis and Morris 1986).

### Egg Viability

Eggs collected from freshwater broodstock ponds had a mean percent viability (±SE) of 20.21±0.03% (N=9 batches of eggs collected in the summers of 2012 and 2013).

### Incubation

There was a difference (t-test, N=12/treatment, t= -9.65, d.f.=30, $P<0.001$) in embryo survival during incubation between the two salinity moisture treatments. The samples sprayed with fresh water had lower survival compared to those sprayed with saline water, with 39.7±4.6% survival at 0 ppt and 85.9±1.5% survival at 7 ppt (Figure 2.1).

### Hatching

There were no differences in relative hatching success between treatments incubated in fresh water and hatched in either fresh water (81.6±4.7%) or saline water

(67.4±9.9%) (t-test, N=6, t=1.28, d.f.=10, $P>0.1$) or between treatments incubated in saline water and hatched in either saline water (72.3±8.3%) or fresh water (56.2±10.3%) (t-test, N=6, t=-1.21, d.f.=10, $P>0.1$).

**Survival**

There was no difference in the post-incubation survival (final larval count/final number of viable eggs) data between treatments incubated in fresh water and hatched in either fresh water (74.5±12.4%) or saline water (76.0±8.0%) (t-test, n=6, t=1.13, d.f.=9, $P>0.1$) (Figure 2.2). There was, however, a difference in post-incubation survival between treatments incubated in saline water and hatched in either saline water (78.9±6.2%) or fresh water (50.0±9.7%), with survival reduced after 15 d exposure of larvae to fresh water (t-test, n=6, t=-2.51, d.f.=10, $P<0.05$) (Figure 2.2). At 15 dph, the mean (±SE) weight and length of all treatments combined was 12.7±0.8 mg and 11.1±0.2 mm.

## Discussion

Unlike previous studies, in this study Gulf killifish eggs were spawned and collected in freshwater. It has been suggested that Gulf killifish do not breed well in freshwater ponds (Strawn et al. 1986). In this study, there were a relatively low proportion of viable eggs harvested (mean±SE= 20.4±3.5%), but the sex ratio in the ponds was unknown and could have been a contributing factor. This is much lower than the 56.6±19.6% viability observed in 9.0 ppt static fiberglass tanks with a 4:1 female to male ratio (Gothreaux and Green 2012). The use of freshwater broodstock and spawning possibly acts as an initial selective pressure favoring freshwater tolerant individuals.

These conditions also better mimic potential inland aquaculture conditions than previous research on *F. grandis*.

The results of this study clearly show higher survival in air-incubated Gulf killifish eggs moistened with saline (7 ppt) water. This agrees with Coulon et al. (2012), who recorded their highest embryo survival across all substrates at 7.5 ppt (69.9±3.65% on foam). Coulon et al. (2012) found no effect of salinity on hatching success when foam substrate was used, and observed that there was less water mold growth in the foam treatments. They did not, however, have a freshwater treatment as was used in this study. The 85.9±1.5% embryo survival observed in the 7.5 ppt treatment of this study is higher than any of the survival values measured by Coulon et al. (2012). This may be due to different incubation temperatures (22°C vs. 24°C in this study) and/or different lengths of incubation (19 d vs. 10 d in this study).

Notably, in this experiment the embryo mortality during terrestrial incubation usually was in conjunction with water mold growth on the eggs. Thus, the saline water (7 ppt) either reduced water mold growth directly or reduced mortalities for water mold to colonize. Studies on water mold infected rainbow trout eggs have shown saline solutions (30 ppt) to effectively reduce mold growth and improve hatching success, although not to the extent of hydrogen peroxide or formalin treatments (Schreier et al. 1996).

Interestingly, there were no differences in relative hatching success among treatments in this study. Thus, hatching success in fresh water was no different than in saline water. Similarly, Dimichele and Taylor (1980) observed 100% hatching success of *F. heteroclitus* eggs in freshwater after terrestrial incubation at 15 ppt and 20°C for 16 d.

Further, the similar hatching success of embryos transferred to different salinities indicates that Gulf killifish are euryhaline at very early life history stages.

Post-incubation 2-week survival (final count/final viable embryos) was not different between treatments incubated in freshwater, but did differ between treatments incubated in saline water. Thus, Gulf killifish have similar post-incubation survival in freshwater as in their optimal salinity when exposed to a freshwater conditions as embryos. Similarly, a study by Rao (1975) of a congener, the California killifish *F. parvipinnis,* found that incubation salinity influences freshwater tolerance. This pattern was also found in Nile tilapia *Oreochromis niloticus*, with high salinity tolerance progressively increasing with spawning, incubation, and early acclimation at a higher salinity (Watanabe et al. 1985). This result supports the possibility of completely freshwater culture methods. If water mold growth is the original cause of mortality and can be reduced during freshwater incubation, the high post-incubation survival indicates that freshwater culture may be an option for the production of this species, albeit with lower initial egg viability.

This study shows that viable Gulf killifish eggs can be harvested from broodstock in freshwater. There was high mortality of eggs in freshwater terrestrial incubation treatments, which was may have been caused by increased water mold growth. The saline moisture helped mitigate this mortality. A future study is needed to compare the effectiveness of saline water incubation and freshwater incubation with water mold treatments such as formalin or hydrogen peroxide, in order to determine if the mold growth is the cause or the symptom of the freshwater incubation mortalities. Hatching success was similar regardless of salinity. Post-incubation survival was lower in the

freshwater tanks after saline incubation but did not differ between salinities after freshwater incubation. Still, a saline hatchery environment would have a higher percent of viable eggs and greater embryo survival. However, in fresh water, the high hatching success (81.6±4.7%) and post-incubation survival (74.5±12.4%) shows promise for utilizing fresh water at some point during the production cycle. The survival of the larvae in the freshwater treatments demonstrates that Gulf killifish are able to handle hypo-osmotic environments at very early life stages demonstrating an impressive osmo-regulatory capacity. Future research would benefit from a focus on the optimal timing for transfer of larval-juvenile Gulf killifish from saline to freshwater conditions.

Table 2.1 Hatching tank mean (and Standard Error; SE) water quality conditions of each treatment.

| | **Temperature (°C)** | | **Salinity (ppt)** | | **pH** | | **DO (ppm)** | | **Nitrite (ppm)** | | **TAN (ppm)** | | **$NH_3$-N (ppm)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| **FF** | 26.66 | 0.19 | 0.18 b | 0.06 | 8.23 | 0.05 | 8.25 | 0.06 | 0.33 | 0.09 | 0.16 | 0.06 | 0.033 | 0.018 |
| **FS** | 26.34 | 0.46 | 7.19 a | 0.08 | 8.24 | 0.08 | 8.27 | 0.13 | 0.36 | 0.07 | 0.05 | 0.03 | 0.008 | 0.004 |
| **SF** | 26.23 | 0.43 | 0.16 b | 0.03 | 8.29 | 0.06 | 8.18 | 0.19 | 0.44 | 0.05 | 0.14 | 0.05 | 0.064 | 0.026 |
| **SS** | 26.68 | 0.20 | 7.13 a | 0.08 | 8.20 | 0.07 | 8.13 | 0.09 | 0.53 | 0.07 | 0.04 | 0.02 | 0.016 | 0.009 |

SS: incubated at 7 ppt hatched at 7 ppt, SF: incubated at 7 ppt, hatched at 0 ppt, FS: incubated at 0 ppt, hatched at 7 ppt, FF: incubated at 0 ppt, hatched at 0 ppt.

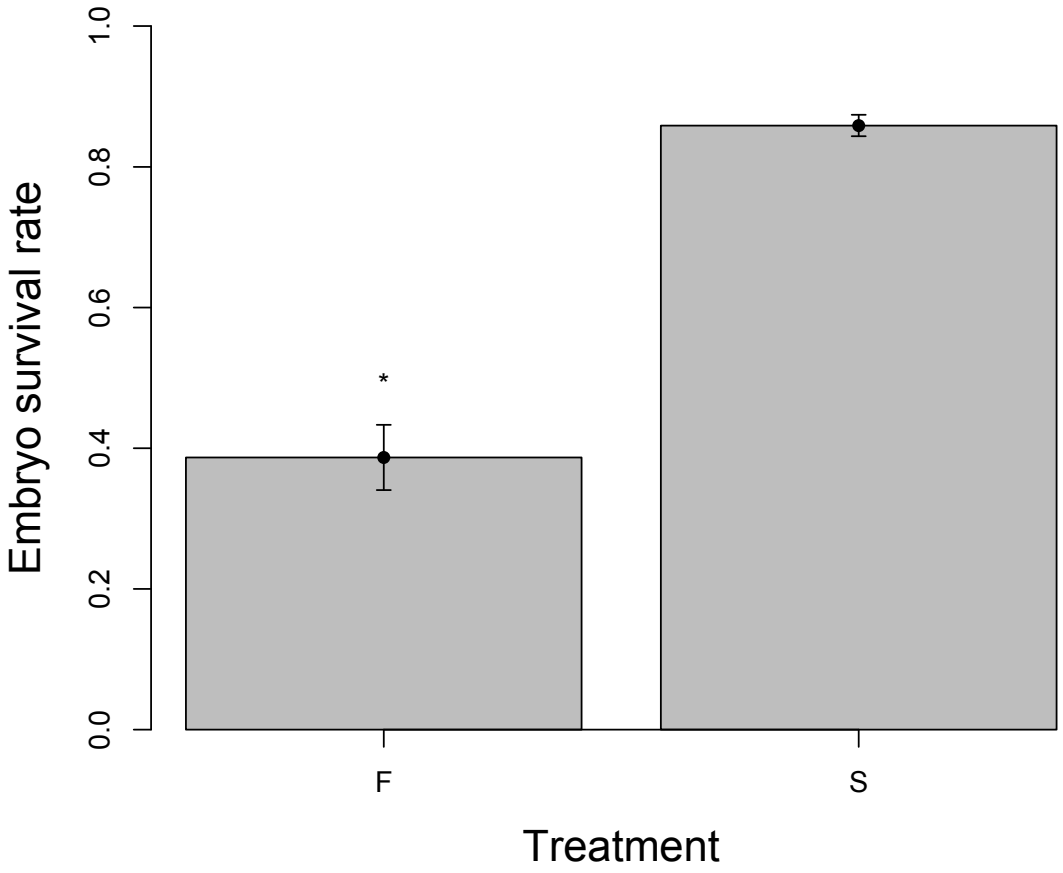


Figure 2.1 Gulf killifish *Fundulus grandis* mean (±SE) embryo survival.

Determined following 10 d of terrestrial incubation in a humidified chamber using either fresh water (F=0 ppt) or brackish water (S=7 ppt). An asterisk above the bars indicates a significant difference (t-test, N=12, $P<0.001$).

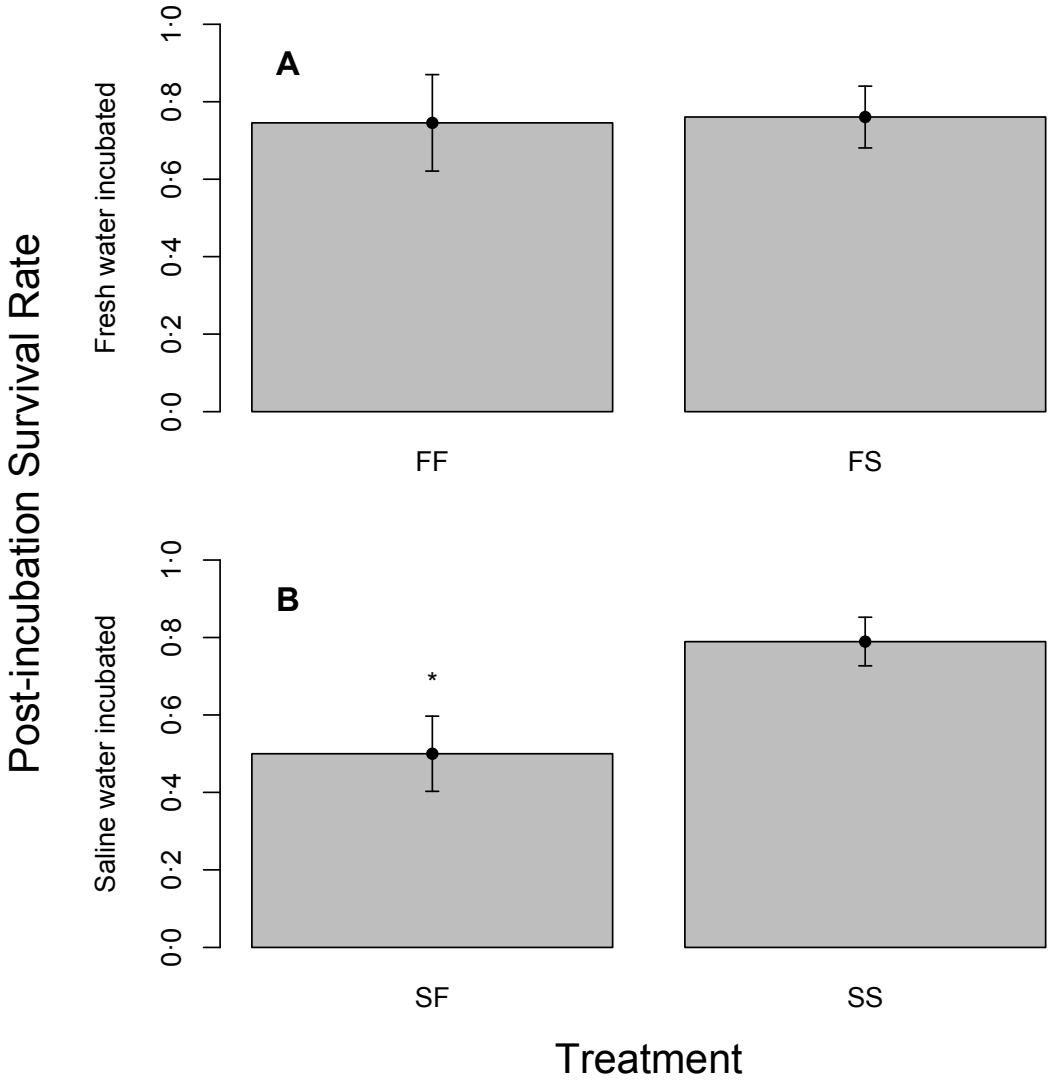


Figure 2.2 Gulf killifish *Fundulus grandis* mean (±SE) post-incubation survival.

Determined 15 d post-hatch in **A)** treatments FS: incubated at 0 ppt, hatched at 7 ppt; FF: incubated at 0 ppt, hatched at 0 ppt and **B)** treatments SS: incubated at 7 ppt, hatched at 7 ppt; SF: incubated at 7 ppt, hatched at 0 ppt. Post-incubation survival was defined as final number of fish/number of viable embryos stocked into each tank for hatching. The asterisk (*) signifies a significant difference in post-incubation survival rate between the SF and SS treatments (t-test, N=6, $P<0.05$).

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# CHAPTER III

# EFFECTS OF LOW SALINITIES ON OSMOREGULATION, GROWTH, AND SURVIVAL IN THREE AGE GROUPS OF JUVENILE GULF KILLIFISH *FUNDULUS GRANDIS*


## Abstract

The Gulf killifish *Fundulus grandis* is a euryhaline fish species native to coastal estuaries of the Gulf of Mexico, and is a popular baitfish. Established culture methods have relied on a saline water source but have not proliferated in the commercial sector. Since this species is highly euryhaline, there is potential for freshwater culture via the utilization of established inland facilities. In order to assess the feasibility of inland culture, changes in *F. grandis* hypo-osmotic tolerance throughout early growth were evaluated. *F. grandis* were challenged with low salinity treatments of 0 (fresh water), 2.5, 5.0, and 7.5 ppt at 2, 7, and 12 weeks post-hatch for 4 weeks to determine growth, survival, gill $Na^+/K^+$-ATPase activity, and whole body $Na^+$ content. Gill $Na^+/K^+$-ATPase activity and whole body ion samples were collected at 0, 2, 14, and 28 days post-transfer (dpt) for each age group. Results indicate *F. grandis* at 2 weeks post-hatch grew and survived as well at 2.5 ppt as at higher salinities but growth was reduced in fresh water. At 7 and 12 weeks post-hatch, freshwater survival and growth rates at 0 ppt were comparable to other salinity treatments. Gill $Na^+/K^+$ ATPase activity increased in *F. grandis* transferred to fresh water at both 7 and 12 weeks post-hatch. Whole body $Na^+$

content was higher in fresh water 28 dpt in the 2 weeks post-hatch age group, and 14 dpt in the 7 weeks post-hatch age group. There were few differences in $Na^+/K^+$ ATPase activity and whole body $Na^+$ content between salinity treatments in the 12 weeks post-hatch age group. These results suggest *F. grandis* develops hypo-osmotic tolerance <2 weeks of age and culture in low salinities including fresh water will be more successful with fish ≥7 weeks post-hatch.

## Introduction

The Gulf killifish *Fundulus grandis* is a common euryhaline species native to the coastal salt marshes and estuaries along the Gulf of Mexico from northern Mexico to the northern Atlantic coast of Florida (Waas et al. 1983; Oesterling et al. 2004; Williams et al. 2008). They are able to tolerate broad ranges of temperature (2-35°C), salinity (0->40 ppt), and temporarily low dissolved oxygen concentrations (Umminger 1971; Perschbacher et al. 1990). *F. grandis* are a popular live baitfish along the Gulf of Mexico's northern coastline for inshore and near-shore sport fish species such as red drum *Sciaenops ocellatus*, speckled trout *Cynoscion nebulosus*, and flounder *Paralichthys lethostigma* (Waas et al. 1983; Green et al. 2010). However, commercial bait dealers are currently reliant on wild caught stocks, which leads to inconsistent availability and sizes of fish (Oesterling et al. 2004; Green et al. 2010).

Culture techniques for *F. grandis* have been developed over the past several decades (Tatum 1982; Waas et al. 1983; Perschbacher and Strawn 1985; Strawn et al. 1986; Wallace and Waters 2004; Green et al. 2010; Anderson and Green 2012). The traditional culture method consists of 3 phases: spawning pond, hatching pond, and grow-out pond. Ideal stocking density, feeding rate, and timing of each stage have been

described by Tatum (1982), Waas et al. (1983), Strawn et al. (1986), Wallace and Waters (2004), and Anderson and Green (2012). The more recent adoption of terrestrial incubation techniques (Perschbacher et al. 1995; Coulon et al. 2012) requires the implementation of an indoor hatchery, replacing the hatching pond in traditional methods. Notably, these production studies all relied on a saline water supply with salinities between 5-18 ppt. *F. grandis* have also been produced in above-ground, static, fiberglass pools between 5-15 ppt (Green et al. 2010; Anderson and Green 2012). Despite this research, *F. grandis* commercial production is very limited, largely due to limited accessibility to saline water sources. If inland low-salinity or freshwater production methods can be developed, it would make commercial culture more feasible. However, there have been few studies conducted on low salinity requirements of *F. grandis*.

Previous osmoregulatory research on the mummichog *F. heteroclitus*, a closely related species, (Potts and Evans 1967; Jacob and Taylor 1983; Marshall et al. 1999; Katoh et al. 2000; Mancera and McCormick 2000; Wood and Laurent 2003; Scott et al. 2004; Kidder et al. 2006a; Kidder et al. 2006b; Laurent et al. 2006; Hyndman and Evans 2009) provides an important basis for the study of the ontogeny of low salinity tolerance of *F. grandis*. Abrupt salinity transfer studies have helped establish how rapidly *F. heteroclitus* can adapt to salinity change, the importance of $Na^+/K^+$ ATPase activity in regulating plasma $Na^+$ (Jacob and Taylor 1983; Marshall et al. 1999; Mancera and McCormick 2000), and the physiological changes that occur (Wood and Laurent 2003). After transfer from 10 ppt to fresh water, *F. heteroclitus* experienced a 4 d reduction in plasma $Na^+$ content and an increase in $Na^+/K^+$ ATPase activity (Scott et al. 2004). In regard to developmental ontogeny, unlike many other teleosts, *F. grandis* are well

developed at hatch, with fully functional mouth parts and gills (Armstrong and Child 1965). The gills of *F. heteroclitus* are well developed and the main site of $Na^+/K^+$ ATPase activity by 4 dph (Katoh et al. 2000).

Past research on *F. grandis* reports that the ideal salinity for survival and growth is between 5 and 20 ppt (Perschbacher et al. 1990; Patterson et al. 2012). This agrees with the general theory that intermediate salinities are ideal for maximizing growth due to reduced standard metabolic rate (Boeuf and Payan 2001). In hypo-osmotic environments, an osmotic gradient between the blood and surrounding water is formed, and active transport of ions into the body is required to compensate for the passive diffusion of ions into the water and combat osmosis of water into the body (Varsamos et al. 2005). Freshwater acclimation has been suggested to have metabolic costs in adult *F. grandis*, based on reduced critical swimming speeds in fresh water compared to 10 ppt (Kolok and Sharkey 1997). However, *F. heteroclitus* respirometry experiments have shown no difference in oxygen consumption between fresh water, 10 ppt, and saltwater (Kidder et al. 2006a). Despite potentially greater metabolic costs of living in fresh water, several studies have reported populations naturally occurring in fresh water (Hillis et al. 1980; Pattillo et al. 1997; Nordlie 2006) and anecdotally, *F. grandis* broodstock have been maintained in freshwater ponds at Mississippi State University for over 3 years (P. Allen, unpublished data). In teleost fishes, the development of tolerance to extreme salinities is often gradual and age or size dependent, with the most dramatic increase in salinity tolerance occurring during the transition to the juvenile phase (Varsamos et al. 2005). *F. grandis* embryonic and larval life stages have been found to be sensitive to fresh water (Perschbacher et al. 1990; Perschbacher et al. 1995; Brown et al. 2011; Coulon et al.

2012). The utilization of an indoor hatchery for embryo incubation, hatching, and early larval growth would allow for a saline environment to be used during these early life stages (Ramee and Allen, in review). If this system were implemented, freshwater or low salinity ponds would be feasible for use in the grow-out phase of production.

In order to successfully implement this production design, more information is needed regarding the ontogeny of *F. grandis* hypo-osmotic tolerance. In this study, *F. grandis* were transferred at 3 ages from 7.5 ppt to 0, 2.5, 5.0, or 7.5 ppt. Survival and growth were monitored along with $Na^+/K^+$ ATPase activity, whole body $Na^+$ content, and percent water content. This information will specifically guide producers on the optimal timing for transferring fish from a saline hatchery environment to a freshwater or low salinity grow-out pond environment. In addition to growth and survival, other physiological indices of osmoregulation were used to help further understand the development of *F. grandis* osmoregulation.

## Methods

### Fish source

*F. grandis* embryos were collected throughout May, June, and July of 2013 from three 0.04 ha freshwater broodstock ponds at the South Farm Aquaculture Facility of Mississippi State University. Ten spawning mats (51 cm X 30 cm, Spawntex, Blocksom & Co., Michigan City, IN, USA) were placed in each pond and left for four nights before collection. Each mat was suspended approximately 15 cm below the surface from PVC floats. Mats were collected, and shaken vigorously against a rigid 0.5 cm steel screen, releasing the deposited embryos into a plastic tub partially filled with fresh water. Consolidated embryos were rinsed with fresh water over a screen sieve (500 micron

nylon screening stretched across a PVC frame), where fertilized embryos were separated out for incubation. Embryos were measured volumetrically in order to estimate the number collected (mean±SE = 113.5±1.2 embryos/mL (Green et al. 2010, Ramee and Allen, in review). Three separate large cohorts of embryos (>20 mL collected on the same day) were used for this study for the three different experimental age groups.

Embryos were incubated at 23°C for 12 d in an incubation chamber (35 cm X 28 cm X 8 cm plastic container with 2 pieces of 2.5 cm thick foam; Poly-Fil True-foam, Fairfield, Danbury, CT, USA). Chambers were misted with a 7.5 ppt saline solution (made with artificial marine salt; Instant Ocean, Mentor, OH, USA) before incubation and every 4 d thereafter, in order to ensure a humid environment. After incubation, embryos were submerged and hatched in 330-L tanks in a 7185-L recirculating system maintained at 7.5 ppt. Larvae were fed *ad libitum* with formulated larval diet (Otohime B1; 250-360 μm; Reed Mariculture Inc., Campbell, CA, USA) for at least 2 weeks before weaning to a larger feed size (Otohime S1; 610-1410 μm). Each cohort was held together in a 330-L tank until the fish were transferred into experimental tanks.

**Experimental design**

Separate cohorts were transferred into experimental tanks at 2, 7, and 12 weeks post-hatch when that cohort reached the initial age of the respective experimental age group. For each cohort, equal numbers of fish were stocked into 16 150-L recirculating tanks at 0, 2.5, 5.0, and 7.5 ppt at the beginning of their relative experimental trial, with 4 replicate tanks per salinity. Each tank was filtered by an individual biological/mechanical canister filter (Ocean Clear, Red Sea, Houston, TX, USA) with activated carbon and recirculated by a 35 Watt magnetic-drive utility pump (Aquatic Ecosystems, Apopka, FL,

USA). Initial stocking densities differed for each age group, depending on the number of fish available from that cohort. The resulting initial stocking densities were 150, 100, and 54 fish/tank (mean weights±SE = 2.71±0.01, 28.23±0.16, and 30.86±0.07 g/tank) for the 2, 7, and 12 weeks post-hatch age groups, respectively. Tanks were held in a common water bath in order to maintain the same temperature in all experimental tanks. Each experimental trial lasted for 4 weeks, resulting in age groups of 2-6, 7-11, and 12-16 weeks post-hatch. Subsamples (N=40 fish/treatment) from each age group were taken before transfer to experimental tanks as well as at 2, 14, and 28 dpt to record individual length (to the nearest 0.1 mm, using calipers), weight (to the nearest 0.0001 g (Scientech SA 210, Scientech Inc, Boulder, CO, USA)), and obtain tissue samples (n=8/treatment) for whole body sodium ($Na^+$) analysis and $Na^+/K^+$ ATPase assays. Individual length and weight data were used to calculate the condition factor for each individual subsampled fish according to Moyle and Cech (2004) using the equation:

$$CF=W/L^3(1) \tag{3.1}$$

where $W$ = wet weight in g, and $L$ = total length in cm.

After 28 dpt, all fish in each tank were counted and weighed in bulk in batches of 10-20 fish at a time. Bulk weights were used to calculate mean individual growth using the equation:

$$\left(\frac{final\ bulk\ weight}{final\ number\ of\ fish}\right)-\left(\frac{initial\ bulk\ weight}{initial\ number\ of\ fish}\right) \tag{3.2}$$

Tanks were fed evenly at approximately 10% of the total initial tank weight (adjusted 14 dpt) daily (via two 5% feedings) with commercial feed (Otohime B1 or S1). Salinity, temperature, and dissolved oxygen (DO) were measured and recorded daily using a YSI ProODO and YSI 85 (YSI Inc., Yellow Springs, OH, USA). Ammonia, nitrite, and pH levels were monitored twice per week with a Hach DR850 colorimeter (Hach Co., Loveland, CO, USA) and a YSI pH10 (Table 3.1).

Due to size differences in fish between the three age groups, the subsampling procedure was slightly different between age groups. In the 2-6 week post-hatch age group fish were too small to effectively remove the gill tissue. For this group, 10 fish/tank were taken at each time point (0, 2, 14, and 28 dpt), individually weighed and measured, and returned to their respective treatment tank. At the end of the 2-6 week old trial, 5 fish were sampled from each tank for whole body $Na^+$ analysis. For the 7-11 week post-hatch age group, 6 fish were collected from each experimental tank at each time point: 2 fish were stored whole in a 2-mL vial or an aluminum foil envelope and flash frozen in liquid nitrogen for whole body $Na^+$ analysis, gills were removed from 4 fish using a dissecting microscope (1× magnification, SteroStar, Reichert, Depew, NY, USA), fine forceps, and micro scissors. Gills were used for subsequent $Na^+/K^+$ ATPase assays with two samples of two pooled sets of gills per tank. For the 12-16 week post-hatch group, fish were large enough that gills did not need to be pooled for the $Na^+/K^+$ ATPase analyses. This resulted in 4 fish being sampled from each tank at each time point, with 2 samples per tank for $Na^+/K^+$ ATPase assays and whole body $Na^+$. In total, there were 8 samples of each type ($Na^+/K^+$ ATPase and whole body $Na^+$) from each time and salinity combination, for a total of 104 samples of each type per age group.

**$Na^+/K^+$ ATPase enzyme assay**

Eight $Na^+/K^+$ ATPase samples/treatment were taken for the 7-11 and 12-16 week post-hatch age groups at each sampling point (0, 2, 14, and 28 dpt). Gill samples were placed in 0.5-mL microcentrifuge tubes that were prefilled with 160-µL of SEI buffer (150 mM sucrose, 10 mM EDTA, and 50 mM imidazole; pH 7.3), flash frozen in liquid nitrogen, and stored at -80°C until analyses following McCormick (1993).

Samples were then thawed and homogenized after the addition of 40-µL of ice-cold SEI buffer containing 0.5% sodium deoxycholate (final concentration 0.1%). Homogenization was done using a tissue homogenizer (VWR PowerMax AHS 200, Pro Scientific, Oxford, CT, USA). Each sample was homogenized for 15-25 s with a clean homogenizer tip and kept on ice immediately before and after homogenization. Samples were centrifuged at 4°C and 5000*g* for 1 min. The supernatant was removed, aliquoted equally between two 0.5-mL vials, frozen in liquid nitrogen, and stored at -80°C.

$Na^+/K^+$ ATPase activity levels were determined in duplicate by monitoring kinetic conversion of nicotinamide adenine dinucleotide (NADH) to $NAD^+$ in the homogenates, and read at 340 nm from 2-10 min in a microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA). Methods were adapted from McCormick (1993) and Allen et al. (2009). In the final reaction, each microwell contained 10-µL of sample and 200-µL of reaction mixture (142 mM NaCl, 31.5 mM KCl, 7.9 mM MgCl2, 1.0 U/mL lactate dehydrogenase (LDH), 1.25 U/mL pyruvate kinase (PK), 2.0 mM phosphoenolpyruvate (PEP), 1.0 mM adenosine triphosphate (ATP), 1.0 mM NADH, and 50 mM imidazole) in the presence or absence of 0.5 mM ouabain at pH 7.5 and 25°C. Protein concentration was measured in duplicate using the bicinchroninic acid (BCA)

method of Smith et al. (1985) via the Pierce® BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) according to manufacturer's instructions. Activities were calculated both as international units (IU)/g protein (Penefsky and Bruist 1984) and as μmol ADP $mg^{-1}$ protein $hr^{-1}$ (McCormick 1993).

**Whole body $Na^+$ content**

In the 2-6 week post-hatch group, whole body $Na^+$ was only measured at 28 dpt. At this time point, 5 fish from each tank were sampled for analysis. In the 7-11 and 12-16 week-old age groups, 8 fish were sampled before transfer and 2 fish were sampled per tank at each sampling point.

During the initial samplings, fish were euthanized with 500 mg/L tricaine methanesulfonate (MS222) in their original treatment water, rinsed with DI water, and flash frozen in plastic 2-mL screw-top tubes or aluminum foil envelopes. Fish were removed from the -80°C freezer and triple rinsed with DI water in a vacuum filtration system in order to remove any external salts remaining from the different salinity treatments. They were weighed to the nearest 0.0001 g on a pre-dried, pre-weighed aluminum weigh-boats. Samples were desiccated for 24 hr at 80°C. Dried samples were weighed, suspended in 5-mL of 50% nitric acid in 15-mL conical polypropylene tubes, and digested in a 100°C water bath for 30 min. One blank sample of 5-mL of 50% nitric acid was also measured for every 15 samples to account for possible contamination. Digested samples were weighed again to account for evaporation. Afterwards, samples were stored at -80°C. Immediately prior to analysis, samples were thawed and diluted so that the resulting concentration was between 10 and 85 μM $Na^+$, and thus well within the range of the standards (0, 25, 50, and 100 μM $Na^+$). Samples were analyzed with an

atomic absorption spectrophotometer (Varian AA240FS, Agilent Technologies, Santa Clara, CA, USA) in flame emission mode for $Na^+$ (gain= 49%, wavelength=589 nm). Dry and wet weights were used to calculate percent water weight for each fish used. An systematic error occurred during the collection of the wet weights of the fish from the 14 dpt time point of the 7-11 weeks post-hatch age group, causing this group of sample to be removed from the final analysis.

**Data analysis**

All analyses were conducted using program R (R Foundation for Statistical Computing, Vienna, Austria) and significance was determined at $\alpha = 0.05$. Due to high nitrite levels (>1.0 mg/L) in one of the 7.5 ppt tanks during the 2-6 week post-hatch age group, this tank had uncharacteristically low growth and survival, and was removed from all analyses. Normality of data was tested using the Shapiro-Wilk normality test and homogeneity of variance was tested for using the Bartlett test. If these two assumptions were met, a one-way analysis of variance (ANOVA) was used to determine differences between treatments. If the assumptions of ANOVA were not met, data were (log or cubically) transformed to meet the normality and homogeneity of variance assumptions when possible. A Fisher's Least Significant Difference (LSD) post-hoc test was used in conjunction with each ANOVA in order to determine treatment level differences. If the data could not be transformed to meet the assumptions of parametric analysis, the non-parametric Kruskal-Wallis rank sum test within the R-package "agricolae" (Mendiburu 2013) was used to compare differences between treatments, and contained a built in *post-hoc* test.

## Results

### Water Quality

Salinities were maintained at their appropriate levels throughout each experiment and were different from the other treatments during each age group trial (Table 3.1). Temperature did not differ between treatments in any age group but did decrease in the older age groups due to seasonal change (Table 3.1). Water pH was higher in the 0 ppt treatment in the 2-6 and 12-16 weeks post-hatch age group. DO was higher in the 7.5 ppt treatment of the 12-16 weeks post-hatch age group. There were differences in nitrite levels between some of the treatments in each age group (Table 3.1) with the highest mean±SE concentration in the 7.5 ppt treatment of the 2-6 week post-hatch age group (0.463±0.067 mg/L). This treatment was below harmful levels for warm water species (Colt et al. 1981; Lewis and Morris 1986), and had growth and survival indices equivalent to the highest levels of that age group (Table 3.2). Total ammonium nitrogen (TAN= $NH_4^+$ and $NH_3$) was higher in the 7.5 and 5.0 ppt salinity treatments of the 2-6 weeks post-hatch age group, however unionized ammonia ($NH_3$) levels were well below harmful levels (Meade 1985) and not different between treatments (Table 3.1).

### Growth and Survival

There were no differences in survival in the 2-6 week post-hatch age group (ANOVA, N=4 tanks/trmt (N=3 for 7.5 ppt) F=0.973, *P*=0.440; Figure 1). Mean (±SE) survival ranged from 76.3 (±10.0)% at 0 ppt to 88.6 (±1.4)% at 2.5 ppt. There were differences in survival in the 7-11 and 12-16 weeks post-hatch age groups (Figure 3.1). In the 7-11 week old age group, the 7.5 ppt treatment had lower survival (78.1±6.9%) than the other 3 treatments (ANOVA, N=4, F=5.57, *P*=0.013), which were not different (0

ppt: 91.9±3.3%, 2.5 ppt: 97.8±1.1%, and 5.0 ppt: 95.9±1.6%). In the 12-16 week old age group, the mean (±SE) survival of the 7.5 ppt treatment (98.8±1.2%) was higher than the 0 ppt and 5.0 ppt treatments (ANOVA, N=4, F=12.16, *P*=0.004) (91.7±0.7% and 94.6±1.8%; respectively).

There were differences in mean total weights (defined as the final total weight of all the fish in a tank) between treatments in the 2-6 and 7-11 week post-hatch age groups, and no differences in the 12-16 week old age group (Table 3.2). In the 2-6 week old age group, the 0 ppt treatment had a lower mean (±SE) final total weight than all the other treatments (Table 3.2). In the 7-11 week old age group, the 7.5 ppt treatment had a lower mean (±SE) final total weight than the 0 ppt and 2.5 ppt treatments. There were no differences among final total weights of the 12-16 week old age group.

There were differences in mean individual growth in the 2-6 and 7-11 week old age groups (Table 3.2). In the 2-6 week old age group, mean (±SE) individual growth of the 0 ppt treatment (95.9±10.0 mg) was less than the other treatments (Table 3.2). In the 7-11 week old age group, mean (±SE) mean individual growth of the 7.5 ppt treatment (410.8±135.9 mg) was less than that of both the 0 ppt and 2.5 ppt treatments. There were no differences in mean individual growth among treatments of the 12-16 week old age group.

There were differences in mean individual final weights in the 2-6 and 7-11 week old age groups (Table 3.2). In the 2-6 week old age group, mean (±SE) final weight of the 0 ppt treatment (95.5±6.6 mg) was lower than the mean (±SE) of all other treatments. In this group, the mean final weights of the 2.5 ppt and 7.5 ppt treatments were higher than the other two treatments. In the 7-11 week old age group, mean final individual weights

of the 0 ppt and 2.5 ppt treatments were both higher than the 5.0 ppt and 7.5 ppt treatments (Table 3.2). Final individual length data followed approximately the same pattern as the weight data. Condition factors were not different across treatments for any age group (Table 3.2).

**2-6 weeks post-hatch whole body $Na^+$ and percent water weight**

There were differences in both whole body $Na^+$ content and percent water weight at the end of the 2-6 week old age group trial (Figure 3.2). At this sampling point, mean (±SE) whole body $Na^+$ concentrations were highest in the 0 ppt treatment (156.5±8.8 μmol $g^{-1}$ dry W), and the mean of the 5.0 ppt treatment was higher than the 2.5 and 7.5 ppt treatments (Kruskal-Wallis rank sum test, N=20 fish/salinity (15 for 7.5 ppt), $\chi^2$=30.58, $P$<0.001; Figure 3.2A). Despite their tight grouping, mean percent water weight of the 7.5 ppt and 2.5 ppt treatments were higher both the 0 ppt and 5.0 ppt treatments (Kruskal-Wallis rank sum test, N=20 fish/salinity (15 for 7.5 ppt), $\chi^2$=42.12, $P$<0.001; Figure 3.2B).

**7-11 weeks post-hatch $Na^+/K^+$ ATPase, whole body $Na^+$, and percent water weight**

In the 7-11 week old age group, there were differences in $Na^+/K^+$ ATPase activity with salinity exposure duration both within a salinity treatment and among salinity treatments (Figure 3.3A). At 2 dpt, the 5.0 ppt treatment had higher mean $Na^+/K^+$ ATPase activity than both the 0 ppt and 2.5 ppt treatments (ANOVA, N=4 tanks/trt, F=4.99, $P$=0.042). At 14 dpt, mean activity of the 2.5 ppt treatment was higher than both the 5.0 ppt and 7.5 ppt treatments (ANOVA, N=4, F=18.15, $P$=0.001). At 28 dpt, the 0 ppt treatment had a higher mean $Na^+/K^+$ ATPase activity than the 5.0 ppt and 7.5 ppt

treatments (ANOVA, N=4, F=11.71, *P*=0.004). In the 0 ppt treatment, activities in the 14 and 28 dpt time points were higher than the activities at the 0 and 2 dpt time points (ANOVA, N=4, F=23.98, *P*<0.001). In the 2.5 ppt treatment, activity was higher at the 14 dpt time point than the 0 or 2 dpt time points (ANOVA, N=4, F=5.28, *P*=0.038). There were no differences between time points within the 5.0 ppt treatment (ANOVA, N=4, F=0.507, *P*=0.488). In the 7.5 ppt treatment, activity at 14 dpt was lower than at 2 dpt (ANOVA, N=4, F=0.563, *P*=0.049).

The 7-11 week old group showed several differences in whole body $Na^+$ content among sampling points, but there were only differences among salinity treatments at 14 dpt (Figure 3.3B). At 14 dpt, the 0 ppt treatment had higher mean (±SE) whole body $Na^+$ content (154.76±15.05 μmol $g^{-1}$ dry W) than all other treatments (ANOVA, N=8, F=8.00, *P*=0.009). The 0 ppt mean $Na^+$ content at 14 dpt was also higher than the $Na^+$ content at any other time point for that treatment (Kruskal-Wallis rank sum test, N=8, $\chi^2$=10.65, *P*=0.014). In the 2.5 ppt treatment, whole body $Na^+$ content at 28 dpt was higher than at 14 dpt (Kruskal-Wallis rank sum test, N=8, $\chi^2$=9.70, *P*=0.021). In the 5.0 ppt treatment, $Na^+$ content of the 2 dpt sampling point was higher that at the 14 and 28 dpt sampling points (Kruskal-Wallis rank sum test, N=8, $\chi^2$=8.88, *P*=0.031). The 7.5 ppt treatment showed no differences between sampling points (Kruskal-Wallis rank sum test, N=8, $\chi^2$=3.40, *P*=0.335).

In the 7-11 week old age group, all treatment groups except the 2.5 ppt treatment showed differences in percent water weight among time points, and there were differences among treatments at the 2 dpt and 28 dpt sampling points (Figure 3.3C). In the 0 ppt treatment, mean percent water weight at 28 dpt was different than the initial two

time points of that treatment (ANOVA, N=8, F=23.73, $P<0.001$). In the 2.5 ppt treatment, there were no differences in mean percent water weight among time points (ANOVA, N=8, F=0.64, $P=0.43$). In the 5.0 ppt treatment, mean percent water weight was higher at 2 dpt than at the initial and final sampling point (Kruskal-Wallis rank sum test, N=8, $\chi^2=9.67$, $P=0.008$). The 7.5 ppt treatment followed the same pattern as the 5 ppt treatment (Kruskal-Wallis rank sum test, N=8, $\chi^2=11.77$, $P=0.003$). At 2 dpt, mean percent water weight of the 0 ppt treatment was lower than all other treatments (Kruskal-Wallis rank sum test, N=8, $\chi^2=18.73$, $P<0.001$). At the 28 dpt sampling point, mean percent water weight of the 0 ppt treatment remained lower than the other three treatments (ANOVA, N=8, F=10.83, $P=0.003$).

**12-16 weeks post-hatch $Na^+/K^+$ ATPase, whole body $Na^+$, and percent water weight**

In the 12-16 week old age group, there were no differences in $Na^+/K^+$ ATPase activity among salinity treatments within the same time point. However, each salinity treatment did show differences among sampling points (Figure 3.4A). In the 0 ppt treatment, $Na^+/K^+$ ATPase activities at 14 and 28 dpt were higher than the activities at 0 and 2 dpt (ANOVA, N=4, F=13.56, $P=0.002$). In the 2.5 ppt treatment, initial activity level was lower than the activity level at the other 3 time points (ANOVA, N=4, F=12.14, $P=0.004$). In the 5.0 ppt treatment, activity at 28 dpt was higher than the activity at 0 and 2 dpt (ANOVA, N=4, F=22.24, $P=0.001$). In the 7.5 ppt treatment, the two later time points had higher activity than the first two time points (Kruskal-Wallis rank sum test, N=4, $\chi^2=11.34$, $P=0.010$).

In the 12-16 week post-hatch age group, the only differences in whole body $Na^+$ content were between salinity treatments at the 14 dpt sampling point (Figure 3.4B). The

mean whole body $Na^+$ content of the 2.5 ppt treatment was higher than the 7.5 ppt treatment (ANOVA, N=8, F=5.571, *P*=0.025).

In the 12-16 week old age group, there were differences in percent water weights across time points in each treatment, but only among treatments at the 2 dpt time point (Figure 3.4C). In the 0 ppt treatment, mean percent water weight of the 0 dpt and 2 dpt time points were lower than the 14 dpt and 28 dpt time points (ANOVA, N=8, F=18.16, *P*<0.001). In the 2.5 ppt treatment, mean percent water weight of the 14 dpt time point was higher than all other sampling points (ANOVA, N=8, F=11.85, *P*=0.002). In the 5.0 ppt treatment, mean percent water weight of the initial time point was lower than the later sampling points, which were not different (ANOVA, N=8, F=4.24, *P*=0.048). The 7.5 ppt treatment followed the same pattern as the 5.0 ppt treatment (ANOVA, N=8, F=12.93, *P*=0.001). At 2 dpt, the 7.5 ppt treatment's mean percent water weight was higher than the 0 ppt and 2.5 ppt treatments (ANOVA, N=8, F=12.9, *P*=0.001).

## Discussion

Results of this study provide specific insight into the ontogeny of juvenile Gulf killifish hypo-osmotic tolerance, and guidance to potential producers on optimal time to transfer *F. grandis* from a saline hatchery environment to a freshwater grow-out pond or tank. The gradient of low salinities used in this study was specifically chosen to test the lower limits of *F. grandis* hypo-osmoregulatory ability at presumably sensitive juvenile ages. All salinities used in this experiment were below isotonic conditions (~10 ppt), requiring fish to actively osmoregulate in order to counteract the osmotic gradient causing passive ion loss and water gain. The gradient of salinity treatments was expected to result in a reciprocal gradient of energy expended in order to actively osmoregulate.

This would result in lower growth rates and higher $Na^+/K^+$ ATPase activity in the 0 and 2.5 ppt treatments. However, it was found that growth was only negatively affected in the 2-week-old age group and $Na^+/K^+$ ATPase activity increased only in 7-week-old age group. In the 12-week-old age group, there were no dramatic differences in growth, $Na^+/K^+$ ATPase activity, whole body $Na^+$ content, or percent water weight. This suggests that *F. grandis* could be stocked into freshwater ponds or tanks as early as 7 weeks post-hatch without reduced growth, and by 12 weeks post-hatch the osmoregulatory system of *F. grandis* is fully developed.

Reduced growth and survival offer broad evidence of chronic hypo-osmotic stress (Boeuf and Payan 2001). Reduced growth rates generally indicate that metabolic resources are being diverted from tissue formation in order to osmoregulate (Boeuf and Payan 2001). In this study, the similar growth and survival among salinities by 7 weeks post-hatch indicates that *F. grandis* growth is not inhibited by fresh water by the time they reach that age and size. In contrast, Patterson et al. (2012) found that growth and survival were lower in 8 month-old *F. grandis* transferred from 7.0 ppt to 0.5 ppt than transferred to 5.0, 8.0, or 12.0 ppt. These contrasting results may stem from different source populations, and the freshwater holding conditions for broodstock and freshly fertilized eggs in this study. The *Fundulus* genus is known for its physiological plasticity and ability to rapidly evolve freshwater tolerance compared to other teleosts (Whitehead 2010). Inland populations of *F. grandis* have been documented 400 km up the Los Brazos River of Texas (Hillis et al. 1980). *F. grandis* fecundity is relatively low in fresh water (Strawn et al. 1986) with freshwater broodstock producing 20.2±0.1% viable embryos (Chapter 2) in contrast to 56.6±19.6% reported from a salinity of 9.0 ppt (with a 4:1

female to male ratio) by Gothreaux and Green (2012). It is possible that selective pressure due to collecting naturally spawned eggs from fresh water held broodstock has caused a change in population structure compared to coastal populations. It is also possible that the age differences between early juveniles utilized in this study and older juveniles by Patterson et al. (2012) contribute to growth and survival differences, although for many fishes osmoregulatory ability increases with size and age (Varsamos et al. 2005; Allen and Cech 2007).

Several other studies have looked at the ontogeny of hypo-osmotic salinity tolerance in euryhaline species (reviewed by Varsamos et al. 2005). For example, out of three size groups (20-29, 30-39, and 40-69 mm) of striped mullet *M. cephalus*, which occupy similar estuarine habitats as *F. grandis*, only the largest group was able to survive in fresh water (Nordlie 2006). In other euryhaline species, it is common to see osmoregulatory capacity increase with age (Varsamos et al. 2001; Bodinier et al. 2010; Fridman et al. 2012). During development, there is often a major increase in osmoregulatory capacity at time points that correspond to hatching, mouth opening, and gill development before maximum osmoregulatory capacity is reached as fish transition into the juvenile stage (Varsamos et al. 2001; Bodinier et al. 2010). *F. grandis* hatch with fully developed mouthparts and gills, conceivably allowing for considerable osmoregulatory ability at early life stages. Immunocytochemistry has shown newly hatched *F. heteroclitus* (15-25 d post fertilization) to already have osmoregulatory chloride cells concentrated on the gills and opercula of larvae (Katoh et al. 2000). Developed mouthparts aid in regulating drinking rate in response to varying levels of diffusional water loss. *F. heteroclitus* and *F. bermudae* can also regulate water intake

embryonically, before the mouthparts develop, through the pharynx via embryonic gill slits (Guggino 1980).

*F. grandis* transferred to fresh water at both 7 and 12 weeks post-hatch dramatically increased their $Na^+/K^+$ ATPase activity, suggesting an increase in energy spent on osmoregulation. Overall, $Na^+/K^+$ ATPase activity was highly variable in the 7-week post-hatch age group. The two higher salinity treatments (5.0 and 7.5 ppt) showed a small spike in $Na^+/K^+$ ATPase activity at 2 dpt, which then leveled off or declined to baseline values as the experiment continued. This pattern is similar to that seen in other salinity transfer experiments (Mancera and McCormick 2000; Scott et al. 2004). The 0 and 2.5 ppt salinity treatments showed a more delayed reaction by spiking later in the experiment at 14 and 28 dpt, respectively. The $Na^+/K^+$ ATPase activity of the 0 ppt treatment spiked at 14 dpt in the 12 week post-hatch age group but the activity of the other treatments increased as well, resulting in no inter-treatment differences. This freshwater transfer pattern is slightly different from adult *F. heteroclitus*, where gill $Na^+/K^+$ ATPase activity increased 1-4 dpt from 10 ppt to 0 ppt, but returned to pre-transfer levels by 14 dpt (Scott et al. 2004). *F. heteroclitus* have been shown to rapidly increase gill $Na^+/K^+$ ATPase activity after abrupt salinity transfer. Mancera and McCormick (2000) witnessed increase in $Na^+/K^+$ ATPase activity 3 hours after transfer from 0.1 ppt to 30 ppt, with a subsequent return to initial levels by 12 hr post-transfer. This spike in $Na^+/K^+$ ATPase activity generally lasts longer after transfer to fresh water as suggested by Scott et al. (2004) and the current study on *F. grandis*. This could be caused by an increased quantity or a change in functionality of chloride cell density in the gills in fresh water. In freshwater adapted *F. heteroclitus*, chloride cells were more

widely distributed across the gill than in seawater adapted fish (Katoh et al. 2001). In addition, distinct freshwater and seawater type chloride cells were found in adult *F. heteroclitus* after at least 1 month of acclimation to fresh water or seawater, but there was no difference in oxygen consumption or $Na^+/K^+$ ATPase activity between freshwater and seawater adapted fish (Katoh et al. 2001). Existing data on *F. grandis* is based on $Na^+/K^+$ ATPase gene expression via real-time PCR. Through this method, Patterson et al. (2012) documented a spike in $Na^+/K^+$ ATPase expression one week post-transfer from 7.0 ppt to 0.5 ppt, with a subsequent drop to levels equivalent to the higher salinity treatments (5.0, 8.0, and 12.0 ppt) by 3 weeks post-transfer.

Whole body $Na^+$ content, an index of osmotic regulatory ability, indicated that *F. grandis* transferred to fresh water were able to maintain or even exceed the internal $Na^+$ levels of fish in more saline environments across age groups and salinity treatments of this study. Changes in plasma $Na^+$ content have been shown to mirror changes in plasma osmolality (Marshall et al. 1999), a common measure of osmoregulation (Varsamos et al. 2005). Interestingly, whole body $Na^+$ content was highest in the 0 ppt treatment at the end of the 2-week age group trial (6 weeks post-hatch) and there was another spike in $Na^+$ content 14 dpt in the 0 ppt treatment of the 7-week-old age group. At these time points, fish in the freshwater treatments seem to be overcompensating for the environmental ion gradient. In the 7-week age group, the $Na^+$ contents of all salinity treatments declined back to pre-transfer levels by 28 dpt. During the 12-week-old trial, the $Na^+$ content of the 0 ppt treatment was no different than the other salinities at any of the sampling points. This suggests that ion-pumping mechanisms were more than capable of adapting to fresh water. Interestingly, the spikes in whole body $Na^+$ content were found in the 0 ppt

treatment, which had the strongest passive osmotic gradient pulling ions out of the body. In contrast, Marshall et al. (1999) observed a spike in plasma $Na^+$ content from 1 to 24 hr after *F. heteroclitus* were transferred from fresh water to seawater, with $Na^+$ returning to pre-transfer levels by 48 hours post-transfer. Fresh water to seawater transfer is also associated with a rapid reduction in drinking rate and permeability to $Na^+$ and $Cl^-$ (Potts and Evans 1967), causing a reduction in $Na^+$ and $Cl^-$ influx and efflux rates (Wood and Laurent 2003).

The percent water weight data serve as another index of overall osmoregulation and support the conclusion that *F. grandis* have the ability to osmoregulate in fresh water. In fresh water conditions, water passively enters the body via osmosis. For example, when saltwater acclimated *F. heteroclitus* were exposed to fresh water at 4°C, they gained 15% of their body weight over 6 d, but when the temperature was raised to 16°C, weights dropped back to original levels within 2 d (Kidder et al. 2006b). This extreme weight gain was caused by osmosis due to the reduced metabolic activity and thus reduced active osmoregulation at low temperatures. Salt water to fresh water transfer at 16°C causes a spike in percent water weight that lasted approximately 24 hrs (Kidder et al. 2006). Kidder et al. (2006) also calculated active $Na^+$ transport of freshwater fish at 16°C to only require 0.94% of the total metabolic budget. Fish in this experiment were sampled along a longer timescale, and some of the initial fluctuations in percent water weight could have been missed. In the instances when percent water weight differed among treatments, the 0 ppt treatment had the lowest or one of the lowest mean percent water weights. This is opposite of what would be expected since the 0 ppt treatment had the largest osmotic gradient pulling water into the body. This indicates that *F. grandis* has

the ability to compensate or even overcompensate its osmoregulatory mechanisms to maintain homeostasis in hypo-osmotic environments.

The physiological data shows a continued increase in osmoregulatory ability between the 7-11 and 12-16 week-old age groups, despite low salinity treatments having equally apt growth and survival indices in these age groups. The 7-11 week post-hatch age group showed reduced growth and survival indices in the two higher salinity treatments. These results are reinforced by the variation in the $Na^+/K^+$ ATPase data, in which the 0 and 2.5 ppt treatments are consistently higher by 14 and 28 dpt. It seems that this increase in $Na^+/K^+$ ATPase activity was sufficient to maintain osmoregulation without negatively affecting growth. In fact, whole body $Na^+$ data from the 7-11 week post-hatch group suggests there may have been some over compensation of the osmoregulatory systems, as indicated by the spike of the 0 ppt treatment at 14 dpt. This reinforces the considerable osmoregulatory capacity of *F. grandis.* On the other hand, the 12-16 week post-hatch group was very consistent among salinity treatments across all growth, survival, and osmoregulatory indices. This suggests *F. grandis* has a highly developed osmoregulatory system by 12 weeks of age. Unfortunately, these data cannot be compared to the 2-6 week old age group, since the fish in the 2-6 week old age group were too small to remove the gills for tissue analysis. The specific processes limiting the osmoregulatory ability of younger fish could be examined in more detail via alternative methods in future studies. Even at this youngest age class, the 2.5 ppt treatment showed growth and survival rates comparable to the 5.0 and 7.5 ppt treatments, which are considered to be in the ideal range for *F. grandis* (Perschbacher et al. 1990; Patterson 2014).

This study demonstrates the extent of *F. grandis'* physiological plasticity in regards to hypo-osmotic tolerance. These results support the potential for freshwater grow-out of juvenile *F. grandis*, after they are at least 7 weeks old. These results also suggest that larvae could be kept at a transitional salinity of 2.5 ppt as early as two weeks post-hatch. Additional research on specific trace ionic requirements and growth and survival of *F. grandis* in freshwater ponds is recommended prior to commercial scale production.

Table 3.1 Mean (SE) water quality data from experimental tanks at four salinity treatments.

| Age group | Treatment | Temp. (°C) | Salinity (ppt) | pH | DO[a] (ppm) | Nitrite (ppm) | TAN[b] ( ppm) | $NH_3$-N[c] (ppm) |
|---|---|---|---|---|---|---|---|---|
| **2-6 weeks post-hatch** | **0 ppt** | 27.1 (0.08) | 0.2 (0.01) z | 7.88 (0.04) y | 7.01 (0.06) | 0.084 (0.013) z | 0.06 (0.01) z | 0.02 (0.00) |
| | **2.5 ppt** | 27.2 (0.09) | 2.6 (0.01) y | 7.54 (0.06) z | 6.95 (0.07) | 0.194 (0.021) yz | 0.15 (0.02) yz | 0.03 (0.01) |
| | **5.0 ppt** | 27.2 (0.08) | 5.0 (0.02) x | 7.51 (0.07) z | 6.85 (0.07) | 0.309 (0.064) xy | 0.16 (0.03) y | 0.03 (0.01) |
| | **7.5 ppt** | 27.1 (0.10) | 7.5 (0.02) w | 7.65 (0.07) z | 6.94 (0.08) | 0.463 (0.067) x | 0.17 (0.03) y | 0.03 (0.01) |
| | **N** | 116 | 116 | 32 | 116 | 27 | 32 | 32 |
| **7-11 weeks post-hatch** | **0 ppt** | 26.3 (0.13) | 0.2 (0.01) z | 7.59 (0.07) | 7.41 (0.05) | 0.107 (0.016) y | 0.06 (0.01) | 0.01 (0.00) |
| | **2.5 ppt** | 26.2 (0.25) | 2.6 (0.01) y | 7.25 (0.10) | 7.39 (0.06) | 0.072 (0.009) z | 0.10 (0.02) | 0.01 (0.00) |
| | **5.0 ppt** | 26.5 (0.09) | 5.1 (0.01) x | 7.22 (0.09) | 7.35 (0.06) | 0.087 (0.012) yz | 0.09 (0.01) | 0.01 (0.00) |
| | **7.5 ppt** | 26.4 (0.09) | 7.6 (0.01) w | 7.41 (0.06) | 7.37 (0.06) | 0.064 (0.010) z | 0.06 (0.01) | 0.01 (0.00) |
| | **N** | 104 | 104 | 36 | 100 | 36 | 36 | 36 |
| **12-16 weeks post-hatch** | **0 ppt** | 23.5 (0.10) | 0.2 (0.00) z | 8.31 (0.09) y | 8.30 (0.03) z | 0.044 (0.009) y | 0.09 (0.02) | 0.05 (0.02) |
| | **2.5 ppt** | 23.5 (0.10) | 2.6 (0.01) y | 8.04 (0.07) z | 8.23 (0.03) z | 0.023 (0.001) z | 0.08 (0.02) | 0.04 (0.01) |
| | **5.0 ppt** | 23.5 (0.10) | 5.0 (0.05) x | 8.01 (0.07) z | 8.25 (0.04) z | 0.026 (0.003) z | 0.08 (0.02) | 0.03 (0.01) |
| | **7.5 ppt** | 23.3 (0.24) | 7.6 (0.02) w | 8.06 (0.05) z | 8.40 (0.03) y | 0.027 (0.004) z | 0.12 (0.07) | 0.05 (0.03) |
| | **N** | 96 | 96 | 32 | 92 | 28 | 27 | 27 |

There were 4 tanks per salinity group for each age group (3 tanks for the 2-6 weeks group at 7.5 ppt) over the 4-week experimental duration. Different letters indicate a significant difference ($P<0.05$) between treatments. All differences were tested with a one-way ANOVA with a Fishers LSD post hoc test. [a]DO: dissolved oxygen, [b]TAN: total ammonium nitrogen, [c]$NH_3$-N: unionized ammonia nitrogen.

Table 3.2 Mean (SE) growth indices of 3 age groups of Gulf killifish *Fundulus grandis* after 4 weeks of growth in four salinity treatments.

| Age group | Treatment | Mean individual growth per fish (mg) | Total final weight per tank (g) | Individual final weight (mg) | Individual final length (mm) | Condition factor |
|---|---|---|---|---|---|---|
| **2-6 weeks** | **0 ppt** | 95.9 (10.1) z | 13.28 (2.48) z | 95.5 (6.7) z | 20.3 (0.46) z | 1.08 (0.03) |
| | **2.5 ppt** | 167.4 (9.9) y | 24.58 (1.30) y | 190.7 (13.0) x | 25.2 (0.55) x | 1.13 (0.02) |
| | **5.0 ppt** | 140.6 (11.3) y | 20.08 (1.56) y | 141.2 (8.0) y | 23.2 (0.43) y | 1.09 (0.02) |
| | **7.5 ppt** | 145.4 (13.2) y | 21.63 (2.12) y | 171.8 (8.4) x | 24.6 (0.41) x | 1.09 (0.03) |
| | | N=4 *P*=0.004 | N=4 *P*=0.008 | N=40 *P*<0.001 | N=40 *P*<0.001 | N=40 *P*=0.869 |
| **7-11 weeks** | **0 ppt** | 822.9 (86.8) y | 81.15 (9.21) y | 1163.7 (67.8) y | 44.6 (0.82) y | 1.26 (0.01) |
| | **2.5 ppt** | 758.9 (60.6) y | 80.76 (5.13) y | 995.7 (52.9) y | 42.7 (0.67) y | 1.23 (0.02) |
| | **5.0 ppt** | 594.6 (73.0) yz | 66.83 (6.65) yz | 801.3 (58.5) z | 39.6 (0.87) z | 1.21 (0.02) |
| | **7.5 ppt** | 410.9 (134.9) z | 46.84 (12.86) z | 808.1 (77.2) z | 38.6 (1.18) z | 1.25 (0.01) |
| | | N=4 *P*=0.025 | N=4 *P*=0.010 | N=40 *P*<0.001 | N=40 *P*<0.001 | N=40 *P*=0.074 |
| **12-16 weeks** | **0 ppt** | 856.9 (46.5) | 54.66 (2.56) | 1095.7 (93.8) | 44.4 (1.13) z | 1.19 (0.02) |
| | **2.5 ppt** | 908.5 (47.1) | 58.72 (3.00) | 1288.4 (110.5) | 46.6 (1.30) yz | 1.21 (0.03) |
| | **5.0 ppt** | 801.8 (77.4) | 53.93 (4.81) | 1341.2 (106.4) | 48.3 (1.20) y | 1.14 (0.02) |
| | **7.5 ppt** | 911.2 (42.7) | 63.32 (2.60) | 1281.5 (110.4) | 47.5 (1.22) yz | 1.14 (0.02) |
| | | N=4 *P*=0.827 | N=4 *P*=0.195 | N=24 *P*=0.285 | N=24 *P*=0.038 | N=24 *P*=0.051 |

Different letters indicate significant differences ($P<0.05$) between treatments. All differences were tested with a one-way ANOVA with a Fisher's LSD post hoc test or with a Kruskal-Wallis rank sum test.

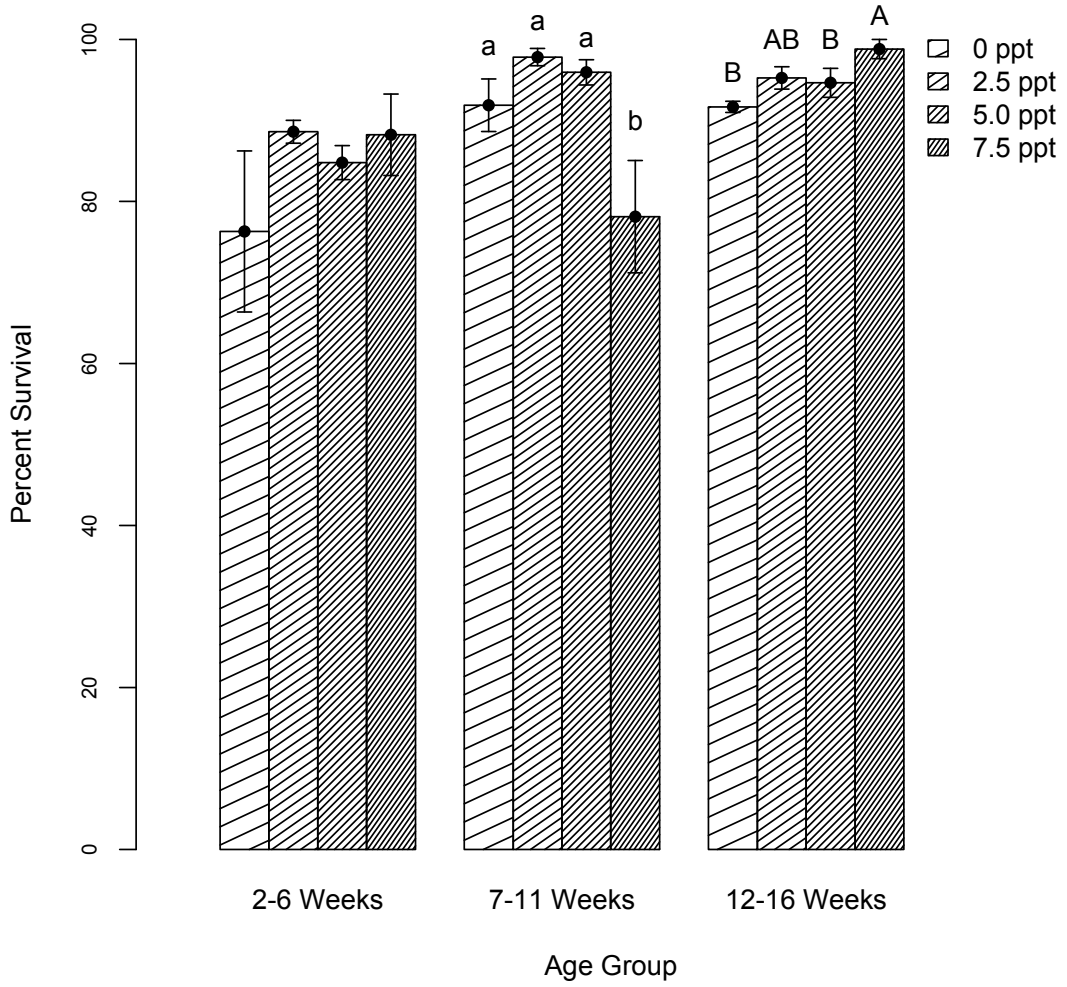


Figure 3.1 Mean (±SE) percent survival of three age groups of Gulf killifish *Fundulus grandis* at low salinities (0, 2.5, 5.0, and 7.5 ppt).

Different letters above the bars indicate significant differences among the treatments and within the age groups (N=4 tanks/trt (N=3 for 7.5 ppt), ANOVA, Fisher's LSD *post hoc*, $P<0.05$).

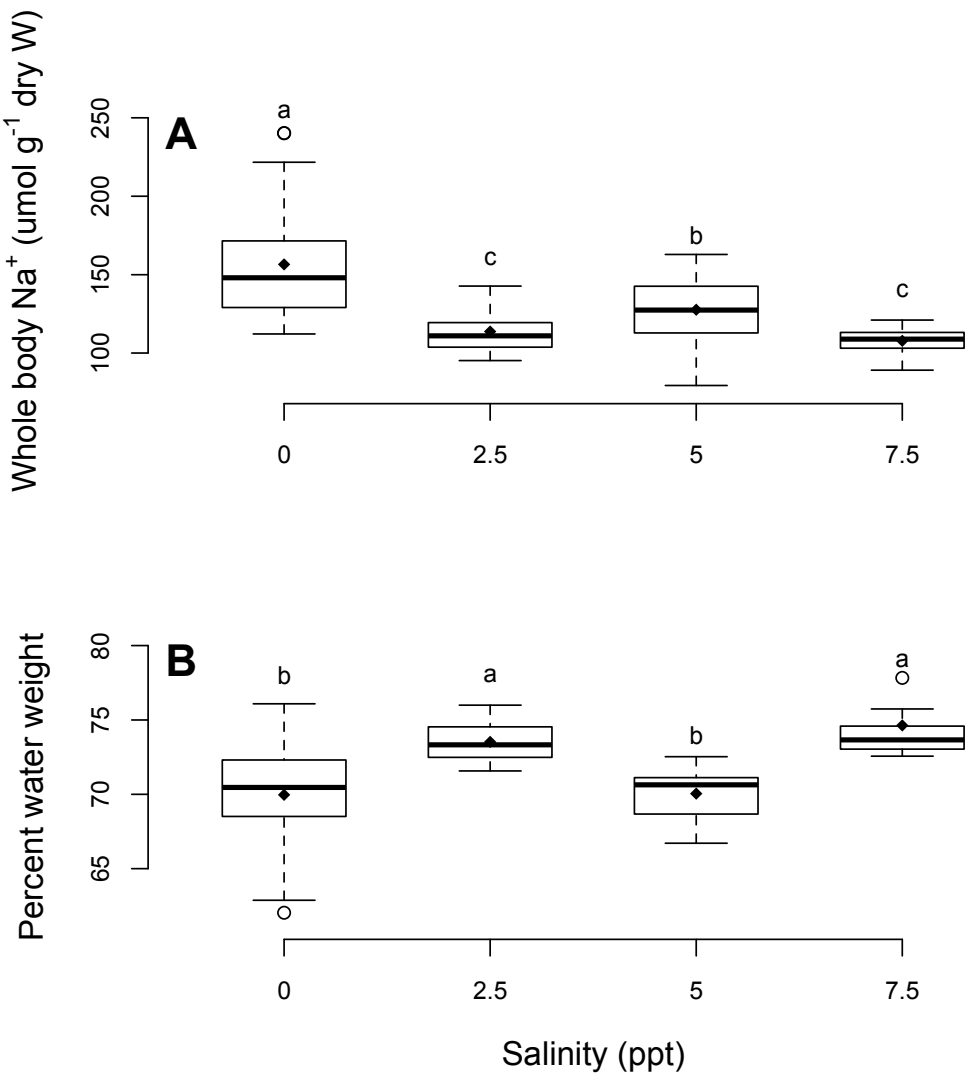


Figure 3.2 Box plot of mean **(A)** whole body $Na^+$ concentration (μmol $g^{-1}$ dry weight) and **(B)** percent water weight of 6-week-old Gulf killifish *Fundulus grandis* after being held for 4 weeks at low salinity (0, 2.5, 5.0, or 7.5 ppt).

The bold black bar represents the median value for each salinity treatment, the black diamond represents the mean of each treatment, the box represents the middle quartiles, the whiskers represent the limits of the data that are within 1.5 interquartile ranges from the mean, and the empty dots are any points outside this range. Different letters above each box represents significant differences between the treatments (N=20/salinity (15 for 7.5), Kruskal-Wallis rank sum test, $P<0.05$).

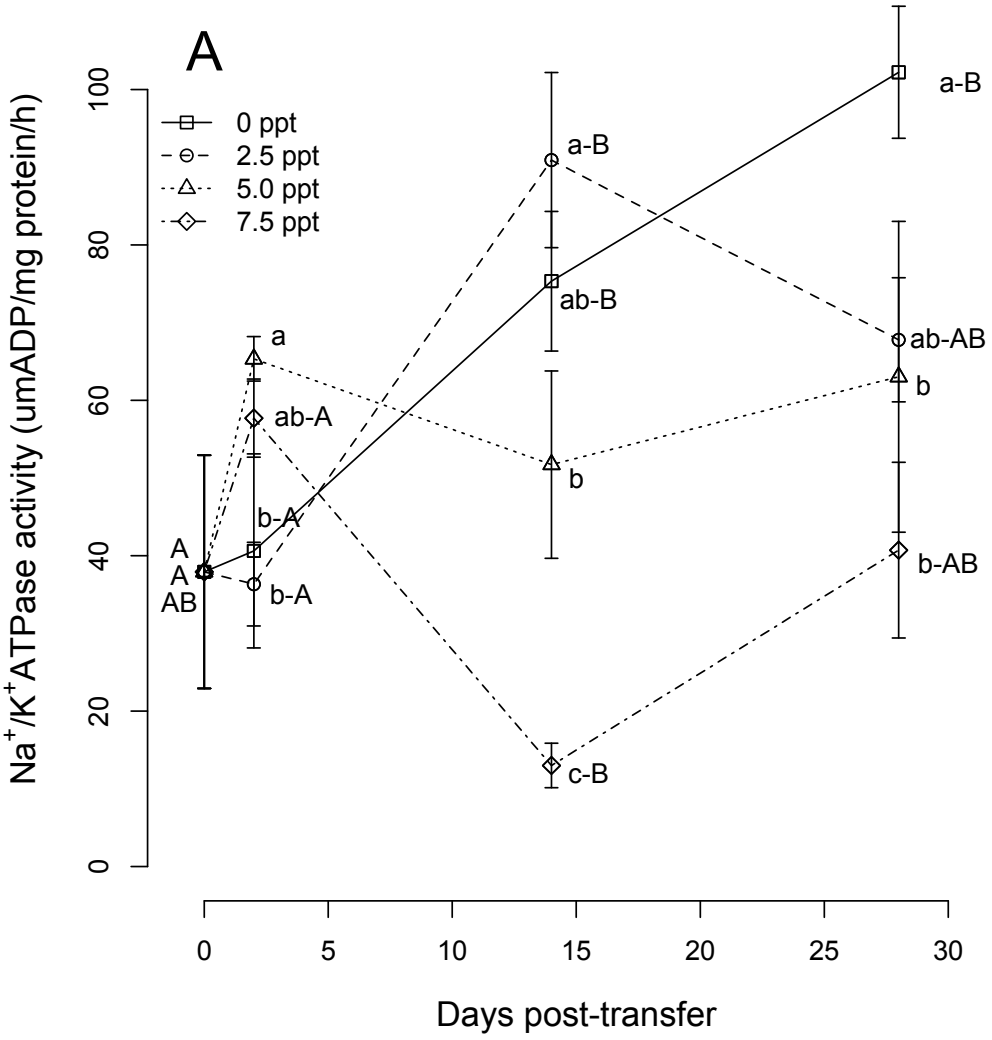


Figure 3.3 Mean (±SE) A) Na+/K+ ATPase activity, B) whole body Na+ concentration (µmol g-1 dry weight), and C) percent water weight of 7-11 week-old Gulf killifish Fundulus grandis following transfer from 7.5 ppt to one of 4 low salinities (0, 2.5, 5.0, or 7.5 ppt).

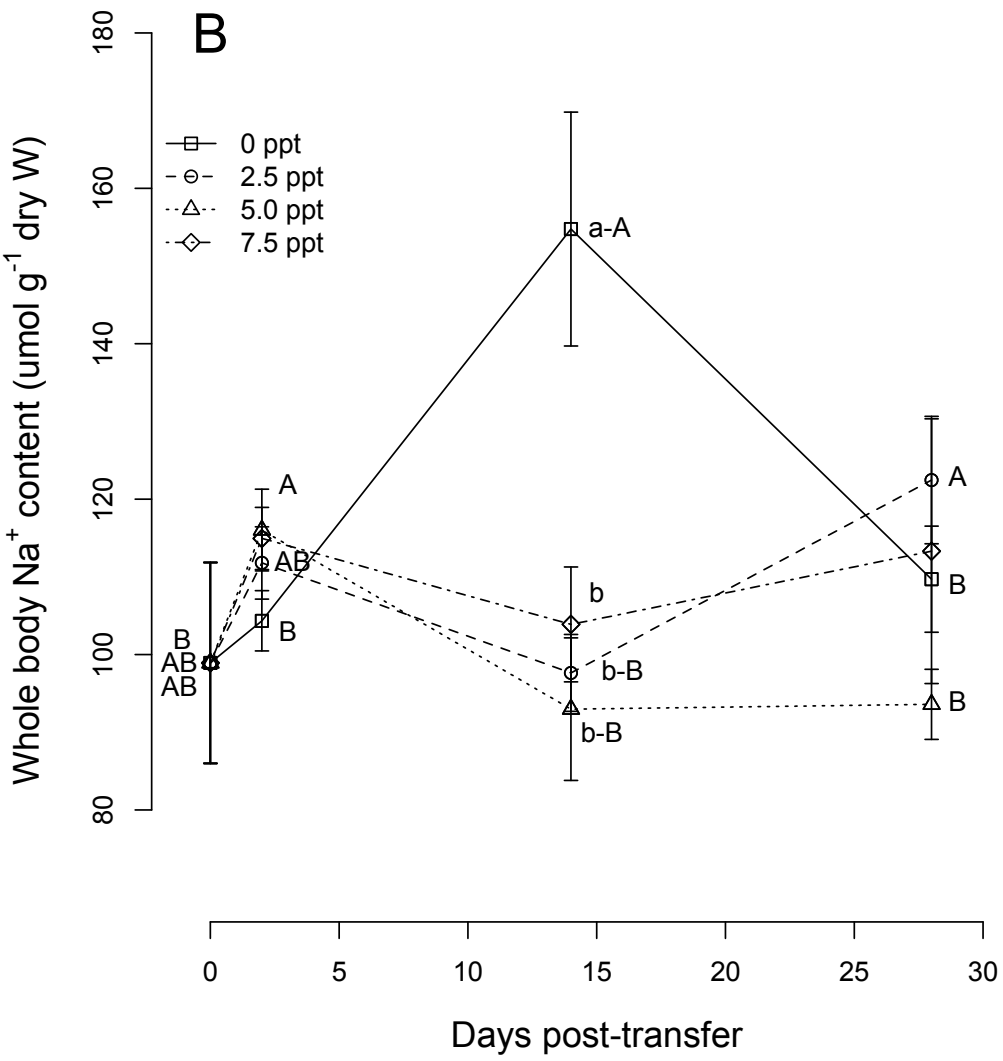


Figure 3.3 (Continued)

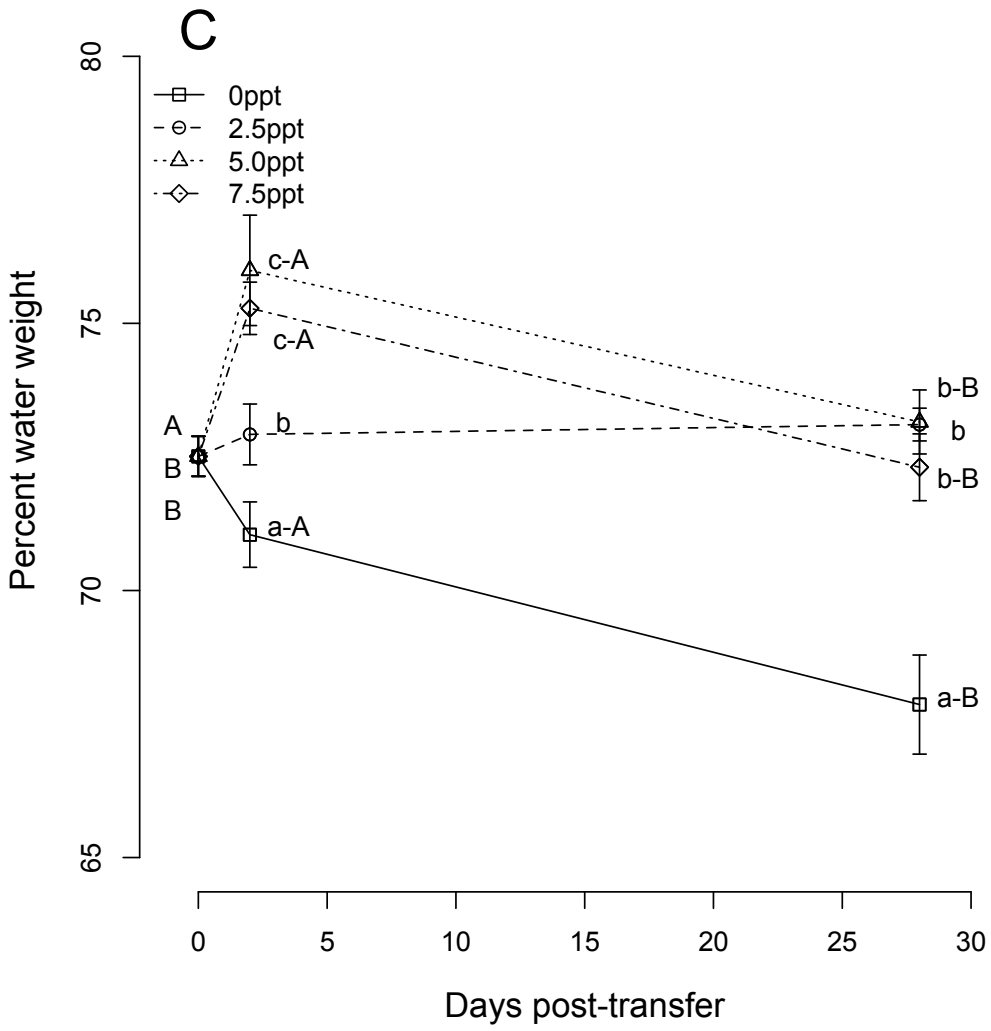


Figure 3.3 (Continued)

Different lowercase letters represent significant differences among salinities at the same time point. Different capital letters represent significant differences among time points but within the same salinity treatment (N=8, ANOVA with Fisher's LSD post hoc or Kruskal-Wallis rank sum test, $P<0.05$).

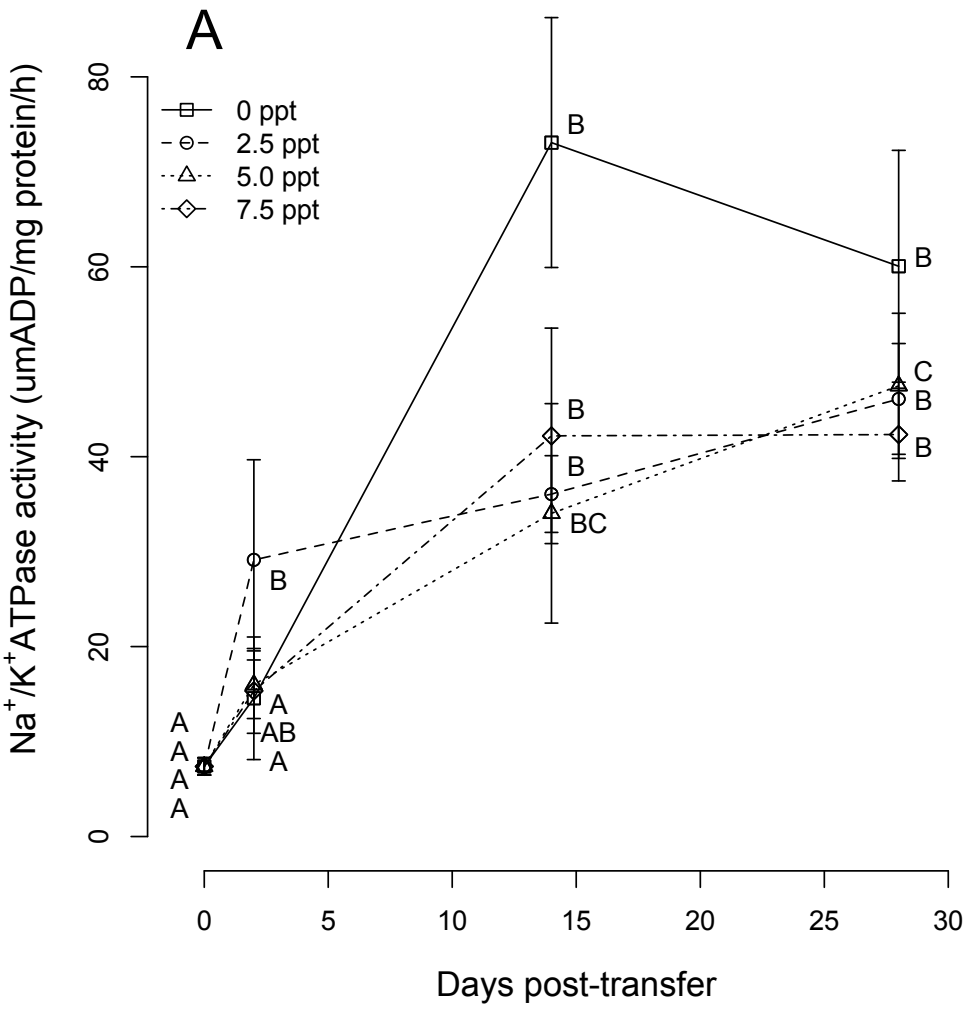


Figure 3.4 Mean (±SE) **A)** $Na^+/K^+$ ATPase activity, **B)** whole body $Na^+$ concentration (μmol $g^{-1}$ dry weight), and **C)** percent water weight of 12-16 week-old Gulf killifish *Fundulus grandis* following transfer from 7.5 ppt to one of 4 low salinities (0, 2.5, 5.0, or 7.5 ppt).

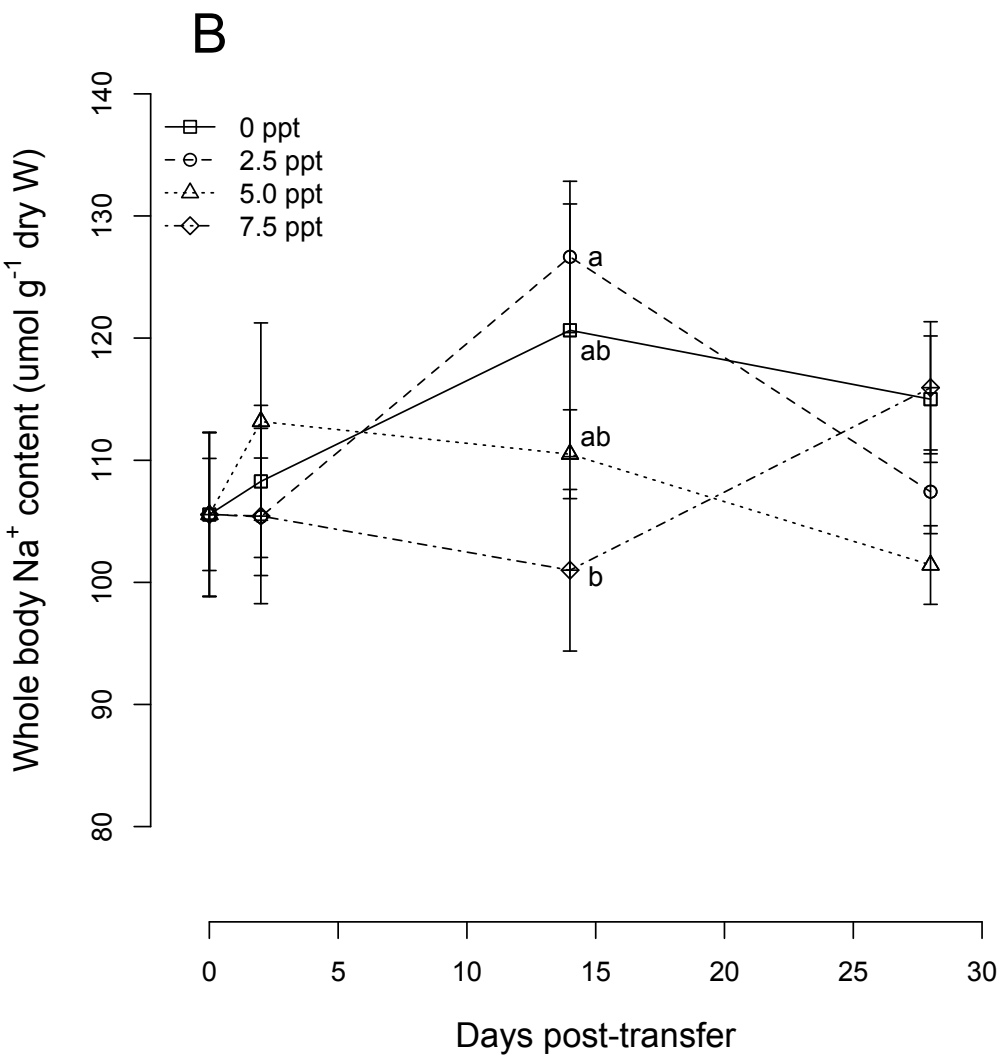


Figure 3.4 (Continued)

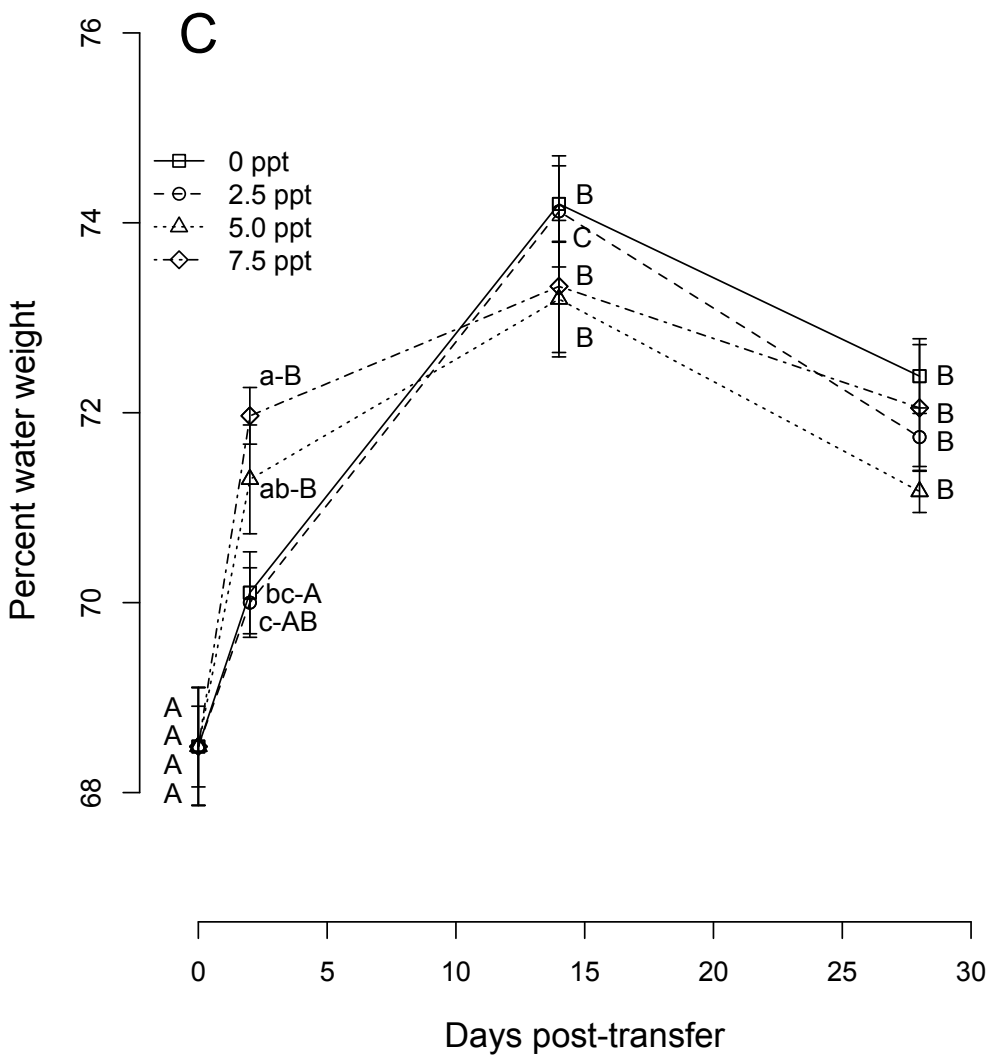


Figure 3.4 (Continued)

Different lowercase letters represent significant differences between salinities at the same time point. Different capital letters represent significant differences among time points but within the same salinity treatment (N=8, ANOVA with Fisher LSD post hoc or Kruskal-Wallis rank sum test, $P<0.05$).

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# CHAPTER IV

# EFFECTS OF HYPO-OSMOTIC SALINITIES ON CARBONIC ANHYDRASE AND $Na^+/K^+$-ATPase GENE EXPRESSION IN JUVENILE *FUNDULUS GRANDIS*


## Abstract

Although the *Fundulus* genus has long been used as a model for the study of euryhalinity, hypo-osmotic acclimation mechanisms have not been extensively investigated. The gills play an important role in hypo-osmotic osmoregulation by facilitating uptake of ions against the natural diffusional gradient. Quantitative real-time Polymerase Chain Reactions (qPCR) can be used to quantify the regulation of genes coding for the enzymes involved in hypo-osmotic acclimation. In this study, the mRNA expressions of two important ion-transporting genes were measured in juvenile Gulf killifish *F. grandis* after transfer from saline water (7.5 ppt) to low salinities (0, 2.5, 5.0, or 7.5 ppt). Carbonic anhydrase 2 (CA2) and $Na^+/K^+$-ATPase (NKA) were measured 2, 14, and 28 dpt. CA2 was up-regulated immediately in the 0 ppt treatment at 2 dpt, whereas NKA up-regulation was delayed and prolonged in the 0 ppt treatment with increases from 14 to 28 dpt. These results demonstrate differing and complementary processes of two enzymatic mechanisms important for ion uptake in the gills following freshwater transfer.

## Introduction

Coastal estuarine habitats offer a unique challenge to resident teleosts due to spatial and temporal variations in salinity. The *Fundulus* genus is well known for its inter- and intra-specific physiological plasticity with regard to osmotic tolerance (Griffith 1974; Whitehead et al. 2011). Killifish, especially the Atlantic killifish *F. heteroclitus*, have long been used as a model for osmoregulation, and have been essential for understanding the gill's role in ion regulation (Potts and Evans 1967; Wood and Marshall 1994; Scott et al. 2004; Prodocimo et al. 2007; Wood 2011). The closely related Gulf killifish *Fundulus grandis* has also been used as a model euryhaline species (Brown et al. 2012; Patterson et al. 2012; Fisher et al. 2013) and is an excellent subject to provide insights into the hypo-osmotic acclimation mechanisms of *Fundulus* and other euryhaline species.

*F. grandis* is native to the coastal salt marshes and estuaries along the Gulf of Mexico; ranging from northern Mexico to the Atlantic coast of North Florida (Kneib 1997; Pattillo et al. 1997; Williams et al. 2008). This species is an important part of the estuarine nekton, linking marsh productivity to larger predatory fish in higher trophic levels (Kneib 1997; Pattillo et al. 1997). *F. grandis* are able to tolerate broad ranges of temperature (2-35°C), salinity (0->40 ppt), and temporarily low oxygen levels ($P_wO_2$=14 torr) (Umminger 1971; Perschbacher et al. 1990; Nordlie and Haney 1998; Virani and Rees 2000; Nordlie 2006) allowing it to thrive in its variable habitat. They also have economic value as a popular baitfish for inshore and near-shore sport fish species along the Gulf of Mexico Coast (Waas et al. 1983; Green et al. 2010).

There is still very little known about the hypo-osmoregulatory mechanisms used as euryhaline fish transition to low salinities. In general, the osmotic capacity of euryhaline fish increases with age or size throughout development (Varsamos et al. 2005). Thus, juvenile specimens can offer insight into the final ontogenetic phases of hypo-osmoregulation. The development of osmoregulatory ability in *F. grandis* is of special interest in the development of procedures for potential inland commercial culture of this baitfish, and to understand the limitations on its natural freshwater distribution (Ramee and Allen, in review). Previous research has revealed that by 7 weeks post-hatch *F. grandis* are able to survive freshwater transfer and have well-developed osmoregulatory function (Chapter 2).

Quantitative real-time polymerase chain reaction (qPCR) has become a valuable tool for assessing the regulation of important osmoregulatory proteins by quantifying the transcription levels of the mRNA of unique protein coding genes in specific tissues (VanGuilder et al. 2008). Several osmoregulatory genes have been examined via qPCR in the gills of *F. grandis* (i.e., $Na^+/K^+$-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), and cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^-$ channel) and *F. heteroclitus* (i.e., NKA, NKCC1, CFTR, glucocorticoid receptor (GR), V-type $H^+$-ATPase, $Na^+/H^+$-exchanger 2 (NHE2), carbonic anhydrase 2 (CA2), cyclooxygenase type 2 (COX2), $Na^+/HCO_3^-$ cotransporter 1, and several endothelin receptors) in low salinity environments (Scott et al. 2004; Scott et al. 2005; Choe et al. 2006; Hyndman and Evans 2009; Patterson et al. 2012).

CA is a zinc metalloenzyme in the gill epithelium that catalyzes the reversible hydrolysis of $CO_2$, and thus plays an important role in osmotic and acid-base regulation

(Conley and Mallatt 1988; Gilmour and Perry 2009). The regulation of CA in osmoregulation is species dependent and seems to be more important in euryhaline species (Conley and Mallatt 1988; Sender et al. 1999). The European flounder *Platichthys flesus* exhibited no difference in CA enzymatic activity when acclimated to either fresh water or saltwater (Sender et al. 1999). In *F. heteroclitus*, the mRNA expression of the CA2 isoform was elevated in freshwater 0.5, 3, and 7 dpt from brackish water (Scott et al. 2005) and also at 3 and 7 dpt from brackish to seawater (Scott et al. 2008), but longer term CA expression has not been studied. Also, branchial CA gene expression has never been quantified in *F. grandis*, and potentially plays an important role in hypo-osmotic acclimation.

NKA is of particular interest due to the high energetic cost associated with increasing NKA activity potentially contributing to reduced growth rates (Sampaio and Bianchini 2002). NKA protein activity has been shown to increase after transfer from brackish to freshwater in *F. heteroclitus* (Scott et al. 2005) and milkfish *Chanos chanos* (Lin et al. 2003). In *F. heteroclitus,* NKA mRNA expression rose twofold from 1 to 4 d post transfer from brackish to freshwater (Scott et al. 2004). In juvenile (~8 month-old) *F. grandis*, NKA mRNA expression was higher at a salinity of 0.5 ppt than 5.0, 8.0 or 12.0 ppt at 1 week post-transfer from 12.0 ppt but showed no difference in expression level at 3 or 7 weeks post-transfer (Patterson et al. 2012). NKA expression has not been quantified in *F. grandis* of younger aged fish and transfer from brackish water to salinities between 0.5 and 5.0 ppt have not been examined

CA and NKA presumably play an important role in freshwater acclimation of juvenile *F. grandis* and other euryhaline fish. Thus, this study adds to the existing

knowledge of the role of these two genes in hypo-osmotic regulation by examining expression levels over a range of low salinity conditions.

## Methods

### Experimental design and sampling protocol

The fish used in this study were also a part of a larger experiment described by in Chapter 2. Experimental recirculating tanks (circular, 92 cm diameter, 150 L) at 4 different salinities (0, 2.5, 5.0, and 7.5 ppt), with 4 tanks per salinity, were stocked with 100 7-week old *F. grandis* (0.282±0.006 g/fish). Each tank was filtered by an individual biological/mechanical canister filter (Ocean Clear, Red Sea, Houston, TX, USA) with activated carbon and recirculated by a 35-W magnetic-drive utility pump (Aquatic Ecosystems, Apopka, FL, USA). At each sampling point (0, 2, 14, and 28 dpt), 4 killifish were sampled from each experimental tank, with the total gill tissue (i.e., 8 gill arches) from 2 fish pooled per sample. This resulted in 2 samples per tank and 8 samples per salinity treatment. Fish were anesthetized in 60 mg/L MS-222 and gills were removed using micro scissors and forceps under a dissecting microscope (Reichert, Depew, NY, USA). All tools were sterilized with 75% ethanol (EtOH) between each sample. Gills were placed in sterile 2-mL screw-cap tubes with 0.5-mL TRI Reagent® (Zymo Research reagent, Irvine, CA, USA), flash frozen in liquid nitrogen, and stored at -80°C.

### RNA extraction and cDNA synthesis

Gill tissue samples were homogenized for 15-30 s using a tissue homogenizer (VWR PowerMax AHS 200, Pro Scientific, Oxford, CT). A fresh homogenizer tip, autoclaved in 0.1% diethyl pyrocarbonate (DEPC), was used for each sample. The

homogenate was centrifuged at 12,000*g* for 5 min, before the supernatant was removed. RNA extraction and purification was performed using a Direct-zol RNA MiniPrep kit (Zymo Research) using the manufacturer's protocol, washing and eluting samples twice to ensure complete RNA extraction. Extracted samples were stored at -80°C.

The quality and concentration of the RNA samples was quantified via spectroscopy (Genesys 10 Bio, Thermo Fischer Scientific Inc., Wilmington, DE, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to synthesize cDNA from 4 ng of total RNA in a 20-µL reaction. RT-PCR was performed using oligo (dT) primers (Thermoscript RT-PCR system, Invitrogen™, Thermo Fischer Scientific Inc., Wilmington, DE, USA).

**Quantitative PCR**

The primers for Elongation Factor 1α (EF1α) and CA2 were designed by comparing GenBank sequences (accession nos. AY430091.1 and AY796057.1, respectively) for *F. heteroclitus* with the sequences of the analogous genes of other teleosts through CLUSTALW alignments using the Biology Workbench 3.2 (San Diego Supercomputer Center, University of California, San Diego, CA, USA). The GenBank primer designer tool was used to generate possible primer combinations that were cross-referenced with the aligned sequences to assure that the primers occurred in highly conserved gene regions. Primers for NKA were reported in Patterson et al. (2012). Candidate primers were ordered from Eurofins Genomics (Huntsville, AL, USA). Final primer combinations (Table 4.1) were chosen and optimized through a series of 20-µL PCR reactions, which were subsequently run via gel electrophoresis in order to insure a single amplicon of the correct size.

The identity of the PCR product of the candidate primer combinations was confirmed via sequencing. Sequencing was performed after purifying the PCR product using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands). The purified PCR product was ligated to pGEM-T easy vectors (Promega, Madison, WI, USA), and transformed into *E. coli* (XL1-Blue cells, Agilent Technologies, Santa Clara, CA, USA) prepared to receive the plasmid via treatment with 0.1 M $CaCl_2$. Transformed cells were incubated at 37°C overnight in Luria Broth (LB)/ampicillin agar plates in the presence of the galactosidase inducer IPTG and galactosidase substrate X-gal. Colonies containing the plasmid transformation were inoculated into 1.5 mL of LB medium and cultured overnight in a shaker at 37°C. Recombinant plasmids were isolated using the Qiagen Spin Miniprep kit per manufacturer's instructions. Recombinant plasmids were first analyzed by gel electrophoresis after digestion with *Eco*RI restriction enzyme. The target genes cloned into the pGEM-T easy plasmids were sequenced using the dideoxynucleotide chain termination method (Sanger and Coulson 1975) with the BigDye terminator V1.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Grand Island, NY, USA). Sequencing was performed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences of all transcripts had >99% identity with the *F. heteroclitus* Genbank sequences.

The expression level of each gene was quantified in duplicate from each sample encompassing four experimental salinities (0, 2.5, 5.0, and 7.5 ppt), three time points (2, 14, and 28 dpt) within each salinity. Samples were run in 20-µL reactions using sybergreen (SsoFast™ EvaGreen® Supermix with low ROX, Bio-Rad, Hercules, CA, USA) per manufacturer's recommendations with 1-µL of 1:3 diluted cDNA template per

reaction in a Stratagene MX3005P QPCR Machine (Stratagene California, La Jolla, CA, USA). Each gene was run using an individually optimized thermocycle program. The cycling conditions consisted of a polymerase activation and DNA denaturation phase (95°C, 60 s), a 40-cycle amplification phase (NKA: 95°C, 5 s; 60°C, 11 s, and CA2: 95°C, 4 s, 56°C, 11 s, 72°C, 6 s), and a melt curve analysis phase (65°C-95°C in 0.5°C increments). EF1α was used as a reference gene (Scott et al. 2005; Tang et al. 2007; Patterson et al. 2012) and was analyzed twice, using the thermocycle profiles of each target gene, to allow for direct comparison with the target genes. Technical replicates that failed to amplify or did not produce expected melt curves were discarded from the analysis (1 NKA and 2 CA2 samples did not replicate). Critical threshold (Ct) values were automatically calculated for each well plate using commercial software (algorithm of the MxPro Mx3005 QPCR Software, Agilent Technologies). Each plate was run with two no template control (NTC) wells for each primer pair to confirm no untargeted transcripts were analyzed. Standard curves were run for each gene on each plate to calculate the efficiency of each qPCR reaction. Standard curves were made by quantifying the purified PCR products of each gene, diluting it to a concentration of 1 ng/μL, and creating serial dilution from this stock solution.

**Data Analysis**

A composite standard curve was made from the logistic regression of the Ct values and standard concentrations of each gene. Each regression consisted of at least 60 points and had an $R^2$>0.88. The efficiency (E) of each qPCR reaction was calculated using the slope of these regressions according to equation (1).

$$E = 2^{\frac{-1}{slope}} \quad (4.1)$$

The efficiencies of the reactions used in this study ranged from 1.50 to 1.77, with an efficiency of 2 representing a complete doubling of transcripts between cycles. The efficiency of each reaction was used to calculate the relative expression ratio, which standardized the expression levels of the target gene relative to the reference gene using equation (2) from Pfaffl (2001).

$$R = \frac{(E_{target})^{\Delta Ct target(mean\ control-sample)}}{(E_{ref})^{\Delta Ct ref(mean\ control-sample)}} \quad (4.2)$$

In this equation, ΔCt is calculated as the difference between mean Ct of the control treatment (7.5 ppt) and the other salinities being analyzed. The ratios of all the samples for each treatment (N=8/treatment) were analyzed for outliers within that respective treatment using a Dixon's Q test (Dean and Dixon 1951) at an $\alpha$=0.05. Outlying samples were removed (≤1 sample removed/treatment) and the mean expression ratio was then taken for each replicate tank (N=4/treatment). All subsequent analyses were done with individual tanks as the unit of replication.

All statistical analyses were done using Program R (R Foundation for Statistical Computing, Vienna, Austria). All means are reported with the standard error (SE), unless otherwise noted. The Ct values of EF1α were found to differ between sampling times (NKA paired samples: ANOVA, df=100, F=19.55, $P$<0.001; CA paired samples: ANOVA, df=92, F=26.02, $P$<0.001) but not between salinities (NKA paired samples: ANOVA, df=100, F=1.399, $P$=0.24; CA paired samples: ANOVA, df=92, F=0.449, $P$=0.504). For this reason, relative expression ratios were compared between treatment salinities within the same time-point. All data were checked for normality and

homogeneity of variance using Shapiro-Wilk and Bartlett's tests, respectively. If data met these assumptions, a one-way analysis of variance (ANOVA) was run along with a Fisher's Least Significant Difference (LSD) *post-hoc* test. If the assumptions of parametric analysis were not met, a non-parametric Kruskal-Wallis rank sum test within the R-package "agricolae" (Mendiburu 2013) was used to compare differences between treatments, and contained a built in *post-hoc* test.

## Results

There were differences in the relative expression of CA2 between salinities at 2 dpt (ANOVA, N=4, df=14, F=18.61, $P<0.001$; Figure 4.1). At this sampling point, the 0 ppt treatment had higher CA2 expression than other salinity treatments. There were no differences between salinities at either the 14 or 28 dpt sampling point (14 dpt: ANOVA, N=4, df=14, F=0.194, $P=0.666$; 28 dpt: Kruskal-Wallis rank sum test, N=4, df=3, $\chi^2=2.49$ $P=0.477$).

There were differences in relative NKA expression between salinities at both 14 and 28 dpt (Figure 4.2). In both cases, expression of NKA in the 0 ppt treatment was higher than in the 5.0 and 7.5 ppt treatments (14 dpt: ANOVA, N=4, df=14, F=10.07, $P=0.007$; 28 dpt: ANOVA, N=4, df=14, F=7.25, $P=0.018$). There were no differences in NKA expression at the 2 dpt sampling point, and overall expression appeared lower at this time point (2 dpt: Kruskal-Wallis rank sum test, N=4, $P=0.123$, df=3, $\chi^2=5.80$, $P=0.123$).

## Discussion

Osmoregulation of euryhaline species in low salinities has not been extensively studied in the *Fundulus* genus, a group with wide osmoregulatory plasticity and the capacity for rapid homeostatic adjustment. Here, two important genes, CA2 and NKA, were examined with specific focus on expression in low salinities in *F. grandis*, a species with hypo-osmotic tolerance from early life history stages (Ramee et al. unpublished, Chapter 2 and 3). In gills of fresh water transferred fish, CA2 response was immediate and short-term, whereas, NKA response was slower and of a longer duration. These data offer specific insight into complementary processes used to cope with freshwater environments over time.

Branchial CA catalyzes the hydration of $CO_2$ resulting in the formation of $H^+$ and $HCO_3^-$, which are in turn exchanged for $Na^+$ and $Cl^-$ from the environment at hypo-osmotic salinities. In this way, CA is integral in both acid-base and ionic regulation and expected to be up-regulated in ion poor environments (Perry and Laurent 1990). The response of CA2 expression to freshwater exposure was a short-term response, increasing 2 dpt to fresh water, but not at any other salinity/time-point combination. These results are similar to those of Scott et al. (2005), who recorded increased CA2 levels in the gills of *F. heteroclitus* 0.5, 3, and 7 dpt to fresh water, but did not sample later time points. Overall, the response of CA to salinity seems to vary with species. For example, CA gene expression of *Danio rerio* was up-regulated 5-6 dpt to ion-poor soft water (Craig et al. 2007). CA expression was up-regulated in the gills of seawater acclimated *Dicentrarchus labrax* compared to freshwater (Boutet et al. 2006) and CA activity increased with salinity in *Oreochromis mossambicus* (Kültz et al. 1992). In *F. heteroclitus* and *Salmo*

*salar*, histochemistry has shown CA staining to be stronger in the chloride cells of fish exposed to saltwater than freshwater (Dimberg et al. 1981; Flügel et al. 1991). Similar levels of CA were seen in osmoregulatory cells of the gills of *Platichthys flesus* acclimated to salt or freshwater (Sender et al. 1999), as would be expected, given the short-term reaction profile seen in this study. Long-term, species-specific CA levels may be more dependent on acid-base regulation, as suggested by Conley and Mallatt (1988), but CA appears to offer a short-term mechanism for osmoregulatory acclimation in *F. grandis*. Although not examined in this study, it is possible that the temporary CA2 increase may be related to changes in gill permeability with freshwater acclimation. Gill permeability is reduced by prolactin after freshwater transfer (Manzon 2002). Initially permeable gills may result in high rates of net water influx in fresh water, which is countered by increased rates of ventilation (Kidder et al. 2006b). This provides increased $CO_2$ as a product of respiration, which is converted by CA to $HCO_3^-$ and $H^+$ for $HCO_3^-/Cl^-$ and $Na^+/H^+$ exchange facilitating $Na^+$ and $Cl^-$ uptake to account for losses (Evans et al. 2005).

NKA is one of the most important and well-studied osmoregulatory enzymes. It is responsible for facilitating the active transport of $Na^+$ from the chloride cell cytoplasm into the blood in exchange for $K^+$, facilitating ion gradients necessary for osmoregulation (Skou and Esmann 1992; Marshall 2002; Evans et al. 2005; McCormick et al. 2013). In fresh water, this basolateral pumping of $Na^+$ into the blood, by NKA, creates an electrochemical gradient in the chloride cell that allows for $Na^+$ uptake on the apical surface of the cell though $Na^+$ channels (i.e., $Na^+/H^+$ exchangers (NHE) and epithelial sodium channel (ENaC)) (Marshall 2002; Hirose et al. 2003). In this study, a delayed

post-salinity transfer up-regulation of NKA occurred, in contrast to the immediate and short-term compensation observed for CA2. Delayed up-regulation following transfer to fresh water was also observed previously in *F. grandis* (Patterson et al. 2012) and *F. heteroclitus* (Scott et al. 2004).

In some species, up-regulated NKA may be a more permanent adaptation to the surrounding environment. In the euryhaline sea bass *Dicentrarchus labrax*, NKA was up-regulated in the gills of both freshwater acclimated (6 month acclimation time) and lagoon sampled fish compared to saltwater and open ocean fish, respectively (Boutet et al. 2006). In studies on *Fundulus*, NKA gene expression was initially up-regulated at 5-7 dpt. In the present study, NKA remained up-regulated in fresh water through the final sampling point at 28 dpt. In contrast, Patterson et al. (2012) observed NKA expression levels return to baseline in *F. grandis* at 21 dpt. Similarly, in *F. heteroclitus* Scott et al. (2004) recorded a return to baseline in NKA expression levels by 30 dpt, and Katoh et al. (2001) found no difference in NKA activity between the gills of fresh water and salt water adapted fish after ≥1 month acclimation time. Therefore NKA up-regulation of the younger juvenile fish in the current study lasted longer compared to past studies, suggesting a slower acclimation to freshwater at this age. The NKA gene expression data from this study are supported by the NKA enzyme activity data with similar increases at 14 and 28 dpt in the fresh water treatment (Chapter 2). Together these data reinforce the delayed and extended up-regulation of NKA in response to fresh water conditions. Interestingly, this pattern contrasts the earlier and shorter duration spike in NKA that occurs after brackish water adapted *F. heteroclitus* are transferred to seawater (Mancera

and McCormick 2000; Scott et al. 2004), suggesting that *Fundulus* physiology may be more adept at adapting to salt water.

CA and NKA are both directly involved in $Na^+$ uptake in the chloride cell along with apical proteins such as NHE, ENaC, and $H^+$-ATPase (Hirose et al. 2003; Scott et al. 2005; Hwang et al. 2011; McCormick et al. 2013). In this model, the uptake of $Na^+$ in exchange for $H^+$ by NHE or the ENaC/$H^+$-ATPase complex on the apical surface of the chloride cell is directly mediated by the supply of $H^+$ from CA and the $Na^+$ gradient mediated by NKA pumping $Na^+$ into the blood on the basal surface of the chloride cell. Though NHE expression was not measured in this study, Scott et al. (2005) recorded an up-regulation of NHE2 in *F. heteroclitus* 12 hours post-transfer from brackish to fresh water. The results of the current study suggest CA2 may be most important initially and later NKA as the driver of $Na^+$ uptake. Future research should focus on the freshwater responses of genes of other ion transporting proteins such as $Na^+/H^+$ exchangers and $Na^+/K^+/2Cl^-$ cotransporters in order to better understand the mechanisms used in adaptation to freshwater.

Table 4.1 Primer sequences (5'-3') used in the qPCR analysis.

| Gene | Sequence (5'-3') | Tm (°C) |
|---|---|---|
| CA2 | F: CTGATGGGCTCGCTGTTGT | 62.3 |
| | R: GAAGTTGTTCACCATGCAGCA | 60.6 |
| NKA | F: ACTGCCAAGGCCATTGCTAA | 60.4 |
| | R: AACGACGCAAGCTTTGGCAT | 60.4 |
| EF1α | F: GAAGCCGCTGAGATGGGAAA | 62.4 |
| | R: ACAGAGCGATGTCGATGGTG | 62.4 |

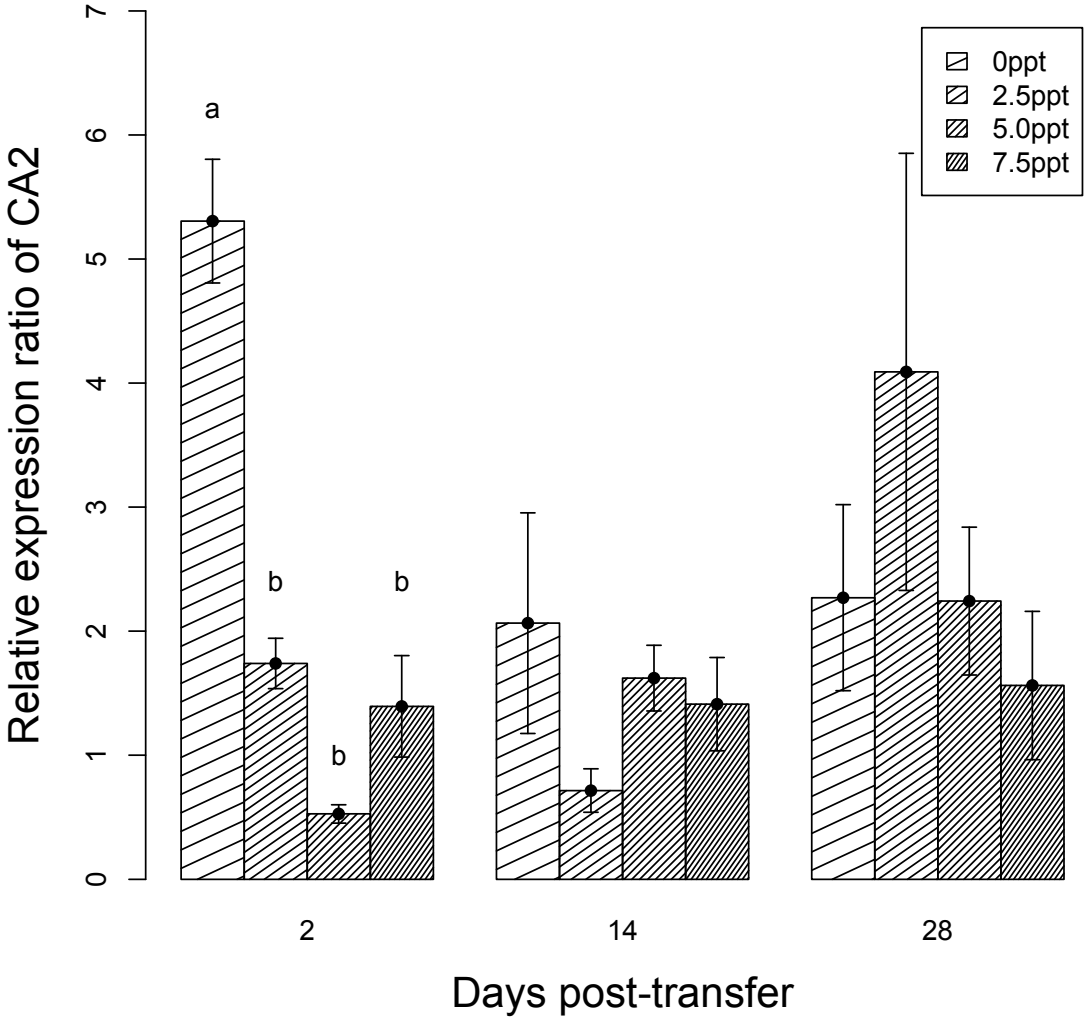


Figure 4.1 Mean (±SE) expression ratios of carbonic anhydrase 2 (CA2) relative to elongation factor 1α (EF1α).

Sampled at 4 different salinities and three different sampling points. Different letters above bars represent significant differences ($P$<0.05) between salinities within a sampling time point (2 dpt: ANOVA, N=4; 14 dpt: ANOVA, N=4; 28 dpt: Kruskal-Wallis rank sum test, N=4).

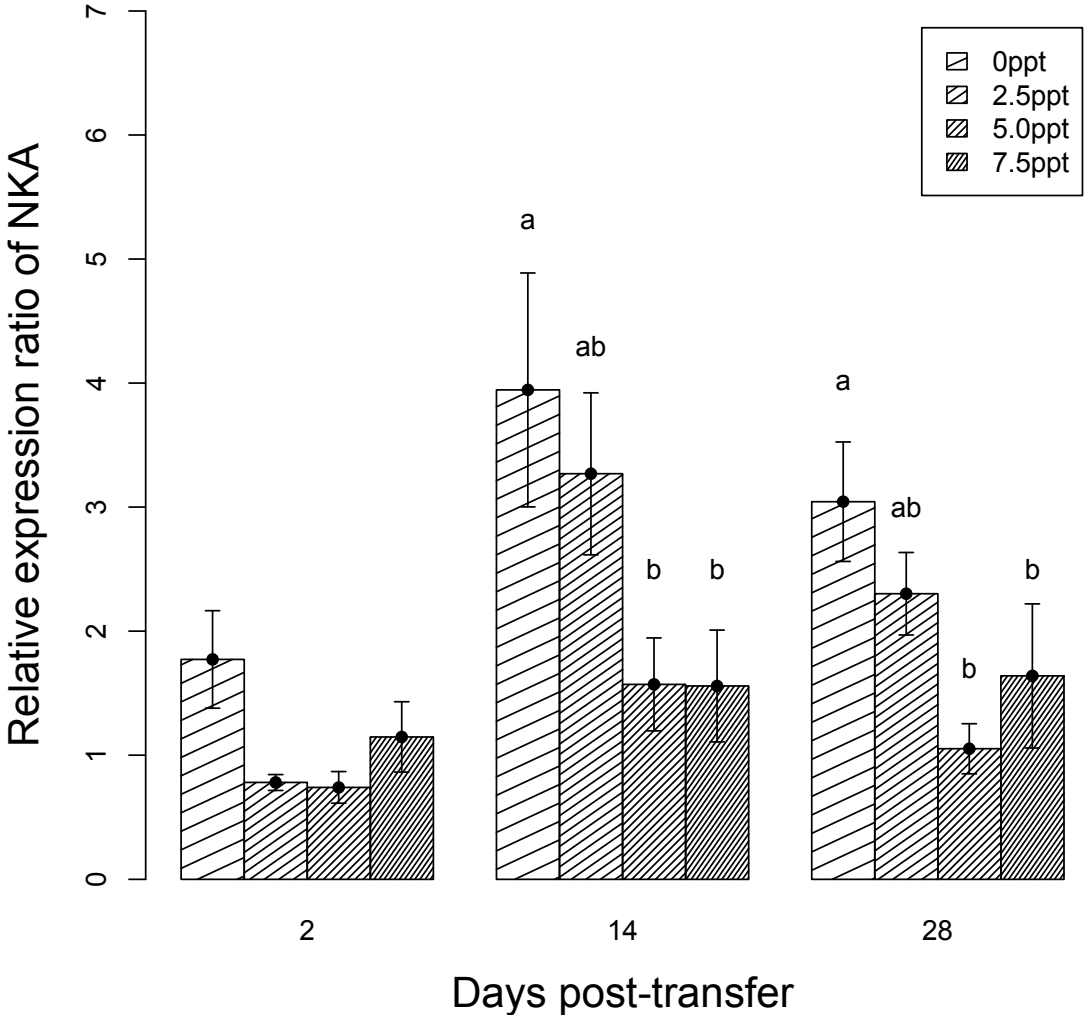


Figure 4.2 Mean (±SE) expression ratios of $Na^+/K^+$-ATPase (NKA) relative to elongation factor 1α (EF1α).

Sampled at 4 different salinities and three different sampling points. Different letters above bars represent significant differences ($P<0.05$) between salinities within a sampling time point (2 dpt: Kruskal-Wallis rank sum test, N=4; 14 dpt: ANOVA, N=4; 28 dpt: ANOVA, N=4).

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# CHAPTER V

# SUMMARY AND CONCLUSION

Gulf killifish *Fundulus grandis* are a highly euryhaline species and able to withstand freshwater at very early ages. Similarly to many other euryhaline species, their freshwater tolerance increases with age (Varsamos et al. 2005). This has important implications on the design of inland production protocols, particularly for minimizing the use of expensive artificial sea salt.

Embryos had the lowest survival in freshwater (mean±SE = 39.7±4.6%). Similarly, Brown et al. (2012) recorded highest embryo mortality at 0.4 ppt. Relative hatch percentage (adjusted for embryo survival) did not differ between embryos incubated and hatched in fresh water (81.6±4.7%) and incubated and hatched in saline water (7.0 ppt: 72.3±8.3%). Post-incubation survival of 15 dph larvae was the same in fresh water (74.5±12.4%) and saline water (76.0±8.0%) after embryos were incubated in fresh water. After incubation in saline water, freshwater larval survival (50.0±9.7%) was lower than saline water (78.9±6.2%). Ability to survive abrupt transfer from saline water (7.5 ppt) to freshwater improves by 2 weeks post-hatch, with a 2-6 week post-hatch survival of 76.3±10.0% in fresh water. By 7 weeks post-hatch, freshwater survival following abrupt transfer from saline water (7.5 ppt) increased to 91.9±3.3%. Freshwater survival remained about the same in the 12-16 week post-hatch age group (91.7±0.7%).

Based on the survival data, *F. grandis* appear to achieve freshwater tolerance by 7 weeks post-hatch.

The physiological data collected during the 7-11 week post-hatch age group helps elucidate the osmoregulatory processes that occur after freshwater transfer. The 7-11 week post-hatch fish had increased whole body $Na^+$ content 14 dpt and decreased percent water weight 2 and 28 dpt compared to the higher salinity treatments. This indicates that *F. grandis* are more than capable of regulating internal ionic content in ion poor environments, and may even be overcompensating during post-transfer freshwater acclimation. One cause may be increased NKA activity, with increases in both NKA enzyme activity levels and relative mRNA expression levels in fresh water 14 and 28 dpt compared to fish at 7.5 ppt. This suggests that there may be greater energetic costs for *F. grandis* to osmoregulate in fresh water at this developmental stage. Yet, growth indices were not decreased in fresh water in the 7-11 week post-hatch age group.

These findings have important implications for the implementation of inland production protocols. This study demonstrated that embryos can be successfully harvested from freshwater held broodstock, though the resulting fertilization rate (20.21±0.03%) was lower than fertilization rates found in other studies that examined spawning in higher salinities (Green et al. 2010; Gothreaux and Green 2012). Further research needs to address whether there are effects of spawning salinity on resulting freshwater tolerance in embryos and larvae. Terrestrial incubation of embryos was more successful with saline moisture. Future research should explore the use of anti-water mold treatments to determine the extent that freshwater embryo mortalities are caused by mold growth or osmoregulation challenges. At this point, it would be prudent for

producers to utilize saline conditions for incubation, hatching, and larval growth, since this method resulted in the highest overall survival from embryo collection to 15 dph (68.8±5.7%). This is still a practical stage for saline water to be used in an hatchery setting, since relatively low volumes of saline water are required. If a saline hatchery system is used to produce *F. grandis* fry, the fry can then be stocked into freshwater ponds or pools ≥7 weeks post-hatch. At this age, mean±SE weight of fish is 0.282±0.006 g, which are easier to work with than smaller sizes. To expand on the encouraging results of the juvenile freshwater growth and survival, pond-scale research needs to be done in freshwater utilizing fish ≥7 weeks post-hatch. In addition, future studies should investigate the impacts of trace ion concentrations and water hardness on the survival and growth of *F. grandis*.

Overall, the ontogeny of *F. grandis* hypo-osmotic tolerance improves with age and life stage, although notably some level of survival and growth exists in fresh water at every life-stage. These results are encouraging for those interested in inland low-salinity culture methods, although more research needs to be conducted before these methods are utilized commercially.

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