Comparisons Among Channel Catfish *Ictalurus punctatus*, Blue Catfish *I. furcatus*, Channel ♀ × Blue ♂ Hybrid Catfish and Transgenic Channel Catfish for Growth, Cold and Salinity Tolerance

by

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Abstract

Two studies were performed related to salinity tolerance and the potential effect of climate change on genetic choices for catfish farming. The first study examined the growth rate and survival rate among channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*), and channel catfish, *I. punctatus*, ♀ × blue catfish, *I. furcatus*, ♂ hybrid catfish at early life stages at 0, 3.0, 6.0, and 9.0 parts per thousand (ppt) salinity. No yolk sac larvae or swim-up fry survived for channel catfish, blue catfish, and hybrid catfish at 9.0 ppt for either stage of development. Channel catfish were more resistant to salinity than hybrid catfish and blue catfish. The resistance of (C×B) hybrid catfish to salinity was intermediate to that of channel catfish and that of blue catfish. NaCl had a negative effect on survival rate of yolk-sac larvae beginning at 3 ppt and for swim-up fry at 6 ppt. Genotype × environment interactions occurred for growth as hybrids grew faster than channel catfish and blue catfish at 0 ppt. Raising salinity to 3 ppt greatly increased the growth rate (50-75%) of channel catfish and blue catfish, but only slightly (10%) for hybrid catfish, which were still larger than the parent species. These results indicate the types of saline environments that might be used long-term for growth of juvenile ictalurid catfish, and the salinities that could be used for long-term disease treatment. This information could be increasingly important for aquaculture planning in the event of global climate change.

The second study examined the survival of fingerlings of channel catfish, channel ♀ × blue ♂ hybrid catfish, channel catfish transgenic for the goldfish *Carassius auratus* glutamate decarboxylase 65 gene driven by the carp β-actin promoter (βA-GAD65) and channel catfish transgenic for the catfish growth hormone gene driven by the antifreeze protein promoter from an ocean pout *Zoarces americanus* (AFP-ccGH) at different temperatures (9.0 °C, 6.0 °C, 3.0 °C, 1.0 °C, 0.5 °C, 0 °C, and −0.5 °C) and different salinities (0 ppt, 2.5 ppt, 5 ppt, and 7.5 ppt). Survival was 98–100% for all genetic groups at all salinities between 0 °C and 9.0 °C. However, large differences were observed at −0.5 °C. At 0 ppt salinity, 100% of AFP-ccGH transgenic (T) fingerlings survived ($P < 0.0001$), but survival of all other genetic groups was 0–2%. Raising salinity to 2.5 ppt at sub-zero temperature had a strong positive impact on survival as survival rates of AFP-ccGH (T), AFP-ccGH control (C), channel catfish, βA-GAD65 (T), βA-GAD65 (C) and hybrid catfish were 100, 100, 98, 76, 100 and 18%, respectively with the hybrid having the lowest survival followed by βA-GAD65 (T) ($P < 0.0001$). Increasing salinity further to 5 ppt decreased overall survival, although it was still higher than at 0 ppt. Survival rankings were altered, as means for βA-GAD65 (T), βA-GAD65 (C), AFP-ccGH (T), AFP-ccGH (C), channel catfish and hybrid catfish were 69, 0, 15, 22, 0 and 0%, respectively with βA-GAD65 (T) having the highest survival ($P < 0.05$). Mortality was 100% in all genetic groups at −0.5 °C and 7.5 ppt demonstrating significant interaction between temperature and salinity.

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List of Abbreviations

WWF	World Wildlife Fund
UNFPA	United Nations Population Fund
EPA	United States Environmental Protection Agency
FAO	Food and Agriculture Organization of the United Nations
NFI	National Fisheries Institute
USAD	United States Department of Agriculture
C × B	Channel Catfish, Female × Blue Catfish, Male
LHRHa	Luteinizing Hormone Releasing Hormone analogue
AFP	Antifreeze Protein
GH	Growth Hormone
CC	Channel Catfish
GAD	Goldfish Glutamate Decarboxylase

CHAPTER ONE

Introduction

Water is the most critical issue for the survival of all living organisms. Only 3% of the world's water is freshwater (WWF, 2016). Increasing pressures on freshwater supplies, agricultural land, aquaculture, industry, and human activity present unprecedented challenges (UNFPA, 2016). Global climate change is altering patterns of water around the world, causing shortage of freshwater and freshwater will be more brackish (EPA 2015, 2016). The amount of water available in many areas is already limited, and demand of freshwater will continue to increase as population grows (EPA, 2016). By 2025, two-thirds of the world's population may face shortage of freshwater (WWF, 2016) and the global population is expected to reach 9.6 billion (FAO, 2016a).

Aquaculture is one of the fastest growing animal food producing industry in the world (FAO, 2016b). Fish are an important source of animal protein and nutrition for people worldwide (FAO, 2014). Catfish is the most important species of aquatic animal commercially cultured in the United States, consecutively ranked from 2006 to 2014 by the National Fisheries Institute (NFI) as one of the top 10 most consumed seafoods in the United States (NFI, 2014). Annual catfish production in the United States has been relatively unstable in recent years peaking at 300 million kg of processed catfish in 2003, declining to 138 million kg in 2011 (Hanson and Sites, 2012) and 150 million kg in 2013, a 50% decrease since the 2003 peak (Hanson and

Sites, 2014). The majority of catfish production occurs in the states of Mississippi, Alabama, Arkansas, and Texas (USAD, 2016).

The hybrid resulting from the mating of ♀ channel catfish, *Ictalurus punctatus*, × ♂ blue catfish, *I. furcatus*, is a superior fish for culture, especially for pond culture ((Dunham et al., 2001). This hybrid is improved growth (Dunham et al., 1987, 1990; Dunham and Brummett, 1999; Jeppsen, 1995; Li et al., 2004), disease resistance (Dunham and Brummett, 1999; Dunham et al., 1990; Jeppsen, 1995; Wolters et al., 1996), tolerance to low dissolved oxygen (Dunham and Smitherman, 1983; Dunham et al., 2014), harvest by seining (Chappell, 1979; Dunham and Argue, 1998), dress out percentage and fillet percentage (Chatakondi et al., 2000), has more uniform growth and body shape (Dunham et al., 1982) compared to the commonly grown channel catfish. However, it has negative heterosis under sub-zero temperature (Abass et al., 2016). The channel catfish ♀ × blue catfish ♂ grows faster than the reciprocal hybrid (blue catfish ♀ × channel catfish ♂) (Dunham et al., 1982). The channel catfish ♀ × blue catfish ♂hybrid catfish now constitutes 50–75% of total production of catfish in the U.S.

The deficiency of freshwater resource in numerous countries leads to a shift to attempt to grow freshwater fishes in brackishwater and seawater (El-Sayed, 2006; Abass at al., 2017). Most researchers agree that salinities, which differ from the internal osmotic concentration of the fish must impose energetic regulatory costs for active ion transport (Swanson, 1998). There is less agreement concerning the

magnitude of these costs (Farmer and Beamish, 1969; Rao, 1971; Potts et al., 1973; Furspan et al., 1984; Febry and Lutz, 1987; Morgan and Iwama, 1991; Nordlie et al., 1991).

Catfish species can withstand higher salinities than most freshwater fishes, including the brook stickleback *Culaea inconstans*, a member of the Gasterosteidae family, which is largely a family of brackish water species (Armitage and Olund, 1962). Kendall and Schwartz (1968) suggested that catfish are able to tolerate greater osmotic stress because their integument is less permeable than the scaled surfaces of most freshwater teleosts.

Effects of environmental salinity on fishes typically found in freshwater are of interest for aquaculture in recirculating systems, where salinity can be adjusted to optimal levels, and in regions where brackish water is available (Forsberg and Neill, 1997). This topic is also important because of the potential exposure of wild fishes to altered salinity resulting from environmental perturbations (Winger and Lasier, 1994).

Exposure to high concentrations of NaCl have been reported to cause physiological problems in fish such as increase in plasma cortisol (Mommsen et al., 1999; Tsuzuki et al., 2001), blood glucose (Carmichael et al., 1983), plasma sodium and chloride (Tomasso et al., 1980) or increased blood ammonia (Tomasso, 1994) due to higher physiological activity to eliminate excess ions. Yolk-sac and swim-up fry may not as yet have effective mechanisms to eliminate excess Na^+ and Cl^- from their

body systems and therefore may be vulnerable to long term exposure to high NaCl concentrations (Magondu et al., 2011).

Fish are subject to stress every day and NaCl is recommended for reducing stress (Velasco-Santamaria and Cruz-Casallas, 2008; Koeypudsa and Kitkamthorn, 2009). Anti-stress compounds accompanied with salt maintains stable blood electrolyte levels by reducing the osmoregulation and decreasing ionic imbalances (Tsuzuki et al., 2001; Harpaz et al., 2005). Andrews et al. (2002) reported that application salt at the level of up to 0.3% could be used to help reduce stress associated with physical damage and high nitrite level. The use of salt in water could reduce the plasma-water gradient and consequently the loss of ions from fish into the environment and increase mucus production (Murphy and Houston, 1974; Wurts, 1995; Gomes et al., 2006; Trumble et al., 2006; Souza-Bastos and Freire, 2009; Yu et al., 2016).

One of the physiological functions clearly influenced by water salinity in fish is growth (Boeuf and Payan, 2001; Engström-Öst et al., 2005). Thus, larval culture at low salinities produces higher growth and survival rates than in freshwater conditions in some freshwater species (Britz and Hecht, 1989). The effects of salinity on growth are complex, vary among species and are not readily predicted (Iwama, 1996). While it is widely accepted that rearing of fish near their iso-osmotic point has an energy saving effect (Gaumet et al. 1995; Boeuf and Payan 2001), few studies have addressed the effects of increased salinity on growth in true freshwater species. In marine fish,

decreasing the salinity towards iso-osmolality often increases growth, which is commonly explained by a reduction in energy expenditure associated with ion regulation (Brett 1979; Jobling 1994). Isosmotic water decreases use of energy for osmoregulation compared to fresh water and salt water. This saved energy is used for growth. Energy expenditures for osmoregulation are supposed to be zero in isosmotic environments (Küçük et al., 2013).

Among the various physical factors affecting the aquatic environment, temperature is one of essential importance and is considered as the 'abiotic master factor' for fishes (Brett, 1979). Low winter temperature can limit the geographical distribution of fish, and mass mortality occurs in several species when exposed to cold temperature shock (Eme and Bennett, 2008). As salt and moderate temperatures can be advantageous to fish, cooling in combination with slight raising of the salinity may have a synergistic benefit for aquaculture.

Stress in catfish was reduced by increasing salinity to 0.1% sodium chloride and lowering temperature, which decreased blood clotting time (Koeypudsa and Jongjareanjai, 2011). Fish become lethargic and can be more sensitive to stress when temperature is too low and especially when coupled with inappropriate salinities (Sun et al., 1995; Ross and Ross, 1999; Alcorn et al., 2002). Lower temperature causes decreased metabolic rate (Buentello et al., 2000; Davis, 2004) which decreases the magnitude of stress responses, blood clotting time. The addition of excess salt could result in excessive water loss, dehydration, and increased viscosity

of the blood (Taylor and Miller, 2001). The immobilized state and blood viscosity of catfish under these conditions could increase blood clotting time.

Global climate change, the change of temperature and salinity, and potential erratic and extreme temperature changes, could have significant impact on aquaculture. Some potential examples of the consequences of such changes have been documented. Fish kills in the relatively undisturbed coastal lake system of KwaZulu-Natal show the importance of combinations of low temperature and salinities. A large-scale mortality of fish at St Lucia in Kwazulu-Natal in 1976 was thought to have been due to lethal combination of low salinities and low temperature leading to osmoregulatory failure. In this instance, an estimated 100,000 fish from at least 11 species died. A lethal temperature-salinity combination occurred in Lake St Lucia in 1987, when an unusually cold spell during the winter caused a drop of 12 °C in water temperature. In contrast to the 1976 situation, the low temperature was coupled with not near freshwater salinities, but near marine salinities (35%). This combination resulted in a kill of at least 250,000 fish of 21 species (Cyrus and Maclean, 1996). The maximum temperature tolerance of many estuarine species is at intermediate salinities (Blaber, 2000).

Sodium chloride is inexpensive and readily available in many forms in rural areas making it a useful tool in small scale hatchery operations. Salt can safely use as prophylactic treatments, restore osmoregulation, enhance growth, and increase survival rate under freezing of freshwater fish (Abass et al., 2016, 2017).

The overall objectives of this research were to conduct studies aimed towards improving production of individuals with improved salinity tolerance will contribute substantially to future utilization in brackishwater aquaculture to face the shortage of freshwater for human usage, assuming global climate change becomes increasingly problematic, and examine the survival of channel catfish, channel ♀ × blue ♂ hybrid catfish, and transgenic channel catfish at different temperatures, and different salinities.

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CHAPTER TWO

Genotype-environment interactions for growth and survival of channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*), and channel catfish, *I. punctatus*, ♀ × blue catfish, *I. furcatus*, ♂ hybrid fry at varying levels of sodium chloride

Abstract

Salinity tolerance of yolk-sac larvae and swim-up fry of channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and hybrid catfish (channel catfish ♀ × blue catfish ♂) was investigated at 0, 3, 6, and 9 ppt salinity. One-hundred percent mortality occurred at 9 ppt. Survivals were different ($P < 0.05$) by day 3 post hatch at 3 ppt, ($P < 0.05$) with blue catfish > hybrid catfish > channel catfish for salinity tolerance. Long-term survival of hybrid fry was better than that of the parent species at 0 ppt salinity, but highest for channel catfish at 3 ppt and 6 ppt with the hybrid being intermediate. NaCl had a negative effect on survival rate of yolk sac larvae starting at 3 ppt and for swim-up fry at 6 ppt. Genotype × environment interactions occurred for growth as hybrids grew faster than channel catfish and blue catfish at 0 ppt. Raising salinity to 3 ppt greatly increased the growth rate (50-75%) of channel catfish and blue catfish, but only slightly (10%) for hybrid catfish, which were still larger than the parent species. 6 ppt was detrimental to yolk-sac larvae and swim-up fry. These results help identify the saline environments that might be suitable for long-term growth and

disease resistance of ictalurid catfish, and reveals changes in salinity tolerance during development.

1. Introduction

Catfish is one of the most important aquatic animals commercially cultured in the United States, consecutively ranked from 2006 to 2014 by the National Fisheries Institute (NFI) as one of the top 10 most consumed seafoods in the United States (NFI, 2014). Annual catfish production in the United States peaked at 300 million kg of processed catfish in 2003, declining to 138 million kg in 2011 (Hanson and Sites, 2012) and 150 million kg in 2013, a 50% decrease since the 2003 peak (Hanson and Sites, 2014). The majority of catfish production occurs in the states of Mississippi, Alabama, Arkansas, and Texas (Hanson and Sites, 2014).

The hybrid resulting from the mating of ♀ channel catfish, *Ictalurus punctatus*, × ♂ blue catfish, *I. furcatus*, is the best genotype for pond culture (Dunham et al., 2001). The hybrid exhibited improved growth (Dunham et al., 1990; Dunham and Brummett, 1999; Li et al., 2004), disease resistance (Dunham and Brummett, 1999; Dunham et al., 1990; Wolters et al., 1996), tolerance to low dissolved oxygen (Dunham et al., 1983), harvest by seining (Dunham and Argue, 1998), dress out percentage and fillet percentage (Li et al., 2004), has more uniform growth and body shape (Dunham et al., 1982) compared to the commonly grown channel catfish. However, the hybrid had negative heterosis for survival under sub-zero temperature (Abass et al., 2016). The channel catfish ♀ × blue catfish ♂ hybrid grows faster than the reciprocal hybrid (blue catfish ♀ × channel catfish ♂) (Dunham et al., 1982).

Shortage of freshwater is a global issue due to the competition for water among agriculture and urban activities, and climate change has made water supply unpredictable in many parts of the world (EPA, 2015). Therefore, there is and will be an increasing need for aquaculture in brackishwater and seawater (El-Sayed, 2006). Salt level is one factor that limits the distribution of many fish species and has effects on survival and growth performance of many fish (Kang'ombe and Brown, 2008; Chand et al., 2015).

Disease, including parasite outbreaks, is a prime agent affecting fish mortality, especially in the early development stages of culture. The outbreaks could be prevented using a number of chemicals including salt (NaCl) (Magondu et al., 2011). The ability of freshwater fish to adapt to salinities depends on their ability to maintain body osmoregulation by producing copious urine as well as by active uptake of ions through gills (Sahoo et al., 2003; Kültz, 2015), thus this ability and tolerance of salt is instrumental in designing some treatments for disease and stress reduction.

In the case of salinity and salt tolerance of channel catfish, different studies have yielded different results. This may be due to hatching technique, differences in other water quality parameters that may affect the toxicity of sodium chloride or genetic differences. Su et al. (2013) observed the highest hatch rate for channel catfish at 4 ppt salinity, Weirich and Tiersch (1997) at 1 ppt and Phelps and Walser (1993) at 2.5 ppt.

Phelps and Walser (1993) tested a variety of salinities between 0–5 ppt and found no significant difference between 1–5 ppt, but 2.5 ppt seemed best for hatching. In a second set of experiments they found that somewhere between 0.5 and 2.5 ppt was best. The regression line they drew suggested that perhaps 3 ppt might give the best hatching rate, but they did not test this level of salinity.

The high salt levels had adverse post-hatch effects as sac-fry exposed to 4 ppt either had high mortality (Su et al., 2013) or 100% mortality (Weirich and Tiersch, 1997). These variable results indicate that research needs to be conducted to evaluate the effect of reducing salt concentration shortly before hatch or immediately after hatch so that the positive effects of 4 ppt salinity can be utilized and the post-hatch mortality avoided. If fry and embryos respond to changes in salt levels in the same manner as older channel catfish (Hargreaves and Tomasso, 2004), initiation of acclimation prior to hatch may be beneficial for survival as well.

Perry and Avault (1970) reported that blue catfish tolerate moderately high levels of salinity and can be grown in coastal water which does not exceed 8 ppt salinity for any extended period of time. However, they can tolerate salinity in estuaries up to 11 ppt (Perry, 1968), and in some waters as high as 14 ppt (Allen and Avault, 1970a). In contrast, channel catfish tolerate salinities <13-15 ppt (Allen and Avault, 1970a). Blue catfish appear to be slightly more tolerant to salinity than channel catfish (Allen and Avault, 1971), but both species can survive for several days at 14

ppt, as can hybrids among blue, channel, and white catfish, *Ameiurus catus*, (Stickney and Simco, 1971).

One of the physiological functions influenced by concentration of salinity in fish culture is growth. Growth and survival rates of larval culture were higher at low salinities in comparison to the freshwater conditions in some freshwater species (Britz and Hecht, 1989). The effects of salinity on growth are complex, and vary among species (Iwama, 1996). It is widely accepted that rearing of fish near their iso-osmotic point has an energy saving effect (Gaumet et al., 1995; Boeuf and Payan, 2001). Growth rate increased when, salinity decreased towards iso-osmolality in marine fish, which, again, is commonly explained by a reduction in energy expenditure associated with ion regulation (Jobling, 1994). Theoretically, energy expenditures for osmoregulation will be zero in isosmotic environments (Küçük et al., 2013).

The objective of this study was to compare the growth rate and survival rate among channel catfish, blue catfish, and channel catfish ♀ × blue catfish ♂ hybrid catfish at early life stages and at variable salinity.

2. Materials and Methods

1.1. Production of experimental embryos

Channel catfish and blue catfish broodstock used for this study were from the Catfish Genetics Research Unit, School of Fisheries Aquaculture and Aquatic

Sciences, Auburn University, AL, USA. Female catfish were selected if they had Well-rounded, soft and distended abdomen, while male catfish showed elongated urinogenital papillae. Channel catfish eggs were hand-stripped from females weighed 1.5 to 3 kg that had been ovulated using luteinizing hormone releasing hormone analogue (LHRHa) implants at 85 µg/kg female body weight. The stripped eggs were fertilized artificially with sperm from channel catfish and blue catfish. The male channel and blue catfish weighed 1.5 to 5 kg. Blue catfish males and females were pen spawned.

Three genetic groups were produced and used. Approximately, 20 families of two distantly related strains should have generated blue catfish of high genetic variability for the experiments.

Kansas random channel catfish, the oldest domesticated strain of channel catfish, originated from the Ninnescha River, Kansas in 1911 and were obtained by Auburn University in 1969 from commercial sources in Kansas. They were initially established at Auburn University in 1976 by randomly mating 6 pairs. Since that time, they have been perpetuated with a minimum of 20 spawns per generation. D&B blue catfish are the primary blue catfish strain used in the catfish industry, originating from fish spawned from multiple rivers at the D&B Fish Farm in Texas. They have been randomly spawned at Auburn University for the last 30 years with about 6 pairings per year, but with overlapping generations. Rio Grande blue catfish originated from the Rio Grande River, Texas, are genetically distinct from D&B, were established at

Auburn University 30 years ago from a commercial farm by randomly mating 6 pairs per year in overlapping generations.

The egg masses were incubated in egg hatching baskets in freshwater. Dead eggs were removed daily. The pH ranged from 7.0 to 7.3 and DO from 6.9 to 7.7 mg/L. Water flow through each tank was maintained at 15 L/min to ensure a renewal rate of at least twice per hour.

1.2. Sodium chloride tolerance test on yolk sac larvae (1-4 dph)

Samples of 120 yolk-sac larvae, 1-day post hatch (dph), from each genetic group were obtained randomly after emergence. Ten fish of each genetic group were stocked in triplicate per treatment in circular 3-L plastic tanks. Salinities tested included 0, 3, 6 and 9 ppt. All fish were initially held at 0 ppt, then sodium chloride was added to increase the salinity by 3 ppt / 30 minutes until the final treatment level was reached. Salt rather than seawater or sea salt was used as in the catfish industry, sodium chloride rather than sea salt is added to hatching tanks or ponds to address disease and water quality issues, and ground water with salinity in this region has low levels of salt. Mortality was monitored daily for 4 days (96 h); dead fish were counted and recorded daily.

1.3. Sodium chloride tolerance test for swim-up fry (7 dph)

A total of 120 swim-up fry from each genetic group (7dph) were randomly divided into groups of 30 fry per treatment for each genetic group. Swim-up fry for a single salt treatment were stocked separately into triplicate screened containers, 10 fry

per screened container, (30) fry from each genetic group in a single trough (60 L) of water having salinity of 0, 3, 6, or 9 ppt. All fry were initially held at 0 ppt then sodium chloride was added to increase the salinity by 3 ppt / 30 min until the final treatment level was reached. Everyday, 70–80% of the total water was replaced with the respective concentration of saline water. Mortality was monitored daily for 45 days. Commencing the day after they were introduced into the different sodium chloride treatments, fry were fed Aquamax fry powder twice a day to satiation (Cat#: 000-7684, Purina Mills, St. Louis, MO). Mean body weight and length of fry were recorded at 7 dph, 21 dph, 36 dph, and 51 dph. Dead fish were counted and recorded daily. Fish weight gain, total length gain, and survival of fry were calculated as follows:

Weight gain = final mean weight – initial mean weight.

Total length gain = final mean total length – initial mean total length.

$$\text{Survival}\ (\%) = \frac{(\text{number of fry survived})}{\text{initial number of fry}} \times 100$$

1.4. Water quality

During the experiment, temperature, dissolved oxygen (DO), pH, and ammonia nitrogen of the water were measured daily. Temperature and dissolved oxygen (DO) were measured with an oxygen meter (YSI-Pro20), pH was measured with an API Freshwater pH Test Kit (Mars Fishcare North America, Inc.), and ammonia nitrogen was measured using a fresh water aquaculture test kit (Model AQ-3, Code 3634-03, LaMotte company, USA).

1.5. Statistical analysis

Data analysis of survival, weight gain, and total length gain were analyzed using SAS software (SAS Institute, 2010). Data were presented as mean ± SEM, and subjected to multiway within-subjects MANOVA (repeated measures) to evaluate the combined effects of days, salinity, and genetic type. Due to a strong interaction among the factors (sodium chloride and days), the effect of each factor was tested at a fixed level of the other factor using one-way ANOVA, and significant differences were determined using Duncan's multiple comparison test (Duncan, 1955) at $p < 0.05$. Statistical analyses were conducted using SAS software (SAS Institute, 2010).

3. Results

1.6. Water quality

The range of water quality in the containers holding the yolk-sac larvae was a water temperature from 24.5 ºC to 25.5 ºC, pH from 6.9 to 7.3, DO from 6.9 to 7.7 mg/L, and total ammonia nitrogen (TAN) near 0 mg/L. The average of water quality parameters for swim-up fry of catfish in all concentrations of sodium chloride are presented in Table 1. All parameters such as DO, pH, and TAN were within the acceptable limit for culturing fishes.

Table 1 Water quality variables measured in the rearing tubs of catfish for a period of 45 days of challenge in different concentrations of sodium chloride as compared to optimum and tolerated levels for growth of channel catfish *Ictalurus punctatus*. Values of the water quality represent the mean ±2SEM.

Water quality variable	Sodium chloride			Optimal level*	Tolerated level*
	0 ppt	3 ppt	6 ppt		
Temperature	24.79±0.18	24.79±0.18	24.79±0.18	27-29	0-40
Dissolved oxygen	7.64±0.11	7.57±0.11	7.69±0.12	5-15	2.00
pH	7.04±0.03	7.04±0.03	7.03±0.03	6-9	5-10
TAN	0.05±0.01	0.07±0.01	0.06±0.01	0 as un-ionized ammonia	<0.2

Units: temperature = °C, Dissolved oxygen = mg/L, pH = hydrogen ion concentration, and total ammonia nitrogen (TAN) = mg/L.

* Tucker and Robinson (1990).

1.7. Sodium chloride tolerance of yolk sac larvae (1–4 dph)

Channel catfish, blue catfish, and hybrid catfish were able to absorb their yolk, and had 100% survival for up to 4 days post-hatch in 0 ppt sodium chloride (Figs. 1, 2). At 3 ppt, channel catfish and hybrid catfish started dying on the second day. Survival among the genetic groups was different ($P = 0.01$) by day 3 with 70.0, 96.7 and 83.3% survival for channel catfish, blue catfish and hybrid catfish, and these differences still existed at day 4. By day 2 at 6 ppt, channel catfish (80% survival) and hybrid catfish (70% survival) started dying, and differences ($P < 0.05$) in survival had emerged. On day 3 blue catfish experienced heavy mortality and by day 4, they had experienced 100% mortality, while 60% of channel catfish and 30% of hybrid catfish were still alive. All three genetic types experienced total mortality within 24 hours at 9 ppt. Days, and genetic group all affected survival ($P < 0.0001$). Additionally, days × NaCl, days × genetic group and days × NaCl × genetic group interactions occurred ($P < 0.0001$) (Fig. 2).

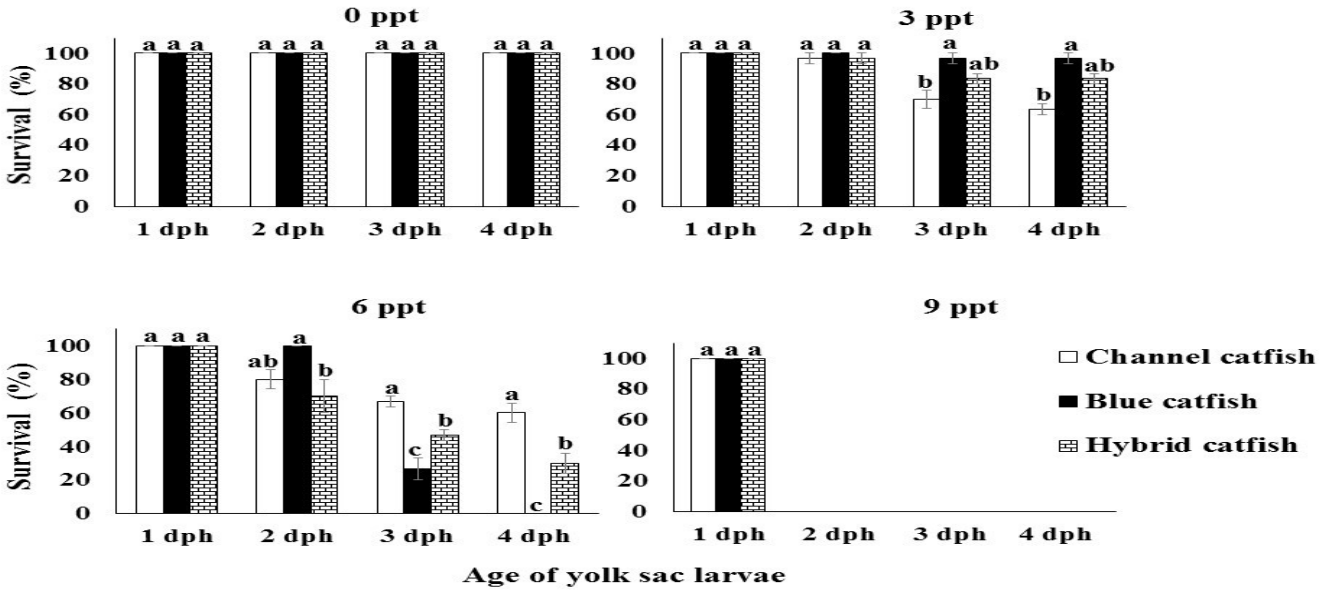

Fig. 1. Mean (±2SEM) percent survival of yolk-sac larvae of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish in different concentrations of NaCl for 4 days (N=3 replicate experimental units). Means that do not differ at the $P = 0.05$ are followed by the same small letter (Duncan's multiple range test) among different genetic groups at each day post-hatch (dph) within each NaCl concentration.

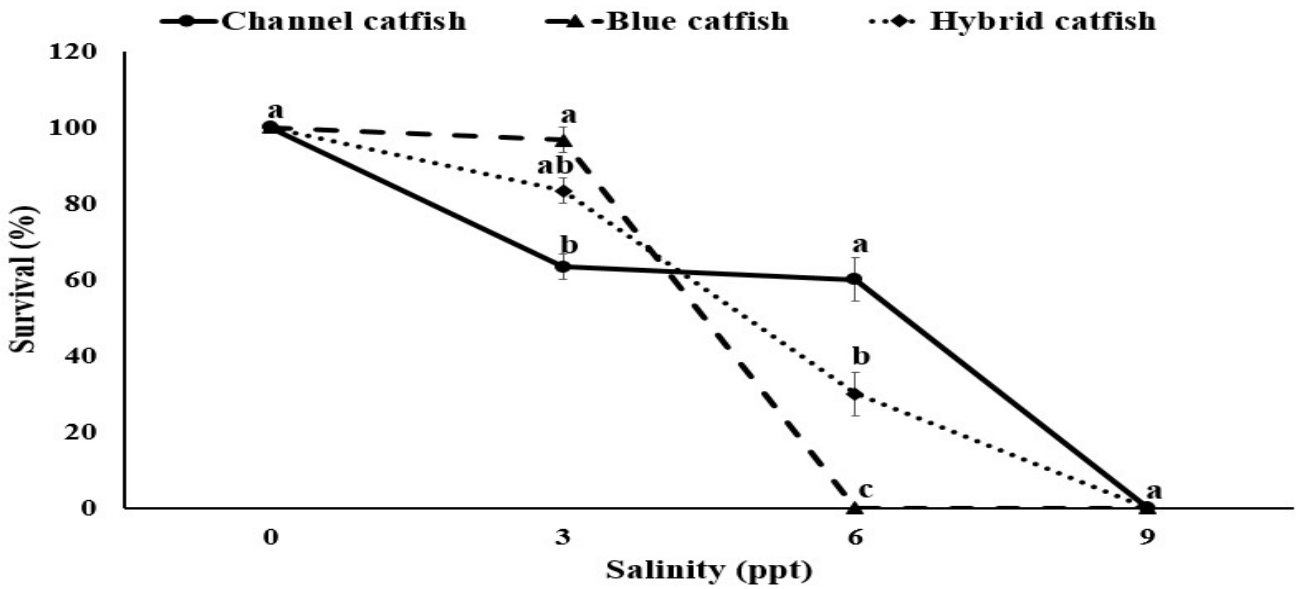

Fig. 2. Mean (±2SEM) percent survival of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish yolk-sac larvae of at different concentrations of NaCl at 4-days post hatch (dph). Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at a fixed level of sodium chloride except differences were at 6 ppt ($^{**}p < 0.01$).

1.8. Sodium chloride tolerance of swim-up fry (7 dph)

Survival of channel catfish, blue catfish, and hybrid catfish fry exposed to varying concentrations of NaCl for 45 days is shown in Fig. 3 and Table 2. At 0 ppt, there was no mortality for the first 30 days. The percent survival, 76.67%, 73.33%, and 80% after 45 days at 0 ppt was not different ($P = 0.9$) for channel catfish, blue catfish, and hybrid catfish, respectively. At 3 ppt, survival was not different, 93.33%, 83.33%, and 86.67%, for channel catfish, blue catfish, and hybrid catfish, respectively ($P = 0.5$). While at 6 ppt survival was different, 53.33%, 0%, and 10% after 45 days for channel catfish, blue catfish, and hybrid catfish, respectively ($P = 0.0001$) (Table 2; Figs. 3 and 4). Blue catfish suffered 100% mortality by the end of day 9 in 6 ppt and all genetic groups had suffered complete mortality by the end of day 1 in 9 ppt (Fig. 3).

Growth was also affected with increase of sodium chloride for ictlaurid catfish. There were significant differences in final body weights among genetic groups at 0 ppt ($P = 0.005$) and 6 ppt ($P = 0.004$) (Table 3A; Figs. 5 and 6), but no significant differences in final body weights were observed among genetic groups at 3 ppt ($P = 0.12$) (Table 3A; Fig. 7). Weight gain at 21 dph was different at 3 ppt among groups ($P = 0.045$) (Fig. 7). No significant differences in final body lengths were observed among genetic groups at different levels of sodium chloride ($P > 0.05$) (Table 3B; Figs. 5-7) at 21 dph, 36 dph, and 51 dph (end of the experiment). Days, sodium chloride level, and genetic group all affected survival ($P < 0.0001$).

Table 2 MANOVA (repeated measures) showing the effect of days, genetic groups and their interaction on survival rate of different genetic groups (channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and hybrid catfish = channel catfish female × blue catfish male hybrid catfish at different test levels of sodium chloride for 45 days. Data are mean ± 2SEM. Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at a fixed level of sodium chloride.

Sodium chloride	Source of variation	DF	MS	*F*	Mean survival ± 2SEM		
					Channel	Blue	Hybrid
0 ppt	Days	44	4.47	12.15**	76.67±14.53[a]	73.33±6.67[a]	80±11.55[a]
	Genetic group* Days	88	0.11	0.30NS			
	Error (Days)	264	0.37				
3 ppt	Days	44	1.48	8.06**	90.33±3.33[a]	83.33±8.81[a]	86.67±3.33[a]
	Genetic group* Days	88	0.16	0.86 NS			
	Error (Days)	264	0.18				
6 ppt	Days	44	40.94	86.00**	53.33±3.33[a]	0.00±0.00[b]	10.00±5.77[b]
	Genetic group* Days	88	7.30	15.34**			
	Error (Days)	264	0.48				

Data were arcsine-transformed to meet ANOVA assumptions.

**$p < 0.001$, and NS: not significant.

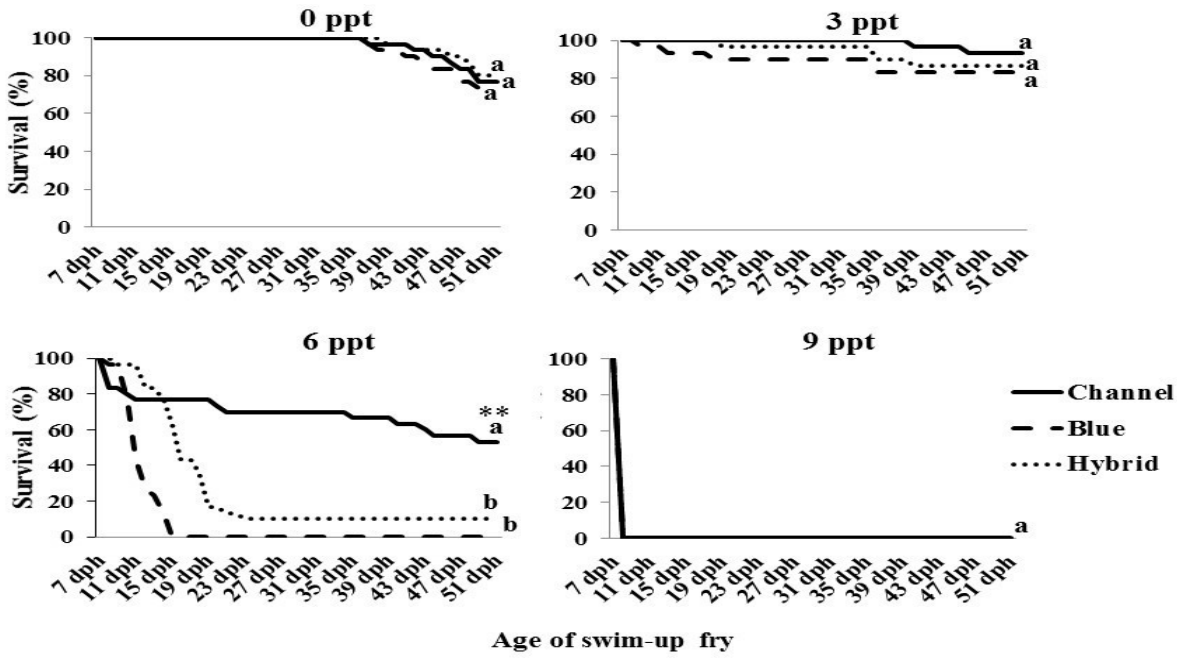

Fig. 3. Mean percent survival of swim-up fry of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish in different concentrations of NaCl for 45 days. (N = 3 replicates per experimental unit). Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at this point except at 6 ppt ($^{**}p < 0.01$).

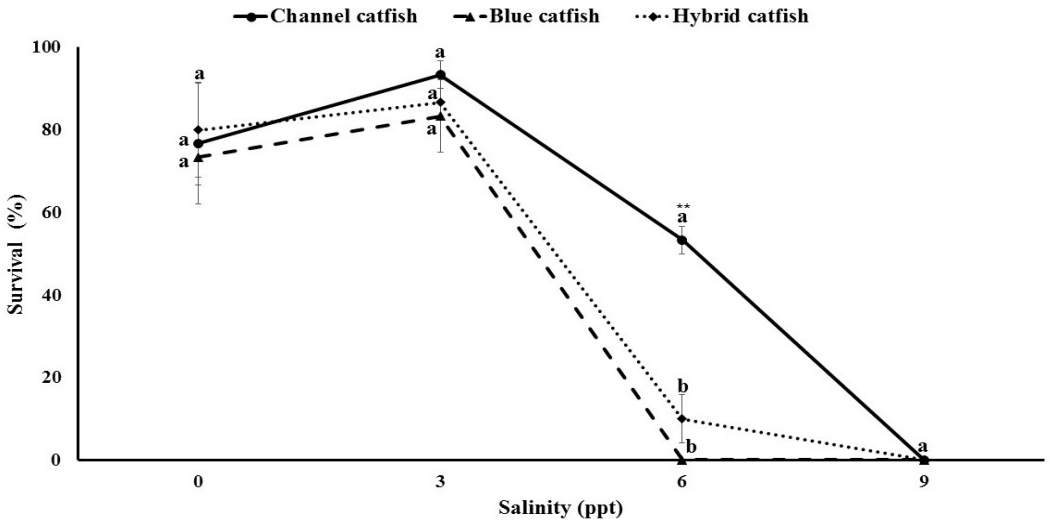

Fig. 4. Mean (±2SEM) percent survival of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish swim-up fry at different concentrations of NaCl at 51-day post hatch (dph). Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at fixed level of NaCl except at 6 ppt ($^{**}p < 0.01$).

Additionally, days × sodium chloride level, days × genetic group and days × sodium chloride level × genetic group interactions occurred ($P < 0.0001$) (Fig. 4).

At the end of the experiment, the channel, blue, and hybrid catfish raised in freshwater (0 ppt) had gained 0.17±0.02, 0.21±0.01, and 0.33±0.02 g body weight respectively, and at 3 ppt, 0.31±0.01, 0.32±0.02 and 0.36±0.04 g body weight, respectively.

Table 3 MANOVA (repeated measures) showing the effect of days, genetic group and their interaction on final body weight gain (g) or loss (A) and length gain (cm) (B) of different genetic groups (channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish swim-up fry at 7-days post-hatch exposed to different test levels of sodium chloride for 45 days. Data are mean ± 2SEM (N=3 replicate experimental units). Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at a fixed level of sodium chloride.

A)

Sodium chloride	Source of variation	DF	MS	F	Mean body length (cm) ± 2SEM		
					Channel	Blue	Hybrid
0 ppt	Days	2	2.10	130.30^{**}	1.80 ± 0.06^{b}	2.13 ± 0.09^{a}	1.97 ± 0.09^{ab}
	Genetic group* Days	4	0.07	4.16^{NS}			
	Error (Days)	12	0.02				
3 ppt	Days	2	5.12	99.48^{**}	2.37 ± 0.12^{a}	2.33 ± 0.07^{a}	2.63 ± 0.20^{a}
	Genetic group* Days	4	0.04	0.71^{NS}			
	Error (Days)	12	0.05				
6 ppt	Days	2	1.53	515.85^{**}	1.53 ± 0.03^{a}	-	1.65 ± 0.05^{a}
	Genetic group* Days	2	0.01	4.65^{NS}			
	Error (Days)	6	0.00				

B)

Sodium chloride	Source of variation	DF	MS	*F*	Mean Body weight (g) ± 2SEM		
					Channel	Blue	Hybrid
0 ppt	Days	2	0.07	193.76**	0.17±0.02[b]	0.21±0.01[b]	0.33±0.02[a]
	Genetic group* Days	4	0.00	14.51**			
	Error (Days)	12	0.33				
3 ppt	Days	2	0.13	100.18**	0.31±0.01[a]	0.32±0.02[a]	0.36±0.04[a]
	Genetic group* Days	4	0.00	0.58[NS]			
	Error (Days)	12	0.001				
6 ppt	Days	2	0.00	17.17**	0.18±0.03[a]	-	- 0.01±0.00[b]
	Genetic groups* Days	2	0.00	10.58**			
	Error (Days)	6	0.00				

Data were arcsine-transformed to meet ANOVA assumptions.

** p = or < 0.001, and NS= not significant.

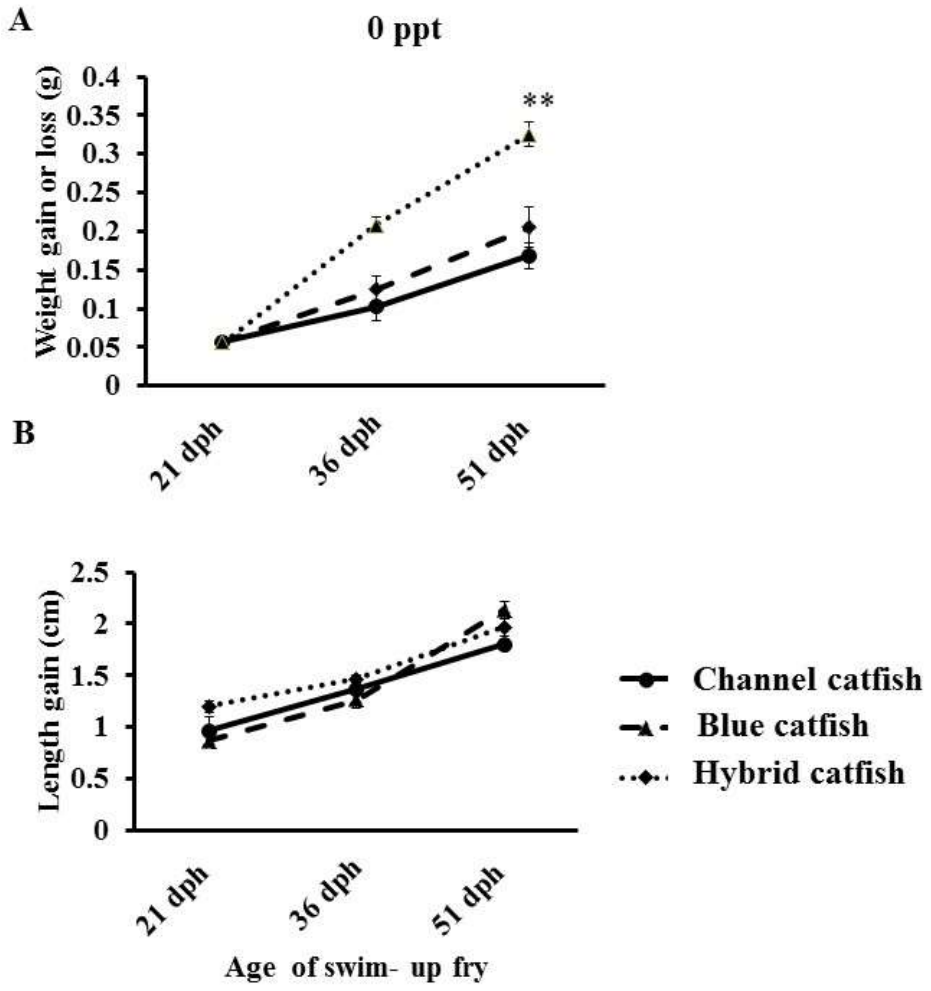

Fig. 5. Change in body weight gain or loss (g, graph A) and length gain (cm, graph B) of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish swim-up fry from 21 to 51 days post-hatch (dph) in 0 ppt sodium chloride. Data are mean ± 2SEM. ** denotes statistically significant differences between groups at a time point (MANOVA (repeated measures), $^{**}P < 0.01$).

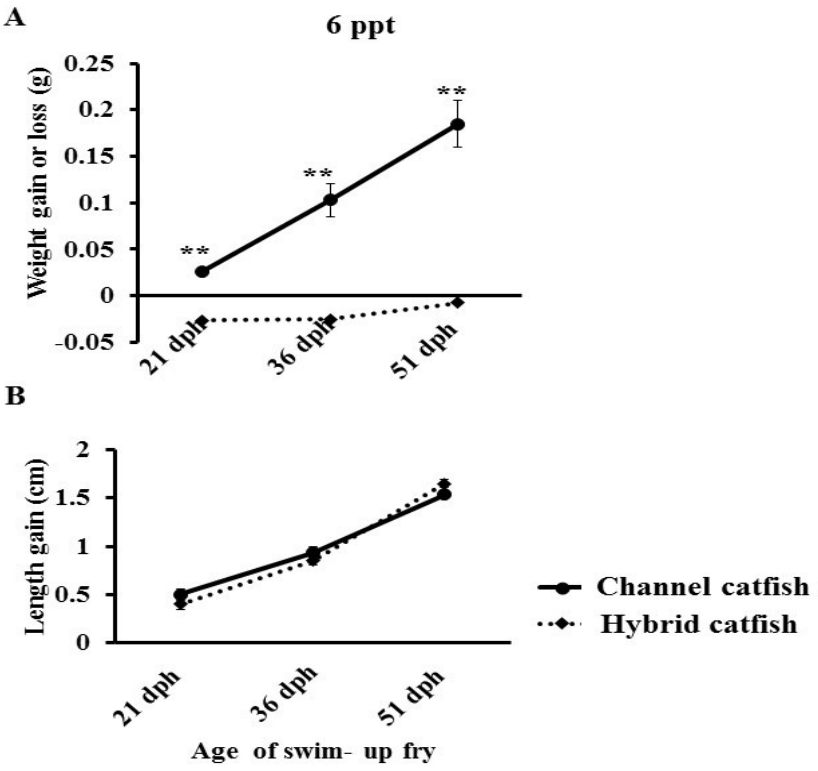

Fig. 6. Change in body weight gain or loss (g, graph A) and length gain (cm, graph B) of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish swim-up fry from 21 to 51 days post-hatch (dph) at 6 ppt sodium chloride. Data are mean ± 2SEM. ** denotes statistically significant differences between groups at this time point (MANOVA (repeated measures); $^{**}P < 0.01$). Blue catfish experienced 100% mortality after 9 days in 6 ppt sodium chloride, thus no growth data is available.

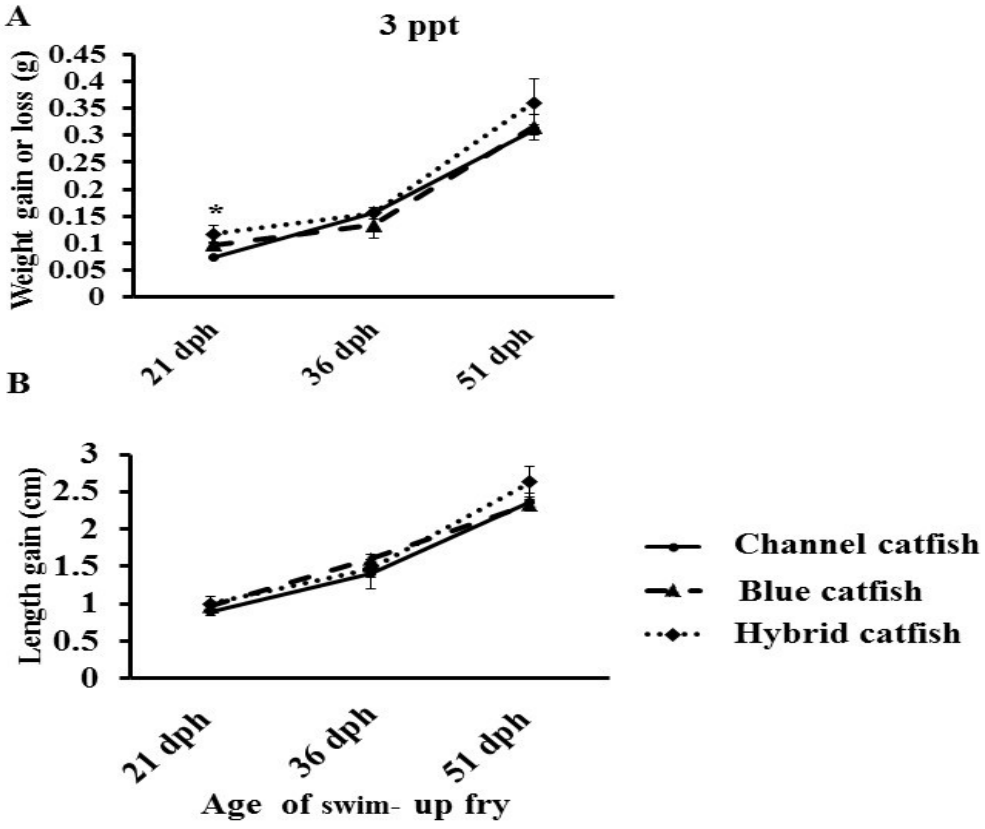

Fig. 7. Change in body weight gain or loss (g, graph A) and length gain (cm, graph B) of channel catfish, *Ictalurus punctatus*, blue catfish, *I. furcatus*, and channel catfish female × blue catfish male hybrid catfish swim-up fry from 21 to 51 days post-hatch (dph) at 3 ppt sodium chloride. Data are mean ± 2SEM. * denote statistically significant differences between groups at this time point (MANOVA (repeated measures); $^{*}P < 0.05$).

However, hybrid catfish lost weight, -0.01±0.00g, and channel gained 0.18±0.03 g body weight at 6 ppt (Table 3A; Figs. 5-7). At the end of experiment, the channel, blue, and hybrid catfish raised in freshwater (0 ppt) had gained 1.80±0.06, 2.13±0.09, and 1.97±0.09 cm body length, respectively, in 3 ppt 2.37±0.12, 2.33±0.07, and 2.63±0.20 cm body length, respectively and in 6 ppt channel catfish gained 1.53±0.03 cm body length, while hybrid catfish gained 1.65±0.05 cm body length (Table 3B; Figs. 5-7). Rate of growth peaked at 3 ppt and decreased at higher levels of sodium chloride.

4. Discussion

When the growth and survival of channel catfish, blue catfish and hybrid catfish was evaluated as hatchlings and over a two-month period and at different levels of sodium chloride, genotype-environment interactions were prevalent. Different genetic groups were the best at different life stages and at different sodium chloride levels. The hybrid catfish, which is now used by > 50% of the U.S. catfish industry, exhibited expected heterosis at 0 ppt. The heterotic growth was mostly gone when sodium chloride reached 3 ppt, and channel catfish had retarded growth and survival at 6 ppt, but had the highest means.

Freshwater is emerging as the most critical resource issue facing humanity. Ongoing climate change will reduce freshwater supplies (EPA, 2015). The world's population and demand for the resource continues to expand rapidly. Tolerance of high sodium chloride or salinity will determine which genetic groups will be most valuable in this changing environment. Additionally, salt can be intentionally used to enhance growth or to treat disease. It has not been previously studied how different genotypes or genetic groups could react in a variable fashion to exposure to salt for aquaculture or potential disease application.

Our results and those of Borode et al. (2002) and Su et al. (2013) indicated that high concentration of NaCl has adverse post-hatch effects on catfish as yolk sac larvae in 6 ppt had difficulty absorbing their yolk sac and experienced massive mortality. The

absorption of yolk was significantly faster in the control and 2 ppt salt treatments, but slower in 4, 6, and 8 ppt for African catfish, *Clarias gariepinus*, (Borode et al., 2002).

In the current study, yolk sac fry of different genetic groups at 6 ppt were active for the first day, but at the end of the challenge both sac larvae and fry became cachectic and moribund. Complete mortality for all genetic groups occurred by the end of day 1 of 9 ppt, probably due to ion imbalance (Enayati et al., 2013). In the early stages of development, fish may not as yet have effective mechanisms to eliminate excess Na^+ and Cl^- from their body systems (Magondu et al., 2011).

The higher mortality of swim-up fry in the control (0 ppt) compared to 3 ppt was probably due to parasitic infection and possible bacterial contamination. Towards the end of the experiment, *Ichthyophthiriasis*, (Ich) was observed on the fry in the 0 ppt treatment. In this case, the hybrid had the highest observed survival when exposed to Ich, which has been the case in some, but not all circumstances for comparisons of fingerling channel catfish, blue catfish and hybrid catfish (Xu et al., 2011; Elaswad, 2016). The survival rate for swim-up fry in 3 ppt was better than at 0 and 6 ppt. Perhaps, at 3 ppt disease organisms were better controlled than at 0 ppt, and 6 ppt was too elevated for sodium chloride causing some stress and death.

The salinity tolerance of these different groups is important for determining salt treatment levels for Ich and other diseases. The ictalurid swim-up fry had a wider tolerance of sodium chloride than the yolk-sac larvae.

However, NaCl concentrations of 6 ppt and 9 ppt appeared to elicit a toxic effect on the yolk sac larvae and swim-up fry stages regardless of the exposure duration. Some pronounced benefit can be gained by exposing catfish to low salt levels. The use of sodium chloride concentrations of 2 ppt in catfish culture water has been shown to protect the fish against the protozoan parasite *Ichthyophthirius multifiliis* (Allen and Avault, 1970b). However, some Ich strains cause high mortality in 2 ppt of NaCl or even higher levels (Dunham et al., Auburn University, unpublished data).

Allen and Avault (1971) found that blue catfish (11.5 to 14.9 cm total length, 7.4 to 17.3 g in weight) were more tolerant to 14 ppt salinity than were channel catfish from two different locations (9.9 to 21.5 cm total length, 5.2 to 55.4 g in weight). All the fish were approximately 1-year-old. Size of the fish did not influence the tolerance of salinity of either species. Fingerling of both species showed signs of distress early in the experiment, but showed some signs of recovery near the middle or end of experiment. All the test fish lost weight indicated that neither species was able to adapt at 14 ppt. The results in the current study were not in agreement with those of Allen and Avault (1971). Blue catfish were only more resistant to high sodium chloride than channel catfish during the sac fry stage, and channel catfish were more tolerant of sodium chloride once they reached swim-up fry stage.

Several possible explanations exist. Relative salinity tolerance of channel catfish and blue catfish appears to change with age and size.

Perhaps if older, larger fish were compared the results of the two studies would be similar. Alternatively, strain effects could explain different results obtained for the two species or other water quality factors interact with and affect salinity tolerance, potentially including the levels and balance of different salts. The salinity tolerance of catfish varies depending on the genetic group, in this case, two species and their hybrid. The average resistance of hybrid catfish to elevated sodium chloride was intermediate to that of channel catfish and that of blue catfish and the hybrid did not exhibit heterosis for survival as sodium chloride increased. Genotype-environment interaction also occurred among channel catfish, transgenic channel catfish and hybrid catfish for survival under at various levels of sodium chloride at the fingerling stage, but under cold conditions with channel catfish usually having higher tolerance (Abass et al., 2016).

Weight gain for hybrid catfish was better, 94% heterosis, than channel and blue catfish at 0 ppt sodium chloride ($P = 0.005$). Genotype-environment interactions were prevalent and no significant difference was observed for weight gain among genetic groups at 3 ppt ($P = 0.12$), and the heterosis of the hybrids was reduced to 16%. Growth rate at 3 ppt was better than at 0 ppt for all genetic groups, but benefitted the parent species much more than the hybrid. Energy demands for osmoregulation must have been diminished for the parent species at 3 ppt, increasing their growth potential, but

hybrids must differ substantially regarding physiology and salt regulation. Culture of freshwater fishes in brackishwater or seawater might require lower protein levels for optimum growth than culture in freshwater (El-Sayed, 2006). At 6 ppt, growth and survival continued to decrease, genotype-environment interactions increased and heterosis for growth became negative, - 100%.

In the current study, it appears that the geographic range and the areas where hybrid catfish and blue catfish could be grown would be more restricted in the event of global warming with increased habitats having higher salinity compared to channel catfish. However, the performance of hybrid catfish has the possibility of being improved through strain selection (Dunham et al., 2014) to cope with environmental change. On the other hand, fish in an estuarine environment would in all likelihood be presented to continuous changes in saltiness, as opposed to sudden changes, as displayed in direct exchange poisonous quality tests with flathead catfish, *Pylodictus olivaris,* (Bringolf et al., 2005). Some fish can adapt to higher salinities with steady increments in salinity than with direct move into salty waters (Eddy, 1981; Anyanwu, 1991; Bringolf et al., 2005). If the salt levels were to increase slightly, it may present more and better aquaculture opportunities as the catfish in the current study had improved growth at 3 ppt. However, water and areas suitable for spawning and hatching might be reduced.

Our and many other studies found that fishes' growth rate was higher in brackishwater environments than freshwater and saltwater or seawater environments

(Vonck et al., 1998; Imsland et al., 2001; Rubio et al., 2005; Resley et al., 2006; Kearney et al., 2008; Overton et al., 2008; Arjona et al., 2009; Mylonas et al., 2009; Küçük, et al., 2013). However, some studies showed that fishes growth rate were higher in freshwater than saltwater (Wang et al., 1997; Altinok and Grizzle, 2001a). The effect of salinity on growth of freshwater fish appears to vary among species, and is affected by feed consumption, digestion, utilization, and metabolic rate (El-Sayed, 2006). Similar to our results, salinities of 0·85 to 4 ppt have been reported to enhance growth of juvenile channel catfish, although results were inconsistent (Allen and Avault, 1970a; Lewis, 1972). Additionally, the tolerance of freshwater fishes to different concentrations of salinity appears to be dependent on species, strain, genetics, sex, size, life stage, adaptation time, and method and environmental factors (Watanabe et al., 1985; Britz and Hecht 1989; Fashina-Bombata and Busari, 2003; El-Sayed, 2006; Luz et al., 2008; Enayati et al., 2013).

An additional benefit of culture of freshwater fishes in brackishwater would be reduction of disease outbreaks that occur in freshwater (Altinok and Grizzle, 2001b; Magondu et al., 2011). The current study provides data that is useful for determining the level of NaCl that can safely used as prophylactic treatments in early stages of catfish and the possible toxicity of the same during longer exposure. We conclude that NaCl can be used in prophylaxis for swim-up fry of catfish and the concentration should not exceed 3 ppt. However, additional study is needed to determine any variation to this recommendation regarding additional life stages, the existence of

strain effects, or other factors that limit survival rate of catfish in low salinities. The negative effects of NaCl above 3 ppt for fry were decreased survival, appetite, growth rate, and even weight loss for hybrid catfish. The growth rate in 3 ppt saline water was better than in freshwater. Sodium chloride is inexpensive and readily available in rural areas making its usage useful in small scale hatchery operations. Hopefully, production of individuals with improved salinity tolerance will contribute substantially to future utilization in brackish water aquaculture to face the shortage of freshwater for human usage, assuming global climate change becomes increasingly problematic.

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CHAPTER THREE

Genotype-Environment Interactions for Survival at Low and Sub-Zero Temperatures at Varying Salinity for Channel Catfish, Hybrid Catfish and Transgenic Channel Catfish

Abstract

Organisms exposed to sub-zero temperatures are at risk of freezing damage. Fingerling channel catfish, *Ictalurus punctatus*, hybrid catfish (channel catfish female × blue catfish, *I. furcatus*, male), channel catfish transgenic for the goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter (βA-GAD65), and channel catfish transgenic for the catfish growth hormone gene driven by the antifreeze protein promoter (AFP-ccGH) were compared for survival at different temperatures (9.0 °C, 6.0 °C, 3.0 °C, 1.0 °C, 0.5 °C, 0 °C, and - 0.5 °C) at different salinities (0 ppt, 2.5 ppt, 5 ppt, and 7.5 ppt). The two transgenes were of interest as growth hormone not only affects growth, but also affects osmoregulation, and GAD65 construct could alter gonadotropin with the potential consequence that GnRH affects growth hormone production. Survival was 98-100% for all genetic groups at all salinities between 0 °C and 9.0 °C. However, large differences were observed at - 0.5 °C. At 0 ppt salinity, 100% of AFP-ccGH transgenic (T) fingerlings survived, but survival of all other genetic groups was 0-2%. Raising salinity to 2.5 ppt at sub-zero temperature had a

strong positive impact on survival as survival rates of AFP-ccGH (T), AFP-ccGH control (C), channel catfish, βA-GAD65 (T), βA-GAD65 (C) and hybrid catfish was 100, 100, 98, 76, 100 and 18%, respectively. Increasing salinity further to 5 ppt decreased overall survival, although it was still higher than at 0 ppt. Survival rankings were altered, with means for βA-GAD65 (T), βA-GAD65 (C), AFP-ccGH (T), AFP-ccGH (C), channel catfish and hybrid catfish of 69, 0, 15, 22, 0 and 0%, respectively. Mortality was 100% in all genetic groups at - 0.5 °C and 7.5 ppt demonstrating significant interaction between temperature and salinity. Negative heterosis was observed for the hybrids at low temperature at the respective salinities.

1. Introduction

Cultured fish can be exposed to many stressors, such as poor water quality (Sobhana, 2009), hypoxia, and salinity and temperature fluctuations (Walters et al., 1980; Robertson et al., 1987). If these stressors are prolonged, they can cause fish mortality or pre-dispose cultured fish to secondary infections (Walters et al., 1980; Robertson et al., 1987). Changes of temperature and salinity, and potential erratic and extreme temperature changes, could have significant impacts on aquaculture. Although not directly linked to climate change, mass fish kills have been associated with combinations of low temperature and low or high salinities (Cyrus and Maclean, 1996, Blaber, 2000) in coastal lake systems in South Africa.

The tolerance of channel catfish, *Ictalurus punctatus*, to a broad range of water quality, temperature and salinity is one of the key attributes of the fish as an aquaculture species. Ongoing climate change will reduce fresh water supplies, coastal water will become more brackish and brackish water will likely increase in abundance (EPA, 2015); the ability of ictalurid catfish to tolerate varying water quality could influence production, production efficiency and sites where they are grown in the future.

Salinity tolerance of channel catfish changes with the stage of ontogenetic development. Allen and Avault (1970) reported that channel catfish can tolerate 16 ppt salinity as eggs, 8 ppt at hatch, 10 ppt after yolk absorption, and 11 to 12 ppt from 5

to 6 months to older ages. Growth, feed consumption, feed conversion and survival of catfish fingerlings (42 to 148 days old) acclimated to 5 ppt are similar to that of fingerlings cultured in freshwater (Allen and Avault, 1970). Subsequent studies with channel catfish embryos and fry had different results (Weirich and Tiersch, 1997; Su et al., 2013a); embryos tolerated upwards to 4-6 ppt salinity, but sac-fry died at this salinity. Significant strain differences may exist for salinity tolerance or there may be other water quality parameters that can affect the toxicity of higher salinity levels.

The CB hybrid between channel catfish ♀ × blue catfish *I. furcatus* ♂ is reported to be the best catfish genotype for pond culture (Dunham et al., 2008). Several researchers have reported that the CB hybrid exhibits several commercially desirable characteristics, including faster growth, better feed conversion, tolerance of low oxygen, increased resistance to some diseases, better carcass yield, seinability and tolerance to crowded growth conditions in ponds (Bosworth, 2012; Bosworth et al., 2004; Dunham et al., 2008; Gima et al., 2014).

Stickney and Simco (1971) reported that nearly 100% of CB hybrid fingerling, channel catfish fingerlings and hybrid catfish backcrossed to channel catfish survived for 96 h at sublethal salinities (14.1–15.0 ppt). Again, this result was in contrast to the findings of Allen and Avault (1970), which indicated that the salinity tolerance of non-hybrid catfish was somewhat less than 14 to 15 ppt for extend period. These apparent contradictions may be a result of genetic or environmental factors, or genotype-environment interactions.

Growth hormone (GH) is a pituitary hormone, which is fundamental for normal somatic growth, development, and linear growth in vertebrates (Chen et al., 1994; Yowe and Epping, 1995; Forsyth and Wallis, 2002). In fish, GH also plays a role in osmoregulation (Sangiao-Alvarellos et al., 2005; Varsamos et al., 2005; Sakamoto and McCormick, 2006); thus, salinity tolerance of GH-transgenic fish might be expected to be altered. Treatment of juvenile salmonids with GH or insulin-like growth factor-I protein (IGF-I) enhanced seawater adaptability by increasing numbers of gill chloride cells and increasing Na^+, K^+, ATPase and enzyme activities (Komourdjian et al., 1976; McCormick et al., 1991; Madsen et al., 1995). Tang el al. (2001) showed that exposure of channel catfish to brackish water resulted in an increase in pituitary GH mRNA, suggesting that GH may possess some hypo-osmoregulatory actions. However, Eckert et al. (2001) could not demonstrate any hypo-osmoregulatory actions of GH in channel catfish injected with heterologous (ovine) GH prior to exposure to brackish water.

In contrast, GH-transgenic zebrafish were more sensitive to salinity (Almeida et al., 2013) as mortalities of non-transgenic and GH-transgenic zebrafish, *Danio rerio*, 96 h post transfer to 11 ppt salinity were 63 and 100 %, respectively. Transgenic coho salmon, *Oncorhynchus kisutch*, reach a stage that allows them to tolerate seawater faster than control; however, this tolerance appears to be size rather than age related (Devlin et al., 2000), and not a direct effect of GH transgene expression.

In regards to the relationship between GH and temperature tolerance, GH and control coho salmon had the same thermal maxima (Chen et al., 2015). Fry survival of

GH transgenic coho salmon and controls was not different for several different temperatures. However, none of these studies addressed survival at lethal sub-zero temperatures.

GH expression is influenced by gonadotropin-releasing hormone (GnRH). The regulatory interactions and between GH and GnRH are complex (Murthy et al., 1994, Melamed et al., 1998, Hull and Harvey, 2014). Theoretically, the overexpression of glutamic acid decarboxylase (GAD) in channel catfish would alter production of GABA (γ-aminobutyric acid), GnRH neuron migration (Fueshko et al., 1998; Bless et al., 2000), and thus, GnRH production. Fish deficient of GnRH would be expected to have altered GH levels and regulation, and perhaps alteration in osmoregulation. Trudeau et al. (2000) found that GABA reduces growth hormone (GH) release in goldfish (*Carassius auratus*). GABA, GnRH, follicle stimulating hormone, luteinizing hormone, dopamine, testosterone, estradiol and GH interact in a complex regulatory fashion (Trudeau et al., 2000; Bernier et al., 2009), thus alterations in GABA or GnRH production could have several potential effects on growth and sexual development. The primary purpose for producing GAD transgenic channel catfish was to produce a transgenically sterile catfish. When genes are inserted into a fish, pleiotropic effects are common (Dunham, 2011).

Genetically modified fish should not be released to private industry unless they are fully evaluated for all relevant economic traits. Environmental risk of the genetically modified fish cannot be fully determined without full phenotypic

evaluation. In our context, GH- or GAD-transgenic fish might have altered temperature tolerance or salinity tolerance, which could be relevant to commercial performance and environmental risk.

Few fish are able to survive water temperatures below 0 °C without antifreeze protein production. Many marine teleosts avoid freezing by producing small antifreeze proteins (AFPs) that adsorb to ice and halt the growth of ice crystals, lowering the freezing point of their tissues (Fletcher et al., 2001). We did not use the AFP gene in the current study, but did utilize the AFP promoter, which might drive gene expression at low temperatures.

The objective of this study was to examine the survival of channel catfish, channel female × blue male hybrid catfish, channel catfish transgenic for the goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter (βA-GAD65) and channel catfish transgenic for catfish growth hormone gene driven by the antifreeze protein promoter from an ocean pout *Zoarces americanus* (AFP-ccGH) at variable salinities and low temperature levels. Hybridization has resulted in heterosis for several traits in the CB hybrid (Dunham et al., 2008; Dunham et al., 2014a, 2014b; Gima 2014), but the tolerance of salinity at low temperatures has not been examined. Temperature and salinity tolerance of GH- and GAD-transgenic channel catfish should be examined to determine any pleiotropic effects of the gene expression of aquaculture or environmental importance. Alteration of GnRH and GH expression via transgenesis could impact salinity tolerance in catfish.

2. Materials and Methods

2.1. Construction of plasmids

2.1.1. AFP-ccGH construct

The AFP-ccGH construct consisted of the growth hormone cDNA from channel catfish (accession number: NM_001200245) whose expression was regulated by the 5′ promoter and 3′ termination regions derived from an ocean pout, *Zoarces americanus*, antifreeze protein (AFP) promoter. The growth hormone cDNA was 603 bp, the antifreeze protein promoter was 2,120 bp, and total size of the construct was 6,741 bp (Fig. 1a).

2.1.2. β-Actin Glutamate Decarboxylase (βA-GAD65) construct

The βA-GAD65 construct consisted of the βA-GAD65 gene (accession number: AF045594) from goldfish placed into the BsrGI, which is an isoschizomer of KpnI, site of pFV-3 Cat (an expression vector that is driven by the carp β-actin promoter obtained from Zhanjiang Liu, Auburn University). GAD65 replaced the chloramphenical acetyl transferase (CAT) gene in this plasmid. These two components were fused to make the βA-GAD65 construct used in this study. The GAD65 gene was 2,653 bp and the pFV-3 Cat carp β-actin promoter was 6,782 bp (Fig. 1b).

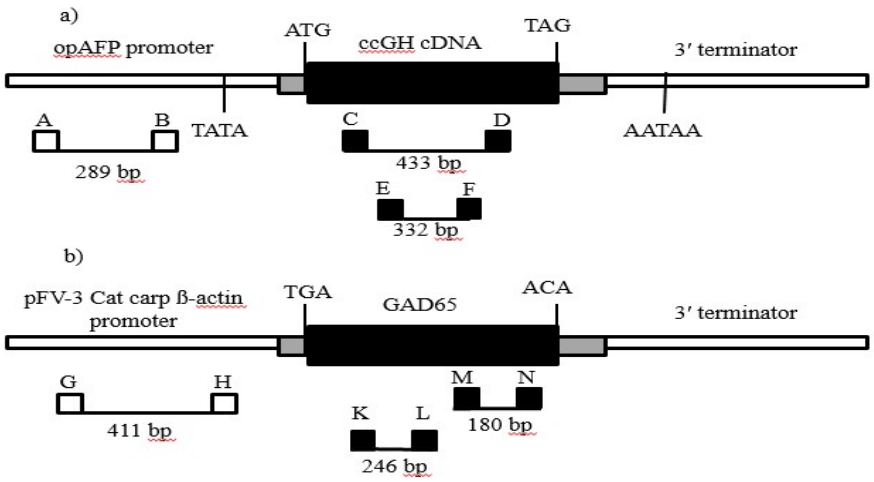

Fig. 1. Design of transgene constructs and allocated PCR-based detection strategies: (a) Structure of the opAFP-ccGH: ▭ ocean pout AFP gene promoter and 3' terminator region; ▬, channel catfish*, Ictalurus punctatus,* (growth hormone) GH cDNA, ▬ 5' and 3' untranslated regions. Three sets of primers were used to detect the presence of the transgene, primers A/B, C/D, and E/F, with the expected sizes of the PCR products indicated. Primers for region A and B are specific for the ocean pout antifreeze protein promoter, and primers for region C, D, E, and F are specific for channel catfish growth hormone cDNA. (b) Structure of the goldfish glutamate decarboxylase 65 (GAD65): ▭ pFV-3 CAT Common carp β-actin promoter and 3' terminator region; ▬, goldfish glutamate decarboxylase 65 gene, ▬ 5' and 3' untranslated regions. Three sets of primers were used to detect the presence of transgene, primer pairs G/H, M/N, and K/L, with the expected sizes of the PCR products indicated. Primer pairs G and H are specific for the carp β-actin promoter, and primer pairs M, N, K, and L are specific for GAD65 gene.

2.2. Broodstock and fry production

The broodstock utilized were from the Catfish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, AL, USA. The P_1 transgenic fish were produced via electroporation (Powers et al. 1992, Su et al. 2015). Broodstock were tested to determine if they were transgenic prior to spawning. One F_2 (F_1 transgenic female mated with a control male) family of channel catfish transgenic for the catfish growth hormone gene driven by the antifreeze protein promoter (AFP), and one F_2 (two F_1 individuals mated together) family of channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter (βA-GAD65) were induced to spawn by injection with luteinizing hormone-releasing hormone analog (LHRHa) implants at 85 μg/kg female body weight (Su et al., 2013b). Several families of mixed strain channel catfish were produced by hormone-induced spawning. Tombigbee (TBB) blue catfish males were used to fertilize Kansas select (KS) channel catfish females to produce 4-5 hybrid catfish families (Table 1). All experimental fish were produced over a few days in June of 2013.

2.3. Fish handling and pre-challenge conditions

A total of 180 fingerlings from each genetic group-channel catfish (*Ictalurus punctatus*) (non-transgenic) (mean weight: 8.28 ± 0.04 g; mean length: 10.68 ± 0.13 cm), CB hybrid (non-transgenic) (mean weight: 8.34 ± 0.05 g; mean length: 11.16 ±

Table 1 Genetic treatments and control groups to determine cold and salinity tolerance of fingerling ictalurid catfishes. A total of 180 fish per genotype were divided into 45 fish per salinity treatment, with 15 fish per replicate except for a total of 82 βA-GAD65 (T), 98 βA-GAD65 (C), 31 AFP-ccGH (T), and 149 AFP-ccGH (C).

Genotype[1]	Mean (±SD) Weight (g)	Mean (±SD) Length (cm)
Channel catfish	8.28 ± 0.04	10.68 ± 0.13
CB hybrid	8.34 ± 0.05	11.16 ± 0.12
βA-GAD65 (T) channel catfish	4.31 ± 0.26	8.42 ± 0.06
βA-GAD65 (C) channel catfish	3.67 ± 0.14	8.17 ± 0.08
AFP-ccGH (T) channel catfish	4.57 ± 0.06	8.25 ± 0.09
AFP-ccGH (C) channel catfish	4.16 ± 0.04	8.03 ± 0.04

[1]Channel catfish, *Ictalurus punctatus* (several families from a mixture of strains) and the primary control for the CB hybrid (also the secondary control for the transgenic individuals), CB hybrid = channel catfish, *I. punctatus*, female × blue catfish, *I. furcatus*, male hybrid catfish (4-5 families mixed together from TBB strain of blue catfish crossed with Kansas Select strain of channel catfish), AFP-ccGH (T) = channel catfish transgenic for channel catfish growth hormone gene driven by the ocean pout antifreeze protein promoter (one family), and AFP-ccGH (C) = channel catfish full-sibling non-transgenic (control) for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter, βA-GAD65 (T) = channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter (one family), βA-GAD65 (C) = channel catfish full-sibling non-transgenic (control) for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter.

0.12 cm), βA-GAD65 (T) channel catfish (mean weight: 4.31 ± 0.26 g; mean length: 8.42 ± 0.06 cm), βA-GAD65 full-sibling control (C) channel catfish (mean weight: 3.67 ± 0.14 g; mean length: 8.17 ± 0.08 cm), AFP-ccGH (T) channel catfish (mean weight: 4.57 ± 0.06 g; mean length: 8.25 ± 0.09 cm), and AFP-ccGH full-sibling control (C) channel catfish (mean weight: 4.16 ± 0.04 g; mean length: 8.03 ± 0.04 cm) were used in this experiment. There were four salinity treatments (0 ppt, 2.5 ppt, 5 ppt, and 7.5 ppt) with 45 fish per treatment for each genetic group. The AFP-ccGH and βA-GAD65 aspects of the experiment were blind, as the transgenic and non-transgenic full-siblings were not identified by PCR until the conclusion of the experiment. Fish for a single salinity treatment were stocked separately into small triplicate cages, 15 fish per cage, 45 fish from each genetic group in a single 60-L trough, and the water depth was approximately 12.5 cm. The test for the AFP-ccGH and βA-GAD65 transgenic fish was blind (controls and transgenic full-siblings were mixed and not identified until the conclusion of the experiment) and thus, N for AFP-ccGH and βA-GAD65 transgenic fish ranged from 4-13 and 13-33, respectively (Tables 3, 4).

The fish were at ambient temperature and photoperiod during the experimental period, as well as from the time of hatch until the experiment began. Fingerlings were at ambient conditions of 12 ± 0.5 °C, dissolved oxygen 9.72 ± 1.2 mg/l, and pH 7 ± 0, and 0 ppt salinity for 9 d before challenge at varying concentrations of salinity.

All fish were initially held at 0 ppt, and then sodium chloride was added to the culture vessels to increase the salinity by 2.5 ppt / 3 days until the final treatment level was reached. Oxygen was maintained with compressed air.

2.4. Challenge experiment

To evaluate the effects of various low temperature-salinity combinations on the survival rate of transgenic and non-transgenic catfish, hybrid catfish and channel catfish, triplicate groups were held at 0 ppt, 2.5 ppt, 5 ppt, and 7.5 ppt for 14 days and daily temperature recorded (Fig. 2). Air and water temperatures were ambient; water temperature progressively dropped during each day, and was 9 °C, 6 °C, 3 °C, 1 °C, 0.5 °C, 0.0 °C and - 0.5 °C for about 2 days each, with 2.5 cm of ice forming when the water dropped below 0.0 °C and then thawing on day 15 when the temperature increased back to 0.0 °C.

Every day, 70-80% of the total water was exchanged to maintain ammonia near 0 except for the two days of ice cover. Fish were not fed during this period, and water quality parameters including temperature, pH and dissolved oxygen were monitored. Mortality was monitored daily for 14 days.

2.5. Genomic DNA extraction

Excised fin-clips (200 mg) from each potentially transgenic fingerling were placed into a 1.5-ml tube on ice for molecular identification. Thereafter, the samples were held at - 80 °C until DNA extraction. DNA was extracted using proteinase K

digestion followed by protein precipitation and ethanol precipitation of DNA as described in the protocol of Kurita et al. (2004).

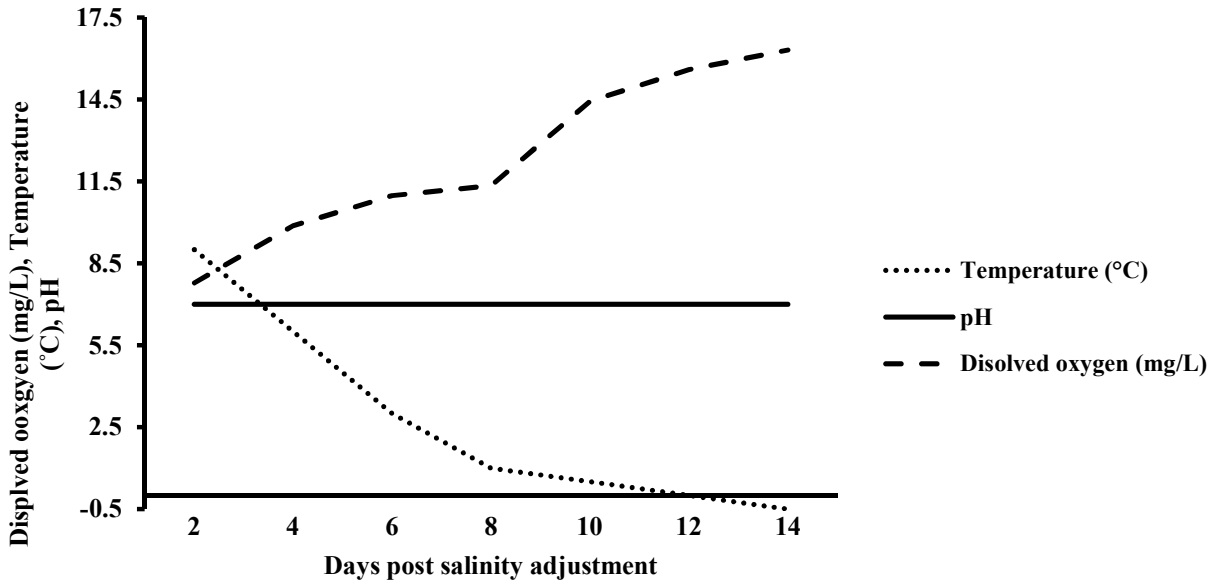

Fig. 2. Water quality parameters during the salinity challenge.

2.6. Transgene identification

Potentially transgenic fingerling samples were screened by PCR with specific forward and reverse primers (Table 2). PCR amplification was done using 1–2 µl DNA template (200 ng) in 6.5 µl PCR water, 1 µl 10× PCR reaction buffer, 0.35 µl $MgCl_2$ (50 mM), 0.8 µl dNTPs (2.5 mM), 0.3 µl forward and reverse primers (10 pmol/µl), and 0.04 µl *Taq* polymerase (Invitrogen). Initial denaturation occurred at 94 °C for 4 min; followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s; followed by 72 °C for 15 min. PCR product was used as template for the second round

of PCR using the same primers that were used in the first round. Primary and secondary PCR products were visualized on an ethidium bromide 1% TAE agarose gel. A PCR product was sought of the expected size of 289 bp for the ocean pout antifreeze protein promoter, 433 bp and 332 bp for ccGH cDNA, 411 bp for the carp β-actin promoter, and 180 bp and 246 bp for the βA-GAD65 gene. (Figs.1,3).

Table 2 Primer sequences used for identifying βA-GAD65 and AFP-ccGH transgenic fingerling channel catfish, *Ictalurus punctatus*.[1]

Primer name	Primer direction[2]	Primer sequence (from 5' to 3')	Target region
A	F	TGA CCC GAC CTC AGA TAA GC	Ocean pout antifreeze
B	R	CAA AGG TCT TAA GCG CAT CC	protein promoter
C	F	GCC AAG ATG ATG GAC GAC TT	Channel catfish
D	R	ACC ACG CTC AGA TAG GTC TC	growth hormone
E	F	AGG AAG CTC TGT TGC CTG AA	cDNA
F	R	CCT CGC TCA AGG TCT GGT AG	
G	F	GTT GCA CAC TTG ATG GAT GG	Carp β-actin
H	R	ATC CTC AGC CCA TTC ATT TG	promoter
K	F	TTC TCT GTC GCT GCT CTG AT	GAD65 gene
L	R	CTC TCG GCT GTA GAC CCA T	
M	F	GGA TAC GTG CCG TTC TTT GT	
N	R	CTC GAC TCC ATT CAG CTT CC	

[1]βA-GAD65 = channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter, AFP-ccGH (T) = channel catfish transgenic for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter.

[2] F = Forward primer, R = Reverse primer.

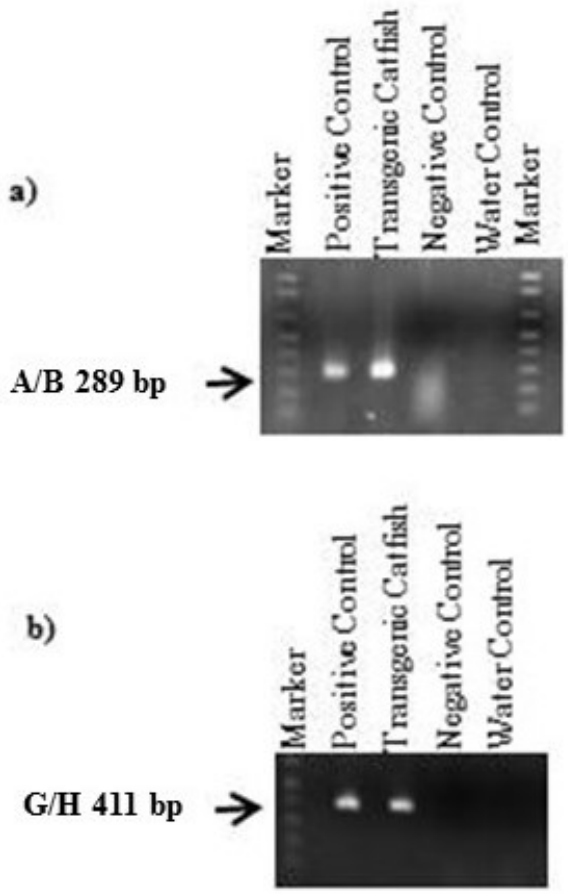

Fig. 3. PCR analyses of transgenic and non-transgenic channel catfish, *Ictalurus punctatus*, total DNA. (a) Analysis of the ocean pout antifreeze protein promoter, 289 bp using primers A/B. (b) Analysis of the carp β-action promoter, 411 bp by primers G/H. Positive control: plasmid DNA containing transgenic P_1 sequence for positive control; negative control: DNA sample from non-transgenic control fish; water control: PCR reaction with water serving as negative control; and marker lanes contain 1 Kb plus DNA ladders (Invitrogen).

2.7. Sequence analysis

Amplified PCR products were purified using the Qiagen PCR Product Extraction Kit (cat# 28104). A total of 200 ng/µl of amplified product from F_2 channel catfish transgenic for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter (AFP-ccGH (T)) was used for sequencing with the A and D primers. From F_2 channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter (βA-GAD65 (T)) amplified product was used for sequencing with the H primer. All sequencing was done by the Auburn University Genomics and Sequencing Lab (AU-GSL) using the Life Technologies POP7 technology. DNA sequences were analyzed with the BLASTN program available from NCBI (http://www.ncbi.nlm.nih.gov). Alignments of DNA sequences were performed with the program Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.8. Statistical analysis

Data were expressed as mean ± 2SEM, and subjected to multiway within-subjects MANOVA (repeated measures) to evaluate the combined effects of temperature and salinity. Due to a strong interaction among the factors (salinity and temperature), the effect of each factor was tested at a fixed level of the other factor using one-way ANOVA, and significant differences were determined using Duncan's multiple comparison test (Duncan, 1955) at $p < 0.05$. Statistical analyses were conducted using SAS software (SAS Institute, 2010).

3. Results

3.1. Water quality

Dissolved oxygen values ranged from 7.45 mg/l to 14.62 mg/l, 8.29 mg/l to 13.92 mg/l, 9.48 mg/l to 15.62 mg/l, and 7.78 mg/l to 16.32 mg/l in 0 ppt, 2.5 ppt, 5 ppt, and 7.5 ppt salinity, respectively, while pH values were 7 and ammonia-nitrogen values were near 0 at different salinities. These values are all in the optimum ranges for ictalurid catfishes (Tucker and Robinson, 1990).

3.2. Influence of temperature and salinity on survival rate

Temperature, salinity and genetic group all affected survival ($P < 0.001$). Additionally, temperature × salinity, temperature × genetic group and temperature × salinity × genetic group interactions occurred ($P < 0.001$) (Fig. 4).

No significant mortality was recorded for the respective genetic groups between 9 and 0 °C at different salinities. However, large differences were observed at - 0.5 °C (Table 3). At 0 ppt salinity, 100% of AFP-ccGH transgenic (T) fingerlings survived, which was higher ($P < 0.0001$) than survival of all other genetic groups, 0-2%. Raising salinity to 2.5 ppt at sub-zero temperature had a strong positive impact on survival, as survival rates for AFP-ccGH (T), AFP-ccGH control (C), channel catfish, βA-GAD65 (T), βA-GAD65 (C) and hybrid catfish were 100, 100, 98, 76, 100 and 18%, respectively (Fig. 4) with the hybrid having the lowest survival followed by βA-GAD65 (T) ($P < 0.0001$).

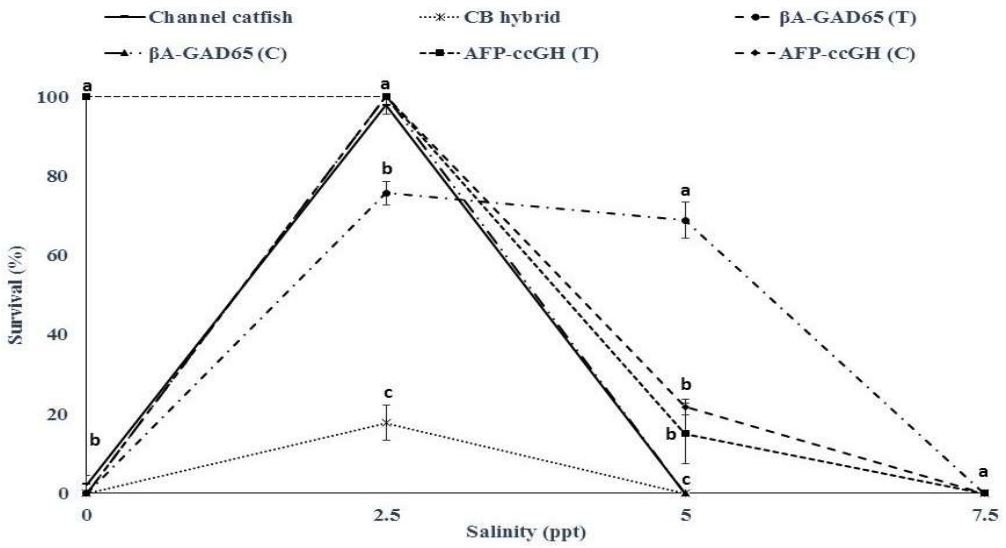

Fig. 4. Mean (±2SEM) percent survival of different genetic groups of fingerling channel catfish, *Ictalurus punctatus*, CB hybrid catfish, βA-GAD65 (T), βA-GAD65 (C), AFP-ccGH (T), and AFP-ccGH (C) at different concentrations of NaCl at - 0.5 ˚C.

CB hybrid = channel catfish, *I. punctatus*, female × blue catfish, *I. furcatus*, male hybrid, βA-GAD65 (T) = channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter, βA-GAD65 (C) = channel catfish full-sibling non-transgenic (control) for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter, AFP-ccGH (T) = channel catfish transgenic for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter, and AFP-ccGH (C) = channel catfish full-sibling non-transgenic (control) for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter. Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at fixed level of salinity at - 0.5 °C.

Table 3

Mean (±2SEM) percent cumulative survival of different genetic groups of fingerling channel catfish, *Ictalurus punctatus*, CB hybrid catfish, βA-GAD65 (T), βA-GAD65 (C), AFP-ccGH (T), and AFP-ccGH (C) throughout study period in different concentrations of NaCl and temperature for 14 days.

Salinity (ppt)	Genetic groups[1] (N)[2]	Temperature (°C)						
		9	6	3	1	0.5	0	- 0.5
0	Channel catfish (45)	100±0	100±0	100±0	100±0	100±0	100±0	2.22±2.22[b]
	CB hybrid (45)	100±0	100±0	100±0	100±0	100±0	100±0	0±0 [b]
	βA-GAD65 (T) (20)	100±0	100±0	100±0	100±0	100±0	100±0	0±0 [b]
	βA-GAD65 (C) (25)	100±0	100±0	100±0	100±0	100±0	100±0	0±0 [b]
	AFP-ccGH (T) (4)	100±0	100±0	100±0	100±0	100±0	100±0	100±0[a]
	AFP-ccGH (C) (41)	100±0	100±0	100±0	100±0	100±0	100±0	0±0 [b]
2.5	Channel catfish (45)	100±0	100±0	100±0	100±0	97.78±2.22[3]	97.78±2.22[3]	97.78±2.22[3a]
	CB hybrid (45)	100±0	100±0	100±0	100±0	100±0	100±0	17.78±4.44[c]
	βA-GAD65 (T) (33)	100±0	100±0	100±0	100±0	100±0	100±0	75.64±2.96[b]
	βA-GAD65 (C) (12)	100±0	100±0	100±0	100±0	100±0	100±0	100±0 [a]
	AFP-ccGH (T) (6)	100±0	100±0	100±0	100±0	100±0	100±0	100±0 [a]
	AFP-ccGH (C) (39)	100±0	100±0	100±0	100±0	100±0	100±0	100±0 [a]
5	Channel catfish (45)	100±0	100±0	100±0	100±0	100±0	100±0	0±0 [c]
	CB hybrid (45)	100±0	100±0	100±0	100±0	100±0	100±0	0±0 [c]
	βA-GAD65 (T) (16)	100±0	100±0	100±0	100±0	100±0	100±0	68.81±4.52 [a]

	βA-GAD65 (C) (29)	100±0	100±0	100±0	100±0	100±0	100±0	0±0[c]
	AFP-ccGH (T) (13)	100±0	100±0	100±0	100±0	100±0	100±0	15±7.64[b]
	AFP-ccGH (C) (32)	100±0	100±0	100±0	100±0	100±0	100±0	21.8±1.98[b]
	Channel catfish (45)	100±0	100±0	100±0	100±0	100±0	100±0	0±0
	CB hybrid (45)	100±0	100±0	100±0	100±0	100±0	100±0	0±0
	βA-GAD65 (T) (13)	100±0	100±0	100±0	100±0	100±0	100±0	0±0
7.5	βA-GAD65 (C) (32)	100±0	100±0	100±0	100±0	100±0	100±0	0±0
	AFP-ccGH (T) (8)	100±0	100±0	100±0	100±0	100±0	100±0	0±0
	AFP-ccGH (C) (37)	100±0	100±0	100±0	100±0	100±0	100±0	0±0

[1]Channel catfish = channel catfish, *Ictalurus punctatus*, CB hybrid = channel catfish, *I. punctatus*, female × blue catfish, *I. furcatus*, male hybrid catfish, βA-GAD65 (T) = channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter, βA-GAD65 (C) = channel catfish full-sibling non-transgenic (control) for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter, AFP-ccGH (T) = channel catfish transgenic for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter, and AFP-ccGH (C) = channel catfish full-sibling non-transgenic (control) for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter. Means that do not differ at the $P = 0.05$ are followed by the same superscript (Duncan's multiple range test) among different genetic groups at fixed level of salinity at - 0.5 °C.

[2]N= Number of fingerling ictalurid catfishes. [3]One fish died at 0.5 °C.

Increasing salinity further to 5 ppt decreased survival rate, although it was still higher than at 0 ppt; as survival rates for βA-GAD65 (T), βA-GAD65 (C), AFP-ccGH (T), AFP-ccGH (C), channel and hybrid catfishes were 69, 0, 15, 22, 0 and 0%, respectively (Fig. 4) with βA-GAD65 (T) having the highest survival

($P < 0.05$). Because of the interaction between temperature and salinity, 7.5 ppt at -0.5 °C was the only treatment that resulted in mortality of 100% of the individuals in all genetic groups (Table 3; Fig. 4). Repeated measure MANOVA revealed that the factors temperature and salinity had significant effects on survival rates of the respective genetic groups, with significant interaction among these factors ($P < 0.001$). Survival was significantly different between 2.5 ppt and other salinities under freezing conditions (- 0.5 °C) for channel catfish, hybrid, and βA-GAD65 (C). Survival for AFP-ccGH (T) was not different between 0 ppt and 2.5 ppt. Among all genetic groups, only AFP-ccGH (T), had a significant difference in survival between 0 ppt and 7.5 ppt ($P < 0.0001$) (Table 4).

Table 4

Results of one-way ANOVA and comparison test showing the effect of different concentrations of salinity on mean survival rate (%) at fixed level of temperature (- 0.5 ºC) for each genetic group of fingerling channel catfish *Ictalurus punctatus*, CB hybrid catfish, βA-GAD65 (T), βA-GAD65 (C), AFP-ccGH (T), and AFP-ccGH (C).

Genetic groups[1]	Source	MS	*F*	R^2	Duncan's Multiple Range Test (N)[2] Mean % (2SEM) 0 ppt	2.5 ppt	5ppt	7.5 ppt
Channel catfish	Salinity Error	7065.22 7.41	952.85**	0.99	(45) 2.22[b] (2.22)	(45) 97.78[a] (2.22)	(45) 0[b] (0)	45 0[b] (0)
CB hybrid	Salinity Error	237.01 14.83	15.98**	0.86	(45) 0[b] (0)	(45) 17.78[a] (4.45)	(45) 0[b] (0)	45 0[b] (0)
βA-GAD65 (T)	Salinity Error	5239.56 21.90	239.25**	0.99	(20) 0[b] (0)	(33) 75.64[a] (2.96)	(16) 68.81[a] (4.52)	13 0[b] (0)
βA-GAD65 (C)	Salinity Error	7500 0	∞**	1	(25) 0[b] (0)	(12) 100[a] (0)	(29) 0[b] (0)	32 0[b] (0)
AFP-ccGH (T)	Salinity Error	8668.75 43.75	198.14**	0.99	(4) 00[a] (0)	(6)100[a] (0)	(13) 5[b] (7.64)	(8) 0[c] (0)
AFP-ccGH (C)	Salinity Error	6766.43 2.94	2301.43**	0.99	(41) 0[c] (0)	(39) 100[a] (0)	(32) 21.8[b] (1.98)	(37) 0[c] (0)

For temperature and salinity effect, only temperature (- 0.5 °C) data were used. Data were arcsine-transformed to meet ANOVA assumptions of normality. Means within the same row that do not differ at the 0.05 level are followed by the same superscript (Duncan's multiple range test).

$^{**}p < 0.001$.

[1]Channel catfish = channel catfish, *Ictalurus punctatus*, CB hybrid = channel catfish, *I. punctatus*, female × blue catfish, *I. furcatus*, male hybrid catfish, βA-GAD65 (T) = channel catfish transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-actin promoter, βA-GAD65 (C) = channel catfish full-sibling non-transgenic (control) for goldfish glutamate 65 gene driven by the carp β-actin promoter, AFP-ccGH (T) = channel catfish transgenic for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter, and AFP-ccGH (C) = channel catfish full-sibling non-transgenic (control) for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter.

[2]N= Number of fingerling ictalurid catfishes.

3.3. Sequence analysis for transgenic genotypes

Transgenic genotypes were confirmed by both PCR and sequence analysis. A 330-bp nucleotide sequence was obtained from sequencing of the AFP-ccGH (T) amplicon with the A primer. BLASTN analysis indicated that the nucleotide sequence had a 97% homology to the ocean pout AFP-antifreeze protein promoter (GenBank accession no. S65567). The sequence is shown, and alignments with the homologous sequence of the antifreeze protein promoter of ocean pout (GenBank accession no. S65567), generated by Clustal Omega in Fig. 5. A 356-bp nucleotide sequence was obtained from sequencing of the βA-GAD65 (T) amplicon with the H primer. BLASTN analysis indicated that the nucleotide sequence had a 98% homology to the common carp β-actin gene (GenBank accession no. M24113). The sequence is shown, and alignments with the homologous sequence of the common carp β-actin gene (GenBank accession no. M24113), generated by Clustal Omega in Fig. 6. A 458-bp nucleotide sequence was obtained from sequencing of the ccGHcDNA amplicon with the D primer. BLASTN analysis indicated that the nucleotide sequence had a 99% homology to the *Ictalurus punctatus* growth hormone mRNA (GenBank accession no. NM_001200245). Sequences were short of 100% homology likely because of sequencing errors at the ends of the PCR products.

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S65567          GGATCCCCCAGAATGAGCTGGAACATGTTGCGGGGAGAGGGAAGTCTGGGTCAGCCTGCT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TGGCCTGCTGCCACCGTGACCCGACCTCAGATAAGCGGAGGAAAATGGATGGATGGATTG
AFP-ccGH(T)     ----------------------------------------TGCGACGCGAGGGCGGACCCG
                                                        *  *   ** **  *    *

S65567          AATCACAGAATGTTTCTGAAGACAGATATCACCTTCGCTTCAAAGAGGTGCGCACCTGGG
AFP-ccGH(T)     ACTCAGAGAGCTGACCCGACCTCAGATAAGCTGACCC----GACCTCAGATAAGCTGACC
                * *** ***      * **   ******       *      *           *

S65567          CAGGCACCCACACAGCCACACAAATGGCATATGAATCAACCAAGAAGACGGTTGGAACTG
AFP-ccGH(T)     CGGCCTCCACATCAGCCACACCAATGGGATATGAATCAACCAAGAAGACGGTTGGAACTG
                * * * **    ********* ***** ******************************

S65567          GTCAAAACCTTCACTATACCATGTGTGACAGTTGTTTGTCACAGTGTATAAAAGACAGGG
AFP-ccGH(T)     GTCAAAACCTTCACTATACCATGTGTGACAGTTGTTTGTCACAGTGTATAAAAGACAGGG
                ************************************************************

S65567          ACTTAGAGACAGAGCTCTGAGCAGCTATGAGATTGTAGTTTGGCCAGGATGCGCTTAAGA
AFP-ccGH(T)     ACTTAGAGACAGAGCTCTGAGCAGCTATGAGATTGTAGTTTGGCCAGGATGCGCTTAACC
                ********************************************************

S65567          CCTTTGTGATGAAAAGTTATCAAATTCGTGAGTTTTCATGGAAGAACCTTGACGTGGCGT
AFP-ccGH(T)     TTTGGAGCTTATCTGAGGTCGGGGCAGCTTATCTGAGTCGGGGATTATCTGAGGGGGGGA
                  *      *                  * *  *     **        *** * ** *

S65567          GGTGGCCATTTTGCGTCATTCGGCATGGAAAAGGAAGTCGTTATAACTCCCAGGTACATT
AFP-ccGH(T)     AGGGGGGGGGGGG----------------------------------------------
                 * **       *

S65567          ATCTTATCTACACAAAATGTCTAATGCATGATACTACTTAAAGCCTGAGCATATTTCAAG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GCCAGCACTTTTCAATAACTCATAGGCCACCTGCTGGCAAAAGGAAATGCCACATTTTAT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ACTTTTATTTACTCCTAGACAGTTGACCTGATCAGTCTCAAATTTGGTAAGGATAGCCTT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AAGACAATGAAGATGCTTCATCAGGAATATTGTGAGTTGTCGTTGAACGTTGTTGCCGTG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GCAACGCATCATTCGCCATGAAAAAGAAGCTGATGGTTCAGTGGCTTGGGATGCTCAAAA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AGTCATGGAACTTTGTACATGTGTCATAATTGATGGGAAGTTGTATGGGTTTTTGGCTTG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CTTGTTATAAATTGTCTCCATAGCGCCCCCTACAATATTTCAAAAGAGCAGCCCCAGTGC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TACGTACATGTATGAAACTTAGTAGCCAGATGTACCATATAGAGACTTACAAAAAGGTAT
AFP-ccGH(T)     ------------------------------------------------------------
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S65567          CTTGGCCATGCTCTCAACCGTACTGGAAGTCGGCCATTTTGATTTTTGCATAATTTTTCA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ATAGATTTTTGCACATTTGTAATCGCTATACTTTAACGAACTCCTCCAAGGAACTTTGTC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TAATCAATTTCAAATTTTGTCAGTACAATCTCAGTACTACAGTACCAAATCTACAGTTCT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GCATCTCGTAGCTGCTCAGAGGTCTGTCTCTAAGTCCCTGTCTTTTATACACTGTGACAA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ACAACTGTCACACATGGTATAGTGAAGGTTTTGACCAGTTCCAACCGTCTTGTTGGTTGA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TTCATATGCCATTCGTGTGGCTGTGTGGGTGCCTACCCAGATGCGCACCTCTTTGAAGCG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AATGTGATATCTGTCTTCATAAACATTCTGTTATTAGCAAGTTCATATGAGAATGAAGGC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TGTATGCAAACAGGTGCACAGTCTGTTTCTAAGCATCATGGAAAAGTACAAGCAATTTGC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ACAAATCATTCTGTATTTTTCCAATAGCTAACAATGTCACCGGGACATTGTGCTATTGGA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TAGAAGAGACCAGCTGATCTAGACAGTTGATATCATGATCAACAGCCCCAAACAACAAGT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GTGCATGCGCGAGGAGTGATTGGCAGATGTATGAGAACTAAACCACTGACTGAACTTGCA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CTAGAGGCATCTATTTTGTCTTTTCTCATATGATGTTGGGATGGCACATGGGAGTTTTTC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CCCTGTCTCAGCTTGCTTTTTACCCCAAATATTGTATATCTATTAGAACCGTTGTCACAG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GGTTCAAATTAACGTTTTAGTTTAGTTTTGATCATGATATACACATTTTATCCGTAAAGC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ATGTGCATATACAGTAAGGGCTTGTTATTCGACAGCAAGAAGAAGAGGATATGTGTGCAG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GCAGTCAGCTAATGCATGGATCACAAGTTATAGAATGCAAGCTTGTGATAGTTTGGACAA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AAACAAGTTATACTTTACTTATAAGAATATAAAATTTCCATTGCAATTGGCATAAGGAGG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TGTGACACAGTGACCTACTTTCAGGCCAATAGGAAACGGGATATGCCGGTTAAGTCCTCC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CACATACTGTATATTAGATGCAGCACATGGACCTGTCCTGTCAGAAGTCTCAGCTACAGC
AFP-ccGH(T)     ------------------------------------------------------------
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S65567          TTTCACTTCGATCCAGATCTTTTCACTTCGATCTCCGATAATTAATTAATTAATTAATTA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          TTAATTAATTAAGTCTCAGCCACCGTTAACTGAACATGTCAAAACCTGTGGAGACTGTTG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AGATTTGATGTTCTGAAAAGATAAAGCCTATAAATAAAATGTTGCCCAAATTTCCTGCCT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GATGTTTTTCTTTGTCTTTGCTACATGGCTTTGCTGCTCGGATCGGCTCACTCTGTGTAT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GCCACGTTCACTTTGTACTCTCCTTCTCACGGTAGGTTTATTATTTTTAGATGTGCAGTT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AGTTTCTGTGAAATAACACACCACACACTGATATTGTCTGTGCATTGACTTGGTGAGTGC
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ACATTGTTTTTGATCTTGACATATTTATATTTGATTGATCAGGTGAACTGTGTGAATCTA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AAGTGCTCCATACAGATGTTCTGCATTGAAAATATTCTCATTTTATTAGTGGAAGTGAGT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GTATGCCACATCCAATCAATTTCAGCAAACACCCCAGTATGATTTAATGCAAAAAAATGA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AGGTATCAAACACGCATTACTACTTTGCAGTTAAATATTTAACATTTATTCCAACACGAA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          AAAAAGCAGTAAATAACACTTTGACAAACACGTCAGGACATCTTATTTTTGTCACCCTCA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CAGGCAATTTAGTATAATATATTATATATATATATATATCATATAATAATATTCAGTATA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ATATATATATATATATCATATTATAATATTCAGTATAATATAAAACACAAACACATATAT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GTATAATATAATATAACATTTTTATTTATTGAGATGCCTCTATGGACCGTGTTATAAGAA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GTAAAGATCAGGAGAAGTAAACATGAAGTGTAATTATGAATACTGATGTTAAATTAAGCT
AFP-ccGH(T)     ------------------------------------------------------------

S65567          ATGATGAGTTTTCACTGTTAATTTACCATCTCAATTAAATGTTGATGCCTCCATGACCAA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GTTAAGCAGATGAGACTGAGACAACTGTAGAAGACAAGATGTTCACTTTGCTGAATATAG
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CTGGCTTGACAGTTATCTATGACTCTATAAATATATATATATTTTTTTTTTTATAAAATG
AFP-ccGH(T)     ------------------------------------------------------------
```

```
S65567          ATTTATTTATAACTATATATCCATTTCTCAGACAGGTGCTTCATATCCCTCACTCCCGTA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          GCTGTCCATGCTGGATCTGTCCCCGTTGTTTTTAAAAAGCTAAATAAGTTATTAACATGA
AFP-ccGH(T)     ------------------------------------------------------------

S65567          CTGCATCCAGCGAGCCAAACCTGTCTGGTGTACAGCTACCAGAGAAGCTT
AFP-ccGH(T)     -------------------------------------------------
```

Fig. 5. Alignment of sequencing result for PCR product from F_2 channel catfish, channel catfish, transgenic for catfish growth hormone gene driven by the ocean pout antifreeze protein promoter (AFP-ccGH (T)), with antifreeze protein {promoter} of ocean pouts (GenBank accession no. S65567), generated by Clustal Omega. Identities and similarities are indicated by star (*) and gaps are indicated by dashes (-).

```
M24113          TTTGATGAAAATCGCTTAGGCCTTGCTCTTCAAACAATCCAGCTTCTCCTTCTTTCACTC
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCAAGTTGCAAGAAGCAAGTGTAGCAATGTGCACGCGACAGCCGGGTGTGTGACGCTGGA
βA-GAD65(T)     ------------------------------------------------------------

M24113          CCAATCAGAGCGCAGAGCTCCGAAAGTTTACCTTTTATGGCTAGAGCCGGCATCTGCCGT
βA-GAD65(T)     ------------------------------------------------------------

M24113          CATATAAAAGAGCGCGCCCAGCGTCTCAGCCTCACTTTGAGCTCCTCCACACGCAGCTAG
βA-GAD65(T)     --------------CGATCGGTCTA----CCAGCATGGTTCCCCCTCCACACAATTATAA
                              **  * *  *     **    *     * *********     **

M24113          TGCGGAATATCATCTGCCTGTAACCCATTCTCTAAAGTCGACAAACCCCCCCAAACCTAA
βA-GAD65(T)     GTCACCATTTC-------------------------------AAACCCAACCAGTACTTT
                  *   ** **                                ******  ***   **

M24113          GGTGAGTTGATCTTTAAGCTTTTTACATTTTCAGCTCGCATATATCAATTC-GAACGTTT
βA-GAD65(T)     --TCACCAGTTATTAAAAA--CTGACTACGTTACCTTACGTTTAGTAACGCGTTAATAGT
                  * *   * * ** **     * **    * * **  * * **  **  *   *    *

M24113          AATTAGAATGTTTAA---ATAAAGCTAGATTAAATGATTAGGCTCAGTTACCGGTCTTTT
βA-GAD65(T)     TAATAAAATGATTGAATAATCAGCCTAAATGAAAGACTGAAGGGCTGCACACTGCGCTAT
                 * ** **** ** *   ** *  *** ** ***   * * *  * *    * *   * *

M24113          TTTTCTC-ATTTACGTGCGAACTCTGCTTAAACTCTAGTTATTCTTTATTAATATGTGGT
βA-GAD65(T)     TTTTCACTACAGTTGGTTAAAGTGAGCTAACAGACGTGTCTTTGATCACTT------CGA
                ***** * *     *    ** *  *** * *  *  **  **  * * *        *
```

```
M24113          TATTTTTATATATGTATGTTATCATAACTGTA-CTGGCTATGTCAGGTGGTAATGACTGT
βA-GAD65(T)     TAT-CACATGAGAGAATATTAGACTCAAGTTACCGGCAGATGAATGCTGGTTAGCCGGCT
                ***    **    * ** ***   * *   ** * *   ***   * **** *      *

M24113          AACGTTACGTTACTCGTTGTAGGCACGACATTGAATGGGCCGGTGTTGAAATAAGTCTTC
βA-GAD65(T)     AACGTTAGCTAA-----AGTAGGTCAC------------ACCCTGCTGAAACA-------
                *******  * *      *****                *  ** ***** *

M24113          AACCCCTTTTAACCTCAAAATGTGCTCTGGTTAACAAGGATTTTAACAGCTATCAGTATG
βA-GAD65(T)     ------------------------------------------------------------

M24113          ACTGTGCGGTTTTAAAGCCGTTAGTGAGGCACGTTGCACACTTGATGGATGGCCGGAATG
βA-GAD65(T)     ------------------------------------------------------------

M24113          GGAAGTTCTTTATGCAGGCAGTGCTGCAGCAGGGTGTGACCTACTTTAGCTAACGTTAGC
βA-GAD65(T)     ------------------------------------------------------------

M24113          CGGCTAACCAGCATTCATCTGCCGGTAACTTGAGTCTAATATTCTCTATGTGATATCGAA
βA-GAD65(T)     ------------------------------------------------------------

M24113          GTGATCAAAGACACGTCTGTTAGCTCACTTTAACCAACTGTAGTGAAAAATAGCGCAGTG
βA-GAD65(T)     ------------------------------------------------------------

M24113          TGCAGCCCTTCAAGTCTTTCATTTAGGCTGATTATTCAATCATTTTATTAACTATTAACG
βA-GAD65(T)     ------------------------------------------------------------

M24113          CGTTACTAAACGTAAGGTAACGTAGTCAGTTTTTAATAACTGGTGAAAAGTACTGGTTGG
βA-GAD65(T)     ------------------------------------------------------------

M24113          GTTTAAATGGTGACTTATAATTGTGTTGGAGGGGGAAACCTTTTTGATAAAGGCTATATA
βA-GAD65(T)     ------------------------------------------------------------

M24113          ATCTCAAATGAATGGGCTGAGGATGGTGTTCACAGGTGCTTTAGTGAAGTCCGCTCGTGA
βA-GAD65(T)     ------------------------------------------------------------

M24113          AGAGTCGCTGAAGTGACTGCAGATCTGTAGCGCATGCGTTTTGGCAGACGGCCGTTGAAA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTCGGTTGAGTAATTGATACCAGGTGAGGCTAGAGGATGTAGAAATTCATTTGTGTAGAA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTTAGGGAGTGGCCTGGCGTGATGAATGTCGAAATCCGTTCCTTTTTACTGAACCCTATG
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCTCTGCTGAGTGCCACACCGCCGGCACAAAGCGTCTCAAACCATTGCCTTTTATGGTAA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TAATGAGAATGCAGAGGGACTTCCTTTGTCTGGCACATCTGAGGCGCGCATTGTCACACT
βA-GAD65(T)     ------------------------------------------------------------

M24113          AGCACCCACTAGCGGTCAGACTGCAGACAAACAGGAAGCTGACTCCACATGGTCACATGC
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCACTGAAGTGTTGACTTCCCTGACAGCTGTGCACTTTCTAAACCGGTTTTCTCATTCAT
βA-GAD65(T)     ------------------------------------------------------------
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M24113          TTACAGTTCAGCCATGGATGATGAAATTGCCGCACTGGTTGTTGACAACGGATCCGGTAT
βA-GAD65(T)     ------------------------------------------------------------

M24113          GTGCAAAGCCGGATTCGCTGGAGATGATGCTCCCCGTGCCGTCTTCCCATCCATCGTGGG
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCGCCCCAGACATCAGGTGAGAAACTGAGGATTAATTTCAGGCTGCCCAATAATTTGGAA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTTTTTTTTTTTAACTTCCTAGTTCCTTAATGTTTATCTAATTAACACTTGTCTTGTTAC
βA-GAD65(T)     ------------------------------------------------------------

M24113          AGGGTGTCATGGTTGGTATGGGACAGAAGGACAGCTACGTTGGTGATGAGGCTCAGAGCA
βA-GAD65(T)     ------------------------------------------------------------

M24113          AGAGAGGTATCCTGACCCTGAAGTACCCCATCGAGCACGGTATTGTCACCAACTGGGATG
βA-GAD65(T)     ------------------------------------------------------------

M24113          ACATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGCGTGTCGCCCCAGAGGAGC
βA-GAD65(T)     ------------------------------------------------------------

M24113          ACCCCGTCCTGCTCACAGAGGCCCCCCTGAACCCCAAGGCCAACAGGGAAAAGATGACAC
βA-GAD65(T)     ------------------------------------------------------------

M24113          AGGTTGGTTTTTGGCTGGCGACTGGTGCTTGGAAGTCTCTTGTCTGTCCTGTTACCTCAT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTTAAGTTCTCCTTTTCATTCACTTCATCCAGGCTTTGTTCTCTGAGCTCCTGAGTTTCT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCCTCTTTTGCTGGAAGGCAGGTTATCTATACATTGCCTGCTTGTTTTGCAGTTTTGACA
βA-GAD65(T)     ------------------------------------------------------------

M24113          CACCTATTCTTTCTGCACTTATTTCTTTACTCCGGATATTCAACTAACCTCTGCATGGAT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTGATGGATGGTGTGCTGTAGCTGTTTTTAGCATAAAGTTAACTTCTCAAACGCTCTCTT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCCAGATCATGTTTGAGACCTTCAACACCCCCGCCATGTACGTTGCCATCCAGGCTGTGC
βA-GAD65(T)     ------------------------------------------------------------


M24113          TGTCCCTGTATGCCTCTGGTCGTACCACTGGTATCGTGATGGACTCTGGTGATGGTGTCA
βA-GAD65(T)     ------------------------------------------------------------

M24113          CCCACACTGTGCCCATCTACGAGGGTTACGCCCTGCCCCATGCCATCCTCCGTCTGGACT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TGGCTGGCCGTGACCTGACAGACTACCTCATGAAGATCCTGACCGAGAGAGGCTACAGCT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCACCACCACAGCCGAGAGGGAAATTGTCCGTGACATCAAGGAGAAGCTCTGCTATGTGG
βA-GAD65(T)     ------------------------------------------------------------
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M24113          CTCTTGACTTCGAGCAGGAGATGGGCACTGCTGCTTCCTCCTCCTCCCTGGAGAAGAGCT
βA-GAD65(T)     ------------------------------------------------------------

M24113          ATGAGCTGCCTGACGGACAGGTCATCACCATTGGCAATGAGAGGTTCAGGTGCCCAGAGG
βA-GAD65(T)     ------------------------------------------------------------

M24113          CCCTGTTCCAGCCATCCTTCTTGGGTAGGTATCCTGACAAACACTTCCTGGTCTGTGTAT
βA-GAD65(T)     ------------------------------------------------------------

M24113          GTACTTCTAGATTTTAGTTGGAGAACATCTGAAGAACTTTTCATCTTCCTTTTGCAGGTA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TGGAGTCTTGCGGTATCCATGAGACCACCTTCAACTCCATCATGAAGTGTGACGTCGACA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCCGTAAGGACCTGTATGCCAACACTGTATTGTCTGGTGGTACCACCATGTACCCTGGCA
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTGCTGACAGGATGCAGAAAGAGATCACATCCCTGGCCCCCAGCACAATGAAAATCAAGG
βA-GAD65(T)     ------------------------------------------------------------

M24113          TGAGCTAGGCCTTGAGCTATGACTGTTACCTCTCCTATCAGTCTAATAACTGAACATTTG
βA-GAD65(T)     ------------------------------------------------------------

M24113          TCAGCTCTGCATCTTGATAATCGTTCATCTCTCTCTACAGATCATTGCCCCACCTGAGCG
βA-GAD65(T)     ------------------------------------------------------------

M24113          TAAATACTCTGTCTGGATCGGAGGTTCCATCCTGGCCTCCCTGTCCACCTTCCAGCAGAT
βA-GAD65(T)     ------------------------------------------------------------

M24113          GTGGATTAGCAAGCAGGAGTATGATGAGTCTGGACCATCCATCGTCCACCGCAAATGCTT
βA-GAD65(T)     ------------------------------------------------------------

M24113          CTAAACGGACTGTTACCACTTCACGCCGACTCAACTGCGCAGAGAAAAACTTCAAACGAC
βA-GAD65(T)     ------------------------------------------------------------

M24113          AACATTGGCATGGCTTTTGTTATTTTTGGCGCTTGACTCAGGATCTAAAAACTGGAACGG
βA-GAD65(T)     ------------------------------------------------------------

M24113          CGAAGGTGACGGCAATGTTTTGGCAAATAAGCATCCCCGAAGTTCTACAATGCATCTGAG
βA-GAD65(T)     ------------------------------------------------------------

M24113          GACTCAATGTTTTTTTTTTTTTTTTTCTTTAGTCATTCCAAATGTTTGTTAAATGCATT
βA-GAD65(T)     ------------------------------------------------------------

M24113          GTTCCGAAACTTATTTGCCTCTATGAAGGCTGCCCAGTAATTGGGAGCATACTTAACATT
βA-GAD65(T)     ------------------------------------------------------------


M24113          GTAGTATTGTATGTAAATTATGTAACAAAACAATGACTGGGTTTTTGTACTTTCAGCCTT
βA-GAD65(T)     ------------------------------------------------------------

M24113          AATCTTGGGTTTTTTTTTTTTTTGGTTCCAAAAAACTAAGCTTTACCATTCAAGATGTA
βA-GAD65(T)     ------------------------------------------------------------
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M24113          AAGGTTTCATTCCCCCTGGCATATTGAAAAAGCTGTGTGGAACGTGGCGGTGCAGACATT
βA-GAD65(T)     ------------------------------------------------------------

M24113          TGGTGGGGCCAACCTGTACACTGACTAATTCAAATAAAAGTGCACATGTAAGACATCCTA
βA-GAD65(T)     --------------------CTGCCTGCATAATATATAAA--------------------
                                    *** **   * * *** **

M24113          CTCTGTGTGATTTTTCTGTTTGTGCTGAGTGAACTTGCTATGAAGTCTTTTAGTGCACTC
βA-GAD65(T)     ------------------------------------------------------------

M24113          TTTAATAAAAGTAGTCTTCCCTTAAAGTGTCCCTTCCCTTATGGCCTTCACATTTCTCAA
βA-GAD65(T)     ------------------------------------------------------------

M24113          CTAGCGCTTCAACTAGAAAGCACTTTAGGGACTGGGATGC
βA-GAD65(T)     ---------------------------------------
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Fig. 6. Alignment of sequencing result for PCR product from F_2 channel catfish, **channel catfish,** transgenic for goldfish glutamate decarboxylase 65 gene driven by the carp β-action promoter (βA-GAD65 (T)), with common carp β-actin gene (GenBank accession no. M24113), generated by Clustal Omega. Identities and similarities are indicated by star (*) and gaps are indicated by dashes (-).

4. Discussion

All genetic groups of catfish exhibited 100% survival at all combinations of temperature and salinity from 0-9 ºC and 0-7.5 ppt salinity. When the temperature reached - 0.5 ºC, however, heavy mortality occurred, but the lethality of the sub-zero temperature varied depending upon salinity level and genotype. Genotype × environment interactions were apparent when survival rates of the different genetic groups were compared among salinity levels at - 0.5 ºC (Fig. 4).

Once sub-freezing temperatures were reached in the current study, massive mortality occurred for most genotypes. A salinity level of 2.5 ppt was ideal for counteracting the - 0.5 ºC temperature, and survival was very high at this salinity. Changes of 2.5 ppt up or down from this point decreased the ability of the fish to cope with the sub-zero temperature and survive. AFP promoter-ccGH transgenic channel catfish exhibited the best survival at - 0.5 ºC at 0-2.5 ppt salinity.

Poor survival at - 0.5 ºC, particularly at extreme ends of the examined salinities for control and most genotypes of catfish would likely be due to osmoregulatory failure at the extreme low temperature or the initiation of freezing of blood or other cell types. At - 0.5 ºC and 0 ppt salinity, virtually all fish died except for the GH- transgenic fish with the AFP promoter. GH plays a role in osmoregulation (Sangiao-Alvarellos et al., 2005; Varsamos et al., 2005; Sakamoto and McCormick, 2006; Hallerman et al., 2007; Almeida et al., 2013), including channel catfish (Tang et al., 2001). The ability AFP-ccGH transgenic channel catfish to survive under these conditions is likely because of increased production and activity of GH driven by the AFP promoter that was functional under cold conditions compared to the host fish's GH promoter (Miao, 2001). To definitively support this hypothesis, survival of GH-channel catfish with different promoters needs to be compared to elucidate the relative importance of promoter function in producing this increased survival. Regulation of the AFP protein is complex, but it does include a cold temperature component (Miao, 2001) thus, utilization of this promoter may allow expression of genes at lower than

normal temperatures. The relationship of promoter function and the GH transgene to extreme temperature susceptibility needs further exploration.

When salinity was raised to 2.5 ppt, most of the fish were rescued from the mortality at - 0.5 ºC, with all genetic groups having 75-100% survival except the CB hybrids, which only had 17.8% survival. Hence, there was no heterosis for cold tolerance at - 0.5 ºC, in contrast to other survival traits (Dunham et al., 2008). Hybrid catfish probably would not perform well during the winter in more northerly climates. Blue catfish should be tested under these conditions to better understand the lack of (and perhaps negative) heterosis of hybrids for subzero cold tolerance.

As the salinity increased to 5 ppt, genotype × environment interactions continued. Survival dramatically decreased for all genotypes, except that βA-GAD65 transgenics still had high survival. There are potential explanations that might explain why fish overexpressing glutamate decarboxylase or having altered production of GABA could have an advantage when facing decreasing temperatures and osmotic stress. In the case of plants, cold stress, heat stress, osmotic shock and other stressors up-regulate GAD expression (Lee et al., 2010). This phenomenon has not been studied in fish, thus we do not know the response of native catfish βA-GAD65 to these stressors. The cold-shock challenge of the plants was limited to 4ºC, higher than the minimum temperature observed in our catfish experiment.

In response to decreasing temperature, one common homeostatic strategy is to increase tissue enzymatic activity by inducing synthesis of more copies of the enzyme

in order to compensate for the effects of low temperature on enzymatic rates (Somero, 2004). Fish under freezing conditions exhibit a comatose state and become inactive. The β-actin promoter for βA-GAD65 transgenic channel catfish may allow continued production of glutamate decarboxylase even though there is the possibility that the native catfish βA-GAD65 promoter is off due to the severe cold. Due to its constant expression across high, normal and cold temperatures, β-actin was used as the reference gene in common carp gene expression studies for cold acclimation (Gerlach et al., 1990) and in freeze-resistance studies for rainbow smelt *Osmerus mordax* (Liebscher et al., 2006). The role of β-actin promoter in response to cold stress should be more thoroughly studied in fish as actin genes are up-regulated in northern house mosquitos that are cold-stressed (Kim et al., 2006).

Presumably, goldfish GAD can function at extreme cold since goldfish are active under the ice (Whoriskey and Brown, 1994), and can tolerate a wide range of temperatures (Whoriskey and Brown, 1994; Sardana et al., 2006). Under cold anoxic conditions, the goldfish brain activates ion channel arrest in order to reduce ion flux through the release of inhibitory g-aminobutyric acid (GABA) and the upregulation of GABAA receptors (Lutz and Nilsson, 1997). βA-GAD65-transgenic catfish may also activate channel arrest to reduce ion flux in a similar manner in order to adapt in a cold, but aerobic environment, and this possibility should be explored in the future.

Water intake of 200g Japanese eel *Anguilla japonica* (a catadromous species) in seawater was inhibited by intracranial injection of a variety of proteins including

GABA. The inhibitory effects were dose-dependent (Kozaka et al., 2002). If βA-GAD65-transgenic channel catfish were able to produce more GABA at the sub-freezing temperatures, it may have helped them to survive the combination of cold and high salinity compared to the non-transgenic controls.

At 5 ppt, non-transgenic full-siblings of the AFP-GH family had 21% survival, which was a bit higher than that of their transgenic full-siblings. All other control (channel catfish, hybrid catfish, and βA-GAD65 (C)) died. This result may be indicative of family or strain effects for cold and salinity tolerance in channel catfish. This is not a surprising result as strain and family effects on relative genetic gain have been observed in all genetic enhancement programs in each case when strain and family effects were measured (Dunham, 2011; Dunham et al., 2014a, 2014b). Heritabilities should be estimated; perhaps tolerance of these adverse conditions could be improved through selection. No fish were able survive 7.5 ppt coupled with sub-zero temperatures.

The genetic differences and genotype interactions for tolerance of freezing temperature did not appear to be affected by differing body sizes of the respective genetic groups, which were significantly different in the current study. Larger fish of various species sometimes tolerate temperatures extremes more effectively than smaller fish (Charo-Karisa et al., 2005; Recsetar et al., 2012; Wuenschel et al., 2012). However, trends were variable, and the most common result was no relationship between temperature and body weight (Ospina and Mora, 2004; Recsetar et al., 2012)

including tolerance of high temperature for channel catfish, as the relationship between temperature tolerance and body weight appears to be species dependent.

Our results indicated that any relationship between body size and tolerance of freezing temperatures in channel catfish did not affect survival. Non-transgenic channel catfish and hybrid catfish were the largest, yet had the poorest combinations of cold and salinity tolerance compared to the transgenic genotypes. The growth hormone-transgenic and βA-GAD-transgenic channel catfish were larger than full-sibling, non-transgenic controls and smaller than the unrelated channel catfish control groups, yet still had equal or greater combinations of cold and salinity tolerance across environments.

Our results open the possibility that there might be positive applications of the AFP promoter and/or the GH transgene, which could be highly beneficial to the aquaculture industry in countries with freezing or sub-zero and brackish water conditions, although the AFP-ccGH transgene only conferred survival advantage below 2.5 ppt salinity at -0.5 C. Transgenic fish may benefit aquaculture production in the future if they are adaptable to extreme environmental conditions such as cold. However, altering the temperature tolerance of a species of fish would potentially allow it to expand its geographic range, similar to release of an exotic species. The environmental risk and its management must be considered. Because of such concerns about transgenic aquatic organisms, research on food safety and potential environmental impact; including the measurement of fitness traits such as predator

avoidance and reproduction, are needed to allow for informed decisions on the risk of utilizing specific transgenic fish (Hallerman and Kapuscinski, 1990, 1995). The first transgenic fish to be commercially applied (GloFish, fluorescent zebrafish) was for ornamental purposes. Recently, triploid GH-transgenic Atlantic salmon embryos were approved in Canada for export (Fisheries and Oceans Canada, 2013 (http://www.dfo-mpo.gc.ca/csas-sccs/Publications/ScR-RS/2013/2013_023-eng.pdf); Dunham and Winn, 2014) and in the United States for human consumption (FDA, 2015). Applications for approval and commercialization of other transgenic aquaculture species are pending in China and Cuba. The decision of the United States regarding the regulation of transgenic fish may influence how other countries or international bodies such as the Food and Agriculture Organization or World Health Organization approach the issue.

Climate change with variable weather patterns, changes in sea level and thus, changes in brackish water areas and their salinities, are predicted for the future. Catfish genetic variation and catfish genetic enhancement programs may be applied with the aim of helping farmers cope with production problems that may be associated with these climate changes. The results of this study may also be relevant to other fish species, both in aquaculture and in the natural environment in the future.

To face human population growth expected by 2050 with a concentration in coastal urban areas, we must meet the huge challenge of feeding our planet while

safeguarding its natural resources for future generations (FAO, 2014). Wise use of transgenic fish could contribute to solving the problem.

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