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Developmental responses to abiotic conditions during aquatic and air incubatoin of the Gulf killifish (Fundulus grandis): subtitle

Charles Alexander Brown
Louisiana State University and Agricultural and Mechanical College

DEVELOPMENTAL RESPONSES TO ABIOTIC CONDITIONS DURING AQUATIC AND AIR INCUBATION OF THE GULF KILLIFISH (*FUNDULUS GRANDIS*)

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Charles A. Brown
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ABSTRACT

The Gulf killifish (*Fundulus grandis*) is a topminnow native to the tidal marshes of the Gulf of Mexico. The species is prized by anglers as effective bait for catching popular sportfish and is of interest to aquaculturists and bait dealers due to its hardy nature. Limited seasonal availability and aquaculture of this species due to low fecundity and larval cannibalism of fry has led to increased incentive to improve cultural techniques. The purpose of this study is to determine the influences of temperature, salinity, and air incubation on embryogenesis of the Gulf killifish.

Temperature was found to have a negative relationship with time to hatch and size at hatch when *F. grandis* eggs were incubated in 10 and 20 g/L saline treatments. Temperature did not significantly affect percent hatch at 10 g/L, but percent hatch displayed a positive relationship with temperature at 20 g/L. When incubated in 0.4, 7, 15, and 30 g/L salinity treatments, a negative relationship between salinity and rate of embryogenesis was detected. While larvae incubated in these salinities hatched at about the same size, reduced hatching percentages were reported in the 0.4 and 30 g/L treatments. A positive relationship between the rate of embryogenesis and temperature was observed in air incubated embryos. After reaching a stage of development when embryos are documented to hatch, temperature was observed to have a negative relationship with the extent of time hatch could be delayed. The results of this study can be used to improve culture practices for Gulf killifish. Larval size at hatch, percent hatch, and time to hatch can now be predicted across a range of temperatures and salinities. Warmer temperatures can be used to accelerate embryogenesis of air incubated embryos while colder temperatures can be used to extend the period of time hatch can be delayed.

CHAPTER 1: INTRODUCTION

Marine baitfish aquaculture is a relatively new industry compared to the well-established freshwater baitfish aquaculture industry. Salt water anglers spent approximately $11 billion in the marine fishing industry in the southern region of the United States in 2006 (Southwick Associates 2007). Currently, the majorities of marine baitfishes supplied to anglers from bait dealers are wild caught and are subject to inconsistent availability and size (Oesterling et al. 2004). Gulf killifish (Actinopterygii, Cyprinodontiformes, *Fundulidae, Fundulus grandis*) has been identified as one of the top candidate species for cultruing in the emerging marine baitfish aquaculture industry (Oesterling et al. 2004).

The Gulf killifish is prized by anglers as an effective bait for catching popular sportfish and is of interest to culturists and bait dealers due to its hardy nature. The culture of this species has been developed through a number of investigations over the last twenty-five years involving the collection of spawning mats in brood ponds for transfer to nursery ponds (Tatum et al. 1982, Waas et al. 1983, Perschbacher and Strawn 1986). The mat transfer method is effective for this fractional spawning species; however, small batches of eggs are continuously fertilized throughout a protracted period increasing the opportunity for different hatch times and increased size variance within cohorts. Investigations of Gulf killifish reproduction have characterized fecundity at approximately 0.9 eggs per g female per day, which is low compared to the fathead minnow *Pimephales promelas* at a daily rate of approximately 6 eggs per g female (Clemment and Stone 2004, Landry et al. 2007, Green et al. 2010). The incubation period for Gulf killifish embryos has been observed to range between 14 and 25 days at a salinity of 15 g/L (Perschbacher et al. 1990). This results in precocial larvae with a considerable degree of morphological development occurring prior to hatch (Armstrong and Child 1965, Noakes and

Godin 1988). The wide range in time-to-hatch (TH) is a concern due to the extent of development within this species and the resulting effects of potential cannibalism. Fishes with protracted spawning seasons and/or high TH variance are particularly vulnerable to intracohort cannibalism, leading to significant losses in aquaculture or potential aquaculture species during early life history stages (Fessehaye et al. 2006, Puvanendran et al. 2008). These problems have prevented large-scale Gulf killifish operations from reaching full economic potential.

Gulf killifish are commonly found in tidal estuaries and coastal areas throughout the Gulf of Mexico and southern Atlantic United States. There are a variety of regional names for this species such as: cocahoe minnow, mudminnow or bullminnow (Tatum et al. 1982, Oesterling et al. 2004). Gulf killifish are able to tolerate many diseases, a wide range of salinities (from 0.5 g/L up to 76.1 g/L), and temporarily, low concentrations of dissolved oxygen (Tatum et al. 1982, Oesterling et al. 2004, Nordlie 2006). Within their natural environment Gulf killifish are reported to spawn throughout the summer months, with peaks observed in early summer and late fall (Nordlie 2000). Spawning events are timed to semilunar tidal cycles when eggs are deposited at the high water mark of marsh grasses during spring tide and are exposed to air once the tide recedes (Taylor et al. 1977, Lipcius and Subrahmanyam 1986). During this period, referred to as air incubation, embryogenesis occurs at an accelerated rate compared to embryos incubated in typical aquatic conditions (Martin 1999, Tingaud-Sequeira et al. 2009). Embryos decrease the rate of development upon reaching a stage of embryogenesis where hatching would occur during aquatic incubation, and remain in the chorion until hatch is initiated by submersion in the next high tide (Dimichele and Taylor 1980, Martin 1999).

Generally, embryos are known to have periods of increased vulnerability to abiotic factors such as temperature, salinity, and low dissolved oxygen (Petereit et al. 2008). Many

marine teleost species are vulnerable to extremes of environmental osmotic pressure prior to gastrulation (Laurence and Rogers 1976). Osmotic pressure has also been determined as an abiotic factor affecting rate of embryogenesis, resulting in diversion of energy to maintenance of osmotic balance that otherwise could be invested in growth (Sampaio and Bianchini 2002). Temperature is also an important factor in poikilotherm developmental rates. Colder temperatures may decrease the rate of embryogenesis but may lead to increased mortalities if below physiologically tolerable ranges (Small and Bates 2001, Lin and Lu 2006). Warmer temperatures may accelerate embryogenesis, but temperatures outside of tolerable ranges can lead to morphological deformities and increased mortalities (Laurence and Rogers 1976, Das et al. 2006).

Nitrogenous metabolic waste in the form of ammonia (NH_3) is produced as yolk proteins are catabolized for growth and development (Mommsen and Walsh 1992; Randall and Tsui 2002). Ammonia is toxic due to its ability to upset biochemical reactions, raise cellular pH, and disrupt transcellular ion movments (Walsh 1998). Ammonia toxicity may cause mortalities at lethal concentrations and delay embryogenesis at sublethal concentrations (Mommsen and Walsh 1992). Urea is another nitrogenous metabolite produced as a byproduct of arginine catabolism, purine catabolism, and the ornithine urea cycle (OUC; Walsh 1998). Urea production through the OUC is prominent in elasmobranchs and a few mature teleosts, and is believed to occur in larval teleosts, with the genes involved in the cycle silenced as the larvae mature (Walsh 1998). Ammonia and urea production as metabolic outputs are important components in incubation studies since the amount of nitrogenous waste produced can serve as a rough estimate of metabolic activity (Essex-Fraser et al. 2005). Measuring release of nitrogenous waste throughout

development may provide insight into effects of temperature, salinity, and air incubation on embryonic development.

The majority of Gulf killifish supplied to anglers originate from wild harvest with availability dependent on seasonal population fluctuations (Fivizzani and Meier 1978, Nordlie 2000, Oesterling et al. 2004). Seasonality (Nordlie 2000) and unreliability of wild harvests have encouraged stronger emphasis on the development of practical aquaculture techniques for the Gulf killifish. However, optimization of large scale Gulf killifish production practices has been inhibited by low fecundity and the high rate of size heterogeneity at hatch. The purpose of this research was to determine how abiotic factors can be used to manipulate Gulf killifish embryogenesis. Experiments controlling size heterogeneity of fry have been shown to significantly limit intracohort cannibalism (Baras and Jobling 2002, Fessehaye et al. 2006). Knowing how temperature and salinity incubation parameters influence embryogenesis can result in better estimation of fry conditions at hatch. Manipulation of these parameters could allow for greater control of time to hatch and size at hatch.

CHAPTER 2: EFFECTS OF TEMPERTURE AND SALINITY DURING INCUBATION ON HATCHING AND YOLK UTILIZATION[1]

2.1 Introduction

Gulf killifish (*Fundulus grandis*) are a popular baitfish commonly found in tidal estuaries and coastal areas throughout the Gulf of Mexico and southern Atlantic United States. There are a variety of regional names for this species, such as: cocahoe minnow, mudminnow, or bullminnow (Tatum et al. 1982, Oesterling et al. 2004). It is prized by anglers as an effective bait for catching popular sportfish and is of interest to culturists and bait dealers due to its ability to tolerate disease, a wide range of salinities (from 0.5 g/L up to 76.1 g/L) and temporarily low dissolved oxygen (DO; Tatum et al. 1982, Oesterling et al. 2004, Nordlie 2006). The majority of Gulf killifish supplied to anglers originate from wild harvest with availability dependent on seasonal fluctuations in populations (Fivizzani and Meier 1978, Nordlie 2000, Oesterling et al. 2004). Investigations of Gulf killifish reproduction have characterized fecundity at approximately 0.9 eggs per g female per day, which is low compared to the fathead minnow (*Pimephales promelas*) at a daily rate of approximately 6 eggs per g female (Clemment and Stone 2004, Landry et al. 2007, Green et al. 2010). Within the natural environment Gulf killifish are reported to spawn throughout the summer months, with peaks observed in early summer and late fall when water temperatures are between 19 and 24°C (Greeley and Macgregor 1983, Nordlie 2000).

The ability to alter abiotic conditions, such as temperature or salinity, during Gulf killifish embryogenesis could assist in coordinating time-to-hatch and size-at-hatch. Fishes with protracted spawning seasons and/or high time-to-hatch variance are particularly vulnerable to intracohort cannibalism, leading to significant losses in aquaculture or potential aquaculture

[1] Reprinted by permission of Aquaculture

species during early life history stages (Fessehaye et al. 2006, Puvanendran et al. 2008). Experiments controlling size heterogeneity of fry have been shown to significantly limit intracohort cannibalism (Baras and Jobling 2002, Fessehaye et al. 2006). Previous research investigating the effects of salinity on egg hatch and survival for Gulf killifish has shown that the greatest number of viable embryos resulted from incubation at salinities between 15 and 35 g/L (Perschbacher et al. 1990). During embryogenesis there is increased vulnerability to abiotic factors such as temperature, salinity, and DO (Petereit et al. 2008). Salinity affects embryogenesis due to the diversion of energy from growth to the maintenance of osmotic balance (Sampaio and Bianchini 2002). Temperature impacts poikilotherm developmental rates in several ways. Lower than optimal temperatures result in embryos developing at slower rates (Small and Bates 2001, Lin et al. 2006), while higher than optimal temperatures may result in embryos develop faster (Das et al. 2006). However, sub-optimal conditions can increase the occurrence of fatal deformities and mortalities as an effect of stress caused by altered developmental timing (Laurence and Rogers 1976, Lindén et al. 1979, Das et al. 2006). The influences of temperature on hatch viability and size at hatch have not been documented in Gulf killifish.

The objective of the current study was to investigate the ability to alter incubation period in Gulf killifish. Specifically, this study characterizes the effects of temperature and salinity on embryo survival, incubation period, and size at hatch to determine optimal incubation environments. Morphometric parameters related to yolk content at hatch were also measured in addition to embryo length at hatch. Modifications of temperature and salinity could allow for altered incubation duration and size at hatch leading to better cohort management.

2.2 Materials and Methods

Animals and Sourcing of Gametes

Twelve females from a population maintained in a recirculation system at 24°C and 8.8 g/L salinity were strip spawned by gently placing pressure on both sides of the abdominal cavity with resulting eggs extruded into a plastic container. Approximately 450 and 240 eggs were obtained for 10 and 20 g/L salinity treatments, respectively. Four males were euthanized by cervical dislocation and pithing prior to surgical removal of the testes. Testes from each male were combined and malleated in 10 ml of Hanks balanced saline solution (350 mOsmol/L at 24°C). The resulting suspension was mixed with eggs with a pipette. Water from a separate recirculation system maintained at a salinity of 15 g/L was added to activate spermatozoa. Eggs were allowed to water harden for 15 min prior to placement in a 2 g/L solution of sodium sulfite for at least 5 min to decrease egg adhesion (Stone and Ludwig 1993).

Two salinity trials were run sequentially, the first at 10 g/L followed by a 20 g/L trial. In each trial, triplicate groups of eggs (n=30 for 10 g/L; n=20 for 20 g/L) were placed in containers of 50 mL of the salinity treatment water. Incubation chambers were set to 20, 21.6, 23, and 29°C and controlled using an externally adjustable thermostat (Glenn and Tiersch 2011). Each was fitted with Dickson SK100 data loggers (Dickson, Addison, IL, USA) set to record temperatures hourly. Eggs were checked a minimum of twice daily (0900 and 1700 hours) for the presence of newly hatched individuals. If larvae were present, they were enumerated, removed and placed in 10% neutral buffered formalin and stored at 4°C prior to analysis.

Water Quality

Treatment water was mixed to salinities of 10 and 20 g/L using Coralife Salt Mix (Energy Savers Unlimited Inc., Carson, CA, USA) and measured with a YSI 30

salinity/conductivity meter (YSI Inc., Yellow Springs, OH, USA). Water quality testing kits (Hach Co., Loveland, CO, USA) were used to test alkalinity and hardness (reported as $CaCO_3$) of the treatment water by titration. An Accumet® Basic AB15 pH meter (Fisher Scientific, Pittsburgh, PA, USA) was used to determine pH. Total ammonia nitrogen (TAN) and nitrite-nitrogen (nitrite) were determined with a Hach DR 4000 Spectrophotometer using the salicylate and diazotization methods, respectively. Alkalinity and hardness were determined at the beginning of each salinity trial while salinity, pH, TAN, and nitrite were measured twice daily. Nitrite levels were not recorded for the second trial due to the large ratio of chloride ions to nitrite. Complete replacement of treatment water in each replicate was conducted twice daily during observations for newly hatched individuals. A reservoir of treatment water (500 mL) was maintained in each incubation chamber to reduce changes in water temperature during water exchanges.

Image Analysis

Images of newly hatched Gulf killifish larvae were captured with a Photometrics Coolsnap *cf* ® camera (Photometrics®, Tuscon, AZ, USA) interfaced with a computer for analysis of morphometric parameters. MetaVue (Molecular Devices, Inc.© , Silicon Valley, CA, USA) software was used to view and save image files. Individuals were placed on their sagittal axis in a solution of glycerol for image capture. The magnification of the microscope was set at 7.5× and a micrometer slide was used for calibrating the images. The Image analysis software Image Tool® (Version 3.0, University of Texas Health Science Center, San Antonio, TX, USA) was used to record total length (TL), depth at vent (DV), yolk length (YL), yolk depth (YD), and yolk area (YA). Yolk volume (YV) was determined with the formula to calculate volume for a prolate spheroid ($YV = (\pi/6)\times YL\times YD^2$).

Statistical Analysis

All tests were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA). Mixed model analysis of variance (ANOVA) in a randomized block design was used to test for variance among replicate trays within incubation chambers. Replicates were set as a fixed effect. If no variance was found among trays, a general linearized model two-way ANOVA in randomized block design was performed to test for significant differences in embryo morphometrics and time-to-hatch (TH; hours) among salinity and temperature treatments. The Ryan-Einot-Gabriel-Welsch (REGWQ) *post-hoc* tests were used for pairwise comparisons among treatment groups. Retrospective power analysis was used to determine an adequate sample size for the 20 g/L trial based on the means ± standard deviation of the 10 g/L treatment variables. Logistic regression procedure was used to determine the frequency of hatch among treatments. Hatch percentages were arcsine transformed and analyzed with general linearized model ANOVA. A correlation procedure was used to test for a relationship between TL and TH. Principal component analysis was used to reveal relationships among morphometric and yolk characteristics. Results are shown as means ± standard error of the mean (SEM). All hypotheses were tested at a significance level of α=0.05.

2.3 Results

Water Quality

The recorded mean salinity among the incubation chambers for the nominal 10 and 20 g/L salinity trials was 9.3 ± 0.05 and 20.9 ± 0.1 g/L, respectively. Mean temperatures for the four incubation chambers during the 10 g/L salinity trial were 19.89 ± 0.02, 21.61 ± 0.02, 22.94 ± 0.06, and 28.34 ± 0.16°C. Mean temperatures for the four incubation chambers during the 20 g/L salinity trial were 20.15 ± 0.02, 21.67 ± 0.01, 23.19 ± 0.05, and 29.14 ± 0.04°C. Remaining

water quality parameters (pH, TAN, nitrite, alkalinity, and hardness) are summarized in Table 2.1.

Table 2.1 Mean water quality parameters ± standard error of the mean. Salinity, pH, total ammonia nitrogen (TAN), and nitrite were tested twice daily. Alkalinity and hardness were tested at the initiation of the trial.

	Nominal salinity (g/L)	
Water quality	10	20
Salinity (g/L)	9.32 ± 0.05	20.94 ± 0.1
pH	8.38 ± 0.01	8.45 ± 0.00
TAN (mg/L)	0.57 ± 0.04	0.57 ± 0.04
Nitrite (mg/L)	0.05 ± 0.00	NA
Alkalinity (mg/L)[2]	119.7	264
Hardness (mg/L)[2]	444.6	3,950

[2]Reported as $CaCO_3$

The number of viable embryos at hatch was independent of temperature for the 10 g/L salinity trial ($P > 0.05$). The highest proportion of viable embryos at hatch was observed in the 20 g/L salinity trial within the 29°C treatment (Table 2.2). The number of viable embryos at hatch was dependent on temperature for the 20 g/L salinity trial ($P \leq 0.05$). A significant positive relationship between the proportion of viable embryos at hatch and temperature was present for the 20 g/L salinity trial ($r^2 = 0.42$, $P \leq 0.05$).

Table 2.2 Mean percent hatch ± standard error of the mean of embryos reared at two different salinities. Superscripts within columns depict significant differences among treatments (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$).

	Salinity (g/L)	
Temperature (°C)	10	20
20	61.1 ± 2.2	58.3 ± 3.3
21.6	73.3 ± 3.3	65.0 ± 10.4
23	76.6 ± 5.0	60.0 ± 7.6
29	77.7 ± 10.0	85.0 ± 8.6

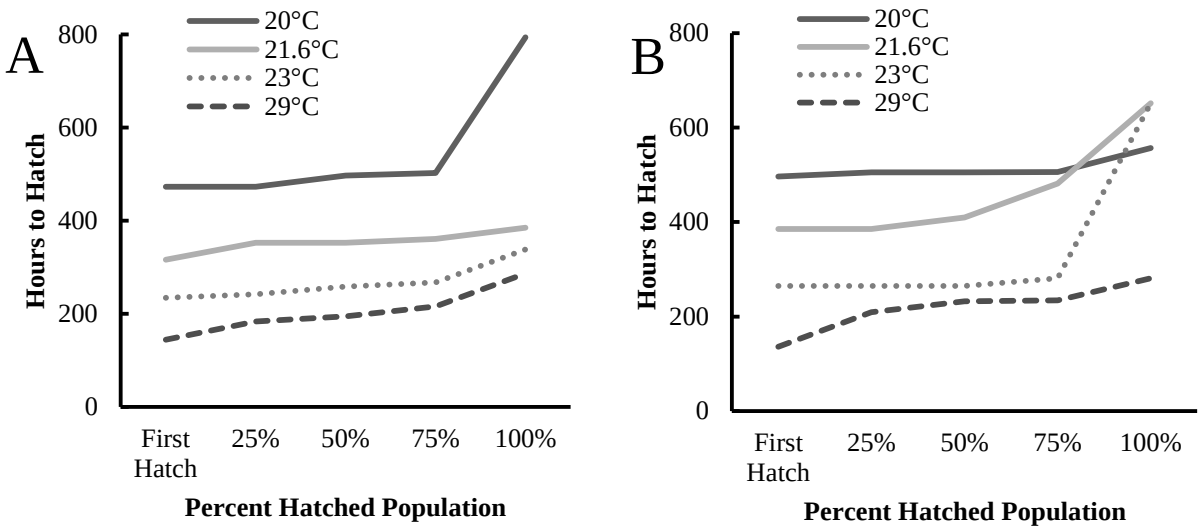

Figure 2.1 Duration of hatch for 10 g/L (A) and 20 g/L (B) salinities. Mean percent duration of hatch (hours) is expressed in a quartile range on the x-axis. Lines depict incubation temperatures: 20, 21.6, 23, and 29°C.

Time-to-hatch

Temperature and salinity had significant effects on TH (salinity, $P \leq 0.01$; temperature, $P \leq 0.01$). Two-way ANOVA indicated that salinity by temperature interactions were significant for TH ($P \leq 0.01$). Hatch durations for salinity and temperature incubation treatments are detailed in Figure 2.1. *Post-hoc* examination of TH demonstrated that embryos incubated at 21.6°C and 23°C in the 10 g/L trial required significantly less incubation time when compared to the 20 g/L trial (Table 2.3). There was no significant difference in incubation time for the 20°C or 29°C treatments at 10 g/L when compared with the 20 g/L trial (Table 2.3).

Size at Hatch

Retrospective power analysis determined that a sample size of 30 embryos per treatment replicate surpassed a power of 0.80 for the 20 g/L trial based upon the mean and standard deviations of size at hatch analysis of the 10 g/L trial. Temperature had a significant effect on TL at hatch during the 10 g/L trial ($P \leq 0.01$) with the 20°C treatment resulting in the longest mean

TL at hatch (Table 2.3). During the 20 g/L trial temperature had a significant effect on TL at hatch ($P \leq 0.01$). *Post-hoc* analysis determined that across all incubation temperatures, mean TL of larvae at hatch reared at 20 g/L were significantly greater than the corresponding temperatures for the 10 g/L salinity trial. Two-way ANOVA showed that salinity by temperature interactions were significant for TL at hatch ($P \leq 0.01$). *Post-hoc* analysis determined that embryos incubated in 10 g/L at 20°C did not have a significant difference in TL from embryos incubated in 20 g/L at 23°C. Regression analysis indicated a positive significant relationship with TH and TL for both salinity trials (10 g/L: $P \leq 0.01$, r^2=0.26; 20 g/L: $P \leq 0.01$, r^2=0.56).

Temperature had a significant effect on BD for both salinity trials (10 g/L, $P \leq 0.01$; 20 g/L, $P \leq 0.01$). Two-way ANOVA showed that salinity by temperature interactions were significant for BD at hatch ($P \leq 0.01$). For both salinity trials, BD was larger at lower temperatures. *Post-hoc* analysis determined that mean BD was significantly greater for every thermal treatment in the 20 g/L trial as compared to the 10 g/L treatment equivalents (Table 2.3).

A PCA of the size at hatch data found a principal component that appeared to measure 92% of the variance of the data. The first principal component is related to the length of the yolk sac, depth of the yolk sac, YA, and YV. Temperature was determined to have a significant effect on YA at hatch (mm^2; $P \leq 0.01$). Temperature also had a significant effect on YV at hatch (mm^3; $P \leq 0.01$) for the 10 g/L salinity trial (Table 2.3) with the larvae reared at 29°C having a significantly greater YV than the other temperature treatments. Temperature did not have a significant effect on yolk morphometrics at hatch for the 20 g/L salinity trial (Table 2.3). Regression analysis indicated a negative significant relationship with TH and YV for both salinity trials (10 g/L: $P \leq 0.01$, r^2=0.17; 20 g/L: $P \leq 0.01$, r^2=0.08).

Table 2.3 Mean morphometric parameters ± standard error of the mean at hatch for embryos reared in varying salinities and temperatures. Columns with different superscript are significantly different (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$).

Salinity (g/L)	Temperature (°C)	Larvae (n)	Total Length (mm)	Body Depth at Vent (mm)	Yolk Volume (mm^3)	Time-to-Hatch (hours)
10	20	68	6.37 ± 0.05^{c}	0.64 ± 0.007^{b}	$0.44 \pm 0.04^{c,d}$	500.4 ± 6.5^{a}
	21.6	69	6.17 ± 0.03^{d}	$0.62 \pm 0.005^{b,c}$	$0.47 \pm 0.04^{b,c,d}$	354.6 ± 1.0^{c}
	23	66	5.96 ± 0.04^{e}	$0.60 \pm 0.005^{c,d}$	$0.51 \pm 0.04^{b,c}$	259.0 ± 2.3^{e}
	29	55	5.88 ± 0.03^{e}	0.57 ± 0.004^{d}	0.77 ± 0.04^{a}	206.9 ± 3.9^{f}
20	20	51	6.86 ± 0.03^{a}	0.70 ± 0.006^{a}	$0.43 \pm 0.08^{c,d}$	508.3 ± 2.3^{a}
	21.6	36	6.69 ± 0.02^{b}	0.69 ± 0.006^{a}	0.41 ± 0.08^{d}	429.7 ± 9.3^{b}
	23	39	$6.25 \pm 0.03^{c,d}$	0.63 ± 0.006^{b}	$0.44 \pm 0.08^{c,d}$	282.1 ± 10.7^{d}
	29	35	6.17 ± 0.03^{d}	$0.62 \pm 0.005^{b,c}$	0.55 ± 0.07^{b}	215.0 ± 5.5^{f}

2.4 Discussion

This study investigated the influence of temperature and salinity on Gulf killifish development. Salinity was determined to change the effect of temperature on embryogenesis of the Gulf killifish. The interaction of temperature and salinity can influence embryogenesis by altering biological responses to abiotic factors typically encountered (Lough and Gonnor 1973, Lough 1975, Ponce-Palafox et al. 1997). Temperature significantly influenced percent hatch in the 20 g/L trial, but did not in the 10 g/L trial, with mortality likely occurring prior to gastrulation when embryos are susceptible to osmotic and thermal pressures (Tay and Garside 1975, Tandler et al. 1995). The 20°C and 21.6°C treatments in the 20 g/L salinity trial were the largest size overall and took the least time to hatch. Lindén et al. (1979) determined that the effect of temperature on development increases as the salinity of the incubation medium increases in the mummichog (*Fundulus heteroclitus*). Incubation of Gulf killifish eggs in higher salinity water and lower temperatures did produce the largest fish. There was overlap of the effects of temperature and salinity on TL where embryos incubated in 10 g/L at 20°C and 21.6°C are similar in size to embryos incubated in 20 g/L at 23°C and 29°C. Within both salinity trials, the two lowest temperature treatments resulted in larvae with the largest TL and BD at hatch and the two highest temperature treatments resulted in larvae with the smallest TL and BD at hatch.

The resulting smaller size at hatch among higher temperature treatments could have resulted from relatively accelerated embryo development and premature hatching. Once development of tissues responsible for the production of hatching enzymes is complete, hatching is independent of stage of development with development continuing after hatch (Lindén et al. 1979, Luczynski and Kolman 1987). Though mortalities are higher for larvae while still in the yolk sac stage, embryos hatch prematurely due to lower oxygen concentrations associated with

increased temperatures (Taggart and Leggett 1987, Yamagami 1988, Martin et al. 2004). Rapidly growing larvae require more oxygen to meet the increasing metabolic demand for increasing biomass (Finn et al. 2002). DiMichele and Taylor (1980) found that high oxygen concentrations inhibited hatching and that hatch occurred for mummichog when oxygen concentrations fell at or below approximately 4.3 mg/L DO. Water at 29°C would not be able to hold as much oxygen as the colder treatments, so it may be advantageous for larvae to hatch prematurely and move to more oxygenated areas to maximize yolk absorption for development (Finn et al. 1995). The transition from yolk sac stage to exogenous feeding behavior may trigger hatching also, as rapid changes in gut structure correspond to the development of feeding behavior (Noakes and Godin 1988).

Rao (1974) found a similar inverse relationship between larval size and yolk volume at hatch when examining the California killifish (*Fundulus parivipinnis*). Atlantic cod (*Gadus morhua*) embryos incubated in warmer temperatures hatched with large amounts of yolk volume, suggesting that temperatures outside the optimal thermal range caused improper development and premature hatching (Jordaan et al. 2006). Temperature has also been determined to influence the level of fat stores in breeding Gulf killifish (Weld and Meir 1983), with the amount of fat stores available affecting yolk deposition (Weigand 1996). The ambient temperature of the parental stock has been shown to affect the optimal incubation temperature for gilthead bream (*Sparus aurata*; Camus and Koustikopoulos 1984). Yolk volume at hatch was not statistically different between the corresponding thermal treatments of the salinity trials except for the 29°C treatment, which could be attributed to an increased amount of energy being invested into metabolic regulation at the higher osmotic pressure (Sampaio and Bianchini 2002).

This experiment demonstrated the influence of temperature and salinity on embryogenesis. Temperature affected critical biological aspects of the Gulf killifish at hatch, such as percent hatch, TH, body size, and yolk volume. The effect of temperature on teleost embryogenesis has been documented for the mummichog (Linden et al. 1979, DiMichele and Westerman 1997) with Gulf killifish in the current study displaying similar developmental responses. While some work has been done on development of the Gulf killifish (Ernst et al. 1977, Greeley and Macgregor 1983, Hsiao and Meier 1986, Perschbacher et al. 1990), little is known about the effect of abiotic factors on the embryology of this species. In contrast, extensive research has been conducted measuring the developmental responses for a closely related species the mummichog (Tay and Garside 1975, Linden et al. 1979, DiMichele and Taylor 1980, DiMichele and Westerman 1997). The results of this study may be used to create developmental markers with morphological traits to compare development of separate cohorts (Martínez-Lagos and Gracia-López 2009). This research can be used to manipulate developmental trajectories to produce large and healthy cohorts and to estimate environmental effects on natural populations.

CHAPTER 3: EMBRYONIC DEVELOPMENT AND DEVELOPMENTAL COSTS OF EMBRYOS EXPOSED TO VARYING ENVIRONMENTAL SALINITIES

3.1 Introduction

Embryonic osmoregulation is important in salt marsh species of teleosts that produce sessile eggs. The Gulf killifish (*Fundulus grandis*) is a euryhaline cyprinodontid native to the coastal salt marshes of the Gulf of Mexico and southern Atlantic coast of the United States (Nordlie 2000, Oesterling et al. 2004). This species is a fractional spawner with a protracted spawning period, peaking in the spring and fall months (Nordlie 2000). Throughout the year, most populations of Gulf killifish reside and spawn in marshes where salinities range from 5 to 39 g/L, with some populations found in entirely freshwater conditions (Simpson and Gunter 1956, Nordlie 2000, Nordlie 2006). While adults have been shown to relocate to more favorable salinities depending on temperature, embryos continue to develop throughout a wide range of salinities (Greeley and MacGregor 1983, Miller et al. 1983).

Recent work on ion regulation and gas exchange at early stages of life-history has demonstrated that the gills of embryonic and larval fish play a more critical role in ion regulation than in gas exchange (for review see Rombough 2007). Developing embryos can rely solely on passive diffusion of oxygen across their body, when the external surface area to biomass ratio is high (Kranenbarg et al. 2000). Osmoregulation is also performed on the body surface due to the localization of ion-transporting cells during early development. Embryonic mummichog (*Fundulus heteroclitus*) use mitochondrion-rich cells (MRCs) located in the basolateral membrane of the yolk sac to actively osmoregulate in hypertonic environments until the gills form and MRCs begin to emerge on the gill surface of embyros immediately before hatch (Evans et al. 1999, Katoh et al. 2000). Despite active regulation of ionic movements, salinity has not been found to influence oxygen demand of mummichog and larval striped mullet *(Mugil*

cephalus; Walsh et al. 1989, Kidder et al. 2006a). Prior to spawning, eggs retain maternal osmotic balance but rapidly adjust to the surrounding osmotic pressure by absorption of water into the perivitelline space upon fertilization (Holliday and Blaxter 1960, Davenport et al. 1981, Finn 2007). Water permeability and passive transfer of osmolites between the embryo and its environment is minimized by the chorion and perivitelline space after water hardening (Alderdice 1988, Finn 2007).

Metabolic byproducts in the form of ammonia and urea are produced during growth, maintenance, and osmotic balance. Ammonia is the primary metabolite produced, however it is highly toxic and must be removed immediately (Mommsen and Walsh 1992, Walsh 1998, Randall and Tsui 2002). Ammonium is eliminated by means of apical Na^+/NH_4^+ exchange in addition to passive diffusion of NH_4^+ through paracellular gap junctions (Wilkie 1997, Wilkie 2002). Ammonia likely passively diffuses out of the gills via a Rhesus glycoprotein, RhCG, which acts to alkalinize the apical gill boundary layer, facilitating the movement of protons out of the cells (Tsui et al. 2009). Conversion of ammonia into urea allows developing embryos an alternative method of toxicity avoidance in comparison to passive diffusion of ammonia across the chorion (Depeche et al. 1979, Wright and Land 1998). Several pathways of urea production and transport exists in teleosts, including arginine catabolism, purine catabolism, and the ornithine-urea cycle (OUC; Walsh 1998). The OUC is the most active pathway of ammonia conversion to urea for embryonic and larval teleosts (Griffith 1991). Urea production via the OUC is energetically costly but may be critical to survival in situations where ammonia may not be readily expelled, such as environments that are alkaline with high buffering capacities or with high external ammonia concentrations (Wood et al. 1989, Walsh 1998). Urea production is also a product of growth due to its liberation by arginase during catabolism of endogenous free amino

acids (FAAs; Watford 2003). Hypersaline conditions have also been shown to influence urea excretion in adult mangrove killifish (*Rivulus marmoratus*) as urea and non-essential FAAs are retained for osmoregulatory purposes at the cost of decreased rates of FAA catabolism (Frick and Wright 2002).

Increased gene expression of two enzymes involved in the OUC pathway, carbamoyl-phosphate synthase III (CPSaseIII) and ornithine carbamoyltransferase (OCTase), have been found in larval rainbow trout (*Oncorhynchus mykiss*) and CPSaseIII in larval African catfish (*Clarias gariepinus*; Wright et al. 1995, Terjesen et al. 2001). With few exceptions, as teleosts mature into adulthood, the OUC pathway is no longer utilized as genes controlling CPSaseIII and OCTase are silenced (Wright et al. 1995, Walsh 1998). Arginase is expressed throughout embryogenesis but expression decreases post hatch until total yolk absorption (Terjesen et al. 2002).

Embryogenesis has been shown to be influenced by environmental salinity in euryhaline teleosts. Though adult California killifish (*Fundulus parvipinnis*) have been found in isolated pools of salinities of up to 70 g/L, embryos display high rates of mortality due to sensitivity of high osmotic pressure prior to gastrulation (Rao 1974). Upper salinity tolerances among larval teleosts reflect environmental salinities typically encountered during development (see review in Bunn et al. 2000). Annual killifish (*Austrofundulus limnaeus*) embryos typically develop in hypersaline ephemeral pools at salinities in excess of 50 g/L and maintain a physiological osmolarity of 290 mOsmol/kg H_2O due to low egg permeability to salts and water (Machado and Podrabsky 2007). Salinities outside of the optimal osmoregulatory ranges of embryos have negative impacts on embryos during incubation, which are usually manifested in the forms of

decreased growth and rate of development (Dushkina 1973, Bunn et al. 2000, Boeuf and Payan 2001, Sampaio and Bianchini 2002).

The purpose of this study was to determine the influence of salinity on embryogenesis in Gulf killifish. This study determined to what degree environmentally relevant salinities ranging 0.4 to 30 g/L, influence embryogenesis (Simpson and Gunter 1956, Nordlie 2000, Nordlie 2006). To test this hypothesis, we measured the rates of embryogenesis, time to hatch, percent hatch, morphological development at hatch, heart rate, and production of ammonia and urea for developing embryos.

3.2 Materials and Methods

Water Quality, Sourcing of Gametes, and Experimental Design

Broodstock Gulf killifish were obtained from Grand Isle, Louisiana and held in outdoor pools maintained at 29.0 ± 0.1°C and a salinity of 7.6 g/L located on the Louisiana State University AgCenter's Aquaculture Research Station (Baton Rouge, LA, USA). Initial alkalinity and hardness (reported as mg/L $CaCO_3$) for each treatment was tested with water quality testing kits (Hach Co., Loveland, CO, USA) and determined by titration. Salinity, temperature, and DO were measured with a YSI 85 (YSI Inc., Yellow Springs, OH, USA). An Accumet® Basic AB15 pH meter (Fisher Scientific, Pittsburgh, PA, USA) was used to measure pH. Total ammonia nitrogen and nitrite nitrogen were measured with a Hach® DR 4000 Spectrophotometer using the salicylate (Hach® Method 10023) and diazotization (Hach® Method 8507) methods, respectively.

To obtain embryos for this study, 40 females and 5 males were collected from the brood stock population and were strip spawned. Eggs were fertilized as described by Brown et al. (2011). Newly fertilized eggs were soaked in a 2 g/L solution of sodium sulfite for ~15 minutes to decrease adhesion (Stone and Ludwig 1993). Embryos were then enumerated and divided into

12 baskets with mesh screen ports (39 embryos per basket) in a flow-through recirculating system in the Life Sciences Aquatic's Facility in the Department of Biological Sciences (LSU). Baskets were placed in triplicate into the four independent recirculating systems. Four salinity treatments were previously mixed to nominal salinities of 0.4, 7, 15 and 30 g/L using Coralife® Salt Mix (Energy Savers Unlimited Inc., Carson, CA, USA). A photoperiod of 12:12 light:dark cycle was maintained for the treatment systems. Each system was outfitted with a LogTag Temperature Recorder (LogTag Recorders, Auckland, New Zealand). Twenty-nine additional newly fertilized eggs were placed in triplicate into static trays at 7 g/L to obtain a control fertilization rate.

Six females and seven males were obtained from the same brood stock population to determine the effect of salinity on percent hatch. Forty-four eggs were placed in triplicate into four recirculating systems, each set at 0.4, 7, 15 and 30 g/L salinity. Eggs were allowed to develop until hatch and percent hatch was calculated. Baseline fertilization for this experiment was determined by placing 40, 41, and 54 eggs into three static trays at a salinity of 7 g/L.

Staging for Embryogenesis

The duration of time from fertilization through five distinct stages of development were recorded to determine the rate of embryogenesis among salinity treatments. Staging was based on embryological development described by Armstrong and Child (1965) for the mummichog. Relevant stages were selected based on ease of visual confirmation and significance to metabolite processing in the forms of ammonia and urea. Stage 15 was characterized by the earliest formation of the germ ring around the blastoderm. Stage 19 was characterized by formation of eye buds on the embryonic keel, which had formed definitive margins. Stage 25 was characterized by the onset of circulation, although blood cells did not appear to be

pigmented at this stage. Ocular structures appeared to be fully formed but lacked any pigmentation. Melanophores were present but had not fully expanded. Stage 28 was characterized by retinal pigmentation. Blood cells had developed pigmentation and melanophores had begun to expand. Pronephrose have previously been reported to be active from stage 27. Stage 34 was characterized by a fully-formed and open lower jaw. Hatching can occur at this stage due to the liberation of chorionase from hatching glands located in the gill and buccal cavity (Dimichele and Taylor 1981). Occasional movements of the jaw and pectoral fins occurred at stage 34. The caudal fin was also well developed with blood vessels radiating parallel to the rays of the fin. Stage 35 was characterized by the extension of the head, with eyes that moved and responded to light stimuli. Five embryos were randomly selected from each replicate salinity treatment to determine developmental stage and were subsampled for ammonia, urea, and heart rate when 80% of embryos from a replicate were at a target stage.

Metabolite Sampling

Ammonia and urea production were determined from four embryos per treatment replicate in 1.5 mL of sampling medium in triplicate. A fourth well was filled with 1.5 mL of sample water without embryos to serve as a blank. Embryos remained in the sample water for four hours at which time they were placed back into their respective treatment and replicate systems. Sample water was stored at -20°C prior to analysis. To test samples for urea, a portion of sample water was lyophilized with a Lyph-Lock18® (Labconoco®, Kansas City, MO, USA) to concentrate the sample and reconstituted with 500 µL of de-ionized water. The remaining original sample was tested for ammonia with the spectrophotometric technique described by Verdouw et al. (1978). Water samples for urea analysis were prepared in triplicate for spectrophotometric assay with a QuaniChrom™ Urea Assay Kit as per manufacturer instructions

(BioAssay Systems, Hayward, CA, USA; Jung et al. 1975). Spectrophotometric assays were read with a BioTek® Synergy 2 Multi-Purpose Microplate Reader (BioTek®, Winooski, VT, USA) and interpreted using Gen5™ analysis software (BioTek®, Winooski, VT, USA).

Heart Rate Sampling

From the onset of circulation (stage 25), heart rates of embryos (*n*=3) were recorded for each treatment replicate (n=9 total) at target stages for salinity treatments and reported as beats per minute (BPM). Heart rate was recorded by videotaping the hearts of individual embryos for a minute with a digital video camera (Sony Digital Handycam, NY, USA) interfaced with a dissecting microscope at a magnification of 20×.

Size at Hatch

Treatment groups were monitored twice daily for hatched larvae. Larvae were preserved in 10% buffered formalin and time-to-hatch (TH) from fertilization was recorded. Total length (TL), Body-depth-at-vent (BD) and body cavity area (BCA) were determined for these larvae with techniques described by Brown et al. (2011). Body cavity area was measured instead of yolk volume due to the inability to distinguish yolk from the abdominal cavity.

Statistical Analysis

A general linearized model analysis of variance (ANOVA) in randomized block design was performed to test for significant differences in embryo morphometrics, time to hatch from fertilization, and urea and ammonia production among salinities, stage, and the interaction of salinity and stage. The Ryan-Einot-Gabriel-Welsch (REGWQ) *post-hoc* test was used for multiple comparisons among treatment groups. Logistic regression analysis was used to determine the dependence of hatch on salinity. Hatch percentages were arcsine transformed and analyzed with a general linearized model ANOVA. Multivariate analysis of variance

(MANOVA) was used to determine morphometric variance among salinity treatments. Principal component analysis was used to test for multicolinearity among yolk morphometric measurements. All tests were performed with Statistical Analysis Software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Results are shown as means ± standard error of the mean (SEM). All hypotheses were tested at a significance level of $\alpha = 0.05$.

3.3 Results

Percent Fertilization and Percent Hatch

Mean fertilization was 69.7 ± 3.2% at 7 g/L salinity. Salinity significantly affected percent hatch with mean hatch percentages of 39.1 ± 2.5, 80.0 ± 1.5, 45.4 ± 2.6, and 36.3 ± 6.9% for salinity trials of 0.4, 7, 15, and 30 g/L, respectively ($P \leq 0.05$). Mean water quality parameters throughout the embryo incubations are presented in Table 3.1.

Table 3.1 Mean water quality parameters ± standard error of the mean for the salinity trials.

	Nominal salinity (g/L)			
Water quality	0.4	7	15	30
Salinity (g/L)	0.53 ± 0.01	7.13 ± 0.05	14.95 ± 0.11	27.85 ± 0.20
DO (mg/L)[1]	7.29 ± 0.08	7.02 ± 0.07	6.62 ± 0.06	6.07 ± 0.05
Temperature (°C)	25.12 ± 0.09	24.51 ± 0.07	24.59 ± 0.08	24.61 ± 0.08
pH	7.70 ± 0.04	7.62 ± 0.02	7.81 ± 0.02	7.89 ± 0.01
TAN (mg/L)[2]	ND[4]	ND	ND	ND
Nitrite (mg/L)	ND	ND	ND	ND
Alkalinity (mg/L)[3]	40	68	80	118
Hardness (mg/L)[3]	92	1,610	2,990	5,300

[1]Dissolved Oxygen
[2]Total Ammonia Nitrogen
[3]Reported as $CaCO_3$, at initiation of study
[4]Not Detectible

Time-to-hatch and Rate of Development

General linearized model ANOVA indicated that salinity had a significant effect on TH ($P \leq 0.05$). First appearance hatch was about 245 hours after fertilization for the 0.4, 7, and 15

g/L treatments and 287 hours for the 30 g/L treatment (Figure 3.1). The shortest mean TH was 292.3 ± 29.3 hours post fertilization (hpf) for the 0.4 g/L treatment. *Post-hoc* analysis determined that mean TH was significantly longer for 30 g/L at 368.2 ± 30.6 hpf (Table 3.2). The interaction between salinity and stage of embryogenesis was significant ($P \leq 0.05$). No statistical inference occurred regarding rate of development due to the lack of variance among replicates in their treatment response (Figure 3.2).

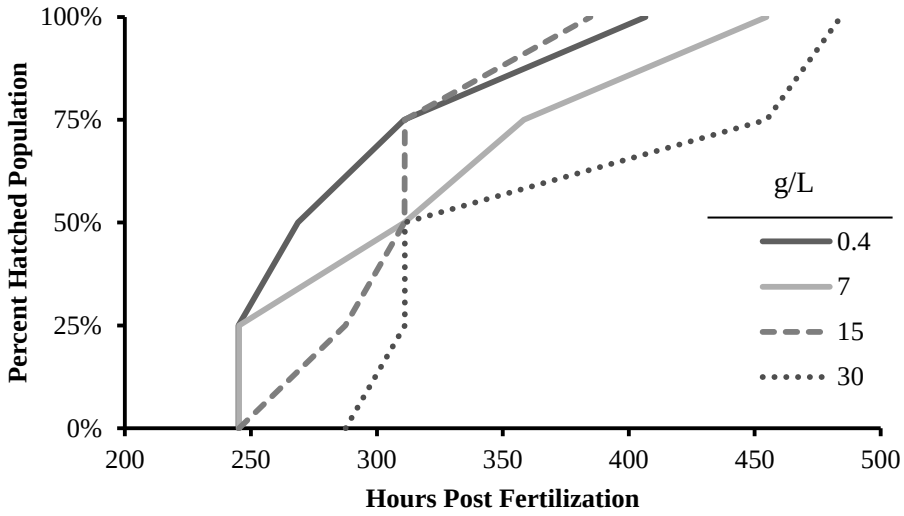

Figure 3.1 Duration of hatch for the nominal incubation salinities. Mean percent duration of hatch (hours) post fertilization exposed to treatment salinities. Lines depict incubation salinities: 0.4, 7, 15, and 30 g/L.

Total length, Body-depth-at-vent, and Body Cavity Area

A statistically significant effect of salinity on TL at hatch was determined ($P \leq 0.05$), and mean TL for the 15 g/L treatment was significantly shorter than the other salinity groups (Table 3.2). Salinity had a statistically significant effect on BD ($P \leq 0.05$), with BD generally demonstrated to increase as salinity increased (Table 3.2). A PCA of the size at hatch data found a principal component that appeared to measure 80% of the variance of the data. The first principal component is related to the length of the yolk sac, depth of the yolk sac, and BCA. A

Table 3.2 Morphometric parameters at hatch and mean time to hatch (hours ± standard error of the mean) for embryos reared in varying salinities. Columns with different superscript are significantly different (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$).

Salinity (g/L)	Larvae (*n*)	Length (mm)	Body-Depth-at-vent (mm)	Body Cavity Area (m^2)	Mean Time to Hatch (hours)
0.4	27	6.57 ± 0.11^{a}	0.63 ± 0.01^{b}	0.71 ± 0.04^{ab}	292.3 ± 29.3^{b}
7	21	6.55 ± 0.12^{a}	0.64 ± 0.01^{ab}	0.66 ± 0.04^{b}	312.2 ± 29.6^{b}
15	33	6.18 ± 0.11^{b}	0.60 ± 0.01^{c}	0.79 ± 0.04^{a}	301.7 ± 29.1^{b}
30	15	6.72 ± 0.12^{a}	0.66 ± 0.01^{a}	0.49 ± 0.05^{c}	368.2 ± 30.6^{a}

statistically significant effect of salinity on BCA was determined ($P \leq 0.05$). *Post-hoc* analysis found that BCA at hatch was significantly smaller for the 30 g/L treatment than the remaining salinities. MANOVA results indicated a significant effect of salinity on morphometric parameters ($P \leq 0.05$), with salinity explaining 58% of the variation among morphometric variables.

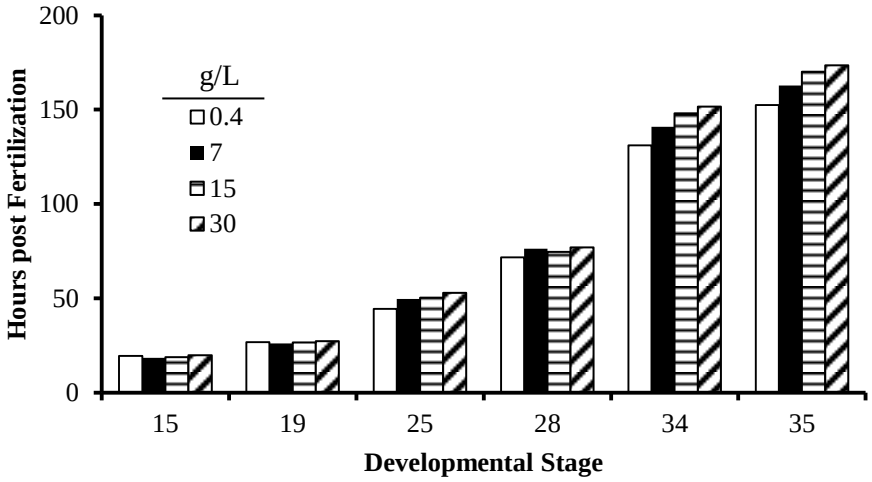

Figure 3.2 Hours post fertilization required for Gulf killifish embryos to reach stages 15, 19, 25, 28, 34, and 35 according to the characterization by Armstrong and Child (1965). Columns depict incubation salinities: 0.4, 7, 15 and 30 g/L.

Heart Rate

Salinity did not have a significant effect on heart rate ($P \geq 0.05$); however, stage of development did have a significant effect on heart rate ($P \leq 0.05$). BPM increased as stage increased for all salinity treatments except 15 g/L. *Post-hoc* analysis determined a significant increase in mean heart rate at stage 28, followed by a significant decrease in BPM at stage 34 for the 15 g/L salinity treatment (Figure 3.3).

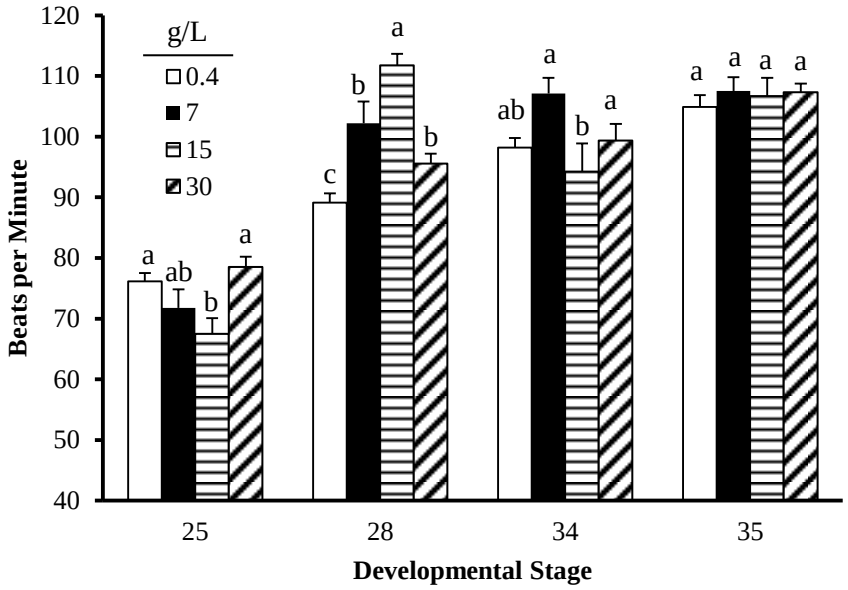

Figure 3.3 Mean heart rate (beats per minute ± standard error of the mean) for embryos at different stages of development. Letters above columns depict significant differences among salinity treatments at stages 15, 19, 25, 28, 34, and 35 of development according to the characterization by Armstrong and Child (1965) (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$). Columns depict incubation salinities: 0.4, 7, 15, and 30 g/L.

Stage and Metabolite Production

Ammonia production statistically significantly affected interaction between salinity and stage ($P \leq 0.05$). Ammonia production was high at stage 15 among all 4 salinity treatments, but decreased significantly as embryogenesis progressed (Table 3.3). Significant main and interaction affects for salinity and stage were detected for urea production ($P \leq 0.05$). Urea production remained low throughout embryogenesis for the two lowest salinity treatments of 0.4 and 7 g/L, except at stages 25 and 28 where production significantly increased (Table 3.3). Urea production varied throughout the 15 g/L incubation treatment and reached the highest recorded levels among treatments. No urea was detected throughout the 30 g/L treatment except at stage 25.

Table 3.3 Mean metabolites produced ± standard error of the mean from embryos reared in four different salinities. Capital letters depict statistical differences in metabolite production among stages (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$). Lowercase letters depict statistical differences in metabolite production among salinities (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$).

Metabolite (µmol/L/Hr)	Salinity (g/L)	Stages					
		15	19	25	28	34	35
Ammonia	0.4	0.54 ± 0.36^{Aa}	0.00 ± 0.00^{Ba}	0.00 ± 0.00^{Ba}	0.00 ± 0.00^{Bb}	0.00 ± 0.00^{Bb}	0.00 ± 0.00^{Ba}
	7	0.22 ± 0.11^{Aa}	0.07 ± 0.01^{Aa}	NA[5]	0.02 ± 0.02^{Ab}	0.04 ± 0.04^{Ab}	0.00 ± 0.00^{Aa}
	15	0.27 ± 0.16^{Aa}	0.09 ± 0.09^{Aa}	0.21 ± 0.14^{Aa}	0.16 ± 0.10^{Ab}	0.27 ± 0.13^{Aab}	0.01 ± 0.01^{Aa}
	30	0.51 ± 0.11^{ABa}	0.04 ± 0.04^{Ba}	0.03 ± 0.03^{Ba}	0.63 ± 0.05^{Aa}	0.59 ± 0.12^{Aa}	0.25 ± 0.25^{Aba}
Urea	0.4	0.17 ± 0.12^{Aa}	0.07 ± 0.06^{Aa}	0.15 ± 0.15^{Aa}	0.15 ± 0.15^{Aab}	0.10 ± 0.10^{Aa}	0.15 ± 0.15^{Ab}
	7	0.03 ± 0.03^{Aa}	0.06 ± 0.06^{Aa}	0.26 ± 0.26^{Aa}	0.26 ± 0.15^{Aab}	0.05 ± 0.05^{Aa}	0.00 ± 0.00^{Ab}
	15	0.03 ± 0.03^{Ca}	0.20 ± 0.00^{BCa}	0.00 ± 0.00^{Ca}	0.58 ± 0.09^{ABa}	0.34 ± 0.15^{BCa}	0.80 ± 0.13^{Aa}
	30	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.31 ± 0.31^{Aa}	0.00 ± 0.00^{Ab}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ab}

[5]Not Available

3.4 Discussion

The effect of salinity on the rate of embryogenesis has been studied in many teleost species, with an overall observation that ontogenic responses and the extent of these responses are species-specific, indicating that species have adapted to native salinity ranges (Laurence and Rogers 1976, Fonds 1979, Imsland et al. 2001, Cook et al. 2005). In the present study, the rate of embryogenesis was most affected by salinity given stage at the onset of circulation (i.e., stage 25) and beyond, tending to decrease as salinity was increased beyond isotonic levels (Figure 3.2). When examining the effect of salinity on mummichog embryogenesis, Tay and Garside (1975) did not find a change in the rate of development in salinities across 0 to 30 g/L. However, they reported that hypersaline conditions (60 g/L) began to slow embryogenesis at stages 25 and above (Figure 3.2). Perschbacher et al. (1990) demonstrated that mean TH did not statistically differ for salinities across 0 to 30 g/L when examining Gulf killifish incubation. In the current experiment, only the 30 g/L treatment had significantly longer mean TH. Salinities outside optimal osmotic range may also reduce percent hatch. Rao (1974) determined that California killifish embryos had a longer mean TH and reduced hatching percentages of 33.3% and 51.4%, respectively in freshwater compared to 76.8% and 75.8% for 5 g/L. Despite a uniform mean TH for the three lowest salinity treatments in the current study, we observed a hatch percent of 39.1% ± 4.3% for the 0.4 g/L treatment compared to 80.0% ± 2.6% for 7 g/L.

Minimum osmotic effort occurs most often when the incubation medium is isotonic with the yolk and perivitelline fluids (Alderdice et al. 1979). At tonicities outside of this optimal range, energy may be diverted away from growth and development of embryos (Rao 1974, Boeuf and Payan 2001, Sampaio and Bianchini 2002). In isotonic conditions, mummichog are estimated to devote less than 1% of total energy use to osmotic regulation compared to a ~10%

energy expenditure when mummichog were transferred to a higher salinity medium and a ~1% energy expenditure when mummichog were transferred to a lower salinity medium (Kidder et al. 2006b). Tilapia (*Oreochromis mossambicus*) embryos reared in saltwater produced an increased number and size of MRCs located on the yolk-sac membrane compared to freshwater reared embryos (Ayson et al. 1994). In this study, the larvae in the 30 g/L treatment had a significantly smaller BCA at hatch compared to lower salinity treatments. An increase in salinity would result in additional energy used above that required for embryogenesis as a result of an increased number and size of MRCs. The decreasing surface area of BCA throughout embryogenesis would limit the ability of MRCs to meet osmoregulatory demand, however, proliferation of MRCs begins 6 dpf on the body and at 10 dpf on the gills of mummichog (Katoh et al. 2000). Although there was a significant influence of salinity on BCA at hatch in the current study, there was no significant influence of salinity on TL at hatch. Gulf killifish embryos hatched within a narrow range of mean TL among the four salinity treatments, which has also been demonstrated in mummichog when incubated across a similar wide range of salinities (Fortin et al. 2008). Though hatching with greater yolk volume would afford larvae more time between endogenous and exogenous feeding (Miller et al. 1988), a decrease in heterogeneity of TH and TL could lead to decreased occurrences of intracohort cannibalism (Baras and Jobling 2002, Fessehaye et al. 2006).

During embryogenesis, one of the earliest functioning organs is the heart (Pelster 2002). However, the onset of heart contractions occurs well before the need of a recirculating system to meet oxygen demand of tissues in embryonic zebrafish (*Danio rerio*), as adequate amounts of oxygen can diffuse through the skin (Pelster and Burggren 1996, Barrionuevo and Burggren 1999, Rombough 2007). Pelster and Burggren (1996) suggested that the onset of heart

contractions would facilitate transport of nutrients and metabolites. In this experiment, during the 15 g/L trial, urea production rapidly increased at stage 28, decreased at stage 34 before increasing at stage 35 (Table 3.3). In this study, heart rate for the 15 g/L salinity trial mirrored this trend, possibly in an effort to remove urea, however, adult gulf toadfish exhibited no changes in rate of ventilation or arterial blood pressure during pulsatile urea excretion when compared to ammonia excretion (Gilmour et al. 1998), suggesting no relation between heart rate and urea excretion in fish.

Metabolite production was found to be influenced by salinity and stage of development. A gradual shift from urea to ammonia production occurred as embryogenesis progressed across all incubation salinities. The embryos in the 0.4 and 7 g/L salinity treatments continuously produced and released urea into the incubation medium. Decreased osmotic pressure would allow developing embryos to invest more energy into growth resulting in urea produced as a byproduct of arginase catabolism. Arginase catabolism produces ornithine, which can subsequently be diverted into polyamine biosynthesis through the coordinated action of ornithine decarboxylase (Pegg 2006, Montanez 2007). Polyamines are a family of highly cationic molecules implicated in numerous physiological processes including cell proliferation, growth, and apoptosis. Ornithine decarboxylase has been shown to be amongst the most highly differentially expressed transcripts following hypoosmotic transfer in a wide array of biota (Davis and Ristow 1995, Watts et al. 1996, Mitchell et al. 1998, Henry and Watts 2001) including fishes (Hascilowicz et al. 2002; Whitehead et al. 2010; Whitehead et al. 2011). Conversely, in this study embryos in 15 and 30 g/L predominantly excrete ammonia. Adult mangrove killifish increased physiological FAA levels and retained more urea in an osmoregulatory response as the fish were transferred to increasing salinities (Frick and Wright

2002). Three adult euryhaline species, including striped bass (*Morone saxatilis*), rainbow trout, and brown trout (*Salmo trutta*), all release decreasing amounts of urea as salinity increases (Altinok and Grizzle 2004). Frick and Wright (2002) found that although urea production for the mangrove killifish was significantly lower for fish acclimated to hypersaline conditions (45 g/L) compared fish acclimated to salinities of 0, 15, and 30 g/L, total nitrogen excretion (ammonia and urea) was not significantly different among salinity treatments. Urea production has been found to be influenced by the stage of embryogenesis for the guppy (*Poecilia reticulata*) and rainbow trout increasing as embryogenesis progressed but decreasing immediately before hatch (Depeche et al. 1979).

One inconsistent aspect of the current study was the 15 g/L treatment, which produced the smallest larvae at hatch in addition to erratic heart rates and urea levels throughout the study. Mean TH of the 15 g/L treatment was about the same as both 0.4 and 7 g/L salinities, but had significantly smaller TL and BD than the other three salinity treatments. Hatching is independent of stage of embryogenesis once development of tissues that release hatching enzymes is completed (Dimichele and Taylor 1981); and external influences such as hypoxic conditions and frequent agitation to eggs can induce premature hatching (Warkentin 2007). DO levels in the 15 g/L treatment did not deviate from other treatments (Table 3.1) and embryos from the 15 g/L treatment were handled similarly. Fluctuations in heart rate observed at stages 28 and onward may be a cause or result of the urea levels that varied in concert with heart rate. The highest recorded urea levels were in the 15 g/L salinity at stages 28 and 35 (Table 3.3). Cortisol has been shown to increase expression of glutamine synthetase, an enzyme involved in the ornithine urea cycle (OUC), and urea transporter proteins in gulf toadfish (Mommsen et al. 1999, McDonald et al. 2000). The influence of cortisol in this experiment is only speculative as concentrations of

cortisol were not tested in either embryos or maternal broodstock, nor do we know whether OUC enzymes are ever expressed during embryogenesis in Gulf killifish.

This experiment demonstrated the embryological responses of Gulf killifish to salinities typically encountered in the wild. Hatching within a concise mean TH range and at similar sizes reduces the effects of cannibalism, however, reduced hatch percentages in 0.4 and 30 g/L and a protracted mean TH for 30 g/L indicate the peripheral ranges of salinity tolerance during embryogenesis. Salinity and stage of development were found to significantly influence ammonia and urea production for embryonic Gulf killifish. Increased ammonia released at higher salinities may be a result of higher concentrations of Na^+ available for ion exchanges (Wilkie 1997), resulting in a decreased need for urea production as a method of ammonia detoxification. Future studies comparing changes in plasma ion content across a range of salinities, genetic regulation of enzymes involved in the OUC, and urea transporter proteins would prove beneficial in determining the magnitude of urea's role in ion and metabolite regulation during embryogenesis.

CHAPTER 4: METABOLIC AND EMBRYOGENIC RESPONSES TO AIR INCUBATION OF EMBRYOS ACROSS A TEMPERATURE GRADIENT

4.1 Introduction

The Gulf killifish (*Fundulus grandis*) is a euryhaline cyprinodontid native to the coastal marshes of the Gulf of Mexico and southern Atlantic coast of the United States (Oesterling et al. 2004). This species is a fractional spawner with a protracted spawning period, peaking in spring and fall (Greeley and MacGregor 1983, Nordlie 2000). Spawning events are timed to semilunar tidal cycles where eggs are deposited at the high water mark of marsh grasses during spring tide and are exposed to air once the tide recesses (Taylor et al. 1977, Lipcius and Subrahmanyam 1986). During this period, referred to as air incubation, embryogenesis occurs at an accelerated rate compared to incubation in typical aquatic conditions (Martin 1999, Tingaud-Sequeira et al. 2009). Embryos decrease the rate of development upon reaching a stage of embryogenesis where hatching has been documented to occur during aquatic incubation (Martin 1999). Embryos remain in the chorion until hatch is initiated by submersion into the returning high tide (Dimichele and Taylor 1980).

Hand and Podrabsky (2000) define diapause as obligatory metabolic depression that is part of the ontogenetic process, and regard quiescence as facultative metabolic depression in response to an environmental stimulus. Once fully developed, Annual killifish (*Austrofundulus limnaeus*) embryos show continuously decreasing levels of oxygen consumption and adenosine trisphosphate (ATP) concentrations as diapause progresses. Compared to diapause and quiescence, delayed incubation is not marked by a depressed metabolism following the completion of embryonic maturation, but of active and stabilized metabolic rates (Darken et al. 1998). The advantage of an actively maintained metabolism allows embryos to hatch quickly

within the narrow range of time once immersed during the returning high tide (Dimichele and Taylor 1981, Darken et al. 1998, Podrabsky and Hand 1999).

Air incubated mummichog (*Fundulus heteroclitus*) embryos develop at an accelerated rate compared to aquatically incubated embryos presumably due to a large concentration of oxygen available for respiratory demand (Tingaud-Sequeira et al. 2009). Once embryos are developed, hatch occurs due to a drop in the partial pressure of oxygen during the submersion of embryos at the returning high tide due to the respiratory demands of the embryo being higher than concentrations of oxygen available (Dimichele and Taylor 1981, Dimichele and Powers 1984). A decrease in the partial pressure of oxygen before embryos have developed to a stage where hatch can occur may result in the production of lactate as the major metabolic endproduct of anaerobic glycolysis (Finn et al. 1995).

The production of end product metabolites is associated with embryogenesis (Wright and Land 1998). Ammonia (NH_3) is produced as a byproduct as yolk proteins are catabolized for growth and energy (Mommsen and Walsh 1992, Randall and Tsui 2002). Recent work has demonstrated the role of Rhesus proteins (Rh) as ammonia transporters and exporters in embryonic, larval, and adult teleosts (Wright and Wood 2009). Throughout embryogenesis embryonic zebrafish (*Danio rerio*) utilize Rh B glycoproteins (Rhbg) and Rh C glycoproteins (Rhcg) to transport ammonia through the basolateral and apical membranes of cells, respectively, of the gills, operculum, and yolk sac (Braun et al. 2009). When the external concentration of ammonia is greater than physiological concentrations, adult rainbow trout (*Oncorhynchus mykiss*) upregulate expression of Rhcg 48 hours after the start of ammonia influx into the body (Nawata et al. 2007). Ammonia elimination is thought to be maintained by a partial pressure gradient facilitated by acidification of the unionized form in the boundary layer surrounding the

egg as it diffuses through the pores of the chorion (Rahaman-Noronha et al. 1996). Alternatively, air incubation may present another strategy of ammonia elimination by volatilization.

Urea is an end product metabolite that embryonic teleosts produce during development (Terjesen et al. 2001, Barimo et al. 2004, Monzani and Moraes 2008). Urea production is believed to be limited to elasmobranchs, embryonic and larval teleosts, and a few adult teleost species (Walsh 1998). When the rate of ammonia production exceeds the rate of diffusion across the chorion or when embryos are in environments that are alkaline, conversion of ammonia into urea via the Orinthine Urea Cycle (OUC) is a method of avoiding ammonia toxicity (Walsh 1998, Wright and Land 1998, Walsh et al. 2001). Ammonia is combined with glutamate to form glutamine, which is the main substrate used in the OUC to create urea (Walsh et al. 1994). Urea is produced in the liver and muscles diffuses through urea transporters (UT) where it is bound to UTs located in red blood cells and eliminated at the gills and kidneys (McDonald et al. 2006, Braun et al. 2009). Another route of urea production is through arginine catabolism as developing embryos utilize endogenous free amino acids (FAA), particularly arginine, for protein anabolism, creating urea as a byproduct (Terjesen et al. 2001, Watford 2003).

The goal of this experiment was to determine biological and developmental responses of the Gulf killifish to air incubation at temperatures of 20, 23, 26, and 30°C. Specifically, this investigation measured rate of development, ammonia and urea production, heart rate, lactate concentrations, ATP concentrations, and size at hatch throughout embryogenesis and extended incubation period.

4.2 Materials and Methods

Sourcing of Gametes and Experimental Set Up

Gulf killifish broodstock were obtained from Terry's Bait Shop in Leeville, Louisiana. Broodstock were held at 28.3 ± 0.1°C (mean ± standard error of the mean; SEM) in 12 outdoor tanks located on the Louisiana State University Agricultural Center Aquaculture Research Station. Twelve spawning mats as described by Green et al. (2010; Spawntex®, Blocksom and Co., Michigan City, IN, USA) were soaked overnight from June 7th, 2010 at 17:00 hours to June 8th, 2010 at 09:30 hours in pools containing brood stock to obtain embryos for the study. Eggs were manually removed and dead and pigmented eggs were discarded. Dead eggs were characterized by a white opaque appearance and pigmented eggs were considered overdeveloped for the purpose of the study. Live embryos were volumetrically quantified in a graduated cylinder. The total volume of embryos collected was 163.3 mL at 130 ± 2 eggs per mL. A subsample of 85 eggs was staged according to developmental descriptions detailed by Armstrong and Child (1965) to ensure uniformity of development. This assessment determined that 82% of the subsampled embryos were at stage 15.

Replicates consisted of 10 mL or about 1,300 embryos sandwiched between two pieces of polyurethane hobby foam in triplicate for each respective temperature treatment (Premium Poly Foam, American Excelsior Company, Arlington, TX, USA; Coulon et al. *In Press*). Each piece of foam was cut to a width, length, and height of 11.4, 14.6, and 2.5 cm, respectively. A solution of 7.6 g/L saline water was mixed using Crystal Sea® (Marine Enterprises International, Inc., Baltimore, MD, USA) and was used to moisten the foam. A salinity of 7.6 g/L was chosen based on previously tested salinity conditions (Coulon et al. *In Press*). Embryos and hobby foam were then covered with plastic to prevent saturation of the hobby foam from condensation in the

incubation chambers. Incubation chambers were set to nominal values of 20, 23, 26, and 30°C with externally adjustable thermostats as described by Glenn and Tiersch (2011). Temperature and humidity were recorded at 10 minute intervals in each chamber with a LogTag Humidity and Temperature Recorder (LogTag Recorders, Auckland, New Zealand). Dead eggs were removed daily throughout the study to prevent fouling.

Staging of Embryogenesis and Sampling

Time required for embryos to progress through five stages of development was recorded to determine the rate of embryogenesis. Staging was based upon descriptions detailed by Armstrong and Child (1965) for the mummichog. Stages were selected based on ease of visual confirmation and relationship to metabolite production, heart rate, and hatching. Twelve embryos were randomly selected from each thermal-treatment triplicate to determine stage of development. If ≥75% of embryos were at a target stage, treatments were sampled for ammonia, urea, heart rate, and lactate levels.

Stages selected as developmental markers were defined using anatomical development. Descriptions of stages can be found in Chapter 3.2. Embryos began air incubation for this study at stage 15, which was characterized by the earliest formation of the germ ring around the blastoderm. Stage 35 marked the end of development and the transition into delayed hatch. Replicates were sampled in 48 hour delayed hatch intervals after reaching stage 35 until embryos could no longer be sampled due to increasing mortalities. Embryos were sampled at 48 hour intervals for ammonia, urea, heart rate, lactate, ATP concentrations, and morphometric parameters at hatch. When embryos reached stage 25, 30 embryos were randomly selected from each treatment replicate to determine percent survival.

Metabolite Sampling

Embryos were evaluated for ammonia and urea production at previously described targeted stages. Four eggs were subsampled from each treatment replicate in 1.5 mL of a 7.6 g/L salinity sampling medium (0 µmol/L ammonia; 0 µmol/L urea) in triplicate. A fourth well was filled with 1.5 mL of sampling medium and was left empty to serve as a blank. Embryos were placed back into their respective incubation chambers and left in the sampling medium for four hours, after which time embryos were removed from the study and sample water was collected and stored at -20°C for later analysis. After reaching stage 35, embryos were evaluated for ammonia and urea production in a similar manner as described above. However, embryos were not discarded after the four hour incubation period and the incubation medium was replaced with a new volume of 1.5 mL to allow embryos to hatch. If larvae hatched within five hours of immersion, they were preserved in 10% buffered formalin for morphometric analysis. Total length (TL), Body-depth-at-vent (BD) and body cavity area (BCA) were determined for these larvae with techniques described in Chapter 2.2. Body cavity area was measured instead of yolk volume due to the inability to distinguish yolk from the peritoneal cavity.

A portion of sample water was lyophilized to concentrate the sample and reconstituted with 500 µL of de-ionized water to test for urea. The remaining original sample was tested for ammonia with the spectrophotometric technique described by Verdouw et al. (1978). Water samples for urea analysis were prepared in triplicate for spectrophotometric assay using the QuaniChrom™ Urea Assay Kit as per manufacturer instructions (BioAssay Systems, Hayward, CA, USA; Jung et al. 1975). All absorbance and luminescence assays were performed with a BioTek® Synergy 2 Multi-Purpose Microplate Reader (BioTek®, Winooski, VT, USA) and interpreted with Gen5™ analysis software (BioTek®, Winooski, VT, USA).

Heart Rate Sampling

At the onset of circulation (stage 25), heart rates of embryos (n=3) were recorded for each treatment replicate at target developmental stages and post stage 35 delayed intervals across treatments and reported as beats per minute (BPM). Heart rate was recorded by videotaping the hearts of individual embryos for 30 seconds using a digital video camera (Sony Digital Handycam, NY, USA) interfaced with a dissecting microscope at a magnification of 20×. The rate observed was doubled to obtain BPM.

Lactate Sampling

Lactate samples were tested at every target developmental stage and post stage 35 delayed interval. At each sampling period, one embryo from each temperature replicate was stored at -80°C for later analysis. Embryos were prepared for spectrophotometric analysis in triplicate wells as per manufacturer instructions for the Eton Bioscience™ L-Lactate Assay kit (Eton Biosciences™, San Diego, CA, USA).

ATP Sampling

ATP samples were tested at every post stage 35 delayed interval. At each sampling period, one embryo from each temperature replicate was stored at -80°C for later analysis. Samples were prepared for luminescent analysis with the Promega CellTiter-Glo® Luminescent Cell Viability Assay kit (G7570; Promega Corporation, Madison, WI, USA). Frozen embryos were kept on ice until weighed on a Discovery (Model DV114C; Ohaus® Corporation, Pine Brook, NJ, USA) analytical balance. Embryos were homogenized on ice with a 1-mL Dounce tissue grinder (Wheaton Industries Inc., Millville, NJ, USA) in a solution of 2.5% trichloroacetic acid (TCA) and 0.0005% p-Xylenol Blue pH indicator (Xu et al. 2001). The homogenized solution was centrifuged at 10,000g for 15 minutes in 4°C (Menze et al. 2010). The supernatant

was collected and neutralized with a 0.1M tris buffer solution that was pH adjusted to 7.75 with 1M glacial acetic acid and stored at -20°C (Webster et al. 1980, Nichols et al. 1981). Samples were prepared in triplicate according to instruction for Promega CellTiter-Glo® Luminescent Cell Viability Assay. Sample reading was set to a one second integration time with a photomultiplier tube sensitivity of 135.

Statistical Analysis

A general linearized model analysis of variance (ANOVA) in randomized block design was performed to test for significant differences in the maximum amount of time hatch could be delayed among temperatures. Separate two-way general linear model ANOVAs were performed to test for statistically significant differences in the maximum amount of embryo morphometrics, BPM, lactate, ATP, urea, and ammonia among temperatures and stage during embryogenesis. Separate two-way general linear model ANOVAs were performed to test for statistically significant differences in the maximum amount of embryo morphometrics, BPM, lactate, ATP, urea, and ammonia among temperatures and delayed hatch hours during delayed hatch. Multivariate analysis of variance (MANOVA) was used to examine changes in BPM, lactate, and ATP in response to temperature treatments. The Shapiro-Wilk test was used to test for normality. MANOVA profile analysis was used to determine statistical significance of overall polynomial model trends for the BPM, lactate, and ATP variables. Logistic regression was performed to determine the frequency of survival among treatments at stage 25. The Ryan-Einot-Gabriel-Welsch (REGWQ) *post-hoc* test was used for multiple comparisons among treatment groups. All tests were performed with Statistical Analysis Software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Results are shown as means $\pm$ standard error of the mean (SEM). All hypotheses were tested at a significance level of $\alpha = 0.05$.

4.3 Results

Incubation Quality

Temperature did not have a significant influence on percent of viable embryos at stage 25 ($P \geq 0.05$). Percent of viable embryos were 59 ± 2%, 62 ± 3%, 58 ± 8%, and 75 ± 1%, for 20, 23, 26, and 30°C, respectively, at stage 25. Mean temperatures for incubation chambers were 19.8, 22.4, 25.2, and 29.0°C with SEM of the measurements for each treatment (± 0.001). Mean relative humidity was 89.7% ± 0.1%, 87.3% ± 0.2%, 91.1% ± 0.2%, and 83.1% ± 0.4%, and for 20, 23, 26, and 30°C thermal treatments, respectively.

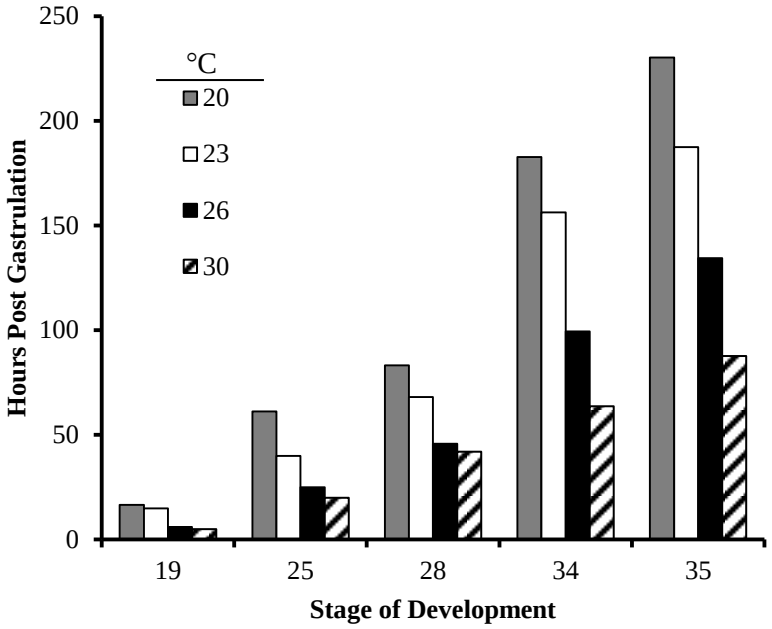

Figure 4.1 Hours post gastrulation required for Gulf killifish embryos to reach stages 19, 25, 28, 34, and 35 according to the characterization by Armstrong and Child (1965). Columns depict incubation temperatures: 20, 23, 26, and 30°C.

Rate of Embryogenesis and Extent of Delayed Incubation

No statistical analysis was performed on the rate of embryogenesis due to the lack of variance among replicates within their response treatments (Figure 4.1). General linearized

model ANOVA indicated that temperature had a significant effect on the longest extent of time that delayed hatch embryos remained viable ($P \leq 0.05$). Chorions became increasingly weak as delayed hatch progressed leading to hatch occurring on the incubation substrate. Embryos began to hatch on the substrate beginning at 96 delayed hatch hours in the 26 and 30°C treatments, but did not hatch on the substrate in the 23 and 20°C treatments. The longest extent of delayed hatch was 320 hours post stage 35 for the 20°C treatment, followed by 272, 224, and 176 hours for 23, 26, and 30°C treatments, respectively. Embryos developed an orange color and began fouling towards the end of air incubation.

Total Length, Body-depth-at-vent, Body cavity area, and First Hatch

Morphometric parameters associated with TL, BD, and BCA are detailed in Table 4.1. Temperature and hours of delayed hatch exhibited a statistically significant interaction for BD ($P \leq 0.05$). Therefore, the effect of temperature on BD was dependent on the hours delayed. The sampling period in which ≥75% of individuals within a replicate hatched within 5 hours of immersion occurred within 240-336, 144-288, 96-240, and 96-192 hours of delayed hatch for the 20, 23, 26, and 30°C treatments, respectively. Hours of delayed hatch was statistically significantly related to TL ($P \leq 0.05$). Size at hatch (TL) and BCA were not statistically significantly related to temperature or hours of delayed hatch.

Metabolite Production during Embryogenesis and Delayed Hatch

Ammonia production was not significantly influenced by temperature given stage, however, there was a significant influence by stage of embryogenesis and a significant interaction of temperature and stage on ammonia production (Figure 4.2; $P \leq 0.05$). Ammonia concentrations were highest at stages 25 and 35 for the 20 and 23°C treatments, respectively, and

highest at stages 28 and 34 for the 26 and 30°C treatments, respectively. Urea production was not significantly influenced by temperature or stage of embryogenesis ($P \geq 0.05$).

Temperature and hours of delayed hatch exhibited a statistically significant interaction for ammonia production (Figure 4.2; $P \leq 0.05$). *Post-hoc* analysis determined that the 20°C treatment produced the highest concentrations of ammonia throughout delayed hatch (Figure 4.2 [A]). Temperature and hours of delayed hatch exhibited a statistically significant interaction for urea production ($P \leq 0.05$). *Post-hoc* analysis determined that the 30°C treatment produced the highest concentrations of urea throughout delayed hatch (Figure 4.2 [D]). Significant increases of urea concentrations were detected at 192 hours of delayed hatch across all thermal treatments (Figure 4.2).

Heart Rate during Embryogenesis and Delayed Hatch

Temperature and stage of embryogenesis had a statistically significant interaction on heart rate (Figure 4.3; $P \leq 0.05$). *Post-hoc* analysis determined that heart rate is lowest upon the onset of circulation at stage 25 across all temperature treatments and plateaus after stage 28 until reaching stage 35. Embryos within the 23°C treatment displayed a significantly lower heart rate throughout embryogenesis. The 20, 26, and 30°C treatments were statistically similar. Temperature and hours of delayed hatch had a statistically significant interaction on heart rate ($P \leq 0.05$). The 20 and 30°C treatments had significantly lower heart rates than 23 and 26°C. Heart rate increased until peaking at 96 hours of delayed hatch. BPM declined to levels similar to the onset of circulation at 192 hours of delayed hatch before increasing (Figure 4.3).

Lactate Concentrations during Embryogenesis and Delayed Hatch

Lactate production was significantly influenced by temperature during embryogenesis (Figure 4.4; $P \leq 0.05$). Significantly higher lactate levels were detected in the 20 and 30°C

Table 4.1 Morphometric parameters at hatch (mean ± standard error of the mean) for embryos reared in varying temperatures at 20°C (*n*=27), 23°C (*n*=39), 26°C (*n*=39), and 30°C (*n*=31). Absence of a superscript indicates no significant difference and rows with different superscript are significantly different (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$)

Morphometry	Temperature (°C)	Delayed Hatch Hours						
		48	96	144	192	240	288	336
Total Length (mm)	20	NH[1]	NH	NH	NH	6.38 ± 0.13	6.02 ± 0.14	6.44 ± 0.15
	23	NH	NH	6.21 ± 0.09	5.99 ± 0.25	6.55 ± 0.12	6.57 ± 0.16	NA[2]
	26	NH	6.06 ± 0.17	6.10 ± 0.05	6.07 ± 0.10	6.49 ± 0.12	NA	NA
	30	NH	6.27 ± 0.10	6.36 ± 0.22	6.82 ± 0.20	NA	NA	NA
Depth-at-vent (mm)	20	NH	NH	NH	NH	0.62 ± 0.02^{A}	0.57 ± 0.03^{A}	0.64 ± 0.02^{A}
	23	NH	NH	0.57 ± 0.02^{A}	0.57 ± 0.04^{A}	0.60 ± 0.03^{A}	0.62 ± 0.02^{A}	NA
	26	NH	0.52 ± 0.04^{B}	0.62 ± 0.02^{AB}	0.53 ± 0.02^{B}	0.66 ± 0.02^{A}	NA	NA
	30	NH	0.59 ± 0.02^{B}	0.58 ± 0.03^{B}	0.70 ± 0.01^{A}	NA	NA	NA
Body cavity area (mm^2)	20	NH	NH	NH	NH	0.96 ± 0.07	0.87 ± 0.09	0.81 ± 0.08
	23	NH	NH	0.80 ± 0.10	0.80 ± 0.11	0.77 ± 0.05	0.70 ± 0.03	NA
	26	NH	1.07 ± 0.18	0.94 ± 0.09	0.76 ± 0.09	0.76 ± 0.06	NA	NA
	30	NH	0.83 ± 0.03	0.69 ± 0.04	0.75 ± 0.03	NA	NA	NA

[1]NH - No Hatch- 75% of fry did not hatch out within 5 hours of immersion
[2]NA - Not Available – Fry did not hatch or died during air incubation

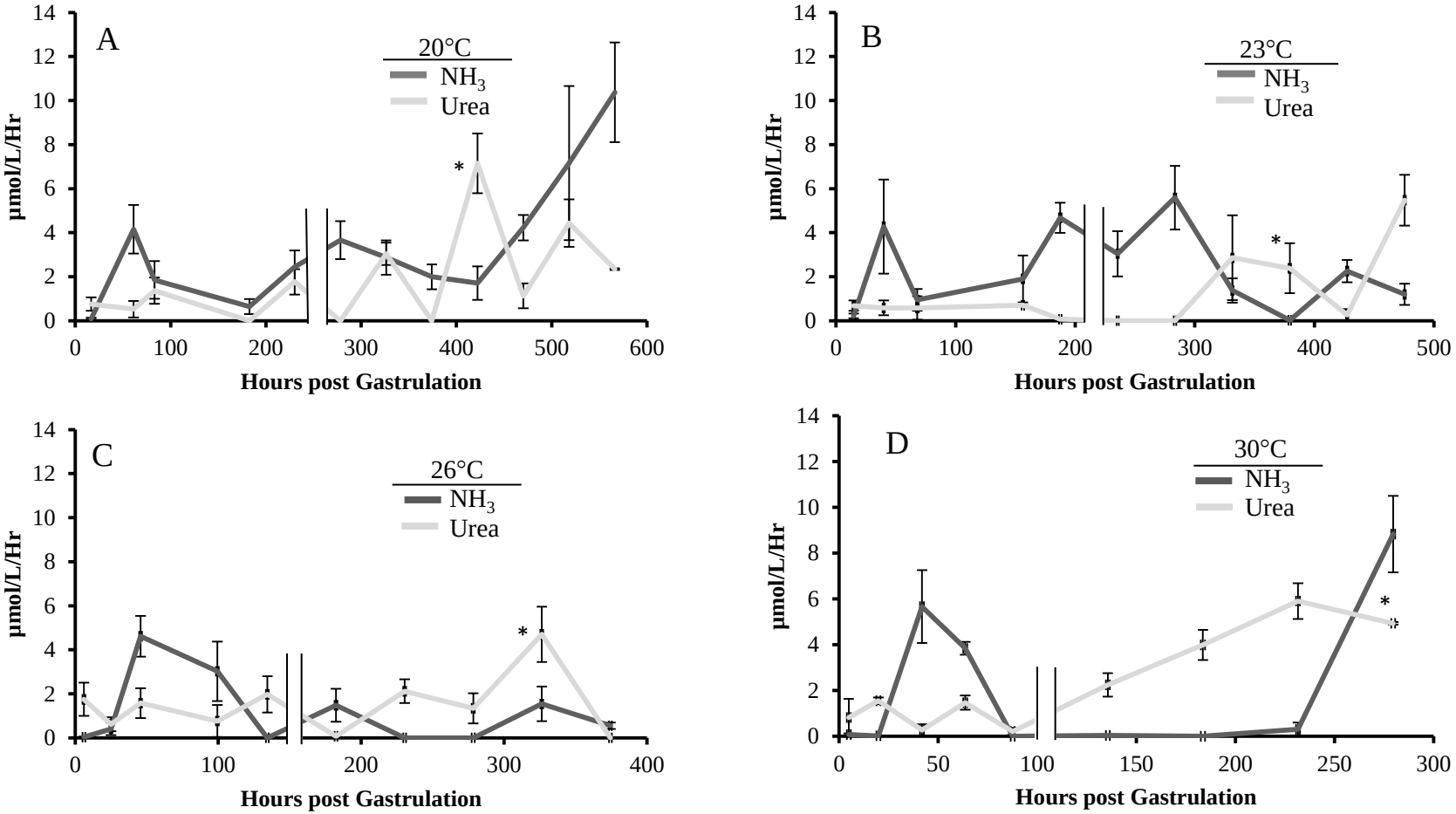

Figure 4.2 Mean metabolites produced ± standard error of the mean from embryos reared at four different temperatures from the onset of gastrulation and hours of delayed hatch. The break in the line represents the transition from embryogenesis to delayed hatch. Asterisks indicate when urea increased significantly at 192 delayed hatch hours. Incubation temperatures are: 20°C (A), 23°C (B), 26°C (C), and 30°C (D).

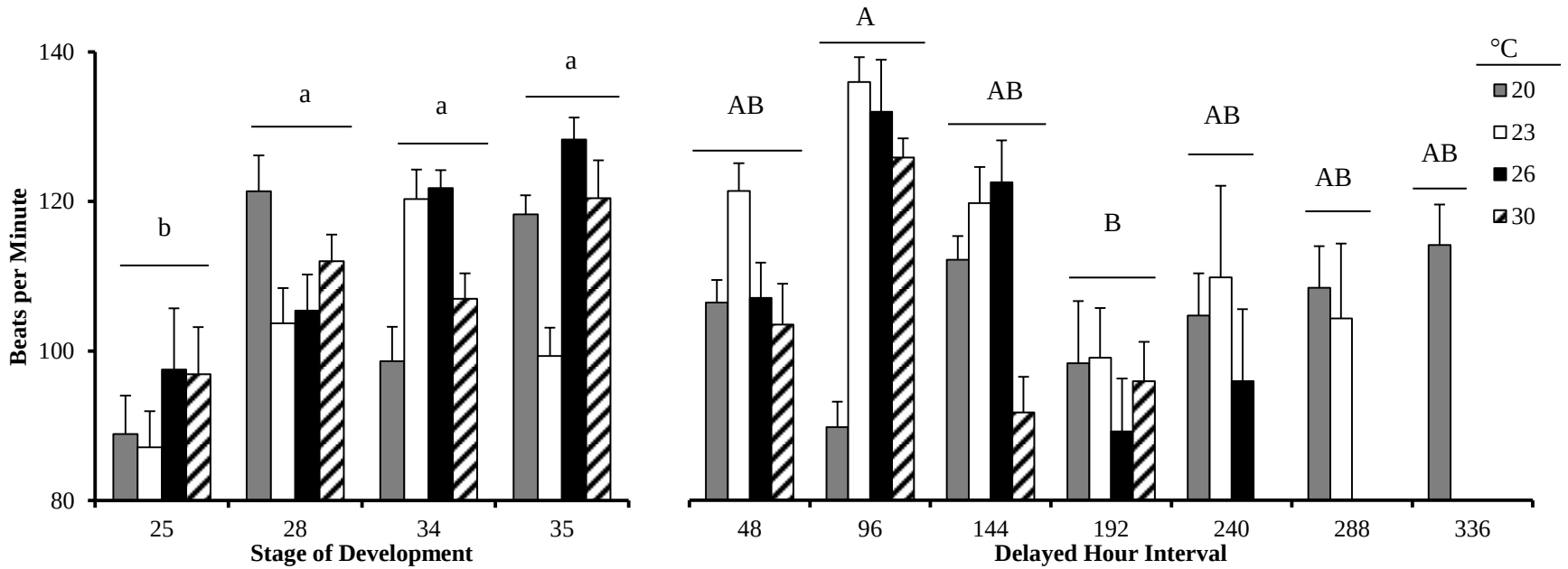

Figure 4.3 Mean heart rate (beats per minute ± standard error of the mean) for embryos at different stages of development and delayed hatch intervals. Lower case letters above columns depict significant differences among stages of development according to the characterization by Armstrong and Child (1965) and capital letters depict differences among delayed hatch intervals (Ryan-Enoit-Gabriel-Welsch-studentized range; $P < 0.05$). The break in the x-axis represents the transition from embryogenesis into delayed hatch. Columns depict incubation temperatures: 20, 23, 26, and 30°C.

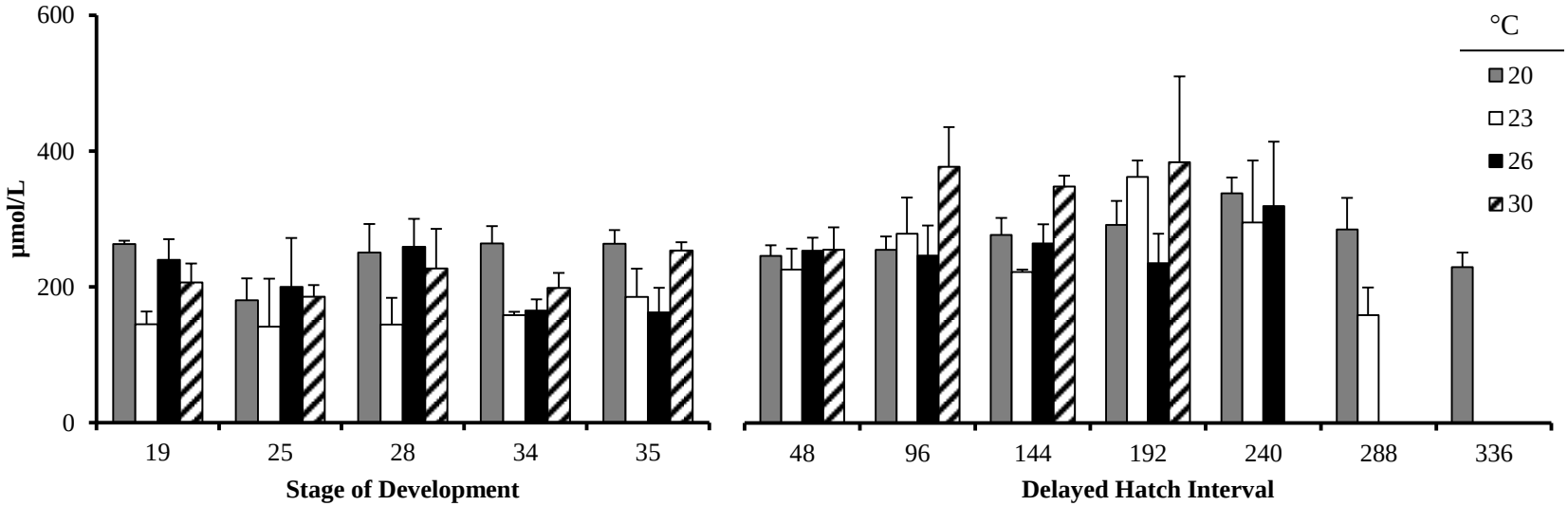

Figure 4.4 Mean lactate concentrations ± standard error of the mean for embryos at different stages of development according to the characterization by Armstrong and Child (1965) and delayed hatch intervals. The break in the x-axis represents the transition from embryogenesis into delayed hatch. Columns depict incubation temperatures: 20, 23, 26, and 30°C.

treatments during the course of the study. Temperature significantly influenced lactate production throughout delayed hatch ($P \leq 0.05$), with the 30°C treatment producing significantly higher lactate than the remaining treatments. Lactate production was not significantly different across stage of development or hours of delayed hatch.

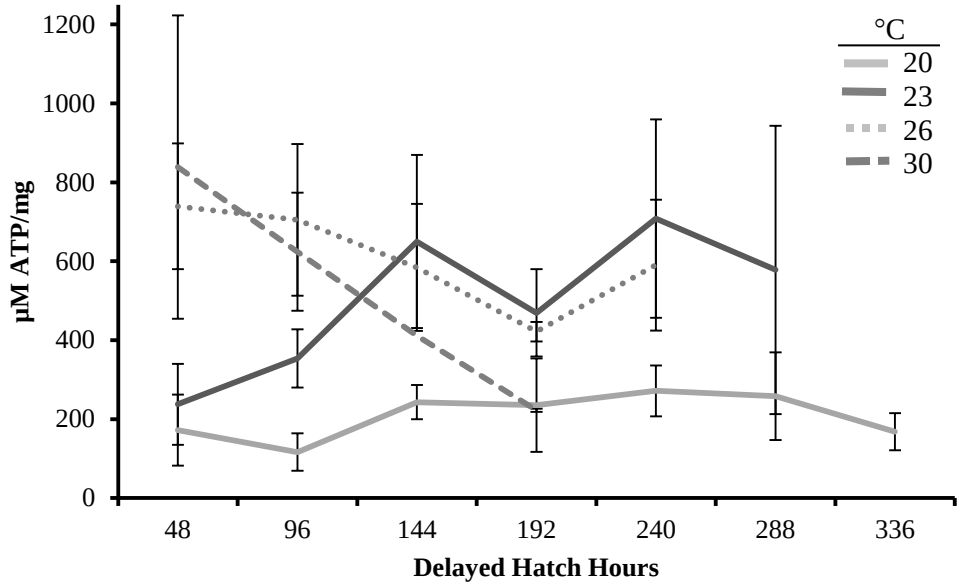

Figure 4.5 ATP concentrations ± standard error of the mean for embryos across delayed hatch intervals. Lines depict temperatures: 20°C, 23°C, 26°C, and 30°C.

ATP Concentrations during Delayed Hatch

Temperature significantly influenced ATP concentrations during delayed hatch (Figure 4.5; $P \leq 0.05$). There was no significant influence of hours of delayed hatch on ATP concentrations ($P \geq 0.05$). ATP concentrations in the 20°C treatment were significantly lower than the remaining treatment temperatures (Figure 4.5). ATP concentrations began to decline in the 30°C treatment at 48 hours post fertilization.

Multivariate Analysis of ATP, BPM, and Lactate

Multivariate analysis of variance found an overall significant cuboidal effect of the predicted ATP, BPM, and lactate values under the influence of temperature (Figure 4.6; Wilks' Lambda 0.69, $P \leq 0.05$). Predicted values for ATP, BPM, and lactate were normal (Shapiro-Wilk $P \leq 0.05$). Peak predicted values occurred at decreasing hours as treatment temperature increased. Peak values occurred at the 240, 192, 96, and 48 delayed hatch hours for the 20, 23, 26, and 30°C treatments, respectively (Fig 4.6 [A-D]).

4.4 Discussion

This experiment demonstrated the Gulf killifish's ability to develop in a terrestrial environment and extend incubation by delaying hatch. Oxygen concentrations in aquatic conditions depend on abiotic factors such as temperature and salinity, and biotic factors such as biological oxygen demand, and will vary according to these dynamic conditions. The site of ovoposition by the Gulf killifish may be a strategy to ensure sufficient oxygen concentrations for embryogenesis and avoid developmental perturbations related to oxygen concentrations associated with delayed rates of embryogenesis and mortalities (Martin et al. 2004).

Higher temperatures tend to accelerate metabolism and development in teleosts (Das et al. 2006, Lin et al. 2006). In the current study, we observed a positive relationship between rate of embryogenesis and temperature (Figure 4.1). Sub-optimal temperatures can slow development, lead to increased rates of developmental deformities, and cause mortalities (Lindén et al. 1979, Das et al. 2006, Jordaan et al. 2006). Though no deformed individuals were documented in this study, a negative relationship was observed between first hatch and

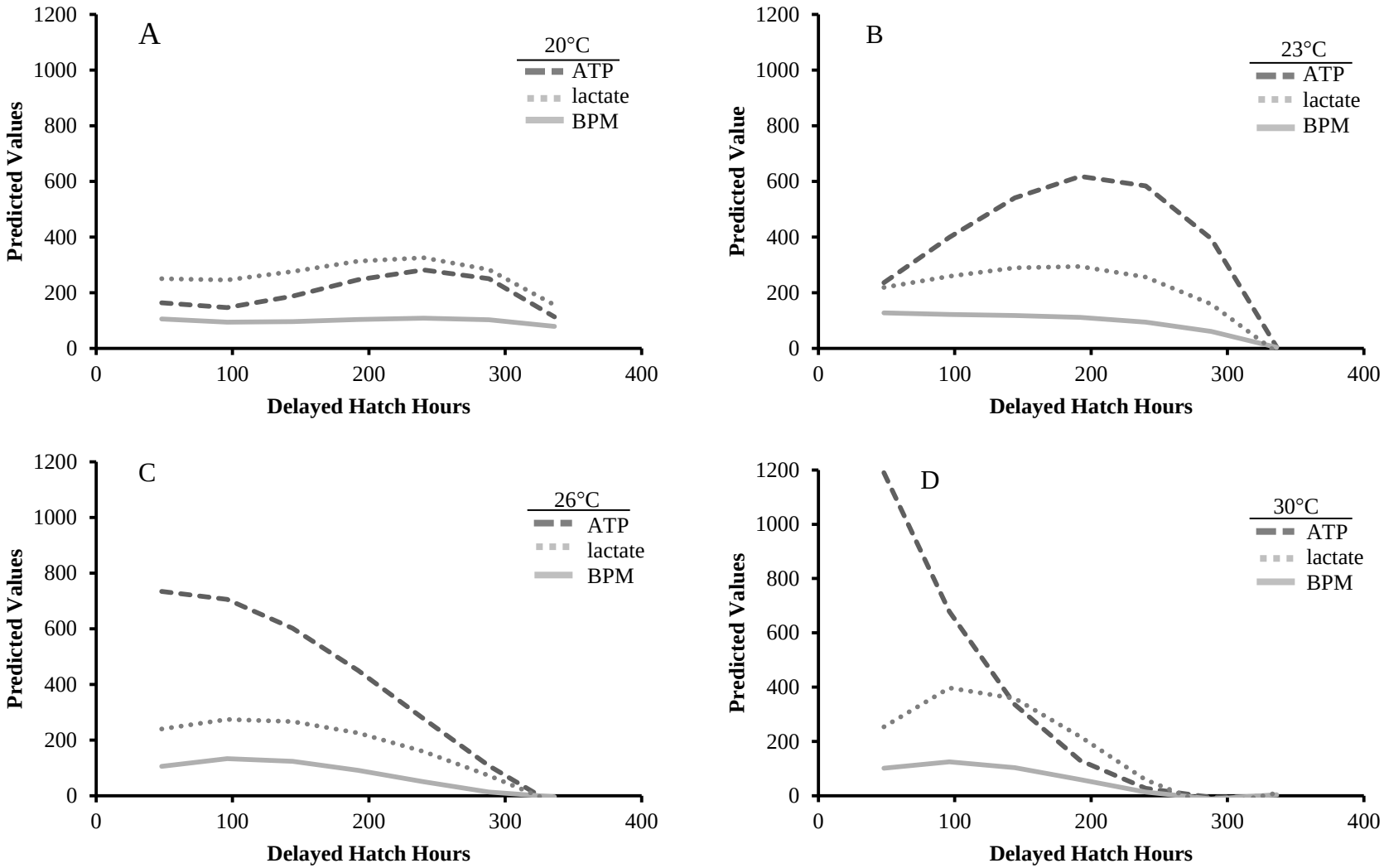

Figure 4.6 Predicted values across delayed hatch hours. Lines depict predicted values for ATP, lactate, and beats per minute (BPM). Incubation temperatures are: 20°C (A), 23°C (B), 26°C (C), and 30°C (D).

temperature. Gulf killifish embryos incubated in water at a similar range of temperatures as this study showed decreasing percent hatch as incubation temperature decreased (Brown et al. 2011).

The strategy of air incubation may have another advantage in improving size and development of larvae at hatch with risk of predation by terrestrial organisms (Warkentin 1995). In the current study, there was no significant influence of temperature on TL or BD at hatch however TL and BD at hatch increased as the delayed hatch interval progressed (Table 4.1). Continuing to develop and grow during delayed hatch allows larvae to increase their ability to find food and escape predation upon hatch (Miller et al. 1988, Huss et al. 2007). Despite the significant relationship of delayed hatch interval on larval size at hatch, we found no significant influence of delayed hatch interval on BCA at hatch, suggesting a depression in metabolic rate. In addition to being well developed, embryos within a regional tidal marsh would have been fertilized at the same peak high tide, and would hatch synchronously at the same returning peak high tide, overwhelming aquatic predators (Colbert et al. 2010). The ability of larvae to hatch within a concise range of size allows for viable larvae to be produced throughout a protracted spawning season that may be characterized by a wide range of temperatures (Smyder and Martin 2002). This is evident through observations in the current study, with viable embryos hatching across a temperature gradient ranging between 20 and 30°C.

Temperature also significantly influenced the length of time hatch could be delayed for air incubated embryos. In the current study, predicted MANOVA results indicated a decreasing shift in time of embryo viability as treatment temperature increased. While the 20° and 23°C treatments saw increasing values that peaked at 240 and 192 delayed hatch hours, respectively, before exhibiting a decline, the 26 and 30°C treatments displayed a decline in predicted values beginning approximately at 48 delayed hatch hours (Figure 4.6). The number of air incubated

viable grunion (*Leuresthes tenuis*) embryos over time decreased as treatment temperature increased (Smyder and Martin 2002). In temperatures exceeding 28°C, the quantity and quality of Gulf killifish eggs has been observed to decrease (Green et al. 2010). Greeley and MacGregor (1983) observed increasing gonadosomatic index values in Alabama populations of Gulf killifish when temperatures warmed to 19°C from 9°C, and observed a decline in reproductive activity when temperatures in the summer months ranged between 25-32°C. The temperature driven decline in viability would negatively affect embryos incubating at 30°C in the current study, as their extent of incubation was approximately 11 days. Embryos incubating at 26°C also displayed a sharp decline in viability after 96 delayed hatch hours, though the extent of incubation in this treatment was about 15 days. The average periodicity of the tidal cycle in the northern Gulf of Mexico is 13.6 days (Greeley and MacGregor 1983), suggesting temperatures above 26°C may be outside thermal tolerance limits of Gulf killifish embryos.

Metabolic rates were observed to have a temporal relationship during extended incubation in addition to a thermal relationship. After an active metabolism during development, metabolic rates began to slow down after 96 delayed hatch hours, at which point anaerobic metabolism may have been used to supplement energetic needs (Finn et al. 1995). Heart rates reached their highest levels across all treatments, except for 20°C, at 96 delayed hatch hours, followed by a decrease in BPM at 144 delayed hatch hours (Figure 4.3). Though temperature significantly influenced heart rate, oxygen may easily diffuse into embryos during air incubation as a result of the high ratio of surface area to biomass (Kranenbarg et al. 2000). The lowest recorded ammonia and lactate levels were at 144 delayed hatch hours. The metabolic depression observed at 144 delayed hatch hours may be intrinsically related to heart type lactate dehydrogenase (LDH), which has been found to be an important enzyme regulating the rate of

development, metabolism, and hatch in the mummichog (Podrabsky et al. 2010). Individuals with the LDH-B^aB^a variant were found to develop faster and hatch sooner than individuals with LDH-B^bB^b (Dimichele and Powers 1982). The LDH-B^bB^b variant has a greater ability to bind ATP, which is an allosteric modifier that decreases the erythrocyte oxygen binding affinity (Dimichele and Powers 1982). This decreased oxygen binding affinity could lead to respiratory stress that would trigger the hatching mechanism. Upon missing an optimal temporal window at 96 delayed hatch hours, LDH expression may have decreased in an effort to decrease the chance of premature hatching.

End product metabolite concentrations, as measured by ammonia and urea release, varied throughout embryogenesis. Ammonia concentrations were significantly lower during the formation of the embryonic keel (stage 19) than the remaining sampled stages of embryogenesis. This is unusual because rates of respiration increase continuously with the formation of the embryonic keel (Walsh et al. 1989, Finn et al. 1995). Embryonic Atlantic halibut (*Hippoglossus hippoglossus*) have been shown to store ammonia in the yolk during early developmental stages when ammonia cannot be readily and rapidly expelled or converted into urea (Steele et al. 2001, Terjesen et al. 2002). Ammonia levels increased significantly upon the onset of circulation (stage 25) in the 20 and 23°C treatments (Figure 4.2). The peak in ammonia levels at this stage may be a result of newly mobilized erythrocytes removing stored ammonia from the yolk in addition to direct ammonia removal through Rh glycoproteins (Pelster and Burggren 1996, Braun et al. 2009). However, no peak was observed at the onset of circulation in the 26 and 30°C treatments for ammonia, possibly due to the increased thermal energy available for ammonia volatilization through the chorion. Ammonia was intermittently released in large concentrations in the remaining stages of embryogenesis at 26 and 30°C. Throughout air incubation embryos would

need to maintain a hyperosmotic environment to maintain hydration and prevent desiccation. Embryos would be unable to take up ions from the environment and would be utilizing a decreasing ionization resource of FAA as development progressed. Ammonium (NH_4^+) may serve as an osmolite when stored in the yolk sac, potentially preventing dehydration (Finn et al. 2000, Terjesen et al. 2002, Skoblina 2010). In the current study, we observed alternating ammonia and urea release, indicating a shunting of end nitrogenous waste down a pathway of elimination. Production of glutamine is a method of ammonia detoxification and elevated levels of glutamine synthatase have been observed in embryonic rainbow trout (Essex-Fraser et al. 2005). Teleost embryos then utilize glutamine to produce urea via the OUC for use as an osmolite or to avoid ammonia toxicity (Wright et al. 1995). Wilkie et al. (2007) observed increased urea and associated water levels in the muscles of the African Lungfish (*Protopterus dolloi*). Reduced gene expression of *Fundulus heteroclitus* aquaporin 3 (*FhAqp3*), a protein involved in water, glycerol, and urea permeability, has been observed to decrease in air incubated mummichog embryos compared to aquatically incubated embryos (Tingaud-Sequeira et al. 2009). Urea may be produced and retained during embryogenesis as a strategy to prevent desiccation until development is completed. Urea is also produced from arginase catabolism during embryogenesis, and could be a result of embryonic growth and tissue anabolism (Watford 2003).

During delayed incubation there was a significant influence of temperature and delayed hatch interval on ammonia production. The potential inability of ammonia to volatilize at 20°C could be a factor in the large concentrations recorded. However, microbial organisms may have introduced exogenous sources of ammonia. In the current study we observed thinning of the chorion beginning at 48 delayed hatch hours across all thermal treatments. As the chorion of

embryos began to break down, microbes may have begun to feed and proliferate on their surface (Hansen and Olafsen 1999). Perschbacher et al. (1995) also observed thinning of the chorion as air incubation proceeded. Urea levels were significantly influenced by temperature and delayed hatch interval during delayed incubation. Higher temperatures incur greater metabolic costs as evident of the large concentrations of urea released by embryos in the 30°C treatment. The rate of ammonia volatilization may not have been sufficient to avoid toxic concentrations, resulting in production of urea via the OUC. The release of urea upon immersion may be a result of anticipated hatch as we observed a significant increase in urea released at 192 delayed hatch hours. Increased expression of UT's was observed in rainbow trout upon hatch (Hung et al. 2008). The 192 delayed hatch hours sampling period could represent the end of an anticipated hatch window that began at 144 delayed hatch hours, subsequently this window in time encompasses half a tidal cycle of approximately 164.4 hours in the northern Gulf of Mexico (Greeley and MacGregor 1983).

Air incubation and associated biological processes of the Gulf killifish appear to have a strong temporal relationship possibly related to the tidal cycles that regulate spawning. Other species with a tidal cycle influenced developmental relationship include the California grunion (Smyder and Martin 2002), Gulf grunion (*Leuresthes sardine*; Moffatt and Thomson 1978), and the mummichog (Taylor 1986). However, the thermal effect on embryogenesis and delayed hatch strongly influences the nature of the temporal relationship (Smyder and Martin 2002). Gulf killifish embryos displayed an ability to depress their metabolism, but still maintain an active rate compared to annual killifish embryos that enter diapause (Podrabsky and Hand 1999).

CHAPTER 5: SUMMARY AND CONCLUSIONS

There is a lack of research on embryogenesis and developmental responses of Gulf killifish (*Fundulus grandis*) to abiotic conditions during embryogenesis. In contrast, extensive research has been conducted measuring embryogenesis and developmental responses for a closely related species, the mummichog (*Fundulus heteroclitus*; Tay and Garside 1975, Lindén et al. 1979, DiMichele and Taylor 1980, DiMichele and Powers 1984, DiMichele and Westerman 1997, Katoh et al. 2000, Fortin et al. 2008, Tinguad-Sequeira et al. 2009). While some work has been done on developmental responses of the Gulf killifish (Ernst et al. 1977, Perschbacher et al. 1990, Brown et al. 2011, Coulon et al. *In press*), little is known about the influence of abiotic factors on specific aspects of embryology.

The effect of temperature on teleost embryogenesis has been documented for the mummichog (Lindén et al. 1979, DiMichele and Westerman 1997) and Gulf killifish in the current study displayed similar developmental responses. Incubation water temperature was observed to have a positive relationship with percent hatch and a negative relationship with size at hatch and time to hatch. The amount of time embryos spent in the chorion appeared to increase the rate of mortality, as embryos incubated in 20°C were largest, but had a reduced hatch percent. The incubation temperature of 20°C may be suboptimal for Gulf killifish embryogenesis, however, culturists must decide if a larger size at hatch at a reduced hatch percentage is acceptable.

Salinity was not observed to influence size at hatch, however, other biological parameters of embryogenesis were influenced. The peripheral range of Gulf killifish embryologenic osmotic tolerance was determined to be at 0.4 and 30 g/L. Accelerated embryogenesis was documented as salinity decreased, but reduced hatch percentages were observed in the 0.4 and 30 g/L

treatments. Hypersaline conditions (>32 g/L) were not documented in this thesis, but may prove fatal to embryos based on the results of this study. Nitrogenous metabolites, in the form of ammonia or urea, and concentration also depended on salinity with embryos releasing mainly ammonia in higher incubation salinities. Incubation in salinities ranging approximately from 5 to 15 g/L may be optimal for Gulf killifish embryogenesis.

An accelerated rate of embryogenesis was observed during air incubation relative to aquatic incubation of this species. Metabolic waste concentrations were higher in air incubation compared to aquatic incubation (Chapter 3.3). Air incubation has been demonstrated to cause increased expression of proteins associated with oxidative stress (Podrabsky et al. 2010). Temperature associated stresses were also observed in addition to stresses caused by air incubation. Embryogenesis for the 30°C treatment was relatively brief compared to the remaining treatments and first hatch occurred at 96 delayed hatch hours, but embryo viability began to decrease upon the initiation of delayed hatch and high urea concentrations were reported throughout delayed hatch. The maximum extent of delayed hatch was observed in the 20°C treatment compared to the remaining treatments, but first hatch did not occur until 240 delayed hatch hours and high ammonia levels were reported throughout delayed hatch, which may not be acceptable in commercial hatchery conditions.

This thesis demonstrated the influence of temperature, salinity and air incubation on embryogenesis. Temperature and salinity affected critical biological aspects of the Gulf killifish at hatch, such as percent hatch, rate of embryogenesis, body size and the quantity of yolk. The modification of these parameters can improve incubation of this species. Current practices utilize spawning mats that are placed in broodstock pools (MacGregor et al. 1983, Waas et al. 1983). Mats containing fertilized eggs are transferred to growout ponds to allow fry to develop.

However, Gulf killifish embryos may be fertilized at different times and fry within the same clutch can hatch out over a protracted length of time ranging in days. This could lead to intracohort larval cannibalism as it is a frequent occurrence among species of teleosts with increased larval size heterogeneity (Baras and Jobling 2002). These results may be applied to hatcheries culturing the Gulf killifish and mummichog. Manipulation of temperature can be used to control the rate of embryogenesis (Tay and Garside 1975). Cohorts fertilized at different times may be incubated at specific temperatures to coordinate a simultaneous hatch date and could result in more homogeneously sized populations. The results of this thesis may also be used in pond production. Knowledge of incubation parameters would allow culturists to estimate size at hatch, time to hatch, and rates of mortalities of pond raised larval cohorts. The use of additional molecular tools and formulation of bioenergetics models to detail effects of temperature, salinity, and air incubation on embryogenesis is still needed. Recommendations from this thesis can be used to optimize embryogenesis and improve the culture of this species within hatchery settings through the controlled modification of incubation conditions.

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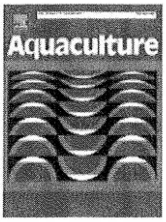

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VITA

Charles A. Brown was born in Augusta, Georgia, in June of 1984. After graduating Evans High School in 2002, he attended Augusta State University. Charles graduated from Augusta State University in 2008 with a Bachelor of Science in biology and a minor in chemistry. Charles began his pursuit of his Master of Science degree in fisheries and aquaculture at Louisiana State University in the summer of 2008 under Dr. Christopher Green as a graduate student at the School of Renewable Natural Resources and plans to graduate in the spring of 2011.