EFFECT OF WATER HARDNESS ON THE SURVIVAL OF RAINBOW SHARKMINNOW (*Epalzeorhynchos frenatum)* EGGS AND LARVAE

By

MICHAEL ANDREW ABERNATHY

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2004

To my parents and fiancé.

ACKNOWLEDGMENTS

I would like to thank:

Dr. Frank Chapman for the intellectual and financial support that made this project possible.

Dr. Charles Cichra for his statistical help as well as his guidance and support.

Dr. David Evans for his advice and support.

John and Kim Skidmore for opening up their farm and home to provide anything needed for the project and my aquaculture education.

All of the people at the UF/IFAS Department of Fisheries and Aquatic Sciences Tropical Aquaculture Laboratory in Ruskin for their time and the use of their facilities.

My father and late mother for their never ending support of my education.

My fiancee Vanessa Wallach for her emotional and financial support.

The faculty and students of the UF Department of Fisheries and Aquatic Sciences for their ideas and friendship.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iii

LIST OF TABLES v

ABSTRACT vi

CHAPTER

1 INTRODUCTION 1

2 METHODOLOGY 6

3 RESULTS 11

4 DISCUSSION 14

LIST OF REFERENCES 26

BIOGRAPHICAL SKETCH 29

LIST OF TABLES

Table		page
4-1	Replicate design for trials 1 and 2	20
4-2	Composition and chemistry for all treatment and control incubation waters.	21
4-3	Comparison of mean square root percent hatch (+ s.d.), mean square root percent larval survival (+ s.d), and mean egg volume (+ s.d.) among incubation waters within treatments .	22
4-4	Comparison of mean square root percent hatch (+ s.d.) among treatments within incubation waters	23
4-5	Comparison of mean square root percent larval survival (+ s.d.) among treatments within incubation waters.	24
4-6	Comparison of mean egg volume (+ s.d.), in cubic millimeters, among treatments within incubation waters.	25

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

EFFECT OF WATER HARDNESS ON THE SURVIVAL OF RAINBOW SHARKMINNOW (*Epalzeorhynchos frenatum*) EGGS AND LARVAE

By

Michael Andrew Abernathy

May 2004

Chair: Frank Chapman
Major Department: Fisheries and Aquatic Sciences

Florida's highest grossing aquaculture product is ornamental fish for the aquarium trade. The native waters of many of the species are soft in terms of water hardness due to the fact that they are low in dissolved divalent cation concentrations. Some of those species have produced low hatch rates and larval survival when spawned in the typically hard water from the Floridan aquifer. This study used the rainbow sharkminnow (*Epalzeorhynchos frenatum*), a species native to soft water and one with consistently poor hatch (50%) and larval survival (70%), to test the effects of incubating eggs in waters with varying levels and types of water hardness. Four treatments, one with calcium and magnesium hardness, one with calcium only hardness, one with magnesium only hardness, and one with no water hardness, were tested. This study found that very soft waters (6.4-7.0 mg/L as $CaCO_3$) produced the highest hatch and larval survival. It also found that calcium is a necessary component of incubation water, and that

magnesium is not a necessary component of incubation waters for the rainbow sharkminnow.

CHAPTER 1
INTRODUCTION

Freshwater ornamental fish for the home aquarium are Florida's most valuable aquaculture commodity. The most recent survey reported 160 producers with farm gate sales totaling $42.4 million in 2001 (Florida Agricultural and Statistics Service [FASS] 2002). One of the more difficult aspects of ornamental aquaculture is husbandry during the early life stages. The most sensitive stage in the life cycle of a teleost is generally considered to be the developing egg and larva (Von Westernhagen 1988). One abiotic parameter having a major effect on egg development, and egg and larval survival is water hardness (Brown and Lynam 1981; Ketola et al. 1988; Spade and Bristow 1999).

Water hardness is the measure of all divalent cations and is expressed in mg/L as calcium carbonate ($CaCO_3$). Water hardness enters the aquatic environment through the leaching of sedimentary rock, containing sources of divalent cations, such as limestone and gypsum. In most natural bodies of freshwater, calcium and magnesium are the major constituents of water hardness (Boyd 1979). Historically, water hardness has been related to the capacity of water to produce lather from soap. Softer water produces more lather than harder water. Water hardness is often split into two categories; permanent and temporary. Temporary hardness is the part that is chemically associated with carbonate, such as $CaCO_3$. It is called temporary hardness because it can be boiled or precipitated out of a solution. Permanent hardness is the amount of hardness in excess of the carbonate hardness and cannot be boiled or precipitated out of solution.

Water hardness has been shown to have a direct effect on the swelling of newly fertilized eggs, which is an important process during the early development of the teleost egg (Spade and Bristow 1999). The process of egg swelling, as described by Redding and Patino in *The Physiology of Fishes* (1993), is the uptake of extracellular water into the perivitelline space. The perivitelline space is located between the outer chorion of the egg and the vitelline membrane that surrounds the developing embryo. In a fertilized egg, the fluid filled perivitelline space provides room and protection for embryonic development (Eddy 1974). According to Rudy and Potts (1969) and Alderdice (1988), the egg draws in extracellular water due to the fact that it has greater osmotic pressure than the extracellular water it is bathed in. Osmotic pressure is greater in the perivitelline space of the egg because the vitelline membrane releases cortical substances into the perivitelline space which increases the osmotic pressure (Redding and Patino 1993). According to this theory, the greater the difference between the osmotic concentration of the perivitelline space and the extracellular water, the greater the swelling of the egg. Typically, egg swelling increases when water hardness decreases because low water hardness usually means low osmotic concentration. Other ions, both mono and multivalent, also play a role in egg swelling. The greater the valence of the ions, the greater the egg swelling is reduced (Eddy and Talbot 1983).

Previous research has investigated the effect of water hardness on eggs and larvae of cultured fish. Spade and Bristow (1999) examined the effects of increasing water hardness on egg diameter and hatch rates of striped bass (*Morone saxatilis*) eggs. They found that increased water hardness of the incubation water reduced swelling (egg volume), which in turn stopped the eggs from rupturing and reduced buoyancy. The

decrease in buoyancy was enough to stop eggs from floating out of hatching jars and being lost. Another study found that calcium, which is a component of water hardness, increased mortality of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and brook trout (*Salvelinus fontinalis*) eggs when in high concentration of approximately 520mg/L in the incubation water (Ketola et al. 1988). In a similar study, Gonzal et al. (1987) found that over a range of water hardness from 100-600 mg/L as $CaCO_3$, silver carp (*Hypophthalmichthys molitrix*) eggs had the greatest hatch and viability when incubated and hatched in 300-500 mg/L as $CaCO_3$ of hardness. These three studies demonstrated that water hardness has a direct effect on egg swelling and survival of developing eggs and larvae and that different species of fish have specific concentrations of water hardness for optimal egg and larval survival.

Using the finding of these three studies, this study was designed to investigate the effects of varied concentration and type (calcium and/or magnesium) of water hardness on the survival of rainbow sharkminnow (*Epalzeorhynchos frenatum*) eggs and larvae. The rainbow sharkminnow is one of the popular species cultured for the ornamental fish trade. Typically, percent hatch for rainbow sharkminnow eggs is 50% and larval survival is approximately 70% (J. Skidmore, Golden Pond Tropicals, pers. comm. 2003). In Florida, the most common freshwater ornamental fish aquaculture practice is to raise fish in outdoor earthen ponds. Fish either spawn in the ponds or are brought indoors and spawned in water from the Floridan aquifer. Water from the Floridan aquifer in Hillsborough County, where most of the ornamental fish farms are located, is typically hard with average concentrations of 180mg/L of hardness as $CaCO_3$ (Shattles 1965). The rainbow sharkminnow was selected for this study because it met a number of criteria. It

has low hatch and larval survival (50% and 70% respectively), and is native to soft water systems like many of the ornamental fish cultured in Florida (Froese and Pauly 2003; Rainboth 1966). It can also be hormonally induced to spawn and has high fecundity, with females producing around 10,000 eggs per spawn (Shireman and Gildea 1989), which provided sufficient numbers of eggs in a timely manner for the study. Of particular interest was the fact that the rainbow sharkminnow is native to waters of low hardness such as the Mekong River. One hypothesis for the cause of the low hatch rate and larval survival is that the eggs and larvae of the rainbow sharkminnow have evolved to develop in soft water, and therefore have low hatch rates and larval survival when incubated and hatched in hard water from the Floridan aquifer.

Water hardness can be increased by the addition of salts containing divalent cations such as calcium chloride (Yeager 1994) and can be decreased by precipitating carbonates such as calcium carbonate and magnesium carbonate out of a solution or via ion exchange or reverse osmosis. Therefore, hatchery systems can be adjusted to provide optimal water hardness levels for the incubation and hatching of different species of fish.

Increasing hatch rates and larval survival would reduce the number of rainbow sharkminnows, or any species of cultured fish, needed to reach desired production levels. The reduction in broodstock numbers would decrease maintenance and breeding costs for a production facility.

To test the effects of water hardness on rainbow sharkminnow eggs and larvae, eggs were incubated and hatched in reconstituted freshwater, of varying levels and type of water hardness. Hatch rate and larval survival were determined. Egg volume was measured to see if there was a relation between egg volume and hatch rates as previously

documented for striped bass (Spade and Bristow 1999). As mentioned above, it is known that water hardness is directly related to egg swelling, which provides physical protection and room for the developing embryo, and egg and larval survival. Therefore, it is hypothesized in this study that a specific concentration of water hardness can be found for optimal hatch rates and larval survival of the rainbow sharkminnow.

CHAPTER 2
METHODOLOGY

Two trials were conducted during the course of the experiment and both used the same methodology. A trial consisted of incubating rainbow sharkminnow eggs in waters of varying concentrations and types of water hardness. The first trial started on July 30, 2004 two days before the second trial started on August 1, 2004. Four females and eight males were used for each trial, each female was considered a replicate, providing four replicates per trial. The four females used in the first trial were different fish than those used in the second trial. The experimental design for each trial is given in Table 1.

The reconstituted waters used for incubation were prepared based on the formulations provided by Marking and Dawson (1973) and varied principally on the concentration, presence, or absence of the calcium and magnesium ions. Marking and Dawson (1973) categorized their reconstituted freshwaters by level of hardness, measured as mg/L of calcium carbonate ($CaCO_3$), with very soft being 10-13, soft 40-48, moderately hard 80-100, hard 160-180, and very hard 280-320, abbreviated as VS, S, MH, H, and VH, respectively. The different incubation waters in this study, which varied in levels of sodium bicarbonate ($NaHCO_3$), calcium sulfate ($CaSO_4$), magnesium sulfate ($MgSO_4$), potassium chloride (KCl), and glucose ($C_6H_{12}O_6$) were categorized using the VS, S, MH, H, and VH abbreviations. The amount of added constituents increased from the VS incubation waters which had the least, up to the VH incubation waters which had the most (Table 2). The incubation waters were prepared using Fisher

Brand certified chemical reagents (Fisher Scientific Inc.). The solutions were first mixed then measured for water hardness, alkalinity, pH, and osmolality. Hardness measurements were made using the ManVer 2 Buret Titration method from the Hach water analysis handbook 3rd edition (Hach, Co. 1997). Alkalinity measurements were made using the buret titration method from the Hach water analysis handbook 3rd edition (Hach, Co. 1997). pH was measured using a Corning 120 pH meter (Corning, Inc.), and osmolality was measured using a Vapro vapor pressure osmometer (Wescor, Inc.). Incubation water composition and chemistry is provided in Table 2.

Four treatments (CAMG, CA, MG, and Z) were developed to test the effects of varying levels and different types of water hardness on egg development. Each treatment consisted of six incubation waters; VS, S, MH, H, VH, and a control. The CAMG treatment, named for having both calcium and magnesium hardness, was based solely upon the reconstituted waters developed by Marking and Dawson (1973). Therefore, the incubation waters of the CAMG treatment were of approximately equal chemistry and composition to the reconstituted freshwaters developed by Marking and Dawson (1973). The CAMG treatment was developed to test the effects of varying concentrations of calcium and magnesium hardness on egg swelling and egg and larval survival. The CA and MG treatments, named for having calcium and magnesium only hardness, respectively, were made to measure the effects of varying levels of calcium only and magnesium only hardness on egg swelling and egg and larval survival. The Z treatment was made to test the effects that hardness-free water had on egg swelling and egg and larval survival. Hardness levels remained approximately equivalent among the respective incubation waters of the CAMG, CA, and MG treatments. For the CA

treatment, this was accomplished by adding molar equivalents of $CaSO_4$ for the $MgSO_4$ removed. The Mg treatment replaced $CaSO_4$ with molar equivalents of $MgSO_4$. The Z treatment, which had no water hardness, was made to measure the effect of hardness-free water on egg swelling and egg and larval survival. Each category of incubation water (i.e., VS, S, MH, H, and VH) had approximately equivalent osmolalities so that egg development could be compared within and among treatments without the confounding variable of differing osmolality. For the Z treatment, glucose replaced hardness constituents to keep the osmolality of the incubation waters equivalent to those of the other three treatments. Glucose was used because it is a non-electrolyte macromolecule that does not have any inhibitory effects on egg swelling and cannot pass through the chorion of the egg (Potts and Rudy 1969; Eddy 1974).

Water pumped from the Floridan aquifer up to the Golden Pond Tropicals fish farm was used as the control so that egg development in the four test treatments could be compared to current on-farm hatch rates and larval survival. Control water hardness, alkalinity, pH, and osmolality were measured, using the same methods as were used for the incubation waters, and are provided in Table 2.

Rainbow sharkminnows were seined from earthen ponds and placed into 160-L foam holding vats filled with pond water. Females were sedated with 80mg/L of tricaine methanesulfonate (Western Chemical Inc.) and then catheterized to attain egg samples. Only females whose eggs had the germinal vesicle near the periphery were considered mature and selected for the study. Two males were selected for every female to ensure there was enough milt to fertilize all of the eggs. Males were chosen by size, with 10 cm (total length) or larger fish considered sexually mature % (J. Skidmore, Golden Pond

Tropicals, pers. comm. 2003). Within an hour of collection, the females were sedated a second time with 80mg/L of tricaine methanesulfonate and injected at the base of the dorsal fin with a primer dose, 20% of the total dose of one microgram (1μg) ovaprim (Syndel International, Inc.) to one gram (1g) of fish. Six hours after the primer, the remainder of the dose was injected into the females after they were sedated a third time with 80mg/L of tricaine methanesulfonate. Males were sedated with the same dose of tricaine methanesulfonate as the females and injected at the base of the dorsal fin with a full dose of ovaprim (1μg ovaprim/ 1g of fish) immediately after the females were given their second injection. The injection sequence was designed so that the eggs and milt would be ready for stripping at the same time.

Females and males were stripped of eggs and milt four to six hours after the final injection. Eggs from each female were placed into separate glass dishes. Milt from the two males was mixed with the eggs in each dish using a feather. A small plastic spoon was used to place approximately 100 eggs into a glass jar with 10ml of incubation water for activation. After swirling the eggs, milt, and water together for one minute to allow for fertilization, eggs were placed into glass dishes with 150ml of incubation water where they remained for the duration of the experiment. Fifty percent water changes were made every six hours to reduce water contamination by egg metabolites. Incubation temperature was 26.6 °C and all tests were run in a dimly lit room.

Egg development was observed and photographs taken over a 37-hour period after the eggs were placed into the dishes. Dead eggs, those which had ruptured or appeared cloudy, were removed after each photo set. Egg diameter was measured after 13.5-14.5 hours of incubation, at which time swelling was considered complete. Egg volume

comparisons between and within CAMG and CA treatments were made. Treatments MG and Z were not part of the comparison because all eggs in those treatments were dead and it was impossible to obtain good measurements due to their poor condition. Eggs were counted and diameters measured using image analysis software (Motic Images 2000, version 1.3) installed in a personal computer. Egg volume was calculated using the equation $\pi D^3/6$, where D is the egg diameter. Percent hatch was measured by dividing the number of hatched eggs by the number of fertilized eggs. Larval survival was determined 12 hours after the eggs hatched by dividing the number of surviving larvae by the number of hatched eggs.

Percent hatch and percent larval survival were calculated for each dish because the number of eggs varied from dish to dish. This allowed for a comparison of incubation waters independent of the number of eggs in each dish. Percents for hatch and larval survival were transformed to square root percent hatch and larval survival before being analyzed statistically. The data were transformed to increase the R^2, which is the proportion of the variability that the analysis could explain. Separate one-way ANOVAs were performed to determine if the type and concentration of water hardness had an effect on egg volume, hatch rate, and larval survival. If significant effect was found, differences among treatment means were determined via a Duncan's Multiple Comparison Test. The square root percent hatch and larval survival were regressed on the volume of the eggs from the CAMG and CA treatments to determine if there was a relation between volume and hatch or volume and larval survival. A significance level of alpha=0.05 was used for all tests. All analysis were conducted using the Statistical Analysis System (SAS 1985).

CHAPTER 3
RESULTS

Within the CAMG treatment, the hardness of the H incubation water (154mg/L as $CaCO_3$) was more similar to the control water hardness (180mg/L as $CaCO_3$) than the VS, S, MH, and VH incubation waters (Table 2). The control water, which was pumped from the Floridan aquifer, was the same water that was used on the fish farm where the study was conducted. There was no significant difference in egg volume, hatch, or larval survival between the H incubation water of the CAMG treatment and the control water (Table 3). Therefore, the CAMG treatment and all other treatments which were derived from the CAMG treatment were considered appropriate test waters to compare to the hard water from the Floridan aquifer.

The VS incubation waters of the CAMG and CA treatment had significantly greater egg volume than all other incubation waters of the CAMG and CA treatments, respectively (Table 3). These data also show that egg volume decreased as hardness increased from the VS incubation water to the VH incubation water in both the CAMG and CA treatments. Table 2 shows that the amount of added constituents increased going from the VS incubation waters to the VH incubation waters. This suggests that there was a relationship between the amount of added constituents in the incubation waters and egg volume. Table 3 also shows that the VS incubation waters of the CAMG and CA treatments had significantly higher larval survival than the H, VH, and control incubation waters of the CAMG and CA treatments. The regression analysis of the square

root percent larval survival (SRPERLRV) on egg volume within the CAMG treatment shows that there was a positive relationship between egg volume and larval survival with greater volume resulting in greater larval survival (SRPERLRV = -3.06 + 0.26 [egg volume], N = 24, $R^2 = 0.36$, $p < 0.01$). The regression analysis of the SRPERLRV on egg volume within the CA treatment also shows that there was a positive relationship between egg volume and larval survival with greater volume resulting in greater larval survival (SRPERLRV = -3.33 + 0.33 [egg volume], N = 24, $R^2 = 0.44$, $p < 0.001$).

The CA treatment tested the effects of removing magnesium from the CAMG incubation waters. The similar results for square root percent hatch and square root percent larval survival for the CAMG and CA treatments demonstrate that magnesium is not a necessary component in the incubation and hatch waters for rainbow sharkminnow eggs (Tables 4 and 5).

The MG treatment tested the effects of removing calcium from the CAMG incubation waters. All of the eggs in the VS, S, MH, H, and VH incubation waters of the MG treatment died. The results suggest that calcium is an essential component of incubation waters for rainbow sharkminnow eggs.

The Z treatment tested the effects of removing all hardness (calcium and magnesium) from the CAMG incubation waters. As a result of omitting the calcium and magnesium, the sulfate was also omitted from the incubation waters (VS, S, MH, H, and VH) of the Z treatment and was therefore the only treatment without sulfate. All of the eggs in the Z treatment VS, S, MH, H, and VH incubation waters died. These results suggest that the absence of calcium, magnesium, and/or sulfate were the cause of egg death.

There were no significant differences in hatch, larval survival, or volume among the eggs from all eight females incubated in the control water (Tables 4, 5, and 6). Due to the consistency among the eggs in the control water, it was possible to consider all females from both trials as equal (replicates) and to analyze the data for hatch, larval survival, and volume among and within all treatments and incubation waters.

CHAPTER 4
DISCUSSION

Egg mortality, due to a lack of calcium in incubation waters, has been previously documented by Lee and Hu (1983) with grey mullet (*Mugil cephalus*) and Brown and Lynam (1981) with brown trout (*Salmo trutta trutta*). They attributed the mortality to the fact that calcium is needed for normal hardening of the chorion (Zotin 1958) and that calcium is an important factor in controlling membrane permeability. Calcium ions decrease membrane porosity by allowing the closer packing of polar organic molecules in the membrane (Maetz 1974). A more porous or permeable membrane could result in osmoregulatory stress and subsequently death for the developing embryo. Calcium has also been shown to play an important role in the development of sea urchin eggs (*Lytechinus pictus*). At the instant of sperm contact, there is an initial depolarization of the plasma membrane which is followed by an extracellular calcium dependent action potential (Alderdice 1988). The similarities of sea urchin egg and fish egg development allow for the probability of a calcium-dependent electrical function following sperm contact in the rainbow sharkminnow egg. The mortality of the rainbow sharkminnow eggs, incubated in the MG and Z treatments calcium-free incubation waters, show that there is at least one extracellular calcium-dependent function during their development. Whether it was a disruption in normal egg swelling, of membrane permeability, of an action potential, or some other calcium dependent function, the disruption caused by the absence of calcium in the case of the Z and MG treatments caused 100% mortality of the

rainbow sharkminnow eggs. The absence of sulfate from the Z treatment is not considered a cause of egg mortality because sulfate is not considered a necessary component of teleost egg development (Alderdice 1988).

Lee and Hu (1983) found that magnesium is not a necessary component of incubation water for grey mullet eggs. Their findings are supported by the results from the magnesium-free incubation waters of the CA treatment of this experiment. Although magnesium is known to be necessary for some enzymatic functions in the metabolic cycle, the data from this study show that there are no extracellular magnesium dependent functions for egg development or for newly hatch larvae of the rainbow sharkminnow.

All added constituents of the CAMG, CA, and MG treatments incubation waters were ions. Thus, solute concentration (osmolality) was directly proportional to the ionic concentration (increased solute concentration = increased ionic concentration). Increasing the solute/ionic concentration of the incubation waters increased the osmotic concentration (increased solute/ionic concentration = increased osmotic concentration). Egg volume in the CAMG and CA treatments, which was the measurement of the amount of egg swelling, decreased with increased osmotic concentration. This can be explained by the fact that egg swelling is an osmoticaly driven process. When osmotic concentration is greater inside the perivitelline space than the extracellular water surrounding the egg, extracellular water moves into the perivitelline space via osmosis causing the egg to swell. The fact that all eggs, in the CAMG and CA treatment incubation waters, increased in volume means that the CAMG and CA treatment incubation waters were hypoosmotic to the perivitelline space of the rainbow sharkminnow eggs.

The swelling of the egg causes the internal hydrostatic pressure to increase (Alderdice et al. 1984; Kao and Chambers 1953). Alderdice (1984) explains that the flow of extracellular water into the perivitelline space of the egg (swelling) ceases when the hydrostatic pressure in the perivitelline space reaches a level which prevents further movement into the perivitelline space. As the hydrostatic pressure increases, so does the tension of the plasma membrane, which decreases its permeability to ions and water (Alderdice 1988). Decreased permeability would allow for greater protection against osmotic stress for the developing embryo. Therefore, one likely cause for greater egg and larval mortality, for eggs with intact protective chorions, found in the incubation waters with higher osmotic concentration and lesser egg volume was osmotic stress due to increased membrane permeability.

The data (Table 2) also show that pH increased with greater osmotic concentration, and was therefore the lowest in the VS incubation waters of the CAMG and CA treatments. The significantly higher larval survival in the VS incubation waters compared to the H, VH, and control incubation waters is not believed to be related to the lower pH, nor is the higher pH in the other incubation waters believed to be related to increased larval mortality. pH has been shown to be a factor in increased egg and larval mortality, but only when eggs were incubated and hatched at low (4.0-5.0) levels of pH (Ingersoll et al. 1990; Von Westernhagen 1988). Low pH impairs the function of the hatching enzyme (chorionase), can be toxic to newly hatched larva, and can inhibit egg swelling (Von Westernhagen 1988). The range of pH in this experiment (6.7-8.3) did not reach levels that are normally considered toxic to fish eggs and larva (Von Westernhagen

1988), and were therefore not considered to have a significant effect on the survival or mortality of rainbow sharkminnow eggs and larvae.

There are many possible factors for why the highest hatch and larval survival in the control incubation waters of this experiment were substantially lower than those experienced on the farm (21.8% hatch and 7.8% larval survival versus 50% hatch and 70% larval survival, respectively). Considering that the control and farm water were the same, on-farm breeding practices are equivalent to this experiment up to the point where eggs and milt were divided into the different incubation waters for the experiment. Handling of the eggs was increased in this experiment compared to on-farm practices during the spawning due to the experimental design, which could be one reason for increased egg and larval mortality. Another possible factor is that on-farm hatching is done in McDonald jars which have water pumped in at an approximate rate of 1 liter per minute. The constant flow of water changes the water in the McDonald jars hundreds of times a day ensuring that dissolved oxygen remains high and toxins, such as ammonia and carbon dioxide, remain low in the incubation water. Due to practical constraints, it was not possible to change the water in the incubation dishes during this experiment as quickly. It is therefore likely that oxygen levels were lower or toxin levels were higher resulting in increased egg and larval mortality. One other possible factor was that this experiment was conducted in August which is late in the spawning season (May through August) for rainbow sharkminnows. Hence, it is possible that the initial egg quality had begun to deteriorate, which would have decreased egg and larval survival. Experimental methods were consistent throughout the experiment. Therefore, any factors effecting egg

volume, hatch, and larval survival were equal among all incubation waters, which validates the comparison of data from the trials and treatments of this experiment.

The premise of this experiment was to investigate the effects of concentration and type (calcium and/or magnesium) of water hardness on egg and larval survival. It was found that calcium, which contributes to water hardness, and the total osmotic concentration are key factors in egg and larval survival for the rainbow sharkminnow. Although calcium and magnesium both contribute to water hardness, calcium, not magnesium, is necessary for the development and survival of rainbow sharkminnow eggs. Calcium, although necessary, was found to have a detrimental effect at higher concentrations due to its increasing the osmotic concentration of the incubation water which directly effected the swelling of the eggs, reducing larval survival. All other constituents of the incubation waters, such as magnesium, increased osmotic concentration of the incubation water, which reduced larval survival. Water selected for the spawning of rainbow sharkminnows should have an osmotic concentration similar to that of the VS incubation waters of the CAMG, CA, and MG treatments. It is also essential that the incubation water contain calcium ions for the proper development of the egg. Therefore, tests that measure the total hardness of water are not sufficient when determining the potential use of that water for the incubation of rainbow sharkminnow eggs. Instead, testing for the presence and concentration of calcium and the total osmotic concentration of the incubation water would be more effective. More research is needed to further examine the hydromineral ion criteria for the incubation of rainbow sharkminnow eggs and many other cultured and non cultured species of fish. For cultured fishes, research should use on-farm spawning and incubation practices so that

the experimental data can be compared directly to data from on-farm practices. Ketola et al. (1988) found similar responses of Atlantic salmon, rainbow trout, and brook trout eggs to high levels of calcium (water hardness). It is therefore likely that the results from this experiment can be applied to closely related species that share the same native waters such as the redtail sharkminnow (*Epalzeochynchos bicolor*). Further research should be conducted to confirm or dismiss this idea.

Table 4-1. Replicate design for trials 1 and 2

Trial [a] #2	Incubation waters for treatments [b] CA and MG					
Female [c] #5	VS	S	MH	H	VH	Control
Female #6	VS	S	MH	H	VH	Control
Female #7	VS	S	MH	H	VH	Control
Female #8	VS	S	MH	H	VH	Control

[a] Trials consisted of incubating rainbow sharkminnow eggs in each of two treatment's incubation waters. Trial 1 was conducted two days before and with four different females than trial 2.

[b] Treatments consisted of calcium and magnesium water hardness (CAMG), no water hardness (Z), calcium only water hardness (CA), and magnesium only water hardness (MG). Each treatment was divided into five incubation waters; VS, S, MH, H, and VH which correspond to very soft, soft, moderately hard, hard, and very hard, respectively. Very soft had the least amount of added constituents increasing up to very hard which had the most. Water from the Floridan aquifer was used as the control.

[c] Each female was considered a replicate.

Table 4-2. Composition and chemistry for all treatment and control incubation waters.

	Treatment	Incubation water	Hardness [a]	Alkalinity [a]	$CaSO_4+2H_2O$ [b]	$MgSO_4$ [b]	KCl [b]	$NaCO_3$ [b]	Glucose [b]	Osmolality [c]	pH
Trial [d] 1											
	CAMG	VS	6.4	8.0	7.5	7.5	0.5	12.0	N/A	46	6.7
		S	34.0	26.0	30.0	30.0	2.0	48.0	N/A	47	7.2
		MH	80.0	49.6	60.0	60.0	4.0	96.0	N/A	49	7.7
		H	154.0	104.0	120.0	120.0	8.0	192.0	N/A	51	8.0
		VH	310.0	210.0	240.0	240.0	16.0	384.0	N/A	56	8.2
	Z	VS	0.0	6.0	N/A	N/A	0.5	12.0	72.0	47	6.7
		S	0.0	24.8	N/A	N/A	2.0	48.0	290.0	47	7.1
		MH	0.0	50.0	N/A	N/A	4.0	96.0	580.0	50	7.5
		H	0.0	102.0	N/A	N/A	8.0	192.0	1159.0	54	8.0
		VH	0.0	208.0	N/A	N/A	16.0	384.0	2318.0	56	8.3
		Control [e]	180.0	150.0	N/A	N/A	N/A	N/A	N/A	55	7.2
Trial [d] 2											
	CA	VS	6.8	7.2	21.5	N/A	0.5	12.0	N/A	47	6.7
		S	36.0	24.0	86.0	N/A	2.0	48.0	N/A	48	7.1
		MH	76.0	52.0	171.5	N/A	4.0	96.0	N/A	51	7.4
		H	162.0	106.0	346.0	N/A	8.0	192.0	N/A	52	8.0
		VH	292.0	164.0	686.0	N/A	16.0	384.0	N/A	57	8.2
	MG	VS	7.0	8.0	N/A	10.5	0.5	12.0	N/A	49	6.8
		S	34.0	24.0	N/A	42.0	2.0	48.0	N/A	50	7.0
		MH	74.0	54.0	N/A	84.0	4.0	96.0	N/A	51	7.5
		H	150.0	106.0	N/A	167.5	8.0	192.0	N/A	56	8.1
		VH	310.0	218.0	N/A	335.0	16.0	384.0	N/A	60	8.3
		Control [e]	180.0	150.0	N/A	N/A	N/A	N/A	N/A	55	7.2

[a] Hardness and alkalinity are given in mg/L as $CaCO_3$.

[b] Chemical compounds were added to deionized water and are given in mg/L.

[c] Osmolality is given in mmol/Kg of H_20.

[d] Trials consisted of incubating rainbow shark eggs in each of two treatment's incubation waters. Trial 1 was conducted two days before and with four different females than trial 2.

[e] Control water was pumped up from the Floridan aquifer.

Table 4-3. Comparison of mean square root percent hatch (+ s.d.), mean square root percent larval survival (+ s.d), and mean egg volume (+ s.d.) among incubation waters within treatments .

Trial [b] 1

CAMG treatment [a]				Z treatment			
Incubation water	Hatch [c]	Larval survival [d]	Volume [e]	Incubation water	Hatch	Larval survival	Volume
VS	4.52 ± 1.08 A	3.13 ± 1.59 A	22.95 ± 1.36 A	VS	0.00 ± 0.00 B	0.00 ± 0.00 A	NA [f]
S	4.49 ± 1.75 A	2.14 ± 2.81 AB	19.78 ± 0.68 B	S	0.00 ± 0.00 B	0.00 ± 0.00 A	NA
MH	4.18 ± 1.30 A	1.93 ± 2.45 AB	17.68 ± 1.32 B	MH	0.00 ± 0.00 B	0.00 ± 0.00 A	NA
H	2.83 ± 2.00 A	0.18 ± 0.36 B	13.72 ± 1.50 C	H	0.00 ± 0.00 B	0.00 ± 0.00 A	NA
VH	2.35 ± 1.91 A	0.00 ± 0.00 B	11.69 ± 1.49 C	VH	0.00 ± 0.00 B	0.00 ± 0.00 A	NA
Control	1.85 ± 2.16 A	0.00 ± 0.00 B	12.23 ± 2.18 C	Control	1.85 ± 2.16 A	0.00 ± 0.00 A	NA

Trial [b] 2

CA treatment				MG treatment			
Incubation water	Hatch	Larval survival	Volume	Incubation water	Hatch	Larval survival	Volume
VS	6.67 ± 0.78 A	5.05 ± 1.74 A	23.77 ± 1.02 A	VS	0.00 ± 0.00 A	0.00 ± 0.00 A	NA
S	5.37 ± 1.07 A	3.30 ± 2.25 AB	19.49 ± 1.41 B	S	0.00 ± 0.00 A	0.00 ± 0.00 A	NA
MH	4.99 ± 1.47 A	2.76 ± 1.67 ABC	16.65 ± 2.49 C	MH	0.00 ± 0.00 A	0.00 ± 0.00 A	NA
H	4.77 ± 1.39 A	0.00 ± 0.00 C	13.58 ± 0.39 D	H	0.00 ± 0.00 A	0.00 ± 0.00 A	NA
VH	4.38 ± 1.72 A	1.19 ± 2.38 BC	12.12 ± 0.50 D	VH	0.00 ± 0.00 A	0.00 ± 0.00 A	NA
Control	1.82 ± 2.26 B	0.69 ± 1.39 BC	11.77 ± 2.09 D	Control	1.82 ± 2.26 A	0.69 ± 1.39 A	NA

[a] Treatments consisted of calcium and magnesium water hardness (CAMG), no water hardness (Z), into five incubation waters; VS, S, MH, H, and VH which correspond to very soft, soft, moderately hard, hard, and very hard, respectively. Very soft had the least amount of added constituents increasing up to very hard, which had the most. Water from the Floridan aquifer was used as the control.

[b] Trials consisted of incubating rainbow shark eggs in each of two treatment's incubation waters. Trial 1 was conducted two days before and with four different females than trial 2.

[c] Means of square root percent hatch within each treatment not sharing a common letter differ significantly.

[d] Means of square root percent larval survival within each treatment not sharing a common letter differ significantly.

[e] Means of egg volume within each treatment not sharing a common letter differ significantly. Significance determined by Duncan's New Multiple Range Test; $P<0.05$.

[f] Volumes of dead eggs not measured. All eggs in Z and MG treatments died.

Table 4-4. Comparison of mean square root percent hatch (± s.d.) among treatments within incubation waters.

Treatment [a]	Incubation water [b c] VS		S		MH		H		VH		Control	
Trial [d] 1												
CAMG	4.52 ± 1.08	B	4.49 ± 1.75	A	4.18 ± 1.30	A	2.83 ± 2.00	B	2.35 ± 1.91	B	1.85 ± 2.16	A
Z	0.00 ± 0.00	C	0.00 ± 0.00	B	0.00 ± 0.00	B	0.00 ± 0.00	C	0.00 ± 0.00	C	1.85 ± 2.16	A
Trial [d] 2												
CA	6.67 ± 0.78	A	5.37 ± 1.07	A	4.99 ± 1.47	A	4.77 ± 1.39	A	4.38 ± 1.72	A	1.82 ± 2.26	A
MG	0.00 ± 0.00	C	0.00 ± 0.00	B	0.00 ± 0.00	B	0.00 ± 0.00	C	0.00 ± 0.00	C	1.82 ± 2.26	A

[a] Treatment waters consisted of a calcium and magnesium water hardness (CAMG), no water hardness (Z), calcium only water hardness (CA), and magnesium only water hardness (MG) treatment.

[b] Each treatment was divided into five incubation waters; VS, S, MH, H, and VH which corresponded to very soft, soft, moderately hard, hard, and very hard water, respectively. Very soft had the least amount of added constituents increasing up to very hard, which had the most. Water from the Floridan aquifer was used as a control.

[c] Treatment means, within each incubation water not followed by the same letter, differ significantly. Significance determined by Duncan's New Multiple Range Test; $P \leq 05$.

[d] Trials consisted of incubating rainbow shark eggs in each of two treatment's incubation waters. Trial 1 was conducted two days before and with four different females than trial 2.

Table 4-5. . Comparison of mean square root percent larval survival (± s.d.) among treatments within incubation waters.

		Incubation water [b c]											
	Treatment [a]	VS		S		MH		H		VH		Control	
Trial [d] 1													
	CAMG	3.13 ± 1.59	B	2.14 ± 2.81	AB	1.93 ± 2.45	AB	0.18 ± 0.36	A	0.00 ± 0.00	A	0.00 ± 0.00	A
	Z	0.00 ± 0.00	C	0.00 ± 0.00	B	0.00 ± 0.00	B	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
Trial [d] 2													
	CA	5.05 ± 1.74	A	3.30 ± 2.25	AB	2.76 ± 1.67	AB	0.00 ± 0.00	A	1.19 ± 2.38	A	0.69 ± 1.39	A
	MG	0.00 ± 0.00	C	0.00 ± 0.00	B	0.00 ± 0.00	B	0.00 ± 0.00	A	0.00 ± 0.00	A	0.69 ± 1.39	A

[a] Treatment waters consisted of a calcium and magnesium water hardness (CAMG), no water hardness (Z), calcium only water hardness (CA), and magnesium only water hardness (MG) treatment.

[b] Each treatment was divided into five incubation waters; VS, S, MH, H, and VH which corresponded to very soft, soft, moderately hard, hard, and very hard water, respectively. Very soft had the least amount of added constituents increasing up to very hard, which had the most. Water from the Floridan aquifer was used as a control.

[c] Treatment means, within each incubation water not followed by the same letter, differ significantly. Significance determined by Duncan's New Multiple Range Test; $P \leq 05$.

[d] Trials consisted of incubating rainbow shark eggs in each of two treatment's incubation waters. Trial 1 was conducted two days before and with four different females than trial 2.

Table 4-6. Comparison of mean egg volume (± s.d.), in cubic millimeters, among treatments within incubation waters.

		Incubation water [b c]											
	Treatment [a]	VS		S		MH		H		VH		Control	
Trial [d] 1													
	CAMG	22.9 ± 1.36	A	19.78 ± 0.68	A	17.68 ± 1.32	A	13.72 ± 1.50	A	11.68 ± 1.49	A	12.23 ± 2.18	A
	Z	NA [e]		NA		NA		NA		NA		12.23 ± 2.18	A
Trial [d] 2													
	CA	23.77 ± 1.02	A	19.49 ± 1.41	A	16.65 ± 2.49	A	13.58 ± 0.39	A	12.12 ± 0.50	A	11.77 ± 2.09	A
	MG	NA [e]		NA		NA		NA		NA		11.77 ± 2.09	A

[a] Treatment waters consisted of a calcium and magnesium water hardness (CAMG), no water hardness (Z), calcium only water hardness (CA), and a magnesium only water hardness (MG) treatment.

[b] Each treatment was divided into five incubation waters; VS, S, MH, H, and VH which corresponded to very soft, soft, moderately hard, hard, and very hard, water respectively. Very soft had the least amount of added constituents increasing up to very hard, which had the most. Water from the Floridan aquifer was used as a control.

[c] Treatment means, within each incubation water not followed by the same letter, differ significantly. Significance determined by Duncan's New Multiple Range Test; $P \leq 05$.

[d] Trials consisted of incubating rainbow shark eggs in each of two treatment's incubation waters. Trial 1 was conducted two days before and with four different females than trial 2.

[e] Volumes of dead eggs not measured. All eggs in Z and MG treatments died.

LIST OF REFERENCES

Alderdice, D. F. 1988. Osmotic and ionic regulation in teleost eggs and larvae. Pages 163-252: Hoar, W. S. and Randall, D. J. (Editors), Fish Physiology, Vol. XIA, Eggs and Larvae. Academic Press, New York.

Alderdice, D. F., Jensen, J. O., and Velsen, F. P. J. 1984. Measurement of hydrostatic pressure in salmonid eggs. Canadian Journal of Zoology. 62: 1977-1987.

Boyd, C. E. 1979. Water Quality in Warmwater Fish Ponds. Auburn University, Agricultural Experiment Station, Auburn, AL, 359pp.

Brown, D. J. A. and Lynam, S. 1981. The effect of sodium and calcium concentrations on the hatching of eggs and the survival of the yolk sac fry of brown trout, *Salmo trutta trutta* at low pH. Journal of Fish Biology. 19: 205-211.

Eddy, F. B. 1974. Osmotic properties of the perivitelline fluid and some properties of the chorion of Atlantic salmon eggs (*Salmo salar*). Journal of Zoology, London. 174: 237-243.

Eddy, F. B. and Talbot, C. 1983. Formation of the perivitelline fluid in Atlantic salmon eggs (*Salmo salar*) in fresh water and in solutions of metal ions. Comprehensive Biochemistry and Physiology. 75C: 1-4.

Florida Agricultural Statistics Service (FASS). June 2002. Aquaculture. 1-4.

Froese, R. and Pauly, D. Editors. 2003. FishBase. World Wide Web electronic publication. www.fishbase.org, version 09 December 2003.

Gonzal, A. C., Aralar, E. V., and Pavico J. M. F. 1987. The effects of water hardness on the hatching and viability of silver carp (*Hypophthalmichthys molitrix*) eggs. Aquaculture. 64: 111-118.

Ingersol, C. G., Mount, D. R., Gulley, D., La Point, T. W., and Bergman, H. L. 1990. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). Canadian Journal of Fisheries and Aquatic Sciences. 47: 1580-1592.

Kao, C. Y. and Chambers, R. 1954. Internal hydrostatic pressure of the Fundulus egg. 1. The activated egg. Journal of Experimental Biology. 31: 139-149.

Ketola, G. H., Longacre D., Greulich A., Phetterplace, L., and Lashomb R. 1988. High calcium concentration in water increases mortality of salmon and trout eggs. The Progressive Fish-Culturist. 50: 129-135.

Lee, C. S. and Hu, F. 1983. Influences of Ca and Mg ions on the egg survival of grey mullet, *Mugil cephalus* L. Journal of Fish Biology. 22: 13-20.

Maetz, J. 1974. Aspects of adaptation to hypo-osmotic and hyper-osmotic environment. Pages 1-165: D.C. Malins and J. R. Sargent (Editors), Biochemical and Biophysical Perspectives in Marine Biology, Vol. 1. Academic Press, London, pp. 1-165.

Marking, L. L. and Dawson, V. K. 1973. Toxicity of quinaldine sulfate to fish. Investigative Fish Control. No. 48, U. S. Fish and Wildlife Service, Department of the Interior, Washington, D. C., 8pp.

Potts, W. T. W. and P. P. Rudy, Jr. 1969. Water balance in the eggs of the Atlantic salmon, *Salmo salar*. Journal of Experimental Biology. 50: 223-237.

Rainboth, W. J. 1966**.** Fishes of the Cambodian Mekong. FAO species identification field guide for fishery purposes. United Nations Food and Agriculture Organization, Rome, Italy, 265 pp., 27 color plates.

Redding, M. J. and Patino, R. 1993. Reproductive physiology. Pages 503-534: Evans, D. H. (Editor), The Physiology of Fishes. CRC press Inc., Boca Raton, Florida.

Rudy, P. P., Jr. and Potts, W. T. W. 1969. Sodium balance in the eggs of the Atlantic salmon, *Salmo salar*. Journal of Experimental Biology. 50: 239-246.

Shattles, D. E. 1965. Quality of water from the Floridan aquifer in Hillsborough County Florida. Florida Board of Conservation, Division of Geology. Map Series No. 9.

Shireman, J. V. and Gildea, J. A. 1989. Induced spawning of rainbow sharks (*Epalzeorhynchos frenatum*) and redtail black sharks (*Epalzeochynchos bicolor*). The Progressive Fish-Culturist. 51: 104-108.

Spade, S. and Bristow, B. 1999. Effects of increasing water hardness on egg diameter and hatch rates of striped bass eggs. North American Journal of Aquaculture. 61: 263-265.

Von Westernhagen, H. 1988. Sublethal effects of pollutants of fish eggs and larvae. Pages 253-347: Hoar, W. S. and Randall, D. J. (Editors), Fish Physiology, Vol. XI A, Eggs and Larvae. Academic Press, New York.

Yeager, D. M. 1994. A System for Increasing Water Hardness in Culture Water at a Soft-Water Striped Bass Production Facility. The Progressive Fish-Culturist. 56: 56-57.

Zotin, A. I. 1958. The mechanism of the hardening of the salmonid egg membrane after fertilization or spontaneous activation. Journal of Embryology and Experimental Morphology. 6: 546-568.

BIOGRAPHICAL SKETCH

Michael Andrew Abernathy was born August 9, 1976, in Van Nuys, California. His youth was spent playing sports and fishing the coastal waters of southern California and northern Baja California, Mexico. He began pursuing his interests in marine biology as early as junior high school, and during his senior year of high school, co-founded a marine science club. His interests in marine biology did not wane, and after high school, he set off to the University of California at Santa Cruz to pursue a Bachelor of Science in marine biology. While acquiring his degree he developed an interest in aquaculture after completing an independent study on the topic. After four enjoyable years in Santa Cruz, he moved back to southern California with his degree in hand. After a few years of working, traveling, and caring for family, he decided to further his education and pursue his interest in aquaculture. In August 2002, he moved to Florida with his fiancee and enrolled as a graduate student under Dr. Frank Chapman at the University of Florida in the Department of Fisheries and Aquatic Sciences. He looks forward to receiving his Master of Science degree and moving back to California to start an ornamental aquaculture business, get married, and continue what has been a great life.