PARASITOLOGICAL AND OSMOREGULATORY EVALUATIONS OF THE SEMINOLE KILLIFISH, *Fundulus seminolis,* A CANDIDATE SPECIES FOR MARINE BAITFISH AQUACULTURE

By

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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

2008

To my father, who fostered my love of learning.
To my mother, who has given me new perspective.
I love you both.

ACKNOWLEDGMENTS

The following study would not have been possible without the help of numerous individuals. I thank Edsel E. Redden and Greg Harris for their help in procuring research specimens and Dr. Ilze Berzins and the University of Florida Tropical Aquaculture Laboratory for use of vital instrumentation. I thank Scott Grabe, Shawn DeSantis, John Marcellus, and Tina Crosby for their tireless assistance throughout the course of numerous experiments. I recognize Dr. Pamela Schofield for her statistical assistance and Dr. Andrew Rhyne for his helpful suggestions and innovative solutions.

I express my gratitude to committee member, Dr. Jeff Hill, for his time and suggestions; and to my cochair, Dr. Denise Petty, whose thirst for knowledge and desire to impart it to others has been an inspiration. I would like to especially thank my advisor Dr. Cortney Ohs for countless hours spent analyzing data and editing manuscripts. His guidance and example have helped me to appreciate the nuances of research.

To my late grandfather, a man who inspired my love for the ocean and taught me to fish, I will forever be grateful. I also thank my parents for their encouragement in my pursuit of higher education. My success is a direct reflection of their self sacrifice and dedication to their family. Finally, I thank my wife, whose loyalty and devotion led her across the country with me. Without her continued love and support, my aspirations might never have been realized.

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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

PARASITOLOGICAL AND OSMOREGULATORY EVALUATIONS OF THE SEMINOLE KILLIFISH, *Fundulus seminolis,* A CANDIDATE SPECIES FOR MARINE BAITFISH AQUACULTURE

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August 2008

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U.S. baitfish production had a 2005 farm gate value of $38 million. Freshwater species currently comprise the majority of all cultured baitfish. Aquaculture of marine baitfish species is still in its relative infancy and the increasing value of coastal property is forcing marine aquaculture inland. *Fundulus seminolis*, a freshwater species endemic to Florida, has shown economic potential for use as a marine baitfish, with a small number of commercial operations currently in production. The objectives of this study were a parasitological survey of wild *F. seminolis* broodfish, characterization of the salinity tolerance of the species, evaluation of a point-of-care blood analyzer for use with *F. seminolis,* and elucidation of the physiology associated with their gradual seawater acclimation.

In adherence with responsible aquaculture practices, a parasitic survey of the wild caught broodstock from Lake George, Florida was conducted to identify potential health problems with the species. This is the first comprehensive parasitic survey of *F. seminolis* and the Lake George region. Thirteen distinct taxa were identified as parasites of *F. seminolis*. Eight parasitic taxa were elucidated which had never before been recorded on *F. seminolis*.

Two separate acute acclimation experiments, natural seawater and sodium chloride, were carried out to determine if survival was influenced by the salinity source. *F. seminolis* were able to tolerate acute transfer to 0, 8, and 16 g/L using both salinity sources but only specimens in natural seawater were able to survive in 24 g/L. No survival was observed in either salt source at 32 g/L. A gradual seawater acclimation was also investigated to examine survival at various acclimation rates. A survival rate of 100% was achieved when salinity was changed from 0 to 32 g/L over 24, 48, 72, and 96 h.

The i-STAT® point-of-care blood analyzer was evaluated against conventionally accepted instrumentation for determination of hematocrit, sodium, potassium, and chloride in *F. seminolis*. Whole blood and heparin diluted whole blood aliquots were analyzed. Results analyzed by t-test, correlation coefficients, and the Bland-Altman method all indicated results obtained with the i-STAT® unit were unreliable when compared with accepted conventional methodologies.

A gradual seawater acclimation from 0 to 32 g/L over 24, 48, 72, and 96 h was conducted. Body weight, muscle water content, hematocrit, sodium, potassium, chloride, and plasma osmolality were analyzed. Generated data revealed physiological stress manifested in multiple variables analyzed after acclimation times of 24, 48 and 72 h. Generally, data gathered from the 96 h acclimation suggest the initiation of physiological acclimation as select analytes began to migrate back towards reference values derived from controls.

Results of these experiments provide information pertinent to the fields of physiology, ecology, and aquaculture regarding this rarely studied species. Additionally, experimental outcomes will help to diversify aquaculture within Florida and shape the marketing and distribution strategies for this economically valuable killifish.

CHAPTER 1
INTRODUCTION

Florida's $7.5 billion annual economic impact for its recreational fishery is the highest of any state in the U.S. according to the 2006 national survey of fishing, hunting, and wildlife-associated recreation (Wattendorf and Sieber, 2008). The Florida Fish and Wildlife Conservation Commission reports that Florida's recreational saltwater fishery had an economic impact of $5.2 billion in 2006 and was responsible for 51,500 jobs (Wattendorf and Sieber, 2008). Despite these overwhelming statistics establishing Florida as a premier fishing location, of the 257 baitfish farms recorded in the 2005 USDA census of aquaculture, only 2 were located in Florida (USDA, 2005). This disparity clearly illustrates the potential for expansion and diversification of aquaculture within the state of Florida.

Saltwater fishing practices and associated equipment have made dramatic advances in technology and efficiency in recent times. Interestingly, methods of marine baitfish procurement have experienced little change over the past 75 years. The use of nets and traps are commonly used to harvest these sometimes elusive organisms. Today the majority of marine baitfish sold in stores are wild caught. Availability of most species is seasonal yet demand remains relatively constant. Aquacultured marine baitfish can potentially provide fishermen with a consistent supply of sought after species in desired sizes, regardless of season. Additionally, development of baitfish aquaculture within Florida would help to diversify the existing aquaculture industry and potentially alleviate collection pressure on targeted wild populations.

The seminole killifish, *Fundulus seminolis*, is an endemic Florida freshwater killifish that has recently emerged as a potential candidate for marine baitfish aquaculture. Its ability to complete its reproductive life cycle in freshwater is appealing for aquaculture in Florida as value of coastal property increases and access to seawater becomes more limited. Culture of this

species in freshwater would allow production to occur away from coastal resources. Its large size and tolerance of poor water quality conditions make it a candidate worthy of investigation for baitfish aquaculture. Additionally, if this species is able to acclimate to full strength seawater, potential for disease transmission from the culture environment to the wild should be diminished because the seawater acclimation would ostensibly act as a prophylactic treatment for freshwater ectoparasites and other salinity sensitive pathogens.

The objectives of this study were a parasitological survey of wild *F. seminolis* broodfish, characterization of the salinity tolerance of the species, evaluation of a point-of-care blood analyzer for use with *F. seminolis,* and elucidation of the physiology associated with its gradual seawater acclimation.

Results from the parasitological investigation will provide data regarding the ecological diversity of parasites occurring on a wild population of *F. seminolis* from Lake George, Florida. Additionally, parasite identification and enumeration are crucial for proper quarantine and biosecurity procedures within an aquaculture production setting.

Salinity tolerance determinations will provide essential information influencing the marketing and distribution of this species as a marine baitfish. Information gathered from salinity tolerance experiments may also help to discern the role of salinity as a barrier to the species' geographic dispersion.

The use of point-of-care blood analyzers for physiological determinations in fish has recently become more prevalent. Validation of new technologies against standard techniques is essential for generation of reliable data. Evaluation of the i-STAT® point-of-care unit against conventionally accepted instrumentation will allow for the determination of agreement between

the two methods and ultimately the validity of the i-STAT® unit for use in fish physiology studies.

Physiological studies should provide insight into underlying processes allowing the species to acclimate to seawater as well as establishing an osmoregulatory time frame within which it is capable of doing so. Experimental results may also contribute to the development of standardized acclimation protocols for use in commercial production settings.

There is a noticeable paucity of published literature dealing with *F. seminolis*. The consequent studies are intended to inform the scientific community and aquaculture industry regarding this emerging *Fundulus* species. Experimental results will have immediate impact for Florida aquaculture and help to substantiate marine baitfish production as a viable aquaculture crop for the state and the region.

CHAPTER 2
THE PARASITIC FAUNA OF THE SEMINOLE KILLIFISH, *FUNDULUS SEMINOLIS*, FROM LAKE GEORGE, FLORIDA

Introduction

The seminole killifish, *Fundulus seminolis*, is an endemic Florida killifish with a geographic range within peninsular Florida from the St. Johns and New River drainage basins to just south of Lake Okeechobee (Page and Burr, 1991). Populations reaching as far south as Nine-Mile Bend have been reported by Tabb and Manning (1961). This species, commonly referred to as a "bullminnow" or "mudminnow", is one of the largest members of the genus, reaching total lengths of 20 cm (Hoyer and Canfield, 1994). Its popularity as a local baitfish for largemouth bass, *Micropterus salmoides*, and other piscivorous game fish has generated interest in this species as a potential candidate for aquaculture. Relatively little is known regarding the life history of *F. seminolis*, with only one publication by DuRant et al. (1979) devoted entirely to the species. It has been referenced anecdotally or as a component in a larger study or survey in several publications (McLane, 1955; Phillips and Springer, 1960; Tabb and Manning, 1961; Gunter and Hall, 1963; Gunter and Hall, 1965; Foster, 1967; Griffith, 1974A; Nordlie, 2006).

To date there are no publications that have extensively examined the parasitic fauna of *Fundulus seminolis*. Although Bangham (1940) included *F. seminolis* in his parasite survey, his sample size was only 14 individuals and most of the specimens were preserved in formalin prior to examination, likely altering the detectable parasite burden. Dillon's (1966) effort at compiling a list of parasites occurring on *Fundulus* spp. merely referenced Bangham's work with no new additions. The most recent and extensive checklist of parasites occurring on *Fundulus* spp. compiled by Harris and Vogelbein (2006) excluded *F. seminolis* altogether. Therefore, the objective of this study is to elucidate and enumerate the various protistan and metazoan parasites found within a population of seminole killifish from Lake George, Florida.

Methods

A total of 140 *F. seminolis* were collected from the eastern shore of Lake George (29° 17′ 12″ N 81° 35′ 53″ W) in Volusia County Florida. This broad and shallow lake is part of the St. Johns River system and is the second largest freshwater lake in the state of Florida. Fish were collected with a seine net (24.4 m X 1.2 m, 0.8 cm mesh) on three separate occasions from March through May 2007. A sample size of 100 fish was determined to be suitable for the experiment based on previous work by Ossiander and Wedemeyer (1973) and Simon and Schill (1984). This sample size would allow detection with a 95% confidence level one carrier fish in a population greater than 1,000,000 with a 3% incidence of disease (Ossiander and Wedemeyer, 1973). Fish were captured with a seine net and transported to the laboratory alive in water obtained from the collection site. A dissolved oxygen saturation of approximately 90% was maintained during transport. Water samples were collected prior to seining and were stored for later analysis. Water temperature was determined at collection sites. Dissolved oxygen (DO) and pH were both measured using Hach's HQ-20 meter whereas total ammonia nitrogen (TAN), nitrite, total hardness, total alkalinity, CO_2, and free and total chlorine were measured using standard techniques (Hach Co., Loveland, Colorado). Salinity was determined using a refractometer.

Upon arrival fish were individually weighed and measured and subsequently examined externally for gross signs of parasitism. If no gross signs of parasitism were evident, a skin biopsy was collected from the entire length of the left lateral body wall of the fish, a gill biopsy (~3mm^2) was collected from the specimens left second gill arch and a fin biopsy (~5mm^2) was collected from the specimen's caudal fin. Active lesions, erosions, erythemic tissues, and visible parasites were given precedence and the area in question was biopsied instead. Wet mounts of all biopsied tissues were prepared for further analysis. Fish were subsequently euthanized in

buffered tricaine methanesulfonate (MS-222, Argent Laboratories, Finquel, C-FINQ-UE-100G) and each specimen's intestine was excised. Wet mounts of the complete intestine were prepared for further inspection. Skin, fin, gill, and intestinal biopsies were performed utilizing techniques described by Noga (1996). All wet mounts were prepared within 24 hours of capture and examined immediately thereafter.

All parasitological terminology utilized adhere to the recommendations of Bush et al. (1997). Parasites were identified utilizing previously published literature (Noga, 1996; Woo, 1995; Stoskopf, 1993) and with the help of B. Denise Petty, DVM, University of Florida. Slide preparations were examined using light microscopy at three different magnifications (40x, 100x, and 400x). Parasites were enumerated individually with the exceptions of Nematoda, Digenea, *Myxobolus* sp., and sessile ectocommensal ciliates of the order Sessilida (SECs). These four parasite groups were quantified utilizing a system similar to the one implemented by Bravo et al. (2007). Three gradations, light, moderate, and heavy, were determined for each parasite relative to numbers observed per field in five random fields of view at a predetermined magnification (Table 2-1). Prevalence, mean abundance, intensity range, and mean intensity were calculated where appropriate. For categorical data, mean intensity was calculated by assigning numerical values to parasite descriptors.

Results

Thirteen distinct taxa were identified as parasites of *F. seminolis*, six of which were identified down to genus and one to species. The most common parasitic group encountered in this survey was the subclass Digenea. Found in all four of the tissues examined, digeneans were responsible for the highest prevalence recorded in this study, 95% in the intestine of sampled organisms (Table 2-2). Skin and gill biopsies yielded the greatest diversity of parasites with 8 taxa represented in each (Table 2-3 & Table 2-4). Monogeneans accounted for the greatest

prevalence on both the skin and gill biopsies, with 14% prevalence for *Gyrodactylus* sp. and 46% prevalence for Dactylogyrida respectively. Hirudinea were the most common of all parasites found on the fin, with a prevalence of 39%, a mean abundance of 0.52, and a mean intensity of 1.3 (Table 2-5). The maximum mean intensity recorded was 5.00 for *Ichthyobodo* sp. on the fin and 5.00 for *Piscinoodinium* sp. on the gill. *Trichodina* sp. found on the gill biopsies demonstrated the broadest intensity range, 1-12 organisms per specimen analyzed. The largest calculable mean abundance of 0.73 was displayed by Dactylogyrida on the gill biopsies.

The mean total length of *F. seminolis* collected was 106 ± 2 mm with a range from 70 – 157 mm. The body weight of the study specimens ranged from 2.84 – 38.00 g, with a mean body weight of 13.02 ± 0.71 g. Mean collection site water quality parameters were as follows; DO = 6.2 ± 0.3 mg/L; pH = 7.6 ± 0.1; temperature = 18.3 ± 1.3 °C; TAN = 0 mg/L; nitrite = 0 mg/L; salinity = 1 g/L; total hardness = 256.5 ± 19.7 mg/L; total alkalinity = 96.9 ± 5.7 mg/L; CO_2 = 8.3 ± 3.3 mg/L; free chlorine = 0 mg/L; total chlorine = 0 mg/L.

Discussion

This study provides the first comprehensive description of the naturally occurring parasite fauna of *F. seminolis.* Additionally, to the authors' knowledge this is the first published parasitological survey of fish collected from Lake George, Florida. As Florida's second largest water body and an important component of the St. Johns River system, this survey provides valuable knowledge into the composition of the resident parasite community.

Bangham's 1940 survey of *F. seminolis* recorded six distinct taxa and their corresponding quantities from which we were able to calculate the prevalence of each parasite. Interestingly, Bangham reports the digenean, *Neascus vancleavei*, as the most common of the parasites observed (71% prevalence). This finding is consistent with results from the present survey, as digeneans were found on all four of the tissues analyzed and exhibited the highest prevalence

recorded of 95% in the intestinal biopsies. Conversely, Bangham failed to report the presence of *Myxobolus* sp., SEC's, Hirudinea, *Ichthyophthirius multifiliis*, *Tetrahymena* sp., *Ichthyobodo* sp., *Piscinoodinium* sp., and *Trichodina* sp.; the present study represents the first recorded accounts of these parasites infecting *F. seminolis*. These results however cannot be accurately compared due to the small sample size used by Bangham as well as unclear diagnostic techniques and preservation of samples in formalin prior to parasite enumeration. The high prevalence of digenetic trematodes observed in the Lake George population of *F. seminolis* is possibly a direct result of the fish's diet. Upon intestinal excision it was noted that a predominant number of the specimens contained multiple gastropods in various stages of digestion.

Similarities of parasite taxa found in the genus *Fundulus* to parasite taxa observed in the current study are evident in previous literature. Yoshino (1972) reported a wide array of digeneans in *Fundulus parvipinnis,* including *N. vancleavei* previously reported in *F. seminolis* (Bangham, 1940). Barse (1998) reported ten taxa found on the gills of *F. heteroclitus* in Chesapeake Bay, slightly more than the eight taxa we found on the gills of *F. seminolis.* Barse also examined the effect of seasonality, locality, and host sex and size. These factors were not investigated in this study, but could provide valuable data if analyzed in future studies. Of note, Lake George had a salinity of 1 g/L during experimental collections. This salinity could have influenced the richness and abundance of parasite species recorded during the survey. Adams (1985) reported six taxa infesting the gills of *Fundulus kansae*, three of which were found on the gills of our study specimens. *Trichodina* spp. prevalence of 59% reported by Adams (1985) is considerably higher than the 5% prevalence on the gills of *F. seminolis* in the present study. Despite differences in Myxosporea genera, it is noteworthy that the parasite was found in both *F.*

kansae and *F. seminolis.* Additionally, 4 *Myxobolus* spp. have been reported in the banded killifish, *Fundulus diaphanus,* (Cone et al., 2006).

Parasitological survey results of wild *F. seminolis* brood fish are integral in the establishment of effective quarantine and subsequent biosecurity procedures unique to aquaculture production facilities. Prevention of pathogen introductions both into and out of the culture environment must be ensured through implementation of responsible aquaculture practices. These findings will dictate treatment therapy options instrumental in the captive husbandry and culture of this emerging marine baitfish. To date, the most comprehensive checklist of parasite taxa infesting *Fundulus* spp. has been compiled by Harris and Vogelbein (2006). Ideally, future checklists will incorporate the data generated from this study and include *F. seminolis* with the other members of the genus.

Table 2-1. Classification of parasite intensity per field of view at predetermined magnifications on the skin, fin, gill and intestine of *Fundulus seminolis.*

Parasite	# of fields of view (FOV)	Magnification	Light (Per FOV)	Moderate (Per FOV)	Heavy (Per FOV)
Digenea	5	40x	1-10	11-25	≥26
Myxobolus sp.	5	400x	1 Xenoma or Individual Myxosporea	2-10 Xenomas	≥11 Xenomas
Nematoda	5	40x	1-10	11-25	≥26
SEC's	5	400x	1-10	11-25	≥26

Table 2-2. Parasite fauna observed on 100 intestinal biopsies of *Fundulus seminolis*.

Parasite	Prevalence (%)	Mean abundance	Intensity range	Mean intensity
Cestoda	2	0.03	1-2	1.50
Digenea	95	-	L-H[a]	L[a]
Myxobolus sp.	8	-	L-M[a]	L[a]
Nematoda	26	-	L[a]	L[a]

[a] Parasite descriptors per field of view (Table 2-1) L = Light; M = Moderate; H = Heavy

Table 2-3. Parasite fauna observed on 100 skin biopsies of *Fundulus seminolis.*

Parasite	Prevalence (%)	Mean abundance	Intensity range	Mean intensity
Dactylogyrida	1	0.01	1	1.00
Digenea	2	-	L[a]	L[a]
Gyrodactylus sp.	14	0.29	1-8	2.07
Hirudinea	5	0.06	1-2	1.20
Ichthyophthirius multifiliis	3	0.03	1	1.00
Myxobolus sp.	2	-	L[a]	L[a]
SEC's	2	-	L-H[a]	M[a]
Tetrahymena sp.	1	0.01	1	1.00

[a] Parasite descriptors per field of view (Table 2-1) L = Light; M = Moderate; H = Heavy

Table 2-4. Parasite fauna observed on 100 gill biopsies of *Fundulus seminolis.*

Parasite	Prevalence (%)	Mean abundance	Intensity range	Mean intensity
Dactylogyrida	46	0.73	1-11	1.60
Digenea	12	-	L[a]	L[a]
Gyrodactylus sp.	1	0.01	1	1.00
Ichthyobodo sp.	1	0.01	1	1.00
Myxobolus sp.	1	-	L[a]	L[a]
Piscinoodinium sp.	1	0.05	5	5.00
SEC's	1	-	L[a]	L[a]
Trichodina sp.	5	0.16	1-12	3.20

[a] Parasite descriptors per field of view (Table 2-1) L = Light; M = Moderate; H = Heavy

Table 2-5. Parasite fauna observed on 100 fin biopsies of *Fundulus seminolis.*

Parasite	Prevalence (%)	Mean abundance	Intensity range	Mean intensity
Digenea	27	-	L[a]	L[a]
Gyrodactylus sp.	2	0.03	1-2	1.50
Hirudinea	39	0.52	1-3	1.30
Ichthyobodo sp.	4	0.20	2-8	5.00
Myxobolus sp.	4	-	L-M[a]	L[a]

[a] Parasite descriptors per field of view (Table 2-1) L = Light; M = Moderate; H = Heavy

CHAPTER 3
EXPERIMENTAL SALINITY TOLERANCE DETERMINATIONS FOR THE SEMINOLE KILLIFISH, *FUNDULUS SEMINOLIS*

Introduction

The seminole killifish, *Fundulus seminolis* (Girard, 1859), is an endemic Florida killifish with a geographic range within peninsular Florida from the St. Johns and New River drainage basins to just south of Lake Okeechobee (Page and Burr, 1991). Relatively little is known regarding the life history of *F. seminolis*, with only one publication by DuRant et al. (1979) devoted entirely to the species. It has been referenced anecdotally or as a component in a larger study or survey in several publications (McLane, 1955; Phillips and Springer, 1960; Tabb and Manning, 1961; Gunter and Hall, 1963; Gunter and Hall, 1965; Foster, 1967; Griffith, 1974A; Nordlie, 2006). This species, commonly referred to as a "bullminnow" or "mudminnow", is one of the largest members of the genus, reaching total lengths of 20 cm (Hoyer and Canfield, 1994). Its popularity as a local freshwater baitfish for largemouth bass, *Micropterus salmoides*, and other piscivorous game fish has generated interest in this species as a potential candidate for aquaculture.

With previous data placing the upper salinity tolerance of *F. seminolis* at 23.4 g/L (Griffith, 1974A), culture of this species for use as a saltwater baitfish warrants further investigation. If the species is able to acclimate to full strength seawater, it could be produced exclusively in freshwater ponds or recirculation systems only needing to be acclimated to saline water prior to marketing and distribution. Additionally, with coastal property values at a premium and limited access to seawater, baitfish producers would be able to utilize inland resources for the culture of marine baitfish.

Determination of salinity tolerance following gradual and acute transfer is necessary to evaluate the species' physiological limitations which will influence culture and marketing

practices. Similar studies have been conducted by Lotan (1971), Griffith (1974A), Stanley and Fleming (1977), Chervinski (1983), Nordlie (1987), Crego and Peterson (1997), Nordlie (2000) and Fuller (2008) on salinity tolerance of various members of the order cyprinodontiformes. For the most recent review of cyprinidontoid salinity tolerance consult Nordlie (2006). The previous studies examined multiple salinity ranges utilizing various salt sources (seawater and synthetic sea salts) and freshwater dilutions to achieve experimental salinities both hypo and hypersaline to natural seawater (32-35 g/L). Distinct salinity thresholds defined by survival and select hematological indices were then characterized for the species in question. Experimental salinity determinations such as these may be a more accurate representation of the organism's true salinity tolerance than maximum reported field salinities (Kefford et al., 2004). This can be seen in Phillips and Springers' (1960) record of *F. seminolis* inhabiting waters with a salinity of 13.5 g/L, the highest recorded salinity from published field observations. Griffith's (1974A) subsequent experimental salinity determination for this species placed the upper salinity tolerance much higher, with an experimental salinity range of 19.3 – 33.4 g/L. However, Griffith (1974A) reported high mortalities in maintaining this species in captivity and his sample size for salinity tolerance determinations was only four individuals. Although the lower lethal temperature has yet to be established for this species, the experimental water temperature of 15°C might have exacerbated the physiological stress caused by the acclimation. Taken together, these circumstances call into question the accuracy of the salinity range reported by Griffith (1974A).

Investigations into the salinity tolerance of freshwater species have shared a predominant ecological motivation. Investigations by Bringolf et al. (2005) and Schofield et al. (2006) assessed salinity tolerances as barriers to invasion for the flathead catfish, *Pylodictis olivaris*, and

goldfish, *Carassius auratus*, respectively. While the impetus for the current study is the evaluation of *F. seminolis* as an aquaculture candidate, investigations into the salinity tolerance of *F. seminolis* should provide valuable information about the species ability to handle osmoregulatory stressors and may further be extrapolated for ecological applications.

Salinity tolerance is an important consideration in the culture of marine and freshwater organisms. It provides information about basic husbandry requirements necessary for the species to thrive in captivity as well as potential applications for the cultured organisms. Additionally, economic considerations associated with culture of marine or brackish water species make low saline or freshwater culture an attractive alternative. Research into low salinity aquaculture of marine species is common, but few studies have been conducted on acclimation of freshwater species to seawater. Experiments examining abrupt transfer of black sea bass, *Centropristis striata,* to low salinities have helped to identify a salinity threshold for the successful culture of this species (Young et al., 2006). Similarly, gradual acclimation experiments with Nile tilapia, *Oreochromis niloticus*, and blackchin tilapia, *Sarotherodon melanotheron*, (Lemarie et al., 2004) as well as larval salinity tolerance experiments with striped mullet, *Mugil cephalus*, thick-lipped grey mullet, *Chelon labrosus* (Hotos and Vlahos, 1998), and cobia, *Rachycentron canadum* (Faulk and Holt, 2006), have provided valuable evidence regarding the osmoregulatory ability of a species for use in conventional aquaculture conditions.

The purpose of this study was to characterize the salinity tolerance of *F. seminolis*, a potential candidate for marine baitfish aquaculture. Abrupt and gradual salinity acclimations were evaluated as well as salinity sources (sodium chloride vs. seawater). This investigation represents the first comprehensive study focused on the salinity tolerance of *F. seminolis*.

Methods

F. seminolis were collected by seine net from the eastern shore of Lake George, in Volusia County Florida and transported to the University of Florida Indian River Research and Education Center in Fort Pierce. Fish were assessed for pathogens and treated accordingly to ensure healthy research specimens for the subsequent salinity experiments. The acute salinity tolerance of *F. seminolis* to varying concentrations of sodium chloride (NaCl) and natural seawater (NSW) were investigated as well as survival following gradual NSW acclimation.

Sodium Chloride Acute Salinity Tolerance

Thirty five fish were transferred from a 6900 L recirculating system to 85 L glass aquaria with one fish per aquarium during the entire acclimation and experimental periods. Specimen's weight and total length (TL) were recorded prior to transfer. Length and weight ranges of 120 - 145 mm and 17.0 - 29.4 g were recorded with means of 131.2 ± 6.3 mm and 23.2 ± 3.6 g, respectively. Aquarium systems were maintained at < 1 g/L salinity well water and recirculated through biofilter media during the 96 h acclimation period. Dissolved oxygen (DO), pH, temperature, salinity, total ammonia nitrogen (TAN), and nitrite were recorded daily during the acclimation and experimental periods with total alkalinity and total hardness recorded on days one and three of acclimation and daily during the course of the experiment. DO and temperature were measured using a YSI 550A meter (YSI Inc., Yellow Springs, Ohio). Salinity was determined using a handheld refractometer, pH was measured using a Hach sensION1 portable pH meter and total alkalinity and total hardness were determined using standardized titration techniques (Hach Co., Loveland, Colorado). TAN and nitrite were evaluated spectrophotometrically using a Hach DR 4800 spectrophotometer (Hach Co., Loveland, Colorado). Aquaria were held at ambient temperature with a range of 19.9 - 26.5°C and a mean

temperature of 22.7°C during the experimental period. Temperature differences among aquaria never exceeded 2°C. An ambient photoperiod of 11 L : 13 D was used during the experiment. Fish were fed once a day to satiation on days two and three of acclimation and food was withheld on days one and four of acclimation and during the entire 96 h experimental period.

Following the 96 h freshwater acclimation, aquaria were made static and individual aquariums were randomly assigned to one of five treatments with seven replicates per treatment. Differences among treatment group's lengths and weights were not significant ($F_{4, 30} = 0.68$, $p = 0.610$; $F_{4, 30} = 0.20$, $p = 0.939$, respectively). Treatment salinities examined were 0 (control), 8, 16, 24, and 32 g/L. Salinities were abruptly changed by removing the appropriate amount of fresh water and adding a predetermined volume of a concentrated brine solution made by dissolving 99.5% NaCl (Morton White Crystal Solar Salt, Morton International Inc., Chicago, IL) into well water. Control aquaria had a predetermined volume of fresh water removed and subsequently replaced to maintain similar treatment of control and experimental groups. Tanks were aerated to thoroughly mix the water, then salinities were remeasured to confirm the desired concentrations were attained. Individual biofilters which had been preconditioned to treatment salinities were placed in each tank to control nitrogenous wastes. Aquaria were examined for mortalities as follows: once every hour from 0 – 12 h, once every 6 h from 12 – 48 h, and once every 12 h from 48 – 96 h. A final weight was recorded upon discovery of a mortality or upon the termination of the 96 h exposure period. Mortality was defined as loss of opercular movement and no response to physical stimulus.

Natural Seawater Acute Salinity Tolerance

Methods for the NSW acute salinity toxicity trial adhere to the previous methods listed for the NaCl acute salinity toxicity trial with exceptions as noted. Treatment salinities were abruptly changed by removing a predetermined volume of fresh water and adding a known volume of

natural seawater collected from the Atlantic Ocean and filtered through a 1 micron cartridge filter. Control aquaria were treated as previously stated. Aquaria were held at ambient temperature with a range of 20.5 - 25.6°C and a mean temperature of 23.4°C during the experimental period. Temperature differences among aquaria never exceeded 2°C. A ambient photoperiod of 11.5 L : 12.5 D was used during the experiment. Length and weight ranges of 122 - 146 mm and 16.6 – 31.2 g were recorded with means of 129.7 ± 5.8 mm and 21.9 ± 3.5 g, respectively. Differences among treatment group's lengths were not statistically significant ($F_{4, 30} = 1.12$, $p = 0.368$). Differences among treatment group's weights were statistically significant ($p = 0.010$) but were not considered to be biologically significant for the purpose of this experiment with a mean treatment weight range of 18.7 – 23.8 g. Differences among treatment weights did not violate any assumptions of subsequent survival analysis.

Natural Seawater Gradual Acclimation Survival

Sixty-four fish were transferred from a 6900 L recirculating system to 32, 85 L glass aquaria, divided in half by aquarium partitions (TDSU, Penn-Plax Inc., Happauge, NY USA) with one fish per aquarium subdivision during the entire acclimation and experimental periods. Fish weight and total length (TL) were recorded prior to transfer. Length and weight ranges of 124 - 152 mm and 21.7 – 38.3 g were recorded with means of 136.3 ± 6.3 mm and 27.5 ± 4.5 g, respectively. Aquarium systems were maintained at < 1 ppt salinity well water and recirculated through biofilter media during the 96 h acclimation period. Dissolved oxygen (DO), pH, temperature, salinity, total ammonia nitrogen (TAN), and nitrite were recorded daily during the acclimation and experimental periods with total alkalinity and total hardness recorded on days one and three of acclimation and at the initiation and cessation of experimental treatment periods. Methods for water quality analysis adhere to previously stated techniques with the exception of salinity, which was measured using a YSI 30 salinity/conductivity meter (YSI Inc.,

Yellow Springs, OH USA). Aquaria were held at ambient temperature with a range of 17.5 - 27.2°C and a mean temperature of 22.6°C during the experimental period. Temperature differences among aquaria never exceeded 2°C. A ambient photoperiod of 13 L : 11 D was used during the experiment. Fish were fed once a day to satiation on days two and three of acclimation and food was withheld on days one and four of acclimation and during the entire experimental period.

Following the 96 h freshwater acclimation, aquaria were made static and individual tanks were randomly assigned to one of eight treatments with eight replicates per treatment. Differences among treatment group's lengths and weights were not significant ($p = 0.784$; $F_{7, 56} = 0.38$, $p = 0.908$, respectively). Among the treatment groups, four underwent a gradual salinity change from 0 to 32 g/L over predetermined time periods with the remaining four treatments serving as corresponding controls. Acclimation times of 24, 48, 72, and 96 h were chosen with approximate salinity increases of 5.3, 2.7, 1.7, and 1.3 g/L, respectively, every 4 h. The final salinity of 32 g/L was achieved 4 h preceding the termination of the treatment group in question. Salinities were gradually changed every 4 h via addition of NSW equally dispersed between aquaria subdivisions until the desired salinity was achieved. Excess water during salinity changes was allowed to flow out of the aquarium's standpipe. A single air stone within each subdivision provided adequate mixing within and between subdivisions ensuring a homogeneous environment within each aquarium. Control aquaria had corresponding volumes of freshwater added in the same fashion as their saline counterparts. Aquaria were examined for mortalities as follows: once every hour from 0 – 12 h and once every 4 h from 12 – 96 h. A final weight was recorded upon discovery of a mortality or upon the termination of the acclimation exposure

period. Mortality was defined as loss of opercular movement and no response to physical stimulus.

Statistical Analysis

Survival was estimated using a Kaplan-Meier product limit estimator (Kaplan and Meier, 1958). Log-rank tests were then performed to compare generated survivorship curves among treatments and within treatments between experiments. Length, weight, and water quality data were analyzed using a one-way ANOVA with a Tukey's HSD means separation test. Nonparametric data were analyzed with a Kruskal-Wallis test. Kaplan-Meier analysis and subsequent log rank tests were performed in SPSS® version 12.0 (SPSS Inc. Chicago, IL USA). All other statistical analyses were performed in SAS® version 8.02 (SAS Institute Inc., Cary, NC USA) All numerical data are represented as the mean ± SD unless otherwise stated. Statistical differences were considered significant if $p \leq 0.05$.

Results

Sodium Chloride Acute Salinity Tolerance

Survival was 100% at salinities of 0 (control), 8, and 16 g/L throughout the entire 96 h experimental period (Table 3 – 1). Acute transfer to higher salinities yielded 100% mortality with mean survival times of 6 h (95% Confidence Interval [CI], 5 – 7 h) at 24 g/L and 2 h (95% CI, 2 – 2 h) at 32 g/L (Table 3 – 1). Despite the complete mortality observed in these two treatments, survival times were significantly different ($p \leq 0.0001$) from each other as well as from all other treatments examined ($p \leq 0.0001$) (Table 3 – 2).

No biologically significant differences among treatments in the measured water quality parameters were observed throughout the course of this experiment. During the 96 h acclimation period the following ranges and means ± SD were recorded, respectively: DO, 6.38 – 7.57, 7.11 ± 0.42 mg/L; pH, 8.33 – 8.52, 8.43 ± 0.06; TAN, 0.00 – 0.02, 0.01 ± 0.01 mg/L; nitrite, 0.0006 –

0.0039, 0.0025 ± 0.0008 mg/L; alkalinity, 136.80 – 153.90, 139.65 ± 6.98 mg/L $CaCO_3$; hardness, 188.10 – 239.40, 202.35 ± 19.99 mg/L $CaCO_3$. During the 96 h experimental period the following ranges and means were recorded, respectively: DO, 7.14 – 8.35, 7.73 ± 0.33 mg/L; pH, 8.28 – 8.58, 8.43 ± 0.08; TAN, 0.00 – 0.09, 0.03 ± 0.03 mg/L; nitrite, 0.0125 – 0.3102, 0.0753 ± 0.0565 mg/L; alkalinity, 119.70 – 153.90, 135.80 ± 9.80 mg/L $CaCO_3$; hardness, 171.00 – 256.50, 193.90 ± 18.90 mg/L $CaCO_3$.

Natural Seawater Acute Salinity Tolerance

Survival was 100% at salinities of 0 (control), 8, 16, and 24 g/L throughout the entire 96 h experimental period (Table 3 – 3). Acute transfer to 32 g/L yielded 100% mortality with a mean survival time of 13 h (95% CI, 11 – 16 h) (Table 3 – 3). This group was significantly different (p = 0.0002) from all other treatments (Table 3 – 4).

No biologically significant differences among treatments in the measured water quality parameters were detected throughout the course of this experiment with the exception of total hardness. Due to experimental dilutions of seawater, total hardness varied by treatment salinity. During the 96 h acclimation period the following ranges and means were recorded, respectively: DO, 6.66 – 7.90, 7.13 ± 0.41 mg/L; pH, 8.19 – 8.53, 8.37 ± 0.11; TAN, 0.00 – 0.06, 0.02 ± 0.02 mg/L; nitrite, 0.0000 – 0.0043, 0.0011 ± 0.0017 mg/L; alkalinity, 153.90 – 205.20, 171.00 ± 18.73 mg/L $CaCO_3$; hardness, 153.90 – 239.40, 190.95 ± 33.19 mg/L $CaCO_3$. During the 96 h experimental period the following ranges and means were recorded, respectively: DO, 7.50 – 8.24, 7.87 ± 0.19 mg/L; pH, 8.06 – 8.63, 8.36 ± 0.13; TAN, 0.00 – 0.10, 0.03 ± 0.02 mg/L; nitrite, 0.0018 – 0.0356, 0.0107 ± 0.0074 mg/L; alkalinity, 119.70 – 171.00, 144.00 ± 9.80 mg/L $CaCO_3$. Hardness ranges and means by salinity are as follows: 0 g/L, 153.90 – 222.30, 182.60 ± 21.40 mg/L $CaCO_3$; 8 g/L, 1250.00 – 2570.00, 1557.10 ± 300.00 mg/L $CaCO_3$; 16 g/L, 2700.00

– 3090.00, 2860.00 ± 95.10 mg/L $CaCO_3$; 24 g/L, 3830.00 – 5580.00, 4282.10 ± 295.40 mg/L $CaCO_3$; 32 g/L, 5700.00 – 6060.00, 5808.60 ± 117.80 mg/L $CaCO_3$.

Sodium Chloride vs. Natural Seawater

When survival was analyzed within treatment salinity between salt source, NSW and NaCl were significantly different ($p < 0.0001$) in the 24 and 32 g/L salinity treatments.

Natural Seawater Gradual Acclimation Survival

For all treatment groups, survival was 100%. Therefore, no analysis of the data was required. With the exception of total hardness, no biologically significant differences among treatments in the measured water quality parameters were observed throughout the course of the experiment. During the 96 h freshwater acclimation period the following ranges and means were recorded, respectively: DO, 7.23 – 7.98, 7.63 ± 0.29 mg/L; pH, 8.03 – 8.41, 8.29 ± 0.11; TAN, 0.00 – 0.02, 0.01 ± 0.01 mg/L; nitrite, 0.0011 – 0.0036, 0.0025 ± 0.0010 mg/L; alkalinity, 180 mg/L $CaCO_3$; hardness, 188.10 – 205.20, 199.50 ± 8.83 mg/L $CaCO_3$. During the 96 h experimental period the following ranges and means were recorded, respectively: DO, 7.56 – 8.35, 7.86 ± 0.19 mg/L; pH, 8.12 – 8.46, 8.31 ± 0.07; TAN, 0.00 – 0.07, 0.03 ± 0.02 mg/L; nitrite, 0.0014 – 0.0200, 0.0040 ± 0.0022 mg/L; alkalinity, 160.00 – 180.00, 170.00 ± 10.1 mg/L $CaCO_3$. Due to experimental addition of seawater, total hardness varied between control and treatment groups. Hardness ranges and final means by salinity are as follows: 0 g/L, 205.20 mg/L $CaCO_3$; 32 g/L, 205.20 – 5840.00, 5717.50 ± 116.02 mg/L $CaCO_3$.

Discussion

Survival analysis for NaCl and NSW acute salinity tolerance experiments revealed *F. seminolis* can tolerate direct transfer from freshwater to salinities of 16 and 24 g/L. Daily water quality analysis during the 96 h experimental periods helped to eliminate potentially confounding variables in an effort to identify salinity as the driving force behind observed mortality. Upon

completion of the 96 h experimental exposure in acute transfer experiments, surviving fish were left in their respective aquaria and monitored for 2 weeks. No mortalities were observed during this time period for any surviving treatments regardless of salinity source. This is evidence that results of 96 h survival experiments are useful indicators of a species' ability to survive long term in selected treatment conditions. Additionally, although 100% survival was noted at 16 g/L in both NaCl and NSW experiments, fish in the NSW treatment generally appeared healthier. *F. seminolis* at 16 g/L in NaCl exhibited multiple areas of hyperemia, overall pallor, decreased activity, and increased mucus production. Excessive mucus has been previously reported in *Fundulus kansae* exposed to low calcium "artificial seawater" (Potts and Fleming, 1970).

Differences between survival due to salinity source were evident in 24 and 32 g/L treatments (Tables 3 – 1 and 3 – 3). Due to lack of physiological data it is only possible to speculate as to the underlying causes for the recorded differences. The role of calcium in osmoregulation and ionic transport has been well studied (Potts and Fleming, 1970; Carrier and Evans, 1976; Isaia and Masoni, 1976; Pic and Maetz, 1981; Hunn, 1985). Although calcium was not measured directly, total hardness levels (mg/L $CaCO_3$) were recorded throughout both experiments. Significant differences ($p < 0.0001$) in hardness were noted among NSW treatment salinities of 8, 16, 24, and 32 g/L. Additionally, hardness values from these treatment groups were also significantly different ($p < 0.0001$) from all NaCl treatment groups and the NSW control. It can be inferred from this data that calcium and other divalent ions were in a greater abundance in the NSW treatment groups than their NaCl counterparts. Low external calcium in an environment hypersosmotic to fish has been shown to increase ion efflux in *Lagodon rhomboides* (Carrier and Evans, 1976) and alter net fluxes of both ions and water in *Anguilla anguilla* (Isaia and Masoni, 1976). Potts and Fleming (1970) reported a 35% reduction in the gill

permeability of *F. kansae* in NSW when compared with fresh water, thereby decreasing ion efflux and drinking rate. Conversely they also reported *F. kansae* to have an increased drinking rate and water exchange rate when transferred to low calcium synthetic seawater. Although not directly tested, findings from the aforementioned studies support the hypothesis that the mortality observed in the NaCl experiment relative to the NSW experiment was a result of loss of hydro-mineral regulation due to lower environmental ion concentrations, particularly calcium. However, environmental deficiencies of other divalent ions such as magnesium, should not be dismissed because these ions also play a functional role in salinity acclimation (Isaia and Masoni, 1976).

Results of gradual NSW acclimation were encouraging for this marine baitfish candidate. *F. seminolis* was able to easily acclimate to 32 g/L in all 4 time intervals with no mortalities in any treatment. Modest lethargy in most replicates of the 24 h treatment group was the only outward sign of physiological stress observed among any of the treatments upon completion of the acclimation process.

Salinity tolerance of the predominantly euryhaline *Fundulus* genus has been extensively investigated yet almost no experimental evidence exists regarding *F. seminolis* until the present study. Griffith's (1974A) seminal investigation on salinity tolerance of the genus is the only study that has evaluated this emerging aquaculture candidate, but due to confounding experimental factors these data are potentially unreliable. An additional study by Griffith (1974B) attempted to elucidate the role of pituitary control in the freshwater acclimation of the genus, but only one *F. seminolis* was used, again limiting the usefulness of the data. Salinity tolerance results from this experiment confirm *F. seminolis* can acclimate to the upper end of Griffith's (1974A) reported range. Survival by *F. seminolis* after acute transfer to 24 g/L

indicates superior salinity tolerance when compared to *Fundulus nottii*'s acute tolerance of 17 g/L (Crego and Peterson, 1997). However, no upper level salinity tolerance has been determined to date but it is unlikely *F. seminolis* will tolerate salinities of 95+ g/L commonly reported for *F. grandis* (Nordlie, 2000), *F. heteroclitus* (Griffith, 1974A), *F. confluentus* (Griffith, 1974A; Nordlie, 2000), *F. similis* (Nordlie, 2000), *F. parvipinnis* (Feldmeth and Waggoner, 1972), *Aphanius dispar* (Lotan, 1971), and *Adenia xenica* (Nordlie, 1987).

F. seminolis is widely considered to be a freshwater killifish, with upper field salinities reported at 2.4 (Gunter and Hall, 1963), 7.3 (Gunter and Hall, 1965), and 13.5 g/L (Phillips and Springer, 1960). Results from our experiments clearly indicate *F. seminolis* is capable of tolerating brackish and full strength seawater for extended periods of time. Experimental results substantiate the potential of *F. seminolis* as a candidate for marine baitfish aquaculture following seawater acclimation. Ability to tolerate acute transfer from 0 - 24 g/L and gradual acclimation to 32 g/L NSW over a broad time period allows for flexibility in the development of a salinity acclimation protocol. Additionally, a mean survival time of 13 h after acute transfer to full strength seawater allows for marketing and use of freshwater acclimated fish in the marine environment if so desired. NSW acclimated fish provide an additional option for bait retailers utilizing saline holding facilities as well as anglers who wish to store their bait in saline livewells. Demands of bait retailers and sportsman will ultimately dictate the acclimation regime for this new marine baitfish species.

Table 3 – 1. Kaplan-Meier survival analysis for acute NaCl transfer.

Treatment (g/L NaCl)	Mean survival (h ± SE)	Survival (%)
(Control) 0	*	100
8	*	100
16	*	100
24	6 ± 1	0
32	2	0

* Statistic cannot be calculated for comparison of treatments with 100% survival for both.

Table 3 – 2. Log-Rank analysis among treatments for NaCl acute transfer.

Treatment	0 g/L	8 g/L	16 g/L	24 g/L
8 g/L	*			
16 g/L	*	*		
24 g/L	$p<0.0001$	$p<0.0001$	$p<0.0001$	
32 g/L	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$

* Statistic cannot be calculated for comparison of treatments with 100% survival for both.

Table 3 – 3. Kaplan-Meier survival analysis for acute NSW transfer.

Treatment (g/L NSW)	Mean survival (h ± SE)	Survival (%)
(Control) 0	*	100
8	*	100
16	*	100
24	*	100
32	13 ± 1	0

* Statistic cannot be calculated for comparison of treatments with 100% survival for both.

Table 3 – 4. Log-Rank analysis among treatments for NSW acute transfer.

Treatment	0 g/L	8 g/L	16 g/L	24 g/L
8 g/L	*			
16 g/L	*	*		
24 g/L	*	*	*	
32 g/L	$p<0.0002$	$p<0.0002$	$p<0.0002$	$p<0.0002$

* Statistic cannot be calculated for comparison of treatments with 100% survival for both.

CHAPTER 4
EVALUATION OF A POINT OF CARE BLOOD ANALYZER FOR USE IN DETERMINATION OF SELECT HEMATOLIGICAL INDICES IN *FUNDULUS SEMINOLIS*

Introduction

In fishes, determinations of hematocrit and plasma electrolyte values are valuable tools for diagnostic and research purposes. The osmoregulatory abilities of fishes are commonly examined using these hematological indices. Fluctuations in plasma electrolyte and hematocrit levels also provide clinicians and researchers with valuable knowledge regarding the physiological impacts of environmental and pathogenic stressors. Conventional methods of hematocrit and electrolyte determination, microhematocrit centrifugation, flame photometry, and chloride titration, are gradually being replaced by new technologies. Point-of-care (POC) blood analyzers are both efficient and user friendly. POC analyzers have undergone extensive testing and validation by the U.S. Food and Drug Administration and independent researchers for use in humans and similar testing has occurred for use in veterinary applications with canine, feline, equine and poultry models (Grosenbaugh et al., 1998; Looney et al., 1998; Acierno and Mitchell, 2007; Steinmetz et al., 2007). As the use of such technologies becomes more pervasive in current literature, investigations into the accuracy and reliability of POC analyzers for evaluating hematological indices of fish is warranted. There is currently little research validating POC analyzers against conventionally accepted instrumentation (CAI) for use in fish. The evaluation of a POC blood analyzer for use in rockfish, *Sebastes* spp., (Harrenstien et al., 2005) is the most comprehensive validation to date.

The seminole killifish, *Fundulus seminolis,* is a freshwater killifish with a geographic range within peninsular Florida. This species, commonly referred to as a “bullminnow” or “mudminnow”, is one of the largest members of the genus, reaching total lengths of 20 cm (Hoyer and Canfield, 1994). Its popularity as a local baitfish for piscivorous game fish has

generated interest in this species as a potential candidate for aquaculture. Acclimation of this species to the marine environment for use as a baitfish will result in numerous physiological changes. Collection of limited blood quantities makes determinations of hematological indices problematic for this species. The blood volume requirement and rapid analysis time of the i-STAT® unit makes it an attractive option for hematological evaluations with this species.

The purpose of this study was to evaluate a POC blood analyzer (i-STAT®), and chosen cartridge (E3+) (Heska Corp., Fort Collins, CO, USA) against CAI for use in determination of hematocrit, sodium, potassium, and chloride values in *F. seminolis*. Operating procedures following manufacturer's specifications and alternative protocols using heparin diluted blood were analyzed.

Methods

F. seminolis were collected by seine net from the eastern shore of Lake George in Volusia County Florida and transported to the University of Florida Indian River Research and Education Center in Fort Pierce, Florida. Fish were assessed for pathogens and treated accordingly to ensure healthy research specimens. Fish were held for a minimum of 6 months in an outdoor 6900 L recirculating system with biofilter media, a UV sterilizer, and a 100 micron bag filter to maintain optimal water quality. Salinity was maintained at 2 g/L with an ambient temperature and photoperiod during the experimental period of February 2 – March 20, 2008. On days of blood analyzation research specimens were collected from the recirculating system and transported to the laboratory (~5min) where they were held in aerated 37.85 L aquaria filled with water from the recirculating system. All blood collection occurred within 24 h of transport. Experiments were performed to validate the accuracy of the i-STAT® handheld clinical blood analyzer and the E3+ cartridge (Heska Corp., Fort Collins, CO, USA) against conventional hematological instrumentation. The E3+ cartridge is designed to measure sodium, potassium,

chloride, hematocrit, and hemoglobin from whole blood aliquots. Hemoglobin measurements were not examined in this study because hemoglobin is not directly measured by the i-STAT®. Instead hemoglobin is automatically calculated by multiplying a human blood conversion factor (0.34) by the measured hematocrit percentage. The E3+ cartridge was chosen because it is one of the most basic cartridges produced by the manufacturer yet its analyzed parameters have application across numerous disciplines. Whole blood was used to measure the remaining parameters in an initial experiment. A second experiment implemented a whole blood heparin dilution prior to analysis with the POC unit. No attempt was made to establish reference ranges for blood parameters in *F. seminolis*.

Whole Blood Analysis

Twenty four *F. seminolis* were used in this experiment ranging in length from 126 – 154 mm with a mean length of 139.1 ± 7.0 mm. Weights ranged from 20.7 – 37.4 g with a mean weight of 27.0 ± 4.7 g. None of the fish sampled exhibited any signs of gross parasitism or infection. Aquaria water did not contain anesthetic to prevent alteration of hematological indices of interest (Reinitz and Rix, 1977). Fish were individually removed from the aquaria and weighed and measured. The specimen's caudal peduncle was rinsed with nanopure water and blotted dry then promptly severed. A 100 µl lithium heparinized capillary tube (7-000-1000, Drummond Scientific, Broomall, PA, USA) was filled first by placing the tube against the severed caudal vessel. Multiple ammonium heparinized microhematocrit capillary tubes were then filled in the same fashion until blood flow ceased. Fish were then euthanized by pithing. All blood collection was completed within 5 minutes after removal from the holding aquaria. The 100 µl lithium heparinized sample was immediately used to fill the E3+ cartridge to its prescribed level after which it was inserted into the i-STAT® unit and analyzed. The manufacturer's operating procedures for the i-STAT® were adhered to throughout the

experiment (i-STAT system manual, 1997). Twenty four E3+ cartridges were utilized in this experiment. Microhematocrit tubes were capped with sealant and centrifuged at 13,460 g (11,500 rpm) for 6 minutes, after which the hematocrit was determined. A mean hematocrit for each fish was calculated from the total number of microhematocrit tubes collected. Plasma was then separated from the red blood cells and frozen in microcentrifuge tubes at -20°C for later analysis. Due to the small size of the fish sampled, blood volume and thus plasma volume was limited, allowing each sample to be run only once through the various analyzers. All samples were run within 30 days of freezing. Thawed plasma samples were analyzed for sodium and potassium concentrations utilizing a Jenway PFP7 flame photometer (Bibby Scientific Ltd., Essex, England) according to the manufacturer's specifications. Plasma chloride concentration was determined utilizing a Labconco digital chloridometer (Labconco, Kansas City, MO, USA) according to the manufacturer's directions.

Whole Blood Heparin Dilution Analysis

Methods for the validation of the i-STAT® using a heparin diluted whole blood sample adhere to the methods previously listed for the whole blood analysis except as noted below. Dilution of whole blood aliquots has not been validated by the manufacturer but was used by Suski et al. (2007). Fifteen *F. seminolis* were used in this experiment ranging in length from 129 – 155 mm with a mean length of 140.5 ± 8.5 mm. Weights ranged from 18.4 – 37.7 g with a mean weight of 29.2 ± 5.0 g. The specimen's caudal peduncle was rinsed with nanopure water and blotted dry after which it was promptly severed. A 90 µl sample of whole blood was drawn up in a lithium heparinized capillary tube from the severed caudal vessel. The blood was then expelled into a sterile microcentrifuge tube into which 10µl of 150 usp/ml lithium heparin (H0878, Sigma-Aldrich, St. Louis, MO, USA) dissolved in nanopure water was added. The sample was mixed by gently pipetting to avoid hemolysis and then transferred to the E3+

cartridge and analyzed. The final heparin concentration of 15 usp/ml blood achieved was within the range of 14 – 16 usp/ml blood used by Harrenstien et al. (2005). Results obtained from the i-STAT® were then corrected for the dilution factor. Fifteen E3+ cartridges were utilized in this experiment. Remaining blood samples were collected and processed undiluted as previously stated for the whole blood experiment.

Statistical Analysis

Data generated by the i-STAT® and E3+ cartridge were compared with conventional instrumentation employed to analyze the indices under investigation. Numbers of data points per analyte varied due primarily to cartridge error or results outside the reportable range of the POC analyzer. The Bland-Altman method for assessing agreement between two methods of clinical measurement was used (Bland and Altman, 1986) as well as calculations of correlation coefficients. A two tailed paired student t-test was also used to compare values generated by both methods of analysis. Bias was defined as the mean percent difference between values generated by both methods. The limits of agreement (LA) were defined as the bias ± 2 SD. Differences were considered significant when $p \leq 0.05$.

Results

Whole Blood Analysis

A 37.5% failure rate, defined as no recordable results, was experienced with the POC analyzer. The error code "unable to position sample" was the most frequent code encountered. Of the remaining 15 cartridges not all yielded results deemed useful for our analysis. Three cartridges reported hematocrit (< 10%) values outside the reportable range for this unit. Additionally, four cartridges reported chloride (> 140mmol/L) values outside the instrument's reportable range as well. These values were not considered in subsequent comparisons. Analysis by CAI was 100% successful with no results above or below the instrumentations thresholds for

all samples analyzed. Mean values by parameter for each method of analysis are presented in Table 4 – 1. Significant differences ($p \leq 0.05$) were noted between methods for every parameter mean analyzed by t-test (Table 4 – 1). Correlation between methods were weak ($r < 0.9$) and varied broadly with r values ranging from -0.52 – 0.79 (Table 4 – 1). Bland-Altman analysis revealed hematocrit to have a value lying outside the limits of agreement (Figure 4 – 1). LA values of ± 34.8, ± 14.6, ± 25.2, and 16.0% and bias values of -44.5, -26.0, -10.3, and 12.8% were calculated for hematocrit, sodium, potassium, and chloride respectively.

Whole Blood Heparin Dilution Analysis

A 33.3% failure rate, defined as no recordable results, was experienced with the POC analyzer. The error code "unable to position sample" was the most frequent code encountered. Of the remaining 10 cartridges, all yielded results deemed useful for analysis. Analysis by CAI was 100% successful with no results above or below the instrumentations thresholds for all samples analyzed. Mean values by parameter for each method of analysis are presented in Table 4 – 2. Significant differences ($p \leq 0.05$) were noted between methods for every parameter mean analyzed by t-test (Table 4 – 2). Correlation coefficients were generally weak with the exception of hematocrit ($r = 0.96$). Coefficients again varied broadly with r values ranging from -0.02 – 0.96 (Table 4 – 2). Bland-Altman analysis revealed hematocrit and chloride to have values lying outside the limits of agreement (Figure 4 – 2). LA values of ± 40.0, ± 17.1, ± 23.3, and ± 17.0% and bias values of -29.0, -16.0, 15.5, and 17.7% were calculated for hematocrit, sodium, potassium, and chloride respectively.

Discussion

Generally, mean values obtained from CAI were higher when compared with the POC analyzer using both whole and diluted blood samples. Chloride values generated in both experiments as well as potassium values resulting from heparin diluted blood were exceptions.

Significant differences observed between all mean parameter values analyzed by t-tests agree with previously reported results by Harrenstien et al. (2005) in which all twelve parameters generated by the POC analyzer except potassium and glucose were found to be significantly different from their CAI counterpart. Bias and LA values varied considerably throughout the experiments. Hematocrit bias (-44.5% and -29.0%) agreed with the trends observed by Harrenstien et al. (2005) in rockfish and the approximate -25% bias reported by Howard and Wack (2002) in birds. All LA values from both whole blood and heparin dilutions fell well outside Clinical Laboratory Improvement Act (CLIA) guidelines governing accepted discrepancies in testing methodologies for hematocrit (± 6%), sodium (± 4 mmol), potassium (± 0.5 mmol), and chloride (± 5%) (United States Department of Health and Social Services, 1992). While these guidelines were developed to ensure accuracy and precision in a clinical setting, it should be a goal of individuals in the research field to adhere to such stringent scientific standards. Differences between the POC values and CAI values can not be easily explained. As the i-STAT® is made to function with mammalian blood and uses the Nernst equation to relate potential with concentration, possible temperature effects of poikilothermic blood on the potentiometric determination of electrolytes could account for observed discrepancies.

Numerous limitations of the POC unit and the E3+ cartridge were identified through the course of this experiment. Successful sample analysis was hindered by the rapid clotting of collected blood despite immediate transfer. Similar problems in sample analysis have been previously noted by Harrenstien et al. (2005), Olsvik et al. (2007) and Steinmetz et al. (2007) but not to the degree seen in this study. Dilution of samples with lithium heparin in subsequent experiments did not ameliorate this problem. Hematocrit and chloride values outside the instrument's reportable range were an additional difficulty encountered. Suski et al. (2007) and

Olsvik et al. (2007) reported similar problems with hemoglobin and sodium respectively. Interestingly, Harrenstien et al. (2005) was not able to evaluate chloride in their experiment because all generated values were above the i-STAT's reportable upper limit of 140 mmol/L. Brill et al. (2008) reported POC mean sodium values of 257 ± 6 and 278 ± 4 mmol/L and POC mean chloride values of 210 ± 2 and 216 ± 2 mmol/L for *Carcharhinus plumbeus*, far exceeding the reportable range for either parameter. Suski et al. (2007) reported diluting blood samples by 25% and it can be inferred from values reported by Brill et al. (2008) dilutions of the same magnitude or greater were used to circumvent out of range values. Heparin dilution of whole blood samples in our experiment brought values within the reportable range of the unit but overall, fared no better in regards to bias or LA values (Figure 4 – 2) than whole blood analysis.

Despite only one validation study evaluating the i-STAT® against CAI for use in rockfish (Harrenstien et al., 2005), a myriad of species and broad array of blood parameters have been subsequently analyzed and reported in scientific literature. bonefish, *Albula vulpes* (Suski et al., 2007), cod, *Gadus morhua* (Foss et al., 2006; Remen et al., 2008), Atlantic salmon, *Salmo salar* (Olsvik et al., 2007; Petri et al., 2008), spiny dogfish, *Squalus acanthias* (Mandelman and Farrington, 2007), Nile tilapia, *Oreochromis niloticus* (Choi et al., 2007), sandbar sharks, *Carcharinus plumbeus* (Brill et al., 2008), amur sturgeon, *Acipenser shrenckii* (Lu et al., 2005), and turbot, *Scophthalmus maximus* (Foss et al., 2007) comprise the increasing number of species whose blood has been analyzed by the unit. Recommendations by Harrenstien et al. (2005) against the use of the i-STAT® for determination of chloride, potassium, glucose, total CO2, and HCO3 in fish as well as calls for additional studies on multiple species have been ignored. Foss et al. (2006), Choi et al. (2007), Foss et al. (2007), Olsvik et al. (2007), Brill et al. (2008), Petri et al. (2008), and Remen et al. (2008) used the i-STAT® to analyze hematological parameters

deemed unreliable and inaccurate with no effort to provide further methodological validation or correct the reported parameters for bias. Furthermore, the dilution of blood prior to analysis (Suski et al., 2007) is not recommended by the manufacturer and has yet to be evaluated against CAI. Choi et al. (2007) acknowledges the need for validation of the POC analyzer as its popularity increases, yet does not corroborate experimental POC values with CAI. However, Mandelman and Farrington (2007) made an effort to inform readers that parameter values are not absolute and should be evaluated with caution.

Method validation studies (Grosenbaugh et al., 1998; Looney et al., 1998; Acierno and Mitchell, 2007; Steinmetz et al., 2007) examining new technologies or new species are essential for the generation and dissemination of reliable data to the scientific community. Results from our analyses support the findings of Harrenstien et al. (2005) and we reiterate the need for further validation studies using multiple species. This POC unit may be useful for elucidation of trends within analyzed parameters but results should be interpreted carefully as further testing is still needed. None of the blood parameters analyzed by the i-STAT® in this experiment could be considered reliable. Use of unvalidated instrumentation must be avoided to prevent publication and distribution of potentially erroneous data. Methodological validation must be considered paramount for the introduction of new technologies in research applications.

Table 4 – 1. Mean ± SD values for analyzed hematological parameters using both the POC analyzer (i-STAT®) and conventionally accepted instrumentation (CAI) generated from whole blood aliquots. Significant results (*p* values) from paired t-tests and calculated correlation coefficients (*r*) comparing both methods of analysis are reported.

Parameter	N	POC analyzer	CAI	t-test (*p*)	*r*
Hematocrit (%)	11	16 ± 4	25 ± 6	<0.0001	0.79
Sodium/Na^+ (mmol/L)	15	140 ± 2	182.3 ± 12.1	<0.0001	-0.52
Potassium/K^+ (mmol/L)	14	6.2 ± 0.8	6.8 ± 0.6	0.0118	0.36
Chloride/Cl^- (mmol/L)	11	137 ± 2	121.0 ± 10.4	0.0003	0.35

Table 4 – 2. Mean ± SD values for analyzed hematological parameters using both the POC analyzer (i-STAT®) and conventionally accepted instrumentation (CAI) generated from heparin diluted whole blood aliquots. Significant results (*p* values) from paired t-tests and calculated correlation coefficients (*r*) comparing both methods of analysis are reported.

Parameter	N	POC analyzer	CAI	t-test (*p*)	*r*
Hematocrit (%)	10	22 ± 8	29 ± 6	<0.0001	0.96
Sodium/Na^+ (mmol/L)	10	142 ± 5	166.5 ± 12.7	0.0003	-0.03
Potassium/K^+ (mmol/L)	10	6.6 ± 0.9	5.7 ± 0.8	0.0038	0.6
Chloride/Cl^- (mmol/L)	10	135 ± 7	113.3 ± 7.9	<0.0001	-0.02

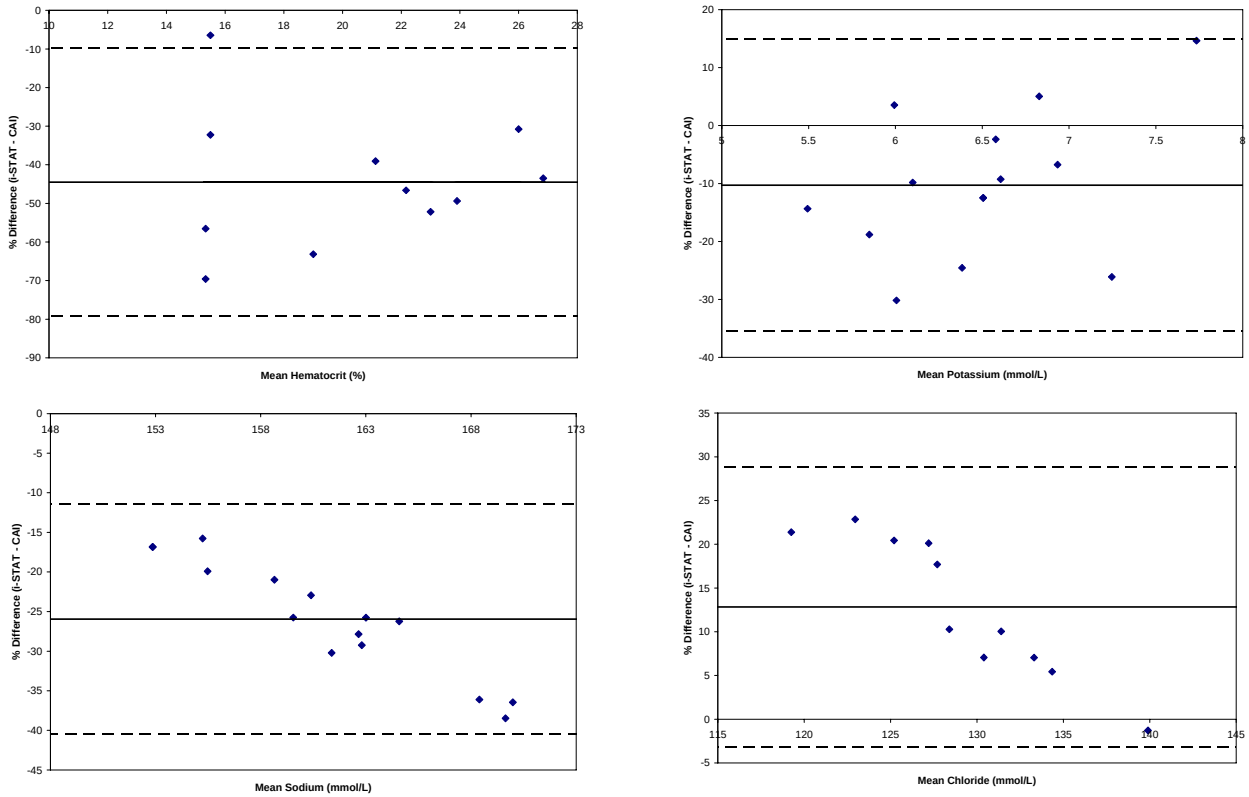

Figure 4-1. Bland-Altman plots of blood parameter percent difference (i-STAT® - conventionally accepted instrumentation [CAI]) versus overall mean blood parameter concentration ([i-STAT® + CAI]/2) for hematocrit, sodium, potassium, and chloride values generated from whole blood aliquots. Bias (mean % difference between the i-STAT® and the CAI) is represented by the solid line. Limits of agreement (bias ± 2SD) are represented by the dashed lines. Each point represents values generated from an individual *F. seminolis*.

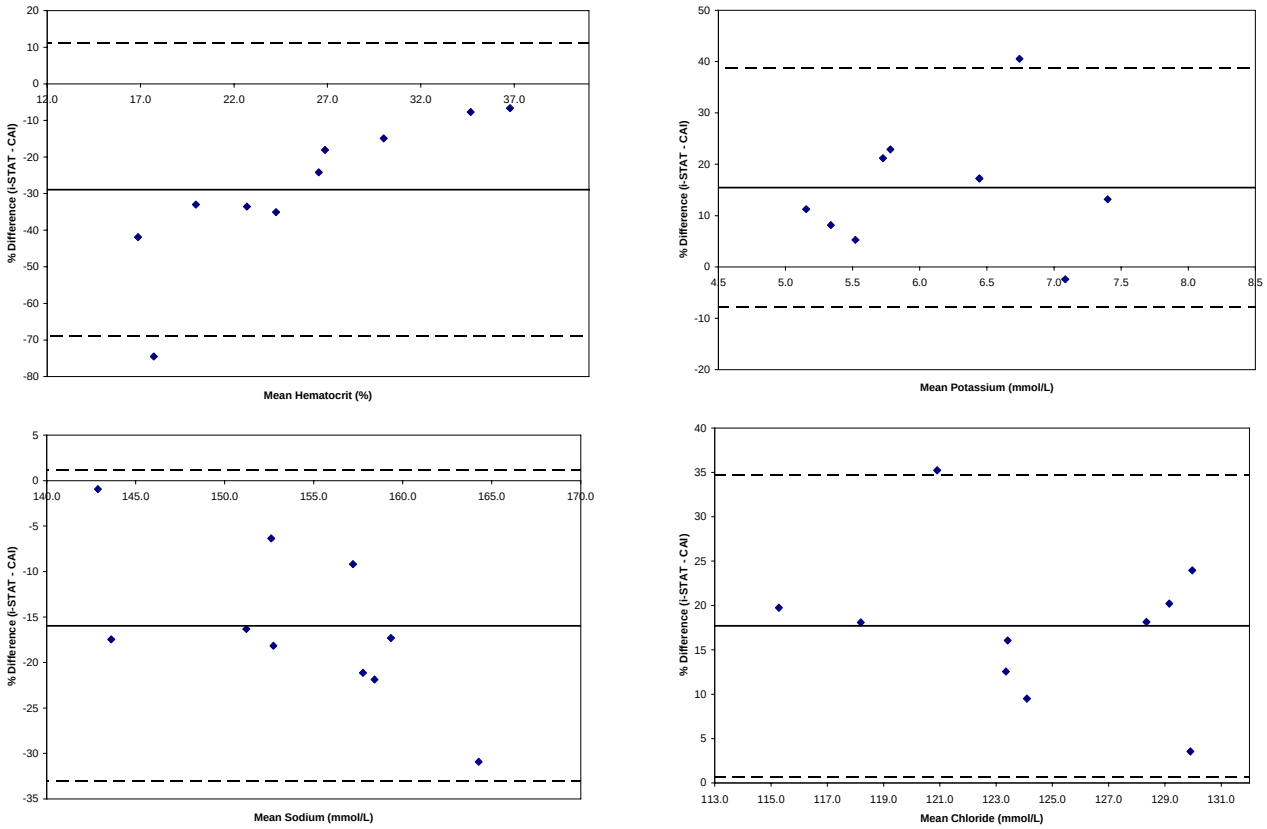

Figure 4-2. Bland-Altman plots of blood parameter percent difference (i-STAT® - conventionally accepted instrumentation [CAI]) versus overall mean blood parameter concentration ([i-STAT® + CAI]/2) for hematocrit, sodium, potassium, and chloride values generated from heparin diluted whole blood aliquots. Bias (mean % difference between the i-STAT® and the CAI) is represented by the solid line. Limits of agreement (bias ± 2SD) are represented by the dashed lines. Each point represents values generated from an individual *F. seminolis*.

CHAPTER 5
PHYSIOLOGICAL EVALUATION OF *FUNDULUS SEMINOLIS* FOLLOWING FOUR SEAWATER ACCLIMATION PROTOCOLS

Introduction

Seawater acclimation in fishes is a culmination of a myriad of physiological processes; a current understanding of its various components is best summarized by Evans et al. (2005). Research into ion secretion and uptake, water balance, and morphological and enzymatic changes have elucidated a multitude of processes allowing this acclimation. The strongly euryhaline killifish, *Fundulus heteroclitus*, has been the focus of a majority of those examinations (Epstein et al., 1967; Karnaky et al., 1976; Jacob and Taylor, 1983; Wood and Marshall, 1994; Marshall et al., 1999; Marshall et al., 2000; Katoh et al., 2001; Mancera and McCormick, 2002). A natural euryhalinity has been a commonality of other experimentally utilized species. The gilthead sea bream, *Sparus auratus* (Laiz-Carrion et al., 2005), Mozambique tilapia, *Oreochromis mossambicus* (Foskett et al., 1981), and rainbow trout, *Oncorhynchus mykiss* (Madsen and Naamansen, 1989) are just several of the previously examined species. Few studies have assessed the osmoregulatory functions of relatively stenohaline fishes acclimated to atypical salinities. Evaluation of low saline aquaculture of marine fishes have provided an impetus for these investigations (Faulk and Holt, 2006; Resley et al., 2006; Young et al., 2006; Fielder et al., 2007).

The ability to culture marine fishes in a low saline or freshwater environment has many implicit advantages yet reproductive and physiological limitations may impede success. As fish are transferred from a hypoosmotic to hyperosmotic environment, osmoregulatory complications may manifest in the form of increased plasma electrolyte concentrations and thus increased plasma osmolality. During seawater acclimation of *F. heteroclitus*, Marshall et al. (1999) reported increased plasma sodium concentrations from 1 – 24 h post transfer with a peak

osmolality occurring 24 h after transfer. Jacob and Taylor (1983) reported significant sodium and osmolality elevations up until 48 h post transfer. Investigations by Pickford et al. (1969) on *F. heteroclitus* blood serum showed an average decrease in sodium, potassium, and chloride of 9% in fish held in freshwater versus seawater. Similar acclimation experiments conducted on the cyprinodontiform fishes *F. kansae* (Stanley and Fleming, 1976) and *Aphanius dispar* (Lotan, 1971) again described an increased elevation of plasma osmolality and ion influx. Loss of ionic homeostasis due to salinity acclimation is well established and has been previously reported in commonly aquacultured species (Imsland et al., 2003; Resley et al., 2006; Young et al., 2006; Fielder et al., 2007). Elevated hematocrit percentage is an indicator of increased water efflux and hemoconcentration (Marshall et al., 2005). Water efflux following hyperosmotic transfer may also be quantified in the calculated water content of a muscle tissue sample. Significant loss of muscle water content, such as that recorded by Altinok et al. (1998) in seawater acclimated Gulf of Mexico sturgeon, *Acipenser oxyrinchus desotoi*, or seawater acclimated medaka, *Oryzias latipes,* (Sakamoto et al., 2001) can provide valuable osmoregulatory information and confirm observed trends in hematocrit values.

Osmoregulatory limitations of candidate marine aquaculture species need to be evaluated to determine feasibility of production and marketing. The seminole killifish, *Fundulus seminolis,* is a naturally stenohaline freshwater killifish (Phillips and Springer, 1960; Gunter and Hall, 1963; Gunter and Hall, 1965) which has recently emerged as a candidate for marine baitfish aquaculture. Griffith's (1974A) experimental salinity determination for this species placed the upper mean salinity tolerance at 23.4 g/L, with an experimental salinity range of 19.3 – 33.4 g/L. If the species is able to acclimate to full strength seawater, it could be produced exclusively in freshwater ponds or recirculation systems, only requiring acclimation to saline water prior to

marketing and distribution. Decreased reliance upon saltwater and coastal access would allow for the utilization of inland resources for the culture of this species.

Evans et al. (2005) recognized that molecular and biochemical events accompanying acute salinity transfers may be species specific. Osmotic evaluation of *F. seminolis* as a stenohaline freshwater analogue to *F. heteroclitus* could generate data which may elucidate physiological responses not previously observed in euryhaline *Fundulus* species. Therefore, the objective of this study was to measure the osmoregulatory effects (plasma osmolality, sodium, potassium, chloride, hematocrit, and muscle water content) of four different rates of seawater acclimation on *F. seminolis*.

Methods

F. seminolis were collected by seine net from the eastern shore of Lake George, in Volusia County, Florida and transported to the University of Florida Indian River Research and Education Center in Fort Pierce. Fish were assessed for pathogens and treated accordingly to ensure healthy research specimens for the subsequent salinity experiments

Natural Seawater Acclimation

Seventy two fish were transferred from a 6900 L recirculating system to 36, 85 L glass aquaria, divided in half by aquarium partitions (TDSU, Penn-Plax Inc., Happauge, N.Y.) with one fish per aquarium subdivision during the acclimation and experimental periods. Specimen's weight and total length (TL) were recorded prior to transfer. Length and weight ranges of 124 - 152 mm and 21.7 – 38.3 g were recorded with means of 136.1 ± 6.4 mm and 27.5 ± 4.5 g, respectively. Aquarium systems were maintained at < 1 g/L salinity well water (Na^+ = 6.1, K^+ = 0.3, Cl^- = 3.3 mmol/L) and recirculated through biofilter media during the 96 h acclimation period. Dissolved oxygen (DO), pH, temperature, salinity, total ammonia nitrogen (TAN), and nitrite were recorded daily during the acclimation and experimental periods with total alkalinity

and total hardness recorded on days one and three of acclimation and at the initiation and cessation of experimental treatment periods. DO and temperature were measured using a YSI 550A meter and salinity was determined using a YSI 30 salinity/conductivity meter (YSI Inc., Yellow Springs, Ohio). pH was measured using a Hach sensION1 portable pH meter and total alkalinity and total hardness were determined using standardized titration techniques (Hach Co., Loveland, Colorado). TAN and nitrite were evaluated spectrophotometrically using a Hach DR 4800 (Hach Co., Loveland, Colorado). Aquaria were held at ambient temperature with a range of 17.5 - 27.2°C and a mean temperature of 22.6°C during the experimental period. Temperature differences among aquaria never exceeded 2°C. A ambient photoperiod of 13 L : 11 D was used during the experiment. Fish were fed once a day to satiation on days two and three of acclimation and food was withheld on days one and four of acclimation and during the entire experimental period.

Following the 96 h freshwater acclimation, aquaria were made static and randomized among nine treatments with eight replicates per treatment. Differences among treatment group's lengths and weights were not significant ($F_{8, 63} = 0.45$, $p = 0.885$; $F_{8, 63} = 0.32$, $p = 0.954$, respectively). Among the treatment groups, the precontrol (time 0) group was sacrificed immediately after the freshwater acclimation period; four treatments were subjected to a gradual salinity change from 0 to 32 g/L over predetermined time periods with the remaining four treatments serving as controls for each corresponding time period. Acclimation times of 24, 48, 72, and 96 h were chosen with approximate salinity increases of 5.3, 2.7, 1.7, and 1.3 g/L, respectively, every 4 h. The final salinity of 32 g/L was attained 4 h prior to the termination of each treatment group. Salinities were gradually changed every 4 h via addition of NSW equally dispersed between aquaria subdivisions until the desired salinity was achieved. Excess water

during salinity changes was allowed to flow out of the aquarium's standpipe. A single air stone within each subdivision provided adequate mixing within and between subdivisions ensuring a homogeneous environment within each aquarium. Control aquaria had similar volumes of freshwater added in the same manner as saline treatment tanks.

Once the specified acclimation time had been reached the treatment group and its corresponding control were sacrificed and blood and tissues were collected. Fish were individually removed from their respective tanks and subsequently weighed. The fish's caudal peduncle was rinsed with nanopure water and blotted dry after which it was promptly severed. Ammonium heparinized microhematocrit capillary tubes were then filled by placing the tube against the severed caudal vessels until blood flow ceased. This method was employed to prevent contamination of the blood sample by any exogenous or endogenous fluids other than blood. Fish were then euthanized by pithing. Weights and blood collection were completed within 5 minutes after removal from tanks. Microhematocrit tubes were capped with clay and centrifuged at 13,460 g (11,500 rpm) for 6 minutes, after which the hematocrit determined. A mean hematocrit for each fish was calculated from the total number of microhematocrit tubes collected. Plasma was then separated from the red blood cells and frozen in microcentrifuge tubes at -20°C for later analysis. Additionally, a muscle tissue sample (mean weight = 0.804 ± 0.227 g) was removed from each specimen to determine muscle water content (MWC). Samples were weighed in tared aluminum weigh boats before and after drying at 105°C for 24 h (Altinok et al., 1998). Due to the small size of the fish sampled, blood volume and thus plasma volume was limited, allowing each sample to be run only once through the various analyzers. All plasma samples were run within 30 days of freezing. Thawed samples were analyzed for sodium and potassium concentrations utilizing a Jenway PFP7 flame photometer (Bibby Scientific Ltd.,

Essex, England) according to the manufacturer's specifications. Chloride concentration was determined utilizing a Labconco digital chloridometer (Labconco, Kansas City, MO, USA) according to the manufacturer's accepted protocols and osmolality was analyzed using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA).

Statistical Analysis

All data were analyzed with a randomized block ANOVA to elucidate any positional effects resulting from tank subdivisions followed by a Tukey's HSD means separation test using SAS® version 8.02 (SAS Institute Inc., USA). All percentage data was arcsine square root transformed prior to analysis. All numerical data are represented as the mean ± SD. A p value ≤ 0.05 was considered statistically significant for all analyses.

Results

For all analyses, a randomized block ANOVA was used to elucidate any positional effects resulting from subdividing aquaria. No significant block effect was recorded for any of the quantified parameters in any of the subsequent analyses with the exception of osmolality when comparing the precontrol and control groups. This effect was marginally significant ($p = 0.0425$) and not observed in any other analyses, thus each subdivision was considered an independent experimental unit.

Analyses of hematocrit, sodium, potassium, and chloride among the precontrol and control experimental groups showed no significant differences. The precontrol osmolality and MWC were significantly different ($p \leq 0.0117$, $p \leq 0.0008$) when compared to all other control groups. Additionally, a significant difference ($p = 0.0425$) in body weight change (BWC) between the precontrol and 96 h control was also noted.

Plasma sodium (Figure 5 – 1), chloride (Figure 5 – 2), and osmolality (Figure 5 – 3) values were all significantly ($p < 0.0001$) elevated for all seawater acclimated fish when compared with

their corresponding freshwater controls. BWC values (Figure 5 – 4) revealed variable weight loss across all control and saline acclimated groups; however, NSW acclimated BWC values were significantly higher ($p < 0.0001$) when compared with values obtained from control fish. MWC (Figure 5 – 5) percentages corroborated the BWC data, showing significantly less water ($p < 0.0001$) in the muscle samples of NSW acclimated fish compared with their freshwater counterparts. The hypertonic NSW exposure significantly ($p \leq 0.0007$) elevated plasma potassium (Figure 5 – 6) concentrations in all but the 48 h acclimation treatment ($p \leq 0.1000$). Conversely, hematocrit percentages (Figure 5 – 7) were variable and only the 96 h acclimation treatment differed significantly ($p = 0.0337$) from the 96 h control. Table 5 – 1 summarizes all hematological values calculated from this experiment.

MWC, BWC, and hematological parameters were compared among NSW acclimation rates of 24, 48, 72, and 96 h. Plasma potassium concentration, 11.5 ± 0.9 mmol/L (Table 5 – 1), was highest following 24 h NSW acclimation and was significantly different ($p < 0.0001$) from all other acclimation times (Figure 5 – 6). Mean osmolality ($p = 0.0106$, $p = 0.0414$) and chloride ($p = 0.0446$, $p = 0.0038$) values from the 24 and 72 h acclimation periods were significantly elevated when compared with values from those acclimated to NSW over 96 h (Figures 5 – 2 and 5 – 3). MWC percentages exhibited a similar trend, with the 96 h acclimation muscle samples containing a significantly ($p = 0.0081$, $p = 0.0083$) larger proportion (1.9%) of water than both the 24 and 72 h treatments (Figure 5 – 5). Plasma sodium concentrations from 72 and 96 h acclimation treatments were appreciably lower ($p \leq 0.0438$) than those recorded in 24 and 48 h acclimation periods. Analysis of hematocrit and BWC demonstrated no significant differences among any of the NSW acclimation periods.

With the exception of total hardness, no biologically significant differences among treatments were detected in any of the measured water quality parameters throughout the course of the experiment. During the 96 h freshwater acclimation period the following ranges and means were recorded, respectively: DO, 7.23 – 7.98, 7.63 ± 0.29 mg/L; pH, 8.03 – 8.41, 8.29 ± 0.11; TAN, 0.00 – 0.02, 0.01 ± 0.01 mg/L; nitrite, 0.0011 – 0.0036, 0.0025 ± 0.0010 mg/L; alkalinity, 180 mg/L $CaCO_3$; hardness, 188.10 – 205.20, 199.50 ± 8.83 mg/L $CaCO_3$. During the 96 h experimental period the following ranges and means were recorded, respectively: DO, 7.56 – 8.35, 7.86 ± 0.19 mg/L; pH, 8.12 – 8.46, 8.31 ± 0.07; TAN, 0.00 – 0.07, 0.03 ± 0.02 mg/L; nitrite, 0.0014 – 0.0200, 0.0040 ± 0.0022 mg/L; alkalinity, 160.00 – 180.00, 170.00 ± 10.1 mg/L $CaCO_3$. Due to experimental addition of seawater, total hardness varied between control and treatment groups. Hardness ranges and final means by salinity were: 0 g/L, 205.20 mg/L $CaCO_3$; 32 g/L, 205.20 – 5840.00, 5717.50 ± 116.02 mg/L $CaCO_3$.

Discussion

Classic salinity acclimation experiments using *F. heteroclitus* (Jacob and Taylor, 1983; Marshall et al. 1999; Mancera and McCormick, 2000) have focused on physiological effects resulting from abrupt transfer to NSW with analyses usually occurring over a predetermined "time course". Inability of *F. seminolis* to survive acute transfer to full strength NSW precludes replication of these studies. If effects of full strength NSW are to be elucidated, investigations into salinity acclimation of *F. seminolis* are thus restricted to physiological evaluations of abrupt salinity transfer into a maximum tolerated salinity or analysis of a gradual acclimation. Four gradual acclimation periods were investigated because full strength NSW is the terminal salinity of interest for marine baitfish.

F. seminolis was able to tolerate NSW acclimation times of 24, 48, 72, and 96 h with varying physiological results. Hematocrit values in the 72 and 96 h acclimation groups were

significantly lower than their corresponding controls. Although every effort was made to treat fish equally, unknown stressors may account for the elevated control hematocrit values. Osmotic efflux of water would contribute to hemoconcentration and increased hematocrit values in more rapid acclimation periods. NSW acclimated fish lost significantly more weight than their controls throughout the course of seawater acclimation although no significant differences in BWC was seen among acclimation times. Similarly, the greatest decline in MWC was exhibited by NSW acclimated *F. seminolis*, with a mean loss of 5.6 ± 1.0% for all acclimation times when compared with freshwater controls, again suggesting increased gill permeability and water efflux in response to the hyperosmotic environment. Investigation of *O. latipes* by Sakamoto et al. (2001) revealed an 8% decrease in MWC apparent 2 h after transfer to NSW and requiring seven days to return to freshwater values. Comparable MWC values in response to salinity acclimation have also been reported in *F. heteroclitus* (Marshall et al., 2005) and *A. oxyrinchus* (Altinok et al. 1998). Katoh and Kaneko (2003) reported a decrease in plasma sodium 12 h after freshwater transfer of saltwater acclimated *F. heteroclitus.* Conversely, freshwater *F. seminolis* acclimated to NSW showed significantly higher plasma sodium concentrations than their controls, regardless of acclimation time. Plasma sodium concentrations were highest among 24 h NSW acclimated fish and gradually diminished with increased acclimation time suggesting superior ion extrusion capabilities in the slower acclimation rates (Figure 5 – 1). In a time course experiment involving *F. heteroclitus*, Marshall et al. (1999) reported a similar peak sodium concentration of 250 mmol/L 24 h after abrupt transfer, closely paralleling sodium concentrations of *F. seminolis* in the 24 and 48 h NSW acclimations (Table 5 – 1). Plasma potassium (Figure 5 – 6) peaked sharply in the 24 h NSW acclimation group while plasma chloride (Figure 5 – 2) exhibited its highest concentration in the 72 h acclimation but was not

significantly different from 24 and 48 h NSW chloride values. Ion fluctuations such as these reflect the ongoing physiological restructuring of *F. seminolis* as it attempts to decrease gill permeability and actively excrete ions against a hyperosmotic gradient. Plasma osmolality is potentially the most useful of all quantified hematological parameters in osmoregulatory studies, delineating ionic and osmotic fluxes in a single value. Abrupt environmental salinity increases in *F. kansae* (Stanley and Fleming, 1976) elicited a peak plasma osmolality occurring 20 h post transfer, similar to the 370 mmol/kg peak osmolality recorded 24 h after the abrupt salinity transfer of *F. heteroclitus* (Marshall et al., 1999). Additionally, Pickford et al. (1969) reported an approximate decrease in osmolality of 8% in freshwater *F. heteroclitus* versus their saline counterparts. Mean osmolality values for NSW acclimated *F. seminolis* ranged from 490 ± 40 – 505 ± 23 mmol/kg for 24, 48, and 72 h acclimation periods and displayed a marked decrease, 447 ± 27 mmol/kg, in fish acclimated to seawater over 96 hours.

Few studies have examined NSW acclimation of fish that normally are found in freshwater stenohaline conditions. Experimental results clearly demarcate the physiological ramifications of seawater acclimation on *F. seminolis* and similar effects on other cyprinodontoids have been summarized by Nordlie (1987, 2000). Cumulatively, BWC, MWC, and hematological data suggest an initiation of hydromineral regulation in the 96 h NSW acclimation treatment. Evans et al. (2005) proposed a potential deficiency in requisite extrusion proteins in mitochondria rich cells or more subtle hormonal, renal, or intestinal deficiencies to explain the osmoregulatory difficulty experienced when freshwater fish are confronted with a hyperosmotic environment. Although no histological or enzymatic evaluations were carried out in this experiment, future studies incorporating these techniques would provide more specific information regarding the physiology of gradual seawater acclimation in *F. seminolis.*

Although significant decreases among NSW acclimated *F. seminolis* were observed in BWC, MWC, plasma osmolality, and plasma ion concentration, none of these measured parameters returned to experimentally normal ranges as defined by their corresponding freshwater controls, regardless of acclimation rate (Table 5 – 1). Furthermore, Marshall et al. (1999) reported elevated plasma osmolality concentrations in *F. heteroclitus* 30 days after salinity transfer. Further salinity experiments with *F. seminolis* utilizing a "time course" approach and extending well beyond 96 h would help to elucidate an osmoregulatory time frame for this species in response to NSW acclimation.

Despite the ability of *F. seminolis* to tolerate NSW acclimation over 24, 48, 72, and 96 h, results indicate that even during the most gradual acclimation (96 h) the fish has not overcome osmotic stressors of the hypertonic environment. It is not yet clear whether further exogenous stressors, such as shipping or high density holding facilities, will exacerbate this transitional physiological status, potentially manifesting in immunocompromise or decreased survival. However, results from preliminary shipping and long term holding experiments show no such indications. Results of this investigation will contribute to the development of salinity acclimation protocols for the species use in commercial aquaculture as well as elucidate physiological responses to NSW acclimation in this freshwater killifish.

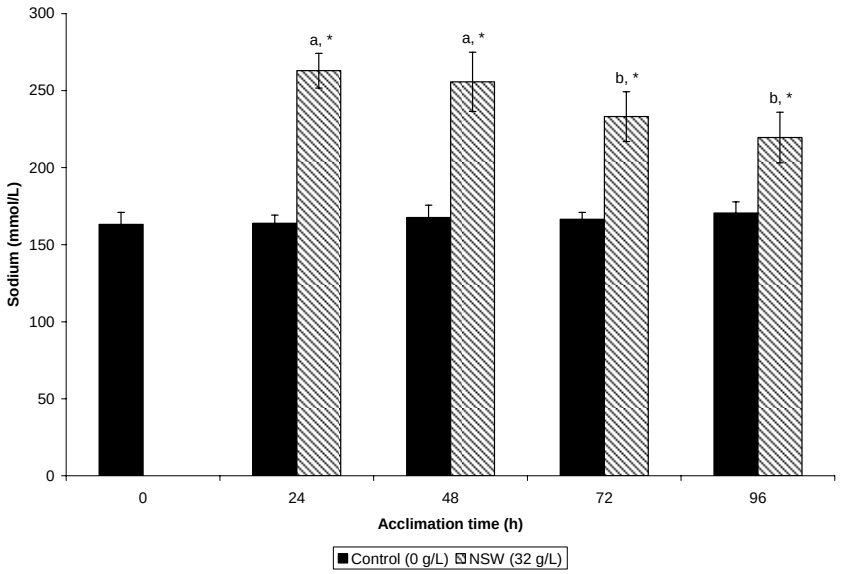

Figure 5 – 1. Mean plasma sodium by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

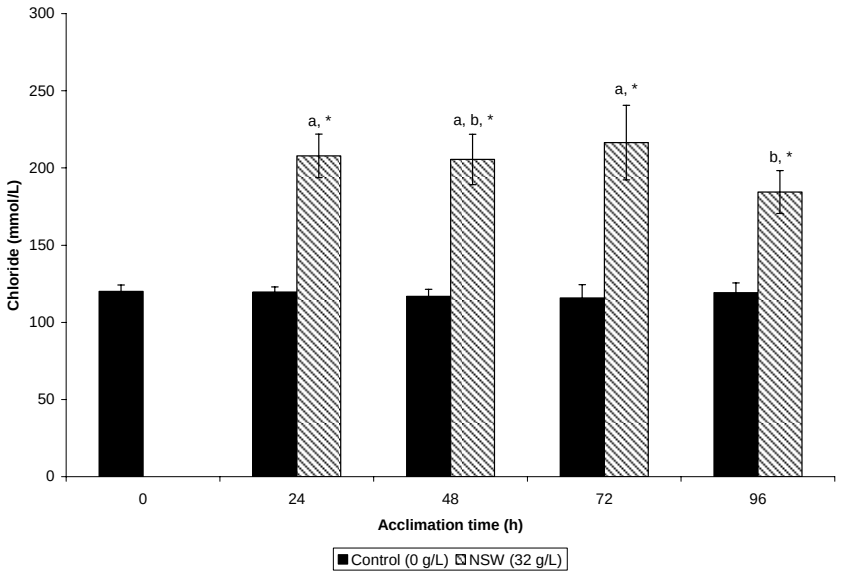

Figure 5 – 2. Mean plasma chloride by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

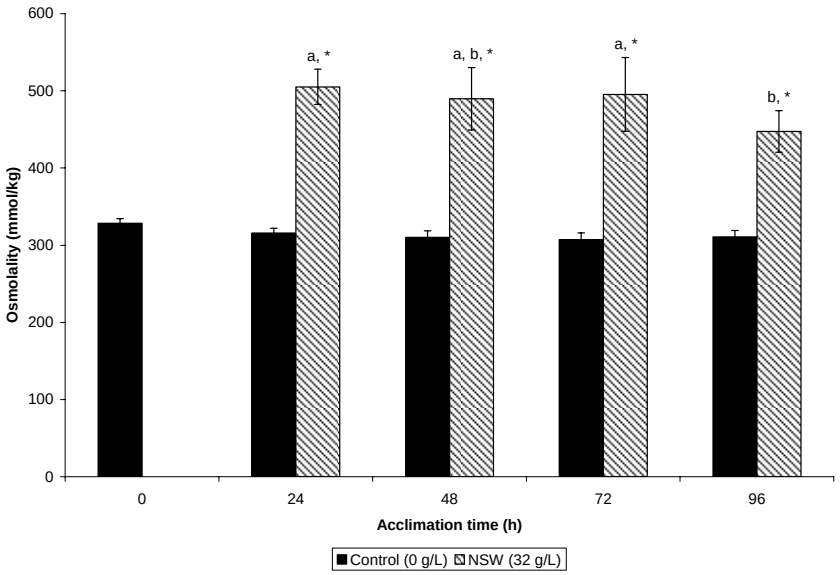

Figure 5 – 3. Mean plasma osmolality by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

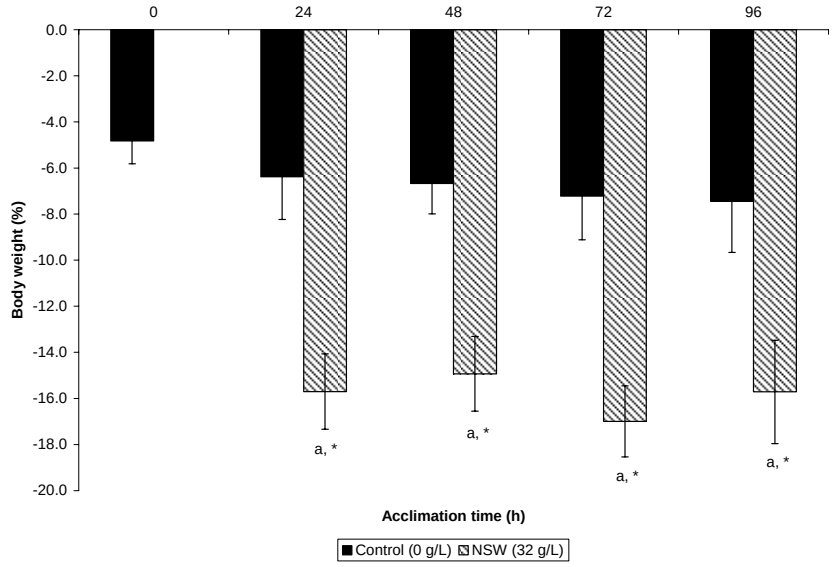

Figure 5 – 4. Mean percent body weight change (BWC) by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD and untransformed for ease of interpretation. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

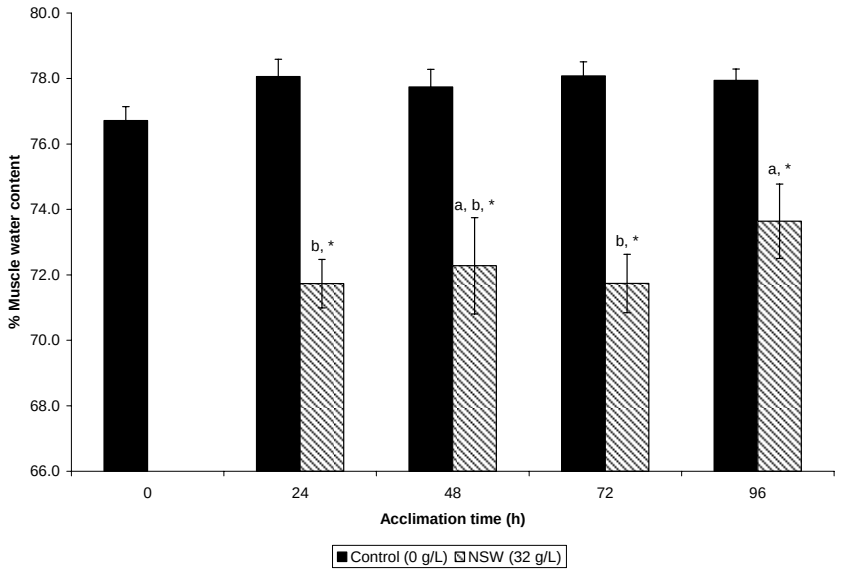

Figure 5 – 5. Mean percent muscle water content (MWC) by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD and untransformed for ease of interpretation. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

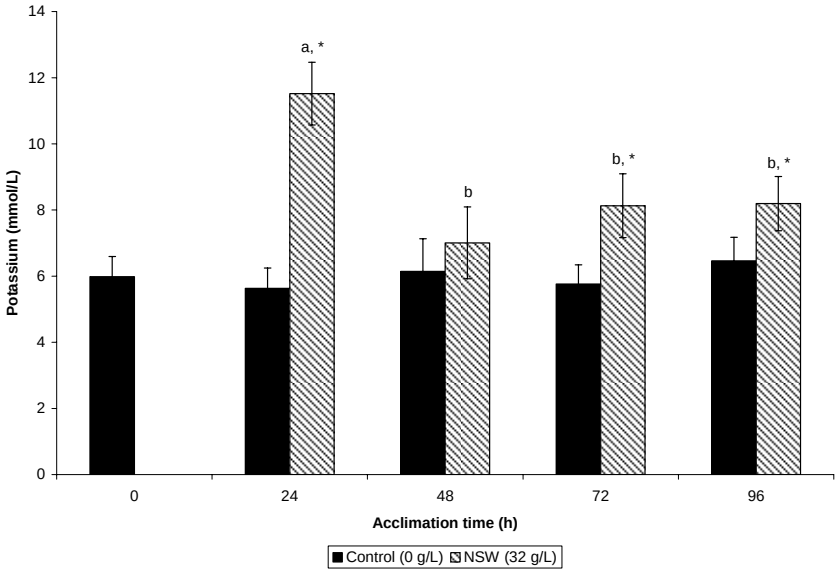

Figure 5 – 6. Mean plasma potassium by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

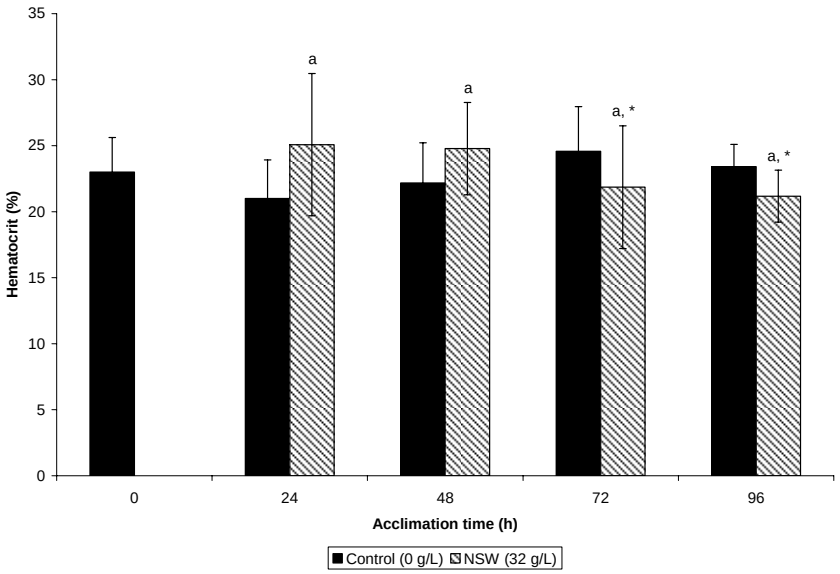

Figure 5 – 7. Mean hematocrit by treatment (control, 0 g/L; NSW, 32 g/L) over experimental acclimation periods. Data are represented as means ± SD. * denotes significant differences (Tukey's HSD, $p \leq 0.05$) between individual NSW treatments and their corresponding controls. Different letters denote significant differences (Tukey's HSD, $p \leq 0.05$) among all NSW treatments. Time 0 (precontrol) sample was taken prior to initiation of the acclimation experiment.

Table 5 – 1. Mean (± SD) hematological parameters of *F. seminolis* acclimated to control (0 g/L) or NSW (32 g/L) over experimental time periods of 24, 48, 72, and 96 h.

Treatment	Hematocrit (%)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Osmolality (mmol/kg)
Time 0 / 0 g/L	23 ± 3	163.2 ± 7.7	6.0 ± 0.6	120.0 ± 4.2	328 ± 6
24 h / 0 g/L	21 ± 3	163.9 ± 5.4	5.6 ± 0.6	119.6 ± 3.3	316 ± 6
48 h / 0 g/L	22 ± 3	167.7 ± 8.0	6.1 ± 1.0	116.8 ± 4.5	310 ± 8
72 h / 0 g/L	25 ± 3	166.4 ± 4.5	5.8 ± 0.6	115.9 ± 8.6	307 ± 9
96 h / 0 g/L	23 ± 2	170.6 ± 7.3	6.5 ± 0.7	119.2 ± 6.4	311 ± 8
24 h / 32 g/L	25 ± 5	263.0 ± 11.3	11.5 ± 0.9	207.9 ± 14.1	505 ± 23
48 h / 32 g/L	25 ± 3	255.8 ± 19.2	7.0 ± 1.1	205.6 ± 16.3	490 ± 40
72 h / 32 g/L	22 ± 5	233.2 ± 16.2	8.1 ± 1.0	216.5 ± 24.1	495 ± 48
96 h / 32 g/L	21 ± 2	219.6 ± 16.4	8.2 ± 0.8	184.4 ± 13.9	447 ± 27

CHAPTER 6
CONCLUSION

Development of marine baitfish aquaculture in Florida is predicated upon a strong consumer demand and identification of technologically and economically viable candidate species. With 2.7 million licensed anglers, identification of a consistent consumer base within Florida seems intuitive. Preliminary evaluations of prospective culture species have identified *F. seminolis* as a marine baitfish candidate with economic potential. Experiments carried out in this study evaluated potential barriers, both environmental and pathogenic, that may impede the culture and marketing of this unique baitfish species.

Parasitological survey results of wild *F. seminolis* brood fish are integral in the establishment of effective quarantine and subsequent biosecurity procedures unique to aquaculture production facilities. Additionally, prevention of pathogen introductions both into and out of the culture environment must be ensured through implementation of responsible aquaculture practices. Thirteen distinct taxa were identified as parasites of *F. seminolis*. Eight parasitic taxa never before recorded on *F. seminolis* were elucidated. This survey represents the first comprehensive examination of the parasitic fauna of *F. seminolis*. These findings will dictate treatment therapy options instrumental in the captive husbandry and culture of this emerging marine baitfish.

Analyses of select hematological indices are of great diagnostic value to clinicians as well as researchers. Recent technological advancements have resulted in more efficient, portable, and operator friendly instrumentation. The i-STAT® is a point-of-care (POC) blood analyzer whose use is becoming increasingly prevalent for hematological analysis of fishes. Validation of new technologies against conventionally accepted instrumentation (CAI) is crucial to prevent dissemination of erroneous data. Results from validation experiments in *F. seminolis* were highly

variable and the accuracy of the unit was questionable when compared with CAI. Calculated bias was inconsistent thus precluding use of a "correction factor". Experiments using larger sample sizes and numerous species are needed to ascertain the reliability of this POC unit. Validation results were inaccurate and excluded the use of this analyzer in consequent experimental analyses.

Determination of acute salinity tolerance and the physiological manifestations of natural seawater (NSW) acclimation were additionally investigated. Acclimation and survival of *F. seminolis* in full strength NSW is essential for feasibility as a marine baitfish candidate. Two salt sources, NaCl and NSW, were used to assess acute salinity tolerance. *F. seminolis* was able to tolerate abrupt transfer into 16 g/L NaCl and 24 g/L NSW with 100% survival in both salinities. Even though no mortalities were observed in 16 g/L NaCl, poor physical appearance and atypical behavior suggested NSW to be far superior as an acclimation salinity source. Gradual NSW acclimation experiments contradicted previously published salinity thresholds for this species. *F. seminolis* exhibited 100% survival when acclimated to a salinity of 32 g/L over 24, 48, 72, and 96 h. Physiological analyses of NSW acclimation rates yielded elevated plasma ion and osmolality concentrations accompanied by decreases in body weight and muscle water content. Although all of the NSW acclimated physiological endpoints measured remained significantly different from control values, a general trend signaling the initiation of osmoregulatory compensation was noticed in 96 h values. Taken together, these results validate *F. seminolis* as a marine baitfish candidate and provide valuable data regarding the species salinity tolerance and underlying physiological processes. Additionally, relatively few studies have examined the physiological adaptation of a freshwater stenohaline fish to a marine environment. *F. heteroclitus*, a euryhaline analogue of *F. seminolis,* has been the focus of extensive studies

elucidating various physiological mechanisms of freshwater and saline acclimation in fishes. Future osmoregulatory studies involving *F. seminolis*, a true freshwater killifish, may elucidate physiological mechanisms not employed by euryhaline members of the genus. Furthermore, results from salinity experiments will have immediate application for Florida baitfish producers and help to develop effective and efficient acclimation protocols.

The culmination of this study provides a valuable assessment of an emerging *Fundulus* bait species with potential application in a marine environment. Diversification of Florida's aquaculture industry is vital to its continued longevity. Marine baitfish production is a logical and potentially lucrative endeavor for Florida aquaculturists. Through continued research into candidate species and novel production methods, marine baitfish culture could soon establish itself as a viable aquaculture crop for the state and the region.

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BIOGRAPHICAL SKETCH

Matthew A. DiMaggio was born in Brooklyn, NY, moving to Staten Island, NY, shortly after. Summers were spent on Block Island, RI, where Matthew developed a passion for the ocean. He attended the State University of New York at Geneseo, where he graduated in 2003 with a B.S. in biology and a minor in environmental sciences. The next three years were spent working in the urology research lab at the University of Rochester / Strong Memorial Hospital, investigating various urological cancers and concomitantly developing fundamental research skills necessary for his further educational aspirations. Matthew moved to Florida in 2006 where he was accepted to a master's program in the Department of Fisheries and Aquatic Sciences at the University of Florida. Matthew will complete his Master of Science in August 2008 and continue on with his graduate studies in pursuit of his Ph.D.