Evaluation of aquaculture techniques to improve growth and health of Ohio sport fish, sunshine bass (*Morone chrysops x M. saxatilis*) and walleye *Sander vitreus*

THESIS

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Abstract

Reducing operational costs and avoiding transmission of diseases continue to be critical areas of study in aquaculture. Two experiments were designed to address these issues. The first experiment examined a major cost associated with sunshine bass (Morone chrysops x M. saxatilis) production, feeding. The effects of four diets on the survival, growth, and body composition of sunshine bass were examined in a feeding experiment, followed by a fasting period. The diets consisted of a high nutrient commercial diet, a practical diet (wheat gluten based), a semi-purified diet (standard nutrient requirements), and a frozen natural diet. Fish were fed by hand three times a day at a rate of 4% of their body weight for 51 days. Survival was significantly lower for fish fed the semi-purified diet. Maximum growth was found in fish fed the commercial diet. To our knowledge, no previous studies with sunshine bass conducted a fasting experiment (14 days) aimed at replicating the conditions of transport and acclimation of farmed fish to new environments. Body composition was significantly affected by diet, and protein depleted faster than lipid during the fasting period. Analysis suggests that sunshine bass utilized protein and glycogen prior to lipid during fasting. At the end of fasting, samples of juveniles were collected for histological analysis of the liver and posterior intestine. The liver contained hepatocytes with larger deposits of lipids and nucleus pushed to the cell wall in fish fed the AN diet than the fish fed the FC diet. The posterior intestine revealed significant differences in the height of folds and number of goblet cells.
The second experiment was designed to eliminate the transmission of pathogens, specifically viral hemorrhagic septicemia (VHS), in hatchery operations. Avoiding viral disease transmission is vital for sport fish hatchery-based stocking programs in regions affected by VHS. Iodine compounds are nonselective antiviral substances that have been used widely as disinfectants, but their effects on embryo survival and early ontogeny of fish are less known. Two groups of walleye (*Sander vitreus*) were collected from the Maumee River, Perrysburg, Ohio in March 2009. Gametes were collected on site, and transported to the aquaculture laboratory in the School of Environment and Natural Resources, The Ohio State University. Eggs from each individual female were fertilized with the combined sperm of 3-4 males. Following fertilization, a tannic acid treatment for egg de-adhesion was used. Then embryos were exposed to iodine at 0 (control) or 100 mgL\(^{-1}\) (in triplicate) for 30 min., and incubated in California trays at 10-12 °C. Survival was estimated at the eyed-stage. Larvae were sampled prior to first feeding at 9 days post hatch (DPH) and approximately every 10 days until 48 DPH to assess growth. The results suggest that embryo survival following iodine treatment appears to be affected by the condition of the parents, but the growth of embryos was unaffected by the treatment. Histological analysis of larval swim bladder morphology revealed that non-inflated swim bladders had features of an underdeveloped organ with hyperplastic epithelium and vacuolarized cells.
Dedication

This document is dedicated to friends, family, and mentors who contributed encouragement, insight, and guidance throughout my life.
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Chapter 1: Aquaculture overview and need for research related to reducing aquaculture costs and avoiding disease transmission

1.1 Aquaculture Overview

Although the oceans have long been considered to be inexhaustible sources of fish (Goldburg and Naylor 2005), current fisheries statistics suggest that global annual fish catches have capped at approximately 90 million metric tons per year (FAO 2002). Many of the world’s fisheries are overfished or heading in that direction (Hilborn et al. 2003). These same trends are seen in freshwater rivers and lakes across the world. Commercial fishing has primarily exploited large, predaceous fish because of their demand as food, but as demand increases for small fish for use in aquaculture feeds as nutrient sources in fishmeal and fish oil, exploitation of these fisheries is likely to increase as well. According to Myers and Worm (2003), commercial fishing has decimated 90% of large marine fish including swordfish, cod, marlin, and sharks. The current trends in fish production increasingly put pressure on marine and freshwater ecosystems.

Aquaculture is dominated by the Asia-Pacific region which accounts for 89 percent of production and 77 percent of commercial value (FAO 2009). Globally, fisheries and aquaculture play a major role, directly or indirectly, in the livelihoods of millions of people. In 2006, an estimated 43.5 million people were directly connected to the fish production industry as full or part time employees (FAO 2009). Production, in terms of species, quantity, and end products, is diverse in each region of the world.
Aquaculture has increasingly become an important source of food, management tool (controlling shad populations in U.S. reservoirs with pelagic predators like hybrid striped bass), and source of sport fishing opportunities (production of saugeye or muskellunge for anglers). Aquaculture and capture fisheries supplied the world with 106 and 110 million tons of food fish in 2004 and 2006, and aquaculture accounted for 43 and 47 percent, respectively (FAO 2007; FAO 2009). While the supply from capture fisheries has become stagnant, primarily due to exploitation of the fisheries, aquaculture production continues to grow. This growth has made it the fastest growing animal food processing sector in the world, and production has even outpaced population growth (FAO 2007). This increase in production has allowed the per capita food fish supply to continue to grow while the supply from capture fisheries has remained relatively constant. However, this trend is beginning to slow.

Exploited and declining fisheries have created a new impetus to expand production through aquaculture (Goldburg and Naylor 2005; Naylor et al. 2009). Aquaculture development is occasionally promoted as a way to alleviate pressure on wild fisheries, and often cited as a method to increase the supply of fish when wild catches have peaked and cannot meet demand. Aquaculture production can also serve as a management tool and provide sport fishing opportunities. However, aquaculture is no different than farming activities on land because production is largely dependent upon the supply of nutrients (Tacon and Metian 2008), and these inputs have their own set of problems such as excess nitrogen pollution, costs, and availability. These nutrients are generally supplied through the consumption of natural food organisms or prepared diets,
and represent the greatest operating cost of most aquaculture operations. The most important inputs are fishmeal and fish oil.

Aquaculture is dependent on these inputs as the dominant sources of protein and lipid in formulated feeds. However, the price of fishmeal and fish oil has progressively increased and the continued growth in aquaculture production threatens to outstrip the current supply jeopardizing the industry’s economic sustainability (Goldburg and Naylor 2005; Naylor et al. 2000; Naylor et al 2009; Delgado et al. 2003). Since feed accounts for a large portion of variable costs in aquaculture operations (45-55%), it has increasingly replaced expensive fish meal and fish oil with cheaper poultry byproducts or plant-based ingredients as the dominant protein and lipid source (D’Abramo et al. 2008). The Food and Agricultural Organization of the United Nations (2010) reported that fishmeal cost approximately $1,700 per ton while soymeal only cost approximately $400 per ton. The replacement of fishmeal and fish oil as the dominate protein and energy sources have been met with varied success in the growth, survival, and desired fatty acid profiles (Nematipour et al 1992; Pine et al. 2008; Brown et al. 2008; Trushenski and Boesenberg 2009). The general reasons for the price increase in fishmeal and fish oil can be attributed to a combination of many factors that include static global supplies, strong market demand, and increasing costs to capture and process fish (Tacon and Metian 2008; FAO 2008).

As wild fish stocks continue to be depleted, a greater emphasis will be placed on aquaculture to meet the demand for food fish and sport fishing opportunities. However, this will not eliminate the need for wild fish. But all efforts must be made to conserve
this resource. Viable future fisheries will require integrating management for fisheries, aquaculture, and conservation. Even if aquaculture begins to displace wild stocks fisheries as the major component of production, likely a gradual process, the wild populations will continue be an important facet of the industry. Improvements in management strategies of these common resources are critical for the sustainable development. Thus, as aquaculture grows, a more ecosystem-based approach will be essential for balancing competing demands, although we are only beginning to understand the important factors of such an approach (Pikitch et al 2004). In addition to better management practices, economic and political factors will play a major role in shaping these resources. Fishermen lack the financial incentive to conserve or leave fish in the water for the future (NRC 1999). Thus, incentives, education programs, conservation practices, and restoration efforts will need to be created to produce a more sustainable industry.

1.2 Aquaculture and fish propagation in the Midwest United States: Background and justification

The focus of this thesis is on the production of sunshine bass and walleye for the purpose of sport fishing and management of inland water bodies in Ohio. These projects were designed to address problems in hatching techniques with a view to improve current rearing methods of important Ohio sport fish. The focal points are related to the growth, survival, and disease control of sunshine bass and walleye in the state fish hatcheries. Our efforts are to help protect Ohio’s inland valuable fisheries resources.
1.2.1. Viability and body composition of sunshine bass following a feeding and fasting experiment

Hybrid striped bass (HSB) is one of the most rapidly expanding fish species in recreational fisheries and production sectors, and the information on dietary requirements is inadequate. Many state and federal agencies have identified striped bass as well as HSB as a priority species for fish culture research, yet much needs to be accomplished in the formulation of optimum cost-effective diets for all life stages (Hughes 1992). Currently, hatchery operations use commercial trout and salmon feeds to grow this species (Harrell et al. 1990). Both researchers and producers have produced substantial information (Nematipour et al. 1992; Brown et al. 1993; Lewis and Kohler 2008; Brown et al. 2008; Trushenski and Boesenberg 2009).

HSB became a desirable species in aquaculture because it shows superior growth due to lower metabolic costs, higher survival, disease resistance, and tolerance of a wide range of temperatures and salinities (Tuncer et al. 1990; Harel and Place 2003). There are two main crosses, the palmetto bass (Morone saxatilis ♀ x M. chrysops ♂) and the sunshine bass (M. chrysops ♀ x M. saxatilis ♂). Sunshine bass is the focus of this study. Sunshine bass is becoming the dominant hybrid due to the difficulty in obtaining striped bass females from the wild and the relative ease with which white bass females can be acquired and handled, i.e., much smaller size of mature fish. The increasing importance of sunshine bass as a food and sport fish continues to generate interest in experimental,
semi-purified diet formulation as well as in formulation of practical, inexpensive diets for
grow out of HSB.

Sunshine bass uses endogenous nutrients from their yolk sac for approximately 4-
5 days after hatching as they undergo metamorphosis and the digestive tract develops.
They are carnivorous throughout the rest of their lives (Gatlin 1997). A variety of
zooplankton species provide the major food items at the beginning of exogenous feeding.
Thus, production of sunshine bass in aquaculture facilities generally requires the use of
live feed before they can be transitioned to a formulated dry feed (Ludwig et al. 2010).
Transitioning to a dry feed as quickly as possible is desirable for production because of
the labor involved with maintaining and producing live feed (Ludwig and Lochmann
2007).

The intent was to assess whether sunshine bass with an initial weight of 0.5-1.0 g
raised in farm ponds fed with zooplankton could be transitioned to formulated feeds in
captivity, and to evaluate the growth, survival, and body composition of the fish after a
51 d feeding experiment and a 14 d fasting experiment. Although feeding experiments
have been conducted with sunshine bass for optimal energy, protein, and lipid levels in
diets (Nematipour et al. 1992; Brown et al. 2008; Trushenski and Boesenberg 2009), to
our knowledge no experiments have simulated fasting conditions of farmed fish during
transportation and acclimation to new environments. This study provided an initial
evaluation of energy use, survival, and body composition of sunshine bass during a 14
day fasting experiment.
1.2.2. Viability of early-life walleye and hybrid striped bass after iodine treatments

Maintaining fish health and avoiding viral disease transmission is paramount in culture operations and fishery management. It is critical to determine exactly which prophylactic treatments are effective and which antiviral treatments are most appropriate at fertilization (i.e., prior to transferring fish to hatchery ponds or inland reservoirs). In addition, broodstock maintained in hatchery ponds may require treatment in every spawning season. If needed, broodstock also can be isolated and treated with antiviral substances.

Preventive measures for rhabdovirus infections must include complex treatment strategies, as many fish species are in contact with potential target species (Costanzi 2005). Rowley et al. (2001) indicated that rhabdoviruses isolated from healthy cultured rainbow trout were severely pathogenic to cyprinid fish. However, povidone-iodine (PVI) has been reported to inactivate HIV and avian influenza viruses in vitro (Sabracos et al. 2007) and parental application may be equally effective.

Direct toxicity results, unfortunately, may be confounded by accompanying infections. Investigations of post-treatment mortality using iodine compounds are unclear due to anti-fungal (Khodabandeh and Abtahi, 2006), anti-bacterial (Cipriano et al. 2001), and antiviral (Bullock et al. 1976; Drennan et al. 2006) activity of these compounds. In common carp embryos, iodine treatments at concentrations of 50-200 mgL⁻¹ (ppm) did not prevent *Saprolegnia* (fungus) infection at water temperatures of 19-22 ºC and a hardness of 25 mg CaCO₃/L. However, hatching rate increased from 8% (control) to 32.5% after flash iodine treatment twice a day at 50 mgL⁻¹.
Several experiments with rainbow trout eggs exposed to iodine have been conducted by Alderman (1984). Unfertilized eggs were mixed with sperm and then exposed to iodine 10 min after “water hardening”. Mortality of larvae among progenies from individual females varied 0-85% when exposed to a range of iodine concentration from 100 to 800 mgL\(^{-1}\). In addition, variation in sensitivity to iodine did not decrease among egg batches from individual females when exposed at 800 mgL\(^{-1}\) for 30 min after “water hardening”. Eyed embryos of rainbow trout tolerated a dose in excess of 3,000 mgL\(^{-1}\) at pH 7 (LD 25). In general, survival of embryos following an immediate fertilization did not decline in response to iodine treatment. The “aging of eggs,” storage of unfertilized eggs prior to fertilization for several days, increased iodine sensitivity.

Treating eggs with iodine should prevent vertical transmission of rhabdoviruses from adult walleye, white bass, and striped bass to their progeny. Based on a review of the literature on iodine toxicity and antiviral doses for Atlantic salmon, white sturgeon, and walleye embryos, we used relatively moderate duration and concentrations of iodine treatments. We quantified how iodine affects the earliest stages of embryonic development of walleye and sunshine bass, as well as late stage (prior to hatching) survival. We also measured female size, fecundity, and advancement of ovulation as well as storage conditions of unfertilized eggs prior to iodine exposure as a means to better understand effects of variation among maternal broodstock.

Our hypothesis is that post-adhesion treatment of eggs will be an effective procedure for preventing vertical transmission of rhabdoviruses from adult fish to its progeny. Fertilization rate and survival of larvae will be monitored to determine the
effect of iodine. We also hypothesize that egg quality is related to fish age, fecundity, phospholipid composition of egg yolk reserves, and also may relate to variation in resistance to iodine toxicity. Because recent work with walleye parental attributes (related to lipid and fatty acid composition) revealed a relationship to embryo survival (Dabrowski et al. unpublished report), determination of how these attributes may be related to viral transmission and effectiveness of iodine treatments in walleye and HSB is necessary.

The goal of this study is to establish procedures for preventing introduction of viral hemorrhagic septicemia virus (VHS) and other pathogens into fish hatcheries. We will quantify how iodine concentration and duration influences viability, as indicated by survival and growth, of walleye *Stizostedion vitreum* and sunshine bass (*Morone chrysops x M. saxatilis chrysops*), embryos, larvae, and juveniles compared to a control group. Given that treatment of adult fish used as broodstock also may become important, exploring the use of iodine intra-peritoneal administration to broodstock white bass and walleye as a means to prevent virus transfer via eggs or sperm during fertilization needs investigation.

Due to difficulties in producing sunshine bass in the spring of 2009 and 2010, we were only able to conduct the experiment with walleye. Fertilization techniques for producing sunshine bass continue to be reviewed to increase survival by the lab in The School of Environment and Natural Resources, The Ohio State University, Columbus, Ohio (hereafter the lab) and the Ohio Department of Natural Resources, Division of Wildlife. Significant strides have been made over the last two years.
1.3 References


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Trushenski, J. T. and J. Boesenberg. 2009. Influence of dietary fish oil concentration and finishing duration on beneficial fatty acid profile restoration in sunshine bass


Trushenski, J. T. and J. Boesenberg. Influence of dietary fish oil concentration and finishing duration on beneficial fatty acid profile restoration in sunshine bass


Chapter 2: Growth, survival, and body composition of sunshine bass following a feeding and fasting experiment

2.1 Abstract

There is continuous interest in experimental, semi-purified diet formulation as well as in formulation of practical, inexpensive diets for grow out to market size of sunshine bass hybrids (*Morone chrysops* x *M. saxatilis*). The effects of four diets on the growth, survival, and body composition of sunshine bass were examined, followed by a fasting period. The diets consisted of a high nutrient commercial diet (AN), a practical diet (wheat gluten based), a semi-purified diet (meeting standard nutrient requirements), and a frozen natural diet (FC). Fish were fed by hand three times a day at a rate of 4% of their body weight for 51 days. To our knowledge, no previous studies with sunshine bass followed a feeding experiment with a fasting experiment (14 days) aimed at replicating the conditions of transport and acclimation of farmed fish to new environments. Survival was significantly lower for fish fed the semi-purified diet than the other three diets. The greatest growth was achieved by fish fed the commercial diet. Body composition was significantly affected by diet, and protein depleted faster than lipid during the fasting period. Analysis suggests that sunshine bass utilized protein and glycogen prior to lipid during this short fasting period. At the end of the feeding period, samples of juveniles were collected for histological analysis of the liver and posterior intestine. Sections of the liver contained hepatocytes with larger deposits of lipids and nucleus pushed to the cell wall in fish fed the AN diet compared to fish fed the FC diet. Sections of the
posterior intestine revealed significant differences in the height of folds and number of goblet cells.

2.2 Introduction

Although the first artificial culture of *Morone* was initiated in the early 1880s, the expansion of hybrid striped bass (HSB) production as a management tool, recreational sport fish, and a commercially important species is relatively recent compared to other traditional fish species (Harrell and Webster 1997). Thus, exploration of dietary requirements for HSB is still in its infancy, and data on nutritional requirements compared to other species are inadequate. Most early diets for HSB were modifications derived from salmonid and catfish diets (Brown et al. 1993).

The two most common crosses of *Morone* are palmetto and sunshine bass. The sunshine bass is a cross between a female white bass and a male striped bass (*Morone chrysops x M. saxatilis*). This makes diet formulation particularly complex for sunshine bass because these fish are crosses between freshwater and marine species that have different essential fatty acid requirements due to a varying ability to elongate and desaturate shorter-chain/18-carbon essential fatty acids (Watanabe 1982). Studies continue to be conducted to determine appropriate dietary requirements specifically for sunshine bass (Griffin et al. 1994; Brown et al 1992; Gaylord et al. 2004; Rawles et al. 2008).

Production of HSB as a food fish in 1987 was 180 tons, but by 1999, production was estimated at 4,600 tons (Webster 2002). These estimates do not include HSB
produced in state and federal hatcheries for stocking in public lakes and rivers as game fish. Artificial hybridization programs from state and federal hatcheries have been recognized as a potential tool for improving fish stocks and management practices (Kerby 1986). Although, HSB are generally stocked in freshwater, their ability to tolerate a wide range of salinities from 0 to 25 g/L allows them to survive in a variety of habitats (Harel and Place 2003). Their superior growth, survival, and disease resistance in the first two years of life has also made them appealing in aquaculture (Kerby 1986). Tuncer et al. (1990) reported that the heterotic effect expressed through faster growth in HSB was not due to a better assimilation efficiency or appetite, but instead, HSB juveniles have a lower metabolism that allow them to grow faster. However, modern techniques of intensive rearing conditions often lead to stressed, immunologically compromised animals (Trushenski and Kohler 2008).

Protein tends to be the most important nutrient in fish feeds because fish generally have a higher protein requirement than land animals (Keembiyehetty and Gatlin 1992). Protein is also the most expensive component of diet formulation. However, increasing dietary lipid levels can spare a considerable portion of dietary protein from energy use while improving feed efficiency and reducing waste production (Ruohonen et al. 1998; Skalli et al. 2004). This is in agreement with the recommendation by Bureau et al. (2008) that salmonid and perhaps other fish diets can be improved by increasing the proportion of lipids. Diet formulation is important because feed is a major cost in aquaculture. Reducing costs of feed can make aquaculture more profitable by substituting expensive ingredients (fishmeal and fish oil) for comparable more cost effective ingredients (plant
based protein and oil sources). However, fishmeal is still the most desired source of protein because it contains a relatively high protein content, favorable amino acid profile, high nutrient digestibility, and high palatability (Naylor et al. 2009), but due to the increasing cost of fishmeal (~$1,700 per ton), alternative cost effective ingredients are being pursued such as soybeanmeal (~$400 per ton) (FAO GLOBEFISH 2010). Due to the recent importance of HSB, studies aimed at quantifying their dietary requirements of proteins, lipids, vitamins, and minerals may result in increased growth, decreased costs, increased survival, and a better end product.

Our study evaluated the growth, body composition, survival, and histological features of the liver and posterior intestine of sunshine bass fed four diets that varied in nutritional content cost during a feeding experiment and a fasting period that simulated transport and acclimation of stocked fish to new environments. We intended to explore the means of reducing feed costs by using plant protein sources while maintaining high production. We also intended to evaluate a new dimension, changes in body composition of sunshine bass during a 14 day fasting period since this has previously not been explored. Our results indicate that, although the commercial feed based on marine protein sources provided the best growth and survival, utilization of plant protein based diets resulted in comparable growth, good health, and high survival. Our data from the fasting experiment suggests that protein and glycogen are the primary energy sources during fasting, and that lipids continue to be synthesized and not used for energy until perhaps extended starvation.
2.3 Methods

Sunshine bass juveniles (45-60 days old) were obtained from Keo Fish Farms (Keo, Arkansas), where they were reared in ten one-acre outdoor ponds. Ponds were fertilized to promote zooplankton growth as a source of feed for the sunshine bass before they were switched to a dry diet of Silver Cup salmon feed (Silver Cup Fish Fed Murray, Utah) containing 42% protein. Juveniles were collected by seining and immediately air shipped in oxygen filled bags to the lab. Before experimentation, two groups of 5-6 juveniles were collected and frozen for analysis of initial body composition. The remaining juveniles were separated into twelve tanks and starved for one day prior to diets being presented to initiate the experiment.

2.3.1. System design

The feeding and fasting experiments were conducted in a semi-closed recirculating-water system composed of twelve 30-L glass aquaria, and a filtration system that used a cartridge filter and a biomass bead filter. Each aquarium was supplied with continuous aeration and dechlorinated tap water at a flow rate of 1 L per min. Water temperatures throughout the experiment ranged between 21-26 °C, while the photoperiod remained constant (12 h light:12 h dark). The aquaria were cleaned and monitored daily for temperature and mortality. Fish were distributed randomly among the 12 aquaria (N=19, except two aquaria N=20) and assigned one of the diets with three aquaria per dietary treatment.
2.3.2. Feeding experiment

The feeding experiment was designed to quantify the effects of four diets on the growth, survival, and body composition of sunshine bass: two experimental [a semi-purified, casein-gelatin based diet (CG), and a practical, fish meal and wheat gluten based diet (PC), formulation details are in Table 2.1], one commercial diet [Aglo Norse Stavanger, Norway (AN)], and one natural diet [frozen chironomids (FC)]. The AN, PC, and CG diets were sieved, and particles between 0.5-0.7 mm were used for feeding. The thawed chironomids were given to fish whole; a daily ration was prepared in wet weight. Fish were fed by hand three times a day at a rate of 4 % of their body weight/day for 51 days.

Feeding rate was re-adjusted every 3 days for each individual aquarium based on estimated weight increases. Approximately every 10 days, fish from all aquaria were weighed and counted, and the feeding rate readjusted to actual biomass. At the end of the feeding experiment, survival, weight gain (WG; \( WG = \frac{\{\text{final weight} - \text{initial weight}\} \times 100}{\text{initial weight}} \)), and average fish weight were calculated. Five or six randomly sampled fish from each aquarium were also anesthetized in an ice slurry, frozen on dry ice, and then stored at -80 °C for subsequent analysis of body composition. Remaining fish were returned to their aquarium for the fasting experiment.

The analysis of crude protein, moisture, and ash of homogenous fish tissue samples and diets were performed in accordance to AOAC International 2003. Diets and fish lipid analyses were performed according to the procedure of Folch et al. (1957). A correction factor was used in the lipid analysis due to initial non-homogenous samples of
the whole body of fish. The correction factor was calculated from reanalyzing three samples that contained enough remaining material to accurately run the analysis in order to extract at least 100 mg of lipids. This factor was then applied to all of the lipid samples for the whole body of fish.

2.3.3. Fasting experiment

The fasting experiment was a continuation of the feeding experiment. These fish were not fed for 14 days to simulate conditions that may occur during transport and post-stocking acclimation in reservoirs. After the fasting period, these fish were weighed, sampled, and stored at -80 °C, and subjected to the same analysis as the samples from the feeding experiment. Metabolic losses during fasting were calculated for protein, lipid, and ash. Mean protein, lipid, and ash content (mean fish weight x dry matter (%) x nutrient (%) / 100) was calculated to determine rate (mg/fish/day) and percent decrease ((mean absolute amount of nutrient after feeding minus the same after fasting) / 100) of protein, lipid, and ash for each diet.

2.3.4. Histological analysis

At the termination of the feeding phase, three fish from each tank and treatment were collected (3 fish x 3 replicates x 4 groups 17 h after final feeding) for histological analysis of the posterior intestine and liver. The liver from each fish was removed and weighed to calculate hepatosomatic index (HSI). HSI was calculated according to the formula: HSI (%) = 100 x LW/TW (LW - weight of the liver (g), TW - total body weight
of the fish (g)). The liver and digestive tract were preserved in formalin for histological examination in accordance to Ostaszewska et al. (2010a). After fixation, fish were transferred to 70% ethanol until further processing. All samples were paraffin-embedded after dehydration with a graded ethanol series and treated with xylene. Histological sections were cut into complete serial sections (5 μm) using a microtome Leica RM 2265 (Leica Microsystems, Nussloch, Germany). The morphology of the cell, the presence of glycogen in hepatocyte cytoplasm, and presence of acid and neutral carbohydrates were examined using a combined alcian blue-periodic acid Schiff (AB/PAS) stain (Ostaszewska et al. 2010b). The measurements of intestinal fold height and number of goblet cells were done in longitudinal intestine sections of 9 fish from each group (15 folds x 3 fish x 3 replicates for all dietary groups). Basophilic granulocytes were identified in the lamina propria of the posterior intestine folds in slides stained according to Crossman (Urán et al. 2008).

Proliferating hepatic cells were identified using antibodies directed against proliferating cell nuclear antigen (PCNA) (Ostaszewska et al. 2008). PCNA-positive hepatocyte nuclei were counted in 10 liver cross-sections of 35,000 μm² in 9 fish from each feeding group, and calculated per 1 mm².

Morphometric measurements (posterior intestine fold height, number of goblet cells per 100 μm of posterior intestine fold area, number of PCNA-positive hepatocytes) were taken using a Nikon ECLIPSE 90i microscope connected to the digital camera Nikon DS5-U1 and equipped with the image analysis system NIS-Elements AR (Nikon Corporation, Tokyo, Japan).
2.3.5. **Statistical analysis**

Results are expressed as means ± SDs for each diet (n=3). Data for mean fish weight, mean weight gain, survival, body composition, nutrient loss, and histological parameters were subjected to analysis of variance using ANOVA, and differences between dietary treatments were determined using Tukey’s test. Significance of difference in survival was determined at the end of the feeding experiment. One of the CG diet tanks was not used in the analysis of survival because of high mortality not attributed to diet. Homogeneity of variance was verified for all data using Hartley’s test (Dagnelie 1975). Statistical significance was accepted when P < 0.05.

2.4 Results

2.4.1. **Fish weight, weight gain, and survival**

Nutritional composition (Table 2) of the diets varied, and analysis of growth and survival at the conclusion of the feeding experiment revealed significant differences among dietary treatments. The greatest growth was achieved with the AN diet, followed by the PC, CG, and finally the FC diet fed fish had the lowest growth (Table 2.3). Similar results were observed in percent weight gain. During the 14 day fasting period, mean fish weight decreased by 0.6-1.0 gram i.e. 14.5-27.4 % of the initial weight.

Survival was significantly affected by dietary treatment during the feeding experiment. The casein-gelatin (CG) diet fed fish experienced significantly higher mortality compared to the other three diets (Table 2.3 and Figure 2.1). One tank was
removed from the analysis of survival in the CG tanks due to significant fish loss unrelated to diet. Survival was 100% in the AN and PC diets while the FC diet fed fish had 98.2% survival. There were no mortalities during the fasting experiment for any dietary treatment.

2.4.2. Body composition

Examination of body composition following the feeding and fasting experiment showed differences between dietary treatments. Whole body composition of protein and lipid (% dry matter) were not significantly different between dietary treatments at the conclusion of the feeding experiment (Table 2.4). However, fish fed the FC diet had a significantly higher percent protein following the fasting experiment. The percent ash in the whole body of the fish fed the FC diet was also significantly higher than in the other treatments. The AN diet fed fish had a higher moisture content. The ratio of mean protein content to mean lipid content was approximately 2:1 at the completion of the feeding and fasting experiments (Table 2.4).

During the fasting experiment, protein decreased faster than lipid, 9.5-20.3 mg/fish/day and 3.1-5.6 mg/fish/day (P < 0.01), respectively (Table 2.5). However, the ratio of mean protein content to mean lipid content remained at approximately 2:1 which was the ratio at the end of the feeding experiment (Table 2.4). There was not a significant difference in the percent decrease of protein or lipid, 26.5-46.3 % and 10.0-37.6 %, respectively (Table 2.5), between groups, except the FC diet fed fish lost protein faster (P < 0.05).
2.4.3. **Histological examination of the liver and posterior intestine**

Histological examination of the liver from fish sampled at the end of the feeding experiment showed hepatocytes containing larger deposits of lipids and nucleus pushed to the cell wall in fish fed the AN diet compared to the fish fed the FC diet (Figure 2.2). The presence of absorptive vacuoles in the posterior intestine was observed in all feeding groups. However, a significant difference occurred in the height of the posterior intestine folds and in the number of goblet cells. Fish fed the plant protein-based diet (PC) showed the longest folds, while the shortest folds were observed in group fed CG (Table 2.6), and the differences were statistically significant. Goblet cells were more numerous in fish fed FC and PC as compared to those fed AN and CG diets (Figure 2.3 A and B, Table 2.6).

The smallest cell area and higher glycogen than lipid storage occurred in hepatocytes of fish fed the FC diet (Figure 2.4 A). On the contrary, hepatocytes of fish fed AN and CG contained more lipid than glycogen (Figure 2.4 B). In the hepatocyte cytoplasm of PC fed group both of the substances were present in similar amounts. Livers of fish fed the CG diet showed twice the amount of PCNA-positive hepatocyte nuclei than in other experimental groups (Figure 2.4 C and D, Table 2.6). It was also observed that hepatocytes of this group of fish showed irregular shape and poorly discernable cell membranes (Figure 2.4 C).
2.5 Discussion

Many studies have explored the replacement of fish meal and oil in sunshine bass diets with plant or poultry by-product sources (Brown et al. 1993; Greenberg and Harrell 1998; Lewis and Kohler 2008a). However, our study adds a new dimension to these results, which is a resistance to fasting following different dietary treatments. The results of other studies indicate that a substantial amount of fish meal and oil in the diets of sunshine bass can be replaced by plant or poultry by-product sources without affecting production, but these other sources have caused changes in the fatty acid (FA) profiles of the fish (Pine et al. 2008; Trushenski and Boesenberg 2009; Lewis and Kohler 2008b). However, Trushenski and Boesenberg (2009) showed that implementing a finishing feed with fish oil can considerably restore desired long-chain polyunsaturated fatty acid levels in the fillets of sunshine bass in as little as 4 weeks.

The high nutrient commercial diet (AN), which contains fish meal, provided the best growth and survival. The plant protein based practical diet (PC) resulted in comparable survival, but growth was decreased by 35.0%. The decreased growth in our study can likely be attributed to differences in the availability of indispensible amino acids in plant proteins compared to animal proteins as reported previously by Gaylord et al. (2004). Another likely reason is the energy content of the diets. Nematipour et al. (1992a) reported that the maximum weight gain of sunshine bass was achieved with fish fed diets containing an energy:protein ratio of 6, 8, and 9 kcal energy/g protein. It has been documented that lipids in sunshine bass diets over 7.0% slightly depressed growth when the diet contained 35% protein and 3.38 kcal available energy/g with a percent
carbohydrate:lipid ratios of 31:7.5 or 25:10, respectively (Nematipour et al 1992b). However, Burr et al. (2006) demonstrated that sunshine bass juveniles fed a high protein (49 %) and lipid (20 %) formulated diet grew better than those on a standard (37.5 % crude protein, 10.5 % crude lipid, and 19.6 kJ/g energy on a dry matter basis) warm-water fish commercial diet.

Our results demonstrate that it is possible to raise sunshine bass on the practical, low fish meal containing diet, but growth may be slower with the current formulation. The semi-purified diet (CG) formulation needs to be improved for the rearing of sunshine bass since growth was depressed compared to the AN and PC diets and survival was significantly lower than the other three diets. For instance, Griffin et al. (1994) used purified diets based on casein (9 %), gelatin (1.8 %), and free amino acids (24 %) to achieve a growth rate of 0.9-0.15 g/day and survival of 93-100 % over a four week feeding experiment with an initial fish weight of 6.1-6.6 g. The FC diet fed fish had high survival, but growth was severely diminished indicating low nutrient content and perhaps nutrients leaking from the wet diet.

To our knowledge, no previous studies with hybrid striped bass have ended a feeding experiment with a fasting period to simulate conditions of stocking programs during the transportation and acclimation of fish to new environments. However, fasting experiments have been conducted with other species. For instance, Simpkins et al. (2003) showed an increase in moisture, increase in protein, and a decrease in lipid when expressed as the percent body composition of fasting rainbow (*Oncorhynchus mykiss*) trout juveniles. A comparison of the percent body composition of fed fish to fasted fish
revealed a general pattern of an increase in moisture, decrease in protein, increase in lipid, and increase in ash (Table 2.4). As expected, fish weight decreased during the fasting experiment as well as mean protein and lipid content (Table 2.3 and Table 2.4). In our study, protein depleted faster than lipid (Table 2.5). This is likely attributed to fasting sunshine bass using proteins and glycogen as an energy source. Rawles et al (2008) demonstrated that a large portion of the liver in sunshine bass can be partitioned for glycogen and lipid storage. Our data suggest that lipids in fasting fish continue to be synthesized and not converted to energy prior to utilization of glycogen. However, this needs to be investigated further.

By the end of the fasting period, the amount of mean protein content in fish among dietary treatments decreased by approximately three times the amount of lipid (Table 2.4). However, the ratio of mean protein content to mean lipid content remained at about 2:1. Higher lipid content in the whole body of fish (AN group in comparison to FC group) corresponded to higher lipid deposition in liver according to histological analysis (Figure 2.2). Gallagher (1996) demonstrated that lipid content in the liver of sunshine bass increased significantly when the dietary protein level in diets increased from 33 to 43 % indicating that dietary protein in excess of requirements resulted in increased lipid deposition. Brown et al (1992) determined the optimal protein level for sunshine bass fed fish meal diets to be approximately 41 % of dry diet. The diets in our study contained 51.0-57.5 % protein, which would be in excess of protein requirements for striped bass according to Millikin (1983). However, fish used in our study were significantly smaller, earlier ontogenetic stage, and excess of lipid deposition in the liver was only observed in
the AN diet but not in the FC diet. The FC diet produced significantly smaller fish than the AN diet, which could have been a result of inadequate nutrient concentration. It is also evident that the percent lipid was not significantly different among either the fed or fasted groups. This suggests that sunshine bass have a high protein requirement and perhaps a marginal capacity of lipid sparing effect on protein utilization. Thus, diets should contain a higher proportion of available proteins compared to lipids.

Glycogen stored in hepatocytes is a cellular energy source (Green and McCormick 1999), while hepatic lipid storage indicates good nutritional condition of fish and is a long-term energy reserve (Caballero et al. 1999). Optimum and balanced feeding of fish results in utilization of food lipids for energetic purposes. The largest hepatocytes, and the highest lipid content were observed in fish fed AN diet. The smallest hepatocytes and low lipid level in fish fed FC might have resulted from fast utilization of nutrient reserves during prolonged diminished nutrient intake. Feeding also affected the values of hepatosomatic indices. Lower content of lipid in the PC diet resulted in a low HSI. Similar results were observed by Rosenlund et al. (2004) in Atlantic cod (Gadus morhua). Nogales Mérida et al. (2010) reported that HSI decreased with an increase in vegetal protein content in the diet of juvenile sharpsnout sea bream (Diplodus puntazzo).

A slightly elevated number of proliferating hepatocytes was observed in the livers of fish fed the CG diet. According to Varedi et al. (2001), an increase in hepatocyte proliferation rate may result from nutritional stress and starvation. Morphology of the posterior intestine and liver of fish indicates that feeding AN, PC, and FC diets did not adversely affect digestive system functions, except for the CG group. Increased
proliferation of hepatocyte nuclei indicates hepatic disturbances caused by components of the CG diet. When the number of matured, functional hepatocytes changes in favor of new, proliferating cells, it may indicate a higher rate of cell renewal due to the inflammatory response, metabolic overburden. Bekke-McKellep et al. (2007) observed in Atlantic salmon (*Salmo salar*) posterior intestine a significant increase of proliferating cells’ compartment length due to the replacement of a fish meal-based diet with a soybean meal containing feed. However, in common carp (*Cyprinus carpio*) juveniles fed the same diet (AN) as used in the present experiment with sunshine bass, Ostaszewska et al. (2010b) found the number of PCNA in hepatic tissue to decrease almost 10 fold in comparison to the plant protein (wheat gluten-based) diet. A predictable pattern in all those experiments was the association between a growth depression and increased PCNA index.

The smaller size of folds in the posterior intestine of fish fed the CG and FC diets as compared to the PC group might have resulted from malnutrition (Gisbert and Doroshov, 2003). The number of goblet cells may vary and depends on feeding level (De Silva and Anderson 1995). In the present study, the number of goblet cells was elevated in fish fed PC and FC diets. The results of our earlier studies showed that abundance of goblet cells increased in starved tench (*Tinca tinca*) (Ostaszewska et al. 2006). Similar changes were observed in herbivorous and predatory fish fed diets containing soybean meal (van den Ingh et al. 1991; Uran et al. 2008). This indicates that vegetal protein in the PC diet probably caused alterations in mucosal metabolism which resulted in an increase in abundance of goblet cells. However, no basophilic granulocytes were
observed in the lamina propria of intestinal mucosa, thus no inflammation occurred as demonstrated in salmonids (Bakke-Mckellep et al. 2007).

We conclude that the comparably high nutrient, animal protein-based commercial diet (AN) provided the highest growth and survival while likely containing the most desired fatty acid profile. This study also demonstrates that sunshine bass can be intensively grown in high density culture systems with plant-protein based diets starting at 0.4 g individual weight without sacrificing survival but slightly compromising growth. These results are similar to previous dietary studies with sunshine bass. However, none of the previous feeding experiments with sunshine bass finished with a fasting period. This study provides insight into the effects of fasting on sunshine bass. Analysis of fasting conditions suggests that sunshine bass utilized protein and glycogen more rapidly than lipid during the fasting period. Thus, lipids continue to be synthesized and not converted to energy suggesting sunshine bass likely have a marginal capacity of lipid sparing effect on protein. The percent body composition of fasted fish remained similar to fed fish after our simulated transport and acclimation period to new environments. Further research is still necessary to understand the impact of fasting in sunshine bass as well as replacing fish meal and fish oil in their diets, which is still the ideal source of protein and lipid. Identifying changes in sunshine bass from fasting during transportation and acclimation to new environments may potentially lead to improved stocking techniques that increased survival, and knowing specific dietary requirements for sunshine bass will help identify alternatives to fish meal and oil.
2.6 Acknowledgements

Financial support for this project was provided by the Ohio Department of Natural Resources, Division of Wildlife. Larval sunshine bass were obtained from Keo Fish Farms (Keo, Arkansas). Mineral and protein analysis was conducted by STAR Lab located in Wooster, Ohio. We are also grateful for our lab co-workers, Bong-Joo Lee, Tim Parker, and David Fullard, at The Ohio State University for aiding in cleaning tanks and feeding the larvae and juveniles.
2.7 References


2.8 Tables and Figures
Table 2.1: Formulation of PC (wheat gluten based) and CG (casein-gelatin semi-purified) experimental diets.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>PC</th>
<th>CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>12.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>37.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>21.6</td>
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<tr>
<td>Casein (vitamin free)</td>
<td>0.0</td>
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</tr>
<tr>
<td>Gelatin</td>
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</tr>
<tr>
<td>Starch</td>
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<td>10.0</td>
</tr>
<tr>
<td>Fish oil</td>
<td>8.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Soy-lecithin</td>
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<td>15.0</td>
</tr>
<tr>
<td>Dextrin (80% water soluble)</td>
<td>0.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Mineral mixture&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mixture&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Amino acid mixture&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5</td>
<td>2.7</td>
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<tr>
<td>CPSP&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>0.0</td>
</tr>
<tr>
<td>Ca-mono P</td>
<td>1.5</td>
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</tr>
<tr>
<td>Choline chloride (60%)</td>
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<td>0.2</td>
</tr>
<tr>
<td>Rovimix Stay-C 35&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>α-cellulose&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mineral Mixture (mg/kg diet): CaHPO<sub>4</sub>, 3,000.00; CaCO<sub>3</sub>, 15,500.00; CuSO<sub>4</sub>, 8.00; FeSO<sub>4</sub>·7H<sub>2</sub>O, 300.00; MnSO<sub>4</sub>·H<sub>2</sub>O, 40.00; KI, 6.00; KH<sub>2</sub>PO<sub>4</sub>, 25,000.00; NaH<sub>2</sub>PO<sub>4</sub>, 3,000.00; NaCl, 3,000.00; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 140.00; NaF, 11.00; Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.85; CoCl<sub>2</sub>·6H<sub>2</sub>O, 2.00 (Sigma Aldrich, St. Louis, MO, USA).

<sup>b</sup>Vitamin mixture (mg/kg diet) sources were Rovimix series: retinyl acetate, 2.00; cholecalciferol, 0.10; DL- α-tocopheryl acetate, 125.00; menadione niacinamide bisulfite, 5.00; nicotinic acid, 25.00; riboflavin, 20.00; pyridoxine hydrochloride, 15.00; D-calcium pantothenate, 50.00; biotin, 1.00; folic acid, 5.00; cyanocobalamin, 0.05; myo-inositol, 500.00 (Aquaculture Research Group, DSM Nutritional Products France, Animal Nutrition & Health Research, Saint-Louis, France).

<sup>c</sup>Free amino acid mixture composition (g/kg diet; all L-form amino acids): proline, 2.0; alanine, 8.0; arginine, 5.0; methionine, 0.4; lysine, 8.0 (MP Biomedicals, Irvine, CA, USA).

<sup>d</sup>Concentrate of fish soluble protein (CPSP 90: crude protein, 82-84% WW; crude lipid, 9-13% WW), Sopropêche S.A., Boulogne-sur-mer, France.

<sup>e</sup>L-Ascorbic acid monophosphate (35%).
Figure 2.1 continued

α-Cellulose was equally replaced by thiamine mononitrate (20 mg/kg diet) and/or either magnesium oxide (700 mg/kg diet) or calcium oxide (700 mg/kg diet) in diet treatments (Rovimix or Sigma-Aldrich, St. Louis, MO, USA).
<table>
<thead>
<tr>
<th>Diet Composition</th>
<th>AN</th>
<th>PC</th>
<th>CG</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>52.7</td>
<td>53.6</td>
<td>51.0</td>
<td>57.5</td>
</tr>
<tr>
<td>Lipid</td>
<td>16.7</td>
<td>11.7</td>
<td>15.8</td>
<td>46.3</td>
</tr>
<tr>
<td>Ash</td>
<td>5.4</td>
<td>3.8</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Moisture</td>
<td>12.8</td>
<td>10.5</td>
<td>8.1</td>
<td>98.2</td>
</tr>
</tbody>
</table>

**Table 2.2:** Composition of diets fed to sunshine bass juveniles in a feeding experiment (51 days).
<table>
<thead>
<tr>
<th>Diet</th>
<th>Fish weight (g)</th>
<th>Weight gain/loss (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>End of feeding</td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>6.8 ± 0.3z</td>
<td>1506.7 ± 59.1z</td>
<td>100.0 ± 0.0z</td>
</tr>
<tr>
<td>PC</td>
<td>4.6 ± 0.5y</td>
<td>978.9 ± 149.2y</td>
<td>100.0 ± 0.0z</td>
</tr>
<tr>
<td>CG</td>
<td>3.7 ± 0.1x</td>
<td>467.0 ± 18.0x</td>
<td>81.6 ± 3.7y</td>
</tr>
<tr>
<td>FC</td>
<td>2.2 ± 0.1w</td>
<td>413.3 ± 20.6x</td>
<td>98.2 ± 3.0z</td>
</tr>
<tr>
<td></td>
<td></td>
<td>End of fasting</td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>5.8 ± 0.2z</td>
<td>-14.7 ± 1.9z</td>
<td>100.0 ± 0.0z</td>
</tr>
<tr>
<td>PC</td>
<td>3.9 ± 0.5y</td>
<td>-14.5 ± 11.6zy</td>
<td>100.0 ± 0.0z</td>
</tr>
<tr>
<td>CG</td>
<td>2.7 ± 0.2x</td>
<td>-19.6 ± 6.4zy</td>
<td>100.0 ± 0.0z</td>
</tr>
<tr>
<td>FC</td>
<td>1.6 ± 0.1w</td>
<td>-27.4 ± 0.5y</td>
<td>100.0 ± 0.0z</td>
</tr>
</tbody>
</table>

**Table 2.3:** Mean final weight and survival of sunshine bass fed four diets in a feeding (51 d) and fasting (14 d) experiment. Weight gain for feeding experiment is based on total tank biomass while weight loss in the fasting experiment is based on average fish weight. Different letters indicate significant difference at P < 0.05.
<table>
<thead>
<tr>
<th>Diet</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
<th>Mean protein content (g/fish)</th>
<th>Mean lipid content (g/fish)</th>
<th>Mean ash content (g/fish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>74.5 ± 2.0z</td>
<td>55.2 ± 3.1z</td>
<td>26.1 ± 7.1z</td>
<td>7.8 ± 0.2z</td>
<td>0.96 ± 0.02z</td>
<td>0.45 ± 0.02z</td>
<td>0.14 ± 0.01z</td>
</tr>
<tr>
<td>PC</td>
<td>77.0 ± 0.5zy</td>
<td>60.8 ± 1.7z</td>
<td>29.3 ± 10.0z</td>
<td>7.8 ± 0.1z</td>
<td>0.64 ± 0.02y</td>
<td>0.31 ± 0.10y</td>
<td>0.08 ± 0.00y</td>
</tr>
<tr>
<td>CG</td>
<td>76.5 ± 2.7zy</td>
<td>57.2 ± 2.6z</td>
<td>28.3 ± 6.1z</td>
<td>8.5 ± 0.8zy</td>
<td>0.49 ± 0.04x</td>
<td>0.24 ± 0.04yx</td>
<td>0.07 ± 0.01x</td>
</tr>
<tr>
<td>FC</td>
<td>79.2 ± 0.4y</td>
<td>62.0 ± 3.2z</td>
<td>24.9 ± 3.9z</td>
<td>9.2 ± 0.7y</td>
<td>0.29 ± 0.01w</td>
<td>0.11 ± 0.00x</td>
<td>0.04 ± 0.00w</td>
</tr>
</tbody>
</table>

Table 2.4: Body composition of sunshine bass fed four diets following a feeding (51 d) and fasting (14 d) experiment. AN = commercial diet (high nutrient), PC = practical diet formulation (wheat gluten based), CG = casein gelatin semi-purified diet (standard nutrient content), and FC = natural diet (frozen chironomids). Calculations are based on dry matter except percent moisture. Different letters indicate significant difference at P < 0.05.
<table>
<thead>
<tr>
<th>Diet</th>
<th>Protein (mg/fish/day)</th>
<th>Lipid (mg/fish/day)</th>
<th>Ash (mg/fish/day)</th>
<th>Protein (%)</th>
<th>Lipid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>20.3 ± 3.7z</td>
<td>3.5 ± 6.8z</td>
<td>1.0 ± 0.3z</td>
<td>29.5 ± 5.1z</td>
<td>10.0 ± 20.5z</td>
</tr>
<tr>
<td>PC</td>
<td>12.1 ± 4.6zy</td>
<td>5.0 ± 7.8z</td>
<td>0.4 ± 1.0z</td>
<td>26.5 ± 10.5z</td>
<td>17.3 ± 25.7z</td>
</tr>
<tr>
<td>CG</td>
<td>12.7 ± 1.9zy</td>
<td>5.6 ± 3.6z</td>
<td>1.7 ± 0.3z</td>
<td>37.1 ± 2.6z</td>
<td>29.4 ± 15.5z</td>
</tr>
<tr>
<td>FC</td>
<td>9.5 ± 0.7y</td>
<td>3.1 ± 1.0z</td>
<td>1.1 ± 0.2z</td>
<td>46.3 ± 1.7y</td>
<td>37.6 ± 10.7z</td>
</tr>
</tbody>
</table>

**Table 2.5:** The mean weight (mg) of protein, lipid, and ash lost per fish per day and percent decrease in protein and lipid for each diet during the fasting experiment (14 days). Different letters indicate significant difference at P < 0.05.
<table>
<thead>
<tr>
<th></th>
<th>AN</th>
<th>PC</th>
<th>CG</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Folds length (µm)</strong></td>
<td>288.5±39.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>322.8±84.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>220.5±39.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>229.3±45.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Number of goblet cell /100 µm length intestinal fold</strong></td>
<td>27.0±3.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46.8±5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.9±4.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62.8±6.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Hepatocyte area (µm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>130.0±30.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120.0±27.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125.0±36.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113.0±16.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Number of positive PCNA cell nuclei/mm&lt;sup&gt;2&lt;/sup&gt; liver tissue</strong></td>
<td>314.3±20.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>228.6±31.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>600.0±36.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>257.1±42.0&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>HSI</strong></td>
<td>2.3±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 2.6:** Histomorphometry of the intestine and the liver of sunshine bass after the end of the experiment.
Figure 2.1: Survival of sunshine bass during feeding experiment. Different letters indicate significant difference at $P < 0.05$. $z^*$ indicates that the AN, PC, and FC diets are statistically not significantly different. CG = mean of two tanks because of significant loss in one tank unrelated to diet.
**Figure 2.2:** Sections of the liver with hepatocytes containing larger deposits of lipids (white spaces marked with arrows) and nucleus pushed to the cell wall in fish fed AN diet (A) as compared to the fish fed FC diet (B). Fish sampled at the end of the feeding trial.
**Figure 2.3:** Longitudinal section of sunshine bass intestine mucosal folds: A) fed CG, B) fed FC, showing goblet cells (arrow). Scale bars: 10 μm. AB/PAS staining.
**Figure 2.4:** Section of sunshine bass liver: A) fed FC, B) fed AN, AB/PAS staining. C) fed CG, D) fed PC showing immunohistochemical detection of PCNA-positive nuclei (arrows). Scale bars: 10 µm.
Chapter 3: Evaluation of the viability and growth of walleye *Sander vitreus* embryos/larvae following antiviral iodine treatment for viral hemorrhagic septicemia (VHS)

3.1 Abstract

Avoiding viral disease transmission and securing health of fish populations are vital for sport fish hatchery-based stocking programs in regions affected by viral hemorrhagic septicemia (VHS). Iodine compounds are nonselective antiviral substances that have been widely used as disinfectants, but their effects on embryo survival and growth during early life stages of fish are less known. Two groups of walleye were collected from the Maumee River, Perrysburg, Ohio in March 2009. Gametes were collected on site, and transported (3-4 h) to the lab. The eggs from each individual female were fertilized with the combined sperm of 3-4 males. After fertilization, a tannic acid treatment for egg de-adhesion was used. Then embryos were exposed to iodine at 0 (control) or 100 mgL\(^{-1}\) (in triplicate) for 30 min. Fertilized eggs were incubated in California trays at 10-12 °C. Survival was estimated at the eyed-stage. Larvae were sampled prior to first feeding at 9 days post hatch (DPH) and approximately every 10 days following first feeding (live brine shrimp nauplii) until 48 DPH to assess growth. The results suggest that embryo survival following iodine treatment appears to be affected by the condition of the parents, but the growth of embryos was unaffected by the iodine treatment. Histological analysis of swim bladder morphology revealed that larvae
with non-inflated swim bladders had features of an undeveloped organ with hyperplastic epithelium and vacuolarized cells.

3.2 Introduction

Viral hemorrhagic septicemia (VHS) is a severe disease affecting wild fish populations in northern temperate regions and aquaculture programs in Europe (Dale et al. 2009). The virus is also considered to be one of the most serious viral diseases in wild populations, and has been responsible for large-scale fish kills in the Greats Lakes (Winton et al. 2007; Elsayed et al. 2006; Groocock et al. 2007). Securing fish health and avoiding viral disease transmission are critical for management of stocking programs and fisheries in regions affected by VHS.

VHS is a member of the family *Rhabdoviridae*, which includes infectious hematopoietic necrosis a highly virulent fish pathogen (Winton et al. 2007; Walker et al. 2000). It has been isolated in wild fish populations in both freshwater and marine environments throughout the northern hemisphere. Genetic analysis indicates that VHS isolates from the Great Lakes are distinct from other isolates found in Europe, North America, Japan, or Korea, and it is, therefore, classified as Genotype IVb (Winton et al. 2008; Bowser 2009). It is a particularly stable virus in cold freshwater water (Hawley and Garver 2008). The virus is also infectious to all age groups of susceptible species, but it often infects young or stressed individuals. VHS poses a serious problem because RNA viruses generally have high mutation rates that give them the potential to adapt quickly to new environmental conditions and infect new species (Dale et al. 2009).
virus is primarily found in the urine and reproductive fluids of infected individuals, but it can also be found in their fecal material. Transmission of the virus is believed to occur through the gills, food, open wounds, or contact with an infected individual.

The virus is reported to be carried by at least 50 fish species worldwide affecting each to a varying degree (Harper 2007). Some infected individuals experience severe anemia and hemorrhaging leading to death while others are asymptomatic. Due to its high virulence, it is important to understand which prophylactic treatments are the most reasonable for antiviral procedures and effective for management of sport species (Dabrowski et al. 2009). This study will assess the effects of iodine treatment on the embryos of an economically important sport fish in the Great Lakes region, walleye *Sander vitreus*.

Since the 1970s, iodophor disinfectants have been part of standard hatchery practices for salmonid eggs to reduce the risk of pathogen transfer through ovarian and seminal fluids during gamete collection operations (Tuttle-Lau et al. 2010). This positive record has made iodine an ideal disinfectant to test for reducing the transmission of VHS. Iodine, a microbial disinfectant, has been shown to have relatively low toxicity to eggs of several freshwater fishes while being highly toxic to fish pathogens (Amend and Pietch 1972; Inoue et al. 1991). However, high concentrations and prolonged exposure to iodophor treatments resulted in increased egg losses of walleye (Dabrowski et al. 2009) and Chinook salmon (Fowler and Banks 1991).

A critical period for treatment of VHS occurs at the time of fertilization before fish are raised and transferred from hatchery ponds to inland systems. Thus, the effects
of iodine treatment following fertilization on the survival, early embryonic development, and larval growth of walleye needs to be examined. Since the VHS virus affects a variety of species, a complex strategy of treatment is necessary to prevent horizontal transfer between introduced fish from hatchery ponds and susceptible species within the stocked bodies of water.

In the present work, we propose to 1) quantify the viability of embryos and growth of walleye larvae that were treated with iodine following fertilization as a method for disinfecting potentially contagious gametes, and 2) assess the development of the gills and swim bladder inflation through histological analysis. Parental attributes, such as fecundity and egg size, were also quantified to determine if they influenced the growth of progeny or predisposed them to increased toxic effects of iodine treatment.

3.3 Methods

Two groups of walleye were collected by electrofishing from the Maumee River, Perrysburg, Ohio during the spring (2009) spawning run. The first group of walleye, six females and three males, were collected mid-morning. The second group of walleye, 20 females and 4 males, was collected that night. Electrofishing was conducted by the Ohio Department of Natural Resources Division of Wildlife, and the water temperature was approximately 6 °C.

Gametes from group 1 were collected on site (without using anesthesia) by stripping fish within 10 min of arriving to shore. The gametes were transported in individual, flat-bottom containers at 5-7 °C (2-3 layers of eggs) or 0 °C (1-2 mm of
sperm) to the lab (Rinchard et al. 2005). The second group of walleye was captured and transported by truck to the Division of Wildlife office in Toledo, Ohio to be held overnight. Gametes from ten females and four males were collected the following morning, and transported to the lab by the same methods previously described. Females were stripped of all their eggs in order to estimate fecundity. Some eggs were separated prior to fertilization for VHS testing and to determine egg size. The length (mm) and weight (g) of each female were also recorded. The experiment was divided into two phases. The first phase of the experiment included early rearing, VHS testing, growth, egg survival, and larvae mortality (described in detail below). Phase 1 began March 30, 2009 with the collection of parental walleye from the Maumee River, and concluded May 21, 2009. Phase 2 was a continuation of phase 1.

3.3.1 Phase 1

When the gametes arrived to the lab, fertilization began immediately (i.e., 3-4 h after gamete collection). Approximately 2 g of eggs (about 800 eggs) were placed in small plastic cups and inseminated with 10 μL of sperm from 3-4 males. An optimum sperm/egg ratio for fertilization was estimated, based on known mean concentrations of walleye sperm (50 x 10^3 spermatozoa/egg; Rinchard et al. 2005). Eggs and sperm were activated by adding 10 mL of hatchery water. One minute after fertilization, we exposed the eggs to 100 mL of a tannic acid solution (400 mg tannic acid/L water) for 3 min exchanging the tannic acid solution once during this time. Fertilized eggs from each female were washed with fresh hatchery water and poured into small baskets for
treatment (3 controls and 3 iodine-treated replicates per female). Eggs were then exposed to a bath of 0 (control) or 100 mgL$^{-1}$ of a pre-buffered stock solution (pH 7.0) of iodophor (Argentyne, Argent Chemical Laboratories, Redmont, WA) for 30 min with continuous aeration. Following the 30 min bath, the eggs were rinsed in fresh hatchery water, and then transferred to California trays for incubation. The water temperature during incubation ranged between 10-12°C. The survival rates were estimated on April 8, 2009 (group 1) and April 9, 2009 (group 2), at the “eye-cap early” stage (106 °D, degree-days), prior to retina pigmentation. Survival was also estimated at the forced-hatching stage (Czesny et al. 2005). Forced-hatching of group 1 and group 2 (216 °D and 219 °D respectively) were carried out by transferring baskets to containers without water flow and high aeration for 24 hours. The survival of larvae for each female and treatment was estimated volumetrically and compared to a known number of eggs (dead) in the baskets following forced hatching.

After observing variability in survival between the two groups of 6 and 10 females, larvae from group 1 (6 females) were selected to be transferred to 60 L tanks (6 controls and 6 iodine-treated). Following the absorption of their yolksac, exogenous feeding began (9 DPH). Larvae were fed nauplii of brine shrimp *Artemia* spp. three times per day at 30 % total tank biomass from the beginning of exogenous feeding to 35 DPH before being transitioned to a dry feed (Bio Oregon) in phase 2. As the fish grew, the size of brine shrimp offered as feeding also increased. A clay (Moore et al. 1994) and sodium chloride (Ribi 1992; Bein and Ribi 1994; Victoria et al. 1992) solution was added to increase larvae survival by reducing clinging behavior and aiding in osmoregulation.
The clay (0.1 ppt) and salinity (2.0 ppt) were maintained in each tank daily (beginning 10 DPH, 334 °D) by adding 600 mL of a prepared solution in the morning, and then adding 300 mL of the solution twice more throughout the day to sustain that concentration. Sprinklers were used to break surface tension and aid in the inflation of the swim bladder (Regier and Summerfelt 1998; Egloff 1996)

3.3.2 Phase 2

Larvae from the twelve tanks in phase 1 (6 controls and 6 iodine-treated) were evenly and randomly transferred to six 60 L tanks (3 controls and 3 iodine-treated) based on treatment for further growth on May 21, 2009 (34 DPH). Sprinklers maintained flow to tanks while turbidity and salinity were gradually reduced until discontinued completely a few days later. Walleye were transitioned to the dry feed by 39 DPH. A belt feeder dispensed approximately 0.5 g of feed every 2 hrs during the day (5x daily). Walleye were transferred June 4, 2009 to 30 L glass aquaria for final grow out. Samples of healthy juveniles (48 DPH) from each tank were taken for growth analysis, while moribund individuals (51 DPH) were taken for VHS testing. Mortality was recorded daily throughout phase 2.

3.3.3 Histological Procedures

Samples of larvae with inflated and non-inflated swim bladders were collected for histological analysis of the gills and swim bladder 9, 20, 32, and 48 DPH. The samples were collected from several females and treatments during each sampling period. A
summary (number of larvae collected, number of females sampled, and total body length of larvae) of each sampling period is provided: 9 (4 larvae from 2 females at 7.82-8.12 mm), 20 (25 larvae from 4 females at 9.30-11.27 mm), 32 (21 larvae from 4 females at 11.42-15.26 mm), and 48 DPH (7 larvae from 2 females at 16.78-22.69 mm). Samples were set for 36-48 hours in a fixative prepared according to Dabrowski and Bardega (1982). After fixation, fish were transferred to 75% ethanol until further processing. All larvae were paraffin-embedded after dehydration with a graded ethanol series and treated with xylene. Histological sections were cut longitudinally into a complete series of serial sections (5 μm) and mounted on albumin-coated slides. Sections were stained with Mayer’s hematoxylin and eosin (H & E) for topographic histological analysis. This procedure was carried out to describe the swim bladder morphology and the presence of the pneumatic duct, macrophages, and food detritus in the swim bladder lumen, and the structure of its epithelium (following Marty et al. 1995). The gill structure was described based on the development and appearance of gill arches, gill filaments, and secondary lamellae (Phillips and Summerfelt, 1999). The histological sections were observed under a light microscope (Olympus BX41). Micrographs were taken with a microscope (Zeiss Axioscope, Zeiss, Germany) and a digital camera (Olympus MagnaFire Digitial).

3.3.4 Statistical Analysis

Statistical analysis of data was conducted using SPSS software. Egg weight, embryo survival, larvae survival during feeding, and larvae growth were subjected to one-way ANOVA analysis, and significance was accepted at P < 0.05. Data are
presented as means ± SD. Analyses of growth and survival to the eye-embryo stage were performed between and within female groups as well as treatment groups. A correlation analysis of the percent survival at the eyed-embryo stage and the mean egg weight was performed to determine the effects of egg size on embryo survival. Swim bladder inflation percent was not statistically analyzed in this experiment.

3.4 Results

3.4.1 Female and Egg Characteristics

Characteristics of the parental females are shown in Table 3.1. The mean length for group 1 and group 2 females were 619 and 685, respectively. Group 2 females had a greater total egg mass than group 1 females, but there was not a significant difference in the mean weight of an individual egg. The mean weight of an egg from female 16 (4.19 ± 0.13 mg) was not included in the calculation. The relationship between egg weight (unfertilized) and embryo survival (eyed-embryo stage) showed a significant correlation for eggs treated with iodine. However, there was no significant relationship in the control group (Figure 3.1). Larger eggs had higher survival at the eyed-embryo stage than smaller ones in the iodine treated group. The relative egg weight for group 2 females comprised 19.8 ± 4.4 % of their body weight.

3.3.2 Walleye Survival at the Eyed-Embryo Stage

The mean survival (%) of walleye embryos to the eyed-embryo stage is shown in Figure 3.2. In group 1 females, only female 2 had a significant difference in survival at
the eyed-embryo stage with higher survival in the iodine treated group, but in group 2 females, six of ten females had significantly higher survival in the control group. When comparing the survival between the control and iodine treated embryos for all 16 females, the control group (77.8 %) had significantly higher survival than the embryos exposed to iodine (54.4 %). However, comparing survival of embryos from group 1 females alone shows no significant difference in survival between the control (75.3 %) and iodine treated embryos (68.5 %) (Figure 3.3, A). Survival of iodine treated embryos (45.9 %) in group 2 was approximately half of the control (79.7 %) (Figure 3.3, B). Gametes from group 1 females were collected on site immediately following the electrofishing session, while gametes from group 2 females were collected the following day after they were stressed by transport and holding overnight in tanks.

Within each group of females, there was variation in the survival to the eyed-embryo stage. This was particular evident in group 2 females. Within group 1, only female 2 showed a significant difference in survival between treatments (Figure 3.2). The iodine treated embryos had higher survival. However in group 2, six of the ten females (Females: 8, 9, 11, 13, 15, and 16) showed a significant difference between treatments. All of the control groups for these females had a higher mean survival.

### 3.4.3 Larvae/Juvenile Growth

The mean larval length and weight for 9, 20, 32, and 48 DPH are shown in Table 3.2. Growth (length or weight) of walleye larvae was not significantly different between treatments throughout this experiment. At 32 DPH, the mean length of the control and
iodine treated group was 14.1 ± 0.3 and 14.3 ± 0.5 mm while the mean individual weights of fish in these groups were 17.3 ± 3.4 and 18.6 ± 3.7 g, respectively. However, progenies from individual females did express significant differences in growth between the control and iodine treated embryos at 9, 20, and 32 DPH (Table 3.2). At 9 and 32 DPH, the length of larvae ranged from 8.1-8.7 and 13.5-14.5 mm in the control group, and 8.2-8.8 and 13.8-14.9 mm in the iodine treated group, respectively.

3.4.4 Histological Analysis of Gills and Swim Bladder Development

Histological analysis of gill development at 9, 32, and 48 DPH showed that clay did not interfere with normal growth of this organ. At 9 DPH, the gill arches can be distinguished and filaments are beginning to develop. Differentiated filaments and the beginning of secondary lamellae development can be observed at 32 DPH. By 48 DPH, gill structures are clearly visible and distinct with no pathological changes in epithelial cells (Figure 3.4).

The percent of walleye larvae with partially or completely inflated swim bladders are shown in Table 3.2. At 9 DPH, the swim bladder is still developing in walleye larvae. At 20 and 32 DPH, swim bladder inflation ranged from 45-90 % and 53-93 % in the control group while 60-80 % and 73-100 % was observed among progenies from individual females in the iodine treated group, respectively. Properly inflated and non-inflated swim bladders are shown in Figure 3.5. Juveniles from 20, 32, and 48 DPH were split into groups (inflated and non-inflated swim bladders) for histological analysis. Individuals from both groups at 20 DPH showed columnar epithelium with vacuoles in
the wall of the swim bladder. The analysis of juveniles collected from 32 and 48 DPH revealed that individuals with non-inflated swim bladders have features of an underdeveloped organ with vacuolarized hyperplastic epithelium consisting of vacuolated cells. This feature resembles epithelium from swim bladders of 20 DPH individuals, while vacuoles were absent from the epithelium in the individuals with properly inflated swim bladders. In some non-inflated swim bladders, there was a presence of macrophages in the lumen. In individuals with an inflated swim bladder at 48 DPH, the pneumatic duct was not found. However, the remnants of this organ could be detected in individuals with non-inflated swim bladders of the same age. Detritus from food was not found in the swim bladder of any larvae.

3.5 Discussion

3.5.1. Iodine egg disinfection

Previous reports have shown iodine to be an effective viral disinfectant. Considering that the iodine treatment time and duration exceeded or was comparable to the conditions reported below, our conditions of egg disinfection were effective. Batts et al. (1991) showed complete inactivation of infectious hematopoietic necrosis virus within 7.5 seconds at iodine concentrations of 0.11, 0.22, and 0.42 mgL$^{-1}$. Viral hemorrhagic septicemia virus was inactivated with iodine at 8 mgL$^{-1}$ for 2.5 min (Amend and Pietch 1972). Kasai et al (2005) demonstrated that the minimum concentration of iodine necessary for 100 % plaque reduction of koi herpesvirus at 30 seconds and 20 min was 200 mgL$^{-1}$ at 15 and 25 °C. Higher concentrations and durations of iodine treatment have
been practically applied for fish egg disinfection in other studies. In general, the higher concentrations and longer durations have resulted in increased mortality of eggs and larvae. Fall Chinook salmon *Oncorhynchus tshawytscha* eggs and alevins treated with 75 mgL⁻¹ iodine for 30 min showed significant increase in mortality (Fowler and Banks 1990). Hirazawa et al (1999) established the safe and effective conditions for iodine disinfection of spotted halibut *Verasper variegates* (75 mgL⁻¹ iodine for 15 min) and red sea bream *Pagrus major* (100-200 mgL⁻¹ iodine for 5 min) eggs, respectively. However, in the rearing of red sea bream eggs early in the spawning period and spotted halibut eggs with low buoyancy and cleavage, the authors noticed iodine treatment had a harmful effect on survival. Thus, egg quality is an important factor. The results of these previous studies indicate that the safe and effective conditions for egg disinfection should be determined for each fish species separately. However, these studies need to be extended to perform experimental infection with VHS where any negative impacts of iodine treatment can be compared with the benefits gained from disinfection of eggs on walleye survival.

### 3.5.2. Embryo survival and egg quality

Walleye embryos treated with iodine (100 mgL⁻¹) for 30 min had significantly lower survival than the control group when considering all 16 females (group 1 and 2 combined). Embryos from females in group 1 showed no statistical differences in survival when treated with 0 (control) or 100 mgL⁻¹ iodine, but embryos treated with iodine from group 2 females showed a significant decrease in survival. This suggests that
the embryos had an increased sensitivity to iodine treatment. Females from group 2 were exposed to stress (confined holding tanks for 8-14 hrs and transport) in the final stages prior to ovulation which could account for the increased sensitivity to iodine treatment due to a decrease in egg quality. The success of reproduction is ultimately determined by the survival of progeny, and fertilization and development of offspring depends heavily on the quality of the gametes produced by the parents. A variety of factors influence egg quality. Factors affecting egg quality are determined by the intrinsic properties of the egg and the environment in which the egg is fertilized and the embryo develops (Brooks et al. 1997). Factors that affect the intrinsic properties of the egg include the physiological condition of the female. Campbell et al. (1992) demonstrated that stress during the final stages of gamete production in rainbow trout reduced the quality of gametes produced and significantly decreased the survival of offspring. Rainbow and brown trout exposed to stress from confined holding tanks in the final stages of reproductive development significantly reduced survival rates of progeny (Campbell et al. 1994).

Embryos from larger eggs exposed to iodine treatment (100 mgL$^{-1}$) following fertilization experienced higher survival than embryos from smaller eggs. Although there was not a significant difference in the mean size of eggs between females, it does appear that slightly larger eggs were less sensitive to iodine exposure in this experiment. However, this observation should be interpreted cautiously since the effect of egg size on quality is undetermined. Eggs obtained from females subjected to a prolonged (chronic) stress condition produced significantly smaller eggs (Campbell et al. 1994), and thus, smaller eggs could be of lower quality. However, uncertainty surrounding the effect of
egg size on survival has continued to be debated because of differences in age and size of parental fish, uncontrolled variation in the ripeness of eggs, and varying culture conditions (Bromage et al. 1992, Brooks et al. 1997). We conclude that the sensitivity of smaller eggs to iodine treatment needs to be explored further.

3.5.3. Juvenile growth

Growth of walleye larvae and juveniles was not significantly affected by iodine treatment at 9, 20, 32, or 48 DPH. Individual females expressed significant differences in growth between treatments, but the overall trend showed no difference. Growth of walleye juveniles in this experiment was considerably less compared to the results of Clayton et al. (2009) and Johnson et al. (2008). In both of those experiments, larvae were fed exclusively a dry feed while live brine shrimp were used in the present experiment. The growth of larvae in this experiment is equivalent to the results of Dabrowski et al. (2009), who followed similar feeding procedures for walleye.

3.5.4. Histological analysis of gills and swim bladder

Development of the gills was unaffected by iodine treatment or the presence of clay during rearing. The structures of the gills (arch, filaments, and secondary lamellae) are clearly visible and distinct by 48 DPH. Phillips and Summerfelt (1999) observed the beginning of gill filament development at 3 DPH and secondary lamellae at 10 DPH. In the present study, gill filaments were observed at 9 DPH and secondary lamellae at 32 DPH. However, samples were not taken as frequently in this study compared to Phillips
and Summerfelt (1999). The delayed development of gill filaments and secondary lamellae in this study can be attributed to the slower growth. Qualitative observations indicate that in our studies the increased turbidity by clay aided in foraging by reducing the clinging behavior of larvae. Clayton et al. (2009) reported that the major benefit of turbid water is a reduction in the clinging behavior of larval walleye which is associated with reduced feed intake, survival, and growth. The authors suggest that turbidity should be maintained through 28 DPH to maximize these beneficial effects. In our experiment, turbidity was maintained until 35 DPH before being completely discontinued a few days later.

Since swim bladder non-inflation is widely reported in hatchery operations (Trotter et al., 2004), sprinklers were added to break surface tension to aid swim bladder inflation. In a study by Sanabria et al. (2009), common disinfectants adversely affected swim bladder inflation in freshwater angelfish, *Pterophyllum scalare*. At 9 DPH, the swim bladder of larval walleye is still developing, but by 20 DPH, normally developing juveniles have successfully inflated their swim bladder. Walleye that fail to inflate their swim bladder experience poor growth and deformities (Rieger and Summerfelt 1998). The conditions leading to swim bladder non-inflation include temperature, total dissolved gases, and the presence of viscous film on the surface of the water (Sanabria et al. 2009).

The features described in walleye with non-inflated swim bladders, i.e. loss of columnar organization of the epithelium that was hyperplastic and frequently folded inward, were previously reported for freshwater angelfish (Sanabria et al. 2009). According to the Perlberg et al. (2008) in angelfish the vacuoles in the epithelial cells
appear near the time of initial inflation and disappeared upon completion of inflation. Thus, these vacuoles are assumed to have a role in swim bladder inflation, and this interpretation seems to fit the data in the present study. The studied walleye larvae with non-inflated swim bladders consisted of vacuolated epithelial cells, features of an undeveloped organ, and an abnormal appearance in older individuals. However, such interpretation should be open to further examination. For instance, it is known that in the case of striped bass (*Morone saxatilis*) no distinct vacuoles were found in the columnar epithelium of inflated swim bladders (Bulak and Heidinger 1980). Since the frequency of non-inflated swim bladders filled with macrophages was very low in our study where live food was offered, and there was no food debris in the swim bladders, we suppose that the reason of non-inflation was different from that reported by Marty et al (1995). Those authors found that the lack of inflation was the result of ingestion of bacteria and organic debris into the swim bladder when dry, formulated diets were used. In the present study, we showed an atrophied pneumatic duct in the swim bladders of the oldest studied individuals (48 DPH) with inflated swim bladders which is consistent with the data on striped bass (Bulak and Heidinger 1980). According to the latter authors, such results suggest that inflation of the gas bladder stimulates the atrophy of the pneumatic duct.

### 3.5.5. Conclusions

In conclusion, we have shown that the survival of walleye embryos and viability of larvae are not compromised when exposed to 100 mgL⁻¹ iodine for 30 min when eggs are collected from unstressed females, and growth of larvae and juveniles was unaffected
by treatment. Histological analysis revealed that the gills developed normally in the presence of the clay and salt solution, and that the swim bladder from non-inflated individuals contained vacuolated hyperplastic epithelial cells. Thus, walleye embryos potentially infected with VHS (or other viruses) can be treated with 100 mgL⁻¹ iodine for 30 min without compromising survival or growth.

3.6 Acknowledgements

Financial support for this project was provided by the Ohio Department of Natural Resources Division of Wildlife. The Division of Wildlife was also responsible collecting the parental walleye used in this experiment. Micrographs of the gills and swim bladder were taken using a microscope (Zeiss Axioskope, Zeiss, Germany) and a digital camera (Olympus MagnaFire Digitial) at Campus Microscopy and Imaging Facilities of the Ohio State University, Columbus, USA. We are also grateful for our lab co-workers, Bong-Joo Lee, Tim Parker, and David Fullard, at The Ohio State University for aiding in fertilization, hatching, and feeding larvae and juveniles.
3.7 References


3.8 Tables and Figures
<table>
<thead>
<tr>
<th>Female number</th>
<th>Length (mm)</th>
<th>BW (g)</th>
<th>Total egg mass (g)</th>
<th>Relative egg weight (%BW)</th>
<th>Mean weight of 1 egg (mg)</th>
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<td>296 ± 217</td>
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<td>2865 ± 754</td>
<td>574 ± 218</td>
<td>19.8 ± 4.4</td>
<td>2.59 ± 0.29*</td>
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</table>

**Table 3.1:** Characteristics of 16 female walleye, length, body weight (BW), relative egg weight (% BW), and mean (±SD) weight of 1 eggs. *Female 16 (4.19 ± 0.13 mg) was not included in the calculation.
<table>
<thead>
<tr>
<th>Iodine Treatment (mg/L)</th>
<th>Length (mm)</th>
<th>Weight (mg)</th>
<th>Length (mm)</th>
<th>Weight (mg)</th>
<th>Swim bladder inflation (%; n=10)</th>
<th>Length (mm)</th>
<th>Weight (mg)</th>
<th>Swim bladder inflation (%: n=15)</th>
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<tr>
<td>0</td>
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<td>2.3 ± 0.3</td>
<td>10.6 ± 0.5</td>
<td>6.5 ± 1.3</td>
<td>73</td>
<td>14.0 ± 0.5</td>
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<td>10.8 ± 0.3</td>
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<td>19.0 ± 4.0</td>
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<td>3.1 ± 0.4</td>
<td>11.1 ± 0.4</td>
<td>7.8 ± 0.9y</td>
<td>80</td>
<td>14.3 ± 0.8</td>
<td>19.3 ± 4.2</td>
<td>87</td>
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<td>16.6 ± 2.7</td>
<td>87</td>
</tr>
</tbody>
</table>

**Table 3.2:** Larval length (mean ± SD), weight (mean ± SD), and frequency of gas bladder inflation (presented as % of gas bladders that were partially or completely inflated) at 9, 20, and 32 days post hatch (DPH) for walleye embryos treated with iodine at 0 (control) or 100 mg/L for 30 min. Different letters indicate significant differences based on treatment (P<0.05).
Figure 3.1: Relationship between egg weight (unfertilized) and embryo survival (eyed-embryo stage) in walleye treated with 0 (control) or 100 mg iodine/L for 30 min following fertilization. Linear regression was fitted to the iodine-treated data and is significant at $P < 0.05$. Analysis of linear regression for the control group was not significant, and therefore, not included in the figure. Female 16 was not used in calculations.
Figure 3.2: Survival of control and iodine treated walleye embryos at the eyed-embryo stage for individual walleye females with different letters indicating a significant difference between treatments for individual females (P < 0.05). Bars indicate standard deviation.
Figure 3.3: Mean survival of fertilized walleye eggs to the eyed-embryo stage following treatment with iodine at 0 (control) or 100 mg/L. A. Mean survival to the eyed-embryo stage of the unstressed group 1 females ($N=6$). B. Mean survival to the eyed-embryo stage of stressed group 2 females ($N=10$). Different letters indicate significant differences between treatment groups ($P < 0.05$), and bars represent standard deviation.
Figure 3.4: The gills of the walleye from 9 dph larvae have gill arches (A) and developing filaments (dF) (A). Differentiated filaments (F) and secondary lamellae (sL) are observed in fish at the age of 32 and 48 dph.
Figure 3.5: The histological depiction of normal and pathological processes of swim bladder (SB) inflation in walleye larvae and juveniles.
Chapter 4: Conclusions

The objectives of this thesis were to control disease transmission, increase growth, increase survival, and improve hatching and rearing techniques in the early developmental stages of two important sport species, sunshine bass and walleye, in Ohio. These projects were performed in collaboration with the Ohio Department of Natural Resources (ODNR), Division of Wildlife (DOW). The projects expanded on previous methods for fertilization and controlling disease transmission.

4.1 Sunshine bass

4.1.1 Propagation

As a pelagic predator, sunshine bass have become a desired sport fish and an important management tool in Ohio reservoirs. However, there have been difficulties raising this species in Ohio hatcheries. Direct evidence demonstrating the difficulty of producing sunshine bass in Ohio can be seen in the failed attempts and low survival of our lab (spring 2009 and 2010) and the state fish hatcheries over the past few years (D. Sweet, Ohio Division of Wildlife, personal communication). Personal communication with successful sunshine bass producers in Illinois and in the southeastern U.S. indicates that there may be a geographic component to the difficulties in Ohio. Despite this potential factor, in our experience there were three key difficulties in raising sunshine bass: 1) adhesiveness of eggs, 2) disinfectant techniques, and 3) condition of parents and gametes.
One of the major problems with the production of sunshine bass is the severe adhesiveness of the eggs. Following fertilization, eggs clump together which results in low survival rates. Tannic acid and a cleansing solution of urea and sodium chloride were explored to remove the adhesiveness of the eggs, but these have been met with varying success. Another important aspect of sunshine bass production in Ohio is controlling pathogen transmission. The female white bass used as broodstock are often collected from waters containing a variety of pathogens, including VHS. Thus, to reduce the possibility of transporting these pathogens to water bodies around the state, the fertilized embryos must be disinfected. Iodine is often used as the disinfectant, but iodine treatment may compound the effects of already relatively ineffective fertilization techniques that result in poor survival. Lastly, the condition of the parents and their gametes seems to be an important factor from our experience. In the first year (2009), female white bass were collected late in the spawning season, and were in poor health. This could have contributed to our poor results. In the second year (2010), female white bass were collected early in the spawning season when they were not fully mature, and once they were placed in holding tanks and injected with a releasing hormone in the lab, the eggs did not seem to develop properly or the females would not release them. In addition, maintaining healthy striped bass males for fresh sperm is difficult. Thus, coordinating the spawning of white bass females and striped bass males for fresh gametes may be a critical factor in production.

Considerable research is still required to improve fertilization and disinfectant techniques in Ohio to increase survival and early development of sunshine bass. It was
our intention to improve these techniques, but due to the failed fertilizations in 2009 and 2010, we were only able to make observations to difficulties we encountered, and hypothesize possible solutions. However, a substantial amount of knowledge was still discovered in our failed attempts. The experience gained from attempting to produce sunshine bass will help develop better techniques for this valuable sport fish.

4.1.2. Diet formulation

Fishmeal and fish oil are still the ideal source of protein and lipid in formulated feeds which can be seen in the high growth rate and survival of the high nutrient, fishmeal-based commercial diet in our experiment, but with the price of fishmeal and fish oil rising, comparable cost-effective alternatives in feeds are highly desirable (Naylor et al. 2009). A viable alternative must meet certain criteria: nutritionally suitable, readily available, easily processed and shipped, minimal ecosystem stress, and competitively priced (Naylor et al. 2009). Plant–based and terrestrial animal-based (such as poultry by-products) protein diets have shown promise, but desired fatty acid profiles are often compromised (Trushenski and Boesenberg 2009). However, a finishing feed that contains fishmeal and fish oil can restore these desired traits (Trushenski and Boesenberg 2009). The end product of fish in aquaculture must be considered. If the end product is food fish, fish fed a plant-protein based diet need to include a finishing feed that contains fishmeal and fish oil prior to harvest to restore desired fatty acid profiles that consumers demand, but this is much less important if the fish are being released into reservoirs for sport fishing or as a management tool. Further research is still necessary to understand
the impact of replacing fishmeal and fish oil in feeds. Continued research of specific
dietary requirements for sunshine bass will help identify alternatives to fishmeal and fish
oil, and potentially improve productivity and operating costs.

4.1.3. Sport fishing

Sunshine bass is a popular fish stocked in rivers and reservoirs for sport fishing
across the United States, but it is particularly favored in reservoirs because of its
management potential, usually controlling forage populations such as gizzard shad (Ney
et al 1990). Reservoirs are constructed for a variety of reasons, although seldom built for
fishing and other recreational activities (Ney et al. 1990). However, these recreational
benefits and activities are often cited to increase public acceptance of these projects.
Some of these projects have produced trophy sunshine bass fisheries, but stocking
success has been highly variable (Axon and Whitehurst 1985). Self-sustaining
populations have rarely been established, and thus, agencies maintain fisheries on a put-
grow-take basis through annual stocking programs (Sutton and Ney 2001). Even with the
difficulty of establishing satisfactory sport fisheries of sunshine bass in some regions,
these programs are still pursued by agencies because of angler demands. The sunshine
bass is a favored sport fish because of its fighting capability, aggressive behavior, and
ability to reach sizes over ten pounds. It has become a particularly important species in
areas where anglers have become accustom to catching it and where trophy fisheries have
been established.
4.2 Walleye

4.2.1. Propagation

The project focused on a pathogen, viral hemorrhagic septicemia virus (VHS), which could be transported to various Ohio water bodies through the state’s walleye stocking programs. VHS is a severe disease that can result in large scale fish kills. It has been in the Great Lakes since 2003, at least, and it has shown an ability to affect a wide variety of species. Concerns of large scale fish kills of economically import fish species, including walleye, forced the ODNR to take action. A top priority of the ODNR was securing the health and avoiding disease transmission of walleye broodstock used in stocking and management programs. Thus, the project was designed to find a safe disinfectant for stocking programs to treat walleye broodstock and their gametes for VHS.

Walleye rearing techniques are much more developed than sunshine bass, and few difficulties were experienced. Standardized methods have been developed for fertilization and removing the adhesiveness of walleye eggs, and obtaining healthy broodstock for stocking programs is accomplished much more easily. The experiment focused on disinfecting fertilized walleye eggs with iodine to protect the valuable sport fishing industry associated with this species in Ohio. The results indicated that walleye embryos from unstressed females can be treated with iodine to eliminate VHS without compromising survival or growth. This is an important step in protecting the popular sport fish.
4.2.2. Sport fishing

Walleye have become a major recreational industry in Ohio on Lake Erie as well as in the rivers and reservoirs throughout the state. Walleye angling generates a substantial economic impact directly (licenses and fishing gear) and indirectly (purchases at local businesses: boat gas, hotels, and meals) in Ohio. All of these activities create a significant amount of income for local businesses in this region. With the growing popularity of walleye as a sport fish and the economic impact the industry has on local businesses, pressure from anglers and local businesses that depend on this industry forced the ODNR to carefully manage this valuable resource. In response, the ODNR began funding projects to protect this important industry.

4.3 Closing thoughts

The information within this thesis can be used by hatchery facilities to aid in the control of disease transmission and increase production of sunshine bass and walleye. To improve hatchery operations, future studies will need to develop better fertilization techniques for sunshine bass in Ohio, improve diet formulations for sunshine bass, and explore swim bladder non-inflation in walleye. Collaborative projects will continue to be a focal point and an effective tool for improving aquaculture procedures to protect our valuable fisheries resources.
4.4 References


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