THE EFFECTS OF HYDROGEN PEROXIDE TREATMENTS ON THE HATCHING SUCCESS OF WALLEYE EGGS INFECTED WITH OOMYCETE PATHOGENS AND THE GROWTH OF THOSE PATHOGENS IN A GEORGIA AQUACULTURE SYSTEM

by

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(Under the Direction of Cecil A. Jennings)

ABSTRACT

Infections of walleye eggs by organisms of the family Saprolegniaceae have been implicated in instances of poor hatching success experienced by the Georgia Department of Natural Resources. In 2018 and 2019, the effectiveness of various hydrogen peroxide treatment regimens on the hatching success of incubating walleye eggs was tested. Three hydrogen peroxide concentrations (100, 250 or 500 mg/L) and two exposure frequencies (once or twice daily) were tested. Results showed improved hatching success in systems treated with 100 mg/L hydrogen peroxide in 2018 but not in 2019. There was no effect of treatment frequency and no interaction effect between concentration and frequency in either year. Quantification of zoospores over the course of both experiments based on qPCR methodologies was dissimilar to observed hyphal growth and was unaffected by any hydrogen peroxide treatment. DNA sequencing of hyphae revealed that *Aphanomyces laevis* is a pathogen associated with walleye for the first time.

INDEX WORDS: *Aphanomyces*, Fish Eggs, Fungicide, Hatchery, Larval Fish, , Parasiticide, Quantitative PCR, *Saprolegnia*, Saprolegniasis

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....iv

TABLE OF CONTENTS.....v

LIST OF TABLES.....vii

LIST OF FIGURES.....viii

CHAPTER 1: INTRODUCTION.....1

CHAPTER 2: LITERATURE REVIEW.....6

An introduction to walleye.....6

Walleye reproduction.....7

Walleye in Georgia.....11

Saprolegniasis: “fungal” infections in hatching jars.....14

Treatments for saprolegniasis.....16

Hydrogen peroxide treatments for saprolegniasis in walleye eggs.....20

Flow-through vs recirculating systems.....21

Evaluation of pathogen growth.....22

CHAPTER 3: METHODS.....26

Facilities and system design.....26

Experimental design.....27

Walleye spawning.....28

Hydrogen peroxide treatment of walleye eggs.....30

Quantification of hatch success.....33

Quantification of pathogen density and growth.....34
Pathogen identification.....39
Statistical analyses.....40
CHAPTER 4: RESULTS.....41
Hatching success and egg viability.....41
Standard curve generation.....43
Pathogen density and growth.....44
Pathogen identification.....45
CHAPTER 5: DISCUSSION.....47
Hatching success and egg viability.....47
Pathogen density and growth.....52
Summary and Conclusions.....58
LITERATURE CITED.....60
TABLES AND FIGURES.....78
APPENDICES.....100

LIST OF TABLES

Table 1. Hydrogen peroxide treatments that have been reported in peer-reviewed literature to increase hatching success in the eggs of a variety of freshwater fish species.....78

Table 2. Treatment combinations randomly assigned to specific hatching units in 2018 (rows 2-22) and 2019 (rows 23-46) to evaluate the efficacy of hydrogen peroxide concentration and dosing frequency of hatch success of fertilized walleye eggs.....79

Table 3a. Data collected on female walleye used for spawning at the Go Fish Center in Perry, GA. Eggs from these females were used to evaluate the effects of hydrogen peroxide concentration and dosing frequency on the hatch success of fertilized walleye eggs during 2018 and 2019.....81

Table 3b. Data collected on eggs produced by female walleye collected at the Go Fish Center in Perry, GA. These eggs were used to evaluate the effects of hydrogen peroxide concentration and dosing frequency on the hatch success of fertilized walleye eggs during 2018 and 2019.....81

Table 4. Primers and probes used for qPCR quantification of *Saprolegnia* abundance in hatching systems being used to evaluate the effects of hydrogen peroxide concentrations and dosing frequencies on the hatching success of fertilized walleye eggs. The primers are based on those used by Rocchi et al. (2017).....82

Table 5. The top 10 GenBank® sequence comparisons based on total BLAST score for a DNA sequence amplified using universal ITS primers 1 and 4 and DNA extracted from a pathogen cultured from walleye eggs that were used in an experiment evaluating the effectiveness of hydrogen peroxide concentration and dosing frequency on hatching success of those fertilized eggs.....83

LIST OF FIGURES

Figure 1. Schematic diagram of a hatching system used to incubate fertilized walleye eggs during 2018-2019. Each individual hatching system consisted of a McDonald-style hatching jar (A), a 5-gallon sump (B), a mesh basket (C) designed to catch larval fish, plastic bioconversion balls (D) and a pump (E). The cycle of water-flow through the system is indicated by arrows.....84

Figure 2. Spatial orientation of experimental walleye hatching systems are shown for 2018 (top) and 2019 (bottom). Large rectangles represent chilled water baths in which the sumps for each hatching system, represented by large circles, were immersed. Numbers marked on the sumps represent their unit number determined randomly by four flips of a coin. Small circles represent the hatching jars associated with each system.....85

Figure 3. Daily water temperatures recorded in each walleye hatching system every morning during the experiments. Horizontal lines at 9 °C and 15 °C represent the lower and upper temperature limits for optimal walleye production as described by Bozek et al. (2011b).....86

Figure 4. Daily dissolved oxygen concentrations recorded in each walleye hatching system every morning during the experiments. The horizontal line at 6 mg/L represents the lower dissolved oxygen limit for optimal walleye production as described by Bozek et al. (2011b).....87

Figure 5. Daily pH values recorded in each walleye hatching system every morning during the experiments. Horizontal lines at pH values of 6 and 9 represent the lower and upper pH limits for optimal walleye production as described by Bozek et al. (2011b).....88

Figure 6. The mean percent hatch (± SE) of walleye eggs for each treatment regimen for the 2018 experiment. Points marked with different letters are significantly different.....89

Figure 7. Mean percent hatch (± SE) of fertilized walleye eggs in four treatment concentrations from the 2018 experiment. Bars marked with different letters are significantly different.....90

Figure 8. The mean percent viability (i.e., eyed eggs and hatched larvae at the end of the experiment; ± SE) of walleye eggs for each treatment regimen for the 2018 experiment. Points marked with different letters are significantly different.....91

Figure 9. The mean percent viability (i.e., eyed eggs and hatched larvae at the end of the experiment; ± SE) of fertilized walleye eggs in four treatment concentrations from the 2019 experiment. Bars marked with different letters are significantly different.....92

Figure 10. The mean percent hatch (± SE) of walleye eggs for each treatment regimen for the 2019 experiment. None of the treatment combinations differed significantly from one another (all $p > 0.05$).....93

Figure 11. The mean percent viability (i.e., eyed eggs and hatched larvae at the end of the experiment; ± SE) of walleye eggs for each treatment regimen for the 2019 experiment. None of the treatment combinations differed significantly from one another (all $p > 0.05$).....94

Figure 12. The mean $\log_{10}$ of starting quantities (± SE) of DNA from filters with known, 10-fold dilutions of zoospores is depicted. A best fit line for a linear regression of these data was generated and is displayed along with its equation, $R^2 = 0.9777$.....95

Figure 13. The $\log_{10}$ adjusted mean number of zoospores per 100 mL (± SE) for each treatment combination and sampling day during the 2018 experiment to evaluate the effects of hydrogen peroxide on pathogen growth on fertilized walleye eggs.....96

Figure 14. The $\log_{10}$ adjusted mean number of zoospores per 100 mL (± SE) for each treatment combination and sampling day during the 2019 experiment to evaluate the effects of hydrogen peroxide on pathogen growth on fertilized walleye eggs.....98

CHAPTER 1:

INTRODUCTION

Walleye (*Sander vitreus*) are large, cool-water sportfish popular among anglers throughout most of their range (Mitchill 1818; Nelson et al. 2003; Quinn 1992). Although most commonly associated with the inland waters of the northern United States and Canada, and thermally limited at the southern end of its range, walleye are native to parts of North Georgia (Page and Burr 2011; VanderKooy and Peterson 1998). Historically, walleye in Georgia inhabited rivers in the Tennessee and Coosa river basins, but these native fish declined significantly in numbers because of environmental changes that led to a loss of spawning habitat and overfishing (Georgia Department of Natural Resources Fisheries Management Section 2018). However, over the past six decades the Georgia Department of Natural Resources (GADNR) has been responsible for operating walleye stocking programs to establish and maintain recreational walleye fisheries in mountain reservoirs (Bednarski et al. 2010; Rabern 1998). As of 2019, 11 impoundments are stocked with walleye by GADNR, both inside and outside the walleye's native range in the state (Georgia Department of Natural Resources Fisheries Management Section 2018). The current stocking program was established in 2002 and, in addition to focusing on recreational fishing goals, was intended to aid in the control of illegally introduced blueback herring populations. As a result of the program, interest in walleye angling has grown in the state. Anglers frequently target fish as they prepare to spawn in rivers during the spring runs, but walleye can be caught in the lakes throughout the year (Georgia Department of Natural Resources Fisheries Management Section 2018).

Each spring GADNR biologists collect broodfish from reservoirs with sufficiently large walleye populations. Following collection, walleye are shipped to a hatchery in Perry, GA where they are spawned and the resulting fertilized eggs hatched in McDonald style hatching jars. Hatched fry are then shipped to other GADNR hatcheries (various combinations of Walton, Burton, Summerville, McDuffie and Richmond Hill depending on the year) where they are reared in grow-out ponds until they reach stocking size of 35-40 mm and can be transported to reservoirs for stocking. Unfortunately, in the past, hatching success at GADNR facilities has been inconsistent. This issue has been attributed to saprolegniasis, a microbial infection that is common in aquaculture operations and which can be devastating for hatching facilities if not properly controlled (Clint Peacock, GADNR, personal communication).

Most fish species and their eggs are easily infected by organisms of the family Saprolegniaceae, commonly of the genera *Saprolegnia*, *Achyla*, *Dictyuchus* and *Aphanomyces* (Alderman and Polglase 1984; Gaikowski et al. 2003; Van Den Berg et al. 2013). Walleye eggs have only been reported to be infected by organisms of the genus *Saprolegnia*. These organisms, once considered to be fungi because of superficial similarities between the hyphal structures of Saprolegniaceae and true fungi, are ubiquitous in freshwater throughout the world (Johnson Jr. et al. 2002). In natural environments, Saprolegniaceae species are a natural and important part of the ecosystem. These organisms break down dead and unfertilized walleye eggs that are scattered across the benthos, aiding in nutrient cycling (Van Den Berg et al. 2013). Under these circumstances, live eggs will not typically be infected. However, when eggs are kept in high densities in hatcheries, the hyphal growth on dead embryos and unfertilized eggs can spread to live embryos and kill them (Smith et al. 1985). In the worst-case scenarios entire hatching jars of eggs can perish.

There are existing methods for the treatment of saprolegniasis in hatcheries. The most straightforward of these is the removal of dead and infected eggs, though this is both labor intensive and not as effective as chemical treatments (Barnes et al. 2003; Piper et al. 1982). Malachite green, an industrial dye, was once considered the standard for chemical control of saprolegniasis (Van West 2006). However, once studies revealed that malachite green had carcinogenic and teratogenic properties, it was banned by the Food and Drug Administration (FDA) (Fitzpatrick et al. 1995). Currently, formaldehyde and hydrogen peroxide are the only two chemicals approved by the FDA as parasiticides for walleye eggs (United States Food and Drug Administration 2019).

GADNR uses a recirculating aquaculture system to hatch walleye eggs. All hatching jars are set up in parallel and use the same water source which is held in a single large head tank housed above the hatching jars. The water flows through by gravity to pipes where the water is diverted to each jar of eggs. Once the water flows out of the jar, it is collected in a tank with the water from approximately four other jars. The water from these tanks flow into a communal sump. Water from the sump is then filtered, undergoes a bioconversion process and is passed through an ultraviolet light in an attempt to kill remaining pathogens (Clint Peacock, GADNR, personal communication).

GADNR uses hydrogen peroxide as a treatment agent for their walleye eggs. Because hydrogen peroxide breaks down into non-toxic materials quickly, it is preferred to formalin which would persist in the system longer, possibly resulting in toxicity to. During the hatching period, walleye eggs are exposed to hydrogen peroxide on a regular basis as needed (Clint Peacock, GADNR, personal communication). Hydrogen peroxide is added to the head tank and sump to be distributed to the jars of eggs. Because the jars are set up in parallel, the water

entering each of the jars has the same effective dose of hydrogen peroxide as every other. Hydrogen peroxide is not diluted or decomposed at different rates depending on jars' relative positions. Treatment continues until hatching begins, after which it ceases because the hydrogen peroxide would be toxic to the newly hatched larvae. Because the system is composed of a single water source, jars cannot be treated individually. Therefore, eggs spawned later in the season will not receive hydrogen peroxide treatment after hatching of previously incubated eggs begins and will likely experience greater losses to saprolegniasis.

Although walleye are a commonly cultured and studied species, relatively few studies have evaluated the effects of various concentrations of hydrogen peroxide on hatching success of walleye eggs (Gaikowski et al. 2003; Rach et al. 1998; Soupir and Barnes 2006). Additionally, none have evaluated the effects of treating eggs with hydrogen peroxide at a frequency greater than once per day. Despite walleye occupying a large range in North America, the studies that have examined the effects of hydrogen peroxide treatments on walleye hatching success all have taken place in the heart of the species' range in the states of Illinois, South Dakota and Wisconsin (Gaikowski et al. 2003; Rach et al. 1998; Soupir and Barnes 2006). Such studies have not evaluated the effects of hydrogen peroxide on walleye hatching success anywhere on the fringe of the walleye's range, such as Georgia. Because walleye are limited in their southern distribution partially by temperatures too warm for consistent reproduction, southern walleye may experience sufficiently warm water temperatures or other environmental conditions to cause gametes to develop sub-optimally when compared to their northern counterparts. This disparity in egg quality could affect the efficacy of hydrogen peroxide treatments of walleye eggs. For instance, there may be an initial percent viability threshold that a set of eggs must exceed for hydrogen peroxide treatments to reduce pathogen growth to an extent that will improve hatching

success. Hatcheries in these regions may be more important for maintaining walleye fisheries because of reduced capacities for wild spawning. Further, whereas the direct purpose of hydrogen peroxide treatment is to reduce the growth of Saprolegniaceae, hardly any studies (of any species) attempt to evaluate growth of the pathogen in any way (Barnes et al. 1998; Straus et al. 2019; Thoen et al. 2016). In the few studies that have tried to evaluate pathogen growth, outputs were qualitative or only semi-quantitative. Therefore, this work was undertaken to evaluate the effects of hydrogen peroxide treatment concentration and frequency on both walleye hatching success and pathogen growth in hatchery operations in Georgia.

CHAPTER 2:

LITERATURE REVIEW

An Introduction to Walleye

The walleye (*Sander vitreus*) is a large, predatory sportfish that is popular among anglers throughout most of its range (Mitchill 1818; Nelson et al. 2003; Quinn 1992). Native to the United States and Canada, walleye naturally occur in an extensive range that includes fresh waters north of Hudson Bay reaching south to warmer waters in parts of the U.S. states of Georgia, Alabama and Mississippi (Hartman 2009; Page and Burr 2011; Scott and Crossman 1973; Trautman 1981; VanderKooy and Peterson 1998). Walleye require sufficiently cool water, discussed in greater detail later in this chapter, that is high in dissolved oxygen to thrive, a factor which limits their southern range (Bozek et al. 2011b; Colby and Nepszy 1981; Hokanson 1977). Due largely to their delectable meat, walleye have been introduced widely outside of their native range to support sport fisheries (Georgia Department of Natural Resources Fisheries Management Section 2018; Kerr 2011). These introductions have been made both by state agencies seeking to establish fisheries and illegally by anglers hoping to do the same (Kerr 2011; McMahon and Bennett 1996). Walleye are also introduced on occasion to control populations of other sportfishes, such as yellow perch (*Perca flavescens*) and sunfishes (*Lepomis spp*.), through predation with the intention of increasing the average size of these sportfish (Kerr 2011; Rabern 1998). Historically, some walleye populations have supported commercial fisheries, primarily in the Great Lakes (Locke et al. 2005; Smith Jr. and Krefting 1954). However, overfishing of

walleye on a commercial scale has led to the extinction of populations and subspecies (e.g., blue pike) and has resulted in significantly reduced commercial pressure (Parsons 1967). Nevertheless, walleye are relatively abundant throughout their range and were classified as a species of least concern by the International Union for the Conservation of Nature when last assessed in 2012 (NatureServe 2013).

Walleye naturally inhabit both lentic (still, fresh water) and lotic (flowing fresh water) systems (Bozek et al. 2011b). Optimum water temperatures for walleye growth exist between 18 °C and 24 °C; 31 °C is the reported thermal maximum for the species (Christie and Regier 1988; Hokanson 1977; Wismer and Christie 1987). Also, water must reach temperatures below 12 °C for walleye gametes to mature properly (Collette et al. 1977). These thermal requirements are primary factors restricting the southern distribution of walleye (Bozek et al. 2011b). Dissolved oxygen is related to water temperature and adult walleye prefer dissolved oxygen concentrations above 5 mg/L, although this requirement may be higher in warmer waters (Bozek et al. 2011b). Walleye can survive for long periods in water with dissolved oxygen concentrations as low as 3 mg/L, but concentrations below 1 mg/L are lethal (Barton and Taylor 1996; Scherer 1971).

Walleye Reproduction

Walleye are one of the earliest spawning fishes each spring, typically making runs to spawning grounds soon after ice-off in regions where ice occurs and occasionally spawning under ice in northern latitudes (Bozek et al. 2011a). Walleye males mature at an earlier age and size than do females in the same population (Barton and Barry 2011). However, there is great variability in the age of maturation among populations with reported ages of maturity ranging from 2 to 15 years (Morgan et al. 2003; Venturelli et al. 2010). In the southern end of their range, spawning can begin as early as February and run as late as July in the most northern latitudes

(Becker 1983; Hokanson 1977; Malison and Held 1996a; Scott and Crossman 1973). Spawning timing is dictated by both water temperature and photoperiod, which combine to spur annual gamete maturation in adult fish; however, temperature alone is responsible for cueing the final spawning event (Bozek et al. 2011a; Malison and Held 1996a). Optimal temperatures for spawning initiation vary among stocks but generally range from 3 °C to 7 °C (Barton and Barry 2011; Becker 1983). Peak spawning activity also varies among populations and ranges from 6 °C to 11 °C (Barton and Barry 2011; McPhail and Lindsey 1970; Nelson and Paetz 1992; Scott and Crossman 1973).

Incubation temperatures from 9 °C and 15 °C optimize hatch success with hatching reaching its peak on the warmer end of that range (Engel et al. 2000; Koenst and Smith Jr. 1976). Dissolved oxygen concentrations above 5-6 mg/L are optimal for the incubation of walleye eggs. Development occurs best in waters with pH values ranging from 6.0 to 9.0 (Bergerhouse 1992; Bozek et al. 2011b; Holtze and Hutchinson 1989; Hulsman et al. 1983; Oseid and Smith Jr. 1976). pH values below 6.0 are associated with high rates of embryonic walleye mortality (Hulsman et al. 1983; Lynch and Corbett 1980; Peterson et al. 1983; Rahel and Magnuson 1983).

When temperatures and day lengths cue spawning, walleye will migrate to suitable spawning habitat. Lake-resident walleye may spawn off rocky shores, points or reefs in the lake or migrate into a river to do so (Eschmeyer 1950; Johnson 1961; Raabe 2006). River-resident walleye will not migrate to lakes to spawn but will move to preferred spawning habitat that is characterized by rapid water with large gravel substrate (Scott and Crossman 1973; Stevens 1990). There are exceptions to this trend in areas where such habitat is not present (Ickes et al. 1999; Priegel 1970). Typically, male walleye will arrive at spawning grounds before females and individual males will stay longer than will females (Becker 1983; Ellis and Giles 1965). Walleye

do not build nests or provide parental care for eggs, larvae or fry; instead, they broadcast their 1.3-2.1 mm diameter eggs over rocky substrates (Colby et al. 1979; McElman 1983; Smith 1941). Low first-year survival (<1%) is offset by high female fecundity (60,000 - 120,000 eggs per kilogram of body weight) which can equate to over 600,000 eggs from a given female (Baccante and Colby 1996; Nickum 1986; Wolfert 1969). Walleye typically are nocturnal spawners and some may exhibit spawning site fidelity to their natal sites (Colby et al. 1979; Ellis and Giles 1965; Eschmeyer 1950).

In the wild, walleye embryos typically hatch in 10 - 27 days (Engel et al. 2000; Johnson 1961; Priegel 1970). In a laboratory setting, hatching time is variable, with a range of 5 - 30 days depending on water temperature (Hurley 1972; Koenst and Smith Jr. 1976; McElman and Balon 1979). The time required for walleye eggs to hatch is related to the sum of thermal units (time units spent at various temperatures, e.g., degree days) that the eggs have experienced. When eggs are incubated at a constant 15 °C, hatching will occur after 135.0 degree days (McElman and Balon 1979). Meanwhile, eggs incubated in temperatures that fluctuated between 7.8 °C and 11.1 °C only began to hatch after 194.9 degree days, which illustrates great variability in the actual number of thermal units required to hatch walleye eggs among systems (Hurley 1972). Walleye larvae become free swimming within a couple days of hatch and will begin exogenous feeding quickly thereafter, before the yolk sac is completely absorbed (Becker 1983; Engel et al. 2000). The availability of sufficient food (e.g., zooplankton) soon after hatch is essential for their survival (Eschmeyer 1950; Mathias and Li 1982). Without enough zooplankton, probability for a weak year class increases (Jonas and Wahl 1998). Fry will become cannibalistic if there is insufficient food and enough size variation among them to allow this to take place (Chevalier 1973).

Most state and provincial agencies intercept walleye in the wild as they prepare to spawn and collect broodstock that will be spawned artificially in hatchery conditions, reared to various sizes and stocked into the wild to improve recreational fisheries (Summerfelt et al. 2011). Stocking can fall under one of three categories described by Laarman (1978). Introductory stocking occurs in waters where walleye do not exist; maintenance stockings occur in waters where walleye are present but are unable to reproduce; and supplemental stockings take place in waters where walleye do spawn but where more fish are desired to support the number of anglers seeking to catch the fish (Laarman 1978).

A typical stocking program will collect male and female walleye each spring proportional to the number of eggs required to produce a desired number of fry. Common methods of collection include fyke nets, gill nets, trap nets and electrofishing (Satterfield Jr. and Flickinger 1996). Male walleye will usually produce milt easily during the spawning season; however, females will not always have mature eggs (i.e., be ripe) upon collection (Summerfelt et al. 2011). Whereas some agencies will wait until they are catching ripe females, others may opt to hold the fish in captivity until they are ready to spawn, particularly if the population being sampled is small (Malison and Held 1996b; Satterfield Jr. and Flickinger 1996). The final development of eggs in females may be increased by using intramuscular injections of hormones including human chorionic gonadotropin, luteinizing hormone releasing hormone and carp pituitary extract (Barry et al. 1995; Barton and Barry 2011; Hearn 1980; Lessman 1978; Malison and Held 1996b).

Although artificial propagation procedures may vary among hatcheries, the following briefly describes a representative hatching procedure (Malison and Held 1996b; Summerfelt et al. 2011). Eggs from one or more female walleye are stripped into a dry bowl to which milt from

three or four male walleye is added. The milt and eggs will be gently mixed by stirring to increase the chances of fertilization. At some point in the mixing, water will be added to the mixture to activate the sperm and simultaneously begin the closing of the micropyle of the egg. Diatomaceous earth (historically, "pond muck") will then be added to prevent the eggs from clumping together (Nevin 1900).

Hardened eggs will be rinsed, subsampled for enumeration, and the rest placed in a McDonald-style hatching jar (Summerfelt et al. 2011). Tens to hundreds of thousands of eggs (volume usually 1-3 liters) will tumble gently in the jar for 1-2 weeks (Malison and Held 1996a; Summerfelt et al. 2011). Once eggs begin to hatch, larvae will swim to the surface to inflate their swim bladders and will be swept out of the jar by water flow. Larval walleye will be collected in tanks below the jars. Once yolk sacs are absorbed, larvae hatched at the same time will be stocked into ponds to be fed and grown to stocking size (Summerfelt et al. 2011). Walleye are stocked at sizes ranging from post yolk sac fry to adult fish over three years old (Kerr 2011). This process, though not 100% efficient, should result in survivorship many hundreds of times higher than would be expected of eggs spawned in the wild.

Walleye in Georgia

Walleye once occurred naturally in the rivers that flow into the Tennessee River and Coosa River systems of North Georgia. These fish were likely extirpated because of habitat degradation and modification, and possibly overfishing (Georgia Department of Natural Resources Fisheries Management Section 2018). However, in the 1960s broodstock from Ohio, Pennsylvania and Tennessee were used to produce walleye fry for reintroduction in waters across the northern part of the state, both within and outside the native range (Bednarski et al. 2010; Rabern 1998). In most cases, walleye were stocked into reservoirs that had been created in the

southern Appalachian Mountains to generate hydropower (Rabern 1998). In theory, these reservoirs provided deep, cool, well-oxygenated water that could support walleye throughout the sweltering southern summers. However, of the 12 reservoirs into which walleye were introduced, only three managed to continually support populations (Rabern 1998). In these three reservoirs, stocking occurred from 1960-1969. Walleye were caught by anglers with relatively high frequency in these reservoirs during the next decade-and-a-half until the populations crashed in the mid-1980s because of a loss of spawning habitat for walleye in the systems (Fatora and England 1982; Rabern 1998).

In the early 1990s, another attempt was made to create walleye fisheries in North Georgia reservoirs (Bednarski et al. 2010; Rabern 1998). Lake Burton and Lake Seed on the Tallulah River were stocked with walleye, which were unintentionally introduced to Lake Rabun (also on the Tallulah River) during a major flooding event. Walleye did not become established in Lake Burton, but they did become established in Lake Seed with some natural reproduction documented in the tailrace of Lake Burton. Walleye introduction was intended to improve yellow perch fishing in the lakes by reducing perch density and allowing more individuals to reach catchable size (Rabern 1998). Walleye initially consumed the perch exclusively, but their primary diet switched to blueback herring (*Alosa aestivalis*) after they were illegally introduced to the systems. As a result, the yellow perch fishery was unaffected by the presence of walleye (Rabern 1998).

Throughout the 1990s, blueback herring continued to be introduced to waters across northern Georgia. This trend, along with continued declines in walleye numbers, prompted GADNR to launch its current walleye stocking program in 2002 (Georgia Department of Natural Resources Fisheries Management Section 2018). Although walleye were not able to control

blueback herring populations, the stocking program continued and the popularity of walleye as a sportfish continues to grow in Georgia. As of 2019, walleye are stocked annually in 11 impoundments inside and outside of their native range across the northern part of the state. Reservoirs in the walleye's native range include lakes Chatuge and Blue Ridge in the Tennessee River drainage and Carter's Lake and two reservoirs in the Rocky Mountain Public Fishing Area in the Coosa River drainage. Walleye were introduced in lakes Seed, Rabun, Tugalo, Yonah and Hartwell in the Savannah River drainage and Lake Lanier in the Chattahoochee River drainage (Georgia Department of Natural Resources Fisheries Management Section 2018).

Personnel from GADNR use electrofishing boats to collect walleye in late February or early March for use as spawning broodstock (Zach Moran, GADNR, personal communication). These fish are shipped to the Go Fish Education Center in Perry, GA where they are spawned. Spawning procedures at the Go Fish Center are as described earlier in this section. A primary difference between most walleye hatching operations and that of the GADNR is that most hatcheries use a flow-through water source and the Go Fish Center hatches walleye on a single, large recirculating system (Clint Peacock, GADNR, personal communication). This difference is noteworthy because it affects how parasiticidal treatments of walleye eggs are applied. Two to four days after walleye are hatched, the larvae are enumerated using a fry-counter and shipped to hatcheries around the state. At these facilities, walleye are stocked in ponds where they will be grown for approximately 4 weeks to a size of 35-40 millimeters before being stocked into reservoirs across the northern part of the state. Typical annual targets for GADNR walleye production range from 1.1 to 1.3 million fry per year. Hatching success of eggs is usually around 20-30 percent, but hatching does not occur at all in some years (Clint Peacock, GADNR, personal communication).

Saprolegniasis: “Fungal” Infections in Hatching Jars

One of the many differences between wild and artificial hatching conditions experienced by walleye eggs is that eggs are kept at much higher densities in hatcheries than in the wild (Bozek et al. 2011a; Corbett and Powles 1986; Summerfelt et al. 2011). Additionally, despite hatchery personnel’s best efforts to optimize fertilization, there will almost certainly be eggs that are not fertilized. Along the way, there also will be many growing embryos that die for various, often unclear reasons. In natural environments, these dead and unfertilized eggs will become colonized by saprotrophic organisms that decompose them and recycle nutrients in the ecosystem (Van Den Berg et al. 2013). Oomycetes, commonly known as “water molds,” is a group of organisms commonly involved in this process are the. Aquaculturists sometimes refer to these fungal-like organisms as “cotton-wool” pathogens or incorrectly as “fungus.” Though frequently reported to not infect living tissues or eggs under normal conditions, exceptions exist (Hulvey et al. 2007; Johnson Jr. et al. 2002; Van Den Berg et al. 2013). For example, zoospores of *Saprolegnia diclina* have been reported to infect live brook trout eggs (Rand and Munden 1993). Circumstances in hatcheries are different from the wild because the hyphae can spread from dead eggs to living eggs in close proximity that become engulfed, die and perpetuate the infestation (Smith et al. 1985; Van Den Berg et al. 2013). In the worst cases, all the eggs in a hatching jar can be lost and result in a complete hatching failure.

Although long considered to be fungi, the taxonomy of these organisms has become confused and disputed in recent decades (Dieguez-Uribeondo et al. 2007; Johnson Jr. et al. 2002). The genus *Saprolegnia* belongs to the family Saprolegniaceae of the order Saprolegniales (Earle and Hintz 2014; Hulvey et al. 2007; Johnson Jr. et al. 2002). The Saprolegniales fall under class Oomycota which contains other parasites including the one responsible for the Irish potato

famine (Hulvey et al. 2007; Rossman and Palm 2006). Oomycetes are classified as Heterokonts (which also includes kelps and diatoms) which is variably considered to be either a phylum or infrakingdom (Rossman and Palm 2006).

The most common "cotton wool" fish pathogens in the temperate world belong to the genus *Saprolegnia* and are most frequently part of the *S. parasitica-S. diclina* complex (Dieguez-Uribeondo et al. 2007; Neish 1976; Van Den Berg et al. 2013). However, other organisms from closely related genera, including *Achyla* and *Aphanomyces*, can cause similar signs. Identifying the particular pathogen responsible for the infection can be difficult without using genetic techniques or in-depth microscopic evaluation by a well-trained mycologist. For the remainder of this thesis, all organisms of these genera will be referred to as *Saprolegnia*.

Saprolegnia has a life cycle that includes sexual and asexual reproduction at different stages (Van Den Berg et al. 2013; Van West 2006). Asexual reproduction consists of a zoospore being released from sporangia on the ends of the *Saprolegnia* hyphae. Zoospores can recognize signals in the water, including chemical traces from fish eggs, and migrate toward these sources (Rand and Munden 1993; Van Den Berg et al. 2013). However, the zoospore may not find a host, in which case it will encyst and produce a secondary zoospore. This new zoospore may encyst and release another zoospore, with this process (repeated zoospore emergence) continuing until a suitable substrate, such as a dead walleye egg, is found (Van Den Berg et al. 2013). Once this occurs, hair-like structures surrounding the zoospore will lock into the substrate and hyphae (the fibrous part of the organism from which the common name "cotton mold" is derived) will begin to grow.

At this point, *Saprolegnia* can either repeat the process of asexual reproduction or proceed with sexual reproduction. To initiate sexual reproduction *Saprolegnia* produce male

antheridia and female oogonia that will use fertilization tubes to fuse to one another. These structures are often the only reliable way to distinguish among *Saprolegnia* species without resorting to the use of molecular techniques and can be difficult to produce in a laboratory (Bly et al. 1992; Coker 1923; Van Den Berg et al. 2013). For this reason, even a trained mycologist may experience extreme difficulty determining which species of *Saprolegnia* is responsible for a particular infection. Following fertilization, a zygote, called an oospore, is created. This oospore is equipped with a thick wall that allows for survival over longer periods, potentially through relatively harsh environmental conditions, before germination occurs (Beakes and Bartinicki-Garcia 1989).

Saprolegnia are ubiquitous pathogens in the world's freshwater ecosystems and infect fish and other aquatic organisms at all life stages both in the wild and in captivity (Van Den Berg et al. 2013; Van West 2006). As mentioned above, dead organisms and necrotic tissues on living organisms are more likely to be infected first, but living tissues can be killed and consumed by *Saprolegnia* after the infection has begun. *Saprolegnia* infections can occur in water temperatures up to 30 °C, but show up with increasing frequency around 10 °C; reports have been made of infections occurring at even lower temperatures (Bly et al. 1992; Bly et al. 1993; Kitancharoen et al. 1996). Quick reductions in temperature and available nutrients are factors that promote increased zoospore production (Bly et al. 1992; Fuller and Jaworski 1987; Van Den Berg et al. 2013). Infections can also be associated with other environmental stressors, physical trauma or improper handling of fish.

Treatments for Saprolegniasis

Several methods can be used to reduce the growth of these pathogens in hatcheries (Burrows 1949; Marking et al. 1994; Summerfelt et al. 2011; Van Den Berg et al. 2013). The

most straightforward of these is the physical removal of dead eggs and clumps of infected eggs when they are observed in the system. Doing so will remove most hyphal growth from the hatching system and prevent the organisms from killing any live embryos; however, this process will not remove zoospores that will continue to infect embryos as they die during the hatching process (Piper et al. 1982). This egg-picking, as it is called, is labor intensive, time consuming and impractical for most large-scale hatcheries if not combined with other techniques to keep infestations at bay (Malison and Held 1996b). Further, egg-picking alone does not improve hatch success to the degree that chemical treatments will (Barnes et al. 2003; Gaikowski et al. 2003).

In addition to limited egg-picking, most hatcheries employ other methods of pathogen treatment, most of which involve exposing the eggs and pathogen to a chemical agent. For decades, the most common and most effective treatment chemical was malachite green, an industrial dye used to color silk, wool and paper, which also possesses antimicrobial properties (Burrows 1949; Foster and Woodbury 1936; Lone and Manohar 2018; O'Donnell 1947; Van Den Berg et al. 2013; Van West 2006). However, research later showed that malachite green and leucomalachite green (the metabolically reduced form which persists in fish tissues) have carcinogenic and teratogenic properties (Lone and Manohar 2018; Srivastava et al. 2004; Zhou et al. 2019). As a result, the use of malachite green in aquaculture was banned by the FDA in 1991 and has since been discontinued in aquaculture throughout much of the world (Fitzpatrick et al. 1995; Lone and Manohar 2018). However, because of its effectiveness and cheap production cost, malachite green is still used to reduce pathogen growth in parts of the world with less-stringent regulations (Srivastava et al. 2004; Zhou et al. 2019).

For most of the last century, copper sulfate has been known to have parasiticidal properties, though it was used less frequently than malachite green (O'Donnell 1947;

Schneberger 1941). Following the bans on the use of malachite green, however, interest in copper sulfate as a means of controlling pathogens in hatchery systems has increased, as has evidence of its efficacy. For example, a treatment of 40 mg/L copper sulfate produced optimal hatch success in channel catfish (*Ictalurus punctatus*) eggs being hatched in a flow-through system (Straus et al. 2009). Similarly, the highest hatching success of sunshine bass (female *Morone chrysops* x male *Morone saxatilis*) eggs was achieved with a 20 mg/L copper sulfate treatment for 10-minutes, twice-daily (Straus et al. 2016). Attempts to semi-quantitatively analyze the effects of copper sulfate on the growth of *Saprolegnia* on mats of largemouth bass (*Micropterus salmoides*) eggs suggest that increasing concentrations of the chemical likely reduce *Saprolegnia* growth up to a maximum tested concentration of 40 mg/L (Straus et al. 2019). Copper also has long been known to reduce infections by Oomycete pathogens of the genus *Phytophthora* in plant roots (Bangemann et al. 2014; Leach 1966). Technological methods that use water and electrolysis to introduce copper to treat these pathogens in hydroponic systems have been developed (Pettitt 2015; Toppe and Thinggaard 1998). However, copper sulfate and other copper-based treatments are not approved by the FDA for microbial control in finfish and is therefore not an option for state agencies hatching fish that will be available for public consumption (United States Food and Drug Administration 2019).

The FDA currently permits formalin (Formalin-F, Formacide-B and Parasite-S) and hydrogen peroxide (35% Perox-Aid) for use to control saprolegniasis in finfish in the United States, all of which are approved for use in walleye and their eggs (United States Food and Drug Administration 2019). Formalin exposure at specific concentrations improves hatch success optimally in rainbow trout (1000-1500 uL/L), common carp (1500-7500 uL/L), white sucker (1500-4500 uL/L), channel catfish (1500 uL/L) and many other aquaculturally important species

(Rach et al. 1997; Schreier et al. 1996; Watanabe 1940). In walleye, formalin improves hatching success at concentrations as low as 834 mg/L and as high as 1667 mg/L (Soupir and Barnes 2006). However, Rach et al. (1997) reported that 45-minute every-other-day treatments of formalin at concentrations of 1500 uL/L, 4500 uL/L and 7500 uL/L did not improve hatching success of walleye eggs compared to untreated controls . However, Rach et al. (1997) may not have observed improved hatch success because untreated eggs hatched at a relatively high percentage (mean = 63%). Formalin poses some human and environmental health risks, so some hatcheries prefer to use hydrogen peroxide instead (Masters 2004; Pedersen and Pedersen 2012; Pedersen et al. 2010; Wooster et al. 2005). Formalin also reduces the concentration of dissolved oxygen in a waterbody and should be used with caution in closed systems and at elevated water temperatures (Francis-Floyd 1996; Leal et al. 2018; Pedersen et al. 2010).

Hydrogen peroxide is the simplest substance possessing an O-O single bond and its antimicrobial properties result from its ability to dissociate into reactive hydrogen and hydroxyl radicals that attack lipids, proteins and nucleic acids (Bandyopadhyay et al. 1999). Because hydrogen peroxide breaks down naturally into oxygen and water, its proper use in aquaculture does not pose long-term environmental threats (Block 1991; Pedersen and Pedersen 2012). Additionally, while physical contact with hydrogen peroxide can be unpleasant, it is generally safer than formalin (Arvin and Pedersen 2015). For these reasons, hydrogen peroxide can be a good disinfectant for use in fish hatcheries as it can improve hatching success of eggs in several species of fish by reducing saprolegniasis (Gaikowski et al. 2003; Mitchell et al. 2009; Rach et al. 2004).

Many different treatment regimens of hydrogen peroxide improve hatch success in a variety of cultured fishes (Table 1). For example, Chinook salmon (*Oncorhynchus tshawytscha*)

eggs hatched at higher rates when treated daily for 15 minutes at a concentration of 700 mg/L hydrogen peroxide (Barnes et al. 2003). Largemouth bass eggs treated with 100 mg/L hydrogen peroxide twice daily had significantly higher hatch percentages than untreated controls (Matthews et al. 2012). In eggs of species other than walleye, hydrogen peroxide treatments ranging from less than 100 mg/L to over 1,000 mg/L have been shown to improve hatch success (Table 1).

Hydrogen Peroxide Treatments for Saprolegniasis in Walleye Eggs

Three studies have examined hydrogen peroxide treatment and its relationship to hatching success in walleye eggs (Gaikowski et al. 2003; Rach et al. 1998; Soupir and Barnes 2006). All used the percent hatch and percent viability of eggs as the measure for success. Saprolegniasis was observed in hatching jars, and the pathogen identified as *S. parasitica* in one study, but none of these researchers attempted to quantify *Saprolegnia* concentrations in egg hatching systems. Potential deleterious effects of hydrogen peroxide on embryos also have not been considered in these reports, and the optimal dosage level that balances hydrogen peroxide treatment success against its toxicity to embryos is unknown.

Rach et al. (1998) evaluated the optimal dosing of hydrogen peroxide for improving hatch probability of the eggs of several fish species, including walleye; hatch probability was similar across all species tested. The experimental system in Rach et al. (1998) used flow-through well water maintained at a temperature of 12 °C ± 2 °C. The authors concluded that 483 mg/L hydrogen peroxide, the lowest concentration tested, was optimal for increasing walleye hatch probability under their test conditions.

Gaikowski et al. (2003) evaluated hydrogen peroxide treatment effects on hatch rates of walleye eggs at four different facilities as part of an Investigational New Animal Drug efficacy

study for hydrogen peroxide. There were two experiments, the first of which was replicated with modifications at all four facilities. Results of the first experiment demonstrated that treating fertilized walleye eggs with 500 mg/L of hydrogen peroxide produced better hatching success than egg picking alone. In the second experiment, performed at only one of the facilities, hydrogen peroxide concentrations as low as 283 mg/L significantly increased walleye hatch. Based on the results, Gaikowski et al. (2003) hypothesized that 500-1000 mg/L treatments of hydrogen peroxide daily are likely to be more effective in hatcheries than lower concentrations. However, neither experiment had the statistical power to uncover any hydrogen peroxide dose-dependency of hatch rates (likely due to inter-facility variability). The authors observed infested embryos and confirmed the diagnosis of *Saprolegnia parasitica* in only one center. Quantification of *Saprolegnia* organisms was not attempted.

Soupir and Barnes (2006) evaluated the effects of formalin (834 and 1,667 mg/L) and hydrogen peroxide (200 mg/L) on walleye eggs at a single hatching center over four years. Formalin and hydrogen peroxide treatments consisted of a daily 15-minute flow-through application. A negative control (no formalin or hydrogen peroxide) was included in some years, but not others. Hatching success of eggs treated with 200 mg/L hydrogen peroxide did not significantly differ from those treated with 834 mg/L formalin. Both of these treatments resulted in hatching successes greater than those produced by the control and lesser than those treated with 1,667 mg/L formalin.

Flow-Through vs Recirculating Systems

Most hatcheries historically have operated with flow-through systems in which a constant supply of fresh water is delivered to the system while the water that was in the systems flows off and is not reused (Scheffer 1969; Tahar et al. 2018). By contrast, recirculating systems reuse the

same water after it has been filtered and treated, solid waste removed and dissolved nitrogenous waste converted to a harmless form (Bregnballe 2015; d'Orbcastel et al. 2009). As environmental regulations become more stringent throughout the world, recirculating aquaculture has become more common than flow-through systems because of its reduced water consumption and effluent discharge (Bregnballe 2015). Hatcheries that use a flow-through system will treat their eggs by stopping the flow of water to the eggs and introducing the preferred treatment chemical to the hatching chamber (e.g., McDonald jar) at the desired concentration. Flow will be returned to the system after a specified length of time (usually 10-15 minutes), flushing the treatment agent from the system (Barnes et al. 2003; Rach et al. 2004; Schreier et al. 1996). Meanwhile, additional factors must be considered when using recirculating systems. Because the treatment is not removed from the system immediately, hatchery managers must consider the rate of decay of the treatment chemical and how long it may remain in the system (Arvin and Pedersen 2015). Treatment concentrations may need to be lower in recirculating systems because of the potential effects of extended exposure to the treatment chemical on nitrifying bacteria as well as the cultured species (Pedersen and Pedersen 2012; Pedersen et al. 2010).

Evaluation of Pathogen Growth

Most studies that have investigated the effects of chemical treatments on *Saprolegnia* in fish hatcheries have focused exclusively on hatch success as a measured endpoint (Gaikowski et al. 2003; Rach et al. 1998; Soupir and Barnes 2006). However, although percent hatch or percent viability at the end of the study is what is of most importance to hatcheries, saprolegniasis is what is supposed to be directly affected by the added agent (Summerfelt et al. 2011). Despite this, few studies have attempted to quantitatively evaluate the abundance of the pathogen in this context. Some studies have counted the number or size of colonies formed on eggs, and although

this is a step in the right direction, the resultant information is not particularly useful (Barnes et al. 1998; Straus et al. 2019).

Methods for accurately measuring *Saprolegnia* are still being developed. There have been a few attempts to quantify *Saprolegnia* in water samples, though these studies have not investigated hatching success relative to anti-saprolegniasis treatments. Waterstrat (1997) and Celio and Padgett (1989) both used peptone-yeast-glucose agar to culture water samples from hatcheries and used the number of colony forming units that appeared as a measure of *Saprolegnia* abundance. Thoen et al. (2016) evaluated the concentration of zoospores in water samples collected in and around Norwegian salmon hatcheries. The authors used a protocol that estimated *Saprolegnia* concentration by quantifying colony forming units grown in an antibiotic glucose-yeast broth (Thoen et al. 2010; Thoen et al. 2016).

The use of quantitative polymerase chain reactions (qPCR) is a promising method for the quantification of *Saprolegnia* in water samples (Rocchi et al. 2017). Like end-point PCRs, qPCR amplifies sections of DNA from a target template with a DNA polymerase (usually derived from *Thermus aquaticus*), deoxyribonucleotide triphosphates (dNTPs; nucleotides), magnesium (as a buffering agent) and specific primers (Arya et al. 2005; Garibyan and Avashia 2013; Mullis 1990; Roux 2009). However, qPCR also uses fluorescent material to measure, in real time, the quantity of double stranded DNA produced from cycle to cycle over the course of a PCR amplification (Arya et al. 2005). There are two common methods of qPCR: dye-based and probe-based (Arya et al. 2005; Cardullo et al. 1988; Lee et al. 1993; Morrison et al. 1998). Dye-based qPCR uses a dye, such as SYBR Green, that fluoresces when bound to double-stranded DNA. As the amount of DNA is increased through PCR, the recorded fluorescence is proportionally increased and can be measured (Arya et al. 2005; Morrison et al. 1998). The number of

replication cycles required to reach detectable fluorescence will be higher for lower initial concentrations of DNA and can be used to back-calculate the starting concentration of DNA in an unknown sample when compared to a standard curve (Arya et al. 2005).

Probe-based qPCR requires the use of hydrolysis probes designed specifically to bind within the target sequence being amplified (Heid et al. 1996; Holland et al. 1991; Lee et al. 1993). These probes are affixed with a fluorophore on the 5' end and a quencher on the 3' end (Arya et al. 2005). Some probes use multiple quenchers at different distances along the probe (Wilson et al. 2011). As long as the probe is intact, the relative position and proximity of the quencher(s) to the fluorophore ensures that the fluorescence emitted by the fluorophore will not be detected by the qPCR machine but will instead be absorbed by the quencher through fluorescence resonance energy transfer (Arya et al. 2005; Heid et al. 1996). However, when Taq polymerase encounters the probe during an extension phase of the PCR, exonuclease activity will lyse the probe, separating the fluorophore and quencher(s) (Arya et al. 2005; Holland et al. 1991). Once these two pieces of the probe are separated, the fluorescence can be detected by the qPCR machine (Arya et al. 2005). Just as with dye-based qPCR, the amount of fluorescence is proportional to the amount of DNA copied throughout the PCR process and is recorded with each copying cycle.

Recent work has employed qPCR techniques to detect the presence of organisms in aquatic systems and in some cases quantify their densities (Bohmann et al. 2014; Griffin et al. 2009; Rocchi et al. 2017). In fact, these techniques have been used recently to properly identify Oomycetes at low concentrations and to quantify the abundance of other fish pathogens (Rocchi et al. 2017; Strepparava et al. 2014; Vralstad et al. 2009). One study by Rocchi et al. (2017) successfully used probe-based qPCR methodology to develop an assay to quantify *Saprolegnia*

from river water. *Saprolegnia* zoospores from hatching jars should be quantifiable using this method (Straus et al. 2016; Thoen et al. 2016; Willoughby et al. 1983). Given that quantification of *Saprolegnia* zoospores is possible using qPCR, quantitatively evaluating the amount of *Saprolegnia* in a system by using the number of zoospores as a proxy should be possible and would provide a measure of treatment efficacy.

CHAPTER 3:

METHODS

Facilities and System Design

Experiments evaluating the effects of hydrogen peroxide concentrations and dosing frequency were performed at the University of Georgia's Aquatic Biology and Ecotoxicology Lab (ABEL) at Whitehall Forest. In 2018, 21 individual hatching systems were constructed based on a design that simulated the facilities of GADNR's walleye hatching operation at the Go Fish Center in Perry, GA (Figure 1). Each hatching system consisted of a 6.8-liter McDonald-style hatching jar, a 18.9-liter bucket, one Sicce Synchro Nano® pump, approximately 101.6 cm of 1.3 cm inner diameter vinyl tubing, 30.5 cm of 7.6 cm diameter PVC pipe used as a pump guard, approximately 9.5 liters of bioconversion media ranging in size from approximately 1 to 3 centimeters in diameter, a mesh basket intended to catch hatched larvae, approximately 91.4 cm of rope and approximately 21.0 liters of dechlorinated water.

Water cycled from the hatching jar to the sump via the spill channel and spilled directly into a mesh basket designed to catch larval walleye (Figure 1). Surrounding the catch basket were the bioconversion balls, which were kept from interfering with the pump by a standpipe. Water was then pumped from the bottom of the sump to the center pipe of the hatching jar via the vinyl tubing to suspend and individualize the incubating eggs. Because water flow was powered by a pump and not gravity-flow, care had to be taken to ensure that a siphon effect would not extract the eggs from the hatching jars. In 2018, a power strip failed on the first night

before treatment and eggs had to be recollected from the sump and returned to their jars. In 2019, stoppers made from washers and rubber bands were used to elevate the vinyl tubing to prevent the siphon effect. Water in each system was cooled to approximately 12 °C by immersing the sump in one of two chilled water baths (Figure 2). A Hach multiprobe® water quality meter was used to measure daily temperature, dissolved oxygen and pH in each system before the first treatment. Recorded water quality conditions were always within acceptable limits throughout both experiments (temperature from 9.0 °C – 15.0 °C, dissolved oxygen above 6.0 mg/L and pH from 6.0 – 9.0) (Figures 3, 4 and 5). In 2019, hatching units were constructed in the same way, though there were 24 constructed to allow for an extra test treatment.

Experimental Design

A 2x3 factorial experiment with a randomized complete block design was used to examine the effects of two treatment frequencies (once daily and twice daily) and three concentrations (100 mg/L, 250 mg/L and 500 mg/L) on the hatching success of fertilized walleye eggs. There were three replicates of each treatment combination. The concentrations selected were chosen based on the results of previous investigations of the effects of hydrogen peroxide on hatching success of walleye eggs (Gaikowski et al. 2003; Rach et al. 1998; Soupir and Barnes 2006). In 2018, a daily treatment of water was used as a control. In 2019, this same water control was used along with a control that was not manipulated for treatment purposes. Each year, eggs from three female walleyes were used for the experiment. Each replicate of a given treatment combination received eggs from only one of the females. In this way, eggs from each female were represented exactly once for each treatment and acted as a blocking factor.

Treatments and controls were randomly assigned to systems by pairing a list of the treatments with a randomized list of numbers (from 1-21 in 2018 and 1-24 in 2019) generated in

R (R Core Team 2017). The treatments assigned to specific hatching units for 2018 and 2019 can be found in Table 2. A series of coin flips was used to determine the order in which systems would be numbered in space. Specifically, the order in which jars were arranged was determined by 1) flipping a coin to select whether the systems were arranged consecutively horizontally or vertically, 2) flipping a coin to determine whether the consecutive order of jars was in a uniform direction or doubled back at the end of the row, and 3) flipping a coin twice to establish a starting corner from which the previously determined sequence would be arranged. The relative spatial placements of hatching units for 2018 and 2019 are shown in Figure 2.

Walleye Spawning

Walleye broodstock were captured by GADNR biologists using electrofishing boats; the broodstock were shipped to the Go Fish Education Center in Perry, GA. Females used for the 2018 experiment were collected from Lake Hartwell; females used for the 2019 experiment came from unspecified reservoirs other than Lake Hartwell (Table 3a). Females and males were held in separate tanks before they were spawned. All females in both 2018 and 2019 were injected with 1 mL of human chorionic gonadotropin (1,000 International Units per mL, Merck Animal Health) to induce spawning.

On the day of spawning, females were inspected in the morning for free-flowing eggs (i.e. ripe). If the female was ripe, she was spawned (see description below); if not, she was left to spawn on a subsequent day. Prior to spawning, hatchery personnel at the Go Fish Center would prepare a solution of diatomaceous earth in a ratio of one cup of earth to one liter of water that would be stirred into a slurry. Ripe females were netted from their holding tanks and patted dry with a towel to prevent contact between water and unfertilized eggs. Hatchery personnel stripped eggs from female walleye by exerting gentle pressure on the fish's abdomen while holding the

fish's tail at a slightly upward angle relative to the fish's orientation. Starting at the pectoral fins and running the fingers towards the vent, eggs were expelled into a dry metal mixing bowl. This process was repeated for each female until the abdomen felt empty of eggs or blood appeared with the eggs.

While eggs were being stripped from the female walleye, milt from at least three males was stripped simultaneously into the same bowl. The male fish were dried and stripped in a manner similar to the females. Hatchery personnel then proceeded to stir the dry mixture of eggs and milt for 30 seconds with a turkey feather to homogenize the mixture for the best chances of fertilization. Water was then added to the mixture to activate the sperm; the amount of water was enough to just cover the eggs. After the water was added, the mixture was stirred with the turkey feather for another minute to increase contact between milt and eggs. Next, the diatomaceous earth slurry was added to the fertilized eggs and stirred with the same turkey feather for 3 minutes until the eggs no longer clumped. Care was taken by hatchery personnel to ensure that eggs did not stick to either the bottom or the side of the bowl.

After eggs were coated with diatomaceous earth, a two-step process was used to remove as much of the earth from the eggs as possible. First, the bowl containing the eggs with the diatomaceous earth was placed below a controlled water source and was slowly decanted off of the bowl by flushing it out of the eggs. Next, the eggs and what remained of the diatomaceous earth solution was poured into a basket with fine mesh (1.59 mm) that would catch the eggs but allow the diatomaceous earth to pass through. After the diatomaceous earth was removed, the eggs were allowed to harden for 2 hours by being placed in a mesh basket and positioned under gently flowing water. Following hardening, the eggs were passed through a sieve (3.18 mm) to remove any debris or clumped eggs. A subset of eggs would then be collected by hatchery

personnel to determine the quantity of eggs per volume while the rest were placed in hatching jars.

Eggs were quantified by Go Fish Center personnel using a method described by the Wisconsin Department of Natural Resources (WIDNR) and used by the St. Croix Tribal Natural Resources Department (Clint Peacock, GADNR, personal communication). A sample of eggs (1-2 mL) was lined up in a measuring trough in a row one egg wide, such that the row of eggs was 15.24 cm long. The eggs in the row were then counted and the number per volume determined using a table produced by the WIDNR (Appendix A). This process was completed for two samples of eggs from each female and the average was used to determine the number of eggs per liter for that female. The number of eggs per liter and other characteristics recorded for each female's eggs by GADNR are reported in Table 3b.

Based on the mean number of eggs per liter, an appropriate volume of eggs was collected from each of three females during each year of experimentation to allow for 10,000 eggs in each treatment. Fertilized eggs were placed with water in plastic bags that lined Styrofoam coolers. Bags were then inflated with oxygen to tightly fill the cooler and sealed tightly with duct tape. Ice was then added to the cooler to fill any gaps. The fertilized eggs were then transported in the coolers to University of Georgia facilities where appropriate volumes of eggs were added to experimental hatching jars the evening of the same day they were spawned. Experimental treatments started the next morning.

Hydrogen Peroxide Treatment of Walleye Eggs

Although the estimated number of eggs differed slightly between females, the number of eggs for any given female was consistent across jars. Specifically, in 2018, systems assigned

eggs from the first female received an estimated 11,607 eggs; systems assigned eggs from the second female received an estimated 10,128 eggs; and systems assigned eggs from the third female received an estimated 11,752 eggs. In 2019, systems assigned eggs from the first female received an estimated 10,553 eggs; systems assigned eggs from the second female received an estimated 12,142 eggs; and systems assigned eggs from the third female received an estimated 9,870 eggs.

In 2018, following the introduction of eggs to the hatching systems, each system was inoculated with five plugs (approximately 3 mm in diameter) of *Saprolegnia* isolated from a channel catfish infection and growing on Sabouraud's dextrose agar. These introductions were made to ensure that the pathogen was present in the system. However, in 2019 the systems were not inoculated with *Saprolegnia* at the onset of the experiment to better simulate what hatching success may be seen in a hatchery setting where the parasite is not introduced intentionally.

The dosing procedures used in our study were similar to those of GADNR's Go Fish Center. This method called for initially treating the eggs with a dose of hydrogen peroxide to the sump and the system was left to cycle with the expectation that the hydrogen peroxide would decay. The dosing concentration by treatment were as follows: systems receiving 100 mg/L hydrogen peroxide were dosed with 5.34 mL 35% Perox-Aid; systems receiving 250 mg/L hydrogen peroxide were dosed with 13.36 mL 35% Perox-Aid; and systems receiving 500 mg/L hydrogen peroxide were dosed with 26.72 mL 35% Perox-Aid. The water control treatments were given a sham "dose" of 5-mL water from a different graduated cylinder from that used to administer hydrogen peroxide. Systems were treated in their numeric order starting at one.

Unfortunately, after several hours on the first day of experimentation the hydrogen peroxide was not decaying as rapidly as expected. The issue was identified by large quantities of

eggs floating to the surfaces of jars and spilling into the mesh baskets; therefore, continued treatment with this method was not possible. This phenomenon has not been reported at the Go Fish Center, likely because GADNR's system has a longer circulation time and more organic matter than my systems. These factors may increase the decay of hydrogen peroxide that occurs in the water before it returns to the eggs. Each system underwent a complete water change and eggs lost to catch baskets were returned to jars. The water change process took place in the same order that the jars were initially treated. As a result, the second daily treatments were not provided on the first day to jars that were supposed to receive them.

A new exposure procedure was implemented on the second day of treatment in 2018. The flow of water from the sump to the hatching jar was stopped and the rope connecting the sump to the hatching jar was untied. The hatching jar was removed and placed above a floor drain. Jars were treated with their assigned concentration of hydrogen peroxide by adding the appropriate volume of hydrogen peroxide with a graduated cylinder and using the center tube of the hatching jar to gently mix the water. The jars only had a volume of 6.8 liters, so those jars receiving 100 mg/L hydrogen peroxide were dosed with 1.73 mL 35% Perox-Aid; jars receiving 250 mg/L hydrogen peroxide were dosed with 4.33 mL 35% Perox-Aid; and jars receiving 500 mg/L hydrogen peroxide were dosed with 8.65 mL 35% Perox-Aid. Water controls were dosed as described previously. After 15 minutes of exposure, the water/hydrogen peroxide mixture in the hatching jar was flushed with fresh water for 5 minutes and a strainer was used to collect any eggs that were expelled from the jar. After being flushed, any eggs caught in the strainer were returned to the jar. Finally, the hatching jar was reconnected to its sump and flow was restored. Systems were again treated in their numeric order starting at one. This new method was used for the dosing exposures from the onset of the 2019 experiment.

All jars were treated in the morning. Jars assigned to be treated twice a day were treated 11-13 hours after their morning exposure. Exposures continued every day for each jar until hatched walleye larvae were observed in that jar. Following observation of hatching, systems continued to cycle for 72 hours (or as soon as possible after the 72nd hour) without any hydrogen peroxide treatment to allow for hatching of eggs. This procedure prohibited cannibalism among larval walleye, which would negatively affect evaluation of hatch success. If hatching was not observed, eggs were treated until day 21.

Quantification of Hatch Success

Seventy-two hours after hatched larvae were observed in a given hatching system, it would be taken down and all larvae, eyed eggs, hatched eggs and *Saprolegnia* would be collected. A 250 mg/L solution of the anesthetic Tricaine-S (tricaine methanesulfonate, Western Chemical, Inc.) was used to euthanize all living eggs and larvae. A tea strainer was used to collect most eggs, larvae and *Saprolegnia* from the hatching jars, the mesh basket and the sump.

All euthanized walleye and *Saprolegnia* were preserved in plastic bottles (250 mL in 2018; 500 mL in 2019) for later quantification of hatch success. In 2018, sampled organisms were frozen with water, and in 2019 they were preserved in 10% buffered formalin. Following preservation, the number of eyed eggs and hatched larvae for each system were counted manually. Counting involved placing samples in a Pyrex® baking dish marked with a grid and systematically examining the dish square by square for both eyed eggs and larvae, which were recorded on a counter as they were removed from the dish. After the entire dish was examined, the dish was gently swirled to move the contents into a new position and counting would resume. This process continued until three successive passes did not produce additional eggs or larvae. The percent hatched for any system was calculated by dividing the number of hatched larvae

recovered from that jar by the estimated number of eggs placed in the system. The percent viability of the eggs in each jar was calculated by dividing the sum of larvae and eyed eggs by the estimated number of eggs placed in the system.

Quantification of Pathogen Density and Growth

Water Collection and Filtration

During the hatching experiments, water samples were taken from each system every third day. The first samples were taken on treatment days three, six, nine and twelve. A 1-liter plastic bottle was used to collect water from the spillway of the hatching jar. One liter of dechlorinated water was then added to the system to replenish the water that was taken for sampling. Whatman® Nuclepore™ Track-Etched Membranes with 1-µm pores were used with a vacuum filtration system to filter three, 100-mL subsamples from the initial 1-L sample. Following filtration, each filter membrane was folded in half inwards on itself three times so that the folded filter appeared to be a one-eighth slice of the original round filter membrane. The folded filter was then sealed in a 2-mL polypropylene microvial. At the end of the sampling day, all the day's filtration membranes were placed together in a minus 80 °C freezer.

Zoospore Production for Standard Curve Generation

Saprolegnia zoospores were produced and collected to create a standard dilution of known concentration with which to compare the experimental filters. The protocol for zoospore production was based loosely off of the methodologies of Willoughby et al. (1983) and Dieguez-Uribeondo et al. (1994).To produce zoospores, five 3-mm plugs of the pathogenic hyphae collected from the 2019 experiment (the culture from 2018 had since expired) and grown on glucose-yeast agar were placed into Erlenmeyer flasks containing 125-mL autoclaved glucose-

yeast-peptone broth. The flasks of broth were premade within 3 days of use by dissolving 1.25 g peptone, 1.25 g yeast and 3 g glucose in 1 L deionized water. Once dissolved, the solution was distributed evenly to each of eight Erlenmeyer flasks that were subsequently covered with aluminum foil. The flasks were then sterilized in an autoclave with slow exhaust for 30 minutes.

Once added to the flasks, each set of five plugs were covered and placed at room temperature to grow for approximately 24 hours. The next day, with hyphae growing off the plugs in all directions and forming a spherical shape, the plugs were rinsed with sterilized water. Rinsing consisted of pouring the contents of the flask through a tea strainer lined with autoclaved cheese cloth, rinsing the flask with sterilized water and pouring through the plugs caught in the strainer, and finally pouring sterilized water for one to two more seconds over the plugs. The cheese cloth containing the plugs was then inverted back over the flask and rinsed back into the flask with filtered, autoclaved lake water from Lake Allyn M. Herrick (Athens, Georgia, USA). The flask was then filled to approximately 150 mL with the sterilized lake water. After the plugs in each flask were rinsed, the flasks containing the plugs were placed in a 10 °C incubator for 24 hours.

After incubation was completed, zoospores were present in large numbers. On occasion, observation under a dissecting microscope revealed less-than-desired zoospore density. In these instances, the flasks were left to incubate at 10 °C for an additional 2-3 hours after which there were usually enough zoospores to harvest. Zoospores in the flasks containing the plugs in lake water were collected by emptying the flask into a tea strainer lined with cheese cloth over a large beaker. The plugs were washed once with approximately 5 mL of sterilized lake water before being discarded.

A glass rod was used to thoroughly stir the water caught in the beaker to homogenize the zoospore solution. Immediately following stirring, a 10-µL sample of water was pipetted and placed onto a hemocytometer for zoospore density evaluation. Under 100x magnification, zoospores were counted within the standard dimensions of the hemocytometer's grid. This process was repeated four times and an average number of zoospores per reading was calculated. The number of zoospores per mL was then calculated by dividing this mean number of zoospores by 0.0009, a scaling coefficient for the area counted on the hemocytometer. With the concentration of zoospores in the beaker calculated, Whatman ® filters identical to those used for filtration of experimental water were prepared in duplicate with known concentrations of zoospores. The highest concentration of zoospores used for standard curve generation was one million zoospores per filter. Five successive 10-fold dilutions were created by transferring an appropriate volume of stirred water from the zoospore beaker to a 150 mL total volume of water and filtering that solution. All filters used for the standard curve came from the same day of zoospore production, whereas other production days were used to prepare filters on which to practice DNA extraction methods. During the DNA extraction process, DNA from one filter with 1,000 zoospores was lost because of a broken microcentrifuge tube.

DNA Extraction from Filters

DNA was extracted from filters by using a modified process similar to that described by Brewer and Milgroom (2010). Sterilized tweezers were used to remove each folded filter from its polypropylene microvial. The filter was held over a weigh-boat and was opened; sterilized scissors were used to cut the unfolded filter into eight approximately equal pie-shaped sections. These pieces were then stacked on top of one another and five parallel, equidistant cuts were made through all pieces in the stack. The pieces of cut filter were then placed into a 1.5 mL

microcentrifuge tube. A 10% by mass slurry of Chelex®100 (ion-exchange resin beads which chelate Mg^{2+} ions which are necessary DNase cofactors) was prepared by adding appropriate portions of Chelex® 100 and sterilized DNA free water to a small, sterilized beaker. A sterilized stir bar was then added to the mixture and used to keep the Chelex® 100 beads in suspension.

The Chelex® 100 slurry (900 µL) was added to the microcentrifuge tube containing the cut filter. The tube was then capped, vortexed for 15 seconds, and spun down for 30 seconds at 8000 rpm. Following centrifugation, the tube was incubated in a 95 °C water bath for 10 minutes before being vortexed for another 15 seconds. After the second vortexing, the tube was returned to the 95 °C water bath for 10 more minutes and vortexed for 15 more seconds. Approximately 500 µL of supernatant containing DNA were then pulled from the tube and deposited in a new microcentrifuge tube. Care was taken to ensure that Chelex® 100 beads were not transferred along with the supernatant. Samples were then stored at minus 20 °C.

Quantitative PCR

Quantitative PCR was used to evaluate the concentration of *Saprolegnia* DNA in samples generated from each filter. The primers used for the reactions were designed to be genus specific for *Saprolegnia* spp. by Rocchi et al. (2017) and are located within the 18S rRNA sequence. A hydrolysis probe was used to quantify amplification and was modified from the probe used in the previously mentioned study. Both the primers and the probe were manufactured by Integrated DNA Technologies and the sequences for each are presented in Table 4.

A Bio Rad CFX96 Touch Real-Time PCR Detection System was used to run reactions in 20 µL final volumes. Primer concentrations were 250 nM, while the concentration of the probe in each reaction was 200 nM. Five µL of DNA solution extracted from the filters were used for

quantification. The remaining reaction volume consisted of 10 µL Bio Rad SsoAdvanced Universal Probes Supermix and DNA-free water. The amplification protocol used was also taken from Rocchi et al. (2017) and began with 3 minutes at 95 °C followed by 45 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Each run of samples included a standard dilution series repeated in triplicate decreasing 10-fold from 10^{-2} ng DNA to 10^{-8} ng DNA as well as three no-template controls to determine starting quantities of DNA in the unknown samples from their quantification cycles (Cq values).

Filters with known concentrations of zoospores were used to derive an equation to relate the amount of DNA in solutions made from experimental filters to numbers of zoospores that were on those filters. Quantitative PCRs were run on triplicate samples of solutions of DNA generated from two sets of filters with known zoospore concentrations. Each set contained filters with zoospores in 10-fold reduction ranging from 10^6 zoospores to 10^1 zoospores, with the exception of one set that was missing a filter with 10^3 zoospores that was lost to a malfunctioned microcentrifuge tube. Each set also included a filter without added zoospores. Data recorded from these filters were used to determine the number of zoospores on each experimental filter.

Standard Curve Generation

R statistical software was used to fit a best fit regression line to the qPCR data from filters with known zoospore concentrations (R Core Team 2017). The linear relationship between the $\log_{10}$ transformed values of starting DNA concentrations and the $\log_{10}$ transformed numbers of zoospores from filters with known quantities was used to generate an equation to calculate the number of zoospores on unknown filters. This equation was used to determine the number of zoospores per 100 mL filtration based on the starting quantity of DNA as determined using qPCR methods.

Pathogen Identification

A sample of hyphal tissue cultured from the naturally occurring infection in jars from the 2019 experiment on Sabouraud's dextrose was sequenced to identify the isolated organism. DNA was extracted as described previously. One µL of this DNA solution, along with 1 µL each of universal primers ITS1 and ITS4, were added with 22 µL of water to an illustra™ PuReTaq™ Ready-To-Go™ PCR Bead tube. The ITS amplification reaction heating cycle consisted of an initial step of 5 minutes at 94 °C followed by 34 cycles of 94 °C for 1 minute, 53 °C for 1 minute and 72 °C for 1 minute. A final extension step at 72 °C for 5 minutes concluded the program. Following agarose gel electrophoresis to confirm amplification, 5 µL of PCR product were transferred to a new PCR tube. Two µL ExoSAP-IT was then added to the tube and the mixture was heated in a thermal cycler for 15 minutes at 37 °C followed by a 15-minute heating at 80 °C. The DNA concentration of this product was then evaluated using a NanoDrop™ 1000 spectrophotometer, and an appropriate amount of DNA was sent for sequencing to Eurofins Genomics in Louisville, Kentucky. Sequence results were compared to known sequences published in GenBank® with a basic local alignment search tool (BLAST). Unfortunately, the *Saprolegnia* used in 2018 was unavailable for sequencing. However, samples from 2018 were examined by two pathologists at the time of the experiment and determined to be a *Saprolegnia* sp. based on the morphology of their terminal zoosporangia. Attempts were made to use the universal ITS primers to sequence DNA from experimental filters from the 2018 experiment; however, sequencing results were inconclusive likely because of the amplification of DNA from multiple species (e.g., walleye) along with that of the target organism.

Statistical Analyses

Hatch Success and Percent Viability

A two-way analysis of variance was used to evaluate differences in treatment means for percent hatch (hatched larvae / estimated initial number of eggs) and percent viability (hatched larvae + eyed eggs / estimated initial number of eggs) among treatment combinations. A Tukey's Honestly Significant Differences post-hoc test was used to identify which means differed from which. All statistical tests were conducted using R software and an alpha value of 0.05 was used to evaluate significance in all cases. The block effect was not of primary interest in this study but was used to determine whether variability in hatch success or percent viability was related to the female fish while also being able to reduce extraneous variability that might be associated with using eggs from multiple females.

Zoospore Density and Growth

Two-way analyses of variance were used to determine if the concentration of zoospores differed between treatment regimens at each sampling day for both the 2018 and 2019 experiment. Additionally, two-way analyses of variance were employed to determine whether either treatment concentration and/or frequency affected the change in zoospore density between successive sampling days, and whether an interaction existed between treatment concentration and frequency. As in the analyses of hatch success and percent viability, all statistical tests were conducted using R software and an alpha value of 0.05 was used to evaluate significance in all cases.

CHAPTER 4:

RESULTS

Hatching Success and Egg Viability

2018 Experiment

Hatching success of walleye eggs among various treatment concentrations ranged from 0.02% in systems treated twice daily with 500 mg/L hydrogen peroxide to 22.00% in systems treated twice daily with 100 mg/L hydrogen peroxide (Figure 6). The concentration of hydrogen peroxide to which the infected eggs were exposed affected hatching success of the eggs (p = 2.65e-4) and neither treatment frequency (p = 0.262) nor the interaction between the two factors (p = 0.486) had an effect (Figure 6). The female walleye that produced the eggs did not have a significant effect on hatching success (p = 0.287). Given the lack of effect of treatment frequency or interaction effect on hatching success, the frequency data were pooled by treatment concentration for further analysis of the pooled data. Tukey's Honestly Significant Difference test revealed that systems treated with 100 mg/L hydrogen peroxide had significantly higher hatching percentages (18.51% ± 3.69) than every other treatment concentration (0 mg/L = 4.58% ± 2.28, 250 mg/L = 4.44% ± 0.90, 500 mg/L = 0.02% ± 0.01; Figure 7). None of the other treatment concentrations differed significantly from each other (all $p > 0.05$; Figure 7).

Percent viability results were similar to those of percent hatch. Percent viability of walleye eggs among various treatment concentrations ranged from 0.03% in systems treated

twice daily with 500 mg/L hydrogen peroxide to 44.94% in systems treated once daily with 100 mg/L hydrogen peroxide (Figure 8). Treatment concentration significantly affected percent viability of the eggs (p = 2.05e-6) but treatment frequency (p = 0.240) and the interaction between the two factors (p = 0.286) did not (Figure 8). However, the female walleye that produced the eggs did have a significant effect on percent viability (p = 1.29e-2). Specifically, eggs from one female (total length = 534 mm, mass = 1,292 g, ID# = 15) reached the eyed stage in a significantly greater percentage than eggs from another female (total length = 512 mm, mass = 1,018 g, ID# = 14). The third female (total length = 455 mm, mass = 812 g, ID# = 16) was not different from either the first or second female. As treatment frequency or interaction did not affect percent viability, those data were pooled based on treatment concentration for further analysis. Tukey's Honestly Significant Difference test revealed that systems treated with 100 mg/L hydrogen peroxide had significantly higher percent viability (39.15% ± 6.02) than every other treatment concentration (0 mg/L = 8.59% ± 5.23, 250 mg/L = 7.03% ± 1.89, 500 mg/L = 0.04% ± 0.02; Figure 9). Meanwhile, none of the other treatment concentrations differed significantly from each other (all $p > 0.05$; Figure 9).

2019 Experiment

Hatching success of walleye eggs among various treatment concentrations ranged from 0.69% in systems that received a daily water control treatment to 5.36% in systems treated once daily with 250 mg/L hydrogen peroxide (Figure 10). Neither treatment concentration (p = 0.103), treatment frequency (p = 0.227) nor the interaction (p = 0.348) between the two factors had an effect on hatching success of walleye eggs (Figure 10). The individual female walleye that produced the eggs (blocking factor) had a significant effect on hatching success (p = 6.24e-3). Specifically, eggs from one female (total length = 492 mm, mass = 962 g, ID# = 33) had

significantly lower hatch success than both of the other females (total lengths = 529 mm and 579 mm, mass = 1,308 g and 1,596 g, ID# = 34 and 35), as revealed by Tukey's Honestly Significant Difference test.

Percent viability results were similar to those of percent hatch. Percent viability of walleye eggs among various treatment concentrations ranged from 3.05% in systems treated twice daily with 100 mg/L hydrogen peroxide to 6.49% in systems treated once daily with 500 mg/L hydrogen peroxide (Figure 11). Neither hydrogen peroxide concentration (p = 0.573), dosing frequency (p = 0.570) nor the interaction (p = 0.978) between those two factors affected the percent viability of walleye eggs (Figure 11). The individual female walleye that produced the eggs had a significant effect on percent viability (p = 7.35e-5). Specifically, eggs from one female (total length = 529 mm, mass = 1,308 g, ID# = 34) had a significantly higher percent viability than both of the other females (total lengths = 492 mm and 579 mm, mass = 962 g and 1,596 g, ID# = 33 and 35), as revealed by Tukey's Honestly Significant Difference test.

Standard Curve Generation

A linear relationship was observed between the $\log_{10}$ transformed values of starting DNA concentration and the $\log_{10}$ transformed values of zoospore quantity at the higher concentrations of zoospores. The linear relationship through the four highest concentrations had a coefficient of determination R^2 = 0.9777 (Figure 12). However, the relationship degraded at concentrations below 1,000 zoospores per filter. These lower values were therefore not used when calculating the conversion because of concerns of accuracy in filter preparation and sensitivity of the qPCR machine at those low concentrations. The equation used for conversion was as follows:

$$Z = 10^{\frac{\log_{10} D + 9.094}{0.411}}$$

where Z is the number of zoospores on an experimental filter and D is the starting quantity of DNA in nanograms as determined by qPCR.

Pathogen Density and Growth

2018 Experiment

The lowest zoospore density recorded was 138 zoospores per 100 mL on day 6 in systems treated twice daily with 500 mg/L hydrogen peroxide (Figure 13). On day 9, control systems had zoospore densities averaging 30,134 zoospores per 100 mL, the highest concentration recorded during the experiment. There was great variability in zoospore densities across treatments and days. Results of two-way analyses of variance revealed that treatment concentration of hydrogen peroxide did not affect the density of zoospores in systems on day 3 ($p = 0.474$), day 6 ($p = 0.731$), day 9 ($p = 0.304$), or day 12 ($p = 0.559$). Similarly, there was no effect of treatment frequency on any sampling day (p-values ranging from 0.167 on day 12 to 0.787 on day 9) and no interaction between concentration and frequency (p-values ranging from 0.306 on day 3 to 0.834 on day 9).

The greatest changes in zoospore densities, both positive and negative, occurred in control systems (Figure 13). Zoospore density increased by 29,152 zoospores per 100 mL from day 6 to day 9. During the next time interval from day 9 to day 12 the zoospore density in these systems dropped by 28,699 zoospores per 100 mL. There were no significant differences in the change in zoospore concentration between successive sampling days with respect to concentration or frequency and, again, no interaction existed between concentration and frequency (p-values ranging from 0.268 to 0.827).

2019 Experiment

Zoospores were not detected in multiple treatments on day 3 of the 2019 experiment (Figure 14). The highest zoospore concentration, 4.16 x 10^9 zoospores per 100 mL, was detected on day 12 in systems treated twice daily with 100 mg/L hydrogen peroxide. Statistically, results from the 2019 experiment were the same as the 2018 experiment. Two-way analyses of variance show that there was no effect of treatment concentration on the concentration of zoospores in systems on day 3 (p = 0.698), day 6 (p = 0.562), day 9 (p = 0.558), or day 12 (p = 0.558). Similarly, treatment frequency did not affect zoospore density on any sampling day (p-values ranging from 0.258 on day 9 to 0.274 an day 3) and no interaction existed between concentration and frequency (p-values ranging from 0.280 on day 6 to 0.443 on day 3).

The greatest increase in zoospore density occurred in systems treated twice daily with 100 mg/L hydrogen peroxide between days 9 and 12 (Figure 14). Over this period, zoospore concentration increased by 3.45 x 10^9 per 100 mL. The greatest decrease in zoospore density was observed in systems treated once daily with 100 mg/L hydrogen peroxide, also between days 9 and 12. During this period, zoospore density decreased by 5,880 zoospores per 100 mL. There were no significant differences in the change in zoospore concentration between successive sampling days with respect to concentration or frequency and, again, no interaction existed between concentration and frequency (p-values ranging from 0.235 to 0.561).

Pathogen Identification

The sequenced code was most similar to species of the genus *Aphanomyces*, which, like *Saprolegnia*, belongs to the family Saprolegniaceae and has a similar life cycle. Specifically, the sequence aligned with over 99% similarity to sequences of various strains of *A. laevis*, a

pathogen known to infect fishes and result in a condition that resembles infections by *Saprolegnia* (Table 5).

CHAPTER 5:

DISCUSSION

Hatching Success and Egg Viability

At the onset of experimentation, I hypothesized that an intermediate concentration of hydrogen peroxide (hereafter peroxide) would result in optimum hatching success of walleye eggs and percent viability to the eyed stage because of the combined effects of toxicity on pathogen growth and on the survivorship of walleye eggs. I also hypothesized that there would be a difference in hatching success and viability between treating the eggs with peroxide once or twice daily, though I was unsure which would produce greater hatch success and viability to the eyed stage. The results were mixed between factors in the 2018's experiment and similar in 2019.

Specifically, peroxide concentration affected hatching success under some conditions, but exposure frequency did not. Peroxide concentration improved hatching success and survivorship to the eyed stage in 2018 as systems treated with 100 mg/L peroxide had eggs hatch and reach the eyed stage in percentages approximately four times greater than other concentrations. However, such differences were not seen in 2019. Although many of the experimental conditions were similar between experiments A and B, there were a couple of differences between the two that might explain the observed results. Additionally, exposure frequency was similar for both exposure frequencies within and between experiments.

In 2018, but not in 2019, I observed the maximum hatching success at a peroxide concentration of 100 mg/L, the lowest non-zero concentration tested in my study. This concentration is lower than any previously tested on walleye eggs, though lesser or equal concentrations have been effective in treating eggs of largemouth bass and channel catfish (Table 1). The lowest previously reported peroxide treatment concentration used on walleye eggs was a daily, 15-minute treatment of 200 mg/L, which was the only concentration used in that study (Soupir and Barnes 2006). The treatment increased hatching success 27 - 40% (Soupir and Barnes 2006). Rach et al. (1998) observed improved hatching success at peroxide concentrations of 483 mg/L, 1,450 mg/L and 2,900 mg/L when treated for 15-minute intervals (presumably once daily but not specified). The highest hatching percentage was observed for the eggs treated with 483 mg/L peroxide, which was the lowest tested concentration in their study. Gaikowski et al. (2003) compared hatching success at low (283 mg/L), medium (565 mg/L) and high (1,130 mg/L) concentrations of peroxide and reported similar rates of increased hatching success for all three concentrations. Although the historic treatments of non-walleye eggs have successfully used concentrations of peroxide of 500 mg/L and greater, these concentrations may be higher than optimal for walleye eggs even though the treatments produce better hatching success than no treatment at all (Gaikowski et al. 2003; Rach et al. 1998). In 2018, peroxide treatments of 100 mg/L produced the best hatching results when compared to other peroxide concentrations of 250 mg/L and 500 mg/L in my study. Though not evaluated by my study or any others, peroxide concentrations lower than 100 mg/L may produce better results than the 100 mg/L used in my study. I likely did not observe a benefit of treatment at concentrations higher than 100 mg/L in my first year of experimentation because of toxicity to the eggs during the period of extended exposure.

There was a procedural difference between my experimental methods in experiments A and B that may have contributed to the different results between the two. In 2018, my experimental systems experienced an unintended extended exposure to peroxide for several hours on the first day of experimentation; this extended exposure did not occur in 2019. Perhaps exposure durations longer than 15 continuous minutes used in this study increases hatching success and eye-up of eggs. Unfortunately, eggs treated in 2019 may not have been in contact with peroxide long enough to affect hatching success or provide a test of this hypothesis. Following the unintended extended exposure in 2018, most of the eggs treated with 500 mg/L peroxide died during this initial exposure. This suggests that exposure to 500 mg/L for multiple hours may be too long for walleye eggs to survive regardless of the peroxide's effects on pathogen growth. However, the other systems treated with the unintended extended exposure saw progressively increasing hatching success with lower concentrations of peroxide.

Some of the results from this study suggest that peroxide exposure durations longer than 15 minutes at a time may improve hatching success compared to the 15-minute exposure duration used in my study. This line of inquiry along with peroxide concentrations less than 100 mg/L would be a logical follow up to the present study. The experimental design would be similar to that described herein but with a single exposure per day instead of two and exposure durations that could increase in 15-minute intervals up to an hour. Recommended peroxide concentrations could range from 25 mg/L to 125 mg/L in 25 mg/L intervals to determine if there is an interaction effect between concentration and duration.

Differences in baseline egg viability between experiments also could have produced the disparity in results observed between 2018 and 2019. For instance, 2018's female broodfish came from Lake Hartwell, whereas 2019's broodfish came from other reservoirs and the walleye

from Lake Hartwell may have higher quality eggs than walleye from the other reservoirs. Alternatively, there may have been inter-annual variability in egg quality between experiments (without regard to broodstock source) because weather or other environmental conditions may have affected egg development. The walleye run in North Georgia in 2018 occurred earlier and was much stronger than in 2019, and the factors that contributed to these differences may also have affected gamete development. There could also have been differences in baseline egg viability between the set of individuals used in Experiments A and B.

I acknowledge that samples of the walleye eggs used in my experiments were not evaluated to assess egg quality and the suggestion that baseline egg quality may have differed between experiments is conjecture based on reduced hatching success observed between experiments. If characteristics of the eggs had been evaluated, determination of baseline egg viability may not have included every possible factor that affects hatching success. However, if there was reduced baseline viability in 2019, considering how this difference might have affected efficacy of peroxide treatment is reasonable. There may be a threshold of viability that must be met before peroxide can significantly increase hatch success. If the percent viability within a jar is too low, the pathogen may take over and reduce hatch success regardless of peroxide treatment. Even if there were effects of hydrogen peroxide, detecting them would be difficult if the baseline egg viability is too low.

Investigations into characteristics (e.g., length of female, age of female, mean egg diameter and mean egg mass) associated with baseline egg viability would provide guidance for procuring the best broodstock and gametes to increase hatching success of hatchery-fertilized eggs. The percent of viable eggs at the end of these experiments, ranging from 0% - 39% in 2018 and 3% - 6% in 2019, were much lower than results of similar studies conducted in the heart of

the walleye's range. Mean hatch percentages of walleye eggs treated with peroxide were seldom lower than 50% in published studies and often were higher than 70% (Gaikowski et al. 2003; Rach et al. 1998; Soupir and Barnes 2006). In experiments from two of these studies, mean hatching success of untreated jars was never below 36%. However, the relatively lower hatch percentages seen in both experiments of my study were similar to the hatching success experienced by GADNR, who have reported hatching percentages that range from 3% to 25% (Clint Peacock, GADNR, personal communication). Determining if the egg quality differs between walleye from Georgia and walleye from more northerly stocks would be valuable. If differences are found, determining what characteristics may contribute to this disparity in viability would also be worthwhile. If possible, determining which batches of eggs were more likely to survive from the onset of incubation would allow greater resources to be dedicated to high quality eggs rather than using valuable space and resources on eggs that have little chance of hatching. Evaluation of egg quality would also be valuable to determine whether this baseline viability affects the efficacy of peroxide treatment of the eggs.

The results of my experiments have produced valuable information that may help GADNR to improve its walleye production. For example, the observation that peroxide persists in recirculating systems and can cause continual exposure beyond what was intended and have negative effects on hatching success was especially noteworthy. This situation is exacerbated at high concentrations. Further, the phenomenon of longer-than-expected exposures can occur even when the initial treatment concentrations are within those recommended by the manufacturer (i.e., 500 mg/L) or other relevant studies performed in flow-through systems. Based on the results of the first experiment, treatment concentrations lower than 250 mg/L peroxide may provide better hatch success for GADNR using their current treatment methodology. Further, an

evaluation of the rate of decay of peroxide in GADNR's recirculating system may better elucidate the peroxide concentrations to which walleye eggs are actually being exposed and would allow for better dosing to increase hatching success.

Pathogen Density and Growth

I hypothesized that pathogen growth would be negatively affected by increasing treatment concentration and increasing treatment frequency. I also hypothesized that all treatments would experience positive pathogen growth over the course of the experiments (i.e., increasing zoospore density at successive time points). The results of these experiments were contradictory and could not address these questions conclusively. Neither treatment concentration nor exposure frequency seems to affect the density of the pathogen zoospores or their change in number over time. Additionally, the expected increase in zoospores over time across all systems was not observed. In several instances, zoospore density decreased or fluctuated over the course of the experiment (Figures 13 and 14). However, this observation is inconsistent with the continuous hyphal growth observed while treating the systems throughout both experiments. Therefore, the treatments may have affected pathogen growth or density, even if though it wasn't detected.

There are several possibilities for why there was a discordance between the observed hyphal growth and the lack of change in the number of zoospores. For example, the assumption that zoospore density was directly proportional to the amount of hyphal growth may be invalid. During the process of zoospore production for standard curve generation, the same procedures appeared to result in similar amounts of hyphal growth (though this was not quantified) but produced zoospores at densities ranging from 5,555 to 13,611 zoospores per mL. This result suggests that equal amounts of hyphae may produce unequal concentrations of zoospores. Even

if the total number of zoospores produced was proportional to the amount of hyphal growth, the production may have come in bursts rather than continuously. The production also could have been related to the number of dead eggs in the systems, which may have varied. If that were the case, detecting differences in zoospore density would not be possible unless each jar happened to be sampled following a mass production event.

Additionally, methods used for collecting and evaluating zoospore density may not have worked properly. Water was collected at the spillway of the hatching jar where the zoospores were assumed to have been washed into the sump with the flow of the water. However, the presence and density of zoospores in the water were never confirmed with microscopy. The 1-µM filters used to collect *Saprolegnia* and *Aphanomyces* zoospores should have done so easily as those zoospores are typically larger than 5 µM in diameter (Schoulties and Yang 1971). Further, standard curve generation confirmed that zoospores of *A. laevis* could be collected on the filters. DNA preparation using Chelex® 100 has a reputation for being exacting to use. As a result, my DNA extractions could have been inefficient or failed. However, because this possibility was a concern at the onset of extraction, work was done with filters specifically prepared to test the technique. Proper amplification with qPCR was observed in all instances before committing to using Chelex® 100 for DNA extraction, and the qPCR machine likely was recording data correctly. Standard dilutions of known quantities of DNA derived from the culture of *A. laevis* were run in triplicate with each amplification run of samples and stayed consistent between runs. In sum, the qPCR assay was reproducible and working well and may point to a possible failure in the collection of zoospores or the extraction of DNA therefrom.

If the zoospores were sampled in a representative fashion and the DNA extraction procedures used were efficient, there are some possible reasons for the observed results. Though

the hyphae in the hatching jars were always treated with peroxide, any zoospores that were in the sump would not have been treated and therefore would not have been affected by peroxide treatments. As a result, a large enough percentage of the zoospores may not have been treated to see an effect of peroxide treatments. Additionally, qPCR only detects copies of DNA and cannot discern between viable and non-viable zoospores. Accordingly, the peroxide concentration and/or treatment frequency may have affected the number of viable zoospores, but that the joint quantification of live and dead zoospores made detecting an effect impossible.

The primers and probe used for this qPCR analysis were designed by Rocchi et al. (2017) to be genus specific for *Saprolegnia*. However, my results demonstrate that DNA of *A. laevis* can be amplified using the primers. In designing the primers and probe, Rocchi et al. (2017) analyzed the sequences of six genre including *Saprolegnia* and *Aphanomyces*, though they only considered *A. astaci* and not *A. laevis*. Additionally, experimental evaluation of the primers and probe only showed specificity for *Saprolegnia* when compared to *A. astaci*. In defense of the authors, a review of GenBank® reveals only 9 sequences of the *A. laevis* 18S rRNA, all of which are partial sequences and do not include the corresponding sequence where the primers would anneal. Regardless, these primers were considered to be genus specific, but they clearly are not.

A method for properly measuring the growth of fungal-like pathogens on fish eggs is needed to evaluate the effects of peroxide treatments on the density and growth of these pathogens. Although the amount of hyphae is typically the measurement of interest in the few studies that have attempted quantification, zoospore concentration may be a more appropriate measurement. Zoospores are the colonizing bodies of the pathogenic organisms and are the primary target of peroxide treatments because they are more sensitive to peroxide than hyphae. Additionally, although measuring hyphal growth in experimental systems where egg-picking is

not performed may be valuable, quantifying hyphal growth in real-world systems where infected eggs are commonly removed would be appropriate. Thoen et al. (2010) developed a promising method for zoospore enumeration that employs the culture of viable spores from samples in microwell plates. According to the authors, though this method underestimates zoospore concentration, it could reliably distinguish between samples with known differing zoospore concentrations. This technique was later used by Thoen et al. (2016) to evaluate the relationship between zoospore concentrations and hatching success in Norwegian salmon hatcheries, but a relationship between the two was not found. Although the qPCR methods described herein may not be successful for evaluating pathogen growth, they could be refined to better assess growth in the future.

Factors related to pathogen growth may have contributed to the differing hatching results in experiments A and B. For example, I used the same water source for both experiments, but pH differed significantly (i.e., 2018 = 7.87 vs 2019 = 6.96) between years. While both of these values are comfortably within the pH requirements for walleye egg incubation, and individual systems never had a pH recorded outside of acceptable limits ranging from 6.0-9.0, differences in pH may have affected the pathogen or the peroxide activity in a way that reduced the efficacy of peroxide treatments. Increased pH is associated with increased peroxide breakdown (Yazici and Deveci 2010). However, if this was the case, there would have been higher effective peroxide concentrations in 2019, which would be predicted to have a more, rather than less, pronounced effect. Additionally, treatment times of 15 minutes probably were not enough to allow for significant decomposition of peroxide. Zoospore production of *S. parasitica* occurs in water with pH ranging from 4.0 to 8.3 (Lee 1962). If this range is similar for other *Saprolegnia*

and *Aphanomyces*, the observed difference in pH between years should not have affected the efficacy of peroxide treatment.

The presence or absence of a pathogen introduction at the onset of the experiments was another difference between experiments A and B. Specifically, a semi-standard quantity of an organism believed to be *Saprolegnia* was introduced into each system at the onset of the first experiment; this procedure was not followed for the second experiment. As a result, there was likely more initial pathogen growth in 2018 than in 2019. This could have affected hatching success, but the experiment with more pathogen growth would be expected to have lower hatching success and greater effects of peroxide. Interestingly, this expected outcome did not occur. Instead, mean hatching success was lower and not statistically different in 2019, the year when *Saprolegnia* was not added to the systems. The introduced pathogen may not have been responsible for the observed infections in 2018. Although the starting concentration of the pathogen did not likely produce the observed differences in hatching results, there is evidence that the pathogen in 2019 was not *Saprolegnia* and was not as susceptible to peroxide, which could have affected the outcome of the experiment.

The unexpected identification of *A. laevis* as a pathogen associated with walleye eggs in 2019 suggests a possibility that the organisms causing the infections were different in the first and second experiments. If this was the case, the differences in hatching results between experiments could be related to the two pathogens' differing responses to peroxide. I would expect that if a difference in pathogens was responsible for the observed difference in results, a similar difference in results would be seen with respect to pathogen growth. Unfortunately, the methodologies used to quantify this growth provided inconclusive results. Published accounts describing the effects of peroxide on eggs infected with *A. laevis* are lacking, but this species

may have higher resistance to peroxide treatments and that resistance may be responsible for the observed differences.

A. laevis is an infrequently studied pathogen that, until now, has not been reported to be associated with walleye or their eggs. However, the organism has infected other species of fish including Nile tilapia (*Oreochromis niloticus*) and blue panchax (*Aplocheilus panchax*) (Ali et al. 2011; Mondal and De 2001). *A. laevis* belongs to the same genus as the highly parasitic *A. invadans* that is responsible for epizootic ulcerative syndrome in fishes (Iberahim et al. 2018). Another congeneric species is the crayfish plague pathogen, *A. astaci*, that is responsible for mass mortalities of crayfish (Alderman et al. 1990; Iberahim et al. 2018). Although this finding was unexpected, it may prove to be pivotal for improving the efficacy of treatment of Oomycete infections in walleye hatching operations. Most publications that have reported on the effects of peroxide on the hatching success of walleye eggs have assumed that the responsible pathogen was *S. parasitica*. This assumption is made for most studies evaluating chemical parisiticide treatments of fish eggs of various species. Yet, most studies fail to properly identify the pathogen because of the difficulty associated with identification. The results of my study indicate that assuming the pathogen responsible for saprolegniasis is *S. parasitica*, or even *Saprolegnia*, is inappropriate. My results also suggest that different pathogens may respond differently to peroxide treatments. This finding could have significant implications for GADNR and other walleye hatching operations. If there are large differences in the treatment regimens required to effectively treat different Saprolegniaceae, hatcheries may need to tailor their treatment procedures to the organism or organisms infecting their eggs in a particular year. An experiment to determine if *A. laevis* reacts differently to chemical treatments than other commonly studied Saprolegniaceae would be beneficial for evaluating this hypothesis.

Summary and Conclusions

This investigation has provided useful information for improving the hatching success in GADNR's walleye hatching program. Specifically, exposing fertilized walleye eggs to a single dose of 100 mg/L of peroxide for 15-minute daily exposures may improve hatching success of their walleye production program. Further, properly characterizing the rate of peroxide decay in their recirculating walleye hatching system would help achieve the targeted treatment concentration and avoid overdosing the eggs.

Additionally, the results of this study suggest that increasing the duration of peroxide treatment and using high-quality walleye eggs may improve hatching success. Evaluating these factors would provide the specifics of how long (e.g., 15 to 60 minutes in 15-minute intervals) to expose the eggs and the minimum egg viability needed to produce high hatching success. Evaluating the condition of female walleye and characteristics of their eggs (e.g., egg size, egg buoyancy) that may be associated with high baseline egg viability may improve efficiency of broodstock and egg selection and increase the likelihood of high hatching success regardless of Oomycete infection.

Prior to this study, the pathogen *A. laevis* had never been previously associated with any walleye life stage. I identified the pathogen naturally colonizing walleye eggs. This finding suggests that various Oomycete pathogens may be responsible for losses of walleye eggs at GADNR facilities (and others) in different years. If that is the case, and if different pathogens respond differently to peroxide treatment, identifying the responsible organism and adjusting treatment based on this information may improve treatment efficiency. However, given the difficulty identifying organisms within Saprolegniaceae, the effort to identify which organism is

responsible for the infection may be worthwhile only if there is a large difference in the efficacy of peroxide treatment between species.

Finally, my results support Rocchi et al. (2017) findings that demonstrated that qPCR technology and the primers they designed can be used successfully to amplify *Saprolegnia sp.* DNA. However, I found that the primers reported by Rocchi et al. (2017) to be genus-specific for *Saprolegnia sp.* are not so because they also amplified DNA of another Oomycete *Aphanomyces laevis*. A proper method of quantifying Oomycete growth on fish eggs is still needed. Refinement of qPCR techniques for this purpose may provide a solution to this problem in the future.

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TABLES AND FIGURES

Table 1. Hydrogen peroxide treatments that have been reported in peer-reviewed literature to increase hatching success in the eggs of a variety of freshwater fish species.

Species	Treatment Duration	Treatment Frequency	Treatment Concentrations	Source
Walleye (*Sander vitreus*)	15 minutes	Every other day	200, 283, 565 and 1130 mg/L	Gaikowski et al. (2003); Soupir and Barnes (2006)
White Sucker (*Catostomus commersonii*)	15 minutes	Every other day	283, 565 and 1130 mg/L	Gaikowski et al. (2003)
Paddlefish (*Polyodon spathula*)	15 minutes	Every other day	1130 mg/L	Gaikowski et al. (2003)
Largemouth Bass (*Micropterus salmoides*)	Unrecorded, flow-through	Twice daily	100 mg/L	Matthews et al. (2012)
Channel Catfish (*Ictalurus punctatus*)	15 minutes	Daily	70 and 750 mg/L	Rach et al. (2004); Small and Wolters (2003)
Chinook Salmon (*Oncorhynchus tshawytscha*)	15 minutes	Daily	700 mg/L	Barnes et al. (2003)
Rainbow Trout (*Oncorhynchus mykiss*)	15 minutes	Daily	500 and 1000 mg/L	Schreier et al. (1996)

Table 2. Treatment combinations randomly assigned to specific hatching units in 2018 (rows 2-22) and 2019 (rows 23-46) to evaluate the efficacy of hydrogen peroxide concentration and dosing frequency of hatch success of fertilized walleye eggs.

Year	Unit Number	Hydrogen Peroxide Concentration (mg/L)	Frequency per Day	Female
2018	1	250	Twice	14
2018	2	250	Once	15
2018	3	500	Once	15
2018	4	100	Twice	14
2018	5	500	Twice	14
2018	6	0.0	Once	15
2018	7	0.0	Once	16
2018	8	250	Twice	16
2018	9	500	Twice	16
2018	10	250	Once	16
2018	11	500	Twice	15
2018	12	100	Twice	15
2018	13	100	Twice	16
2018	14	100	Once	15
2018	15	500	Once	16
2018	16	100	Once	14
2018	17	250	Twice	15
2018	18	0.0	Once	14
2018	19	500	Once	14
2018	20	250	Once	14
2018	21	100	Once	16
2019	1	250	Twice	35
2019	2	500	Twice	34
2019	3	100	Once	33
2019	4	250	Once	35
2019	5	100	Once	34
2019	6	500	Once	35
2019	7	0.0	Once	35
2019	8	No Interference Control		34
2019	9	500	Twice	33
2019	10	500	Once	34
2019	11	500	Twice	35
2019	12	100	Once	35
2019	13	250	Once	33
2019	14	500	Once	33
2019	15	250	Once	34
2019	16	No Interference Control		35
2019	17	No Interference Control		33
2019	18	100	Twice	34
2019	19	100	Twice	33

Year	Unit Number	Hydrogen Peroxide Concentration (mg/L)	Frequency per Day	Female
2019	20	100	Twice	35
2019	21	250	Twice	34
2019	22	250	Twice	33
2019	23	0.0	Once	33
2019	24	0.0	Once	34

Table 3a. Data collected on female walleye used for spawning at the Go Fish Center in Perry, GA. Eggs from these females were used to evaluate the effects of hydrogen peroxide concentration and dosing frequency on the hatch success of fertilized walleye eggs during 2018 and 2019.

GADNR Fish ID	Date Spawned (mm-dd-yyyy)	HCG Injection	Number of Males	Total Length (mm)	Weight of Female Post-Spawn (g)	Source Reservoir
14	03-02-2018	Yes	3	512	1,018	Hartwell
15	03-02-2018	Yes	3	534	1,292	Hartwell
16	03-02-2018	Yes	3	455	812	Hartwell
33	03-15-2019	Yes	4	492	962	Not Hartwell
34	03-15-2019	Yes	4	529	1,308	Not Hartwell
35	03-15-2019	Yes	4	579	1,596	Not Hartwell

Table 3b. Data collected on eggs produced by female walleye collected at the Go Fish Center in Perry, GA. These eggs were used to evaluate the effects of hydrogen peroxide concentration and dosing frequency on the hatch success of fertilized walleye eggs during 2018 and 2019.

GADNR Fish ID	Weight of Eggs (g)	Number of Eggs per 6” (Trough)	Number of Eggs per Liter	Total Volume of Eggs (L)	Total Number of Eggs
14	282	76	145,086	0.8	116,069
15	228	73	129,851	0.7	90,896
16	174	76	145,086	0.6	87,052
33	246	81	191,871	0.6	115,122
34	368	76	145,086	1.0	145,086
35	278	78	156,681	0.7	109,677

Table 4. Primers and probes used for qPCR quantification of *Saprolegnia* abundance in hatching systems being used to evaluate the effects of hydrogen peroxide concentrations and dosing frequencies on the hatching success of fertilized walleye eggs. The primers are based on those used by Rocchi et al. (2017).

Primer / Probe	Sequence
F Primer	5'-GCATTCAAGTTTGTGGGAAC-3'
R Primer	5'-CGGAAACCTTGTTACGACTTC-3'
Probe	5'/56-FAM/TCCTTAACC/ZEN/TCGCCATTTAGAGGAAGG/31ABkFQ/-3'

Table 5. The top 10 GenBank® sequence comparisons based on total BLAST score for a DNA sequence amplified using universal ITS primers 1 and 4 and DNA extracted from a pathogen cultured from walleye eggs that were used in an experiment evaluating the effectiveness of hydrogen peroxide concentration and dosing frequency on hatching success of those fertilized eggs.

Species	Percent Similarity	Query Cover	GenBank® Accession ID
Aphanomyces laevis	99.86%	99%	AY310497.1
Aphanomyces laevis	99.70%	97%	AY683885.1
Aphanomyces laevis	99.85%	96%	AM947028.1
Aphanomyces sp.	99.85%	95%	HQ643123.1
Aphanomyces laevis	100.00%	92%	HQ111469.1
Aphanomyces laevis	98.01%	93%	FM999236.1
Aphanomyces cochlioides	95.84%	99%	AY647191.1
Aphanomyces sp.	96.65%	94%	AB533289.1
Aphanomyces laevis	96.76%	93%	KP006463.1
Aphanomyces sp.	100.00%	77%	GU014281.1

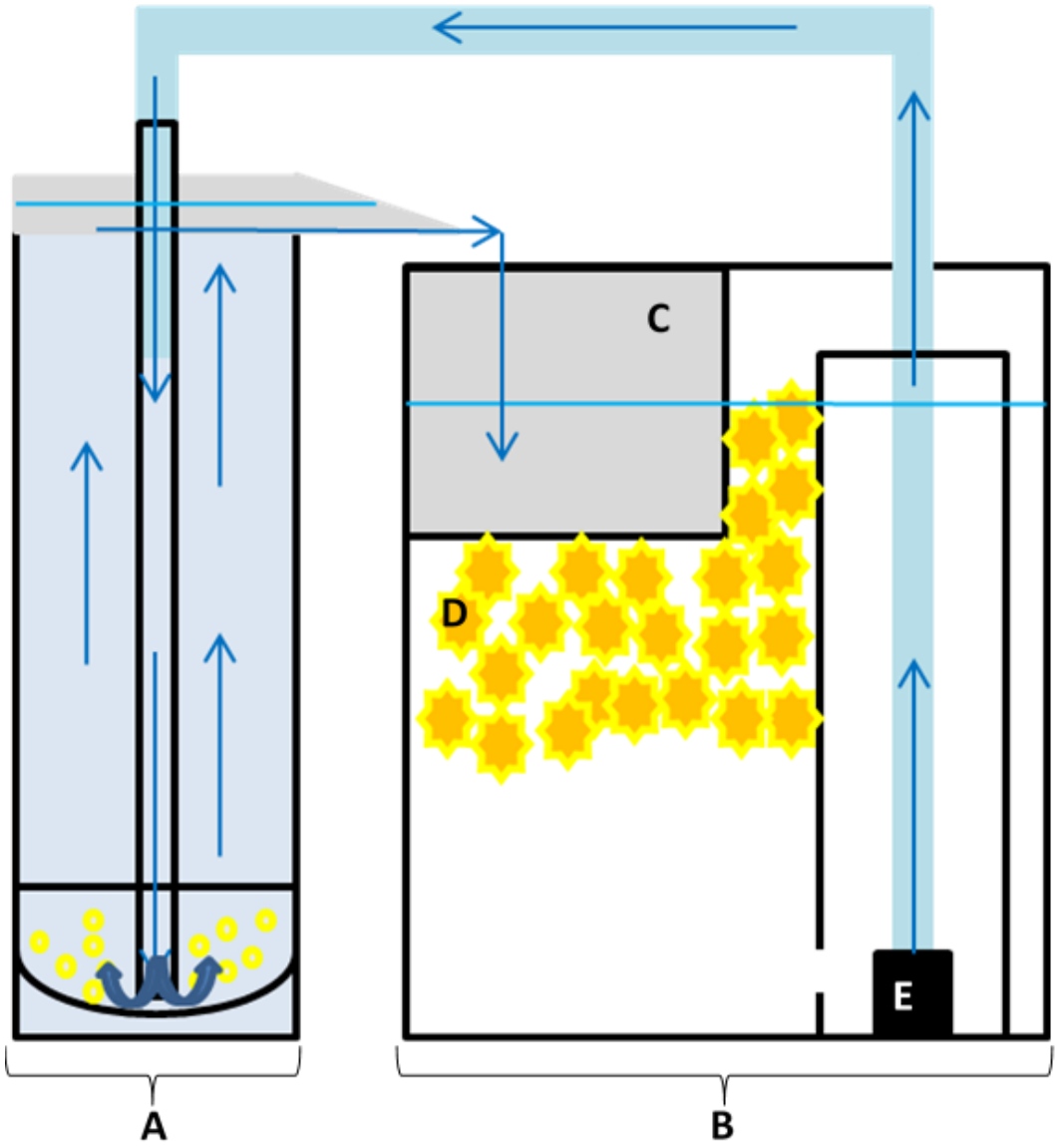


Figure 1. Schematic diagram of a hatching system used to incubate fertilized walleye eggs during 2018-2019. Each individual hatching system consisted of a McDonald-style hatching jar (A), a 5-gallon sump (B), a mesh basket (C) designed to catch larval fish, plastic bioconversion balls (D) and a pump (E). The cycle of water-flow through the system is indicated by arrows.

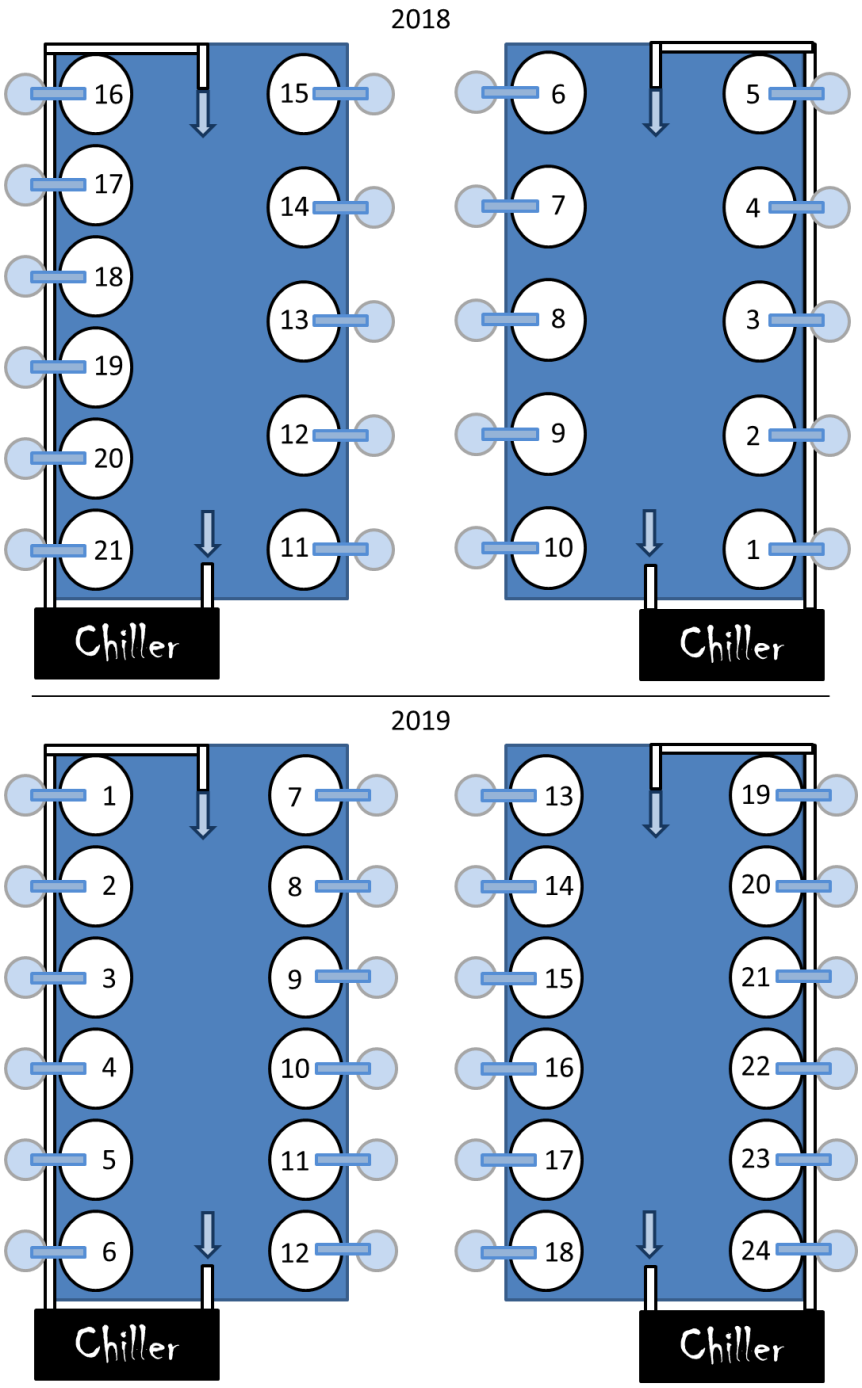


Figure 2. Spatial orientation of experimental walleye hatching systems are shown for 2018 (top) and 2019 (bottom). Large rectangles represent chilled water baths in which the sumps for each hatching system, represented by large circles, were immersed. Numbers marked on the sumps represent their unit number determined randomly by four flips of a coin. Small circles represent the hatching jars associated with each system.

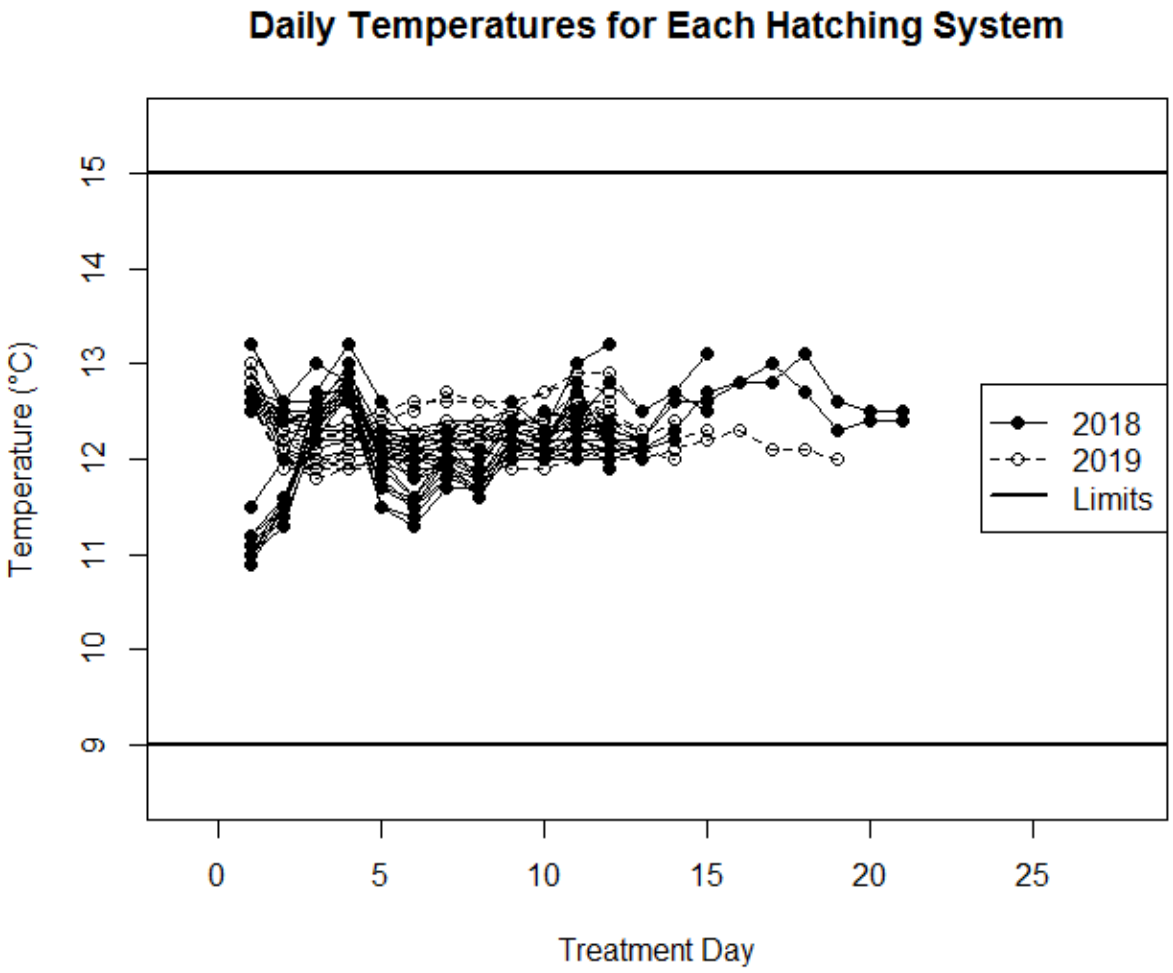


Figure 3. Daily water temperatures recorded in each walleye hatching system every morning during the experiments. Horizontal lines at 9 °C and 15 °C represent the lower and upper temperature limits for optimal walleye production as described by Bozek et al. (2011b).

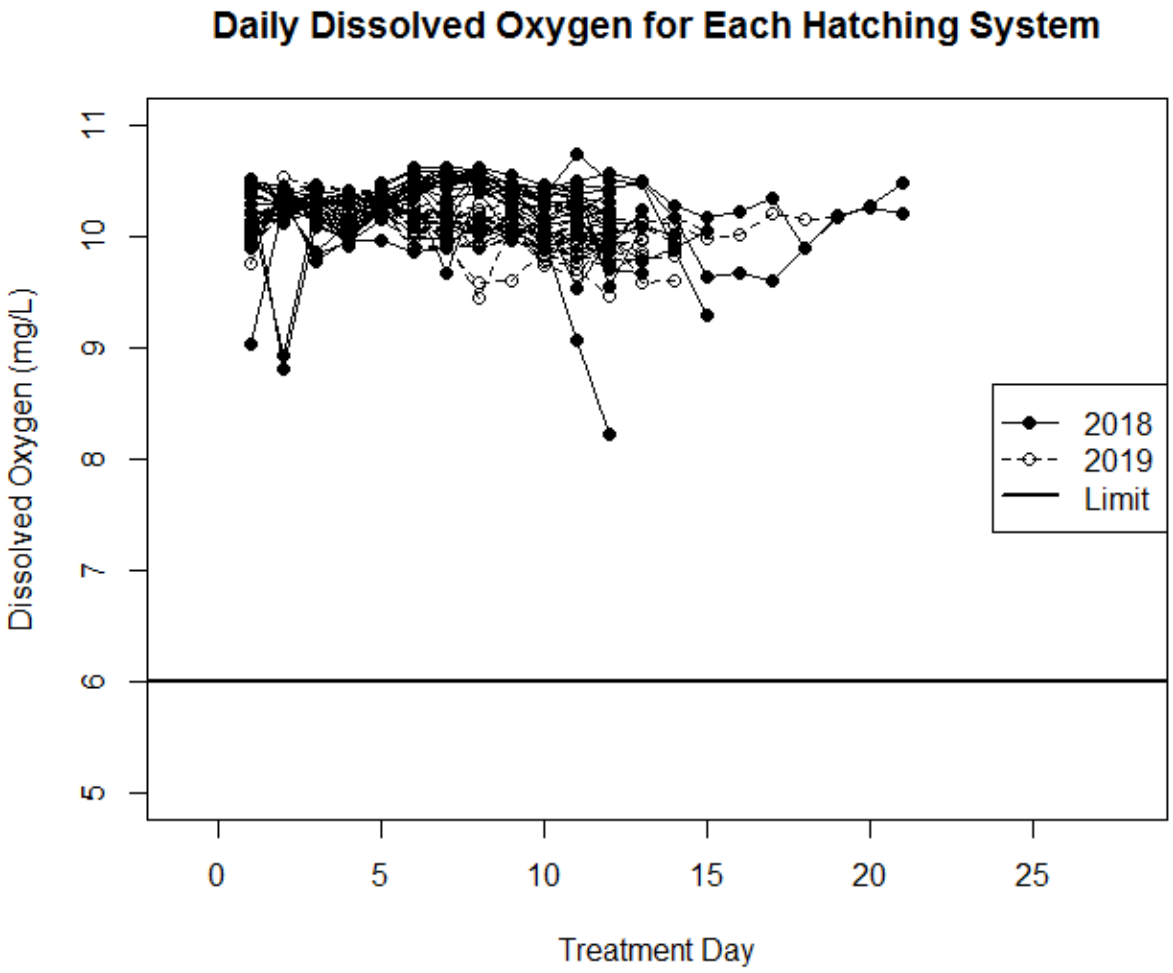


Figure 4. Daily dissolved oxygen concentrations recorded in each walleye hatching system every morning during the experiments. The horizontal line at 6 mg/L represents the lower dissolved oxygen limit for optimal walleye production as described by Bozek et al. (2011b).

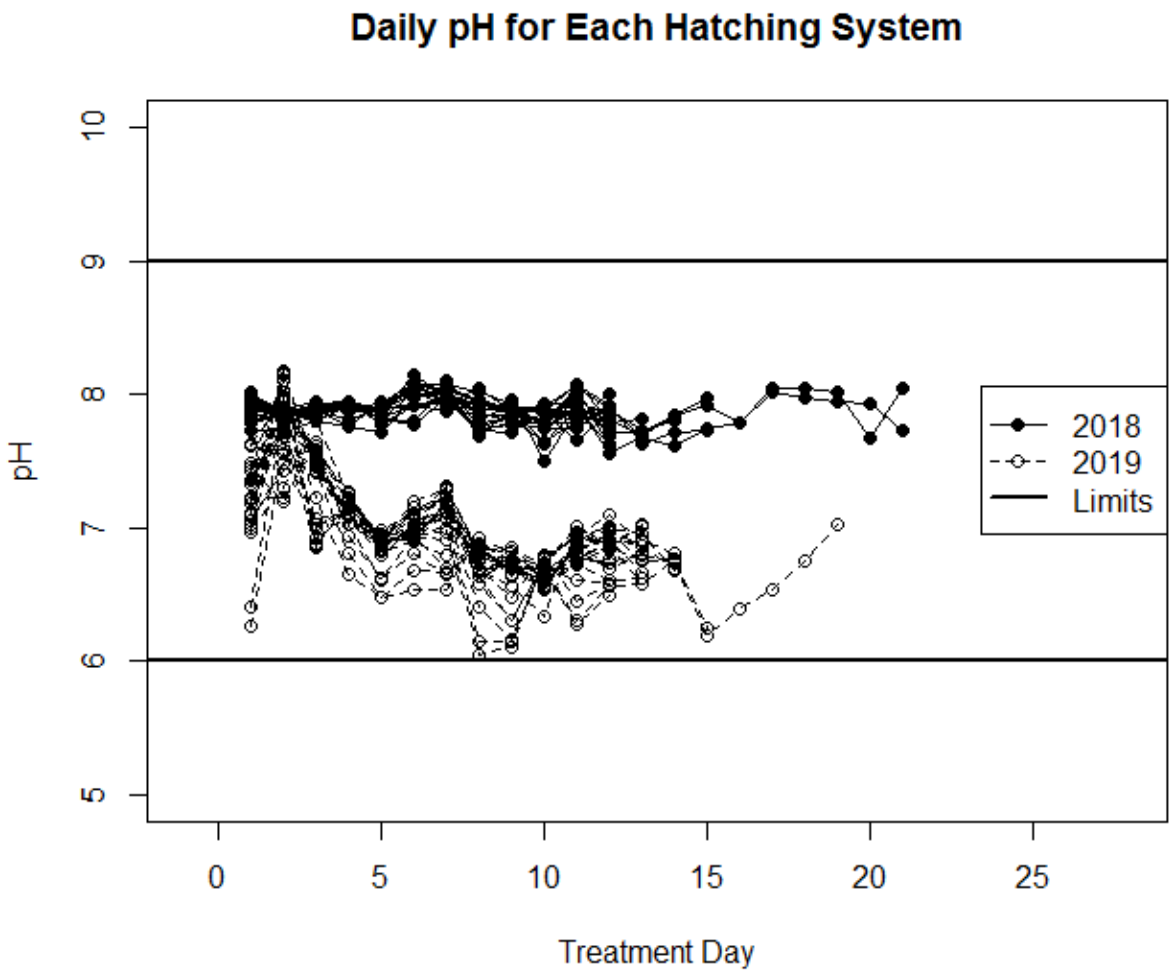


Figure 5. Daily pH values recorded in each walleye hatching system every morning during the experiments. Horizontal lines at pH values of 6 and 9 represent the lower and upper pH limits for optimal walleye production as described by Bozek et al. (2011b).

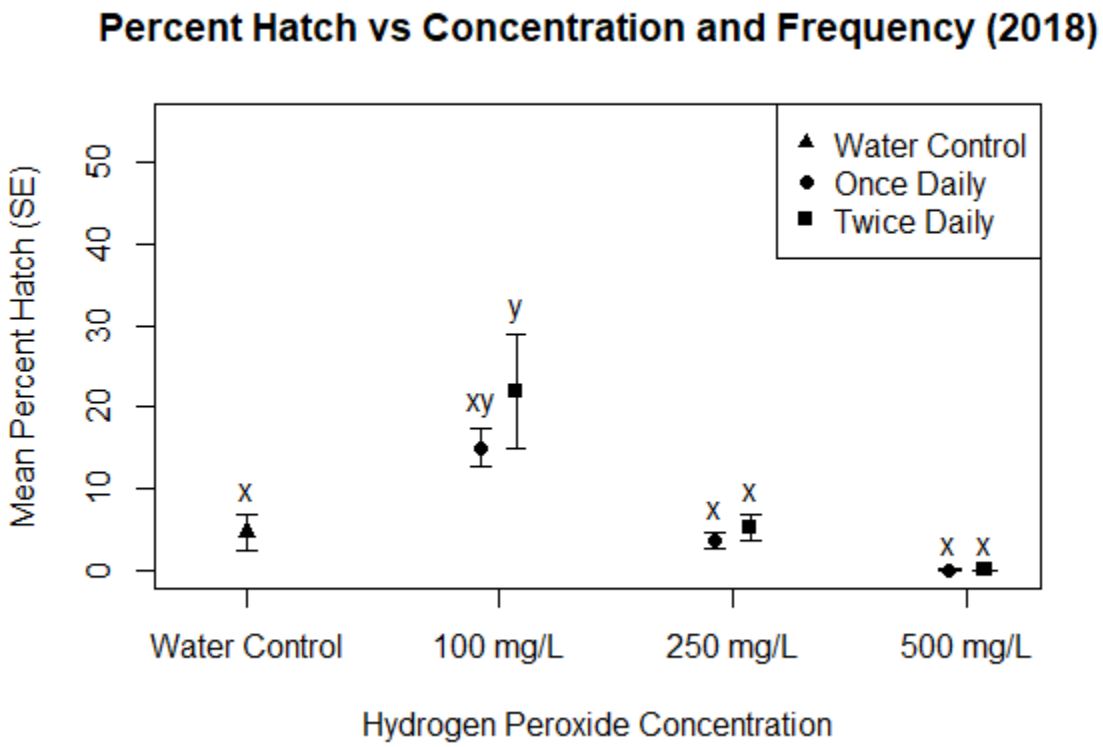


Figure 6. The mean percent hatch (± SE) of walleye eggs for each treatment regimen for the 2018 experiment. Points marked with different letters are significantly different.

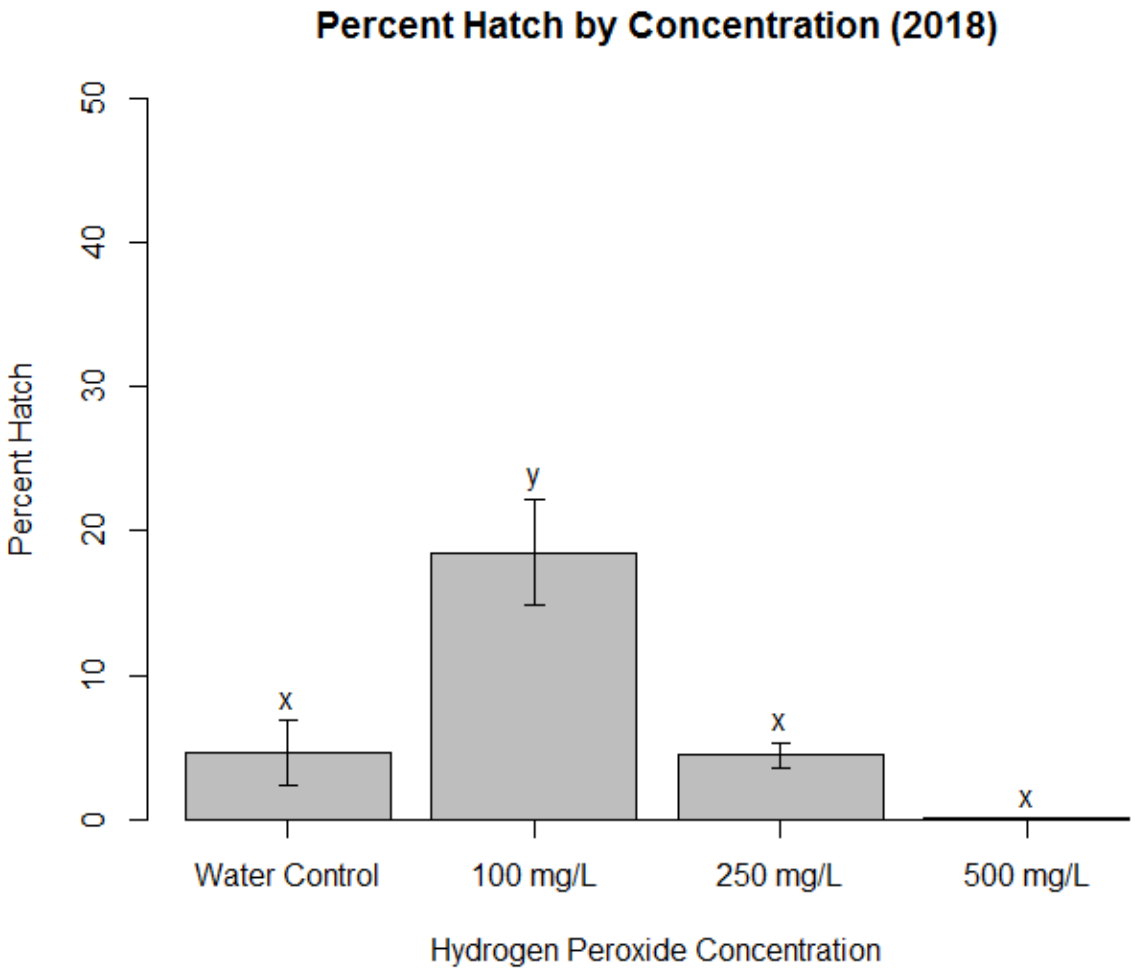


Figure 7. Mean percent hatch (± SE) of fertilized walleye eggs in four treatment concentrations from the 2018 experiment. Bars marked with different letters are significantly different.

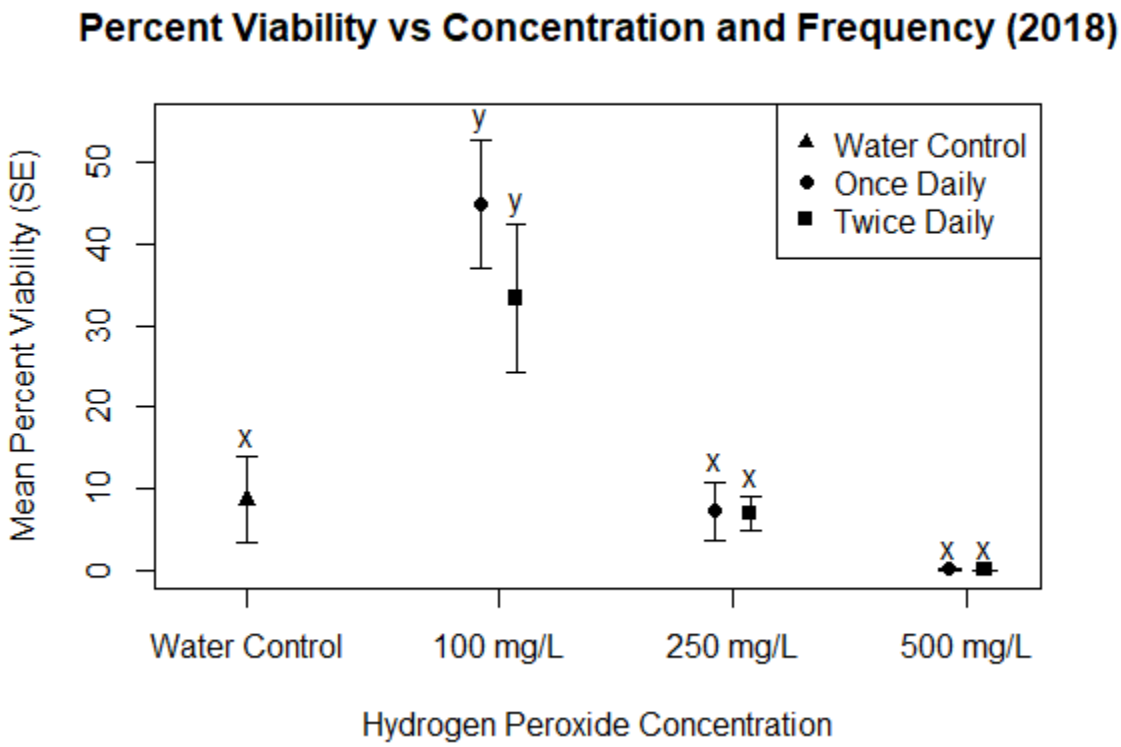


Figure 8. The mean percent viability (i.e., eyed eggs and hatched larvae at the end of the experiment; ± SE) of walleye eggs for each treatment regimen for the 2018 experiment. Points marked with different letters are significantly different.

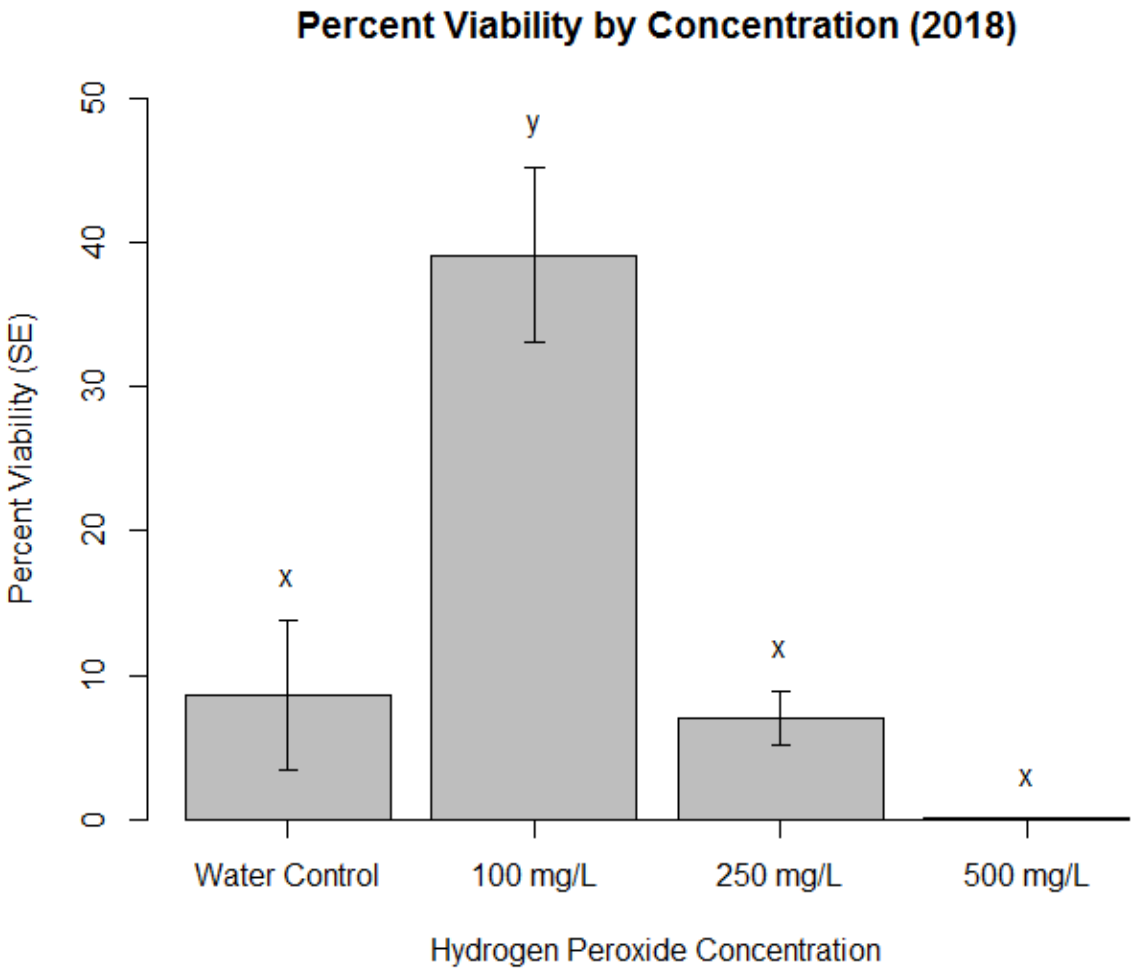


Figure 9. The mean percent viability (i.e., eyed eggs and hatched larvae at the end of the experiment; ± SE) of fertilized walleye eggs in four treatment concentrations from the 2019 experiment. Bars marked with different letters are significantly different.

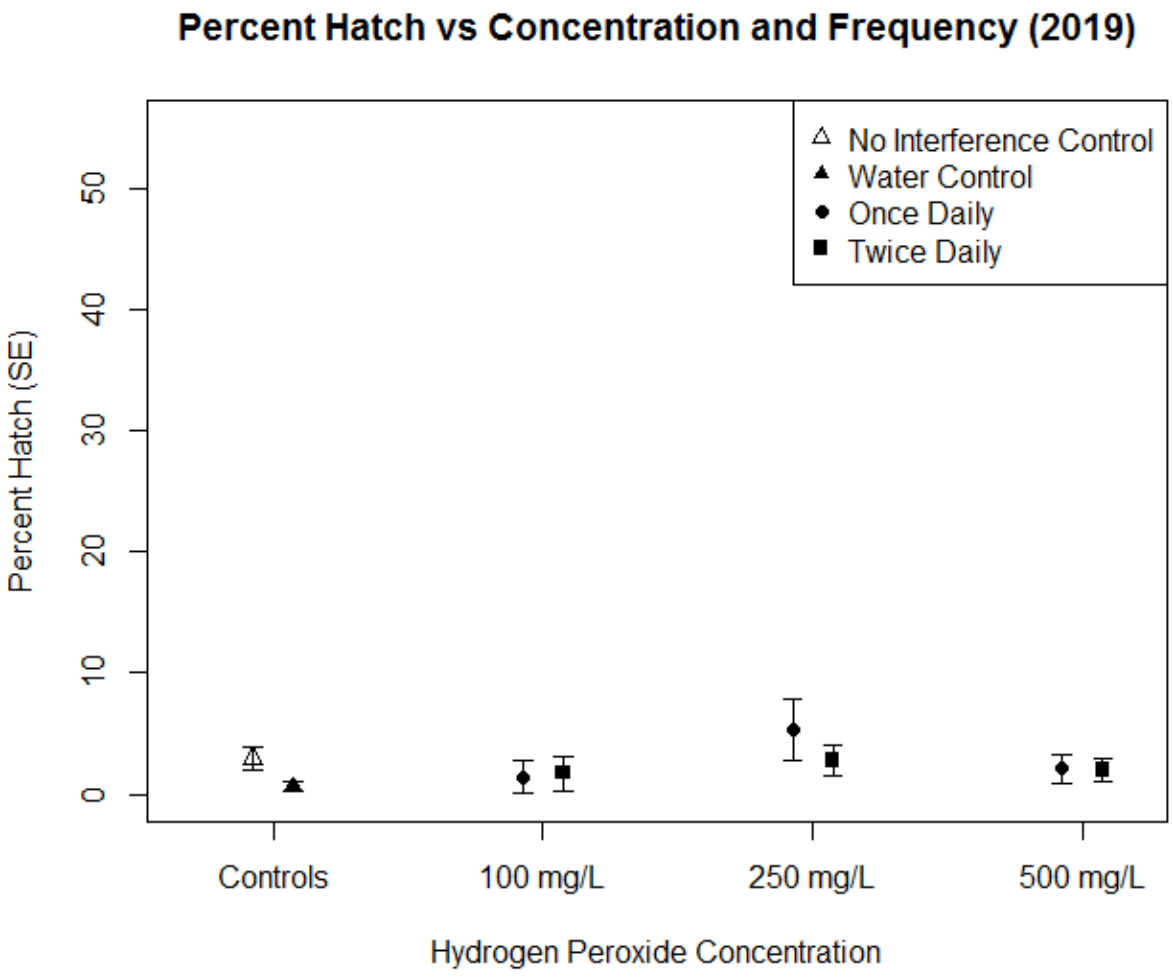


Figure 10. The mean percent hatch (± SE) of walleye eggs for each treatment regimen for the 2019 experiment. None of the treatment combinations differed significantly from one another (all $p > 0.05$).

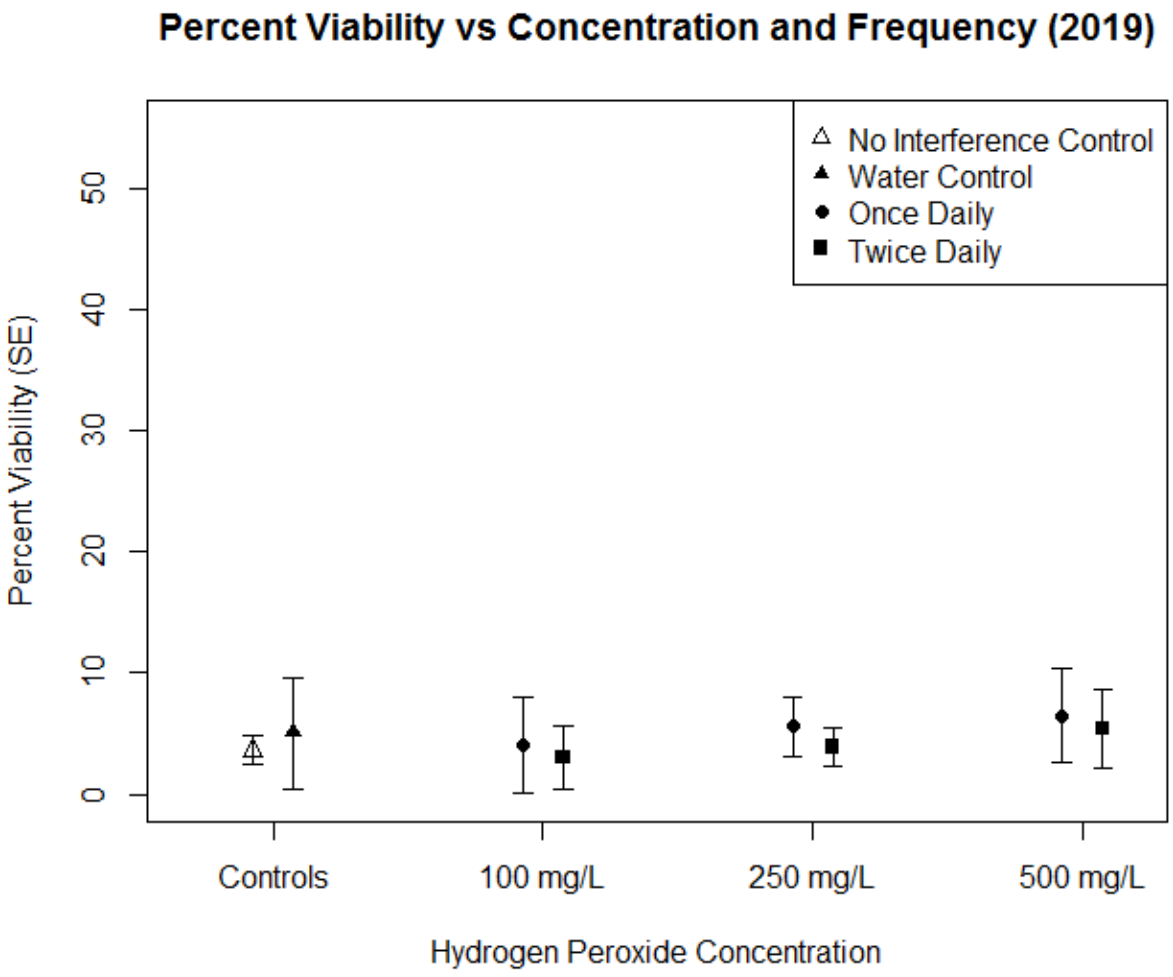


Figure 11. The mean percent viability (i.e., eyed eggs and hatched larvae at the end of the experiment; ± SE) of walleye eggs for each treatment regimen for the 2019 experiment. None of the treatment combinations differed significantly from one another (all $p > 0.05$).

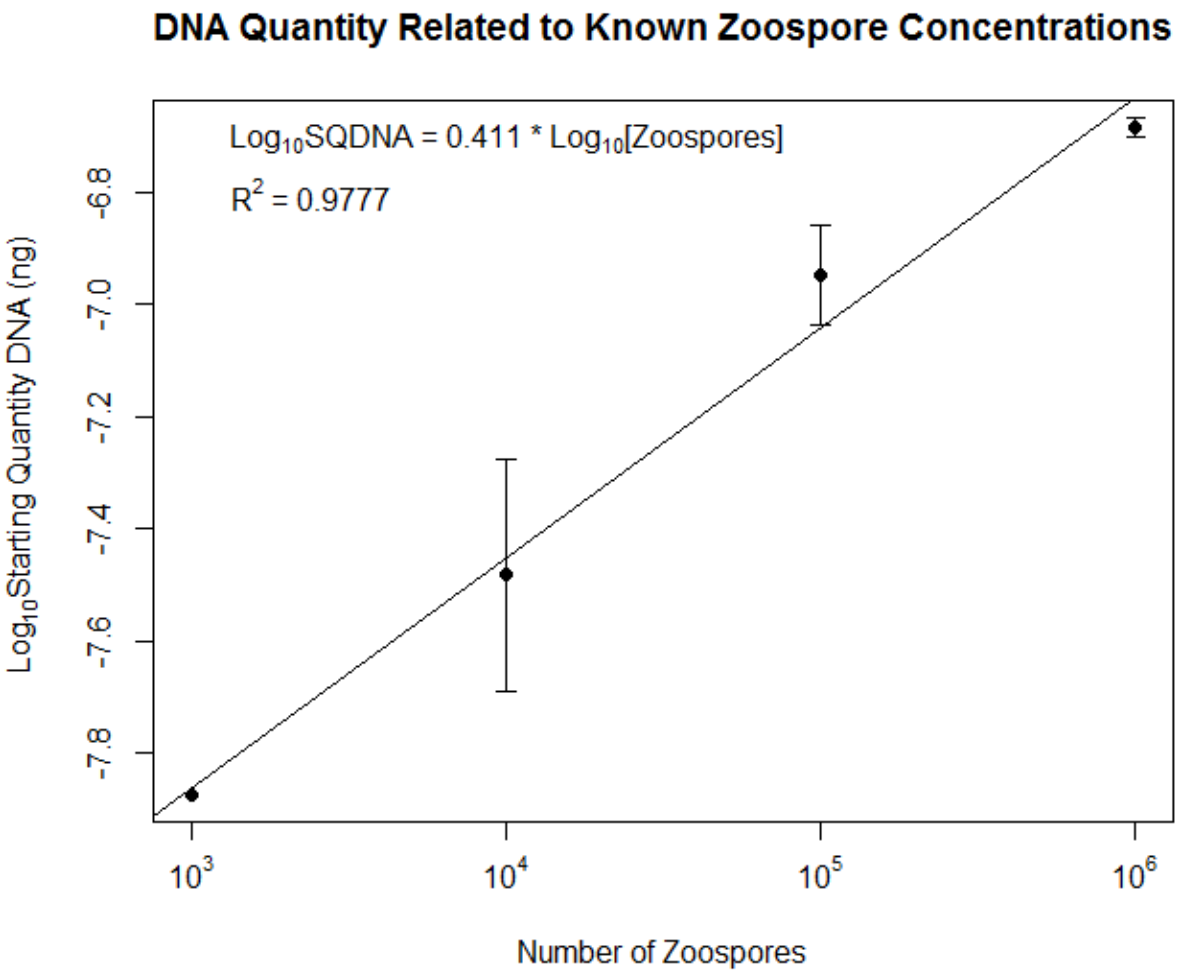
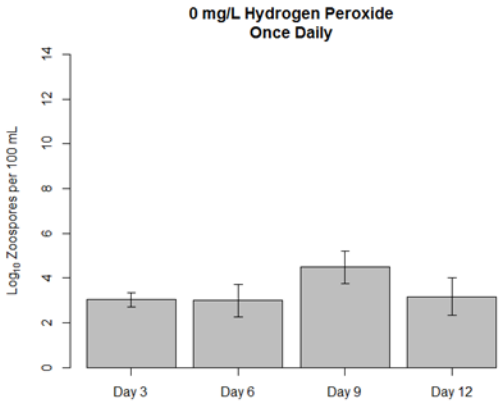
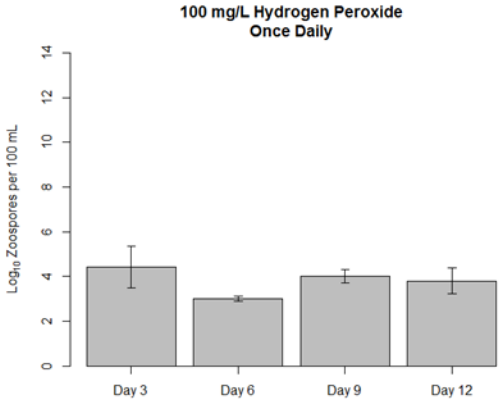


Figure 12. The mean $\log_{10}$ of starting quantities (± SE) of DNA from filters with known, 10-fold dilutions of zoospores is depicted. A best fit line for a linear regression of these data was generated and is displayed along with its equation, $R^2 = 0.9777$.


0 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


100 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
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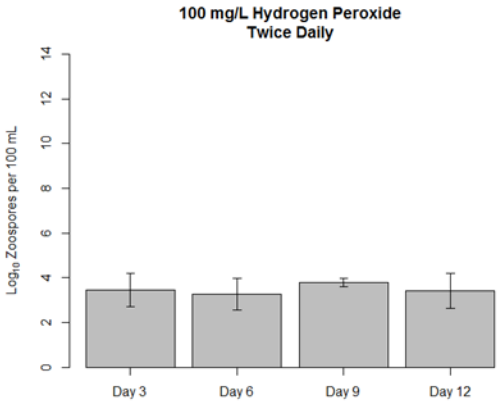
12
14
Day 3
Day 6
Day 9
Day 12


100 mg/L Hydrogen Peroxide
Twice Daily
Log10 Zoospores per 100 mL
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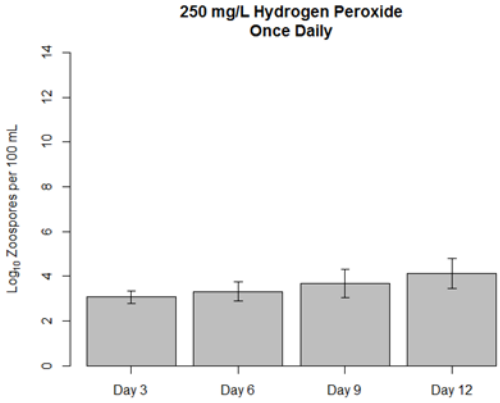
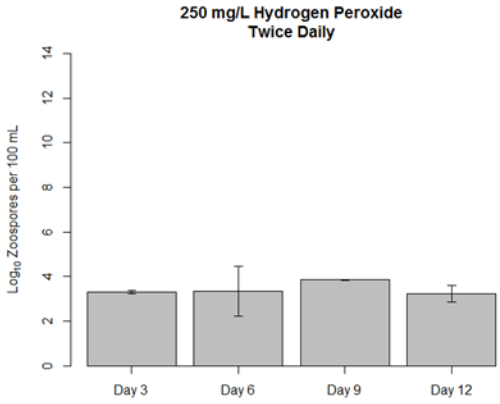
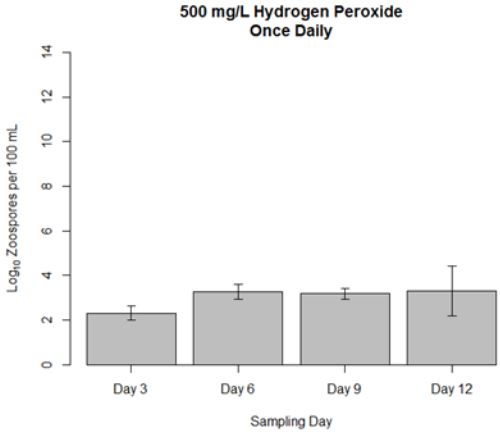
Day 3
Day 6
Day 9
Day 12


250 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


250 mg/L Hydrogen Peroxide
Twice Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


500 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12
Sampling Day

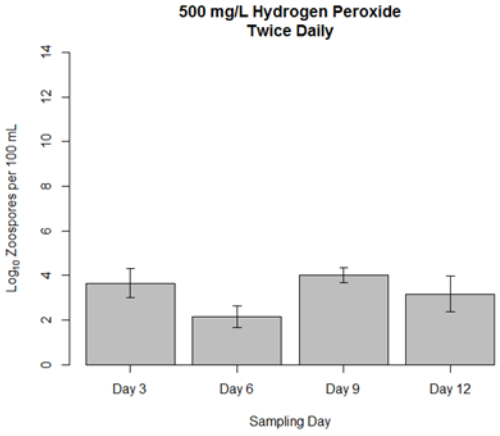
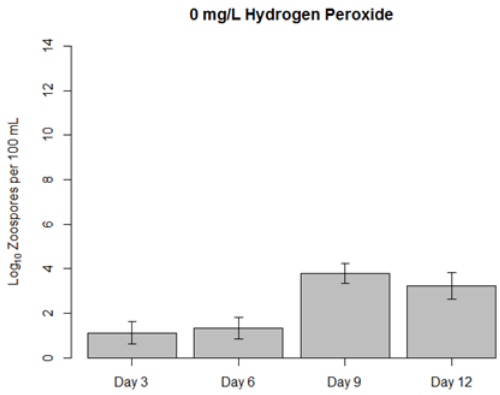
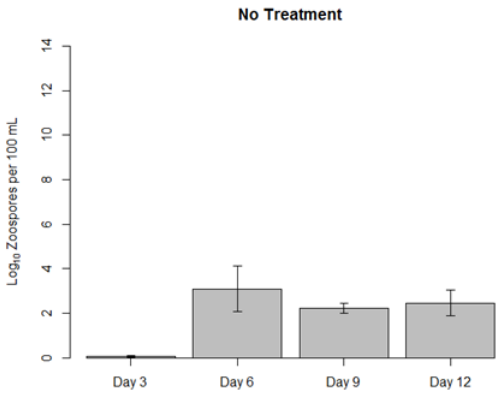
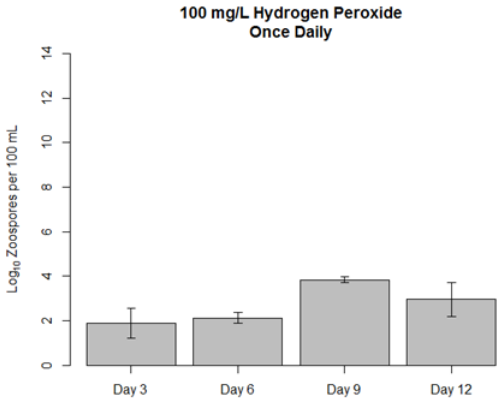
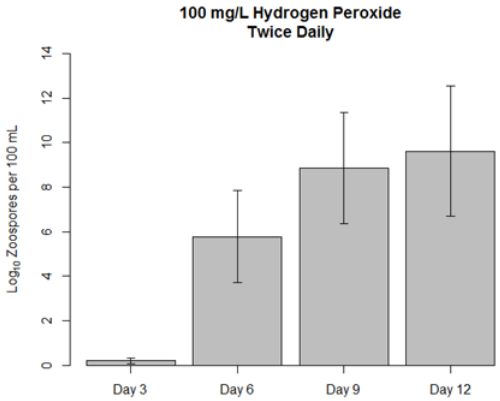

500 mg/L Hydrogen Peroxide
Twice Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12
Sampling Day

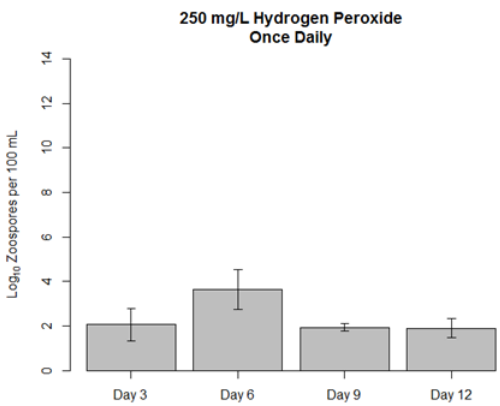
Figure 13. The $\log_{10}$ adjusted mean number of zoospores per 100 mL (± SE) for each treatment combination and sampling day during the 2018 experiment to evaluate the effects of hydrogen peroxide on pathogen growth on fertilized walleye eggs.

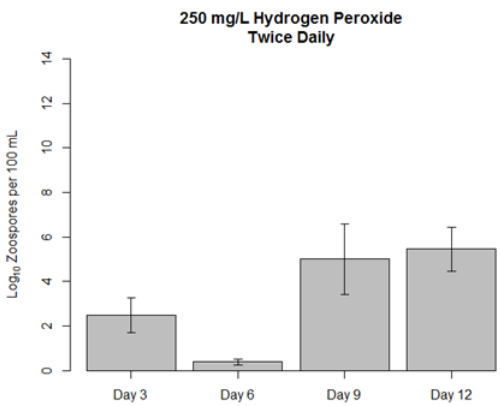

0 mg/L Hydrogen Peroxide
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12

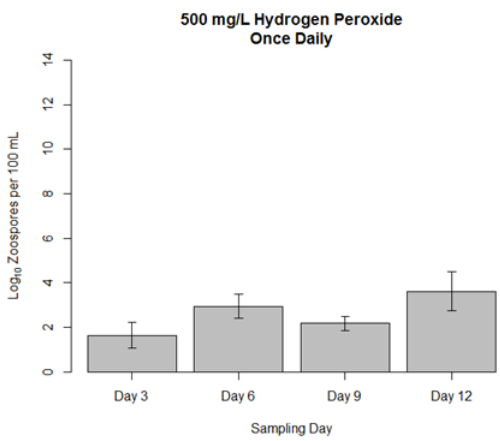

No Treatment
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


100 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


100 mg/L Hydrogen Peroxide
Twice Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


250 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


250 mg/L Hydrogen Peroxide
Twice Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12


500 mg/L Hydrogen Peroxide
Once Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12
Sampling Day

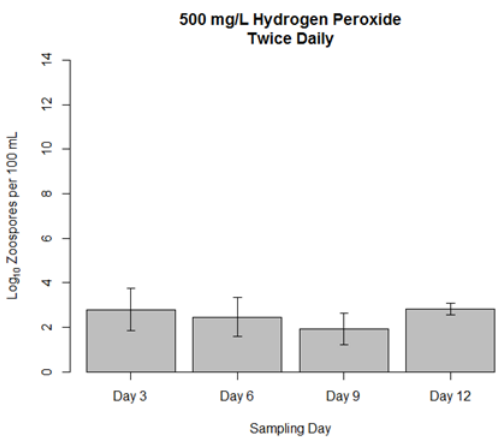

500 mg/L Hydrogen Peroxide
Twice Daily
Log10 Zoospores per 100 mL
0
2
4
6
8
10
12
14
Day 3
Day 6
Day 9
Day 12
Sampling Day

Figure 14. The $\log_{10}$ adjusted mean number of zoospores per 100 mL (± SE) for each treatment combination and sampling day during the 2019 experiment to evaluate the effects of hydrogen peroxide on pathogengrowth on fertilized walleye eggs.

APPENDICES

Appendix A. Walleye hardened egg count chart produced by the Wisconsin Department of Natural Resources and used by the Georgia Department of Natural resources for calculating the number of fertilized walleye eggs spawned.

Number in 6-inch trough	Number per Quart	Number per Liter	Number in 6-inch trough	Number per Quart	Number per Liter
38	16,960	17,928	58	61,925	65,460
39	18,240	19,281	59	65,680	69,429
40	19,680	20,803	60	67,670	71,533
41	21,744	22,985	61	71,899	75,993
42	23,140	24,461	62	74,146	78,378
43	25,197	26,635	63	78,927	83,432
44	26,901	28,436	64	84,130	88,932
45	28,764	30,406	65	86,904	91,865
46	30,801	32,559	66	92,826	98,125
47	32,268	34,110	67	95,990	101,469
48	34,647	36,625	68	99,297	104,965
49	37,265	39,392	69	102,762	108,628
50	39,161	41,396	70	110,190	116,480
51	41,186	43,537	72	118,346	125,101
52	44,494	47,034	74	127,333	134,601
53	46,899	49,576	76	137,251	145,086
54	49,480	52,304	78	148,220	156,681
55	52,254	55,237	80	160,400	169,556
56	55,239	58,392	82	173,950	183,879
57	58,456	61,793	84	189,070	199,862