Spring 5-2017

Pathobiology of Water Molds in Fish: An Insight into Saprolegniasis

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PATHOBIOLOGY OF WATER MOLDS IN FISH: AN INSIGHT INTO SAPROLEGNIASIS

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Marine Sciences)

The Honors College

University of Maine

May 2017

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ABSTRACT

Saprolegnia is an aquatic pathogen with a fishy appetite—it develops on farmed and wild fish populations as a notoriously destructive 'water mold'. The etiologic agent of Saprolegniasis, *Saprolegnia* is an opportunistic oomycete that is of significant interest in the aquaculture industry due to its financial impact and widespread effect. Previously, infection models studying the effects of *Saprolegnia* utilized methods that were injurious to fish and did not mimic a natural outbreak, thus making it difficult to use to evaluate new treatments for the disease. By developing a novel zebrafish (*D. rerio*) egg infection model, a new insight into the pathogenesis and progression of the disease *in vivo* is possible. Once completed, the model could serve as a platform to quickly identify and test alternative treatments to formalin. This study compared existing culture techniques of *Saprolegnia* and developed a novel staining method to view the pathogen during host invasion. Zebrafish eggs were infected with zoospore suspensions and monitored carefully. It was found that in all treatments, the zoospore concentrations were too low to infect healthy zebrafish eggs reproducibly. However, almost all zebrafish that were exposed to *Saprolegnia* colonized hemp seeds developed an infection within 24 hours. Different concentrations of Fluorescent Brightener 28 were assessed to develop an efficient staining protocol to visualize the disease progression. This staining technique could potentially be utilized to help quantify and track the infection in fish eggs. Future studies should consider the use of zebrafish as model organisms in *Saprolegnia* research.

ACKNOWLEDGEMENTS

I would like to thank everyone who has helped me with the development of this project, and for providing help and support throughout my college career. My sincerest appreciation to my advisor, Dr. I. Bricknell for being my mentor and friend throughout the project, and many thanks to the Bricknell lab for helping wherever possible. A million thanks to Dr. S. Barker; as an integral part of this project, she helped guide me throughout the whole infection model process and many other laboratory procedures. Thanks are due to the following persons, either for their advice or their generous assistance: Dr. R. Wheeler, Dr. R. Gratacap, the Wheeler lab, Ms. D. Bouchard and lab, Dr. T. Bowden and lab, and Mr. M. Nilan. I would also like to thank my advisory committee for this learning opportunity, and for their time and patience throughout this experience.

I am also indebted to Fish Vet Group, Inc., University of Maine Center for Undergraduate Research (CUGR), and the Aquaculture Research Institute for support through internships and undergraduate research grants. I am grateful to the University of Maine Honors College which provided this unforgettable learning experience.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

Saprolegnia is an aquatic oomycete that affects many organisms, particularly freshwater salmonids in the aquaculture industry. Although oomycetes (commonly referred to as 'water molds') appear to have fungal characteristics, they are classified as Stramenopiles and are more closely related to chromophyte algae (van West 2006; Sarwowar *et al.* 2013). Oomycetes are some of the most destructive pathogens responsible for diseases in freshwater fish, crustaceans, amphibians, and insect larvae (van West, 2006). However, the role and impact of *Saprolegnia* and other oomycete pathogens is often overlooked due to the paucity of data on the pathobiology of water molds in fish (van den Berg *et al.* 2013).

Saprolegnia is the etiologic agent of Saprolegniasis, a disease characterized by white or gray patches of mycelium growing on the body and into the epidermis of fish, with severe cases covering the entire body of the fish. *Saprolegnia* is also responsible for 'winter kill' in catfish (*Ictalurus punctatus*) raised in the aquaculture industry (Quiniou *et al.* 1998).

Aquaculture is a growing industry, and currently accounts for over 50% of the seafood supply within the United States (NMFS, 2016). Fungal infections of freshwater fish eggs, particularly salmonids, are a major economic problem in the aquaculture industry, causing millions of dollars in losses annually (Fregeneda-Grandes *et al.* 2007; Jiang *et al.* 2012). These pathogens pose a significant threat to food security worldwide (Van den Berg *et al.* 2013).

Saprolegniasis is usually a secondary infection incited by the fish's depressed immune system, due to sickness, poor water quality, or change in temperature, and causes

large wounds on the skin (Sarowar *et al.* 2013). When *Saprolegnia* invades the gills, it impairs respiration and osmoregulation as well as causing hemodilution; extensive gill infections can cause acute respiratory failure leading to death (Sarowar *et al.* 2013).

Previously, *Saprolegnia* infections were treated successfully with the alanine dye Malachite Green (4-{[4(dimethylamino)phenyl](phenyl)methylidene}-*N*, *N*-dimethylcyclohexa-2,5-dien-1-iminium chloride) until the substance was banned in many countries including the US for its known carcinogenic, mutagenic, and teratogenic properties (Culp & Beland 1996). No fully effective alternative treatment to Malachite Green has been found (Pottinger & Day 1999). Other substances such as formalin and hydrogen peroxide are used to treat the pathogen, but the safety and environmental impacts of these chemicals are questionable (Pottinger & Day 1999). That leaves us with the question of 'how do we effectively combat Saprolegniasis in a safe and cost-effective way?'

To answer this important question, it is proposed to develop an *in vivo* zebrafish model to test the pathogenesis of *Saprolegnia* using a fish model organism susceptible to natural infections with *Saprolegnia.* Zebrafish (*D. rerio* (Hamilton, 1822)) are being increasingly used to assess the effects of chemical compounds in the biomedicine and chemical industries (Kanungo *et al.* 2014). Zebrafish are perfect research animals for this investigation for several reasons. The first is that zebrafish take a relatively short time to culture in comparison to salmonids allowing research to progress quickly. *D. rerio* is also a natural host of *Saprolegnia* (Ke *et al*. 2009). The embryos and eggs are used because of their microscopic size and optical transparency, which allows for non-invasive visualization of the development of disease *in vivo*. The genome of *D. rerio* has been

sequenced, which makes for easier identification and genomic work (Kanungo *et al.* 2014), as well as the fact that several transgenic lines are available to be able to study host-pathogen interactions. The use of zebrafish eggs and embryos also exemplifies the principles of the three R's in animal research: reduce, replace and refine (Goodman, Chanda, and Roe 2015). By replacing adult animal with the egg and yolk sack larvae and refining the experiments by providing a standardized challenge method, a more humane infection model can be used to study the pathobiology of water molds in fish.

It is imperative that an alternate treatment for *Saprolegnia* be developed since Saprolegniasis is not only the leading cause of freshwater fish kills but a significant cause of economic loss in the aquaculture industry. With global food demand on the rise, aquaculture is becoming increasingly important to the world food supply. With the development of a safe and cost-effective treatment of Saprolegniasis, not only will profits increase but also, more importantly, the aquaculturists employing the treatment, as well as the environment, will be safer.

In this study, the pathobiology of several species of *Saprolegnia* was investigated by the optimization of *in vitro* culture methods, and by developing an *in vivo* zebrafish model to study the progression of the disease in real-time. This infection model may serve as a new platform to rapidly screen chemical agents for anti-fungal properties against *Saprolegnia* infections in the future.

METHODS

Obtaining and purifying cultures

The American Type Culture Collection (ATCC) wild type strain of *Saprolegnia parasitica* (Coker, ATCC® 200233™ strain NMJ8761) was acquired and cultured per the distributor's instructions, then subsequently cultured onto 2% Peptone Yeast agar (Atlas, 2004), 3% V-8 (ATCC medium 2040), or 2% Glucose Yeast agarose plates (Atlas, 2004). Infected zebrafish eggs (Zebrafish facility, University of Maine, Orono, ME) and infected juvenile salmonids (Cooke Aquaculture) were obtained to isolate *Saprolegnia*. Dead *D. rerio* eggs were disinfected in 70% ethanol, and one or two eggs were plated onto low-nutrient water agar (2%) (12g BD bacto agar, 1L Nanopure water). Plugs of agar (~5mm²) were excised from the leading edge of the mycelia and subsequently placed onto fresh agar plates, and this process was repeated until pure isolates were obtained. Sterile cotton swabs were aseptically rubbed over the gills, the dorsal fin, or other afflicted areas on juvenile salmonids and either plated onto water agar or inoculated into sterile distilled water baited with hemp seeds. Samples taken from fish were initially treated with Gentamicin, Trimethoprim, and Sulfamethoxazole-trimethoprim antimicrobial Sensi discs (BBL). After subculturing *Saprolegnia* isolates several times, antibiotic use was discontinued. This was to ensure that antibiotics did not inhibit vegetative growth and to prevent microbial antibiotic resistance. Additionally, lake water was obtained and seeded with hemp seeds (Willoughby, 1962; Willoughby & Pickering, 1977) and grown on water agar until pure isolates were obtained.

Water samples from the Penobscot River and Pushaw Lake (Orono, ME) were obtained, filtered, and sterilized for culture media. The *Saprolegnia* cultures were

incubated at room temperature (~22º C) on Glucose Yeast Agar, V-8 agar, and a modified Peptone Yeast Agar for several days. Different media were utilized to 1) ensure that there were plenty of viable samples to use for the experiment and 2) determine which medium is optimal for survival and growth. Single spore isolations (Ho & Ko, 1997) were conducted to further isolate *Saprolegnia* from other aquatic fungi and bacteria in cultures before genomic identification. Spore suspensions were plated onto agar plates subdivided into 100 marked sections. The agar plates were then closely monitored under a dissecting microscope, and when a single germinated spore was identified, a 5mm agar plug was excised with sterile needles and plated onto a new LNA plate and incubated at 22ºC.

Samples of *Saprolegnia* were also prepared for long-term preservation by following established protocols for the preservation of *Phytophthora* and *Pythium* (Dr. J. Hao lab protocols kindly provided by N. Marangoni, 2016). Colonized plugs of agar were excised from cultures and placed into 2mL bijous (Sterilin) and incubated at 4ºC for up to several months. Additionally, colonized hemp seeds were placed into sterile Nanopure water and incubated at room temperature (22ºC). Table 1 provides a description of *Saprolegnia*

isolates.

Identification of cultures

Morphological identifications were made where possible; not all *Saprolegnia* cultures produced sexual structures *in vitro.* Cultures were allowed ample time (at least three days) to grow in GY broth (10g glucose, 2.5g yeast extract, 1L Nanopure water) before portions of mycelia were extracted aseptically with sterile forceps. Approximately

40mg of fresh mycelia was harvested and lysed with 3mm glass beads and nuclei lysis solution (Promega) on a tissue lyser (Qiagen) at 28Hz for 2 minutes.

The gDNA was then extracted following a plant and yeast gDNA extraction protocol (Promega). The concentration of gDNA for each sample was measured with a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). 35 cycles of polymerase chain reaction (PCR) were then performed utilizing International Transcribed Spacers (ITS) 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGA AGTAAAAGTCGTAACAAGG-3') with a denaturation temperature of 95ºC for 0.5min, annealing at 57ºC 0.5min, and extension at 73ºC for 5min with a final hold at 4ºC (Eissa *et al.,* 2013). The purified PCR products were then visualized on a 1.5% agarose electrophoresis gel with ethidium bromide. Singular bands were excised and purified with a gel extraction kit (Qiagen), and then they were sent to the University of Maine DNA sequencing lab for sequencing and editing. Sequences were then analyzed and compared to an online nucleotide database (BLAST).

Optimization of staining techniques

Wild-type (AB strain) zebrafish eggs were collected and treated with methylene blue for several hours and then washed in 1x E3. The eggs were then divided into five different Petri dishes with n=50 eggs in each dish (2 controls, 3 infected with *Saprolegnia*) and inoculated accordingly. Either a single autoclaved hemp seed or a colonized hemp seed was washed three times with autoclaved well water and placed in the petri dish, incubating at 26ºC overnight. Infected eggs were then collected and

euthanized in a lethal dose of tricaine (0.4g/L) buffered with tris base (pH 9) for 10 minutes and then fixed in 10% neutral buffered formalin (NBF) at 4ºC overnight.

Eggs were then subjected to different staining conditions for the optimization of the fluorescent staining techniques. Eggs were either treated with PBS, 25µM CW, 2.5µM CW, or 0.25µM CW and 1.5% DMSO for a given time point (15min, 30min, 1hr). After the time point had passed, eggs were rinsed three times with PBS and mounted with 80% glycerol in a 24-well glass viewing plate for fluorescent microscopy and imaging. The stain was qualitatively assessed for its efficacy by the clarity of images, the brightness of the stain, and whether the mycelia were stained or not.

Lactophenol Cotton Blue (LCB) stain droppers (BD) were utilized to identify Saprolegnia samples *in vitro* morphologically. Small bundles of mycelia were aseptically removed from colonized hemp seeds and placed onto a glass slide. A drop of LCB was added to each slide to stain the *Saprolegnia.* Samples were viewed with an inverted compound light microscope (Zeiss).

Zebrafish egg infection model development

Wild-type strain zebrafish (*D. rerio*) eggs were obtained from the University of Maine zebrafish facility (Orono, ME). Eggs were placed into 150mL sterile Petri dishes containing 1x E3 media (5mM NaCl, 0.17mM KCl, 0.33mM$CaCl_2$, 0.33mM $MgSO_4$, 1L water, pH 7.0) and 0.013% methylene blue to disinfect the water. In each container that zebrafish eggs were kept, egg densities did not exceed one egg per mL of E3 media to ensure proper oxygenation of eggs (Dr. Remi Gratacap and Dr. Sarah Barker, personal comm., 2014). Eggs were incubated at 26ºC and kept no longer than 48 hours post-

fertilization (hpf) per IACUC regulations. The W-4 strain *Saprolegnia* was utilized for all infection assays (Table 1).

After at least 6 hours of disinfection with methylene blue, eggs were washed with fresh 1x E3 media three times, and the culture media was then replaced. Eggs were portioned into 50mL Petri dishes and incubated overnight at 26ºC, which is close to the natural temperature at which zebrafish live (*circa*. 28º C). The next day, unfertilized or dead eggs were removed.

Eggs were portioned into 12-well plates (n=6 per well), and for each infection assay, one plate was designated for mortality assessments alone, and one plate was designated for staining and viewing eggs. This was to ensure that eggs did not die due to handling stress or staining. The eggs were monitored over two days for mortalities and the presence or absence of the pathogen as well as its dissemination.

To determine the concentration of zoospores necessary to cause an infection of Saprolegniasis at a prevalence of 60-70%, a preliminary infection assay utilizing zoospores was performed. Motile zoospores were obtained following established sporulation protocols (Diéguez-Uribeondo, Cerenius, & Söderhäll, 1994). Spores were isolated by gently pipetting from the top of sporulating samples and then pooled into 20mL portions and enumerated on a cell counter. Spore concentrations were adjusted by diluting with zebrafish media (1x E3 media) for infection trials.

Wild-type zebrafish eggs were collected and disinfected with methylene blue and washed as described previously. The eggs were then plated into 12-well plates containing different dilutions of zoospore suspensions (10 spores/mL, 100 spores/mL, 500 spores/mL, and 1000 spores/mL E3) and a negative control of E3. The eggs were then

incubated overnight at 26°C and then washed again in the morning. The eggs were monitored for visible signs of infection characterized by white or gray mycelia as well as mortalities.

Interpretation of results

Zebrafish mortalities and infection prevalence were quantified over the course of two days post-infection. An ANOVA test was conducted (GraphPad Prism 6 software) for the different zoospore concentration treatments to assess if there was any significant difference between mortalities in treatments. Additionally, mortality curves were plotted for the infection trial to evaluate how the disease progressed over time.

RESULTS

Obtaining and purifying cultures: culture method efficacy

Most *Saprolegnia* samples were not very selective about growth medias (apart from the PL-1 isolate) and achieved growth rates of approximately 10mm per day on agarose plates under the culture conditions used there. Low nutrient water agar (LNA) allowed for sparse mycelial growth, but bacteria did not grow readily on it. LNA was best suited for sub-culturing *Saprolegnia* directly from the fish and preventing bacterial contamination without the use of antibiotics. Peptone yeast glucose (PYG) broth supplemented with salmon sterols facilitated vegetative growth, but GY broth had similar effects.

V-8 agar plates were optimal for preserving cultures for long-term, as the mycelium did not penetrate the agarose as quickly as in other medias. Colonized plugs of agar stored in 2mL bijous were viable for up to 6 months and could be revitalized by adding 1mL sterile water and split hemp seeds and subsequently subcultured onto fresh agar plates. Samples stored in sterile ddH_2O with split hemp seeds remained viable if the mycelia remained moist (an excess of 6-8 months, ongoing). After some time, samples stored in the ddH_2O sporulated spontaneously. Some isolates were more viable than others after long-term storage; PL-1 and ZF-1 isolates did not recover well after being stored at 4ºC but remained viable after storage at room temperature (23ºC).

Identification of cultures

The Mystic Aquarium isolate was a species of *Ceratobasidium,* an opportunistic fungus (Table 1). It was found that Floyd's baitfish farm isolates contained a minuscule amount of *Saprolegnia parasitica* mixed with *Phoma ascomycetes,* a freshwater fungus (Table 1). It was also found that the W-4 Cooke Aquaculture isolate was a pure strain of *Saprolegnia salmonis* (Table 1). The W-4 and ATCC-2 isolates were kept for studies of pathogenicity of *Saprolegnia,* as well as for the development of the zebrafish model.

The PL-1, H1-L, and ZF-1 isolates morphologically appear to be *Saprolegnia sp.* However, molecular identification of those samples was not assessed yet (Table 1). The unidentified samples were cultured and preserved as previously described for future identification and use in pathogenicity studies.

The W-4 and ATCC-2 strains had pyriform primary zoospores with two subapical flagella, and reniform, laterally biflagellate secondary zoospores. Both isolates grew luxuriantly on sterile hemp seeds in water at room temperature (~23°C). Some zoosporangia had formed on W-4 isolates, and zoospores were discharged between 12-16h after incubating in GY broth. In both isolates, hyphae were sparingly branched, stout, and aseptate. Sporangia were straight, fusiform, and terminal for both W-4 and ATCC-2 strains. No other strains produced sporangia or zoospores *in vitro.*

Isolate	Location	Source	gDNA ID
Cooke	Cooke Aquaculture, Bingham, ME	Juvenile Atlantic Salmon (*Salmo salar*) dorsal fin	*S. parasitica, Aspergillus sp.*
W-4	Cooke Aquaculture, Bingham, ME	Juvenile Atlantic Salmon (*Salmo salar*) dorsal fin	*Saprolegnia salmonis*
Floyd's	Floyd's Baitfish Farm, ME	Rainbow smelt (*Osmerus mordax*) dorsal fin	*Phoma ascomycetes*, *S. parasitica*
ATCC-2	Miyagi, Japan	Coho salmon (*Oncorhynchus kisutch*)	*S. parasitica*
PL-1	Pushaw Lake, Orono, ME	Lake water (hemp seed bait)	NA
ZF-1	Zebrafish facility, University of Maine, Orono, ME	Zebrafish eggs (*D. rerio*)	NA
H1-L	Cooke Aquaculture, ME (raised at University of Maine, Orono, ME)	Juvenile Atlantic Salmon (*S. salar*)	NA
Mystic	Mystic Aquarium, Stonington, CT	Newt food pellets	*Ceratobasidium sp.*

Table 1: Molecular identification of *Saprolegnia* cultures.

Optimization of staining techniques

It was found that the hyphae stained with CW fluoresced brightly and was visible at most concentrations of CW utilized. Calcofluor White effectively distinguished mycelia from the egg chorion, but in some eggs, the yolk and chorion auto-fluoresced. In the negative control treatments, CW did not fluoresce (Fig. 1). It was found that 25µM CW was the minimum concentration that fluoresced adequately to distinguish different

mycelia and fluoresce brightly (Fig. 2). 250µM of CW was also found to stain the mycelia well (Fig. 3), but it was not necessary as the 25µM CW concentration also worked efficiently but used less stain.

Eggs that were infected with *Saprolegnia* died overnight and appeared 'fluffy' and overwhelmed with dense mycelia. By the time infected eggs were imaged, much of the embryonic tissues had been degraded by *Saprolegnia* (Fig. 2). There was an infection prevalence of approximately 60%, and of those eggs that were infected, 100% of them died within 24 hours post-infection (hpi). The nature of the rapid and widespread infection indicates that zebrafish are indeed susceptible hosts to *Saprolegnia* at early life stages and that zebrafish eggs can be used in future studies for *Saprolegnia* infection trials.

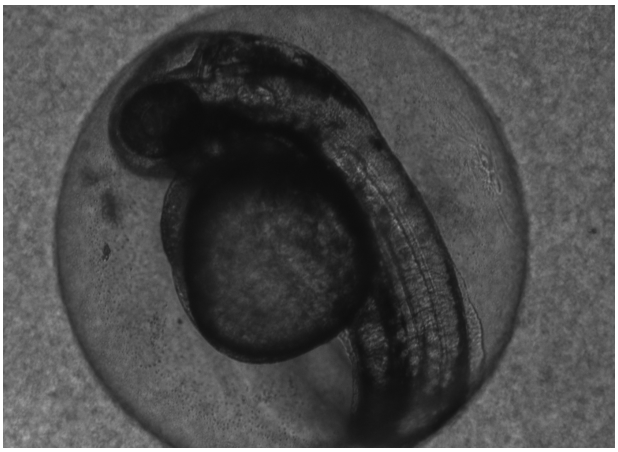

Fig. 1: Zebra fish 24hpi at 10x magnification. The mycelia were stained for 15min with 250µM fluorescent brightener 28 (CW).

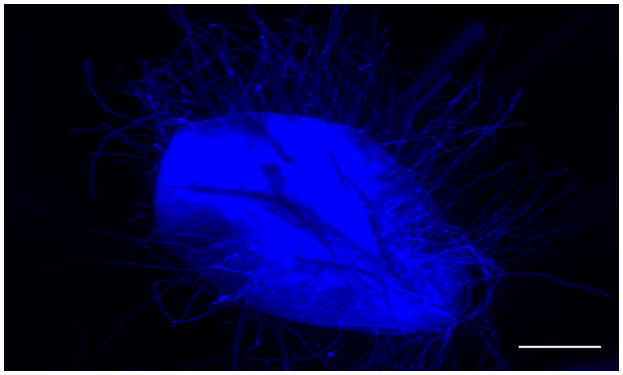

Fig. 2: Zebra fish egg infected with *S. parasitica* 24hpi at 10x magnification. The mycelia were stained for 15min with 25µM fluorescent brightener 28 (CW). Scale bar is 200uM.

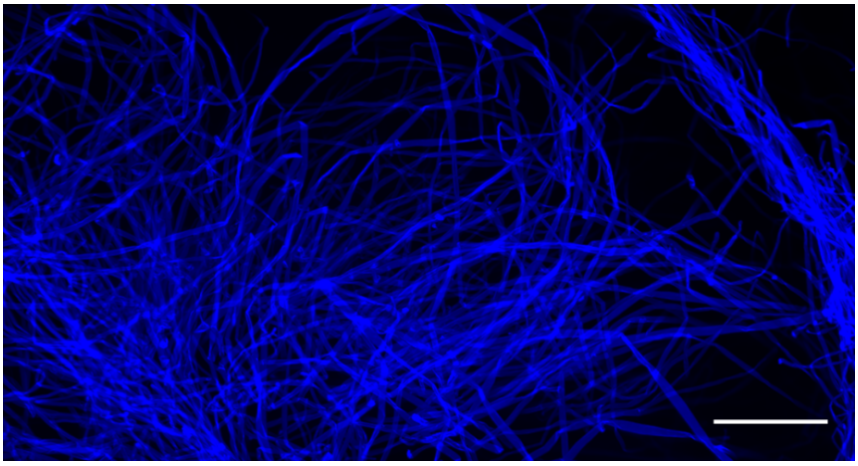

Fig. 3: Zebra fish egg infected with *S. parasitica* 24hpi at 10x magnification. The mycelia were stained for 15min with 250µM fluorescent brightener 28 (CW). Scale bar is 200µM.

The LCB stained mycelia effectively (Fig. 4), but should be tested on the oogonia and antheridia of Saprolegniales. However, no sexual structures formed on any of the *Saprolegnia* cultures, making a morphological identification difficult. Any traces of hemp seed that remained in the subsample of *Saprolegnia* were stained with LCB (Fig. 4). Despite efforts to prevent pieces of hemp

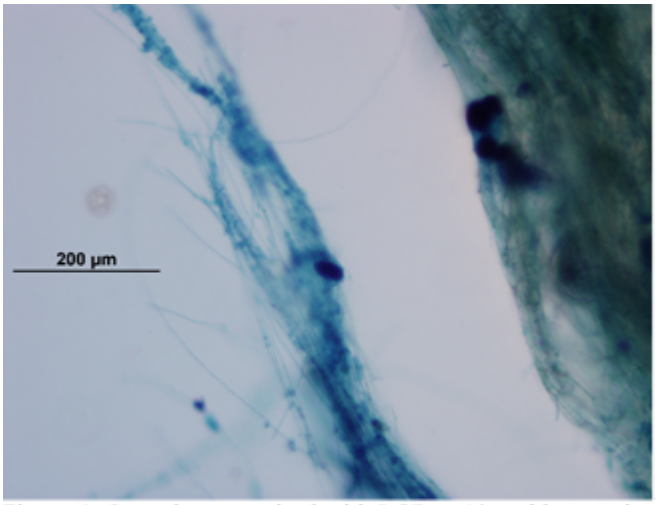

Figure 4: *Saprolegnia* stained with LCB at 10x with organic matter. Scale bar = 200µm.

from being mixed with the sample, we were unable to extract all the hemp seed material from the sample.

Zebrafish egg infection model development

In preliminary zebrafish infection trials, infection prevalence was 0% for all treatments (0, 10, 100, 500, 1000spores/mL E3). There were no mortalities within any treatment (Fig. 5, 6). There was no significant difference between the mortalities in *Saprolegnia* zoospore treatments (ANOVA, $p>0.05$).

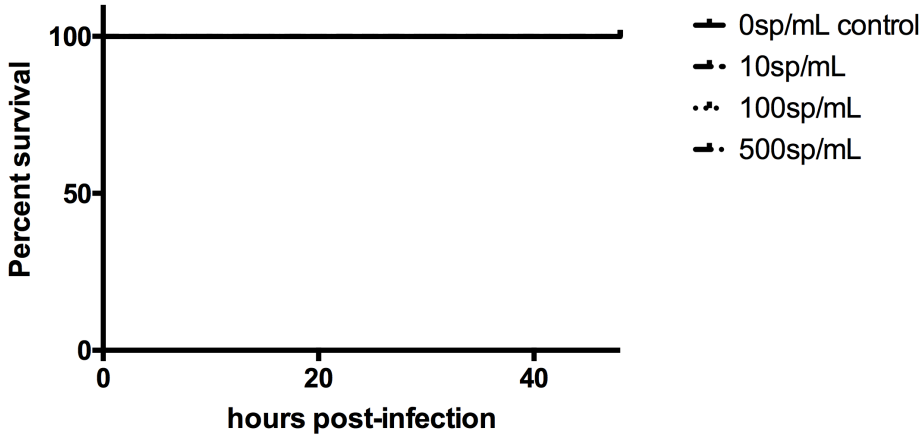

Figure 5: Zebrafish egg mortality assessment curve. Eggs were treated with different concentrations of spore/cyst suspension and incubated overnight. N= 144 eggs. Eggs were infected at 6-8hpf.

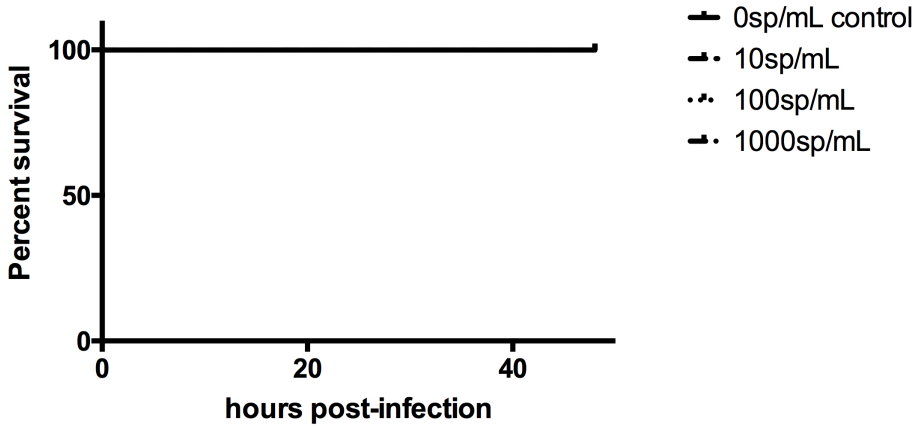

Figure 6: Zebrafish egg mortality assessment curve. Eggs were treated with different concentrations of spore/cyst suspension and incubated overnight. N=288 eggs (72 eggs per plate). Eggs were infected at 6-8hpf.

DISCUSSION

Most of the *Saprolegnia* samples were not selective of their growth media (except for the PL-1 isolate, which grew very sparsely on almost all medias), and demonstrated similar radial growth rates. Long term storage of *Saprolegnia* isolates yielded variable results for the viability of cultures. W-4, ATCC-2, and H1-L isolates remained viable after storage at both 4ºC and at 23ºC, whereas the ZF-1 and PL-1 isolates were not viable at 4ºC. This may be indicative of the isolates' natural habitats, though *Saprolegnia* is found in nearly every temperate freshwater environment globally.

None of the *Saprolegnia* strains produced sexual structures (oogonia, antheridia) *in vitro,* and as such, molecular identification of samples was necessary for the project. ATCC-2 and W-4 samples produced primary zoospores and cysts, but did not produce secondary spores and cysts, and did not exhibit repeated zoospore emergence (RZE). Saprolegniales are primarily identified by morphological features, typically sexual structures (i.e. oogonia, antheridia, oospores), despite the variability of the morphology of these structures (Coker, 1923; Seymour, 1970). However, morphologically identifying Saprolegniales can be problematic, as many isolates do not form sexual structures *in vitro* (Stueland, Hatai, & Skaar, 2005; Diéguez-Uribeondo *et al.* 2007). Moreover, some species of *Saprolegnia* appear similar at the gross morphological level and even produce similar zoospores (Ke, Wang, Gu, Li, & Gong, 2009).

The Mystic and Floyd's baitfish farm samples were not pure strains of *Saprolegnia* (Table 1) and were subsequently not used in this study. W-4 samples were identified as *Saprolegnia salmonis* (Table 1), a part of the *S. parasitica/S. diclina* species complex (clade III; parasitic Saprolegniales) (Diéguez-Uribeondo *et al.,* 2007). ATCC-2

isolates were pure strains of *S. parasitica* (Table 1) and were used to compare to DNA samples of *Saprolegnia sp.* The W-4 samples infected zebrafish eggs via co-incubation readily, and produced zoospores *in vitro,* unlike the ATCC-2 strain, which produced few spores.

The hyphae of *Saprolegnia* isolates stained readily with both LCB and CW stains (Fig. 2-4). CW effectively distinguished individual hyphae from the egg chorion despite some minor auto-fluorescence in the fish ova (Fig. 2). Additionally, LCB was an effective stain for viewing *Saprolegnia* cultures *in vitro* (Fig. 4). Calcofluor White is a stain that binds to chitin primarily but also binds to cellulose (Ali *et al.* 2013). Structurally, oomycete hyphae are comprised of cellulose (Seymour, 1970; Ali *et al.* 2013). LCB staining techniques have also been previously utilized for preparing samples of preserved *Saprolegnia* for viewing (Seymour, 1970). Although none of the cultures produced sexual structures *in vitro,* LCB stain should be utilized to study *Saprolegnia* morphology in the future. Additionally, CW stain may have a novel application in the future to qualitatively assess the effectiveness of chemical treatments against *Saprolegnia* in a zebrafish infection model. Given how CW effectively distinguishes hyphae from the transparent zebrafish chorion, there is a potential for computer applications to quantify hyphal growth and density from images rapidly. Additionally, CW allows an observer to track the progression of Saprolegniasis *in vivo.*

Zebrafish eggs that were infected via co-incubation for the staining optimization died rapidly and suffered a high infection prevalence (approximately 60%). The nature of the rapid infection indicates that zebrafish are susceptible hosts to *S. salmonis* at early life stages and that zebrafish eggs can be utilized to study *Saprolegnia* infections in future

research. However, it should be noted that co-incubation may not be a suitable method of infecting fish ova. This is because when colonized hemp seeds are left in fish aquaria for extended periods of time, the actual infectious dose of *Saprolegnia* administered to the fish cannot be quantified. *Saprolegnia* has been shown to exhibit RZE (Diéguez-Uribeondo, Cerenius, & Söderhäll, 1994a), form secondary zoosporangia (Seymour, 1970), and form biofilms (Ali *et al.* 2013), which may enable the pathogen to continue infecting hosts continuously while the hyphae remain in the aquaria and long after its removal. The lack of a quantifiable inoculum in previous infection models necessitates the utilization of an inoculum that can be quantified and optimized for the study.

In the zebrafish infection model (via zoospore inoculation), it was surprising that *S. parasitica* (ATCC-2) did not infect any zebrafish eggs in a reproducible manner; however, it is important to note that the culture originated from coho salmon (*O. kisutch*) and had repeatedly been subcultured over several years. This *Saprolegnia* strain may not be particularly pathogenic to zebrafish, or it may have lost its virulence over time. More importantly, none of the zoospore treatments produced any mortalities (Fig. 5, 6). Several eggs had superficial infections that were shed with the chorion upon hatching, but there were no severe or fatal *Saprolegnia* infections within any treatment groups. This suggests that ATCC-2 has become culture adapted over many sub-cultures and does not resemble its wild phenotype anymore.

Not all *Saprolegnia* strains are species-specific—some Saprolegniales infect a variety of hosts. *S. parasitica* has been shown to infect Atlantic salmon, brown trout, crayfish, amphibians, and many other organisms (Wood, Willoughby, & Beakes, 1986; Diéguez-Uribeondo, Cerenius, & Söderhäll, 1994b). Additionally, the role of zoospores

in the pathogenicity of *Saprolegnia sp.* is unclear, though it is thought that the secondary cysts may play an principal role in infecting fish (Liu *et al.* 2014). Despite this, previous infection models have utilized zoospores as inocula (Willoughby & Pickering, 1977; Wood, Willoughby, & Beakes, 1988; Pottinger & Day, 1999)

The structure of the egg chorion and the thickness of the mucus layer enveloping it play a significant role in the occurrence of infections in fish eggs (Songe *et al.* 2016). Fish ova have immature adaptive immune systems and are entirely reliant on their innate immune system as a defense against pathogens (Liu et al., 2014). *Saprolegnia sp.* can potentially infect all freshwater fish and eggs, and it has been shown that different *Saprolegnia* strains have significantly different pathogenicity (Stueland, Hatai, and Skaar, 2005). There are no previous records of *S. salmonis* infecting zebrafish eggs; even though *S. salmonis* has demonstrated that it is an opportunistic pathogen with a very diverse taste in hosts.

Additionally, the nature of the microbiome of the chorion is unclear, as is the relationship between the natural fauna that cohabitates there (Liu *et al.* 2014). Liu *et al.* (2014) examined the chorions of Atlantic salmon ova for their microfauna and the role they may play with defense against *Saprolegnia.* The authors found that while some naturally occurring bacteria on fish ova were pathogenic to fish (i.e. *Vibrio sp.*), other species of bacteria had significant inhibitory effects on the hyphal attachment of *Saprolegnia* (*Frondihabitans*). The inhibitory effects of *Frondihabitans* were as effective as malachite green treatments (Liu *et al*. 2014).

The mechanism behind this inhibitory effect of *Frondihabitans* upon *S. diclina* is unclear, however (Liu *et al.* 2014). Moreover, the nature of the zebrafish egg chorion

microbiome is unclear, and there may be other factors at play involved in *Saprolegnia* infections. *Saprolegnia* has been shown to form biofilm associations, and biofilms may be considered as a significant factor in the reemergence of Saprolegniasis in aquaculture even after treatments, as biofilms can provide protection from chemical treatments (Ali et al., 2013).

This model needs further optimization to produce an infection prevalence of 50% or greater. While the model necessitates the use of zoospores as inocula, the concentrations were too low to achieve the ideal infection prevalence. Future directions for this project should include pathogenicity studies of *Saprolegnia sp.* in zebrafish as well as an assessment of chemical treatments against Saprolegniasis. Additionally, it is possible that CW could be used to track the progression of the disease in real time. Computer software (i.e. Image J) may be utilized to quantify hyphal densities, diameters, or length on a fish ova through image analysis. This application of CW stain is currently being assessed.

CONCLUSIONS

In this study, it was concluded that for some strains of *Saprolegnia,* culture media should be carefully selected to ensure optimal growth. However, some strains are not particular of their culture media. Additionally, long-term storage methods of *Saprolegnia* cultures should be further optimized to accommodate different species.

CW and LCB were both effective stains for visualizing *Saprolegnia.* CW has a new application in zebrafish infection models to track the progression of *Saprolegnia* within an egg. CW has the potential to be utilized as a qualitative assessment of Saprolegniasis infections.

The zebrafish infection model needs to be optimized further to infect fish ova at an ideal infection prevalence reproducibly. With some modifications, this model has the potential to rapidly screen *Saprolegnia* treatments in a high-throughput manner. Future studies should look to the zebrafish egg to study the progression of Saprolegniasis infections.

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APPENDIX

Zebrafish toxicity assay

The zebrafish infection model requires the use of chemicals to test against *Saprolegnia* infections, and as such, it was necessary to determine whether mortalities in the model resulted from a *Saprolegnia* infection or from the chemical treatment itself. The chemical controls for the model were proposed to be chemicals already utilized in the aquaculture industry to combat oomycete infections, such as formalin. Methods for treatment and chemical concentrations for the toxicity assay were determined based on treatments used in Atlantic salmon hatcheries (personal correspondence with Dr. Michael Pietrak, USDA facility, Franklin, ME). Since zebrafish are utilized in the model rather than salmon, it was necessary to test the concentrations of formalin used for the chemical control to determine if the formalin treatments should be different than that of salmon.

Wild-type *D. rerio* eggs were collected and disinfected and sorted as previously described. Eggs were separated into different formalin treatment groups in 50mm Petri dishes (0ppm neg. control, 1000ppm, 1500ppm, and 2000ppm formalin) and bath treated with formalin for 15min. The eggs were then washed 3x with E3 water before plating in 96-well microplates and incubating at 26°C for 2d. Every 4h, the eggs were checked for mortalities due to formalin toxicity. At 3dpf, the eggs were checked once more for mortality and then euthanized with an overdose of tricaine.

Eggs were examined carefully both at a macro and micro scale for mortalities. An initial scan of the plate by eye detected any dead, whitish eggs followed by a closer observation under a dissecting microscope. Each egg was examined for signs of life (i.e. beating heart, flowing blood vessels in the tail, movement, etc.).

It was found that none of the treatments suffered any mortality for any of the time points. This indicates that treatments utilized as a flow-through treatment for salmon are still safe for use for closed-system bath treatments with zebrafish. However, concentrations of treatments may still need to be adjusted to have a significant effect on *Saprolegnia* once introduced into the model.

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Kathryn A. Liberman (Katie) was born in Leonardtown, Maryland on February 14th, 1995. She was raised in Sumner, Illinois and graduated from Red Hill High School in 2013. Majoring in marine sciences, Katie has a minor in aquaculture. She is a member of the scuba club and marine sciences club. Katie enjoys spending her free time practicing calligraphy and writing novels. She has received a Center for Undergraduate Research (CUGR) fellowship and a Charlie Slavin Research Fund fellowship. Upon graduation, Katie plans to gain experience working in marine sciences before returning to graduate school to study chondrichthyan behavior.