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Sensitivity of a LAMP Assay for Detection of the Dinoflagellate Amyloodinium ocellatum in Simulated Field Conditions and Freeze Tolerance of the Parasite

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SENSITIVITY OF A LAMP ASSAY FOR DETECTION OF THE DINOFLAGELLATE AMYLOODINIUM OCELLATUM IN SIMULATED FIELD CONDITIONS AND FREEZE TOLERANCE OF THE PARASITE

by

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ABSTRACT

The obligate parasitic dinoflagellate *Amyloodinium ocellatum* causes amyloodiniosis in warm water marine fishes. The prolific parasite, which has a direct, three-stage life cycle, is highly infectious and can cause heavy losses in aquaculture. Prevention, biosecurity, and early detection are vital for control. In this work, microscopy and a loop-mediated isothermal amplification (LAMP) assay were compared for early diagnosis of *A. ocellatum* in cultured stocks, and the freeze tolerance of tomonts was assessed to determine if frozen wild fish used as fish food can serve as a potential vector for the parasite.

The lowest dinospore concentration that could be detected by the LAMP assay in water samples was 0.5 dinospores/L, while positive detections occurred only at 5 dinospores/L or higher when using LAMP or microscopy on gill samples. Thus, LAMP of water samples is a superior diagnostic tool. Simulations of replicated assays indicated that a 95% probability of detection was achieved with 10 replicated assays of water samples when the dinospore concentration is as low as 1 dinospore/L.

All tomonts frozen for either 0, 24, 36, 48, or 72 hrs sporulated in *in vitro* experiments. Dinospore production decreased as freeze duration increased. Heavy infections were produced in *in vivo* experiments from tomonts frozen for all freeze durations, but tomonts frozen for 72 hrs took longer to establish a heavy infection. Tomont viability was negatively correlated with freeze duration suggesting longer freezing durations may successfully inactivate tomonts, a hypothesis that will require further experimental evaluation.

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LIST OF ABBREVIATIONS

AIC	Akaike information criterion
AO-LAMP	*Amyloodinium* LAMP
BIP	Backwards inner primer
B3	Backwards outer primer
℃	Degrees Celsius
D_j	Fixed effect of day of examination
T_i	Fixed effect of freeze duration
FIP	Forward inner primer
F3	Forward outer primer
g	Grams
hr	Hour
L	Liter
LAMP	Loop-mediated isothermal amplification
m	Meter
ppt	Parts per thousand
ppm	Parts per million
psu	Practical salinity unit
tr_k	Random effect of replicate trial
ε_{ijkl}	Residual error
y_{ijk}	Observation of an individual

CHAPTER I – GENERAL INTRODUCTION

1.1 Importance of Aquaculture

The global human population is rapidly growing and expected to reach approximately 9.7 billion by the year 2050 (FAO, 2018a). This rapid population growth translates into a steady increase in the demand for protein. In developing countries, fish is an important food source for populations that lack access to other animal protein. In industrialized countries, the demand for fish also is increasing due to the health benefits provided by fish products. Historically, wild fisheries have been the dominant source of fish, but the harvest from capture fisheries has been stagnant since the 1980s, and, thus, cannot support the growing demand. In contrast, aquaculture is currently the fastest growing food production sector, averaging 5.8% growth annually, and can provide a sustainable and cost-effective source of fish products to fill the gap between the demand and the supply from wild fisheries (FAO, 2020).

Aquaculture, until now, has focused primarily on freshwater species that account for about two thirds of current worldwide production. However, almost 80% of fish products consumed in the United States is of marine origin, to which aquaculture contributes little beyond shrimp and salmon (FAO, 2018b). In the United States, in 2019, total seafood production for human consumption was about 4.1 million tons, 93 percent of which came from wild fisheries (NOAA, 2021).

All forms of aquaculture seek to maximize production per unit area or volume. As such, the density of animals typically is high and can reach hundreds per liter in intensive larval systems and up to 100 $kg.m^{-3}$ in intensive recirculating grow out units. The consequence of this high density is increased vulnerability to disease. Indeed, disease is a

major limiting factor in the growth of aquaculture. *White spot syndrome virus*, for example, has cost the global shrimp aquaculture industry over $15 billion since the virus emerged in the early 1990s (Lightner, 2012). Between 1990 and 2000, the virus substantially slowed the growth of shrimp aquaculture (FAO, 2012). During that time, however, researchers worked to develop the diagnostic tools required to reliably identify cases, elucidate the transmission dynamics of the virus, and coordinate strategies to mitigate the spread of the disease. Since 2000, production has recovered, and shrimp aquaculture continues to lead growth in the aquaculture sector. Diseases including parasitic infections also plague finfish aquaculture. As an example, the economic losses due to *Lepeophtheirus salmonis*, an ectoparasitic copepod affecting salmonids, were estimated at $436M in Norway alone in 2011 (Abolofia et al., 2017). Therefore, research that increases the understanding of diagnosis, treatment, and prevention of disease is critical to the continued development of the aquaculture industry.

1.2 *Amyloodinium ocellatum*

Amyloodinium ocellatum is a cosmopolitan, warm water, ectoparasitic dinoflagellate that infects a wide variety of marine and brackish-water fishes causing amyloodiniosis or marine velvet disease (Brown, 1931; Paperna, 1984a; Noga and Levy, 2006). The life cycle of *A. ocellatum* (Figure 1.1) consists of three stages: the trophont which attaches to and feeds on the host, the tomont which is free-living and reproductive, and the dinospore which hatches from the tomont and infects new hosts (Brown, 1934; Nigrelli, 1936; Brown & Hovasse, 1946; Paperna, 1984a). Because of its tolerance to a wide range of temperatures and salinities and its ability to use the majority of temperate and warmwater marine finfish species as hosts, it is considered the most troublesome

parasite in warmwater marine aquaculture. Infections typically occur through introduction of contaminated live fish, feed, water, and fomites, though the relative importance of the different routes can vary.

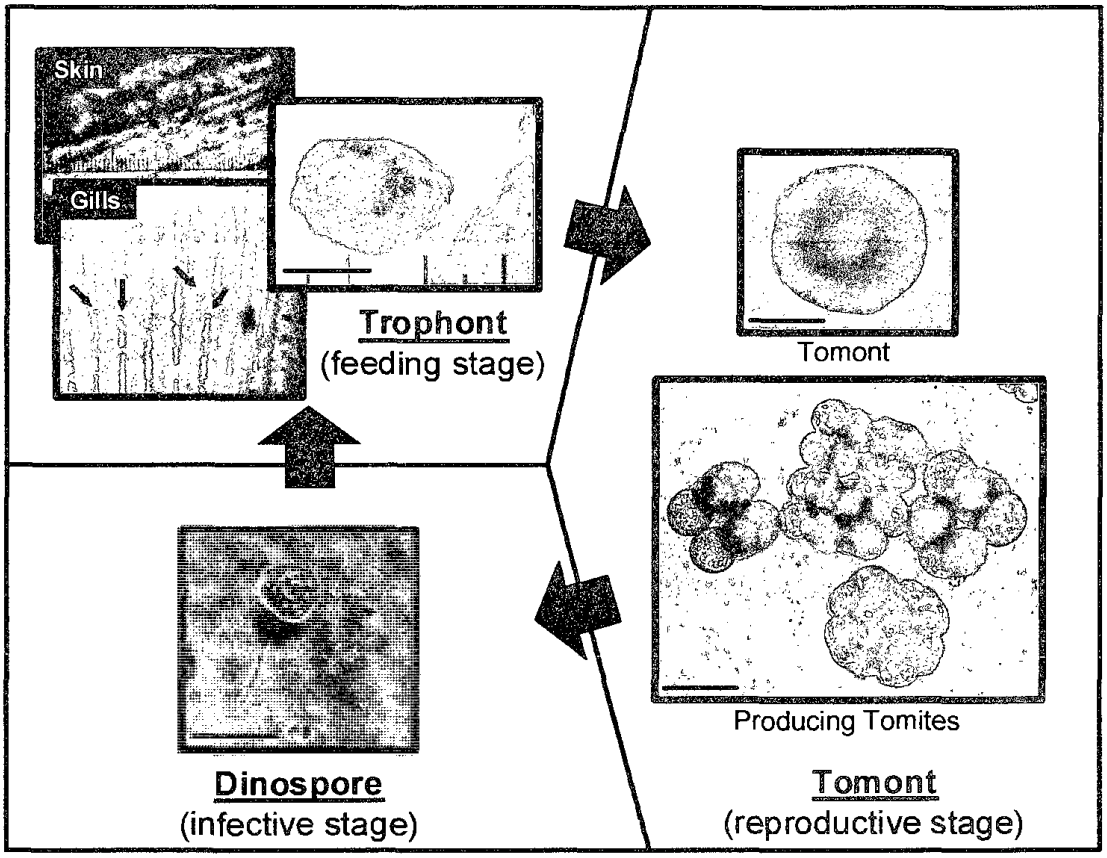

Figure 1.1 *The life cycle of Amyloodinium ocellatum, Scale bar: 25 µm (from Masson, 2009)*

1.3 Classification

Amyloodinium ocellatum, according to botanical classification, falls within the Phylum Dinoflagellida, Class Blastodiniphyceae, Order Blastodiniales, Family Oodiniaceae (Cachon and Cachon, 1987). Dinoflagellates also have been classified as zoological taxa as they share characteristics with plants and animals (Noga and Levy, 2006). According to this classification, *A. ocellatum* falls in the Phylum

Sarcomastigophora, Subphylum Mastigophora (flagellates), Class Phytomastigophorea (phytoflagellates), Order Dinoflagellida (Levine et al., 1980). More recently Guiry and Guiry (2015) classified *A. ocellatum* in Phylum Myzozoa, Subphylum Dinozoa, Infraphylum Dinoflagellata, Class Dinophyceae, Order Blastodiniales, and Family Oodiniaceae. Of the approximately 2,000 known dinoflagellates, only about 140 are known to be parasitic in nature, mainly targeting invertebrates (Drebes, 1984). *Amyloodinium* is one of five genera of dinoflagellates containing species that use fish as their host (Noga and Levy, 2006). These parasitic dinoflagellates are a polyphyletic group formed by representatives of three botanical orders, Blastodiniales, Dinococcales, and Syndiniales, that are similar due to convergent evolution (Cachon and Cachon, 1987). Molecular studies have shown that, based on small subunit ribosomal RNA, *A. ocellatum* is closely related to *Pfiesteria piscicida,* the *Pfiesteria*-like dinoflagellate and cryptoperidiniopsoid sp., all of which belong to the Class Blastodiniphyceae (Litaker et al., 1999).

Occurrence of multiple species or sub species of *A. ocellatum* was assessed by Levy et al. (2007) who characterized isolates from the Red Sea (Israel), the eastern Mediterranean Sea (Israel), the Adriatic Sea (Italy), the Gulf of Mexico (Florida), and a fifth isolate from an unknown origin using the internal transcribed spacer region of the ribosomal DNA. The sequence variants obtained for each isolate did not reveal significant geographic clustering, providing no support for the designation of a new species or of sub-species within *A. ocellatum*. However, Levy et al. (2007) also noted that difference in environmental tolerances among geographic isolates had been reported by multiple authors (e.g., Paperna, 1984a; Kuperman and Matey, 1999), and may reflect

occurrence of subspecies or locally adapted populations that could differ in pathogenicity and immunogenicity.

1.4 Management and Control

Current management of *A. ocellatum* is based on biosecurity to prevent infections and chemical treatments after infections are detected (Francis-Floyd and Floyd, 2011). Chemicals treatments, which kill the dinospores, include copper sulfate, chloroquine diphosphate, hydrogen peroxide, and formalin. The effectiveness of management, however, is dependent on the ability to control the risks of new introductions through appropriate routine husbandry practices and reliable diagnosis of the infection. The parasite has a high reproductive capacity (i.e., each tomont can produce up to 256 infectious dinospores). Therefore, early detection and prevention are key to preventing outbreaks in aquaculture facilities. To determine and select a reliable detection method, candidate diagnostic tests can be compared by assessing their sensitivity at various stages of an infection along with their specificity, practicability, and cost of implementation.

1.5 Charactristics of Diagnostic Methods

Sensitivity and specificity are key characteristics of a diagnostic test. Sensitivity is the ability of a test to correctly classify an individual as infected (Parikh et al., 2008). Therefore, a sensitive test is expected to generate a low rate of false-negative results. Specificity is the ability of a test to correctly classify an individual as uninfected. A highly-specific test is expected to have a low rate of false-positive results. The current gold standard for diagnosis of amyloodiniosis is microscopy, but the applicability of this method to early detection is limited. First, microscopy can only be accomplished using fish tissue, so the parasite cannot be detected in the water prior to establishment on the

fish host. Second, because small trophonts can be misidentified, early infections can be missed (or incorrectly identified as positive leading to unnecessary treatment). Additionally, trophonts can be missed entirely by microscopy at the earliest stage of infection when they are present only in very small numbers. As a result, the parasite can remain undetected until mortalities occur, at which point treatment is of limited value. Thus, methods that improve diagnostic capabilities are required.

1.6 Current Molecular Diagnostic Tools

A conventional polymerase chain reaction (PCR) assay was developed by Levy et al. (2007). The assay is sensitive and specific, but the technical complexities and labor involved limit its use in the field. Picon-Camacho et al. (2013) developed a loop-mediated isothermal amplification (LAMP) based assay as an alternative to the standard PCR.

The LAMP assay is an amplification reaction that targets a DNA sequence with a high rate of sensitivity and specificity (Mori and Notomi, 2009). The mechanisms of the LAMP assay contain three steps: an initial step that employs a DNA polymerase with strand displacement activity to form a dumbbell structure that serves as the starting structure for amplification, an amplification step, and an elongation step. The amplification requires a forward inner primer (FIP), a backwards inner primer (BIP), and a pair of outer primers (F3 and B3). In the *Amyloodinium ocellatum* assay, these primers are designed from 6 specific regions of the target gene (the 5′ end of the Small Subunit (SSU) rDNA region): the FIP is designed from the F2 and F1c region sequences and the BIP is designed from the B2 and B1c regions, while the outer primers (F3 and B3) are designed from the F3 or B3 regions (Figure 1.2, Picón-Camacho et al., 2013). During the

initial step, the double stranded template DNA is invaded by the FIP primer and amplification occurs using both the outer primer and the FIP using the displacement polymerase. This reaction releases single stranded fragments bearing the complementary sequences F1 and F1c needed to form a loop. The same process is used to synthesize the loop formed by annealing of B1 and B1c at the other end of the structure. The new product is a dumbbell structure that consists of the 3' ends of the open loops and annealing sites for both inner and loop primers. Amplification occurs at these sites and forms concatemers that allow for more amplification due to the increase in iniation sites (Figure 1.2).

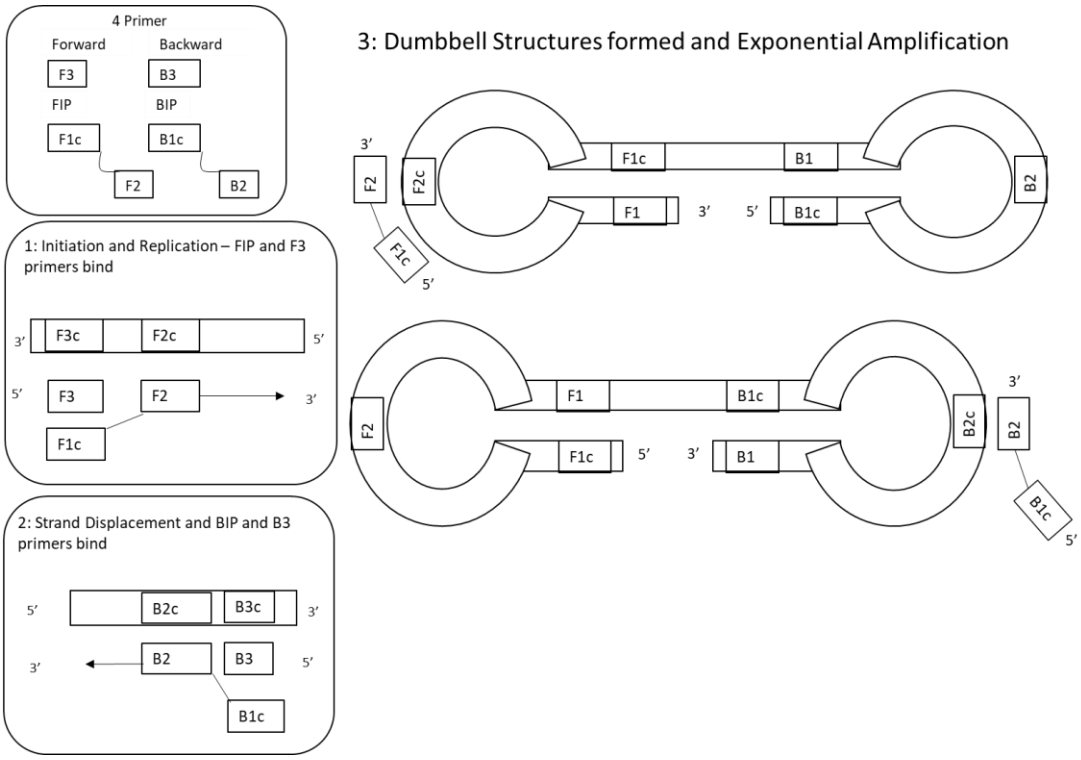

Figure 1.2 *Simplified overview of the LAMP primers and the LAMP reaction*

The LAMP assay, which has been tested for a variety of human and animal diseases, is potentially superior to the PCR assay for practical detection due to its

simplicity, rapidity, and improved sensitivity. It can be completed in less than an hour, is performed under isothermal conditions (requiring only a water bath or a small oven), and can be easily interpreted via observation of a color or turbidity change. The *Amyloodinium* LAMP (AO-LAMP) assay was shown to be highly specific (i.e., did not cross-react with 4 closely related species), exceptionally sensitive, and its accuracy was unaffected by potentially inhibitory substances in water or tissue (Picon-Camacho et al., 2013). At a small, benchtop scale, the AO-LAMP assay could detect single organisms in gill and water samples spiked with known quantities of the parasite as well as in naturally infected gill tissue. However, the assay was not formally compared to microscopy for early detection of *Amyloodinium*. Therefore, quantification of the sensitivity of the assay in comparison to that of the standard microscopic diagnosis is required. Further, information on assay sensitivity is needed to define a robust sampling program that will enable reliable early detection of the parasite in field operations.

1.7 Prevention

With respect to prevention, most standard biosecurity protocols recognize the risk of contaminated water, live fish, and people. While disinfection protocols are in place for water and live fish, transmission also could occur during normal hatchery activities that do not involve these vectors. Nets and water quality equipment, for example, alternate between wet and dry environments and have the potential to transfer the infection among tanks even when dried. This source of contamination may be contained through rigorous cleaning protocols or simply by avoiding the shared use of instruments and equipment among multiple tanks or culture rooms. Quite notably, frozen wild fish, which may carry encysted tomonts capable of producing viable dinospores, are used by many hatcheries as

a food source. Cyst forming dinoflagellates are known to survive cold-storage and proliferate (Montresor and Marino, 1996). Preliminary evidence (Haley Dutton, personal communication) demonstrated that gills from a fish infected with *Amyloodinium* frozen in a domestic freezer could produce an infection in uninfected fish following thawing. However, there has been no formal testing of the effect of freezing on the viability of *Amyloodinium ocellatum*. Therefore, research to elucidate the potential of frozen wild food as a vector for transmission of *Amyloodinium* is warranted.

1.8 Objectives

The first objective of this project is to compare the sensitivity of light microscopy to that of the LAMP assay for detection of *Amyloodinium ocellatum* in gill tissue and water in simulated field conditions. I will test the hypothesis that the LAMP assay is superior to light microscopy for detection of *A. ocellatum* in gill tissue and that detection in water samples can be successful for levels of infection lower than those leading to detection in gills. Further, this project will assess the probability of detecting the parasite using microscopy and LAMP in both water and tissue samples under varying levels of infection to determine the sample size required to detect the parasite or conclude that a culture is free of it with quantified statistical confidence. These values will serve as a basis for establishing sampling protocols for routine monitoring of aquaculture systems.

The second objective is to assess the tolerance of *Amyloodinium ocellatum* to freezing. I will test the hypothesis that freezing inactivates the parasite (i.e., removes its ability to infect fish). The inactivation will be studied following exposure to various durations of freezing. The findings will allow assessment of the risk of introduction of the parasite through frozen food in practical situations.

CHAPTER II – SENSITIVITY OF THE LAMP ASSAY IN SIMULATED FIELD CONDITIONS

2.1 Introduction

Infections usually occur through introduction of contaminated live fish, feed, water, fomites, or various combination of these vectors. Diagnosis typically involves microscopic examination of live gill tissue. Treatments such as copper sulfate and chloroquine diphosphate, which kill dinospores, are available (Dempster, 1955; Lawler, 1977; Paperna, 1984b; Noga & Levy, 2006). However, because of the fast proliferation rate of *A. ocellatum*, the parasite often escapes detection until mortalities occur, at which point treatments are of little use. The best form of disease management is prevention. Prevention is achieved through strict biosecurity protocols to prevent introduction and transfer within a facility. However, the ability to efficiently diagnose the infection prior to the onset of mortalities could significantly improve outcomes.

The current gold standard for diagnosis involves micoscopic examination of gill tissue, but the method has limited applicability for the detection of infections at early stages. First, microscopy can only be accomplished using fish tissue, so the parasite cannot be detected in the water prior to establishment on the fish host. Second, because of the small size of the trophonts and their paucity at early stages of an infection, trophonts can be missed entirely leading to false-negative outcomes. As a result, the parasite can remain undetected until mortalities occur. False-positive identifications also may occur at early infection stages leading to unnecessary treatment. Thus, methods that improve diagnostic capabilities are required.

The effectiveness of candidate alternative methods needs to be formally compared to microscopy. Key metrics of a practical diagnostic test include sensitivity and specificity. Sensitivity is the ability of a test to correctly classify an individual as infected (Parikh et al., 2008). Therefore, a sensitive test is expected to generate a low rate of false-negative results. Specificity is the ability of a test to correctly classify an individual as uninfected. A highly-specific test is expected to have a low rate of false-positive results. Additional aspects under consideration are related to the applicability of the methods in the field (e.g., the need for specific laboratory equipment and facilities or the duration of the laboratory phases of the assay).

Two molecular assays are available to detect *A. ocellatum*. A conventional PCR assay was developed by Levy et al. (2007). The assay is sensitive and specific, but it requires significant labor and utilizes equipment that is not available in fish hatcheries, making the protocol of limited use in the field. Picon-Camacho et al. (2013) developed the LAMP-based assay as an alternative to the standard PCR. The amplification reaction in this assay employs a DNA polymerase with strand displacement activity and primers designed to form a dumbbell structure that serves as the starting structure for further amplification of the target DNA sequence. The process results in high sensitivity and specificity (Mori and Notomi, 2009). The LAMP assay can be completed in less than an hour, is performed under isothermal conditions (requiring only a water bath or a small oven), and the results are easily interpreted in the field via observation of a color or turbidity change. The AO-LAMP assay was shown to be highly specific (i.e., did not cross-react with 4 closely related dinoflagellate species) and exceptionally sensitive in comparison to the PCR test (AO-LAMP detected 10 fg compared to 1 pg for the PCR).

Tests conducted using environmental water and gill tissue showed that the performance of the assay was unaffected by potentially inhibitory substances in these samples (Picon-Camacho et al., 2013). However, while the assay was shown to be superior to traditional PCR by Picon-Camacho et al. (2013) and more applicable in the field, it was not formally compared to microscopy for early detection of *Amyloodinium*. Therefore, quantification of the sensitivity of the assay in comparison to that of the standard microscopic diagnosis is required. Further, information on assay sensitivity and specificity is needed to define a robust sampling program that will enable reliable early detection of the parasite in field operations.

In this chapter, the sensitivity of the LAMP assay was compared to that of light microscopy for detection of *Amyloodinium ocellatum* in gill tissue and water samples under varying infection loads. Further, the probability of detecting the parasite at different concentrations was assessed for both assays to determine the minimum concentration that can be detected in a monitoring program. The applicability of the two methods in practical infection scenarios is discussed.

2.2 Materials and Methods

2.2.1 Production of naïve Spotted Seatrout

Experimental animals were derived from *A. ocellatum*-free broodstock held in captivity at the Thad Cochran Marine Aquaculture Center and volitionally spawned according to the methods described in Arnold et al. (1976). Newly hatched larvae were held for approximately 25 days and fed a succession of rotifers, *Artemia*, and prepared microdiets until fully transitioned to dry, pelleted food as described in Blaylock et al. (2021). Weaned fish were transferred to nursery systems (2 m^3 tanks connected to

recirculating systems) where they were fed a sequence of dry pellets (Skretting, Gemma Diamond – 57% protein and 15% lipid) of increasing particle size at approximately 5% body weight per day until required for experiments. These naïve spotted seatrout were kept in a biosecure building to ensure the absence of *A. ocellatum* prior to experimental exposure to the parasite. Fish were held in 25 psu artificial seawater (Crystal Sea® Marinemix, Marine Enterprises International Inc, Baltimore, MD, mixed with well water). Temperature, salinity, pH, ammonium, nitrate, nitrite, dissolved oxygen, and alkalinity were monitored and recorded daily. The target ranges for each of these water quality parameters were 23-27 ℃, 20-25 psu, pH 8.0-8.5, 0-0.25 ppm, 50 ppm, 0-1.0 ppm, 7.0-10.0 mg/L, and 180-240 ppm $CaCO_3$, respectively. As a precaution, systems holding the naïve fish were maintained on 0.250 ppm $CuSO_4$ chelated with citric acid to ensure they remained free of *A. ocellatum*.

2.2.2 Parasite Culture

The parasite culture was maintained in a dedicated building located on the Halstead campus of the Gulf Coast Research Laboratory to prevent spreading the infection to biosecure facilities of the Thad Cochran Marine Aquaculture Center. The culture system comprised two 750-L tanks filled with approximately 375 L of water prepared by mixing well water with Crystal Sea® Marinemix salt (Marine Enterprises International Inc, Baltimore, MD). Water quality parameters were monitored and managed as described above for the nursery systems. This system had no mechanical filtration, but biological filtration was accomplished using a moving bed bioreactor. Temperature was controlled through a combination of ambient air regulation and titanium

rod heaters in the tanks set to 27°C. Water quality parameters were measured daily. Target parameters were the same as those for the naïve culture tanks described above.

The initial infection was obtained from the heavily infected gills of a Greater Amberjack, *Seriola dumerili*, provided by Dr. Roy Yanong (University of Florida). The parasite culture was maintained using naïve lab-reared Spotted Seatrout, *Cynoscion nebulosus*, as well as wild-caught Striped Mullet, *Mugil cephalus*, Atlantic Croaker, *Micropogonias undulatus*, and Pinfish, *Lagodon rhomboides* collected by cast-net or hook and line fishing. Following the initial infection, gills of fish in the system were examined every week for trophonts to verify that the infection had taken place. As fish died, their gills were removed and returned to the system. To maintain the *A. ocellatum* culture, either naïve seatrout or wild fish of the species noted above were regularly added to replace deceased fish in the infected system and ensure that a host for the parasite was always available. Generally, fish were added when there was two or fewer fish left alive in the system. Dry, pelleted food and frozen shrimp was provided three times a week to satiation.

2.2.3 Dinospore Collection

Dinospores used in experiments were collected from heavily infected fish. Fish showing signs of heavy infection (e.g., gasping for air, aimless swimming, listlessness) were killed by double pithing. The gills were removed, placed in a Petri dish with artificial seawater, and examined using a dissecting microscope. The trophonts were dislodged from the gills using a stream of artificial seawater or through agitation using forceps. The trophonts were then transferred by pipetting to 5-mL tubes prefilled with 5 mL of water and allowed to settle for 15-30 minutes. The excess water was decanted and

the trophonts washed, re-suspended, and allowed to resettle. This process was repeated three times. Following the third washing and settling cycle, the density of trophonts was determined by volumetry, which was achieved by counting trophonts in five 0.5 mL samples and extrapolating the average count to the total volume. Twelve aliquots of approximately 100 trophonts, as determined by volumetry, were pipetted into the wells of a 12-well plate (100 trophonts per well, final well water volume adjusted to 2 mL) and allowed to incubate for 72 h at 25 ℃. After the incubation period, the dinospores were transferred to a container filled with 1 L of seawater. Following the method described by Masson et al. (2013), a Sedgwick-Rafter counting cell was used to estimate the number of dinospores in the volume of water. Water in the 1 L container was stirred to homogenize dinospores in the water column and 0.5 mL was sampled by pipetting and mixed with 0.5 mL formalin to kill the dinospores prior to counting on the Sedgwick-Rafter cell. This procedure was repeated three times, and the number of dinospores in the known volume of water was calculated as the average of the three replicate counts.

2.2.4 Exposure Experiment

Forty-two 10-gallon aquariums were filled with 20 L of freshly prepared 25 psu artificial seawater. These tanks were bleached and cleaned prior to the trial to ensure they were free of parasites. Tanks were inoculated with either 0, 1, 10, 100, 1000, 5000, or 10,000 dinospores (six tanks for each dose), resulting in concentrations of 0, 0.05, 0.5, 5, 50, 250, and 500 dinospores/L, respectively. The desired numbers of dinospores introduced in each aquarium was added from the dinospore stock by volumetry. Water in three tanks for each dosage (21 tanks total) was then agitated to homogenize the distribution of dinospores. Following agitation, ten 1-L aliquots from each of the 21 tanks

were removed and filtered on a 0.22 µm 47 mm nitrocellulose filter membrane using vacuum filtration. Following filtration, each membrane was placed in a Ziploc bag and stored at -80 ℃. The other 21 tanks (3 per dosage) received four naïve spotted seatrout each. After 7 days or upon death, whichever came first, the fish were removed, and their gills were examined via microscopy. Presence or absence of trophonts on the gills was recorded for 4 gill arches per fish (2 from each side of the fish). These gill arches were preserved in 95% ethanol for DNA extraction and analysis with the LAMP assay. Seven days was chosen as an end-point to determine infection based on data from Masson (2009) who showed that the lifespan of dinospores did not exceed this duration at 25 ℃. This experiment was repeated three times.

2.2.5 DNA Extraction

DNA from the membrane filters and the gill tissues from challenged spotted seatrout was extracted using Qiagen PowerWater kits and Qiagen Blood and Tissue kits following the protocol from the manufacturer for each of the kits. The obtained DNA solutions were frozen at -80℃ until use.

2.2.6 AO-LAMP Assay

The LAMP assay was performed using the DNA extracted from both the filter and the gill samples following the protocol developed by Picon-Camacho et al. (2013). The 4 LAMP primers designed by Picon-Camacho et al. (2013) used for amplification are presented in Table 2.1. The successive steps of the assay were performed in separate locations to minimize the risk of contamination. All replicate water samples taken from tanks dosed with 10 dinospores or higher were assayed (10 samples per tank x 3 replicates tanks per trial x 3 trials, 90 assays per dose-treatment). Similarly, all gill

samples taken from fish in tanks dosed with 100 dinospores or higher were assayed (4 gills per fish x 4 fish per tank x 3 replicate tanks per trial x 3 trials, 144 assays total per dose-treatment) (Figure 2.1). Fewer tests were performed on samples from tanks dosed with the lowest numbers of dinospores to conserve assay supplies based on early findings that showed no positive tests were recorded in any of the initial replicate assays. Thirty LAMP assays were performed on water samples from tanks dosed with 0 and 1 dinospores (3 or 4 replicates per tank) and 36 assays were performed on gill tissue samples from fish held in tanks dosed with 0, 1, and 10 dinospores (one gill sample per fish).

Table 2.1 *Sequence of the Amyloodinium ocellatum LAMP primers. These primers were originally designed by Picon-Camacho et al. (2013).*

Primer	Length	Sequence (5'-3')
F3	22	CGAAAAGCTCATTAAAACAGTT
B3	19	TCTATCGCCGATTGAGATC
FIP	45	TGGAAGCGTGTATTAGCTCTAGGATTTATTTGATGGTTGTTCCCT
BIP	43	ATTAGTTACAGAACCAACCCAAGTCATGCGATTCGTACGGTTA

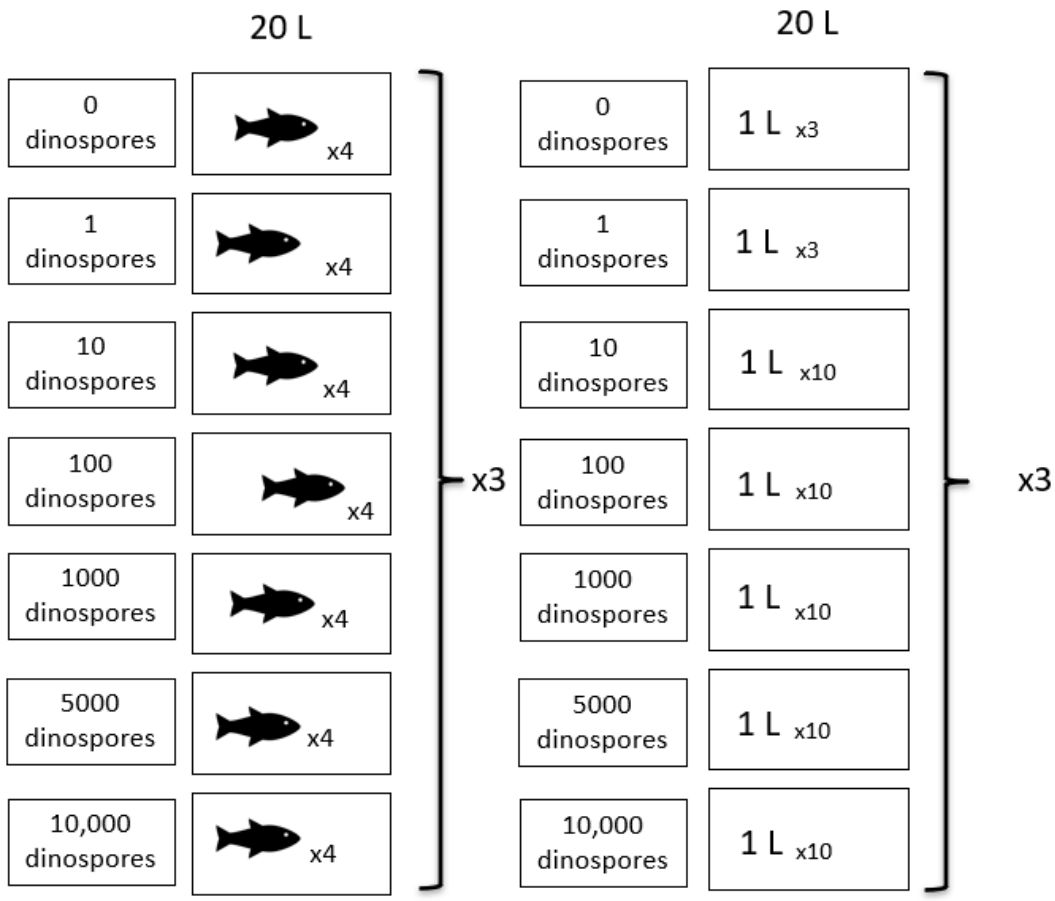

Figure 2.1 *Experimental design for total number of water samples and gill tissue samples for microscopy and the LAMP assay. Forty-two 20-L aquariums filled with 25 psu artificial seawater were dosed with either 0, 1, 10, 100, 1000, 5000, or 10,000 dinospores (6 tanks per dose). For each dose, four naïve Spotted Seatrout were added to three tanks (left panel) and three tanks were left with just water (right panel).*

A positive LAMP reaction was inferred by fluorescence under UV light. Fluorescence is observed following addition of a calcein-dye in a positive reaction. Calcein binds with manganese ions in a reaction that quenches fluorescence. In a positive LAMP assay, the polymerase reaction produces pyrophosphate that binds with Calcein-bound manganese ions as well as magnesium ions. This reaction restores fluorescence of the Calcein complex and produces a precipitate of manganese phosphate that can be

detected in the reaction medium (Tomita et al., 2008). The positive LAMP reactions were confirmed by agarose gel detection. Three gel detections were performed per dosage level per trial (63 total). Positive reactions are expected to produce multiple specific bands ranging in size from 180 to 300bp and several heavier bands arranged in a ladder-like pattern (Picon-Camacho et al., 2013).

2.2.7 Data Analysis

Sensitivity values for the LAMP assay were calculated using a 2x2 contingency table analysis for paired data obtained by assaying the same sample of gill tissue with both the microscopy identification and the LAMP assay. The McNemar's test for paired data was used to compare the two assays at each infection load tested.

Generalized linear models were used to model the probability of detection as a function of parasite concentration per liter for both water samples and gill tissue samples and test the effect of replicate trial, replicate tank within trial, and replicate fish within tank on the inferred probability function. Models were fit using the GLM function of R and employed a logit link function. Dinospore concentration was log-transformed to improve model fitting. Optimization by the Newton-Raphson algorithm yielded estimates of regression coefficients for each factor (dinospore dose, replicate trial, tank, or fish) and the associated standard error and probability that the regression coefficient was significantly greater than zero. Model selection was based on the assessment of significance of individual factors (non-significant factors removed from the model) and comparisons of overall model fit based on the Akaike information criterion (AIC) when replicate trial, replicate tank or replicate fish were excluded.

Optimized logistic probability functions (P) followed the equation

$$P = \frac{e^{(b+dx)}}{(1 + e^{(b+dx)})}$$

where b is the intercept value, d is the coefficient of the log (dinospore dosage), and x is the log (dinospore dosage).

2.3 Results

The sensitivity values of the LAMP assay varied across the different dinospore dosage levels. Sensitivity was calculated starting at the 100 dinospore dosage (Figures 2.2, 2.3). The McNemar test indicated that the LAMP assay performed on gills had a significantly higher sensitivity than microscopy at the 100 (χ^2_1=5.1428, p=0.0234), 1000 (χ^2_1=12.0714, p=0.0005), and 5000 (χ^2_1=4.1667, p=0.0412) dosages. Specificity could not be formally estimated based on the results, but we note that there were no false positives (no positive recorded in any of the controls used for the methods used).

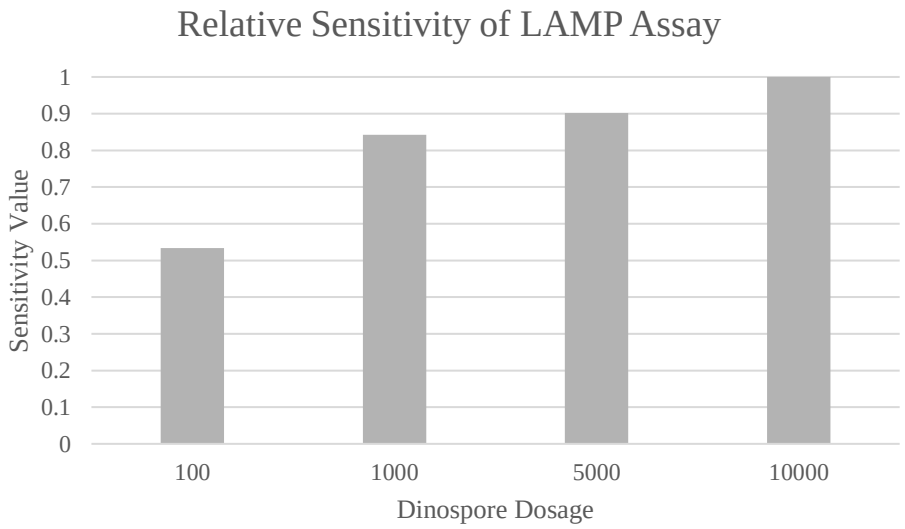

Figure 2.2 *Sensitivity values of the microscopy of gill tissue with reference to the LAMP assay of the same samples starting at the 100 dinospore dosage level. Values start at the 100 dinospore dosage level because the sensitivity could not be calculated for lower doses.*

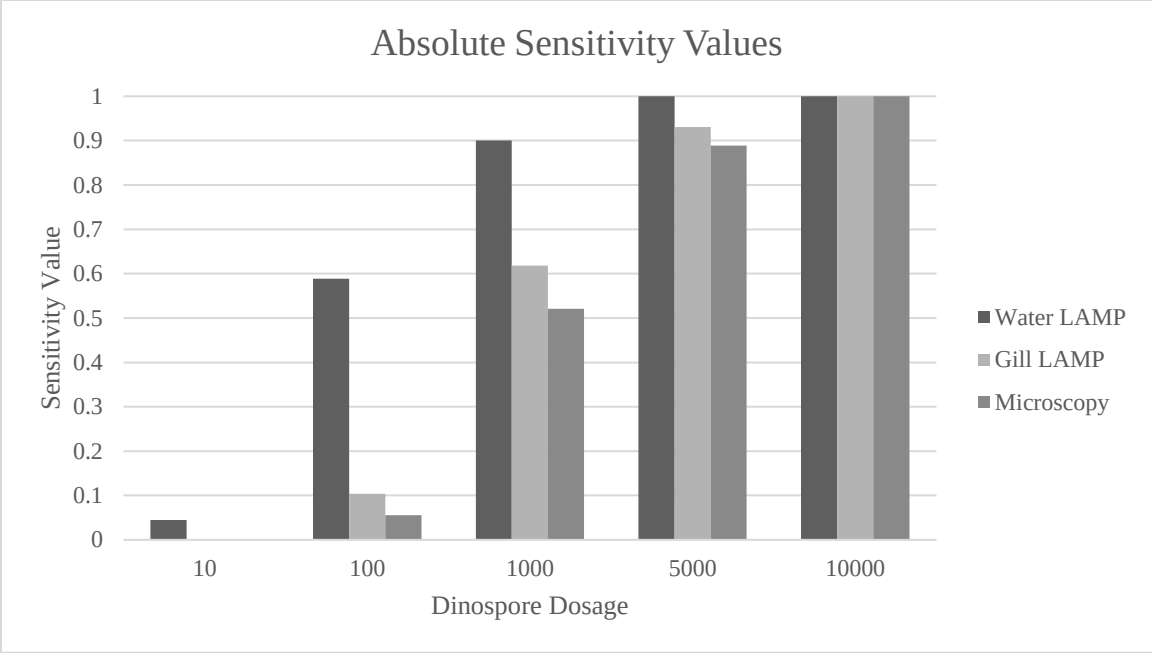

Figure 2.3 *Absolute sensitivity values of the LAMP assay on water samples, LAMP assay on gill tissue, and microscopy, starting at the 10 dinospore dosage level. Values start at the 10 dinospore dosage level because the sensitivity for lower doses could not be calculated.*

Models fitted on the water sample data showed highly significant predictor coefficients for the dinospore load, while the coefficients associated with replicate trials or replicate tanks within a trial were not significantly different from zero except for the predictor optimized for one replicate tank in trial B. However, the model with the lowest AIC was the simple model accounting for the dinospore load only. The probability function was therefore modeled accounting for the dinospore load only (i.e., data pooled across trials and replicate tanks, Equation 1).

$$P = \frac{e^{(-5.601+2.84x)}}{(1+e^{(-5.601+2.84x)})} \text{ Equation 1.}$$

Predicted values and their 95% Confidence Intervals are presented in Figure 2.4. The first positive detections using the LAMP assay in water samples were recorded in tanks with a concentration of 0.5 dinospores/L (10 dinospores per tank) (empirical proportion of

positive tests 4.4%, Table 2.2, Figure 2.4). Detection rapidly increased as the dosage level increased. At the concentration of 50 dinospores/L (1,000 dinospores per tank), the proportion of positive tests was 90%. All the tests performed at the 250 dinospores/L (5,000 dinospores per tank) were positive (90/90) (Figures 2.4 and 2.5).

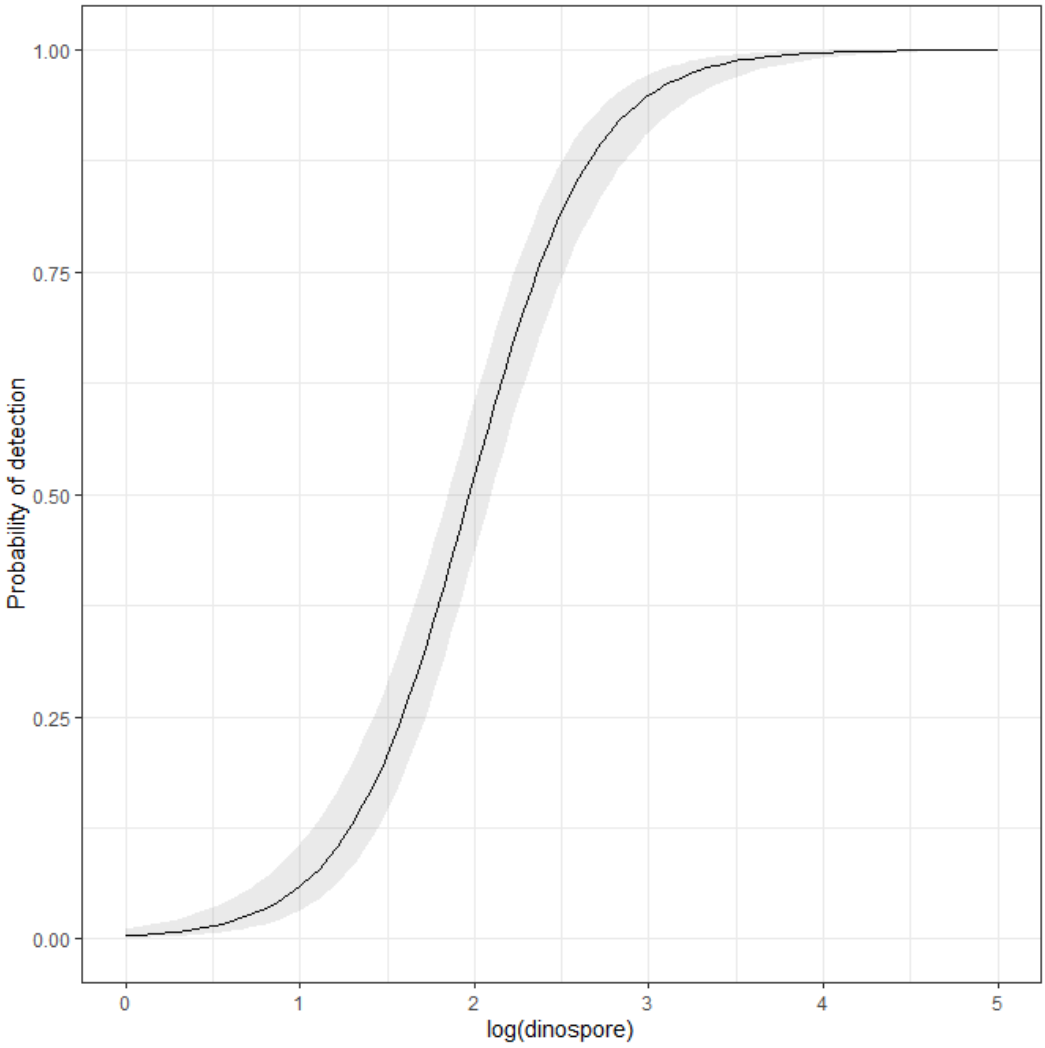

Figure 2.4 *The probablity of detecting dinospores in 1 L water samples following filtration using the LAMP assay as a function of log(dinospore), where log(dinospore) represents total dosage level transformed to a logarithmic scale.*

Table 2.2 *Number of positive results and the positivity rate for microscopy, the LAMP assay on gill tissue, and the LAMP assay of water samples per dinospore dosage level. Each test corresponds to a single gill arch. Four gill arches were checked using microscopy for all dosages, LAMP assays were performed on 2 of these gill arches for low dosage levels (0, 1, and 10) and all 4 gill arches for higher dosage levels (100, 1000, 5000, and 10000). All positive microscopy tests were also positive using the LAMP assay.*

Dinospore Dosage Level (# dinospores/L)	Positive Microscopy Identification / Positivity Rate (Gill Arches)	Positive LAMP Results (Gill Tissue) / Positivity Rate	Positive LAMP Result (Water Sample) / Positivity Rate	Total Number of Tests (Microscopy/LAMP-Gill Tissue/LAMP Water Sample)
0 (0/L)	0 / 0.0	0 / 0.0	0 / 0.0	144/36/30
1 (0.05/L)	0 / 0.0	0 / 0.0	0 / 0.0	144/36/30
10 (0.5/L)	0 / 0.0	0 0.0	4 / 0.044	144/36/90
100 (5/L)	8 / 0.055	15 / 0.104	53 / 0.589	144/144/90
1000 (50/L)	75 / 0.521	89 / 0.618	81 / 0.9	144/144/90
5000 (250/L)	128 / 0.889	134 / 0.931	90 / 1.0	144/144/90
10000 (500/L)	144 / 1.0	144 / 1.0	90 / 1.0	144/144/90

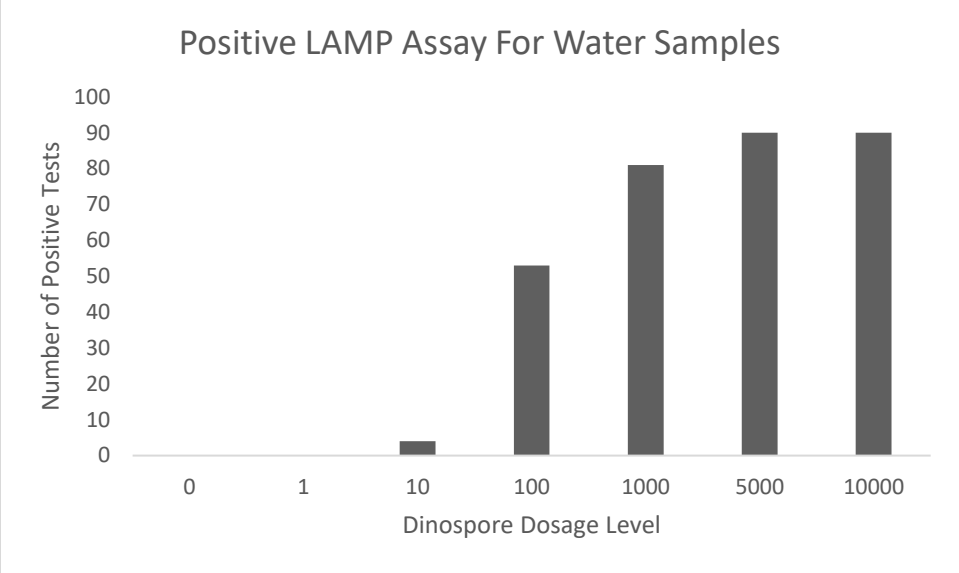

Figure 2.5 *Number of positive LAMP assay results obtained on 1-L water samples at various dinospore dosage levels. Sample sizes were n = 30 tests for dinospore dosages 0 and 1, and n = 90 for all other dosage levels.*

Models fitted on the gill sample data (LAMP assay and microscopy) also showed highly significant predictor coefficients for the dinospore load while the coefficients associated with replicate trials or replicate tanks within a trial were not significantly different from zero except for the predictor optimized for one replicate tank in trial C. The model with the lowest AIC was the simple model accounting for the dinospore load only. The probability functions were therefore modeled accounting for this factor only (data pooled across trials and replicate tanks). Equations 2 and 3 provide the parameters of the regression of the LAMP assay and microscopy detection data, respectively.

$$P = \frac{e^{(-8.4023+3.0042x)}}{(1+e^{(-8.4023+3.0042x)})} \text{ Equation 2}$$

and

$$P = \frac{e^{(-9.7078+3.2867x)}}{(1+e^{(-9.7078+3.2867x)})} \text{ Equation 3}$$

Predicted probability values and 95% CIs are presented in Figures 2.6 and 2.7. The first positive detection using both microscopy and the LAMP assay was recorded in tanks at 5 dinospores/L (5.5% positives for LAMP, 10.4% positives for microscopy), and detection rates with both methods increased as dosage level increased (Table 2.2). For both the LAMP assay and microscopy, models fitted accounting for individual fish within replicate tank revealed several significant predictor coefficients associated with variation among individual fish highlighting strong variations in infection loads among fish.

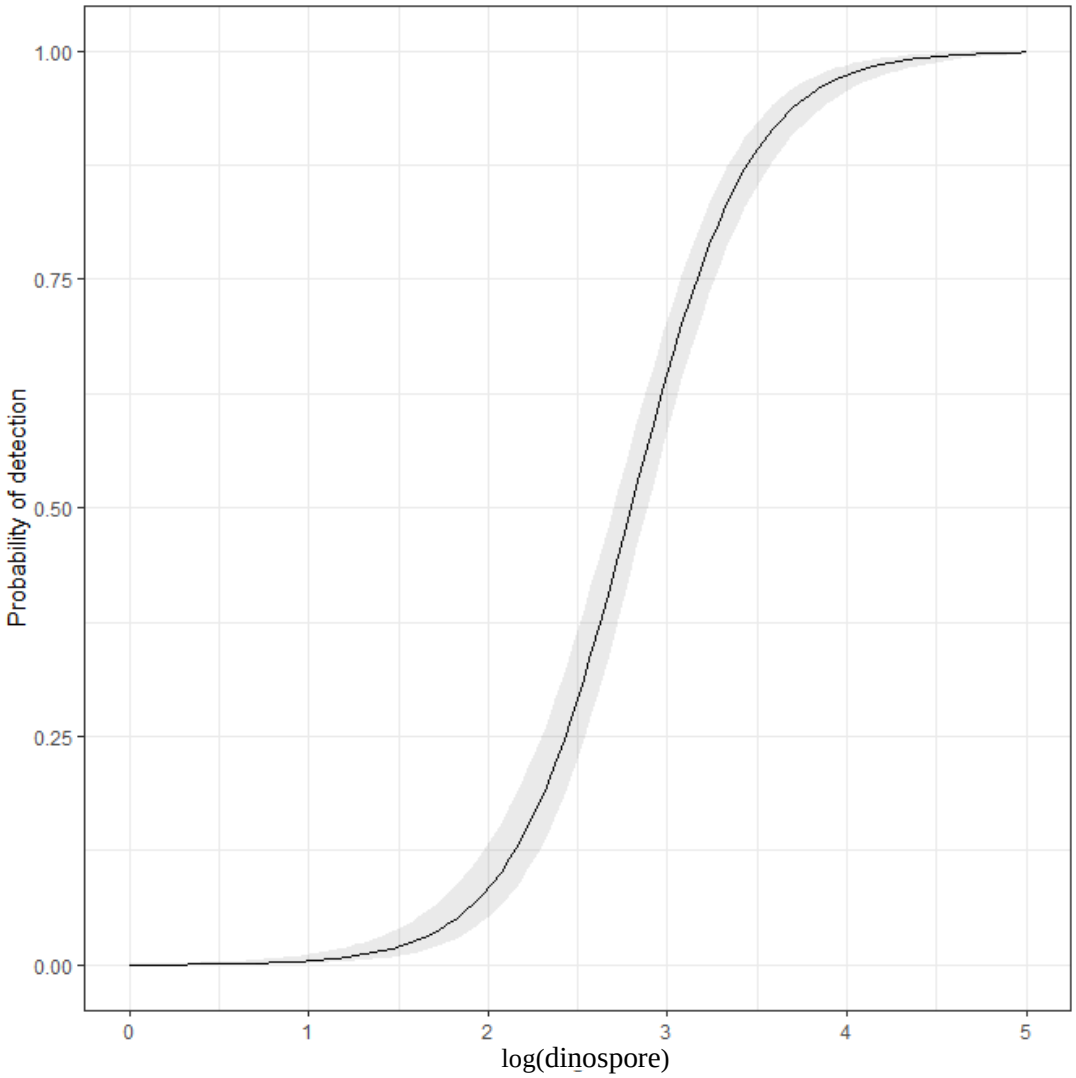

Figure 2.6 *The probablity of detecting the parasite on gill tissue using the LAMP assay as a function of log(dinospore) where log (dinospore) represents total dosage level transformed to a logarithmic scale.*

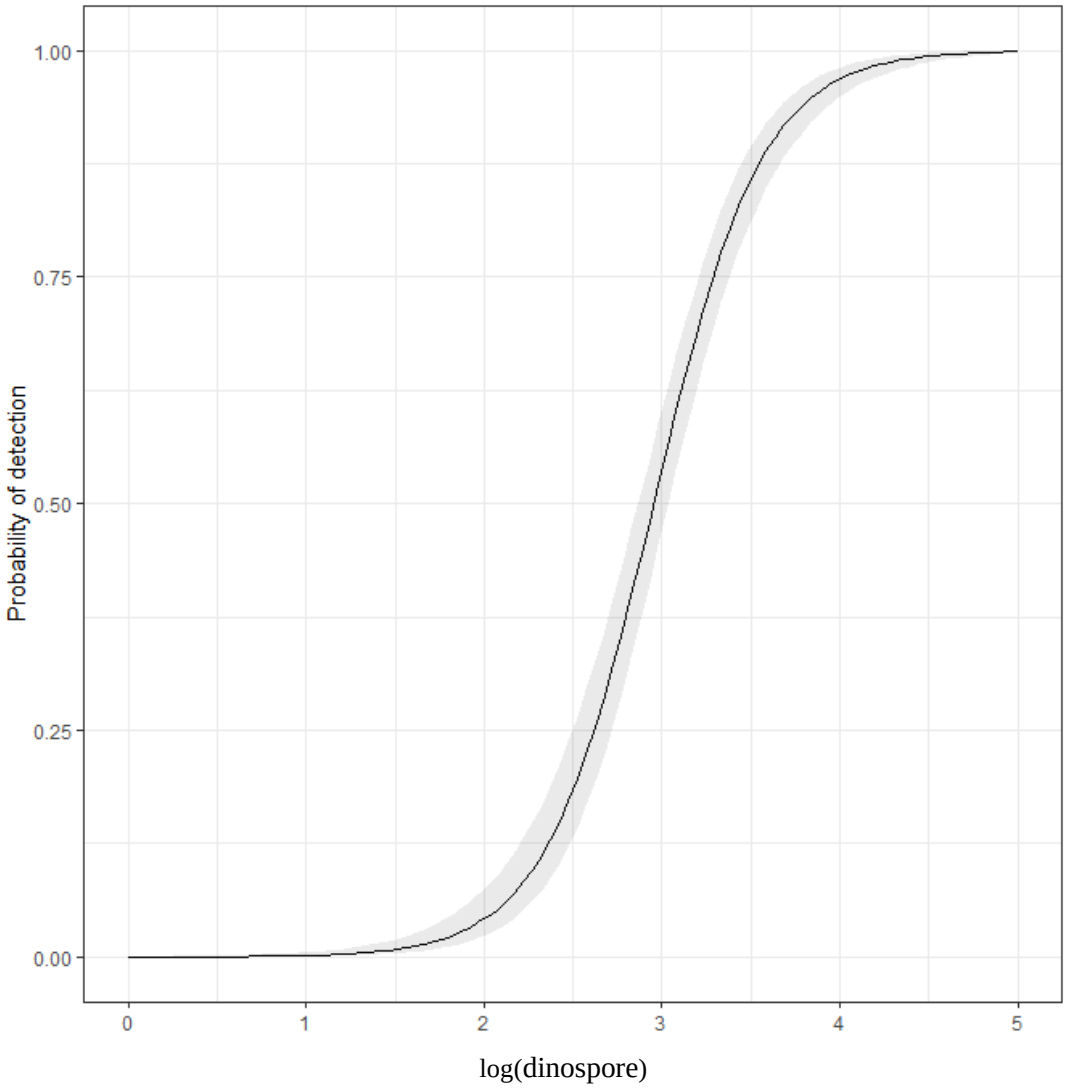

Figure 2.7 *The probablity of detecting the parasite on gill tissue using microscopy as a function of the log(dinospore) where log(dinospore) represents the dosage level transformed to a logarithmic scale.*

Using the predictive equations optimized above, the probability of obtaining at least one positive result in 10 independent trials was estimated at different dinospore concentrations to simulate a practical detection scenario in the field where 10 samples would be taken and assayed (Table 2.3). Results indicate that assays of 10 water samples

would lead to at least one positive detection with a 95% probability for concentrations of 50 dinospores/L or higher. The same outcome (at least one positive detection in 10 assays) would be achieved by running microscopy or LAMP assays on gill samples only for dinospore concentrations of 500 dinospores/L or higher.

Table 2.3 *A simulation of the probabilty of a single positive result after 10 tests using the LAMP assay on water samples, the LAMP assay on gill tissue, or microscopy on gill tissue.*

Dinospore Concentration per L	Water Samples (LAMP)	Gill Tissue (LAMP)	Gill Tissue (Microscopy)
1	0.805	0.125	0.052
5	1.0	0.603	0.365
10	1.0	0.885	0.695
25	1.0	0.997	0.979
50	1.0	1.0	1.0

2.4 Discussion

2.4.1 Comparison of assays performed on water samples using the LAMP method and the gills using microscopy of the LAMP method

The minimum detection concentration for the LAMP assay performed on water samples was one order of magnitude lower than that for assays performed on fish gill tissue samples with 4.4% positive detections at 0.5 dinospore/L during tests on water versus 10.4% positive detection at 5 dinospore/L when assays were performed on fish gills (Table 2.2). This result indicates that either dinospores did not successfully infect fish at the lowest doses tested (1 and 10 dinospore per aquarium) or that the sensitivity of the assay on gills is lower than that for the test on water samples. Gills were sampled at 7 days post-introduction of the dinospores, which is the maximum lifespan of this stage

(Masson, 2009). Accordingly, our sampling of gills was expected to capture the maximum initial attachment rate and, in cases of successful attachment early after inoculation, a possible second and even a third generation of trophonts considering that the cycle can be completed in as little as 3 days (Paperna, 1984a). The lowest dose tested in this experiment (1 dinospore per tank) could infect, at best, one of the 4 fish. If the dinospore attached to a gill, it would have been detected, on average, in only half of the cases by sampling 4 of the 8 gill arches as per the protocol for this experiment unless the new trophont produced a second generation of trophonts from the initial infection. Considering the productivity of a single tomont (up to 256 dinospores, see chapter 2 and Brown, 1981; Nigrelli, 1936; Brown and Hovasse, 1946), it seems likely that a second generation would have been detected on gills. The complete lack of detection at this density suggests that either no or only one initial infection occurred in the 1 dinospore treatment and that it was not detected.

The second treatment dose had, on average, a ratio of 2.5 dinospores per fish. Considering the high infectivity of *A. ocellatum* (Bower et al., 1987), it is likely that attachment did occur at least on some fish. However, the infection may have been limited to an initial infection and a very low infection load per fish (e.g., one or two trophonts) that could have been missed on the 4 gill arches sampled. The complete lack of detection on gills in that treatment is consistent with the hypothesis that only initial infections occurred and were not detected. Determining if fish were infected at the low dinospore densities would provide information on the probability of an outbreak occurring in the instance where only a few dinospores are introduced into a tank. Considering the challenges in detecting low loads per fish, which would require thorough assessment of

all gill arches and the skin to ensure infection is not missed, one strategy could be to allow more time after inoculation for two or more cycles of the parasite to be completed, which may lead to detectable levels.

With regards to detection, this work showed that the LAMP assay of water samples, in the conditions of this experiment, provides the highest sensitivity. An added benefit of testing water samples instead of gill tissue is that it avoids loss of the fish and the resulting stress on remaining stocks associated with sampling. This work also compared the LAMP assay and microscopy for detection in gill tissue. The LAMP assay was significantly more sensitive, although the difference between the two methods was moderate (first detection at 5 dinospores/L for both methods with 5.5% positive tests with microscopy versus 10.4% with the LAMP assay).

Sensitivity and specificity values close to 1.0 are generally considered good in diagnostic tests (Šimundić, 2009). In this study, the LAMP assay produced an absolute sensitivity value above 0.93 when the dinospore dosage was 250 dinospores/L on gill tissue, while assays on water samples yielded a sensitivity of 0.9 at the dosage of 50 dinospores/L. Specificity was not formally reevaluated in this work, but Picon-Camacho et al. (2013) screened four other dinoflagellate species and found no cross-reaction. I do note that in this experiment, no false positive was detected in any of the control group samples.

There was a significant effect of individual fish on the detection probability functions, which reflects variable infection rates among individual fish. This variation is expected to be particularly important at a low infection load when only some fish are infected, and the infection can be missed or underestimated (as discussed above).

Accordingly, several fish would need to be sampled and examined in practical situations to improve the probability of a positive detection. It is possible that variations also occur among water samples. For example, water circulation in large volume culture units may lead to some non-random distribution of dinospores. Such variation could not be evaluated in the small volume used in this study as water could be mixed and representative samples (5% of the volume) were filtered to extract DNA for assay. It is not clear if the variation among water samples from a replicate tank recorded at low dinospore density were due to presence/absence of a dinospore in individual 1-L samples or if some positive tests were obtained due to some free DNA shed by the spores in the water. If positive outcomes were obtained only when dinospores were present in the tested 1-L sample, non-random distribution of dinospores in a full-size culture tank could impact detection probabilities as 1-L water samples would represent a negligible fraction of the total culture volume. Future studies could evaluate reproducibility by repeating assays on different samples of water taken from different parts of large commercial-scale systems inoculated with *A. ocellatum* at different densities.

2.4.2 Probability of detection and sampling interval

Using the results from this work, a simulation was conducted to determine the probability of obtaining one or more positive results out of ten tests at a given dinospore concentration. Ten replicate tests were considered in this simulation as an upper bound of the number of tests that could be performed in practical situations. The simulations showed that 10 assays of water samples would produce at least one detection with a probability greater than 0.95 for a concentration of 2 dinospores/L, based on the logistic probability function optimized based on the data obtained in this study (Figure 2.4).

While the minimum concentration of dinospores required to start an infection has not been formally established, the minimum concentration that can be detected (2 dinospores/L or 60,000 dinospores in a 30 m^3 tank) suggests that if fish were exposed to that concentration of dinospores, an infection likely would result. The assays do provide information on the minimum load of *A. ocellatum* in the water or on the fish. Based on this information, population modelling can be used to estimate the time until the lethal load on fish is reached, and mortality should be expected, which, in turn, can be used to determine a sampling frequency for monitoring. In small volume experiments, Masson et al. (2011) found that the lethal dose for Spotted Seatrout was 237,243 dinospores per fish or 94,897 dinospores/L. The estimate of population growth rate at 25°C in optimal conditions was 1.9 individuals per day (Masson et al., 2013). Based on this growth rate, the concentration of dinospores would reach lethal levels in approximately 17 days if the first detection was exactly at 2 dinospores/L (the current detection threshold in the water). Using this hypothetical scenario, testing seatrout every 7-10 days would leave a one-week window after a positive detection to implement treatment before mortalities occur. Masson et al. (2011; 2013) also applied the model to data from the Red Snapper, *Lutjanus campechanus*. Results were slightly different (i.e., lethal dose was lower at 141,010 dinospores per fish or 56,404 dinospores/L), but the population growth rate of *A. ocellatum* was similar at 1.92 individuals per day such that at the detection threshold of 2 dinospore/L, it would take approximately 16 days to reach the lethal dose. This would suggest implementing a similar testing scheme for Red Snapper, sampling once a week, that would provide about a week to begin the proper treatment and prevent mortalities from occurring. While the calculation might vary depending on the fish species in

culture, this general testing scheme might be practical because the LAMP assay is rapid and simple. Financial considerations related to assay cost may guide implementation decisions, but this sampling regime is likely to be cheaper than the cost of losing a crop of fish or shutting down a system for cleaning and disinfection. Brock et al. (1999) established a protocol to monitor subclinical infections. Their protocol consisted of five gill biopsies per week. The fish were asymptomatic and behaving normally while the infection load was below an average trophont count/field (per gill sample) of 5 or less. When average trophont counts increased to an average of 20 trophont/field, there was an elevated risk of an outbreak and they treated with hydrogen peroxide to prevent mortalities. The suggested monitoring program from this study indicates that treatment should begin regardless of the stage of infection. The LAMP as a diagnostic tool is sensitive enough to detect the parasite in water samples at the levels Brock et al. (1999) saw on the gills. Both methods have flaws and do not account for other stages of the life cycle, i.e., tomonts and trophonts for the LAMP assay of water samples, so the parasitic load would likely be higher than inferred from assays on water samples. Overestimating the population of the parasite would be advisable and beginning treatment upon first detection would have a higher chance at preventing mortalities.

This work showed that the LAMP assay is superior to the gold standard microscopy method as a diagnostic tool for detecting *A. ocellatum* in both water samples and gill tissue samples. The AO-LAMP has improved sensitivity and is a non-invasive procedure that can be performed on water samples. While other molecular assays exist, the LAMP assay is more suited for farms and facilities because it is more sensitive, just as specific, easier to run, and requires minimal equipment. Practical application in culture

systems could consist of periodic monitoring, particularly following a possible exposure, to determine if the culture was exposed to the parasite, and to detect infection early enough to allow time for proper treatment before mortalities occur.

CHAPTER III – FREEZE TOLERANCE OF *AMYLOODINIUM OCELLATUM*

3.1 Introduction

Most forms of aquaculture stock animals at a high density to maximize production efficiency and cost effectiveness. However, the proximity of animals in high-density systems can promote the transmission of diseases and ultimately lead to large outbreaks and heavy mortalities (Pillay and Kutty, 2005). Because of the losses they cause, diseases act as a major limiting factor in the expansion of aquaculture. Controlling infectious diseases is, therefore, vital for the continued development of aquaculture. Methods employed to control diseases include prevention of outbreaks through implementation of biosecurity protocols, vaccinations to protect stocks (where possible), and treatments when outbreaks occur. When these approaches are ineffective or not possible, aquaculturists often need to remove infected stocks and sanitize affected systems or, in some cases, entire facilities resulting in large economic losses. Because vaccination is not always available and treatment can be costly, of limited effectiveness (treatment usually occurs once the system is already infected and does not prevent some losses), and affect marketing of treated stocks, optimizing prevention is of paramount importance. Understanding the pathogen's life cycle, epidemiology and potential transmission routes is essential in order to develop effective prevention strategies.

Amyloodinium ocellatum is a cosmopolitan, warm water, ectoparasitic dinoflagellate that infects a wide variety of marine and brackish-water fishes causing amyloodiniosis or marine velvet disease (Brown, 1931; Paperna, 1984a; Noga and Levy, 2006). The life cycle of *A. ocellatum* consists of three stages: the trophont which attaches to and feeds on the host, the tomont which is free-living and reproductive, and the

dinospore which hatches from the tomont and infects new hosts (Brown, 1934; Nigrelli, 1936; Brown & Hovasse, 1946; Paperna, 1984a). The life cycle can be completed in as little as 3 days, and a single individual tomont can undergo several divisions to produce up to 256 infective dinospores. This short, proliferative life cycle leads to rapid increases in the parasite population that result in acute mortality, in some cases before any clinical signs are detected (Francis-Floyd & Floyd, 2011). Because the preferred site of infection is the gills, clinical signs of the disease are most commonly respiratory and include a rapid respiratory rate, "piping", and gathering in areas of higher dissolved oxygen concentration. If the infection is on the skin of the fish, a white or brownish discoloration on the skin will appear and fish may be seen rubbing on the sides of the tank. Because of its tolerance to a wide range of temperatures and salinities and its ability to use the majority of temperate and warmwater marine finfish species as hosts, *A. ocellatum* is considered the most troublesome parasite in warmwater marine aquaculture. Infections typically occur through introduction of contaminated live fish, feed, water, or fomites, though the relative importance of the different routes is unknown.

Management of this parasite consists of prevention, diagnosis, and treatment when infections do occur. The current 'gold standard' method for diagnosis of amyloodiniosis is the observation of trophonts on the tissue of infected fish using light microscopy. This method detects the parasite only when trophonts appear on the gills or skin. Therefore, treatments are typically not initiated until late in the infection and at least some level of mortality cannot be avoided. In addition, because small trophonts can be difficult to detect and only a portion of the gill tissue of a fish is typically examined in a diagnostic procedure, early infections can be missed. If an outbreak does occur, a

chemical treatment using copper sulfate or chloroquine diphosphate can be applied (Paperna, 1984b; Francis-Floyd & Floyd, 2011). Because both treatments target the dinospore stage of the life cycle exclusively, a therapeutic concentration must be maintained continuously for a duration that exceeds the life cycle of the parasite which is quite long (close to 1 month at 25 ºC) to ensure all the cohorts of parasites present at the initiation of the treatment are exposed to the treatment as dinospores.

Because of the constraints associated with treatment, the best form of management for the disease is prevention. Prevention is achieved through proper biosecurity protocols that control sanitation practices for water, live fish, and human traffic. Protocols typically prohibit movement of water and/or equipment between systems or locations unless strict sanitation procedures are followed. All live animals entering the site must observe a quarantine period during which prophylactic treatments are applied to ensure fish are parasite free before being transferred to biosecure facilities. Finally, human activities must proceed from the 'cleanest' most biosecure area to the least biosecure area when the same person visits multiple areas during a working day. While protocols to prevent the spread of *A. ocellatum* through water and live fish are in place in most marine hatcheries as discussed above, other vectors could transmit the parasite during normal hatchery activities. For example, frozen wild fish are often used as food for cultured stock, in particular broodstock, and may carry encysted tomonts susceptible of producing viable dinospores, the infectious stage of *A. ocellatum*. Thawing of the frozen tissue in tank water effectively restores favorable conditions for the parasite and could lead to the production of dinospores by tomonts. Indeed, cyst forming dinoflagellates are known to survive cold-storage and proliferate following return to a

favorable temperature (Montresor and Marino, 1996). Moreover, preliminary evidence (Haley Dutton, personal communication) demonstrated that gills from a fish infected with *A. ocellatum* frozen in a domestic freezer could produce an infection in uninfected fish following thawing. These preliminary findings indicate that research to elucidate the potential of frozen wild food as a vector for transmission of *Amyloodinium* is warranted.

The goal of this chapter was to assess the tolerance of *A. ocellatum* to freezing at the temperature of common household freezers (-20°C) applied for 0 to 72 hrs. *In vivo* experiments assessed the ability of trophonts to infect naïve Spotted Seatrout, *Cynoscion nebulosus*, and reproduce following freezing. *In vitro* experiments assessed the sporulation rate and reproductive capacity of individual tomonts following freezing for different durations.

3.2 Materials and Methods

3.2.1 Production of naïve Spotted Seatrout

Experimental naïve Spotted Seatrout were obtained from *A. ocellatum*-free broodstock held in captivity at the Thad Cochran Marine Aquaculture Center (see Chapter II).

3.2.2 Parasite Culture

The parasite culture was maintained in a dedicated building on the Halstead campus of the Gulf Coast Research Laboratory (see Chapter II).

3.2.3 Experiment 1: Exposure of naïve fish to frozen infected tissue

Eight gill arches were collected from recently deceased fish or infected fish immediately after euthanizing the fish by double pithing, and the trophonts present on each gill arch were counted to verify that heavily infected fish were used for the

experiment. The eight gill arches from each heavily infected fish were then placed in a Ziploc sandwich bag, 16.5 cm x 14.9 cm, with approximately 125 mL of 25 psu artificial seawater and frozen at -20°C for 24, 36, 48 or 72 hrs. There were 5 treatments, each replicated three times in three consecutive trials. For each trial, 15 bags with gills from 15 different infected fish were prepared for the experiment. Three bags of gills were not frozen and acted as controls (freezing duration '0'). For the other groups, following each of the protocol freezing period (24, 36, 48 or 72 hrs), the contents of three bags were thawed under warm running water for approximately 5 minutes. Once thawed, the gill tissue was placed in a 10-gal aquarium filled with 20 l of 25 psu artificial seawater along with ten naïve juvenile Spotted Seatrout ranging from 125g to 200g. The resulting five treatment-aquaria were placed in table water baths containing titanium rod heaters set to 27°C. Each aquarium was aerated by air stones connected to an air blower.

On day 0 prior to the introduction of the frozen and thawed material, 4 gill arches from 2 fish were examined to confirm the absence of the parasite. On days 4 and 7, four fish were removed and euthanized by double pithing. Four gill arches, two from each side, from euthanized fish were examined by microscopy for the presence of trophonts using a light microscope at 40x magnification. Trophonts were counted on each gill arch.

3.2.4 Experiment 2: Production of dinospores by tomonts derived from frozen infected tissue

A sample of trophonts was obtained from the thawed gill tissue used in each freeze duration treatment tested in experiment 1. The sample was collected by agitating a portion of the gills in a petri dish partially filled with 25 psu artificial seawater to dislodge the trophonts, which were then collected from the bottom of the petri dish via

pipetting. The trophonts from each of the freeze durations were then individually stocked into the wells of 12-well tissue culture plates (one trophont per well, 8 trophonts per freeze duration for each replicate trial) filled with 5 mL of 25 psu artificial seawater.

Trophonts were observed every 6 hrs for 6 days until either successful hatch or the termination of divisions. Following the successful hatches, viable dinospores were counted for each tomont and recorded. Temperature was maintained at 25℃ through the ambient air temperature. This experiment was repeated three times.

3.2.5 Data analysis

For experiment 1, an ANOVA was used to test the effects of freezing duration and date of examination on the number of trophonts per gill arch. For each exposure, the four fish samples acted as four replicate trophont counts, and the counts on each of the gill arches that were examined were averaged to characterize the level of infection of each fish.

The ANOVA model used was

$$y_{ijk} = T_i + D_j + tr_k + (TD)_{ij} + (Ttr)_{ik} + (Dtr)_{jk} + \varepsilon_{ijk} \text{ Model 1}$$

where y_{ijk} is an observation in an individual, T_i is the fixed effect of freezing duration, D_j the fixed effect of day of examination (4 or 7), and tr_k the random effect of replicate trial. $(TD)_{ij}$, $(Ttr)_{ik}$, $(Dtr)_{jk}$ are the three first order interaction terms between freezing duration, day of examination and replicate trial, and ε_{ijkl} is the residual error.

For experiment 2, an ANOVA was used to test the effect of the fixed factors freeze duration and that of the random effect of replicate trial (n=3) on the number of dinospores. Variables assessed were the number of tomonts that successfully hatched and the number of dinospores produced per tomont. The ANOVA model used was

$$y_{ijk} = T_i + tr_j + (Ttr)_{ij} + \varepsilon_{ijk} \text{ Model 2}$$

where y_{ijk} is an observation of an individual tomont, T_i is the fixed effect of freezing duration and tr_k the random effect of replicate trial. $(Ttr)_{ij}$ is the interaction terms between freezing duration, and replicate trial and ε_{ijk} is the residual error. A linear regression was used to model the number of dinospores produced per tomont as a function of freeze time.

3.3 Results

3.3.1 Experiment 1

Trophonts were observed in the gills of fish from all freeze duration groups beginning at the day 4 observation point. The number of trophonts per gill arch varied significantly among examination days ($F_{2,691}$ = 8861.86, P = <0.0001) and freeze duration treatments ($F_{4,691}$ = 422.48, P = <0.0001). As expected, there were no trophonts on gill arches before introduction of the contaminated tissue to aquaria (day 0, Figure 3.1). The average number of trophonts per gill arch rose rapidly to 253.2 at day 4 and 719.6 (a 184% increase) at day 7. The number of trophonts per gill arch observed at day 4 and 7 decreased with increasing freeze duration. All treatment groups had a significantly lower number of trophonts per gill arch than controls. The 24, 36 and 48 hr groups were not statistically different, but followed a decreasing pattern in the number of trophonts per gill arch with increasing freeze duration. The 72 hr group had a substantially and significantly lower number of trophonts than all the other treatment groups. There was a significant interaction between the freeze duration and the examination day ($F_{8,691}$ = 119.01, P = <0.0001), which was due to (i) an increase in the magnitude of the difference between the intermediate treatments (24, 36, and 48 hr) between day 4 and day 7 (Figure

3.1) and (ii) a substantial rise in the number of trophonts per gill arch in the 72 hr treatment between day 4 and day 7 (1,775% increase), which reduced the gap between this and the other treatments.

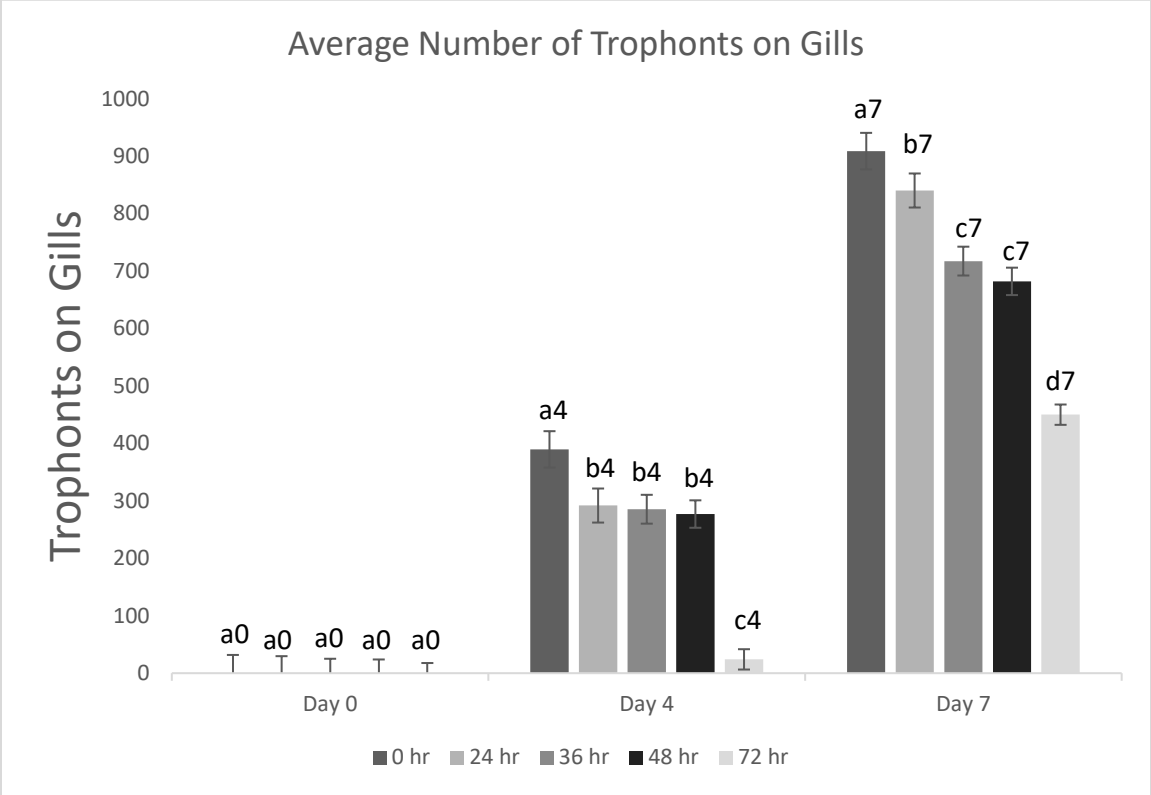

Figure 3.1 *Average number of Amyloodinium ocellatum trophonts per gill arch observed at 0, 4, and 7 days post-introduction of infected gills frozen for 0, 24, 36, 48, and 72 hrs. Groups labeled with different letters for a given day of observation are significantly different from each other.*

The number of trophonts per gill arch also differed among trials ($F_{2,691} = 4.29$, $P = 0.0141$). On average counts per gill arches were 4.3% higher during trial 3 than during the first two trials.

3.3.2 Experiment 2

The sporulation rate was almost 100% across all freeze durations and replicates. The percentage of tomonts that sporulated in the 48 and 72 hr freeze durations was marginally lower (22 out of 24 in these two treatments across the three replicates) than in the 24 and 36 hr groups where it was 100%, but the differences were not statistically significant ($F_{4,119} = 3$, $P = 0.0723$) (Table 3.1; Figure 3.2). However, the tomonts from the 72 hr freeze duration underwent the initial division approximately 24 hours later than the tomonts of the other freeze durations following the thawing process. The number of dinospores produced per hatched tomont differed significantly among freeze durations ($F_{4,105} = 26.35$, $P = <0.0001$). The 48 and 72 hr treatments were significantly different from each other and from the other groups, reflecting a decrease in the longest freeze duration treatments (Figure 3.3). Linear regression analysis of the number of dinospores per hatched tomont as a function of freeze duration revealed a strong linear relationship (percent of variance explained by the model $R^2 = 0.9372$) which was modelled with the following equation

$$y = -1.0559x + 250.31 \ (P = <0.0001) \text{ Equation 1}$$

Table 3.1 *Number of successfully hatched Amyloodinium ocellatum tomonts and total sporulation rate in freeze duration and replicate groups.*

	Replicate 1	Replicate 2	Replicate 3	
Freeze Duration	Hatched Tomonts	Hatched Tomonts	Hatched Tomonts	Total Sporulation Rate
0 hr	8	8	8	100%
24 hr	8	8	8	100%
36 hr	8	8	8	100%
48 hr	8	7	7	91.67%
72 hr	7	8	7	91.67%

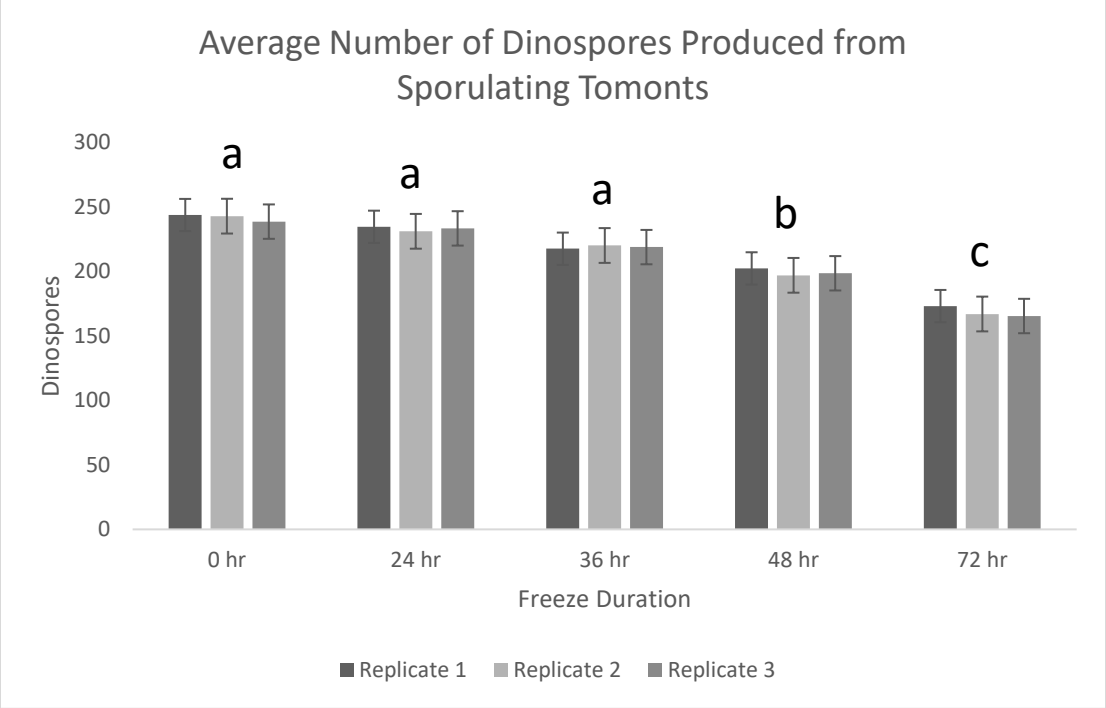

Figure 3.2 *Average number of dinospores produced in groups treated with different freeze durations. Groups labeled with different letters are significantly different.*

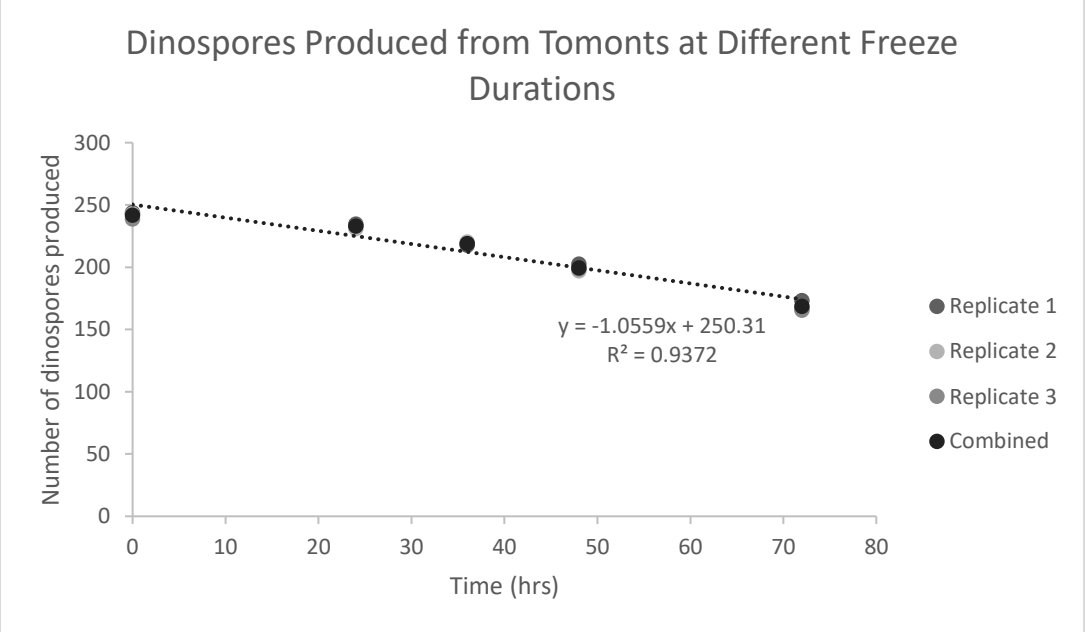

Figure 3.3 *Average number of Amyloodinium ocellatum dinospores produced from successfully hatched tomonts after 0, 24, 36, 48, and 72 hrs of freezing for the three replicate trials and a regression line modelling the mean dinospore production in relation to freeze duration.*

3.4 Discussion

This study showed that naïve fish became infected in the presence of thawed infected frozen fish tissue and that the resulting infections progressed rapidly producing large numbers of trophonts per gill arch in all fish after only 7 days of incubation in the conditions of experiment 1. Therefore, this work confirms that frozen food, if frozen for 72 hours or less at -20°C, can act as an effective transmission vector for the parasite.

Paperna (1984a) reported that *Amylodinium ocellatum* tolerates a wide range of environmental conditions, particularly extreme ranges in salinity and temperature for at least short periods, an observation consistent with short-term tolerance to freezing in this study. However, Paperna (1984a) also suggested that extended periods of time at cold temperatures inhibited reproduction. In his work, only a few tomonts sporulated at 15°C, and the yield of dinospores after 10-16 days was very low. Reproduction resumed after returning to a temperature of 20°C, but sporulation occurred at a reduced level compared to tomonts that had not experienced the drop in temperature (Paperna, 1984a). In the same study, tomont divisions stopped at 8°C. Division resumed upon return to 20°C, but the time required to resume division was directly related to the time spent at 8°C. In Experiment 2 of this study, even though nearly 100% of the tomonts sporulated in all treatments and the dinospores that were produced infected naïve fish, short-term freezing affected sporulation of the parasite and the time to initiate divisions. As freeze duration increased, tomonts took longer to initiate divisions and produced fewer dinospores per tomont. Dinospore viability also decreased as freeze duration increased. These negative impacts translated into a reduced rate of infection for naïve fish and a delay in the

progression of infection for the longer freeze duration treatments in experiment 1. The delay in the initiation of the tomont divisions observed in the longer freeze duration treatments suggests that freezing affects the parasite's ability to initiate division, although further development of the tomonts and dinospore release still occurred once division began. The decreasing trend in the number of dinospores per tomont as freeze duration increased could be due to a reduction of the viability of the dinospores following sporulation or a reduced division rate within the tomont prior to hatch. Non-viable dinospores were not recorded during observation and counting during experiment 2, but the delay in initiating divisions observed in the longer freeze duration treatments suggests that a lower rate of division prior to hatch of the tomont may be a factor explaining the reduction of the production of dinospores with increasing freeze durations.

The reduction of dinospore production and infectivity with increasing freeze duration suggests that freezing could provide some degree of parasite control if the freezing temperature is applied for longer periods than the range tested in experiments 1 and 2. Assuming the decrease in the number of dinospores produced per tomont follows the linear model fitted in this study and extrapolates to longer freeze durations, equation 1 predicts that no viable dinospore would be produced after ~237 hours of freezing. However, the slope of the decay may change as freeze time increases beyond the range tested in this experiment. Extending the freeze duration to ensure frozen fish tissue is not infectious may, therefore, be a viable approach in practice as frozen fish can have a lengthy shelf life at the temperature used in this study (-20℃) (Gogou et al., 2015). The infection potential of infected fish tissue after longer freeze durations will need to be formally tested to determine a protocol for successfully inactivating all tomonts. The rate

of decay of dinospore production estimated above suggests that freeze durations of 10 days or more should be tested in future studies.

The apparent tolerance of *A. ocellatum* to short-term freezing at -20C is somewhat surprising considering the range of temperature experienced by the species in its natural temperate and subtropical habitats (Brown, 1931; Paperna, 1984b; Bower et al., 1987). The isolate used in this work was from the Gulf of Mexico where the average low water temperature during the winter months is around 13-14℃ (Moore, 1975). Winter cold fronts do occur in the region and can bring temperatures several degrees below 0℃ to coastal waters (Moore, 1976), although freezing conditions are typically maintained only for a few hours or a few days. The temperature of -20℃ used in domestic freezers and tested in our experiments never occurs in the Gulf of Mexico. While there is little information on the range of environmental tolerance of the cysts of *A. ocellatum* or other dinoflagellates, the cysts of other encysting organisms such as *Artemia franciscana* (Ramløv and Hvidt, 1992; Yoshida et al., 2011) have been shown to tolerate cold conditions far below -20℃ and largely exceeding the range encountered in natural habitats.

It is important to note that this study was done using an *A. ocellatum* isolate sourced in the Gulf of Mexico. To date, information on the genetic structure of *Amyloodinium* is limited. Levy et al. (2007) looked at 5 isolates of *Amyloodinium ocellatum* from geographic areas including the Red Sea (Israel), the eastern Mediterranean Sea (Israel), the Adriatic Sea (Italy), the Gulf of Mexico (Florida), and an unknown locality. Across the five isolates, Levy et al. (2007) did not find sufficient variation in the sequence of the internal transcribed spacer region of the ribosomal DNA

to justify considering any of the isolates separate species or subspecies. However, the assay did not provide enough resolution to determine the level of genetic differentiation and potential divergence at adaptive genes. Paperna (1984a) and Kuperman and Matey (1999) found different environmental tolerances among different isolates of *A. ocellatum*. Thus, Levy et al. (2007) did not rule out the possibility that additional research would reveal divergent populations or subspecies of the parasite. This suggests that different isolates of *A. ocellatum*, particularly their non-encysted life stages, may be adapted to environmental conditions in the specific region they are located. In terms of management, this could alter the appropriate temperature and freeze duration required to successfully inactivate the parasite. However, it should be noted that the temperature tolerated by cysts in this study is well below the natural range of temperature in the Gulf and thus might be widespread and unrelated to local conditions.

Because of the reproductive capacity of the parasite, a single viable dinospore produced from a tomont could result in an outbreak of amyloodiniosis. This underscores the importance of preventing the introduction of the parasite and understanding biosecurity protocols, including those for freezing fish used for food. Until further studies determine the adequate freeze duration and temperature that completely inactivates the parasite, aquaculture facilities that use frozen wild caught fish face an inherent risk of introducing the parasite. While it may not be currently practical for facilities to freeze food fish below -20°C, freezing at the coldest attainable temperature for the maximum amount of time combined with a routine surveillance program may reduce the risk of infection by this vector. Until further studies are done, this study suggests that a frozen

fish used for food should be frozen for a minimum of 237 hrs (10 days) to inactivate *Amyloodinium* tomonts and prevent infection.

CHAPTER IV – GENERAL CONCLUSION

Amyloodinium ocellatum is considered one of the most important parasites constraining the culture of warm water marine finfish. The parasite is of significant concern because of the rapidity at which it reproduces and causes mortalities. The life cycle can be completed in as little as three days, with single individuals producing up to 256 infectious dinospores. Any degree of control relies on early diagnosis to provide the maximum opportunity for treatment as well as prevention of infections through elimination of potential vectors. This thesis sought to contribute to the development of methods and practices to control the parasite through improvement in diagnostic techniques and elucidation of the risk of frozen, wild-caught fish as a potential vector.

The first objective of this study was to assess how a LAMP assay developed by Picon-Camacho (2013) compared to the current standard light microscopy technique for the detection of *A. ocellatum* in gill tissue and in culture water. The LAMP assay performed on water samples allowed detections of dinospore concentrations as low as 0.5 per liter and was substantially more sensitive than the LAMP assay or microscopy on gill tissue that recorded positive detections only when inoculation concentration was 5 dinospores/L or higher. The LAMP assay on water samples, therefore, seems the most promising option for early detection of the parasite in culture systems. However, several questions remain to be addressed to fully assess this method. The lack of detection on gills at the low inoculation concentrations (below 5 dinospores/L) may have been due to low success of attachment of dinospores to fish gills and/or infected areas of the gills could have been missed during sub-sampling of gill arches for observation or assay. In addition, a low concentration of dinospores in water could correspond to a high average

trophont load per fish if fish are cultured at low density e.g., in a broodstock tank. Therefore, better information on the relationship between the density of trophonts on fish gills and the density of dinospores in the water in infection scenarios with varying fish density, life stage and culture parameters would be useful to provide additional context on the sensitivity of the two tests in real situations. My experiments were conducted in small volume (20 L) aquariums and clear water. Water circulation in large systems may influence the distribution of dinospores in the water and detection probabilities. Further study in commercial-scale tanks that examine practical implementation aspects such as the specific location of sampling (outflow, surface, different depths) and the detection sensitivity when varying numbers of replicates may be useful for optimizing a sampling protocol that maximizes the sensitivity of the assay. In addition, temporal changes in dinospore abundance may occur with periods of low dinospore concentration alternating with periods of higher abundance when new cohorts of dinospores are released, especially at early stages of an outbreak. Temporal surveys during the initial phases of an infection may be useful to determine if such fluctuations occur and their potential effect on detection and assessment of the status of an outbreak. Finally, the dynamics of outbreaks in a culture system are still not well understood. Modelling of the evolution of parasite abundance as a function of culture variables such as temperature and fish density would be useful to better predict the status of an infection based on LAMP assay results and the timeline of its evolution towards lethal outcomes.

This study also sought to estimate the probability of detecting the parasite at various concentrations to form the basis for development of a competent monitoring scheme. For this purpose, the probability of one positive result out of ten measurements

was estimated across three methods: LAMP on water samples, LAMP on gill tissues, and microscopy of gill tissue. The probability of having at least one positive test out of ten was 100% at 5 dinospores/L, 50 dinospores/L, and 50 dinospores/L for each of the methods, respectively (Table 2.3). Based on previous models established by Masson et al. (2013), testing every 7-10 days was predicted to afford a week's time to establish effective treatment before lethal doses would be reached on fish. However, this was calculated from a model derived for Spotted Seatrout, *Cynoscion nebulosus*, in small volume systems. Additional data on the dynamics of infections in large-scale systems as a function of some critical variables including fish density and temperature are warranted as discussed above. The appropriate timeframe for testing also may vary to some degree depending on the fish species in culture based on their tolerance to *A. ocellatum*.

The second objective of this work was to investigate the freeze tolerance (-20℃) of *A. ocellatum* at different short-term durations, 24, 36, 48, and 72 hrs. *In vivo* experiments showed that fish exposed to frozen then thawed infected tissue were infected across all freeze durations, indicating that short-term freezing does not inactivate *A. ocellatum*. There was a lag in infection at the 72 hr freeze duration, but once infections established (at 4 days post-inoculation), the number of trophonts present on gills at 7 days post-inoculation in this group approached that found in other treatment durations, suggesting that freezing only delayed developmental processes. Tomonts were competent to resume divisions and produced spores when returned to ambient temperature. Due to the rapid life cycle of *A. ocellatum*, the increased number of trophonts detected at 7 days post-inoculation could reflect the production of a new generation of the parasite between day 4 and day 7, particularly considering the warm temperature of the trial, but this

hypothesis would need to be formally explored experimentally. The *in vitro* experiment showed that there was a significant decrease in the number of viable dinospores produced per tomont at the freeze durations of 48 and 72 hrs, possibly reflecting inhibition of divisions in the tomont, which were delayed approximately 24 hours in the 72 hr freeze duration. Testing of longer freezing durations is warranted to determine if dinospore production and infections can be prevented entirely. This work provided information that can be used to determine the duration of future candidate treatments via the modeling of the reduction of the number of dinospores produced as a function of freezing duration. Extrapolation of the regression model obtained in this study predicts that a freeze duration of approximately 237 hours would result in production of no viable dinospores. While this is only hypothetical at this point, a formal test of freeze durations at, below, and beyond this value would be informative. Frozen, wild-caught fish are relatively shelf-stable and can be stored for extended periods, thus such a protocol would be compatible with implementation in aquaculture facilities. This project did not test the infectivity of subsequent generations of the parasite following freezing which could be also studied in future research.

In summary, it would be advisable for commercial farms to evaluate an *A. ocellatum* monitoring and testing plan. A monitoring program would allow for time to establish treatment and prevent mortalities. Continuous, regular, weekly testing for systems would be ideal; however, regular testing may not be an option for some farms. In those situations, initiation of weekly monitoring could begin after a potential exposure event and continue for at least a month. Longer freeze durations need to be assessed to determine the point at which the parasite's ability to reproduce can be inactivated. In the

meantime, however, frozen food brought to a facility needs to be kept at a minimum of -20°C for the longest duration possible. Finally, the freezing temperature examined in this study is far lower than that encountered by the parasite in the northern Gulf of Mexico and other parts of its range. Despite this, the parasite was able to reproduce. The mechanisms and evolutionary significance of these observations deserve further examination.

APPENDIX A – IACUC Approval Letter 2017-2020

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	17103001
PROJECT TITLE:	Development of aquaculture and stock enhancement for marine finfish at the Thad Cochran Marine Aquaculture
PROPOSED PROJECT DATES:	11/2017 – 09/2020
PROJECT TYPE:	Modification – addition of species
PRINCIPAL INVESTIGATOR(S):	Eric Saillant
DEPARTMENT:	Division of Coastal Sciences
FUNDING AGENCY/SPONSOR:	N/A
IACUC COMMITTEE ACTION:	Designated Review Approval
PROTOCOL EXPIRATON DATE:	September 30, 2020

June 15, 2018

Jake Schaefer, PhD
IACUC Chair

Date

APPENDIX B – IACUC Approval Letter 2020-2022

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	17103001.2
PROJECT TITLE:	**Development of Aquaculture & Stock Enhancement for Marine Finfish at the Thad Cochran Marine Aquaculture Center**
PROPOSED PROJECT DATES:	11/2020 – 09/2022
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Eric Salliant**
DEPARTMENT:	**Coastal Sciences**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Designated Review Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2022**

November 24, 2020

Jake Schaefer, PhD
IACUC Chair

Date

APPENDIX C – Figure Permission from Dr. Ignacio Masson

Re: Permission to Use Figure

IM

Ignacio Masson <imasson.ihlla@gmail.com>

2/25/2022 3:04 PM

To: Robert Gonzales

Hello Robert. Yes, sure, go ahead. Good luck with your thesis. Hope everybody is doing fine at GCRL. Send my greetings please.
Kind regards, Ignacio

El vie, 25 feb 2022 a las 13:00, Robert Gonzales (<R.Gonzales@usm.edu>) escribió:

> Good morning Dr. Masson,
>
> I am currently a student of Reg Blaylock and I am in the process of finishing up my thesis. Reg suggested contacting you to see if I could have your permission to use the Amyloodinium ocellatum life cycle figure from your dissertation to use in my thesis. I would greatly appreciate it.
>
> Thank you,
>
> Robert Gonzales

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