Faculty of Science and Technology

MASTER'S THESIS

Study program/ Specialization: Environmental technology with specialization in water science and technology	Spring semester, 2013 Restricted access
Writer: Ida Renate Øglænd	………………………………………… (Writer's signature)

Faculty supervisor: Torleiv Bilstad, PhD

External supervisor(s): Andrew S. Kane, PhD, at University of Florida

Title of thesis:

Prevalence, Distribution and Environmental Factors Associated with *Perkinsus marinus* Infection in Eastern Oyster, *Crassostrea virginica*, in Apalachicola Bay, Florida, USA

Credits (ECTS): 30

Key words: Dermo, *Perkinsus marinus*, Eastern oyster, *Crassostrea virginica*, Apalachicola Bay, Florida, prevalence, infection intensity, distribution	Pages: 61 + enclosure: 9 Stavanger, 14.06.2013 Date/year

Prevalence, Distribution and Environmental Factors Associated with *Perkinsus marinus* Infection in Eastern Oyster, *Crassostrea virginica,* in Apalachicola Bay, Florida, USA

By

Ida Renate Øglænd (Oeglaend)

A thesis submitted in partial fulfillment of the requirements for the degree of Master in Environmental Technology

Faculty of Science and Technology

Department of Mathematics and Natural Sciences

University of Stavanger, Norway

June 2013

Written under the direction of

Andrew S. Kane

Associate Professor of Environmental and Global Health

Aquatic Pathobiology Laboratories

University of Florida

Gainesville, Florida, USA

Abstract

Perkinsus marinus is a protozoan parasite causing Dermo disease in the Eastern Oyster, *Crassostrea virginica*. The parasite spreads from oyster to oyster through the water column, and can cause extensive oyster mortalities, especially after periods with high temperature and salinity. This study investigated the distribution and weighted prevalence (prevalence and infection intensity) of *P. marinus* in *C. virginica* in Apalachicola Bay (Franklin County, Florida, USA), as a part of a larger project that investigates the decline of oyster populations and fishery collapse in the bay. The relationship between oyster health and *P. marinus* infections were also investigated.

No differences were found in weighted prevalence of *P. marinus* infections in oysters within oyster bars, between oyster bars, nor between November 2012 and February 2013 sampling time points. Mean weighted prevalence of *P. marinus* infections in Apalachicola Bay was 1.01 ± 0.11 and 0.90 ± 0.05 (mean ± SE) for November 2012 and February 2013, respectfully. A negative relationship between oyster meat condition and *P. marinus* infection intensity was found. No other relationships were found between different internal and external oyster health condition indices and *P. marinus* infection intensity.

Results from this study compared with other studies suggest that weighted prevalence of *P. marinus* in Apalachicola Bay oysters has increased since 2005. Drought periods in the Apalachicola River watershed are associated with reduced freshwater flow into Apalachicola Bay and elevated salinity, fostering conditions favorable for *P. marinus* infection in oysters.

Acknowledgements

I would like to thank Torleiv Bilstad, who made it possible for me to travel to Florida to work on my master's project. Thanks to Patti Anderson, who kindly took care of me and made sure I got to experience different parts of Florida.

I also express my deepest thanks to my advisor, Andrew Kane, for giving me the opportunity to work on this project, and for his guidance and support. Thanks to all members of the Aquatic Pathobiology Laboratories, especially Ross Brooks, who let me include some of his work in my thesis.

Special thanks go to my fiancé, Espen Christensen, for patience and continuous support through these busy months.

Stavanger, June 2013

Ida Renate Øglænd

Table of Contents

Abstract 3

Acknowledgements 4

Table of Contents 5

Abbreviations 7

List of Figures 8

List of Tables 11

1 Introduction 12

1.1 The Eastern Oyster, *Crassostrea virginica* 12

1.2 Perkinsus marinus 14

1.2.1 Life Cycle of *P. marinus* 15

1.2.2 Transmission of *P. marinus* 16

1.2.3 Infection mechanism 17

1.2.4 Effect of *P. marinus* on Oysters 18

1.2.5 *P. marinus* Infection Intensity in Oysters of Different Sizes 19

1.2.6 Environmental Factors Affecting *P. marinus* Prevalence and Intensity in Oysters 19

1.3 Overview of Historical Data on *P. marinus* Infections in Apalachicola Bay 22

1.4 Methods of *Perkinsus marinus* Detection and Monitoring 24

1.4.1 RFTM Oyster Tissue Assay 25

2 Aims of Thesis 28

3 Methods 30

3.1 Oyster Sampling Locations and Water Quality 30

3.2 Ranking of Oyster Health Condition Indices 31

3.2.1 External Rankings of Oyster Shells 32

3.2.2 Internal Rankings of Oyster Shells and Meat Condition Index 32

3.3 Tissue Collection 34

3.4 Tissue Analysis 34

3.5 Calculations 35

3.6 Statistical Analyses 35

4 Results 37

4.1 Temperature and Salinity in Apalachicola Bay 37

4.2 Spatial Distribution of *P. marinus* 39

4.2.1 Weighted Prevalence of *P. marinus* Infections within Oyster Bars 39

4.2.2 Weighted Prevalence of *P. marinus* Infections between Oyster Bars and Time Points 42

4.3 Weighted Prevalence of *P. marinus* Infections in Oysters of Different Sizes 43

4.4 Linear Regression between Infection Intensity and Oyster Health Condition Indices 44

4.5 Comparisons with Historic Data from Apalachicola Bay 45

5 Discussion 46

5.1 Weighted Prevalence of *P. marinus* Infections in Apalachicola Bay 46

5.1.1 Temperature and Salinity in Apalachicola Bay 46

5.1.2 Spatial and Temporal Distribution of *P. marinus* Infections in Apalachicola Bay Oysters 46

5.1.3 *P. marinus* Infections in Oysters of Different Sizes 47

5.2 *P. marinus* Infections Versus Oyster Health Indices 48

5.3 Comparisons with Historical Data 48

5.4 Evaluation of RFTM Tissue Assay 49

5.4.1 General Problems Encountered 49

5.4.2 Mackin Rank Photo Guide 50

5.4.3 Variability and Sample Size: 51

5.4.4 Appearance of Tissue Samples: 51

6 Conclusion 52

7 Future Prospects 53

8 References: 54

9 Appendix 62

Abbreviations

APL – Aquatic Pathobiology Laboratories

g – gram

h – hour

L – liter

mL – milliliter

mm – millimeter

NERRS – National Estuarine Research Reserve System

ppt – parts per thousands (‰)

RFTM – Ray's fluid thioglycollate medium

SD – Standard deviation

SE – Standard error

List of Figures

Figure 1. Eastern Oyster, *C. virginica*, from Apalachicola Bay. Photo credit: A. Kane. 12

Figure 2. Global distribution of natural occurring *C. virginica* [3]. Yellow asterisk indicates study site location of Apalachicola Bay in the Gulf of Mexico. 13

Figure 3. Global capture (left) and aquaculture (right) production for *C. virginica* [3]. 14

Figure 4. *P. marinus* life cycle. (1) Trophozoites and zoospores in the water column (2) enters the oyster during filter-feeding, where they are directed toward the gills and mouth. (3) Some cells may be released into the water again with the pseudofeces, feces or from the decaying tissue of an oyster (see section 1.2.2). (4) Trophozoites enlarge and form hypnospores, which develop a discharge tube and after successive bipartitioning, hundreds of zoospores are released into the water column. Figure taken from Fernández Robledo *et al.* (2011) [41]. 16

Figure 5. 28-day average discharge in Apalachicola River from January 2005 to April 2013, relative to historical flow in the past 89 years. Figure was created using the USGS Streamflow Duration Hydrograph Builder (http://waterwatch.usgs.gov/index.php) for USGS station 02358000, 1 km below Woodruff Dam. Flow line indicates drought periods in 2006-2008, and in 2010-2012. 21

Figure 6. Oyster Sentinel and Petes *et al*. sampling locations in Apalachicola Bay. 23

Figure 7. Left: Reported weighted prevalence ± SE of *P. marinus* infections in oysters at Dry Bar and Cat Point by Petes *et al.* (2012). Infections were ranked using a modified ranking system originally described by Ray (1954). Figure taken from Petes *et al.* (2012) [78]. Right: Petes *et al*. ranks converted to Mackin ranks. 24

Figure 8. Reported weighted prevalence of *P. marinus* infections in oysters at Dry Bar and Jetties from November 2005 to September 2012, by Oyster Sentinel (SE was not available). Data downloaded from www.oystersentinel.org (retrieved 14.01.13). Notice a higher weighted prevalence in 2007 and 2010. Could be a result of the drought mentioned in section 1.2.6 (Figure 5). Also, X-axis does not show time linearly, only the specific months sampled. 24

Figure 9. Examples of infected oyster tissue samples analyzed in this project, ranked from 0 to 5 at 4x magnification. 27

Figure 10. Map of sampling locations in Apalachicola Bay on November 5th 2012. Parenthesis indicates individual site number, where two to three non-adjacent sample sets were harvested. 30

Figure 11. Map of sampling sites in Apalachicola Bay on February 16th 2013. Parenthesis indicates individual site number, where two to four non-adjacent sample sets were harvested. 31

Figure 12. General anatomy of an oyster viewed from the right side with the right valve removed. Original figure from Galtsoff (1964) [88], as presented in VanderKooy (2012) [105]. 32

Figure 13. Shell parasites found on oyster shells in Apalachicola Bay. Left photo: Whole oyster shell with parasitic damage from boring clams (blue arrow) and boring sponges (numerous small holes). Right photo panel: (A) Two boring clams seen at the edge of a shell that was fractured to reveal the parasites. Note the black spot (yellow arrow) associated with the clam's activity on the inner nacreous layer of the shell. 8B) Close up of exterior shell holes bored by *Cliona* sponge. In life, this sponge organism is yellow and protrudes from the shell holes as shown in panel C. (D) *Polydora* worm. Photo credit: A. Kane. 33

Figure 14. Internal shell observations associated with parasites from Apalachicola Bay Oysters. Observations are described by numbers: (1) Yellowing. (2) Black *Diplothyra* clam spots. (3) Burrowing tubes at periphery of shell. These are points of access of boring *Polydora* worms. (4) Enlarged borrows of *Polydora* worm holes within shell. (5) Mud blisters. (6) Long-standing mud blisters with thicker layer of nacre walling off the worm. (7) White chalky deposits. Photo credit: A. Kane. 33

Figure 15. Examples of meat condition indices from Apalachicola Bay Oysters. Meat rank of oyster in A) 4.5, B) 3.5, C) 2.0 and D) 1.0. Photo credit: A. Kane. 34

Figure 16. Mean monthly temperature ± SD from September 1st 2012 to February 28th 2013 at Cat Point and Dry Bar. 38

Figure 17. Mean monthly salinity ± SD from September 1st 2012 to February 28th 2013 at Cat Point and Dry Bar. 38

Figure 18. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at Eastpoint Channel. Samples 907A-B were collected in November 2012, whereas samples 913A-B were collected in February 2013. Non-adjacent replicated samples were collected in an East to West direction along the shore line. 40

Figure 19. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at Cat Point. Samples 906A-C were collected in November 2012, whereas samples 914A-D were collected in February 2013. Non-adjacent replicated samples were collected in a North to South direction. 40

Figure 20. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at the Jetties. Samples 908A-B (Jetties East) and 909A-B (Jetties West) were collected in November 2012. Non-adjacent replicated samples were collected in a South to North direction. 41

Figure 21. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at St. Vincent Sound. Samples 911A-B were collected in November 2012, whereas samples 923A-B were collected in February 2013. Non-adjacent replicated samples were collected in a South to North direction. 41

Figure 22. Weighted prevalence ± SE of *P. marinus* infections in oysters at Eastpoint Channel, Cat Point, the Jetties and St. Vincent Sound in November and February. 42

Figure 23. Weighed prevalence ± SE of *P. marinus* infections in adult and juvenile oysters at Eastpoint Channel (913A) and Cat Point (914A, 914D). 43

Figure 24. Mean weighted prevalence of *P. marinus* infections in Apalachicola Bay, from pooled historical data available from 6-month intervals (September to February), based on data from Oyster Sentinel, Petes *et al*. and Oeglaend (error bar = SE). Error bars are not presented for historical data sets since raw data were not available. 45

Figure 25. Conversion of Petes *et al.*'s ranking system to Mackin ranking system. Conversion factor: Mackin rank = (Petes *et al*'s rank -0.4283)/1.0651 64

Figure 26. Mackin rank from 0 to 2+ viewed at 4x magnification in a light microscope. 67

Figure 27. Mackin rank from 3- to 5 viewed at 4x magnification in a light microscope. 68

Figure 28. Example of hypnospores in oyster tissue that did not stain well, at 4x magnification. Unstained hypnospores are seen as white see-through spheres (left), which eventually (with enough iodine solution present) changes to black spheres (right). 69

Figure 29. Thick tissue samples did not stain well in the middle of the tissue. Left photo: Iodine solution has penetrated the outer edge of the tissue (red color), while there is not much iodine further in on the tissue where it is thicker (4x magnification). Right photo: Hypnospores close to the surface stain better (top of photo) than cells deeper down in the tissue (bottom of photo, 10x magnification). 69

Figure 30. Components that at 4x magnification could look like hypnospores, but at 20x (shown here) it is clear that these are not hypnospores. 69

Figure 31. Left photo show a cluster of hypnospores in an oyster tissue sample (4x magnification). Right photo show how hypnospore cell size varies within a sample and between samples (10x magnification). 70

List of Tables

Table 1: Taxonomic Classification of *P. marinus* (www.ncbi.nlm.nih.gov, retrieved 29.04.13)......... 15

Table 2: Semi-quantitative scale of infection intensity of *Perkinsus marinus*. Adapted from Mackin (1962) by Craig *et al.* (1989) [21], with an additional APL ranking designation included.................. 26

Table 3: Mean bottom temperature and salinity measurements at sampling locations in November and February at the time of each sampling. Data are mean ± SE.. 38

Table 5: Results of linear regression between infection intensity of all oyster samples and their internal/external condition indices. 44

Table A1: Site name, number and GPS location of sampling sites on November 5th 2012 and February 16th 2013. 62

Table A2: RFTM Tissue Assay - Chemicals 63

Table A3: RFTM Tissue Assay - Equipment 63

Table A4: Overview of data collected at four locations in Apalachicola Bay in November 2012 and February 2013.. 65

Table A5: Overview of data used when comparing historical data from Oyster Sentinel and Petes *et al.* with results from this study. 66

1 Introduction

This project describes a survey of *Perkinsus marinus*, a pathogen of Eastern oyster (*Crassostrea virginica*), in Apalachicola Bay, Florida's Gulf of Mexico coast, United States. Apalachicola Bay produces 90% of Florida's oyster for the domestic US seafood market, and is known for premium, top quality oysters. *Perkinsus spp.* is historically associated with high prevalence and mortalities of oysters and other bivalves globally. The current investigation focuses on *P. marinus* infections in *C. virginica* in Apalachicola Bay, in association with apparent declines in the bay's oyster fisheries. This introductory section will provide a literature overview of the biology and ecology of the host (*C. virginica*), the parasite (*P. marinus*), environmental variables relevant to the host-parasite relationship, overview of recent historical reports on parasite prevalence and intensity in Apalachicola Bay, and information on RFTM tissue assay used for identification and ranking of infection intensity.

1.1 The Eastern Oyster, *Crassostrea virginica*

The Eastern oyster, *Crassostrea virginica*, (Figure 1) is an ecologically important species of bivalve mollusk, as well as an important economic and cultural resource to coastal inhabitants [1, 2]. *C. virginica* naturally occurs in estuarine and inshore waters from the Republic of Panama to Florida in the Gulf of Mexico, along the Atlantic coast of the U.S.A. and in the Gulf of St. Lawrence (Figure 2) [3]. Prior to 1939, *C. virginica* was imported to Europe, and colonies can still be found from the British Islands to Bay of Biscay and in the Adriatic Sea (Mediterranean Sea) [3].

Figure 1. Eastern Oyster, *C. virginica*, from Apalachicola Bay. Photo credit: A. Kane.

Figure 2. Global distribution of natural occurring *C. virginica* [3]. Yellow asterisk indicates study site location of Apalachicola Bay in the Gulf of Mexico.

Although *C. virginica* is most sought after in the United States, it is distributed throughout the global seafood market where hundreds of tonnes of oysters are harvested each year (Figure 3). In 2011, global capture production had a total of 120 795 tonnes and aquaculture production had a total of 71 355 tonnes [3].

Apalachicola Bay is known for its abundance of rapid growing, high quality oysters [4]. The bay supports an important commercial oyster fishery for the entire Gulf of Mexico, where Apalachicola Bay oysters accounts for up to 90 % of Florida's annual oyster landings, and 10 % of the harvest nationwide [5]. In the last decades, however, natural growing oyster populations have decreased due to overharvest, loss of habitat, poor growth and mortality from stressful environmental conditions (e.g. sub-optimal water quality, contamination), predation and disease [6-9]. Reduced availability of oysters can lead to tremendous losses in the oyster industry and cause damage to estuarine ecosystems [6, 10, 11]. Oyster reefs provide food, shelter and habitat for numerous species, as well as improving the overall water quality by filtering large quantities of water when they are feeding [1, 2, 11-15].

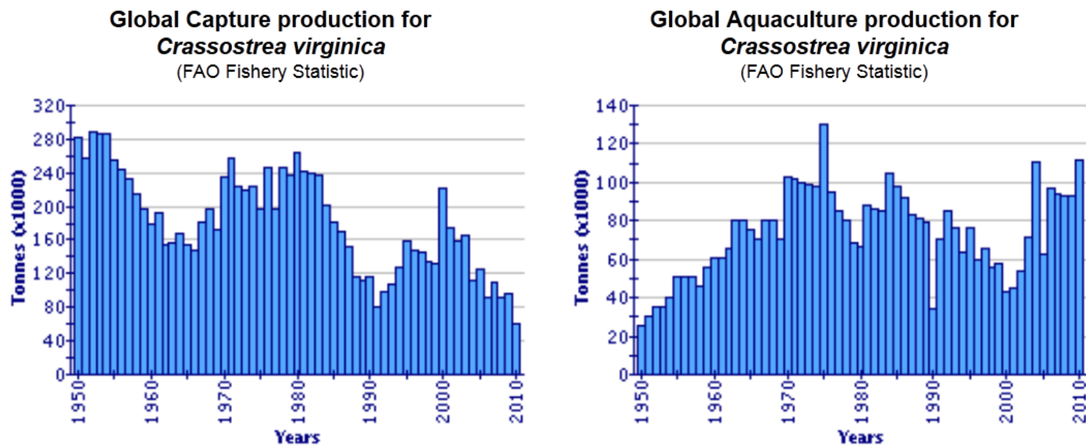


Figure 3. Global capture (left) and aquaculture (right) production for *C. virginica* [3].

1.2 Perkinsus marinus

Perkinsus marinus infection is one of the most common diseases in *C. virginica*, and has accounted for serious, wide-spread, periodic oyster mortalities on the Atlantic and Gulf of Mexico coasts of the U.S. (e.g. [16-20]), however it is not known to be harmful to humans. Prevalence of *P. marinus* infections in *C. virginica* can in some areas be as high as 100%, and can have a marked effect on the commercial harvest of *C. virginica*, both wild and cultured. A survey of 49 oyster-growing areas from Florida to Texas found only one site with a prevalence of *P. marinus* less than 50 % [21]. Intensive *P. marinus* infections have been associated with massive mortalities in oyster populations during summer and fall [17, 19].

P. marinus, is a single-celled protozoan parasite which causes a disease in *C. virginica* commonly called Dermo disease. The disease is called Dermo, as the organism initially was classified as a fungus, *Dermocystidium marinum* [22]. *P. marinus* was classified for a long time as a member of the phylum Apicomplexa [23], however recent phylogenetic studies have shown that *P. marinus* is more closely related to Dinoflagellates than Apicomplexans [24-26], and has recently been classified as a member of the phylum Perkinsozoa, branching close to the node shared by Dinoflagellates and Apicomplexans [27]. The complete, current, taxonomic classification is listed in Table 1.

Table 1: Taxonomic Classification of P. marinus (www.ncbi.nlm.nih.gov, retrieved 29.04.13).

Taxonomic Classifications	
Domain	Eukaryota
Kingdom	Chromalveolata
Superphylum	Alveolata
Phylum	Perkinsozoa
Class	Perkinsea
Order	Perkinsida
Family	Perkinsidae
Genus	*Perkinsus*
Species	*Perkinsus marinus*

P. marinus is found in bivalves along the East coast of the US from Maine to Florida and in the Gulf of Mexico to the Yucatan Peninsula [28, 29]. Recently it has also been reported as far south as Paraíba, Brazil, and on the pacific coast of Mexico [30, 31].

P. marinus infection is highly contagious and is transmitted directly from oyster to oyster [17]. A study in Chesapeake Bay revealed that *P. marinus* almost exclusively infects *C. virginica* in a benthic community where this oyster lived in close proximity to six other clam species [32]. However, recent studies identified the pathogen by molecular diagnostics in free growing *Crassostrea rhizophorae* (Mangrove cupped oyster), *Saccostrea palmula* (Mangrove oyster), *Crassostrea gigas* (Pasific oyster), and *Crassostrea cortenziensis* (Cortez oyster) [30, 31, 33, 34]. Laboratory experiments have shown that *P. marinus* infection is also possible in *Crassostrea ariakensis* (Suminoe oyster), *Mya arenaria* (Softshell clam), and *Macoma balthica* (Baltic macoma clam) [35, 36]. Common for these infected species is that *P. marinus* infection intensity is usually lighter than what it is in *C. virginica*, in other words, *C. virginica* appears to be the most susceptible species.

1.2.1 Life Cycle of *P. marinus*

P. marinus has three main life stages; zoospore, trophozoite and hypnospore [37]. Although not observed, it is believed that biflagellated zoospores move around freely in water until they are ingested by the host, and then transforms to trophozoites (2-12 µm) [38]. In the infected host tissue and within the hosts' hemocytes, trophozoites grow and develop a large vacuole and a displaced nucleus, giving the cell an appearance of a signet ring. Mature trophozoites proliferate by undergoing successive bipartitioning (cycle of karyokinesis followed by cytokinesis) and one cell can yield from 8 to 32 daughter cells (often 8 to 16), which are released by rupture of the mature trophozoite [38]. These

daughter cells may continue to grow and infect the remaining tissues of the host, or they may be released into the water again (see section 1.2.2).

During unfavorable conditions (e.g. when the host has died), trophozoites transforms into hypnospores (sometimes called enlarged trophozoites), which are enlarged and thick walled cells (20-80 µm) [38, 39]. When conditions improve, hypnospores begin zoosporulation [40]. Hundreds of zoospores can form within the original cell wall of the hypnospore. Biflagellated zoospores leave the hypnospore, which is also called a zoosporangium at this stage, through a single (occasionally two) discharge tube, which appears on each hypnospores before any cell division has occurred (Figure 4 shows a summary of *P. marinus* lifecycle) [38]. The speed at which zoosporulation occurs varies with temperature. It takes 2 to 4 days to develop at 28 °C and 2 to 12 days at 20 °C [40].

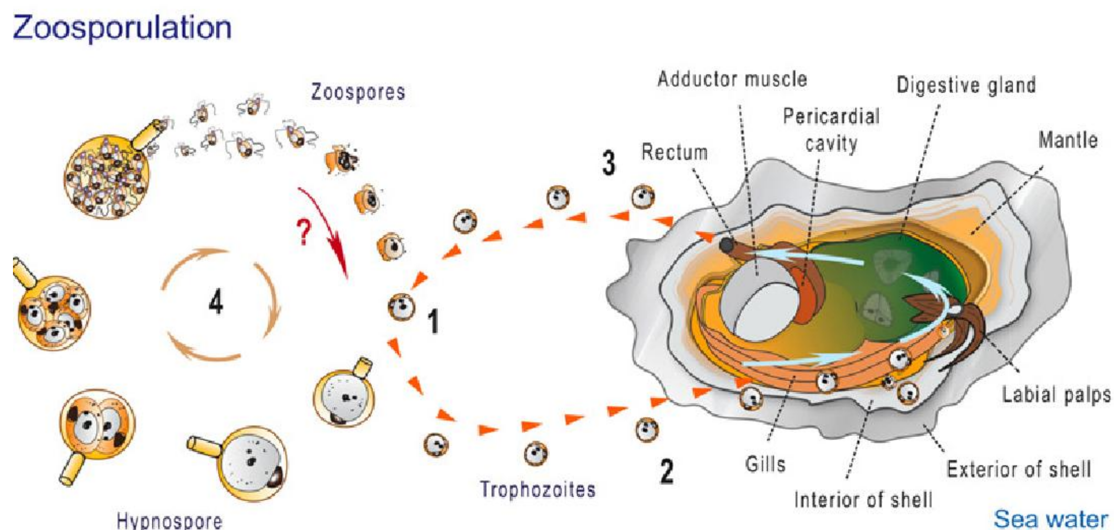


Figure 4. *P. marinus* life cycle. (1) Trophozoites and zoospores in the water column (2) enters the oyster during filter-feeding, where they are directed toward the gills and mouth. (3) Some cells may be released into the water again with the pseudofeces, feces or from the decaying tissue of an oyster (see section 1.2.2). (4) Trophozoites enlarge and form hypnospores, which develop a discharge tube and after successive bipartitioning, hundreds of zoospores are released into the water column. Figure taken from Fernández Robledo *et al.* (2011) [41].

1.2.2 Transmission of *P. marinus*

All three life stages of *P. marinus* have been shown experimentally to cause infections in oysters [37, 42-44], where trophozoites are the most effective infection agent in laboratory experiments [45, 46]. However, it is not known which stage is the most effective in the natural environment. Motile biflagellated zoospores are presumably the primary life-stage involved in water-borne transmission

but naturally occurring zoospores, nor trophozoites, have not yet been observed in the water column [40].

The main transmission route of *P. marinus* is through death of heavily infected oysters and disintegration of tissue, which release high cell concentrations into the aquatic environment where other oysters are filtering water [16-18, 47]. Maximum transmission rates of *P. marinus* have been observed during periods of maximum *P.marinus*-associated host mortality, typically in late summer, however transmission can also occur when host mortality is low or absent [18].

A minor route is release of infective cells through diapedesis or from feces and pseudofeces of live (moderately to heavily) infected oysters which make infected oysters a continuous source of infective cells [47-49]. High density oyster bars can hasten the transmission and development of the disease [50].

Laboratory experiments have shown that it is possible to have intermediate vectors in transmission of the parasite, e.g. *Boonea impressa* (ectoparasitic snail) [51, 52]. An intermediate vector, however, it is not required for *P. marinus* transmission [17].

1.2.3 Infection mechanism

Infection of oysters with *P. marinus* mostly happens during the feeding process. Invasion through the gut epithelium has been considered as the primary portal of entry [53]. However, recent experiments have shown that infection more commonly invades gills, mantle and labial palps before reaching the mouth and digestive system [43, 48].

Laboratory experiments have shown the importance of infective cell density in transmission [45]. It has been found that an infective dose of 10-100 cells are required for infection by shell cavity injection [45]. This number may be higher in the natural environment in order to initiate infection in oysters, since some cells are released as feces and pseudofeces [45]. Laboratory experiments have also shown that the infection intensity is higher in oysters exposed to *P. marinus* incorporated in aggregates than in freely suspended cells [48]. Long distances between infected and healthy oysters and continuous flowing fresh water can effectively dilute infective cell density and protect healthy oysters from infection.

Currently the molecular and cellular mechanism of the interaction between *P. marinus* and oysters are not fully understood [54, 55]. The severity of *P. marinus* infections depends on the host’s immune system’s ability to overcome the parasite’s evasion mechanisms [37]. *P. marinus* infect oysters by penetrating tissues of the oyster and efficiently evade the humoral and cellular immune defenses of the

host [55, 56]. Humoral components include lysosomal enzymes, lectins and antimicrobial peptides that aid in the recognition of pathogens and parasites by marking them for destruction via opsonizing or direct killing. Cellular components include hemocytes, which play a central role in immune response of the oyster. Hemocytes are responsible for the respiratory burst, apoptosis and most importantly phagocytosis, where parasitic cells are encapsuled by hemocytes [55-58]. Some *P. marinus* cells appear to be destroyed within the phagocytes [57], but others continue to develop within the host cells and eventually destroy them [38, 53, 55, 59].

P. marinus secrete extracellular products (e.g. proteases) which can suppress host immune defense and facilitate internalization of the parasite [49, 54, 59-63], making the host more susceptible to secondary infections [64].

1.2.4 Effect of *P. marinus* on Oysters

The effects of *P. marinus* infection in oysters depend on the infection intensity, and the general health condition of the oyster. Light *P. marinus* infections have little measurable impact on the host, but with a heavy infection they usually exhibit a reduction in feeding rate [17], reduced shell and tissue growth rate [65-67], reproduction capacity [68-70], and a reduced condition index [45, 65, 71].

Heavy infections are characterized by massive hemocytic infiltration of epithelia, connective tissue, muscle fascicles and blood spaces, with parasite cells occurring both inside hemocytes and free. The parasite load in the haemolymph can exceed several hundred thousand per mL [59]. Oyster death occurs only when infection intensity has become extensive, which is usually 1 to 2 years after infection [20], however the disease can become lethal within a few weeks of infection [17]. The parasite proliferation causes oyster tissue degradation (due to extracellular products excreted by *P. marinus*) and occlusion of major hemolymph vessels [17, 53, 57], which will likely result in organ dysfunction [37]. During *P. marinus* epizootics, 100 % of the adult oysters on a bed are likely to be infected and up to 90 % may die from those infections [19, 20].

Oysters undergoing spawning, exposed to environmental stress, predation or contaminants are more likely to get infected and have an increased risk for mortality due to synergistic effects [43, 72-76]. Weakened condition of oysters post-spawning may also facilitate disease progression [37]. A possible synergistic effect between *Haplosporidium nelsoni* infections and *P. marinus* infections in oysters have also been reported [18, 68].

1.2.5 *P. marinus* Infection Intensity in Oysters of Different Sizes

Mortality in oysters due to *P. marinus* infections is in general size dependent. Juvenile oysters (less than 6-11 months, depending on growth area) generally show lower prevalence and intensity of disease [53, 77]. These factors increase in the second year, and the epizootiological pattern of disease development follows temperature and salinity trends (see section 1.2.6) [16, 53, 78, 79]. Higher filtration rates and longer exposure to infective cells could explain why *P. marinus* prevalence is higher in adult than in juvenile oysters living in the same area [45].

Populations of juvenile oysters in enzootic areas can acquire disease prevalence and intensity that exceed those of the adult population due to an increased mortality among larger oysters, especially during summer months [17].

Due to the rapid growth rate of oysters from the Gulf of Mexico, compared to oysters from northern estuaries, they are to some extent able to "outgrow" the parasite and reach harvestable size ($\geq$ 75 mm) before the infection becomes lethal. Oysters from Apalachicola typically reach harvestable size between 12 and 18 months [4, 80, 81].

1.2.6 Environmental Factors Affecting *P. marinus* Prevalence and Intensity in Oysters

Many factors can dictate disease prevalence and intensity of *P. marinus* infections in *C. virginica* and other bivalves. These include temperature, salinity, water quality, density of oysters, patterns of water movement, oyster age/size, genetic strains, physiological condition, food availability and numbers and levels of other parasitic species causing stress on the oysters [72, 79].

Temperature and salinity are considered the main environmental factors that influence the *P. marinus* disease dynamics in oyster populations. These two environmental factors impact the disease progression/regression by modulation the host immune system [82-84], as well as parasite activity [85, 86]. Studies also suggest that the oyster have a higher immune defense capacity during the winter when the water temperature is low [82, 84].

Several studies have shown that *P. marinus* is most prevalent in oysters exposed to conditions of high temperature and salinity for longer periods of time (e.g. [16, 45, 85-87]). It is unclear which factor is most dominant. Authors have indicated that salinity is the most important factor influencing the disease susceptibility of the oyster, while temperature affects the distribution and prevalence in a more large-scale geographic area [37, 45, 88]. Both temperature and salinity varies with season and water depth within estuaries.

The prevalence and infection intensity of *P. marinus* in *C. virginica* have a seasonal cycle, which is mainly governed by temperature. Prevalence and infection intensity are at its minimum during the winter and early spring and at its maximum in late summer and early fall [18, 20, 28]. This is most noticeable in the northern areas of the USA, since there is a clear difference between summer and winter temperatures [16, 20]. In a northern estuary, Chesapeake Bay, Virginia, maximum prevalence and infection intensity was recorded one to two months after maximum summer water temperature, and minimum prevalence and infection intensity after minimum winter water temperatures [20]. A potential lag period for prevalence and infection intensity in the Gulf of Mexico has so far not been reported. In the southern part of USA and in the Gulf of Mexico, high infection intensity and mortality periods are not as discrete as in the northern areas, most likely because temperature is never low enough to suppress activity of either host or parasite [78, 89].

C. virginica has a wide temperature (1 to 36 °C, with optimum at 25 to 26 °C [88, 90]) and salinity (0 to 42 ppt, with optimum at 14 to 28 ppt [88]) tolerance. Optimal temperature and salinity for *P. marinus* growth and proliferation are 28 to 32 °C and 24 to 36 ppt [91-93]. Although *P. marinus* does not have an equally wide temperature and salinity tolerance as the oyster, it can tolerate temperatures as low as 4 °C and salinities as low as 4 ppt for a short period of time [40]. However zoosporulation does not happen at temperatures lower than 18 °C, but can happen at salinity as low as 6 ppt at 28 °C [40]. At 0 to 1 ppt infection intensities are lowered in the oyster tissue, but the parasite is not completely eliminated [87]. The larges decrease in cell viability occurs during combined low temperature and salinity conditions, indicating that there is synergistic effect [94].

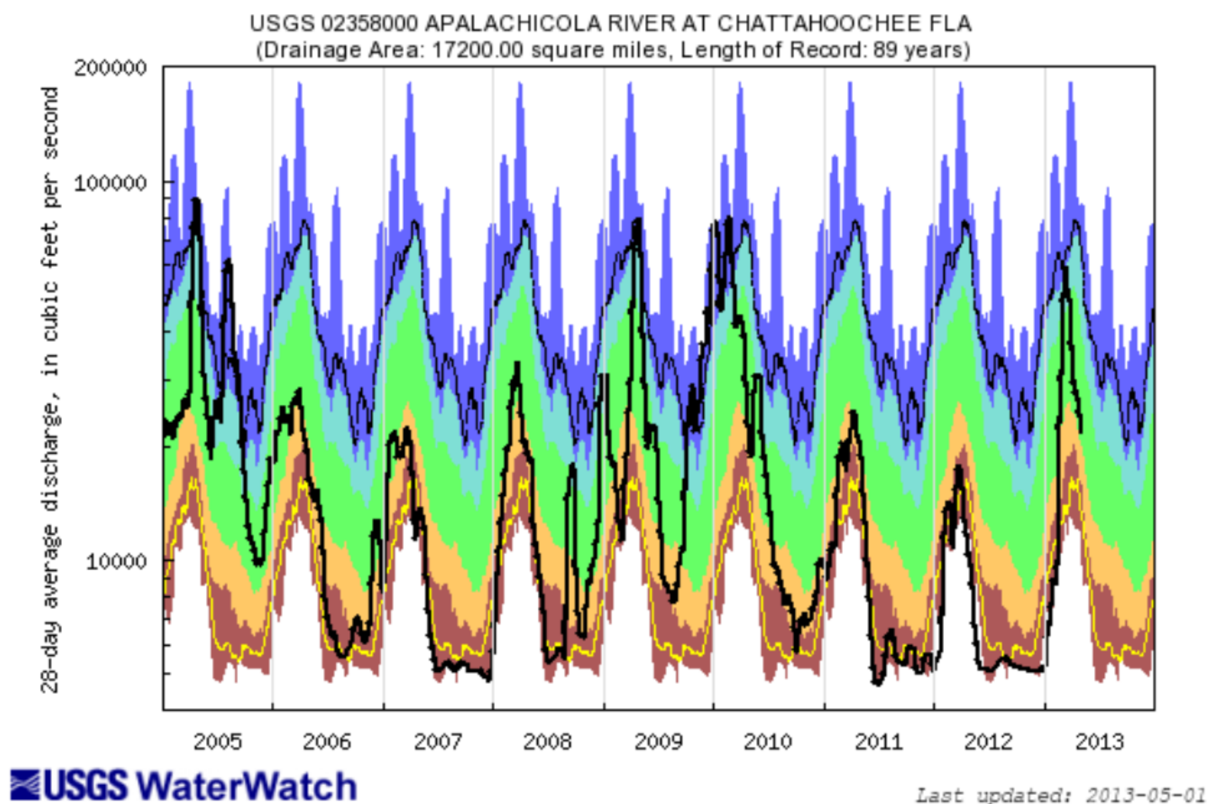
P. marinus in oyster tissue does also have an advantage that it is protected from acute environmental stress (e.g. sudden salinity decrease during heavy rainfall events), since bivalves close their valves tightly to avoid the stress [88]. This may explain the persistence of this parasite for relatively long periods in oysters in low salinity areas [19].

The salinity regime in Apalachicola Bay is governed by the influx of freshwater from the Apalachicola River [5, 78]. Salinity in Apalachicola Bay typically varies from 3 ppt to 33 ppt depending on the season and rainfall conditions [5]. Not only does the freshwater influx bring nutrients for the ecosystem in the bay, but it probably also protect the oysters by diluting planktonic levels of *P. marinus* cells [72, 87].

From 2010 to the present (May 2013) there has been a drought going on in the Apalachicola-Chattahoochee-Flint watershed. This drought has affected the water flow of Apalachicola River draining into Apalachicola Bay (Figure 5). Although the figure shows that the water flow is considered normal in the first few months of 2013, the flow is rapidly declining and the drought could

still continue this summer. It should also be mentioned that the figure shows a drought period in 2006 to the summer of 2008, since some of the data presented in section 1.3 are collected in that period.

With lower freshwater flow into the bay, the salinity increases. High salinity makes it easier for the parasite to establish higher up in the estuary, and can result in increased oyster mortality [95, 96].



Explanation - Percentile classes

lowest-10th percentile	5	10-24	25-75	76-90	95	90th percentile-highest	Flow
Much below Normal		Below normal	Normal	Above normal	Much above normal		

Figure 5. 28-day average discharge in Apalachicola River from January 2005 to April 2013, relative to historical flow in the past 89 years. Figure was created using the USGS Streamflow Duration Hydrograph Builder (http://waterwatch.usgs.gov/index.php) for USGS station 02358000, 1 km below Woodruff Dam. Flow line indicates drought periods in 2006-2008, and in 2010-2012.

1.3 Overview of Historical Data on *P. marinus* Infections in Apalachicola Bay

Before reading this section it is important to know that *P. marinus* infections usually are ranked based on Mackin ranks (Table 2, section 1.4.1), and are mainly described with three terms; prevalence (percent infected oysters), mean infection intensity and weighted prevalence (relative severity of infection in a population). Calculations of these terms are presented in Methods (section 3.5). However, some prefer to use median infection intensity, instead of mean infection intensity, this is likely due to high variability of infection intensity between oyster samples.

A few studies on *P. marinus* prevalence and infection intensity have been done in Apalachicola Bay. Craig *et al*. (1989) reported 63 and 92 % prevalence and a median infection intensity of 0.33 (based on Mackin rank) at Dry Bar and Cat Point, respectively, in January 1986 (n = 20 per site) [21]. Oliver *et al.* (1998) reported 100 % prevalence each month between October 1993 and July 1994 at Cat Point, where minimum average intensity was observed in March and maximum in July. Infection intensity were presented as whole body burden and ranged from Log_{10} 2.15 to 5.52 cells, and a mean of 4.19 cells (n = 20)[28]. It is possible to calculate the Mackin rank of a body burden, using the equation found in Choi *et al.* (1989; Equation 1) [97]. However, it was not possible for me to calculate it for Oliver *et al*.'s (1998) results, due to the lack of information of tissue weight of the oyster samples. Compared to the other places investigated in that article (New York and Virginia), the mean result from Apalachicola Bay were highest.

Equation 1. $Number\ of\ cells = 1409.9\ (10^{0.64296 * Mackin\ rank\ of\ sample}) * g\ wet\ tissue\ weight$

Only two sources are available when it comes to relatively extensive surveys on weighted prevalence of *P. marinus* infections in Apalachicola Bay; Oyster Sentinel, a web-based community created by Ray and Soniat [89], and an article by Petes *et al.* (2012) [78]. Both sources sampled several locations in the bay; some of these locations overlap in the two studies, and the present study (this project). Sampling locations reported by Oyster Sentinel and Petes *et al*. are shown in Figure 6; sampling locations for this project are shown in figures 10 and 11 in Methods (section 3.1).

In this project, samples were collected during the winter (November 2012 and February 2013). In order to make temporal comparisons between this present study and historical studies, annual comparisons were made using 6-month Fall-Winter data sets (September through February). Although September and October are not winter months, and *P. marinus* infection intensities can vary seasonally [20], such a comparison is still useful to look for possible gross increasing/decreasing trend in *P. marinus* infections in Apalachicola Bay.

Some comments on the historical data sets:

- Petes *et al*. collected data once a month from November 2007 to December 2008, except from January 2008 at Cat Point and Dry Bar (n = 28-30, with exception of n = 18 at Dry Bar and n = 20 at Cat Point in November 2007; Figure 7), and Oyster Sentinel collected data one to three times a year from November 2005 to September 2012 at St. Vincent Bar, the Jetties and Porters Bar (n =10; Figure 8). Notice that Figure 7 and 8 show all data available from these sources. Only data collected from September to February will be used later on in this report.

- Petes *et al*. used a modified infection intensity ranking system based on Ray (1954) [77], and it is worth noticing that her ranks are a little higher compared to Mackin ranks. Her data had to be converted to the Mackin ranking system before comparisons could be made. A linear trend line between Petes *et al*. rank and Mackin rank was created and a conversion factor was established (Appendix A.4, Figure 25). Also, since her data is presented with a graph, one should be aware of that my readings of her data might not be a 100% accurate.

- Oyster Sentinel has three stations, St. Vincent Bar, the Jetties and Porters Bar. In this report, St. Vincent has been renamed Dry Bar, as this area is known by locals to be Dry Bar, and Petes *et al*. also use this name for that area. Porters Bar have not been included in this report, since it is located too far East from our sampling locations, and oysters sampled there could be different from those sampled at our locations.

Figure 6. Oyster Sentinel and Petes *et al*. sampling locations in Apalachicola Bay.

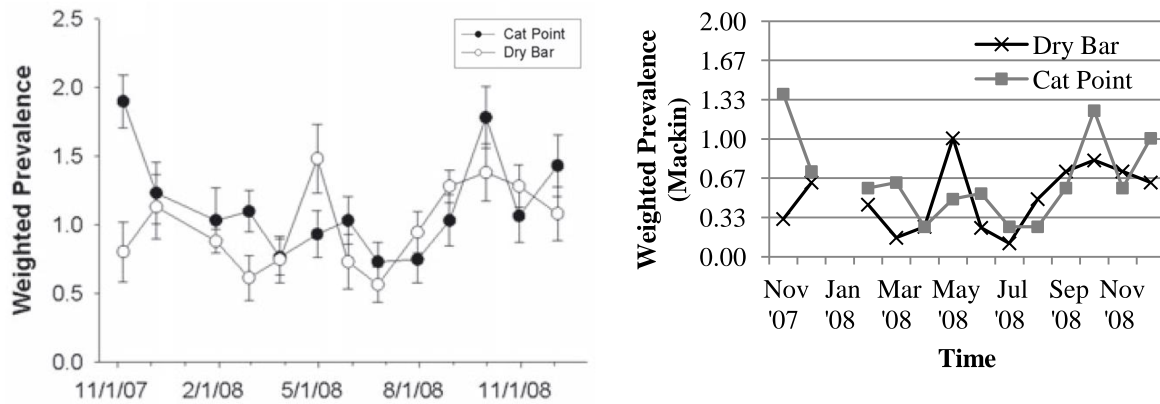


Figure 7. Left: Reported weighted prevalence ± SE of *P. marinus* infections in oysters at Dry Bar and Cat Point by Petes *et al.* (2012). Infections were ranked using a modified ranking system originally described by Ray (1954). Figure taken from Petes *et al.* (2012) [78]. Right: Petes *et al*. ranks converted to Mackin ranks.

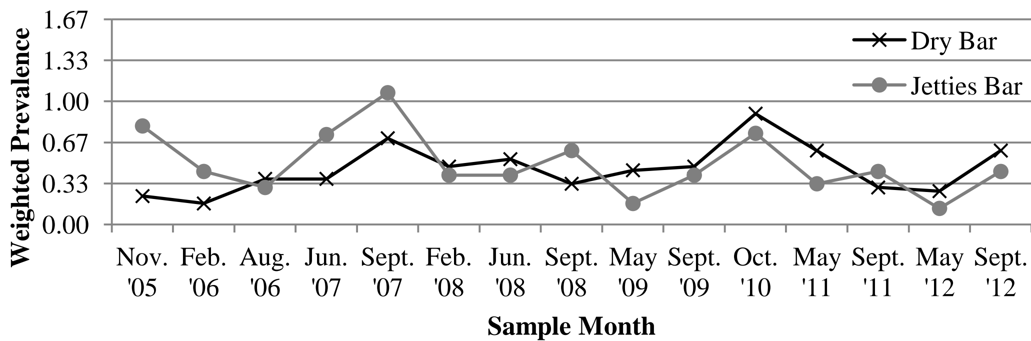


Figure 8. Reported weighted prevalence of *P. marinus* infections in oysters at Dry Bar and Jetties from November 2005 to September 2012, by Oyster Sentinel (SE was not available). Data downloaded from www.oystersentinel.org (retrieved 14.01.13). Notice a higher weighted prevalence in 2007 and 2010. Could be a result of the drought mentioned in section 1.2.6 (Figure 5). Also, X-axis does not show time linearly, only the specific months sampled.

1.4 Methods of *Perkinsus marinus* Detection and Monitoring

The standard method used for identification and enumeration of *P. marinus* in the Eastern oyster is the Ray's fluid thioglycollate medium (RFTM) tissue assay developed Ray (1966) [98]. Modifications of this assay exist for the examination of oyster hemolymph and total body burden of oysters [99]. The tissue assay is more accurate and sensitive than the hemolymph method and less time consuming and expensive than the total body burden method [28, 100]. The RFTM assay is not species specific and will diagnose most *Perkinsus spp.* and Perkinsus-like protozoans (e.g. it does not detect *Perkinsus*

qugwadi) [17, 100, 101]. Care should be taken in areas where more than one species of *Perkinsus* is known to cause infections. Species specific PCR assays have been developed, however until recently it was not practical for quantitative routine diagnosis, but was an important aid in quality control and detection of false negative samples [32, 102-104]. In this study the RFTM tissue assay was used to detect *P. marinus*, and is described below.

1.4.1 RFTM Oyster Tissue Assay

The RFTM tissue assay involves incubation of oyster tissue samples (typically gills, mantle, and/or intestine tissue) in RFTM for 4-7 days in dark, anaerobic conditions at 20-25 °C. This allows the parasite cells (trophozoites) to enlarge and form thick-walled hypnospores with minimal proliferation [17, 98]. Samples are then stained with Lugol's iodine solution and examined microscopically.

The infection intensity is ranked on a semi-quantitative scale from negative (N) to heavy (H), commonly called the Mackin scale (Table 2, examples of infected tissue in Figure 9), which was modified by Craig *et al*. (1989) [21]. The Mackin scale provides a semi-quantitative measure of intensity based on parasite density; parasites are counted in light infections only. Ranks are assigned to heavier infections based on the percentage of tissue occupied by *P. marinus* cells. The number designation in the 1st column (Aquatic Pathobiology Laboratories (APL) Rank) in Table 2 was invented for this project, in order to make it easier to rank and discuss the results. By calculating a mean rank across all oysters tested, a weighted prevalence, indicative of the overall level of parasitism in the population, is obtained. A quantitative parasite estimate can be derived from the Mackin rank using Equation 1.

Diagnosis based on RFTM method makes several assumptions. It is assumed that all life stages of *P. marinus* found in the oyster sample are retrieved and that the number of parasites remains constant during incubation. Furthermore, it is assumed that the distribution of *P. marinus* in the assayed tissues is representative of the distribution of the parasite throughout the oyster. In an evaluation report by Bushek *et al.* (1994) they found that the tissue assay can produce false negatives at low infection levels (at <1000 cells/g wet tissue), and that the parasite cells could be unevenly distributed in the tissue [100]. However, they concluded that the tissue assay provides a reasonable estimate of average infection level in a population. The tissue assay was recommended for monitoring epizootics because of its simplicity and accuracy at the population level.

Table 2: Semi-quantitative scale of infection intensity of Perkinsus marinus. Adapted from Mackin (1962) by Craig et al. (1989) [21], with an additional APL ranking designation included.

APL Rank	Mackin Rank	Mackin Letter Designation	Mackin Severity Description	Observation Description
0	0.00	N	Negative	No hypnospore present
0+	0.33	VL	Very light	1-10 hypnospores
1-	0.67	L-		11-74 hypnospores
1	1.00	L	Light	75-125 hypnospores
1+	1.33	L+		>125 hypnospores but much less than 25% of tissue is hypnospores
2-	1.67	LM-		<25% of tissue is hypnospores
2	2.00	LM	Light/moderate	25% of tissue is hypnospores
2+	2.33	LM+		>25% but much less than 50% of tissue is hypnospores
3-	2.67	M-		>25% but <50% of tissue is hypnospores
3	3.00	M	Moderate	50% of tissue is hypnospores
3+	3.33	M+		>50% but much less than 75% of tissue is hypnospores
4-	3.67	MH-		>50% but <75% of tissue is hypnospores
4	4.00	MH	Moderately heavy	75% of tissue is hypnospores
4+	4.33	MH+		>75% but much less than 100% of tissue is hypnospores
5-	4.67	H-		>75% of tissue is hypnospores but some oyster tissue is still visible
5	5.00	H	Heavy	Nearly 100% of tissue is hypnospores

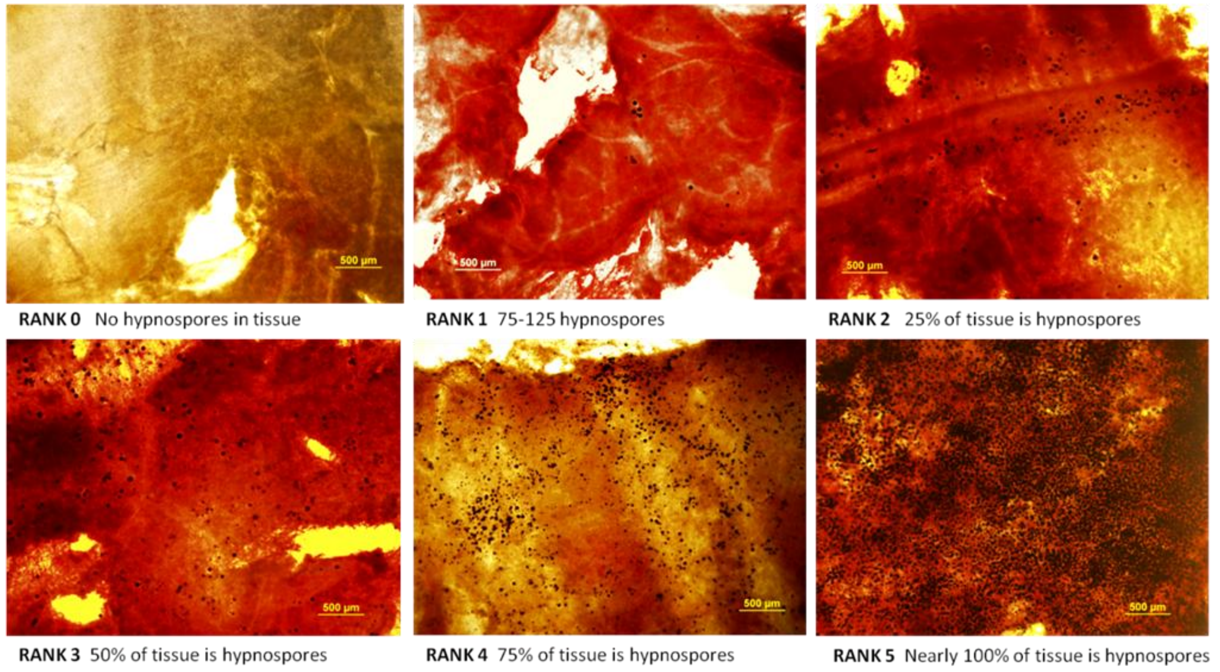


Figure 9. Examples of infected oyster tissue samples analyzed in this project, ranked from 0 to 5 at 4x magnification.

2 Aims of Thesis

The overall objective of this thesis is to investigate the prevalence and infection intensity of *P. marinus* infections in *C. virginica* harvested from Apalachicola Bay in November 2012 and February 2013. Analysis of *P. marinus* weighted prevalence is important in the determining the health of the Apalachicola Bay oyster population, its potential contribution to the ongoing oyster fishery collapse, and potential variability of infection prevalence based on spatial distribution throughout the bay, within and between oyster bars, and differences in water quality.

As such, the following specific aims and hypotheses have been developed:

Specific Aim 1: Determine the weighted prevalence of *P. marinus* infections in oysters from multiple oyster bars in Apalachicola Bay

Ho_1: Variability of *P. marinus* infection in oysters is similar between replicate samples within the same oyster bar during the same season (i.e., within-oyster bar variability is negligible).

Ho_2: Variability of *P. marinus* infection in oysters is similar between oyster bars in different locations in Apalachicola Bay during the same season (i.e., between-oyster bar variability is negligible).

Ho_3: Variability of *P. marinus* infection in oysters is similar between oyster specimens sampled in November 2012 and those sampled in February 2013 (i.e., short temporal and potential water quality differences have negligible effect on *P. marinus* infection).

Specific Aim 2: Determine the weighted prevalence of *P. marinus* infections in oyster of different sizes in order to discern if age is a determining factor in *P. marinus* infection (as described in the literature).

Ho_1: *P. marinus* infection in juvenile oysters is similar to infection in adult oysters. We will test this hypothesis by examining weighted prevalence of infection in oysters <70 mm and ≥70 mm height.

Ho_2: *P. marinus* infection in oyster is not associated with oyster height (all samples pooled together).

Specific Aim 3: Examine the association between oyster health condition and *P. marinus* infection intensity. Stressed oysters may be more susceptible to *P. marinus* infections. It is expected to find a relationship between infection intensity of *P. marinus* and oyster health conditions, such as shell parasite indices and meat condition index.

Ho_1: *P. marinus* infection in oysters is associated with oyster meat condition index.

Ho_2: *P. marinus* infection in oysters is associated with shell condition indices and/or shell parasite loading.

This thesis also compares my results with historic data from Oyster Sentinel Project and Petes *et al.* (2012), and provides my evaluation of the RFTM tissue assay.

3 Methods

This section provides information on oyster sampling in Apalachicola Bay, and processing in the laboratory afterwards (oyster health rankings and RFTM tissue assay).

3.1 Oyster Sampling Locations and Water Quality

Oysters were collected at four oyster bars in Apalachicola Bay. These locations include Eastpoint Channel, Cat Point, the Jetties and St. Vincent Sound. Oysters were harvested from these locations on November 5th 2012 (Figure 10) and February 16th 2013 (Figure 11, GPS locations for each site are provided Table A1 in Appendix A.1).

Oysters were harvested by using hand tongs (long scissor-like tool with metal rakes on the ends) from small motor boats. A minimum of 10 oysters were collected from each non-adjacent replicate sampling site on each oyster bar. Two to four replicates were sampled for each oyster bar at each sampling time point.

At each replicate sampling site water temperature and salinity were measured just above the bottom using a Hydrolab sonde Model Quanta G (Hach Hydromet, Austin (TX)). Oysters were transported live, and kept cool until processing at the Aquatic Pathobiology Laboratory at the University of Florida within 48 hours.

Figure 10. Map of sampling locations in Apalachicola Bay on November 5th 2012. Parenthesis indicates individual site number, where two to three non-adjacent sample sets were harvested.

Figure 11. Map of sampling sites in Apalachicola Bay on February 16th 2013. Parenthesis indicates individual site number, where two to four non-adjacent sample sets were harvested.

3.2 Ranking of Oyster Health Condition Indices

The height (dorsal to ventral maximal length, see Figure 12) of each oyster was measured using manual calipers before they were shucked open, and the adductor muscle was carefully cut at its connection with the upper shell. External and internal condition ranks were assessed and recorded for each oyster, in order to evaluate the overall health of the oysters and to examine possible relationship with *P. marinus* infections. External ranking were done for boring sponge (*Cliona spp.*) and boring clam (*Diplothyra smithii*) holes on the outside of the shell. Internal rankings were done for yellowing, white chalky deposits, dark clam (*Diplothyra smithii*) spots, *Polydora* worm tubes, mud blisters, and boring sponge (*Cliona spp.*) spots on the inside of the shell. The overall appearance of the oyster soft tissue (meat condition index) was also recorded. These rankings were done by Ross Brooks and Dr. Kane at the Aquatic Pathobiology Laboratory.

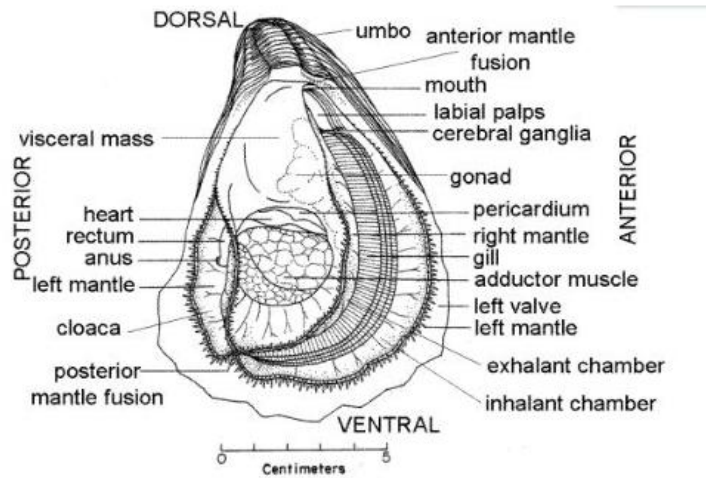


Figure 12. General anatomy of an oyster viewed from the right side with the right valve removed. Original figure from Galtsoff (1964) [88], as presented in VanderKooy (2012) [105].

3.2.1 External Rankings of Oyster Shells

Boring sponges (*Clinoa* spp.), which creates small holes on the oyster shell, were ranked from 0 (no holes) to 5 (heavily attacked). Boring clams (*Diplothyra smithii*), which creates larger holes on the oyster shell, were counted (Figure 13).

3.2.2 Internal Rankings of Oyster Shells and Meat Condition Index

Yellowing, white chalky deposits, sponge spots was rank from 0 (no imperfections) to 5 (heavily affected). Mud blisters was ranked from 0 (no mud blisters) to 3(several mud blisters). Number and size of imperfections were included together in determining the rank for each of these factors. Dark clam spots and *Polydora* worm tubes were counted (Figure 13A and 14). Yellowing of the nacreous layer in the shell and white chalky deposits are deposited by the mantle under stressful conditions. Mud blisters are formed when *Polydora* worms penetrate the nacreous layer of the shell and forms tubes within the shell. The oyster expends energy to secrete more nacre to wall-off the invader [9].

Meat condition index was ranked from 5 (perfect) to 1 (bad looking, Figure 15), based on observation of the oyster meat. Perfect meat is considered plump, not watery or translucent, with a uniform tan-creamy appearance and fills out the shell completely.

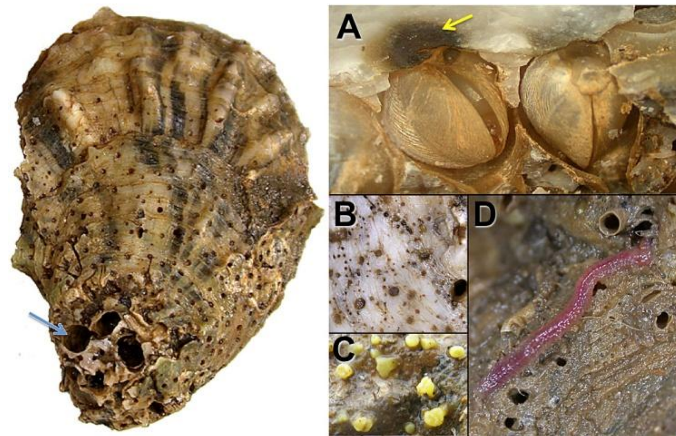


Figure 13. Shell parasites found on oyster shells in Apalachicola Bay. Left photo: Whole oyster shell with parasitic damage from boring clams (blue arrow) and boring sponges (numerous small holes). Right photo panel: (A) Two boring clams seen at the edge of a shell that was fractured to reveal the parasites. Note the black spot (yellow arrow) associated with the clam's activity on the inner nacreous layer of the shell. 8B) Close up of exterior shell holes bored by *Cliona* sponge. In life, this sponge organism is yellow and protrudes from the shell holes as shown in panel C. (D) *Polydora* worm. Photo credit: A. Kane.

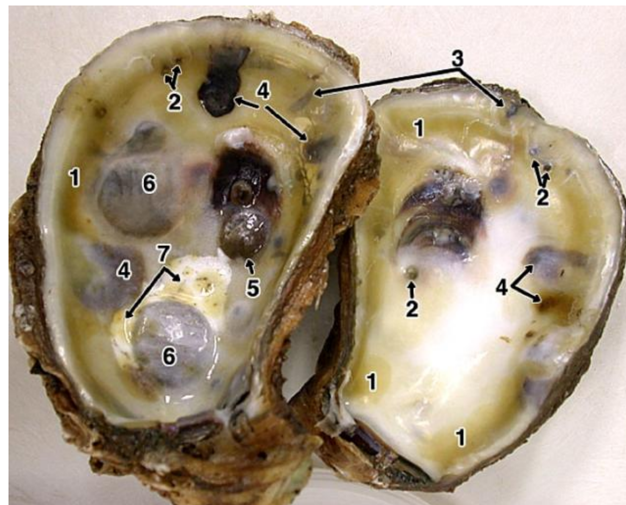


Figure 14. Internal shell observations associated with parasites from Apalachicola Bay Oysters. Observations are described by numbers: (1) Yellowing. (2) Black *Diplothyra* clam spots. (3) Burrowing tubes at periphery of shell. These are points of access of boring *Polydora* worms. (4) Enlarged borrows of *Polydora* worm holes within shell. (5) Mud blisters. (6) Long-standing mud blisters with thicker layer of nacre walling off the worm. (7) White chalky deposits. Photo credit: A. Kane.

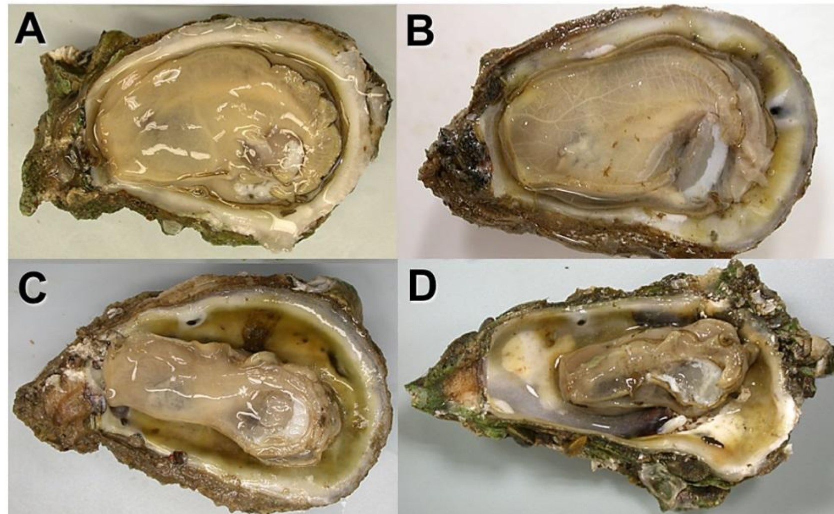


Figure 15. Examples of meat condition indices from Apalachicola Bay Oysters. Meat rank of oyster in A) 4.5, B) 3.5, C) 2.0 and D) 1.0. Photo credit: A. Kane.

3.3 Tissue Collection

Table A2 and A3 in appendix A.2 lists chemicals and equipment needed to perform the tissue collection and analysis. Recipes for the different solutions are provided in appendix A.3.

Sterile dissecting scissors and forceps were used to cut a 5 x 5 mm piece of mantle-edge tissue from just above the labial palps (Figure 12). If the sample size was slightly larger, e. g. 7 x 7 mm, it was normalized down to 5 x 5 mm during tissue analysis (section below).

The tissue was placed in a culture tube containing 5 mL of sterile RFTM to which 0.5 mL Penicillin-Streptomycin solution had been added. Culture tubes were placed in the dark at room temperature and incubated for four to seven days. If the tissue had not been analyzed by the end of day seven, the tubes were placed in the refrigerator in the dark where they could be kept for up to three months without deterioration.

3.4 Tissue Analysis

After incubation, oyster mantle tissue was removed from the RFTM, using a sterile probe and placed on a microscope slide. The tissue sample was teased apart using sterile needle tips to assure even staining with Lugol's iodine solution. One to two drops of Lugol's iodine solution was added to the tissue with a syringe fitted with a 45 µm filter. The tissue was covered with a cover slip, and the sample was examined using light microscopy on an Olympus BX51microscope (Olympus America Inc., Center Valley, PA).

P. marinus hypnospores appeared as blue/black spheres 5-300 µm in diameter when viewed at 40-100x magnification. Infection intensity was assigned to each sample based on the number and coverage of *P. marinus* cells observed in the tissue using the modified Mackin scale (Table 2, photos of all ranks in Appendix A.6.1). Blind assays were conducted among two slide readers in order to maintain quality control.

I was involved in the oyster harvesting and tissue collection with Dr. Kane in February, while harvesting and tissue collection done in November was done by Dr. Kane and others in his team in the Aquatic Pathobiology Laboratories at the University of Florida. All microscopic tissue analysis of *P. marinus* prevalence and intensity was done by me, where Dr. Kane conducted the blind assays to provide verification of results. Microscopic analyses were done within three months after sampling in November (after samples were incubated at ambient temperature for five days, and then refrigerated) and seven days after sampling in February.

3.5 Calculations

Two observations were used to describe *P. marinus* distribution in oyster bar replicates: (1) infection intensity (mean rank of each data set), and (2) infection prevalence (presence/absence).Combined, these data permit calculation of the weighted prevalence (Equation 2-4). The weighted prevalence, or mean abundance as it is also called, gives a measure of the relative severity of *P. marinus* in a population.

Equation 2. $Mean\ infection\ intensity = \frac{Sum\ of\ infection\ rankings\ (Mackin\ value, Table\ 2)}{Number\ of\ oysters\ infected}$

Equation 3. $Prevalence = \frac{Number\ of\ infected\ oysters}{Total\ number\ of\ oysters\ analyzed}$

Equation 4. $Weighted\ prevalence = Mean\ infection\ intensity * Prevalence$

3.6 Statistical Analyses

To test for differences in infection intensity within sampling sites (oyster bar), and between sampling sites (different oyster bars) and sampling time points (November 2012 and February 2013), data were examined using one-way ANOVA and two-way ANOVA (p-level 0.05). One-way ANOVA when three or more data sets were compared (comparison within one oyster bar with three or more replicate samples and between different oyster bars at one time point), and two-way ANOVA when site and time points were included in the data analysis (comparison between different oyster bars at two time

points). In the event of a significant difference observed in an ANOVA, Student's t-test was used to discern differences between different groups, or when only two data sets were compared (comparison within one oyster bar with two replicate samples, comparison of adult and juvenile oysters).

Relationships between Mackin rank and internal and external oyster health ranks, and Mackin rank and oyster height were examined using linear regression analysis.

At the University of Florida statistical analyses were done using StatPlus:mac 5.8.0.0 (AnalystSoft Inc., Alexandria, VA) software, and at the University of Stavanger SPSS Statistics 20.0.0.1 (IBM Corp., Armonk, NY) software was used.

4 Results

The first section below shows temperature and salinity data collected on sampling days in Apalachicola Bay, and downloaded data starting two months before sampling. Following sections present *P. marinus* data collected in this project, and show comparisons of spatial distribution, adult versus juvenile and linear regression analysis of sample Mackin ranks and oyster health condition indices. The total number of oysters sampled for *P. marinus* infections was 240, with height ranging from 44 – 156 mm, and a mean of 86 mm. An overview of data collected in this project is provided in Table A4 in Appendix A.5. Finally, a comparison of *P. marinus* data collected in this project with historical data sets is presented.

4.1 Temperature and Salinity in Apalachicola Bay

Due to a possible lag period, it is important to analyze temperature and salinity data that are measured before the oyster are harvested. Measurements taken on the sampling day do not functionally account the relationship between *P. marinus* infection levels and temperature and salinity, but they can be used to quality check temperature and salinity data downloaded from National Estuarine Research Reserve System (NERRS, http://cdmo.baruch.sc.edu/).

Table 3 shows the mean measured water temperature and salinity of each site on the day that the oysters were harvested. There was unusually rough, windy weather on February 16th and salinity measurements at the top and bottom of the water column revealed that the water was well mixed that day. The data presented in Table 3 was used to verify the data presented in Figure 16 and 17.

Figure 16 and 17 illustrates the mean monthly temperature and salinity in the Eastern (Cat Point) and Western (Dry Bar) part of the bay from September 1st 2012 to February 28th 2013. Data were downloaded from NERRS (retrieved 21.03.13), where water quality was measured every 15 minutes at each station, and a mean was calculated for each month. Our measurements were similar to the NERRS data.

Table 3: Mean bottom temperature and salinity measurements at sampling locations in November and February at the time of each sampling. Data are mean ± SE.

	Water Temperature (°C)		Salinity (ppt)	
Oyster Bar sites	**November 2012**	**February 2013**	**November 2012**	**February 2013**
Cat Point	20.3 ± 0.3	15.7 ± 0.04	25.0 ± 0.4	16.5 ± 0.9
Eastpoint Channel	21.9 ± 0.3	15.1 ± 0.0	24.2 ± 0.2	28.0 ± 0.0
The Jetties	21.4 ± 0.1	-	22.8 ± 2.9	-
St. Vincent Sound	20.8 ± 0.3	14.7 ± 0.05	34.9 ± 0.1	24.8 ± 0.2

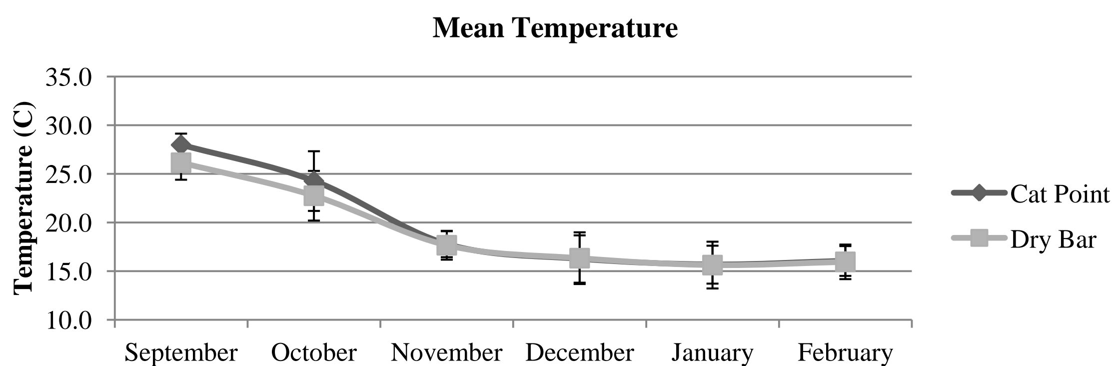


Figure 16. Mean monthly temperature ± SD from September 1st 2012 to February 28th 2013 at Cat Point and Dry Bar.

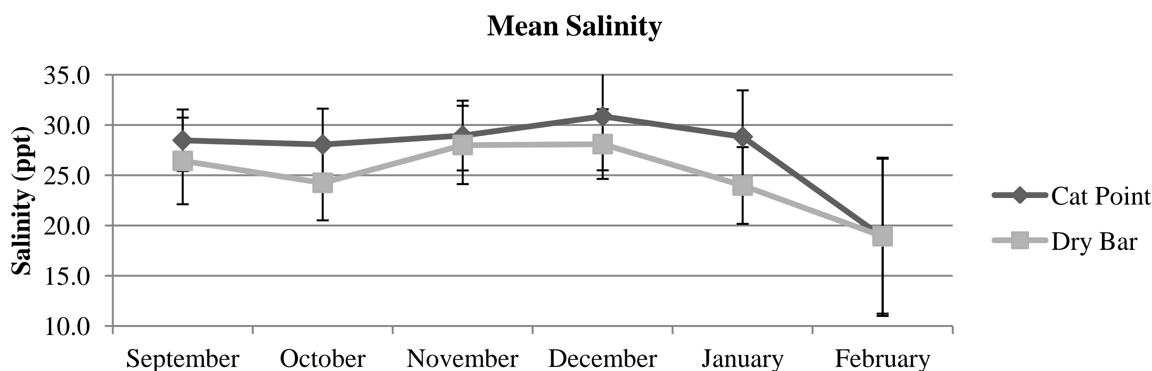


Figure 17. Mean monthly salinity ± SD from September 1st 2012 to February 28th 2013 at Cat Point and Dry Bar.

4.2 Spatial Distribution of *P. marinus*

4.2.1 Weighted Prevalence of *P. marinus* Infections within Oyster Bars

Figure 18-21 show the weighted prevalence of *P. marinus* infections of each site sampled at Eastpoint Channel, Cat Point, the Jetties and St. Vincent Sound, respectively, where light gray columns represent sampling in November and dark grey columns February. The Jetties were not sampled in February due to bad weather conditions on sampling day.

Empirical data suggest that there could be a difference in weighted prevalence e.g. within Cat Point oyster bar in February (site 914A-D) and the Jetties in November (site 908A-B). However, statistical analysis (Students t-test and One-way ANOVA) revealed no significant differences between sites sampled within oyster bars at any time point (Table 4).

Table 4: Statistical analysis of differences within oyster bars.

Oyster Bar	Statistical Results
Eastpoint Channel	Students t-test indicated no differences between sample replicates in November ($t(18) = 0.043$, $p = 0.925$), nor in February ($t(21) = 0.157$, $p = 0.689$).
Cat Point	One-way ANOVA indicated no differences between sample replicates in November ($F_{2,27} = 0.173$, $p = 0.842$), nor in February ($F_{3,49} = 0.792$, $p = 0.504$).
The Jetties	One-way ANOVA indicated no differences between sample replicates in November ($F_{3,36} = 2.001$, $p = 0.131$).
St. Vincent Sound	Students t-test indicated no differences between sample replicates in November ($t(18) = 0.118$, $p = 0.823$), nor in February ($t(18) = 0.567$, $p = 0.800$).

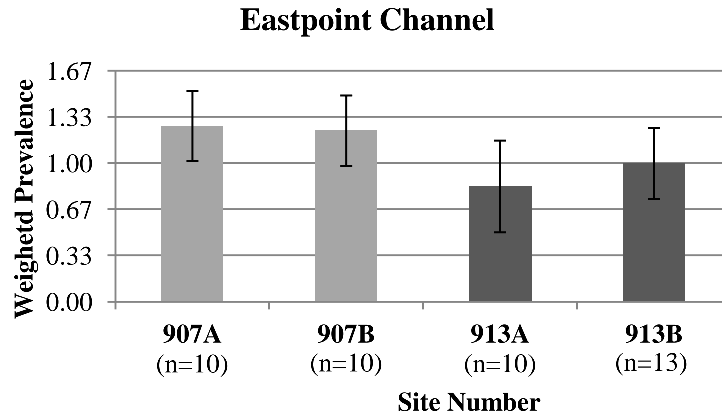


Figure 18. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at Eastpoint Channel. Samples 907A-B were collected in November 2012, whereas samples 913A-B were collected in February 2013. Non-adjacent replicated samples were collected in an East to West direction along the shore line.

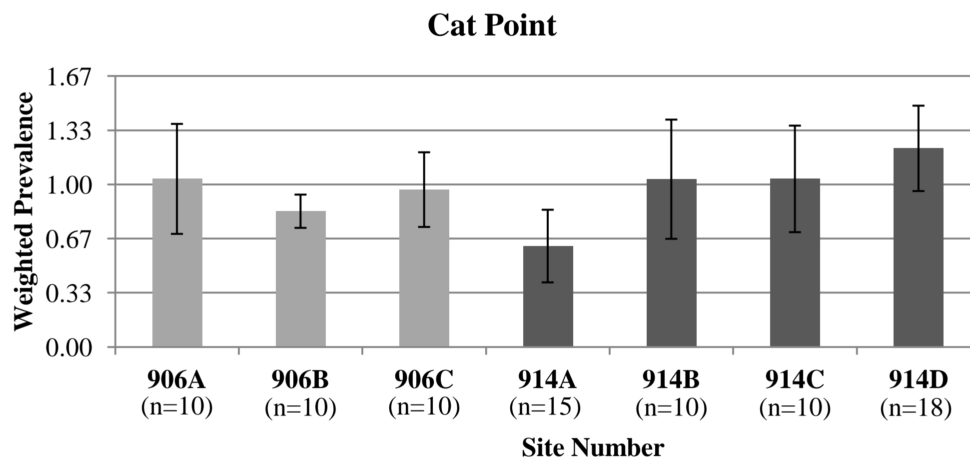


Figure 19. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at Cat Point. Samples 906A-C were collected in November 2012, whereas samples 914A-D were collected in February 2013. Non-adjacent replicated samples were collected in a North to South direction.

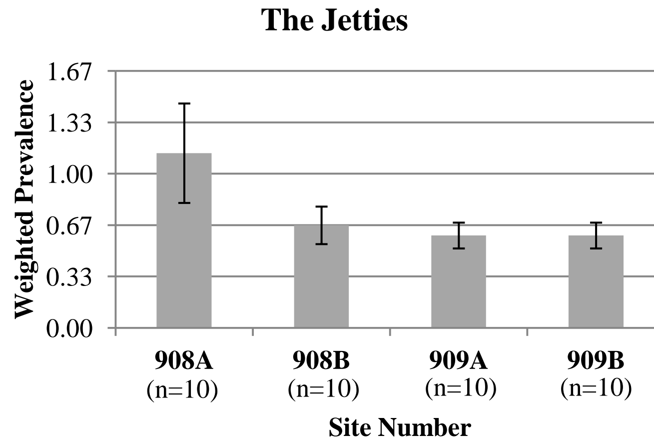


Figure 20. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at the Jetties. Samples 908A-B (Jetties East) and 909A-B (Jetties West) were collected in November 2012. Non-adjacent replicated samples were collected in a South to North direction.

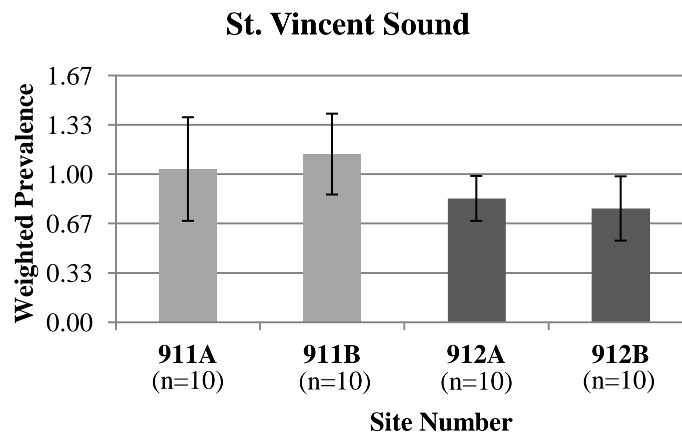


Figure 21. Weighted prevalence ± SE of *P. marinus* infections in oysters sampled at St. Vincent Sound. Samples 911A-B were collected in November 2012, whereas samples 923A-B were collected in February 2013. Non-adjacent replicated samples were collected in a South to North direction.

4.2.2 Weighted Prevalence of *P. marinus* Infections between Oyster Bars and Time Points

Figure 22 shows the weighted prevalence of all four locations, Eastpoint Channel, Cat Point, the Jetties and St. Vincent Sound, in November and February. The Jetties was not samples in February due to bad weather conditions. Two-way ANOVA revealed no significant difference between the four locations nor between February and November ($F_{6,206} = 1.013$, $p = 0.418$).

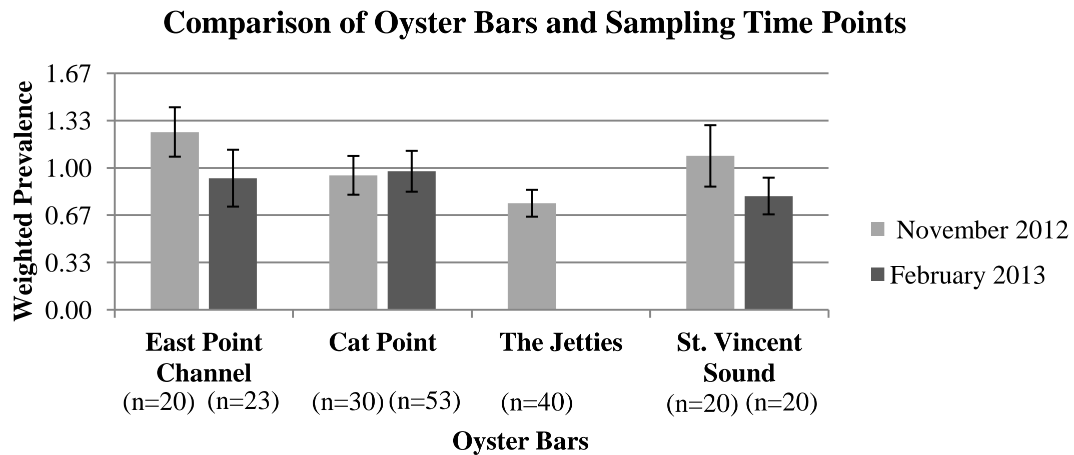


Figure 22. Weighted prevalence ± SE of *P. marinus* infections in oysters at Eastpoint Channel, Cat Point, the Jetties and St. Vincent Sound in November and February.

4.3 Weighted Prevalence of *P. marinus* Infections in Oysters of Different Sizes

Figure 23 shows the comparison of weighted prevalence in adult (≥ 70 mm) and juvenile oysters (< 70 mm) at Eastpoint Channel (913B) and Cat Point (914A, 914D). At each site there was not statistical difference in *P. marinus* weighted prevalence between adult and juvenile oysters; 913B ($t(7) = 15.586$, $p = 0.200$), 914A ($t(28) = 1.853$, $p = 0.446$) and 914D ($t(19) = 15.240$, $p = 0.082$). Comparison of pooled adult and juvenile samples also indicated no difference in weighted prevalence between the two size groups($t(78) = 0.579$, $p = 0.781$).

Since no differences were statistically discerned between sample locations and time points in this study, all samples were pooled for linear regression analysis of oyster height versus infection intensity (Mackin rank) of oysters. The analysis revealed no relationship between these two factors (result presented in Table 5 in next section).

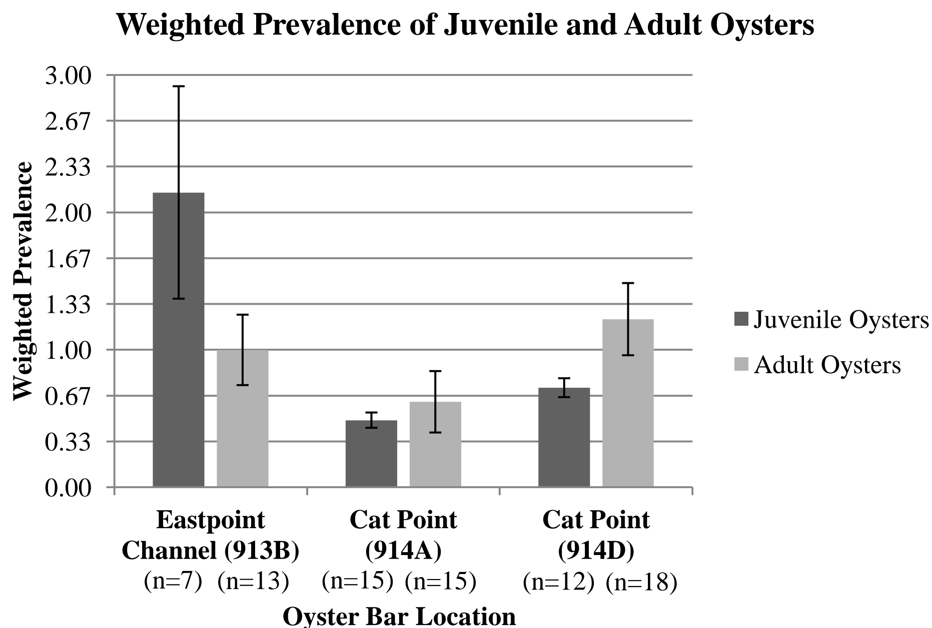


Figure 23. Weighed prevalence ± SE of *P. marinus* infections in adult and juvenile oysters at Eastpoint Channel (913A) and Cat Point (914A, 914D).

4.4 Linear Regression between Infection Intensity and Oyster Health Condition Indices

All samples from November 2012 and February 2013 were pooled together for linear regression analysis. The analysis revealed no relationship between infection intensity and height, or between Mackin rank and internal/external ranks, except for between infection intensity and meat condition index (summary of results presented in Table 5). Although the R^2-value (0.03) for the relationship between infection intensity and meat condition index is not optimal, this result indicates that the weighted prevalence of *P. marinus* is negatively associated with the meat condition index.

Table 5: Results of linear regression between infection intensity of all oyster samples and their internal/external condition indices.

Condition Indices Tested Against Mackin Rank	**Linear Regression Results**					
	R^2	**SE**	***df***		***F***	***p*-value**
Height (mm)	0.00027	0.89	1	238	0.063	0.802
External Sponge Hole Rank (0-5)	0.00449	0.88	1	238	1.073	0.301
Number of External Clam Holes	0.00451	0.76	1	108	0.489	0.486
Meat Condition Index (5-0)	0.03804	0.88	1	232	9.175	0.003
Internal Yellow Rank (0-5)	0.01018	0.88	1	238	2.448	0.119
Internal White Rank (0-5)	0.00001	0.89	1	238	0.003	0.956
Number of Internal Clam Spots	0.00186	0.89	1	237	0.003	0.507
Number of Internal *Polydora* tubes	0.00296	0.88	1	238	0.706	0.402
Internal Sponge Rank (0-5)	0.00578	0.88	1	238	1.384	0.241
Mud Blister Rank (0-3)	0.01391	0.88	1	238	3.356	0.068

4.5 Comparisons with Historic Data from Apalachicola Bay

In order to look for a possible increasing/decreasing trend in weighted prevalence of *P. marinus* infections in Apalachicola Bay, results from this study were compared to data reported by Oyster Sentinel and Petes *et al*. For simplicity and to include the limited historical data available, the mean of a six months period from September to February was calculated for each year, where possible (Figure 24).

These data, together, suggest that the weighted prevalence of *P. marinus* infection in AP Bay oysters may be increasing. These increases, although not dramatic, may be associated with climatological and hydrological conditions (see Figure 5, drought conditions in the bay). From 2008-2009 to 2012-2013 there is a high variability in the columns representing Oyster Sentinel. This is because there was only one sample month available in those years. Table A5 in Appendix A.5 provides the actual numbers used to create Figure 24.

Data are pooled means from one or more sample sets from each source, for each year presented.

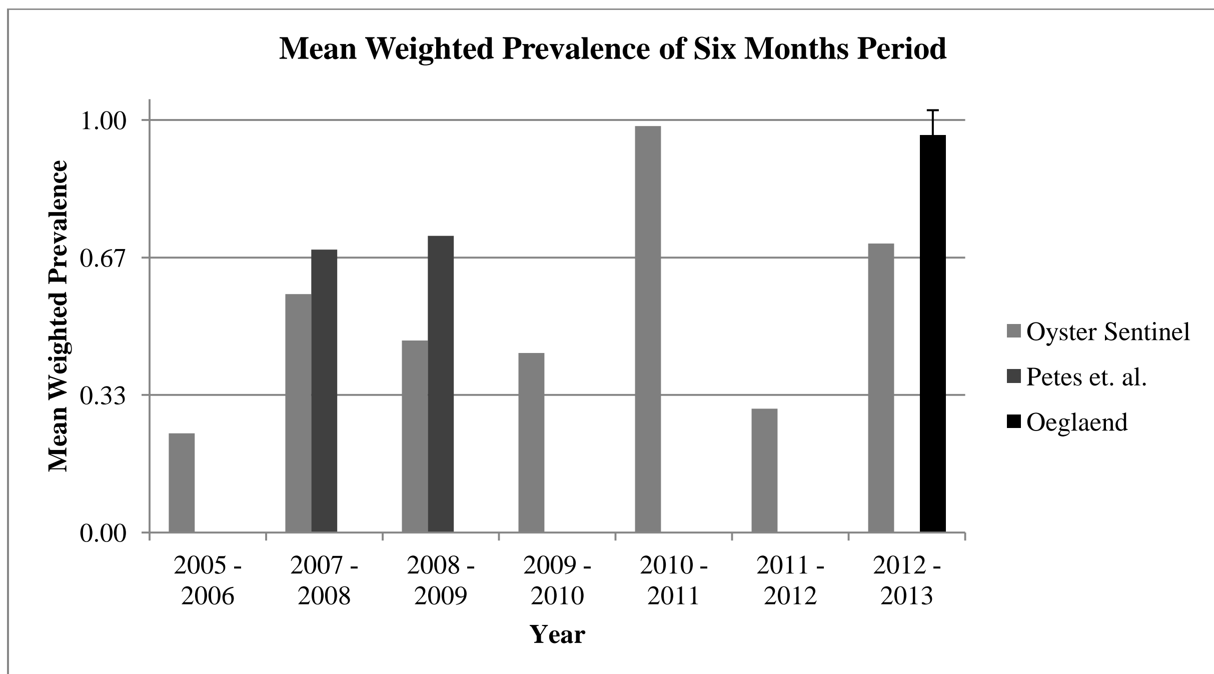


Figure 24. Mean weighted prevalence of *P. marinus* infections in Apalachicola Bay, from pooled historical data available from 6-month intervals (September to February), based on data from Oyster Sentinel, Petes *et al*. and Oeglaend (error bar = SE). Error bars are not presented for historical data sets since raw data were not available.

5 Discussion

5.1 Weighted Prevalence of *P. marinus* Infections in Apalachicola Bay

5.1.1 Temperature and Salinity in Apalachicola Bay

Based on NERRS long-term monitoring data, the mean monthly temperatures from September to November dropped from 26.2 °C and 28.0 °C, to 17.7 °C and 17.8 °C, at Dry Bar and Cat Point, respectively (Figure 16). Between November and February the temperature drop was not that noticeable, decreasing down to 16.0 °C at both locations.

Mean monthly salinity was relatively stable at Cat Point from September to December, ranging from 28.1 to 30.9 ppt, before it rapidly decreased in February to 18.9 ppt (and it continued to drop even further in March due to heavy rainfall events; Figure 17). The mean monthly salinity was generally lower at Dry Bar than at Cat Point, and a bit more variable. From September to December salinity ranged from 24.2 to 28.1 ppt, and dropped down to 18.9 ppt in February.

5.1.2 Spatial and Temporal Distribution of *P. marinus* Infections in Apalachicola Bay Oysters

The first specific aim was to determine the weighted prevalence of *P. marinus* infections in oysters from multiple oyster bars in Apalachicola Bay, with the following hypotheses:

> Ho_1: Variability of *P. marinus* infection in oysters is similar between replicate samples within the same oyster bar during the same season (i.e., within-oyster bar variability is negligible).
>
> Ho_2: Variability of *P. marinus* infection in oysters is similar between oyster bars in different locations in Apalachicola Bay during the same season (i.e., between-oyster bar variability is negligible).
>
> Ho_3: Variability of *P. marinus* infection in oysters is similar between oyster specimens sampled in November 2012 and those sampled in February 2013 (i.e., short temporal and potential water quality differences have negligible effect on *P. marinus* infection).

Hypotheses 1 to 3 failed to be rejected in favor of alternate hypotheses. Data from this project revealed no difference in weighted prevalence of *P. marinus* infections in *C. virginica* within oyster bars (Ho_1), between oyster bars (Ho_2), nor between November and February (Ho_3).

Based on the temperature pattern one could expect to find a higher *P. marinus* weighted prevalence in November, compared to February. Salinity, however, did not change much and probably had little impact on differences in weighted prevalence.

As mentioned in section 1.2.6, optimal conditions for *P. marinus* growth and proliferation are 28 to 32 °C and 24 to 36 ppt, where minimum for proliferation is 18 °C and 6 ppt. Both temperature and salinity requirements are met in the months before sampling in November. In the months before February the temperature is below 18 °C, but salinity requirements are still met. Overall, the temperature and salinity differences between the two sampling times might not be big enough to see a clear difference in *P. marinus* weighted prevalence. There is also a chance that the heavily infected oysters that we expected to find in November had already died before samples were collected, since data from Oyster Sentinel and Petes *et al.* indicates that the weighted prevalence is highest in October (Figure 7 and 8).

A difference in weighted prevalence may become evident based on planned sampling and analyses in summer 2013 (follow up efforts with Dr. Kane after this thesis has been evaluated).

5.1.3 *P. marinus* Infections in Oysters of Different Sizes

The second specific aim was to determine weighted prevalence of *P. marinus* in oyster of different sizes in order to discern if age is a determining factor in *P. marinus* infections. The following hypotheses were developed:

> Ho_1: *P. marinus* infection in juvenile oysters is similar to infection in adult oysters. We will test this hypothesis by examining weighted prevalence of infection in oysters <70 mm and ≥ 70 mm height.
>
> Ho_2: *P. marinus* infection in oyster is not associated with oyster height (all samples pooled together).

Hypotheses 1 and 2 failed to be rejected in favor of alternate hypotheses. There were found no differences between adult and juvenile oysters at the three sites analyzed (Eastpoint Channel and two Cat Point sites, Ho_1). These results were consistent when analyzed by location, as well as baywide.

Due to a mix-up of the chosen cut-off limit between adult and juvenile oysters, Eastpoint Channel 913B ended up with 7 juvenile and 13 adult samples, not 10/10 as planned (15/15 and 13/18 were sampled Cat Point 914 A and D). The juvenile samples from 913B had three samples with unusually high infection intensity, which had a large impact on the weighted prevalence and SE. This is probably why the empirical results revealed a higher weighted prevalence in juvenile oysters, the opposite of what was hypothesized (Figure 24). Potentially, an increase in sample size may have revealed a stronger size-weighted prevalence relationship.

No relationship between oyster size (height) and infection intensity were found when all samples were pooled together (Ho_2). By graphing infection intensity and height data (not shown in this report), one

could see a potential increase in infection intensity with height. The problem was that there were too few samples (n < 10) in the lowest (< 50 mm) and highest height size range (> 130 mm), that the observed results were too uncertain. Also at the highest sizes (> 130 mm), the mean infection intensity was 1 to 1.5 times lower than the mean of samples between 60 and 130 mm. It is speculated that most oysters with high infection intensity die before they reach a size of ≥ 130 mm, and that this might happen in October (highest weighted prevalence was reported in October, both by Oyster Sentinel and Petes *et al.*).

5.2 *P. marinus* Infections Versus Oyster Health Indices

The third, and last, specific aim was to examine the association between oyster health/condition and *P. marinus* infection intensity. Stressed oysters may be more susceptible to *P. marinus* infections. It was expected to find a relationship between infection intensity of *P. marinus* and oyster health conditions, such as shell parasite indices and meat condition index. The following hypotheses were investigated:

> Ho_1: *P. marinus* infection in oysters is associated with oyster meat condition index.
>
> Ho_2: *P. marinus* infection in oysters is associated with shell condition indices and/or shell parasite loading.

Hypothesis 1 failed to be rejected in favor of alternate hypotheses. Hypothesis 2, however, can be rejected.

No relationship between Mackin rank and external oyster health indices (sponge and clam holes), and Mackin rank and most of the internal health indices (yellow and white color, clam spots, *Polydora* tubes, sponge and mud blisters), based on the linear regression analysis. A linear relationship was found, however, between Mackin rank and meat condition index. As the meat condition decreased, the Mackin rank of samples increased, as mentioned in the literature (section 1.2.4). If it is the *P. marinus* infection that is affecting the meat condition, or if oysters with poor meat condition are more susceptible for infection, was not investigated in this project.

5.3 Comparisons with Historical Data

The comparison of my data with historical data from Oyster Sentinel and Petes *et al.* proved more difficult than initially thought. One of the main problems was that we sampled in different parts of the bay, and that the sampling was not done consistently at specific time points. That made it difficult to find the best way to present the data and draw conclusions from it. Also, since the ranking systems

used (Mackin rank and Petes *et al.* Rank) are open to personal interpretation, one has to consider that when compared to each other, over-/under- estimations might have happened.

By comparing my weighted prevalence of *P. marinus* infections with Oyster Sentinel and Petes *et al.*, there is an indication that the mean weighted prevalence of Apalachicola Bay has increased since September 2005 – February 2006 to September 2012 – February 2013, from 0.24 to 0.96. As mentioned in section 1.2.6, there have been two periods of drought in the Apalachicola River watershed (2006-2008 and 2010-present), and it was expected that the weighted prevalence would increase in these periods due to the increased salinity in the bay. Unfortunately, there is only one measurement from 2009 (in September by Oyster Sentinel), the year between the two droughts, and we do not know if the weighted prevalence stayed high or was reduced in that year.

As mentioned previously, there has not been reported lag-period for *P. marinus* infection in Apalachicola Bay, while Chesapeake Bay has the most severe infections one to two months after the highest summer temperature and salinity [20]. Interestingly, by looking at Petes *et al.*'s graphs of temperature, salinity (not shown in this report) and *P. marinus* weighted prevalence, one can see that the highest weighted prevalence appears between one to two months after the highest summer temperature and salinity (in October 2008) [78]. This could be interesting to investigate later on, by doing more extensive sampling to include fall months.

5.4 Evaluation of RFTM Tissue Assay

5.4.1 General Problems Encountered

Most scientific methodology has strengths and challenges. As mentioned in section 1.4.1, the RFTM tissue assay method has inherent strengths and weaknesses. As a recently trained user of this method, it was sometimes difficult to tell the difference between an actual hypnospore and other similar black, spherical particles (iodine precipitate, debris) (Figure 30, Appendix A.6.2). The size of hypnospores also appears to vary within a sample and between samples, and the hypnospores sometimes appeared as clusters in the oyster tissue (Figure 31, Appendix A.6.2). These observations may confound the ranking process. Should large hypnospores count the same as small ones, and did the cell cluster appear before sampling, or did it appear during incubation? It is assumed that these clusters are trophozoites which have just proliferated. Nevertheless, all samples in this study were treated similarly, and any bias introduced, based on the above observations, should be relatively uniform across all samples.

Most literature discussing *P. marinus* detection using the RFTM method, describes the hypnospores as dark blue-black spheres. However, it was discovered that the hypnospores did not always stain well.

Unstained and poorly stained cells appeared as white see-through or light-grey spheres in the tissue, and did not resemble the typical description. This was especially apparent in thick tissue samples (Figure 28 and 29, Appendix A.6.2). Unfortunately, this fact was not known when the microscopic tissue analysis started, and not all of these cells were counted. Therefore, the ranks of samples from four sites at four different locations in Apalachicola Bay sampled in October 2012 were likely under-estimated and the results were not included in these analyses or this report.

Also, the RFTM tissue assay is not sensitive to low infections (<1000 cells/g tissue). Some of our samples were a bit larger than 5x5 mm (approximately 7x7 mm), and several times these slightly larger samples were ranked of 0.33 (1-10 hypnospores), but they could have been ranked as 0 if the sample had been only 5x5 mm.

A total of 300 samples were analyzed in this project, where 240 of the samples are presented in this report, and only two samples were *P. marinus* free. Samples that were given a rank of 0.33, were examined extra carefully to make sure hypnospore-like particles were not counted.

5.4.2 Mackin Rank Photo Guide

One of the components in being confidently trained to consistently rank hypnospores was the determination of the percent of hypnospores covering heavily infected tissue samples (in other words determining ranks above 1 or >125 hypnospores). Taking photos of our ranked samples and conducting blind assays between two sample readers was helpful to give fair ranking of samples. To our knowledge, a good or complete photo guide of the ranking system has not been published. Therefore, a photo guide was created based on our samples, such that it will be easier for others interested in our results, to get an idea of how we did the ranking (Figure 26 and 27, Appendix A.6.1). Hopefully it will be helpful for other researchers that are using the same method.

In the process of creating a photo guide of the different infection intensities on oyster tissue, we tried to preserve some samples by using Cytoseal™ XYL, a xylene –based mounting medium. This method was not effective since stained hypnospores lost much of their color. For example, a sample which was ranked 4.33 got a new rank of 1.67 after mounting the sample using Cytoseal. The effect of the mounting medium was not as severe in stained samples that had dried out over night. However, some weakly colored hypnospores disappeared, but the rank in heavily infected samples did not change more than one rank. We do not recommend using this mounting medium for preserving tissue samples in the RFTM tissue assay.

5.4.3 Variability and Sample Size:

The relatively small sample size (n = 10, with exception of two Cat Point sites in February where n = 15 and 18) used in this project made the results vulnerable to high variance.

Both median and modus of the samples collected at all sites in this project mainly had a rank of 0.33 or 0.67), while mean infection intensity was ranked between 0.67 and 1.33 (Table A4, Appendix A.5). As the slightly higher mean infection intensity portrays that each site had some sample outliers that were at least two ranks higher than the median and modus of the site, and therefore greatly affected the mean infection intensity at each site.

Based on an idea, the weighted prevalence of *P. marinus* infection and standard error (SE) of individual sites were added together, and the number of samples that had a higher ranking than this were counted. For each site in both sample months, the percentage varied from 10 to 40 % above weighted prevalence +SE. The averages of each location at each time point were between 20 and 30 % (Table A4, Appendix A.5). This show that the few samples that have an unusual high rank may greatly affect the mean of the result, especially when the sample size is as small as 10, which is recommended in the methodology description [99]. A higher sample size (e.g. n = 15-20) could reduce the variability, and might give results of differences between sites that can be statistically supported.

5.4.4 Appearance of Tissue Samples:

Tissue samples from October and November were analyzed after 2.5 to 3 months, and February samples after seven days.

The appearances of the tissue samples of these three time points were quite different. Samples from October and November had a thin, peach-colored, necrotic appearance, while the February samples were thick and had creamy beige color. It is not known if the incubation time in the RFTM media affected this. It could potentially also be associated with post-spawning conditions in the oyster samples from October and November, and the pre-spawning condition in the oysters from February.

6 Conclusion

Mean weighted prevalence of *P. marinus* infections for November 2012 and February 2013, was 1.01 ± 0.11 and 0.90 ± 0.05 (mean ± SE), respectfully. No differences were found for weighted prevalence within oyster bars, between oyster bars, nor between the November and February samples. It is assumed that the temperature and salinity did not vary enough between these winter months to have a notable effect. Sampling is planned for this summer to look for trends and possible differences.

No relationship was found between oyster height and infection intensity. However we did not have enough samples in the highest and lowest size range, and since two other sources have reported the highest intensity in October, there is a chance that the large oysters with high infection died before sampling in November, and hence were not represented in the present data set.

A negative relationship was found between oyster meat condition index and infection intensity. This suggests that either are oysters in relatively poor condition are more susceptible to *P. marinus* infection, and/or that *P. marinus* infection is associated with reduced meat condition index. No other relationship between oyster health and infection intensity was found.

Comparison of data amassed in the present study with historical data indicate that the weighted prevalence in Apalachicola Bay may have increased since 2005. This may be associated with two severe droughts (2006-2008, 2010-present) in the Apalachicola River watershed. However, the data available is not sufficient to say for sure.

7 Future Prospects

The next step in this project is to collect summer samples to further investigate whether there is a difference in *P. marinus* weighted prevalence between winter (February, November) and summer.

It could be interesting to increase the sample size from each site to determine if the high variability in *P. marinus* weighted prevalence can be reduced sufficiently such that any differences between oyster bars, seasons, water quality, etc, may be realized.

Species specific PCR should also be run on samples from Apalachicola Bay, to confirm the presence of *P. marinus*, and to exclude the presence of other *Perkinsus* spp. or *Perkinsus*-like organisms that may also be detected with the RFTM method.

In the future, it could be interesting to get a more continuous investigation of *P. marinus* infections in the Bay, e.g. once or twice a month, in order to look for a temperature and salinity effect on a lag period. Sampling only a few times a year makes it difficult to discover a potential lag period. Further, such monitoring would support discerning relationships between environmental factors (i.e., temperature, water flow, salinity) and the health of oysters in important fisheries, such as Apalachicola Bay.

8 References:

1. Coen, L.D., et al., *Ecosystem services related to oyster restoration.* Marine Ecology Progress Series, 2007. **341**: p. 303-307.
2. Arnold, W.S., M.L. Parker, and S.P. Stephenson, *Oyster monitoring in the northern estuaries.* Status report, Florida Fish and Wildlife Research Institute, 2008: p. 1-156.
3. Food and Agriculture Organization of the United Nations - Fisheries and Aquaculture Department. *Crassostrea virginica*. [Species fact sheets] 2013 Retrieved 28.03.13]; Available from: http://www.fao.org/fishery/species/2669/en.
4. Ingle, R.M. and C.E. Dawson Jr, *Growth of the American Oyster, Crassostrea Virginica (Gmelin) in Florida Waters.* Bulletin of Marine Science, 1952. **2**(2): p. 393-404.
5. Livingston, R., et al., *Modelling oyster population response to variation in freshwater input.* Estuarine, Coastal and Shelf Science, 2000. **50**(5): p. 655-672.
6. Newell, R.I.E., *Ecological changes in Chesapeake Bay: Are they the result of overharvesting the American oyster, Crassostrea virginica.* Understanding the estuary: advances in Chesapeake Bay research, 1988. **129**: p. 536-546.
7. Menzel, W.R. and F.E. Nichy, *Studies of the Distribution and Feeding Habits of Some Oyster Predators in Alligator Harbor, Florida.* Bulletin of Marine Science, 1958. **8**(2): p. 125-145.
8. Rotschild, B., et al., *Decline of the Chesapeake Bay oyster population: a century of habitat destruction and overfishing.* Marine Ecology Progress Series, 1994. **111**: p. 29-39.
9. Havens, K., et al., *Apalachicola Bay Oyster Situation Report*, T. Nordlie, Editor 2013, University of Florida: Gainesville. p. 1-32.
10. Volety, A.K., et al., *Eastern oysters (Crassostrea virginica) as an indicator for restoration of Everglades Ecosystems.* Ecological Indicators, 2009. **9**(6, Supplement): p. S120-S136.
11. Gutiérrez, J.L., et al., *Mollusks as ecosystem engineers: the role of shell production in aquatic habitats.* Oikos, 2003. **101**(1): p. 79-90.
12. Wells, H.W., *The fauna of oyster beds, with special reference to the salinity factor.* Ecological Monographs, 1961: p. 239-266.
13. Eastern Oyster Biological Review Team, *Status review of the eastern oyster (Crassostrea virginica).* Report to the National Marine Fisheries Service, Northeast Regional Office, 2007: p. 1-105.
14. Coen, L.D., M.W. Luckenbach, and D.L. Breitburg. *The role of oyster reefs as essential fish habitat: a review of current knowledge and some new perspectives*. in *American Fisheries Society Symposium*. 1999.
15. Haven, D.S. and R. Morales-Alamo, *Filtration of particles from suspension by the American oyster Crassostrea virginica.* The Biological bulletin, 1970. **139**(2): p. 248-264.

16. Andrews, J.D. and W.G. Hewatt, *Oyster mortality studies in Virginia. II. The fungus disease caused by Dermocystidium marinum in oysters of Chesapeake Bay.* Ecological Monographs, 1957. **27**(1): p. 2-25.
17. Ray, S.M., *Biological studies of Dermocystidium Marinum*, 1954, Rice Institute: Huston. p. 1-135.
18. Ragone Calvo, L.M., et al., *Systematic evaluation of factors controlling Perkinsus marinus transmission dynamics in lower Chesapeake Bay.* Diseases of aquatic organisms, 2003. **56**(1): p. 75-86.
19. Andrews, J.D., *History of Perkinsus marinus, a pathogen of oysters in Chesapeake Bay 1950-1984.* Journal of Shellfish Research, 1996. **15**(1): p. 13-16.
20. Burreson, E.M. and L.M. Ragone Calvo, *Epizootiology of Perkinsus marinus disease of oysters in Chesapeake Bay, with emphasis on data since 1985.* J. Shellfish Res., 1996. **15**: p. 17-34.
21. Craig, A., et al., *Distribution of Perkinsus marinus in Gulf Coast oyster populations.* Estuaries, 1989. **12**(2): p. 82-91.
22. Mackin, J.G., H.M. Owen, and A. Collier, *Preliminary Note on the Occurrence of a New Protistan Parasite, Dermocystidium marinum n. sp. in Crassostrea virginica (Gmelin).* Science, 1950. **111**(2883): p. 328-329.
23. Levine, N.D., *Perkinsus gen. n. and other new taxa in the protozoan phylum Apicomplexa.* Journal of Parasitology, 1978. **64**: p. 549.
24. Reece, K.S., et al., *Phylogenetic analysis of Perkinsus based on actin gene sequences.* The Journal of parasitology, 1997. **83**(3): p. 417-23.
25. Siddall, M.E., et al., *Total evidence refutes the inclusion of Perkinsus species in the phylum Apicomplexa.* Parasitology, 1997. **115**(02): p. 165-176.
26. Gornik, Sebastian G., et al., *Loss of Nucleosomal DNA Condensation Coincides with Appearance of a Novel Nuclear Protein in Dinoflagellates.* Current Biology, 2012. **22**(24): p. 2303-2312.
27. Fernández-Robledo, J.A., et al., *The search for the missing link: A relic plastid in Perkinsus?* International Journal for Parasitology, 2011. **41**(12): p. 1217-1229.
28. Oliver, L., et al., *Perkinsus marinus tissue distribution and seasonal variation in oysters Crassostrea virginica from Florida, Virginia and New York.* Diseases of aquatic organisms, 1998. **34**(1): p. 51-61.
29. Reece, K., et al., *Geographic distribution of Perkinsus marinus genetic strains along the Atlantic and Gulf coasts of the USA.* Marine Biology, 2001. **139**(6): p. 1047-1055.
30. da Silva, P.M., et al., *First report of the protozoan parasite Perkinsus marinus in South America, infecting mangrove oysters Crassostrea rhizophorae from the Paraíba River (NE, Brazil).* Journal of Invertebrate Pathology, 2013. **113**(1): p. 96-103.

31. Cáceres-Martínez, J., et al., *Natural and cultured populations of the mangrove oyster Saccostrea palmula from Sinaloa, Mexico, infected by Perkinsus marinus.* Journal of Invertebrate Pathology, 2012. **110**(3): p. 321-325.
32. Reece, K.S., C.F. Dungan, and E.M. Burreson, *Molecular epizootiology of Perkinsus marinus and P. chesapeaki infections among wild oysters and clams in the Chesapeake Bay, USA.* Diseases of aquatic organisms, 2008. **82**(3): p. 237-248.
33. Enriquez-Espinoza, T., et al., *Perkinsus marinus in Crassostrea gigas in the Gulf of California.* Diseases of aquatic organisms, 2010. **89**(3): p. 269-273.
34. Cáceres-Martínez, J., et al., *Perkinsus marinus in pleasure oyster Crassostrea corteziensis from Nayarit, Pacific coast of México.* Journal of Invertebrate Pathology, 2008. **99**(1): p. 66-73.
35. Moss, J.A., E.M. Burreson, and K.S. Reece, *Advanced Perkinsus Marinus Infections in Crassostrea Ariakensis Maintained Under Laboratory Conditions.* Journal of Shellfish Research, 2006. **25**(1): p. 65-72.
36. Dungan, C.F., et al., *Experimental cross-infections by Perkinsus marinus and P. chesapeaki in three sympatric species of Chesapeake Bay oysters and clams.* Diseases of aquatic organisms, 2007. **76**(1): p. 67-75.
37. Villalba, A., et al., *Perkinsosis in molluscs: A review.* Aquatic Living Resources, 2004. **17**(04): p. 411-432.
38. Perkins, F.O., *The structure of Perkinsus marinus (Mackin, Owen & Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of Perkinsus spp.* Journal of Shellfish Research, 1996. **15**(1): p. 67-88.
39. Casas, S.M. and J.F. La Peyre, *Identifying factors inducing trophozoite differentiation into hypnospores in Perkinsus species.* European journal of protistology, 2013. **49**(2): p. 201-209.
40. Chu, F.L.E. and K.H. Greene, *Effect of temperature and salinity on in vitro culture of the oyster pathogen, Perkinsus marinus (Apicomplexa: Perkinsea).* Journal of Invertebrate Pathology, 1989. **53**(2): p. 260-268.
41. Fernández Robledo, J.A., et al., *The search for the missing link: A relic plastid in Perkinsus?* International Journal for Parasitology, 2011. **41**(12): p. 1217-1229.
42. Ford, S.E., M.M. Chintala, and D. Bushek, *Comparison of in vitro-cultured and wild-type Perkinsus marinus. I. Pathogen virulence.* Diseases of aquatic organisms, 2002. **51**(3): p. 187-201.
43. Chintala, M.M., D. Bushek, and S.E. Ford, *Comparison of in vitro-cultured and wild-type Perkinsus marinus. II. Dosing methods and host response.* Diseases of aquatic organisms, 2002. **51**(3): p. 203-216.

44. Perkins, F. and R.W. Menzel. *Morphological and cultural studies of a motile stage in the life cycle of Dermocystidium marinum*. in *Proceedings of the National Shellfisheries Association*. 1966.
45. Chu, F.L.E. and A.K. Volety, *Disease processes of the parasite Perkinsus marinus in eastern oyster Crassostrea virginica: minimum dose for infection initiation, and interaction of temperature, salinity and infective cell dose.* Diseases of aquatic organisms, 1997. **28**: p. 61-68.
46. Volety, A.K. and F.-L.E. Chu, *Comparison of infectivity and pathogenicity of meront (trophozoite) and prezoosporangiae stages of the oyster pathogen Perkinsus marinus in eastern oysters, Crassostrea virginica (Gmelin, 1791).* Journal of Shellfish Research, 1994. **13**(2): p. 521-528.
47. Bushek, D., S.E. Ford, and M.M. Chintala, *Comparison of in vitro-cultured and wild-type Perkinsus marinus. III. Fecal elimination and its role in transmission.* Diseases of aquatic organisms, 2002. **51**(3): p. 217-225.
48. Allam, B., et al., *Early host-pathogen interactions in marine bivalves: Evidence that the alveolate parasite Perkinsus marinus infects through the oyster mantle during rejection of pseudofeces.* Journal of Invertebrate Pathology, 2013. **113**(1): p. 26-34.
49. Sunila, I. and J. LaBanca, *Apoptosis in the pathogenesis of infectious diseases of the eastern oyster Crassostrea virginica.* Diseases of aquatic organisms, 2003. **56**(2): p. 163-70.
50. Andrews, J.D., *Infection experiments in nature with Dermocystidium marinum in Chesapeake Bay.* Chesapeake Science, 1965. **6**(1): p. 60-67.
51. White, M.E., et al. *Host-to-host transmission of Perkinsus marinus in oyster (Crassostrea virginica) populations by the ectoparasitic snail Boonea impressa (Pyramidellidae).* 1987. National Shellfisheries Association.
52. Diamond, E.A., *Do scavengers influence dermo disease (Perkinsus marinus) transmission?: experiments in oyster parasite trophic interactions*, 2012, Rutgers University-Graduate School-New Brunswick.
53. Mackin, J.G., *Histopathology of Infection of Crassostrea virginica (Gmelin) by Dermocystidium marinum Mackin, Owen, and Collier.* Bulletin of Marine Science, 1951. **1**(1): p. 72-87.
54. Hughes, F.M., et al., *Apoptosis as a host defense mechanism in Crassostrea virginica and its modulation by Perkinsus marinus.* Fish & shellfish immunology, 2010. **29**(2): p. 247-57.
55. Gauthier, J.D. and G.R. Vasta, *Effects of plasma from bivalve mollusk species on the in vitro proliferation of the protistan parasite Perkinsus marinus.* Journal of Experimental Zoology, 2002. **292**(3): p. 221-230.
56. Sokolova, I., *Apoptosis in molluscan immune defense.* Invertebrate Survival Journal, 2009. **6**: p. 49-58.

57. La Peyre, J.F., F.-l.E. Chu, and W.K. Vogelbein, *In vitro interaction of Perkinsus marinus merozoites with eastern and pacific oyster hemocytes.* Developmental & Comparative Immunology, 1995. **19**(4): p. 291-304.
58. Tasumi, S. and G.R. Vasta, *A Galectin of Unique Domain Organization from Hemocytes of the Eastern Oyster (Crassostrea virginica) Is a Receptor for the Protistan Parasite Perkinsus marinus.* The Journal of Immunology, 2007. **179**(5): p. 3086-3098.
59. Anderson, R.S., *Perkinsus marinussecretory products modulate superoxide anion production by oyster (Crassostrea virginica) haemocytes.* Fish & shellfish immunology, 1999. **9**(1): p. 51-60.
60. Garreis, K.A., J.F. La Peyre, and M. Faisal, *The effects of Perkinsus marinus extracellular products and purified proteases on oyster defence parameters in vitro.* Fish & shellfish immunology, 1996. **6**(8): p. 581-597.
61. Oliver, J.L., et al., *Analysis of the Effects of Perkinsus marinus Proteases on Plasma Proteins of the Eastern Oyster (Crassostrea virginica) and the Pacific Oyster (Crassostrea gigas).* Journal of Invertebrate Pathology, 1999. **74**(2): p. 173-183.
62. La Peyre, J.F., H.A. Yarnall, and M. Faisal, *Contribution of Perkinsus marinus extracellular products in the infection of eastern oysters (Crassostrea virginica).* Journal of Invertebrate Pathology, 1996. **68**(3): p. 312-313.
63. Volety, A.K. and F.-L.E. Chu, *Suppression of chemiluminescence of eastern oyster (Crassostrea virginica) hemocytes by the protozoan parasite Perkinsus marinus.* Developmental & Comparative Immunology, 1995. **19**(2): p. 135-142.
64. Tall, B., et al., *Perkinsus marinus extracellular protease modulates survival of Vibrio vulnificus in Eastern oyster (Crassostrea virginica) hemocytes.* Applied and environmental microbiology, 1999. **65**(9): p. 4261-4263.
65. Paynter, K.T. and E. Burreson, *Effects of Perkinsus marinus infection in the eastern oyster, Crassostrea virginica: II. Disease development and impact on growth rate at different salinities.* Journal of Shellfish Research, 1991. **10**(2): p. 425-431.
66. Menzel, R.W. and S.H. Hopkins, *The growth of oysters parasitized by the fungus Dermocystidium marinum and by the trematode Bucephalus cuculus.* The Journal of parasitology, 1955. **41**(4): p. 333-342.
67. Ray, S., J.G. Mackin, and J.L. Boswell, *Quantitative Measurement of the Effect on Oysters of Disease Caused by Dermocystidium Marinum.* Bulletin of Marine Science, 1953. **3**(1): p. 6-33.
68. Barber, B.J., *Gametogenesis of eastern oysters, Crassostrea virginica(Gmelin, 1791), and Pacific oysters, Crassostrea gigas(Thunberg, 1793) in disease-endemic lower Chesapeake Bay.* Journal of Shellfish Research, 1996. **15**(2): p. 285-290.

69. Dittman, D., S. Ford, and D. Padilla, *Effects of Perkinsus marinus on reproduction and condition of the eastern oyster, Crassostrea virginica, depend on timing.* Journal of Shellfish Research, 2001. **20**(3): p. 1025-1034.
70. Kennedy, V., et al., *Reproductive capacity of the eastern oyster Crassostrea virginica infected with the parasite Perkinsus marinus.* Diseases of aquatic organisms, 1995. **23**: p. 135-144.
71. Paynter, K.T., *The effects of Perkinsus marinus infection on physiological processes in the eastern oyster, Crassostrea virginica.* Journal of Shellfish Research, 1996. **15**(1): p. 119-126.
72. Lenihan, H.S., et al., *The influence of multiple environmental stressors on susceptibility to parasites: an experimental determination with oysters.* Limnology and Oceanography, 1999. **44**(3): p. 910-924.
73. Chu, F.L.E. and R.C. Hale, *Relationship between pollution and susceptibility to infectious disease in the eastern oyster, Crassostrea virginica.* Marine Environmental Research, 1994. **38**(4): p. 243-256.
74. Fisher, W.S., et al., *Decreased resistance of eastern oysters (Crassostrea virginica) to a protozoan pathogen (Perkinsus marinus) after sublethal exposure to tributyltin oxide.* Marine Environmental Research, 1999. **47**(2): p. 185-201.
75. Wilson, E.A., et al., *The Distribution of Perkinsus marinus in Gulf Coast Oysters: Its Relationship with Temperature, Reproduction, and Pollutant Body Burden.* Internationale Revue der gesamten Hydrobiologie und Hydrographie, 1990. **75**(4): p. 533-550.
76. Winstead, J.T. and J.A. Couch, *Enhancement of protozoan pathogen Perkinsus marinus infections in American oysters Crassostrea virginica exposed to the chemical carcinogen n-nitrosodiethylamine (DENA).* Diseases of aquatic organisms, 1988. **5**(3): p. 205-213.
77. Ray, S. *Studies on the occurrence of Dermocystidium marinum in young oysters*. in *Convention Addresses National Shellfisheries Association 1953*. 1954.
78. Petes, L.E., A.J. Brown, and C.R. Knight, *Impacts of upstream drought and water withdrawals on the health and survival of downstream estuarine oyster populations.* Ecology and Evolution, 2012. **2**(7): p. 1712-1724.
79. Volety, A., et al., *Progression of diseases caused by the oyster parasites, Perkinsus marinus and Haplosporidium nelsoni, in Crassostrea virginica on constructed intertidal reefs.* Journal of Shellfish Research, 2000. **19**(1): p. 341-348.
80. Hayes, P.F. and R.W. Menzel, *The Reproductive Cycle of Early Setting Crassostrea Virginica (Gmelin) in the Northern Gulf of Mexico, and its Implications for Population Recruitment.* The Biological bulletin, 1981. **160**(1): p. 80-88.
81. Soniat, T.M., *Epizootiology of Perkinsus marinus disease of eastern oysters in the Gulf of Mexico* Journal of Shellfish Research, 1996. **15**(1): p. 35-44.

82. Feng, S., W. Canzonier, and S. SNIESZKO. *Humoral responses in the American oyster (Crassostrea virginica) infected with Bucephalus sp. and Minchinia nelsoni*. in *A symposium on diseases of fishes and shellfishes.* 1970.

83. Fisher, W.S. and M. Tamplin, *Environmental Influence on Activities and Foreign-Particle Binding by Hemocytes of American Oysters, Crassostrea virginica.* Canadian Journal of Fisheries and Aquatic Sciences, 1988. **45**(7): p. 1309-1315.

84. Chu, F.-L.E. and J.F. La Peyre, *Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (Crassostrea virginica).* Journal of Invertebrate Pathology, 1989. **54**(2): p. 224-232.

85. Burreson, E.M., et al., *Acute osmotic tolerance of cultured cells of the oyster pathogen Perkinsus marinus (Apicomplexa:Perkinsida).* Comparative biochemistry and physiology. Part A, Physiology, 1994. **109**(3): p. 575-582.

86. Chu, F.-L.E. and J.F. La Peyre, *Perkinsus marinus susceptibility and defense-related activities in eastern oysters Crassostrea virginica: temperature effects.* Diseases of aquatic organisms, 1993. **16**: p. 223-223.

87. La Peyre, M.K., et al., *Environmental significance of freshets in reducing Perkinsus marinus infection in eastern oysters Crassostrea virginica: potential management applications.* Marine Ecology Progress Series, 2003. **248**: p. 165-176.

88. Galtsoff, P.S., *The American oyster, Crassostrea virginica Gmelin*. Fishery bulletin of the Fish and Wildlife Service Vol 64. 1964, Washington D.C: U.S. G.P.O. p. 67, 397-416.

89. Ray, S.M. and T.M. Soniat. *Oyster Sentinel*. 2007-2013 Retrieved 14.01.2013]; Available from: http://www.oystersentinel.org/.

90. Ingle, R.M., *Spawning and setting of oysters in relation to seasonal environmental changes.* Bulletin of Marine Science, 1951. **1**(2): p. 111-135.

91. Ragone, L. and E. Burreson, *Effect of salinity on infection progression and pathogenicity of Perkinsus marinus in the eastern oyster, Crassostrea virginica (Gmelin, 1791).* Journal of Shellfish Research, 1993. **12**: p. 1-7.

92. Peyre, J.F.L., *Propagation and in vitro studies of Perkinsus marinus.* Journal of Shellfish Research, 1996. **15**(1): p. 89-102.

93. Gauthier, J.D. and G.R. Vasta, *In Vitro Culture of the Eastern Oyster Parasite Perkinsus marinus: Optimization of the Methodology.* Journal of Invertebrate Pathology, 1995. **66**(2): p. 156-168.

94. La Peyre, M.K., et al., *The combined influence of sub-optimal temperature and salinity on the in vitro viability of Perkinsus marinus, a protistan parasite of the eastern oyster Crassostrea virginica.* Journal of Invertebrate Pathology, 2010. **105**(2): p. 176-181.

95. Soniat, T.M., et al., *Understanding the Success and Failure of Oyster Populations: Periodicities of Perkinsus marinus, and Oyster Recruitment, Mortality, and Size.* Journal of Shellfish Research, 2012. **31**(3): p. 635-646.

96. Burreson, E.M. and J.D. Andrews. *Unusual intensification of Chesapeake Bay oyster diseases during recent drought conditions*. in *OCEANS '88. A Partnership of Marine Interests. Proceedings*. 1988.

97. Choi, K., et al., *The energetic cost of Perkinsus marinus parasitism in oysters: quantification of the thioglycollate method.* Journal of Shellfish Research, 1989. **8**(1): p. 125-131.

98. Ray, S.M., *A review of the culture method for detecting Dermocyctidium marinum, with suggested modifications and precautions.* National Shellfisheries Association, 1966. **54**: p. 55-69.

99. Kim, Y., K.A. Ashton-Alcox, and E.N. Powell, *Histological Techniques for Marine Bivalve Molluscs: Update*, 2006, NOAA Technical Memorandium NOS NCCOS 27: Silver Spring, MD. p. 63-75.

100. Bushek, D., S.E. Ford, and S.K. Allen Jr, *Evaluation of methods using ray's fluid thioglycollate medium for diagnosis of Perkinsus marinus infection in the eastern oyster, Crassostrea virginica.* Annual Review of Fish Diseases, 1994. **4**(0): p. 201-217.

101. Blackbourn, J., S.M. Bower, and G.R. Meyer, *Perkinsus qugwadi sp.nov. (incertae sedis), a pathogenic protozoan parasite of Japanese scallops, Patinopecten yessoensis, cultured in British Columbia, Canada.* Canadian Journal of Zoology, 1998. **76**(5): p. 942-953.

102. Remacha-Triviño, A., et al., *Numerical Quantification of Perkinsus marinus in the American Oyster Crassostrea virginica (Gmelin, 1791) (Mollusca: Bivalvia) by Modern Stereology.* Journal of Parasitology, 2008. **94**(1): p. 125-136.

103. Russell, S., et al., *Application of a multiplex PCR for the detection of protozoan pathogens of the eastern oyster Crassostrea virginica in field samples.* Diseases of aquatic organisms, 2004. **59**(1): p. 85-91.

104. Faveri, J.D., R.M. Smolowitz, and S.B. Roberts, *Development and Validation of a Real-Time Quantitative PCR Assay for the Detection and Quantification of Perkinsus marinus in the Eastern Oyster, Crassostrea virginica.* Journal of Shellfish Research, 2009. **28**(3): p. 459-464.

105. Oyster Technical Task Force, *The Oyster Fishery of the Gulf of Mexico, United States: A Regional Management Plan*, S.J. VanderKooy, Editor 2012: Ocean Springs. p. 1-376.

9 Appendix

A.1 GPS Location of Sampled Sites in Apalachicola Bay

Table A1: Site name, number and GPS location of sampling sites on November 5th 2012 and February 16th 2013.

Date	Site	Site Number	Lat, Long
05.11.2012	Cat Point	906A	29.71865, -84.87786
		906B	29.71571, -84.87559
		906C	29.71012, -84.87643
	Eastpoint Channel	907A	29.74007, -84.87056
		907B	29.73900, -84.87229
	Jetties East	908A	29.69047, -84.97121
		908B	29.69190, -84.97190
	Jetties West	909A	29.69046, -84,97571
		909B	29.69154, -84.97605
	St. Vincent Sound	911A	29.69894, -85.12941
		911B	29.69892, -85.12930
16.02.2013	Eastpoint Channel	913A	29.74032,-84.87012
		913B	29.73992,-84.87074
	Cat Point	914A	29.72094,-84.88206
		914B	29.71733,-84.87998
		914C	29.70236,-84.87942
		914D	29.71161,-84.87728
	St. Vincent Sound	912A	29.69592,-85.12632
		912B	29.69521,-85.12632

A.2 Chemicals and Equipment

Table A2-A3 lists chemicals and equipment needed to perform RFTM mantle tissue assay on oysters.

Table A2: RFTM Tissue Assay - Chemicals

Materials	Product number [CAS]	Supplier
Ethanol, 190 proof (95 %) (C_2H_6O)	2805 [64-17-5, 99.90-100.00%], [7732-18-5 (purified water)]	Decon Labs Inc., King of Prussia (PA)
Iodine (I_2)	I3380 [7553-56-2]	Sigma-Aldrich, St. Louis (MO)
Penicillin G sodium salt ($C_{16}H_{17}N_2NaO_4S$)	P3032-10MU [69-57-8]	Sigma-Aldrich, St. Louis (MO)
Potassium iodide (KI)	60399 [7681-11-0]	Sigma-Aldrich, St. Louis (MO)
Sodium chloride (NaCl)	S-9888 [7647-14-5]	Sigma-Aldrich, St. Louis (MO)
Streptomycin sulfate ($C_{42}H_{84}N_{14}O_{36}S_3$)	BP910-50 [3810-74-0]	Fisher BioReagents, New Jersey
Thioglycollate medium (FTM)	T9032	Sigma-Aldrich, St. Louis (MO)

Table A3: RFTM Tissue Assay - Equipment

Equipment
Alcohol lamp
Autoclave
Bacti-cinerator IV (McCormick Scientific)
Cover slips
Culture tubes with screw cap
Dissecting tools (scissors, probes, forceps)
Heater with stirring mechanism
Inoculation loop
Microscope slides
Microscope, BX51 (Olympus America)
Oyster knives
Volumetric pipette, 10 mL
Syringe filter, 45 µm
Syringe, 1 mL

A. 3 Solution Recipes

Ray's (1952) Thioglycollate medium preparation (RFTM)

22.0 g NaCl and 29.3 g dehydrated fluid thioglycollate medium (FTM) were added to 1.0 L of distilled water and heated while stirring until the medium dissolved and the solution became transparent golden-yellow color. After cooling 5.0 mL portions of the solution was dispensed into 15.0 mL culture tubes, which were subsequently autoclaved and sealed. They were stored in the dark until they were used. Unused, autoclaved tubes could be stored for many months in the dark without deterioration.

RFTM maintains anaerobic conditions in the culture tubes as well as providing needed nutrients and appropriate osmotic environment. Therefore, tubes should be sealed tightly and opened only briefly when necessary, and returned immediately to the dark afterwards.

Antibiotic solution

0.330 g streptomycin sulfate and 0.159 g penicillin G were added to 500 mL of sterilized, deionized water and shaken lightly until the powder was dissolved. The solution was kept refrigerated until used, and could be stored safely for several months.

Lugol's iodine solution

4.0 g KI and 2.0 g I_2 were dissolved in 100 mL distilled water, and allowed to stand for 24 h before it was filtered. The solution was stored in room temperature in a dark bottle, and could remain stable for weeks but should be filtered occasionally to remove particles that may precipitate. These particles may be confused with *P. marinus* hypnospores by less experienced slide reads [100].

A.4 Conversion of Petes *et al.*'s Ranking System to Mackin Ranking System

Figure 26 show how Petes *et al.*'s ranking system was converted to the Mackin ranking system used in this project. Based on the description of their ranking system, the best matches in the Mackin ranking system were found. A trend line was created on the graphed results.

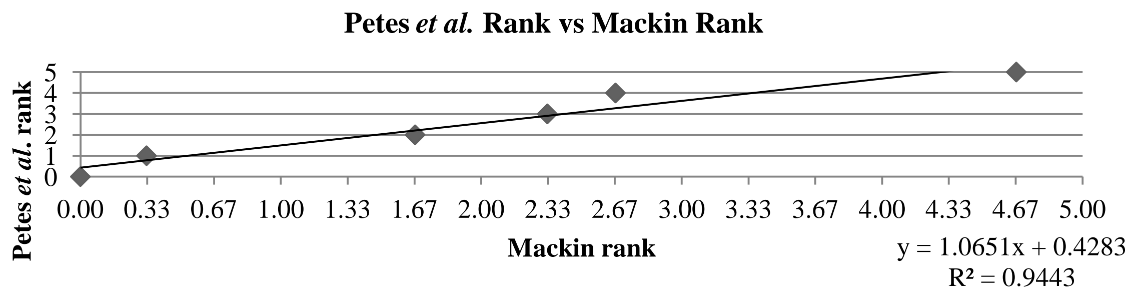


Figure 25. Conversion of Petes *et al.*'s ranking system to Mackin ranking system. Conversion factor: Mackin rank = (Petes *et al*'s rank -0.4283)/1.0651

A.5 Overview of Collected Data Used in This Report

Table A4: Overview of data collected at four locations in Apalachicola Bay in November 2012 and February 2013.

Location	Date	Site	Count Total	Count Infected	Infection intensity	Prevalence	Mean Weighted Prevalence	SE	Variance	Median	Min	Max	Modus	Oysters with High Mackin Rank			
														WP+SE	Count from	# Higher Rank	% of total
Cat Point	05.11.2012	906A	10	10	1.04	1	1.04	0.34	1.15	0.67	0.33	4.00	0.67	1.37	1.67	1	0.10
	05.11.2012	906B	10	10	0.84	1	0.84	0.10	0.10	0.67	0.67	1.67	0.67	0.94	1	3	0.30
	05.11.2012	906C	10	10	0.97	1	0.97	0.23	0.53	0.67	0.67	3.00	0.67	1.20	1.33	1	0.10
	16.02.2013	914A	15	14	0.67	0.93	0.62	0.22	0.75	0.33	0.00	3.67	0.33	0.85	1	2	0.14
	16.02.2013	914B	10	10	1.03	1	1.03	0.37	1.35	0.67	0.33	4.00	0.33	1.40	1.33	2	0.20
	16.02.2013	914C	10	10	1.04	1	1.04	0.33	1.07	0.67	0.33	3.67	0.67	1.36	1.67	2	0.20
	16.02.2013	914D	18	18	1.22	1	1.22	0.26	1.24	0.67	0.33	4.00	0.67	1.49	1.67	5	0.28
East-point Channel	05.11.2012	907A	10	10	1.27	1	1.27	0.25	0.64	0.84	0.67	3.00	0.67	1.52	1.67	4	0.40
	05.11.2012	907B	10	10	1.24	1	1.24	0.25	0.64	0.84	0.67	2.67	0.67	1.49	1.67	2	0.20
	05.11.2012	913A	10	10	0.83	1	0.83	0.33	1.10	0.33	0.33	3.67	0.33	1.16	1.33	2	0.20
	16.02.2013	913B	13	13	1.00	1	1.00	0.26	0.85	0.67	0.33	3.00	0.33	1.26	1.33	4	0.31
The Jetties	05.11.2012	908A	10	10	1.13	1	1.13	0.32	1.04	0.67	0.33	3.33	0.67	1.46	1.67	2	0.20
	05.11.2012	908B	10	10	0.67	1	0.67	0.12	0.15	0.67	0.33	1.33	0.67	0.79	1	2	0.20
	05.11.2012	909A	10	10	0.60	1	0.60	0.08	0.07	0.67	0.33	1.00	0.33	0.68	1	2	0.20
	05.11.2012	909B	10	10	0.60	1	0.60	0.08	0.07	0.67	0.33	1.00	0.33	0.68	1	2	0.20
St. Vincent Sound	06.11.2012	911A	10	10	1.03	1	1.03	0.35	1.22	0.67	0.33	3.67	0.33	1.38	1.67	2	0.20
	06.11.2012	911B	10	10	1.13	1	1.13	0.27	0.75	0.67	0.33	3.00	0.67	1.41	1.67	3	0.30
	16.02.2013	912A	10	10	0.84	1	0.84	0.15	0.23	0.67	0.33	1.67	0.67	0.99	1	3	0.30
	16.02.2013	912B	10	9	0.85	0.90	0.77	0.22	0.47	0.67	0.00	2.00	0.67	0.98	1	2	0.22

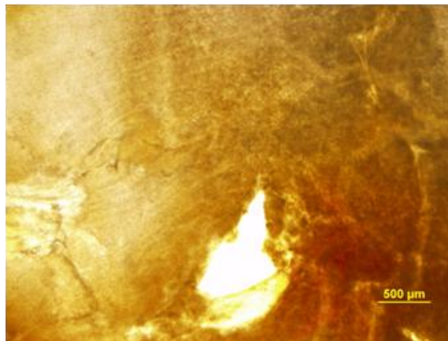
Table A5: Overview of data used when comparing historical data from Oyster Sentinel and Petes et al. with results from this study.

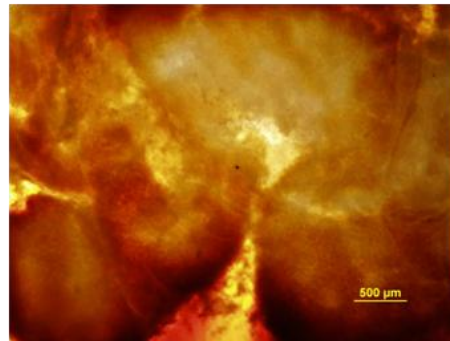
<table>
<tr><th rowspan="2"></th><th colspan="3">Sentinel</th><th colspan="3">Petes et al.</th><th colspan="6">Oeglaend</th></tr>
<tr><th>Dry Bar</th><th>Jetties</th><th>Mean</th><th>Cat Point</th><th>Dry Bar</th><th>Mean</th><th>Eastpoint</th><th>St. Vincent Sound</th><th>Cat Point</th><th>Jetties</th><th>Mean</th><th>SE</th></tr>
<tr><td>Nov. 2005</td><td>0.23</td><td>0.43</td><td rowspan="2">0.24</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Feb. 2006</td><td>0.17</td><td>0.13</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Sep. 2007</td><td>0.70</td><td>0.74</td><td rowspan="4">0.58</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Nov. 2007</td><td></td><td></td><td>1.38</td><td>0.35</td><td rowspan="3">0.69</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Dec. 2007</td><td></td><td></td><td>0.72</td><td>0.63</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Feb. 2008</td><td>0.47</td><td>0.40</td><td>0.58</td><td>0.44</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Sep. 2008</td><td>0.33</td><td>0.60</td><td rowspan="4">0.47</td><td>0.58</td><td>0.16</td><td rowspan="4">0.72</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Oct. 2008</td><td></td><td></td><td>1.24</td><td>0.82</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Nov. 2008</td><td></td><td></td><td>0.58</td><td>0.72</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Des. 2008</td><td></td><td></td><td>1.01</td><td>0.63</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Sep. 2009</td><td>0.47</td><td>0.40</td><td>0.44</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Oct. 2010</td><td>0.90</td><td>1.07</td><td>0.99</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Sep. 2011</td><td>0.30</td><td>0.30</td><td>0.30</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Sep. 2012</td><td>0.60</td><td>0.80</td><td rowspan="3">0.70</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Nov. 2012</td><td></td><td></td><td></td><td></td><td></td><td>1.25</td><td>1.08</td><td>0.95</td><td>0.75</td><td rowspan="2">0.96</td><td rowspan="2">0.06</td></tr>
<tr><td>Feb. 2013</td><td></td><td></td><td></td><td></td><td></td><td>0.93</td><td>0.80</td><td>0.98</td><td>-</td></tr>
</table>

A.6 Photos Taken During Tissue Analysis

A.6.1 Photos of Mackin Ranks

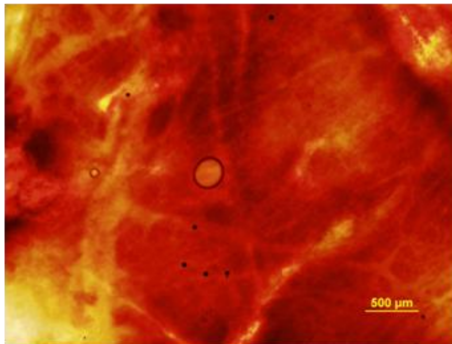
RANK 0
No hypnospores in tissue

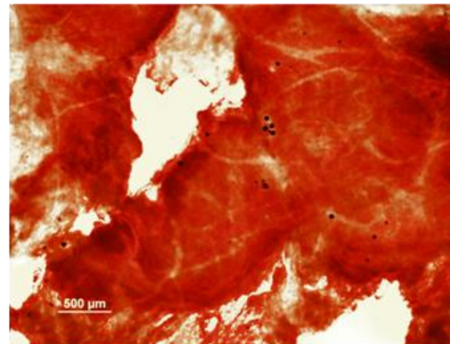




RANK 0+
1-10 hypnospores

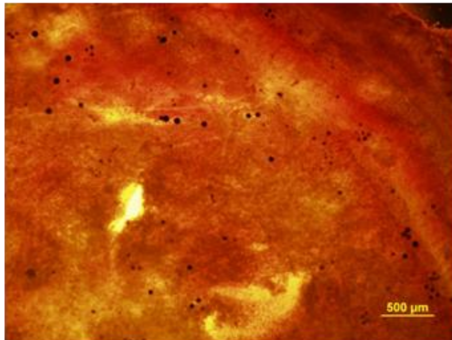
RANK 1-
11-74 hypnospores

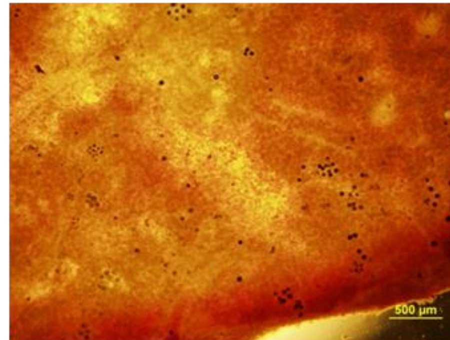




RANK 1
75-125 hypnospores

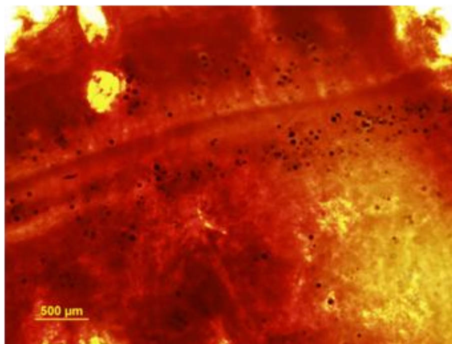
RANK 1+
>125 hypnospores but much less than 25% of tissue is hypnospores

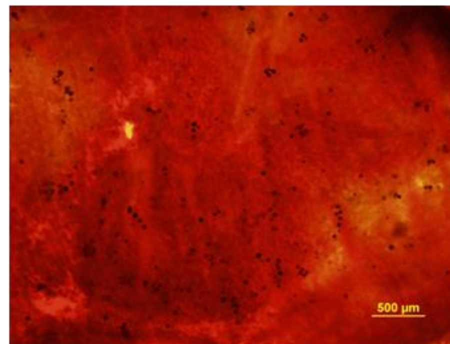




RANK 2-
<25% of tissue is hypnospores

RANK 2
25% of tissue is hypnospores

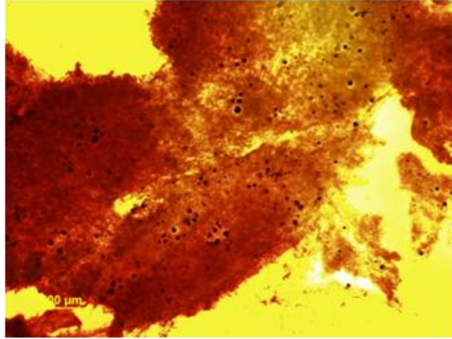


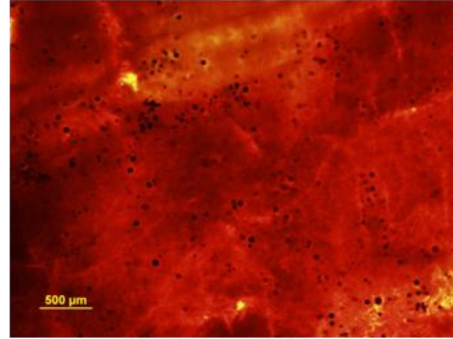


RANK 2+
>25% but much less than 50% of tissue is hypnospores

Figure 26. Mackin rank from 0 to 2+ viewed at 4x magnification in a light microscope.

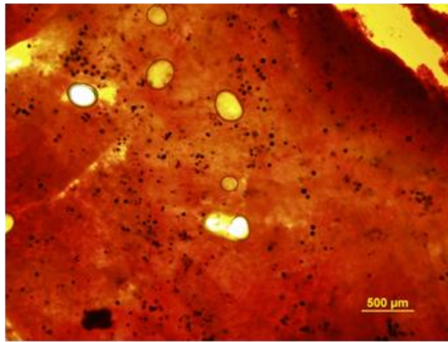
RANK 3-
>25% but <50% of tissue is hypnospores

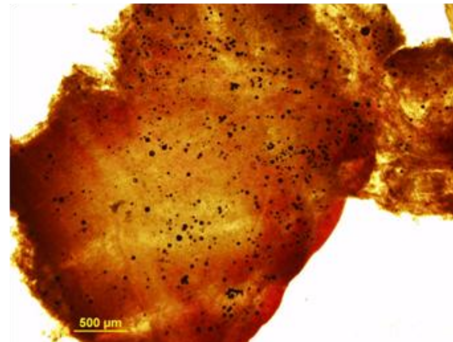




RANK 3
50% of tissue is hypnospores

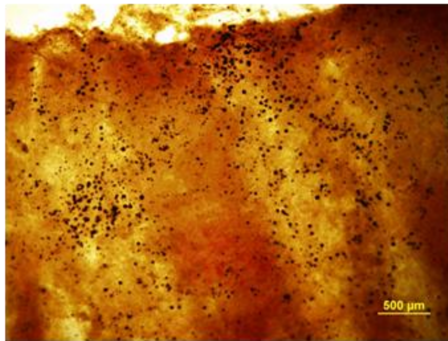
RANK 3+
>50% but much less than 75% of tissue is hypnospores

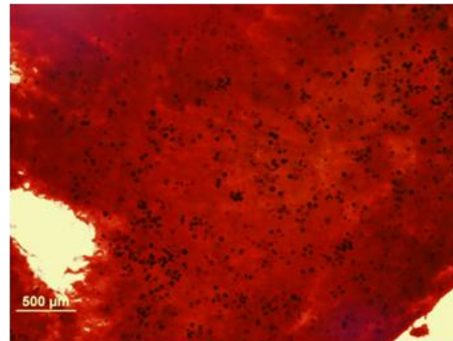




RANK 4-
>50% but <75% of tissue is hypnospores

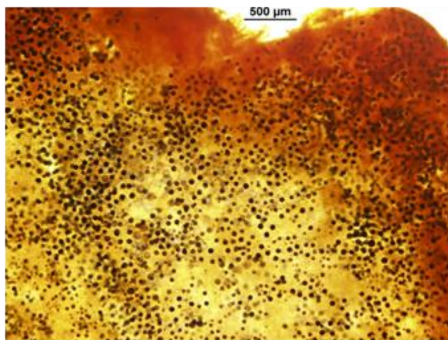
RANK 4
75% of tissue is hypnospores

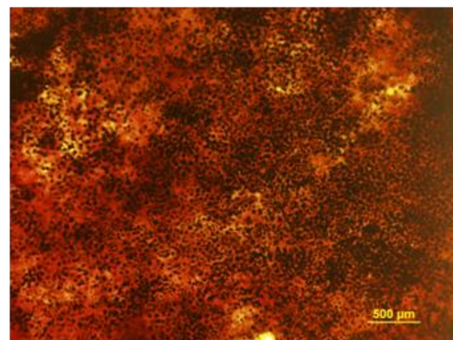




RANK 4+
>75% but much less than 100% of tissue is hypnospores

RANK 5-
>75% of tissue is hypnospores but some oyster tissue is still visible





RANK 5
Nearly 100% of tissue is hypnospores

Figure 27. Mackin rank from 3- to 5 viewed at 4x magnification in a light microscope.

A.6.2 Examples of Observations and pProblems Encountered During Ranking of Infection Intensity in Samples

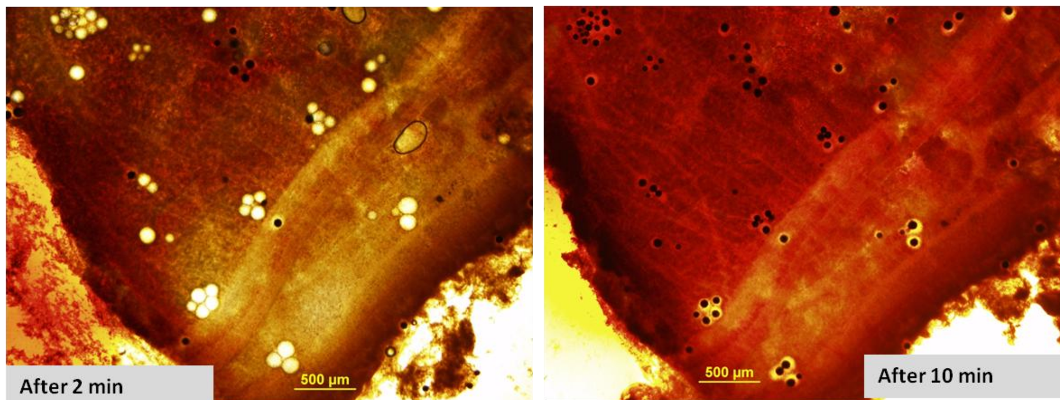


Figure 28. Example of hypnospores in oyster tissue that did not stain well, at 4x magnification. Unstained hypnospores are seen as white see-through spheres (left), which eventually (with enough iodine solution present) changes to black spheres (right).

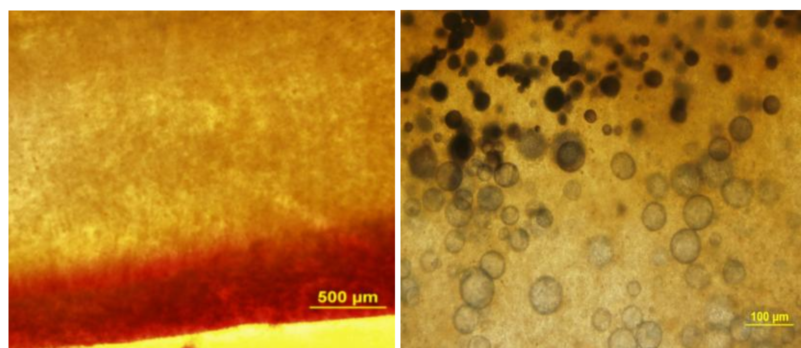


Figure 29. Thick tissue samples did not stain well in the middle of the tissue. Left photo: Iodine solution has penetrated the outer edge of the tissue (red color), while there is not much iodine further in on the tissue where it is thicker (4x magnification). Right photo: Hypnospores close to the surface stain better (top of photo) than cells deeper down in the tissue (bottom of photo, 10x magnification).

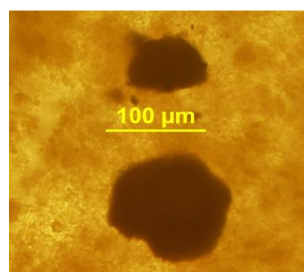


Figure 30. Components that at 4x magnification could look like hypnospores, but at 20x (shown here) it is clear that these are not hypnospores.

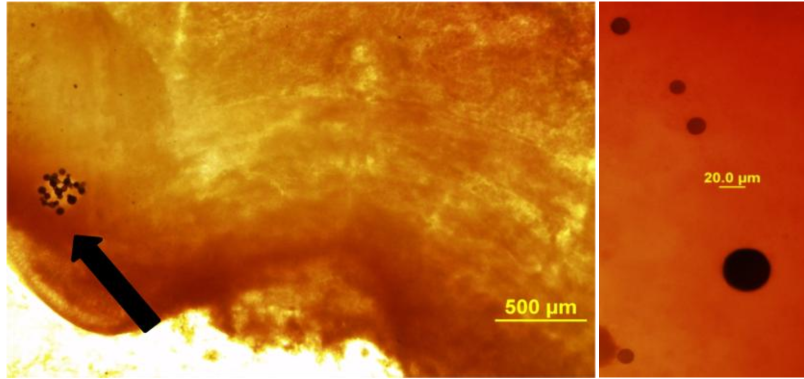


Figure 31. Left photo show a cluster of hypnospores in an oyster tissue sample (4x magnification). Right photo show how hypnospore cell size varies within a sample and between samples (10x magnification).