QUANTITATIVE REAL-TIME PCR DETECTION OF *PERKINSUS MARINUS* AND *HAPLOSPORIDIUM NELSONI* IN TEXAS OYSTERS

A Senior Scholars Thesis

by

JAKE EMERSON HEARE

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Marine Biology

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ABSTRACT

Quantitative Real-time PCR Detection of *Perkinsus marinus* and *Haplosporidium nelsoni* in Texas Oysters. (April 2009)

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Haplosporidium nelsoni (MSX) and *Perkinsus marinus* (Dermo) are protozoan parasites that are traditionally detected using time and labor intensive histological methods. Recently developed traditional PCR assays, specific for these parasites, were used to for initial screening of presence/absence in samples of the eastern oyster, *Crassostrea virginica,* collected from Galveston Bay, Aransas Bay, and Corpus Christi Bay, Texas. *H. nelsoni* (MSX) was not detected in any of the samples. *P. marinus* (dermo) was detected in oysters from all bays. Oysters that tested positive for *P. marinus* were further screened with quantitative PCR assays to enumerate the parasites. These data were directly compared to values obtained by Ray's Fluid Thioglycollate histological method from the same sample. Though these tests have not been "ground-truthed" against the traditional histological methods it is the goal of this project to begin the process of comparing the two methods. There was strong agreement between the PCR and histological determination of *P. marinus* that is promising for eventual transition to PCR assays.

DEDICATION

I would like to dedicate this project to my Mom: she has always believed in me.

ACKNOWLEDGMENTS

I would like to acknowledge Drs. Sammy Ray and Robin Brinkmeyer, who helped me create, write, understand the project, and for giving me the chance to conduct this research. I would like to thank everyone who worked in the Coastal Health and Estuarine Microbiology lab for all their assistance, especially Liz Neyland and all the mistakes she enabled me to understand. I would also like to thank the Texas Institute for Oceanography and the Research and Graduate Studies office at Texas A&M at Galveston for financial support. I also thank Dr. Nancy Stokes at the Virginia Institute of Marine Science for MSX DNA and Dr. Jerome La Peyre at Louisiana State University for the Dermo sample used for the PCR positive controls. I am grateful to Dr. Heath Mills for allowing me to store my samples at his laboratory during the Hurricane Ike evacuation of the Galveston campus to College Station in the fall semester of 2008.

NOMENCLATURE

Dermo	*Perkinsus marinus*
MSX	*Haplosporidium nelsoni*
PCR	Polymerase Chain Reaction
RFTM	Ray's Fluid Thioglycollate Method
QPCR	Quantitative PCR

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CHAPTER I

INTRODUCTION

Haplosporidium nelsoni (multinucleated sphere X: MSX) and *Perkinsus marinus* (Dermo) are spore forming protozoan intracellular parasites that have caused devastating declines on the populations of the eastern oyster, *Crassostrea virginica*, along the coast of the eastern U.S. and the Gulf of Mexico since the 1950's (Bushek and Allen 1996; Ewart and Ford 1993; Ulrich et al. 2007; Gauthier et al. 2006). All three life stages of *P. marinus* (2 to 4 μm; fig. 1 A,B) can induce infection in oysters (Andrews 1988). Oyster death results as hundreds of thousands of dermo cells over grow and eventually lyse tissues. *P. marinus* are transmitted oyster to oyster, to the water column through decomposing tissues of dead oysters, and by the excretions of scavengers that feed on the dead oysters (Audemard et al. 2004). Although not all life stages of *Haplosporidium nelsoni* are known, the predominant life stage is thought to occur in the oyster as a multinucleated plasmodium ranging in size from 5-70 μm (fig. 1 C,D; Hoffman et al. 2001).

The earliest detected presence of *P. marinus* in the Gulf of Mexico was in Louisiana 'prime' oysters that were sent for exhibition to the 1893 World's Fair in Chicago. The oysters' tissues, preserved and stored in New Orleans' Cabildo Museum, were later examined for evidence of the parasite (S. Ray, pers. comm.). *P. marinus* was first described in the environment by Mackin et al. (1950) from Louisiana and other Gulf state oysters and its increase in occurrence in the Gulf and northward along the Atlantic coast in the last 50 years

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This thesis follows the style in the Journal of Invertebrate Pathology.

appears to be a 'home grown' problem. After introduction to the U. S. east coast by import of the Japanese oyster (*C. gigas*) in 1957, *H. nelsoni* has spread to populations of *C. virginica* as far north as the Damariscotta River, Maine and as far south as Biscayne Bay, Florida (Burreson et al. 2000; Ewart and Ford, 1993). The presence of *H. nelsoni* was reported recently by Ulrich et al. (2007) in the Caribbean Sea and Gulf of Mexico from as far south as Venezuela and as far north as Florida, including Texas.

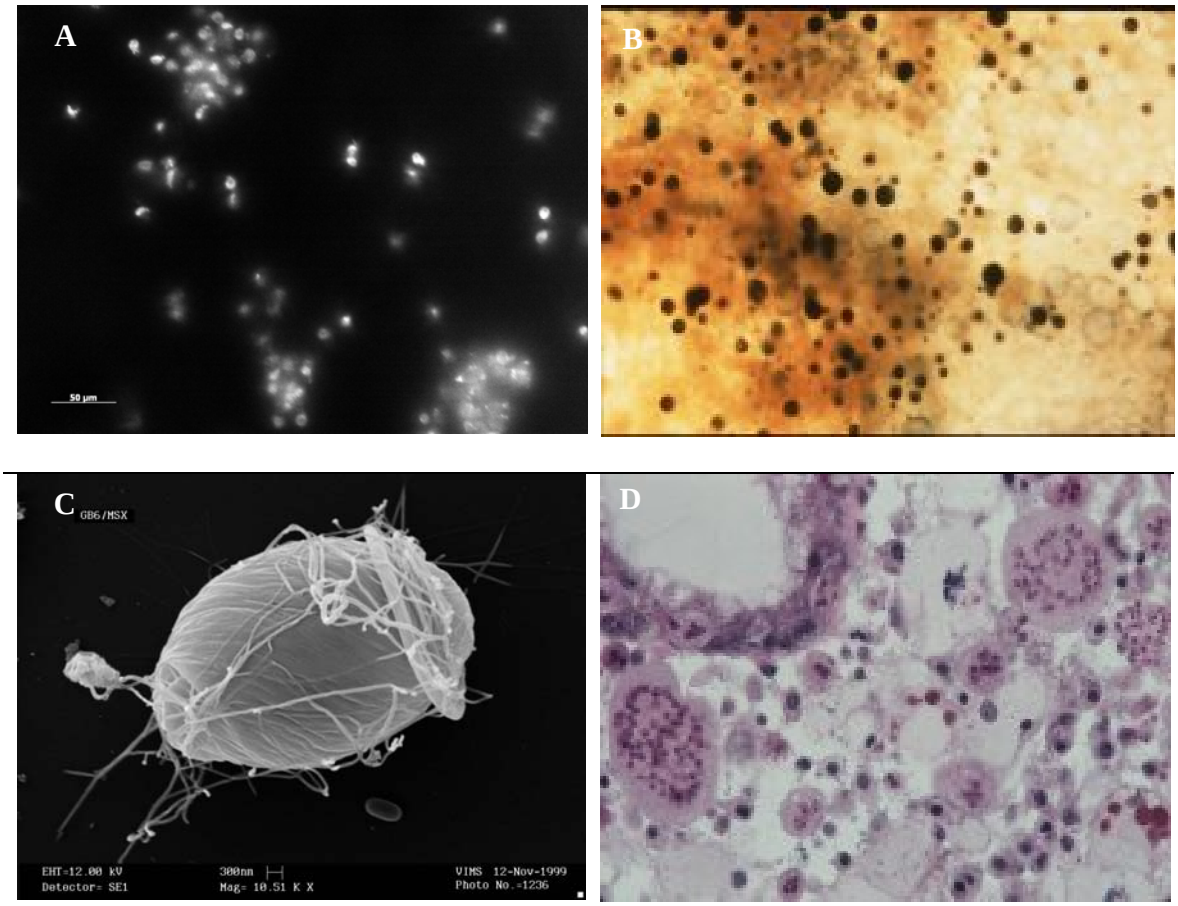

Figure 1. **Images of *P. marinus* and *H. nelsoni*.** (A) *P. marinus* stained with DAPI. Image taken by J. Heare using a Zeiss Axioimager Epifluorescence microscope at the Coastal Health and Estuarine Microbiology Laboratory at TAMUG; (B) *P. marinus* in oyster tissue stained with Lugol's solution (American Museum of Natural History, AMNH.org); (C) Scanning electron micrograph of H. nelsoni (American Museum of Natural History, AMNH.org); (D) H. nelsoni in oyster tissue (American Museum of Natural History, AMNH.org)

Activity and distribution of *H. nelsoni* and *P. marinus* increase at higher salinities (>10 to 12 ppt) and may be linked to reduced freshwater inflows into estuaries as a result of increased water demands by ever-growing urban areas along the Atlantic and Gulf coasts. Dramatic increases in upper water column salinities in the mid and lower latitudes observed in the western Atlantic in the last 50 years may also be responsible (Curry et al. 2003). Temperature plays a role in regulating *P. marinus* (growth is halted below 20°C) and lower temperatures are thought to reduce the number of infective *H. nelsoni* particles by killing the purported intermediate host (Ewart & Ford 1993; Hofmann et al. 2001). Eutrophication caused by population growth along the coasts may also be a factor regulating the activity and distribution of these parasites, however it has not been thoroughly investigated.

Salinity and temperature appear to be the main regulators of *P. marinus* and *H. nelsoni* infection rates and development of best management practices (BMPs), at least for fresh water inflows into Texas bays are necessary. *P. marinus* is a serious problem and occurs throughout the Texas oyster population. However, unlike the Atlantic coast oysters, decimation of the Texas population has not occurred. Differences in virulence among *P. marinus* strains in the Gulf and Atlantic or resistance of Texas oysters may be the cause (Bushek and Allen 1996). Additionally, the growing season for market size oysters (3 inches) in Texas and other Gulf states is shorter, 18 to 24 months, versus the 3 to 4 years along the Atlantic coast, which in turn reduces the reservoir time of *P. marinus* and thus its impact on the Texas populations as a whole. There is also the question of sub-tidal (typical of the upper Atlantic coast) versus inter-tidal growth (more typical of the Gulf states) of

oysters which impacts exposure to *P. marinus*. The recent report of *H. nelsoni* detection in Aransas Bay oysters (Ulrich et al., 2007), leads to many questions about its existence and distribution throughout Texas oyster beds. It may just be a matter of time before *H. nelsoni* is firmly established. Again, Texas oysters may be resistant to *H. nelsoni* and would be an important potential source for 'healthy' oysters to re-establish decimated beds along the east coast of the U.S.

The traditional method for detection and enumeration of *P. marinus* is the fluid thioglycollate method (FTM) developed by Ray (1952) that involves light microscopic examination of infected oyster tissues stained by Lugol's solution. Polymerase chain reaction (PCR) assays have been developed for *P. marinus* (Gauthier et al. 2006), however PCR is not currently employed for diagnosis since there has never been a direct comparison to FTM for validation of PCR. *H. nelsoni* infection is determined with histological examination using light microscopy of paraffin embedded tissue sections. PCR and DNA probe assays are also employed for diagnosis (Day et al. 2000; Stokes et al. 1995).

C. virginica samples collected from three Texas bays were processed with FTM by Dr. Sammy Ray at the Oyster Sentinel Laboratory at TAMUG as well as with traditional and quantitative PCR assays for *P. marinus*. This study is the first direct comparison. This 'ground-truthing' of the PCR methods against the histological data produced by FTM may eventually allow for the replacement of FTM with PCR for detection and enumeration of *P. marinus*. This study also provides the first broad PCR screening for *H. nelsoni* in Texas bays oysters.

CHAPTER II

METHODS

Sample collection

Oyster samples were obtained from Dr. Sammy Ray's Oyster Sentinel Laboratory (www.oystersentinel.org) at Texas A&M University at Galveston (TAMUG). The Oyster Sentinel Laboratory, with the assistance of the Texas Parks and Wildlife Department (TPWD), collects oysters from multiple sites within the seven estuaries on the Texas Gulf of Mexico Coast (fig. 2 A). Typically, ten commercial (larger than 3 inches in diameter) and ten juvenile oysters are collected from each site for histological determination of 'dermo' infection. For my study, I examined commercial oysters from five sites in Galveston Bay (fig. 2 B), collected in October 2007, Corpus Christi Bay (4 sites; fig. 2 C) and Aransas Bay (3 sites; fig. 2 D), collected in September and November 2007 (Table 1).

Oysters were processed for histological and PCR analysis within 7 days of collection. Using sterile conditions that included boiled and autoclaved instruments, oysters were opened and the mantle was removed and split into two subsamples. Histological determination of 'dermo' was performed according to Ray 1952. Subsamples for PCR screening were stored in sterile 2 ml microcentrifuge tubes at -20 °C for DNA extraction.

Table 1. Sampling sites in this study.

Site	Nomenclature Oyster Sentinel Laboratory Site	Latitude/Longitude	Date Collected
GB-1	Redfish Reef	29°30'51.18"N/94°52'9.27"W	10-26-07
GB-2	Hannah's Reef	29°29'12.03"N/ 94°43'9.64"W	10-02-07
GB-4	April Fool's Reef	29°28'38.88"N/ 94°54'51.76"W	10-26-07
GB-5	Frenchy's Reef	29°31'22.11"N/94°36'36.39"W	10-02-07
GB-6	Confederate Reef	29°15'18.93"N/94°55'7.06"W	10-23-07
AB-1	Allyn's Bight Reef	27°57'44.89"N/96°59'22.71"W	9-10-07 11-12-07
AB-2	Long Reef	28° 3'18.01"N/ 96°57'4.95"W	9-10-07 11-12-07
AB-3	Half Moon Reef	28° 4'34.35"N/96°59'7.83"W	9-10-07 11-12-07
CCB-1	East Flats	27°48'37.42"N/ 97° 6'33.32"W	9-09-07 11-11-07
CCB-2	Island Mooring Marine	27°48'40.62"N/97° 5'36.52"W	9-09-07 11-11-07
CCB-3	Port Aransas Mooring	27°50'26.45"N/ 97° 3'8.43"W	9-09-07 11-11-07
CCB-4	Packery Channel	27°39'38.97"N/97°12'51.68"W	9-05-07 11-12-07

DNA extraction

DNA was extracted from 0.15 g of oyster mantle using the DNEasy Blood and Tissue DNA extraction kit (Qiagen, Valencia, CA) according to the protocol for mouse tails modified with an overnight lyses step as described by Robledo et al. (2000). Nucleic acids concentration and purity was determined by $A_{260/280}$ measurements using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) spectrometer.

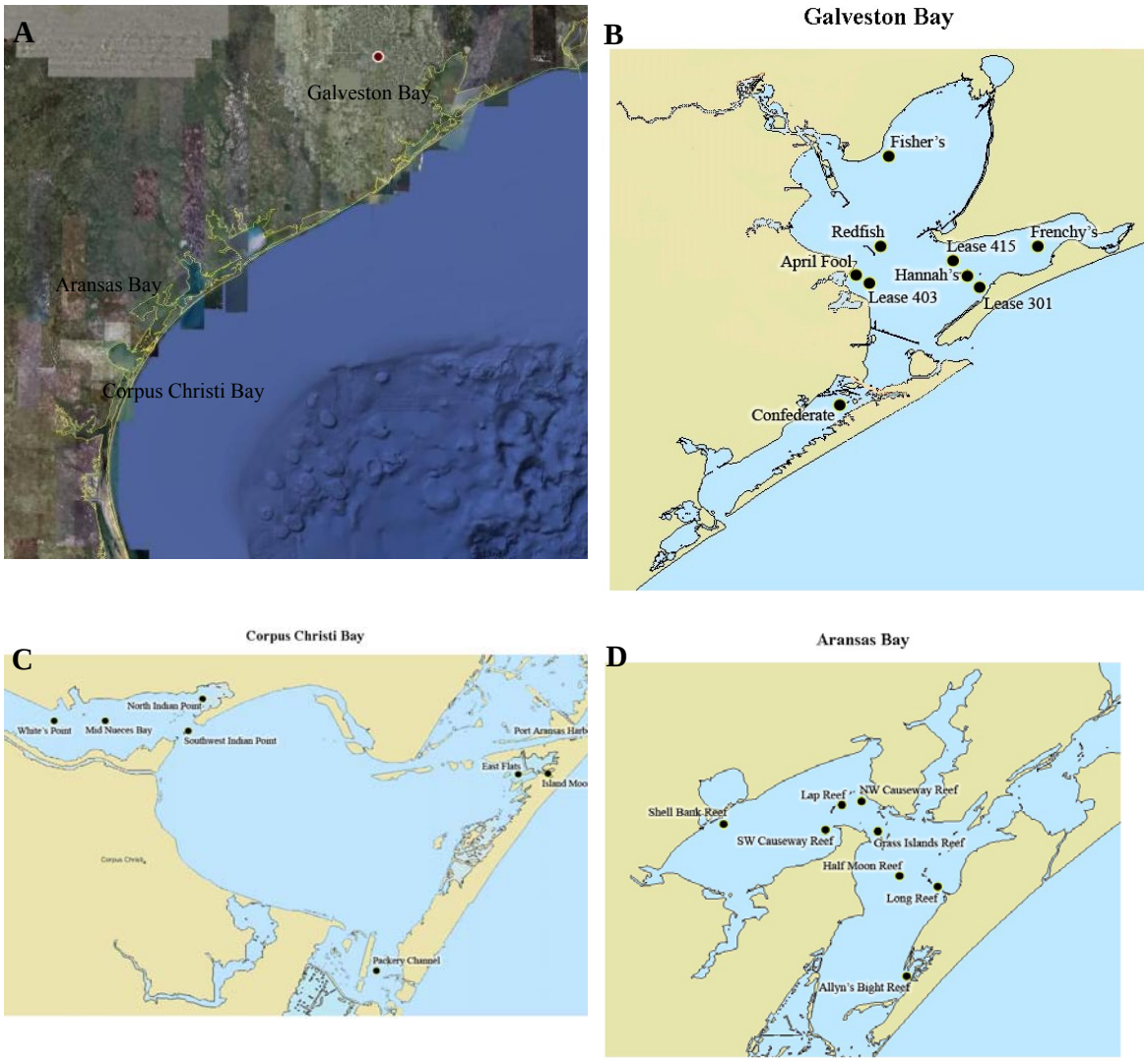

Figure 2. Maps of sampling sites. (A) Texas estuaries sampled for the Oyster Sentinel Laboratory (source: Google Earth); and sites in (B) Galveston Bay, (C) Corpus Christi Bay, and (D) Aransas Bay.

Traditional PCR screening

Samples were screened with traditional PCR to determine presence/absence of MSX and/or dermo prior to quantitative PCR assays.

A 564-bp fragment of the *H. nelsoni* small subunit rRNA gene was amplified from DNA extracts using primers MSX A (*f*) (5'-CGACTTTGGCATTAGGTTTCAGACC-3') and MSX B (*r*) (5'-ATGTGTTGGTGACGCTAACCG-3') (Stokes et al. 1995). PCR was performed with a MasterCycler thermocycler (Eppendorf, Hamburg, Germany) with cycling conditions having an initial denaturation step of 5 minutes at 95°C, and 30 cycles of 94°C for 1 m, 57°C for 1 m, and 72°C for 1 m, and final extension step at 72°C for 5 m according to Day et al. (2000). The 25 µl PCR reaction mixture contained 1 µl DNA (variable concentration), 2.5 µl of 10×PCR Buffer, 0.25 µl of 1×BSA, 18.25 µl of PCR water (Sigma Aldrich), 0.5 µl *Taq* DNA polymerase (1U, Roche Diagnostics, Indianapolis, IN), 1 µl of DNTP mixture (10 mM; Roche Diagnostics), and 0.5 µl of each primer (10 µM; Integrated DNA Technologies, Coralville, IA). PCR amplicons were verified with an MSX DNA positive control, from N. Stokes (Virginia Institute of Marine Science) and a blank reaction mixture with no DNA.

An 86-bp fragment of the intergenic spacer (ITS) region of *Perkinsus* spp. (dermo) was amplified from DNA extracts using primers PMAR-*f* (5'-TTGTTAACGCAACTCAATGCTTTGT-3') and PMAR-*r* (5'-GGTGGTTCGTTATGTGCGCTT-3') according to Gauthier et al. 2006. PCR was performed with a MasterCycler thermocycler (Eppendorf, Hamburg, Germany) with cycling conditions having an initial denaturation step of 5 minutes at 95°C, and 40 cycles of 95°C for 15 s, followed by 60°C for 1 m, and a final extension step at 72°C for 5 m. The 25 µl PCR reaction mixture contained 1 µl DNA (variable concentration), 2.5 µl of 10×PCR Buffer, 0.25 µl of 1×BSA, 18.25 µl of PCR water (Sigma Aldrich), 0.5 µl *Taq* DNA

polymerase (1U, Roche Diagnostics, Indianapolis, IN), 1 µl of DNTP mixture (10 mM; Roche Diagnostics), and 0.5 µl of each primer (10 µM; Integrated DNA Technologies, Coralville, IA). PCR amplicons were verified with a dermo DNA positive control, from J. La Peyre (Louisiana State University Agricultural Center) and a blank reaction mixture with no DNA.

Gel electrophoresis

Traditional PCR amplicons were visualized on 3% agarose gels (100 V for approximately 45 m) stained with ethidium bromide (10 mg/ml stock solution) and documented with the GelDoc System (Biorad, Hercules, CA).

Quantitative real-time PCR

H. nelsoni was not detected with traditional PCR screening in any of the samples therefore, quantitative PCR assays were not conducted.

Quantitative PCR (QPCR) assays for dermo were performed using the same cycling conditions described above for traditional PCR but with a 10 minute initial denaturation step and no final extensions step. The assay developed by Gauthier et al. 2006 was modified and the SYBR green intercalating dye was substituted for the TaqMan® probe. The reaction mixture contained 1 µl DNA (variable concentration), 12.5 µl iQ SYBR Green Supermix (Biorad), 0.25 µl of 1×BSA, 11.25 µl of PCR water (Sigma Aldrich), and 0.5 µl of each primer (10 µM; Integrated DNA Technologies, Coralville, IA). A SmartCycler II (Cepheid; Sunnyvale, CA) quantitative real-time thermocycler was used to enumerate the DNA

template copies in the extractions. QPCR allows quantification of starting amounts of DNA, cDNA, or RNA templates. QPCR is based on the detection of a fluorescent reporter molecule that increases as PCR product accumulates with each cycle of amplification. Fluorescent reporter molecules include dyes that bind double-stranded DNA such as SYBR green used in my assay or sequence–specific probes (i.e. Molecular Beacons or TaqMan® Probes). Quantitative PCR exceeds the limitations of traditional end–point PCR methods by allowing either absolute or relative quantification of PCR product at the end of each cycle. Replicates were not necessary since the SmartCycler consists of 16 I-CORE (intelligent Cooling/Heating Optical Reaction) modules. The modules provide a fluid change in temperature to each individual reaction tube allowing for rapid and precise amplification. The reaction tubes themselves are thin unique design, allowing for a rapid heating and cooling of the reaction and thus rapid amplification. By controlling each reaction individually, there is no inconsistency in temperature like there can be in a multi-sample thermocycler. Therefore, the need for replicates is removed. Dermo concentrations calculated using a standard curve created from QPCR assay analysis of a 6 point dilutions series (10^6, 10^5, 10^4, 10^3, 10^2, and 10^1) of cells/ml. The corresponding DNA concentration for 10^6 cells/ml was determined to be 13 ng/µl after extraction using the DNeasy Blood and Tissue kit following the same protocol that was used for oyster samples.

Analysis of data

Quantitative PCR data represent the correlation of DNA concentration to organisms/ml. To directly compare PCR data to the Mackin Values scale (Ray 1952) used by the Oyster

Sentinel Laboratory to express degree of *P. marinus* infection, the quantitative PCR data was assigned a range of High (100+ cells), Medium (10-100 cells), and Low (10-1 cells), and no detectable cells represented by 0. The Mackin Values scale was adapted by Dr. Sammy Ray as follows: 0 = no observable infection; 1 = slight infection; 3 = moderate infection; and 5 = heavily infected (Ray *pers. comm.*). The Oyster Sentinel Laboratory reports percent infected oysters per site, and the Mackin Values scale calculates the intensity of infection (number of parasites in mantle tissue/ number of infected individual oysters per site), incidence of infection (number of parasites in mantle tissue/ total oysters examined per site). For this study, presence/absence data from traditional PCR were used to determine percent infected oysters and combined with the QPCR data for calculation of intensity and incidence of infection as described for the FTM calculation of the Mackin Values above.

CHAPTER III

RESULTS

Traditional PCR screening for *H. nelsoni* (MSX) determined no presence of this parasite for in any of the bays sampled during September, October, and November 2007 (Fig. 3A; Tables 2,3,4, and 5). Traditional PCR screening detected *P. marinus* (dermo) in 37 out of 50 oysters in Galveston Bay in October 2007, 38 of 60 oysters in Aransas Bay in September and October 2007, and 43 out of 70 oysters in Corpus Christi Bay September and October 2007 (Fig. 3B; Tables 2,3,4, and 5).

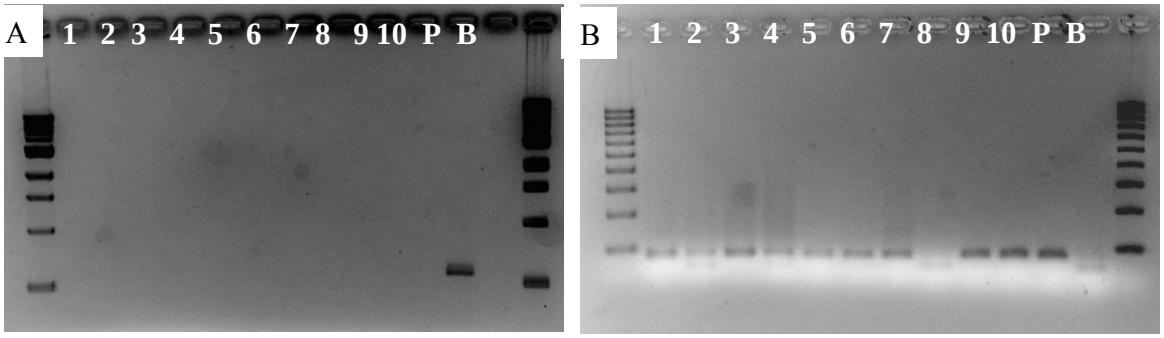

Figure 3. **Verification of traditional PCR assays** for (A) *H. nelsoni* (MSX) and (B) *P. marinus* (dermo). PCR amplicons were visualized on 3% agarose gels stained with ethidium bromide. A 500 bp DNA ladder and 100 bp DNA ladder (Biorad) were used to confirm the size of the *H. nelsoni* (564 bp) and *P. marinus* (86 bp) amplicons, respectively. P = positive control, B = blank.

Traditional PCR detection of *P. marinus* was verified by QPCR (Tables 2,3, 4, 5) but in some cases, the level of amplification was not high enough (below threshold) to be detectable by the SmartCycler camera. Unlike traditional PCR that only determines presence/absence, QPCR amplification curves determine first cycle of first detectable

amplification. Fig. 4 illustrates how sample GB-1 (1-10) and GB-2 (1-3, 6-8) amplifies earlier than the other samples indicating a higher starting concentration of template i.e. number of parasites. The larger the amount of parasites within a sample, the larger the amount of starting dermo DNA which allows for a lower number of amplifications to reach the visible threshold. The number of parasites should be reflected by higher concentrations of DNA after extraction, but we found this to be false. The high quantitative value shown in Figure 4 is for Galveston Bay site 2, sample 6, while the QPCR determined the value of this line to be equivalent to the presence of approximately 5222 cells, the concentration of the extracted DNA for this sample was 25.1 ng/µl. Similarly Galveston Bay site 1 sample 5 quantitative value was around 68 cells, with an extraction concentration of 186.2 ng/µl. This increase in concentration did not increase the quantitative value determined by QPCR. In fact, the Galveston Bay site 1 sample 1, which was non detectable by QPCR, had a very high concentration of 457.4 ng/µl. R^2 values for all QPCR curves were 0.839, low for good correlation.

Table 2. Traditional PCR, Quantitative PCR, temperature and salinity data for Galveston Bay oysters in October 2007.

Site-Sample/	Haplosporidium nelsoni	Perkinsus marinus		Temperature	Salinity
Date Collected	Traditional PCR	Traditional PCR	Quantitative Real-Time PCR	°C	ppt
GB-1/10-26-07				18.1	9.7
1	-	+	<10		
2	-	+	<10		
3	-	+	32		
4	-	+	<10		
5	-	+	68		
6	-	+	<10		
7	-	+	<10		
8	-	+	<10		
9	-	+	<10		
10	-	+	<10		
GB-2/10-02-07				27.6	10
1	-	+	44		
2	-	+	<10		
3	-	+	<10		
4	-	-	0		
5	-	-	0		
6	-	+	5222		
7	-	+	<10		
8	-	+	<10		
9	-	+	<10		
10	-	+	<10		
GB-4/10-26-07			0	17.6	7.6
1	-	+	69		
2	-	-	0		
3	-	+	10>		
4	-	-	0		
5	-	+	160		
6	-	+	10>		
7	-	-	0		
8	-	+	36		
9	-	-	0		
10	-	+	<10		
GB-5/10-02-07				28.1	5.3
1	-	-	0		
2	-	+	<10		
3	-	+	<10		
4	-	+	<10		
5	-	-	0		
6	-	-	0		
7	-	-	0		
8	-	-	0		
9	-	+	<10		
10	-	-	0		
GB-6/10-23-07				19	23
1	-	+	<10		
2	-	+	71		
3	-	+	68		
4	-	+	<10		
5	-	+	<10		
6	-	+	<10		
7	-	+	71		
8	-	-	0		
9	-	+	<10		
10	-	+	65		

Table 3. Traditional PCR, Quantitative PCR, temperature and salinity data for Aransas Bay oysters in September and November 2007.

Site-Sample/	Haplosporidium nelsoni	Perkinsus marinus		Temperature	Salinity
Date Collected	Traditional PCR	Traditional PCR	Quantitative Real-Time PCR	°C	ppt
AB-1/9-10-07				28.6	12.9
1	-	+	<10		
2	-	+	<10		
3	-	+	<10		
4	-	+	<10		
5	-	-	0		
6	-	-	0		
7	-	-	0		
8	-	-	0		
9	-	-	0		
10	-	-	0		
AB-2/9-10-07				29.2	12.8
1	-	-	0		
2	-	-	0		
3	-	-	0		
4	-	-	0		
5	-	+	<10		
6	-	+	<10		
7	-	+	<10		
8	-	+	<10		
9	-	+	<10		
10	-	-	0		
AB-3/9-10-07				29.1	5.7
1	-	+	<10		
2	-	-	0		
3	-	-	0		
4	-	+	<10		
5	-	+	<10		
6	-	-	0		
7	-	+	<10		
8	-	-	0		
9	-	-	0		
10	-	-	0		
AB-1/11-12-07				22.9	20.2
1	-	+	<10		
2	-	+	<10		
3	-	+	<10		
4	-	+	<10		
5	-	+	46		
6	-	+	<10		
7	-	+	<10		
8	-	+	<10		
9	-	-	0		
10	-	+	85		
AB-2/11-12-07				23.2	15.8
1	-	+	<10		
2	-	+	<10		
3	-	-	0		
4	-	-	0		
5	-	+	43		
6	-	+	33		
7	-	+	<10		
8	-	+	<10		
9	-	+	<10		
10	-	+	<10		
AB-3/11-12-07				23.1	12.2
1	-	+	<10		
2	-	+	<10		
3	-	+	80		
4	-	-	0		
5	-	-	0		
6	-	+	<10		
7	-	+	<10		
8	-	+	391		
9	-	+	<10		
10	-	+	<10		

Table 4. Traditional PCR, Quantitative PCR, temperature and salinity data for Corpus Christi Bay oysters in September 2007.

Site-Sample/	Haplosporidium nelsoni	Perkinsus marinus		Temperature	Salinity
Date Collected	Traditional PCR	Traditional PCR	Quantitative Real-Time PCR	°C	ppt
CCB-1/9-09-07					
1	-	+	<10		
2	-	+	<10		
3	-	+	153		
4	-	+	<10		
5	-	+	<10		
6	-	+	76		
7	-	-	0		
8	-	+	55		
9	-	-	0		
10	-	-	0		
CCB-2/9-09-07				30.4	24
1	-	-	0		
2	-	+	60		
3	-	+	193		
4	-	+	35		
5	-	-	0		
6	-	+	54		
7	-	+	<10		
8	-	+	33		
9	-	+	77		
10	-	+	35		
CCB-3/9-09-07				30.1	24
1	-	-	0		
2	-	+	<10		
3	-	+	129		
4	-	-	0		
5	-	+	37		
6	-	-	0		
7	-	-	0		
8	-	-	0		
9	-	+	157		
10	-	-	0		
CCB-4/9-05-07				27.9	29.3
1	-	-	0		
2	-	-	0		
3	-	+	<10		
4	-	-	0		
5	-	-	0		
6	-	-	0		
7	-	-	0		
8	-	+	<10		
9	-	-	0		
10	-	-	0		

Table 5. Traditional PCR, Quantitative PCR, temperature and salinity data for Corpus Christi Bay oysters in November 2007.

Site-Sample/ Date Collected	Haplosporidium nelsoni	Perkinsus marinus		Temperature	Salinity
	Traditional PCR	Traditional PCR	Quantitative Real-Time PCR	°C	ppt
CCB-1/11-11-07				25.2	27
1	-	?	ND		
2	-	?	ND		
3	-	?	ND		
4	-	?	ND		
5	-	?	ND		
6	-	?	ND		
7	-	?	ND		
8	-	?	ND		
9	-	?	ND		
10	-	?	ND		
CCB-2/11-11-07				24.1	27
1	-	+	<10		
2	-	+	<10		
3	-	+	114		
4	-	+	181		
5	-	+	113		
6	-	+	4530		
7	-	+	253		
8	-	+	184		
9	-	+	<10		
10	-	+	<10		
CCB-3/11-11-07				24.1	31
1	-	+	<10		
2	-	+	<10		
3	-	-	0		
4	-	-	0		
5	-	-	0		
6	-	+	<10		
7	-	+	<10		
8	-	+	<10		
9	-	+	<10		
10	-	-	0		
CCB-4/11-12-07				23.7	31.5
1	-	+	<10		
2	-	+	<10		
3	-	+	500		
4	-	-	0		
5	-	+	<10		
6	-	-	0		
7	-	+	<10		
8	-	+	<10		
9	-	-	0		
10	-	-	0		

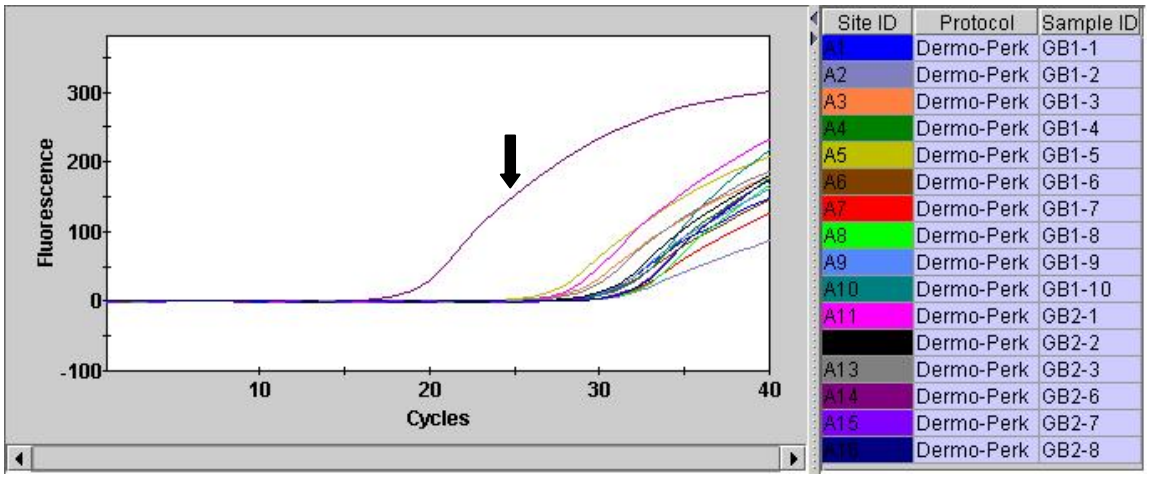

Figure 4. Percent fluorescence generated per cycle by the quantitative PCR method using the Cepheid SmartCycler II and SYBR green intercalating dye in the PCR reaction mixture. Amplicons generated during lower cycles (arrow) indicate a higher concentration of organisms/ml (i.e. DNA template) in the PCR reaction mixture whereas amplicons generated at later cycles represent low starting concentration.

QPCR values of *P. marinus* (dermo) for Galveston Bay Site 1 ranged from <10 (for non detectable samples) to 67.66 cells, Site 2 from 0 to 5221 cells, Site 4 from 0 to 160 cells, site 5 from 0 to <10 cells, and Site 6 from 0 to 71 cells. September Aransas Bay QPCR values for site 1 ranged from 0 to <10 cells, site 2 from 0 to 10> cells, and site 3 from 0 to <10 cells. Sample QPCR values for November from this bay were site 1 from 0 to 85 cells, site 2 from 0 to 43 cells, and site 3 from 0 to 391 cells. September Corpus Christi Bay QPCR values for site 1 ranged from 0 to 153 cells, site 2 from 0 to 193 cells, site 3 from 0 to 157 cells, and site 4 from 0 to <10 cells. Sample QPCR values for November from this bay were site 2 from <10 to 4530 cells, site 3 from 0 to <10 cells, and site 4 from 0 to 500 cells. At all sites, no discernable trend was observed between salinity and temperature and level of QPCR detectable parasites.

There was strong agreement with Ray's Fluid Thioglycollate Method and PCR (Table 6). Only four of the sample sets did not agree for 'incidence', two of which due the lack of data from Dr. Ray's Oyster Sentinel website. For the other sample sets that did not agree, QPCR found a higher incidence than Ray's FTM. The first disparate site, GB-2, had high 'QPCR' incidence while FTM was moderate. Similarly, QPCR incidence for sample AB-3, sampled in November 2007, was higher than the FTM value.

Table 6. Comparison of Ray's Fluid Thioglycollate Method with PCR to score infection of *P. marinus* in Galveston, Aransas, and Corpus Christi Bay oysters. White cells indicate no infection, yellow indicate medium infection, green indicate moderate infection, and red indicate highly infected.

Date	Site	Ray's Fluid Thioglycollate Method[1]			PCR[4]		
		Percent Infected	Intensity[2]	Incidence[3]	Percent Positive	Intensity[5]	Incidence[6]
	GB-1	60	2.17	1.30	100	18	18.0
	GB-2	60	2.84	1.70	80	665	532.6
Oct-07	GB-4	90	1.82	1.63	60	49	29.5
	GB-5	0	0	0.00	40	10	4.0
	GB-6	100	1.87	1.87	90	36	32.5
	AB-1	30	1.45	0.43	40	10	4.0
	AB-2	50	1.13	0.57	50	10	5.0
	AB-3	20	0.33	0.07	40	10	4.0
Sep-07	CCB-1	0	0	0.00	70	46	32.4
	CCB-2	90	1.41	1.27	80	62	50.0
	CCB-3	70	1.47	1.03	40	83.25	33.3
	CCB-4	20	3	0.60	20	10	2.0
	AB-1	69.2	1.71	1.18	90	22.3	20.0
	AB-2	70	1.67	1.17	80	17	13.6
Nov-07	AB-3	40	1.08	0.43	80	66	53.0
	CCB-2	90	2.33	2.10	100	541	541.0
	CCB-3	40	1.34	0.53	60	10	6.0
	CCB-4	70	2.14	1.50	60	91	55.0

[1]Histological (Ray 1952); The Mackin Values scale: 0 = no observable infection; 1 = slight infection; 3 = moderate infection; and 5 = heavily infected (Ray *pers. Comm. 2009.*).
[2]Calculated as number of parasites in mantle tissue according to Mackin Scale/ number of infected individual oysters per site (oystersentinel.org).
[3]Calculated as number of parasites in mantle tissue according to Mackin Scale/ total oysters examined per site (oystersentinel.org).
4Traditional and quantitative PCR. Traditional PCR scale: High (100+ cells), Medium (10-100 cells), and Low (10-1 cells), and no detectable cells represented by 0
[5]Calculated as number of parasites in mantle tissue estimated by QPCR/ number of infected individual oysters per site determined by traditional PCR screening.
[6]Calculated as number of parasites in mantle tissue estimated by QPCR/ total oysters examined per site.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Traditional PCR screening determined that *H. nelsoni* (MSX) was not present in the Texas bays tested during Sept – Nov. 2007. Salinity (<10 ppt) is thought to be the main factor controlling infection of MSX (Burreson and Ford, 2004). Modeling studies of MSX infection in Chesapeake Bay oysters from 1986 to 1995 determined that winters with sustained temperatures of ≤3 °C followed by a year of low salinity (< 15 ppt) were required to prevent or reduce infection by MSX. However, when average environmental conditions returned, so did the MSX infections (Hofmann et al. 2001). Salinity in Galveston and Aransas Bays at time of sampling ranged from 5.3 to 23 ppt. This influence of freshwater may have been enough to control or prevent MSX from infecting these reefs (Mears 2005). However, salinities in Corpus Christi Bay were consistently above 23 ppt, well within the optimal range for growth of MSX. The lack of infection could point to the absence the purported vector (separate organism), but no such vector has been described (Stokes, 1995). MSX was detected by traditional PCR screening in one oyster collected from the Port Aransas mooring, Aransas Bay (Ulrich et al. 2007). Conditions for MSX may have been favorable for MSX at that date, however we found no evidence of this parasite an any samples. A possible explanation for this disparity is that our DNA extraction technique was not rigorous enough to lyse the MSX cells in oyster tissue. Traditional PCR did, however, detect *P. Marinus* (dermo) in all bays

examined. These data were confirmed with histological screening conducted by the Oyster Sentinel lab and occurrence agreed with salinity and temperature conditions. We encountered problems with DNA extraction efficiency using the Qiagen DNEasy DNA extraction kit with modification for oyster tissue and parasites. This extraction method led to a wide range of extraction concentrations from as low as 5 ng/µl to as high as 500 ng/µl when using roughly 0.15 g of tissue. The extraction method does not differentiate between oyster tissue DNA and parasite DNA. Additional problems are associated with the 'raw number' disparities observed between Q-PCR and histological counts of organisms (i.e. dermo) within the tissue. Moreover dermo enters a trophozoite phase that when immature only contains a few nuclei, but in a mature phase can contain 64+ nuclei before it reproduces. The stage of the parasite influences the number of target genes per cell, thus altering the QPCR enumeration.

Traditional PCR screening yields only presence/absence data without influence of nuclei number, however these data alone are not sufficient to gauge level of infection. None-the-less, traditional PCR is a good method for rapid determination of presence/absence within only a few hours rather than few days as with Ray's FTM. The simple presence or absence of the indicator is easy to read and does not take much experience to determine if it is a real or false positive, Ray's FTM requires more experience to determine the existence of a parasite in the sample. This could allow for oyster fisheries to hire fisheries management technicians to perform the simple PCR method rather than sending samples to researchers such as Dr. Ray to determine the presence of the parasite. The

major caveat to using Traditional PCR for screening is the higher cost and potential for cross contamination of samples. While Ray's FTM may be more time and labor intensive, the incidence of cross contamination is very low.

Despite problems with DNA extraction, we had good agreement between our quantitative PCR determination of intensity and incidence of infection with Ray's Fluid Thioglycollate Method used to analyze the same samples. Although this study is not the first to compare QPCR with Ray's method (Gauthier et al. 2006), it is the first to directly compare PCR and Ray's method conducted by S. Ray and hold's promise for ground truthing of QPCR and eventual transition to PCR screening only. The original QPCR assay developed by Gauthier et al. (2006) used TaqMan probes that are expensive. This study used SYBR green intercalating dye that is less expensive and produced similar results. Caveats of QPCR using SYBR green is the lack of detection at very low concentrations of parasites. This deficiency shows that although QPCR is good for determining relative abundance, it can entirely miss the presence of infection. Other downsides to QPCR is the relative ease at which cross contamination can occur, the increased cost of such probes as SYBR green or TaqMan, and the need for very specific instrumentation, i.e. the SmartCycler, whereas Ray's FTM requires only generic test tubes, lugol's solution and a light microscope. QPCR samples must also be prepared in low light conditions to avoid bleaching of the fluorescent probe. Additionally, amplification curves had a low R value of 0.839, optimal is R values of 99 or greater,

which may have been caused by the low extraction yield for the control that was used to create the standard curve for the assay.

The values given by QPCR had clear trends- low intensity infections which were undetectable, moderate intensities that appeared in the 10 to 100 cell range, and then high intensities shown in samples with 100+ cells. These trends were justified when we averaged the QPCR values to find the incidence for QPCR. This study is the first to 'normalize' QPCR data to the Mackin Values Scale used by the Oyster Sentinel Lab. Other studies (e.g. Gauthier et al. 2006) correlated QPCR values with actual counts obtained with FTM. Additional samples require parallel analysis of the PCR technique and FTM to standardize the degree of infection determined by the PCR technique.

Ground truthing QPCR with Ray's FTM is an enormous step in allowing newer, faster molecular methods to replace standard histopathological methods in fisheries and wildlife management. This project shows that even with some difficulties, QPCR does agree with Ray's FTM. The arbitrarily assigned Mackin values for QPCR in this study may need to be refined with a more definite equation for determining this relationship. This project also leaves many unanswered questions such as: are primer specific probes such as the Taqman probe (Gauthier 2006) better at determine quantities than general intercalating dyes such as SYBR green? Are species specific primers better at identifying dermo than genus specific primers such as the PERK primer which was used in this study? Are more studies needed to determine the presence of MSX in Texas

Bays? With more research into these areas we can better determine the health of Texas Bays and of what role molecular methods will play in determining this prognosis.

REFERENCES

Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. Am. Fish. Soc. Spec. Publ. 18:47-63.

Burreson, E. M., Stokes, N. A., Friedman, C. S. 2000. Increased virulence in an introduced pathogen: *Haplosporidium nelsoni* (MSX) in the eastern oyster *Crassostrea virginica*. J. Aquat. Anim. Health 12:1-8.

Burreson, E. M., Ford, S. E. 2004. A review of recent information on the Haplosporidia, with special reference to *Haplosporidium nelsoni* (MSX disease). Aquat. Living Resour. 17:499-517.

Bushek, D., Allen, S.K.J. 1996. Host-parasite interactions among broadly distributed populations of the eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus*. Mar. Ecol. Prog. Ser. 139:127-141.

Curry, R., Dickson, B., Yashayaev, I. 2003. A change in the freshwater balance of the Atlantic Ocean over the past four decades. Nature 426: 826-829.

Day, J. M., Franklin, M. E., Brown, B. L. 2000. Use of competitive PCR to detect and quantify *Haplosporidium nelsoni* infection (MSX disease) in the eastern oyster (*Crassostrea virginica*). Mar. Biotechnol. 1:147-154.

Ewart, J. W., Ford, S. E. 1993. History and impact of MSX and Dermo diseases on oyster stocks in the northeast region. NRAC Fact Sheet No. 200, University of Massachusetts Dartmouth.

Gauthier, J. D., Miller, C. R., Wilbur, A. E. 2006. Taqman®MGB Real-time PCR approach to quantification of *Perkinsus marinus* and *Perkinsus* spp. in oysters. J. Shell. Res. 25:619-824.

Hofmann, E., Ford, S., Powell, E., Klinck, J. 2001. Modeling studies of the effect of climate variability on MSX disease in eastern oyster (*Crassostrea virginica*) populations. Hydrobiologia 460:195-212.

Mears, G., 2005. Oyster Diseases of Chesapeake Bay: MSX fact sheet. http://web.vims.edu/env/research/shellfish/msx.html?svr=www., Department of Environmental and Aquatic Animal Health.

Ray, S. M. 1952. A culture technique for the diagnosis of infection with *Dermocystidium marinum* (Mackin, Owen, and Collier) in oysters. Science. 114:360-361.

Ray, S. M. “2009”. Personal communication, Texas A&M University, Galveston, Texas.

Reece, K.S., D. Bushek, K.L. Hudson, J.E. Graves. 2001. Geographic distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf coasts of the USA, Mar. Biol. 139: 1047-1055.

Stokes, N., Siddall, M., Burreson, E., 1995. Detection of *Haplosporidium nelsoni* (Haplosporidia: Haplosporidiidae) in oysters by PCR amplification. Dis. Aquat. Org. 23: 145-152.

Ulrich, P.N., Colton, C.M., Hoover, C.A., Gaffney, P.M., Marsh, A.G. 2007. Haplosporidium nelsoni (MSX) rDNA Detected in Oysters from the Gulf of Mexico and the Caribbean Sea. April Journ. Shellfish Res. Vol. 26. No. 1; 195-199

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