ABSTRACT

Title of Document:

DEVELOPMENT, VALIDATION AND APPLICATION OF A QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY TO ASSESS ENVIRONMENTAL SAMPLES FOR *HEMATODINIUM PEREZI* PREVALENCE

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Dinoflagellate parasites in the *Hematodinium* genus have emerged as important pathogens of economically important crustaceans worldwide, causing significant economic losses to fisheries and aquaculture. An understanding of the routes of infection in blue crab (*Callinectes sapidus*) populations would be facilitated by an improved knowledge of environmental reservoirs. A previously used PCR assay, based on small subunit rRNA sequences, lacked the specificity needed for *Hematodinium perezi* detection of environmental samples. Therefore a quantitative PCR assay based on the internal transcribed spacer 2 (ITS2) region

of *H. perezi* rRNA genes was developed, validated, and applied to examine temporal and spatial incidences of environmental reservoirs in Delmarva coastal bays. *H. perezi* was detected in sediment and water in several Delmarva coastal bays, as well as the host, *C. sapidus*. Results suggest the existence of localized sediment reservoirs in areas where hydrological and geophysical features allow for the formation of cell deposits.

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Acknowledgements

I owe much gratitude to my primary and co-advisor, Dr. Eric Schott and Dr. Rosemary Jagus. They have worked to make sure that I understood the basic aspects of my research and master my technical skills.

I am grateful for the support, financial and educational, the NOAA-Education Partnership Program Living Marine Resources Cooperative Science Center (LMRCSC) has provided me. The experiences that I have had as part of the program have been very valuable.

I would like to thank Dr. Sook Chung for teaching me basic blue crab biology and physiology and Dr. Feng Chen, Dr. Russell Hill and Dr. Allen Place who have taught me dinoflagellate ecology, principles of molecular techniques and microbiology. While not directly involved in my research, their expertise has helped to enhance my understanding of my research.

I would like to thank all the graduate students, lab techs and post docs for helping with experiments, giving feedback on presentations and explaining techniques. I am also grateful for the assistance that LMRCSC summer interns provided with experiments.

Lastly I want to thank collaborators from Delaware State; Dr. Dennis McIntosh, Dr. Dewayne Fox, Marissa Brady, Tylor, Johnny Moore, UMES; Dr. Joseph Pitula, Whitney Dyson, and Cooperative Oxford Lab, NOAA-NCCOS; Gretchen Messick.

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Chapter One: General Introduction

Blue crab fishery: The blue crab, *Callinectus sapidus*, is an ecologically and economically significant crustacean with a habitat range from Nova Scotia to Uruguay, South America. It is a key benthic-pelagic link in the marine environment where it forages for food in the benthos and is a staple in the diet of many pelagic fish. In the United States the blue crab supports a fishery with an estimated worth of $160 million annually. There is a large commercial fishery from Maryland to the Gulf of Mexico. In the Chesapeake Bay the annual value of harvest is over $50 million [1]. From Massachusetts to Delaware it is an important recreational fishery.

There was a time when the blue crab was so abundant the harvests were described as "inexhaustible" [2], however prior to 2009 the blue crab population in the Chesapeake Bay had seen a decade long decline [3]. According to the 2010 Chesapeake Bay Blue Crab Advisory Report the historically low population sizes have been associated with high levels of exploitation [4]. Other states such as North Carolina, South Carolina and Georgia have also experienced declines in their blue crab populations. In the Chesapeake Bay, the persistently low blue crab broodstock stimulated the formation of a research consortium to study hatchery-based broodstock enhancement and led to the implementation of unprecedented harvest restrictions [5]. One approach of this research consortium was to assess various areas for their suitability as habitat for entry of hatchery reared blue crabs that would grow into future broodstock. Potential habitat needed to be assessed for disease prevalence to ensure greater success of crab

survival. Since 2009, in the Chesapeake Bay harvest restrictions and improved

stock management practices have helped to alleviate the pressure on the blue

crab population, however it is typical to have year-to-year fluctuations in

abundance that cannot be explained by fishing pressure alone [6]. In the context

of efforts to sustain a vigorous fishery, it is critical to monitor disease-causing

agents. It is now agreed that estimates for natural mortality of blue crab are over-

simplified and even though many blue crab diseases have been described, their

influence on mortality and shaping of the population are poorly understood [7–

10].

In mid-Atlantic coastal bays of North America, the blue crab population

suffers episodic mortalities associated with infections by the parasitic

dinoflagellate, *Hematodinium perezi* [11–13]. It is found in both hemolymph and

tissues of the blue crab where the parasites can multiply rapidly, eventually

causing organ failure resulting in crab death. These outbreaks generally recur

seasonally and are geographically localized in "hotspots," which are

characterized by constrained, high salinity, warm waters. Outbreaks most likely

have a varied effect on the blue crab population and most probably go unnoted.

For example, Lee and Frischer [14] reported such high mortality rates in a

Georgia outbreak that the fishery was affected widely for several years, whereas

an outbreak we observed in a Delaware coastal bay(see **Chapter 2**) was highly

localized and transient. The fact that these outbreaks are seasonal and not

ongoing shows that there exists some reservoir of the parasite. The presence of

Hematodinium sp. is of significant concern since hotspots of this pathogen have

been shown to exist in several blue crab fisheries throughout the mid-Atlantic [12, 13, 15]. In particular, release of hatchery crabs should avoid hotspots where this parasite could affect survival. A survey of the parasite in wild blue crabs, blue crab prey, water and sediment samples could help in the understanding of the infection cycle and the impact on survival rates. An understanding of the life cycle, infection dynamics and environmental prevalence and incidence should yield insight into why these outbreaks and recurring infections occur.

Life cycle of *Hematodinium* sp.: The life cycle of *Hematodinium* sp. within the blue crab is still poorly understood, despite several morphologically distinct stages being reported from the hemolymph and tissues [10, 12, 16–18] and from short-term cultures (7–14 days) of the parasite [19, 20]. These findings suggest that *Hematodinium* sp. has a very complex life cycle. Naturally infected blue crabs often die over a period of 14 to 35 days depending on the intensity of the infection [12]. Experimentally infected blue crabs start dying 14 to 17 days post-inoculation (p.i.), with cumulative mortalities of 86 % by 40 days p.i. [10] and 100 % by 55 days p.i. [12]. Cells of the parasite undergo characteristic developmental changes *in vitro,* consistent with stages of *Hematodinium* sp. identifiable in infected crabs: filamentous trophonts, amoeboid trophonts, arachnoid trophonts and sporonts, sporoblasts, prespores and dinospores (macrospores and microspores) (**Figure 1.1**) [21]. In culture, an unusual shunt in the life cycle has been observed as presumptive schizonts derived from arachnoid sporonts developed into filamentous and arachnoid trophonts that can then initiate arachnoid sporonts in new cultures [21]. This may explain the rapid proliferation

of the parasite in blue crab hosts. Li *et al.* observed the effect of temperature and

light intensity on the growth and development of the parasite *in vitro* by

comparing cultures grown at 10 °C, 15 °C, and 23 °C in the dark and at 23 °C in
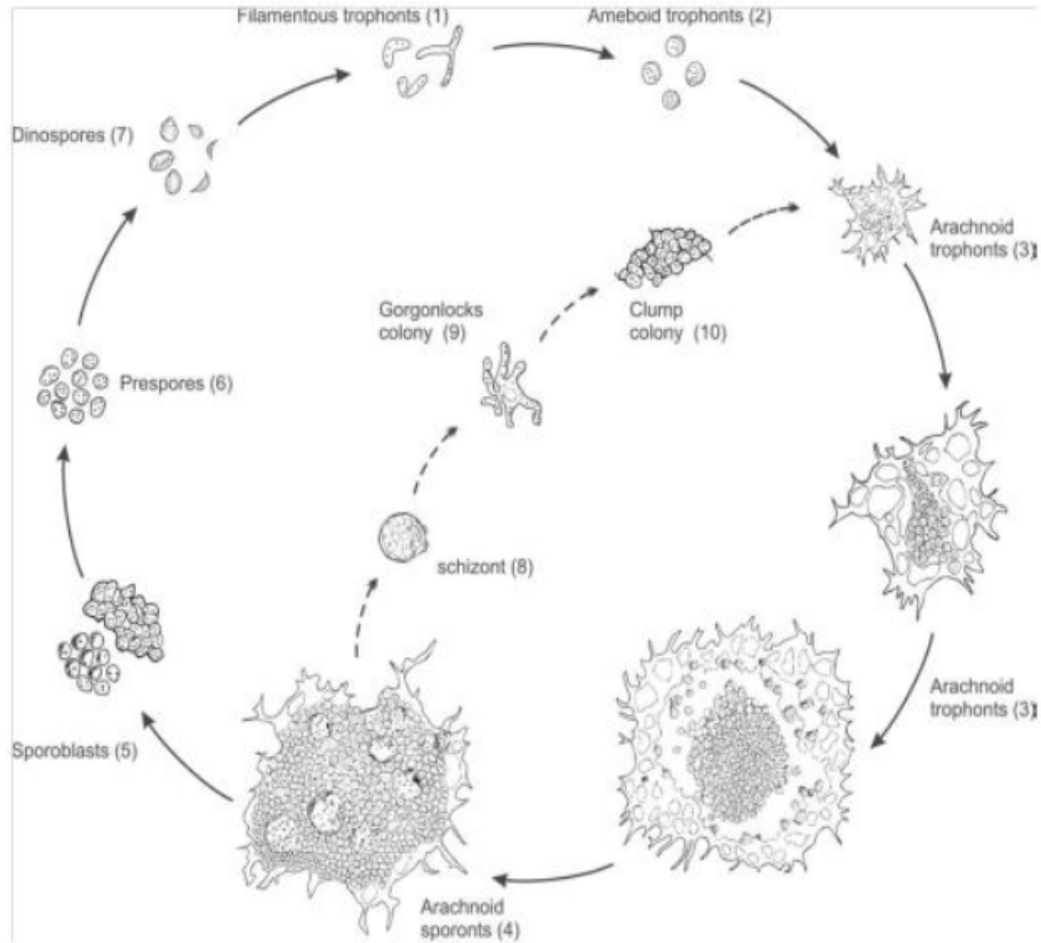
Figure 1.1: Schematic diagram of the *in vitro* life cycle of *Hematodinium* sp. from *Callinectes sapidus*. adopted from Li *et al.* [21].

complete light, light/dark and complete dark cycles [21]. While cultures grew at

each condition, only the culture grown under complete dark at 23 °C completed

the life cycle as depicted in **Figure 1.1**. This may give clues as to what

environmental factors aid in episodic disease outbreaks.

Similar developmental stages have been observed in cultures of the cold water species of *Hematodinium* from the Norway lobster, *N. norvegicus* [22].

Crabs possess a circulatory system described as semi-open or semi-closed. Hemolymph is distributed though the body by a series of arteries and veins and generally accumulate in sinuses where hemocytes and hemolymph diffuse into or from surrounding tissues. As a result, parasites that enter the hemolymph tend to become systemic in nature. Such infections affect hemocyte densities and hemolymph chemistry [10, 23–27]. A common feature of systemic infections, including *Hematodinium*-like infections, is that they are accompanied by severe or complete hemocytopenia. As parasite numbers increase, hemocyte densities decrease [13, 24]. It has been observed that in *Hematodinium* sp. infections trophonts dominate early infections, mixed densities of trophonts and pre-spores are observed in intermediate infections, and pre-spores dominate in late to terminal infections.

Proposed methods of transmission: Multiple studies have shown that *Hematodinium* sp. and *Hematodinium*-like spp. possess a dinospore stage that exits the crab host during sporulation [13, 14, 28, 29]. Dinospores have been observed in terminal infections as white "clouds," of either macro- or micro-dinospores, that escape through the mouth [29]. Love [30] and Tamone *et al*. [31] reported that macrospores and microspores of the *Hematodinium*-like sp. infecting *Chionoecetes bairdi* were able to survive in seawater for 73 and 52 days respectively, while Appleton [32] reported that viable microspores of the *Hematodinium*-like sp. infecting *Nephrops norvegicus* could be maintained in

water for 49 days. As for the *Hematodinium* sp. that infects blue crabs, there is growing evidence that the parasite resides in the water column as short-lived dinospores [33–35]. For example, an aquarium study by Frischer *et al.* [33] showed that *Hematodinium* DNA, presumably from free-living dinospores, could be detected in the water column. These investigators also reported that exposure of *Hematodinium*-free *C. sapidus* to water with *H. perezi* resulted in the transmission of the parasite to the crabs. These observations indicate that recurring infections could be facilitated by transmission of dinospores in the water column. Alternatively, as many dinoflagellates do, *H. perezi* dinospores may require a preparatory cyst phase that is currently unrecognized [36]. Because a small portion of cultured dinospores develop into filamentous trophonts, it has been speculated that the mode of parasite transmission in nature involves infective dinospores [21]. Furthermore, as the filamentous trophont is routinely observed in infected crustaceans from field studies [21, 37, 38], Pitula *et al.* postulated that released dinospores from infected hosts may develop into trophonts in the water column and/or an intermediate vector [36].

Recently the role of alternate host species in recurring infections has been examined [39, 40]. New infections could also be associated with potential zooplankton vectors, such as amphipods and crab larvae [24, 39, 41]. The literature reports higher prevalence of *Hematodinium* sp. in juvenile crabs <30 mm carapace width (CW) [12]. However there is a lack of information concerning the prevalence of *Hematodinium* sp. in the early life stages of blue crabs. Consequently the earliest stage in the blue crabs life cycle that can be infected

has not been established. Also, over-wintering crabs could carry low-level infections that can be transmitted to other blue crabs after emerging from the sediment in the spring. Pagenkopp *et al.* [39] have confirmed infection by the same *Hematodinium* sp. in *C. sapidus* and five alternate host species (*L. dubia, L. emarginata, E. depressus, P. herbstii*, and *P. pollicaris*) from the Delmarva Peninsula and Virginia, including three new hosts for this parasite, (*L. dubia, E. depressus*, and *P. pollicaris*). They also report PCR evidence for *Hematodinium* sp. in the caprellid amphipod, *Caprella geometrica*.

As blue crabs are known to be cannibalistic, this has been proposed to be a mode of transmission. However there have been conflicting results with cannibalism studies in laboratory settings. An earlier study by Walker *et al.* in which naïve crabs were fed infected tissue and obtained infections, supported transmission via cannibalism [42]. However, a more recent study by Li *et al.* using a similar approach has not confirmed these results and concluded that the individuals that developed disease in the earlier study were most likely harboring low-level infections prior to the experiment [21].

Comparison of research methods: Earlier studies looking at the prevalence and level of *Hematodinium* sp. infection in blue crabs have been done using histological techniques. Investigations of the parasite that use this technique involved fixed and stained hemolymph and tissue sections [11, 12] or the use of neutral red dye to stain parasites in hemolymph [13, 19, 43]. While this approach is good for *in vivo* morphology studies, it is impractical for environmental samples. More recently developed, DNA-based, molecular tools have provided a

sensitive and flexible methodology for detecting parasites in virtually any sample from which DNA can be extracted [44–46]. There have been several advances in using polymerase chain reaction (PCR) based assays in answering several *Hematodinium* sp. questions [34, 36, 39, 47, 48]. Endpoint PCR based assays were initially developed [33, 35], however it was observed that this method is not as sensitive as quantitative PCR (qPCR) and may not provide the sensitivity needed to assess prevalence and incidence in environmental samples. Furthermore, initial assays were designed with primers that targeted the small subunit (SSU) of the ribosomal RNA genes [33, 35, 49]. While this is sufficient to document *Hematodinium* sp. infections in blue crab hemolymph, it is not specific enough for analysis of water and sediment. Primers designed based on the more conserved SSU region have been shown to potentially detect other *Hematodinium* sp., *Hematodinium*-like spp., and *Syndiniales* [34]. **Table 1.1** shows BLAST analysis using the amplicon generated by the Nagle *et al.* [49] qPCR primers. These primers returned several Genbank entries for free living dinoflagellates whose amplicon sequence shares 100 % identity with the primer and probe sequence. Furthermore, additional sequences in the top 100 BLAST hits showed only a single nucleotide substitution within the 5' primer, raising the possibility that a false positive could occur from imperfect annealing. Given that environmental samples can harbor hundreds of free-living dinoflagellate species, that may possess similar SSU sequences [50, 51], which can co-occur with *Hematodinium* sp. potentially yielding false positives, a new qPCR assay targeting a different region of the rRNA gene had to be developed to provide the

sensitivity and specificity needed to detect the *Hematodinium* sp. that infects blue crabs in environmental samples.

Table 1.1: BLAST analysis against amplicon generated by Nagle primers		
GB number	**Species**	**Habitat**
HQ438156	Uncultured marine alveolate clone North_Pole_S.	North pole
JF791095	*Leucocryptos marina*	Hypoxic gulf of mexico
JF275463	Uncultured dinoflagellate clone plate95f11.g1	Columbia River estuary
GU825000	Uncultured eukaryote clone AA3F14RJ1B04	Cariaco Basin, Caribbean
FJ914413	Uncultured marine dinoflagellate clone B11	southeastern North Carolina
EF675764	*Hematodinium* sp. ex *Carcinus maenas* Cm887	Hamilton et al 2010.
GU825563	Uncultured eukaryote clone BCI5F14RJ3H06	Cariaco Basin, Caribbean

Taxonomic identification of *Hematodinium* sp. infecting blue crabs: The recent increase in available *Hematodinium* sp. DNA sequences has advanced our understanding of its global diversity and distribution. Historically there are only two formally described species; *H. perezi* and *H. australis*. *H. perezi* was first described by Chatton & Possion infecting the portinids, *C. maenas* (green crab) and *Liocarcinus depurator* (swimming crab) both collected from the French coastline [43]. *H. australis* has a somewhat more recent description from Hudson and Shields where it was observed in *Portunus armatus* (Australian sand crab)

collected from Moreton Bay, Australia [52]. For many years the lack of detail in descriptions of morphological, ultrastructure and sequence data of the parasite described in over 38 infections, has led to confusion over speciation [47]. As a result the *Hematodinium* species described infecting blue crabs on the east coast of the United States have been referred to as *Hematodinium* sp. and *H. perezi* [11, 14, 16]. Due to more molecular studies from described infections and the recent rediscovery of *H. perezi* infection in the type host *L. depurator* originally described by Chatton & Possion, some clarity has developed in the issue of taxonomy [47, 48]. Analysis of 18S rDNA and internal transcribed spacers 1 and 2 (ITS1, ITS2) loci suggests that at least two clades exist in crustaceans from the North Pacific and Atlantic Oceans [20, 35, 48, 53]. Morado [54] describes the presence of one clade/species residing in deep, stenohaline waters infecting *C. opilio, C. bairdi, C. tanneri* and *N. norvegicus* and another clade/species residing in shallow, euryhaline waters infecting *C. sapidus, L. depurator* and *C. maenas*. Jensen *et al.* characterize them as clade B and clade A respectively [48]. Small *et al.* [47] further describes clade A as being broken down further to include three subtly different genotypes which appear to be classified by infection of hosts from three geographic locations. Based on ITS1 sequences, *Hematodinium* sp. infecting blue crabs from the eastern seaboard of the United States is characterized as being *H. perezi* genotype 3 [47, 55]. The assay we have developed, described in **Chapter 2**, has been designed to be specific to clade A *Hematodinium* based on an alignment of our ITS2 sequences and those deposited in GenBank. For this reason, I refer to the parasite studied as *H. perezi*

belonging to genotype 3. Because of inter-strain differences in the ITS1 region, the ITS2 region was chosen for the target sequence. At the time the assay was designed there were no ITS2 sequences available. In this thesis, when speaking generally, I make reference as *Hematodinium* sp. or *Hematodinium*-like sp.

The vast majority of *Hematodinium* sp. infections in the Chesapeake Bay region occur in the predominant crustacean species, *C. sapidus*, which has been classified as a clade A host species [48]. Based on the high similarity of the ITS1 rRNA region sequences obtained (98 %), and the similarity in morphology and pathology between host species, Pagenkopp *et al.* [39] provide evidence that only one species of *Hematodinium* infects a broad range of crustaceans in the high salinity waters of the Delmarva Peninsula, Virginia. *H. perezi* is a host generalist in the region and probably occurs in a wide range of hosts throughout the temperate western Atlantic.

In summary, research on the blue crab was prompted by a decline in the population of this socio-economical and ecological significant crustacean. The parasitic dinoflagellate, *Hematodinium* sp., was given much attention as being the causative agent for several mass mortality events that included the crash of localized blue crab fisheries. However, although *Hematodinium* sp. was emerging as a significant pathogen, little was known about it. Questions about the complete life cycle, such as how reinfections occur, and the role of alternate hosts, needed to be answered in order to understand how the pathogen was affecting the blue crab population. The main focus of the research was to use molecular tools to track incidence of *H. perezi* in environmental samples. The

following chapter (**Chapter 2**) shows the development of a qPCR assay for *H. perezi*, the validation of the assay, as well as its application to assess *H. perezi* incidence in environmental samples.

Chapter Two: Variation in Temporal and Spatial Incidence of the *Hematodinium perezi* in Environmental Samples from Atlantic Coastal Bays

Abstract:

Background: Along the Atlantic and Gulf coasts of the United States, the blue crab, *Callinectes sapidus*, suffers recurring mortality associated with infections of *Hematodinium perezi*. Whether the annual infections of this parasitic dinoflagellate come from biotic or environmental reservoirs has not been determined. An understanding of the potential for transmission from such putative reservoirs would be facilitated by an improved knowledge of where and when the parasite can be detected in environmental samples. A previously used PCR assay for *H. perezi*, based on the small subunit rRNA gene sequence, was found to lack adequate species specificity to discriminate non-*Hematodinium* sp. dinoflagellate species in environmental samples. We therefore sought to develop and apply a quantitative PCR assay based on the internal transcribed spacer 2 (ITS2) region of *H. perezi* rRNA genes. Primers for the ITS2 region were based on an alignment of rRNA gene sequences of *H. perezi* infecting *C. sapidus* from Atlantic and Gulf coasts. The ITS2-targeted *H. perezi* assay was used to examine the variation of temporal and spatial incidence in environmental reservoirs in Delaware and Maryland coastal bays.

Results: To develop and validate methods for sediment DNA extraction and qPCR detection of the parasite, we used a bacterial surrogate in place of *H. perezi* cells. A standard curve made from DNA extracted from sediment spiked

with *E. coli* carrying the *H. perezi* rDNA genes displayed limits of detection and qPCR efficiency comparable to detection of purified cloned plasmid. Application of the method to environmental samples identified a potential *H. perezi* hotspot in sediment in Indian River Inlet, DE. *H. perezi* was not, however, detected in co-occurring shrimp or snails, even during an outbreak of the parasite in *C. sapidus*. Application of the ITS2-targeted assay to DNA from water/plankton and sediment from Chincoteague Bay revealed that the parasite was detectable at various times from April through November, in 15 of 18 sites throughout the Bay. The abundance of *H. perezi* in the water column in the spring was unexpected, as crab infections with the parasite typically peak in the fall. In Chincoteague Bay, a potential hotspot was also detected, as defined by a location that was positive for *H. perezi* in 6 of the 9 months sampled.

Conclusions: *H. perezi* is present in water and sediment samples in Maryland and Delaware coastal bays from April through November with a wide spatial and temporal variability in incidence of the parasite. Sampling sites with high levels of *H. perezi* DNA in both bays share characteristics of silty, organic sediments and low tidal currents. The detection of *H. perezi* in spring months was unexpected, pointing to gaps in our understanding of the development of the parasite prior to infection in crabs as well as the mode of environmental transmission. These findings emphasize the need for a better understanding of the *H. perezi* life cycle, as well as the need for monitoring the parasite in habitats as well as hosts. A better understanding of potential environmental transmission to crabs will facilitate the development of disease forecasting.

Background

The blue crab, *Callinectes sapidus*, is an ecologically and economically significant crustacean with a geographic distribution from maritime Canada to Uruguay. It is a key epibenthic link in the near shore marine environment where it feeds on invertebrates and fish, and is a component of the diet of many coastal fish species. In the Atlantic and Gulf coast of the United States the blue crab supports a commercial fishery with an estimated worth of $165 million [56] and a substantial recreational fishery of undetermined magnitude that extends as far North as Massachusetts. There was a time when the blue crab was so abundant the harvests were described as "inexhaustible" [2], however the blue crab population in the Chesapeake Bay has been at historic lows for most of the past decade [3], most likely due to levels of exploitation [4]. Other states such as North Carolina, South Carolina and Georgia have also experienced declines in their blue crab population and harvests [57]. In the Chesapeake Bay, the persistently low blue crab broodstock stimulated the formation of a research consortium to study hatchery-based broodstock enhancement and motivated Maryland and Virginia to implement unprecedented harvest restrictions [5, 9]. Though harvest restrictions have helped alleviate the strain on the blue crab population, it is typical to have year-to-year fluctuations in abundance that cannot be explained by fishing pressure. It is now understood that estimates for natural mortalities of blue crab are over simplified and even though many blue crab diseases have been described, their influence on mortality and shaping the population are poorly understood [7, 8, 10, 23].

In mid-Atlantic coastal bays of North America, blue crab populations suffer episodic mortalities associated with infections by a parasitic dinoflagellate in the genus *Hematodinium* sp. [13, 14]. Currently, there are two formally described species in the genus: *H. perezi* and *H. australis*. *H. perezi* was first described by Chatton and Poisson infecting the portunid crabs *C. maenas* (green crab) and *Liocarcinus depurator* (swimming crab) on the French Mediterranean coast [43]. In Australia, *H. australis* was described in *Portunus pelagicus*, the sand crab (also confusingly known as "blue swimmer crab") [52]. Crustaceans residing in deep, stenohaline waters, such as *Chionoecetes opilio, C. bairdi, C. tanneri* and *Nephrops norvegicus*, are infected by yet another genotype of *Hematodinium* sp. distinct from *H. perezi* and *H. australis* [48, 54]. For many years, data gaps in descriptions of morphology, host range and sequence data, led to uncertainty in species designations, [47] resulting in the *Hematodinium* species infecting blue crabs on the east coast of the United States being referred to as *Hematodinium* sp. [11, 14, 16]. The recent work by Small *et al.* confirms that the *Hematodinium* sp. infecting blue crabs on the US Atlantic coast is *H. perezi* [47, 55].

Epizootics of *H. perezi* in blue crab can recur seasonally and are geographically localized in "hotspots", characterized by constrained, high salinity, warm waters, suggesting that there are environmental or biotic reservoirs of the parasite [12]. The parasite is found in the hemolymph and tissue of the blue crab where the it can multiply rapidly, eventually causing organ failure resulting in crab death [13]. *Hematodinium* spp. have complex life cycles that include trophont and sporont cell types within the host and flagellated dinospores that are

occasionally observed within and emerging from infected hosts [22, 33, 58]. Recently, Li *et al*. reported on the *in vitro* culture of the entire *H. perezi* life cycle [21].

Detection of *Hematodinium* sp. can be achieved histologically, by looking for the parasite in preserved and stained hemolymph or tissue sections [11, 13], or using neutral red dye to stain live parasites in fresh hemolymph [13, 19, 43]. Although histology is useful in confirming the presence of *Hematodinium* sp. cells in host tissues, it is impractical for detecting the parasite in environmental samples. Efforts have been made to develop molecular techniques for rapid detection of *Hematodinium* sp. in several commercially important species [35, 45, 46, 48, 49]. DNA-based molecular tools provide a sensitive and flexible methodology for detecting parasites in virtually any sample from which DNA can be extracted. Earlier research, using either endpoint polymerase chain reaction (PCR) or quantitative PCR (qPCR), targeted the SSU region of the parasite ribosomal RNA gene to document *Hematodinium* sp. infections in blue crab hemolymph [35, 49]. Although these primers were sufficient for detection within blue crab, the sequences targeted were not specific enough for analysis of environmental samples, which may contain hundreds of free-living dinoflagellate species that possess SSU gene sequences similar to those of *Hematodinium* sp. [34, 45]. Furthermore, assays based on end-point PCR may not provide the sensitivity or quantitative abilities needed to assess prevalence in environmental samples in comparison to qPCR.

There are two frequently encountered challenges when using qPCR to look for organisms in the environment. First, the accuracy of a qPCR assay rests on the ability to produce a reliable standard curve, which is usually based on DNA extracted from a pure culture of the organism itself or on plasmid DNA carrying the target sequence of the organism of interest. Until recently [21], *H. perezi*, cultures were not readily available for use in generating standard curves, and reliable passage of infections from wild isolates posed significant technical hurdles. The second challenge is that, in practice, accuracy of a qPCR method also depends on reproducible extraction of target DNA from environmental samples. An assay based on purified or cloned target DNA may not be representative of how parasite DNA is recovered from environmental samples in which there is a heterogeneous mixture of DNA from other species, in addition to potential qPCR inhibitory compounds. Therefore, to develop methods suitable for environmental assessment and prevalence studies, we sought to design and validate a qPCR assay targeting the species-specific ITS2 region of the rRNA gene, and establish a standard curve using a surrogate for *H. perezi* cells mixed with environmental sediment. We then used this assay to look for *H. perezi* in various environmental samples on a temporal and spatial scale.

Methods:

Sources of *Hematodinium* sp. infected blue crabs and DNA extractions

Hematodinium sp.-infected blue crabs and ethanol-fixed blue crab hemolymph from the Gulf of Mexico were kindly provided by colleagues at the Gulf Coast Research Lab (GCRL; Noah Zimmerman, Jeff Lotz) and shipped to the Institute

of Marine and Environmental Technology (IMET) by overnight courier. Blue crabs infected with *Hematodinium* sp., obtained from the MD coastal bays, were driven to IMET in coolers and frozen after arrival. Originally identified by the appearance of parasites in fresh hemolymph, all *Hematodinium* sp. infections were verified by PCR amplification using the primers described in Nagle *et al.* [49] or Gruebl *et al.* [35].

DNA was extracted from hemolymph of confirmed *Hematodinium* sp.-infected crabs using the Qiagen DNAEasy Blood and Tissue Kit (Qiagen, Valencia, CA, Cat. # 69506). For DNA extractions from field-collected samples of crabs and other invertebrates, the MoBio Tissue Kit was employed (MoBio, Carlsbad, CA, cat. # 2334). DNA was extracted from water and sediment samples using the MoBio Ultraclean Soil kit (cat. #12800), which includes mechanical tissue disruption. DNA was quantified by spectrophotometry (Nanodrop Inc., Wilmington, DE).

Amplification and cloning the ITS1, 5.8S, and ITS2 regions of the ribosomal gene cluster

Using Taq polymerase (ExTaq, Takara Bio, Inc., Otsu, Shiga, Japan), and primers 1487 and D2C (**Table 2.1**), amplicons encompassing the ITS1, 5.8S, and ITS2 regions were amplified from genomic DNA preparations of a Gulf coast and Atlantic coast *H. perezi-* infected blue crab.

Hematodinium-specific primer 1487 [35] and a generic eukaryotic primer D2C [59] were used to amplify and clone a ~2 kb region of the rRNA gene cluster from DNA extracted from 4 individual *H. perezi* -infected crabs from both Maryland

and Mississippi. Thermocycling conditions were 95 °C 5 min, followed by 30

cycles of 94 °C, 25 sec; 54 °C, 30 sec; 72 °C, 3 min. The resulting ~2 kb

fragment was ligated to pGEM-T according to manufacturer's instructions

(Promega Corp., Madison, WI) and transformed into *E. coli* strain JM109

(Promega Corp.).

Table 2.1: Oligonucleotides used in this study			
Oligo-nucleotide	Sequence (5' to 3')	Purpose/target	Source
D2C	CCTTGGTCCGTGTTTCAAG	Dinoflagellate LSU	Scholin et al. (1994)
1487F	CCTGGCTCGATAGAGTTG	Hematodinium SSU	Greubl et al. (2002)
1654R	GGCTGCCGTCCGAATTATTCAC	Hematodinium SSU	Greubl et al. (2002)
PlaceF	GGGTAATCTTCTGAAAACGCATCGT	Hematodinium SSU	Nagle et al. 2009
PlaceR	GTACAAAGGGCAGGGACGTAATC	Hematodinium SSU	Nagle et al. 2009
ITS2for	AGGTCTAATGCTTGTTGGCC	Hematodinium ITS2 qPCR	This study
ITS2Rev	CACTAGTCCGAAAACCTGTG	Hematodinium ITS2 qPCR	This study
HemITSF2	TCGTAACAAGGTTTCCGTAGG	Hematodinium SSU	This study
HemITSR2	GACCCAGCCTTCACGATAAA	Hematodinium LSU	This study
HemITS2 probe	6-FAM-ACCGCTACTCT-TCTTCCGCCCT-BHQ1a	Hematodinium ITS2 qPCR	This study
M13 F	CGCCAGGGTTTTCCCAGTCACGAC	pGEM-T vector	
M13 R	TCACACAGGAAACAGCTATGAC	pGEM-T vector	

Sequencing was performed on a minimum of 3 clones from each infected crab

(Gulf or Atlantic), using a combination of primers originating in the pGEM-T

vector sequence and cloned *H. perezi* DNA (see **Table 2.1**: primers 1487, M13F,

M13R, ITS2for, ITS2rev, HemITSF2, and HemITSR2). Sequencing was

conducted using Big Dye Terminator reagents (Applied Biosystems, Inc.,

Carlsbad, CA) and analyzed with the ABI 3130 XL Genetic Analyzer (Applied

Biosystems, Inc.). Sequences were aligned using Sequencher (Genecodes

Corp, Ann Arbor, MI) and CLC DNA Workbench (CLC Bio, Aarhus, Denmark).

From assembled consensus sequences, additional primers were designed to

amplify specific segments of the ITS1-5.8S-ITS2 region to provide additional

sequence depth [36]. A consensus sequence of 2056 nucleotides was derived

from the assembly and has been submitted to GenBank as accession JQ815886.

Development of ITS2-targeted qPCR assay

BLAST analyses of the consensus sequence were used to identify boundaries

between SSU, ITS1, 5.8S, ITS2 and LSU regions, as compared to cognate

sequences in GenBank [60]. Based on the ~280 bp ITS2 region, PCR primers

ITS2For and ITS2Rev (**Table 2.1**) were designed (CLC bioinformatics software).

A TaqMan probe (HemITS2probe) was designed, carrying 6-FAM and BHQ1 as

reporter and quencher dyes, respectively. Using the above primers at 400 nm,

probe at 300 nm, Taq-pro complete (Denville Scientific, Metuchen, NJ) with 2.5

mM final $MgCl_2$ and 0.25 mg/ml BSA (Idaho Technologies, Salt Lake City, Utah),

thermocycling was carried out using the following conditions: initial heating to 95

°C, 5 min; followed by 95 °C, 15 sec; 58 °C, 30 sec, 45 cycles. For routine

analyses, thermocycling was performed on 1 μl of DNA (10 to 50 ng) using an

Applied Biosystems Fast7500 thermocycler (Indian River Inlet samples) or Bio-

Rad iCycler iQ Optical Model (Chincoteague Bay samples). Cross checking

confirmed that the assay performed with the same sensitivity on both iCycler and

Fast7500 thermocyclers.

Sensitivity and Specificity of the ITS2 assay

The sensitivity of the ITS2 assay was investigated by conducting qPCR on a serial dilution of plasmid pES103, which carries the 2 kb amplicon produced from primers 1487 and D2C, encompassing the 3' end of the SSU gene as well as complete ITS1, 5.8S, ITS2 and partial LSU genes. A subclone of pES103 was produced by PCR amplification using primers ITS2for and ITS2rev (0.4 µM each), to produce pES146 (in pGEM-T). Based on spectrophotometric measurements of plasmid concentration, a series of samples from 13 to 1.3x 10e6 copies were prepared in nuclease free water. Quantitative PCR was conducted on 1 µl of each dilution in triplicate and the results plotted as Ct versus copy number. Slope and R^2 of the standard curve were calculated using the ABI software (Applied Biosystems 7500 Fast Real-Time PCR system).

Validation of the qPCR assay using *Hematodinium* sp. cell surrogates

Because *H. perezi* cells were not easily obtainable, it was necessary to develop an *H. perezi* surrogate to use in the validation of the assay. This cell surrogate consisted of *E. coli* strain JM109 carrying the plasmid pES103, carrying the *H. perezi* ITS2 region. The *H. perezi* surrogate was grown in LB+AMP liquid broth medium overnight. Based on OD_{A600} of 1.9 = 1 x 10^9 cells* ml^{-1} we made a 10 fold serial dilution of the overnight culture that was calculated to have from 3 to 3 x 10^5 bacteria/µl to spike into sediment. The bacteria cell number was verified by plating overnight culture on LB+AMP plates. DNA extraction of the *H. perezi* surrogate was done using the MoBio Tissue and Cells DNA extraction kit per manufacture instructions. For spiked sediment, the serially diluted *H. perezi*

surrogate was added to sediment from Baltimore Harbor (and shown to be negative for *H. perezi* by ITS2 qPCR). DNA was then extracted from each spiked sediment sample using the MoBio Soil DNA extraction kit per manufacture instructions. We used the qPCR assay to compare slopes and limits of detection using 1 μl of DNA for qPCR.

Environmental sample collection and DNA extraction

Sampling locations and seasons: Sampling was conducted at 12 sites in the Indian River Inlet during July of 2008 and 2009 (Figure 3A). Sampling was conducted at 18 sites in Chincoteague Bay, Newport Bay, and Sinepuxent Bay (Figure 3B) between April and November of 2010, in conjunction with the National Park Service water quality monitoring program. The only exception was September, when only three sites were sampled: Verrazano Bridge (Site 2), Newport Bay (Site 3), and Public Landing (Site 5).

Biotic Samples: Estuarine invertebrates were collected 07.29.08 in Indian River Inlet near sites 1, 5, 6, and 7 at low tide by hand or using long handled nets. In the field, individual animals were placed in labeled ziplock bags and kept on ice until transport to the lab, where they were stored at -20 ˚C. DNA was extracted using the MoBio Cell and Tissue Kit. From snails (*I. obsoleta*), DNA was extracted from a cross section of the viscera that included the stomach or intestine. From grass shrimp (*P. pugio*), DNA was extracted from a cross section of the abdomen that included the intestine. From crab species (*C. sapidus*, *C. maenas*, *O. ocellatus*), DNA was extracted from walking leg or back fin muscle tissue.

Sediment: The top 3-5 cm of sediment was collected within the DE (Indian River Inlet) and Chincoteague Bay, MD, and VA coastal bays. In shallow water (DE), sediment was collected directly with a 50 ml conical tube. In water over 1.5 m deep, a petit ponar grab was used to obtain sediment, and the top layer collected with a 50 ml conical tube and maintained on ice for transport to the laboratory. Sediment samples were mixed with an equal volume of 15 ppt sterile artificial seawater (Crystal Sea, Marinemix, Baltimore, MD) and 0.5 ml aliquots were frozen at -20 °C for up to 6 mo until DNA extractions were performed.

DNA was extracted from Indian River Inlet sediment using the MoBio UltraClean Soil DNA kit, following manufacturer's recommendations, with the exception that DNA was eluted from the purification matrix in two aliquots of nuclease free water rather than a single elution. DNA preparations were divided into two aliquots and stored at -80 °C. DNA was extracted from MD and VA coastal bays sediment using the Sureprep (Fisher Bioreagents) soil isolation kit.

Water: In Indian River Inlet, surface water samples were collected using 1, 2, and 4 L bottles. Water was maintained at 0-4 °C until further processing. For 2008 samples, 60 ml aliquots were filtered onto 2.5 cm diameter 1 μM filters (Millipore) using a Swinnex cartridge. DNA was extracted from filters using the MoBio UltraClean Soil DNA kit. For 2009 samples, water was centrifuged at 500 xg for 30 min to sediment particles, and the entire pellet was extracted with the MoBio UltraClean Soil DNA as described above.

In Chincoteague Bay, water was sampled using a 30 cm diameter plankton net, towed at ~3 knots for 3 min, resulting in a sampling of ca. 25 m^3. The collected

material was saved in 500 ml seawater, refrigerated, and transported to the

laboratory where 200 µl was used for DNA extractions using the Illustra tissue

and cells genomicPrep™ DNA kit (GE Healthcare).

Results:

Design of an ITS2-targeted PCR assay for *H. perezi* ex *C. sapidus*

Initial efforts to detect DNA of *H. perezi* in environmental DNA samples using an

SSU-targeted qPCR assay [49] indicated that DNA of non-target organisms may

produce spurious signal. When the Nagle et al. [49] SSU primers (PlaceF,

PlaceR, **Table 2.1**) were used to amplify DNA extracted from sediment from

Parsons Creek, MD (N38.5017°, W76.2669°), a low salinity subestuary of the

Chesapeake Bay with no record of *H. perezi* infections, a positive qPCR signal

was nevertheless obtained (data not shown). The product displayed the

expected mobility on agarose gels, but its sequence was an imperfect match to

the available sequences for *H. perezi* such as JQ815886, [49], showing a single

base substitution relative to *H. perezi*. A BLAST database search using the

predicted *H. perezi* SSU amplicon returned at least 10 non-*Hematodinium* sp.

protozoan species that are identical in sequence to the primers and probe, and

therefore would be expected to generate positive results. Furthermore the list of

matching organisms included species found in mid-Atlantic and Gulf coast

waters, in areas that also harbor *H. perezi*, which demonstrated the need to

develop a new primer set (BLAST analysis of primers and probe is shown in

Table 1.1).

Based on the likelihood that amplification of DNA other than *H. perezi* would occur with DNA samples from water or sediment using the Nagle *et al.* [49] primers, we designed a more specific set of primers, targeting the internal transcribed spacer (ITS2) region. To first asses variability in the ITS regions, a 2 kb amplicon of the *H. perezi* ribosomal DNA gene cluster, encompassing the 5' end of the SSU, entire ITS1-5.8S-ITS2 and partial LSU regions was cloned and sequenced from 4 infected crabs, 2 from Maryland and 2 from Mississippi. Sequences of all clones were very similar, with the exception of a 3 base insertion/deletion (ATA) in the ITS1 region and two single nucleotide polymorphisms in ITS1 and 5.8S regions. A consensus sequence of the region is deposited in GenBank as accession JQ815886, with annotations to identify the locations of inter-crab polymorphisms. A schematic of the locations of individual single nucleotide polymorphisms (SNPs) and the insertion/deletion (indel) is depicted in **Figure 2.1**. Sequence polymorphisms did not correlate with geographic origin of the *H. perezi* infections. For example, both forms of the (ATA) indel were found in infections from Maryland and Mississippi. In fact, both variants were found in the DNA extracted from a single infected crab from MD. Direct sequencing of the PCR products obtained from a single infected crab showed mixed bases (double peaks) present at nucleotide positions that showed SNPs or indel between cloned amplicons.

Figure 2.1: Schematic representation of *H. perezi* rDNA locus, showing locations of PCR primer pairs, derived from GenBank entry JQ815886. PlaceF/R primers are from Nagle *et al.* [29], and target the SSU gene. Primers Schott F/R are from Pitula *et al*. [38], and amplify the 5.8S gene. Primers Bowers F/R (ITS2For/Rev **Table 2.1**) were developed in the current study. The star and diamonds indicate locations of indel and single nucleotide polymorphisms, respectively, between clones of *H. perezi* derived from independent infections of *C. sapidus*. Polymorphisms are annotated in GenBank JQ815886.

Because we observed SNPs and an indel in the ITS1 and 5.8S regions (even within a single infected crab) but not in the sequences of the ITS2, we targeted the ITS2 region for development of a qPCR assay. Application of a computer algorithm from the CLC Workbench program and visual inspection led to the design of primers ITS2For and ITS2Rev, which produce an amplicon of 99 bp, as well as the HemITS2probe (**Table 2.1**) carrying a FAM fluorophore and BHQ quencher (see Methods). When used to amplify a serial dilution of purified plasmid (pES146) carrying the *H. perezi* ITS2 rRNA region, the assay had a typical efficiency of at least 93 % and a sensitivity of 13 copies.

Validation of the qPCR assay using *Hematodinium* sp. cell surrogates

In order to develop useful standard curves applicable to environmental samples, an *H. perezi* surrogate was used, which consisted of the cloned *H. perezi* ITS2

region carried in *E. coli* (see Methods). A qPCR standard curve of plasmid DNA was compared to DNA extracted from a dilution series of the *H. perezi* surrogate, and to DNA extracted from sediment spiked with a dilution series of the *H. perezi* surrogate. The slopes of each dilution series were -3.39 (purified plasmid), -2.70 (*H. perezi* surrogate), and – 3.22 (sediment with *H. perezi* surrogate) (**Figure 2.2**). In order to determine if sediment type and background DNA would have an effect on the efficiency; we also spiked two different types of sediment, sandy and silty, and found that this did not affect the efficiency or sensitivity of the qPCR assay (data not shown). Prior to spiking, these sediment samples were shown to be negative for *H. perezi* by ITS2-targeted qPCR.

In recent years there has been evidence showing that using linearized target DNA could amplify more than 1 cycle earlier than circular cDNA for a given amount of input DNA, resulting in a miscalculation of gene copy number in unknown samples [61]. To address this issue we compared the slopes and limits of detection of plasmid pES146 in the linear and circular conformations. We found that the linearized target and intact plasmid gave rise to similar Ct numbers compared to those from use of circular DNA. Therefore we conclude that the standard curve target conformation would have only a minimal effect on the sensitivity of the assay (data not shown).
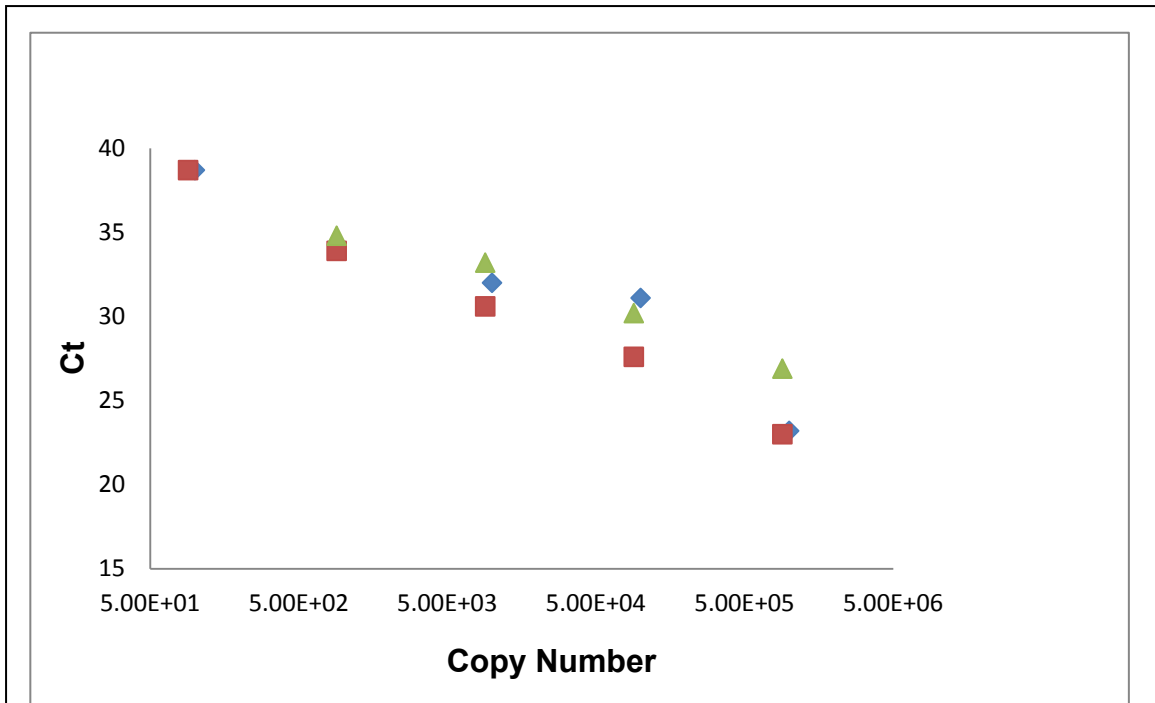
Figure 2.2: Comparison of the performance of the *H. perezi* qPCR assay with purified target DNA, and DNA from sediment spiked with a surrogate for the parasite. Standard curves were generated from 10-fold serial dilutions of DNA corresponding to purified cloned plasmid pES103 DNA (symbol ◆slope of -3.39), DNA prepared with the MoBio kit, from *E. coli* containing plasmid pES103 (symbol ▲,slope of -2.70), and DNA prepared with the MoBio kit from sediment spiked with *E. coli* containing plasmid pES103 (symbol ■, slope of -3.22).

Using the qPCR assay to detect *H. perezi* on a spatial scale in sediment in Indian River Inlet

On three dates in 2008 (07.01.08, 07.29.08, 12.09.08), eight sites within the Indian River Inlet were sampled (**Figure 2.3**). Water and sediment were collected on both July trips; sediment only was collected on the December trip. DNA was extracted from water and sediment, and qPCR assays for *H. perezi* were conducted as described in **Methods**.

Of the 07.01.08 samples, only sediment DNA from site IR7 produced signal by

qPCR (**Table 2.2, 2008 section**). From the 07.29.08 sampling, three sites

produced positive qPCR signal; IR1, IR2, and IR4. There were no positives

among the December sediment samples. None of the DNA extracted from 0.1

liter water samples from any 2008 site produced ITS2 qPCR signal.

Figure 2.3: Map of Indian River Inlet sampling sites. In 2008, sites
sampled were 1, 2-1, 2-2, 3, 4, 5, 7, and 12. In 2009, sampling was
expanded to all locations (1 through12). Symbols (▲, ■, X) refer to 2009
data only. As noted in **Table 2.2**, sediment from 2008 sites numbered 2-1,
2-2, 3, and 7 were positive for *H. perezi* DNA. In 2009, sediment sampling
was expanded to include all 13 locations, and triplicate samples were taken
at each site. Sites from which none of the triplicate samples were positive
are indicated by (**X**), sites with one of the triplicate samples positive are
indicated with triangles (▲), and sites from which all three triplicates were
positive are indicated with squares (■).

Indian River sediment and water samples positive for *H. perezi* by the ITS2 assay				
	Sampling date	Positive sites	Sediment type	gene copies/g sediment
Sediment	**2008**			
	07.01.08	IR7	sandy clay	1.0E+04
	07.29.08	IR2-1	silty sand	5.9E+03
		IR2-2	sand	4.4E+03
		IR3	silty sand	5.8E+03
	2009			
	07.15.09	IR1a	silty sand	2.0E+02
		IR2-1c	silty sand	1.5E+02
		IR3a	silt	1.7E+03
		IR3b	silt	9.5E+02
		IR3c	silty sand	2.3E+02
		IR4a	silty sand	1.7E+02
		IR4b	silty sand	1.1E+03
		IR4c	silty sand	3.6E+02
		IR7a	sand	1.5E+03
		IR8c	silt	2.2E+02
		IR10b	silt	2.3E+02
		IR12b	silt	4.0E+02
	2009			
				gene copies/L water
Water	07.15.09	IR1		6.1E+02
		IR3		4.0E+02
		IR11		1.3E+02
		IR12		8.2E+ 01

Table 2.2: Levels of *H. perezi* from Indian River Inlet sediments: DNA was extracted from sediment samples and qPCR was used, as described in Methods, to detect *H. perezi* in environmental samples of sediment from described locations in Indian River Inlet, DE, from July 2008 and 2009. Results are displayed as *H. perezi* ribosomal gene copy number per g sediment.

During the 07.29.08 sampling trip, many dead and dying *C. sapidus* were

observed in Indian River Inlet. No mass mortality of other species was detected.

Twenty dead or moribund *C. sapidus*, as well as apparently healthy individuals of

two additional crab species (*Ovalipes ocellatus* and *Carcinus maenas*) were collected from the eastern end of Indian River Inlet. DNA was extracted from leg muscle of all crabs and tested for *H. perezi* by the ITS2 qPCR assay (**Table 2.3**). Sixteen of the *C. sapidus*, as well as both the *C. maenas* and *O. ocellatus* were found to be positive for *Hematodinium* sp. The *C. sapidus* infections displayed from 1.01E+06 to 1.63E+08 gene copies per g tissue, while the *C. maenas* and *O. ocellatus* infections were

Levels of *H. perezi* DNA in *C. sapidus* on 07.29.08 in Indian River Inlet		
Crab ID	Species	gene copies/g tissue
2-1A	*C.sapidus*	<10
2-1B	*C.sapidus*	<10
2-1C	*C.sapidus*	3.76E+07
2-1D	*C.sapidus*	<10
2-1E	*C.sapidus*	1.63E+08
2-1F	*C.sapidus*	-*
5A	*C.sapidus*	2.99E+07
5B	*C.sapidus*	3.59E+07
5C	*C.sapidus*	1.01E+06
1A	*C.sapidus*	2.53E+06
1B	*C.sapidus*	4.55E+07
1C	*C.sapidus*	4.05E+06
1D	*C.sapidus*	4.05E+06
1E	*C.sapidus*	1.16E+07
1F	*C.sapidus*	1.43E+08
1G	*C.sapidus*	1.04E+07
1H	*C. maenas*	1.79E+06
7A	*C.sapidus*	7.54E+07
7B	*C.sapidus*	9.88E+06
7C	*C.sapidus*	1.08E+08
7D	*O. ocellatus*	4.79E+05

* PCR inhibition prevented assessment of this sample.

Table 2.3: Levels of *H. perezi* in blue crab during an outbreak in Indian River Inlet, DE: DNA was extracted and qPCR was used, as described in <u>Methods</u>, to determine *H. perezi* sp. copy number per g tissue from crabs collected at Indian River Inlet, DE, on 07.15.08 during a *H. perezi* outbreak.

at 1.79E+6 and 4.79E+05 gene copies per g tissue, respectively. Two of the *C. sapidus* DNA samples showed strong PCR inhibition (failure to amplify even when spiked with 10e5 copies of the cloned target).

During the apparent *H. perezi* outbreak, snails (*Ilyanassa obsoleta*) were found to be foraging amongst dead and dying crabs. In addition, abundant grass shrimp (*Palaemonetes pugio*) were present in the waters in which the outbreak occurred. Both species were assessed for *H. perezi* using the qPCR assay. Despite being amongst crabs that were positive for *H. perezi*, none of the non-crab species were found to produce a positive signal (**Table 2.4**).

Incidence of *Hematodinium* sp. in Indian River invertebrates 07.29.08.			
Year	**Species**	**N**	**% Prevalence**
2008	*Callinectes sapidus*	19	79
	Ovalipes ocellatus	1	100
	Carcinus maenas	1	100
	Palaemonetes pugio	12	0
	Ilyanassa obsoleta	12	0
2009	*Orchestia grillus*	7	0
	Palaemonetes pugio	10	0

Table 2.4: Levels of *H. perezi* in biotic samples from Indian River collection sites: DNA was extracted and qPCR was used, as described in Methods, on biotic samples collected during the July 2008 and 2009 sampling trips to give a percent prevalence of *H. perezi* in each species.

Based on the findings from 2008 sediment samples, a modified sampling plan was designed for 2009. In particular, 2009 sampling focused effort in the vicinity of sites 3 and 4, expanding a transect along an East-West line and conducting sampling in triplicate at each location. Additional sample sites were also added

in the western end of Indian River Inlet, shortly downstream of a coal-fired electric generating station (sites IR9-12 in **Figure 2.3**). In 2009 two sampling trips were conducted: 06.01.09 and 07.15.09. None of the samples from 06.01.09 were positive for *H. perezi* by the ITS2 assay. In contrast, 8 of the 12 sites during the 07.15.09 sampling trip yielded positive signal in at least one of the triplicate samples (**Table 2.2, 2009 section**). Six of the sites had only one of three replicates positive (sites 1, 2-1, 7, 8, 10, 12). However sites IR3 and IR4 produced positive signal from all three of the samples within the transect.

In 2009, at each location, 1 L of water (instead of 0.1 L as in 2008) was collected and later centrifuged in the laboratory to collect particulates. DNA extracted from the pelleted material was then tested for *H. perezi* using the ITS2 qPCR assay. Four of the sites (IR1, IR3, IR11, IR12) produced signal in this assay (**Table 2.2, 2009 section**).

Unlike the 07.29.08 sampling trip, in 2009 *C. sapidus* mortality was not observed, and no moribund or dead *C. sapidus* were encountered. Therefore, no *C. sapidus* were collected during the 2009 sampling trips. However, two non-crab species were opportunistically collected: the amphipod *Orchestia grillus* and grass shrimp *P. pugio*. Neither of these species yielded positive results in the ITS2 *H. perezi* assay (**Table 2.4**).

Using qPCR to investigate *Hematodinium* sp. incidence on a seasonal scale in Chincoteague Bay

Figure 2.4: Map of Chincoteague Bay sampling sites: From April through November of 2010 and 2011, sediment and water/plankton samples were collected from 18 sites monitored by the National Park Service water quality program. DNA was extracted from these samples for subsequent PCR analysis to monitor the presence of *H. perezi*. (Map is courtesy of the National Park Service). Sites are represented by numbers in open circles: 1: Commercial Harbor; 2: Verrazano Bridge; 3: Newport Bay; 4: Trappe Creek; 5: Public Landing; 6:Whittington Point; 7: Taylor's Landing; 8: Wildcat Point; 9: Greenbackville; 10: Sinnickson; 11: Chincoteague Channel; 12: Assateague Channel; 13: Tom's Cove; 14: Johnson's Bay; 15: Cedar Island; 16: South Point; 17: Ocean City Inlet; 18: Snug Harbor.

In a study to follow the temporal and large-scale spatial occurrence of *H. perezi* in a coastal bay ecosystem, eighteen sites within Chincoteague Bay were sampled on a monthly basis (in conjunction with the National Park Service water quality monitoring program) from April to November of 2010 (**Figure 2.4**). Both plankton and sediment were collected at each location, and DNA was extracted for analysis by the ITS2-targeted qPCR assay. Overall, 34 of the 324 Chincoteague Bay water and sediment samples (10.5 %) were positive for *H. perezi* DNA by the ITS2-targeted qPCR assay (**Table 2.5A & B**).

Chincoteague Bay Sediment Samples				
	Sampling Date	Positive Sites	Sediment Type	Gene copies/g sediment
Sediment	04.10	Verrazano Bridge (a)	sandy silt	3.9E+3
		Snug Harbor (b)	sandy	1.3E+4
	05.10	Sinnickson (a)	sandy silt	7.1E+3
		Sinnickson (b)	sandy silt	9.8E+3
		Sinnickson (c)	sandy silt	1.7E+4
	06.10	Newport Bay (a)	silt loam	1.4E+4
		Newport Bay (b)	silt loam	2.7E+3
	07.10	Verrazano Bridge (b)	sandy silt	5.4E+3
		Sinnickson (a)	sandy silt	1.5E+4
		Sinnickson (b)	sandy silt	8.7E+3
		Sinnikson (c)	sandy silt	9.4E+3
	08.10	Whittington Point (a)	sandy clay	1.3E+4
		Newport Bay (a)	silt loam	1.3E+4
		Tom's Cove (a)	silt clay	5.4E+4
		Tom's Cove (b)	silt clay	2.2E+4
		Trappe Creek (a)	silt loam	2.1E+4
	09.10	Newport Bay (a)	silt loam	3.7E+4
		Newport Bay (b)	silt loam	1.7E+4
	10.10	Sinnickson (a)	sandy silt	1.5E+4
	11.10	Tom's Cove (b)	silt clay	7.4E+4

Table 2.5A: List of Chincoteague Bay sediment samples positive for *H. perezi* DNA 2010-2011: Listed are sites at which *H. perezi* was detected in the sediment over the course of a two-year survey. Three samples of sediment were collected at each site. The letters a, b, and c represent which sample from a specific site tested positive. Dates are indicated by the month, followed by the year. Soil type was determined by personal observation and the Cornell University Fact Sheet.

There was no obvious geographic pattern or concentration of sediment signal in

any one region or month. Fifteen of the sites were positive at least once during

the 9-month study. The three sites that never tested positive for *H. perezi* DNA

(Assateague Channel (12), Ocean City Inlet (17), and South Point (16) are in areas with high tidal flux and have mostly sandy sediment. Ocean City and Assateague Channel Inlet are at the constricted north and south

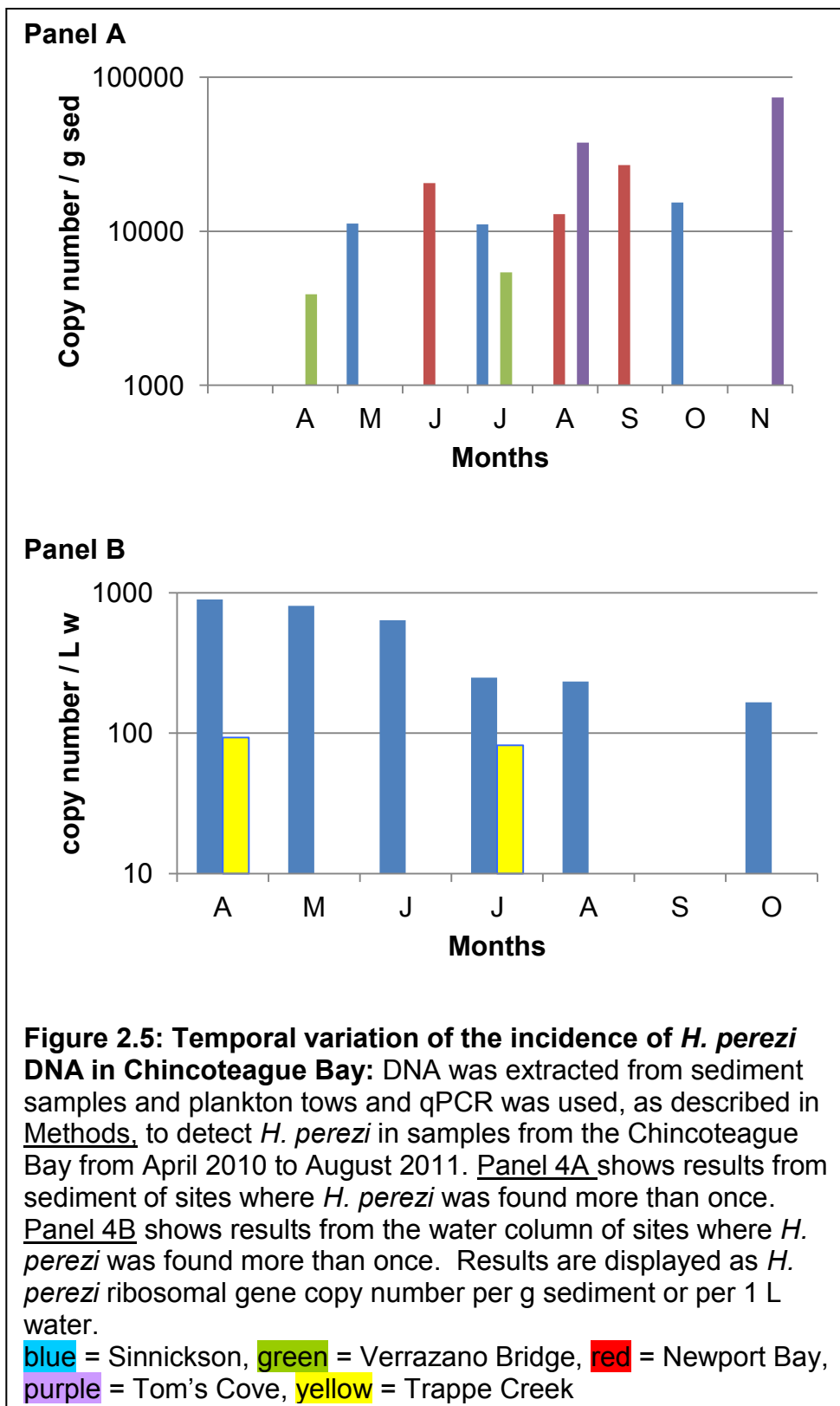| Chincoteague Bay Water Samples			
	Sampling Date	**Positive Sites**	**Gene copies/L water**
Water	04.10	Trappe Creek	9.3E+1
		Sinnickson	9.0E+2
		Johnson's Bay	6.4E+1
	05.10	Sinnickson	8.1E+2
	06.10	Newport Bay	2.7E+2
		Sinnckson	6.4E+2
		Taylor's Landing	3.3E+2
	07.10	Sinnickson	2.5E+2
		Trappe creek	8.2E+1
		Ocean City Inlet	6.9E+1
		Cedar Island	62.E+2
	08.10	Sinnickson	2.3E+2
	09.10	Public Landing	5.3E+2
	10.10	Sinnickson	1.7E+2

Table 2.5B: List of Chincoteague Bay water samples positive for *H. perezi* DNA 2010-2011: Listed are sites at which *H. perezi* was detected in the water over the course of a two-year survey. Dates are indicated by the month, followed by the year.

ends of the Bay, where water velocity is expected to be highest. Sediment from

4 sites tested positive for *H. perezi* in two or more months (**Figure 2.5, Panel A)**.

These 4 sites (Verrazano (2), Newport Bay (3), Sinnickson (10), Tom's Cove

(13)), encompass most of the north-south axis of Chincoteague Bay, as well as

the east-west axis. The physical characteristics of the 4 sites are quite diverse: two are near freshwater inputs (Sinnickson and Newport Bay), Tom's Cove is a shallow bay with no freshwater input, and Verrazano is near deep water in a wide channel between Ocean City inlet and the main Chincoteague Bay. One common feature of all four sites is that the sediment contained silt in addition to clay, loam or sand.

In contrast to the results for sediment, which showed at least one positive sample at most sites, analysis of plankton samples revealed that just eight of the locations were positive by the qPCR assay during the 9-month study (**Table 2.5B**). Plankton samples from six of these sites tested positive only once, plankton samples from one site tested positive twice (Trappe Creek (4)), and plankton samples from Sinnickson (10), a site on the western side of Chincoteague Bay, were positive for *H. perezi* in 6 of the 9 months sampled in 2010 (**Figure 2.5, Panel B**).

Panel A

Panel B

Figure 2.5: Temporal variation of the incidence of *H. perezi* DNA in Chincoteague Bay: DNA was extracted from sediment samples and plankton tows and qPCR was used, as described in Methods, to detect *H. perezi* in samples from the Chincoteague Bay from April 2010 to August 2011. Panel 4A shows results from sediment of sites where *H. perezi* was found more than once. Panel 4B shows results from the water column of sites where *H. perezi* was found more than once. Results are displayed as *H. perezi* ribosomal gene copy number per g sediment or per 1 L water.
blue = Sinnickson, green = Verrazano Bridge, red = Newport Bay, purple = Tom's Cove, yellow = Trappe Creek

Discussion:

Along the US Atlantic coast, *H. perezi* infections in blue crabs display an annual peak of prevalence and intensity in late summer or autumn [12]. Conversely, *H. perezi* infections disappear between peaks, and therefore raise the question of whether there is a source (reservoir) of new *H. perezi* infections. Possible (not mutually exclusive) *H. perezi* reservoirs include other blue crabs, alternate hosts, and the environment. There have been conflicting reports as to the role of cannibalism in transmission [34, 42]. *Hematodinium* sp. has been detected in numerous alternate hosts sympatric with (and possible prey for) blue crab [40], but only at low prevalence and intensity [39]. *H. perezi* is a dinoflagellate, and the existence of a water-borne dinospore stage is well documented [33, 34]. Additionally, like other dinoflagellates, *Hematodinium* spp. may have a resting stage as (either a true cyst, or another overwintering stage) which could form the equivalent of cyst beds and could be a source of re-infection. Recent work by several researchers supports a model in which dinospores released into the environment are a likely source of infection [33, 34, 36, 41].

A straightforward approach to investigating whether environmental reservoirs of *H. perezi* exist is to use genome-targeted PCR assays to explore when and where DNA of *H. perezi* is found in Atlantic coastal bays. A number of PCR assays have been developed to detect *Hematodinium* spp. DNA, all of which target one of the regions within the ribosomal RNA locus [20, 34, 35, 45, 49]. The SSU region of the rDNA locus shares high sequence identity between

different species of *Hematodinium* (including the various clades described by others [20, 48, 62]). The *Hematodinium* sp. SSU also shares significant identity to other, free-living, dinoflagellates, as we discovered before the initiation of the present study. As reflected in **Table 1.1,** ,-BLAST analysis revealed that the primers and probe used in Nagle *et al.* [49] are predicted to amplify the SSU sequences from presumptive free-living dinoflagellates on both the Gulf and Atlantic coasts. More species-specific sequences are found in ITS1 [21] and ITS2 regions, which are transcribed as part of the polycistronic rRNA product, but are not part of the mature ribosomal RNAs (SSU, 5.8S and LSU).

For our qPCR assay we chose the ITS2 region for two reasons: firstly, when rDNA clones derived from several *H. perezi* infections in MD and MS were compared, the ITS2 region displayed fewer clone-to-clone SNPs and length polymorphisms, as compared to the ITS1 region; and secondly, the inter-species variation of the sequence of the ITS2 region is constrained by its role in processing of the final rRNA molecules. The ITS2 region of the rRNA transcript folds into a cruciform structure that interacts with splicing enzymes. Coleman [63] and others [64] have reported on the advantages of the ITS2 as a molecular feature for taxonomic comparisons between congeneric species. Our finding that a single *H. perezi* -infected crab had two versions of an ITS1 indel could be interpreted to mean that a single strain of *H. perezi* carried both versions, or that the crab carried a dual infection of two strains that differ in that nucleotide position. There is precedent for the former possibility: Matsumoto *et al.* [65] have reported within-strain length variation of ITS1 sequences (indels) in insects.

In this study we validated DNA extraction and qPCR methods to detect *H. perezi* in environmental samples, based on primers targeted to the ITS2 portion of the rRNA locus of *H. perezi* DNA from environmental samples is frequently seen to carry PCR inhibitors, so it was important to demonstrate that the assay performed well with DNA extracted from environmental sources (sediment and water). Because the hypothetical sediment or water-borne forms of *H. perezi* were not available for 'spiking' sediment, and because the stages of *H. perezi* found in blue crab hemolymph were not readily available, we used sediment spiked with bacteria carrying the cloned PCR target as a surrogate to validate the DNA extractions and PCR assay. Using this approach, we estimated that the PCR method has a limit of detection (in DNA extracted from sediment) of 9 gene copies per qPCR reaction, which translates to a lower limit of 900 copies per gram sample of sediment. It is still not certain, however, how few parasite cells can be detected by the method. The uncertainty derives from disagreement in the literature as to how many rRNA gene copies there are per *H. perezi* cell. Nagle *et al*. [49], targeting the SSU, reported up to 10,000 copies/cell, while Li *et al*. [34], targeting the ITS1 region, reported rRNA gene copies on the order of 300 copies/cell. Interestingly, in a recent study of the rRNA gene copy number in a free living dinoflagellate, Perini *et al.* [66] reported that, depending on isolate and stage in culture, *Ostreopsis cf. ovata* may possess from 81 to 8488 copies/cell of the LSU gene. This example provides one possible explanation for why the two estimates of *H. perezi* rRNA gene copy number may be so divergent: that different isolates and different culture stages of the same species may indeed

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have widely different copies of the rRNA genes. It would be worthwhile to assess the number of rRNA genes per *H. perezi* cell from a number of different isolates of the parasite and different developmental stages within the host. The recent development of *in vitro* culture for *H. perezi* by Li *et al*. [21] may provide very valuable material for a standardized assessment of rRNA gene copy number through the different life stages.

Although the question of how many copies of the rRNA locus are present per cell of *H. perezi* was not addressed in the current study, the field data we obtained is pertinent to the issue. Many of the environmental samples in the current study contained from several hundred to several thousand ITS2 targets per gram sediment or per liter water/plankton. These values are more consistent with the gene copy numbers of Li *et al*. [34] unless we postulate that DNA recovery was poor, or that naked *H. perezi* DNA (representing less than one cell/genome) was present in environmental samples. Using dilutions of ethanol-preserved *H. perezi* cells, we have also generated qPCR data that are more consistent with the lower number of gene copies/cell (Hanif and Schott, unpublished).

There are several findings we would highlight from the application of the qPCR *H. perezi* assay to the coastal bay study. 1) *H. perezi* signal was widely detected throughout both coastal bays, and a potential sediment hotspot was detected in Indian River Inlet. 2) *H. perezi* DNA was detected in water samples from geographically dispersed areas in both coastal bays as well as temporally prevalent in Chincoteague Bay, being detected at Sinnisckson during all three

seasons in which samples were collected. 3) *H. perezi* DNA was not found in alternate invertebrates examined, even though they were collected in the vicinity of a mass mortality of blue crabs infected with *H. perezi* in Indian River Inlet.

Consistent detection of *H. perezi* DNA in sediment is consistent with the hypothesis that there is a sediment reservoir for the parasite. Hamilton *et al.* [67] suggested that, like other dinoflagellates, *Hematodinium* spp. may have a resting stage outside of its host as a part of its life cycle. Though a cyst or resting stage has not been observed by microscopy, molecular methods have been used to detect parasite DNA in sediment samples [21, 29]. There is mounting evidence that dinospores, the free-living stage of *H. perezi* are probably involved in transmission. Frischer *et al.* [33] presented evidence that the dinospore stage could establish infections in naïve blue crabs upon *in situ* exposure to water and sediment. Li *et al.* [68] showed that *H. perezi* amoeboid trophonts begin to lose viability after 24 h in seawater; however dinospores have been shown to survive up to 7 days in aquaria [34]. This stage could accumulate in the sediment if left undisturbed or deposited by hydrological forces after a mass mortality. By analogy with free-living dinoflagellates, *H. perezi* dinospores may be a precursor to a resting or cyst stage. Survival time of dinospores and hypothetical resting stages may vary according to factors such as predation [69], or destruction by bacteria [29].

Plankton DNA samples from Sinnickson were positive for *H. perezi* in 6 of the 9 months sampled (**Table 2.5B and Figure 2.4B**). Finding evidence of the parasite in the water/plankton, which is highly mobile, implies the existence of a

persistent environmental source. It is therefore suggestive that sediment from Sinnickson was positive 3 of the 9 dates sampled, and in all seasons, unlike sediment samples from the other areas tested. The other site with multiple positive plankton samples (2 of 9) was Trappe Creek, which, like Sinnickson, has a sizeable freshwater input. Trappe Creek is close to, and upstream of, Newport Bay, which had positive sediment 3 of the 9 months sampled. A possible mode of *H. perezi* DNA accumulation in sediments and water of some areas and not others is through tidal cycles that cause water to oscillate in/out of the small rivers feeding into coastal bays. This is in contrast to the channel areas that may have faster moving water and have shorter slack water periods than may be found in the mouths of rivers. This is reflected in the sediment types in each area: only sand is present under the fast moving water in the channels while silt-containing sediments (with more organic detritus) are adjacent to river outlets where tidal currents oscillate back and forth.

The presence of *H. perezi* DNA in 2009 water and sediment samples obtained from Indian River Inlet was also consistent with observations (above) in Chincoteague Bay in 2010. Most of the Indian River Inlet *H. perezi* signal (including the hotspot) was present in silty sediment with high organic matter content, located in a backwater that experienced relatively little tidal current/flushing. The lack of *H. perezi* detected in 2008 water from Indian River Inlet was somewhat surprising, as the sampling coincided with what appeared to be a *H. perezi* outbreak during the 07.29.08 DE sampling date. However, the volume of water collected for analysis in 2008 (100 ml) was probably insufficient

for detection of low amounts of parasite DNA. In 2009, when the volume of water sampled was increased to 1 L qPCR analysis yielded 82 to 613 targets per liter. If the 2008 samples had contained parasite DNA at this abundance, a 100 ml water sample would have had been below the limit of detection for the qPCR assay.

In 2008, the presence of scores of dead and dying crabs that contained *H. perezi* gave us a unique opportunity to examine the water as well as other aquatic species during a parasite outbreak. We think it is meaningful that none of the grass shrimp (*P. pugio*), amphipods, or snails (*O. grillus*) grazing on shells of dead *C. sapidus* yielded positive results. We did find parasite DNA in both lady crab (*O. ocellatus*) and green crab (*C. maenas*), in agreement with previous reports [39, 40]. Both of these crabs were collected (and appeared healthy) during the 07.29.08 outbreak, however, and may represent incidental carriers of the parasite rather than reservoir hosts. The lack of parasite in small invertebrates that could serve as crab prey, even during an outbreak, argues that these are not a major *H. perezi* reservoir. This is consistent with the large-scale study by Pagenkopp *et al.* [39], who also found no *H. perezi* in over 200 individuals of *Palaemonetes* sp. shrimp, and only a low (3 %) prevalence in caprellid amphipods. It was concluded that these were not likely sources of blue crab infections of *H. perezi.* Johnson [71] and Messick and Shields [12], using histological methods, also reported *Hematodinium* sp. infections in amphipods on the U.S east coast. Pagenkopp *et al.* [39] did however suggest that spider crabs (*Libinia dubia*) could serve as a biotic reservoir based on a prevalence of 17 %.

Our sampling did not, unfortunately, include *Libinia* spp. so we have no data for comparison.

Lastly another possibility is that blue crabs with non-patent *H. perezi* infections can act as reservoirs of the parasite. Blue crabs collected from winter trawls in Virginia, USA have been reported to be infected with *H. perezi* [12, 39]. Given that blue crabs are highly cannibalistic and feed heavily after hibernation, infections could be transmitted via this mode. However, there have been conflicting results with cannibalism studies in laboratory settings. When Walker et al. [42] fed blue crabs fed infected conspecific tissue, they successfully transmitted the parasite to 7 of 11 blue crabs. In contrast to these results, a more recent study by Li *et al.* [68] showed a lack of transmission to naïve blue crabs and amphipods via cannibalism. More research is needed to elucidate if transmission may be from ingestion of infected crab tissue, entry of dinospores through carapace, gills, breaks in integument while scavenging on infected dead or dying crab or from environmental sources such as the water column or sediments.

Conclusions:

Application of a species-specific *H. perezi* qPCR assay to DNA extracted from coastal bay sediment, plankton, and water samples suggests that *H. perezi* is continuously present in Delmarva coastal bays, and may present a continuous source of transmission. These assays show that *H. perezi* DNA in sediments and water in these coastal bays is both temporally and spatially variable. The accumulation of parasite DNA in sediments or "hotspots" may be a function of

hydrodynamic features and sediment type. There are gaps in our understanding of the natural history of the parasite as well as the mode of environmental or biological transmission. A better understanding of the parasites life cycle and modes of transmission will assist managers to provide more effective fishery and ecosystem management. Filling some of those information gaps can be accomplished by using the qPCR methodology in a more focused study of the presence of parasite DNA in the water and sediment in the vicinity of one or more hotspots.

Chapter Three: Conclusions and Overview

Determining reservoirs of *H. perezi* at Indian River inlet, DE: An enduring

question in *Hematodinium* sp. research is where re-occurring infections come

from. Thus far research determining biotic and abiotic reservoirs, studying modes

of transmission, and establishing the life cycle have given insight into the answer

of this question. The term "reservoir" has been loosely defined throughout the

literature (see Small and Pagenkopp 2011 for review). To be considered an

environmental reservoir transmission from the host to the environment and back

to the host must occur. Similarly, alternate hosts need to demonstrate this

sequence of transmission. Small and Pagenkopp [40] defined a reservoir host to

be any invertebrate or vertebrate that is capable of carrying and transmitting a

damaging pathogen to an economically important crustacean. They also defined

an environmental reservoir as being any environmental component, other than a

living organism, where the pathogen can survive and be readily transmitted. With

the main focus on gathering evidence for the existence of *H. perezi* in the

environment, in **Chapter 2** we developed, validated, and applied a qPCR assay

and other methods designed to look for the pathogen in environmental and biotic

samples collected from Indian River Inlet, DE. Additionally, our collaborators

used this assay to assess *H. perezi* prevalence and incidence temporally in

Maryland and Virginia coastal bays*.*

The *H. perezi* assay was developed to target the ITS2 portion of the rRNA

gene of *H. perezi* and had a limit of detection of 9 gene copies. However,

consistent amplification from dilutions containing fewer than 9 gene copies has

been observed. Since *H. perezi* was not easily obtainable and difficult to culture we used a surrogate for *H. perezi* cells, consisting of *E. coli* carrying a cloned copy of the *H. perezi* rRNA gene, to validate the assay. Using the ITS2 assay to assess incidence and prevalence in environmental and biotic samples, *H. perezi* was detected in samples collected from Indian River Inlet, DE in 2008 and 2009. In 2008, parasite DNA was not detected in any of the water samples collected, even those taken during a mass mortality of crabs infected with *H. perezi*. The surprising absence of qPCR signal for *H. perezi* may reflect the small volume of water sampled in 2008 (~100 ml), or could indicate that the combination of hydrological conditions and the short lifespan for the water-borne stage of *H. perezi* quickly remove possible dinospores (or other stages) from the water column. It is interesting, however, that the number of positive sediment samples from 2008 increased at the time of the parasite outbreak in crabs from that prior to the outbreak. These observations are consistent with (but do not prove) a model in which free-floating forms of *H. perezi* (possibly dinospores) are liberated from moribund/diseased crabs, and then rapidly settle into the sediment.

In 2009, when 10 fold larger water samples were taken, *H. perezi* DNA was detected in various locations in Indian River Inlet, even in the absence of a disease outbreak. Sediment sampling in 2009 was expanded to new sites, as well as focused on a shallow area that consisted of silty sediment with a high percentage of fine organic detritus. Parasite DNA was sporadically detected throughout Indian River Inlet, but there was a higher incidence of positive samples observed at two adjoining sampling locations. The existence of a

concentrated zone in which all sediment cores were positive for *H. perezi* suggests that a sediment reservoir can indeed form. It is unknown whether such *H. perezi*-laden sediment contributes to disease transmission. One feasible experiment to test this would be to move *H. perezi*-positive sediment to the aquaculture lab, expose disease-free hatchery-reared crabs to it, and use the qPCR assay to assess whether the crabs acquire *H. perezi* infections.

As discussed in **Chapter 2** our collaborators used the assay to assess *H. perezi* prevalence and incidence in Chincoteague Bay. In comparison to Indian River Inlet results for sediment and water samples, the intensity of signal for *H. perezi* in sediment and water Chincoteague Bay samples were higher by approximately one order of magnitude. While a reason for this difference for the water results can be explained by the difference in water sampling technique, there was no difference in sediment sampling or DNA extraction techniques. Assuming that DNA extraction efficiency and qPCR conditions were the same across labs, other reasons can be postulated for the higher intensity observed in Chincoteague Bay. The topography and hydrology of these two bays are very different which may result in different environmental conditions that favor higher *H. perezi* prevalence in one bay over the other. For example, less freshwater input into Chincoteague Bay results in high salinity, similar to that of the surrounding coastal ocean, which is more favorable for intense *H. perezi* infections. Also the blue crab fishery in Chincoteague Bay is more commercial which may result in a larger population of juvenile blue crabs that are known to have a higher prevalence of *H. perezi* infections.

Influence of environmental stressors on seasonal outbreaks: *Hematodinium*

sp. infection patterns show that there are seasonal peaks of prevalence and

intensity in commonly studied hosts. However, environmental stressors can also

enhance the effects of an outbreak by providing a weakened host or ideal

conditions for disease proliferation that may result in a long term or chronic

outbreak. For example, the two-year collapse of the blue crab fishery in Georgia,

caused by high prevalence of *Hematodinium* sp. infections, has been attributed

to a period of drought induced high salinity waters [14]. Due to the seasonal and

yearly fluctuations in recurring *Hematodinium* sp. outbreaks, the possibility of

environmental factors influencing outbreaks needs to be looked at more closely.

For example, long term monitoring of the parasite in environmental samples

coupled with collection of environmental data is needed to help understand the

dynamics of an outbreak. Anecdotally, environmental data show that in 2008,

when an outbreak occurred in Indian River inlet, conditions were warmer and

dryer than in 2009. This may have caused salinity in 2008 to be higher than in

2009, which is more favorable for *H. perezi.*

Possibility of alternate hosts as reservoirs: Given the abundant evidence in

the literature that *Hematodinium* sp. has been observed in crustaceans and

amphipods [40], it has been speculated that alternate hosts may act as a

pathogen reservoir [39]. The results presented in **Chapter 2** showed two

additional crab species positive for *H. perezi* but provided no evidence of the

parasite in the other species collected. Even the snails (*I. obsolete* **Table 2.4**)

that were seen grazing on dead crabs during the 2009 outbreak did not appear positive for the parasite.

Identifying life stages in environmental samples: Given the nature of our assessment methods we can assay for the presence of *Hematodinium sp, but* cannot determine what life cycle stage of the parasite is present. The qPCR assay allows for detection of DNA sequences unique to the pathogen of interest, but it does not guarantee the detection of a viable pathogen or established infection [40]. Therefore, more research is needed in order to determine which life stages are being detected in environmental samples. Other methods such as fluorescence *in situ* hybridization (FISH) could be used, in conjunction with qPCR, to determine parasite cell morphology in environmental samples. For example, Li *et al.* used FISH to assess the presence of *Hematodinium* dinospores in aquaria water holding diseased blue crabs [34]. These protocols could be adapted to look for parasite cells in environmental samples, such as water and sediment, to help determine the mechanism of transmission as well as location of important reservoirs.

Including disease mortality in fishery models: In 1931 when Chatton & Poisson [43] first described *Hematodinium* sp., they predicted that the parasite was not going to be a significant pathogen in the two crab species they examined. However, subsequent research has indicated the opposite. High prevalence in several significant crustaceans fisheries, such as *C. opilio* (snow crab), *N. norvegicus* (Norway lobster), and *C. sapidus* (blue crab), has stimulated research to understand this parasitic dinoflagellate and the parasite-host

relationship [10, 12, 34, 39, 72, 73]. As seafood is a high valued food commodity, direct losses due to pathogens can have dramatic effects on a crustacean fishery. Farmed and wild-caught crustaceans contribute a significant portion of the market exceeding 10 M tons annually with a value of $40 billion worldwide [74]. Shrimp aquaculture is an example of disease having a huge impact on production; current estimates for pathogen-associated mortalities of shrimp can be as high as 40 % (>$3 bn) [74]. *Hematodinium* sp. has been responsible for losses in several fisheries in northern hemisphere. Outbreaks of the parasite caused a catastrophic loss in the velvet crab (*Necora puber*) fishery off France and significant damage by recurrent epizootics in the Norway lobster off Scotland, the blue crab in the mid-Atlantic region of the US, as well as Tanner and snow crabs off the Alaskan coasts [13, 75]. Most studies only focus on direct losses because indirect losses caused by pathogens can be more difficult to assess. For example impacts of pathogens that cause indirect losses, such as castration, stunting, or morbidity of animals, require ongoing sampling and monitoring of the population [75]. Though difficult to obtain, this information is valuable as it provides insight on the loss of new recruits into the fishery.

Current fishery models do not account for year-to-year fluctuations in disease related mortalities caused by environmental factors. With the crash of the blue crab fishery in Georgia, Lee and Frischer proposed to include this factor in abundance models [14]. Correlating mortality events with environmental factors could help fishery managers and modelers predict times when there will be a high probably of disease outbreaks. An example of this is the Dermowatch

program established to monitor *Perkinsus marinus* infections in the oyster, *Crassostrea virginica*, in the Gulf of Mexico. This was a web based program that included an embedded model to explore the dynamics of disease progression and help managers control disease prevalence [76–78]. As discussed by Morado *et al*. [54], monitoring programs need to apply more sophisticated analytical techniques to field data. To better understand and predict disease epizootics for natural mortality, inferential statistics such as logistic regression [79] or Bayesian methods [80] need to be applied and the contribution of *Hematodinium*-associated disease to natural mortality requires mathematical modeling. For example, Siddeek *et al*. [81] used a rebuilding analysis using the criteria of age – sex – size in structured simulations that incorporated the then recent changes in the definition of overfishing in the US federal fisheries management plan (FMP). These investigators included a comparison of the potential effect of additional mortality that cold water *Hematodinium* sp. could have on rebuilding performance of light and heavy infected Alaska Tanner crab stocks. Precise *Hematodinium* sp. monitoring offers the unique and rare opportunity to model disease in a wild aquatic population with the added advantage of improving stock abundance estimates.

Conclusions and future studies: In conclusion, the development of DNA based techniques have helped to answer some of the pressing questions that the histological technique could not. At the start of this research, previous PCR assays were only qualitative and did not provide the specificity or sensitivity needed for environmental studies. With the development of the current assay,

which has a sensitivity of as few as 10 gene copies per PCR reaction, we succeeded in detecting *H. perezi* DNA in environmental samples of water and sediment. It is still not certain, however, how few parasite cells can be detected by the method. The uncertainty derives from disagreement in the literature as to how many rRNA gene copies there are per *H. perezi* cell. Nagle *et al.* [49] reported up to 10,000 copies/cell, while Li *et al.* [34] reported gene copies on the order of 300 copies/cell. Interestingly, in a recent study of the rRNA gene copy number in a free living dinoflagellate, *Ostreopsis cf. ovata*, Perini et al. [66] reported that the cells possess from 81 to 8488 copies/cell of the rRNA gene, depending on the isolate and the growth phase in culture. Such variability in rRNA copy number with growth phase has also been postulated for certain marine planktonic dinoflagellates [82]. This important question should therefore be re-addressed in *H. perezi* by additional investigations of more parasite isolates from blue crab. The copy number per cell can be re-assessed by methods already used in earlier studies [34, 49]. The recent development of *in vitro* culture for *H. perezi* by Li *et al.* [21] can provide very valuable material for a standardized assessment of rRNA gene copy number through the different life stages. Furthermore, any method to quantify rRNA gene copy should also be conducted on a well-characterized positive control organism for which gene copy numbers have already been determined.

Given that this qPCR assay is now available, further experiments that require DNA based techniques can be tackled. Transmission studies could provide a better understanding of parasite-host dynamics. A possible way to do

this would be to expose naïve crabs to putatively infectious sediment under conditions that are optimal for *H. perezi* proliferation in blue crabs. The infectious sediment could also be used to determine how long the parasite can remain viable in the sediment. With the recently reported ability to culture *H. perezi* cells *in vitro*, determining the infectious stage can also be included in such a study. Furthermore the qPCR assay will facilitate the investigation of whether alternate hosts are a necessary part of the *H. perezi* life cycle. Disease monitoring is inconsistent largely because of the effort needed to adequately conduct long-term monitoring programs. Monitoring programs also vary with respect to detection protocol, resulting in differences in reporting from lab to lab. It would be good practice for researchers working on the same pathogen to agree on and use a single diagnostic assay that has been well validated. This would make it much easier to compare results from different laboratories.

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