Available online at www.sciencedirect.com
ScienceDirect
Harmful Algae 5 (2006) 548–557
HARMFUL ALGAE
www.elsevier.com/locate/hal

Evidence for a free-living life stage of the blue crab parasitic dinoflagelate, *Hematodinium* sp.

M.E. Frischer [a,*], R.F. Lee [a], M.A. Sheppard [a,b], A. Mauer [a], F. Rambow [a], M. Neumann [a], J.E. Brofft [a], T. Wizenmann [a], J.M. Danforth [a]

[a] *Skidaway Institute of Oceanography, 10 Ocean Science Circle, Savannah, Georgia 31411, USA*
[b] *Savannah State University, Savannah, Georgia 31404, USA*

Received 1 September 2005; received in revised form 18 September 2005; accepted 12 November 2005

Abstract

Hematodinium sp. is a parasitic dinoflagellate reported to cause disease and death in a variety of crustacean species including the blue crab (*Callinectes sapidus*). However, because of difficulties in the culture of *Hematodinium* sp. associated with blue crabs, little is known about its life cycle or mode of transmission. Here, we report the first detection of this organism outside of a metazoan host and provide evidence that this life stage can act as an infective agent. Observations of dinospores in crab hemolymph samples suggest that dinospores may be responsible for waterborne disease transmission. Additionally, we developed and validated a quantitative Real Time PCR assay for the detection of *Hematodinium* sp. inside and outside of a host organism that will be useful for future investigations of *Hematodinium* biology and *Hematodinium* sp.-infection etiology. Based on the observations of a free-living form of *Hematodinium* sp. and the association of this parasite with a widespread epizootic in blue crab populations, we propose that *Hematodinium* sp. be considered a Harmful Algal Bloom species.

Keywords: *Hematodinium* sp.; Blue crab; Quantitative PCR; South Atlantic Bight

1. Introduction

Hematodinium perezi is a parasitic dinoflagellate first reported in 1931 in two crab species, the green shore crab, *Carcinus maenas*, and the harbor crab, *Liocarcinus depurator*, along the French coast (Chatton and Poisson, 1931). Since this early work, *Hematodinium* sp. infection has been reported in a variety of other crustaceans. Crabs infected with *Hematodinium* sp. have been observed in Australia, Alaska, Britain, France, Scotland, Canada, and the eastern United States (Bower et al., 2003; Pestal et al., 2003; Stentiford et al., 2002; Greubl et al., 2002; Shields and Squyars, 2000; Wilhelm and Mialhe, 1996; Taylor and Khan, 1995; Hudson and Shields, 1994; Messick, 1994; Field et al., 1992; Messick and Sindermann, 1992; Shields, 1992; Meyers et al., 1987; MacLean and Ruddell, 1978; Newman and Johnson, 1975). *Hematodinium* sp. infected blue crabs *Callinectes sapidus* have been found in estuaries along the east and gulf coasts of the United States with very high incidences of *Hematodinium* sp. in coastal bays of Maryland and Virginia (Shields and Squyars, 2000; Messick and Shields, 2000; Messick et al., 1999; Messick, 1994; Newman and Johnson, 1975). We have reported high prevalence and intensities of *Hematodinium* sp. in blue crabs from salt marsh estuaries in coastal Georgia (Sheppard et al., 2003; Greubl et al., 2002). *Hematodinum* spp. infection was also observed in the spider crab (*Libinia*

* Corresponding author. Tel.: +1 912 598 2308; fax: +1 912 598 2310.
E-mail address: frischer@skio.peachnet.edu (M.E. Frischer).

1568-9883/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.hal.2005.11.002

emarginata), a xanthid crab (*Nepanope sayi*), and the stone crab (*Menippe mercenaria*) in the estuaries of the South Atlantic Bight (Sheppard et al., 2003). In a recent review, Stentiford and Shields (2005) report *Hematodinium* sp. and *Hematodinium*-like infections in 42 crustacean species including crabs, lobsters, and amphipods.

Crabs infected by this parasite exhibit a spectrum of disease ranging from asymptomatic carriage to death. *Hematodinium* sp. proliferates in crustacean hemolymph, consuming hemocyanin, along with other hemolymph proteins and possibly hemocytes (Field and Appleton, 1995; Love et al., 1993; Field et al., 1992). Our work and others has also demonstrated the ability of this parasite to infiltrate other tissues, including cardiac and skeletal muscle (Sheppard et al., 2003; Shields and Squyars, 2000; Hudson and Shields, 1994). Other reports in the literature suggest the possibility of latent *Hematododinium* sp. infection of other tissue types including the hepatopancreas (Stentiford et al., 2001; Field and Appleton, 1996), gill, eye stalk, and gut connective tissues (Field and Appleton, 1995; Meyers et al., 1987). Our preliminary observations also suggest that *Hematodinium* sp.-infected crabs suffer higher incidence of secondary bacterial infections (Frischer, unpublished data). Heavily infected crabs become lethargic, possibly due to hypoxemia, compromised cardiac and skeletal muscle, and secondary bacterial infection. If not preyed upon, high mortality is associated with *Hematodinium* sp.-infection.

The blue crab *C. sapidus*, supports an important fishery along the Atlantic and Gulf coasts of the United States. This fishery suffered a dramatic decline in 1998–2003 on the East coast of the USA, with catch declines ranging from 9.7 to 51% compared to the previous 10 year average (Georgia Department of Natural Resources, 2004). In coastal Georgia, blue crab landings in 2003 were 0.8 million kg, only 20% of the previous 45 year average landings (3.9 million kg) (Lee and Frischer, 2004). Five years of observation suggest a causal relationship between the current decline of blue crab populations and a disease caused by the parasitic dinoflagellate, *Hematodinium* sp. triggered by a 5-year drought (Lee and Frischer, 2004).

The life cycle of *Hematodinium* sp. in blue crabs is complex, most likely involving several different life history stages of the parasite including dinospores, prespores, trophonts, and plasmodia (Sheppard et al., 2003; Messick, 1994; Shields, 1994). Shields (1994) speculated on the relationship between the different forms in the crab host and the outside water/sediment environment. Based on this work and our own observations we have developed a hypothesized life history model suggesting that cannibalism of infected crabs by healthy crabs and exposure to water containing dinospores are important transmission modes for this parasite (Lee and Frischer, 2004). A similar life history model was also recently proposed by Stentiford and Shields (2005). However, until it is possible to culture *Hematodinium* sp. and study each of these life stages specifically, this model must be considered as speculative.

In this study we report the first detection of *Hematodinium* sp. in water samples collected from an estuary along the Georgia, US coast and the development of a quantitative PCR-based assay for *Hematodinium* sp. We also present microscopic observations of *Hematodinium* sp. dinospores and evidence that *Hematodinium* sp.-infections can be transmitted via contact with water presumably containing *Hematodinium* sp. These observations support the hypothesis that *Hematodinium* sp. produces a free-living dinospore that may be responsible for waterborne disease transmission.

2. Methods

2.1. Collection of blue crabs and water

Blue crabs (*C. sapidus*) were collected biweekly from October 1999 through August 2000 from the Wassaw Sound estuary, Georgia USA (31.9N 80.9W) with 10 standard commercial traps baited with menhaden (Fig. 1). Surface water samples were collected synoptically with crabs in November 1999 and from March through July, 2000.

2.2. Purification of DNA from hemolymph and water samples

Hemolymph (0.5–1.0 ml) was collected from living crabs at the hemal sinus with a 1-ml syringe using a sterile chilled syringe and transferred to sterile 1.5 ml microfuge tubes. Anticoagulant was not required if the hemolymph was kept cool. Total DNA was extracted and purified from hemolymph samples as previously described by Greubl et al. (2002) using the DNeasy™ Tissue Kit (Qiagen Inc., Valencia, CA).

Total DNA from water was purified from 5 l surface water samples. Surface water samples were collected using a clean bucket and prefiltered through a 63-μm sieve to remove large particles and debris prior to collection on 0.8 μm Supor filters (PALL Life Sciences,

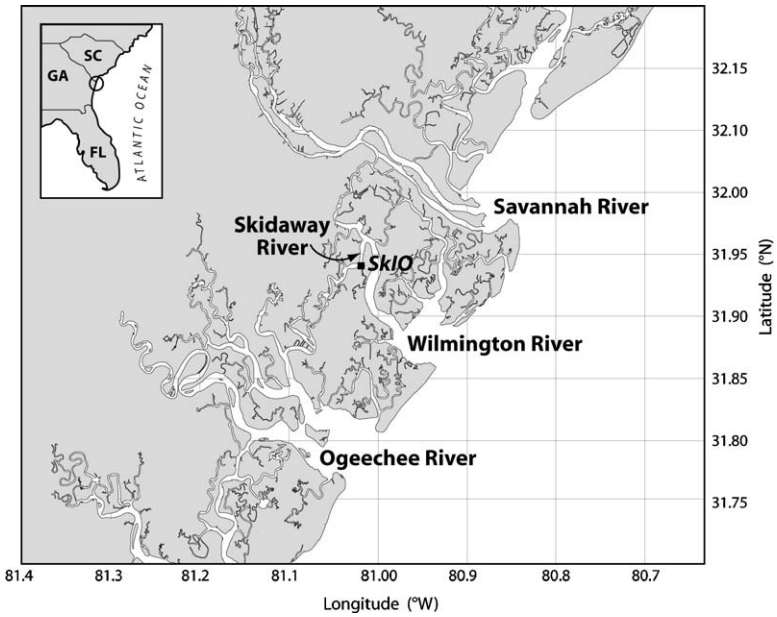

Fig. 1. Location of study sites in coastal Georgia, USA.

East Hills, NY). Total DNA was extracted from the filters using the UltraClean™ Soil DNA Mega Prep kit following the manufactures instructions (Mo Bio Laboratories, Inc., Solana Beach, CA). Purified DNA was resuspended in 300 μl of sterile nuclease free water and stored at −20 °C.

2.3. Molecular diagnostics

The specific diagnosis of *Hematodinium* sp. in crabs and water samples was routinely made using a polymerase chain reaction (PCR) assay and the *Hematodinium*-specific primers Hemat-F-1487 and Hemat-R-1654 (Greubl et al., 2002). To confirm the presence of *Hematodinium* sp. in water samples, a larger fragment of the 18S rRNA gene was amplified and sequenced from several representative water samples. A 1273 bp fragment of the *Hematodinium* sp. 18S rRNA gene was amplified using a nested PCR strategy. The first round of PCR utilized a general 18S rRNA targeted primer (Univ-F-15) paired with a *Hematodinium* sp.-specific reverse primer (Hemat-R-1654). This primer set amplifies a 1682 bp fragment of the *Hematodinium* sp. 18S rRNA (Greubl et al., 2002). However, as was previously observed, this primer set often did not yield PCR amplicons easily visualized by agarose gel electrophoresis and occasionally produced non-specific amplification products if *Hematodinium* sp. was absent or at low concentration in a sample (Greubl et al., 2002). Thus, a second round of PCR using the product of the first reaction (5 μl) as the template and a pair of *Hematodinium* sp.-selective primers Hemat-F-232 and Hemat-R-1487 that amplify a 1273 bp fragment of the *Hematodinium* sp.18S rRNA gene internal to the amplicon produced during the first round of PCR. All primer sequences and PCR reaction conditions are provided in Table 1. PCR was performed using GenAMP 9700 or 2400 PCR thermal cyclers (PerkinElmer, Inc., Wellesley, MA). Amplified gene fragments were visualized and sized by agarose gel electrophoresis in 1.2% gels stained with GelStar® nucleic acid stain (Cambrex).

Sequencing of the 1273 bp fragment produced from water samples with Hemat-F-232 and Hemat-R-1487 was facilitated by cloning the gel purified amplified 1273 bp 18S rRNA gene fragments into the PCR 2.1-TOPO cloning vector using a TOPO™ Cloning Kit, Version J (Invitrogen) following the manufacturer's instructions. The correct sized amplicon was excised from an agarose gel and purified using the Quantum Prep™ Freeze 'N Squeeze DNA Gel Extraction Spin Column (Bio-Rad Laboratories Inc.) following the manufactures instructions. After cloning into the plasmid vector, the plasmid was isolated and purified from *E. coli* using the High Pure Plasmid Isolation Kit (Boehringer Mannheim) following the manufacturer's instructions. Plasmid concentrations were estimated by fluorometry after staining with PicoGreen® (Molecular Probes) using a TD-700 fluorometer (Turner Designs). Sequencing was accomplished by automated

Table 1
18S rRNA targeted PCR primer sets and reaction conditions used in this study

Primer	PCR assay	Primer sequence (5′–3′)
Hemat-F-1487	1[a], 2[a,b]	cct ggc tcg ata gag ttg
Hemat-R-1654	1[a], 2[a,b]	ggc tgc cgt ccg aat tat tca c
Hemat-F-232	2[a,b]	ggt gat tca gaa taa ccg tac
Univ-F-15	2[a,b]	ctc cca gta gtc ata tgc

Standard PCR reaction conditions: 40–400 μg total DNA; 30 ng each forward and reverse primers (primer stock concentrations 100 ng ul^{-1}); Taq Master Mix Buffer (Qiagen, the Qiagen Taq Master Mix Buffer System contains 10 U Taq DNA polymerase/100 μl, 6 mM $MgCl_2$, and 800 μM dNTP mix), double-distilled sterile water to 25 μl. 1, Routine diagnostic use for *Hematodinium* sp., primers Hemat-F-1487 and Hemat-R-1654. Produces an expected 187 bp fragment.
2, nested PCR for amplification of large (1272 bp) fragment from water samples. First reaction Univ-F-15 and Hemat-R-1654. Second nested PCR reaction, Hemat-F-232 and Hemat-R-1487.
[a] PCR reactions conditions: initial template denaturation (94 °C for 3 min); 30 amplification cycles (94 °C, 15 s; 56 °C, 15 s; 72°, 30 s). Following the completion of amplification cycles, a final extension step (72 °C, 10 min) was completed and samples stored at 4 °C until analysis.
[b] PCR reaction conditions (Hemat-F-232 & Hemat-R-1487): initial template denaturation (94 °C for 3 min); 30 amplification cycles (94 °C, 15 s; 53 °C, 15 s; 72 °C, 30 s). Following the completion of amplification cycles, a final extension step (72 °C, 10 min) was completed and samples stored at 4 °C until analysis.

sequencing using the sequencing primers described in Greubl et al. (2002) with a Beckman CEQ 8000XL DNA Analysis System. Sequencing reactions were facilitated by using a CEQ DTCS dye terminator cycle sequencing quick start kit, following the protocols recommended by the manufacturer (Beckman Coulter). Sequence analysis was accomplished using the Beckman CEQ 2000XL Sequence Analysis software, version 4.3.9.

2.4. Quantitative PCR (qPCR)

A quantitative real time PCR assay targeted to the *Hematodinium* sp. 18S rRNA gene was developed and validated in this study. Real time PCR was performed in 25 μl reaction volumes using the *Hematodinium* sp.-specific primer pair Hemat-F-1487 and Hemat-R-1654. Real time PCR was performed using a Bio-Rad iCyclerIQ Real-Time Detection System (Bio-Rad Laboratories, Inc.). Reactions were performed in 96-well plates, with each reaction well containing 12.5 μl of 2× QuantiTech SYBR Green Master Mix (Qiagen), 0.1 μM of each primer, and 0.8–200 ng DNA extracted from crab hemolymph. The appropriate amount of template DNA was generally achieved using 1–5 μl of a 1–10 dilution of the final elution from the DNeasy™ hemolymph purification eluted in 100 μl of PCR grade water. Amplification cycle conditions were 95° for 13.5 min, followed by 40 cycles of 94 °C for 15 s, 58.2 °C for 15 s, and 72 °C for 30 s. Amplification cycles were followed immediately by a melt-curve thermal profile from 65 to 90 °C to confirm the amplification of a single *Hematodinium* sp.-specific PCR product with a melting temperature of ca. 83.1 °C. In each 96-well plate, a dilution series of purified plasmid DNA containing a cloned 18S rRNA gene was run along with the unknown samples. Each sample and standard was run in triplicate, and all reactions were repeated at least three times independently to evaluate the reproducibility of the results.

2.5. Cytological detection of Hematodinium sp.

For determination of infection intensity, cytological techniques were used. Crabs were bled at the hemal sinus with a 1-ml syringe. Hemolymph samples were applied to poly-L-lysine-coated microscope slides as described by Messick (1995), fixed in Bouin's fluid and stained with Mayer's hematoxylin and eosin (Luna, 1968). Fixed and stained slides were examined at 1000× with a Nikon Eclipse 6400 microscope equipped with a Nikon 100× 1.2NA oil objective. *Hematodinium* sp. was identified based on morphologic similarities to *Hematodinium* sp. on slides authenticated by G. Messick (NOAA, Oxford, MD). Identification of different forms of *Hematodinium* sp. including the trophont, plasmodium, sporont, and dinospore forms were based on our own observations and the description by others, including Appleton and Vickerman (1998), Hudson and Shields (1994), Shields and Squyars (2000). The taxonomy and life history of *Hematodinium* spp. was recently reviewed by Stentiford and Shields (2005). Infection intensity was calculated as the percentage of *Hematodinium* sp. cells counted among a total of 300 cells from the hemolymph of an individual crab.

The number of parasites was also quantified in crab hemolymph after staining with the vital stain neutral red using a procedure developed by J. Shields and recently described by Stentiford and Shields (2005). Briefly, 4 μl of 0.5 M EDTA was added to hemolymph samples (500 μl) to prevent coagulation. An aliquot of hemolymph (350 μl) was stained with 4 μl of a 3% neutral red (Fisher Scientific, N-129-25) solution and allowed to settle for 5 min. Total hemocyte and parasite cells were counted in triplicate 100 μl subsamples in a Palmer Maloney counting chamber using a Nikon

Eclipse E800 microscope equipped with a Nikon long working distance 40× N.A. 0.55 objective. Under these conditions, parasite cells were preferentially stained and could be differentiated from crab hemocyte cells. A minimum of 40 parasite cells or 30 fields per sample were counted in each replicate.

2.6. Water transmission studies

Three variants of experimental studies were conducted to determine whether *Hematodinium* sp. infections could be contracted after exposure to *Hematodinum* sp. when exposure routes did not involve the ingestion of infected crabs.

2.6.1. Caged studies

Healthy PCR-certified disease-free crabs (8) were collected from the Ogechee River, Georgia (5–10 psu), placed in wire cages (1 crab per trap), and held in the Wilmington River (Wassaw Sound) where *Hematodinium* sp. infected crabs were present. Cages were either suspended in the water attached by hanging ropes from a pier or placed on the bottom. Crabs were removed daily over a 3-day period, hemolymph sampled, and each crab was assayed for *Hematodinium* sp. by PCR, the neutral red assay, and the cytological method (i.e. fixed and stained). Exposure to water studies (hanging cages) were conducted May, 2000, and exposure to sediments and water studies were conducted October 2000.

2.6.2. Water exposure

Healthy PCR-certified disease-free crabs and *Hematodinium* sp.-infected crabs were placed in 40 l flow through aquaria (1 each) but separated from each other by a 1 cm mesh size plastic screen. This screening prevented the crabs from directly interacting with each other, but allowed free water exchange between them. Water (salinity 24–28 psu) was supplied from the Skidaway River, Georgia at a flow rate of 5 l min^{-1}. Water from the Skidaway River was assayed daily by PCR for *Hematodinium* sp. over the 4-day course of the experiment. *Hematodinium* sp. was not detected in the inflowing water. Each crab was assayed daily by PCR, the neutral red assay, and cytologically (fixed and stained).

2.6.3. Exposure to Hematodinium sp. dinospores

On one occasion we detected an adult male crab that appeared to harbor *Hematodinium* sp. dinospores based on the appearance of fixed and stained preparations (Fig. 2). This crab was maintained in a 30-l aquarium for 24 h after which the infected crab was removed and two healthy PCR certified *Hematodinium* sp.-free crabs were placed into the aquarium and assayed for *Hematodinium* sp. over a 4-day period. Each crab was assayed daily by PCR, the neutral red assay, and the cytological procedure.

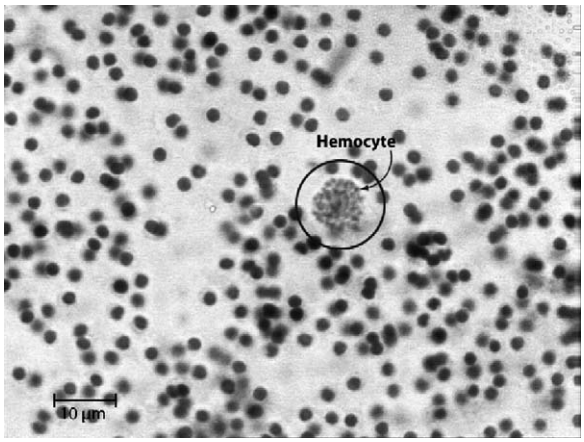

Fig. 2. Photomicrograph of presumptive *Hematodinium* sp. dinospores. Hemolymph was fixed and stained with hematoxylin and eosin and viewed by light microscopy (40× magnification). A normal crab hemocyte is circled and all other objects in photomicrograph are presumptive *Hematodinium* dinospores. Scale bar 10 μm.

3. Results

The prevalence and intensity of *Hematodinium* sp. infection and the average crab catch (crabs per standard crab trap) was monitored weekly in Wassaw Sound, GA from October 1999 through August 2000. In addition, during the periods when *Hematodinium* sp. was present in crabs (Fall 1999 and Spring 2000) the occurrence of *Hematodinium* sp. in water samples was estimated using the routine PCR assay. The results of these studies are shown in Fig. 3. Crab catches averaged 4.3 ± 0.9 crabs per trap from October 1999 through May 2000. However, following the Spring 2000 emergence of *Hematodinium* sp. infection, crab catches declined to the point where no crabs were recovered in traps from June through August, 2000. When detected, *Hematodinium* sp. was present in crabs at relatively high prevalence and intensity over the course of these studies. The principle form of *Hematodinium* sp. found in infected blue crabs was the trophont or vegetative form, although in a few infected crabs the plasmodia or dinospore forms were observed. Parasite prevalence ranged from 20 to 68% with intensities of 46 to 94% (Fig. 3). *Hematodinium* sp. was detected by the PCR assay in water samples following both the Fall and

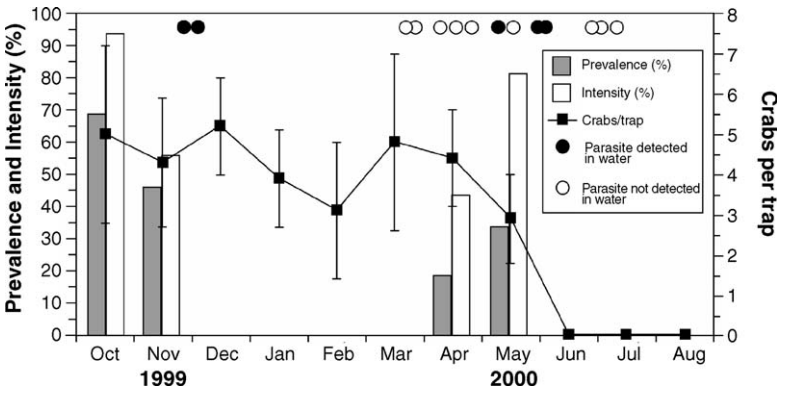

Fig. 3. Blue crab catches (–■–), *Hematodinium* sp. presence or absence in the water (●/○) and in crabs (▣), and infection intensity in crabs (□) in Wassaw Sound, Georgia, USA from October 1999–August 2000. The presence of *Hematodinium* sp. in water samples was investigated starting November 1999 when the *Hematodinium* sp.-specific PCR assay became available. Error bars indicate one standard deviation around the mean. Water samples were not screened from January 2000–March 2000.

Spring appearance of *Hematodinium* sp.-infection in crabs. Interestingly, *Hematodinium* sp. was not detected in surface waters in the early Spring (March–April) prior to the Spring emergence of the disease, but was present in 3 of 4 samples collected in May when parasitized crabs were detected. Unfortunately, samples were not collected prior to December 1999 when the assay was first developed. During the period when crabs were not present in Wassaw Sound (June–August, 2000), *Hematodinium* sp. was not detected in the water. These results indicate the presence of a waterborne form of *Hematodinium* sp. associated with the presence of *Hemtodinium* sp.-infections in blue crab populations.

To confirm that the small 187 bp 18S rDNA PCR amplicon diagnostic for *Hematodinium* sp. in crab hemolymph was indeed derived *Hematodinium* sp. and not a related organism present in the water samples, a larger fragment (1273 bp) of the *Hematodinium* sp. 18S rRNA gene was amplified from three of the water samples identified as containing parasite DNA. All three of the 1273 bp amplicons sequenced were identical and shared 99.8% nucleotide similarity (1270 bp) with the *Hematodinium* sp. 18S rRNA gene previously identified in blue crabs from these waters (Grubel et al., 2000; Genbank AF286023) confirming the identity of *Hematodinium* sp. The sequence recovered from the water samples was deposited in Genbank (accession number AF421184). These results support the hypothesis that *Hematodinium* sp. was present in the water.

Although not systematically applied to all samples, during these studies we developed a quantitative Real Time PCR assay for *Hematodinium* sp. utilizing the primers Hemat-F-1487 and Hemat-R-1654. The number of copies of *Hematodinium* sp. 18S rRNA gene was standardized against a copy of the *Hematodinium* sp. 18S rRNA gene cloned into a bacterial plasmid vector. The quantitative validity of the real time PCR assay was evaluated by comparing estimates of parasite abundance (cells ml^{-1} hemolymph) in crab hemolymph from infected crabs determined by counting in a counting chamber after staining fresh hemolymph with neutral red with estimates of 18S rRNA copy abundance (Fig. 4). *Hematodinium* sp. infection in all of these crabs was also confirmed cytologically (hematoxylin and eosin staining) and by PCR. Infection intensities in these crabs varied from 5–12%. The estimated number of *Hematodinium* sp. 18S rDNA copies increased linearly ($r^2 = 0.88$) with

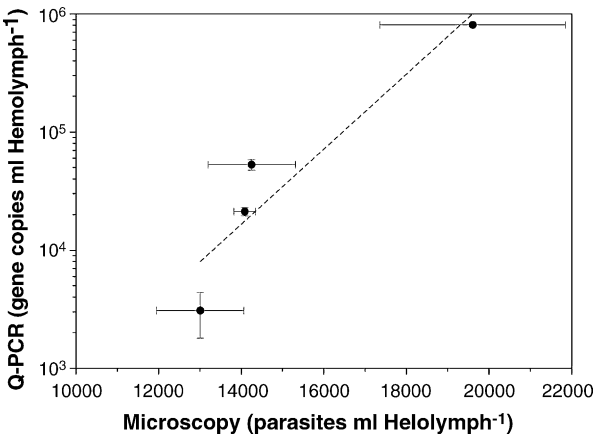

Fig. 4. Validation of a *Hematodinium* sp.-specific quantitative real time PCR (qPCR) assay. A significant correlation between qPCR estimates of *Hematodinium* sp. 18S rRNA gene copies and direct microscopic estimates of parasite abundance were observed ($r^2 = 0.88$). Error bars indicate one standard deviation around the mean.

direct microscopic estimates of parasite abundance indicating successful quantification of the parasite based on the real time PCR assay. The ratio of 18S gene copies per parasite cell was 11.69 ± 1.9, suggesting a ribosomal copy number of approximately 11–12 rRNA genes per *Hematodinium* sp. cell. During our studies, many examined crabs had slight infections, often only detected by the routine PCR assay. In crabs with low parasite concentrations, obtaining accurate estimates of parasite abundance by direct microscopy was difficult because so few parasite cells were present. Often in these samples less than 10 parasitic cells in total were counted. Consequently, in most of these samples, Q-PCR based and direct microscopy based comparisons were not significantly correlated (data not shown).

Although a free-living form of *Hematodinim* sp. has to date never been observed directly in water samples, on one occasion we observed what appeared to be dinospores in the hemolymph collected from a diseased crab (Fig. 2). Although only speculative, it may be this form that persists outside host organisms and is detected in water samples with the PCR assay. Unfortunately, at the time when this sample was detected it was not possible to confirm that these cells were dinospores by electron microscopy, though this would be useful in the future. Attempts to culture *Hematodinium* sp. from this hemolymph sample in various media including diluted crab hemolymph and a variety of different tissue culture medias were unsuccessful. Exposure of two healthy *Hematodinium* sp.-free crabs to water that had contained a crab infected with the dinospore stage of *Hematodinium* sp. tested positive for *Hematodinium* sp. as determined by cytological assay. Infections were confirmed by PCR (Table 2). Both crabs exposed to water with presumptive *Hematodinium* sp. dinospores were infected after 2 days and died within 4 days. These observations confirm that these dinospores are a life stage of *Hematodinium* sp. and indicate that they are highly infective.

A second line of evidence consistent with the presence of a waterborne form of *Hematodinium* sp. was the observation that exposure of *Hematodinium* sp.-free animals to water with *Hematodinium* sp. resulted in the transmission of the parasite (Table 2). When *Hematodinium* sp.-free crabs were placed in cages either suspended in the water or on top of sediments in areas where *Hematodinium* sp. was present, 100% ($n = 8$) of the crabs were infected after 1 day of exposure. In laboratory experiments where naïve crabs were held in flow-thru systems in proximity to, but physically separated from infected crabs, 20% ($n = 5$) were infected by *Hematodinium* sp. after 4 days as determined by cytological assay and confirmed by PCR. Although these studies do not completely eliminate the

Table 2
Water transmission of *Hematodinium* sp.

Experimental design	Date	Exposure time (days)	% Infection intensity[a]				
			Crab 1	Crab 2	Crab 3	Crab 4	Crab 5
Cage studies							
Hanging cage	May 2000	1	10	15	12	6	
Hanging cage	May 2000	2	24	17	21	26	
Hanging cage	May 2000	3	30	29	45	35	
Bottom cage	Oct. 2000	1	24	35	27	17	
Bottom cage	Oct. 2000	2	36	52	48	37	
Bottom cage	Oct. 2000	3	47	Dead	67	65	
Water cxposure							
Flow thru aquarium	Sept. 2000	1	0	0	0	0	0
Flow thru aquarium	Sept. 2000	2	0	0	21	0	0
Flow thru aquarium	Sept. 2000	3	0	0	27	0	0
Flow thru aquarium	Sept. 2000	4	0	0	37	0	0
Dinospore exposure							
Static aquarium	May 2001	1	0	0			
Static aquarium	May 2001	2	35	25			
Static aquarium	May 2001	3	48	55			
Static aquarium	May 2001	4	Dead	Dead			

The description of the exposure conditions are described in Section 2. Initial infection intensities of the crabs used for the water exposure experiment and of the crab with dinospores were 65 and 74%, respectively.

[a] Infection intensity was estimated by the cytological assay after hematoxylin and eosin staining and confirmed by PCR.

possibility that *Hematodinium* sp. might have been transferred by exposure to sediments, the consumption of small organisms capable of entering crab cages, or developed from an undetected latent infection, these observations are consistent with the presence of *Hematodinium* sp. in the water column and suggest that such forms are capable of causing *Hematodinium* sp. infections in blue crabs.

4. Discussion

In this study several independent lines of evidence suggested the existence of a free-living life stage of the parasitic dinoflagellate *Hematodinium* sp. associated with the blue crab *C. sapidus*. Ribosomal gene sequences specific to *Hematodinium* sp. were recovered from several independent size fractionated water samples (<63 μm) by PCR amplification of *Hematodinium* sp.-specific fragments of the 18S rRNA gene. Strengthening this observation is that *Hematodinium* sp. was detected in water samples synoptically with detection of *Hematodinium* sp. infections in blue crabs. *Hematodinium* sp. was not detected in the water when the disease was absent in the crabs. Furthermore, *Hematodinium* sp. infections could be transmitted to healthy crabs after exposure to water suspected to contain *Hematodinium* sp. To our knowledge this is the first reported evidence of a free-living infectious form of *Hematodinium* sp.

Interestingly, during our routine examination of crab hemolymph samples we occasionally observed what appeared to be dinospores. Although in this study we were unable to culture *Hematodinium* sp. from the blue crab, *Hematodinium* sp. has been cultured from the Norwegian lobster (*Nephrops norvegicus*) (Appleton and Vickerman, 1998). Based on this culture work Appleton and Vickerman (1998) reported 8 life cycle forms of *Hematodinium* sp., including flagellated uninucleate dinospores. In other parasitic dinoflagellates, including the HAB species *Pfiesteria piscicida* and related crypotoperidiniopsoids, it has been shown that motile free-living dinospores are capable of actively infecting their respective hosts (Parrow and Burkholder, 2003; Vogelbein et al., 2002). Thus, although *Hematodinium* sp. dinospores have never been observed in the field, it seems quite plausible that *Hematodinium* sp. can form free-living dinospores that act as waterborne transmission agents of blue crab *Hematodinium* sp. infections.

The existence of a free-living infective form of *Hematodinium* sp. may have important implications for the etiology of *Hematodinium* sp. infections. The recent epizootic of *Hematodinium* sp.-like infections in Southeastern Georgia, USA suggested that it was associated with drought conditions and increased estuarine salinities (Lee and Frischer, 2004; Sheppard et al., 2003; Greubl et al., 2002). Several other investigators have also reported the association of *Hematodinium*-like infections and increased salinities (Messick et al., 1999; Messick and Shields, 2000). The relationship between salinity and *Hematodinium* sp.-infections has been difficult to understand since hemolymph and hemocyanin function in blue crabs is relatively unaffected by the large external water salinity variations that *C. sapidus* regularly encounters (Weiland and Mangum, 1975). However, a free-living dinospore might be affected by changes in water salinity. Successful culture of this species would allow exploration of this hypothesis.

One of the major obstacles associated with the study of dinoflagellates is their ambiguous identification in field samples (Bowers et al., 2000). In the case of a parasitic dinoflagellate species, this problem is further confounded since traditional cytological-based diagnostic methods are limited in their ability to recognize the organism outside of well-characterized host organisms. In the case of *Hematodinium* sp. specifically, where many of the transmission modes and vectors may be unknown, the lack of alternative diagnostic methods has significantly limited the study of this organism in nature.

Molecular techniques have become wide-spread in their use as diagnostic tools to assess the presence of cryptic parasitic and non-parasitic organism in diverse settings including protist pathogens of marine invertebrates. For example, PCR-based diagnostic approaches have been used to detect the protist parasites *Haplosporidium nelsoni* and *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* (Ford et al., 2000). PCR primers have also been designed to detect the parasite *Bonamia ostreae* in the flat oyster (*Ostrea edulis*), the agent of QPX in the hard clam *Mercenaria mercenaria* (Carnegie et al., 2000; Stokes et al., 2000), and for *Hematodinium* sp. in blue crabs (Greubl et al., 2002). As is evident from this study, an advantage of genetic based diagnostic tools over cytological methods for studying parasitic organisms with complex life histories is that it is not necessary for the infection to be symptomatic in the host. Additionally, molecular detection obviates the need to recognize the parasite morphologically which, especially in the case of parasitic dinoflagellates with complex life histories, can be a difficult challenge requiring highly trained personnel and complete life history information (Burkholder and Glasgow, 1997).

However, despite the usefulness of the PCR-based *Hematodinium* sp.-specific assay, it only provides semi-quantitative information about the pathogen. In the present study, we developed and validated a real-time quantitative PCR assay format utilizing our previously published *Hematodinium* sp.-specific PCR primer set. Comparison of quantitative PCR results to direct microscopic counts from hemolymph collected from heavily infected crabs indicated a direct relationship between the two techniques quantitatively validating the assay. As is true when comparing the standard PCR method to cytology methods, the application of the quantitative PCR method increases the sensitivity of detection by several orders of magnitude.

In a recent review of the effects of Harmful Algal Blooms, Landsberg (2002) defines HAB species as those that ''produce toxins or cause harm, directly or indirectly, to aquatic organisms or to terrestrial organisms associated with aquatic habitats or their products''. By this broad definition, *Hematodinium* sp. should be considered to be a HAB species. However, traditionally microalgal parasites and pathogens have been excluded from most definitions of HAB species which generally have been applied to free-living species that can reach concentrations in excess of 1 million cells per liter (IPHAB, 1993). Although such high concentrations of *Hematodinium* sp. have not yet been reported outside of laboratory studies (Shields and Squyars, 2000), the detection of a free-living form of *Hematodinium* sp. that is apparently capable of acting as a vector for *Hematodinium* sp.-like infection raises the question of whether this species should be reconsidered as a HAB species. In this study we report the existence of a free-living infective life stage of *Hematodinium* sp. and corroborate earlier observations that suggest a direct linkage between a recent decline in blue crab populations in the Eastern US and an epizootic of *Hematodinium* sp.-like infections (Lee and Frischer, 2004). Based on these observations, we propose that *Hematodinium* sp. is a HAB species.

Acknowledgements

The work was supported by NOAA National Sea Grant College Marine Environmental Biotechnology Program (NA 06RG0029) and by awards from the US Department of Energy (FG02-88ER62531 and FG02-98ER62531) and the US National Science Foundation (OPP-00-83381 and OCE 99-82133). We also acknowledge the advice and help of J. Shields (Virginia Institute of Marine Sciences) and G. Messick (Center for Coastal Environmental Health and Biomolecular Research, Cooperative Oxford Lab, Oxford, MD). A. Boyette helped to prepare the figures and V. Patrick prepared the final manuscript.[SS]

References

Appleton, P.L., Vickerman, K., 1998. In vitro cultivation and developmental cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters. Parasitology 116, 115–130.

Bower, S.M., Meyer, G.R., Phillips, A., Workman, G., Clark, D., 2003. New host and range extension of bitter crab syndrome in *Chionoecetes* spp. caused by *Hematodinium* sp. Bull. Eur. Assoc. Fish Pathol. 23, 86–91.

Bowers, H.A., Tengs, T., Glasgow Jr., H.B., Burkholder, J.M., Rublee, P.A., Oldach, D.W., 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscidida* and related dinoflagellates. Appl. Environ. Microbiol. 66, 4641–4648.

Burkholder, J.M., Glasgow, H.B., 1997. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates; behavior, impacts, and environmental controls. Limnol. Oceanogr. 42, 1053–1075.

Carnegie, R.B., Barber, B.J., Distel, D.L., Culloty, S.C., 2000. Development of a PCR assay for detection of *Bonamia ostreae* in flat oysters, *Ostrea edulis*. J. Shellfish Res. 19, 643.

Chatton, E., Poisson, R., 1931. Sur l'existence, dans le sang des crabes, de peridiniens parasites: *Hematodinium perezi* n.g., n.sp. (Syndinidae) CR Seances Soc. Biol. Paris 105, 553–557.

Field, R.H., Appleton, P.L., 1996. An indirect fluorescent antibody technique for the diagnosis of *Hematodinium* sp. infection of the Norway lobster *Nephrops norvegicus*. Dis. Aquat. Org. 24, 199–204.

Field, R.H., Appleton, P.L., 1995. A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops Norvegicus* – Observations on pathology and progression of infection. Dis. Aquat. Org. 22, 115–128.

Field, R.H., Chapman, C.J., Taylor, A.C., Neil, D.M., Vickerman, K., 1992. Infection of the Norway lobster *Nephropos norvegicus* on the west coast of Scotland. Dis. Aquat. Org. 13, 1–15.

Ford, S., Xu, Z., DeBrosse, G., 2000. Acquisition and prevention of MSX and Dermo in a hatchery and land-based nursery: A DNA assay investigation. J. Shellfish Res. 19, 571.

Georgia Department of Natural Resources, 2004. Historical Monthly Hard Blue Crab Landings for Georgia. Georgia Department of Natural Resources, Coastal Reources Division, Brunswick, GA.

Greubl, T., Frischer, M.E., Shepperd, M., Neumann, M., Lee, R.F., 2002. Development of a 18S rRNA gene targeted PCR based diagnostic for the blue crab parasite, *Hematodinium* sp. Dis. Aquat. Org. 49, 61–70.

Hudson, D., Shields, J.D., 1994. Infection of *Trapezia* spp. (Decapoda: Xanthidae) by *Hematodinium* sp. (Duboscquodinida: Syndinidae): a new family record of infection. J. Fish Dis. 16, 273–276.

IPHAB, 1993. The IOC harmful algal bloom programme. www.ioc.unesco.org/ioc/oslr/hab.htm (accessed August 2005).

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10, 113–390.

Lee, R.F., Frischer, M.E., 2004. Where have the blue crabs gone: Evidence for a drought induced epidemic of a protozoan disease. Am. Sci. 92, 547–553.

Love, D.C., Rice, S.D., Moles, D.A., Eaton, W.D., 1993. Seasonal prevalence and intensity of Bitter Crab dinoflagellate infection and

host mortality in Alaskan Tanner crabs (*Chionoecetes bairdi* from Auke Bay, Alaska, USA). Dis. Aquat. Org. 15, 1–7.

Luna, L.G., 1968. Manual of histologic staining methods of the armed forces institute of pathology, third ed. McGraw-Hill, New York.

MacLean, S.A., Ruddell, C.L., 1978. Three new crustacean hosts for the parasitic dinoflagellate *Hematodinium perezi* (Dinoflagellata: Syndinidae). J. Parsitol. 64, 158–160.

Messick, G.A., Shields, J.D., 2000. The epizoogiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue crab *Callinectes sapidus*. Dis. Aquat. Org. 43, 139–152.

Messick, G.A., Jordan, S.J., Van Heukelem, W.F., 1999. Salinity and temperature effects of *Hematodinium* sp. in the blue crab *Callinectes sapidus*. J. Shellfish Res. 18, 657–662.

Messick, G.A., 1994. *Hematodinium perezi* infections in adult and juvenile blue crabs *Callinectes sapidus* from coastal bays of Maryland and Virginia, USA. Dis. Aquat. Org. 19, 77–82.

Messick, G.A., 1995. Laboratory techniques to detect parasites and diseases of blue crab, *Callinectes sapidus*. In: Stolen, J.S., Fletcher, T.C., Smith, A., Selikoff, J.T., Kaatari, S.L., Anderson, R.S., Sőderhäll, K., Weeks, B.A. (Eds.), Techniques in Fish Immunology-4. SOS Publications, Fair Haven, pp. 187–198.

Messick, G.A., Sindermann, C.J., 1992. Synopsis of principal diseases of the blue crab, *Callinectes sapidus*. U.S. Dept. Commerce, NOAA Technical Memorandum NMFS-F/NEC-88.

Meyers, T.R., Koeneman, T.M., Botelho, C., Short, S., 1987. Bitter crab disease: a fatal dinoflagellate infection and marketing problem for Alaskan Tanner crabs *Chionoecetes bairdi*. Dis. Aquat. Org. 3, 195–216.

Newman, M.W., Johnson, C.A., 1975. A disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. J. Parasitol. 63, 554–557.

Parrow, M.W., Burkholder, J.M., 2003. Estuarine heterotrophic cryptoperidiniopsoids (Dinophyceae): Life cycle and culture studies. J. Phycol. 39, 678–696.

Pestal, G.P., Taylor, D.M., Hoenig, J.M., Shields, J.D., Pickavance, R., 2003. Monitoring the prevalence of the parasitic dinoflagellate *Hematodinium* sp. in snow crabs *Chionoecetes opilio* from Conception Bay, Newfoundland. Dis. Aquat. Org. 53, 67–75.

Sheppard, M., Walker, A., Frischer, M.E., Lee, R.F., 2003. Histopathology and prevalence of the parasitic dinolflagellate, *Hematodinium* sp. in crabs (*Callinectes sapidus*, *Callinectes similis*, *Neopanope sayi*, *Libinia emarginata*, *Menippe mercenaria*) from a Georgia Estuary. J. Shellfish Res. 22 (3), 873–880.

Shields, J.D., Squyars, C.M., 2000. Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi*. Fish Bull. 98, 139–152.

Shields, J.D., 1994. The parasitic dinoflagellates of marine crustaceans. Ann. Rev. Fish Dis. 4, 241–271.

Shields, J.D., 1992. The parasites and symbionts of the blue sand crab, *Portunus pelagicus*, from Moreton Bay, Australia. J. Crust. Biol. 12, 94–100.

Stentiford, G.D., Shields, J.D., 2005. A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. Dis. Aquat. Org. 66, 47–70.

Stentiford, G.D., Neil, D.M., Coombs, G.H., 2001. Development and application of an immunoassay diagnostic technique for studying *Hematodinium* infections in *Nephrops norvegicus* populations. Dis. Aquat. Org. 46, 223–229.

Stentiford, G.D., Green, M., Bateman, K., Small, H.J., Neil, D.M., Feist, S.W., 2002. Infection by a *Hematodinium*-like parasitic dinoflagellate causes Pink Crab disease (PCD) in the edible crab *Cancer pargurus*. J. Invert. Path. 79, 179–191.

Stokes, N.A., Calvo, L.M.R., Burreson, E.M., 2000. DNA-based molecular diagnostics for the hard clam parasite QPX (quahog parasite unknown). J. Shellfish Res. 19, 649.

Taylor, D.M., Khan, R.A., 1995. Observations on the occurrence of *Hematodinium* sp. (Dinoflagellata: Syndinidae), the causative agent of bitter crab disease in Newfoundland snow crab (*Chionoecetes opilio*) J. Invert. Pathol. 65, 283–288.

Vogelbein, W.K., Lovko, V.J., Shields, J.D., Reece, K.S., Mason, P.L., Haas, L.W., Walker, C.C., 2002. *Pfiesteria shumwayae* kills fish by micropredation not exotoxin secretion. Nature 418, 967–970.

Weiland, A.L., Mangum, C.P., 1975. The influence of environmental salinity on hemocyanin function in the blue crab, *Callinectes sapidus*. J. Exp. Zool. 193, 265–273.

Wilhelm, Mialhe, E., 1996. Dinoflagellate infections associated with the decline of *Necora puber* crab populations in France. Dis. Aquat. Org. 26, 213–219.