Vol. 84: 79–87, 2009
doi: 10.3354/dao02027

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 9

Real-time PCR-based assay for quantitative detection of *Hematodinium* sp. in the blue crab *Callinectes sapidus*

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ABSTRACT: *Hematodinium* sp. is a parasitic dinoflagellate infecting the blue crab *Callinectes sapidus* and other crustaceans. PCR-based assays are currently being used to identify infections in crabs that would have been undetectable by traditional microscopic examination. We therefore sought to define the limits of quantitative PCR (qPCR) detection within the context of field collection protocols. We present a qPCR assay based on the *Hematodinium* sp. 18S rRNA gene that can detect 10 copies of the gene per reaction. Analysis of a cell dilution series vs. defined numbers of a cloned *Hematodinium* sp. 18S rRNA gene suggests a copy number of 10 000 per parasite and predicts a sensitivity of 0.001 cell equivalents. In practice, the assays are based on analysis of 1% of the DNA extracted from 200 µl of serum, yielding a theoretical detection limit of 5 cells ml^{-1} hemolymph, assuming that 1 cell is present per sample. When applied to a limited field survey of blue crabs collected in Maryland coastal bays from May to August 2005, 24 of 128 crabs (18.8%) were identified as positive for *Hematodinium* sp. infection using qPCR. In comparison, only 6 of 128 crabs (4.7%) were identified as positive using traditional hemolymph microscopic examination. The qPCR method also detected the parasite in gill, muscle, heart and hepatopancreas tissues, with 17.2% of the crabs showing infection in at least one of these tissues. Importantly, it is now possible to enumerate parasites within defined quantities of crab tissue, which permits collection of more detailed information on the epizootiology of the pathogen.

KEY WORDS: *Callinectes sapidus* · *Hematodinium* sp. · Quantitative real-time PCR

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INTRODUCTION

The blue crab *Callinectes sapidus* supports economically significant fisheries along the Gulf of Mexico and Atlantic coasts of the USA. Unfortunately, these fisheries have suffered significant declines in recent years. For example, annual harvests of Georgia blue crabs declined from ~9.3 million lb (~4218 metric tons, t) in 1995 to 1.8 million lb (816 t) in 2002 (Georgia Department of Natural Resources 2004). In Chesapeake Bay, the 2007 blue crab harvest of 23.7 million lb (10 750 t) is the second lowest data recorded (Chesapeake Bay Stock Assessment Committee 2008). This dramatic decline, while partly dependent on fishing pressure, may be multi-factorial. Interestingly, it has coincided with an increase in the prevalence of infection by the parasitic dinoflagellate *Hematodinium* sp., highlighting a need for more intensive monitoring of the onset of infection within blue crab populations (Shields 2003, Lee & Frischer 2004).

Hematodinium species are known to parasitize other crustaceans inhabiting waters within the North Atlantic, mid-Atlantic, North Pacific, and the east coast of Australia (Newman & Johnson 1975, Love et al. 1993, Hudson & Shields 1994, Squires & Dawe 2003, Stentiford et al. 2003, Stentiford & Shields 2005). These crustaceans include the swimming crab *Portunus pelagicus*, Tanner crabs *Chionoecetes bairdi* and the

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Norway lobster *Nephrops norvegicus*. In the blue crab, pathogenicity is principally observed in salinities >11, with highest infection rates found in salinities between 26 and 30 (Newman & Johnson 1975, Messick & Shields 2000, Shields & Squyars 2000, Stentiford & Shields 2005). In Chesapeake and its associated coastal bays, there is a correlation between infection and season, with most infections being reported following warmer summer months (Messick & Shields 2000). Parasitism is manifested in an opaque hemolymph and sluggishness in captured crustaceans, often with changes in exoskeleton and gill color (Messick 1994, Messick & Shields 2000, Stentiford et al. 2001).

Microscopic observation of hemolymph smears is the traditional method for detection of *Hematodinium* sp. parasitism that yields a clear diagnosis in cases of relatively heavy infections (Messick 1994, Wilhelm & Boulo 1996). However, this method can be time consuming, particularly when low-level infections are suspected. Furthermore, since trophic stages resemble hemocytes, a high degree of investigator skill/experience is required and detection is problematic at low infection densities (<50 cells ml^{-1}). In addition, the infection cycle within the host has not been fully delineated (Eaton et al. 1991, Field & Appleton 1996, Appleton & Vickerman 1998); thus, hemolymph smears may miss phases in which the parasite is relatively absent in the hemolymph but persists within other tissues (Sheppard et al. 2003, Small et al. 2006).

Molecular-based assays have been developed to increase the sensitivity of *Hematodinium* sp. detection including an ELISA-based assay for use with *Nephrops norvegicus* (Small et al. 2002) and a PCR-based assay for use with blue crabs (Gruebl et al. 2002). In an adaptation of the PCR assay to real-time PCR instrumentation, *Hematodinium* sp. was detected in the water column leading to speculation that dinospores may play a role in environmental transmission (Frischer et al. 2006). Most recently, Small et al. (2007) reported a sensitive (3 parasites ml^{-1} hemolymph) endpoint PCR-based assay, targeting differences in the internal transcribed spacer region of the *Hematodinium* sp. ribosomal gene cluster.

Despite these advances, the use of PCR to assay for *Hematodinium* sp. infection depends on its ability to isolate sufficient quantities of parasite DNA from a background of host tissue, and is increasingly difficult for low-level infections. Estimates of the sensitivity level of PCR assays have relied upon harvesting parasites from heavily infected hemolymph, purifying the DNA, and then diluting the DNA to levels predicted to be found in low-level infections. Although this is valid from the vantage of assay development, it does not address the empirical issue of quantifying low parasite numbers expected to be encountered in sub-patent infections of blue crab populations.

To meet the need for a quantitative, sensitive, and specific detection of *Hematodinium* sp. in field-collected samples, we have developed a real-time quantitative PCR (qPCR) assay targeting the 18S rRNA gene. This refined method permits the quantification of infection directly from hemolymph samples containing as few as 7.5 *Hematodinium* sp. cells ml^{-1}. We validated the technique in crabs harvested from the lower Chesapeake Bay and associated Delmarva coastal bays, and present evidence for low-intensity infections in blue crabs that did not appear infected by microscopy.

MATERIALS AND METHODS

Sources of naïve and *Hematodinium* sp.-infected crabs. Crabs heavily infected with *Hematodinium* sp. were collected from Maryland coastal bays by the Cooperative Oxford Lab (NOAA/NCCOS). Naïve *Hematodinium*-free crabs were provided by the UMBI-COMB blue crab hatchery (Zmora et al. 2005). Additional crabs from the Gulf of Mexico were kindly provided by the Gulf Coast Research Laboratory (J. Lotz, R. Overstreet, N. Zimmerman). Infected crabs were maintained in 57 l aquaria (S = 28, 22 to 24°C) in a Biosafety Level 2 laboratory space at UMBI-COMB, in isolation from the crab hatchery.

Generation of standard curves for quantification of *Hematodinium* sp. cells and DNA. To create a standard curve representing a range of parasite numbers in hemolymph, *Hematodinium* sp. trophonts from an infected crab were enumerated on a hemocytometer, and then serially diluted 10-fold into hemolymph from a disease-free hatchery-reared crab. Beginning with 7.5×10^6 parasites ml^{-1}, 100-fold to 1×10^7-fold dilutions were generated in a final volume of 200 µl. DNA was extracted using a Blood and Tissue kit (Qiagen), and eluted in a final volume of 100 µl. Thus, each µl represented 2 µl of hemolymph. The final dilution was calculated to contain 1.5 cells in 200 µl to ensure that at least one cell would be present in each sample. qPCR analyses were conducted in triplicate on 1 µl aliquots of DNA extractions.

An additional set of PCR reactions was performed using defined numbers (10 to 1.0×10^7) of a plasmid carrying the 18S rRNA target gene (M. Frischer, Skidaway Institute of Oceanography, Savannah, GA). Plots of cycle threshold (C_t) vs. gene copy number and C_t vs. cell number are shown in Fig. 2, with best-fit logarithmic curves calculated using Kaleidagraph 4 (Synergy Software). For generation of curves a C_t greater than 38 was considered undetectable, as this was the limit for a diluted plasmid standard to provide a signal above background. For analysis of

field sampling data, a best-fit relationship generated by the software was used to convert C_ts to 'cell equivalents per PCR reaction' through the exponential equation $y = 7.0142e + 6 \times e^{-0.565x}$ (Fig. 2B).

qPCR methodology. A real-time quantitative PCR protocol (Steven et al. 2003) was used for all quantification studies. PCR primers were designed using Primer Express design software (Applied Biosystems) targeting the reported sequence for *Hematodinium* sp. 18S rRNA (accession #AF286023; see Fig. 1): forward primer 5′-GGT AAT CTT CTG AAA ACG CAT CGT-3′; reverse primer 5′-GTA CAA AGG GCA GGG ACG TAA TC-3′. For detection of product by qPCR, an end-labeled fluorescent probe (6FAM-5′-AAT TCC TAG TAA GCG CGA GTC ATC AGC TCG-3′) was monitored during the progression of target amplification. TaqMan Universal Master Mix and FAM-labeled probe were obtained from Applied Biosystems, and qPCR analysis performed on either a BioRad iCyclerIQ or an ABI 7500 Real-Time PCR system. Cycle parameters were: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, repeating the last 2 steps for 40 cycles. qPCR was performed in triplicate on all samples.

Field sampling of crabs. Crabs were collected by trawling from 3 sites in Chesapeake Bay (Deal Island, MD; Occahannock Creek, VA; and Cherrystone Inlet, VA) and 4 sites in the coastal bays (Assawoman Bay, MD; Newport River, MD; Chincoteague Bay, VA; and Sandy Island Channel, VA). Salinity and water temperatures were measured at all sites. Generally, 10 crabs were collected per location, and all sites except Sandy Island Channel were sampled twice. Hemolymph samples (0.5 to 2 ml) were drawn from the 5th pleopod (swimmer fin) using sterile syringes. This hemolymph was used both for analysis by microscopy and for qPCR. In instances where insufficient hemolymph was drawn from the pleopod, hemolymph was isolated directly from the heart using a 20-gauge needle. Crabs were then labeled on the carapace, placed on ice and immediately frozen at –80°C upon return to the laboratory. The size, sex, and location from which each crab was collected were recorded for correlation of possible infection trends.

Microscopic analysis. Hemolymph smears on polylysine-coated slides were prepared using published methods (Luna 1968, Messick 1994). Briefly, the smeared hemolymph was fixed in Bouin's solution, stained with Mayer's hematoxylin and eosin, and examined by light microscopy (40× power) to determine the presence of the parasite. Enumeration of parasites continued until a minimum of 300 hemocytes was counted.

Tissue preparation. DNA for qPCR analysis was isolated from 200 µl of hemolymph samples and ~50 mg of selected tissues from all crabs. DNA from hemolymph and tissues included in the environmental detection survey (gill, muscle, heart and hepatopancreas) was isolated using a FastDNA kit (Qbiogene) according to the manufacturer's protocol. Genomic DNA was eluted in a final volume of 100 µl and the concentration determined by spectrophotometry (absorbance of light with a wavelength of 260 nm, A_{260}). All samples were diluted with deionized water to 4 ng μl^{-1} for qPCR analysis.

RESULTS

Validation of the real-time qPCR methodology

The initial goal of these studies was to assess qPCR as a means to specifically identify *Hematodinium* sp. from the hemolymph of infected blue crabs. As a negative control, DNA was isolated from non-infected crabs reared in the UMBI-COMB aquaculture facility and demonstrated to be *Hematodinium*-free. Using the primers indicated in Fig. 1, an endpoint PCR methodology amplified a single 121 bp product from hemolymph DNA of infected crabs only, as determined by agarose gel electrophoresis (data not shown). Sequencing of a cloned copy of this PCR product verified its identity with the *Hematodinium* sp. 18S rRNA gene sequence (GenBank accession #AF286023). Subsequent qPCR analysis confirmed these results (see below). qPCR was also performed on DNA (20 ng) isolated from a range of cultured dinoflagellate species including *Amphidinium carterae, Karlodinium veneficum, Prorocentrum minimum, Lingulodinium polyedrum*, and *Pfiesteria psicicida*. No qPCR signal was

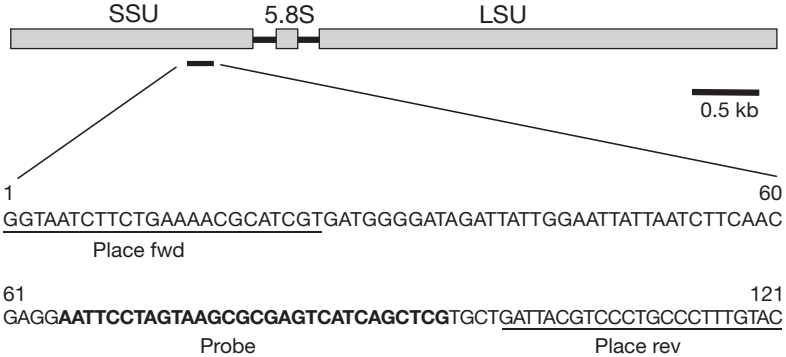

Fig. 1. Schematic representation of the location of quantitive PCR (qPCR) primers and product on a *Hematodinium* sp. ribosomal RNA gene cluster. Forward (fwd) and reverse (rev) primers are underlined; TaqMan probe is in bold text. The region shown is encompassed within the sequence used by Gruebl et al. (2002). SSU: small subunit; LSU: large subunit

detected, demonstrating the species-specificity of the method (data not shown).

The response of the qPCR assay was evaluated by conducting triplicate reactions on serial dilutions of a plasmid carrying a single copy of the *Hematodinium* sp. 18S rRNA gene (M. Frisher). As shown in Fig. 2A (triangles), the assay detected from 1.0×10^7 copies of plasmid down to a dilution representing 10 copies per reaction. The protocol was also evaluated for detection of limiting numbers of *Hematodinium* sp. cells. Parasite dilutions of over 4 $\log_{10}$ magnitude were prepared in 200 µl of naïve hemolymph, containing from 1.5×10^4 cells down to a calculated 1.5 cells. This final dilution represented the assay detection limit (Fig. 2A, circles), with the observed sensitivity of 7.5 cells ml^{-1} reflecting a dilution that increased the likelihood of at least one cell being present for DNA extraction. A DNA extract from the next dilution in the series was generated, but was undetectable by qPCR (data not shown). Comparison of the plasmid copy number detection curve with the diluted *Hematodinium* sp. curve indicates that *Hematodinium* sp. has ~10 000 copies of the 18S rRNA gene $cell^{-1}$ (Fig. 2B). Based on a detection limit of 10 copies, in a reaction using 1% of the DNA from 200 µl of hemolymph, the theoretical maximum sensitivity is calculated to be 5 cells ml^{-1} hemolymph, assuming that one cell must be present per sample.

Comparison of microscopic analysis to qPCR

Having established quantification of parasites by qPCR, we endeavored to validate the method in field monitoring. We used blue crab samples collected during a survey of sites in the lower Chesapeake Bay and associated coastal bays (see Fig. 3) over a 5 mo period from May to October 2005. Results of detection by microscopic analysis were compared with analysis by qPCR (Table 1). From late May to early August 2005, no *Hematodinium* sp. infections were observed by microscopy, in contrast to 6 of 70 harvested crabs (8.6%) showing signs of infection from October and November. Representative analyses from 2 heavily infected crabs are shown in Fig. 4. Collectively, 6 of 128 total sampled crabs (4.7%) were found to be positive by microscopy. Through qPCR analyses, 13 of 128 crabs (10.2%) were found to be positive for infection, and importantly, 4 of these were from the early summer months when all microscopic observations showed negative results.

Enumeration of parasites by qPCR

Table 2 shows quantitative data from individual qPCR-positive blue crabs. The levels of *Hematodinium* are expressed as cell number equivalents per 4 ng

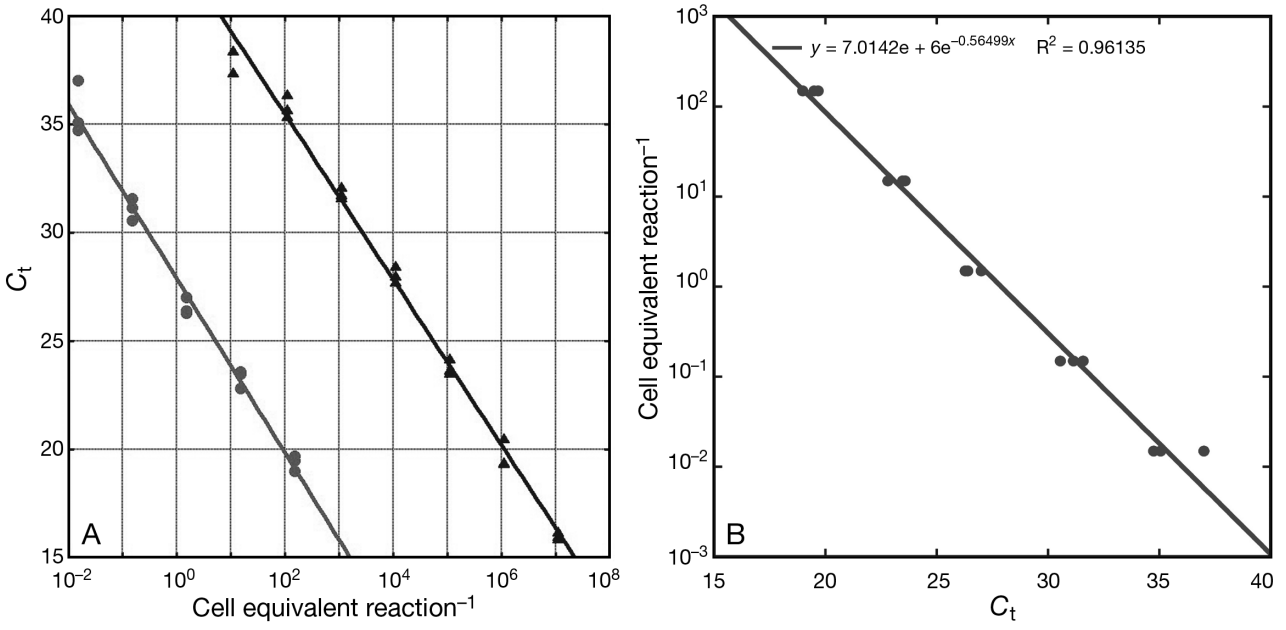

Fig. 2. (A) Parallel standard curves of cycle threshold (C_t) vs. target gene copy and C_t vs. parasite number. Standard quantitive PCR (qPCR) reactions were conducted using a plasmid-borne cloned 18S rRNA target and parasites diluted into naïve hemolymph prior to DNA extraction. Upper and lower *x*-axes are plasmid copies (10 to 1.0×10^7 plasmids; triangles) and cell equivalents (0.015 to 150 cells; circles) per PCR reaction, respectively. (B) Best-fit curve of cell number expressed as a function of C_t. Data was re-plotted and best-fit curve generated using Kaleidagraph (Synergy Software). The exponential equation ($y = 7.0142e + 6 \times e^{-0.565x}$) was used to calculate cell number equivalents for field data, presented in Tables 2 & 3

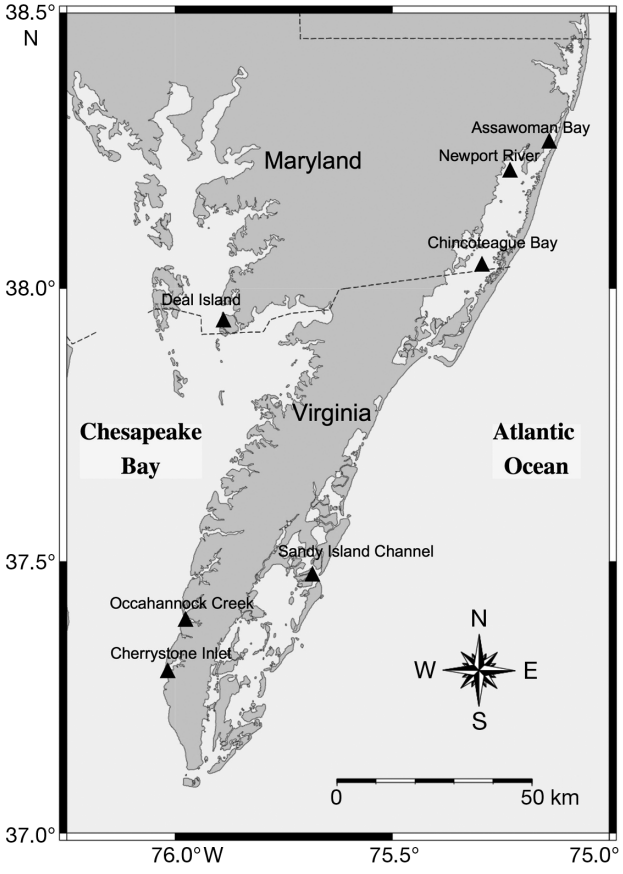

Fig. 3. Blue crab sampling locations on the Delmarva peninsula inclusive of the eastern shore of Maryland and Virginia, showing both Chesapeake and coastal bays

DNA using the relationship established between C_t and cell number in Fig. 2B. The qPCR-positive crabs represented a range of sizes of both males and females, and there appears to be no concentration of infection in any particular group. DNA was isolated from hemolymph, viscera, and leg muscle for analysis. Nearly all crabs that had hemolymph infection by *Hematodinium* sp. (via microscopy or by qPCR) showed infection in other tissues, and several crabs that were found to be negative by microscopy were found to be positive by qPCR. Surprisingly, in 5 crabs in which microscopy revealed heavy infections, the qPCR assay failed to detect parasites in the hemolymph, suggesting the presence of an inhibitor of the reaction.

We also observed *Hematodinium* sp. infection in 6 crabs in which the hemolymph tested negative by both microscopic analysis and qPCR. As shown in Table 3, low-level infection was detected primarily within muscle tissue from these crabs. This indicates that although *Hematodinium* sp. has traditionally been designated as a parasite of the hemolymph, there may exist other phases of infection in which this tissue is devoid of *Hematodinium* sp., with muscle tissue potentially being a repository of the parasite. These results also demonstrate that it is now possible to identify blue crabs carrying the parasite before disease becomes apparent in the hemolymph.

Table 1. Dates and source locations for 128 harvested blue crabs sampled in this survey. Data generated from parasite identification via microscopic examination and quantitative PCR (qPCR) analysis show the comparative detection capabilities of the 2 methods

Date (mo/d/yr)	Location	Water temp. (°C)	Salinity	No. of crabs	Slide positive	qPCR positive
5/26/05	Newport Bay, MD	17.7	14.3	8	0	0
6/08/05	Cherrystone Inlet, VA	25.9	18.9	5	0	0
6/15/05	Occahannock Creek, VA	30.2	12.5	10	0	0
6/24/05	Chincoteague Bay, VA	24.6	28.6	10	0	0
7/14/05	Cherrystone Inlet, VA	26.7	21.1	5	0	0
7/15/05	Assawoman Bay, MD	28.2	21.2	10	0	4
8/05/05	Deal Island, MD	29.7	13.4	10	0	0
10/04/05	Sandy Island Channel, VA	21.7	30.1	10	3	3
10/18/05	Deal Island, MD	16.1	16.2	10	0	0
10/19/05	Assawoman Bay, MD	16.6	29.1	10	1	2
10/19/05	Newport Bay, MD	16.1	27.4	10	0	0
10/27/05	Chincoteague Bay, VA	10.5	30.1	10	1	1
11/1/05	Cherrystone Inlet, VA	12.7	24	10	1	3
11/1/05	Occahannock Creek, VA	11.7	24	10	0	0

Evidence for an inhibitor of qPCR in DNA from infected blue crabs

Because some of the heavily infected crabs, as determined by microscopic examination of hemolymph, showed surprisingly low levels of infection by qPCR, we reasoned that DNA extracts from hemolymph of these crabs might contain an inhibitor of the qPCR reaction (see Table 2). To test for the presence of an inhibitor, we first established a new standard curve, using the plasmid carrying the 18S rRNA gene at 0.0002 to 20 ng per reaction (Fig. 5). qPCR was performed on either 20 or 0.2 ng of control plasmid DNA that was 'spiked' with 4 ng of DNA from crabs containing a suspected inhibitor. An inhibitor, if present, should suppress the reaction and thereby increase the C_t of control amplification.

Due to the paucity of materials, we were only able to perform our analysis using hemolymph from crabs SIC6_10/4 and SIC7_10/4. As shown in Table 4, the 20 and 0.2 ng standards were amplified at an average of 20.2 and 28.9 C_t, respec-

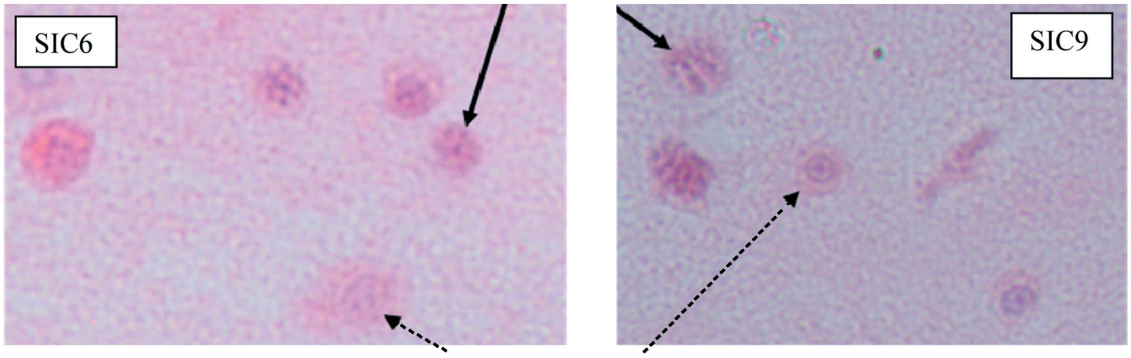

Fig. 4. *Hematodinium* sp. Photomicrograph (40×) in representative hemolymph smears on polylysine-coated slides. *Hematodinium* sp. cells are distinguishable from hemocytes by the lack of a defined nuclear membrane. Solid arrows: *Hematodinium* sp. cells; dashed arrows: hemocytes. SIC: Sandy Island Channel, VA (refers to crab sample source site)

Table 2. *Hematodinium* sp. Identification in hemolymph and other tissues by quantitive PCR (qPCR). Blue crabs identified as positive for infection (Table 1) were subjected to further investigation of selected tissues by qPCR analysis. Calculation of cells per qPCR reaction was accomplished using Kaleidagraph (Synergy Software) as described in the 'Materials and methods'. AB: Assawoman Bay, MD; SIC: Sandy Island Channel, VA; CB: Chincoteague Bay, VA; CI: Cherrystone Inlet, VA

Sample site_date (mo/d)	Crab sex	Crab size (cm)	Slide parasite/hemocyte	qPCR, as cell no. equiv. per 4 ng DNA				
				Hemolymph	Muscle	Heart	Hepatopancreas	Gill
AB1_7/15	F	8.9	–	0.066	–	0.013	–	0.088
AB3_7/15	F	8.9	–	1.205	–	0.713	0.002	0.342
AB8_7/15	F	13.4	–	0.002	–	–	–	–
AB10_7/15	M	13.7	–	2.570	–	0.383	0.008	0.798
SIC2_10/4	F	11.6	–	0.030	0.007	0.003	0.001	0.063
SIC4_10/4	F	10.8	–	–	–	–	–	0.002
SIC6_10/4	F	14.6	1.13	0.001	0.383	0.045	0.018	0.040
SIC7_10/4	M	10.8	0.06	–	31.395	0.038	0.088	0.025
SIC9_10/4	M	14.6	2.03	0.026	0.273	–	0.138	0.602
AB6_10/19	F	13.3	–	3.131	0.218	0.453	0.014	4.598
AB7_10/19	M	14.0	–	0.132	0.305	0.053	0.003	0.003
AB8_10/19	F	9.5	1.68	–	0.164	–	0.013	0.508
CB4_10/27	M	11.4	–	0.135	–	0.004	0.010	–
CB7 10/27	M	9.9	0.02	–	0.040	–	0.009	–
CI4 11/1	M	9.5	–	–	–	0.007	–	0.093
CI5 11/1	F	14	–	0.206	0.010	0.059	0.002	0.088
CI8 11/1	F	12.7	0.01	0.157	0.005	–	0.002	–
CI9 11/1	F	13.3	–	0.001	–	0.013	–	–

Table 3. *Hematodinium* sp. Infection in other tissues in a subset of blue crabs that were negative for infection in hemolymph. Blue crabs identified as negative for infection in hemolymph were screened for infection in non-hemolymph tissues by quantitive PCR (qPCR) analysis. AB: Assawoman Bay, MD; DI: Deal Island, MD; SIC: Sandy Island Channel, VA; NB: Newport Bay, MD

Sample site_date (mo/d)	Crab sex	Crab size (cm)	Slide parasite/hemocyte	qPCR, as cell no. equiv. per 4 ng DNA				
				Hemolymph	Muscle	Heart	Hepatopancreas	Gill
AB9_7/15	M	16.5	–	–	0.014	–	–	–
DI2_8/5	M	8.9	–	–	–	0.001	–	–
SIC5_10/4	F	14.0	–	–	0.001	–	–	0.002
DI2_10/18	M	7.6	–	–	0.002	–	–	–
DI8_10/18	F	13.3	–	–	0.003	–	–	–
NB9_10/19	M	10.2	–	–	0.007	–	–	–

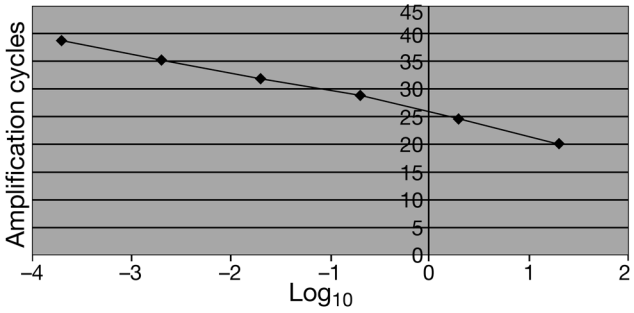

Fig. 5. The $\log_{10}$ of the mass of a plasmid carrying the 18S rRNA gene from *Hematodinium* sp. was calculated and plotted against the average cycle threshold on the *y*-axis. This standard curve was used for evaluation of a suspected inhibitor of quantitive PCR (qPCR) in the hemolymph, found in a subset of blue crabs (see Table 4)

Table 4. For inhibition studies, 20 and 0.2 ng of 18S rDNA plasmid were added to 4 ng of DNA isolated from hemolymph that was positive by smear methodology, but negative by quantative PCR (qPCR) analysis. Shown are the cycle thresholds (C_t) for 9 replicates of standard DNA (std.), and then on the right are averages for 9 replicates of each standard spiked with the corresponding crab hemolymph DNA extract

Plasmid std. (ng)	C_t (mean ± SD)	C_t with 4 ng SIC6_10/4	SIC7_10/4
20	20.2±2.3	24.6	22.7
0.2	28.9±2.3	28.7	29.9

tively. However, the C_t increased when amplified in the presence of 4 ng of DNA from infected hemolymph. This effect was most observable in amplification of the higher concentration (20 ng) of positive control plasmid, with C_t increases ranging from 2.5 to 4.4 cycles. For both SIC6_10/4 and SIC7_10/4, this represented an increase greater than the SD for the 20 ng sample, with SIC6 showing a more dramatic response. Thus, compared to amplifications of unspiked standard (see Fig. 2), the spiked samples underestimated the target by 5- to 20-fold, suggesting that an inhibitor is present in these DNA extracts.

DISCUSSION

We present here the development and validation of a fluorescent probe-based qPCR technique for the quantification of *Hematodinium* sp. infections in blue crabs. The standard method for parasite identification is the histological smear technique in which 20 µl of hemolymph is examined on a microscope slide, with a maximum sensitivity of 1 cell per slide. In contrast, our method detected a signal using 1% of DNA extracted from samples averaging 1.5 cells diluted into 200 µl of hemolymph (Fig. 2), translating into an empirical/practical assay sensitivity of 1 cell per 133 µl of blue crab hemolymph (7.5 cells ml^{-1}). This represents a ~7-fold improvement over the traditional method. Based on our theoretical qPCR sensitivity of 5 cells ml^{-1}, the method can potentially yield a 10-fold improvement. Depending on modifications of the protocol such as DNA extraction from larger volumes of hemolymph, or use of increased volumes of isolated DNA in the qPCR reactions, we predict that the assay sensitivity may be increased as needed.

Frischer et al. (2006) reported the application of a SybrGreen-based qPCR method to the identification of *Hematodinium* sp. in environmental samples. However, this study did not specify a detection limit (cells per volume of hemolymph or per mg of tissue). The PCR-based *Hematodinium* sp. assay reported by Small et al. (2007) has a detection limit of 3 ml^{-1}, based on dilutions of DNA extracted from a single sample of a high-intensity infection. Although this is a valid expression of sensitivity, it does not address the practical issue of detection limit in field samples in which DNA is recovered from small volumes of hemolymph with low numbers of parasites. The sensitivity we report (7.5 ml^{-1}) reflects a practical rather than theoretical limit and delivers quantitative data >4 orders of magnitude.

The lower limit of target gene copies necessary to generate a signal by qPCR showed that we could detect as few as 10 copies of a cloned 18 S rRNA gene. That parasite numbers correlate with rRNA gene copy numbers on the dual curve indicates that our method for extracting *Hematodinium* DNA from parasite-spiked hemolymph is applicable over a broad range of parasite densities. Comparison of the standard curve data in Fig. 2 indicates that each *Hematodinium* sp. cell carries ~10 000 copies of the 18S rRNA gene. We predict therefore that only 0.001 cell equivalents need to be present in individual reactions to generate an observable signal. We should note that DNA recovery is unlikely to be 100%; thus, gene copy number may be slightly larger than the 10 000 we report. This is notably higher than the 1000 copies of the 5.8S rRNA gene in the genome of the dinoflagellate *Alexandrium minutum* (Galluzzi et al. 2004), but is within the range (1 to 12 000) reported for various free-living dinoflagellate species investigated by Zhu et al. (2005).

By applying the qPCR methodology to a limited field survey, we identified a number of crabs that were positive for *Hematodinium* sp. despite being designated as negative by microscopy. In one instance (July 15, 2005, Assawoman Bay, MD; Tables 1 & 3), 5 infected crabs were identified solely by the qPCR method. This contrasts with a similar PCR-based identification of blue crabs infected in July (Gruebl et al. 2002), in

which the 2 PCR-positive crabs were also positive by microscopy. Inclusive of all months of our sampling survey, 13 of 128 crabs (10.2%) were deemed positive by qPCR analysis of hemolymph DNA (Table 2). When this analysis was extended to include all tissues, 22 of 128 crabs (17.2%) had infections in at least one other tissue (Tables 2 & 3). Six crabs from this group displayed infection solely in tissues but not in hemolymph (Table 3). Thus, 24 out of 128 total crabs (18.8%) were identified as positive for *Hematodinium* sp. infection in some tissue using our qPCR methodology. In comparison, only 6 of 128 crabs (4.7%) were identified as infected using traditional hemolymph microscopic examination.

In some crabs, we encountered difficulty in establishing a direct correlation between parasites enumerated in the hemolymph by microscopy and what should have been a quantitative response by qPCR. For example, SIC6 and SIC9 were 2 crabs with very similar microscopy profiles (see Fig. 4) but distinctly different qPCR profiles, as indicated in Table 2. We interpret this as evidence that a PCR inhibitor was present in DNA extracted from a subset of crabs. Although we are uncertain as to what gives rise to the occasional PCR inhibition, we are not the first to report such a phenomenon (Guy et al. 2003, Dumonceaux et al. 2005). In our study, inhibition was most apparent in spiked control amplifications using a higher concentration (20 ng) of standard (see Table 3). Accordingly, we have modified our ongoing qPCR assays by testing for inhibition through inclusion of a plasmid-borne copy of the target gene in parallel sets of reactions.

The presence of parasite cells in other tissues, particularly in muscles, was of particular interest to us. Infection of tissues other than the hemolymph has been reported in various *Hematodinium* sp.-susceptible crustaceans (Newman & Johnson 1975, Field & Appleton 1996, Appleton & Vickerman 1998, Stentiford & Shields 2005, Small et al. 2006). Our study confirms that infection must also involve other tissues, and indicates that parasites may be present in these tissues even when hemolymph infection is low or undetectable. Whether the parasite is dormant or actively dividing in these tissues will be the subject of future investigations, as tissue-resident parasites may represent phases prior to hemolymph appearance. It is not known how *Hematodinium* sp. is transmitted to the blue crab. It has been suggested that the disease may be transmitted by consumption of infected crabs (Messick et al. 1999, Sheppard et al. 2003) or via free-living dinospores of the parasite within the water column (Shields 1994, Frischer et al. 2006). The qPCR assay reported here should be a powerful tool for delineating the parasite threshold required for colonization of tissues and subsequent morbidity/mortality.

One motivation for this effort was the need to screen for *Hematodinium* sp. in hatchery-produced blue crabs destined for release back into Chesapeake Bay as part of a larger restoration effort (Zmora et al. 2005, 2008). The method can be used for rapid screening of broodstock brought into the hatchery to ensure that outgoing hatchery production is not infected. Ongoing environmental studies will also address known hotspots for the disease in coastal bays. Collectively, this will assist in implementing a disease and health management plan for responsible stock enhancement strategies and potentially improve stocks (Blankenship & Leber 1995).

Acknowledgements. We thank C. Steven and K. Hunter-Cevera for their assistance during the optimization of the qPCR assay used in this study, and W. Pecher for his crucial role in graphics and mapping. This work was funded by grants from NOAA Award #NA17FU2841 Blue Crab Advanced Research Consortium Project to A.R.P, contract NA06 NMF4570283 from NOAA-NCCOS to E.J.S., and NOAA Award #NA17AE1626 and #NA060AR4810163 to the LMRCSC. We also thank M. Frischer and R. Lee of the Skidaway Institute for Oceanography for the gift of the cloned copy of the *Hematodinium* sp. 18S rRNA gene fragment, and N. Zimmerman and J. Lotz of the Gulf Coast Research Laboratory for providing *Hematodinium*-infected crabs from the Gulf of Mexico. This is Contribution 09-202 from the University of Maryland Biotechnology Institute, Center of Marine Biotechnology.

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Editorial responsibility: Ken Hasson, College Station, Texas, USA

Submitted: September 17, 2007; Accepted: January 5, 2009
Proofs received from author(s): February 24, 2009