Overwintering of the parasitic dinoflagellate *Hematodinium perezi* in dredged blue crabs (*Callinectes sapidus*) from Wachapreague Creek, Virginia

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**Abstract**
Parasitic dinoflagellates in the genus *Hematodinium* cause disease and mortality in several commercially important marine decapod crustaceans. One species, *Hematodinium perezi*, occurs in blue crabs, *Callinectes sapidus*, along the eastern seaboard and Gulf coast of the USA. The parasite infects blue crabs, other decapods, and amphipods in the high salinity waters of coastal bays. Epizootics of the parasite often reach prevalence levels of 75–80% during outbreaks with diseased crabs dying from the infection. Prevalence of the parasite is bimodal, with a minor peak in late spring or summer, and a major peak in fall, and declining rapidly to nearly zero in late November and December. The rapid decline in infections in the late fall brings up the question of whether the parasite overwinters in crabs or whether it uses an unidentified resting stage, such as a cyst. We report observations on the prevalence of the parasite from winter dredge surveys undertaken in 2011 and 2012. Crabs were examined via hemolymph smears, histology, and PCR diagnosis for the presence of *H. perezi* and other pathogens. Active infections were observed from January through March in 2011 and 2012, indicating the parasite can overwinter in blue crabs. However, several crabs that were positive by PCR had presumptive effete infections that were difficult to diagnose in histological slides and hemolymph smears. These infections did not appear to be active and may have been in subsidence. Dredged crabs with light and moderate active infections were held at 15°C to determine if the parasite was capable of rapid progression. In 8 cases, infections exhibited logarithmic growth progressing rapidly over 8–12 days. We present evidence that overwintering of *H. perezi* occurs in the blue crab hosts, that infections are capable of responding rapidly to increases in temperatures, and that overwintering provides a reservoir of infected animals for transmission to occur in the spring.

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1. Introduction

In 1997 blue crabs (*Callinectes sapidus*) supported the largest commercial fishery in Chesapeake Bay, and the second largest fishery in Virginia (Kirkeley, 1997). During the years prior to 1997, the crab industry annually harvested 80–120 million pounds from Chesapeake Bay; of that, approximately 10–14 million pounds were soft-shell crabs (Johnson et al., 1998). However, major declines in crab catches have occurred from 1998, culminating in recent record lows in bay-wide harvests of approximately 43.5 million pounds in 2007, the lowest recorded since 1945, and the fishery was declared a disaster (NOAA, 2008a,b). Since 2007, coordinated management efforts across bay jurisdictions to reduce female harvests appeared to help, with recent 2010–2012 surveys showing modest increases in crab abundances (NOAA, 2010, 2012). However, the 2013 blue crab survey reported a substantial decline in the overall abundance of blue crabs (from 765 million to 300 million), principally driven by a decline in juvenile crab abundance, from 581 million to 111 million (NOAA, 2013). While predation continues to be cited for the recent declines, other factors such as disease and environmental processes have not been well examined; yet both processes have contributed to declines in other crustacean fisheries (Shields, 2012, 2013).

Parasitic diseases, particularly the parasitic dinoflagellate *Hematodinium perezi*, and other viral and bacterial agents are known to occur in seasonal outbreaks in blue crabs from Chesapeake Bay and the coastal bays of the Delmarva Peninsula (Shields and Overstreet, 2007). Such outbreaks can be damaging, often reaching high prevalence levels of 75–80% or more, and...
resulting in large mortalities (Newman and Johnson, 1975; Messick, 1994; Messick and Shields, 2000; Lee and Frischer, 2004). *Hematodinium perezi* is a syndinid dinoflagellate that infects the blue crab, several other decapods, and even several amphipod species (Messick and Shields, 2000; Stentiford and Shields, 2005; Pagenkopp Lohan et al., 2012; Small, 2012). The parasite can be highly virulent in its blue crab host, with mortality rates up to 87% in naturally and experimentally infected mature blue crabs (Messick and Shields, 2000; Shields and Squyars, 2000). Infected blue crabs die due to malfunction of the hepatopancreas, degradation of the muscle, and loss of respiratory function (Shields et al., 2003). *Hematodinium perezi* was described from *Carcinus maenas* and *Liocarcinus depurator* from France (Chatton and Poisson, 1931). A morphologically identical form occurs in blue crabs from the USA (Newman and Johnson, 1975; Messick and Shields, 2000). Based on molecular evidence, the species in blue crabs has been identified as *H. perezi* genotype III as it is closely related to the type species, *H. perezi* genotype I; thus the parasite in the blue crab is *H. perezi* until additional work separates the species (Small et al., 2012). The life cycle of *H. perezi* has been examined in culture (Li et al., 2011). The parasite has a complex life cycle progressing through two distinct processes – filamentous, ameboid and arachnoid forms that are associated with merogony and sporogony. Through sporogony the dinospores form and sporulate from the crab through the gills and orifices, typically killing the host during this process. All stages have been observed in vitro, but filamentous and ameboid trophonts are the most common stages found in host crabs. In *H. perezi*, macro- and micro-dinospores develop in separate in vitro cultures; but we have not observed possible zygotes, rather spore stages give rise to filamentous trophonts (Li et al., 2011). Dinospores from naturally sporulating crabs and those from cultures survive up to 7 days at 21–23 °C but do not form cysts (Li et al., 2011; Coffey et al., 2012).

Seasonality of infections is a significant feature of all *Hematodinium*–host relationships that have been studied (Stentiford and Shields, 2005). In blue crabs from the Delmarva Peninsula, there are bimodal peaks in late spring or early summer and fall months, with a nadir in winter (Messick and Shields, 2000). This indicates the possibility of an external reservoir or a latency of infection, as well as different temperature requirements for cell growth and proliferation. The lack of observable parasites in hemolymph smears during winter months may result from seasonally low water temperatures (Messick et al., 1999). Infections can be detected again in April, with low prevalence levels rising to higher levels by mid June, but the source of new infections is unknown (Messick and Shields, 2000, Shields, unpubl. data). Thus, our objectives were to (1) determine if *H. perezi* overwinters in crabs from an endemic area; (2) if the parasite is present, explore what stages infect crabs in winter during host hibernation, (3) compare diagnostic methods for assessment during winter months to establish epidemiological sensitivity and specificity, and (4) determine if natural infections in hibernating crabs can become active and proliferate as temperature increases.

2. Materials and methods

2.1. Collection and handling of experimental animals

Blue crabs were collected from Wachapreague Creek (37°37’32. 05”N, 75°40’42.14”W) using a 1.2 m oyster dredge weighted with a metal dive plate. Crabs were dredged in January, February, and March, in 2011 and 2012. A sample size of approximately 100 crabs was attempted in each monthly sample. Crabs were placed in coolers containing ambient seawater, ice packs, and wet towels for transport to VIMS for assessment. At VIMS crabs were either assessed immediately, or they were held overnight in 2 × 500 gal, recirculating saltwater tanks in the Seawater Research Laboratory at VIMS for assessment the following day (typically within 16–20 h of capture).

Crabs were given a visual assessment then the carapace width (with and without epibranchial spines), sex, molt stage (intermolting, pre- or post-molt), maturity status, and presence of injuries or regenerating limbs were noted prior to dissection. A swab with 95% ethanol was used to clean the sample site, then a hemolymph sample was removed from the arthrodial membrane at the juncture of the basis and the ischium of the 5th walking leg using a sterile 27-ga. needle and 1-ml syringe. Two to three drops of hemolymph were mixed approximately 1:1 with neutral red in physiological saline (2.5% w/v) and examined immediately at 100× and 400× with a compound microscope. Hemolymph sub-samples (100 µl) were preserved in 95% ethanol (1:10 ratio) or frozen whole at −20 °C (only in January 2011) for the first fifty crabs each month.

In 2011, the first 50 crabs to be processed were dissected immediately after hemolymph assessment, and selected tissues (heart, hepatopancreas, muscle, epidermis, and gill, and in some cases gonad) were preserved in Boin’s fixative for later histological assessment. In the 2012 sampling, the first 50 crabs were processed as above for histology, except for those that were obviously infected with *H. perezi* as determined by examination of their hemolymph smears. Infected crabs were used in the progression study or in other studies.

In February, 2012, a small-scale progression study was undertaken. Crabs that had been held at 7–8 °C and diagnosed with infections of *H. perezi* via hemolymph smear were placed in individual tanks in a recirculating seawater (artificial seawater, 30 psu) system at 15 °C, a typical spring temperature. Initial life history stages were noted at the beginning of the study and every four days crabs were bled, hemolymph stained with neutral red, and parasite cell densities estimated with a Neubauer hemacytometer. Parasite density estimates continued until the crabs were dead. The study ended after 16 days due to high host mortality.

2.2. Hemolymph assessment

All crabs were screened for *H. perezi* and other hemolymph-dwelling parasites via a hemolymph smear. The hemolymph and neutral red mixture was examined for parasite presence using transmitted light microscopy. Parasites were differentiated from host cells due to the ptake of the neutral red solution, and cell motility and morphology. Crabs infected with *H. perezi* were further assessed for parasite intensity and life history stage. Intensity was based on a semi-quantitative designation related to the number of individual parasite cells in each infected host and was classified as light, moderate and heavy in accordance with Messick and Shields (2000). Additional items of note were also recorded, such as bacterial infections, other protist infections, or unique hemocyte morphologies.

2.3. DNA extraction and PCR

The first fifty crabs assessed each month also had a hemolymph subsample taken for DNA extraction and PCR analysis. For the majority of samples, 500 µl of hemolymph-ethanol suspension (equating to approximately 50 µl hemolymph in 450 µl 95% ethanol) was centrifuged at 15,800×g for 1 min and the excess ethanol removed. The centrifuged samples were placed in a class IIIR biological safety cabinet for two hours to allow for the evaporation of residual ethanol. The dried samples were then extracted using a Qiagen Tissue and Blood Kit (Qiagen, Valencia, California) following the manufacturer’s instructions for animal tissues. The only exception to the above was for the hemolymph samples in January 2011 that were frozen whole. These were thawed
(~100 μl) and extracted as if they were a solid tissue sample following the above Qiagen protocol for animal tissues. All DNA samples were eluted in 100 μl of AE buffer, quantified using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, Florida), and stored at −20°C. All extractions completed within the same day included a blank column extraction which served as a control for extraction contamination in PCR analyses.

A random subset (15) of hemolymph samples from each month was assessed for the presence of amplifiable high molecular weight DNA using PCR primers nSSU A (5'-AACCTGGRTTGATCTGATCCT GCCAGT-3') and nSSU B (5'-GATCTTCGCCAGGTTCACTAC-3'), which targeted the SSU rRNA gene (modified from Medlin et al., 1988). Cycling conditions and reaction concentrations were as described by Pagenkopp Lohan et al. (2012). Ten micro liters of the resulting PCR product was electrophoresed on a 1.5% w/v agarose gel and visualized under UV light after ethidium bromide staining. The expected fragment size was ~1700 bp in size.

All hemolymph samples were screened for the presence of H. perezi DNA using primers previously designed to target the ITS1 rRNA region of the parasite (Small et al., 2007). Cycling conditions and reaction concentrations were as described by Pagenkopp Lohan et al. (2012). Approximately 15–40 ng (1 μł) of template DNA was added per PCR assay. Aliquots of 10 μł of the resulting PCR product was electrophoresed on a 1.5% w/v agarose gel and visualized under UV light after ethidium bromide staining. The expected fragment was 302-bp in size. PCR assays were repeated for selected samples that were positive by only one diagnostic technique (hemolymph smear or diagnostic PCR). Included in all PCR assays was a negative control that consisted of no DNA, and a positive control consisting of a sample of H. perezi DNA that had routinely amplified in past PCR studies. We did not use a quantitative PCR method of Li et al. (2010) because it was optimized for environmental samples, not hemolymph samples.

2.4. Histological assessment

The first fifty crabs assessed each month were dissected and their tissues preserved for histological examination (except for those identified as infected from hemolymph smears in 2012). Tissues from dissected animals were placed in individually labeled cassettes. Soft tissues (heart, hepatopancreas, epidermis, and leg muscle) and gill samples were taken from all dissected animals. All tissues were placed in Bouin’s fixative for 24–72 h before transfer to 70% ethanol for short-term storage. Gill tissues were decalcified in a formic acid–sodium citrate solution for 4–12 h before processing into paraffin (Luna, 1968). Tissues were processed in standard ethanol series, embedded in paraffin, cut at 5–6 μm, and processed in an ethanol series before staining with Mayer’s hematoxylin and eosin (Humason, 1979). All histological slides were examined for the presence and intensity of parasite, as well as other histological features. All of the slides were read by one observer (JDS). The histological assessment was blind to the PCR results, but crabs later identified as positive for H. perezi by PCR were re-evaluated for histological diagnosis. Data from the second histological assessment were not included in the specificity and sensitivity calculations. Crabs that were negative by PCR and histological assessment were used as negative controls to evaluate the presence and quantity of effete or apoptotic cells in histological slides.

3. Results

Over the sample period of two winters, 2011 and 2012, 466 crabs were collected from Wachapreague Creek. Excluded were seven samples from crabs that were dead at the time of assessment and whose hemolymph samples were not adequately obtained (2 in 2011, 5 in 2012). Irrespective of assessment method, a total of 94 (20.1%) crabs were infected with H. perezi. Infections were lower in 2011 than in 2012, with mean prevalence levels of 8.2% and 25.4%, respectively. During winter, 2011, month to month prevalence was similar, ranging from 7.9% to 9.1%. In 2012, the monthly prevalence varied. In January the prevalence was at its highest at 32.7%, followed by 24.7% in February and 25.5% in March (Fig. 1). Water temperatures were markedly different between annual dredge periods. In 2011, at the time of collection water temperatures in January were 2.5°C, in February, 5.6°C, and in March, 10.5°C, whereas in 2012, temperatures were 6.0°C, 7.2°C and 13.9°C, respectively. For the pooled data, female crabs had significantly more infections than males (18.2% vs. 6.4%, Chi-square = 14.95, df = 1, p < 0.001), and infected crabs were somewhat smaller than uninfected crabs (83.9 ± 24.3 mm vs. 96.5 ± 21.8 mm CW, ± sd, respectively) (t-test = 3.663, df = 453, p < 0.001). The presence of injuries was not associated with prevalence of the parasite (Chi-square).

3.1. Diagnostic performance, specificity and sensitivity analyses

The three diagnostic methods were compared against each other using the first 50 crabs sampled in each month (50 crabs per month, total used in comparison = 300). In this assessment, 34 crabs had positive infections (11.3%) by all three methods (Fig. 2). PCR assays and histological preparations accounted for the majority of positive diagnoses (n = 60 and 48, respectively), with PCR assays solely identifying potentially three light infections and histology (and by proxy hemolymph smear) solely identifying two light infections.

From hemolymph smears, 34 (11.3%) of 300 crabs were determined to have H. perezi infections based on presence of the parasite. Of these, 13 were not diagnosed by histology as the infected animals were repeatedly confirmed to have infections in later experimental observations (see below). Note that the data for these animals were included in the histological assessment as their infections are obvious in histology when present in smears. The sensitivity of the hemolymph smears (or the % that were true positives) was 94.1% and the specificity (or % that were true negatives) was 89.4% (Table 1). However, the positive predictive value (% correctly diagnosed as positive) of the hemolymph smears was low, 53.3% that of the PCR assays. In comparison, PCR resulted in the highest number of positive samples, diagnosing 60 or 20.0% of the animals examined using this method. Histological assessment revealed positive diagnoses for 48 animals or 16.0% of the animals

![Fig. 1.](image-url)
Table 1

<table>
<thead>
<tr>
<th>PCR assay</th>
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<tr>
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Table 2

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The sensitivity of the histological method was 96% and the specificity 95.2%. The positive predictive value of the histological assessment was 80.0% of the PCR assay. Note, however, that two crabs had infections via histological assessment (and hemolymph smear) but not by PCR. Therefore, the negative predictive value (% correctly identified as negative) for any single method was high, >99.0% but not 100%, indicating that the methods were very good at properly diagnosing the parasite, but there were minor inconsistencies between all methods.

3.2. Histological assessment

Two types of infections, active and effete, were noted in the histological assessments. Active infections possessed obvious parasite cells in their classical forms (uni- and multinucleate stages, cells with characteristic condensed chromatin). These infections frequently had parasites present in the hepatopancreas, heart, and gill, and rarely in muscle, gonad and epidermal tissues. Active infections consisted primarily of ameboid trophonts and clamp colonies (Fig. 3). They were significantly more prevalent in crabs from the 2012 surveys (84.7% of infections), than in the 2011 surveys (13.0%) (Chi-square = 25.12, df = 1.00, p < 0.001).

After the PCR assays were completed, all histological slides from crabs with PCR-positive assays were rescored to confirm infections. Many cases of atypical, presumptive effete infections were observed (Fig. 4). Effete infections were particularly evident in PCR + crabs from the 2011 samples (87% of infections) (Table 3). Effete cells were not assessed via hemolymph smears but some cells in smears did not have the typical staining pattern observed in neutral red-staining of the parasite. These cells often had a single orange-red vacuole unlike the multiple vacuoles observed in parasites from active infections. Effete infections presented as infrequent rare pylocytic, karyorhectic, or apoptotic-like nuclei in enlarged cells (25 - 30 μm) which were typical of ameboid trophonts, and in many cases these cells had condensed nuclei identical to the trophont stages of H. perezi. In the initial assessments, these most of these crabs initially scored as possessing Hematodinium-like cells, but not a definitive diagnosis. Effete cells, presumably parasites, were also observed in individual crabs with active infections of H. perezi. The low numbers of effete cells present and their inconsistent presentation of distinct diagnostic features made their positive diagnosis difficult via histology, but the effete cells were highly associated with crabs with positive PCR results (Table 3).

Other conditions were also noted in the histological assessments of dredged crabs. Two crabs had microsporidian infections (cf. Plistophora sp.) present in their cardiac and skeletal muscles, four crabs infected with H. perezi had co-infections with an unidentified flagellate (cf. Rhyncopus sp.), and two crabs had crystalline deposits in their tissues consistent with calcinosis (Fig. 5). Two of the co-infections with Rhyncopus sp. were identified in crabs held in the progression study. The identification of Rhyncopus sp. was based on molecular and morphological characterization (Small & Shields, unpubl. data). Morphologically, the flagellates resembled dinospores of H. perezi, except for their smaller, uncondensed nucleus, smaller cell size compared to filamentous trophonts, granularity of the cytoplasm, and presence of two anteriorly projecting flagella seen on live specimens. Plistophora sp. was identified based on the presence of spore packets with >16 spores per packet. Nonspecific granulomas were also present in the tissues of some animals.

3.3. Progression study

Eight crabs with light or moderate infections were held individually at 15 °C and their infections followed over time. Crabs were diagnosed with infections on day 0, but initial parasite densities were not assessed at that time. On day 4 parasite densities ranged from near zero to 4.4 × 10^6 parasites per ml. On day 8 parasite densities ranged from near zero to 1.0 × 10^6 cells per ml. Most crabs experienced an increase in parasite density, but one crab exhibited an approximate 50% decrease in density and another crab had a density near zero (positive, but below the limit of the hemacytometer). Two crabs were removed on Day 8 and processed for histology and PCR because they had rare co-infections with Rhyncopus sp. After Day 12 only four crabs remained alive, and all showed rapid, nearly logarithmic increases in parasite density over time, with two crabs having densities over 7.0 × 10^6 parasites per ml (Fig. 6). All of the crabs were dead by Day 16.

4. Discussion

Hematodinium perezi was observed in active infections overwintering in hibernating blue crabs from a coastal bay in Virginia. While the temperature data are sparse, winter, 2011, was colder...
than that of 2012, and the colder temperatures may have reduced the prevalence and intensity of the parasite in blue crabs in 2011. In 2011, all of the infections were categorized as light intensity or effete, whereas in winter 2012, prevalence was higher and several infections were categorized as moderate intensity and fewer effete cells were observed. In addition, infections were capable of quickly responding to increased temperature in the laboratory, reaching heavy infections after only 12 days at 15 °C.

Few pathogens occur as active infections in temperate decapods during cold winter months. The cold-adapted species of *Hematodinium*, that infects snow crabs and other boreal hosts, is found at temperatures from −1 °C to 8 °C (Meyers et al., 1987; Field et al., 1992; Shields et al., 2005, 2007), and has been cultured at 0 °C (Gaudet et al., 2015) and 8 °C to 10 °C (Appleton and Vickerman, 1998). Outbreaks of a scuticociliate, *Orchitophrya stellarum* have occurred in blue crabs in holding systems during winter months and the pathogen can grow in culture at 4 °C (Small et al., 2013; Miller et al., 2013), but the related species, *Mesanophrys chesapeakensis*, has not been associated with colder temperatures (Messick and Small, 1996). Another scuticociliate, *Anophryoides haemophila*, infects and kills lobsters during winter months (Aiken and Waddy, 1986; Cawthorn, 1997). Active infections of *H. perezi* were found in hibernating blue crabs but many infections appeared effete or quiescent. A previous histological assessment of blue crabs from winter dredge surveys did not observe *H. perezi*, because the survey areas were in moderate salinity (15–25 psu) areas of Chesapeake Bay where the parasite does not occur (Messick, 1998). However, several parasites and pathogens were present at low to moderate levels in the tissues of the overwintering crabs from non-endemic areas in Chesapeake Bay. Our survey area was in the high salinity (~30 psu) waters in a coastal bay where *H. perezi* is endemic (Messick and Shields, 2000).

Effete infections were difficult to diagnose via histological assessment and were not diagnosable via hemolymph smear. Histologically, these infections were identified by an increase in the number of large ameboid cells with pyknotic nuclei present in the gill and hepatopancreas. Because these cells appeared to be in an apoptotic state, it was not clear whether these stages were dead and dying parasites or whether they were host cells. Nonetheless, crabs with effete infections were associated with positive PCR assays for *H. perezi* indicating that the DNA of the parasite had not degraded and that some of the parasites may have survived in the hemolymph of these crabs. Moreover, in some cases, the atypical cells (pyknotic, apoptotic or karyorrhectic nuclei, enlarged cytoplasm) were found in conjunction with parasites exhibiting normal morphologies.

Seasonality is a significant epidemiological feature of all *Hematodinium* – host systems (Stentiford and Shields, 2005). In the coastal bays of Maryland and Virginia, prevalence shows regular sharp peaks in late autumn with rapid declines in winter followed by moderate increases in spring, with a minor peak in late spring or early summer (Messick and Shields, 2000; Sheppard et al., 2003). Epizootics can reach high prevalence levels (80–100%) in juveniles in fall (Messick, 1994), but taper off to nil with the onset of winter temperatures (Messick and Shields, 2000). Messick et al. (1999) showed that survival was low in infected crabs held in decreasing ambient water temperatures (16 °C to 3–4 °C), but the intensity of infection changed little, albeit their experiments ended in early January. Our findings suggest that infections in overwintering crabs can thrive and that these crabs likely serve as a reservoir for transmission of new infections in the spring. Hibernating crabs with light to moderate infections developed high intensity infections within 7–12 days when held at 15 °C. Given that infected crabs were prevalent during a mild
winter, our data indicates that crabs overwintering with active infections may rapidly produce heavy infections that release dinospores to infect new hosts. Although transmission remains an unresolved component in the life cycle of *H. perezi*, the presence of an overwintering reservoir can explain why we have not observed cyst stages in the life cycle of the parasite in culture (Li et al., 2011). It may also explain the relatively rapid onset of new infections in the spring in relation to crab molting, the possible transmission route, when water temperatures are above 15 °C.

In late fall and early winter blue crabs migrate to deeper waters to hibernate, buried 5–10 cm in the benthos (Lippson, 1973). During hibernation they can suffer winter mortalities in low to moderate salinity waters when temperatures drop to 6 °C or below (see Rome et al., 2005). Temperature alone is thought to be the primary driver of winter mortality in blue crabs (Rome et al., 2005). However, few if any studies on winter mortality have examined crabs for the presence of parasitic agents, even though some pathogens have been identified from overwintering crabs (Messick et al., 1999). The results from our winter dredge surveys indicate that *H. perezi* infections may be severely limited by cold temperatures and that the pathogen is unlikely to be the cause of winter mortalities in high salinity zones. Nonetheless, outbreaks of *H. perezi* can damage crab populations in late spring, early summer, and fall (Messick and Shields, 2000).

### Table 3

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<th>Infection status</th>
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Fig. 4. Effete or quiescent infections of *H. perezi* in overwintering blue crabs. Bars = 20 μm. (A) Apparent multinucleate trophont undergoing karyorhexis (arrow) in crab DR225. Another multinucleate trophont possess pycnotic nuclei (arrowhead). (B) Ameboid trophont exhibiting condensed chromatin, possibly with karyorhexis (arrow) in gill of crab DR139. (C) Large, sac-like trophont (arrow) with pycnotic nucleus in a hemal sinus in the hepatopancreas of crab DR123. (D) Large, sac-like multinucleate trophont (arrow) exhibiting pycnosis in the gill of crab DR112. (E) Two effete ameboid trophonts (arrows) in the hepatopancreas of crab DR138. (F) Ameboid trophonts in hepatopancreas of crab DR237. One appears normal (arrow), the other with a pycnotic nucleus (arrowhead). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
4.1. Epidemiological analyses

Several methods have been used to diagnose infections of Hematodinium spp. in their crustacean hosts. Hemolymph smears using vital stains, prepared histological slides, PCR assays and in some cases altered body coloration indicative of advanced infections have been used for many years (see Stentiford and Shields, 2005). Molecular studies have progressed from diagnosis using PCR assays with genus-specific probes (Hudson and Adlard, 1994; Gruebl et al., 2002; Hamilton et al., 2007; Small et al., 2007, 2012), to more refined tools for detection in environmental samples (Frischer et al., 2006; Hamilton et al., 2011), and for real-time PCR in environmental samples (Li et al., 2010; Hanif et al., 2013), as well as the development of more advanced or novel tools, such as the application of high performance liquid chromatography to detect pathogens (Troedsson et al., 2008), use of microsatellite markers for population genetics (Pagenkopf Lohan et al., 2014), and development of a novel dinoflagellate/viral nucleo-protein technique for visualizing the parasite in tissues (Gornik et al., 2013). However, none of these studies have compared methodologies using the epidemiological reference points of sensitivity and specificity.

Two studies have contrasted differences between different diagnostic methods for Hematodinium infections. Pestal et al. (2003) compared microscopic or visual diagnosis with prepared hemolymph smears. They used epidemiological analysis of sensitivity and specificity to determine that macroscopically infected snow crabs represented about half of the infected animals in the sample. Ni Chualáin and Robinson (2011) compared hemolymph smears, histology and PCR assays for Hematodinium sp. infections in Cancer pagurus, but they did not use epidemiological analysis to investigate differences between methods. Hemolymph smears failed to identify one infection and the error rate was calculated as 0.7%, the percentage of incorrectly diagnosed crabs (one of

Fig. 5. Unusual conditions observed in the tissues of hibernating blue crabs. (A) Birefringent crystals (arrows) in the antennal gland of crab DR419. Nomarski, pseudo-darkfield. Bar = 200 µm. (B) Birefringent crystal (arrow) in the gill of crab DR461. Nomarski, pseudo-darkfield. Bar = 200 µm. (C) Multiple non-specific granulomas (arrow) in the hepatopancreas of crab DR085. Bar = 100 µm. (D) A flagellate, cf. Rhyncopus sp. (arrows), in a co-infection with H. perezi in the gill of crab DR101. Bar = 20 µm. (E) A microsporidian sporont, cf. Plistophora sp. (arrow), in the gill musculature of crab DR020. The spores are refractile and do not stain well in H&E. Bar = 20 µm. (F) Focal infection of presumptive Plistophora sp. (arrow) in cardiac muscle of crab DR052. Bar = 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
We examined the sensitivity, specificity, positive predictive value and negative predictive value of three diagnostic methods of screening for *H. perezi*. All three methods use relatively small volumes of host tissues for analysis; thus, there is some possibility of observing false negatives. In addition, each method uses different quantities and types of host tissue, which could account for variations in their diagnostic capability. Thus, comparisons among methods may not reach specificities of 100% due to these differences. Moreover, there may be specific tissues associated with different stages of infection. For example, early stages of *H. perezi* in the blue crab can often be found in the heart (Shields and Squyars, 2000), whereas similar stages of *Hematodinium* sp. in the Norwegian lobster (*Nephrops norvegicus*) occur in the hemal sinuses of the hepatopancreas (Field and Appleton, 1995, 1996).

Initial assessment of diagnostic tools is very important because infections can show seasonal differences, and, thus, should be evaluated for different periods of use (Pestal et al., 2003). For example, hemolymph smears during an October outbreak of *Hematodinium* sp. in blue crabs had a positive predictive value of 97% when compared with PCR (Shields, unpubl. data), but was only 56.7% in crabs from the dredge surveys. The low sensitivity of the hemolymph smears indicates that this method missed about half of the existing infections in the winter surveys. Nonetheless, hemolymph smears are a cost- and time-effective diagnostic method; they should be used as an initial assessment tool because their specificity, sensitivity and negative predictive value are high. Histological assessment and PCR assays gave similarly high sensitivity and specificity values (>95%), but PCR analysis did not differentiate between active and effete infections. Both methods had very high positive and negative predictive values. Finally, the diagnostic method to be used depends on the questions to be addressed. Hemolymph smears and histological assessment can indicate the level and activity of the infection, with the latter providing a permanent record and view of the histopathology, whereas PCR-based assays can provide presence/absence, and, depending on the technique, level of infection.

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**References**


