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Disease ecology of *Hematodinium perezi* in a high salinity estuary: investigating seasonal trends in environmental detection

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ABSTRACT: The blue crab *Callinectes sapidus* has seen a general decline in population levels. One factor influencing mortality is infections by *Hematodinium perezi*, a dinoflagellate parasite. A 2 yr study was conducted in 2014 and 2015 to monitor *H. perezi* DNA within the Maryland (USA) coastal bays, comparing seasonal cycles in the abundance of parasite DNA in environmental samples to parasite presence in host blue crabs. A late summer to early fall peak in *H. perezi* infections in blue crabs was observed, consistent with previous work. Infection intensities matched this trend, showing a slow progression of low intensity infections early in the year, with a peak in moderate and heavy infections occurring between July and September, for both years. It was hypothesized that the peak in water column occurrence would coincide with those months when infection intensities were highest in blue crabs. As the peaks in water column occurrence were in July 2014 and August–September 2015, this is consistent with sporulation being the primary contributor to environmental detection in summer months. An additional peak in environmental detection occurred in both years during the early spring months, the cause of which is currently unknown but may be related to infections in overwintering crabs or alternate hosts. Several new crustacean hosts were identified within this estuary, including grass shrimp *Palaemonetes* spp. and the sand shrimp *Crangon septemspinosa*, as well as the mud crab *Dyspanopeus sayi*. Improved knowledge of this disease system will allow for better management of this important fishery.

KEY WORDS: Blue crab · Crustacean · Environment · DNA · quantitative PCR · qPCR

INTRODUCTION

The blue crab *Callinectes sapidus* makes up one of the largest commercial crustacean fisheries in the USA. In the Chesapeake Bay region alone, the 2014 blue crab catch was worth just over $US 80.5 million (NOAA 2016). However, blue crab populations along the eastern coast of the USA have seen major fluctuations and declines in recent years (Chesapeake Bay Stock Assessment Committee 2015). These declines are likely the result of a combination of factors, including overharvesting, poor larval recruitment, and disease.

Natural mortality rates are difficult to estimate and the impact of disease on blue crab populations is currently unknown. Adverse conditions, such as increasing water temperatures caused by global climate change and elevated pollutants due to human population growth, may also influence disease and mortality. In response to these concerns, the Chesapeake Bay Stock Assessment for the blue crab has recommended investigating incidental sources of mortality, such as disease, to improve these estimates for better management of this valuable fishery (Chesapeake Bay Stock Assessment Committee 2015).

In the mid-Atlantic, the parasite *Hematodinium perezi* is one such cause of disease in the blue crab. This dinoflagellate has the potential to drastically affect blue crab populations, with lab studies showing mortality rates of up to 86% (Shields & Squyars 2000).

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Epizootic events where infection prevalence reached 100% have been observed in the field, after which infections disappeared from the crab population presumably due to high mortality (Messick 1994).

Along the Maryland coastline, there are several oceanic bays where summer mortality events have been attributed to infections by *H. perezi*. For the purpose of this study, these bays are denoted as a unified system known as the Maryland coastal bays (MCB), which includes Isle of Wight, Assawoman, Sinepuxent, and Chincoteague bays, though a portion of Chincoteague Bay is in Virginia. Seasonal cycles of infection in this system suggest higher rates of infection when water temperatures are at their highest or beginning to cool from their summer peak (Messick 1994, Messick et al. 1999, Messick & Shields 2000).

Although the exact mechanism of disease transmission is still unknown, there have been reports of sporulation events, where heavily infected hosts release dinospores into the water column (Shields & Squyars 2000). Despite this known connection to environmental release, limited work has investigated the hypothesis that sporulation in highly infected blue crabs leads to greater detection in the water column, and thus likely represents the dispersal phase in the parasite life cycle. Frischer et al. (2006) reported *Hematodinium* sp. DNA in the water column only when blue crabs were also present in a Georgia estuary, following disease manifestation in the blue crab population. Emergence from a resting life cycle stage or from alternate hosts has also been postulated as a means for parasite entry into the water column, particularly during periods when blue crab infections are not of high intensity (Messick & Shields 2000, Pagenkopp Lohan et al. 2012, Pitula et al. 2012). Consistent with this, in the MCB, *H. perezi* DNA can be found in water and sediment from April to November (Pitula et al. 2012, Hanif et al. 2013). As heavily infected crabs (currently the only known source of spores) are rare in the spring, environmental detection is thus likely a consequence of as yet unknown mechanisms. However, recent evidence has shown that blue crabs are capable of overwintering with viable *H. perezi*, and that infection cycles can rapidly reinitiate in warmer water temperatures (Shields at al. 2015). This represents another potential avenue for entry of the parasite into the water column.

The objective of this study was to investigate this disparity in field observations by comparing the seasonal abundance of *H. perezi* DNA in the environment to that in the blue crab population over a 2 yr period. This directly tested the hypothesis that the abundance of *H. perezi* in the water column would coincide with periods when infection intensity was greatest. Previous evidence in the MCB has also shown that *H. perezi* is detectable in the water column during periods when blue crabs are just emerging from hibernation (Pitula et al. 2012). As other crustacean species are more active than blue crabs in the early spring months, their potential to harbor *H. perezi* at appreciable levels was also explored.

MATERIALS AND METHODS

Environmental sample collection

Environmental samples were collected at 18 different sites according to a randomized sampling regime within the MCB (Fig. 1) in conjunction with the National Park Service's Bay Water Quality Surveys. The sampling method for environmental collection using a 20 µM plankton net is described in Pitula et al. (2012). A concentrated sample of biota was collected in a final volume of approximately 50 ml from

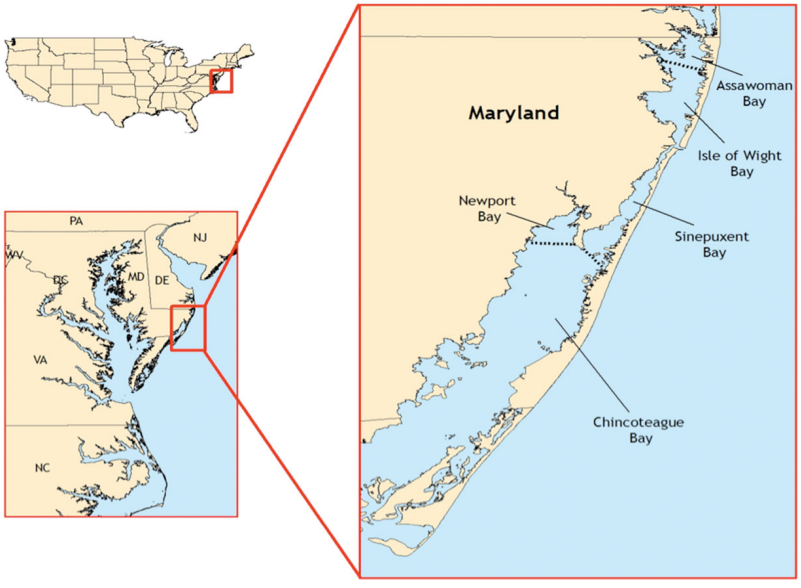

Fig. 1. Sampling areas within the Maryland coastal bay system, on the Delmarva Peninsula. This system includes Assawoman Bay, Isle of Wight Bay, Sinepuxent Bay, and Chincoteague Bay. Black dotted lines: northern borders of Chincoteague Bay. Image generously provided by Rebecca Peters

each tow. Tubes were placed on ice until sampling was completed, after which they were frozen at −50°C and purified for DNA analysis at a later date at the University of Maryland Eastern Shore (UMES). Samples were collected from February to November in 2014 and from March to November in 2015. The change in collection in February 2015 was due to the extremely low temperatures that caused ice to form on the bays and prevented sampling. At each site, environmental data were recorded at the surface using a YSI 6600 with a 650 MDS multi-parameter display and included water temperature, salinity, dissolved oxygen, pH, turbidity, and chlorophyll *a*.

Crab and alternate host species sampling

The blue crab population was sampled via a randomized sampling design in conjunction with the Maryland Department of Natural Resources Coastal Bays Fisheries Investigation Surveys. Trawl surveys were performed at 20 sites in the MCB from April through October and are discussed in detail in Messick & Shields (2000).

During sampling, live crabs were placed in bags labeled with the date and site and stored on ice for transport. All collected crabs were measured and sexed at the UMES Paul. S. Sarbanes Coastal Ecology Center. Larger crabs (>40 mm carapace width [CW]) had hemolymph drawn from the arthrodial membrane of the 5th walking leg using a 1 ml syringe equipped with a 27 gauge needle. Drawn hemolymph was immediately mixed in a 1:1 ratio of anti-clotting buffer (HEPES 10 mM [pH 7.4], NaCl 400 mM, KCl 10 mM, glucose 100 mM, $NaHCO_3$ 10 mM, EDTA 10 mM; Söderhäll & Smith 1983), frozen at −50°C, and processed at a later date. Smaller crabs (<40 mm CW) were frozen at −50°C and 50 mg of muscle tissue was dissected at a later date. All dissections and DNA purifications were performed at UMES.

In 2015, several alternate host species were also collected. Sampling focused on 2 species of shrimp, grass shrimp *Palaemonetes* spp. and the sand shrimp *Crangon septemspinosa*, as well as mud crabs *Dyspanopeus sayi* and *Panopeus herbstii*. All species were frozen and 50 mg of muscle tissue was dissected at a later date. In some cases, mud crabs were too small for muscle tissue alone to be dissected, in which case, internal soft tissue was used. At the time of dissection, shrimp were weighed and their overall length was measured. All crab species were measured for carapace width.

DNA purifications

DNA purifications were performed using the Illustra Tissue and Cells Genomic Prep Kit (GE Healthcare), according to the manufacturer's protocols with several exceptions. For plankton samples, 200 µl of the collected water was used. For tissue purifications, approximately 50 mg of tissue was used. For hemolymph purifications, 50 µl of the 1:1 buffer mix was used. Tissue purifications were incubated at 56°C for 2 to 3 h, instead of the 1 h used for all other samples. Resulting DNA from all purifications was suspended in 100 µl of the provided elution buffer. Each batch of purifications was run with a negative control, which received only the purification kit reagents. Each batch was also run with a positive control, which received 50 µl of a positive control plasmid containing the ribosomal DNA gene of *H. perezi*. This plasmid was generously provided by Dr. Eric Schott at the University of Maryland Center for Environmental Science Institute for Marine and Environmental Technology (UMCES IMET).

Quantitative polymerase chain reaction

Quantitative PCR (qPCR) was used to verify the presence or absence of *H. perezi* DNA in samples. Based on the previous work of Nagel et al. (2009), qPCR detection was used as an indicator of infection status in both hemolymph and muscle tissue. The ITS2 primer set and the protocol described in Hanif et al. (2013) were used for all samples in this study. All qPCR reactions were performed on a Bio-Rad CFX Connect Real-Time System using the Bio-Rad Universal Probe SsoAdvanced Supermix. Following detection in alternate hosts, qPCR results were confirmed by sequence analysis covering the ITS1 region within the ribosomal DNA gene using the forward primer CCT AGT AAG CGC GAG TCA TCA GC and reverse primer TTC ACG GAA TTC TGC AAT TCA CAA TGC TT.

A standard curve for qPCR was created using aliquots of the positive control plasmid. Plasmid concentration was quantified using a NanoDrop spectrophotometer and diluted to 10^9 gene copies. A serial dilution was then created anew for each plate from 10^8 to 10^1 gene copies. The standard curve and no template control (NTC) were run in duplicate and all unknown samples were run in triplicate. Also included in each run were the negative and positive controls from each batch of purifications. The purification negative control was used to determine back-

ground amplification and set the limit for what was considered negative within that batch of DNA purifications.

Raw qPCR numbers were then used to calculate cells l^{-1} under the assumption that 300 copies of rDNA exist per cell (Li et al. 2010). A cell count was performed to test this assumption, using a neutral red stain (as described by Li et al. 2010) to identify parasite cells from a heavily infected blue crab. A comparison was made between the estimated cell count obtained with a hemocytometer and by calculating cells ml^{-1} from the raw copy number generated by qPCR. The cell count estimated with a hemocytometer was 2.74×10^7 cells ml^{-1} hemolymph, whereas that for qPCR was estimated at 2.42×10^7 cells ml^{-1}. This confirms that 300 gene copies per cell is an appropriate estimate. To compare tissue dissections to hemolymph, all qPCR data for tissue samples was standardized as parasite cells g^{-1} tissue (comparable to cells ml^{-1} hemolymph). This included alternate host species and juvenile blue crabs where hemolymph was not drawn. In crab hemolymph and tissue samples, infection status was determined using the following categories as defined by Shields & Squyars (2000): light infections ($<4.0 \times 10^5$ parasites ml^{-1}), moderate infections (4.0×10^5 to 2.0×10^6 parasites ml^{-1}), and heavy infections ($>2.0 \times 10^6$ parasites ml^{-1}).

Statistical analysis

The comparison of environmental occurrence (i.e. the proportion of sites where parasite DNA was detected) between years was analyzed with a contingency table. Parasite prevalence in crabs (e.g. proportion infected) between months within years (separately for each month of 2014 and 2015), and between years was also analyzed with contingency tables. In addition, contingency tables were used to compare the proportion of light infections between years and the proportion of heavy and moderate infections between years (Table S1 in the Supplement at www.int-res.com/articles/suppl/d124p169_supp.pdf). Moderate and heavy infections were grouped due to low sample sizes (McDonald 2014). A Student's *t*-test was performed to compare the environmental abundance of parasite cells between years.

ANOVA tests were performed to compare abiotic variables (dissolved oxygen, pH, temperature, chlorophyll *a*, salinity, and turbidity) between the 2 years of the study (Table S2 in the Supplement). To keep the dataset balanced, data for February 2014 were removed from the analysis. Assumptions of the ANOVA were tested using Shapiro and Levene's tests. In cases where data were non-normal and variance between groups was different, a non-parametric test, the Kruskal-Wallis test, was performed to check the accuracy of the ANOVA. In most cases, ANOVA p-values were less significant than p-values for the Kruskal-Wallis test.

Correlograms (package corrgram in R) were created to explore the relationship between the monthly proportions of infection prevalence and environmental occurrence and the monthly averages of infection intensity, environmental density, and all environmental data collected by YSI for 2014 and 2015. Individual correlation tests were run to investigate the strength of the correlation for infection intensity and environmental occurrence in both 2014 and 2015. Statistical tests were performed in R (version 3.3.0) using R studio (version 0.99.879) (R Core Team 2013, R Studio Team 2015).

RESULTS

Trends in environmental detection

In 2014 and 2015, there were similar seasonal patterns in the number of sites (occurrence) where parasite DNA was detected, with a peak in the early spring and a second peak in the summer (Fig. 2A). However, the summer peak in 2015 was delayed by 1 mo from that observed in 2014. Parasite DNA was detected in plankton tows at a higher proportion of sites in 2014 (106/180 sites, or 59%) than in 2015 (79/162 sites, or 49%). However, there was no significant difference ($\chi^2 = 2.73$, $p > 0.98$) in the environmental occurrence of DNA between 2014 and 2015. The highest occurrence of parasite DNA in environmental samples was seen in July and March of 2014, with 100% occurrence in July and 89% occurrence in March (16/18 sites). In 2015, the highest environmental occurrence was seen in March, August, and September, with 78% occurrence in March (14/18 sites), and 72% occurrence in August and September (13/18 sites).

The average number of parasite cells in 1 l of water was also calculated for each month and is henceforth referred to as environmental density. The highest average monthly densities were seen in July (3.28×10^3 cells l^{-1}) and February (2.55×10^3 cells l^{-1}) of 2014 (Fig. 2B) and in March (1.33×10^3 cells l^{-1}), August (7.54×10^2 cells l^{-1}), and October (7.69×10^2 cells l^{-1}) of 2015. There was a significant difference in the estimated environmental density between the 2 years

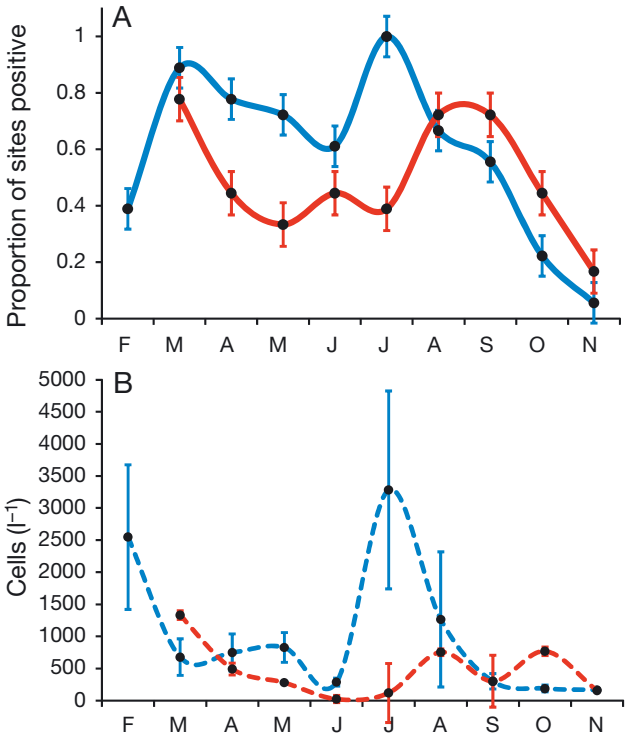

Fig. 2. Monthly occurrence and abundance of *Hematodinium perezi* DNA in environmental samples. (A) Proportion of sites where parasite DNA was detected in plankton samples. Error bars are calculated as the binomial proportion 95% CI. (B) Abundance of *H. perezi* in cells l^{-1} of water (average of all sites where parasite DNA was detected in a given month). Due to the high variation in monthly abundance, error bars are calculated as SE to highlight the accuracy of the calculated mean for a given month. Blue lines: 2014; red lines: 2015

(t = 2.255; df = 227.84, p = 0.025), with 2014 having higher environmental densities. Of the sites that were positive, the average density was 1.24×10^3 cells l^{-1} ($\pm 5.20 \times 10^2$, 95% CI) in 2014, and in 2015 the average density was 5.57×10^2 cells l^{-1} ($\pm 2.29 \times 10^2$, 95% CI).

Infections in blue crabs

Blue crabs were assayed for seasonal changes in infection prevalence and intensity. Prevalence peaked in August 2014, with 40% of all crabs testing positive for parasite DNA (16/40 crabs), and in April and August 2015 at 69 and 67% (27/39 and 22/33 crabs), respectively (Table 1). Parasite prevalence in crabs differed significantly between months in both 2014 and 2015, indicating marked seasonal variation in infections (χ^2 = 17.39, p < 0.01 and χ^2 = 27.45, p < 0.001, respectively). Prevalence across the entire

Table 1. Monthly infection prevalence and average infection intensity for 2014 and 2015. Infection prevalence is the total number of crabs infected, divided by the total number of crabs sampled in a given month. Infection intensity is the average number of cells g^{-1} of tissue or ml^{-1} of hemolymph for all crabs testing positive for *Hematodinium perezi* DNA.
na: not applicable; n = total sampled; Prev.: prevalence

Month	Intensity (cells g^{-1}) Mean	SE	Total positive	n	Prev. (%)
2014					
Apr	3.00×10^5	2.45×10^5	4	30	13
May	2.08×10^5	7.47×10^4	3	22	14
Jun	2.26×10^5	1.73×10^5	10	51	21
Jul	2.09×10^5	7.37×10^4	7	30	23
Aug	1.50×10^6	5.22×10^5	16	40	40
Sep	1.89×10^5	9.32×10^4	5	49	10
Oct	na	na	0	10	0
Total			45	232	19
2015					
Apr	1.17×10^5	3.07×10^4	27	39	69
May	5.72×10^5	5.25×10^5	7	30	23
Jun	1.39×10^5	4.34×10^4	32	85	38
Jul	1.02×10^6	3.91×10^5	30	49	59
Aug	2.35×10^6	1.34×10^6	22	33	66
Sep	1.91×10^6	1.18×10^6	19	36	33
Oct	2.17×10^5	9.24×10^4	9	25	35
Total			146	297	49

sampling season in 2014 was 19% (45/234 crabs), whereas in 2015, it was 49% (146/297 crabs). This difference in yearly prevalence was statistically significant (χ^2 = 48.265, p < 0.00001).

The highest intensities of infection were seen in August of both years (1.50×10^6 and 2.35×10^6 cells g^{-1} tissue, respectively; Table 1). The lowest average infection intensities were seen in May 2014 (2.08×10^5 cells l^{-1}) and April 2015 (1.17×10^5 cells l^{-1}). Infections were grouped according to infection status (light, moderate, and heavy) as defined above (Fig. 3, Table S1). The proportion of moderate and heavy infections was not significantly different between years based on a chi-squared test (χ^2= 1.15, df = 1, p = 0.282), whereas the proportion of light infections was significantly different between years (χ^2 = 44.85, df = 1, p < 0.0001). Similar monthly trends were observed in both years with moderate and heavy infections beginning to build in June to their peaks in August. These moderate and heavy infections then declined through September before disappearing in October. Light infections also displayed peaks in summer months with declines beginning in the fall. However, 2015 saw a much higher proportion of light infections in April, with elevated infections (compared with 2014) continuing throughout the year.

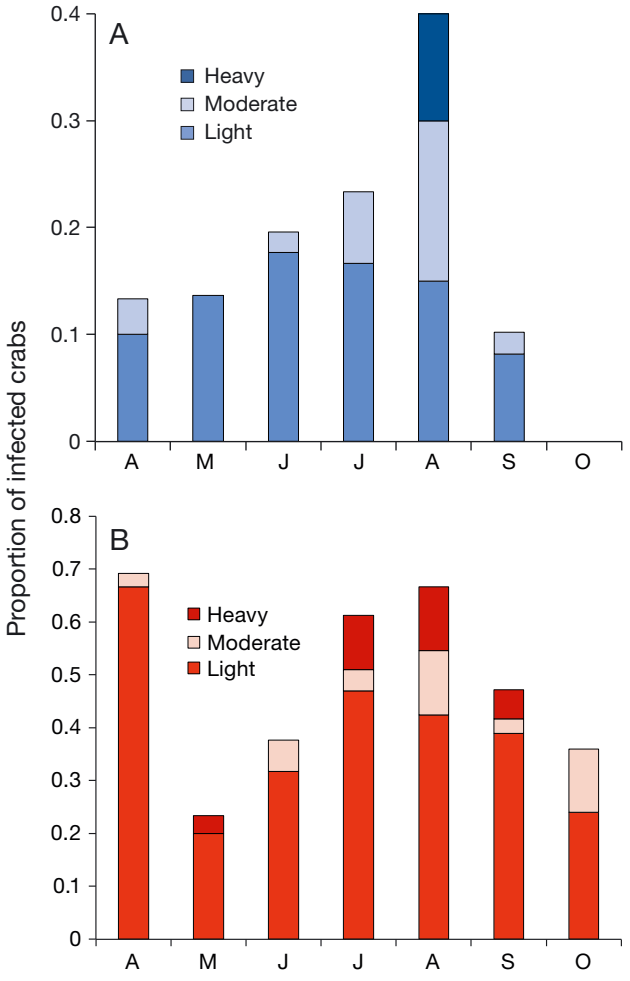

Fig. 3. Infection status of blue crabs *Callinectes sapidus* by month for (A) 2014 and (B) 2015. The monthly proportion of crabs in each infection status (light, moderate, and heavy) is shown, representing the total number of crabs categorized by that level of infection divided by the total number of crabs sampled in that month

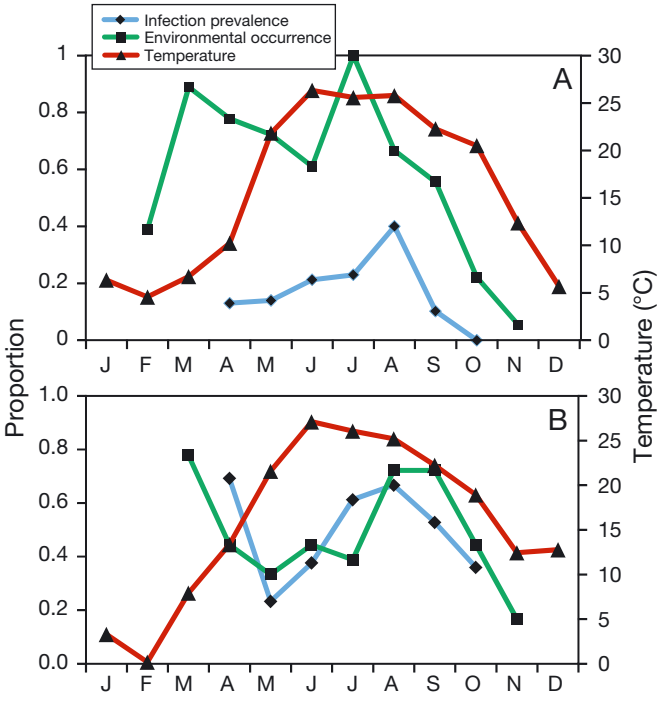

Fig. 4. Monthly infection prevalence, environmental occurrence, and temperature in (A) 2014 and (B) 2015. Proportion of infected blue crabs *Callinectes sapidus* each month and the proportion of sites where *Hematodinium perezi* DNA was detected (environmental occurrence) along with the average monthly temperature (on the secondary axis)

Environmental variation between years

Due to the differences in water column abundance of parasite DNA between 2014 and 2015 (Fig. 2), as well as the observed difference in the prevalence of infection in blue crabs (Table 1), the changes in abiotic factors between the 2 years were investigated via ANOVA. According to the results of these tests, dissolved oxygen, pH, and chlorophyll *a* were all significantly different between the 2 years, and all 3 of these variables were elevated in 2015 (Table S2). There were no significant differences between salinity, temperature, and turbidity between the 2 years.

Temperature showed no statistically significant difference between sites ($F = 0.204$, df = 17, 306, p = 1.0). This implies that these conditions are relatively constant in a given month over the entire MCB system, and it is thus possible to use a system-wide average of all 18 sites to determine monthly temperature. The monthly average temperature for both 2014 and 2015, alongside the monthly proportion of infected crabs and the monthly proportion of sites where parasite DNA was detected, is shown in Fig. 4. The lowest average temperatures occurred in February of both years (4.56°C in 2014 and 0.20°C in 2015) and the highest average temperatures occurred in June of both years (26.33°C in 2014 and 27.10°C in 2015). No obvious relationship was observed between temperature and fluctuations in parasite detection in the environment or in blue crabs.

Comparing environmental factors with infections in blue crabs

A correlogram analysis was used to further explore the correlation between infections in blue crabs and all environmental factors measured in this study (Fig. 5). Infection prevalence was positively correlated with environmental occurrence and environmental

density in both 2014 and 2015, though the strength of that relationship varied between years. A correlation test for infection intensity and environmental occurrence shows that the correlation for these variables was not significant in 2014 (t = 0.39, df = 5, p = 0.71, correlation = 0.17), but was significant in 2015 (t = 3.40, df = 5, p < 0.02, correlation = 0.84). A positive correlation was also seen in both years between chlorophyll *a* and infection prevalence, infection intensity, and environmental occurrence. A slight positive trend was also seen between infection intensity and temperature in both years.

Detection of *H. perezi* in alternate host species

In 2015, other potential host species were collected in an opportunistic manner. Several species of shrimp and 2 species of xanthid mud crab were examined. Detection prevalence for the most abundant species by month is presented in Table 2. Parasite load, as determined by qPCR, is presented in Table 3.

In April, 83% of sand shrimp were qPCR positive for parasite DNA in muscle tissue (5/6 shrimp), which declined in May and June (4/22 and 4/15, respectively). This detection was confirmed by cloning and sequencing of an approximately 600 base pair fragment of the ribosomal DNA gene (data not shown). Sand shrimp were not seen and therefore not collected after June. In both May and June, 5 grass shrimp *Palaemonetes* spp. were collected and in each month, 2 of the 5 were qPCR positive. Detections then declined through the summer and disappeared in the fall.

Parasite DNA was present in *Dyspanopeus sayi* throughout the sampling season, with the highest prevalence of qPCR positive hosts occurring in May (7 of 7 crabs sampled) and June (7 of 10 crabs sampled). Only 6 *Panopeus herbstii* were collected, 3 of which were

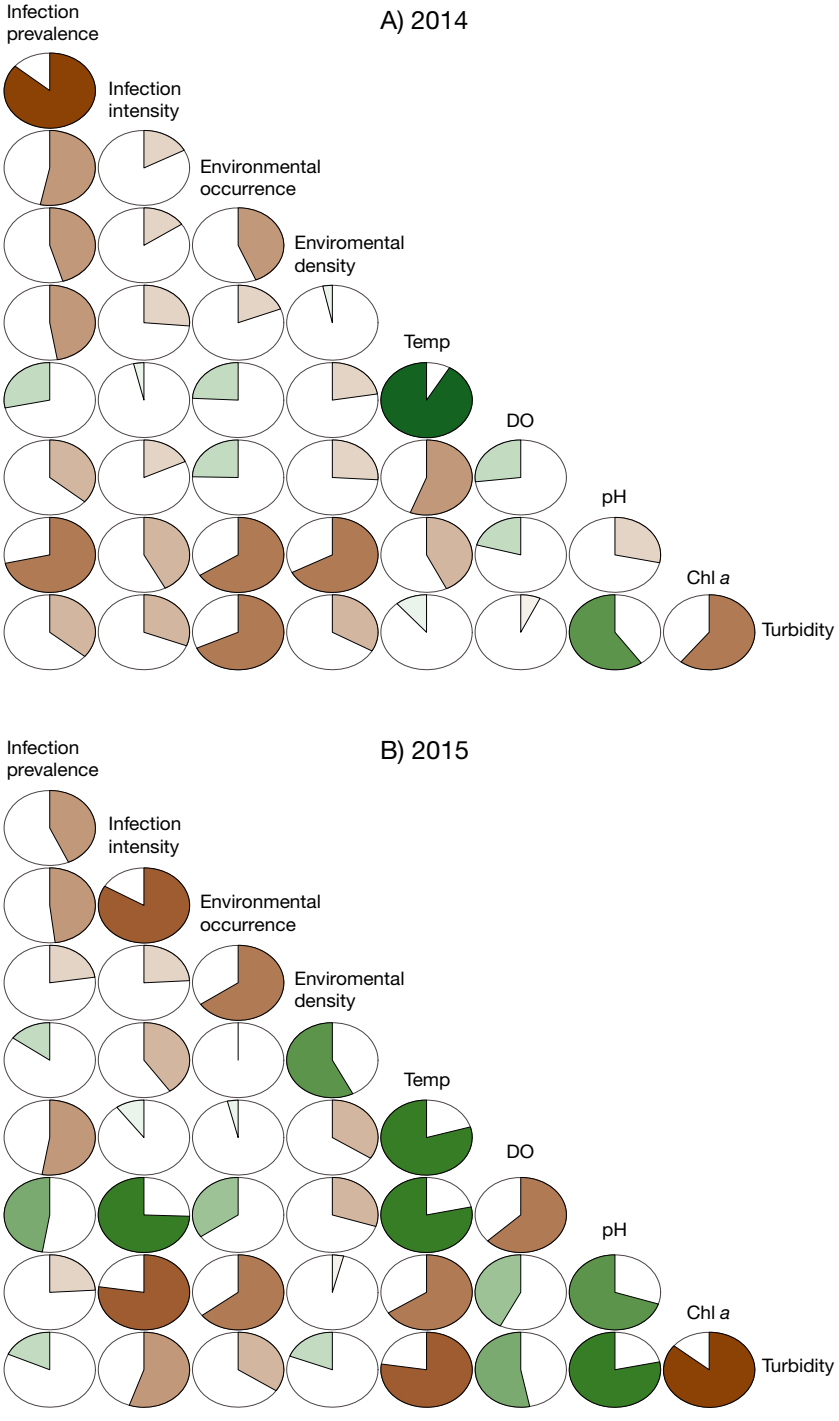

Fig. 5. Correlograms showing the correlation between monthly average or proportion of all variables recorded in this study for (A) 2014 and (B) 2015. Brown indicates a positive relationship between variables, while green indicates a negative relationship. The intensity of the 2 colors indicates the strength of the relationship, with darker shades indicating stronger relationships and lighter shades indicating weaker relationships. The individual pie charts represent the correlation coefficient for a given pairing. For example, in 2014, infection prevalence and infection intensity have a correlation value of 0.86. Of interest are the factors that show the same correlation in both years of the study (either positive correlation for both years or negative correlation for both years)

Table 2. Infection prevalence in 3 alternate host species, mud crab *Dyspanopeus sayi*, sand shrimp *Crangon septemspinosa*, and grass shrimp *Palaemonetes* spp., displayed by month. Not all species were found every month. Not included are 3 *Panopeus herbstii* that tested positive for *Hematodinium perezi* DNA, one each in July, August, and October. n = total sampled; qPCR+: qPCR positive; Prev.: prevalence

Month	*D. sayi*			*C. septemspinosa*			*Palaemonetes* spp.		
	n	No. qPCR+	Prev. (%)	n	No. qPCR+	Prev. (%)	n	No. qPCR+	Prev. (%)
Apr	4	2	50	6	5	83			
May	7	7	100	22	4	18	5	2	40
Jun	10	7	70	15	4	26	5	2	40
Jul	20	8	40				5	1	20
Aug	10	4	40				11	1	9.1
Sep	2	1	50				5	0	0
Oct	2	1	50				2	0	0

Table 3. qPCR data for alternate host species. Sample size and positive detection prevalence is presented here, along with the average estimated *Hematodinium perezi* cells g^{-1} tissue. Each species has all positive samples averaged and the SE is calculated and presented as well. Note the high SE values indicating the range of parasite cells g^{-1} tissue present in the alternate host species samples. The cell counts in these samples range from 3.33×10^2 to 8.63×10^5 cells g^{-1} of tissue. qPCR+: qPCR positive; n = total sampled; Prev.: prevalence

Species	No. qPCR+	n	Prev. (%)	Infection intensity (cells g^{-1} tissue) Mean	SE	Range
D. sayi	31	55	56.4	3.50×10^4	2.77×10^4	$333–8.63\times10^5$
Palaemonetes spp.	8	34	23.5	1.94×10^4	9.21×10^3	$333–5.0\times10^4$
C. septemspinosa	14	43	32.6	1.97×10^4	1.12×10^4	$333–1.57\times10^5$
P. herbstii	3	6	50.0	1.70×10^4	1.65×10^4	$333–7.53\times10^4$

qPCR positive for parasite DNA, one each in July, August, and October (Table 3).

The abundance of parasite DNA in alternate host tissues varied widely both within the same species (high SE) and between different species (much higher average abundance in *D. sayi* than all other species). When an attempt was made to classify parasite abundance in alternate host tissues in terms of infection status, only a single mud crab *D. sayi*, collected in April 2015, was classified as moderate (8.63×10^5 cells g^{-1} tissue). All other detections fell under the light category ($<2.0 \times 10^5$ cells g^{-1} tissue).

DISCUSSION

Seasonal cycles of infection appear to be common in all known *Hematodinium* systems, though the timing of peak infection varies between hosts and climates (Stentiford et al. 2001, Stentiford & Shields 2005, Small et al. 2012). For example, in a Georgia estuary, an April–May peak was observed with infections disappearing from blue crabs in the summer, followed by a strong prevalence of fall infections (Sheppard et al. 2003). Hamilton et al. (2009) also detected a dual peak infection cycle for several, but not all, species of crustaceans found to carry the cold water species of *Hematodinium*. In a multiyear study in the MCB, conducted between 1992 and 1997 and using histological approaches, Messick & Shields (2000) observed strong fall infection prevalence, occasionally preceded by less-pronounced peaks. Early- to mid-spring infections were virtually undetectable, the sole exception being in May 1996 with 5% prevalence. In the work reported here, using qPCR-based analysis, infection prevalence in April varied between years with 13% prevalence in 2014 and 69% prevalence in 2015 (the vast majority of April infections being categorized as light). This is consistent with recent work that has shown overwintering crabs are capable of harboring disease (Shields et al. 2015).

The paradigm of a late summer to early fall peak in *Hematodinium perezi* infections in blue crabs from the MCBs, originally reported by Messick & Shields (2000), is consistent with what was observed in this study (Table 1). Infection intensities matched this trend, showing a slow progression of low intensity infections early in the year, with a peak in moderate and heavy infections occurring between July and September for both years (Fig. 3). Based on previous work in this disease system, it was hypothesized that the peak in water column occurrence would coincide with those months when infection intensities were highest in blue crabs, due to robust sporulation events associated with these types of infections (Shields & Squyars 2000). However, the correlograms show that environmental occurrence has only a weak positive correlation with infection prevalence (Fig. 5). It should be noted that the origin of *H. perezi* DNA in environmental samples potentially includes dinospores, cells in association with micro- and mesoplankton, and/or environmental DNA from dead cells. While each of these aspects of *H. perezi* ecology is relevant to understanding the disease cycle, a direct relationship between infection in blue crabs and environmental presence may be masked from year to year. Never-

theless, summer peaks in environmental occurrence (July 2014 and August and September 2015) and environmental density (July 2014 and August 2015) match peaks in infection prevalence (August 2014 and July and August 2015) and infection intensity (August of both 2014 and 2015).

Parasite DNA was detected in at least one site in all months sampled, even when heavy infections were not seen in blue crabs. This is especially interesting in the colder late-winter and early-spring months when blue crabs are not expected to be active. In temperate regions, blue crabs enter a senescent period below approximately 10°C (Kennedy & Cronin 2007). A temperature minimum of 9°C was also postulated to be required for parasites to proliferate in crabs harboring overwintering infections (Messick et al. 1999). Temperatures below this level are often seen in the MCB from approximately December to March (Fig. 4). Although recent studies have shown that blue crabs can overwinter with infections, many of these infections are not active or are of low intensity, depending on winter temperatures (Messick et al. 1999, Shields et al. 2015). The possibility that overwintering crabs sporulate in early spring cannot be ruled out, as Shields et al. (2015) observed that overwintering crabs rapidly developed heavy infections when moved to the lab and held at 15°C. It should be noted, however, that these temperatures are not typically seen in the MCB until May. This argues against sporulation in blue crabs in the MCB as a means of entry into the water column during this time period. As some of the highest abundances of environmental parasite DNA were seen in February and March in both years of this study, it seems likely that the early-spring detection of parasite DNA comes from another source.

The possibility of environmental (Pitula et al. 2012, Hanif et al. 2013) and biotic (Johnson 1986, Hamilton et al. 2009, Pagenkopp Lohan et al. 2012) reservoirs of *H. perezi* has been postulated. In this study, parasite DNA was detected in DNA isolations from several alternate host species, including a previously unknown host, sand shrimp. Price (1962) posited that sand shrimp were a year-round inhabitant of Delaware Bay, which is likely to be the case in the MCB as well, though adult shrimp may migrate out of the system during summer months in search of cooler waters (Haefner 1969). The high prevalence of parasite DNA detected in sand shrimp in April suggests that this species may be a prime candidate for *H. perezi* to overwinter in, providing a potential reservoir for a spring sporulation event.

Even though *H. perezi* has been detected in a variety of species, it is unclear whether these hosts represent true targets of infection as 'maintenance' hosts from which the pathogen can be transmitted to the primary host, the blue crab (Small & Pagenkopp 2011). Such hosts may influence disease transmission by acting as a transmission vector encouraging sporulation and proliferation, or as prey sources for blue crabs (Thieltges et al. 2008). While there is some debate on whether consumption of infected blue crabs can transmit infection to naïve crabs and amphipods, no work has been done to investigate disease transmission via consumption of alternate host species by blue crabs (Sheppard et al. 2003, Walker et al. 2009, Li et al. 2011). In addition, these alternate hosts may represent 'spillover hosts' that are only infected when extremely high disease prevalence is present in the primary host (Pagenkopp Lohan et al. 2012).

Future work should confirm whether detections in these shrimp and other alternate host species are infections. In this study, parasite abundances in alternate host tissues ranged from an estimated 3.33×10^2 to 8.63×10^5 cells g^{-1} of tissue. It is likely that detections on the lower side of this range are incidental; however, some organisms have parasite loads that are suggestive of infection. In April 2015, a single mud crab was found with parasite DNA levels corresponding to a moderate infection. This needs to be confirmed by histopathology but does have important implications for management of the parasite (Haydon 2002).

Infection prevalence in blue crabs also fluctuates between years (Messick 1994, Pagenkopp Lohan et al. 2012, Gandy et al. 2015, Shields et al. 2015). This held true for the current study as blue crab infection prevalence was significantly higher in 2015 than in 2014. Variations in environmental conditions may play a determining role in host–parasite interactions by influencing host or parasite fitness (Wolinska & King 2009), thus creating the yearly fluctuations in infection prevalence seen in *H. perezi* systems. For example, in a South Carolina estuary, elevated dissolved oxygen and pH levels were associated with increased rates of *H. perezi* infections in blue crabs (Parmenter et al. 2013).

Another environmental condition known to influence *H. perezi* infections in blue crabs is temperature (Messick et al. 1999, Messick & Shields 2000, Gandy et al. 2015, Shields et al. 2015). Colder winter temperatures have been linked to reduced infections (Gandy et al. 2015, Shields et al. 2015). Gandy et al. (2015) presented strong evidence for a link between temperature and *H. perezi* disease prevalence, with temperature optimums between 15 and 18°C. In addition, Messick & Shields (2000) reported higher in-

fection prevalence in fall, after water temperatures cooled from their peaks in summer months. Similarly, Parmenter et al. (2013) saw peaks in infection when water temperatures began cooling. Likewise, the data presented here matched these trends, with infections peaking in July and August, after the peak in water temperatures that occurred in June of both years (Fig. 4).

A variety of environmental conditions are also influenced by climate events such as El Niño and La Niña. In late 204 and early 2015, there was a transition to El Niño conditions (NOAA Climate Prediction Center 2016), leading to a higher April water temperature seen in 2015 (13.36 ± 0.56°C), compared with April 2014 (10.24 ± 0.82°C). In association with this shift, there was also a more rapid warming period between February and April in 2015, compared with 2014 (Fig. 4). Perhaps this influenced the development of the spring peak in infection prevalence observed in 2015 and it may partially explain the elevated prevalence observed throughout 2015. Morado et al. (2010) suggested that the expansion of the parasite into new hosts and geographic areas may also be due to recent warming events, such as the shift in the North Atlantic Oscillation towards warmer atmospheric conditions in 1977. Thus, understanding the influence of climate change may be of ultimate importance in understanding both the parasite life cycle in blue crabs and its potential to proliferate in alternate host species.

Although the influence of temperature on disease has been relatively well studied, the influence of other environmental factors on *H. perezi* is currently unknown. In this work, average monthly chlorophyll *a* levels were positively correlated with the monthly proportion of sites where parasite DNA was detected in both 2014 and 2015 (Fig. 5; Table S2), though the relationship was not statistically significant. This may suggest an influence of the photosynthetic community, including other dinoflagellate species, on survival of *H. perezi* in the water column. It is possible that conditions that favor robust populations of organisms that produce chlorophyll *a* may also favor survival of *H. perezi* dinospores or other free-living life stages. In addition, if a free-living stage does exist, nutritional requirements may involve predation on chlorophyll *a*-producing species. This work represents an early attempt at identifying the wide range of factors that may influence the observed yearly and seasonal fluctuations in *H. perezi* infections in blue crabs. However, future work must determine the complete life cycle of the parasite outside the host: only after this is known, can the potential environmental factors that influence individual life stages be explored.

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LITERATURE CITED

Chesapeake Bay Stock Assessment Committee (2015) 2015 Chesapeake Bay blue crab advisory report. www.chesapeakebay.net/documents/CBSAC_2015_Advisory_Report_6-30_FINAL.pdf

Frischer ME, Lee RF, Sheppard MA, Mauer A and others (2006) Evidence for a free-living life stage of the blue crab parasitic dinoflagellate, *Hematodinium* sp. Harmful Algae 5:548–557

Gandy R, Schott EJ, Crowley C, Leone EH (2015) Temperature correlates with annual changes in *Hematodinium perezi* prevalence in blue crab *Callinectes sapidus* in Florida, USA. Dis Aquat Org 113:235–243

Haefner PA (1969) Temperature and salinity tolerance of the sand shrimp, *Crangon septemspinosa* Say. Physiol Zool 42:388–397

Hamilton KM, Shaw PW, Morritt D (2009) Prevalence and seasonality of *Hematodinium* (Alveolata: Syndinea) in a Scottich crustacean community. ICES J Mar Sci 66: 1837–1845

Hanif AW, Dyson WD, Bowers HA, Pitula JS, Messick GA, Jagus R, Schott EJ (2013) Variation in temporal and spatial incidence of the crustacean pathogen *Hematodinium perezi* in environmental samples from Atlantic coastal bays. Aquat Biosyst 9:11

Haydon DT, Cleaveland S, Taylor LH, Laurenson MK (2002) Identifying reservoirs of infection: a conceptual and practical challenge. Emerg Infect Dis 8:1468–1473

Johnson PT (1986) Parasites of benthic amphipods: dinoflagellates (Duboscquodinida: Syndinidae). Fish Bull 84: 605–614

Kennedy VS, Cronin LE (eds) (2007) The blue crab: *Callinectes sapidus*. Maryland Sea Grant College, College Park, MD

Li C, Shields JD, Miller TL, Small HJ, Pagenkopp KM, Reece KS (2010) Detection and quantification of the free-living stage of the parasitic dinoflagellate *Hematodinium* sp. in laboratory and environmental samples. Harmful Algae 9:515–521

Li C, Wheeler KN, Shields JD (2011) Lack of transmission of *Hematodinium* sp. in the blue crab *Callinectes sapidus* through cannibalism. Dis Aquat Org 96:249–258

McDonald JH (2014) Handbook of biological statistics, 3rd edn. Sparky House Publishing, Baltimore, MD

Messick GA (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 GA, Shields JD (2000) Epizootiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue

crab *Callinectes sapidus*. Dis Aquat Org 43:139–152

Messick GA, Jordan SJ, Van Heukelem WF (1999) Salinity and temperature effects of *Hematodinium* sp. in the blue crab *Callinectes sapidus*. J Shellfish Res 18:657–662

Morado JF, Dawe EG, Mullowney D, Shavey CA, Lowe VC, Cawthorne RJ (2010) Climate change and the worldwide emergence of *Hematodinium*-associated disease: Is there evidence for a relationship? In: Kruse GH, Eckert GL, Foy RJ, Lipcius RN, Sainte-Marie B, Stram DL, Woodby D (eds) Biology and management of exploited crab populations under climate change. Alaska Sea Grant, University of Alaska, Fairbanks, AK

Nagel L, Place AR, Schott EJ, Jagus R, Messick G, Pitula JS (2009) Real-time PCR-based assay for quantitative detection of *Hematodinium* sp. in the blue crab *Callinectes sapidus*. Dis Aquat Org 84:79–87

NOAA (National Oceanic and Atmospheric Administration) (2016) Annual commercial landing statistics. www.st.nmfs.noaa.gov/commercial-fisheries/commercial-landings/annual-landings/index (accessed on 20 January 2016)

NOAA Climate Prediction Center (2016) Historical El Nino/La Nina episodes (1950–present). www.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ensoyears2011.shtml (accessed on 29 April 2016)

Pagenkopp Lohan KM, Reece KS, Miller TL, Wheeler KN, Small HS, Shields JD (2012) The role of alternate hosts in the ecology and life history of *Hematodinium* sp., a parasitic dinoflagellate of the blue crab (*Callinectes sapidus*). J Parasitol 98:73–84

Parmenter KJ, Vigueira PA, Morlok CK, Micklewright JA, Smith KM, Paul KS, Childress MJ (2013) Seasonal prevalence of *Hematodinium* sp. infections of blue crabs in three South Carolina (USA) rivers. Estuaries Coasts 36:174–191

Pitula JS, Dyson WD, Bakht HB, Njoku I, Chen F (2012) Temporal distribution of genetically homogenous 'free-living' *Hematodinium* sp. in a Delmarva coastal ecosystem. Aquat Biosyst 8:16

Price KS (1962) Biology of the sand shrimp, *Crangon septemspinosa*, in the shore zone of the Delaware Bay region. Chesap Sci 3:244–255

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. www.r-project.org

RStudio Team (2015) RStudio: integrated development for R. RStudio, Boston, MA. www.rstudio.com

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

Shields JD, Squyars CM (2000) Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi*. Fish Bull 98:139–152

Shields JD, Sullivan SE, Small HJ (2015) Overwintering of the parasitic dinoflagellate *Hematodinium perezi* in dredged blue crabs (*Callinectes sapidus*) from Wachapreague Creek, Virginia. J Invertebr Pathol 130:124–132

Small HJ, Pagenkopp KM (2011) Reservoirs and alternate hosts for pathogens of commercially important crustaceans: a review. J Invertebr Pathol 106:153–164

Small HJ, Shields J, Reece K, Bateman K, Stentiford G (2012) Morphological and molecular characterization of *Hematodinium perezi* (Dinophyceae: Syndiniales), a dinoflagellate parasite of the harbour crab, *Liocarcinus depurator*. J Eukaryot Microbiol 59:54–66

Söderhäll K, Smith VJ (1983) Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods and prophenoloxidase distribution. Dev Comp Immunol 7:229–239

Stentiford GD, Shields JD (2005) A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. Dis Aquat Org 66:47–70

Stentiford GD, Neil DM, Atkinson RJA (2001) The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to seasonality, moulting and sex. ICES J Mar Sci 58:814–823

Thieltges DW, Bordalo MD, Hernandez AC, Prinz K, Jensen KT (2008) Ambient fauna impairs parasite transmission in a marine parasite–host system. Parasitology 135:1111–1116

Walker AN, Lee RF, Frischer ME (2009) Transmission of the parasitic dinoflagellate *Hematodinium* sp. infection in blue crabs *Callinectes sapidus* by cannibalism. Dis Aquat Org 85:193–197

Wolinska J, King KC (2009) Environment can alter selection in host–parasite interactions. Trends Parasitol 25:236–244

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