The influence of temperature and salinity on mortality of recently recruited blue crabs, *Callinectes sapidus*, naturally infected with *Hematodinium perezi* (Dinoflagellata)

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The parasitic dinoflagellate *Hematodinium perezi* is highly prevalent in juvenile blue crabs, *Callinectes sapidus*, along the eastern seaboard of the USA. Although the parasite is known to kill adult crabs, the mortality rate of naturally infected juvenile crabs remains unknown. We analyzed the influence of temperature and salinity on the mortality of recently recruited blue crabs that were naturally infected with *H. perezi*. Over 492 juvenile crabs (infected, n = 282; uninfected controls, n = 210) were held individually in six-well plates and held at six temperatures (4, 10, 15, 20, 25, and 30 °C) or three salinities (5, 15, and 30 psu) for a maximum of 90 days. Mortality of infected crabs was 10 times higher at elevated temperatures (25 and 30 °C) and salinity (30 psu) compared to uninfected control treatments. By contrast, infected crabs exposed to mild temperatures (10, 15, and 20 °C) showed a high survival (> 80%), no different than uninfected control treatments. Infected crabs at the lowest temperature (4 °C) exhibited a high mortality, but the intensity of infection was lower than in the other temperature treatments. In addition, this study revealed the optimal temperature (25 °C) and salinity (30 psu) for *H. perezi* to progress in its life cycle leading to sporulation in juvenile crabs; 31.6% (19/60) of crabs held under these conditions released dinospores of *H. perezi* after 10 days. Crabs held at other temperatures did not release dinospores over the time course of the experiment. Infected crabs were capable of molting and in most cases molted at the same frequency as uninfected crabs serving as controls. The mortality observed in this study indicates that early benthic juveniles will experience significant mortality due to *H. perezi* with increasing ocean temperatures and that this mortality may be a significant factor in the recruitment of blue crabs to high salinity regions.

1. Introduction

Temperature and salinity are key environmental factors that influence the epidemiology of the pathogenic dinoflagellate *Hematodinium perezi* in blue crabs, *Callinectes sapidus* (Messick and Shields, 2000; Coffey et al., 2012; Shields et al., 2017). Along the Atlantic seaboard of the USA, the parasitic dinoflagellate is highly prevalent in juvenile blue crabs in high salinity areas (> 30 psu) from late summer and early autumn, when the water temperature is > 25 °C (Messick and Shields, 2000). The mortality rate of adult blue crabs (> 120 mm carapace width [CW]) infected with *H. perezi* was 86% over 40 days in adult females inoculated with the pathogen (Shields and Squyars, 2000). In experimental infections with fiddler crabs, *Uca minax*, mortality was 95% over 34 days in adult males inoculated with *H. perezi*, but handling stress contributed to the increased mortality rate and may have been a factor in the earlier study with blue crabs (O’Leary and Shields, 2017). Early benthic juvenile blue crabs often exhibit very high prevalence levels of *H. perezi* (Huchin-Mian et al., 2017), but comparative mortality rates have not been obtained, even though this segment of the crab population has the highest prevalence levels.

Infections of *H. perezi* proliferate rapidly in crabs at high temperatures and salinities. Naturally infected crabs collected during winter (6 °C) showed a logarithmic proliferation of the parasite when held at a 15 °C for 12 days (Shields et al., 2015). In another study proliferation of the parasite showed several bouts of logarithmic growth over three to four weeks (Shields and Squyars, 2000). This rapid proliferation may explain why mortality may reach 100% in naturally and experimentally infected adult crabs over a relatively short time period of 35 days and 55 days, respectively (Messick and Shields, 2000). The cause of death is associated with the proliferation of the parasite in hemolymph and...
tissues, that ultimately induces metabolic exhaustion (Shields et al., 2003) and respiratory dysfunction (Field et al., 1992; Taylor et al., 1996). Although low salinities are not associated with the transmission of the parasite, infected crabs can survive at low salinities and apparently produce prespores and dinospores in experimentally inoculated large juvenile and adult blue crabs (Coffey et al., 2012).

Although the route of transmission remains unknown for H. perezi, recent sentinel studies support that direct transmission via an infectious dinospore is the likely route to blue crabs (Huchin-Mian et al., 2017; Shields et al., 2017). In a process known as sporulation, heavily infected blue crabs release large numbers of dinospores (~1.6 × 10⁸ dinospores ml⁻¹) through their gills into the surrounding seawater, often causing a discoloration of the water in their aquaria (Shields and Squyars, 2000; Wheeler et al., 2007). Sporulation events have been implicated in outbreaks of Hematodinium sp. in Norway lobsters Nephrops norvegicus (Field et al., 1998) and tanner crabs Chionoecetes bairdi (Meyers et al., 1987). Although sporulation is rarely observed under laboratory conditions, sporadic episodes of sporulation have been observed in adult blue crabs C. sapidus held for several months in the laboratory (Li et al., 2010). However, the factors that lead to sporulation, particularly temperature and salinity, have not been examined in detail, due in part to the rarity of observing it.

In the Chesapeake Bay, mature blue crabs mate in low-salinity waters from late spring through summer (Van Engel, 1958). Egg-bearing females migrate to high-salinity areas, near the mouth of the bay, where their eggs hatch and the larval zoae are released to oceanic waters onto the continental shelf (Van Engel, 1958; Epifanio, 2007). Larvae undergo six to seven molts, before migrating back into the bay as post-larval megalopae, where they settle on seagrass beds and metamorphose to the first juvenile instar stage (C1) (Pile et al., 1996; van Montfrans et al., 1995). The early benthic juveniles (< 20 mm CW) recruit into primary nursery habitats, such as seagrass beds in estuaries and their tributaries, where shelter and food resources are available for protection and growth (Pile et al., 1996; Lipeius et al., 2007; Ralph and Lipeius, 2014). The high prevalence of H. perezi in small juvenile crabs from high salinity estuaries (Messick and Shields, 2000), the possibility of rapid acquisition of new infections (Frischer et al., 2006; Huchin-Mian et al., 2017), and the lack of infections in post-larvae entering the bay (Small et al., unpubl. data), indicate that juvenile crabs are mainly infected after their recruitment into high salinity habitats (Huchin-Mian et al., 2017; Shields et al., 2017). Thus there is a strong association between settlement, high salinities, and transmission of the parasite.

The major source of natural mortality in small juvenile crabs (<20 mm CW) has previously been attributed to predation, cannibalism, and habitat variability (Hovel and Lipeius, 2001; Orth and Van Montfrans, 2002; Moksnes and Heck, 2006); nonetheless, the high prevalence and pathogenicity of H. perezi in juvenile crabs suggests that diseases may be a major source of mortality in high salinity systems. Until now, survival studies with early benthic juveniles infected with H. perezi have not been investigated. However, given the high prevalence levels observed in early benthic juveniles and the rapidity with which infections can be transmitted (Huchin-Mian et al., 2017; Shields et al., 2017), we were able to obtain large numbers of naturally infected crabs for survival studies under different conditions. Therefore, the objectives of this study were (1) to examine the mortality rates of naturally infected juvenile crabs held under controlled conditions of temperature and salinity, (2) analyze the outcome and progression of the parasite during these experiments, and (3) examine the occurrence of sporulation events and their association with temperature and salinity conditions.

2. Material and methods

2.1. Collection of naturally infected crabs

Early benthic juvenile crab (6–18 mm CW, including epibranchial spines) naturally infected with H. perezi were collected in Cobb Bay (37°19.234′N, 75°47.120′W), a high endemic site of the parasite on the Eastern Shore of Virginia (Fig. 1). In this high salinity bay (33 psu) the prevalence of H. perezi in small juvenile crabs (<20 mm CW) varies from 80% to 100% during Fall seasons (see Huchin-Mian et al., 2017; Shields et al., 2017). Crabs were sampled on November 9, 2015, and October 13, 2016. Crabs collected in 2015 were used for salinity experiments at 5, 15, and 30 psu; and temperature experiments at 4, 10, 15, 20 and 25 °C. Crabs collected in 2016 were only used for a temperature treatment at 30 °C. Juvenile crabs were collected with dip nets (0.79 mm mesh size) dragged through seagrass beds in shallow subtidal areas of the bay. Crabs were transported in coolers chilled with 1–2 closed containers of ice to the Virginia Institute of Marine Science (VIMS, ~130 km from Cobb Bay). In the laboratory, crabs were held overnight in their respective coolers at room temperature (21–23 °C).

Prior to temperature or salinity acclimation, a subsample of the collected crabs was screened by dissection and neutral red smears as described below to confirm natural infections with H. perezi. Because the prevalence level was 100% (n = 25 crabs) in the 2015 subsample, a minimum sample size of 30 crabs was used in each temperature and salinity treatment described below. Because the prevalence level was lower in 2016 (66.2% (53/80)), a minimum sample size of 42 crabs was used in the 30 °C treatment (for precision estimates, see Shields et al., 2017).

2.2. Collection of control crabs

Uninfected juvenile crabs were collected in Mobjack Bay (37°18.595′N, 76°25.220′W), a non-endemic site for H. perezi on the western shore of Chesapeake Bay (Fig. 1). Mobjack Bay is a low salinity area (~20 psu) where infections do not occur (Messick and Shields, 2000; Huchin-Mian et al., 2017; Shields et al., 2017). Crabs were collected with dip nets, transported to the laboratory in coolers and handled under identical conditions as infected crabs as above. A subsample of 30 crabs was dissected and examined for H. perezi as below prior to the assignment of remaining crabs into control groups.

2.3. Acclimation and experimental set up

Prior to the experiments, all crabs were gradually acclimated to laboratory conditions using short, 3-h time steps. To reduce mortality due to handling, only crabs in the inter-molt stage (hard carapace) were used. For temperature experiments, crabs from the control (n = 120) and infected groups (n = 192) were placed individually in 6-well plates (Falcon®) with 10 ml of artificial seawater (30 psu, Crystal Sea®, Marinemix). Because all of the crabs were initially held at 20 °C before their field collection, the treatment at this temperature did not require acclimation; however, the plates with crabs for treatments at lower temperatures (i.e., 4, 10, and 15 °C) and higher temperatures (i.e., 25 and 30 °C) were gradually changed in 5 °C steps every 3 h, until they reached the required experimental temperatures. Pilot studies indicated that the 3-h time steps were sufficient for initial acclimation to these temperatures based on survival and feeding responses (Shields, unpubl. data). All temperature experiments were performed using artificial seawater at a salinity of 30 psu.

For the salinity experiment, control crabs (n = 90) were placed individually in 6-well plates with 10 ml artificial seawater at 20 psu (similar salinity as Mobjack Bay). The salinity was gradually adjusted by 5 psu every 3 h, until they reached the experimental salinities: 5, 15 and 30 psu. The acclimation of naturally infected crabs (n = 90) that were originally held at 33 psu (same salinity as Cobb Bay) was performed in a similar manner as above using 3-h time steps. The acclimation of crabs in the salinity experiment was conducted in an incubator (VWR®) at 25 °C.
2.4. Temperature experiments

After acclimation, 312 crabs (192 infected and 120 controls) were held individually in 6-well plates with 10 ml artificial seawater (30 psu). Temperature treatments consisted of 30 naturally infected crabs held at 4, 10, 15, 20, and 25 °C, respectively; with 42 crabs held in the 30 °C treatment. The 30 °C treatment had a higher number of crabs due to expected higher mortality. Similarly, 30 uninfected crabs were used as controls for 4, 20, 25, and 30 °C, respectively. Pilot studies indicated that mortality in the 4, 20 and 25 °C groups likely would not be different (Shields, unpubl. data); hence we did not use orthogonal controls for all temperatures. The experiment was carried out in several thermally stable incubators (Thermo Fisher Scientific™, and VWR™), except for the 4 °C treatment which was held in a refrigerator (Gibson™). The temperature variation in incubators and refrigerator were monitored for 3 weeks with a pendant data-logger (Onset HOBO®) placed into a 100-ml beaker with artificial seawater. Crabs were fed twice a week with 1–2 mini pellets (pellet size: 0.5 mm, Omega one®). Six hours after the feeding, food consumption was qualitatively documented as partially, completely, or not eaten according to the amount remaining. Water changes (100%) were performed daily using an electronic pipettor (Eppendorf®). For each treatment, a 1-L bottle of artificial seawater (30 psu) was maintained in each incubator to avoid changes in water temperature during water exchanges. Crabs in each treatment were monitored twice a day for mortality, molting and sporulation events. Dead crabs were removed immediately and screened for the presence of *H. perezi* by neutral red smear (see below). To prevent the unlikely possibility of cross-infection during the experiments, all maintenance items (forceps, pipettes, trays and plastic containers) were assigned to control and infected groups only. The experiments were terminated when the cumulative mortality exceeded 60% within a temperature treatment or after 90 days, whichever came first. Crabs that survived to the end of the experiments were screened for the presence and severity of infection of *H. perezi* (see below).

2.5. Salinity experiments

To examine the effect of salinity on survival of infected crabs, animals were held at salinities of 5, 15 or 30 psu. Sixty crabs (30 infected and 30 controls) were used per treatment. For all treatments, crab maintenance, feeding, water changes, and monitoring, were as described above. The salinity experiment was performed in an incubator (VWR™) at 25 °C.

2.6. Sporulation events

Sporulation events were detected when the water in individual plate wells appeared cloudy due to the high density of released dinospores. All crabs that sporulated were immediately removed, sized, and screened for the presence of *H. perezi* by neutral red smear. In addition,
to confirm the presence of dinospores, an aliquot of 20 μl of water from the well was placed on a clean microscope slide for observation via transmitted light microscopy (200–400x). Dinospores were categorized as micro- or macro-dinospores according to their size, motility, and morphology as in Li et al. (2011). Dinospores were photographed and measured (Olympus cellSens®). The density and survival of dinospores in five sporulation events (three with micro-dinospores and two with macro-dinospores) were estimated using a Neubauer hemacytometer.

To monitor their survival, dinospores were diluted to 5 × 10^4 cells/ml in 1 ml artificial seawater (30 psu) and held in 24-well plates (Falcon*) at 10, 15, and 25 °C. These plates were observed daily with an inverted microscope (Olympus IX50).

2.7. Diagnostic assessment

The diagnosis of H. perezi in small juvenile crabs (< 18 mm CW) using dissection smears was detailed in Huchin-Mian et al. (2017). Briefly, small juvenile crabs were cut in half on a clean microslide and the fresh dissection smear was stained with 1–2 drops of 0.3% neutral red solution (Stentiford and Shields, 2005). Samples were examined via a transmitted light microscope for the presence, relative intensity, and developmental stage of the parasite. The reliable detection of H. perezi in dead crabs depended on the degradation of the host. In recently dead crabs held at cold (4 °C and 10 °C) or moderate (15 °C and 20 °C) temperatures, we were able to diagnose H. perezi and determine the intensity of infection; those held at higher temperatures degraded too quickly and only the presence of the parasite was recorded.

2.8. Histological assessment

A half portion of each dissected crab was placed in a cassette and fixed in Bouin’s solution (Fisher Scientific®). After 48 h, samples were rinsed with tap water for 10 min and decalcified with formic acid-sodium citrate method (Luna, 1968). All samples were processed, sectioned and stained using routine histological techniques (Humason, 1979). The intensity of infection was described as in Wheeler et al. (2007). Briefly, intensity is the relative number of parasites in the tissues visible per microscopic field at 200x: light infection (1–5 parasites), moderate (5–20 parasites), and heavy (20 + parasites). The main life history stages of the parasite detected in the tissues and hemolymph (filamentous trophonts, ameboid thophonts, clump colonies and prespores) were documented as in Shields et al. (2017).

2.9. Statistical analysis

Kaplan-Meier survival curves were calculated for infected and control crabs. Differences between survival curves were detected by a pairwise comparison using the log-rank test in the package survminer. The mean size of crabs that sporulated was compared using one-way ANOVA and Tukey’s multiple comparison test. A 2 × 2 contingency table was used to compare the number of crabs molting between control groups and treatments. All statistical analyses were performed using R 3.2.2 (R Core Team, 2013). Results were considered significant when p < .05.

3. Results

3.1. Pre-sampling of collected crabs

A subsample showed that all crabs collected in Fall, 2015, from Cobb Bay (endemic site) were infected (100%, n = 25) by Hematodinium perezi; but in Fall, 2016, the prevalence level was lower (66.2% n = 53/80). The proportion of light, moderate and heavy infections in crabs in the subsamples were relatively similar (Fig. 2). The majority of naturally infected crabs used in the experiments had moderate and heavy infections (Fig. 2). None of the pre-sampled crabs from Mobjack Bay (non-endemic site) were infected, indicating that all crabs used as controls in the experiments were free of infection. In addition, we have never observed H. perezi from this location in several previous studies.

3.2. Temperature experiments

Increased temperatures had a marked effect on survival of infected crabs (Fig. 3). Juvenile crabs infected by H. perezi died rapidly at 25 °C and 30 °C and had significantly lower survival rates than uninfected crabs (Fig. 3e and f). In both treatments, over 50% of deaths occurred within the first 20 days of exposure. Infected crabs held at 25 °C showed the sharpest decline in survival; over a four-day period survival decreased from 90% (day 5) to 63.3% (day 9). Infected crabs exposed at 4 °C also had significantly lower survival rates compared to uninfected crabs, but only after a longer exposure to this temperature (90 days) than crabs held at the higher temperatures. Conversely, infected crabs exposed at 10 °C, 15 °C, and 20 °C showed a very high survival (> 83%), indicating that the infections were not inducing acute mortality to juvenile crabs held at these moderate temperatures (Fig. 3b–d). The survival of these crabs was not significantly different than the crabs serving as controls at 20 °C (log-rank test, p = .65) (Fig. 3d). Overall, crabs serving as uninfected controls for the different temperature treatments exhibited a high survival (> 76%) over the course of the experiment (Fig. 3).

The screening of surviving crabs (n = 112) revealed that all crabs from the salinity and temperature experiments were infected by H. perezi, except for six survivors from the treatment at 30 °C. These uninfected survivors were from the Fall, 2016, collection, where the prevalence of H. perezi in the pre-sampled crabs was 66.2%. The crabs from the other treatments were from the Fall, 2015 collection where prevalence was 100%. None of the control crabs (n = 120) examined were infected by H. perezi.

Low temperatures apparently limited the proliferation of H. perezi in infected crabs (Fig. 4). Light infections were found in 75% (9/12) of surviving crabs held at 4 °C, whereas, only 16% of those held at 10 °C (4/25) had light infections, and none of the infected crabs held at higher temperatures had light infections. Histological evaluations revealed filamentous trophont stages (early stages of infection) in 33% (4/12) of crabs held at 4 °C; this stage is indicative of early infections.

Molting was examined in relation to temperature and infection status (Table 1). None of the crabs held at 4 °C molted over the 90 days of the experiment. Only 3% of the infected crabs held at 10 °C molted and only 33% held at 15 °C molted, whereas over 60% molted

![Fig. 2. Intensity of Hematodinium perezi in naturally infected crabs collected from Cobb Bay. The total number of crabs examined per sampling are given in parentheses. Crabs were diagnosed by dissection smear using 0.3% neutral red.](image-url)
Fig. 3. Kaplan–Meier survival curves for juvenile blue crabs exposed to different temperatures. Crabs were exposed for 90 days (a–d), and 32 days (e and f). All treatments were performed at high salinity (30 psu). Controls were not included for the 10° and 15 °C treatments. The solid line indicates uninfected crabs serving as controls and the dashed line indicates naturally infected crabs held in their respective treatments.

Fig. 4. Proportion of light, moderate and heavy infections in juvenile crabs that survived until the end of the experiments. The total number of crabs examined is given in parenthesis. Crabs in the salinity treatments were held at 25 °C.
when held at temperatures > 15 °C. At warmer temperatures (20 °C and 30 °C), the frequency of molting between control and infected crabs were not significantly different (χ² test, p > .05); but infected crabs held at 25 °C showed a significantly lower frequency of molting than crabs serving as controls.

### 3.3. Salinity experiments

The survival of infected crabs was negatively impacted by increasing salinity (Fig. 5). After 22 days of exposure, infected crabs held at high salinity (30 psu) showed the lowest survival: 30% (17.4–51.8, 95% CI), which was significantly lower than that for crabs held at the lowest salinity (5 psu), 62.1% (46.7–81.5, 95% CI) (log-rank test, p = .0073). However, for all treatments, infected crabs exhibited a high mortality rate over the first 10 days of exposure (Fig. 5a–c). In comparison, crabs serving as controls showed high survival (> 90%), which did not differ significantly among salinity treatments (log-rank test, p > .05). Histological assessment of surviving crabs showed that none of the control crabs (n = 83) were infected, whereas all of the crabs (n = 42) in the infected treatments harbored heavy infections comprised mostly of ameboid trophonts (Fig. 4). Molting was examined in relation to salinity (Table 1), and after 22 days no differences were found in the frequency of molting between control and infected crabs held at different salinities.

#### 3.4. Sporulation

Only crabs held at 25 °C exhibited gross signs of mass sporulation (Table 2). There were 25 crabs in both the temperature and salinity experiments that sporulated, and they were grouped by salinity for analysis. Eleven crabs released highly motile micro-dinospores (mean length 5.1 ± 0.9 μm SD), and nine crabs released non-motile macro-dinospores (mean length 16.9 μm ± 1.2 μm SD). In addition, five crabs liberated prespores (Fig. 6). Overall, 76% (n = 19) of sporulations occurred at high salinity (30 psu), whereas only one crab sporulated at low salinity (5 psu) (Fig. 6). The mean size of crabs (13.7 ± 1.6 mm) that sporulated did not differ among treatments (one-way ANOVA, p > 0.05). In 96% of the cases, sporulation events occurred within the first 10 days of exposure, indicating a rapid progression of infections in response to the higher temperature of 25 °C (Table 2). Mass sporulation was lethal; all crabs died within a few hours of releasing spores, with the exception of one crab that survived for 24 h post sporulation. A high density of dinospores was released by infected crabs; i.e., one small juvenile crab (12 mm CW) released spores to create a density of $5.7 \times 10^6$ dinospores/ml in the 10 ml volume of the plate (see Fig. 7).

The dinospores did not survive long when held in 30 psu seawater at different temperatures under laboratory conditions. Although spore viability and mortality were not quantitatively assessed over time, mortality was 100% at 10 °C and 15 °C after 24 h, and all dinospores were dead after 5 days at 25 °C. Although these mass sporulation events occurred in crabs maintained at 25 °C, one crab released abnormal “circular” prespores (cell size 7.5 μm) at 10 °C. These prespores did not survive longer than 24 h and did not become motile.

### 4. Discussion

We demonstrated that high temperatures and salinities are likely important drivers of mortality of juvenile crabs naturally infected by *Hematodinium perezi*. The mortality of infected crabs held at warmer temperatures (25 °C and above) and high salinity (30 psu) was ten times higher than that of controls. The frequency of molting between control and infected crabs held at different salinities is shown in Table 1. All salinity treatments were conducted at 25 °C. NC = no control crabs. A significant p-value is given in bold.

### Table 1

Comparison of molting in control and infected crabs in the salinity and temperature experiments. A total of 60 crabs were used per treatment: 30 controls and 30 infected; except the infected group at 30 °C, in which 42 crabs were used.

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All salinity treatments were conducted at 25 °C. NC = no control crabs. A significant p-value is given in bold.
The 25 °C temperature treatment (n = 30) and 30 psu salinity treatment (n = 30) were identical replicates and were pooled for this analysis.

A rapid decline in hemocyte density in relation to infection (Shields and Squyars 2000; Co and Squyars, 2000). Similar logarithmic increases in cell density were noted experimentally infected and held at room temperature (Shields and Squyars, 2000). This supports the finding of Messick et al. (1999) that parasite intensity declines in dinospore viability during sporulation events at salinities below 24 psu (Coffey et al., 2012). Thus, although lower salinities do not hinder the development of infections and movement of infected crabs into low salinity sites, lower salinities effectively limit transmission of the parasite. This explains why prevalence levels approach zero in the low salinity embayments within Chesapeake Bay. We did not examine interactive effects between temperature and salinity. Additional studies would clarify the finer scale of these dynamics in infected juvenile crabs.

Despite the proliferation of the parasite in the temperature experiment, many of the infected juvenile blue crabs were capable of molting at a similar rate as controls. Heavy infections of Hematodinium spp. can impose significant metabolic demands on the crustacean host which can manifest as parasitic castration, reduced molting frequency, anhydroysis, and stunting (Shields, 2012). Molting may lead to a proliferation of Hematodinium-like parasites in the Norway lobster (Field et al., 1992, Stentiford et al., 2001) and prevalence is typically higher in postmolt Tanner crabs (Meyers et al., 1987; Shields et al., 2007). The lower frequency of molting in infected crabs held at 25 °C may be the result of the high parasite-induced mortality in this treatment. Nonetheless, our observations indicate that juvenile crabs at temperatures below 25 °C are able to molt and grow while maintaining active infections.

Low temperatures (4 °C) limited the proliferation of the parasite. This supports the finding of Messick et al. (1999) that parasite intensity declined with low temperature (9–12 °C) in large juvenile crabs. Although the majority of crabs entering our experiment had moderate or heavy infections, 73% (9/12) of surviving crabs held at 4 °C had light infections. This indicates that not only did H. perezi not proliferate at this temperature, but that the low temperature reduced the intensity of infection in these crabs. This finding is supported further by the subsample that indicated that only 20% of crabs entering this treatment were harboring light infections prior to the initiation of the experiment. Moreover, effete parasite cells were observed in these animals and these cells are indicative of quiescent infections in hibernating crabs (Shields et al., 2015). The presence of the filamentous trophont, an early stage of the parasite, after 90 d at 4 °C, supports the finding that low temperatures reduced the natural development rate of the parasite in infected juveniles.

Although low temperature (4 °C) inhibited parasite proliferation, it significantly increased mortality in infected juvenile crabs. The mortality rate of 60% may be explained by a combination of at least two factors. First, the majority of recently dead crabs had moderate and heavy infections (13/18), whereas the survivors mostly had light infections (see above). Second, low temperatures are known to contribute to the mortality of overwintering blue crabs (Van Engel, 1958; Rome et al., 2005). In addition, the crabs did not have a substrate in which to bury for hibernation. In our experiment crabs exposed to 4 °C were in an apparent torpor; they did not feed and were mostly inactive. Rome et al. (2005) found a high mortality rate in early benthic juveniles (< 15 mm CW) over an extended time course of cold temperature, and this may be a function of the animals being held below a critical thermal threshold for homeoestasis; however, they did not consider pathogens or disease in their study. The boreal species of Hematodinium occurs in chronic infections in tanner crabs (Chionoecetes bairdi), snow crabs (C. opilio) and Norway lobsters (Nephrops norvegicus), but these decapods and their parasites are adapted to the cold temperatures (Meyers et al., 1987;
These hosts harbor annual infections that take long periods to develop, and at least for the snow crab, develop at temperatures as low as −1 °C (Shields et al., 2005). Nonetheless, high mortality has been observed after 75 d of infection in Norway lobsters (Field et al., 1992), 158-400 d in infected tanner crabs (Meyers et al., 1987; Love et al., 1993), and 100–200 d in naturally infected and inoculated snow crabs (Shields et al., 2005). Notably, the mortality rates of infected juveniles of these boreal hosts have not been examined, even though prevalence in juveniles is often much higher than in adults (Shields et al., 2005; Shields, 2012).

The dinospores of H. perezi from blue crabs have not been studied in detail. We found that sporulation of H. perezi in juvenile blue crabs was induced at a moderately warm temperature (25 °C) and high salinity (30 psu) after 10 days in crabs evaluated as having mostly moderate and heavy infections. These conditions appear to be optimal for the parasite to develop and undergo sporogony. The released micro-dinospores were highly motile, but their activity declined rapidly over time. Similar results have been reported from cultured dinospores in blue crabs (Li et al., 2011). In contrast, dinospores released from infected Tanner crabs can survive for 73 days in sterile seawater at 5 °C (Meyers et al., 1987). Dinospores are thought to be the infectious stage for H. perezi (Shields, 1994; Frischer et al., 2006; Lycett and Pitula, 2017; Shields et al., 2017). However, we have not been able to transmit infections using naturally released dinospores, despite repeated efforts (Shields et al., unpubl. data). Although it is unclear why the highly active dinospores did not infect naive crabs, we hypothesize that they may require a maturation period to become infectious, or that they are gametes that require fusion and production of a different spore stage for transmission, or that they may need an intermediate host in their life cycle (e.g. amphipods, or macro-zooplankton). Similarly, direct inoculation of prespore stages in tanner crabs failed to induce infection (Meyers et al., 1987), and prespore stages are not infectious to blue crabs (Shields, unpubl. data). Given our success in generating spores from juvenile crabs, detailed experimental studies on the influence of temperature, salinity, and density of spores are now possible.

In the coastal bays of the Delmarva peninsula of Maryland and Virginia, H. perezi exhibits two seasonal peaks in prevalence levels in the adult blue crab population: a minor peak (30–50%) in late spring and early summer (Jun.-Jul.), which are often associated with adult crab mortalities and a high peak in prevalence (70–100%) in fall (Sep.-Nov.) (Messick and Shields, 2000). Prevalence levels decline rapidly with decreases in water temperature in late fall and winter (Shields et al., 2015). In addition, prevalence levels of newly recruited crabs may be affected by differential mortality in infected megalopae that experience high mortality rates with high water temperatures (Sullivan and Neigel 2017), but we have not detected infections in megalopae in Chesapeake Bay (Small et al., unpubl. data). Our results support the field observations for the region by showing an enhanced pathogenicity of the parasite with increasing water temperature as well as the capacity for overwintering of infections in winter months. Increasing water temperatures facilitate transmission (Huchin-Mian et al., 2017; Shields et al., 2017) of new infections, and ultimately contribute to the rapid proliferation and mortality of infected crabs (this study). Conversely, crabs hibernating with light and moderate infections during the winter appear to serve as the primary reservoir for new infections in the following spring (Shields et al., 2015).

Our findings indicate that H. perezi has a significant impact on the survival of juvenile crabs in high salinity areas of the mid Atlantic region during the warmer seasons. The mortality rates observed in this study reflect the natural outcome of the disease in the highly endemic coastal bays of the Eastern Shore of the Delmarva Peninsula. In addition, the results may help to explain the level of natural mortality induced by disease and its possible contribution to the fluctuations of the blue crab population in Chesapeake Bay.

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Fig. 7. Prespores and dinospores of Hematodinium perezi released during sporulation. (a) prespores stages, (b) highly motile microdinospores (arrows), (c) non-active macro-dinospores. Bars = 20 µm.
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Conflict of interest: none.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2018.01.003.

References


