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Parasitic dinoflagellate *Hematodinium perezi* prevalence in larval and juvenile blue crabs *Callinectes sapidus* from coastal bays of Virginia

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ABSTRACT: The parasitic dinoflagellate *Hematodinium perezi* infects the American blue crab *Callinectes sapidus* and other decapods along the Eastern seaboard and Gulf of Mexico coast of the USA. Large juvenile and adult blue crabs experience high mortality during seasonal outbreaks of *H. perezi*, but less is known about its presence in the early life history stages of this host. We determined the prevalence of *H. perezi* in megalopae and early benthic juvenile crabs from multiple locations along the Virginia portion of the Delmarva Peninsula. The DNA of *H. perezi* was not detected in any megalopae collected from several locations within the oceanic coastal bay complex in which *H. perezi* is found at high prevalence levels. However, prevalence levels were high in early benthic juveniles from 2 oceanic coastal embayments: South Bay and Cobb Bay. Prevalence levels were lower at locations within Chesapeake Bay, including Cherrystone Creek, Hungars Creek, and Pungoteague Creek. Sampling over different seasons and several consecutive years indicates that disease transmission occurs rapidly after megalopae settle in high-salinity bays along the Delmarva Peninsula during the late summer and fall. Infected juvenile crabs can overwinter with the parasite and, when subjected to increasing water temperatures in spring, infections progress rapidly, culminating in transmission to other crabs in late spring and early summer. In high-salinity embayments, *H. perezi* can reach high prevalence levels and may significantly affect recruitment of juvenile blue crabs into the adult fishery.

KEY WORDS: Dinoflagellate · Parasite · Crustacea · Endemic · Infection

1. INTRODUCTION

The blue crab *Callinectes sapidus* is an abundant, highly mobile, benthic predator with a complex life history. The species is distributed from Nova Scotia (Canada) to Brazil (Williams 1984) and supports economically important fisheries along the Eastern seaboard and Gulf of Mexico coast of the USA. In the last decade, however, there have been major declines in harvest yields of many blue crab fisheries in the USA. Fishing pressure and habitat loss contribute greatly to those declines, but disease and environmental processes affecting blue crab populations have not been thoroughly researched, although both have contributed to declines and changes in population cycles in other crustacean fisheries (Shields 2012). In particular, few studies have examined the effects of pathogens upon settlement of postlarvae or new recruits, yet pathogens are often most prevalent in and damaging to the early juvenile stages of crustaceans.

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The blue crab serves as a host for a variety of pathogens, including viruses, bacteria, fungi, protozoa, helminths, and other crustaceans (Shields & Overstreet 2007). Most pathogens cause little pathological alteration to the host, but several have the capacity to cause serious disease and significantly affect host populations and their associated fisheries. For instance, the parasitic dinoflagellate *Hematodinium perezi* causes widespread blue crab mortalities during annual seasonal outbreaks (Messick 1994, Messick & Shields 2000, Lee & Frischer 2004). The parasite was first documented in blue crabs by Newman & Johnson (1975) and was later shown to have a broad distribution on the Eastern seaboard of the USA and Gulf of Mexico coast (Messick & Shields 2000, Pagenkopp Lohan et al. 2013). The parasite is endemic in crabs living in areas of high salinity (>20 psu), and infections are rarely found in waters where salinity is below 18 psu (Newman & Johnson 1975, Messick & Sindermann 1992, Messick & Shields 2000). The parasite also infects several other crustacean species, including decapods and amphipods (Johnson 1986, Messick & Shields 2000, Sheppard et al. 2003, Pagenkopp Lohan et al. 2012).

H. perezi can be highly pathogenic in its blue crab host, with mortality rates up to 87% in naturally and experimentally infected mature blue crabs (Messick & Shields 2000, Shields & Squyars 2000). Infections and crab mortalities have historically been reported from large juvenile and adult crabs due to their ease of capture in commercial traps; few studies have examined larval and early benthic juvenile stages. Messick (1994) documented prevalence levels up to 100% in early benthic juveniles sampled in 1992 and 1993 from coastal bays near Ocean City, Maryland, and Franklin City, Virginia. Little is known about whether the parasite can infect larval stages. Sullivan et al. (2016) and Sullivan & Neigel (2017) detected *Hematodinium* DNA associated with blue crab megalopae collected from inshore locations in Louisiana, but their studies did not demonstrate whether the positive PCR assays were associated with internal infections.

The purpose of the current study was to determine if megalopae had infections of *H. perezi* and to assess the prevalence levels in early benthic juveniles from both intermediate- and high-salinity locations along the Virginia portion of the Delmarva Peninsula. Diagnostic PCR was employed to screen initial collections of juvenile crabs and megalopae. Subsequent crab collections were screened by tissue smears that allowed us to semi-quantitatively assign an intensity of infection and document the life stages of the parasite.

2. MATERIALS AND METHODS

2.1. Collection and treatment of experimental animals

In 2008 and 2009, juvenile blue crabs were obtained from colleagues studying the relationship between submerged aquatic vegetation (SAV) cover and juvenile crab density (Ralph et al. 2013). Crabs were collected from Cherrystone Creek and Hungars Creek, 2 moderate-salinity (18–22 psu) locations on the Chesapeake Bay side of the Delmarva Peninsula (Fig. 1), by suction sampling, transported to the Virginia Institute of Marine Science (VIMS), and frozen at −20°C. Samples were then thawed and sorted, and individual crabs were measured (carapace width [CW] including epibranchial spines) prior to their preservation in 70% ethanol. In 2011 and 2012, early benthic juvenile crabs were collected from SAV habitats in Cherrystone Creek and South Bay, a high-salinity (32–34 psu) oceanic coastal embayment on the Delmarva Peninsula. Crabs were captured using dip nets and immediately stored in coolers containing ambient seawater for transport to VIMS for assessment. Upon return to the laboratory, crabs were preserved whole in 95% ethanol. All subsequent collections from SAV habitats (2014–2017) were conducted in the same manner. Over this period, crabs were also collected from Cobb Bay, which abuts South Bay. In fall 2016, juvenile crabs were also collected from Pungoteague Creek and Gwynn's Island, 2 moderate-salinity locations (16–18 psu) on the Chesapeake Bay side of the Delmarva Peninsula and on the western shore of Chesapeake Bay, respectively. At the laboratory, crabs were measured and assessed for obvious injuries prior to dissection. They were then bisected sagittally along the carapace length using a sterile razor blade on a clean glass microslide. Half of the crab was placed into Bouin's solution and the other half was placed into 95% ethanol for later processing. The dissection smear was then stained with 0.3% neutral red in physiological saline and assessed for the presence of the parasite.

Megalopae were collected from several high-salinity (28–34 psu) locations on the Eastern Shore of Virginia (Fig. 1) during late summer and fall 2010 using larval collectors. The collectors were made of 'horsehair' filter material wrapped around and tied to a 1 m section of 15 cm PVC pipe weighted on one end by concrete and buoyed on the other by half a standard crab float. These were deployed in shallow waters tethered by rope to anchors. The collectors were de-

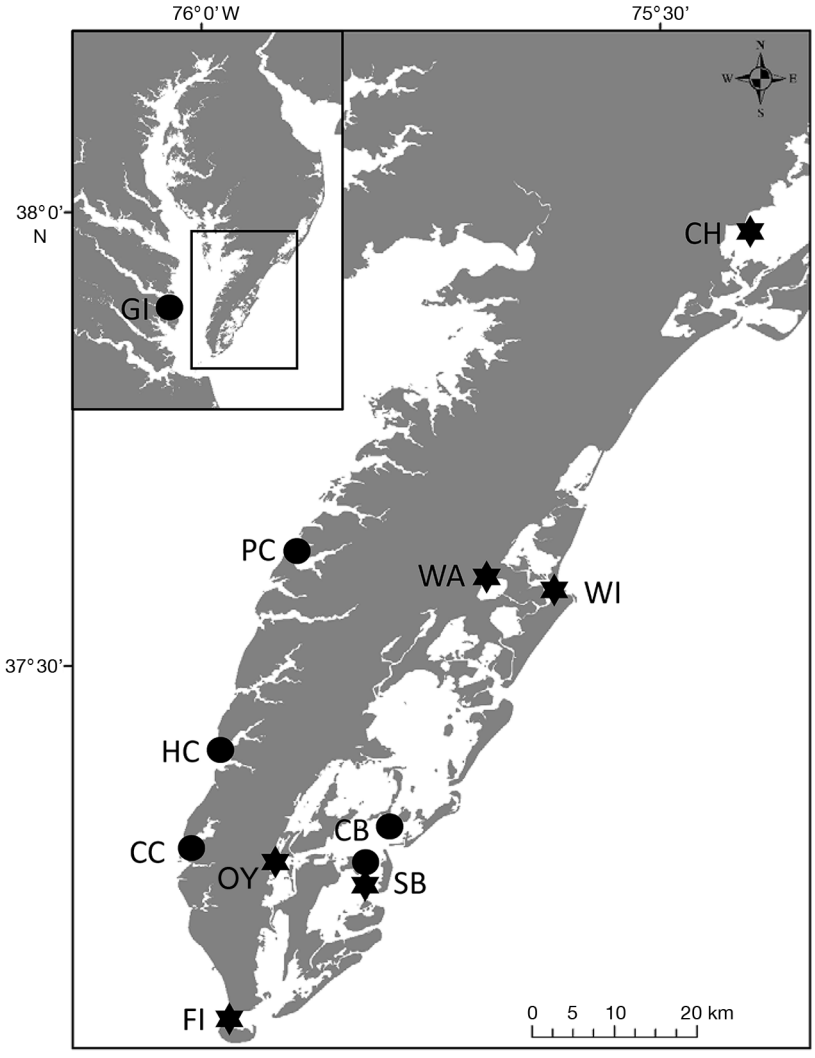

Fig. 1. Eastern Shore of Virginia, USA, showing sample locations. Inset shows Chesapeake Bay. Stars indicate megalopae collections, circles indicate juvenile crab collections. CB: Cobb Bay; CC: Cherrystone Creek; CH: Chincoteague Bay; FI: Fisherman's Island; GI: Gwynn's Island; HC: Hungers Creek; OY: Oyster Harbor; PC: Pungoteague Creek; SB: South Bay; WA: Wachapreague Creek; WI: Wachapreague Inlet

ployed overnight around the period of the full or new moons. Collectors were recovered the morning of the following day and the filter material washed onto a 200 µm sieve. Washed megalopae were carefully recovered from the sieve and preserved in 95% ethanol for later processing.

2.2. Nucleic acid extraction and diagnostic PCR

Ethanol-preserved megalopae (see Section 2.1) and juvenile blue crabs from 2008, 2009, 2011, and 2012 were assessed by PCR for the presence of *Hematodinium perezi*. DNA was extracted from individual megalopae and juveniles. Based on their CW, juvenile crabs were subjected to DNA extraction either as a whole crab (<5 mm CW), half of a crab (5–10 mm CW), or gill tissues that were dissected from larger crabs (>10 mm CW). Prior to extraction, megalopae and crab samples were placed in molecular-grade water for 30 min to allow for removal of residual ethanol. Total genomic DNA was extracted from all samples using a Qiagen DNeasy Tissue Kit following the manufacturer's protocol. Samples were homogenized in ATL buffer and then subject to overnight digestion. DNA was eluted in 100 µl AE buffer, quantified using a NanoDrop 2000 (Thermo Scientific), and stored at −20°C prior to use in PCR assays. All extractions were completed within the same 2 d period and included a blank column extraction that was subjected to PCR analysis and served as a control for extraction contamination.

A random subset (approximately 25%) of all samples were assessed for the presence of amplifiable high molecular weight, genomic DNA using primers nSSU A (5′-AAC CTG GRT TGA TCC TGA TCC TGC CAG T-3′) and nSSU B (5′-GAT CCT TCC GCA GGT TCA CCT AC-3′) (modified from Medlin et al. 1988), which target the small subunit (SSU) rRNA gene of both the parasite and the crab host. Cycling conditions and reaction concentrations were as described by Pagenkopp Lohan et al. (2012). Five microliters of the resulting PCR product were 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. All extracted DNA samples from crabs and megalopae were screened for the presence of *H. perezi* DNA using primers previously designed to target the parasite's internal transcribed spacer 1 (ITS1) rRNA region (Small et al. 2007). Briefly, each 20 µl reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, each dNTP at 0.1 mM, each primer at 0.5 µM, 0.5 units of *Taq* polymerase (Invitrogen), and 1 µl genomic DNA. Amplifications were performed at an initial denaturation temperature of 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s, with a final

elongation step at 72°C for 5 min. Included in all PCR runs were a negative control that consisted of no DNA, a blank column extraction control, and a positive control consisting of a previous blue crab DNA sample from the field that had routinely amplified in the past. Ten microliters of the resulting PCR product were electrophoresed and visualized as above. The expected fragment was 302 bp. Diagnostic PCRs were repeated for all samples that tested positive to confirm diagnoses.

2.3. Microscopic assessment

Juvenile crabs collected in 2014–2017 were screened for the presence of *H. perezi* by light microscopy. Briefly, 2 drops of neutral red solution (0.3% w/v in physiological saline) were added to the wet dissection smear, a cover slip applied, and the sample then immediately assessed by transmitted light microscopy (200–400×) for the presence of life history stages, including filamentous trophonts, ameboid trophonts, clump colonies, and prespores. The intensity of infection was categorized as 'light,' 'moderate,' or 'heavy' following methods used by Shields et al. (2017). Light infections were characterized by the presence of filamentous trophonts (vermiform plasmodia) or few ameboid trophonts per microscopic field (1–3 parasites at 200×). Moderate infections typically had 4–10 parasites (filamentous trophonts, ameboid trophonts, and clump colonies) per field at 200×, and heavy infections had >10 parasites (ameboid trophonts, prespores) per field.

3. RESULTS

3.1. Prevalence of *Hematodinium perezi* by diagnostic PCR

DNA of *H. perezi* was detected in juvenile blue crabs from several locations at various prevalence levels (Table 1). Overall, crabs from Cherrystone and Hungars Creek had relatively low to moderate prevalence levels. DNA of *H. perezi* was not detected in the 293 individual megalopae collected from multiple endemic locations on the Eastern Shore of Virginia between August and October 2010 (Table 2). All DNA samples from megalopae and juvenile crabs collected from 2008, 2009, 2011, and 2012 and assessed for the presence of amplifiable, high molecular weight genomic DNA produced intense banding of the SSU rRNA gene PCR products at ~1700 bp. These data, together with the assessment of individual DNA sample concentrations by NanoDrop quantification (approximately 20–100 ng μl^{-1}), confirmed that amplifiable DNA was present in all assayed samples.

Table 1. Sampling location (abbreviations as in Fig. 1), number sampled (n), size range, and prevalence of *Hematodinium perezi* as determined by PCR and microscopy in juvenile blue crabs collected between 2008 and 2017 from 6 locations in Virginia, USA. CW: carapace width; nd: not done

Date (mo/d/yr)	Sampling location	n	CW range (mm)	Prevalence (%) PCR	Smear
10/24/2008	HC	58	2–14	8.6	nd
10/25/2008	CC	31	2–11	54.8	nd
11/04/2009	HC	23	3–20	4.3	nd
11/04/2009	CC	4	7–12	75.0	nd
11/22/2011	CC	72	3–13	5.6	nd
10/22/2012	CC	50	3–21	18.0	nd
10/22/2012	SB	50	4–16	78.0	nd
10/02/2014	SB	30	5–16	nd	100.0
11/05/2014	SB	34	7–25	nd	97.1
08/26/2015	SB	15	5–10	nd	66.7
09/22/2015	SB	15	7–33	nd	80.0
10/07/2015	SB	10	9–24	nd	100.0
11/09/2015	CB	25	10–16	nd	100.0
04/21/2016	CB	25	8–23	nd	60.0
05/16/2016	CB	42	14–36	nd	59.5
06/20/2016	CB	12	26–58	nd	91.7
09/25/2016	CC	77	5–35	nd	1.3
09/26/2016	HC	80	3–30	nd	5.0
09/27/2016	CB	83	4–36	nd	50.6
09/28/2016	PC	95	8–32	nd	0
09/28/2016	GI	30	11–25	nd	0
10/13/2016	CB	80	6–46	nd	66.3
01/26/2017	CB	30	12–25	nd	63.3
02/08/2017	CB	37	9–42	nd	56.7

Table 2. Sampling location (abbreviations as in Fig. 1) and numbers of blue crab megalopae examined (n) for *Hematodinium perezi* by PCR. Infection prevalence was 0% for all sampled populations

Month	Sampling location	n	Prevalence (%)
August	OY	18	0
	SB	4	0
	WA	62	0
	WI	64	0
	FI	9	0
	CH	21	0
September	OY	7	0
	SB	1	0
	WA	19	0
	WI	35	0
	FI	2	0
October	WA	1	0
	WI	48	0
	CH	3	0

3.2. Prevalence of *H. perezi* by light microscopy

The prevalence of *H. perezi* in juvenile blue crabs from South Bay and Cobb Bay ranged from moderate (50.6%) to high (100.0%) in all months and years examined (October 2014 to February 2017; Table 1). In South Bay, crabs sampled in late fall months (October/November) had the highest prevalence levels, indicative of recent parasite transmission. In November 2015, a significant eelgrass die-back occurred within South Bay, and few crabs were captured. All subsequent crab collections in this region were carried out in nearby Cobb Bay (Fig. 1). Prevalence levels in Cobb Bay in November 2015 (100%) were comparable to what was observed previously in South Bay and continued to be moderate/high in subsequent samplings at this location, even in the winter months of January and February 2017.

In fall 2016, prevalence levels of *H. perezi* infections in crabs were 5.0% for Hungers Creek and 1.3% for Cherrystone Creek. No infections were encountered in samples from Pungoteague Creek or Gwynn's Island on the western shore of Chesapeake Bay (Table 1). Due to small sample sizes in monthly crab collections from South Bay and Cobb Bay, the data on size classes of infected crabs were pooled by season (fall 2015: September–November; spring 2016: April and May; fall 2016: September and October; winter 2017: January and February) for comparison among seasons and host sizes (CW). In fall 2015, 91.5% of *H. perezi* infections were found in small juvenile crabs (1–20 mm CW, Fig. 2). In comparison, 100% of infections were observed in larger crabs (11–30 mm CW) sampled in spring 2016. A similar pattern was observed in juveniles collected in fall 2016, where 89.2% of infections were in smaller crabs (1–20 mm CW) compared to 92.7% of infections in larger juveniles (11–30 mm CW) from the winter 2017 sample.

3.3. Infection intensity and life history stages of *H. perezi*

Light, moderate, and heavy infections were observed in almost all monthly juvenile crab collections from South Bay and Cobb Bay in 2014–2017 (Fig. 3). Although progression of infections may be rapid and ephemeral, light infections were abundant in April, June, August, and September, whereas heavy infections were abundant in October and November. Infection intensity and parasite life

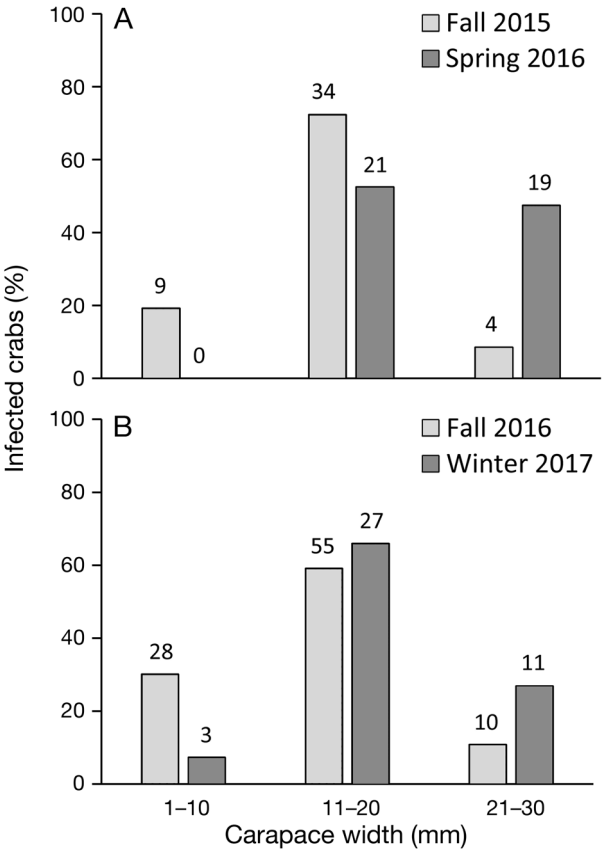

Fig. 2. Comparison of *Hematodinium perezi* infections in juvenile blue crabs of different size ranges (1–10, 11–20, and 21–30 mm carapace width). (A) Crabs collected in fall 2015 (September–November) and spring 2016 (April, May). (B) Crabs collected in fall 2016 (October, November) and winter 2017 (January, February). Sample size is shown above each bar

stage were highly variable and changed considerably among months, indicating rapid development, growth, and differentiation. Ameboid trophonts were the most common stage observed (Fig. 4). Filamentous trophonts, the earliest diagnostic stage in blue crabs, were observed in August and September 2015, and also in April, June, September, and October 2016 (Fig. 4). Of particular note, filamentous trophonts were observed in 93.4% of infected crabs in April 2016. In subsequent monthly samplings, ameboid trophonts and clump colony stages were common. Prespore stages, which are indicative of imminent sporulation and release from an infected crab, were observed in October 2014 and 2015 (16.7 and 10.0%, respectively) and in May, September, and October 2016 (4.2, 26.2, and 5.7%, respectively). Light, moderate, and heavy ameboid trophont infections were observed in crabs sampled in January and February 2017.

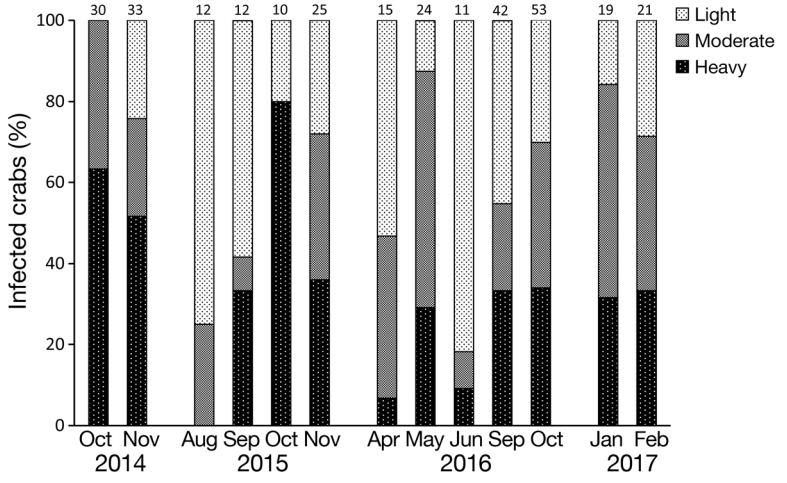

Fig. 3. *Hematodinium perezi* intensity of infection in juvenile blue crabs sampled from South Bay (October 2014–October 2015) and Cobb Bay (November 2015–February 2017). Sample size is shown above each bar

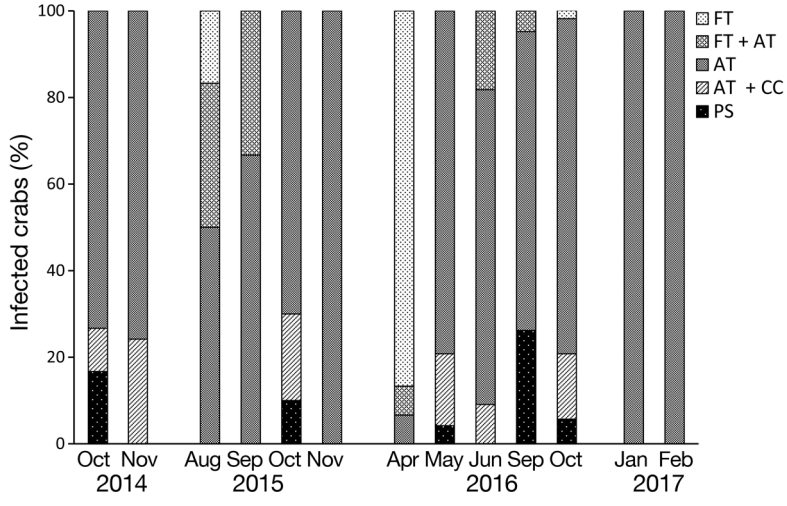

Fig. 4. Life history stages of *Hematodinium perezi* in early juvenile blue crabs sampled from South Bay (October 2014–October 2015) and Cobb Bay (November 2015–February 2017). FT: filamentous trophont; AT: ameboid trophont; CC: clump colony; PS: prespore. Sample sizes are given in Fig. 3

4. DISCUSSION

Hematodinium perezi is endemic in early benthic juvenile blue crabs from the high-salinity, oceanic coastal bays along the southern tip of the Delmarva Peninsula. Prevalence levels ranged from 50.6 to 100%. Infections were found in all size classes examined, the smallest of which were ~2 mm CW, and in all months sampled. The prespore stage, which is indicative of imminent sporulation, was observed in spring (May) and fall (September and October). *H. perezi* DNA was not detected in megalopae transiting through the high-salinity bays, despite doing so during peak periods in parasite infection of early benthic juveniles. Taken together, these data suggest that *H. perezi* rapidly infects early benthic instars of blue crabs in early summer and fall as they recruit into structured habitats (e.g. eelgrass, oyster beds) in these high-salinity embayments. Our data also show that the parasite can overwinter at moderate to high prevalence levels in early benthic juveniles. Given its high mortality rate in both adults and juveniles (Messick & Shields 2000, Shields & Squyars 2000), its high mortality rate associated with warm water temperatures (25°C and above; Huchin-Mian et al. 2018), and high prevalence levels in early instars, *H. perezi* appears to significantly affect recruitment of early juvenile crabs in the high-salinity bays along the Delmarva Peninsula.

As with many pathogens, there is considerable temporal variability in the prevalence of *H. perezi* in the high-salinity bays of the Delmarva Peninsula. These embayments have salinities of ~30–34 psu and are mostly shallow expanses with moderate to high water residence times that serve to amplify transmission of the parasite (Shields 2012). For example, Cobb Bay has a residence time of ~7 d (Herman et al. 2007), a feature that when coupled with the viability of *H. perezi* dinospores at high salinity, likely enhances transmission of the parasite. Transmission occurs rapidly in early fall months in endemic locations, and water temperature appears to be an important driver modulating transmission (Huchin-Mian et al. 2017, Shields et al. 2017). Furthermore, a recent laboratory study with naturally infected early instars indicated that water temperatures around 25°C are optimal for parasite development and sporulation (Huchin-Mian et al. 2018). Infected crabs held at lower temperatures did not sporulate and maintained infections over extended periods (90 d).

We did not find evidence of *H. perezi* infections in megalopae entering into the coastal bays. This finding validated earlier histological examinations which were also negative (J. D. Shields unpubl. data). This is in contrast with recent studies from Louisiana, in

which *H. perezi* DNA was detected in blue crab megalopae (Sullivan et al. 2016, Sullivan & Neigel 2017). Although there are ecosystem differences between the Delmarva Peninsula and the Gulf of Mexico, there is a potential confounding factor in the study by Sullivan et al. (2016) and their later analyses. The PCR primers used by Sullivan et al. (2016) were originally designed to target the conserved 18S rRNA gene of a different *Hematodinium* sp., one that infects Tanner crabs *Chionoecetes bairdi* and snow crabs *C. opilio* from the North Pacific (Friedman et al. 2009). Due to the conserved nature of the SSU rRNA gene, these same primers would detect *H. perezi*; however, molecular assays used to detect dinoflagellates based on this conserved gene have significantly reduced specificity (Litaker et al. 2007, Small et al. 2007). Hanif et al. (2013) succinctly demonstrated this when testing a previously published assay targeting the *H. perezi* SSU gene (Nagle et al. 2009) as they amplified at least 10 protozoan species that were identical in sequence at the primer binding sites, none of which were *Hematodinium* species. The list of organisms with perfect matches to the primer sequences included several species found in mid-Atlantic and Gulf coast waters (Hanif et al. 2013). We therefore recommend that in future studies where parasite DNA is detected in larval stages or microcrustaceans, the amplicons are sequenced or there is definitive proof of infection through *in situ* hybridization techniques. In addition, studies of larval host stages infected with *H. perezi* or pathogens should be careful to rule out external contamination of the cuticle or gills (Burreson 2008, Shields 2017).

We observed *H. perezi* overwintering in early benthic crabs from the high-salinity bays during winter. Infections were observed at all intensities and at moderate prevalence levels (55–60%). We have previously reported *H. perezi* infections in large juvenile and adult blue crabs from winter dredge surveys in the winter months of January, February, and March from nearby Wachapreague Creek (Shields et al. 2015). In that study, active infections were observed in all months, and they rapidly developed into high-intensity infections when held at 15°C. It is therefore likely that early benthic juveniles respond in a similar manner, and thus likely explains the bimodal peaks in prevalence observed in larger juveniles and adults, and the detection of *H. perezi* in environmental samples (Pitula et al. 2012, Hanif et al. 2013, Lycett & Pitula 2017).

Lastly, we observed infections in small juveniles in early fall months, and in larger crabs in subsequent months (e.g. winter or the following spring). At temperate latitudes (e.g. Chesapeake Bay) megalopae settlement generally occurs from August to November. Assuming that movement of juveniles is minimal among adjacent estuaries and sub-estuaries (van Montfrans et al. 1991), our data suggest that juvenile crabs surviving infections with *H. perezi* in late fall can molt, grow, and carry infections forward into spring of the following year. Our field observations are consistent with recent laboratory studies which showed that *Hematodinium*-infected juvenile crabs were capable of molting at frequencies similar to those of uninfected crabs (Huchin-Mian et al. 2018). Huchin-Mian et al. (2018) also showed that infected juvenile blue crabs held at mild temperatures (10, 15, and 20°C) have high survival, but their survival is diminished at low temperature (4°C). Therefore, water temperature during winter months may significantly modulate the survival of parasite and infected host, and ultimately influence the transmission dynamics in the following spring/summer. Further studies over winter and spring months are needed to investigate this hypothesis.

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