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New insight into the transmission dynamics of the crustacean pathogen *Hematodinium perezi* (Dinoflagellata) using a novel sentinel methodology

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ABSTRACT: *Hematodinium perezi* causes disease and mortality in several decapod crustaceans along the eastern seaboard and Gulf coast of the USA. The route of transmission of the parasite is unknown, but infections exhibit a sharp seasonal cycle in its primary host, the blue crab *Callinectes sapidus*, that indicates the possibility of a short transmission period in its life cycle. We developed a sentinel methodology based on the use of naïve, uninfected, early benthic juvenile crabs (instars C1 to C10) to investigate the transmission of *H. perezi*. Crabs were collected from a non-endemic site, held for a short period for evaluation, and then deployed in a highly endemic site for 14 d. Transmission of the pathogen was successful; 12.7 to 25.7% of the crabs deployed at the endemic site became infected over this period. Infections developed rapidly, with 25% of new infections developing into heavy infections during the deployment. The large number of infections that developed using the sentinel methodology allowed for the first estimates of incidence (the proportion of new infections in a population over time) in this system. Incidence varied from 0.9 to 1.8% of the resident crab population per day and accounts for the high prevalence levels observed in the endemic coastal bays of the Delmarva Peninsula. The development of this sentinel methodology has broad application for studying disease ecology in this system and in other pathogens that infect decapods.

KEY WORDS: Life cycle · Seasonality · Infection · Incidence · Prevalence · Parasite · *Callinectes sapidus*

INTRODUCTION

Sentinel studies have been used in disease surveillance for over 70 yr with much of the work focused on pathogen detection in mosquitos or non-human vertebrate hosts (e.g. Moore et al. 1993). They have been used in aquatic systems to investigate pathogen prevalence using naïve hosts, including oysters (e.g. Ragone Calvo et al. 2003, Carnegie & Burreson 2011, Jenkins et al. 2013), freshwater crabs (Katabarwa et al. 2012), spiny lobsters (Moss et al. 2012), freshwater fishes (proliferative kidney disease, Foott & Hedrick 1987; whirling disease, Nehring & Walker 1996), marine fishes (salmonids, Norris et al. 2008, Johansen et al. 2011), and marine mammals (Reddy et al. 2001, Bossart 2011). In all these studies, the time scale for surveillance was relatively long (on the order of several weeks or months), or the objective was to establish presence or absence of a pathogen; thus, they gave little information on the dynamics of transmission events, the establishment of infection, or the progression of a pathogen in early infections.

Sentinel studies have varied approaches and different methodologies depending on whether a contaminant or a pathogen is being investigated. Three types of sentinel studies are used in field studies with pathogens. The most basic type is to simply trap or collect intermediate hosts or vectors (e.g. mosquitoes,

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ticks, snails), pool them, and test them for specific microbial pathogens using appropriately sensitive and specific diagnostic techniques (e.g. immunodiagnostics or PCR). This type of sentinel study gives pooled estimates of prevalence, but not incidence, for a pathogen in the host population. This basic type has been used widely to monitor mosquitoes for the presence of a variety of viral agents. Mitigation actions such as spraying insecticide or enhanced surveillance of livestock or human populations are then implemented at predetermined levels of estimated prevalence (e.g. Moore et al. 1993). A second type of sentinel study is to monitor dead, dying, or putatively affected animals to make inferences about increases in pathogen prevalence or exposure based on sampling surveys (e.g. Reddy et al. 2001). Although these types of studies provide baseline information on the agent or contaminant of interest, they offer little data on incidence or accumulation rates. The third type of sentinel study is to place uninfected (naïve) hosts into areas where the pathogen is endemic and periodically monitor them for specific pathogens of interest (e.g. Foott & Hedrick 1987). This type of sentinel study gives powerful insights into the transmission dynamics of a pathogen, particularly in terms of prevalence, incidence, variability in the timing of transmission, and progression of new infections.

Here we developed a sentinel methodology to study the transmission of the parasitic dinoflagellate *Hematodinium perezi* to naïve juvenile blue crabs *Callinectes sapidus.* Our objectives were to (1) evaluate the sentinel methodology for use in studying the transmission dynamics of the parasite to the blue crab host, (2) determine the incidence of new infections in blue crabs over a short deployment period, and (3) investigate the progression of the parasite in natural infections acquired during sentinel deployments. Incidence—the proportion of new infections in a population over time—is a difficult metric to obtain in many host–pathogen systems. By using naïve hosts that were deployed for short time periods in a highly endemic area, we were able to examine transmission to make direct estimates of incidence of *H. perezi* and examine disease progression in newly infected sentinel hosts.

H. perezi is an endoparasitic dinoflagellate that infects the blue crab, several other decapods, and amphipods along the seaboards of the eastern USA and western Europe (Messick & Shields 2000, Small et al. 2012). The parasite lives in the hemocoelom of its hosts and proliferates rapidly in the hemolymph, resulting in host morbidity and mortality (Shields & Squyars 2000, Stentiford & Shields 2005). Outbreaks of *H. perezi* occur annually in early summer and autumn in blue crabs in the coastal bays of Maryland and Virginia, where prevalence levels can approach 100% in juveniles and >50% in adults (Messick 1994, Messick & Shields 2000). Infections are limited to hosts in high-salinity coastal bays and have not been reported in low salinity sub-estuaries inside Chesapeake Bay.

Although the life cycle of *H. perezi* has been described using *in vitro* studies (Li et al. 2011a), little is known about how it is transmitted to blue crabs in their natural environment. Dinospores are the likely transmissive stage, but attempts to expose blue crabs to dinospores have been logistically difficult and unsuccessful (J. D. Shields unpubl. data). In controlled laboratory studies, the parasite is highly infectious and can be serially transmitted to blue crabs by injection of hemolymph containing filamentous trophonts or ameboid trophonts of the parasite (Messick & Shields 2000, Shields & Squyars 2000). Cannibalism has been posited as a mode of transmission (Walker et al. 2009), but it was ruled out in a comprehensive feeding study (Li et al. 2011b). Transmission occurs around host molting periods in boreal (Meyers et al. 1990, Eaton et al. 1991, Field et al. 1992, Stentiford et al. 2001, Shields et al. 2005, 2007) and temperate hosts (Messick & Shields 2000), leading to speculation that infections occur when the host is soft from ecdysis.

The prevalence of *H. perezi* has a strong seasonal cycle in blue crabs. It exhibits a small peak in late spring, presumably as infections become active as blue crabs leave winter hibernation (Shields et al. 2015), followed by a sharp autumnal peak that overlaps with the settlement, recruitment, and rapid growth and molting of juvenile blue crabs (Messick & Shields 2000, Shields 2003). These peaks arise very quickly from September through November, indicating that transmission is likely occurring at this time. Given that peaks in prevalence occur very quickly in autumn, we investigated the feasibility of using naïve (uninfected) juvenile blue crabs as sentinels to study the transmission dynamics of *H. perezi* in blue crabs from a highly endemic coastal bay.

MATERIALS AND METHODS

Collection and handling of experimental animals

Early benthic juvenile crabs *Callinectes sapidus* (instars C1 to C10) for use in sentinel studies were collected by dip nets in shallow beds of submerged

aquatic vegetation from Mobjack Bay (37° 18′ N, 76° 24′ W), a non-endemic area for the parasite on the western side of Chesapeake Bay (Fig. 1). Mobjack Bay has numerous shallow eelgrass beds and an average salinity of around 20 psu. The Bay has served as a research site for studies on blue crab ecology for many years (e.g. Orth & van Montfrans 1987, Pile et al. 1996, Ralph et al. 2013). In this study, crabs were collected from August to October 2014, as needed for experiments. They were placed in coolers containing ambient seawater and large sheets of nytex screen (~12 mm mesh) for transport 16 km to the Virginia Institute of Marine Science (VIMS) for holding and assessment. At VIMS, the first ~250 crabs were immediately sorted into individual containers and held in a shallow 50 l recirculating system (see below).

Crabs used in sentinel studies were initially held for observation in containers made from 10 cm diameter white PVC tubing cut into 5 to 6 cm long pieces, with one end closed by a piece of 200 µm nytex screen affixed with silicone aquarium sealant (Marineland) to provide access to water circulation. The containers were placed on top of a shallow, baffled piece of plastic within a long, shallow, custom-built 50 l fiberglass aquarium filled with 38 to 45 l of artificial seawater at 30 psu. The system was provided with aeration and a power head (AquaTop) with a 1.5 m long tygon tube to provide water circulation. The containers were covered with pieces of plexiglass to prevent larger crabs from escaping. An additional housing unit capable of holding ~90 crabs was also used. It was made from black molded plastic-formed squares (plastic landscaping paver) with nytex screen on one side to provide water circulation, and housed in the same fiberglass aquarium. Crabs were fed 2 to 3 pellets of commercial shrimp pellets (pellet size 0.5 mm; Omega One™) 3 times wk^{-1} and 50% water changes were done 3 times wk^{-1}.

Crabs were observed daily for 7 to 10 d in the above system prior to each deployment in sentinel studies. During the observation period, crabs were monitored for molting, morbidity, and mortality, and the system was monitored for temperature and salinity. The salinity was gradually adjusted from 20 to 30 psu to approximate salinity at the endemic deployment site. During deployments, crabs serving as laboratory controls for molting and mortality were housed as above in PVC containers in the 50 l recirculating system. These crabs also served as an additional control for the use of uninfected crabs from Mobjack Bay, the non-endemic site of collection.

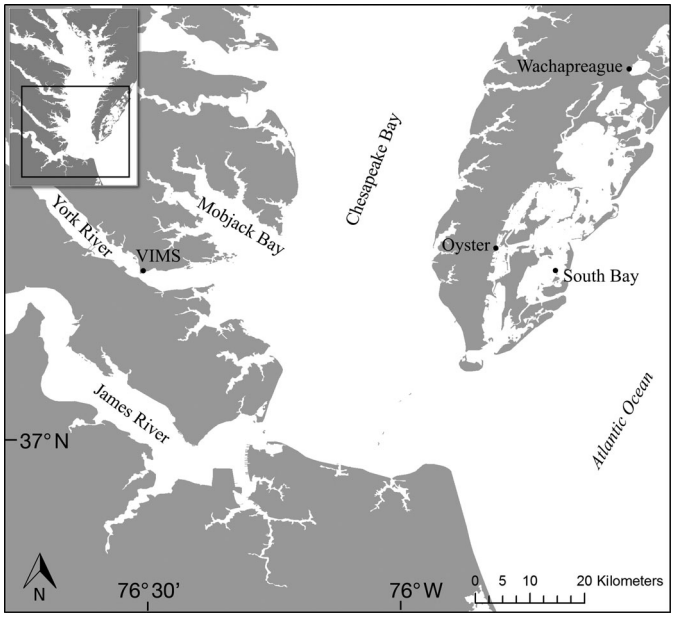

Fig. 1. Lower Chesapeake Bay showing the deployment sites for the sentinel studies in southern Virginia, USA. Blue crabs were collected from a non-endemic site in Mobjack Bay and deployed at an endemic site (experimental sentinels) off the pier in Oyster, VA, or at a non-endemic site (control sentinels) off the Virginia Institute of Marine Science (VIMS) pier. South Bay (background infections) and Wachapreague Creek (temperatures) are also indicated

Sentinel deployments

Urinalysis cups (90 ml, screw-top lid; Fisher Scientific) were used as individual housing units (crab condos) for the sentinel deployments. Cups were modified by either drilling a series of 20 to 24 holes (2.4 mm) in the sides and lids or by cutting out a side panel with a hand-held Dremel rotary tool and using silicone aquarium sealant (Marineland) to glue plastic mesh (200 µm mesh opening size) onto the exposed side. For deployments, dice were used to randomly assign crabs into different condo units and treatment groups. Crabs were placed in condos and transported in seawater (30 psu) with 1 or 2 blue ice bricks to the deployment sites (Oyster or VIMS piers, Fig. 1); the condos were placed in a standard minnow pot (20 condos pot^{-1}), and then deployed off the pier. Replicate minnow pots labeled A–D were deployed at each site using ropes and anchors to keep the pots near the bottom.

Crabs serving as 'experimental sentinels' were deployed in the endemic site at Oyster. These crabs were hung from a pier operated by the University of Virginia's Anheuser-Busch Coastal

Research Center (37° 17.247′ N, 75° 55.517′ W), which is part of the Virginia Coast Reserve Long-Term Ecological Reserve (LTER). The pier is in a small seaside embayment with salinities ranging from 29 to 32 psu. Crabs serving as 'control sentinels' were deployed at the non-endemic site, VIMS pier (37° 14.837′ N, 76° 29.972′ W). They were treated as above, but placed in the coolers for 2 h prior to deployment to simulate the transit conditions and time to Oyster. These animals were deployed directly into the lower salinity of 22 to 24 psu. Deployments lasted for 14 d. Temperature was monitored electronically from a NOAA data buoy (www.ndbc.noaa.gov; Stn wav2) at Wachapreague, (Fig. 1), a coastal bay north of Oyster, with a very similar physiography. The temperature profile of Wachapreague Creek is representative of that at the pier at Oyster (J. P. Huchin-Mian unpubl. data).

Background infections in endemic area

For comparative purposes, background assessments for the prevalence of *Hematodinium perezi* were conducted on crabs collected with dip nets as above from South Bay (37° 15.778′ N, 75° 50.533′ W, Fig. 1), an oceanic coastal bay near Oyster, close to the southern tip of the Delmarva Peninsula. The South Bay estuary has numerous shallow eelgrass beds, an average salinity over 30 psu, and is highly endemic (i.e. hyper-endemic) for transmission of *H. perezi* (J. D. Shields et al. unpubl. data). The estuary is a component of the Virginia Coast Reserve LTER near Oyster, and has served as an important area for numerous studies in marsh and estuarine ecology. Crabs collected from South Bay were transported as above 130 km to VIMS for assessment. At VIMS, crabs were processed immediately for diagnosis of *H. perezi* infections (see below).

Study design

Each sentinel study was conducted with 5 groups: (1) an experimental sentinel group deployed into the endemic area (Oyster pier), (2) a control sentinel group deployed into the non-endemic area (VIMS pier), (3) a preliminary assessment (pre-sample) of crabs from the non-endemic site (Mobjack Bay), (4) an assessment of background infections in crabs from the highly endemic site (South Bay), and (5) a group of crabs from the non-endemic site held as a laboratory control group to control for mortalities in the deployments. Crabs were deployed in 2 separate studies. The first deployment was from 17 September to 2 October, and the second was from 9 to 23 October 2014. Sample sizes ranged from 35 to 92 crabs, depending on the treatment (see Table 1). Crab sizes ranged from 5 to 31 mm carapace width (CW). Background assessments for the highly endemic site were conducted on 2 October and 5 November 2014.

Disease diagnostics and microscopic assessments

All crabs in the sentinel deployments and those serving as laboratory controls were screened for *H. perezi*. Crabs were measured using a ruler, then cut in half lengthwise (along their longest carapace length) with a sterile razor on a glass microslide. Half of the crab was placed in 95% ethanol, the other half was placed in a cassette and fixed in Z-Fix (Fisher Scientific). A drop or 2 of 0.3% (w/v) neutral red dye was placed on the wet dissection smear, which was then mounted with a coverslip and examined for the presence of the parasite using transmitted light microscopy (200 to 400×). Parasites were differentiated from host cells by the uptake of neutral red and the presence of obvious life history stages (filamentous trophont, ameboid trophonts, and clump colonies). Intensity was based on a semi-quantitative designation related to the number of individual parasite cells per field. Light infections were characterized by the presence of filamentous trophonts (vermiform plasmodia) or few ameboid trophonts per microscopic field (1 to 3 parasites at 200×). Moderate infections typically had 4 to 10 parasites (filamentous trophonts, ameboid trophonts and clump colonies) per field at 200×, and heavy infections had >10 parasites (ameboid trophonts, potential sporonts, clump colonies) per field. Smears from all of the crabs were microscopically examined for the parasite.

DNA extraction and PCR

All crabs in the sentinel deployments from Oyster and 10 randomly selected crabs from control groups (non-endemic deployment or laboratory-held controls) were screened for *H. perezi* by diagnostic PCR assays. The entire tissue sample consisting of half of a crab was extracted from crabs ranging from 5 to 14 mm in CW, whereas only one set of gills were extracted in crabs >14 mm CW because of their size. These samples were immersed in sterile, autoclaved H_2O for 60 min to facilitate removal of residual

ethanol. DNA samples were extracted using a Qiagen tissue and blood kit (Qiagen) following the manufacturer's instructions for animal tissues. DNA samples were eluted in 100 µl of AE buffer, quantified using a NanoDrop 2000 (Thermo Scientific), and stored at −20°C. All extractions completed within the same day included a blank column extraction which served as a control for extraction contamination in subsequent PCR analyses.

All DNA samples were screened for the presence of *H. perezi* using primers designed to target the ITS1 rRNA region of the parasite (Small et al. 2007). Cycling conditions and reaction concentrations were as described by Pagenkopp Lohan et al. (2013). Approximately 20 to 100 ng (1 µl) of template DNA was added per PCR assay. Aliquots of 10 µl 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 amplicon for *H. perezi* was 302 bp in size. Included in all PCR assays was a negative control that consisted of no DNA, a blank column extraction, and a positive control consisting of a sample of *H. perezi* DNA that had routinely amplified in prior studies (Shields et al. 2015).

Histological assessment

Crab and tissue samples for histological analysis were processed for histology according to standard procedures (Wheeler et al. 2007, Shields et al. 2012). These samples were fixed in Z-Fix for 24 to 72 h before transfer to 70% ethanol for short-term storage. Samples were decalcified in a formic acid-sodium citrate solution for 6 to 12 h before processing (Luna 1968). Tissues were processed through a standard ethanol series, embedded in paraffin, cut at 5 to 6 µm, and processed in an ethanol series before staining with Mayer's hematoxlyin and eosin (Humason 1979). All histological slides were examined for the presence and intensity of parasite, as well as other histological features. Emphasis was placed on observing the gills, hepatopancreas, and heart, as these organs have comparatively more hemal sinuses and arterioles per unit area than other organs. All of the slides were read by one observer (J. D. Shields). The histological assessment was blind to the PCR results, but the few crabs later identified as positive for *H. perezi* DNA by PCR, but negative by histology or neutral red, were re-evaluated and read twice for histological diagnosis.

Sample size estimation

We used standard statistical methods to estimate the probability of detection of a pathogen given known prevalence levels (e.g. Gu & Novak 2004). Briefly, the probability of detection (P) is equivalent to (1 − precision), where precision is an estimate of the random errors associated with sampling, typically 5%, and 1 − precision is essentially the power of detection. The probability of detection of any infected individual in a sample of hosts (N) can be estimated based on the binomial distribution, where r is the prevalence of infection:

$$P = 1 - (1 - r)^N \quad (1)$$

This is the probability of detecting a specified prevalence based on any given sample of hosts tested. Based on Eq. (1), the minimum sample size needed for a specified probability of detection is then given by:

$$N = \log(1 - P) + \log(1 - r) \quad (2)$$

Using Eq. (2), one can calculate sample sizes needed to gauge the precision of any prevalence level. Contingency tables and chi-squared analyses were performed in Systat v.11.0. Formulas for estimating confidence intervals for population proportions are from Zar (1999).

RESULTS

Sentinel studies

Only crabs surviving in the experimental sentinel group at Oyster and those from the background infections group at South Bay were infected with *Hematodinium perezi*. With a few exceptions, dead crabs were not diagnosable. Empty condos were considered to have a dead crab (they had decayed because escape was not possible), and dead crabs were not included in the analysis. For microscopic diagnosis using neutral red, the prevalence of infection after 14 d varied from 25.7% in the first deployment to 12.7% in the second deployment of crabs at Oyster (Table 1). Over the 14 d deployment, this corresponded to an incidence of 0.9 to 1.8% of the resident crab population per day. Background infections in crabs from South Bay had prevalence levels of 100 and 97% during the deployment periods.

Infections were capable of rapid progression in experimental sentinels. Light infections were common in crabs from both deployment periods (Fig. 2).

Table 1. Survival of sentinel crabs held in deployments and prevalence of infections in crabs using different diagnostics. Treatments consisted of experimental sentinels deployed at the highly endemic site (Oyster), background infections in crabs sampled in the endemic location (South Bay), control sentinels deployed at the non-endemic site (Virginia Institute of Marine Science [VIMS] pier), and crabs held as laboratory controls. Numbers of crabs surviving deployment (numerator) and total number deployed (denominator) or used in each treatment are given. Prevalence levels and 95% CI (in parentheses) are given for each diagnostic technique based on live crabs at the end of each sentinel deployment period. nd: not done

Treatment (deployment period)	Crabs surviving / total deployed	Prevalence (%) by diagnostic method				
		Smear with neutral red	Histological assessment	PCR (all signals)	PCR (strong)	PCR (faint)
Experimental sentinels (1)	74/92	25.7 (16.3–37.4)	24.3 (15.4–35.8)	40.6 (29.3–52.8)	20.3 (11.8–31.4)	20.3 (11.8–31.4)
Experimental sentinels (2)	55/56	12.7 (5.3–24.4)	12.7 (5.3–24.4)	25.5[a] (14.7–39.0)	12.7[a] (5.3–24.4)	12.7 (5.3–24.4)
Background infections (1)	30/30	100.0 (88.4–100)	100.0 (88.4–100)	nd	nd	nd
Background infections (2)	34/34	97.1 (84.6–99.9)	nd	nd	nd	nd
Control sentinels (1)	27/41	0.0	0.0	0.0	nd	nd
Control sentinels (2)	40/40	0.0	0.0	0.0	nd	nd
Laboratory control (1)	33/33	0.0	0.0	nd	nd	nd
Laboratory control (2)	40/41	0.0	0.0	nd	nd	nd

[a]Includes 1 infected, recently dead crab

However, several crabs in the first deployment exhibited moderate and heavy infections after the 14 d deployment period. In the first deployment, 30% of the infections were categorized as moderate and 25% of the infections were heavy. Background infections in crabs from South Bay at approximately the same time as the first deployment had predominantly heavy infections as well as advanced infections containing prespore stages; none were categorized as light infections. In the second deployment, 75% of the infected crabs had light infections, but no heavy infections were observed. Background infections in crabs from South Bay in this time frame were predominantly heavy infections, with the prevalence levels of light and moderate infections roughly equal.

Mortality was assessed for individual crabs and collectively for crabs housed in separate minnow pots. In the first deployment, 2 minnow pots showed excessive mortality (39.1 to 52.3%): one at the experimental sentinels site and one at the control sentinels site (Fig. 3a). In both cases extenuating circumstances occurred. At the experimental sentinels site, 2 pots became entangled on the pier resulting in air exposure of one of the pots (Experimental Sentinel D) at low tide. A pot deployed at control sentinel site (VIMS B) became tangled in a bottom obstruction which resulted in its partial burial. It was re-situated 10 d into the deployment and thereafter exhibited negligible mortality. If these 2 pots are excluded from the analysis, then the overall mortality varied from 8.3 to 15.0% in the deployed treatments (Fig. 3B). Mortality in the laboratory-held controls was negligible, from 0.0 to 2.4%.

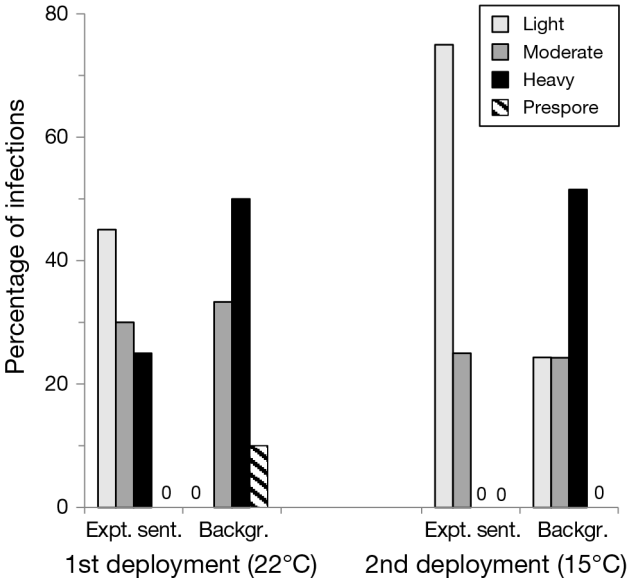

Fig. 2. Percentage of blue crabs infected with *Hematodinium perezi* in relation to the intensity of infection for different deployment periods. Crabs in the experimental sentinel group became infected during each 14 d deployment, while those at the background site had natural infections. Note the difference in the percentage of crabs with light and heavy infections between deployment periods. Mean temperature is given for each deployment period

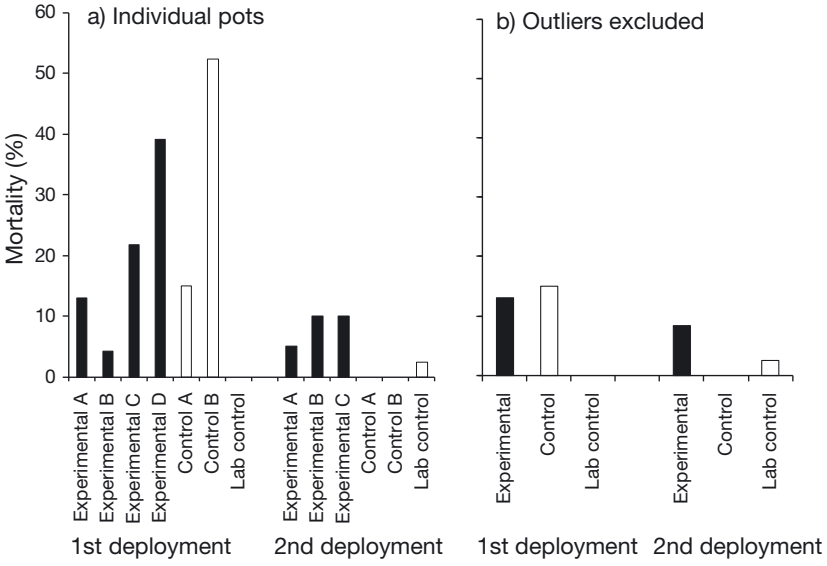

Fig. 3. Mortality (%) of blue crabs in relation to treatment and control groups. (a) Mortality within each replicate minnow pot in the experimental (A, B, C, D) and control (A, B) sentinel groups and the lab control, shown for each deployment period. (b) Mean mortality with outliers excluded for each treatment and control group in different deployment periods. Outliers (Experimental Sentinel D and Control Sentinel B) were excluded due to deployment errors (see 'Results: Sentinel studies')

Molting was assessed daily in the laboratory-held controls (Fig. 4). At least 7 d prior to deployment, crabs were captured and acclimated in the holding system. Molting during this initial period was high, from 3.5 to 8.4% daily molting frequency. During deployments the laboratory-held crabs exhibited a consistent 3.0 to 3.8% daily molting frequency. Molting was not directly assessed in sentinel crabs in the deployments; nonetheless, in the second deployment, crabs were measured (CW, including epibranchial spines) and re-measured after deployment to gauge the number of crabs potentially growing in size as a function of molting. Many of the deployed crabs did not grow, and there was some evidence that they became smaller in size (Fig. 4). This was likely not measurement error as all of the crabs were measured by the same person using the same ruler; rather, it was probably an experimental or container effect. However, significantly more crabs in the control sentinel group showed an increase in post-deployment size than crabs in the experimental sentinel group (χ^2 = 3.903, df = 1, p = 0.048), and significantly more crabs in the laboratory control group had larger post-deployment sizes than those in either sentinel group (χ^2 = 19.104, df = 2, p < 0.001). This was likely not a feature of parasite-induced changes in growth rates because only 2 of the infected crabs from the experimental sentinel group were smaller in their post-deployment measurement.

Patterns in disease progression may be reflected in the differences in water temperature between the deployments (Fig. 5). The mean (±SD) temperature in the first deployment was 22.4 ± 1.3°C and mean daily temperatures were relatively flat, varying from 19.6 to 23.8°C. The mean temperature in the second deployment was 19.5 ± 2.0°C and mean daily temperatures were, with a few exceptions, consistently lower, ranging from 16.2 to 21.9°C. The lower temperature during the second deployment coincided with a lower incidence of infection during that period and a change from heavy to light infections.

Using the method of Gu & Novak (2004), we calculated the sample sizes need to assess different prevalence levels of *H. perezi* given the

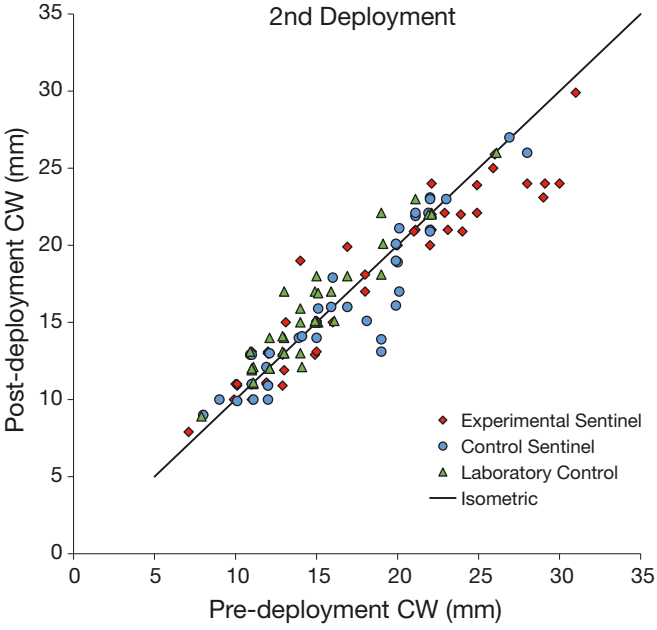

Fig. 4. Pre- and post-deployment measures of carapace width (CW) for blue crabs deployed in the second deployment. Molting crabs would normally show an increase in the post-deployment CW (numbers above the isometric line). Note the variation in size and number of crabs occurring below the isometric line. For statistical comparisons, the frequencies of crabs above and below the isometric were compared via chi-squared analysis. The post-deployment size of several crabs were smaller than expected compared with their pre-deployment size, indicating a container effect

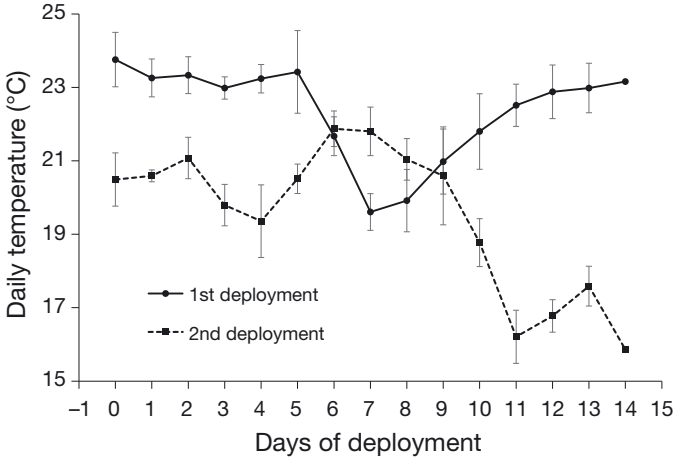

Fig. 5. Mean (±SD) daily water temperatures at Wachapreague, VA, representative of temperatures at the endemic site, Oyster, VA, showing different temperature profiles between the 2 deployment periods at the experimental sentinel site

probability of detection (1 – precision) of the given prevalence (Fig. 6). When prevalence is high (≥10%) and mortality moderate (8 to 10%), only 35 to 40 crabs are needed to establish high precision estimates of the prevalence level, and consequently, incidence (Fig. 6a). Conversely, a larger number of hosts may be needed to accurately assess low prevalence levels when a high precision is required (Fig. 6b).

Diagnostics

For comparisons of detection among diagnostic methods (neutral red, histology, and PCR assays), there were no differences in the prevalence levels between deployment periods (contingency tables, chi-squared tests, p-values > 0.098); therefore, data from the 2 periods were pooled for comparisons between assays. Diagnosis by neutral red, histology and strong PCR positive assays showed good correspondence (Fig. 7). However, the PCR assay indicated the presence of *H. perezi* DNA as faint bands on agarose gels in additional samples, and 16 of 17 of the faint bands were not associated with infections diagnosed by neutral red or histology. These may represent 3 outcomes: (1) very light infections that are not diagnosable via light microscopy; (2) false positives (albeit control PCRs were negative and the method has been well tested, e.g. Shields et al. 2015); or (3) the presence of *H. perezi* externally on crabs or within their gill chambers. Selecting only strong PCR assays, the sensitivity and specificity of the assays were all very high (>95%) as well as the positive and negative predictive values; however, if the faint PCR assays are included, the specificities and positive predictive values decline to 81 and 55%, respectively.

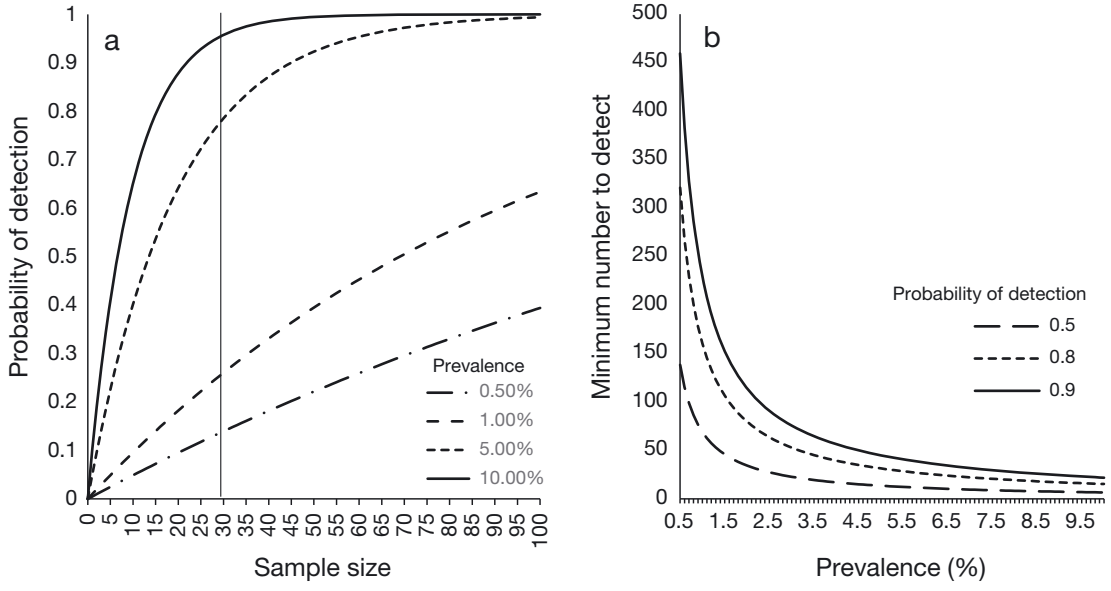

Fig. 6. Sample sizes needed for estimates of detection probabilities (1 – precision) based on different prevalence levels in a representative host population. (a) Probability of detection in relation to sample size for different prevalence levels. Vertical line indicates minimum sample size of 30 is needed to achieve 95% probability of accurately detecting a prevalence level >5% (after Gu & Novak 2004). Trajectories for different prevalence levels are shown. (b) Minimum number of crabs needed to detect infections in relation to prevalence for different probabilities of detection (1 – precision). Trajectories for different probabilities of detection are shown

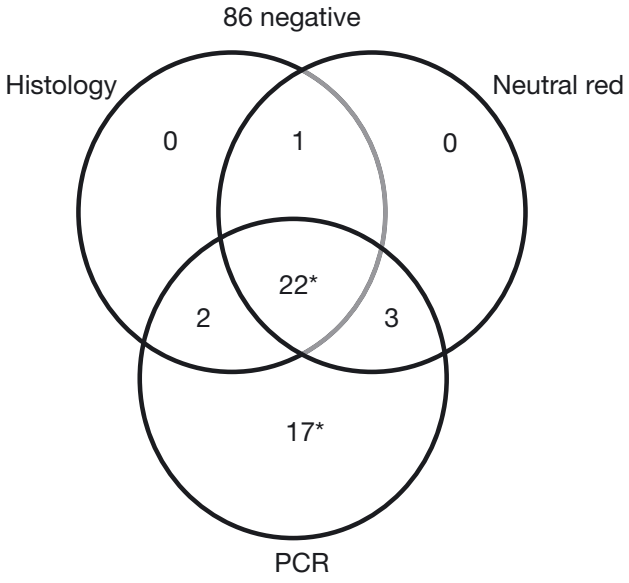

Fig. 7. Venn diagram showing the frequencies of positive diagnoses assigned by different methods. A total of 86 blue crabs were diagnosed as negative by all 3 methods. (*) The PCR diagnosis comprised assays with faint versus strong bands; 17 crabs diagnosed as PCR-positive had faint PCR assays that did not have correspondence with infections using other methods, while 5 crabs with faint bands had correspondence with the other methods

DISCUSSION

We demonstrated the suitability of using sentinel studies for examining key aspects in the ecology of *Hematodinium perezi* infections in the blue crab, *Callinectes sapidus*. Our use of naïve juvenile crabs as sentinels gave perspective on transmission, incidence, and the possible influence of biotic and abiotic factors on disease progression. In our study, transmission of the pathogen occurred quickly, infections developed rapidly and naturally, and the large number of infections over time allowed for the first estimates of incidence in this system. Incidence (the proportion of new infections in a population over time) is a difficult metric to obtain in many host–pathogen systems because the number of exposed hosts is often unknown. It is directly related to the force of infection, a key concept in modeling the epidemiology and ecology of pathogens, as well as the transmission coefficient, which is used in many mass action models of disease. In our initial deployments, incidence ranged from 0.9 to 1.8% of crabs obtaining new infections per day. This is a relatively high incidence for a pathogen and explains why the background prevalence of *H. perezi* approaches 100% in the seaside bays of the Delmarva Peninsula in the autumn.

Incidence is an important variable in disease ecology because it is a component of the transmission coefficient used in many models. The transmission coefficient (β) for mass action models can be estimated as:

$$\beta = 1 - \exp[\ln(S_i/S_0)/I_0] \quad (3)$$

where S_i is the number of susceptible individuals at time i, S_0 is the number of susceptible individuals at time 0, and I_0 is the number of infected individuals at time 0 (e.g. d'Amico et al. 1996, Soto & Lotz 2001, 2003). In cases where $I_0 = 1$, the transmission coefficient equals the estimate of incidence over the course of the study. This equation does not include a time step, which is iterated elsewhere in mass action models. Estimation of the transmission coefficient for *H. perezi* to blue crabs is hampered by the unquantifiable sources of infection (I_0) arising from natural transmission in the field. From this study we can show that transmission is water-borne and likely occurs through the dinospore stage (Frischer et al. 2006, Li et al. 2010). Given that a single infected crab can release 10^8 dinospores ml^{-1} of hemolymph, and that ~30% of the weight of a crab is hemolymph (Shields & Squyars 2000), a single crab releasing dinospores may equate effectively to $I_0 = 1$ in a small estuarine system. Using this value, the transmission coefficient for *H. perezi* varied from 0.009 to 0.018, the number of infections divided by the 14 d time course of each deployment. These values for *H. perezi* range from roughly equivalent to nearly double those estimated for natural routes of transmission of highly infectious viruses in shrimp through ingestion (Soto & Lotz 2001, 2003, Lotz & Soto 2002) or transmission of virus in spiny lobster through waterborne contact (Butler et al. 2008). The transmission coefficient is often modeled as a constant value, but it may be affected by a number of biotic and abiotic factors (d'Amico et al. 1996, McCallum et al. 2001). The factors that lead to this variation in incidence remain the topic of our continued research effort.

The sentinel methodology has broad application to studying disease processes in other aquatic systems. For many aquatic systems there is little quantitative data on transmission of pathogens other than inferences with respect to seasonal increases in prevalence in natural infections (e.g. Shields et al. 2005) or changes in prevalence during an epidemic (e.g. Hallett et al. 2008). Thus, this type of study, which uses naïve hosts as sentinels, can provide important information on transmission, prevalence, and incidence. In addition, data from sentinel studies can provide estimates to calculate required samples sizes for

assessment of different prevalence levels, for calculating detection probabilities, and for determining the cost-effectiveness of the method when used for surveillance. The methods for establishing sample sizes for sentinel studies have been well developed for use in monitoring human and veterinary pathogens in arthropods (e.g. Moore et al. 1993, Gu & Novak 2004, Williams et al. 2007) as well as pathogens in livestock (e.g. Fosgate 2009). Given that natural prevalence levels vary from 10 to 100% in juveniles and 5 to 20% in adult crabs and that incidence levels are high, modest samples sizes (35 to 40) of juvenile crabs are needed to accurately assess incidence and other aspects of the transmission dynamics of *H. perezi* in the coastal bays of the eastern USA.

We exploited several features in developing the sentinel methodology. First, uninfected juvenile blue crabs could be collected from non-endemic zones and maintained in the laboratory under ideal conditions. Second, juvenile crabs exhibited relatively low mortality in the laboratory and during deployments, an exploitable feature because they are capable of suspension feeding (see below). Third, transmission occurred quickly in sentinel crabs and infections developed rapidly, allowing for study of the natural progression of the pathogen over a relatively short time scale (14 d). This also makes it possible to study natural transmission in relation to short-term fluctuations in temperature and other abiotic factors. For example, several crabs had heavy infections at the end of the first deployment period, but not at the end of the second deployment period, which had a lower temperature. This is a key aspect because in other systems, the life cycle of the pathogen may follow an annual cycle (e.g. *Hematodinium* sp. infections in snow crabs; Shields et al. 2007). Fourth, the infections were acquired naturally, and thus studies of the early infection process are now feasible using this sentinel system. Fifth, juvenile crabs were able to molt in the relative security of their confinement; however, the molt increment was likely affected by a container effect. Thus the effect of molting on pathogen transmission can be examined (Huchin-Mian et al. in press). Sixth, in the case of systemic pathogens, it may be feasible to test an autotomized limb prior to deployment to ensure the absence of a specific pathogen of interest, but sample and processing times are critical (Moss et al. 2012). Seventh, this methodology was relatively inexpensive and easily deployed, allowing for rapid follow-up studies. These features could be applicable to sentinel studies on viruses (e.g. *Panulirus argus* virus 1 in spiny lobsters, white spot syndrome virus in shrimp fisheries), other protists (e.g. *Anophryoides haemophila* in clawed lobsters), and possibly metazoan parasites or symbionts (e.g. *Paragonimus* spp. in freshwater decapods, *Simulium* spp. on freshwater decapods).

Finally, juvenile crabs serving as sentinels in our study were capable of suspension feeding as demonstrated by the presence of a detrital food bolus in their digestive tract (fore-, mid-, and hind-guts) in histological samples. As far as we know, this is the first report of suspension feeding in juvenile blue crabs (J. D. Shields et al. unpubl. data). Zoeae and megalopae are raptorial feeders (McConaugha 2002), but it is unknown whether they are capable of suspension feeding. Although juvenile crabs in our deployments did not molt and grow normally, their suspension feeding may have improved their survival over the deployment periods. With further refinements and proper siting of deployments, the deployment period may be reduced, which can further reduce mortality (Huchin-Mian et al. in press).

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LITERATURE CITED

Bossart GD (2011) Marine mammals as sentinel species for oceans and human health. Vet Pathol 48:676–690

Butler MJ, Behringer DC, Shields JD (2008) Transmission of *Panulirus argus* virus 1 (PaV1) and its effect on the survival of juvenile Caribbean spiny lobster. Dis Aquat Org 79:173–182

Carnegie RB, Burreson EM (2011) Declining impact of an introduced pathogen: *Haplosporidium nelsoni* in the oyster *Crassostrea virginica* in Chesapeake Bay. Mar Ecol Prog Ser 432:1–15

d'Amico V, Elkinton JS, Dwyer G, Burland JP (1996) Virus transmission in gypsy moths is not a simple mass action process. Ecology 77:201–206

Eaton WD, Love DC, Botelho C, Meyers TR, Imamura K, Koeneman T (1991) Preliminary results on the seasonality and life cycle of the parasitic dinoflagellate causing bitter crab disease in Alaskan Tanner crabs (*Chionoecetes bairdi*). J Invertebr Pathol 57:426–434

Field RH, Chapman CJ, Taylor AC, Neil DM, Vickerman K (1992) Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the west coast of Scotland. Dis Aquat Org 13:1–15

Foott JS, Hedrick RP (1987) Seasonal occurrence of the infectious stage of proliferative kidney disease (PKD) and

resistance of rainbow trout, *Salmo gairdneri* Richardson, to reinfection. J Fish Biol 30:477–483

Fosgate GT (2009) Practical sample size calculations for surveillance and diagnostic investigations. J Vet Diagn Invest 21:3–14

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

Gu W, Novak RJ (2004) Short report: detection probability of arbovirus infection in mosquito populations. Am J Trop Med Hyg 71:636–638

Hallett TB, Zaba B, Todd J, Lopman B and others (2008) Estimating incidence from prevalence in generalised HIV epidemics: methods and validation. PLOS Med 5:e80

Huchin-Mian JP, Small HJ, Shields JD (in press) Patterns in the natural transmission of the parasitic dinoflagellate *Hematodinium perezi* in American blue crabs, *Callinectes sapidus*, from a highly endemic area. Mar Biol

Humason GL (1979) Animal tissue techniques, 4th edn. WH Freeman, San Francisco, CA

Jenkins C, Hick P, Gabor M, Spiers Z and others (2013) Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis Aquat Org 105: 109–126

Johansen LH, Jensen I, Mikkelsen H, Bjørn PA, Jansen PA, Bergh Ø (2011) Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway. Aquaculture 315:167–186

Katabarwa MN, Walsh F, Habomugisha P, Lakwo TL and others (2012) Transmission of onchocerciasis in Wadelai focus of northwestern Uganda has been interrupted and the disease eliminated. J Parasitol Res 2012:748540

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, Miller TL, Small HJ, Shields JD (2011a) *In vitro* culture and developmental cycle of the parasitic dinoflagellate *Hematodinium* sp. from the blue crab *Callinectes sapidus.* Parasitology 138:1924–1934

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

Lotz JM, Soto MA (2002) Model of white spot syndrome virus (WSSV) epidemics in *Litopenaeus vannamei*. Dis Aquat Org 50:199–209

Luna LG (1968) Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd edn. McGraw-Hill, New York, NY

McCallum H, Barlow N, Hone J (2001) How should pathogen transmission be modeled? Trends Ecol Evol 16: 295–300

McConaugha J (2002) Alternative feeding mechanisms in megalopae of the blue crab *Callinectes sapidus.* Mar Biol 140:1227–1233

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

Meyers TR, Botelho C, Koeneman TM, Short S, Imamura K (1990) Distribution of bitter crab dinoflagellate syndrome in southeast Alaskan Tanner crabs, *Chionoecetes bairdi.* Dis Aquat Org 9:37–43

Moore CG, McLean RG, Mitchell CJ, Nasci RS and others (1993) Western equine encephalomyelitis. In: Guidelines for arbovirus surveillance programs in the United States, Vol 500. US Department of Health and Human Services, Division of Vector-Borne Infectious Diseases, Fort Collins, CO, p 36–40

Moss J, Butler MJ IV, Behringer DC, Shields JD (2012) Genetic diversity of the Caribbean spiny lobster virus, *Panulirus argus* virus 1 (PaV1), and the discovery of PaV1 in lobster postlarvae. Aquat Biol 14:223–232

Nehring RB, Walker PG (1996) Whirling disease in the wild: the new reality in the intermountain west. Fisheries (Bethesda, Md) 21:28–30

Norris A, Foyle L, Ratcliff J (2008) Heritability of mortality in response to a natural pancreas disease (SPDV) challenge in Atlantic salmon, *Salmo salar* L., post smolts on a West of Ireland sea site. J Fish Dis 31:913–920

Orth RJ, van Montfrans J (1987) Utilization of a seagrass meadow and tidal marsh creek by blue crabs *Callinectes sapidus*. I. Seasonal and annual variations in abundance with emphasis on post-settlement juveniles. Mar Ecol Prog Ser 41:283–294

Pagenkopp Lohan KM, Small HJ, Shields JD, Place AR, Reece KS (2013) Conservation in the first internal transcribed spacer (ITS1) region of *Hematodinium perezi* from *Callinectes sapidus.* Dis Aquat Org 103: 65–75

Pile A, Lipcius R, van Montfrans J, Orth R (1996) Density-dependent settler-recruit-juvenile relationships in blue crabs. Ecol Monogr 66:277–307

Ragone Calvo LM, Dungan CF, Roberson BS, Burreson EM (2003) Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Dis Aquat Org 56:75–86

Ralph GM, Seitz RD, Orth RJ, Knick KE, Lipcius RN (2013) Broad-scale association between seagrass cover and juvenile blue crab density in Chesapeake Bay. Mar Ecol Prog Ser 488:51–63

Reddy LM, Dierauf LA, Gulland FM (2001) Marine mammals as sentinels of ocean health. In: Dierauf LA, Gulland FMD (eds) CRC handbook of marine mammal medicine, 2nd edn. CRC Press, Boca Raton, FL, p 3–13

Shields JD (2003) Research priorities for diseases of the blue crab *Callinectes sapidus.* Bull Mar Sci 72:505–517

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, Taylor DM, Sutton SG, O'Keefe PO, Collins PW, Ings DW, Pardy AL (2005) Epizootiology of bitter crabs disease (*Hematodinium* sp.) in snow crabs *Chionoecetes opilio*, from Newfoundland, Canada. Dis Aquat Org 64: 253–264

Shields JD, Taylor DM, O'Keefe PG, Colbourne E, Hynick E (2007) Epidemiological determinants in outbreaks of bitter crab disease (*Hematodinium* sp.) in snow crabs *Chionoecetes opilio*, from Newfoundland, Canada. Dis Aquat Org 77:61–72

Shields JD, Wheeler KN, Moss J (2012) Histological assessment of lobsters in the '100 lobster' project. J Shellfish Res 31:439–447

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, Shields JD, Hudson KL, Reece KS (2007) Molecular detection of the *Hematodinium* sp. infecting the blue crab, *Callinectes sapidus*. J Shellfish Res 26:131–139

Small HJ, Shields JD, Reece KS, Bateman K, Stentiford GD (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

Soto MA, Lotz JM (2001) Epidemiological parameter of white spot syndrome virus infections in *Litopenaeus vannamei* and *L. setiferus*. J Invertebr Pathol 78:9–15

Soto MA, Lotz JM (2003) Transmission, virulence, and recovery coefficients of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) infections in Kona Stock *Litopenaeus vannamei*. J Aquat Anim Health 15:48–54

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

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

Wheeler K, Shields JD, Taylor DM (2007) Pathology of *Hematodinium* sp. infections in snow crabs (*Chionoecetes opilio*) from Newfoundland, Canada. J Invertebr Pathol 95:93–100

Williams CR, Long SA, Webb CE, Bitzhenner M, Geier M, Russell RC, Ritchie SA (2007) *Aedes aegypti* population sampling using BG-Sentinel traps in north Queensland Australia: statistical considerations for trap deployment and sampling strategy. J Med Entomol 44:345–350

Zar JH (1999) Biostatistical analysis, 4th edn. Prentice Hall, Upper Saddle River, NJ

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