

NOTE

Molecular detection of the parasitic dinoflagellate *Hematodinium perezii* from blue crabs *Callinectes sapidus* in Louisiana, USA

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ABSTRACT: The dinoflagellate *Hematodinium perezii* is a prolific pathogen of the blue crab *Callinectes sapidus* along the Atlantic and Gulf of Mexico coasts of North America. High prevalence, sometimes approaching 100%, and outbreaks with high mortality are associated with higher salinities. *H. perezii* has not been reported previously in blue crabs from Louisiana, USA, where salinities in coastal habitats are generally below the parasite's favorable range. However, the possibility that *H. perezii* infects blue crabs in higher salinity habitats offshore has not been investigated. A PCR-based test for *H. perezii* was used to screen blue crabs collected from both high and low salinity areas. These included juvenile and adult crabs from inshore marshes where salinities are relatively low and from higher salinity offshore shoals that are spawning sites for females. *H. perezii* was detected in blue crabs from offshore shoals (prevalence = 5.6%) but not in juvenile or adult crabs from inshore habitats. Megalopae (post-larvae) were also collected from inshore locations. Although megalopae settle inshore where salinities are relatively low, the megalopal stage is preceded by a planktonic phase in higher salinity offshore waters. We detected *H. perezii* in 11.2% of settling megalopae tested. Although the prevalence of *H. perezii* was relatively low within our samples, if spawning females and megalopae are especially vulnerable, the impact on the population could be compounded. This is the first report of *H. perezii* from blue crabs in Louisiana and demonstrates the importance of examining all life stages in determining the prevalence of a harmful parasite.

KEY WORDS: Blue crab · *Callinectes* · Louisiana · *Hematodinium* · Dinoflagellate · Pathogen · Gulf of Mexico

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INTRODUCTION

The dinoflagellate *Hematodinium perezii* is a virulent parasite of the blue crab *Callinectes sapidus* in North America (Shields & Overstreet 2007), where the prevalence of infection is often as high as 30% and can approach 100%. The mortality rate for infected crabs is also high: a study with captive individuals found 87% mortality after 40 d (Shields & Squyars

2000). Not surprisingly, outbreaks of *H. perezii* are considered to be a major factor in regional declines of blue crab fisheries (Lee & Frischer 2004). Symptoms of infection consist of lethargy and changes in hemolymph, including a decline in the number of hemocytes, reduced clotting ability, and discoloration (Shields & Squyars 2000, Shields & Overstreet 2007).

Surveys of infection by *H. perezii* in blue crabs typically report the prevalence of infection (percent-

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age of infected individuals) and occasionally the average intensity of infection (percentage of cells in the hemolymph that are parasites). Both prevalence and intensity vary seasonally and are correlated with salinity and temperature, although there is regional variation in these relationships (Messick & Shields 2000, Gandy et al. 2015). Infection has been observed less frequently in waters with salinities below 11 ppt (Messick & Shields 2000), while severe outbreaks are often associated with high salinities following droughts (Lee & Frischer 2004, Parmenter et al. 2013, Gandy et al. 2015). In all of the previous surveys for *H. perezii* that included blue crabs from Louisiana, USA, none detected the pathogen in Louisiana (Messick & Shields 2000, Pagenkopp Lohan et al. 2013, Rogers et al. 2015a,b) although infected crabs have been reported from the Gulf coasts of Texas, Florida and Mississippi, USA (Newman & Johnson 1975, Messick & Shields 2000, Shields & Overstreet 2007, Gandy et al. 2015). The relatively low salinities of Louisiana's inshore waters, combined with limited sampling from higher salinity offshore habitats could explain the apparent absence of *H. perezii* in Louisiana. However, critical stages of the blue crab's life cycle take place offshore, where salinities tend to be higher. Female blue crabs migrate seaward to spawn because larvae require higher salinities for development (Sandoz & Rogers 1944). Larvae remain offshore for 4–6 wk before they metamorphose to megalopae and begin to migrate inshore where they settle. Thus, even if *H. perezii* does not infect blue crabs in Louisiana's low salinity habitats, it could have significant impacts on reproductive females and larvae in offshore habitats. In the only study that we are aware of in which decapod larvae were tested for the presence of *Hematodinium*, i.e. Hamilton et

al. (2011), prevalence of ~12–15% was reported throughout the summer for the larvae of both langoustines *Nephrops norvegicus* and unidentified crab species from the Clyde Sea in Scotland.

The present study investigates the possibility that *H. perezii* occurs in blue crabs in Louisiana but mainly in offshore habitats with higher salinities. We detected DNA of *H. perezii* in tissues of juvenile and adult crabs from offshore shoals as well as in megalopae settling in low salinity marshes. To our knowledge, these are the first reports of the presence of *H. perezii* from blue crabs in Louisiana.

MATERIALS AND METHODS

Sample collection

Blue crabs were collected during August 2014 in bottom trawls from 3 offshore shoal locations: St. Bernard Shoals, Ship Shoal, and the Tiger-Trinity Shoals Complex (Fig. 1). Trawl transects consisted of 30 min tows with a 7.3 m net pulled at ~3 knots. Size (carapace width from point to point) and weight were measured for each crab, and tissues (muscle, hepatopancreas, and ovary) were removed and freeze-dried. Crabs were collected from inshore habitats throughout the summer of 2014 with crab pots and by handline fishing at 7 coastal locations: Grand Isle, LUMCON, Marsh Island, Freshwater City, Rockefeller Wildlife Refuge, Rutherford Beach Culvert, and Lake Calcasieu (Fig. 1). For these crabs, 1 ml of hemolymph was drained from the cardiac sinus with 100 μ l of sterile Soderhall and Smith's formulation added as an anticoagulant; the second walking leg on the right side was removed with forceps, and both samples were preserved in pre-chilled 95% ethanol at 4°C. Settling megalopae were collected from 4 locations: Grand Isle, LUMCON, Freshwater City, and Rockefeller Wildlife Refuge (Fig. 1) in 2010, 2013, 2014, and 2015. At each location, 5 hog-hair collectors were deployed for ~24 h, after which these passive collectors were rinsed with ambient seawater. In addition, a 253 μ m plankton net (Wildco) was towed between 3 and 5 times over a total distance of ~200 m. Both types of samples were washed on a 200 μ m filter with ambient seawater, and all *Callinectes sapidus* megalopae were individually removed while still alive. The megalopae were then washed a second time with ambient seawater before being preserved whole in pre-chilled 95% ethanol at 4°C. Spot measurements of salinity and water temperature were taken at the time of each collection.

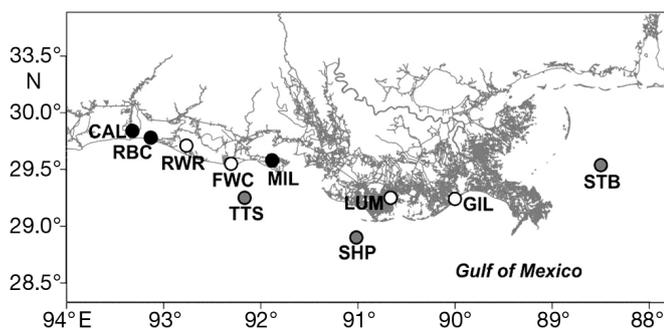


Fig.1. Sampling locations in Louisiana, USA, for coastal adult/juvenile blue crabs (●), coastal adult/juvenile blue crabs and megalopae (○), and offshore adult/juvenile blue crabs (●) evaluated for the presence of *Hematodinium perezii*. See Table 1 for definition of abbreviations

DNA extraction and detection of *Hematodinium perezii* DNA

DNA initially was extracted from ~2 mg of freeze-dried muscle tissue, ~200 μ l of preserved hemolymph, or from whole ethanol-preserved megalopae using NucleoSpin[®] 96 Tissue Kits (Machery-Nagel) with an epMotion 5075 TMX liquid handling workstation (Eppendorf) following the manufacturer's protocol. The presence of DNA of *H. perezii* was detected by PCR amplification of a portion of the 18s ribosomal RNA gene using the primers Hemat-F-18S and Hemat-R-18S developed by Friedman et al. (2009). For samples in which DNA of *H. perezii* was detected, DNA was subsequently extracted from hepatopancreas, ovary and, where available, egg-mass tissue using the same protocol. DNA concentrations were determined with a Nanodrop spectrophotometer (Thermo Scientific). PCR reactions were in 15 μ l with 1 \times AmpliTaq Gold[®] PCR Buffer (Applied Biosystems), 2.5 mM MgCl₂, (1 mM) dNTPs, (1.2 μ M) of each forward and reverse primer, 0.6 units of AmpliTaq[®] Gold (Applied Biosystems), 20 ng of DNA, and Milli-Q[®] water. PCR conditions were as follows: 95°C for 5 min, then 40 cycles of 96°C for 15 s, 56°C for 30 s, 72°C for 45 s, and lastly 72°C for 10 min. Positive and negative controls were included in all reactions. PCR products were electrophoresed on 2% agarose gels with 0.05% ethidium bromide and visualized on a Molecular Imager[®] Gel Doc[™] XR system (Bio Rad).

RESULTS

Among the 54 blue crabs collected from offshore shoals in August 2014, DNA of *Hematodinium perezii* was detected in 3, an overall prevalence of 5.6% (Table 1). All 3 were adult females (1 ovigerous) from St. Bernard Shoals, with point-to-point carapace widths ranging from 130 to 157 mm. In each of these crabs, the DNA of *H. perezii* was detected in muscle and hepatopancreas tissues but not in ovary tissues or in the eggs of the ovigerous female. The PCR prevalence of *H. perezii* for crabs from St. Bernard Shoals (20%) was significantly different from the zero prevalence for crabs from Ship Shoal and the Tiger-Trinity Shoals Complex ($p = 0.04$; 2-tailed Fisher's exact test; Table 1). DNA of *H. perezii* was not detected among the 262 juvenile and adult blue crabs collected from coastal marsh locations (Table 1). Prevalence of *H. perezii* DNA from crabs at the 7 coastal locations was significantly different than for crabs from the 3 offshore locations ($p = 0.0047$; 2-tailed Fisher's exact test). Among the 582 settling megalopae, DNA of *H. perezii* was detected in 65 (11.2%), with prevalence among individual collections ranging from 0 to 35% (Table 2). Prevalence in megalopae was higher in 2014 than 2010, 2013, or 2015 ($\chi^2 = 26.734$, $df = 3$, $p < 0.001$; Table 2), and higher in collections from Grand Isle than from other locations ($\chi^2 = 13.211$, $df = 3$, $p = 0.004$; Table 2).

Table 1. Summary information for adult/juvenile blue crabs collected from the 7 coastal and 3 offshore shoaling areas in Louisiana, USA, including location, date of collection in 2014, latitude (Lat.), longitude (Long.), salinity, temperature, size range, sample size (N), number positive (N_p), and prevalence (Prev.)

Location	Date (mo/d)	Lat. (°N)	Long. (°E)	Salinity (ppt)	Temp. (°C)	Size range (mm)	N	N _p	Prev. (%)
Coastal									
Grand Isle (GIL)	8/1	29.24	90.00	22	30	115–170	19	0	0
LUMCON (LUM)	7/18	29.25	90.66	7	28	106–180	32	0	0
Marsh Island (MIL)	7/16	29.58	91.88	15	29	110–183	33	0	0
Freshwater City (FWC)	6/16, 6/30, 7/29 & 8/12	29.55	92.31	6.3	28.5	63–200	57	0	0
Rockefeller Wildlife Refuge (RWR)	6/16, 6/30, 8/7, & 8/12	29.71	92.77	7	29.7	98–175	38	0	0
Rutherford Beach Culvert (RBC)	6/11, 6/30 & 8/7	29.78	93.13	10	30	50–172	45	0	0
Lake Calcasieu (CAL)	6/10, 7/8	29.84	93.32	15	28	88–182	38	0	0
Coastal total	–	–	–	–	–	50–200	262	0	0
Offshore									
St. Bernard (STB)	8/18	29.54	88.11	36	24.1	130–174	15	3	20
Ship (SHP)	8/20	28.90	91.02	29.6	30.2	162–169	2	0	0
Tiger-Trinity Shoals Complex (TTS)	8/21	29.25	92.17	30.6	29.6	86–179	37	0	0
Offshore total	–	–	–	–	–	86–179	54	3	5.6

Table 2. Summary information for blue crab megalopae including latitude (Lat.), longitude (Long.), date of collection, salinity, temperature, sample size (N), number positive (N_P), and prevalence (Prev.). See Table 1 for definition of abbreviations

Location	Lat. (°N)	Long. (°E)	Date (mo/d/yr)	Salinity (ppt)	Temp. (°C)	N	N _P	Prev. (%)
GIL	29.24	90.00	7/31/2010	12	30	35	8	23
			8/31/2013	18	32	38	3	8
			8/1/2014	22	29.5	43	15	35
			8/18/2015	12	30.5	40	2	5
			Total	–	–	156	28	18
LUM	29.25	90.66	8/29/2010	4	33	36	1	3
			8/21/2013	18	30.5	39	4	10
			7/18/2014	7	28.5	37	3	8
			8/16/2015	10	33.5	40	0	0
			Total	–	–	152	8	5
FWC	29.55	92.31	8/1/2010	20	32	36	5	14
			8/25/2013	22	34	45	1	2
			8/12/2014	10	29	39	11	28
			8/11/2015	19	31.5	32	1	3
			Total	–	–	152	18	12
RWR	29.71	92.77	8/9/2010	17	32	36	4	11
			8/25/2013	22	34	13	0	0
			8/12/2014	0	29	38	6	16
			8/11/2015	16	32	35	1	3
			Total	–	–	122	11	9
Louisiana total	–	–	–	–	–	582	65	11

DISCUSSION

The most significant finding of this study is evidence that the parasitic dinoflagellate *Hematodinium perezii* occurs in blue crabs in Louisiana, as it does elsewhere along the Gulf of Mexico and Atlantic coasts of North America. To our knowledge these are the first reports of *H. perezii* in blue crabs from Louisiana. Our findings agreed with those of previous studies that did not detect this pathogen in blue crabs from Louisiana's coastal marshes. *H. perezii* appears to require salinities above 11 ppt (Messick & Shields 2000, Shields & Overstreet 2007, Coffey et al. 2012); salinities in the coastal marshes of Louisiana are often below 11 ppt (Boesch et al. 1994) but generally higher offshore (Brown 2015). The distribution of infected adults is consistent with a negative effect of low salinities on *H. perezii* in Louisiana's marshes that does not fully extend to higher salinity offshore habitats. Alternatively, the DNA-positive females that we collected from St. Bernard Shoals in Louisiana could have been infected on the coast of Mississippi, where salinities are higher, and subsequently migrated to St. Bernard Shoals. The migration of blue crabs in the Gulf of Mexico could thus be important in the spread of *H. perezii*. Although

infected megalopae were collected in lower salinity marshes, they would have recently encountered higher salinities during their larval phase. A potentially confounding factor in our study is collection method. Crabs were collected from offshore shoals by trawls but in baited traps from marshes. Traps could be less effective than trawls in collecting crabs that have become lethargic as the result of infection by *H. perezii*.

The overall prevalence of *H. perezii* among blue crabs sampled from the Gulf coast of Louisiana was low in comparison to values reported for many other locations. For instance, Gandy et al. (2015) reported prevalence of *H. perezii* in Jacksonville and Panama City, Florida, reached 63 and 68%, respectively, during winter. Messick & Shields (2000) found a cyclic pattern in prevalence from 1992 to 1997 in blue crabs from the lower Chesapeake Bay, with ~90% infected in late-fall peaks. However, it should not be concluded that *H. perezii* has

little impact on blue crabs in Louisiana. Apart from the limited sampling (restricted to summer) on which this report is based, the prevalence of *H. perezii* must be considered in relation to other factors. The prevalence detected is the fraction of individuals that have encountered *H. perezii* but have not died. If the mortality rate is high and death occurs quickly, prevalence represents only a small fraction of all those that may become infected and die. In captive crabs, *H. perezii* kills the majority of infected individuals within 6 wk (Shields & Squyars 2000, Shields et al. 2015). Furthermore, high rates of infection for females spawning on offshore shoals and early life stages prior to settlement could have disproportionately large demographic impacts. Gelpi et al. (2009) showed that offshore spawning female crabs have the potential to spawn 7 times in a spawning season with an average of 2.5 million larvae released per spawn.

Blue crabs support a fishery in Louisiana that accounts for ~80% of hard crab production in the Gulf of Mexico, totaling ~43 million US\$ yr⁻¹ (Perry & VanderKooy 2015). Our findings suggest that infection by *H. perezii* should be a concern for the management of the blue crab fishery in Louisiana and should also be recognized as a factor that could be

amplified by climate change and other anthropogenic disturbances. Ongoing and planned projects intended to preserve and restore Louisiana's coastline make use of large-scale hydrological diversions and sediment mining from offshore shoals (Stone et al. 2004, Moore et al. 2014). Reduction of the shoals, along with the encroachment of hypoxic zones (Bianchi et al. 2010), will reduce critical spawning areas for female blue crabs (Gelpi et al. 2009, 2013, Condrey & Gelpi 2010) and concentrate them in a smaller area where they could be more vulnerable to localized outbreaks by *H. perezii*. Conversely, freshwater and sediment diversions also have the potential to reduce salinity, which could hinder the growth and establishment of *H. perezii*.

Another important finding from our study was that blue crab post-larvae that tested positive for DNA of *H. perezii* were found at multiple locations in each of the 4 years of sampling, which suggests that *H. perezii* is endemic to the region rather than occurring episodically. We also detected significant interannual variation in prevalence for settling megalopae at individual locations. This variation could be related to environmental conditions at the site of settlement, differences in exposure to *H. perezii* prior to settlement, or differences in rates of pre-settlement mortality. At present, we can only speculate about where and when megalopae become infected or how the probability of infection might depend on the particular trajectory a larva follows from spawning site to settlement site. These possibilities should be investigated, as infection of offshore larvae could be both a significant source of mortality as well as a mechanism for rapid spread of disease over large areas. The overall prevalence of *H. perezii* detected in blue crab post-larvae (11.2%) was just below the range of 12–15% reported by Hamilton et al. (2011) for unidentified crab larvae from the Clyde Sea. The absence of crabs with detectable *H. perezii* in marshes as documented in this study and others (Messick & Shields 2000, Pagenkopp Lohan et al. 2013, Rogers et al. 2015a,b) could be explained by a low survival rate for megalopae impacted by *H. perezii*, a lack of transmission in low salinity waters, elimination of the parasite in low salinity habitats or a combination of these mechanisms. More work is needed to assess the importance of *H. perezii* in early life stages of the blue crab.

Our results highlight the importance of considering the environmental tolerances of a pathogen in relation to its host's life history when assessing the pathogen's potential to cause disease. Currently, ~80% of all hard crabs produced in the Gulf of Mexico come

from Louisiana. Infection of blue crabs by *H. perezii* in higher salinity waters represents an unaccounted threat to this profitable fishery, especially as it is likely to have a large impact on spawning females. Additional surveys are needed to determine the spatial and temporal extent of the *H. perezii* range in Louisiana, as well estimates of mortality for all affected life stages. A better understanding also is needed of anthropogenic factors that are likely to change the disease ecology of Louisiana blue crabs.

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