

Disease, parasite, and commensal prevalences for blue crab *Callinectes sapidus* at shedding facilities in Louisiana, USA

Holly A. Rogers¹, Sabrina S. Taylor¹, John P. Hawke², Eric J. Schott³,
Julie A. Anderson Lively^{1,*}

¹School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, USA

²School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803, USA

³IMET, University of Maryland Center for Environmental Science, Baltimore, Maryland 21202, USA

ABSTRACT: Blue crab diseases, parasites, and commensals are not well studied in the Gulf of Mexico, and their prevalence rates have only been sporadically determined. Commercial soft shell shedding facilities in Louisiana experience high mortality rates of pre-molt crabs, and some of these deaths may be attributable to diseases or parasites. During the active shedding season in 2013, we determined the prevalence of shell disease, *Vibrio* spp., *Lagenophrys callinectes*, and *Hematodinium perezii* at 4 commercial shedding facilities along the Louisiana coast. We also detected *Ameson michaelis* and reo-like virus infections. Shell disease was moderately prevalent at rates above 50% and varied by shedding facility, collection month, and crab size. *Vibrio* spp. bacteria were prevalent in the hemolymph of 37% of the pre-molt crabs. *Lagenophrys callinectes* was highly prevalent in the pre-molt crabs, but because it is a commensal species, it may not cause high mortality rates. *Hematodinium perezii* was absent in all pre-molt crabs.

KEY WORDS: Shell disease · *Vibrio* · *Lagenophrys* · Reo-like virus · *Callinectes sapidus* · Molting

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INTRODUCTION

Louisiana has the largest soft shell blue crab industry in the northern Gulf of Mexico. In 2012, approximately 69.0 metric tons (t) of pre-molt and 4.0 t of soft shell crabs were landed in Louisiana and valued at \$430 000 and \$47 000, respectively (National Marine Fisheries Service 2013). The remaining Gulf states landed about 14.7 t of pre-molt and soft shell crabs combined (National Marine Fisheries Service 2013). This valuable Louisiana industry is dependent on low mortality rates at shedding facilities, but an estimated 23% of pre-molt crabs die within 5 d of being held (Chaves & Eggleston 2003). Possible explanations for high mortality rates include diseases and parasites, stress or injury due to handling or crowd-

ing, physiological stress associated with molting, and poor water quality at the facilities.

Diseases, parasites, and commensals that can affect the mortality rate at shedding facilities include shell disease, *Vibrio* spp., *Ameson michaelis* (Microsporida: Pereziiidae), reo-like virus (RLV, Type I Reoviridae), and *Lagenophrys callinectes* (Peritrichida: Lagenophryidae). Shell disease develops after the shell is damaged, and chitinoclastic bacteria colonize the injury and degrade the chitin, leading to discolored spots or lesions on the shell (Cook & Lofton 1973, Shields & Overstreet 2007). Severe, necrotic shell disease can be harmful to pre-molt crabs as it can expose the internal tissues and organs to pathogens present in the water, and then, secondary, fatal infections can occur (Millikin & Williams 1984). Preva-

*Corresponding author: janderson@agcenter.lsu.edu

lence of shell disease may be high at shedding facilities due to stress and crowding (Iversen & Beardsley 1976). *Vibrio* spp. (Vibrionales: Vibrionaceae) are the main chitinoclastic bacteria that cause shell disease (Shields & Overstreet 2007), and infections can cause morbidity and mortality of infected crabs (Bowser et al. 1981, Shields & Overstreet 2007). *Vibrio* spp. infections have been correlated with mortality rates of >80% in North Carolina shedding facilities (Size-more 1985).

Both *A. michaelis* and RLV are hypothesized to cause mass mortalities in shedding facilities, possibly due to crowding and cannibalism, which facilitate transmission (Messick & Sindermann 1992, Bowers et al. 2010, 2011). *A. michaelis* causes muscle lysis in infected crabs (Messick & Sindermann 1992). Crabs infected with *A. michaelis* appear lethargic, and lysed muscle is chalky white (Messick & Sindermann 1992). RLV is an RNA virus that infects hemocytes, hemopoietic, and connective tissues, the nervous system, and gills (Shields & Overstreet 2007). Crabs infected with RLV develop tremors, are lethargic, and eventually become paralyzed (Johnson 1977, Shields & Overstreet 2007). RLV may be the predominant cause of crab deaths in shedding facilities from Delaware to Florida (Bowers et al. 2011).

L. callinectes is an ectocommensalistic ciliate that is prevalent on pre-molt crab gills before it is shed during ecdysis. It is usually harmless to the infested crab. However, it can occasionally cause asphyxiation, especially when a crab is stressed by low dissolved oxygen or poor water quality (Couch & Martin 1982), both of which can occur at shedding facilities.

A parasitic dinoflagellate that indirectly can harm the soft shell industry is *Hematodinium perezii* (Coccoliniaceae: Syndiniaceae). *H. perezii* causes morbidity and mortality of infected crabs after destroying hemocytes and hemocyanin (Taylor et al. 1996, Lee & Frischer 2004). It has not been directly correlated with mortalities in shedding systems because this parasite has only been found in wild blue crabs at salinities greater than 11 ppt, and the potential transmissive state of the parasite is not active below 20 ppt, both higher than typical salinities of shedding systems (Newman & Johnson 1975, Messick & Shields 2000, Coffey et al. 2012). However, *H. perezii* could infect a pre-molt crab that was caught in a high salinity area and transported to the shedding tanks. Additionally, *H. perezii* can significantly decrease the number of crabs in the wild that are actively molting and subsequently available to soft shell facilities.

In Louisiana, the optimal seasons for shedding are when warm water temperatures stimulate molting

from March to October, with pre-molt crabs most abundant in April or May and in September or October (Guillory et al. 2001). During the active shedding season in 2013, we determined the prevalence of shell disease, *Vibrio* spp., *L. callinectes*, and *H. perezii*, as well as occurrences of RLV and *A. michaelis*, at 4 commercial shedding facilities along the Louisiana coast.

MATERIALS AND METHODS

Four shedding facilities in southeastern and south-central Louisiana were selected because they were willing to cooperate and were operational in 2013. The facilities were located in (East to West) Hopedale (HO), Dulac (DU), Port of West St. Mary (SM), and Erath, LA (ER). Three systems—HO, DU, and ER—were open, flow-through systems, while SM was a closed, recirculating system.

Starting in April 2013, pre-molt crabs were collected from each facility every 2 mo throughout their active season (Table 1). Water temperature, salinity (Table 1), and pH were measured with a YSI 63-10FT, and water samples were collected to determine alkalinity, nitrite, nitrate, and ammonia levels with standard aquarium test strips (Mardel and Tetra). Crabs were placed alive on burlap-covered ice for transport to Louisiana State University (LSU) for subsequent processing and dissection.

All pre-molt crabs were processed and dissected alive, with the exception of 20 frozen, post-molt crabs purchased from DU in April. Before dissection, the carapace width (CW) was measured to the nearest millimeter, and crabs were sexed and photographed.

Shell disease diagnosis

Shell disease was diagnosed when the shell was discolored with black or brown spots that did not scrape off and when the shell was discolored and rotted with evident necrosis. Shell disease intensity was determined by scoring the disease from 0 to 2, where intensity score 0 represented no shell disease, 1 represented non-necrotic shell disease spots, and 2 represented necrotic, rotted lesions.

Vibrio spp. detection

Hemolymph was drawn with a sterile needle and syringe from the ethanol-sterilized joint of the swimming leg and carapace. A small amount (approx-

Table 1. Sampling parameters (n: sample size; salinity; water temperature) at 4 shedding facilities in Louisiana in 2013. At the Dulac facility in April, 13 of the crabs collected were pre-molt, and 27 were post-molt. nc: no collection of crabs; cp: crabs collected prior to placement in shedding tanks; n₁: total number of crabs collected and analyzed for shell disease, *Lagenophrys callinectes*, and *Hematodinium perezii* tests; n₂: number of crabs analyzed for *Vibrio* spp.

Parameter	April	June	August	October
Hopedale				
n ₁	0	20	20	0
n ₂	0	20	0	0
Salinity (ppt)	nc	cp	5.4	nc
Temp. (°C)	nc	cp	29.4	nc
Dulac				
n ₁	13 (27)	19	20	20
n ₂	13	0	19	0
Salinity (ppt)	7.9	7.7	5.7	5.5
Temp. (°C)	18.9	29.5	28.8	24.6
Port of W. St. Mary				
n ₁	20	20	20	20
n ₂	20	20	20	19
Salinity (ppt)	4.1	3.3	2.8	2.5
Temp. (°C)	23.0	27.6	28.6	26.6
Erath				
n ₁	20	20	20	20
n ₂	20	20	0	8
Salinity (ppt)	7.3	3.6	3.6	2.2
Temp. (°C)	25.1	27.3	26.8	24.7

mately 400 to 800 µl) of hemolymph was plated on sterile thiosulfate-citrate-bile salts-sucrose (TCBS) agar to determine *Vibrio* spp. colonization (Colwell et al. 1975). Agar plates were prepared per manufacturer instructions (Sigma-Aldrich), and plates were incubated for 48 h at 28°C. After incubation, plates were photographed and preliminarily assessed, and a subset was sent to the LA Aquatic Diagnostic Laboratory (Baton Rouge, LA) for bacterial identification. Identification was done by standard bacteriological techniques and biochemical profiling with the analytical profile index (API) system (bioMerieux, Durham, NC). We omitted samples (n = 88) that were contaminated when the hepatopancreas was punctured. Additionally, 5 samples were omitted because colonies could not be identified by API. We analyzed 179 uncontaminated samples (Table 1).

Dissection

An additional 1 ml of hemolymph was preserved in 95% ethanol for polymerase chain reaction (PCR) analysis (see '*Hematodinium perezii* PCR detection').

One walking leg was removed and frozen at -20°C for RLV reverse-transcription PCR at the University of Maryland Center for Environmental Science (see 'RLV RT-PCR detection'; Bowers et al. 2010). Gill samples were frozen at -20°C for *L. callinectes* detection (see below). Any abnormalities, such as muscle discoloration or lysis, were recorded, and abnormal tissue was fixed in 1.25% glutaraldehyde with 2% paraformaldehyde and 5% sucrose. Fixed tissues were transferred to 0.1 M cacodylate buffer after 24 h and delivered to the LSU School of Veterinary Medicine Microscopy Center for transmission electron microscopy.

Lagenophrys callinectes detection

Detection of active *L. callinectes* infestations depended on the presence or absence of the ciliate's circular lorica on the frozen gills (Couch & Martin 1982, Mayen-Estrada & Aguilar-Aguilar 2012). Thin gill sections (<1 cm thick, on average) were placed on microscope slides and immediately examined under a Micromaster Premier light microscope (Fisher Scientific) at a magnification of ≥10× (Fig. 1). To detect only active infestations, presence of *L. callinectes* was recorded only when an occupied lorica was seen. When an occupied lorica was not observed in the first section, a minimum of 3 sections were examined to confirm absence of the commensal.



Fig. 1. Light microscopy image of *Lagenophrys callinectes* loricae on unstained, frozen gill tissue. Scale bar = 100 µm

***Hematodinium perezii* PCR detection**

DNA extraction, PCR, and gel electrophoresis methodology were adapted from Pagenkopp Lohan et al. (2012). Prior to DNA extraction, approximately 200 μl of ethanol-preserved hemolymph was centrifuged at $1500 \times g$ for 1 min, and excess ethanol was removed (Pagenkopp Lohan et al. 2012). Samples sat for at least 30 min to allow residual ethanol to evaporate and then were lysed overnight when possible (Pagenkopp Lohan et al. 2012). DNA was extracted with Qiagen DNeasy Blood and Tissue kits per manufacturer recommendations with two 5 min elution incubations and two 100 μl elutions. For PCR, 3 primer pairs from Pagenkopp Lohan et al. (2012) were used: (1) the general metazoan primers nSSU A and nSSU B; (2) *Hematodinium* sp. primers HITS1F and HITS1R; and (3) *Hematodinium* spp. primers Hemat-F-1487 and Hemat-R-1654.

Reactions for the nSSU pair included 1 \times standard *Taq* (Mg-free) reaction buffer (New England Biolabs), 1.5 mM MgCl_2 , 0.2 mM dNTPs, 1 μM nSSU A, 1 μM nSSU B, 1 unit of *Taq* polymerase, and 0.4 mg ml^{-1} BSA or RSA (Pagenkopp Lohan et al. 2012). Thermocycling conditions were 95.0°C for 4 min; 45 cycles of 94.0°C for 30 s, 45.0°C for 30 s, and 72.0°C for 2 min; and a final extension at 72.0°C for 5 min (modified from Pagenkopp Lohan et al. 2012).

Reactions for the HITS1 pair contained 1 \times standard *Taq* (Mg-free) reaction buffer, 1 mM MgCl_2 , 0.1 mM dNTPs, 0.5 μM HITS1F, 0.5 μM HITS1R, and 1 unit of *Taq* polymerase (Pagenkopp Lohan et al. 2012). Thermocycling conditions were 95.0°C for 5 min; 40 cycles of 94.0°C for 30 s, 58.0°C for 30 s, and 72.0°C for 90 s; and a final extension at 72.0°C for 5 min (modified from Pagenkopp Lohan et al. 2012).

For the Hemat primers, the PCR reaction contained 1 \times standard *Taq* (Mg-free) reaction buffer, 1.5 mM MgCl_2 , 0.1 mM dNTPs, 0.5 μM Hemat-F-1487, 0.5 μM Hemat-R-1654, 1 unit of *Taq* polymerase, and 1.0 mg ml^{-1} BSA or RSA (Pagenkopp Lohan et al. 2012). Thermocycling conditions were 95.0°C for 10 min; 40 cycles of 94.0°C for 30 s, 56.0°C for 30 s, and 72.0°C for 1 min; and a final extension at 72.0°C for 10 min (modified from Pagenkopp Lohan et al. 2012). All reactions were brought to a volume of 10 μl with Nanopure water. All PCR runs included a negative and positive control.

Amplified products were electrophoresed on a 2% (w/v) agarose gel in sodium borate buffer and visualized under UV light after staining with EZ-Vision DNA Dye (diluted to 1.2 \times concentration; Amresco, Solon, OH). Amplifications were considered successful when

the metazoan primers amplified DNA from each sample (~1700 bp) and all 3 primer pairs amplified the positive control but none amplified the negative control (Pagenkopp Lohan et al. 2012). If a sample did not amplify with the metazoan primers, we extracted and amplified a new sample. Presence of *H. perezii* was recorded when a 302 bp band was present on the gel for the HITS1 primers and a 187 bp band was present for the Hemat primers (Pagenkopp Lohan et al. 2012).

RLV RT-PCR detection

Detection of RLV was accomplished by extraction of total RNA from leg muscle and application of a modification of the RT-PCR assay described by Bowers et al. (2010), with TaqMan® Fast Virus 1-Step Master Mix (Life Technologies), conducted on an Applied Biosystems Fast 7500 thermocycler. Standards for the RLV qPCR assay were prepared from a serial dilution of the RLV dsRNA genome (10 to 10^6 copies). Due to the high testing costs, we were only able to test 9 crabs from DU and 11 crabs from ER collected in June.

Statistical analysis

Aggregate prevalence rates were calculated as the ratio of the total number of infected individuals to the total number of individuals collected at all facilities. Statistical analyses were performed in RStudio (R Development Core Team 2013). A generalized linear model (binomial distribution with a logit link) was appropriate for the presence-absence data and was run in the package *stats* to determine the effect of a predictor variable on the probability of infection by a particular disease, parasite, or commensal. Predictor variables included facility, month, facility \times month interaction, water temperature, sex, and size. For the categorical predictor variables (facility, month, and sex), a reference category was arbitrarily chosen for calculation of parameter estimates. Reference month was different among the facilities because collections were not possible at HO in all 4 months, unlike at DU, SM, and ER (Table 1).

An analysis of deviance chi-square test was performed in the *stats* package to evaluate model fit. To assess model assumptions, the residual and normal Q-Q plots of Pearson's residuals were examined. Violations of model assumptions were few, but noted. Models were run with individual and combinations of predictor variables, and the final model was selec-

ted based on the AIC, deviance chi-square, and significant predictor variables.

Shell disease intensity data scored from 0 to 2 were analyzed in the package *vgam* with a cumulative multategorical logit model that is appropriate for categorical data with >2 outcome categories (Agresti 2007, Yee 2010, 2013). Likelihood ratio tests were also performed in the *vgam* package to analyze cumulative logit model fit (Yee 2010, 2013). For all statistical tests, α was 0.05 for statistical significance.

RESULTS

Water quality

At the facilities, pH ranged from 6.36 to 8.39. Alkalinity typically was <80 ppm. Nitrate ranged from 0 to >200 ppm, and nitrite was <1 ppm with 1 exception

at ER in August when it reached approximately 10 ppm. Ammonia concentrations were also low and ranged from 0 to 1 ppm.

Prevalence of shell disease

The aggregate prevalence of shell disease diagnosed on pre-molt crabs at all 4 facilities was 64.3%. Prevalence ranged from 30% in April at SM to 95% at HO in June (Fig. 2). Shell disease varied significantly by collection month (or water temperature), shedding facility, the facility \times month interaction, and crab size (Table 2), with very slight deviation of the residuals from normality. Shell disease varied significantly by collection month at HO, SM, and ER (Table 3). At HO, prevalence was higher in June than in August (Table 3). At DU, prevalence was marginally higher (84.2%) in June (Table 3). At SM, the

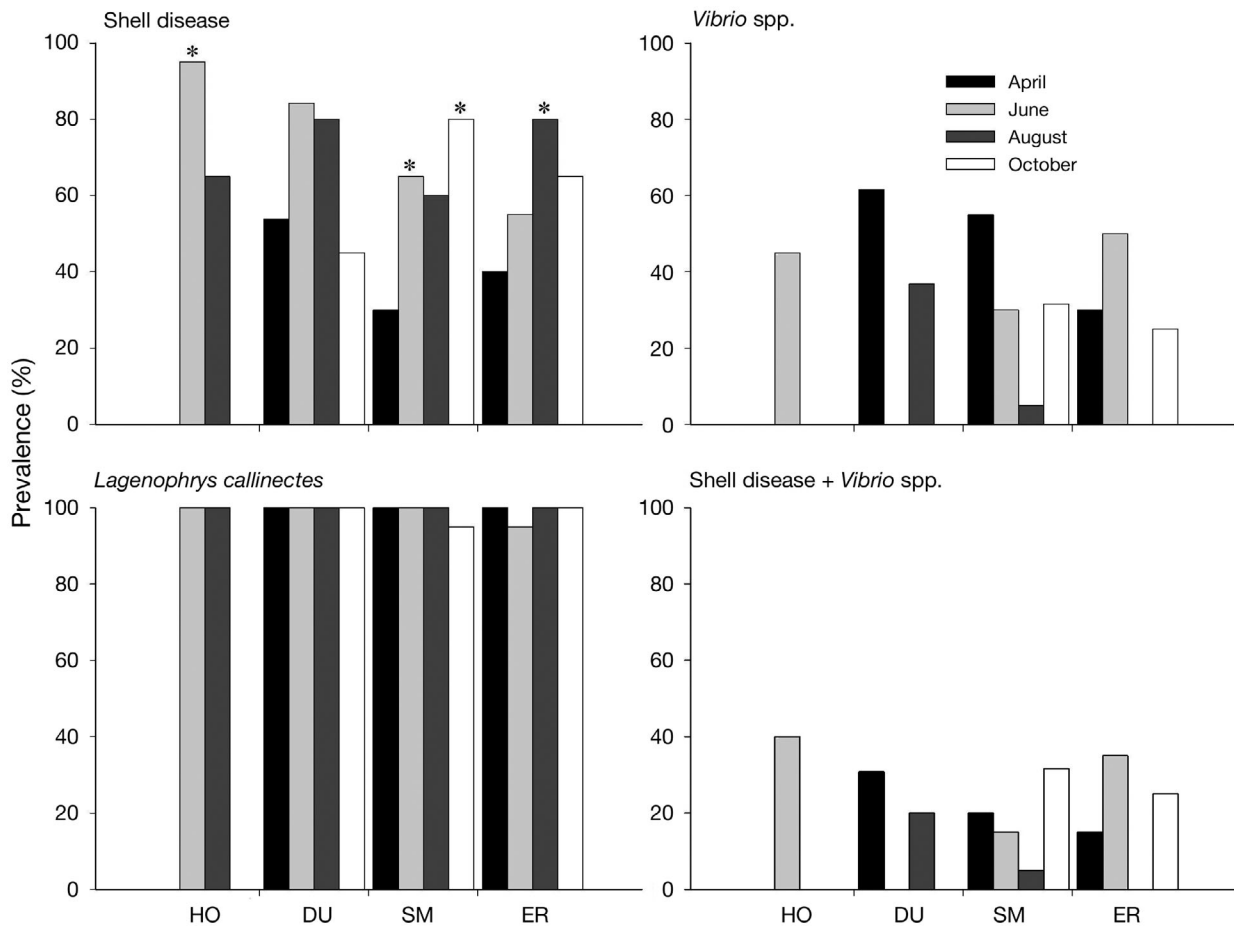


Fig. 2. Prevalence of shell disease, *Vibrio* spp., *Lagenophrys callinectes*, and the co-occurrence of shell disease and *Vibrio* spp. at 4 shedding facilities in Hopedale (HO), Dulac (DU), Port of W. St. Mary (SM), and Erath (ER). Missing bars for *Vibrio* spp. and co-occurrence of shell disease and *Vibrio* spp. are omitted data (due to cross-contamination with the hepatopancreas), with the exception of HO for April and October when no crabs were collected. *for shell disease indicates a significant difference between the respective month and the reference month (HO reference month = August; DU, SM, ER reference month = April)

Table 2. Parameter estimates, standard errors, and p-values for predictor variables in logistic regressions. Unlisted interactions were not significant. Significant predictor variables in **bold**. The reference site category was Erath, and April was the reference month. na: not applicable

Disease, parasite, commensal	Predictor	Category	Parameter estimate	Standard error	p-value
Shell disease	Site	HO	2.7438	1.2101	0.0143
		DU	0.5596	0.7196	0.4368
		SM	-0.4418	0.6682	0.5084
	Month	June	0.6061	0.6406	0.3440
		August	1.7918	0.7217	0.0130
		October	1.0245	0.6543	0.1174
	Site × Month	HO × August	-3.5110	1.3367	<0.01
	Water temperature	na	0.1583	0.0510	<0.01
	Size	na	0.3925	0.1504	<0.01
	Sex	Male	0.3748	0.3701	0.3112
<i>Vibrio</i> spp.	Site	HO	0.5901	0.6279	0.3474
		DU	1.3521	0.6250	0.0305
		SM	-0.2056	0.4195	0.6240
	Month	June	-0.0987	0.4599	0.8301
		August	-2.3783	0.6476	<0.001
		October	-0.5142	0.5353	0.3367
	Water temperature	na	0.3809	0.2662	0.1524
	Size	na	-0.4953	0.1621	<0.01
	Sex	Male	0.1840	0.4719	0.6966
	<i>Lagenophrys callinectes</i>	Site	All sampled	na	na
Month		All sampled	na	na	>0.99
Size		na	-0.4448	0.8982	0.62
Sex		Male	0.1991	1.616	0.902

Table 3. Parameter estimates, standard errors, and p-values for shell disease monthly variation at each of the 4 facilities in Hopedale (HO), Dulac (DU), Port of W. St. Mary (SM), and Erath (ER). Significant results in **bold**. August was the reference month at HO, and April was the reference month at DU, SM, and ER

Site	Month	Parameter estimate	Standard error	p-value
HO	June	2.3254	1.1280	0.0392
DU	June	1.5198	0.8399	0.0704
	August	1.2321	0.7887	0.1182
	October	-0.3548	0.7152	0.6198
SM	June	1.4663	0.6767	0.0302
	August	1.2528	0.6682	0.0608
	October	2.2336	0.7420	<0.01
ER	June	0.6061	0.6406	0.344
	August	1.7918	0.7217	0.013
	October	1.0245	0.6543	0.117

prevalence of shell disease was significantly higher in June and October, and at ER, it was significantly more prevalent in August (Table 3). These monthly variations may be correlated with water temperature variation as the presence of shell disease was more probable as water temperature increased (Table 2). Prevalence also varied significantly among the shed-

ding facilities: crabs at HO had significantly higher probability of infection than at ER (Table 2). When site was included as a predictor variable, the variance was heterogeneous with regard to prevalence at HO.

Sex was not a significant predictor for variation in shell disease prevalence (Table 2). The effect of size was significant; the odds of shell disease increased by 48.07% for every 1 cm increase in CW (Table 2). In this study, the average CW was 13.17 cm. At HO, the average size was the largest at 14.32 cm.

Intensity of shell disease

The average shell disease intensity was 0.89 when scored from 0 to 2. Severe shell disease with necrotic lesions was present on 25% of the pre-molt crabs and was 63.6% of all shell disease cases diagnosed. The cumulative multcategory logit model for shell disease intensity included size and collection month as predictors for intensity score, but not sex or facility based on likelihood ratio tests (Table 4). The probability of severe shell disease was highest in August and lowest in April, and the probability of shell disease (scores 1 and 2) increased with increasing size

(Table 4). Category probabilities for each month indicated that crabs collected in April had the highest probability of score 0, and those collected in August had the highest probability of score 2, necrotic shell disease.

Prevalence of *Vibrio* spp.

In the 179 samples analyzed, *Vibrio* spp. prevalence in the hemolymph ranged from 5 to 61.5%, with an aggregate prevalence of 36.8% (Fig. 2). In the best model, incidence of *Vibrio* spp. in the hemolymph was affected by facility, month, and crab size (Table 2), but there was slight deviation from normality, and variance with month as a predictor variable was heterogeneous. Despite this heterogeneity, month was retained in the model instead of water temperature because 20 samples from HO in June had no corresponding water temperature (Table 1). Size had a negative effect on prevalence of hemolymph infections, and the prevalence was significantly lower in August than in April (Table 2). The only significant site difference was between DU and ER, with the highest prevalence rate at DU (Table 2). Sex did not significantly affect *Vibrio* spp. hemolymph prevalence (Table 2).

Co-occurrence of shell disease and *Vibrio* spp.

Shell disease and *Vibrio* spp. in the hemolymph co-occurred in crabs at rates between 5 and 40%, with an aggregate co-occurrence of 24% (Fig. 2). The presence of *Vibrio* spp. in the hemolymph had no significant effect on the prevalence of shell disease ($\beta_{Vibrio} = 0.6218$, $p = 0.1229$). Shell disease intensity

Table 4. Predictor variables for the cumulative logit model for shell disease intensity. Sex and facility were excluded from the model, and parameters were not estimated because shell disease intensity was not dependent on these variables. The reference month was April. na: not applicable

Predictor	Likelihood ratio statistics			Category	Parameter estimate
	χ^2	df	p-value		
Sex	3.0731	1	0.0796	na	na
Facility	5.3696	3	0.1467	na	na
Size	3.8721	1	0.0491	na	-0.2107
Month	14.4	3	<0.01	June	1.1678
				August	-1.5659
				October	1.1978

was also independent of *Vibrio* spp. prevalence ($\chi^2 = 1.9965$, $df = 1$, $p = 0.1557$). The presence of shell disease had an insignificant effect on the prevalence of *Vibrio* spp. in the hemolymph ($\beta_{Shell\ disease} = 0.5578$, $p = 0.1489$). Additionally, shell disease intensity scores did not significantly affect the prevalence of *Vibrio* spp. ($\beta_{Score\ 0} = -0.7211$, $p = 0.1187$; $\beta_{Score\ 1} = -0.4492$, $p = 0.3212$).

Prevalence of *Lagenophrys callinectes*

Aggregate prevalence of *L. callinectes* in pre-molt crabs from the 4 facilities was 99.3% (Fig. 2). In the 27 post-molt samples collected from DU in April (Table 1), the prevalence was 22.2%. At HO and DU, prevalence in pre-molt crabs was 100% (Fig. 2). At SM and ER, prevalence was 98.75%, with 1 uninfested crab in October at SM and 1 in June at ER (Fig. 2).

There was no significant monthly variation in prevalence at the 4 facilities and no significant variation in prevalence among the 4 facilities (Table 2). Additionally, crab size and sex were not significant predictors for the probability of a crab having *L. callinectes* (Table 2).

Other parasites

All 272 pre-molt and 27 post-molt samples (Table 1) were negative for *H. perezii*. One pre-molt crab from SM in August was infected with *A. michaelis* (Fig. 3). Two of 9 crabs tested for RLV from DU and 1 of 11 crabs tested from ER were infected with RLV. From API results, non-*Vibrio* spp. chitinoclastic bacteria, *Aeromonas* spp. (*A. hydrophila*, *A. sobria*, and *A. caviae*; Aeromonadales: Aeromonadaceae), were found in the hemolymph of 18 pre-molt crabs across all sample months and at HO, DU, and SM. *Shewanella putrefaciens* (Alteromonadales: Shewanellaceae) was also detected by API in the hemolymph of 4 crabs from SM and 1 crab from DU.

DISCUSSION

Prevalence and intensity of shell disease

Shell disease prevalence varies greatly in wild blue crab populations but may be more prevalent in shedding systems due to its conta-

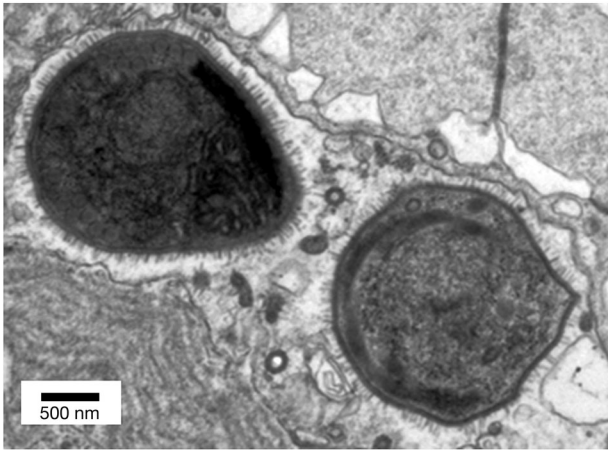


Fig. 3. Transmission electron micrograph of *Ameson michaelis* infection in blue crab muscle collected in August at the shedding facility in Port of W. St. Mary. Thin projections from the microsporidian are characteristic of *A. michaelis*. Photo credit: Yuliya Sokolova, Microscopy Center, Louisiana State University School of Veterinary Medicine, 2013

giousness (Malloy 1978, Sindermann 1989). Pathogens hypothesized to cause shell disease inhabit a wide range of salinities, so the disease is often not correlated with salinity (Shields & Overstreet 2007). The moderately high aggregate prevalence of shell disease (64.3%) in shedding facilities where the salinity was consistently <8 ppt (Table 1) confirms that shell disease can be observed at low salinities. Conversely, shell disease is dependent on ecdysis, which depends on water temperature and crab size (Sandifer & Eldridge 1974). Shell disease is generally more prevalent and intense in older, larger crabs that molt less frequently and during periods of infrequent molting, especially in the winter.

In a healthy system, it is expected that shell disease is predominantly absent but when it is present, it is at low intensities. Given that shedding facility samples were not collected in the winter when shell disease is expected to be highest due to infrequent molting, we hypothesized that shell disease would be most prevalent and intense when water temperatures were highest because the pathogens that cause shell disease, such as *Vibrio* spp. and other bacteria, are more prevalent at warmer temperatures (Huq et al. 1984, Welsh & Sizemore 1985). Results did not indicate that shell disease was consistently more prevalent in June and August versus April and October at all 4 facilities (Fig. 2, Table 3). Despite this inconsistency in monthly variation of prevalence, shell disease was dependent on water temperature: the probability of shell disease infections increased when water tem-

perature increased. Unlike prevalence, shell disease intensities were highest in the warmest month, August, and lowest in the coolest month, April, which may be correlated with higher pathogen loads in the tanks during warmer months.

The probability of shell disease infections also varied significantly among shedding facilities, particularly in June. Higher prevalence at HO may be a geographic difference if crabs brought to this facility are from an area of high shell disease prevalence. Alternatively, crabs at HO were larger than at the other facilities, which is important because molt frequency decreases with increasing size (Smith & Chang 2007) and because the probability of infection increased as size increased (Table 2). The water quality at HO likely is not the explanation for higher prevalence at HO as the parameters measured were within ideal ranges for blue crab shedding facilities (Manthe et al. 1983), and the crabs were only held in the system for a couple of days. The low sample size at HO (Table 1) also may explain the apparent site variation.

Overall, the most significant predictors for shell disease prevalence and intensity are water temperature and crab size. The monthly variation in shell disease is likely due to water temperature changes. The moderate to high prevalence rates could be a cause of concern for commercial blue crab shedders. Shell disease infections can be fatal in some cases, especially when crabs are stressed in crowded tanks or are handled roughly. Facilities may experience increased mortality rates when many pre-molt crabs have severe shell disease because necrotic lesions can expose crabs to bacteria that are often in high densities in shedding tanks (Sandifer & Eldridge 1974). Also, high incidence of shell disease can be important in shedding systems because the causative pathogens are contagious (Sandifer & Eldridge 1974, Sindermann 1989). Fortunately for shedding facilities, if a crab successfully molts, it will shed any shell disease and will have a clean, soft shell that is appetizing and marketable (Vogan et al. 1999).

Prevalence of *Vibrio* spp.

Mortalities caused by *Vibrio* spp. in North Carolina shedding facilities have been recorded as high as 80% (Sizemore 1985). In crowded shedding tanks, especially during warm months, *Vibrio* spp. prevalence is hypothesized to be high and potentially detrimental to shedding crabs (Overstreet & Cook 1972, Huq et al. 1984, Welsh & Sizemore 1985, Shields 1997). High densities of *Vibrio* spp. occur be-

cause of proliferation and rapid reproduction of the bacteria at higher temperatures.

We detected monthly variation that was contrary to our hypothesis as prevalence was lowest in the warmest month, August. However, this result is influenced by the low sample size in August ($n = 39$) and low prevalence at SM in August (Fig. 2).

There was significant variation in prevalence rates among the 4 shedding facilities, with DU having the highest and SM having the lowest aggregate prevalence rates. Interestingly, SM is the only facility of the 4 to have a closed, recirculating system, where we expected *Vibrio* spp. loads to be the highest, especially if there was improper water filtration (Messick & Kennedy 1990). However, a closed system can have better water quality than open systems if open systems obtain water from polluted or degraded sources (Guillory et al. 2001). Different *Vibrio* spp. loads in tank water may explain the variation in *Vibrio* spp. prevalence among the facilities. Another potential explanation for the variation among facilities may be the way that crabs are handled as *Vibrio* spp. infections are more prevalent and intense in injured and roughly-handled pre-molt crabs (Welsh & Sizemore 1985, Givens et al. 2013). Therefore, a potential explanation for the site variation is that crabs going to DU were not as carefully handled as those going to SM.

Vibrio spp. were not more prevalent in males or females, which is consistent with previous studies (Welsh & Sizemore 1985). The 39% decrease in odds of *Vibrio* spp. colonization for every 1 cm increase in CW may be explained by the decreased molt frequency of adults because molting can increase exposure to *Vibrio* spp. (Davis & Sizemore 1982).

High temperatures, injury, and stress from handling and salinity changes are hypothesized to be the main factors causing high *Vibrio* spp. prevalence (Shields & Overstreet 2007). In future studies, presence of injuries, especially autotomized legs, should be recorded to test for correlation between injury and *Vibrio* spp. prevalence. Additionally, determination of colonization intensity is important to differentiate between potentially fatal infections and mild, harmless colonization.

The overall moderate prevalence of 36.8% at the shedding facilities may represent a threat to the facilities' profitability as *Vibrio* spp. have been linked to high mortality rates at shedding facilities (Overstreet & Cook 1972). Some of the deaths in shedding systems may be due to stress that can lead to high bacterial loads in the hemolymph. *Vibrio* spp. and other bacteria are ubiquitous and opportunistic, so preven-

tion of intense infections should focus on minimization of stress and injury.

Co-occurrence of shell disease and *Vibrio* spp.

Co-occurrence of shell disease and *Vibrio* spp. in the hemolymph has been recorded in many studies because of the inherent connection between the syndromes (Sandifer & Eldridge 1974, Iversen & Beardswiley 1976, Malloy 1978, Shields & Overstreet 2007). We expected frequent co-occurrence of these 2 infections, but such co-occurrence was only 24% in the pre-molt crabs. Neither malady significantly predicted occurrence of the other (Fig. 2). We also expected that shell disease intensity and *Vibrio* spp. in the hemolymph would be correlated, with more *Vibrio* spp. present in crabs with necrotic shell disease that could expose internal tissues to ambient *Vibrio* spp. However, shell disease intensity was not a significant predictor of *Vibrio* spp. prevalence in the hemolymph.

If we had swabbed every shell disease lesion, we might have observed a correlation between shell disease and *Vibrio* spp. colonizing the crab's shell. However, other chitinoclastic bacteria, fungi, and other pathogens may have been the etiological agents for the majority of the shell disease cases. For example, at low salinities like those at shedding facilities, *Aeromonas* spp. and *Pseudomonas* spp. have been more prevalent and associated with shell disease more often than *Vibrio* spp. (Noga et al. 1994). At these 4 facilities, this is a possible explanation for low co-occurrence because *Aeromonas* spp. (including *A. hydrophila*, *A. sobria*, and *A. caviae*) were present in the hemolymph.

Prevalence of *Lagenophrys callinectes*

At the 4 shedding facilities, *L. callinectes* prevalence overall was 99.3% in the pre-molt crabs. The lower prevalence rate of 22.2% in the soft shell, post-molt crabs confirms that this ciliate is shed with the old shell, and reinfestation is not immediate in shedding tanks. The lack of variation across months, sites, and sizes indicates that this ciliate is ubiquitous in Gulf blue crabs. Although this ciliate is present at high rates in shedding facilities, it likely does not result in high mortality rates as it is commensalistic. To determine whether stressed pre-molt crabs may die of asphyxiation from this ciliate, future studies should measure the intensity of infestations and associated mortality rates.

Prevalence of *Hematodinium perezii*

Because salinities at shedding facilities in Louisiana are usually <10 ppt (Table 1), transmission of *H. perezii* between crabs in the shedding tanks is not possible (Coffey et al. 2012). Conversely, infected crabs can be transported to the facility where the infection develops until the crab molts and is removed from the tanks. However, during the soft shell shedding season, the majority of large juvenile and adult crabs are in the upper estuaries that have lower salinities in Louisiana (Guillory et al. 2001), so crabs transported to the facilities are from areas where *H. perezii* would not be expected to be prevalent. Our result of 0% prevalence in the pre-molt crabs is consistent with this hypothesis of low prevalence in the fished populations.

In 2011, positive detections of *H. perezii* in pre-molt crabs from Louisiana shedding tanks were reported but never confirmed with PCR (J. P. Hawke unpubl. data). Based on our current results, these positive detections were possibly false positives due to the unfamiliarity with histological identification and the potential unreliability of histology (Gruebl et al. 2002). *Hematodinium perezii* does not directly affect the profitability of Louisiana shedding facilities because mortalities in the tanks are not attributable to this parasite.

Conclusions and future investigations

From the results of this study, we cannot definitively state the cause of most shedding facility mortalities in Louisiana. It is unlikely that high death rates are caused by *L. callinectes* unless the crabs are also highly stressed from low dissolved oxygen and poor water quality. Deaths are also not attributable to *H. perezii*. Additionally, water quality is not a likely explanation for high loss rates because at all 4 facilities, the levels of nitrites, ammonia, and pH were within acceptable ranges, with only 1 exception at ER in August when nitrite reached approximately 10 ppm. Also, previous studies have not linked high mortality rates with poor water quality (Manthe et al. 1983, Chaves & Eggleston 2003).

High death rates may be caused by a combination of harmful parasites and diseases such as shell disease, *Vibrio* spp., *A. michaelis*, and RLV, but more research is needed. *A. michaelis* was only detected in 1 pre-molt crab, and 3 of the 20 crabs tested for RLV were infected. In the future, more extensive examination of all crabs for *A. michaelis* and RLV should be

conducted, especially after a mass mortality event. Several other factors should be investigated, including *Vibrio* spp. loads in tank water, rough handling, injury, and stress from abrupt salinity changes. As this was a 1 yr study and 2013 landings were low, more long-term monitoring is necessary to understand the context of 2013 prevalence rates and the long-term health of the soft shell fishery.

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LITERATURE CITED

- Agresti A (2007) An introduction to categorical data analysis. John Wiley & Sons, Hoboken, NJ
- Bowers HA, Messick GA, Hanif A, Jagus R, Carrion L, Zmora O, Schott EJ (2010) Physicochemical properties of double-stranded RNA used to discover a reo-like virus from blue crab *Callinectes sapidus*. Dis Aquat Org 93: 17–29
- Bowers HA, Carrion L, Hanif A, Messick GA, Zmora O, Schott EJ (2011) A reo-like virus associated with mortalities of blue crab *Callinectes sapidus*: development of tools to improve soft shell crab aquaculture. J Shellfish Res 30:487
- Bowser PR, Rosemark R, Reiner CR (1981) A preliminary report of vibriosis in cultured American lobsters, *Homarus americanus*. J Invertebr Pathol 37:80–85
- Chaves JC, Eggleston DB (2003) Blue crab mortality in the North Carolina soft-shell industry: biological and operational effects. J Shellfish Res 22:241–249
- Coffey AH, Li C, Shields JD (2012) The effect of salinity on experimental infections of a *Hematodinium* sp. in blue crabs, *Callinectes sapidus*. J Parasitol 98:536–542
- Colwell RR, Wicks TC, Tubiash HS (1975) A comparative study of the bacterial flora of the hemolymph of *Callinectes sapidus*. Mar Fish Rev 37:29–33
- Cook DW, Lofton SR (1973) Chitinoclastic bacteria associated with shell disease in *Penaeus* shrimp and the blue crab (*Callinectes sapidus*). J Wildl Dis 9:154–159
- Couch JA, Martin S (1982) Protozoan symbionts and related diseases of the blue crab, *Callinectes sapidus* Rathbun from the Atlantic and Gulf coasts of the United States. In: Perry HM, Van Engel WA (eds) Proceedings of the blue crab colloquium, Biloxi, MS. Gulf States Marine Fisheries Commission, Ocean Springs, MS, p 71–81
- Davis JW, Sizemore RK (1982) Incidence of *Vibrio* species associated with blue crabs (*Callinectes sapidus*) collected from Galveston Bay, Texas. Appl Environ Microbiol 43:1092–1097
- Givens CE, Burnett KG, Burnett LE, Hollibaugh JT (2013) Microbial communities of the carapace, gut, and hemolymph of the Atlantic blue crab, *Callinectes sapidus*. Mar Biol 160:2841–2851

- Gruebl T, Frischer ME, Sheppard M, Neumann M, Maurer AN, Lee RF (2002) Development of an 18S rRNA gene targeted PCR based diagnostic for the blue crab parasite *Hematodinium* sp. *Dis Aquat Org* 49:61–70
- Guillory V, Perry H, Steele P, Wagner T and others (2001) The blue crab fishery of the Gulf of Mexico, United States: a regional management plan. Gulf States Marine Fisheries Commission, Ocean Springs, MS
- Huq A, West PA, Small EB, Huq MI, Colwell RR (1984) Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosms. *Appl Environ Microbiol* 48:420–424
- Iversen ES, Beardsley GL (1976) Shell disease in crustaceans indigenous to South Florida. *Prog Fish-Cult* 38:195–196
- Johnson PT (1977) A viral disease of the blue crab, *Callinectes sapidus*: histopathology and differential diagnosis. *J Invertebr Pathol* 29:201–209
- Lee RFD, Frischer ME (2004) The decline of the blue crab — changing weather patterns and a suffocating parasite may have reduced the numbers of this species along the Eastern seaboard. *Am Sci* 92:548–553
- Malloy SC (1978) Bacteria induced shell disease of lobsters (*Homarus americanus*). *J Wildl Dis* 14:2–10
- Manthe DP, Malone RF, Perry HM (1983) Water quality fluctuations in response to variable loading in a commercial, closed shedding facility for blue crabs. *J Shellfish Res* 3: 175–182
- Mayen-Estrada R, Aguilar-Aguilar R (2012) Track analysis and geographic distribution of some *Lagenophrys* Stein, 1852 (Protozoa: Ciliophora: Peritrichia) species. *J Nat Hist* 46:249–263
- Messick GA, Kennedy VS (1990) Putative bacterial and viral infections in blue crabs, *Callinectes sapidus* Rathbun, 1896 held in flow-through or a re-circulation system. *J Shellfish Res* 9:33–40
- Messick GA, Shields JD (2000) Epizootiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue crab *Callinectes sapidus*. *Dis Aquat Org* 43:139–152
- Messick GA, Sindermann CJ (1992) Synopsis of principal diseases of the blue crab, *Callinectes sapidus*. US Department of Commerce, NOAA, Woods Hole, MA
- Millikin MR, Williams AB (1984) Synopsis of biological data on the blue crab, *Callinectes sapidus*. US Department of Commerce, NOAA, Charleston, SC
- National Marine Fisheries Service (2013) Annual commercial landing statistics. NOAA, Silver Springs, MD
- Newman MW, Johnson CA (1975) Disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. *J Parasitol* 61:554–557
- Noga EJ, Engel DP, Arroll TW, McKenna S, Davidian M (1994) Low serum antibacterial activity coincides with increased prevalence of shell disease in blue crabs *Callinectes sapidus*. *Dis Aquat Org* 19:121–128
- Overstreet RM, Cook DW (1972) An underexploited Gulf coast fishery: soft shelled crabbing. *Am Fish Farmer* 3(9): 12–17
- Pagenkopp Lohan KM, Reece KS, Miller TL, Wheeler KN, Small HJ, Shields JD (2012) The role of alternate hosts in the ecology and life history of *Hematodinium* sp., a parasitic dinoflagellate of the blue crab (*Callinectes sapidus*). *J Parasitol* 98:73–84
- R Development Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, available at www.r-project.org
- Sandifer PA, Eldridge PJ (1974) Observations on the incidence of shell disease in South Carolina blue crabs, *Callinectes sapidus* (Rathbun). In: Amborski RL, Hood MA, Miller RR (eds) Proc Gulf Coast Regional Symp Diseases Aquatic Animals, Louisiana State University, Baton Rouge, LA, p 161–184
- Shields JD (1997) The potential effects of outbreaks of parasitic diseases on the blue crab fishery of Virginia. In: Proc 2nd Virginia Eastern Shore Natural Resources Symp, Oct 31–Nov 2, 1996. The Eastern Shore Institute, Exmore, VA, p 23–29
- Shields JD, Overstreet RM (2007) Diseases, parasites, and other symbionts. In: Kennedy VS, Cronin LE (eds) The blue crab: *Callinectes sapidus*. Maryland Sea Grant College, College Park, MD, p 299–417
- Sindermann CJ (1989) The shell disease syndrome in marine crustaceans. U.S. Department of Commerce (ed), NOAA, Woods Hole, MA
- Sizemore RK (1985) Involvement of *Vibrio* spp. in soft crab mortality. In: Perry HM, Malone RF (eds) National Symp Soft-Shell Blue Crab Fishery, Biloxi, MS, Feb 12–13, 1985. Louisiana Sea Grant, Baton Rouge, LA, p 21–22
- Smith SG, Chang ES (2007) Molting and growth. In: Kennedy VS, Cronin LE (eds) The blue crab: *Callinectes sapidus*. Maryland Sea Grant College, College Park, MD, p 197–254
- Taylor AC, Field RH, Parslow-Williams PJ (1996) The effects of *Hematodinium* sp. infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L.). *J Exp Mar Biol Ecol* 207:217–228
- Vogan CL, Llewellyn PJ, Rowley AF (1999) Epidemiology and dynamics of shell disease in the edible crab *Cancer pagurus*: a preliminary study of Langland Bay, Swansea, UK. *Dis Aquat Org* 35:81–87
- Welsh PC, Sizemore RK (1985) Incidence of bacteremia in stressed and unstressed populations of the crab, *Callinectes sapidus*. *Appl Environ Microbiol* 50:420–425
- Yee TW (2010) The VGAM package for categorical data analysis. *J Stat Softw* 32:1–34
- Yee TW (2013) Package 'VGAM'. University of Auckland. Available at www.stat.auckland.ac.nz/~yee/VGAM/doc/VGAMrefcard.pdf

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