Journal of Invertebrate Pathology 151 (2018) 82–90

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Effects of temperature and salinity on prevalence and intensity of infection of blue crabs, *Callinectes sapidus*, by *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in Louisiana

Timothy J. Sullivan[a,*], Joseph E. Neigel[a]

[a] *Department of Biology, University of Louisiana at Lafayette, Lafayette, LA, USA*

ARTICLE INFO

Keywords:
Blue Crab
Callinectes
Hemolymph
Vibrio
Gulf of Mexico
Louisiana
Prevalence

ABSTRACT

Coastal marine and estuarine environments are experiencing higher average temperatures, greater frequency of extreme temperature events, and altered salinities. These changes are expected to stress organisms and increase their susceptibility to infectious diseases. However, beyond these generalities, little is known about how environmental factors influence host-pathogen relationships in the marine realm. We investigated the prevalence and intensity of infections by *Vibrio cholerae, V. parahaemolyticus,* and *V. vulnificus* in blue crabs, *Callinectes sapidus*, from Louisiana saltmarshes in relation to temperature and salinity. We evaluated relationships for single measurements taken at the time of collection and for more complex measurements representing accumulated exposure to physiologically-stressful environmental conditions for up to 31 days prior to collection. We found that: (1) prevalence of infection varied across the Louisiana coast, (2) prevalence of all three *Vibrio* species was influenced by temperature and salinity, and (3) measurements that represent accumulated exposure to extreme conditions are useful predictors of infection prevalence and can provide insights into underlying biological mechanisms.

1. Introduction

Climate models predict that coastal habitats will experience dramatic environmental changes over the next century (Change, 2014; Harley et al., 2006; Hoegh-Guldberg and Bruno, 2010; Houghton et al., 2001; Huq et al., 1984; Scavia et al., 2002). These changes will include elevated temperatures, more frequent heatwaves, and alterations in salinity regimes (Harley et al., 2006; Houghton et al., 2001). The potential for these changes to reshape marine communities and alter their productivity has made predicting the impacts of these changes an urgent priority (Doney et al., 2012; Harley et al., 2006; Hoegh-Guldberg and Bruno, 2010; Scavia et al., 2002).

Environmental monitoring has become important for predicting diverse ecological outcomes including range expansions, disease outbreaks, and mass mortality events (Baker-Austin et al., 2013; Burge et al., 2014; Groner et al., 2015, 2016; Maynard et al., 2016). For marine diseases, changes in salinity or temperature can alter host-pathogen relationships, favoring one or the other. Comparisons of environmental conditions with spatial and temporal patterns of infection can suggest how environmental factors are influencing marine diseases (Gandy et al., 2015; Harvell et al., 2007; Kelly, 1982; Kuta and Richardson, 2002; Rogers et al., 2015). However, environmental conditions are often measured only at the time of specimen collection, while infections typically develop over a span of time prior to collection. Automated monitoring of marine environments and the deployment of ocean observatory networks (Jannasch et al., 2008; Schofield et al., 2003) offer opportunities to use time-integrated measures of environmental conditions that are more relevant to underlying biological mechanisms and could improve predictions of ecological outcomes.

The blue crab, *Callinectes sapidus*, inhabits marshes and waterways along the Atlantic and Gulf of Mexico coasts of North America. Larval and juvenile blue crabs are important in the diets of fish, invertebrate and avian predators (Guillory and Elliot, 2001). Juvenile and adult blue crabs also act as generalist predators, consuming invertebrates, fish, and crustaceans as well as plant and detrital materials (Alexander, 1986; Darnell, 1958; Lipcius et al., 2007; Meise and Stehlik, 2003). It has been proposed that the blue crab is a keystone species in the marsh because it preys on gastropods that graze *Spartina* and other grasses (Silliman and Bertness, 2002).

Bacteria in the genus *Vibrio* are important marine pathogens that cause disease and mortality in many fishery species including oysters

* Corresponding author at: Department of Biological Sciences, University of Arkansas, SCEN 601, 850 West Dickson Street, Fayetteville Arkansas 72703, USA.
E-mail address: TSulli1988@gmail.com (T.J. Sullivan).

https://doi.org/10.1016/j.jip.2017.11.004
Received 18 May 2017; Received in revised form 22 September 2017; Accepted 2 November 2017
Available online 07 November 2017
0022-2011/

(Lacoste et al., 2001), lobsters (Bowser et al., 1981), shrimp (Karunasagar et al., 1994), rock crabs (Newman and Feng, 1982) and blue crabs (Welsh and Sizemore, 1985). In both nature and aquaculture, blue crabs are often infected by *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Shields and Overstreet, 2007). High densities of these bacteria in the hemolymph reduce respiratory capacity, metabolic activity, and other physiological functions, which renders crabs weak and lethargic and causes or contributes to mortality (Davis and Sizemore, 1982; Krantz et al., 1969; Shields and Overstreet, 2007; Thibodeaux et al., 2009).

The effects of temperature and salinity on the growth and abundance of *Vibrio* in the coastal environment could underlie their effects on the prevalence and intensity of *Vibrio* infections in blue crabs. It is well established that temperature is an important factor in the growth of *Vibrio* (Cook et al., 2002; Duan and Su, 2005; Kaneko and Colwell, 1973; Louis et al., 2003). Growth and abundance of *V. vulnificus* has been found to increase with higher temperatures (Kaspar and Tamplin, 1993; Motes et al., 1998; Randa et al., 2004) and the highest growth of *V. vulnificus* and *V. parahaemolyticus* occurs in summer when temperatures are high (Blackwell and Oliver, 2008). Salinity also influences the growth of *Vibrio*. Field studies suggest that the optimum range of salinity for the growth of *Vibrio* spp. is from near fresh (1 ppt) to brackish (17 ppt) (Jiang and Fu, 2001; Kelly, 1982; Louis et al., 2003). Optimum growth of *V. cholerae* occurs between 15 and 25 ppt (Singleton et al., 1982) and between 5 and 25 ppt for *V. vulnificus* (Kaspar and Tamplin, 1993).

Physiological effects of temperature and salinity on blue crabs could also influence the dynamics of infection by *Vibrio* by altering rates of infection, recovery, or mortality. In marsh habitats, temperature and salinity change rapidly and reach extremes that are stressful and even lethal for blue crabs. The physiological effects of short-term exposures to extremes of temperature and salinity on blue crabs have been investigated in the laboratory (Guerin and Stickle, 1992; Holland et al., 1971; Leffler, 1972) and provided the basis for defining the thresholds for stressful and lethal conditions used in this study.

We used PCR assays to estimate both the prevalence of infection (the proportion of hosts infected) and the relative intensity of infection (the relative density of bacteria in hemolymph (Shields and Squyars, 2000)) by *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in blue crabs. We analyzed these data with respect to two sets of environmental measurements: (1) temperature and salinity measured at the time and place of collection and (2) the accumulated percentage of time that salinities or temperatures recorded at nearby stations of the Louisiana Coastwide Reference Monitoring System (CRMS) crossed physiologically-defined thresholds for stressful or lethal effects on blue crabs. The informativeness of these measurements with respect to the prevalence and intensity of *Vibrio* infections was evaluated by comparing and ranking alternative models with the Akaike Information Criterion (AIC) (Burnham and Anderson, 2003, 2004). In addition, we varied the time span and thresholds used for the second set of environmental measurements to find values that optimized their predictive power and provide insights about the biological mechanisms behind their effects.

2. Materials and methods

2.1. Sample collection

Blue crabs were collected with hand-lines, hoop-nets, and crab-pots (all baited) from seven coastal marsh locations (Fig. 1, Table 1) in the summers (June-August) of 2014 and 2015. Upon collection, individuals were photographed, measured, and sexed. For female crabs, maturity was determined by the shape of the abdominal tergites and/or the presence of a visible egg mass. Males with a carapace point-to-point size over 125 mm were classified as mature. After collection, ~1 ml of hemolymph was drawn aseptically with a sterile needle already containing 200 µl of sterile and cold Söderhäll and Smith's formulation (Söderhäll and Smith, 1983) as an anticoagulant. This solution was mixed and added to a pre-chilled vial of 95% ethanol and stored on ice at 4° C. Spot measurements of salinity and water temperature were taken with a thermometer and a refractometer at the time of each collection.

2.2. DNA extraction and PCR screening

DNA was extracted from 200 µl of hemolymph using NucleoSpin® 96 Tissue Kits (Machery-Nagel) with an epMotion 5075 TMX liquid handling workstation (Eppendorf) following the manufacturer's protocol. DNA concentrations were determined with a Nanodrop spectrophotometer (Thermo Scientific). The presence of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* DNA was assessed by endpoint PCR reactions with previously published primer sequences. For *V. vulnificus*, primers were based on the gene *vvh* (Panicker et al., 2004), for *V. parahaemolyticus*, *tlh* (Nordstrom et al., 2007), and for *V. cholerae*, *ompW* (Bielawska-Drózd et al., 2012). PCR reactions were in 15 µl with 1X AmpliTaq Gold® PCR Buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 1 mM dNTPs, 1.2 µM of each forward and reverse primer, 0.6 units of AmpliTaq® Gold (Applied Biosystems), 10 ng of DNA, and Milli-Q® water. PCR thermal profiles were taken from the publications describing the primers. PCR amplicons were electrophoresed on 2% agarose gels with 0.05% ethidium bromide and visualized on a Molecular Imager ® Gel Doc ™ XR system (Bio Rad).

For crabs that tested positive for any of the three *Vibrio* species, we used real-time PCR to quantify the relative number of copies of that bacterial species' genome (a proxy for cell number) in samples of hemolymph. This measurement, which differs from infection intensity as defined by Rózsa et al. (2000), was based on real-time PCR with Taq-Man probes for the same gene targets as were used to test for presence of pathogen DNA (Bielawska-Drózd et al., 2012; Nordstrom et al., 2007; Panicker et al., 2004). To estimate the relative quantity of the DNA target in a sample, we created a calibration curve using purified PCR product. To create this purified DNA standard, we ran PCR amplicons on 1% agarose gels with a FastRuler MR size standard (Thermo Fisher) and compared intensity of the amplicon bands to this quantified size standard for a preliminary estimate of the relative number of copies present. PCR amplicons were then purified from the gel using Freeze 'N Snap gel purification columns (BioRad). The purified PCR products were then accurately quantified with a Nanodrop spectrophotometer (Thermo Scientific). These standards were diluted to establish 4-point standard curves with a 1:10 dilution series. We used these standards for all subsequent qPCR reactions to estimate relative infection intensity. Real-time PCR reactions were in a total volume of 15 µl with 1X AmpliTaq Gold® PCR buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 1 mM dNTPs, 1.5 µM of the Taq-Man probe, 0.6 units of AmpliTaq® Gold (Applied Biosystems), 10 ng of DNA, and Milli-Q® water. Each 96-well plate included negative controls, with unknown samples run in triplicate. Only standard curve plates with E and $R^2 > 0.90$ were used to estimate relative copy number.

2.3. Exposure to temperature and salinity extremes before capture

We downloaded hourly salinity and water temperature data from the website of the Louisiana Coastwide Reference Monitoring System (www.lacoast.gov/crms2/home.aspx) for stations nearest to each collection location (station codes in Table 1) and used a Perl script to calculate the percentage of hours over which temperatures were above 30 °C or 35 °C and the percentage of hours that salinity was below 2 ppt or above 25 ppt. These *a priori* thresholds for physiological stress and lethality were based on laboratory studies of blue crabs. Short-term exposure to temperatures above 30 °C or salinities either below 2 ppt or above 25 ppt were reported to be stressful, while temperatures over 35 °C resulted in increased mortality (Guerin and Stickle, 1992; Holland et al., 1971; Leffler, 1972). For each collection, we generated a profile

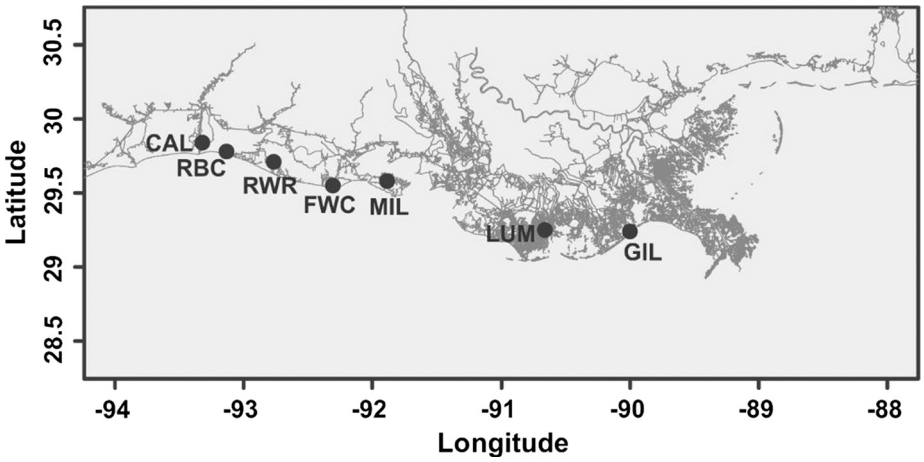

Fig. 1. Map of Louisiana blue crab collection locations. CAL = Lake Calcasieu, RBC = Rutherford Beach Culvert, RWR = Rockefeller Wildlife Refuge, FWC = Freshwater City Locks, MIL = Marsh Island, LUM = LUMCON (Louisiana Universities Marine Consortium), GIL = Grand Isle.

of accumulated hours that exceeded the thresholds over a time span of 21 days prior to collection. Additional profiles were generated with all combinations of time spans between 3 and 31 days, threshold temperatures between 26 and 39 °C (in 1 °C increments) and salinities between 18 and 27 ppt in (1 ppt increments). These variable profiles were used in logistic models (detailed below) to determine the optimum (maximum likelihood) time span before collection (e.g. 3 days, 1 week, 1 month, etc.) and thresholds of temperature and salinity for predicting the prevalence of *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* infections.

Table 1
Summary information for adult/juvenile blue crabs collected from the seven coastal saltmarshes including: year, location, CRMS station ID, latitude (Lat.), longitude (Long.), date of collection, salinity (ppt), temperature (°C), size range (mm), sample size (N), *V. cholerae* prevalence (P_{VC}), *V. parahaemolyticus* prevalence (P_{VP}), and *V. vulnificus* prevalence (P_{VV}).

Year	Location	CRMS ID	Lat.	Long.	Date	Salinity (ppt)	Temp. (°C)	Size range (mm)	*N*	P_{VC} (%)	P_{VP} (%)	P_{VV} (%)
2014	GIL: Grand Isle	0178	29.24	−90.00	8/01/2014	22	29.5	115–175	19	53	21	11
	LUM: LUMCON	0347	29.25	−90.66	7/18/2014	7	28.5	106–180	32	13	6	3
	MIL: Marsh Island	0520	29.58	−91.88	7/16/2014	15	29	110–183	33	0	0	0
	FWC: Freshwater City	0633	29.55	−92.31	6/16/2014	0	27.5	63–200	24	17	33	4
					6/30/2014	10	28	78–180	14	14	21	0
					7/29/2014	5	29	120–156	10	30	30	30
					8/12/2014	10	29	106–134	9	0	0	11
					Total	—	—	63–200	57	26	9	16
	RWR: Rockefeller Wildlife Refuge	0581	29.71	−92.77	6/16/2014	12	31.5	113–175	5	0	20	0
					6/30/2014	17	29.5	109–136	20	5	40	5
					8/07/2014	2	31	98–160	6	0	0	17
					8/12/2014	0	29	108–138	7	0	0	0
					Total	—	—	98–175	38	3	24	5
	RBC: Rutherford Beach Culvert	0614	29.78	−93.13	6/11/2014	19	28	50–172	14	14	14	0
					6/30/2014	21	27.5	93–144	17	18	12	0
					8/07/2014	5	30	126–159	14	36	0	14
					Total	—	—	50–172	45	22	9	4
	CAL: Lake Calcasieu	1743	29.84	−93.32	6/10/2014	14	26	88–182	19	21	26	16
					7/08/2014	12	29	125–142	19	0	5	11
					Total	—	—	88–182	38	11	16	13
	Total		—	—	—	—	—	50–200	262	15	15	7
2015	GIL: Grand Isle	0178	29.24	−90.00	8/18/2015	12	30	80–160	27	44	0	7
	LUM: LUMCON	0347	29.25	−90.66	8/15/2015	8	33.5	88–170	44	18	20	2
	MIL: Marsh Island	0520	29.58	−91.88	8/04/2015	10	32	118–170	30	17	63	3
	FWC: Freshwater City	0633	29.55	−92.31	6/08/2015	7	29	110–168	9	33	11	0
					6/26/2015	3	32	108–154	13	46	38	0
					7/02/2015	16	31	90–130	9	0	22	11
					8/11/2015	19	31	108–190	17	0	6	18
					Total	—	—	90–190	48	19	19	8
	RWR: Rockefeller Wildlife Refuge	0581	29.71	−92.77	6/08/2015	0	29	90–126	9	11	0	0
					7/02/2015	0	30	98–150	14	7	0	0
					8/11/2015	16	32	125–140	16	19	13	0
					Total	—	—	90–150	39	13	5	0
	RBC: Rutherford Beach Culvert	0614	29.78	−93.13	6/08/2015	3	28.5	85–145	9	33	44	0
					6/26/2015	5	30	90–179	19	16	11	0
					7/02/2015	2	29	70–150	11	9	18	9
					8/25/2015	8	31	124–152	18	6	0	0
					Total	—	—	70–179	57	14	14	2
	CAL: Lake Calcasieu	1743	29.84	−93.32	6/22/2014	7	30	90–195	30	23	30	3
					7/07/2015	2	27.5	108–180	27	33	15	0
					Total	—	—	90–195	57	28	23	2
	Total		—	—	—	—	—	70–190	302	21	20	3

2.4. Effects of temperature and salinity on prevalence and intensity

We calculated prevalence of *Vibrio* infection as it is generally defined (Rózsa et al., 2000): the proportion of individuals in which a pathogen was detected. To evaluate the effects of individual and environmental factors on prevalence, we used logistic regression in R 3.2.0 (www.r-project.org). Predictor variables included: location, sex, maturity, size, collection temperature, collection salinity, percentage of the time span below 2 ppt, percentage of the time span above 25 ppt, percentage of the time span above 30 °C, and percentage of the time span above 35 °C. For models that predicted infection intensity, we used general linear models in R 3.2.0 (www.r-project.org). For these models, only data for infected individuals was used. In both prevalence and intensity models, highly collinear variables were evaluated using the *vif* calculation in the *car* package of R 3.2.0 (www.r-project.org). This resulted in the removal of maturity, which was correlated with size; percentage of the time span below 2 ppt, which was correlated with location; and percentage of the time span above 30 °C, which was correlated with percentage of the time span above 35 °C. We chose to retain location rather than percentage of the time span below 2 ppt because location is more likely to include unobserved factors. We used the corrected Akiake Information Criterion (AICc) (Burnham and Anderson, 2003, 2004) for model selection and multimodal inference in the *MuMIN* package (Bartoń, 2013) of R 3.2.0 (www.r-project.org) to evaluate all possible first-order model combinations. Then, individual models from the total group that had ΔAICc values less than 2 from the best model were averaged based on their model weight values into the final averaged models (Burnham and Anderson, 2003; Wagenmakers and Farrell, 2004).

To identify optimum values for thresholds of salinity and temperature as well as the optimum span over which the accumulated time exceeding those thresholds is calculated, we compared logistic models in R 3.2.0 (www.r-project.org). In these models, the dependent variable was the log of the odds ratio (probability of being infected divided by probability of not being infected) and the sole independent variable was the percentage of hourly measurements that salinity (or temperature) was above a threshold for a particular time span before collection. The probability of infection is equivalent to the expected prevalence of infection. Both model log-likelihoods and slopes in relation to different thresholds and time spans were plotted as wireframe plots in R 3.2.0 (www.r-project.org). Plots of log likelihood were used to visualize which thresholds and time spans had the highest likelihoods for the observed infection data. Plots of slope visualized how strongly the probability of infection depended on the percentage of time over the threshold, and whether the probability of infection increased or decreased with greater accumulated time over the threshold.

Wireframe plots of log-likelihood and slope for models that differed in temperature and salinity threshold parameters (detailed above) were used in two ways. First, they were used to find the time spans and thresholds that had the most information about the prevalence of infection. Second, they were used to test predictions of hypotheses about the biological mechanisms that produced the observed effects of temperature and salinity on prevalence. Specifically, we predicted that effects on prevalence mediated by effects on host physiology would exhibit thresholds of temperature and salinity corresponding to thresholds for physiological stress and mortality in blue crabs as determined by previous laboratory studies. Furthermore, we predicted that effects on host physiology would be consistent for all three species of *Vibrio*. In contrast, we predicted that effects on prevalence mediated by effects on pathogen growth and abundance would not exhibit sharp thresholds, but would instead exhibit patterns that paralleled effects on *Vibrio* growth and abundance as reported in the literature. Furthermore, we predicted that pathogen-mediated effects would vary among species of *Vibrio*.

3. Results

3.1. Interannual variation in Vibrio spp. prevalence

In the summers of 2014 and 2015, we sampled 564 blue crabs: 262 in 2014 and 302 in 2015. The sample sizes per location ranged from 19 to 57 in 2014 and 27 to 57 in 2015. Prevalence was not significantly different between 2014 and 2015 for either *V. parahaemolyticus* (15% vs. 20%; $\chi^2 = 3.7$, d.f. = 1, $p = .053$), or *V. cholerae* (15% vs. 21%; $\chi^2 = 2.4$, d.f. = 1, $p = .120$). However, the prevalence of *V. vulnificus* was significantly lower in 2015 (3%) than in 2014 (7%) ($\chi 2 = 4.7$, d.f. = 1, $p = .031$). Seven crabs (3-CAL, 2-FWC, 1-MIL, 1-RWR) tested positive for both *V. parahaemolyticus* and *V. vulnificus* and four for all three *Vibrio* species (1-FWC, 1-GIL, 1-MIL, 1-RBC).

3.2. Modeling effects on prevalence

There were five models within 2ΔAICc of the best model for *V. cholerae* prevalence (Table S1). The most important factors were the percentage of time salinity was above 25 ppt, location, collection salinity, and collection temperature; these factors were included in all five models (Table S1). The AIC-weighted averaged model was:

$$\begin{aligned} Prevalence = {} & -6.9 - 0.20 * FWC + 2.2 * GIL - 0.69 * LUM - 0.81 * MIL \\ & - 0.31 * RBC - 1.21 * RWR + 0.07 * size + 0.47 * male \\ & - 0.10 * collection\ salinity + 0.19 * collection\ temperature \\ & + 0.02 * time > 25ppt + 0.01 * time > 35°C. \end{aligned}$$

V. cholera prevalence was significantly higher at Grand Isle (GIL) and lower at Rockefeller Wildlife Refuge (RWR; Fig. 2; Table 2). Prevalence significantly decreased with collection salinity (Fig. 3A) and increased with collection temperature (Fig. 4A; Table 2). Also, prevalence significantly increased with the percentage of time that salinity conditions were above 25 ppt over the 21 days preceding collection. Trends that were noticeable but not significant ($p > = .05$) were a higher prevalence in males and increased prevalence with both size and

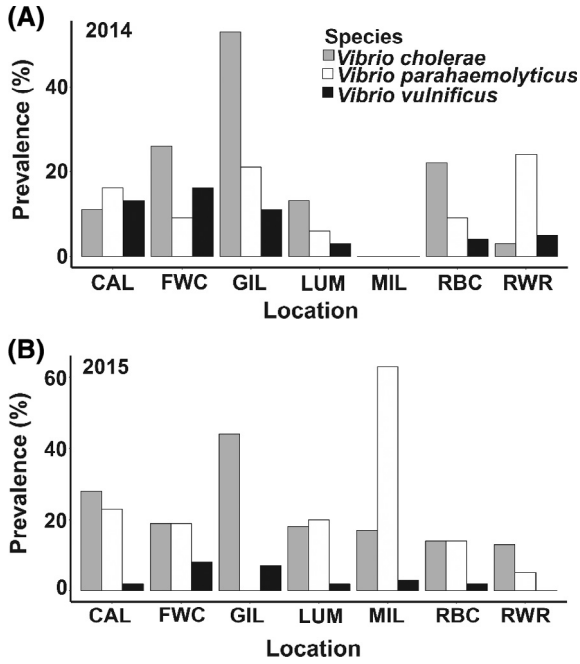

Fig. 2. Bar plot of *Vibrio cholerae* (grey), *V. parahaemolyticus* (white), and *V. vulnificus* (black) prevalence at each sampled location: CAL = Lake Calcasieu, RBC = Rutherford Beach Culvert, RWR = Rockefeller Wildlife Refuge, FWC = Freshwater City Locks, MIL = Marsh Island, LUM = LUMCON (Louisiana Universities Marine Consortium), GIL = Grand Isle in A) 2014 and B) 2015.

Table 2
Summary of the influence of environmental conditions, host stress, and host physical conditions on the prevalence of *Vibrio cholera*, *V. parahaemolyticus,* and *V. vulnificus*. The sign (positive or negative) indicates whether that factor increases or decreases prevalence, and the magnitude of the influence on prevalence is given by the value itself. FWC = Freshwater City, GIL = Grand Isle, LUM = LUMCON, MIL = Marsh Island, RBC = Rutherford Beach Culvert, RWR = Rockefeller Wildlife Refuge, Size = carapace width (point to point), Collection Temperature = temperature measured at collection (˚C), Collection Salinity = salinity measured at collection (ppt), > 25 ppt = the percent of time salinity was above 25 ppt, and > 35 ˚C = the percent of time temperatures were above 35 ˚C. * = significant at $p < .05$.

Comparison	β_0	FWC	GIL	LUM	MIL	RBC	RWR	Size	Sex (male)	Collection Salinity	Collection Temperature	> 25 ppt	> 35 ˚C
V. cholerae	−6.9	−0.02	***2.2****	−0.69	−0.81	−0.31	***−1.21****	0.07	0.47	***−0.1****	***0.19****	***0.02****	0.01
V parahaemolyticus	−7.5	0.05	−0.99	−0.93	0.41	***−0.87****	−0.73	No	−0.01	−0.016	***0.22****	0.008	−0.002
V. vulnificus	−2.7	0.1	0.38	−2.3	1.85	−1.07	***−2.19****	No	−0.63	0.02	No	0.005	***0.04****

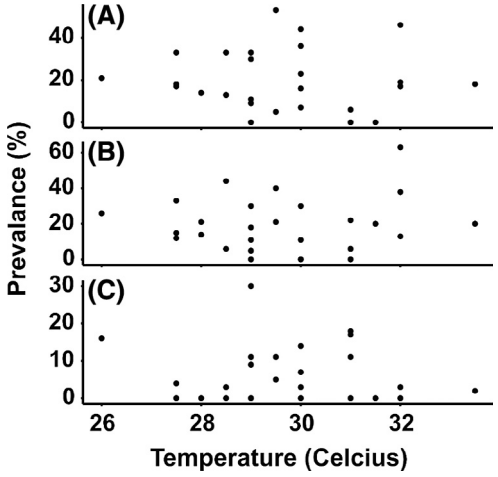

Fig. 3. A-C: Scatterplots showing relationships between prevalence of infection by A) *V. cholerae*, B) *V. parahaemolyticus*, and C) *V. vulnificus* and temperature (˚C) measured at the time of collection.

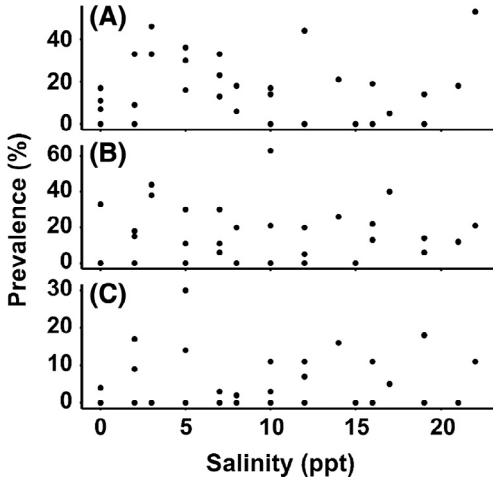

Fig. 4. A-C: Scatterplots showing relationships between prevalence of infection by A) *V. cholerae*, B) *V. parahaemolyticus*, and C) *V. vulnificus* and salinity (ppt) measured at the time of collection.

the percentage of time above 35 ˚C (Table 2).

There were seven models within 2ΔAICc of the best model for *V. parahaemolyticus* prevalence (Table S1). The most important factors were location and collection temperature, which were included in all seven models (Table S1). The AIC-weighted averaged model was:

$$\begin{aligned} Prevalence = {} & -7.5 + 0.05{*}FWC - 0.99{*}GIL - 0.93{*}LUM + 0.41{*}MIL \\ & - 0.87{*}RBC - 0.73\ RWR - 0.01{*}male - 0.016{*}collection\ salinity \\ & + 0.22{*}collection\ temperature + 0.008{*}time > 25ppt \\ & - 0.002{*}time > 35°C. \end{aligned}$$

V. parahaemolyticus prevalence was significantly lower at Rutherford Beach Culvert (RBC; Fig. 2; Table 2) and significantly increased with collection temperature (Table 2; Fig. 3B). Non-significant trends ($p >= 0.05$) were lower prevalence in males, decreased prevalence with both collection salinity (Fig. 4B) and the percentage of time above 35 ˚C, and increased prevalence with the percentage of time above 25 ppt (Table 2).

For the prevalence of *V. vulnificus*, there were four models within 2ΔAICc of the best model (Table S1). The most important factors were the percentage of time above 35 ˚C and location, which were included in all four models (Table S1). The AIC-weighted averaged model was:

$$\begin{aligned} Prevalence = {} & -2.76 + 0.10{*}FWC + 0.38{*}GIL - 2.30{*}LUM - 1.85{*}MIL \\ & - 1.07{*}RBC - 2.19{*}RWR - 0.63{*}male \\ & + 0.02{*}collection\ salinity + 0.005{*}time > 25ppt \\ & + 0.04{*}time > 35°C. \end{aligned}$$

V. vulnificus prevalence was significantly lower at Rockefeller Wildlife Refuge (RWR; Fig. 2; Table 2). Although prevalence did not vary with collection temperature (Fig. 3C) it significantly increased with the percentage of time above 35 ˚C. Non-significant trends ($p >= .05$) were lower prevalence in males and decreased prevalence with both increasing collection salinity (Fig. 4C) and the percentage of time above 25 ppt (Table 2).

3.3. Modeling and optimizing temperature and salinity thresholds

To find specific values for thresholds and time spans that would best predict the probability of infection for each species of *Vibrio*, we used logistic regression models with only a single factor: the percentage of time that either salinity or temperature was above a threshold, and varied both the time span over which the percentage was calculated and the value of the threshold. For all three species of *Vibrio*, there were tall, narrow peaks in the log-likelihood wireframe plots for temperature thresholds between 32 ˚C and 34 ˚C (Fig. 5A–C). These peaks were between 2 and 5 units above the surrounding regions, indicating strong support (i.e. statistically significant). For *V. cholerae*, the maximum likelihood threshold temperature was 32 ˚C, for *V. vulnificus* 33 ˚C and for *V. parahaemolyticus* 34 ˚C, all slightly below our *a priori* threshold of 35 ˚C for lethal effects on healthy blue crabs. The optimal time span over which to calculate time exceeding the threshold was 3 days for *V. vulnificus* and *V. parahaemolyticus* and 10 days for *V. cholerae* (Fig. 5A-5C). This near consistency of the threshold temperatures, independent of pathogen species, suggests an underlying effect on host physiology. Furthermore, the closeness of the optimum threshold temperatures to the lethal temperature for healthy blue crabs (35 ˚C) and the short time spans (3–10 days) over which these effects were produced suggest that differential mortality of the host could be involved. If infected crabs have a lower temperature threshold for heat-induced mortality or experience an increased rate of mortality at the threshold temperature, then prevalence should decrease with a higher accumulated percentage of time over the threshold temperature. The wireframe plots of slope show this expected relationship for *V. cholerae* and *V. parahaemolyticus* (negative slopes) but not for *V. vulnificus* (Fig. 5D–F).

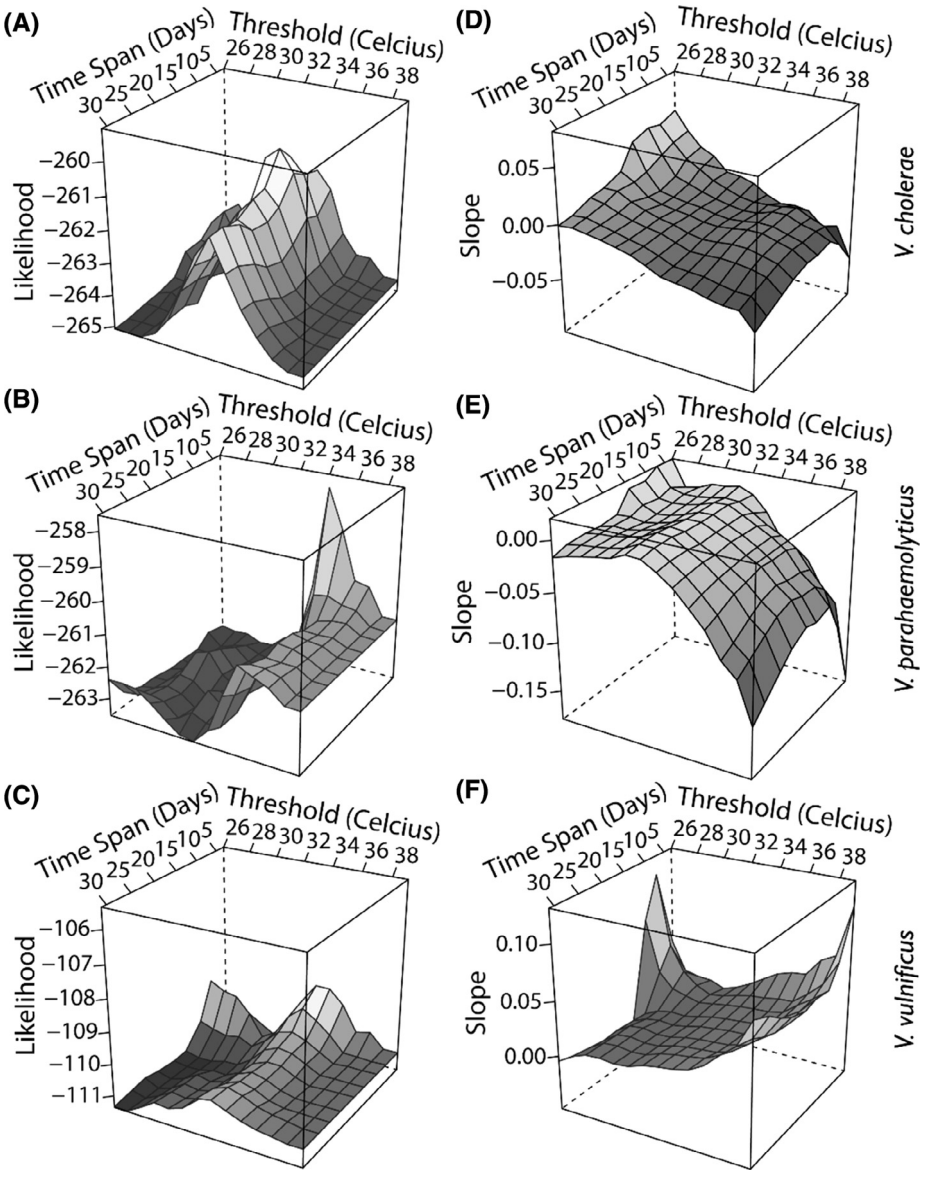

Fig. 5. A-F: Wireframe plots showing the effects of varying timespan and threshold when measuring temperature stress before capture on A) *V. cholerae*, B) *V. parahaemolyticus*, C) *V. vulnificus* log-likelihood values and D) *V. cholerae*, E) *V. parahaemolyticus*, and F) *V. vulnificus* model slope from logistic regressions. Increasing log-likelihood values indicate models where the threshold and timespan used to measure stress results in models with a higher probability of infection. For slope plots, higher values indicate increasing the percent of the timespan above the threshold value has a more positive influence on the probability of infection.

There were no sharply-defined peaks in the log-likelihood wireframe plots for models in which the percentage of time over a salinity threshold was the only factor (Fig. 6A–C). The differences between the highest and lowest regions of these plots were all less than 1.5 units. For *V. cholerae* and *V. parahaemolyticus*, the slope of the relationship between the percentage of time exposed to salinities over the threshold and the probability of infection was negative for salinity thresholds below 22 ppt, but positive for higher thresholds, indicating that a higher probability of infection was associated with longer exposures to salinities above 22 ppt. This pattern was mostly independent of the time span over which the exposure was calculated. However, for *V. vulnificus* the slope was positive over all the combinations of threshold salinity (18–27) and time span that were modeled. This is consistent with field observations that optimum salinity for *V. cholerae* is higher than for *V. vulnificus* (Fig. 5D–F).

3.4. Factors that impact Vibrio spp. relative load

For *V. vulnificus* relative pathogen load, there were 7 models within 2ΔAICc of the best model (Table S2). The most important factor was the percentage of time above 35 °C, which was included in all seven models. The AIC-weighted averaged model was:

$$Relative\ load = -838 + 35.3{*}size + 279.2{*}male + 41.6{*}collection\ salinity + 29.8{*} > 35°C.$$

V. vulnificus relative load significantly increased with increasing exposure to temperatures above 35 °C. *V. vulnificus* relative load also increased with collection salinity and crab size and was higher for male blue crabs, although these trends were not significant at $\alpha = 0.05$. For *V. parahaemolyticus* and *V. cholerae* relative load, there were 5 and 6 models within 2ΔAICc of the best model (Table S2), however in both sets of comparisons one of those models was the NULL model, indicating that no models were significant improvements over the random model for the relative load of these two *Vibrio* species (Table S2).

4. Discussion

4.1. Relation to previous Vibrio spp. surveys

In 1984, Huq et al. examined the effects of temperature, salinity, and pH on the growth of *Vibrio cholerae* on copepods, and found elevated temperature increased bacterial growth. This relationship was also documented in *Vibrio* from oysters and environmental cultures

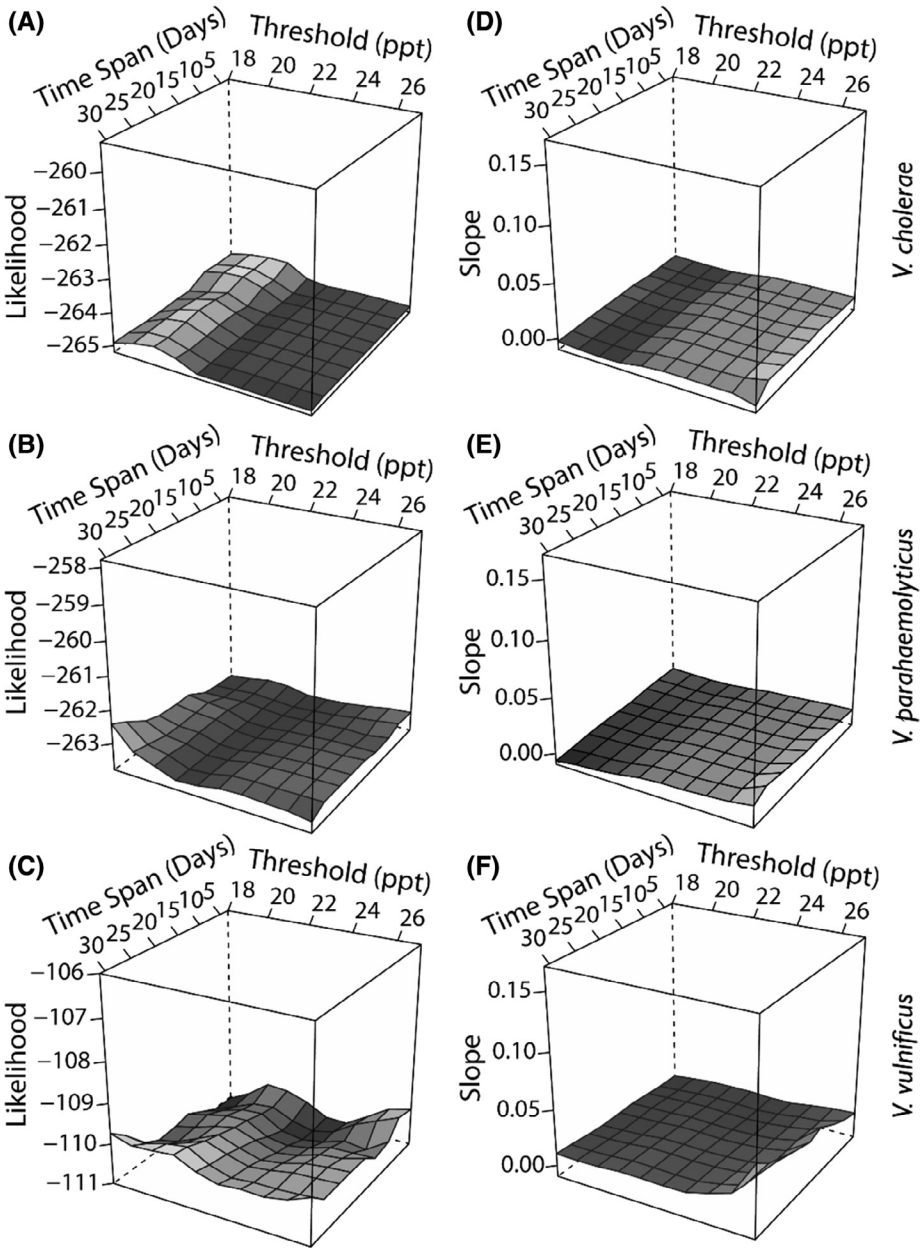

Fig. 6. A-F: Wireframe plots showing the effects of varying timespan and threshold when measuring salinity stress before capture on A) *V. cholerae*, B) *V. parahaemolyticus*, C) *V. vulnificus* log-likelihood values and D) *V. cholerae*, E) *V. parahaemolyticus*, and F) *V. vulnificus* model slope from logistic regressions. Increasing log-likelihood values indicate models where the threshold and timespan used to measure stress results in models with a high probability of infection. For slope plots, higher values indicate increasing the percent of the timespan above the threshold value has a more positive influence on the probability of infection.

(Kaspar and Tamplin, 1993; Randa et al., 2004; Shiah and Ducklow, 1994; Turner et al., 2014). In blue crabs, a positive correlation between temperature and *Vibrio* intensity was documented by Welsh and Sizemore (1985). Consistent with these earlier findings, we observed higher prevalence of *Vibrio* infection with warmer temperatures. In our multi-factor models, prevalence significantly increased with collection temperature for *V. parahaemolyticus* and *V. cholera*, while for *V. vulnificus* prevalence increased with the percentage of time above 35 °C for the 21 days prior to collection.

In our study, salinity also affected the prevalence of *V. cholerae*. Prevalence significantly decreased with higher collection salinity but significantly increased with the percentage of time above 25 ppt for the 21 days prior to collection. These are not contradictory results; in models in which the percentage of time above a threshold salinity was the only factor, the slope between the probability of infection and the time above the threshold was negative for threshold salinities below 22 ppt but positive for thresholds above 22 ppt. This simply indicates that prevalence was highest at salinity extremes, a relationship that is also evident in the scatterplot of prevalence against salinity (Fig. 4A).

There has been one previous survey of diseases in blue crabs from Louisiana. Rogers et al. (2015) conducted a seasonal investigation of symbionts (including *Vibrio* spp.) in blue crabs from four locations in Louisiana, three of which coincide with our study locations. In agreement with our results, they found spatial variability in *Vibrio* spp. prevalence, with blue crabs from Rockefeller Wildlife Refuge having significantly lower prevalences of infection. They also found a lower prevalence for male vs. female blue crabs that were non-significant trends for *V. parahaemolyticus* and *V. vulnificus* in our study. Finally, they found a negative relationship between prevalence and salinity, in agreement with our findings for *V. cholerae* and *V. parahaemolyticus*.

4.2. The significance of temperature and salinity thresholds

In models with all factors that affected prevalence, the effect of the time that temperatures were above the *a priori* threshold of 35 °C for the 21 days prior to collection was significant only for *V. cholerae* (Table 2). In contrast, in single-factor models there was strong support (differences in log likelihood > 2) for specific threshold temperatures

between 32 and 34 °C for all three *Vibrio* species. A reason for this apparent disparity in statistical support is evident in the wireframe plots of log-likelihood for the temperature threshold models. For both *V. parahaemolyticus* and *V. vulnificus*, the maximum-likelihood estimate of the optimal time span was only 3 days—much shorter than the 21 days we used in the multi-factor models. In contrast, for *V. cholerae* the optimal time span was 10 days and likelihoods were relatively high for even longer time spans (Fig. 5A). In addition, the maximum-likelihood threshold temperatures for all three species were slightly (1–3 °C) below the temperature of 35 °C used in the multi-factor models. Considering both threshold temperature and time span, the differences in likelihoods between the values used in the multi-factor models and their optimum values were 3.3 for *V. cholerae*, 3.2 for *V. parahaemolyticus* and 1.4 for *V. vulnificus* (differences of 2 or greater are considered significant). If the effects we detected in the field are due to lethality, as we suspect, the difference between the optimum threshold temperatures and the laboratory-based lethal temperature of 35 °C could reflect a lower threshold for lethality in infected crabs or differences between field and laboratory conditions.

The accumulated hours of exposure to salinities above 25 ppt was a significant factor affecting prevalence of *V. cholerae* in models that included multiple factors, although there was no support for 25 ppt or any other salinity value as a specific threshold for salinity effects in single-factor models. Instead, models that include a positive effect of exposure to salinities above 25 ppt along with a negative effect of collection salinity appear to better fit the bimodal relationship between prevalence of *V. cholerae* and salinity. Prevalence decreased with salinity below 22 ppt but increased with salinity above 22 ppt. The initial decrease could correspond to the 15–25 ppt optimum salinity range for growth of *V. cholerae*, but this does not explain the increase in prevalence at higher salinities. We can speculate that the stressful effect of higher salinities on blue crabs could be a secondary mechanism that strongly favors infection and more than compensates for a lesser negative effect on the growth of *V. cholerae*.

An important distinction among the four *a priori* salinity and temperature thresholds used for this study is that only the high-temperature threshold of 35 °C is associated with lethal effects, the others are for physiological stress. If the effects of environmental extremes on prevalence are caused by host mortality, we might not expect to see thresholds for sublethal effects. The short time spans (3–10 days) we estimated for the effects on prevalence of exposure to temperatures over 32–34 °C are consistent with an acute effect on the host. The decreases in prevalence of *V. cholerae* and *V. parahaemolyticus* with greater accumulated exposure to high temperatures are also consistent with an increase in mortality from infection. However, this relationship was reversed for *V. vulnificus*; prevalence increased with more exposure to high temperatures, suggesting stressfully high temperatures favor infection. This is consistent with effects on infection intensity. For *V. vulnificus* (but not for *V. cholerae* or *V. parahaemolyticus*) the accumulated time of exposure to temperatures over 35 °C had a strong positive effect on intensity. The saddle-shaped plot of slope for this effect has two peaks, one at the lowest threshold temperature threshold of 26 °C for 7 days, and another at the highest threshold temperature of 39 °C for 3 days (Fig. 6F). Such complex relationships between environmental factors and infection suggest that multiple mechanisms are at work, possibly some related to pathogen growth and others to host susceptibility or mortality.

4.3. Environmental sensor data for disease ecology

There has been growing appreciation of the potential for environmental sensor data to advance the study of ecology (Burge et al., 2014; Groner et al., 2015, 2016; Maynard et al., 2016). Sensor data can aid in the prediction of bioinvasions, shifts in species ranges, and marine disease outbreaks (Baker-Austin et al., 2013; Bruno et al., 2007; Goldstein et al., 2016; Johnston and Purkis, 2011; Maynard et al., 2016). The ecological processes that determine the prevalence of infectious diseases in natural populations are dynamic and complex, as are the mechanism by which environmental factors influence those processes (Anderson and May, 1979). Single-point measurements or simple averages of environmental conditions cannot capture rates of change, duration of extreme events, or other complex features of a changing environment. Environmental sensor data opens the prospect of developing time-integrated measures of environmental conditions that reflect underlying physiological mechanisms. As we hope to have demonstrated here, time-integrated measures of exposure to extreme conditions based on *a priori* considerations can be used to test hypotheses about mechanisms that underlie patterns of disease occurrence, while *a posteriori* determination of environmental variables that influence disease can lead to new hypotheses. Our results suggest that the relationships between environmental conditions and prevalence of infection of blue crabs by *Vibrio* bacterial pathogens are complex. Accurate prediction of pathogen outbreaks and advances in understanding the dynamics of marine diseases may be improved by the development of correspondingly complex measures of environmental conditions.

This study benefited from the wealth of research conducted on blue crabs (over 1000 peer reviewed manuscripts as of October 2016), especially studies that characterized conditions stressful to blue crabs (Guerin and Stickle, 1992; Holland et al., 1971; Leffler, 1972). Our analysis of environmental conditions would not have been possible without the Louisiana Coastwide Reference Monitoring System (CRMS), a high coverage sensor network deployed throughout the coastal marshes of Louisiana (Steyer et al., 2003). We hope to have demonstrated that both thorough laboratory studies of physiology and large-scale collection of environmental data are important to understanding the disease ecology of blue crabs, and the long-term value of these approaches for the preservation and management of marine systems.

Acknowledgements

This research was made possible in part by grants from The Gulf of Mexico Research Initiative (GRI-013 and GoMRI2012-II-523) and by a University of Louisiana at Lafayette Doctoral Fellowship to T. Sullivan. Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (DOI: R2.x2.4.000:0011). We thank Sophie Plouviez, Tyson Crouch, Casey Choate, Louisiana Department of Wildlife and Fisheries, and the Army Corps of Engineers for assistance in sampling access.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2017.11.004.

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