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## Prevalence of Blue Crab (*Callinectes sapidus*) Diseases, Parasites, and Symbionts in Louisiana

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PREVALENCE OF BLUE CRAB (*CALLINECTES SAPIDUS*) DISEASES,  
PARASITES, AND SYMBIONTS IN LOUISIANA

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The School of Renewable Natural Resources

by  
Holly A. Rogers  
B.S., University of Cincinnati, 2011  
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## ABSTRACT

Research on blue crab diseases, parasites, and symbionts has been sporadic in the Gulf of Mexico. Understanding the prevalence of diseases, parasites, and symbionts is important for managers to set informed regulations for the commercial industry and to understand the impacts of environmental disasters on aquatic animal health. The objective of this research was to determine the prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, *Hematodinium perezii*, *Loxothylacus texanus*, reo-like virus (RLV), shell rot, and *Vibrio* spp. in crabs collected from four coastal locations and four shedding facilities in 2013 and the beginning of 2014. Additionally, I determined the prevalence of white spot syndrome virus (WSSV) in the wild populations. I also recorded infections by non-*Vibrio* bacteria and by *Ameson michaelis*.

*H. perezii*, *L. texanus*, WSSV, and RLV were detected by polymerase chain reactions. Shell rot and *U. crescens* were detected by gross visual detection. *L. callinectes* and *A. michaelis* were identified by microscopy and *Vibrio* spp. and non-*Vibrio* spp. bacteria were detected by standard microbiological culture techniques and biochemical testing.

No samples were infected with *H. perezii*, *L. texanus*, or WSSV. Based on the low salinities sampled, these results were expected for *H. perezii* and *L. texanus*. Shell rot and *Vibrio* spp. were moderately prevalent in the wild and captive crabs, but infections were more common in the captive crabs. *U. crescens* was never found in crabs from Lake Pontchartrain, the lowest salinity field site. It was also rare in crabs from the low salinity shedding facilities, indicating that this hyperparasite may be limited to moderate to high salinities. *L. callinectes* was ubiquitous with over 90% prevalence in wild and captive crabs. Reo-like virus infections were found in wild and captive crabs in the summer of 2013, and *A. michaelis* was present in two shedding facility pre-molt crabs. Overall, Louisiana's blue crab nearshore populations appear to be healthy with no parasitization by the two most ecologically and economically detrimental parasites, *H. perezii* and *L. texanus*. However, in the future, RLV needs to be extensively studied because it is also capable of decimating blue crab populations.

## CHAPTER 1: INTRODUCTION

Diseases, parasites, and symbionts are ubiquitous in marine environments. Infection and parasitism of some shellfish and crustacean species, such as those of Eastern oysters and of penaeid shrimp, have been thoroughly studied; however, this is untrue for the blue crab (*Callinectes sapidus*), especially in the Gulf of Mexico (Shields and Overstreet 2007). A blue crab can be infected by numerous species of bacteria, fungi, protists, barnacles, worms, and viruses throughout its life cycle.

### 1.1 Life Cycle and Ecology

The blue crab's life cycle begins with a planktonic larval stage as zoeae (Kennedy 2007). After seven or eight zoeal stages, the zoeae metamorphose into megalopae that molt into juvenile crabs. For the first time in the life cycle, juveniles resemble adults and have transitioned to a truly benthic lifestyle (Kennedy 2007). Juveniles molt until they reach sexual maturity and adulthood in their 16<sup>th</sup> to 20<sup>th</sup> instar, which corresponds to 110 to 180 mm carapace width (CW) (Hines 2007). Approximately 50% of male crabs are sexually mature at 107 mm CW, while about half of females are mature at a larger size of 132 mm CW (Jivoff et al. 2007). In studies specifically conducted in Louisiana, 50% of males and females were mature at 110 and 125 mm CW, respectively and 100% of them at 130 and 160 mm, respectively (Guillory et al. 2001).

The molting process in blue crabs is significant ecologically, biologically, and economically. Molting can be highly influential on the symbiont and disease load of crabs as several of these are dependent on ecdysis. The molt cycle consists of a period of intermolt followed by premolt, ecdysis, and brief postmolt (Smith and Chang 2007). As a blue crab prepares to molt into a soft shell crab, it migrates to shallow, vegetated waters for protection from predators. During the late premolt and early postmolt periods, crabs do not move or forage (Smith and Chang 2007). Ecdysis occurs when water osmoses into the shell and creates hydrostatic pressure that ruptures the shell and supports the skeleton of the soft crab (Smith and Chang 2007). Calcification commences within a few hours following ecdysis.

Molting frequency and size increase (molt increment) vary throughout a crab's life cycle. Young postlarval crabs molt every few days while large juveniles go weeks without molting. Eventually, crabs reach anecdysis as growth is determinate (Smith and Chang 2007). Crabs, especially females, have been observed to molt past the hypothesized terminal molt, but this occurrence is rare in the northern Gulf of Mexico (Guillory et al. 2001, Smith and Chang 2007).

Ecdysis depends on water temperature and, to some extent, on salinity and nutrient availability. The intermolt period is shorter for crabs at higher water temperatures than those at colder temperatures (Smith and Chang 2007). This is one explanation for why Gulf crabs reach sexual maturity within a year of postlarval settlement whereas this takes upwards of 18 months in the Chesapeake Bay (Guillory et al. 2001). As temperatures change seasonally, winter anecdysis and subsequent, spring synchronous molting occur (Smith and Chang 2007). Unlike temperature, the effects of salinity and nutrition on ecdysis are less clear and consistent. It has been

hypothesized that at lower salinities the intermolt period increases and/or molt increment decreases, but these results are inconsistent across studies (Smith and Chang 2007). Food availability can also potentially affect molting rates as ecdysis is energetically expensive.

Blue crab habitat preferences change as it progresses through its life cycle. Larval stages require high salinities and water temperatures to fully develop. In the Gulf of Mexico, the megalopae move inshore and settle into nurseries in periodic, high density settlement episodes. Nurseries include seagrass beds, oyster reefs, salt marshes, and woody debris (Lipcius et al. 2007). In the Gulf, marshes are key primary nurseries due to their small, diurnal tides (Lipcius et al. 2007). Ideal habitats for megalopae have salinities around 20 ppt and temperatures around 20 °C (Millikin and Williams 1984). In laboratory experiments as salinities doubled from 20 ppt to 40 ppt, the length of the megalopal stage increased from 34 to 58 days, indicating that moderate salinities of inshore marshes and estuaries are ideal (Millikin and Williams 1984). Metamorphosis into the juvenile stage begins once megalopae encounter cues used for ideal habitat selection. These cues include reduced salinities, appropriate estuarine biota, ideal flow, and absence of predator cues (Lipcius et al. 2007).

Juveniles often migrate from estuarine nurseries to mud or sand flats or salt marshes, with lower population density or with greater food availability (Lipcius et al. 2007). As juveniles grow they migrate to lower salinity areas to facilitate molting. Research on salinity zones in Mississippi shows that: 1) juveniles ranging from 3 to 10 mm CW are common in salinities between 15 and 20 ppt; 2) juveniles between 10 and 20 mm CW are most common in salinities less than 10 ppt; and 3) juveniles from 20 to 40 mm CW are usually in salinities below 5 ppt (Millikin and Williams 1984). Immature males that are approaching maturity move into tidal creeks to use the tidal marsh edge as a protected habitat for molting (Hines 2007). After molting, these males move back to subestuarine basins. Females molt to maturity in estuarine basins and stay in these lower salinity areas to mate (Millikin and Williams 1984). Once inseminated, females migrate to high salinity spawning areas that to promote larval development (Millikin and Williams 1984).

Additionally, the diet of the blue crab is ecologically important and varies by season, region, and age. Young juvenile blue crabs are less cannibalistic than adults and predominantly eat benthic infauna, such as bivalves, crabs, shrimp, amphipods, isopods, worms, gastropods, detritus, and fish carcasses (Lipcius et al. 2007). Adult diets are less diverse than those of juveniles and are composed mainly of molluscs, arthropods, annelids, detritus, xanthid crabs, other blue crabs, and fishes (Hines 2007). The increased cannibalism in adults has major implications for the transmission of diseases and parasites within blue crab populations.

## **1.2 Biology**

The blue crab is a portunid crab with an external skeleton that is characterized by a hard carapace shell with five pairs of pereopods (Williams 2007). The first pair of pereopods terminates with the heterochelic claws, and pereopods two through four are walking legs, while the fifth pereopods are swimming legs (Kennedy and Cronin 2007).



Male crab abdomens are T-shaped with the third through fifth abdominal segments fused, and female abdomens develop from a triangular shape to a broad U-shape at maturity (Kennedy and Cronin 2007). Females typically have red claws while male claws are blue. Internally, males have gonopods and females have gonopores (Kennedy and Cronin 2007, Williams 2007).

The digestive system of the blue crab is a simple tube system with a foregut, midgut, and hindgut. The hindgut gland, or hepatopancreas, produces digestive enzymes, absorbs nutrients, and stores lipids (Johnson 1980). The hepatopancreas often shows physiological abnormalities due to infection and parasitization (Johnson 1980). Another important organ system in the blue crab often targeted by diseases, parasites, and symbionts is the respiratory system, specifically the gills. The blue crab has eight gills that function in respiration and ion regulation and excretion (Kennedy and Cronin 2007). Blue crabs have an open circulatory system with a combination of hemal sinuses and blood vessels that transport hemolymph (Kennedy and Cronin 2007). Hemolymph is often contaminated during infections, and the hemocytes remove pathogens by phagocytosing and encapsulating foreign particles (Johnson 1980).

When hemocytes fail to defend against a pathogen, the crab becomes diseased, and its tissues and organs are infected. Diseases, parasites, and symbionts that are known to infect blue crabs with a variety of accompanying symptoms include: *Loxothylacus texanus*, shell disease or shell rot, *Vibrio* spp., *Lagenophrys callinectes*, *Hematodinium perezii*, *Urosporidium crescens*, *Whispovirus* spp., *Ameson michaelis*, and reo-like virus (RLV).

### 1.3 Diseases, Parasites, and Symbionts

#### 1.3.1 *Loxothylacus texanus*

*Loxothylacus texanus* is an obligate, internal barnacle that can have severe reproductive and developmental consequences for its host. Female cypris larvae of the barnacle use a carbohydrate chemical cue to locate a potential host, such as a freshly-molted crab (Sherman et al. 2008). The barnacle enters the crab through its soft cuticle, and the barnacle's interna attaches to the exterior of the intestinal wall (Guillory et al. 2001). The interna moves along the intestines to the ventral region of the abdomen and develops a rootlike system that penetrates the crab's muscle and hepatopancreas and will nourish the externa of the barnacle (Messick and Sindermann 1992, Bortolini and Alvarez 2008). After the crab molts five to nine times, the externa of the barnacle appears as a brood sac under the crab's abdomen (Tindle et al. 2004).

The barnacle affects the crab's ability to osmoregulate in response to salinity changes and causes lower hemolymph osmolality (Alvarez et al. 2002). *L. texanus* causes sterilization of infected crabs, which can greatly reduce crab abundance when the barnacle is very prevalent. Secondary sexual characteristics of infected crabs resemble those of adult females (Ragan and Matherne 1974).

Due to the termination of ecdysis and preferential infection of juvenile crabs that molt more frequently and have thinner shells than adult crabs (Boone et al. 2003). Infected adult blue crabs are typically undersized with a CW between 30 and 80 mm, with an average size of 58 mm (Adkins 1972, Overstreet et al. 1983). These undersized

crabs never reach legal size for the commercial fishery (Alvarez and Calderon 1996). Infected, undersized crabs are called “button” crabs in the Gulf of Mexico (Noga et al. 1998, Guillory et al. 2001).

Parasitization by *L. texanus* is limited to moderate or higher salinities because larvae of *L. texanus* are not viable in salinities below 12 ppt. The ideal salinity and temperature for this barnacle are 25 ppt to 30 ppt and 21 °C to 25 °C, respectively (Shields and Overstreet 2007). Parasitization by *L. texanus* typically peaks in the late summer and fall (Adkins 1972, Hochberg et al. 1992).

### 1.3.2 Shell Rot

Shell rot is caused by chitinoclastic bacteria, which occur ubiquitously in marine environments. For infections to occur, the crab must have sustained an injury or be stressed (Cook and Lofton 1973, Gemperline et al. 1992). The disease starts as small marks that eventually coalesce to form necrotic lesions (Rosen 1967, Shields and Overstreet 2007).

Shell rot, while aesthetically unappealing, is generally innocuous to blue crabs. However, it can lead to more severe infections by harmful, opportunistic bacteria or fungi when a necrotic lesion exposes underlying tissues to the ambient water (Millikin and Williams 1984). Shell rot can become harmful, and even fatal, when several shell molts adhere to the necrotic lesion site (Vogan et al. 2001).

Shell rot can occur over a wide salinity range, but intensity of the disease is often dependent on water temperature as ecdysis occurs infrequently at cool temperatures. Also, older shells have more time to be exposed to pathogens that cause shell rot. For this reason, shell rot is usually most prevalent in the cooler months and in older, larger crabs (Sandifer and Eldridge 1974). Shell rot can also be prevalent in winter months due to burrowing activities that cause sediments to abrade the shell and expose the crab to high pathogen loads in the sediment (Shields and Overstreet 2007). Incidence of shell rot may also be higher in crabs held in captivity in aquaculture and shedding operations due to stress and crowding (Iverson and Beardsley 1976).

### 1.3.3 *Vibrio* spp.

*Vibrio* spp. are the largest portion of bacterial species infecting blue crabs and are often the primary chitinoclastic bacteria to cause shell rot (Davis and Sizemore 1982). In stressed, hypoxic hypercapnic crabs, clearance of *Vibrio* spp. from hemolymph decreases, and infection is probable (Holman et al. 2004). Infections with *Vibrio* spp. can result in cloudy hemocyte aggregations in gill lamellae and the walking legs (Bowser et al. 1981, Shields and Overstreet 2007). Intense infections can lead to weak, moribund crabs and to mass mortalities, especially in soft shell shedding facilities (Shields 1997). *Vibrio* spp. concentrations in the environment vary seasonally with peak prevalence in warm months (Huq et al. 1984, Welsh and Sizemore 1985). *Vibrio* spp. prevalence appears to be uncorrelated with salinity; however, at high salinities *Vibrio* spp. are the causative agents of shell rot while at low salinities *Aeromonas* spp. and *Pseudomonas* spp. are more prevalent in shell rot lesions (Noga et al. 1994).

#### 1.3.4 *Lagenophrys callinectes*

*Lagenophrys callinectes* is an ectocommensal, sessile, bacteriotrophic ciliate that is recognizable by its protective lorica around its cell body (Couch and Martin 1979). The lorica's attachment to the crab's gills and shell explains why the ciliate is shed with the crab's old cuticle during ecdysis (Mayen-Estrada and Aguilar-Aguilar 2012). Despite the commensalistic relationship, blue crabs can be harmed if this symbiont interferes with respiration and excretion and asphyxiation can occur (Scott and Thune 1986). Infested gill tissue may become brown or black and infested crabs are said to have black gill disease (Couch 1967, Schuwerack et al. 2001).

*L. callinectes* has been found in the Gulf and is capable of tolerating moderate salinities and low temperatures (Messick 1998). In Mississippi, the prevalence and intensity of *L. callinectes* infestations were not correlated with water temperature (Shields and Overstreet 2007). It has been proposed on the East Coast that *L. callinectes* may be a seasonal factor affecting blue crab survival, especially when water conditions are stressful for the crabs due to low oxygen, low temperature, pollution, etc. (Couch and Martin 1979).

#### 1.3.5 *Hematodinium perezii*

Another protozoan that infects blue crabs is the dinoflagellate *Hematodinium perezii*. This parasite is known to cause bitter crab disease in some crab species such as the snow crab and Tanner crab (Shields and Overstreet 2007). Bitter crab disease causes crab meat to have a bitter, aspirin-like taste due to biochemical alterations (Stentiford and Shields 2005). Although *H. perezii* does not cause this biochemical alteration in blue crabs, it does cause mass blue crab mortalities (Albalat et al. 2012). Crab population declines in Maryland and Virginia have been linked to high prevalence of *H. perezii* in the past two decades (Mancinelli et al. 2013). *H. perezii* will eventually kill all infected crabs (Meyers et al. 1996), and cannibalism is a major source of transmission (Hanif et al. 2013).

Infected crabs occasionally have a discolored, pink carapace (Meyers et al. 1990, Messick 1994). The parasite also changes the chemistry of the hemolymph, and hemolymph from infected blue crabs is usually milky or opaque and slow or unable to clot with a decreased hemocyte density (Williams-Ryan 1997). The dinoflagellate consumes hemocytes and hemocyanin, the oxygen transporter protein, causing respiratory complications (Taylor et al. 1996, Lee and Frischer 2004). In advanced stages, *Hematodinium* spp. cause total lysis of the hepatopancreatic tubules and some muscle lysis (Millikin and Williams 1984).

*H. perezii* infections depend on temperature and salinity. Researchers have noticed that infections typically peak in the fall and decline in the winter as water temperatures drop, especially below 9 °C (Messick et al. 1999, Lee and Frischer 2004, Shields and Overstreet 2007). However, only infection intensity, not prevalence, may fluctuate seasonally because subpatent infections may take 10 or more months to become patent infections (Beevers et al. 2012).

*Hematodinium* spp. have only been found in salinities above 11 ppt (Newman and Johnson 1975, Messick and Shields 2000). Reports of infected crabs inhabiting

salinities below 18 ppt are rare because the parasite dinospores, the hypothesized transmissive state, are only active at salinities above 20 ppt (Coffey et al. 2012). Additionally, areas with restricted water flow such as canals and embayments typically have a higher prevalence of the parasite due to limited migration of the crabs, concentrated crab populations, or limited water exchange (Meyers et al. 1996). In addition to the geographical variation of prevalence, infection rates are higher in juvenile blue crabs than in adults due to more frequent molting (Shields and Squyars 2000, Shields et al. 2003).

#### 1.3.6 *Urosporidium crescens*

*Urosporidium crescens* is a hyperparasite of the blue crab that causes pepper spot or buckshot disease. It infects a trematode, *Microphallus basodactylophallus*, and causes the worm to expand and darken (Newman et al. 1976, Messick 1998). *M. basodactylophallus* is commonly found in the thoracic muscles, hepatopancreas, and ventral ganglia of blue crabs and is small and lightly colored when uninfected (Messick and Sindermann 1992). *U. crescens* causes the trematode's encysted larvae to be sensitive to pressure and prone to rupture. If the infected larvae rupture while in a live crab, the hemocytes of the crab likely destroy the hyperparasite (Couch 1974). *U. crescens* can thrive in moderate salinities and can tolerate a wide temperature range of 2 °C to 22 °C or higher (Messick 1998); however, no known data exist on infections at low salinities. Although *U. crescens* does not harm the crab, it does result in visually unappetizing crab meat, and therefore, negatively affects the commercial industry (Couch and Martin 1979).

#### 1.3.7 *Ameson michaelis*

*Ameson michaelis* is a microsporidian pathogen that causes cotton crab or sick crab disease and can be found in the blue crab's muscle and hemolymph. *A. michaelis* causes muscle lysis, in which the muscle turns chalky or opaque and the abdomen turns grayish (Messick and Sindermann 1992, Shields and Overstreet 2007). The hemolymph's ion, amino acid, lactic acid, and glucose concentrations are all affected by this infection (Findley et al. 1981). Usually wild, infected crabs appear healthy. This pathogen has been documented in Lake Pontchartrain and along the Louisiana coast (Overstreet and Whatley 1975, Shields and Overstreet 2007). It can tolerate very low salinities (2 ppt) and thrives at high temperatures (Findley et al. 1981). It has been suggested that this microsporidian is transmitted by cannibalism.

#### 1.3.8 White Spot Syndrome Virus

*Whispovirus* spp. causes White Spot Syndrome Virus (WSSV) disease and has caused mass mortalities in shrimp cultures, as well as other crustacean populations (Shields and Overstreet 2007, Bateman et al. 2012). White spot is not considered a major crab virus; however, the blue crab is generally perceived as an asymptomatic host (Chang et al. 2001, Shields and Overstreet 2007). Some blue crab mortalities have been linked to the virus, but the data are highly varied (Stentiford et al. 2009). This virus

causes white spots associated with calcium deposition to appear on the carapace of some crustacean hosts, especially on shrimp (Stentiford et al. 2009). White spot virus infects the gills, stomach, hepatopancreas, and hemolymph (Browdy et al. 2006).

Prevalence of WSSV is dependent on both temperature and salinity. Rapid salinity changes affect the immunity of crustaceans against the virus and low temperatures prevent virus replication. It has been suggested that a salinity of 35 ppt and a temperature of 30 °C are optimal for WSSV proliferation (Gao et al. 2011). White spot virus has been detected in blue crabs from the Gulf of Mexico (Baumgartner et al. 2009).

The mechanism of transmission of the virus is not fully understood, but some research suggests that ingestion of infected tissues causes the virus to spread (Bateman et al. 2012). Other research suggests that infection is derived from viral particles in the water (Stentiford et al. 2009). Prevalence of WSSV in wild blue crabs is important because crabs transmit the virus to other crustacean populations such as farmed shrimp populations. This can then result in mass shrimp mortalities (Shields and Overstreet 2007).

#### 1.3.9 Reo-like Virus

Reo-like virus (RLV) is a double-stranded RNA virus that infects hemocytes, hemopoietic tissues, nervous system cells, gills, and connective tissue in blue crabs (Shields and Overstreet 2007, Bonami and Zhang 2011). Symptoms of infection include discolored, red or brownish gills and carapace, inability of hemolymph to clot, tremors, lethargy, and eventual paralysis (Johnson 1977, Shields and Overstreet 2007). Reo-like virus often co-occurs with other viruses, such as Rhabdo-like Virus A in blue crabs and *Baculovirus penaei* in white shrimp (Krol et al. 1990, Shields and Overstreet 2007). Experimentally, crabs injected with RLV-infected hemolymph or fed infected tissues experience high mortality rates (Shields 2003, Bowers et al. 2010). Reo-like virus is found at high and low salinities and has caused mass mortalities in soft shell shedding systems from Delaware to Florida (Shields and Overstreet 2007, Bowers et al. 2011). Cannibalism and cohabitation in crowded tanks may be means of transmission (Bowers et al. 2011).

### 1.4 Previous Blue Crab Disease, Parasite, and Symbiont Research

The majority of the research about these diseases, parasites, and symbionts is from the Atlantic Coast. In some instances, epizootics have been correlated to large declines in blue crab landings. *H. perezii* is one parasite known to cause blue crab population declines in Delaware, Maryland, and Virginia (Messick and Shields 2000). In 1991 and 1992 this dinoflagellate's prevalence of 70 to 100% in juveniles in Maryland and Virginia caused significant declines in catch (Shields and Overstreet 2007). Mortalities hypothesized to be caused by *Vibrio* spp. infections have been recorded as high as 80% in North Carolina shedding facilities (Sizemore 1985). *A. michaelis* is relatively rare, but can cause mass mortalities in shedding facilities (Messick and Sindermann 1992). Reo-like virus has been hypothesized to cause mass mortalities in

soft shell shedding systems but its prevalence rates are unknown (Bowers et al. 2010, Bowers et al. 2011).

The Atlantic Coast blue crab commercial industry is also harmed by non-fatal diseases, parasites, and commensalistic symbionts. High prevalence of shell rot can cause economic losses, as the shell rot is visually unappealing. Prevalence of shell rot along South Carolina's coast has been determined as high as 53.1% (Shields and Overstreet 2007). *U. crescens* can also cause economic losses as infected tissues are discolored and unappetizing (Messick and Sindermann 1992).

Research on the prevalence of blue crab diseases, parasites, and symbionts in the Gulf of Mexico has been more limited and sporadic than on the Atlantic Coast. The Atlantic Coast data are not transferrable to the Gulf Coast due to different water conditions such as the higher average water temperatures in the Gulf. Also, the Louisiana fishery includes low salinity ( $\leq 1$  ppt) waters, unlike fisheries on the Atlantic Coast (Guillory et al. 2001). Without consistent, population specific prevalence data, correlation between increased disease, parasite, and symbiont prevalence and decreased commercial landings in the Gulf is nearly impossible. This need for current data came to light after the Deepwater Horizon oil spill in the spring of 2010. After the spill and subsequent freshwater release from diversion openings, reports from fishermen of diseases and parasites in blue crabs increased, and in early 2011, above average die-offs in shedding facilities were reported (J.P. Hawke and J.A. Anderson, pers. comm.). However, with no baseline prevalence rates for Louisiana, no conclusions could be drawn on the potential impact of oil, dispersant, or increased freshwater on the prevalence rates of disease, parasite, and symbiont in blue crabs. Therefore, prevalence data is needed for future comparison if another catastrophic event or significantly decreased landings occur.

Previous research in the Gulf has been conducted for *L. texanus*, *L. callinectes*, *H. perezii*, and *A. michaelis*. *L. texanus* has been found in estuaries and lagoons in the Gulf with a moderate prevalence of 1.4% to 17.6% (Bortolini and Alvarez 2008). In the late 1970s, prevalence of this parasitic barnacle was recorded as high as 38% along Louisiana's coast (Wardle and Tirpak 1991). *L. texanus* is linked to major economic losses of blue crabs in the Gulf (Lazaro-Chavez et al. 1996). However, all previous prevalence data relied on gross visual detection but this combined with polymerase chain reaction (PCR) will more accurately estimate prevalence as infected crabs not yet bearing an externa can be included in the dataset (Sherman et al. 2008).

Research on *L. callinectes* in the Gulf has shown that this symbiont is abundant. In Mississippi, prevalence and intensity do not correlate with water temperatures and intensity of the infestations is often high (Shields and Overstreet 2007). *H. perezii* research has been on very few samples from the Gulf, and it has been found to be abundant in Mississippi (Shields and Overstreet 2007). The use of PCR is fairly new for *Hematodinium* spp. infection detection, and researchers have not used it for many Louisiana blue crabs yet (Shields and Overstreet 2007, Lohan et al. 2012). PCR will increase detection rates and decrease the amount of time to detect the parasite, so *H. perezii* prevalence data for the Gulf will be easier to establish in the future.

*U. crescens* and shell rot have been observed in the Gulf but prevalence rates have not been determined. The LSU Animal Disease Diagnostic Lab has conducted some WSSV testing, but no prevalence has been established and sample sizes have

been low (J.P. Hawke, pers. comm.). The microsporidian in this study, *A. michaelis*, has been found in Lake Pontchartrain during studies conducted during the 1970s and 1980s with little prevalence data collected since (Shields and Overstreet 2007).

## 1.5 Significance for the Louisiana Fishery

Louisiana has the largest blue crab fishery in the USA. In Louisiana in 2012, approximately 20,600 metric tons of blue crab were landed for a value of \$43.1 million, with peelers and soft shells included (National Marine Fisheries Service 2013). For all of the Gulf States, about 24,800 metric tons were landed valued at \$52.9 million, with peelers and soft shells included (National Marine Fisheries Service 2013). However, in order to have a thriving fishery, the wild populations' health must be understood and considered in management decisions.

All of the previously described nine diseases, parasites, and symbionts are known to occur in the Gulf, whether through observational reports or sporadic scientific surveys. However, there was still substantial prevalence research to be conducted, and these prevalence data could help managers of the largest fishery better understand the health of the blue crab populations and future landing trends.

## 1.6 Research Objectives

The objectives of this research were to determine the prevalence of selected diseases, parasites, and symbionts in wild blue crab populations along Louisiana's coast and in populations held at commercial soft shell shedding facilities. Specifically, I determined the prevalence of *L. callinectes*, *U. crescens*, *H. perezii*, *L. texanus*, RLV, shell rot, and *Vibrio* spp. in crabs collected from four coastal locations and four shedding facilities. Additionally, I determined the prevalence of WSSV in the wild populations. I recorded infections by non-*Vibrio* bacteria and by *A. michaelis*. Furthermore, I determined how prevalence of these diseases, parasites, and symbionts varied between and within each collection site and determined significant predictor variables for the prevalence rates. Variables predicted to influence prevalence included collection site, season, salinity, water temperature, crab sex, crab size, and capture method.

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## CHAPTER 2: PREVALENCE OF DISEASES, PARASITES, AND SYMBIONTS IN WILD BLUE CRAB POPULATIONS

### 2.1 Introduction

Diseases and parasites of some marine organisms are well studied and their prevalence rates documented. For example, the Office International des Épidémiologies (OIE) investigates and monitors fish, mollusc, and crustacean diseases such as MSX in the Eastern oyster (*Crassostrea virginica*) and white spot disease in penaeid shrimp (Office International des Epizooties 2009). However, diseases, parasites, and symbionts of the blue crab (*Callinectes sapidus*) are not as well understood, and their prevalence has not been determined in parts of the crab's range, including the Gulf of Mexico.

Blue crab symbionts such as *Lagenophrys callinectes*, *Urosporidium crescens*, and white spot syndrome virus (WSSV) are usually innocuous to the crab, while parasites such as *Hematodinium perezii*, *Loxothylacus texanus*, and reo-like virus (RLV) are harmful (Shields and Overstreet 2007). *H. perezii* has been correlated with mass mortalities and decreased commercial landings of blue crabs on the East Coast (Shields 2003, Mancinelli et al. 2013). *L. texanus* causes sterilization and suppression of ecdysis in its host, which consequently decrease population numbers and the number of legal sized crabs able to be commercially landed (Ragan and Matherne 1974, Shields and Overstreet 2007). Symptoms of RLV infection include tremors, lethargy, and paralysis (Johnson 1977, Shields and Overstreet 2007).

Bacteria such as *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. can also be harmful to infected crabs by causing shell rot and mass mortalities (Noga et al. 1994, Shields and Overstreet 2007). Shell rot is harmless to crabs unless it is an intense infection with necrotic lesions that expose underlying tissues and organs to pathogens in the water (Millikin and Williams 1984).

Some of these diseases, parasites, and symbionts are limited by water temperature and salinity. *L. callinectes* infests blue crab gills and is shed during ecdysis (Shields and Overstreet 2007). Prevalence of this ciliate is therefore hypothesized to be highest when molting is infrequent. However, prevalence in the Gulf has not been highly correlated with water temperature possibly because waters tend to stay warmer year-round as compared to the East Coast (Shields and Overstreet 2007). *U. crescens*, the causative haplosporidian of buckshot or pepper spot crabs, hyperparasitizes trematodes in blue crab muscle, hepatopancreas, and ventral ganglia at moderate salinities but may not tolerate low salinities of freshwater dominated estuaries such as Lake Pontchartrain (Messick and Sindermann 1992, Messick 1998). *H. perezii* is dependent on salinities above 11 ppt with the transmissible dinospores only active above 20 ppt (Newman and Johnson 1975, Messick and Shields 2000, Coffey et al. 2012). *H. perezii* epizootics vary seasonally with a peak in the fall and absence at water temperatures below 9 °C (Messick et al. 1999, Lee and Frischer 2004). *L. texanus* larvae are only viable above 12 ppt, and parasitization by this barnacle typically peaks during the late summer and fall when water temperatures are warm (Adkins 1972, Shields and Overstreet 2007). Bacteria are ubiquitous across a wide salinity range and peak in abundance when waters are warmest (Huq et al. 1984, Welsh and Sizemore 1985). Shell rot prevalence is dependent on ecdysis, which partially depends on water temperature. Shell rot is



typically most prevalent when crabs molt infrequently and burrow more frequently in cold winter months (Sandifer and Eldridge 1974, Shields and Overstreet 2007). No specific salinity and temperature trends have been found for WSSV and RLV; however, infections by both are hypothesized to be most common when waters are warmer due to faster replication.

In Louisiana, little research has been dedicated to blue crab diseases, parasites, and symbionts, despite the state having the largest blue crab fishery with a 2012 dockside value of \$43.1 million (National Marine Fisheries Service 2013). Following the Deepwater Horizon oil spill in 2010, this knowledge gap became evident when processors, fishermen, and shedding facilities reported higher than average die-offs and higher disease and parasite prevalence. However, with no previous prevalence rates for Louisiana established, managers and researchers could not determine the effects of the oil, dispersant, or freshwater diversions on the prevalence rates of blue crab diseases, parasites, and symbionts. To determine effects of natural and anthropogenic disasters on prevalence rates in the future and to more fully understand population health and commercial landing trends, I determined the prevalence of *L. callinectes*, *U. crescens*, WSSV, *H. perezii*, *L. texanus*, shell rot, and *Vibrio* spp. in blue crabs caught along the Louisiana coast in 2013 and the beginning of 2014. I also detected RLV and non-*Vibrio* bacteria in the samples.

## **2.2 Materials and Methods**

### **2.2.1 Sample Sites**

The four field sites for collection of wild crabs were (East to West): Lake Pontchartrain, Grand Isle, Cocodrie, and Rockefeller Wildlife Refuge (Fig. 2.1, Table 2.1). These sites were chosen to represent a salinity gradient from low salinity at Lake Pontchartrain and to moderately-high salinity at Grand Isle (Table 2.2). Both Rockefeller and Cocodrie have moderate salinities ranging from 5 to 20 ppt depending on the specific location (Table 2.2). However, these two locations differ in fishing pressure. Rockefeller is a protected area with no commercial fishing while Cocodrie in Terrebonne Bay experiences high commercial fishing effort.

### **2.2.2 Sample Collection**

I collected crabs at each of the field sites seasonally. Seasons were determined predominantly by water temperature, where winter was 20 °C or colder, spring and fall were 20-25 °C, and summer was greater than 25 °C.

I collected crabs at the four field sites by seining (304.8 cm x 152.4 cm net with 0.635 cm mesh and 304.8 cm x 182.9 cm net with 2.54 cm mesh), trawling (standard 16 ft. otter and bait trawl nets), baited trapping, unbaited trapping, dip netting, and using a baited line. Bait in this study included chicken and Atlantic menhaden. When traps were set, all crabs were in the traps for 48 hours or less to minimize stress that can increase injury and *Vibrio* spp. infections.



Figure 2.1: Sample sites for blue crab collection were (East to West): Lake Pontchartrain, Grand Isle, Cocodrie, and Rockefeller Wildlife Refuge (red stars).

Table 2.1: Specific sampling locations and corresponding GPS locations within the four sample sites, Lake Pontchartrain, Grand Isle, Cocodrie, and Rockefeller Wildlife Refuge.

Site	Sampling Location Name	GPS Location
Pontchartrain	Fontainebleau State Park	30° 20' 5.1684", -90° 2' 42.3738"
	Big Branch Marsh National Wildlife Refuge	30° 15' 3.2826", -89° 51' 56.1198"
	North Shore off LA-433	30° 12' 55.1406", -89° 45' 0.2196"
	Bayou Savage National Wildlife Refuge	30° 9' 13.4526", -89° 51' 34.4916"
Grand Isle	Sea Grant Oyster Hatchery	29° 14' 19.1898", -90° 0' 11.2392"
	Grand Isle beach	29° 13' 46.4298", -89° 59' 46.482"
	Elmer's Island	29° 11' 22.4118", -90° 4' 1.7862"
	Ferblanc Bayou	29° 10' 30.0756", -90° 8' 6.9678"
Cocodrie	Bayou Little Caillou	29° 21' 2.7282", -90° 37' 34.3956"
	Dulac Beach	29° 19' 30.6078", -90° 41' 2.6556"
	Louisiana Universities Marine Consortium Facility	29° 15' 19.8504", -90° 39' 52.8222"
	Houma Navigation Channel	29° 13' 54.2496", -90° 40' 11.9784"
Rockefeller	Headquarters Canal	29° 43' 28.3614", -92° 49' 5.8578"
	Bayous off Price Lake Rd	29° 41' 26.3826", -92° 49' 52.9788"

Table 2.2: Salinity ranges and average water temperatures sampled in 2013 and the beginning of 2014 at Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle (-- denotes unsuccessful collection).

Site	Water Parameter	Winter 2013	Spring	Summer	Fall	Winter 2013-2014
Pontchartrain	Salinity (ppt)	--	1.6-2.1	1.4-5.3	5-7.6	--
	Avg. Temp. (°C)	--	15.78	29.35	24.44	--
Cocodrie	Salinity (ppt)	--	--	3.1-16.6	5.2-16.9	4.7-17.5
	Avg. Temp. (°C)	--	--	30.22	24.44	15.29
Rockefeller	Salinity (ppt)	0.6-3.55	4.7-8.7	10.8-17	10.9-19.5	9.5-16.6
	Avg. Temp. (°C)	11.77	18.91	29.99	25.44	11.87
Grand Isle	Salinity (ppt)	13.6-13.9	18.3-26.7	9.6-25.4	20.8-26.6	13.1-26.3
	Avg. Temp. (°C)	16.89	17.79	29.78	24.42	13.84

I collected large juvenile and adult blue crabs that had a carapace width (CW) of 11 cm or larger, lateral spine to lateral spine. Equal sampling of males and females across all sites and seasons was not possible due to the differing migration patterns of the sexes during different seasons.

During each collection, salinity and water temperature were measured with a YSI 30-10FT or YSI 63-10FT at the completion of the collection (Table 2.2). When traps were set, temperature and salinity were measured only when traps were pulled. To remove daily and diel water temperature variation within a season, I gathered water temperature data from nearby NOAA/USGS buoys and water monitoring stations, including: 1) Rigolets USGS buoy and Lake Pontchartrain Basin Foundation weekly water quality data; 2) Grand Isle NOAA buoy; 3) USGS Houma Navigation Channel buoy in Dulac and Louisiana Universities Marine Consortium Marine Center; and 4) Freshwater Canal NOAA buoy. These water temperature data were averaged starting a maximum of 21 days before the first sampling when temperatures began to shift through the last sampling at that site for that season. From these averaged data, winter water temperatures were less than 16.9 °C; spring were 15.8 to 18.9 °C; summer were 29.3 to 30.3 °C; and fall were 23.9 to 25.4 °C (Table 2.2).

During the winter and spring of 2013, I did not use any baited capture methods and used only seining and trawling. I introduced baited capture in the summer to increase the sample size to 60 crabs per site per season. In total, 768 crabs were collected from the field sites during this study (Table 2.3). Live crabs were placed on top of burlap-covered ice in coolers for transport to LSU. Crabs collected with different methods were kept separate.

A small subset of crabs that were <11 cm CW were brought back to LSU, frozen at -20 °C, and dissected for *Loxothylacus texanus* detection. These crabs were included only for *L. texanus* because infected crabs are typically undersized (Adkins 1972, Overstreet et al. 1983). I specifically collected crabs <10 cm CW in August and September 2013 at the two highest salinity sites, Rockefeller and Grand Isle, using a baited line and miniature baited crab traps (23.5 cm x 22.9 cm x 12.7 cm; Southern Wire, Raceland, LA). The small crabs collected in other months were caught with crabs >11 cm CW in a seine net or baited traps (Table 2.4). Total, I collected 156 small juveniles (Table 2.4).

Table 2.3: Sample sizes of large juvenile and adult (> 11 cm carapace width) blue crabs collected in 2013 and the beginning of 2014 at Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle.

Site	Winter 2013	Spring	Summer	Fall	Winter 2013-2014
Pontchartrain	0	24	60	60	0
Cocodrie	0	0	60	60	60
Rockefeller	17	43	60	64	60
Grand Isle	7	13	60	60	60

Table 2.4: Sample sizes of small (< 10 cm carapace width) blue crabs collected in 2013 for *Loxothylacus texanus* detection at Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle.

Site	Winter 2013	Summer
Pontchartrain	0	32
Cocodrie	0	11
Rockefeller	5	47
Grand Isle	8	53

### 2.2.3 Initial Processing and Dissection

All crabs were dissected alive. I used ice and cold water to reduce crab mobility for dissection. Before any dissection began, I measured the CW to the nearest millimeter with a standard metric ruler and sexed the crab. Every crab was photographed with a Canon camera against a white background with a unique identifier including a designation for the collection site and individual crab (ex. R121 was the 121<sup>st</sup> crab collected from Rockefeller). Photographs included all claws and legs and captured both the dorsal and ventral sides of the crab. These images were later analyzed in the computer program photoQuad for percent coverage of the carapace with shell rot. Close-up photographs of shell rot lesions and spots were also taken when possible. I visually estimated percent coverage of the shell with shell rot and scored it from 0 to 3 based on the estimation (Table 2.5). Shell rot intensity was also scored from 0 to 2 based on the progression of the disease to necrosis (Table 2.6). Any other visual abnormalities or external signs of infection were noted.

Next, I cleaned the right swimming leg (based on dorsal view) at the uncalcified joint near the carapace with a cotton swab and 70% ethanol (Welsh and Sizemore 1985). To ensure complete sterilization of the joint, I sterilized the leg twice. A sterile needle (18 to 25 gauge) with a sterile, 3-cc syringe was inserted into the leg. Hemolymph was drawn without the removal of the needle and was plated on sterile, *Vibrio* spp. selective (Colwell et al. 1975) thiosulfate-citrate-bile salts-sucrose (TCBS) agar prepared per manufacturer instructions (Sigma-Aldrich, St. Louis, MO). After plating <0.5 mL of hemolymph on the agar, an additional 1 mL of hemolymph was drawn and preserved in 95% ethanol for polymerase chain reaction (PCR) analysis.

Table 2.5: Shell rot intensity score based on visual observations and percent coverage. Adapted from: Castro and Somers 2012, Messick 2012.

Visual observations	% Coverage of Shell	Intensity
Clean shell, discoloration easily scrapes off	0%	0
Discolored spot, mild	1-10%	1
Moderate, many discolored spots	11-50%	2
Severe, necrosis, covered in discolored spots	51-100%	3

Table 2.6: Shell rot intensity score based on progression of shell rot as the presence of spots and/or lesions.

Visual observations	Intensity
No shell rot	0
Shell rot spots only (no lesions)	1
Necrotic lesions (deep, shell eroded)	2

After withdrawing the hemolymph, one walking leg was removed and frozen at -20 °C for RLV PCR. Then, the crab was dissected and hepatopancreas, gill, heart, and midgut samples were removed (Appendix A). One sample of hepatopancreas was frozen at -20 °C and a second was preserved in 95% ethanol for *L. texanus* PCR. I removed and froze at -20 °C two samples of gill for *L. callinectes* and WSSV analyses. The heart was frozen at -20 °C and the midgut was preserved in 95% ethanol for *L. texanus* PCR. While removing these tissues, I noted any abnormalities or signs of infection. I checked the muscle underneath the gills for infection by *U. crescens* or *A. michaelis*. I also cracked the abdomen to ensure that there were no *L. texanus* externa. After each dissection, forceps and other dissection tools were sterilized in a 10% bleach solution to minimize DNA transfer between crabs.

During the winter and spring seasons, I fixed samples of hepatopancreas and gill in zinc buffered formalin (Z-Fix) for histological analyses. After histology was determined ineffective, I stopped removing and fixing tissues for standard histology. However, when a particularly abnormal tissue was noticed, I removed that tissue and fixed it in 1.25% glutaraldehyde with 2% paraformaldehyde and 5% sucrose. These fixed tissues were transferred to 0.1 M cacodylate buffer and delivered to Dr. Julia Sokolova at the LSU Veterinary Medicine Microscopy Center.

For crabs <11 cm CW collected for *L. texanus*, I thawed the crabs in warm tap water for a minimum of 1 hr prior to dissection to ease removal of the fragile midgut. Once crabs were thawed, I cut the dorsal carapace, removed samples of hepatopancreas and the midgut, and preserved both tissues in 95% ethanol for *L. texanus* PCR (Appendix A).

#### 2.2.4 Detection of *Lagenophrys callinectes*

I detected *L. callinectes* on frozen gills based on the presence of the ciliate's circular lorica (Couch and Martin 1979, Mayen-Estrada and Aguilar-Aguilar 2012). Thin gill sections (<1 cm thick on average) were cut from the gills with a scalpel blade, placed on microscope slides, and immediately examined under a light microscope at a magnification of 10X or higher. For the majority of the gills collected in the fall and in the



winter 2013-2014, presence of *L. callinectes* was only recorded when an occupied lorica was seen in order to detect only active infestations (Fig. 2.2 and 2.3). In samples collected earlier than the fall, samples were determined to be infested if any lorica, occupied or not, was present (Fig. 2.2 and 2.3). Despite this difference, the presence of many empty loricae co-occurred with occupied loricae, so the potential error in recording presence of *L. callinectes* with no distinction between empty and occupied loricae is likely minimal. When a lorica was not present in the first section, a minimum of three sections were examined to confirm absence of the symbiont.

In the winter and spring of 2013, I tried to detect *L. callinectes* on decalcified gill that I embedded in paraffin and stained with hematoxylin and eosin. The goal was to create a permanent, preserved record of infested gills. However, these histological processes led to loss of the loricae from gill tissue and histology was discontinued.

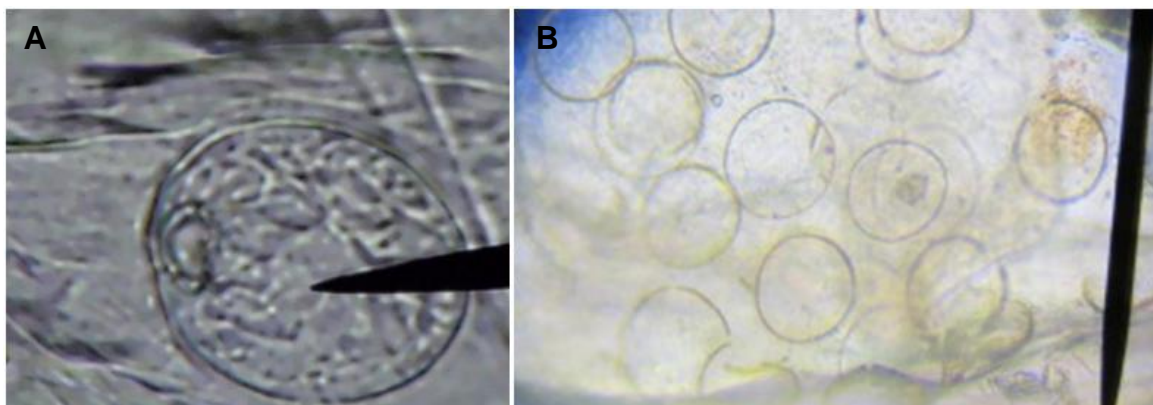


Figure 2.2: A: An occupied *Lagenophrys callinectes* lorica (arrow) present on gill tissue collected in April 2013. The lips of the lorica aperture are visible (left of arrow). This gill had the ectocommensalistic symbiont. B: Unoccupied *Lagenophrys callinectes* circular loricae present on gill tissue collected in April 2013.

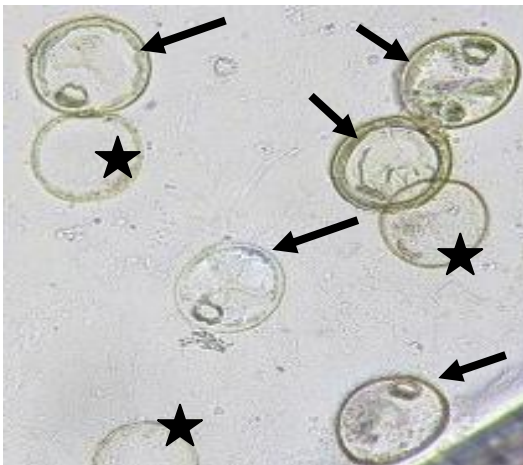


Figure 2.3: Occupied *Lagenophrys callinectes* loricae (arrows) co-occur with empty loricae (stars) on blue crab gills.

### 2.2.5 Detection of *Urosporidium crescens*

Presence of *U. crescens* is very noticeable as black spots in the hepatopancreas or muscle during dissection (Fig. 2.4; Section 2.2.3, Appendix A). I planned to also detect *U. crescens* with histology to include a permanent slide record of the infection. However, due to histology's small field of inspection, this methodology was not useful for *U. crescens*. Therefore, I abandoned histology after the spring season and only detected this hyperparasite visually.



Figure 2.4: *Urosporidium crescens* hyperparasitizes a trematode found in blue crab hepatopancreas and muscle. A: Large, black, *U. crescens*-infected, encysted trematode larvae found in a crab collected at Grand Isle in October 2013 (red circle). B: Smaller, black *U. crescens* spots in the hepatopancreas of a crab collected at Grand Isle in June 2013 (red circles).

### 2.2.6 Detection of White Spot Syndrome Virus (WSSV)

For WSSV, I analyzed all crabs collected during the winter and spring of 2013. However, during the summer, fall, and winter 2013-2014, I performed PCR on a subset of 45 crabs from each site during these three seasons to reduce costs. The 45 crabs were randomly selected from the total set of crabs collected at that site during that season.

Because WSSV is very difficult to detect by histology and requires a trained eye, I only used PCR to determine the presence or absence of WSSV in crab gill tissue (Chang et al. 2001, Galaviz-Silva et al. 2004). The gill was removed during dissection and frozen at -20 °C for DNA extraction (Section 2.2.3, Appendix A). Gill tissue samples were lysed overnight and then extracted with Qiagen's DNeasy Blood and Tissue kits per the manufacturer recommendations with a 5 minute elution incubation and one 200 µl elution. For PCR, I used the general decapod primers 143F (5'-TGCCTTATCAGCTNTCGATTGTAG-3') and 145R (5'-TTCAGNTTTGCAACCATCTCCC-3') to verify that each sample contained amplifiable DNA (Lo et al. 1996b, Baumgartner et al. 2009). In these primers "N" represents any of the four DNA nitrogenous bases. To specifically detect WSSV, I used the primers 146F2 (5'-GTAAGTCCCCCTTCCATCTCCA-3') and 146R2 (5'-TACGGCAGCTGCTGCACCTTGT-3') (Baumgartner et al. 2009, Nunan and Lightner

2011). Both PCR reactions contained 1X standard *Taq* (Mg-free) reaction buffer (New England Biolabs, Ipswich, MA), 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 5 µM forward primer, 5 µM reverse primer, 2.5 units of *Taq* polymerase, and 1 mg/mL BSA or RSA (Baumgartner et al. 2009). The reactions were brought to a volume of 25 µl with Nanopure water. Thermocycling conditions were: 94.0 °C for 3 min; 40 cycles of 94.0 °C for 20 s, 62.0 °C for 20 s, 72.0 °C for 30 s; and a final extension at 72.0 °C for 3 min (Nunan and Lightner 2011). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller.

Amplified products were separated by 1% agarose gel electrophoresis and stained with EZ-Vision DNA Dye (Amresco, Solon, OH; diluted from 6X stock concentration to approximately 1.2X). I stained 2 µl of PCR product with 2 µl diluted EZ-Vision and then ran the electrophoresis at 150 volts in sodium borate (SB) buffer. Gels were visualized with a FisherBiotech 312 nm UV transilluminator and photographed with a Kodak Gel Logic 112. All PCR runs included a negative control and a positive control of DNA extracted from ethanol-preserved shrimp infected with WSSV that was obtained from the OIE WSSV reference lab at University of Arizona. Extraction of the positive control was consistent with extractions of study samples. Amplifications were considered successful when the decapod primers amplified DNA from each sample (848 bp) and both primer pairs amplified the positive control but neither amplified the negative control (Fig. 2.5). If the decapod primers did not amplify a sample, I extracted a new piece of the gill tissue and amplified it again. If the second amplification was unsuccessful, I was unable to assess that particular crab for a WSSV infection. I was unable to assess one sample from Rockefeller in the spring, one from Rockefeller in both the summer and fall, and three from Cocodrie, two from Rockefeller, and four from Grand Isle in the winter of 2013-2014 (12 samples total). Presence of WSSV was recorded when a 942 bp band was present for the 146F2 and 146R2 primers (Fig. 2.5; Lo et al. 1996b, Nunan and Lightner 2011).

### 2.2.7 Detection of *Hematodinium perezii*

*H. perezii* can be detected in stained, fresh hemolymph smears or by PCR. Due to unfamiliarity and inexperience with this parasite, I was unable to identify *H. perezii* by staining hemolymph with neutral red stain, and therefore, I only used PCR to detect the dinoflagellate (Shields 2012). Hemolymph was originally frozen at -20 °C for DNA extraction; however, due to clotting I began to preserve hemolymph in 95% ethanol during the spring sampling (Section 2.2.3).

Prior to DNA extraction, I centrifuged approximately 200 µl of ethanol-preserved hemolymph at 1500 g for 1 min and removed the excess ethanol (Lohan et al. 2012). Samples dried for at least 30 min to allow residual ethanol to evaporate (Lohan et al. 2012). Hemolymph samples were lysed overnight with a few rare exceptions when lysis was limited to approximately 4 hours. To extract DNA from the lysed hemolymph, I used Qiagen's DNeasy Blood and Tissue kits per the manufacturer recommendations with two 5 minute elution incubations and two 100 µl elutions.



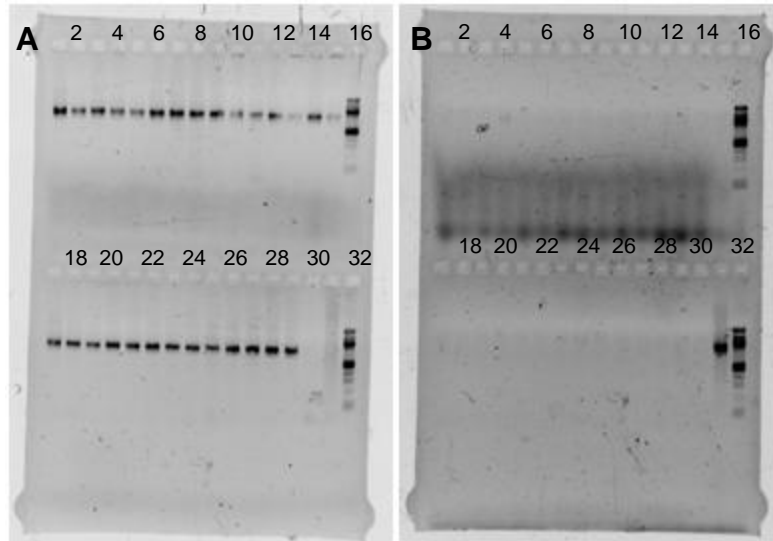


Figure 2.5: Agarose gel for white spot virus (WSSV) gill tissue PCR results. These samples were from the spring of 2013. A: Gel for the decapod primers (143F, 145R). Each band in lanes 1-15 and 17-29 indicates amplifiable DNA in the sample. Lane 30 is the negative control and lane 31 is the positive control. Lanes 16 and 32 are 100 bp ladders. B: Gel for the WSSV primers (146F2, 146R2). Only the positive control lane has a band (lane 31), indicating that the samples (lanes 1-15, 17-29) were not infected with WSSV. Lane 30 is a negative control and lanes 16 and 32 are 100 bp ladders.

For PCR, I used the general metazoan primers nSSU A (5'-AACCTGGRTTGATCCTGATCCTGCCAGT-3') and nSSU B (5'-GATCCTTCCGCAGGTTTACCTAC-3') to verify that each sample contained amplifiable DNA (Lohan et al. 2012). This PCR reaction contained 1X standard *Taq* (Mg-free) reaction buffer (New England Biolabs, Ipswich, MA), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1 μM nSSU A, 1 μM nSSU B, 1 unit of *Taq* polymerase, and 0.4 mg/mL BSA or RSA (Lohan et al. 2012). The reaction was brought to a volume of 10 μl with Nanopure water. Thermocycling conditions were: 95.0 °C for 4 min; 45 cycles of 94.0 °C for 30 s, 45.0 °C for 30 s, 72.0 °C for 2 min; and a final extension at 72.0 °C for 5 min (modified from Lohan et al. 2012). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller.

To detect *Hematodinium* sp., I used the *Hematodinium* sp. primers HITS1F (5'-CATTCACCGTGAACCTTAGCC-3') and HITS1R (5'-CTAGTCATACGTTTGAAGAAAGCC-3') (Lohan et al. 2012). This PCR reaction contained 1X standard *Taq* (Mg-free) reaction buffer, 1.5 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.5 μM HITS1F, 0.5 μM HITS1R, and 1 unit of *Taq* polymerase (Lohan et al. 2012). The reaction was brought to a volume of 10 μl with Nanopure water. After November 1, 2013, PCR reactions contained 1 mM MgCl<sub>2</sub> instead of 1.5 mM in order to optimize the reaction (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, 72.0 °C for 90 s; and a final extension at 72.0 °C for 5 min (modified from Lohan et al. 2012). Thermocycling was performed in an Eppendorf Mastercycler proS thermocycler.

To detect *Hematodinium* spp., I used a second set of primers that target the SSU rRNA gene that is more conserved than the ITS1 region targeted by HITS1 primers (Lohan et al. 2012). This second *Hematodinium* spp. primer pair was Hemat-F-1487 (5'-CCTGGCTCGATAGAGTTG-3') and Hemat-R-1654 (5'-GGCTGCCGTCCGAATTATTCAC-3') (Lohan et al. 2012). This PCR reaction contained 1X standard *Taq* (Mg-free) reaction buffer, 1.5 mM MgCl<sub>2</sub>, 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 BSA or RSA (Lohan et al. 2012). The reaction was brought to a volume of 10 μl with Nanopure water. Thermocycling conditions were: 95.0 °C for 10 min; 40 cycles of 94.0 °C for 30 s, 56.0 °C for 30 s, 72.0 °C for 1 min; and a final extension at 72.0 °C for 10 min (modified from Lohan et al. 2012). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller.

Amplified products were separated by 2% agarose gel electrophoresis and stained with EZ-Vision DNA Dye (Amresco, Solon, OH; diluted from 6X stock concentration to approximately 1.2X). I stained 2 μl of PCR product with 2 μl diluted EZ-Vision and then ran the electrophoresis at 150 volts in SB buffer. Gels were visualized with a FisherBiotech 312 nm UV transilluminator and photographed with a Kodak Gel Logic 112. All PCR runs included a negative control and a positive control of DNA extracted from ethanol-preserved hemolymph infected with *H. perezii* that was obtained from Dr. Shields at Virginia Institute of Marine Science. Extraction of the positive control was consistent with sample extraction methods. Amplifications were only considered successful when the metazoan primers amplified DNA from each sample (~ 1700 bp) and all three primer pairs amplified the positive control but none amplified the negative control (Fig. 2.6). If a sample did not amplify with the metazoan primers, I re-extracted a new sample of hemolymph and amplified it again. If the second amplification was unsuccessful, I was unable to assess that particular crab for a *H. perezii* infection. Due to unsuccessful amplifications, one sample from Rockefeller in the winter of 2013, one from Grand Isle in the spring, one from Rockefeller in the summer, one from Cocodrie in the fall, three from Rockefeller in the fall, and one from Grand Isle in winter 2013-2014 (eight samples total) could not be assessed. Presence of *H. perezii* was recorded whenever a 302 bp band was present on the gel for the HITS1 primers and a 187 bp band was present for the Hemat primers (Fig. 2.6; Lohan et al. 2012).

#### 2.2.8 Detection of *Loxothylacus texanus*

*L. texanus* is rare at salinities below 12 ppt because its larvae are not viable and low salinities can cause loss of barnacle externa (Shields and Overstreet 2007). Because of this salinity limit, I did not use PCR to detect *L. texanus* in any of the crabs collected from Pontchartrain after spring 2013. Also the average size of infected crabs is usually less than 10 cm CW and the largest recorded infected crab had a CW of 13.5 cm (Adkins 1972, Overstreet et al. 1983, Alvarez and Calderon 1996). Therefore, after the spring of 2013, I established a maximum size of 15 cm CW for PCR analysis (Table 2.7). However, all crabs collected were examined for externa. Additionally, I performed PCR on 156 crabs that had a CW <11 cm (Table 2.4, Section 2.2.2).

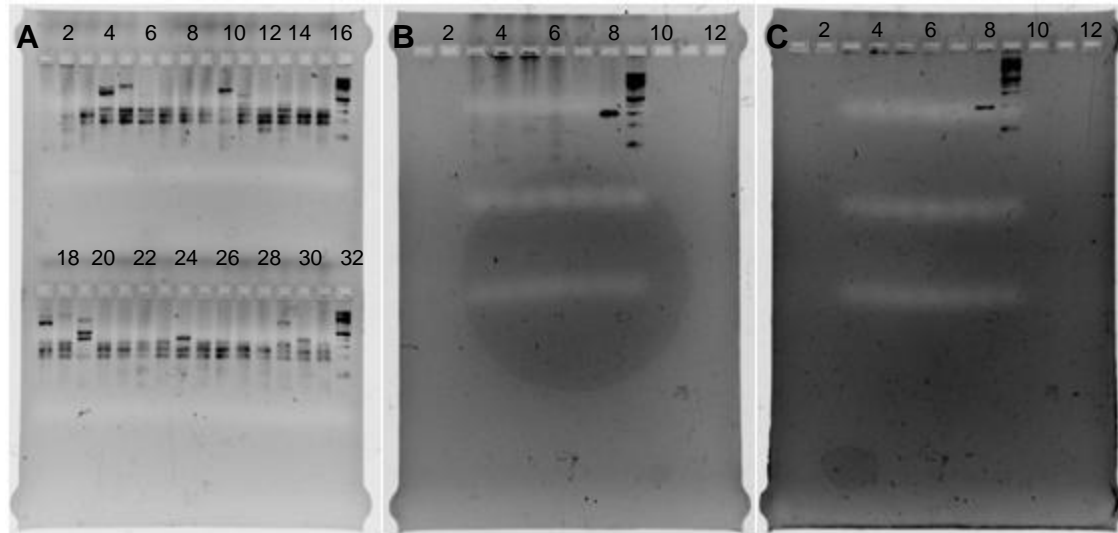


Figure 2.6: Agarose gel results from *Hematodinium perezii* PCR on hemolymph. Samples were collected in the fall of 2013. A: Gel for the metazoan primers (nSSU A, nSSU B). Each lane with distinguishable bands indicates amplifiable DNA in the sample (lanes 1-15, 17-31). The particular band of interest was the largest band ~1700 bp. Lanes 16 and 32 are 100 bp ladders. B: Gel for *H. perezii* specific primers (HITS1F, HITS1R). Only the positive control lane has a band (lane 8), indicating that the samples (lanes 1-7) were not infected with *H. perezii*. Lane 9 is a 100 bp ladder and lanes 10-12 were empty. C: Gel for *H. perezii* specific primers (Hemat-F-1487, Hemat-R-1654). Only the positive control lane (lane 8) has a band, indicating that the samples (lanes 1-7) were not infected with *H. perezii*. Lane 9 is a 100 bp ladder and lanes 10-12 were empty.

To detect infections that had not yet developed externa, I did PCR on hepatopancreas and midgut from each crab. All tissue samples were lysed overnight prior to extraction. To extract DNA from the tissues, I used Qiagen's DNeasy Blood and Tissue kits per the manufacturer recommendations with a 5 minute elution incubation and one 200 µl elution. For PCR, I used the universal crustacean primers HI (5'-GTGCATGGCCGTTCTTAGTTG-3') and 329 (5'-TAATGATCCTTCCGCAGGTTACCTACG-3') to verify that each sample contained amplifiable DNA (Sherman et al. 2008). To detect *L. texanus* infections, I used the same forward HI primer with reverse Loxo3 primer (5'-ACGTTTGATTGCGCGCGCACTGTCTGC-3') (Sherman et al. 2008). Both PCR reactions contained 1X standard *Taq* (Mg-free) reaction buffer (New England Biolabs, Ipswich, MA), 1.75 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.4 µM HI primer, 0.4 µM 329 or Loxo3 reverse primer, 1 unit of *Taq* polymerase, and 0.2 mg/mL BSA or RSA (Sherman et al. 2008). All reactions were brought to a volume of 10 µl with Nanopure water.

Thermocycling conditions were: 95.0 °C for 5 min; 30 cycles of 95.0 °C for 40 s, 66.8 °C for 25 s, 72.0 °C for 3 min; and a final extension at 72.0 °C for 10 min (Sherman et al. 2008). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller. Amplified products were separated by 1% agarose gel electrophoresis after staining with EZ-Vision DNA Dye (Amresco, Solon, OH; diluted from 6X stock concentration to approximately 1.2X). I stained 2 µl of PCR product with 2 µl diluted EZ-Vision and then

ran the electrophoresis at 150 volts in SB buffer. Gels were visualized with a FisherBiotech 312 nm UV transilluminator and photographed with a Kodak Gel Logic 112. All PCR runs included a negative control and a positive control of DNA extracted from a *L. texanus* externa provided by Dr. Sherman from University of South Alabama. Extraction of the positive control was consistent with extraction methods for samples. Amplifications were only considered successful when the crustacean primers amplified DNA from each sample (~ 500 bp) and both primer pairs amplified the positive control but neither amplified the negative control (Fig. 2.7). If neither a crab's hepatopancreas nor midgut amplified with the crustacean primers, I re-extracted new hepatopancreas and midgut samples and amplified them again. If the second amplifications were both unsuccessful, I was unable to assess that particular crab for *L. texanus* parasitization. Presence of *L. texanus* was recorded whenever a 237 bp band was present on the gel for the primer pair HI and Loxo3 (Fig. 2.7; Sherman et al. 2008).

Table 2.7: *Loxothylacus texanus* PCR sample sizes of large crabs (> 11 cm carapace width) collected in 2013 and the beginning of 2014 at Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle.

Site	Winter 2013	Spring	Summer	Fall	Winter 2013-2014
Pontchartrain	0	23	0	0	0
Cocodrie	0	0	33	20	24
Rockefeller	17	39	33	26	6
Grand Isle	7	12	36	32	49

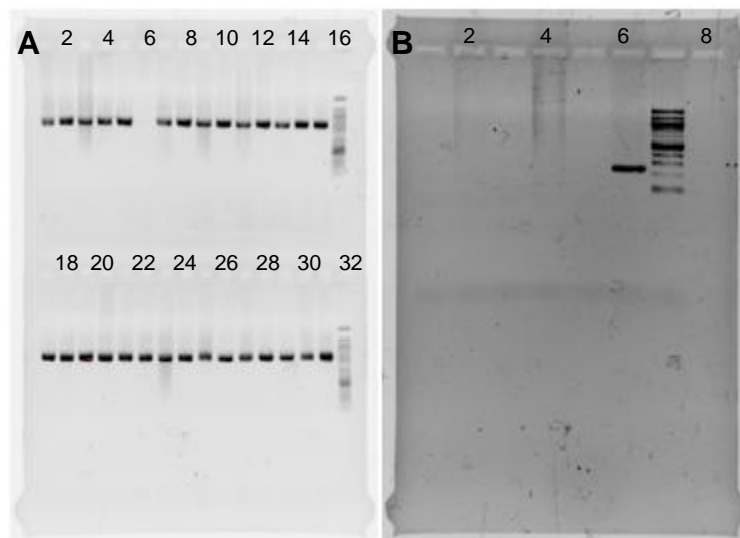


Figure 2.7: Agarose gel results from *Loxothylacus texanus* PCR on midgut and hepatopancreas samples. These samples were collected in the summer of 2013. A: Gel for the crustacean primers (HI, 329). Each lane (lanes 1-15, 17-31) with a distinguishable band indicates amplifiable DNA in the sample. Lanes 16 and 32 are 100 bp ladders. B: Gel for *L. texanus* specific primers (HI, Loxo3). Only the positive control (lane 6) has a band, indicating that the samples (lanes 1-5) were not infected by *L. texanus*. Lane 7 is a 100 bp ladder and lane 8 is empty.

### 2.2.9 Detection of Reo-like Virus

Reo-like virus (RLV) was not originally included in this project. After speaking with other blue crab disease researchers, I began collecting and freezing walking legs during the initial dissection at the end of the spring 2013 season (Section 2.2.3). Legs were placed in labeled whirl-pak bags and were frozen at -20 °C until 20 legs collected from Grand Isle and 19 legs collected from Rockefeller in the summer of 2013 were sent to Dr. Eric Schott at the University of Maryland Center for Environmental Science for reverse-transcription PCR (Bowers et al. 2010).

### 2.2.10 Diagnosis of Shell Rot

Shell rot was the only disease for which I determined both prevalence and intensity. Shell rot was diagnosed as being present or absent during the initial dissection (Section 2.2.3). To diagnose the disease, I examined the dorsal and ventral portions of the carapace as well as all legs and claws (Fig. 2.8). Intensity was determined by percent coverage and by progression of the disease to necrosis (Tables 2.5 and 2.6, Fig. 2.8). I estimated percent coverage during the initial dissection and by using the computer program photoQuad (Trygonis and Sini 2012).

In photoQuad, I outlined a freehand quadrat to eliminate the background because inclusion of the background area would artificially decrease the percent coverage of the carapace with shell rot. Then I placed an 8 pixels by 8 pixels grid over the image and selected each grid square whose area was at least 50% covered by shell rot. In the cell count summary, photoQuad generated a percent coverage based on selected versus unselected grid squares. Because only the dorsal or ventral side of a crab could be analyzed at a time, I divided the percent coverage in half to represent the percent of the total carapace covered with shell rot. As percent coverage alone was not an accurate measure of intensity of shell rot, I developed the scale from 0 to 2 based on the presence of shell rot spots versus presence of necrotic lesions that indicate a more life-threatening, severe case of shell rot (Table 2.6).

### 2.2.11 Detection of *Vibrio* spp.

A small amount of hemolymph (<0.5 mL) was plated on thiosulfate-citrate-bile-salts-sucrose (TCBS) agar during the initial dissection (Section 2.2.3). I placed all plates in an incubator at 28 °C for 48 hours and then removed each plate, photographed it, and made an initial assessment on the presence of *Vibrio* spp (Fig. 2.9). On TCBS agar, *Vibrio* spp. turn yellow, greenish yellow, and green blue. However, other bacteria such as *Pseudomonas* spp., *Aeromonas* spp., and *Photobacterium* spp. can grow on the agar. Due to the inability to use growth and coloration as a definitive method of infection detection, plates with growth were sent to Dr. Hawke at the LSU Veterinary Medicine (LA Aquatic Diagnostic Laboratory, LADL) for further testing (Fig. 2.9). To avoid high testing costs, I did not send any plates that had identical colony growth during the same sampling period. At the LADL, colonies were replated onto blood agar and grown for general bacteriology.

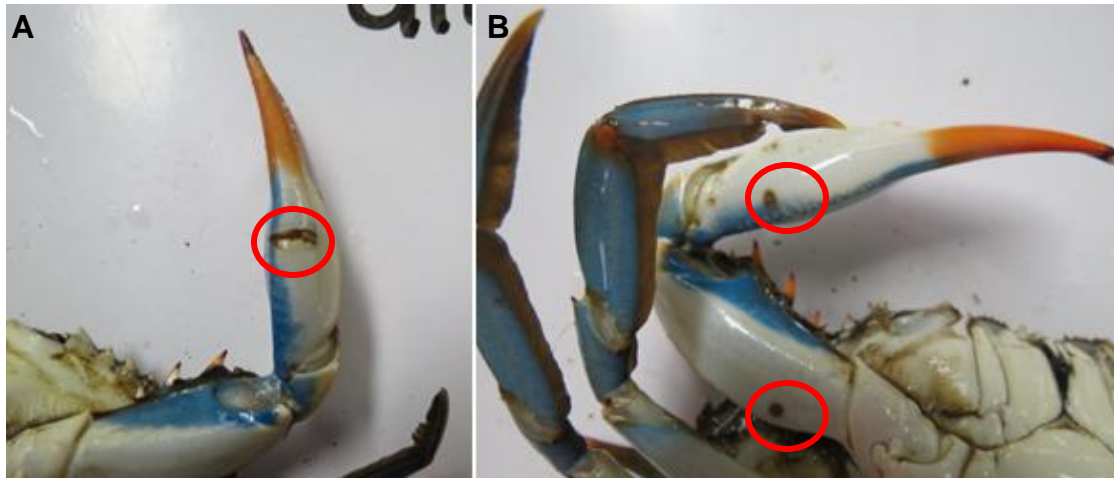


Figure 2.8: Additional shell rot photographs of two crabs collected from Grand Isle in December 2013. A: This crab's claw was likely injured and a necrotic shell rot lesion has formed (red circle). On the shell rot scale from 0 to 2, this shell rot scored as intensity 2. B: This crab's claw has small shell rot spots (red circles). On the shell rot scale from 0 to 2, this shell rot scored as intensity 1 because no necrosis was evident.

The colonies on the blood agar were analyzed with analytical profile index (API) 20E bacteriology strips to identify the genus and species of the colony. If the results from the API strips were not definitive, I did not make an assessment for *Vibrio* spp. and did not include this missing result in analyses. I assessed 140 crabs from Pontchartrain, 174 from Cocodrie, 235 from Rockefeller, and 196 from Grand Isle. Results from API included identifications for non-*Vibrio* spp. bacteria, and those data were also recorded. Due to lab error, bacterial results included bacteria from the hemolymph and internal organs (most likely the hepatopancreas). Of the 744 assessments, 328 were results for infections in the hemolymph only (Table 2.8).

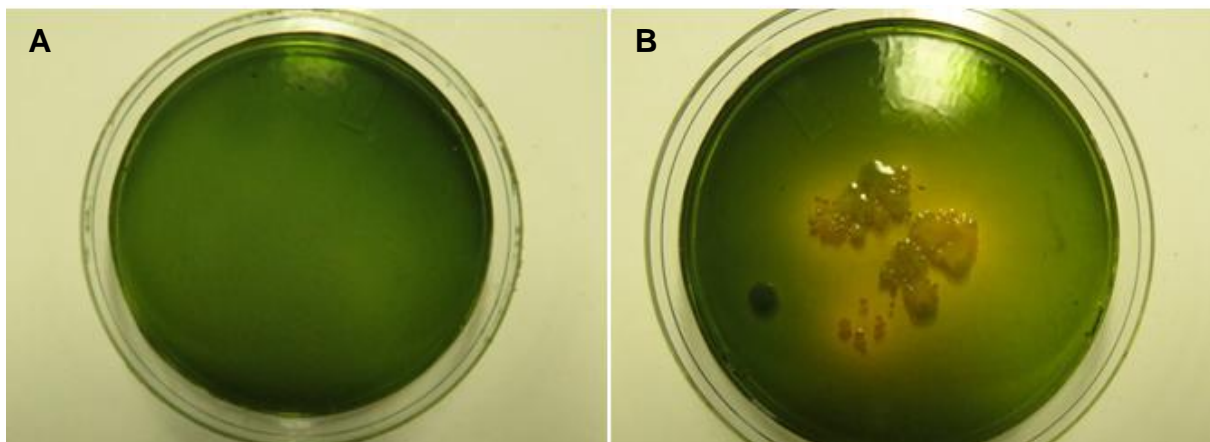


Figure 2.9: Thiosulfate-citrate-bile salts-sucrose (TCBS) agar was inoculated with hemolymph to detect *Vibrio* spp. infections. A: This clean TCBS plate for a crab from Cocodrie in June 2013 indicated that the crab was not infected with *Vibrio* spp. B: This TCBS plate with bacterial growth indicated that this crab from Cocodrie in June 2013 was potentially infected with *Vibrio* spp. and the colony identities were verified by analytical profile index.

Table 2.8: The number of hemolymph samples tested for *Vibrio* spp. from four field sites, Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle (n/a- no samples collected at that site).

Site	Season				
	Winter 2013	Spring	Summer	Fall	Winter 2013-2014
Pontchartrain	n/a	24	0	10	n/a
Cocodrie	n/a	n/a	55	15	29
Rockefeller	17	36	0	1	12
Grand Isle	7	13	23	47	39

## 2.2.12 Statistical Analysis

I performed all statistical analyses in RStudio (R Core Team 2013). For the analysis of the effect of a predictor variable on the presence of a particular disease, parasite, or symbiont, I did a logistic regression as a generalized linear model in the package *stats* in RStudio. Predictor variables included: site, season, the site\*season interaction, crab sex, crab size, water temperature, salinity, and capture method. I also performed an analysis of deviance Chi-square test in the *stats* package to evaluate model fit.

To generate a cumulative multicategorical logit model for shell rot intensity, I used package *vgam* in which the categories were scored from 0 to 2 (Table 2.6; Agresti 2007, Yee 2010, Yee 2013). For cumulative logit models, likelihood ratio tests were performed in package *vgam* to analyze model fit (Yee 2010, Yee 2013).

When multicollinearity between predictor variables was hypothesized, I used function *vif* in package *car* to generate generalized variance inflation factors (GVIFs). Multicollinearity was determined to be an issue when GVIFs were greater than 4.0. For all statistical tests, alpha was set at 0.05 for statistical significance.

## 2.3 Results

### 2.3.1 Prevalence of *Lagenophrys callinectes*

The aggregate prevalence of *L. callinectes* at the four field sites was 93.2% (Fig. 2.10-2.14). Pontchartrain had the highest percent prevalence, 98.6%, while Rockefeller had the lowest, 89.8% (Fig. 2.10). Prevalence at neither Pontchartrain nor Cocodrie varied significantly by season (Fig. 2.11 and 2.12; all  $p > 0.99$ ). At Rockefeller, the summer prevalence rate of 76.7% was significantly lower than other seasons (Fig. 2.13, Table 2.9). At Grand Isle, prevalence during the spring was significantly lower than the other seasons (Fig. 2.14, Table 2.9). For Rockefeller and Grand Isle, model fit improved with season included as a predictor variable (both  $p < 0.01$ ).

In addition to seasonal variation in *L. callinectes*, Rockefeller and Grand Isle were significantly different during the spring (Table 2.9). When each season was analyzed individually for site variation, Grand Isle and Rockefeller again varied during the spring (Table 2.9). In all seasons, comparison between models with and without site as a predictor variable indicated that site is an important predictor for model fit (all  $p < 0.0001$ ).

The low prevalence at Grand Isle in the spring influenced significant results for water temperature and salinity in the model with site, salinity, temperature, and the site\*temperature interaction (Table 2.9). Neither crab size (CW) nor sex were significant predictors for the probability of *L. callinectes* infestations (Table 2.9). Lastly, prevalence did not vary between crabs collected with bait and without bait (Table 2.9). For size, sex, and method, model fit was equivalent with and without these predictors (all  $p > 0.45$ ).

Overall, the important predictor variables for *L. callinectes* prevalence included site, season (or water temperature), the site\*season interaction, and salinity (all GVIFS <3.5) and prevalence was high throughout the study (Tables 2.10 and 2.11).

Table 2.9: Parameter estimates and p-values for predictor variables for prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. infections. Site, season, and site\*season parameter estimates for specific categories not listed were not significant. Category cells are blacked out for continuous variables.

Disease, Parasite, Symbiont	Predictor	Category	Parameter Estimate	p-value
<i>Lagenophrys callinectes</i>	Site	Rockefeller	4.224	< 0.0001
	Season	Spring (Grand Isle)	-3.8430	< 0.0001
		Summer (Rockefeller)	-1.0791	0.0404
	Site*Season	Rockefeller* Spring	4.5948	< 0.001
	Water Temperature		0.0893	0.0482
	Salinity		-0.1066	0.0332
	Size		0.004	0.9687
	Sex	Male	-0.0431	0.8987
<i>Urosporidium crescens</i>	Site	Cocodrie	1.339	0.0109
		Rockefeller	1.592	<0.01
	Season	Summer (Cocodrie)	-2.02	<0.001
	Season	Spring (Rockefeller)	-1.5835	<0.01
		Winter 13-14 (Rockefeller)	-1.4271	<0.01
		Spring (Grand Isle)	-2.2409	0.0392
	Site*Season	Rockefeller*Winter 13-14	-1.446	0.0151
	Water Temperature		0.4825	0.0151
	Salinity		0.1336	< 0.0001
	Size		-0.0535	0.3900
	Sex	Male	-0.1185	0.5576
	Capture Method	Unbaited	-3.2989	< 0.0001
Shell Rot	Site	Rockefeller (Summer)	1.6546	<0.0001
		Rockefeller (Winter 13-14)	1.0845	<0.01
	Season	Spring (Pontchartrain)	-1.3863	<0.01
		Spring (Rockefeller)	-2.3432	<0.0001
		Summer (Rockefeller)	1.0712	<0.01
		Winter 13-14 (Rockefeller)	0.9701	0.0160
		Spring (Grand Isle)	-3.1039	<0.01
		Summer (Grand Isle)	-0.8873	0.0182
	Site*Season	Rockefeller*Summer	1.9585	<0.001
		Rockefeller* Winter 13-14	1.3885	0.0116
	Water Temperature		0.4337	0.0265
	Salinity		0.0227	0.3715



(Table 2.9 continued)

Disease, Parasite, Symbiont	Predictor	Category	Parameter Estimate	p-value
Shell Rot	Size		0.1832	<0.001
	Sex	Male	0.8825	<0.0001
	Capture Method	Unbaited	-0.3429	0.1877
<i>Vibrio</i> spp. Hemolymph and Organ Infections	Site	Pontchartrain	1.8909	<0.0001
		Cocodrie	2.5709	<0.0001
		Rockefeller	2.4039	<0.0001
	Season	Summer (Cocodrie)	-1.3938	<0.001
		Spring (Rockefeller)	1.7086	<0.001
		Summer (Grand Isle)	1.4778	<0.01
		Winter 2013 (Grand Isle)	2.3121	<0.01
	Site*Season	Cocodrie*Summer	-2.8717	<0.0001
		Rockefeller*Spring	-2.0283	0.0382
		Rockefeller*Summer	-1.5209	0.0120
		Rockefeller*Winter 13	-3.0482	<0.01
	Water Temperature		0.0429	0.1685
	Salinity		0.0746	<0.01
	Size		-0.1235	0.0212
	Sex	Male	-0.5947	<0.001
	Capture Method	Unbaited	0.5587	0.0454
<i>Vibrio</i> spp. Hemolymph Infections	Lab Assistant	Assistant 1	-2.209	<0.0001
		Assistant 2	-1.417	0.0293
	Site	All sampled	n/a	All >0.97
	Season	All sampled	n/a	All >0.79
	Site*Season	All possible	n/a	All >0.38
	Water Temperature		0.0164	0.4911
	Salinity		-0.0422	0.1008
	Size		0.0619	0.5079
	Sex	Male	-0.7555	0.0175
	Capture Method	Unbaited	2.3494	<0.0001

Table 2.10: Predictor variables for the prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. infections in wild crabs collected in 2013 and the beginning of 2014 (\* denotes statistically significant predictor).

Predictor variable	Disease, Parasite, or Symbiont				
	<i>L. callinectes</i>	<i>U. crescens</i>	Shell Rot	<i>Vibrio</i> spp. total	<i>Vibrio</i> spp. hemolymph
Site	*	*	*	*	
Season	*	*	*	*	
Site-Season Interact.	*	*	*	*	
Salinity	*	*		*	
Water Temp.	*	*	*		
Size			*	*	
Sex			*	*	*
Capture Method		*		*	*
Lab Assistant	n/a	n/a	n/a	*	n/a

Table 2.11: Salinity and temperature ranges at which infections by *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. were detected in wild crabs collected in 2013 and the beginning of 2014.

Water Quality Parameter	Disease, Parasite, or Symbiont			
	<i>L. callinectes</i>	<i>U. crescens</i>	Shell Rot	<i>Vibrio</i> spp.
Salinity Range (ppt)	0.6-26.7	0.6-26.6*	0.6-26.7	0.6-26.6
Temp. Range (°C)	8.3-33.6	10.6-33.6	8.3-33.6	10.6-33.6

\*Low salinity measured after freshwater event; 90% of infections occurred at salinities above 6.3 ppt and 80% at salinities above 10.8 ppt

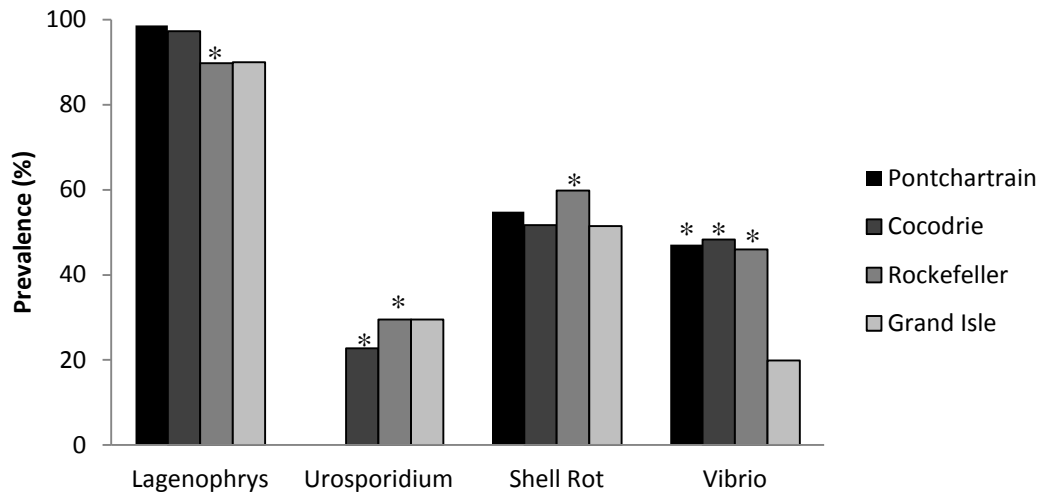


Figure 2.10: Aggregate prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. in blue crabs collected from each field site: Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle (\* denotes statistical significance compared to prevalence at Grand Isle).

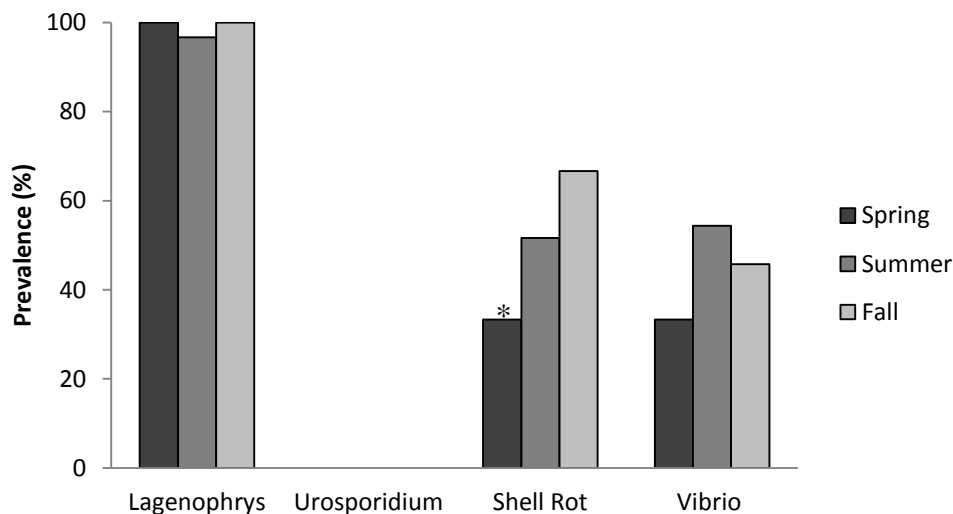


Figure 2.11: Prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. in blue crabs collected from Lake Pontchartrain in the spring, summer, and fall of 2013 (\* denotes statistical significance compared to prevalence in the fall).

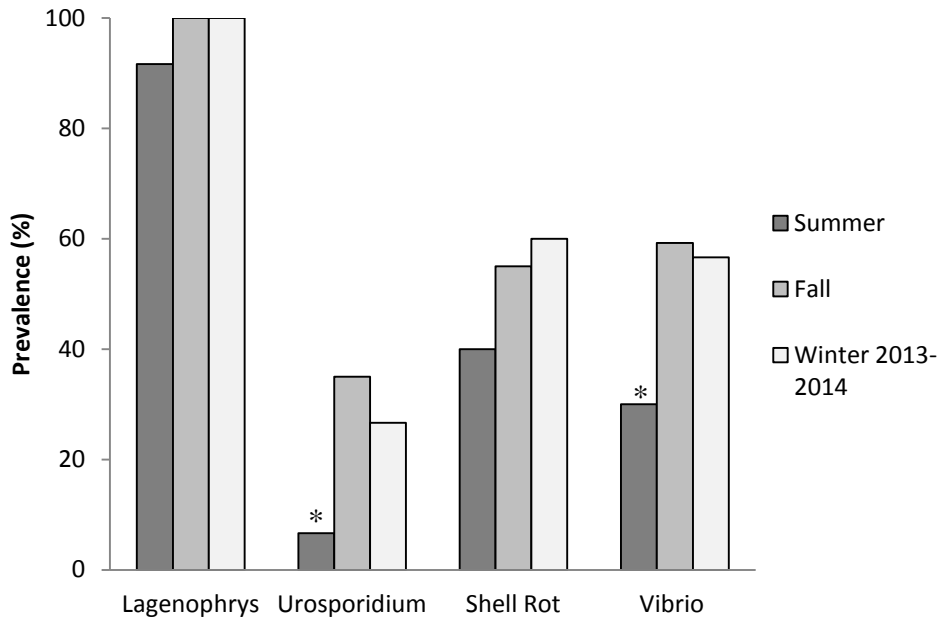


Figure 2.12: Prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. in blue crabs from Cocodrie in summer and fall 2013 and winter 2013-2014 (\* denotes statistical significance compared to prevalence in the fall).

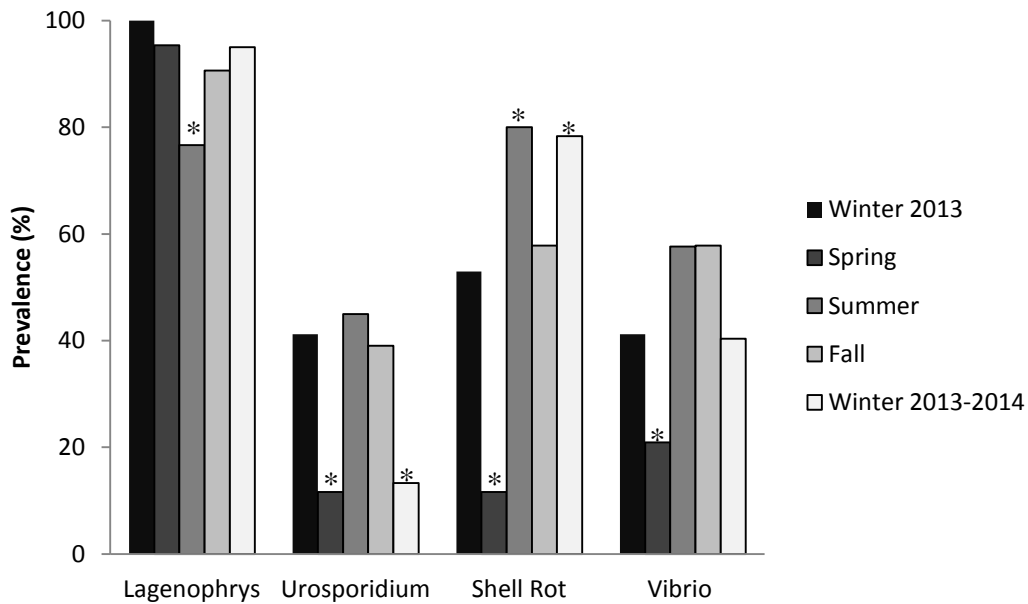


Figure 2.13: Prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. in blue crabs from Rockefeller Wildlife Refuge in the winter, spring, summer, and fall of 2013 and winter 2013-2014 (\* denotes statistical significance compared to prevalence in the fall).

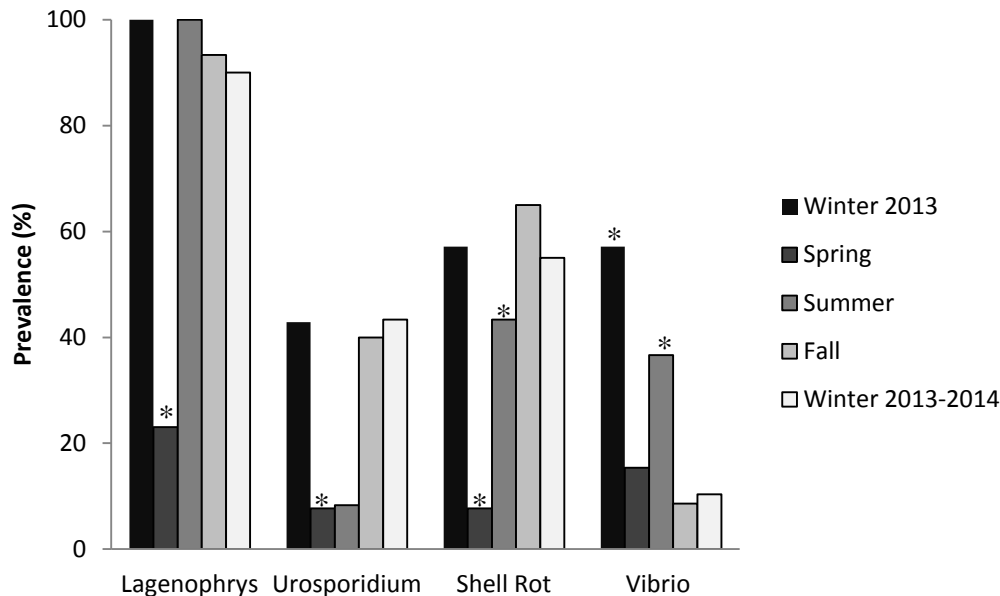


Figure 2.14: Prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. in blue crabs from Grand Isle in the winter, spring, summer, and fall of 2013 and winter 2013-2014 (\* denotes statistical significance compared to prevalence in the fall).

### 2.3.2 Prevalence of *Urosporidium crescens*

The overall prevalence of *U. crescens* at the four field sites was 22.4% (Fig. 2.10). I detected no crabs from Pontchartrain with *U. crescens* (Fig. 2.10 and 2.11). Prevalence rates at Cocodrie, Rockefeller, and Grand Isle ranged from 22.8% at Cocodrie to 29.5% at Rockefeller and Grand Isle (Fig. 2.10 and 2.12-2.14). There was apparent seasonal variation in the prevalence of *U. crescens* at Cocodrie, Rockefeller, and Grand Isle (Fig. 2.12-2.14). At Cocodrie, the summer prevalence of 6.7% was significantly lower than the other seasons (Table 2.9); however, salinity was an important predictor for model fit while season was not (salinity:  $\chi^2 = 35.667$ , d.f. = 1,  $p < 0.0001$ ; season:  $\chi^2 = 2.7086$ , d.f. = 2,  $p = 0.2581$ ). At Rockefeller, the spring and winter 2013-2014 prevalence rates (11.6% and 13.3%, respectively) were significantly lower than those in other seasons (Table 2.9) and unlike at Cocodrie, salinity was not a significant explanatory variable for the seasonal variation ( $\chi^2 = 0.8393$ , d.f. = 1,  $p = 0.3596$ ). Also at Rockefeller when the winter of 2013 was removed due to low sample size, water temperature did not explain the significant seasonal variation ( $\beta_{\text{Temp}} = 0.0176$ ,  $p = 0.8439$ ; model fit:  $\chi^2 = 0.0392$ , d.f. = 1,  $p = 0.8431$ ). At Grand Isle, prevalence during the spring was significantly lower than other seasons (Table 2.9), and both season and salinity were important explanatory variables.

In addition to the seasonal variation, both Cocodrie and Rockefeller were significantly different from Pontchartrain and Grand Isle (Table 2.9). In this model, all four explanatory variables were necessary for the best model fit and lowest AIC (all deviance  $p < 0.03$ ). Multicollinearity was not detected between site and salinity (GVIF<sub>Site</sub> = 3.266, GVIF<sub>Season</sub> = 1.601, GVIF<sub>Salinity</sub> = 3.733). When each season was

analyzed individually for site variation, there was significant site variation in the summer with a higher prevalence rate at Rockefeller and in winter 2013-2014 with a lower rate at Rockefeller (Table 2.9).

Salinity is a significant predictor for the prevalence of *U. crescens* where the odds of infections increase by 14.3% for a 1 ppt increase in salinity (Table 2.9). A model without salinity as a factor had a higher AIC and was significantly different from the regression including salinity (AIC: 691.86 vs. 674.72;  $\chi^2 = 19.139$ , d.f. = 1,  $p < 0.0001$ ). Unlike salinity, size and sex were not significant predictors of *U. crescens* prevalence and did not improve the model fit (Table 2.9; both deviance  $p > 0.38$ ).

As water temperature increased by 1 °C, the odds of *U. crescens* infections increased by 62% (Table 2.9). There was multicollinearity between season and water temperature (GVIF<sub>Season</sub> = 83.798, GVIF<sub>Temperature</sub> = 53.340).

Lastly, capture method was an important indicator for *U. crescens* prevalence with a lower probability of infections in crabs collected without bait (Table 2.9). I caught 245 crabs without bait and 523 with bait where 7.3% and 29.4% of these crabs, respectively, had *U. crescens*. The model with capture method included had a lower AIC than one with only site, season, site\*season interaction, and salinity (AIC: 635.89 vs. 674.72); however, there was multicollinearity when capture method was included in the model (GVIF<sub>Season</sub> = 8.334, GVIF<sub>Site</sub> = 4.254, GVIF<sub>Salinity</sub> = 4.813, GVIF<sub>Method</sub> = 6.181). Overall, *U. crescens* prevalence varied by site, season, the site\*season interaction, salinity, water temperature, and capture method (Tables 2.10 and 2.11).

### 2.3.3 Prevalence of White Spot Syndrome Virus (WSSV)

None of the 587 samples successfully extracted were positive for WSSV according to amplifications with the WSSV specific primer pair, 146F2 and 146R2.

### 2.3.4 Prevalence of *Hematodinium perezii*

None of the 760 samples successfully extracted were positive for *H. perezii* according to amplifications with the HITS1 and Hemat primers.

### 2.3.5 Prevalence of *Loxothylacus texanus*

None of the crabs collected from the field sites had *L. texanus* externa. After PCR amplifications with the HI and Loxo3 primers, none of the 156 small juvenile crabs nor any of the 357 larger (> 11 cm CW) crabs were infected with *L. texanus*.

### 2.3.6 Prevalence of Reo-like Virus (RLV)

One of the 20 crabs from Grand Isle in the summer was infected with RLV. Two of the 19 crabs from Rockefeller in the summer were infected. Therefore, the aggregate prevalence rate for RLV during the summer was 7.7%.

### 2.3.7 Prevalence and Intensity of Shell Rot

The total prevalence of shell rot in the wild populations sampled was 54.8% (Fig. 2.10). At Pontchartrain, prevalence ranged from 33% to 67% with the highest prevalence rate in the fall (Fig. 2.11). Cocodrie's highest rate was in the winter of 2013-2014 at 60% (Fig. 2.12). Prevalence varied more at Rockefeller and Grand Isle with low rates in the spring (Fig. 2.13 and 2.14). At Rockefeller, prevalence was highest in the summer and winter 2013-2014 (Fig. 2.13, Table 2.9). At Grand Isle, shell rot was most prevalent in the fall (Fig. 2.14).

Seasonal variation was detected at Pontchartrain, Rockefeller, and Grand Isle. At Pontchartrain, the spring prevalence rate was significantly different from the fall rate, and season was an important predictor variable for the regression fit (Table 2.9;  $\chi^2 = 8.2178$ , d.f. = 2,  $p = 0.0164$ ). At Rockefeller, the lower spring and higher summer and winter 2013-2014 were all significantly different from fall 2013 (Table 2.9). The model was significantly better with season included as an explanatory variable (AIC: 274.34 vs. 330.75;  $\chi^2 = 64.41$ , d.f. = 4,  $p < 0.0001$ ). At Grand Isle, both spring and summer prevalence percentages were significantly lower than the other seasons' (Table 2.9) and the model at Grand Isle should include season as a predictor variable ( $\chi^2 = 18.089$ , d.f. = 4,  $p < 0.01$ ). The seasonal variations may be explained by the significant effects of water temperature, where odds of shell rot decreased by 35% as water temperature increased by 1 °C (Table 2.9). However, in a model including both season and water temperature, there was multicollinearity ( $\text{GVIF}_{\text{Site}} = 1.762$ ,  $\text{GVIF}_{\text{Season}} = 82.378$ ,  $\text{GVIF}_{\text{Temperature}} = 64.954$ ).

Shell rot prevalence also varied between the four sites, specifically at Rockefeller in the summer and in the winter of 2013-2014 (Table 2.9). When I analyzed each season individually to detect site variation, results were consistent with the full model in that Rockefeller's prevalence of shell rot was significantly higher in the summer and winter 2013-2014 (Table 2.9). This site variation was not explained by salinity differences (Table 2.9).

The odds of shell rot infections increased by 20% as crab CW increased by 1 cm (Table 2.9). Models with and without size as an explanatory variable were significantly different ( $\chi^2 = 13.09$ , d.f. = 4,  $p < 0.001$ ). Additionally, the odds for shell rot infections were 142% higher for males than for females, and sex was an important factor in the full model (Table 2.9;  $\chi^2 = 24.677$ , d.f. = 4,  $p < 0.0001$ ). Unlike size and sex, capture method did not affect prevalence rates (Table 2.9). The full logistic regression for shell rot prevalence included site, season (or water temperature), site\*season interaction, size, and sex as the predictor variables (Tables 2.10 and 2.11).

In addition to shell rot prevalence of 54.8%, the average shell rot intensity was 0.76. Severe shell rot with necrotic lesions was present on 21% of all crabs collected and was 38.2% of all shell rot cases diagnosed. The highest average intensity score, 0.84, was at Rockefeller and at the lowest salinity site, Pontchartrain, the average score was 0.64. The average intensity score for crabs caught with bait was 0.83 (N= 523) and was 0.49 (N= 245) for crabs caught without bait.

The cumulative logit model for shell rot intensity included site, season, size, sex, salinity, and capture method as predictor variables for shell rot intensity score (Table 2.6). Likelihood ratio tests indicated that shell rot intensity is dependent on all six of

these variables (all  $p < 0.05$ ); however, multicollinearity was present between site and salinity ( $\text{GVIF}_{\text{Site}} = 6.617$ ,  $\text{GVIF}_{\text{Salinity}} = 4.964$ ), so salinity was retained in the model and site was not. The logit for shell rot intensity score 0 was (Agresti 2007):

$$3.587 + 0.673(\text{Spring}) - 0.133(\text{Summer}) - 0.798(\text{Winter 2013}) - 0.276(\text{Winter 2013-2014}) - 0.519(\text{Male}) - 0.247(\text{CW}) - 0.005(\text{Salinity}) + 0.747(\text{Unbaited Capture}).$$

The logit for shell rot intensity score 1 was (Agresti 2007):

$$5.274 + 0.673(\text{Spring}) - 0.133(\text{Summer}) - 0.798(\text{Winter 2013}) - 0.276(\text{Winter 2013-2014}) - 0.519(\text{Male}) - 0.247(\text{CW}) - 0.005(\text{Salinity}) + 0.747(\text{Unbaited Capture}).$$

Based on these logit equations, the cumulative probability of a crab not having shell rot (score 0) was (Agresti 2007):

$$P(Y=0) = \frac{e^{3.587+0.673(\text{Spr})-0.133(\text{Sum})-0.798(\text{W13})-0.276(\text{W14})-0.519(\text{Male})-0.247(\text{CW})-0.005(\text{Sal})+0.747(\text{Unbaited})}}{1+e^{3.587+0.673(\text{Spr})-0.133(\text{Sum})-0.798(\text{W13})-0.276(\text{W14})-0.519(\text{Male})-0.247(\text{CW})-0.005(\text{Sal})+0.747(\text{Unbaited})}}$$

The cumulative probability of a crab having no shell rot (score 0) or non-necrotic shell rot (score 1) was (Agresti 2007):

$$P(Y \leq 1) = \frac{e^{5.274+0.673(\text{Spr})-0.133(\text{Sum})-0.798(\text{W13})-0.276(\text{W14})-0.519(\text{Male})-0.247(\text{CW})-0.005(\text{Sal})+0.747(\text{Unbaited})}}{1+e^{5.274+0.673(\text{Spr})-0.133(\text{Sum})-0.798(\text{W13})-0.276(\text{W14})-0.519(\text{Male})-0.247(\text{CW})-0.005(\text{Sal})+0.747(\text{Unbaited})}}$$

Cumulative probability for a crab having shell rot intensity scores 0, 1, or 2 (Table 2.6) was 1.0 (100%).

Based on these logit equations, crabs were most likely to have shell rot in the winter and least likely to have it in the spring. Large males were more likely to have shell rot. Shell rot intensity increased with increasing salinities. Also shell rot was more likely on crabs caught with bait than those caught without bait.

### 2.3.8 Prevalence of *Vibrio* spp.

The aggregate prevalence of *Vibrio* spp. infections in the hemolymph and internal organs was 39.9% (Fig. 2.10). At Pontchartrain, prevalence ranged from 33 to 54%, with highest prevalence in the summer (Fig. 2.11). Cocodrie's *Vibrio* spp. prevalence rate was highest in the fall at 59% and lowest in the summer at 30% (Fig. 2.12). Prevalence at Rockefeller ranged from 21% in the spring to 58% in the summer and fall (Fig. 2.13). At Grand Isle, it was between 9% in the fall and 57% in the winter of 2013 (Fig. 2.14).

There was significant seasonal variation in the probability of *Vibrio* spp. infections at Cocodrie, Rockefeller, and Grand Isle. At Cocodrie, the prevalence in the summer was significantly lower than in the other seasons, and at Rockefeller, the spring's rate of 21% was significantly lower than the other seasons' rates (Table 2.9). Conversely at Grand Isle, the summer and winter of 2013 probabilities were higher than in the other three seasons (Table 2.9). For Cocodrie, Rockefeller, and Grand Isle, models with season as a predictor variable fit significantly better (all  $p < 0.01$ ) and had lower AICs.

In addition to the seasonal variation, there was also significant site variation, in which Pontchartrain, Cocodrie, and Rockefeller rates were all significantly higher than Grand Isle's (Table 2.9, Fig. 2.10). When each season was analyzed individually for site variation, there were significant differences in the summer, fall, and winter 2013-2014. In the summer, prevalence rates at Pontchartrain and Rockefeller were higher than at Grand Isle (Table 2.9). In the fall and winter 2013-2014, Grand Isle prevalence was significantly lower than the other sites (Table 2.9).

The odds of a *Vibrio* spp. infection decreased by 7.2% as salinity increased by 1 ppt (Table 2.9) and there was not multicollinearity in the model including site, season, site\*season interaction, and salinity as independent variables ( $GVIF_{\text{Site}} = 3.373$ ,  $GVIF_{\text{Season}} = 1.843$ ,  $GVIF_{\text{Salinity}} = 3.497$ ). Crab size and sex were also important predictor variables for *Vibrio* spp. prevalence (Table 2.9). In this study, 44% of females (N= 341) and 36% of males (N= 403) were infected. The odds of infection decreased by 11.6% as size increased by 1 cm CW and were 44.8% lower in males than in females. Both of these variables improved model fit (both  $p < 0.05$ ) and lowered the model AIC to 953.89.

Water temperature did not significantly affect the prevalence of *Vibrio* spp. (Table 2.9) in a model that included water temperature instead of season to prevent multicollinearity ( $GVIF_{\text{Season}} = 129.449$ ,  $GVIF_{\text{Temp}} = 58.768$ ). Unlike water temperature, the odds of infection were 42.8% higher for crabs caught with bait than for crabs caught without bait (Table 2.9). When capture method was included in the model, model fit improved (AIC: 951.8 vs. 953.89;  $\chi^2 = 4.0827$ , d.f. = 1,  $p = 0.0433$ ), but moderate multicollinearity was detected ( $GVIF_{\text{Site}} = 6.086$ ,  $GVIF_{\text{Salinity}} = 4.407$ ). If site was removed from the model, the AIC increased from 951.8 to 978.76. If salinity was removed from the model, the AIC increased slightly to 958.26.

Lastly, *Vibrio* spp. prevalence varied from 21 to 57% depending on which lab assistant pulled the hemolymph, and this variation in prevalence rates was significant (Table 2.9). There was moderate multicollinearity between site, season, salinity, and the lab assistant, as not all assistants helped with every site during every season ( $GVIF_{\text{Site}} = 9.213$ ,  $GVIF_{\text{Season}} = 6.996$ ,  $GVIF_{\text{Salinity}} = 4.774$ ,  $GVIF_{\text{Assistant}} = 8.912$ ). This full model had the lowest AIC of 902.18 and included: site, season, the site\*season interaction, salinity, crab size, crab sex, capture method, and lab assistant. However, when some of the multicollinear terms were removed, the AIC decreased to 869.5. This improved model included crab sex, salinity, and lab assistant as predictor variables ( $GVIFs < 1.3$ ) and all predictor variables were significant ( $\beta_{\text{Male}} = -0.774$ ,  $p < 0.0001$ ;  $\beta_{\text{Salinity}} = -0.081$ ,  $p < 0.0001$ ;  $\beta_{\text{Assistant 1}} = -1.783$ ,  $p < 0.0001$ ;  $\beta_{\text{Assistant 2}} = -1.511$ ,  $p < 0.001$ ).

Overall, site, season, the site\*season interaction, salinity, crab sex, crab size, capture method, and the lab assistant were significant predictor variables but the best model included only sex, salinity, and lab assistant (Tables 2.10 and 2.11).

When samples possibly contaminated by internal organ tissue were ignored and hemolymph only infections were considered (Table 2.8), prevalence of *Vibrio* spp. ranged from 0 to 55% and aggregate prevalence was 22.3% (Fig. 2.15). *Vibrio* spp. hemolymph infection prevalence only varied significantly by sex and capture method, where males were less likely to be infected and crabs caught without bait were more likely (Table 2.9). Salinity and temperature were predictors in the model instead of site and season due to multicollinearity ( $GVIF_{\text{Site}} = 9.368$ ,  $GVIF_{\text{Season}} = 10.469$ ); however,



neither salinity nor temperature were retained in the final model because of their insignificance (Table 2.9).

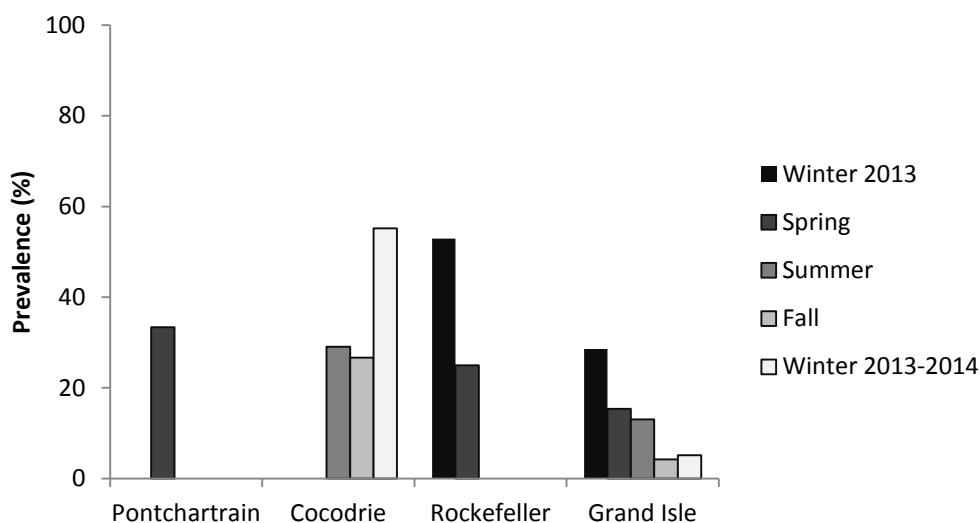


Figure 2.15: *Vibrio* spp. hemolymph infection rates in crabs collected from four field sites (Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle) in 2013 and the beginning of 2014. Missing bars indicate that results are missing due to error or unsuccessful collection.

#### 2.3.9 Co-occurrence of Shell Rot and *Vibrio* spp.

Shell rot and *Vibrio* spp. hemolymph infections co-occurred in crabs at rates between 0% and 28%, with an aggregate co-occurrence of 8% (Fig. 2.16). When a full model including site, season, site\*season interaction, crab size, crab sex, and *Vibrio* spp. hemolymph infections was analyzed for the prevalence of shell rot, the presence of a *Vibrio* spp. infection had no significant effect on the probability of a shell rot infection ( $\beta_{\text{Vibrio}} = -0.1860$ ,  $p = 0.5802$ ). This full model fit the data equally well without *Vibrio* spp. prevalence included as a predictor variable (AIC: 399.22 vs. 397.53;  $\chi^2 = 0.3073$ , d.f. = 1,  $p = 0.5794$ ). Additionally, shell rot intensity was independent of *Vibrio* spp. presence or absence ( $\chi^2 = 0.1117$ , d.f. = 1,  $p = 0.7383$ ). The presence of shell rot infections also did not affect the prevalence of *Vibrio* spp. in the model including crab sex, capture method, and shell rot prevalence as predictors ( $\beta_{\text{Shellrot}} = 0.1912$ ,  $p = 0.5380$ ). Model fit was equivalent with and without shell rot as a predictor variable ( $\chi^2 = 0.3044$ , d.f. = 1,  $p = 0.5811$ ).

#### 2.3.10 Other Bacterial Infections

After API analyses, other bacterial infections found in crab hemolymph and internal organs included: *Aeromonas* spp. (*A. sobria*, *A. hydrophila*, *A. caviae*, *A. salmonicida*), *Pseudomonas* spp. (*P. fluorescens*, *P. putida*, *P. aeruginosa*), *Providentia* spp., *Photobacterium* spp. (including *P. damsela*), *Citrobacter braakii*, *Shewanella putrefaciens*, *Serratia* spp. (*S. liquefaciens*), *Pasteurella* spp. (including *P. multocida*), *Pantoea* spp., *Pasteurella/Mannheimia* spp., *Yersinia enterocolitica*, *Chryseobacterium*

spp. (*C. indologenes*, *C. meningosepticum*), *Chromobacterium* spp., and *Brevundimonas vesicularis*.

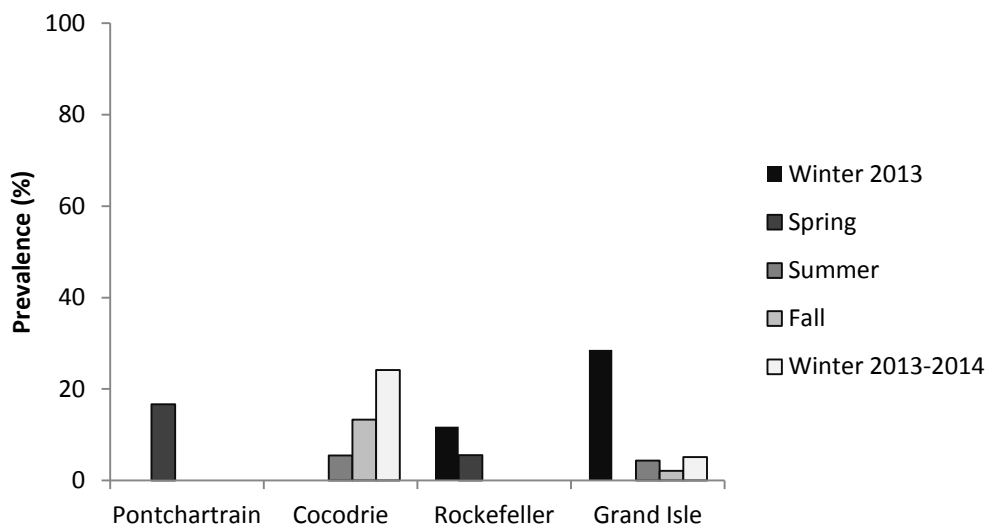


Figure 2.16: Shell rot and *Vibrio* spp. hemolymph infection co-occurrence rates in crabs collected seasonally from Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle in 2013 and 2014. Missing bars indicate that results are missing due to error or unsuccessful collection.

## 2.4 Discussion

### 2.4.1 Prevalence of *Lagenophrys callinectes*

*L. callinectes* is an ectocommensalistic, loricated ciliate that can cause rare cases of impaired respiration (Couch and Martin 1979, Scott and Thune 1986). Infestations are shed during ecdysis and reinfestation does not generally occur immediately after molting (Shields and Overstreet 2007). In the Gulf, prevalence of *L. callinectes* is primarily correlated with the crab's molt cycle not with salinity or water temperature (Shields and Overstreet 2007). However, in this study water temperature and salinity were significant predictors for *L. callinectes* prevalence, but these results were influenced by the low prevalence at Grand Isle in the spring.

In this study, prevalence was 93.2% across all four sites and all four seasons (Fig. 2.10). The seasonal variation at both Rockefeller and Grand Isle is likely correlated with collection of newer molt crabs, but I did not record every crab's molt stage. Collecting fresh molt crabs is very likely in the spring because April and May are peak molting months in the Gulf (Guillory et al. 2001). The lower prevalence at Rockefeller in the summer was less expected. High prevalence of shell rot in the summer at Rockefeller indicated that these crabs were not likely to have recently molted so the lower prevalence of *L. callinectes* is probably not a function of ecdysis. Reinfestation after spring molting may be infrequent at Rockefeller if the symbiont is less prevalent at this site. This site difference may not be evident in prevalence rates during other seasons because of the potentially longer lapse between molts. However, for this lower

prevalence rate to be a site effect at Rockefeller, I would have expected to see low prevalence in the late spring that slowly increased into the summer, fall, and winter. Another possible source of this discrepancy in the summer could be error in the detection if infestations were light in intensity.

The only significant site variation was in the spring between Rockefeller and Grand Isle, with Grand Isle's prevalence at 23% and Rockefeller's at 95%. However, Grand Isle and Pontchartrain should have differed as well but there was no significant difference between these two sites due to the low sample sizes. This significant site variation during the spring is likely correlated with the crab molt cycle. If there was a true site difference, I would expect to see the difference throughout all of the seasons.

The lack of variation in prevalence across different sizes and sexes is consistent with previous data as this symbiont is opportunistic and will infest any crab. Additionally, the capture method did not affect prevalence of *L. callinectes*, which is expected because this symbiont does not affect the foraging behavior of blue crabs and does not derive nutrition from the crab (Shields and Overstreet 2007).

The overall high prevalence rates of this symbiont are not alarming for management of the fishery or for sustainability of the populations because the relationship between *L. callinectes* and blue crabs is usually commensalistic. If a researcher studies *L. callinectes* in the future, it is highly probable that prevalence will be high regardless of water temperature and salinity but may vary based on molt stage. A significant decline in prevalence in the future could indicate that 2013 was atypical or that the *L. callinectes* population is in decline.

#### 2.4.2 Prevalence of *Urosporidium crescens*

*U. crescens* is a harmless hyperparasite of the blue crab that causes the trematode *Microphallus basodactylophallus* to darken and expand (Newman et al. 1976, Messick 1998). This hyperparasite can negatively affect the commercial fishery because infected meat is unappetizing and unappealing (Couch and Martin 1979). Overall the prevalence of *U. crescens* was 22.4% and varied by season, site, salinity, water temperature, and capture method. *U. crescens* has been detected in crabs from moderate salinities and over a wide temperature range from near freezing to over 20 °C (Messick 1998). No known data exist on infections at low salinities so this hyperparasite's potential intolerance of low salinities may affect its prevalence at coastal areas in Louisiana. At Pontchartrain, the lack of infected crabs could be due to low salinities (Tables 2.2 and 2.10). At Cocodrie, prevalence was lowest in the summer; however, this seasonal effect was influenced by the areas sampled during different seasons. In the summer, the majority of the Cocodrie crabs were trawled in a low salinity bayou (5.4 ppt), but in the fall and winter the majority of the crabs were caught in salinities above 12 ppt (Table 2.2 and 2.10). Therefore, this apparent seasonal variation at Cocodrie is likely correlated with salinity and not season.

At Rockefeller, the spring and winter of 2013-2014 infection rates were significantly lower than in the other seasons. Biologically, a reasonable explanation for the low rates during these seasons is that the salinities were slightly lower (less than 10 ppt) than during the other seasons (Table 2.2). One exception to this was the winter of 2013, in which salinities were below 5 ppt and prevalence was high (Table 2.10). The

higher winter 2013 prevalence could be influenced by the low sample size or by abrupt salinity changes due to recent rainfall. However, the logit model did not indicate that salinity was a significant explanatory variable at Rockefeller. After the removal of low sample sizes to eliminate bias, water temperature also did not explain the lower prevalence rates in the spring and winter 2013-2014 at Rockefeller. Therefore, the seasonal variation is unexplainable based on the factors measured.

At Grand Isle, the spring prevalence rate of 7.7% was significantly lower than other seasons but salinity did not correlate with this trend. The summer prevalence was 8.3% with lower salinities than in the spring. However, the spring sample size was low (N=13) compared to the summer (N=60). With such a low sample size in the spring, model predictions are variable and potentially erred.

The probability of *U. crescens* infections varied significantly between the sites and site and salinity were not multicollinear predictors. Cocodrie and Rockefeller prevalence rates were both significantly higher than those at Pontchartrain and Grand Isle. I expected Grand Isle to be significantly different from Pontchartrain but it was not. However, the 0% prevalence at Pontchartrain may skew the parameter estimate results.

Salinity was not the sole explanatory variable for the site variation as evidenced by the summer salinities and prevalence rates (Table 2.2, Fig. 2.13 and 2.14). I hypothesize that habitat type and population density may also be important. Rockefeller has complex marsh edge with dense crab populations, especially in the summer. Sample sites on Grand Isle were in more open water and the populations were not as dense, as evidenced by the intensified sampling effort required there. The differing commercial fishing pressures between Rockefeller, a protected wildlife refuge, and the other three commercially fished sites may influence population densities and subsequently *U. crescens* infection rates. If this symbiont is density dependent and its spores thrive in marsh edge habitats, then these could be undocumented, explanatory factors for the site difference.

Crab sex and size were not significant predictors of the probability of infections, which is consistent with my hypothesis because this hyperparasite opportunistically infects crabs, and no previous research supports its selection of a certain size or sex of crab. Prevalence was lower for crabs caught without bait. Biologically and ecologically, there is no indication that *U. crescens* affects crabs' foraging behavior or attraction to bait. A more likely explanation for capture method's significance was its multicollinearity with site, season, and salinity. The majority of the crabs caught with bait were caught at higher salinities (avg. 13.18 ppt) than crabs caught without bait (avg. 7.79 ppt) and the odds of infection increased with increasing salinities.

The moderate prevalence rates of this hyperparasite are not ecologically alarming because it does not harm the infected crabs (Perkins 1971). However, high incidence of *U. crescens* can have economic impacts because infected meat is unappealing and unappetizing (Couch and Martin 1979). If prevalence rates increase dramatically in commercial crabs in the future, managers should recommend that crabbers fish low salinities areas where *U. crescens* is less likely to be present. The absence of this protozoan in Lake Pontchartrain crabs is a commercial advantage for crabbers in that area.

#### 2.4.3 Prevalence of White Spot Syndrome Virus (WSSV)

White spot virus is a deadly crustacean pathogen that has been linked to global mass mortalities in farmed shrimp populations (Dhar et al. 2001, Shields and Overstreet 2007). It is usually not considered a major crab virus but blue crabs are an asymptomatic host of the virus and WSSV is a natural virus in some wild crab populations (Chang et al. 2001, Galaviz-Silva et al. 2004, Shields and Overstreet 2007). The determination of WSSV prevalence in wild blue crab populations is ecologically important due to possible transmission of the virus from crabs to more susceptible hosts such as shrimp (Shields and Overstreet 2007). Infected blue crabs have been found in the Gulf of Mexico (Shields and Overstreet 2007, Baumgartner et al. 2009).

In this study, I did not detect any WSSV infections in the 587 crabs analyzed. My result is inconsistent with another study on WSSV in blue crabs, in which prevalence was 27.2% in over 200 blue crabs collected from the New York, New Jersey, and Texas coasts (Chang et al. 2001). Prevalence of WSSV was hypothesized to be greater than 20% in other wild blue crab populations (Chang et al. 2001). In another crab species, the mud crab *Helice tridens*, collected from infected Taiwanese shrimp farms, prevalence of WSSV was approximately 36% (Lo et al. 1996a). A congener of the blue crab, *Callinectes arcuatus*, has been found to be infected with WSSV near three Mexican Pacific shrimp farms (Galaviz-Silva et al. 2004). Overall, infections may be limited to higher salinities that promote virus replication (Gao et al. 2011).

The absence of infected crabs in this study could be a result of the preservation of tissues used for the DNA extraction and PCR. Chang et al. (2001) recommended performing extractions on fresh gill tissue as frozen crab tissue was always negative. However, other studies have used frozen crustacean tissues and had positive results (Browdy et al. 2006, Baumgartner et al. 2009, Gao et al. 2011). All assessments were based on results when amplifiable decapod DNA was present so the chances of degradation of only viral and not crab DNA is unlikely. Regardless, ethanol-preserved tissues may be preferential in the future to ensure that freezing does not introduce error.

Another, more likely explanation for the 0% prevalence may be that the majority of Louisiana blue crabs have not been exposed to the virus yet. There have been documented cases of infection in the Gulf, but if the virus is not widely distributed, most crabs randomly collected along the coast would not be infected. If future prevalence rates continue to be low, then transmission of WSSV from Gulf crabs to other crustaceans should be of minor concern.

#### 2.4.4 Prevalence of *Hematodinium perezii*

*H. perezii* is a highly pathogenic dinoflagellate that has caused decreased commercial landings of blue crabs on the East Coast (Shields and Overstreet 2007, Mancinelli et al. 2013). None of the 760 crabs were infected with *H. perezii*. This result was not unexpected because the average salinity throughout this study was 11.5 ppt (Table 2.1). *H. perezii* infections are limited to water temperatures above 9 °C and salinities above 11 ppt, and typically are highest in the fall and lowest in the winter (Newman and Johnson 1975, Messick et al. 1999, Messick and Shields 2000, Lee and Frischer 2004). The dinoflagellate's dinospores are only active at salinities above 20 ppt

so infections at lower salinities are very rare (Coffey et al. 2012). At Grand Isle, the average salinity was only 20.5 ppt (Table 2.2). Although the parasite has been observed at equivalent salinities, its prevalence usually does not reach high numbers until 26-30 ppt (Messick and Shields 2000). Prevalence of *H. perezii* is typically highest in coastal areas with restricted water flow such as canals and embayments because crab migration and water exchange are limited (Meyers et al. 1996). Few crabs were collected from high salinity areas that had restricted water flow and the crabs that were from such areas were collected exclusively during the winter of 2013-2014 when water temperatures are low and *H. perezii* has been least prevalent in other studies (Messick et al. 1999, Messick and Shields 2000).

In the future, higher salinity areas should be sampled more extensively to more accurately estimate the effects *H. perezii* has on Gulf populations. If prevalence is high in areas where gravid females migrate to spawn, larval and adult life stages could be infected and die prematurely. Also, if prevalence is high in high salinity bayous and canals that are intensely fished, decreased commercial catch could be observed in these areas as has been noted on the East Coast (Shields and Overstreet 2007). In order to reduce costs, sites with salinities consistently below 11 ppt could be eliminated from PCR analyses (Shields 2001).

Many researchers suggest validation of PCR results and have relied on hemolymph smears to do so; however, hemolymph smears are often difficult to interpret (Lohan et al. 2012). If there are positive PCR results in the future, new methodology with dinoflagellate viral nucleoproteins (DNVPs) could be used to validate the PCR results (Gornik et al. 2012, Gornik et al. 2013). DNVPs are used in immunofluorescence assays and Western blot methods to sensitively detect *Hematodinium* sp. infections (Gornik et al. 2013). These methods may be more viable options for validating PCR results than hemolymph smears due to their sensitivity.

#### 2.4.5 Prevalence of *Loxothylacus texanus*

*L. texanus* is an internal barnacle that infects freshly molted crabs and causes suppression of the infected crab's molt cycle and castration of the crab (Ragan and Matherne 1974, Tindle et al. 2004, Shields and Overstreet 2007). Crabs are typically infected as small juveniles and their growth is typically stunted at 3 to 8 cm CW (Adkins 1972, Overstreet et al. 1983).

None of the adult or juvenile crabs had externa, and none were infected according to PCR amplifications. This prevalence rate was not unexpected because of the sampled sizes and salinities (Table 2.2). Parasitization by this barnacle is limited to salinities above 12 ppt because of the tolerance associated with the barnacle's larvae (Shields and Overstreet 2007). Infections are most commonly observed at high salinities above 25 ppt in the summer and fall in the Gulf of Mexico (Adkins 1972, Hochberg et al. 1992, Shields and Overstreet 2007). Some salinities sampled at Cocodrie, Rockefeller, and Grand Isle were high enough to support *L. texanus*, but with the focus on large juveniles and adults, I biased the sampling towards uninfected crabs.

To combat this bias, I sampled smaller crabs (<10 cm CW) at Rockefeller and Grand Isle in the late summer (Table 2.7). This barnacle is known to occur in Louisiana waters and infected, stunted crabs have been found in salinities of 15-20 ppt (Adkins

1972, Ragan and Matherne 1974). At Rockefeller the average salinity sampled for the smaller crabs was 15.7 ppt and at Grand Isle it was 21.8 ppt. Although these salinities are both higher than the barnacle's lower tolerance, in the future, more intensive sampling of small crabs in higher salinities may result in a non-zero prevalence rate.

If the prevalence of *L. texanus* is very low even in high salinity, offshore and nearshore sites, then the threat of this barnacle to the fishery is minimal. However, an increase in prevalence may represent a management concern because infected crabs are smaller than legal landing size and are not reproducing, leading to depletion of populations and destruction of the fishery (Christmas 1969, Alvarez and Calderon 1996). For this reason, the prevalence of *L. texanus* should be monitored continually. At a minimum, small crabs should be inspected for externa, and then secondarily, tissues should be extracted and amplified by PCR for internal infections.

#### 2.4.6 Prevalence of Reo-like Virus (RLV)

Reo-like virus is a double-stranded RNA virus that causes discoloration of the gills and carapace, tremors, lethargy, and paralysis of infected crabs (Johnson 1977, Shields and Overstreet 2007). The overall prevalence of RLV in 39 crabs analyzed was 7.7%. At this low prevalence rate, RLV may not pose a major threat to the commercial industry.

However, more samples need to be tested from many sample sites across all seasons to continually monitor this virus's potential population effects. If the prevalence rate from this study is artificially low due to a small sample size, then there may be a substantial threat to Gulf blue crabs because this parasite may pose a more serious threat to population numbers than any of the other diseases, parasites, or symbionts in this study. Reo-like virus has been correlated with high mortalities of crabs in shedding facilities (Bowers et al. 2011). Reo-like virus infections can occur over the wide range of salinities in Louisiana waters and is readily transmissible by cannibalism (Johnson 1977, Shields and Overstreet 2007, Bowers et al. 2010). The prevalence of RLV is usually lower in wild populations than in shedding systems because wild crabs are not as crowded and stressed (Bowers et al. 2010). Detected prevalence may also be lower in the wild because infected crabs may not be as likely to be caught in traps (Bowers et al. 2010).

#### 2.4.7 Prevalence and Intensity of Shell Rot

In wild populations the prevalence of shell rot varies and is often correlated with water quality (Shields and Overstreet 2007). For example, in waters with sewage outflows, shell rot is prevalent (Malloy 1978). On the East Coast, prevalence is usually less than 10% but epizootics have occurred in wild populations (Sindermann 1989, Shields and Overstreet 2007). In this study, the overall prevalence of shell rot was 54.8% (Fig. 2.10).

Prevalence of shell rot is dependent on water temperature and on ecdysis. The lower spring prevalence rates are explainable by the frequent molting in the spring as water temperatures warm (Guillory et al. 2001). At Rockefeller, the summer and winter 2013-2014 rates were significantly higher than the other seasons. In the summer, crabs

molt less frequently when water temperatures are very high (Guillory et al. 2001). Additionally in the winter, molting is infrequent because of low water temperatures and burrowing activities can abrade the shell and cause increased shell rot (Vogan et al. 1999, Shields and Overstreet 2007).

The significantly lower rate at Grand Isle in the summer was unexpected as prevalence was moderate at 43%. This result is not easily explained, but I have hypothesized that this significantly lower rate could be related to the frequent molting in the spring. If crabs molted in the late spring and were not exposed to many pathogens in the time between molting and early summer capture, then shell rot could be lower in the summer. Overall, these seasonal variations can be partially explained by the significant effect of water temperature where the odds of shell rot decreases by 35% as water temperature increases. This explains why the winter had high prevalence rates of shell rot, but does not explain why prevalence is also high in the summer. In the future, molt stage (postmolt, intermolt, premolt) should be recorded for every crab. I predict that a combination of water temperature and molt stage will more fully explain the probability of shell rot infections.

In addition to the significant seasonal variation, there was significant site variation where Rockefeller had the highest probability of shell rot infections, particularly in the summer and winter. Salinity was not a significant explanatory variable for the site variation, which was expected. Pathogens that cause shell rot, particularly chitinoclastic bacteria, occur over a wide salinity range so shell rot is not limited to particular salinity regimes (Shields and Overstreet 2007). The site variation could be explained by water quality and/or sediment substrate differences (Vogan and Rowley 2002). Because Rockefeller is a wildlife refuge, I would not expect it to have poorer water quality with increased fecal coliforms or bacteria than the other sites. However, if the substrates are different at Rockefeller in composition and size, they may more readily hold bacteria or fungi that can cause shell rot. In other studies, areas with higher organic content in the sediments have higher bacterial loads in the sediments (Vogan and Rowley 2002). In the future, sediment samples should be collected to determine whether sediment composition and particle size could explain the variation in the prevalence of shell rot.

There is also variation in prevalence between different sized crabs. This is caused by higher ecdysis frequency in smaller crabs (Sandifer and Eldridge 1974, Vogan et al. 1999). Males were more likely to have shell rot than females, and this can be explained by the aggressive nature and mating behaviors of male blue crabs (Vogan and Rowley 2002). The injuries received during mating and fighting can damage the shell and make it susceptible to pathogens that degrade the shell and cause shell rot (Rosen 1967, Shields and Overstreet 2007). Unlike crab size and sex, the use of bait did not affect the prevalence of shell rot. This result was expected because there is no known link between shell rot and suppressed appetite or foraging that may cause only healthier crabs to be attracted to the bait. Additionally, shell rot is typically harmless so infected crabs would not necessarily be weaker and more attracted than healthy crabs to the shelter of traps.

Overall, I would expect shell rot to be largely absent from wild populations and when present, at low intensities. The shell rot intensity results were consistent with the prevalence results for season, size, and sex variation. However, results for intensity and prevalence were inconsistent for the effects of salinity and capture method. The



increased intensities with increasing salinity are likely a result of the high intensity scores at Rockefeller and the low intensity scores at Pontchartrain, which was true when site was retained in the model instead of salinity. The higher intensity score for crabs caught with bait could be due to aggressive interactions in the traps that cause injury to the shell. However, crabs were also collected with baited lines and subsequent aggressive interactions would be minimal. Therefore, more likely explanations are based on unequal sample sizes and season when each capture method was used. The majority of the crabs caught without bait were caught before I developed the intensity score scale. The retrospective scoring of these crabs could have introduced error, especially in the winter and spring of 2013, when the photographs were of low quality.

Shell rot can be an indicator for crustacean health because at high prevalence rates, it can indicate poor water quality or high stress levels (Noga et al. 1994, Shields and Overstreet 2007). Currently, shell rot likely poses little threat to the populations and fishery, because shell rot is shed during ecdysis and most infections are not necrotic. In the future, if shell rot becomes more prevalent and more intense, then increased mortalities from shell rot and secondary infections can be expected.

#### 2.4.8 Prevalence of *Vibrio* spp.

*Vibrio* spp. are ubiquitous, chitinoclastic bacteria in marine ecosystems and are one of the main pathogens causing shell rot (Sindermann 1989, Noga et al. 1998, Shields and Overstreet 2007). I found that the overall prevalence of *Vibrio* spp. infections in the hemolymph and internal organs was 39.9% (Fig. 2.10). Overall prevalence of *Vibrio* spp. hemolymph infections was 22.3% (Fig. 2.15). The sample set for hemolymph only infections (Table 2.8) was reduced because of lab error that included inconsistent amounts of hemolymph plated and/or puncture of internal organs that contain high bacteria loads.

I hypothesized that season or water temperature would significantly affect the prevalence rates because *Vibrio* spp. prevalence is dependent on water temperature (Huq et al. 1984). At water temperatures above 25 °C, the bacteria survive and replicate more readily (Huq et al. 1984). However, there was no significant variation with water temperature and it is possible that there is no true seasonality to *Vibrio* spp. infections in Louisiana. This trend has been observed in Texas studies and has been attributed to the warmer year-round temperatures as compared to the East Coast (Davis and Sizemore 1982). The lack of variation in hemolymph prevalence rates with salinity is consistent with previous research that has demonstrated no correlation (Huq et al. 1984).

The final model indicated that females were more likely to be infected than males. Other studies' data are largely inconclusive on effects of sex (Shields and Overstreet 2007). Welsh and Sizemore (1985) did not find any difference in infection rates between males and females. I would have expected that males would be more likely to be infected than females because they are more aggressive, and aggressive behavior can cause injury that exposes internal tissues to bacteria in the water. Additionally, crabs caught without bait were more likely to be infected, which is again contrary to my hypothesis. Particularly crabs caught in traps should be more stressed

and more likely to be injured and these factors can promote hemolymph bacterial infections (Welsh and Sizemore 1985).

It is possible that this apparent discrepancy between the sexes and capture methods used is actually a difference in injured versus healthy crabs as injured crabs are more likely to have *Vibrio* spp. infections (Tubiash et al. 1975, Shields and Overstreet 2007). In the future, injuries, especially autotomized legs, should be recorded to examine if injuries are ecologically more important than sex or collection site for predicting *Vibrio* spp. infections. Also a consistent amount of hemolymph should be plated, and infection intensity should be determined. Low intensity infections may not be harmful to crabs so a measure of potential harm to the populations should be included.

Overall, despite the low aggregate prevalence rate of hemolymph infections in the wild, infections may threaten the commercial fishery because infections become more intense and prevalent upon commercial handling of the crabs and may contribute to losses of crabs prior to sale (Welsh and Sizemore 1985). Additionally, heavily infected, weak crabs could be more attracted to shelter that traps provide than healthy crabs. Because *Vibrio* spp. are ubiquitous, the primary recommendation for minimizing infections in the commercial industry is to improve handling of crabs to reduce injury and stress. Also, when possible, trap soak time should be minimized, especially during rain events, because enmeshed crabs that are subjected to abrupt salinity changes often die from intense *Vibrio* spp. infections (Shields and Overstreet 2007).

#### 2.4.9 Co-occurrence of Shell Rot and *Vibrio* spp.

*Vibrio* spp. are the main bacteria that cause shell rot (Shields and Overstreet 2007). Necrotic shell rot lesions can expose internal organs and tissues to *Vibrio* spp. that are ubiquitous in the water (Millikin and Williams 1984). Because of this correlation, many studies have recorded the co-occurrence of shell rot and *Vibrio* spp. infections of the hemolymph and of the shell itself (Sandifer and Eldridge 1974, Iverson and Beardsley 1976, Malloy 1978, Shields and Overstreet 2007). I expected moderate rates of co-occurrence of shell rot and hemolymph infections in this study, but the rate was only 8%. Neither shell rot nor *Vibrio* spp. prevalence rates correlated with the presence of the other.

If I had swabbed all shell rot spots and lesions, the co-occurrence may have been at a higher rate. Additionally, carapace swabs may be of interest to determine what pathogens co-occur with shell rot in the Gulf of Mexico. For example, Noga et al. (1994) found that *Vibrio* spp. were more predominant in shell rot lesions at high salinities and *Aeromonas* spp. and *Pseudomonas* spp. were more prevalent at low salinities.

#### 2.4.10 Other Bacterial Infections

Many non-*Vibrio* spp. bacteria were identified from crab hemolymph and organs. *Aeromonas* spp. and *Pseudomonas* spp. are both common in blue crabs and are often associated with disease (Noga et al. 1998). *Serratia* spp. has been isolated from shell rot lesions (Noga et al. 2000). *Photobacterium damsela* is pathogenic to blue crabs

and may cause some mortalities (Givens et al. 2013). Many bacterial species isolated may explain why the co-occurrence between *Vibrio* spp. and shell rot infections was low because some of these species are known to cause shell rot, especially *Aeromonas* spp., *Pseudomonas* spp., and *Serratia* spp. (Noga et al. 1994, Shields and Overstreet 2007).

In the future, identification of bacteria infecting blue crab hemolymph and organs should be studied more extensively, and intensity of infections should be determined. Also, swabs from shell rot lesions should be included to identify what bacteria may be causing the majority of shell rot infections.

#### 2.4.11 Conclusions and Future Investigations

The results of this study indicate that the nearshore populations of blue crabs in Louisiana are potentially threatened by RLV, shell rot, and *Vibrio* spp. These three were all detected and are capable of causing blue crab mortalities. In future studies, more samples should be analyzed for RLV, and *Vibrio* spp. infection intensity should be determined.

The absence of *H. perezii* and *L. texanus* is a favorable result ecologically and economically as both parasites can cause significant decreases in population numbers and landings. However, the salinities and the minimum size sampled may bias these prevalence rates. In the future, higher salinity areas, especially spawning areas and areas commercially fished, should be sampled for a more accurate estimation of prevalence. Also, smaller juvenile crabs should be extensively sampled for *L. texanus*.

I determined the prevalence rates for the three harmless symbionts, *L. callinectes*, *U. crescens*, and WSSV. Future measurements of prevalence may be important if there are increased reports of internal or external signs of infection attributable to these three symbionts.

Overall, with regards to the seven primary diseases, parasites, and symbionts studied in this thesis, the blue crab populations of coastal Louisiana appear healthy with no widespread infections by fatal diseases or parasites. However, RLV prevalence may be much higher than the 7.7% determined in 39 samples. If this virus is highly prevalent, then the populations and fishery may be in danger of decline and management changes may be necessary.

As this was a one year study, more long-term monitoring is necessary, especially given the low landings of 17575 metric tons in 2013 as compared to average landings of 21052 metric tons (Appendix C). Future studies are important to understand the context of 2013's prevalence rates and the long-term health of the fishery.

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## CHAPTER 3: PREVALENCE OF DISEASES, PARASITES, AND SYMBIONTS IN BLUE CRABS HELD AT SOFT SHELL SHEDDING FACILITIES

### 3.1 Introduction

The soft shell blue crab industry began in the Gulf around 1887 (Guillory et al. 2001). In Louisiana, soft shell crabs were originally shed in and around Lake Pontchartrain in floating boxes (Guillory et al. 2001). The industry slowly spread westward in Louisiana and eventually into shedding facilities in the 1960s (Jaworski 1982, Guillory et al. 2001). Shedding facilities replaced floating boxes and live cars because these facilities were no longer limited to the coastline and were less affected by worsening water quality (Guillory et al. 2001). Shedding facilities are usually either open, flow-through or closed, recirculating systems.

In Louisiana, most pre-molt crabs, often called “redliners” or “peelers”, are caught in hard crab traps (Guillory et al. 2001). Fishermen can determine molt stage based on the coloration of the line at the edge of the swimming leg (Smith and Chang 2007). Ninety-one percent of redliner blue crabs will molt within three days (Oesterling 1982). In Louisiana the peak season for soft shell shedding is usually from March to October when water temperatures are warm and stimulate frequent molting (Guillory et al. 2001). Within this season, soft shell crabs are most abundant during peak molting periods in April or May and in September or October (Guillory et al. 2001).

Louisiana has the largest soft shell industry in the Gulf. In 2012, approximately 69.0 metric tons of peeler crabs and 4.0 metric tons of soft shell crabs were landed in Louisiana for a value of about \$430,000 and \$47,000, respectively (National Marine Fisheries Service 2013). The remaining Gulf states only landed about 14.7 metric tons of peeler and soft shell crabs combined (National Marine Fisheries Service 2013). This valuable Louisiana industry is extremely dependent on mortality rates at shedding facilities, and it has been estimated that about 23% of redliners die within five days of being held in the facilities (Chaves and Eggleston 2003). Possible explanations for this very high mortality rate include diseases and parasites, stress or injury due to handling, physiological stress associated with molting, stress or injury due to crowding, and poor water quality at the facilities.

Diseases and parasites that can affect the mortality rate in shedding facilities include: shell rot, *Vibrio* spp., *Ameson michaelis*, reo-like virus (RLV), and *Lagenophrys callinectes*. Although typically harmless, severe, necrotic shell rot 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 and Williams 1984). Shell rot can also be fatal to peelers because the previous molt can adhere to the new shell at the shell rot lesion (Vogan et al. 2001). Prevalence of shell rot may be high in crabs at shedding facilities due to stress and crowding (Iverson and Beardsley 1976). *Vibrio* spp. infections can cause hemocytes to aggregate around the high densities of bacteria, which can lead to morbidity and mortality of the infected crab (Bowser et al. 1981, Shields and Overstreet 2007). *Vibrio* spp. infections have been correlated with high mortalities in North Carolina shedding facilities and may have caused as much as 80% mortality in pre-molt crabs (Sizemore 1985). *A. michaelis* causes muscle lysis of infected crabs (Messick and Sindermann 1992). Reo-like virus infected crabs often

tremor, are lethargic, and eventually become paralyzed (Johnson 1977, Shields and Overstreet 2007). Both *A. michaelis* and RLV are hypothesized to cause mass mortalities in shedding facilities, possibly due to crowding and cannibalism that allow for easy transmission (Messick and Sindermann 1992, Bowers et al. 2010, Bowers et al. 2011). Reo-like virus has been hypothesized to be the predominant cause of blue 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 but it can occasionally cause asphyxiation. The negative effects of this ciliate generally occur when a crab is stressed due to low dissolved oxygen or poor water quality (Couch and Martin 1979); both of which can occur at shedding facilities.

Other parasites that can indirectly harm the soft shell industry include *Hematodinium perezii* and *Loxothylacus texanus*. *H. perezii* causes morbidity and mortality of infected crabs after destroying hemocytes as well as the oxygen transporter protein (Taylor et al. 1996, Lee and Frischer 2004). *L. texanus* does not kill its host but does castrate its host and suppresses the infected crab's molt cycle (Shields and Overstreet 2007). *H. perezii* has not been directly correlated with mortalities in shedding systems because this parasite has only been found at salinities greater than 11 ppt, and the potential transmissive state of the parasite is not active below 20 ppt (Newman and Johnson 1975, Messick and Shields 2000, Coffey et al. 2012). However, *H. perezii* could infect and kill a pre-molt crab that was caught in a high salinity area and transported to the shedding tanks. *L. texanus* is not usually observed in shedding facilities for several reasons: 1) the barnacle's larvae are only viable at salinities of 12 ppt or higher; 2) crabs that move into lower salinity areas can shed the infection due to the barnacle's intolerance of low salinities; and 3) infected crabs bearing the externa of the barnacle no longer molt and would not exhibit signs indicative of an upcoming molt that are used to select crabs for shedding facilities (Adkins 1972, Overstreet et al. 1983, Shields and Overstreet 2007). Both *H. perezii* and *L. texanus* can decrease the number of crabs in the wild that are actively molting and subsequently available to be shed at soft shell shedding facilities.

One hyperparasitic symbiont that can indirectly affect the soft shell industry is *Urosporidium crescens*. *U. crescens* is innocuous to the blue crab, but it causes large black cysts in the crab's hepatopancreas and muscle (Messick and Sindermann 1992). It has only been recorded at moderate and higher salinities, either because of lack of infections at low salinities or because salinity tolerance research has not been conducted (Messick 1998). The black "pepper spots" or "buckshots" seen in infected crabs can negatively affect the soft shell industry because the meat is unappetizing and some consumers will discard the infected meat (Couch and Martin 1979).

During the active shedding season in 2013, I determined the prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, *Hematodinium perezii*, *Loxothylacus texanus*, and *Urosporidium crescens* at four commercial shedding facilities along the Louisiana coast. *Ameson michaelis*, reo-like virus, and non-*Vibrio* spp. bacterial infections were observed but prevalence rates were not determined.

## 3.2 Materials and Methods

### 3.2.1 Shedding Facilities

I chose four shedding facilities in southeastern and south central Louisiana based on their active shedding season and their willingness to cooperate. The facilities were located in (East to West): Hopedale (KM), Dulac (LU), Port of W. St. Mary (SM), and Erath (HA) (Fig. 3.1).

### 3.2.2 Sample Collection

Starting in April 2013, I collected 20 redliner, pre-molt crabs from each facility every two months throughout their active season. At the time of collection, I measured the water temperature, salinity, and pH (Table 3.1). I also collected water samples to determine alkalinity, nitrite, nitrate, and ammonium levels. I used Mardel standard aquarium test strips to determine approximate nitrate, nitrite, and alkalinity concentrations. For ammonia I used Tetra standard aquarium test strips. More precise measurements using spectrophotometry were not necessary because water quality monitoring was a secondary interest in the event that one facility experienced a mass mortality. The approximate holding time of the crabs was recorded and was consistently less than a week at the facilities.

The 20 crabs were placed alive on burlap-covered ice to be transported back to LSU for processing and dissection. In April 2013 I collected 13 pre-molt and 27 post-molt crabs from LU, 20 pre-molt from SM, and 20 pre-molt from HA. The post-molt crabs were analyzed for diseases, parasites, and symbionts but those results were not included in any of the statistical analyses due to different molt stage. KM was not sampled in April and October because it was not operational during these months. KM was added to the study after another facility near Lake Pontchartrain did not open in 2013 due to low crab landings. After April, all crabs collected were pre-molt. In June I collected 20 from KM, SM, and HA and 19 from LU. In August I collected 20 from all four facilities and in October I got 20 from LU, SM, and HA.

Table 3.1: Salinities and water temperatures sampled in 2013 at four soft shell shedding facilities located in Hopedale (KM), Dulac (LU), Port of W. St. Mary (SM), and Erath (HA) ([--] denotes that crabs were not in a shedding tank at time of collection, -- denotes that crabs were not collected at that facility).

Site	Water Parameter	April	June	August	October
Hopedale (KM)	Salinity (ppt)	--	[--]	5.4	--
	Temp. (°C)	--	[--]	29.4	--
Dulac (LU)	Salinity (ppt)	7.9	7.7	5.7	5.5
	Avg. Temp. (°C)	18.9	29.5	28.8	24.6
Port of W. St. Mary (SM)	Salinity (ppt)	4.1	3.3	2.8	2.5
	Avg. Temp. (°C)	23	27.6	28.6	26.6
Erath (HA)	Salinity (ppt)	7.3	3.6	3.6	2.2
	Avg. Temp. (°C)	25.1	27.3	26.8	24.7

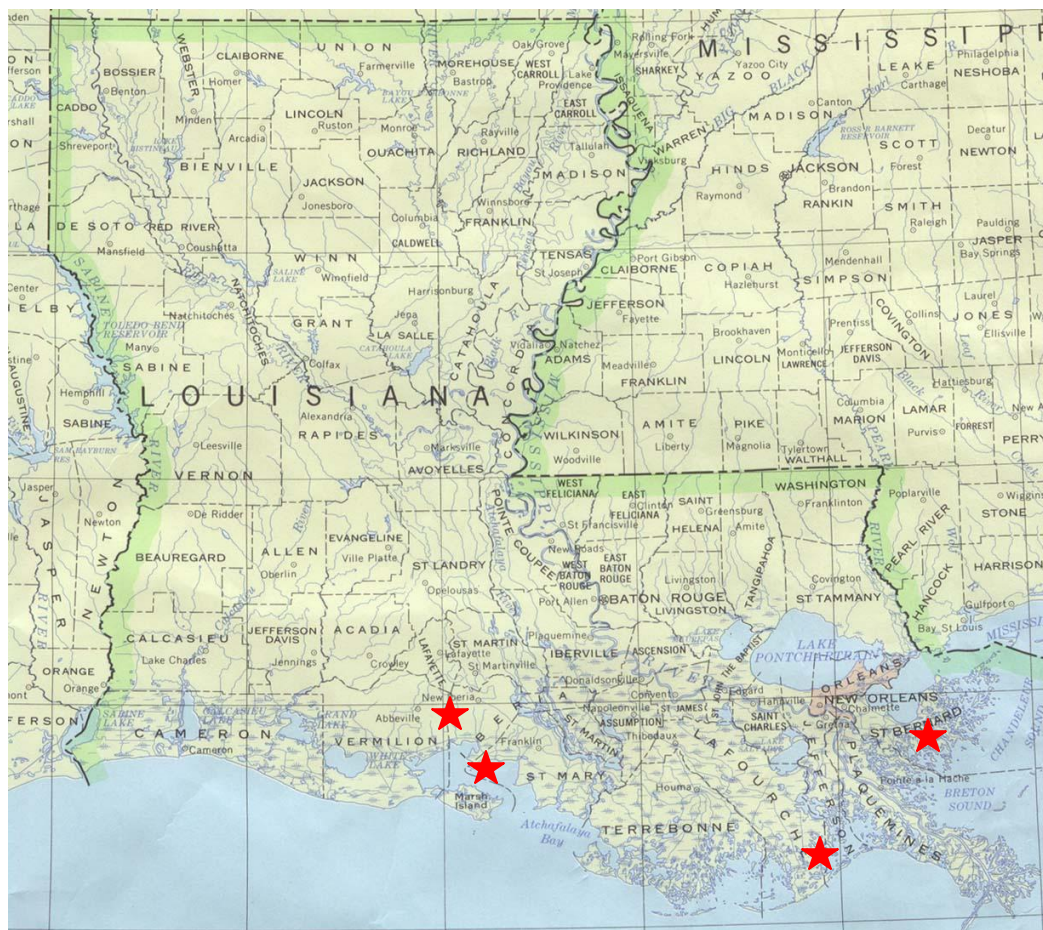


Figure 3.1: Louisiana shedding facilities for blue crab collection were located in (East to West): Hopedale (KM), Dulac (LU), Port of W. St. Mary (SM), and Erath (HA) (red stars).

### 3.2.3 Initial Processing and Dissection

All pre-molt crabs were dissected alive, with the exception of 20 post-molt crabs purchased from LU in April. These post-molt crabs were dead and frozen at the time of purchase. I used ice and cold water to reduce crab mobility for ease of dissection. Before dissection began, I measured the carapace width (CW) to the nearest millimeter with a standard metric ruler and sexed the crab. Every crab was photographed with a Canon camera against a white background with a unique identifier including a designation for the shedding facility, collection month, and individual crab (ex. SM1 #3 was the 1<sup>st</sup> crab collected from the St. Mary's shedder in the third sampling month, August). In the photographs, I included all claws and legs and captured both the dorsal and ventral sides of the crab. These images were later analyzed in the computer program photoQuad for percent coverage of the carapace with shell rot. Close-up photographs of shell rot lesions and spots were also taken when possible for shell rot diagnosis (Section 3.2.4). I visually estimated percent coverage of shell rot and scored it from 0 to 3 based on the estimation (Table 3.2). Shell rot intensity was also scored from

0 to 2 based on the progression of the disease to necrosis (Table 3.3). Any other abnormalities or external signs of infection were noted.

Next, I cleaned the right swimming leg (based on dorsal view) at the uncalcified joint near the carapace with a cotton swab and 70% ethanol (Welsh and Sizemore 1985). In order to ensure complete sterilization of the joint, I sterilized the leg twice. A sterile needle (18 to 25 gauge) with a sterile, 3-cc syringe was inserted into the leg. Hemolymph was drawn without the removal of the needle and was plated on sterile thiosulfate-citrate-bile salts-sucrose (TCBS) agar that was prepared per manufacturer instructions (Sigma-Aldrich, St. Louis, MO). TCBS is selective for *Vibrio* spp. (Colwell et al. 1975). After plating <0.5 mL of hemolymph on the agar, an additional 1 mL of hemolymph was drawn from the crab and was preserved in 95% ethanol for polymerase chain reaction (PCR) analysis.

After withdrawing the hemolymph, one walking leg was removed and frozen at -20 °C for RLV PCR. Then, the crab was dissected, and hepatopancreas, gill, heart, and midgut samples were removed (Appendix A). One sample of hepatopancreas was frozen at -20 °C, and a second was preserved in 95% ethanol for *L. texanus* PCR. I removed and froze at -20 °C two samples of gill for *L. callinectes*. The heart was frozen at -20 °C, and the midgut was preserved in 95% ethanol for *L. texanus* PCR. While removing these tissues, I noted any abnormalities or signs of infection. Then I checked the muscle underneath the gills for infection by *U. crescens* or *A. michaelis*. Before discarding each crab, I also cracked the abdomen to ensure that there were no *L. texanus* externa. After each dissection, forceps and other tools used to dissect the tissues were sterilized in a 10% bleach solution to minimize DNA transfer between crabs.

For the April and June samples, I fixed samples of hepatopancreas and gill in zinc buffered formalin (Z-Fix) for histological analyses. However, after unsuccessful detections from histologically-prepared samples, I stopped removing and fixing tissues for standard histology. However, when I noticed a particularly abnormal tissue, I removed that tissue and fixed it in 1.25% glutaraldehyde with 2% paraformaldehyde and 5% sucrose. These fixed tissues were transferred to 0.1 M cacodylate buffer and delivered to Dr. Julia Sokolova at the LSU Veterinary Medicine Microscopy Center.

Table 3.2: Shell rot intensity score based on visual observations and percent coverage. Adapted from: Castro and Somers 2012, Messick 2012.

Visual observations	% Coverage of Shell	Intensity
Clean shell, discoloration easily scrapes off	0%	0
Discolored spot, mild	1-10%	1
Moderate, many discolored spots	11-50%	2
Severe, necrosis, covered in discolored spots	51-100%	3

Table 3.3: Shell rot intensity score based on progression of shell rot as the presence of spots and/or lesions.

Visual observations	Intensity
No shell rot	0
Shell rot spots only (no lesions)	1
Necrotic lesions (deep, shell eroded)	2



### 3.2.4 Diagnosis of Shell Rot

Shell rot was the only disease for which I determined both prevalence and intensity. Shell rot was diagnosed during the initial dissection (Section 3.2.3). To diagnose the disease, I examined the dorsal and ventral portions of the carapace as well as all legs and claws (Fig. 3.2). Intensity was determined by percent coverage and by progression of the disease to necrosis (Tables 3.2 and 3.3, Fig. 3.2). I estimated percent coverage during the initial dissection and by using the computer program photoQuad (Trygonis and Sini 2012).

In photoQuad, I outlined a freehand quadrat to eliminate the background because inclusion of the background area would artificially decrease the percent coverage of the carapace with shell rot. After that, I placed an 8 pixels by 8 pixels grid over the image and selected each grid square whose area was at least 50% covered by shell rot. In the cell count summary, photoQuad generated a percent coverage based on selected versus unselected grid squares. Because only the dorsal or ventral side of a crab could be analyzed at a time, I divided the percent coverage in half to represent the percent of the total carapace covered with shell rot. As percent coverage alone was not an accurate measure of intensity of shell rot, I developed the scale from 0 to 2 based on the presence of shell rot spots versus presence of necrotic lesions that indicate a more life-threatening, severe case of shell rot (Table 3.3).

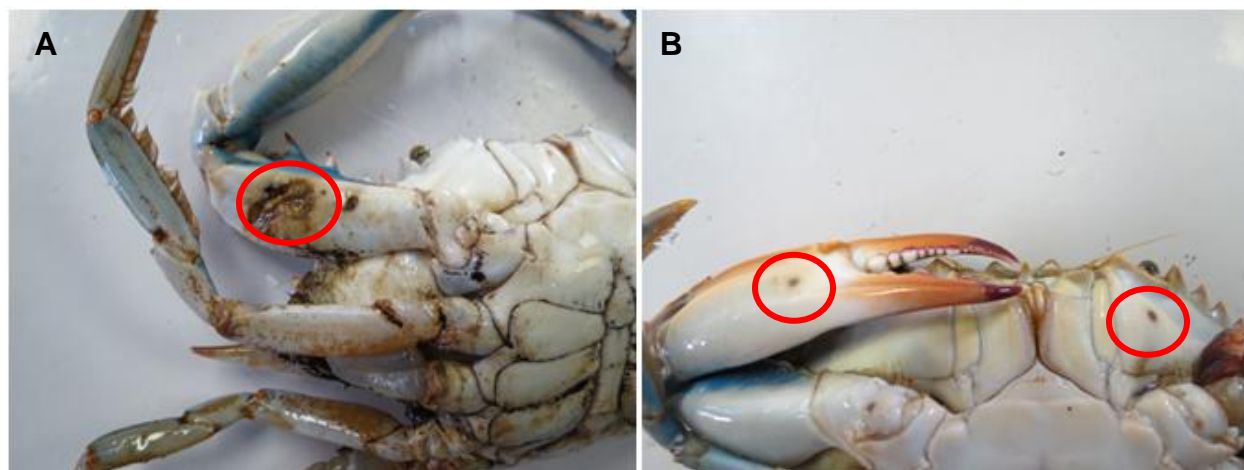


Figure 3.2: Shell rot photographs of two crabs collected from the St. Mary's facility in August and October 2013. A: This crab's claw was likely injured and a necrotic shell rot lesion has formed (red circle). On the shell rot scale from 0 to 2, this shell rot scored as intensity 2. B: This crab's claw and carapace have small shell rot spots (red circles). On the shell rot scale from 0 to 2, this shell rot scored as intensity 1 because no necrosis was evident.

### 3.2.5 Detection of *Vibrio* spp.

Less than 0.5 mL of hemolymph was plated on TCBS agar during the initial dissection (Section 3.2.3). I placed all plates in an incubator at 28 °C for 48 hours and after 48 hours, removed each plate, photographed it, and made an initial assessment on the presence of *Vibrio* spp. (Fig. 3.3). On TCBS agar, *Vibrio* spp. turn yellow, greenish



yellow, and green blue. However, other bacteria such as *Pseudomonas* spp., *Aeromonas* spp., and *Photobacterium* spp. can grow on the agar. Due to the inability to use growth and coloration as a definitive means of detection, plates with growth were sent to Dr. Hawke at the LSU Veterinary Medicine School (LA Aquatic Diagnostic Laboratory, LADL) for further testing (Fig. 3.3). To avoid high testing costs, I did not send any plates that had identical colony growth during the same sampling period. At the LADL, colonies were replated onto blood agar and grown for general bacteriology. The colonies on the blood agar were analyzed with analytical profile index (API) 20E bacteriology strips in order to identify the genus and species of the colony. If the results from the API strips were not definitive, I did not make an assessment for *Vibrio* spp. infection and did not include this missing result in any analyses. I assessed 38 crabs from KM, 71 from LU, 79 from SM, and 79 from HA. Results from API included identifications of non-*Vibrio* spp. bacteria, and those data were also recorded. Due to lab error, results included bacteria from the hemolymph and internal organs. Only 20 samples from KM in June, 13 from April and 19 from August at LU, 79 from SM, and 20 from April and June and 8 from October at HA were considered accurate results for infections of only the hemolymph.

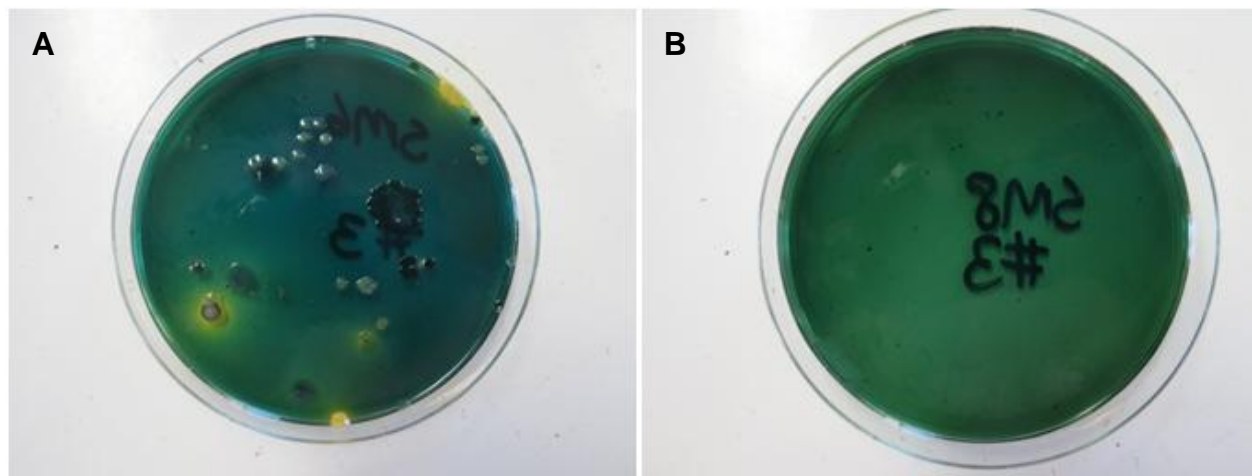


Figure 3.3: Thiosulfate-citrate-bile salts-sucrose (TCBS) agar was inoculated with hemolymph to detect *Vibrio* spp. infections. A: This TCBS plate with bacterial growth for a crab collected from the St. Mary's (SM) facility in August 2013 was potentially infected with *Vibrio* spp. and the colony identities were verified by analytical profile index. B: This clean TCBS plate indicated that this crab collected from SM in August 2013 was not infected with *Vibrio* spp.

### 3.2.6 Detection of *Ameson michaelis*

Any very white muscle was dissected from the crab and fixed in 1.25% glutaraldehyde with 2% paraformaldehyde and 5% sucrose (Section 3.2.3, Fig. 3.4). After 24 hours, I transferred the muscle tissue to 0.1 M cacodylate buffer and delivered it to Dr. Julia Sokolova at the LSU Veterinary Medicine Microscopy Center. Dr. Sokolova prepared the tissues for electron microscopy after embedding, sectioning, and staining the tissues with uranyl acetate and lead citrate. She analyzed the slides with a

transmission electron microscope to identify any *A. michaelis* spores in the muscle (Fig. 3.5).

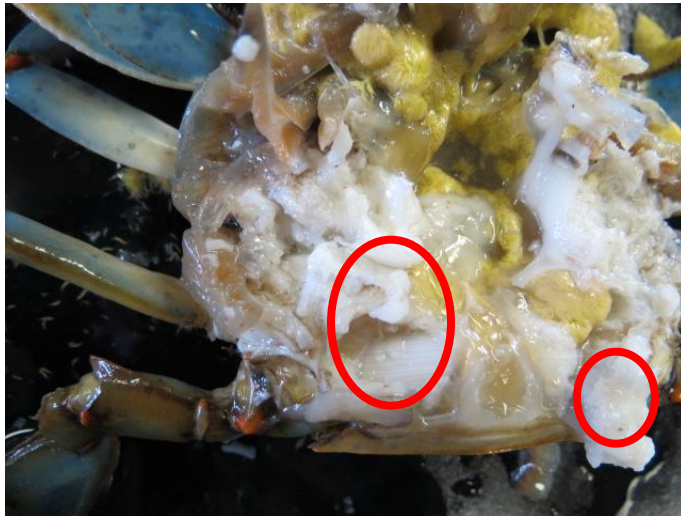
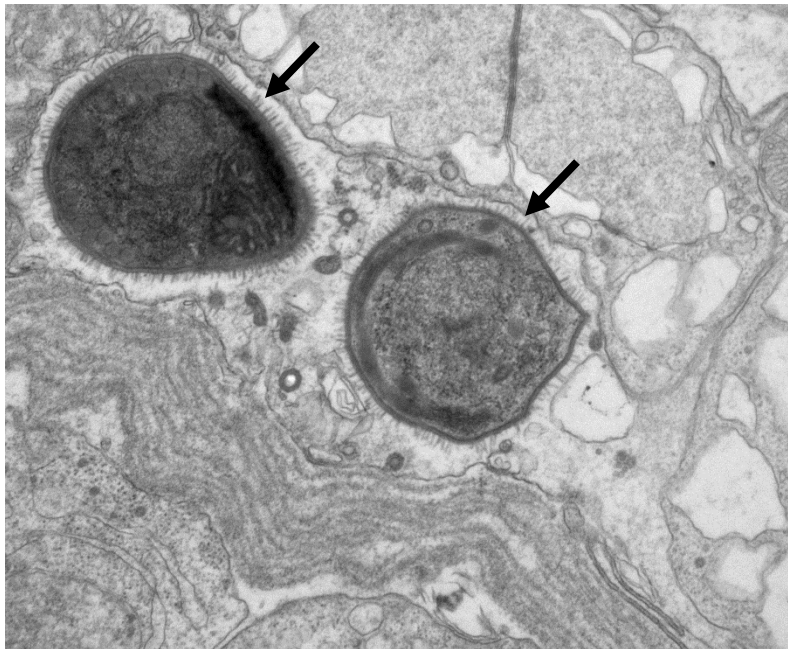


Figure 3.4: Very white muscle (red circles) indicating a possible *Ameson michaelis* infection in a crab collected in August 2013 from the St. Mary's shedding facility.



2013-08-12\_Holly\_Cs-Ameson\_008

Cs-Ameson Aug 3, 2013

500 nm

Figure 3.5: Transmission electron micrograph showing *Ameson michaelis* spores (arrows) and their characteristic, thin projections.

### 3.2.7 Detection of Reo-like Virus

Reo-like virus (RLV) was not originally included in this project. After speaking with other blue crab disease researchers, I began collecting and freezing walking legs during the initial dissection at the end of April 2013 (Section 3.2.3). Legs were placed in

labeled whirl-pak bags and were frozen at -20 °C until 9 legs collected from LU and 11 legs collected from HA during June 2013 were sent to Dr. Eric Schott at the University of Maryland Center for Environmental Science for reverse-transcription PCR (Bowers et al. 2010).

### 3.2.8 Detection of *Lagenophrys callinectes*

I detected *L. callinectes* on frozen gills depending on the presence of the ciliate's circular lorica (Couch and Martin 1979, Mayen-Estrada and Aguilar-Aguilar 2012). Thin gill sections (<1 cm thick on average) were cut from the gills with a scalpel blade, placed on microscope slides, and immediately examined under a light microscope at a magnification of 10X or higher. To detect active infestations, presence of *L. callinectes* was only recorded when an occupied lorica was seen (Fig. 3.6 and 3.7). When I did not see an occupied lorica in the first section, I examined a minimum of three sections to confirm absence of the symbiont.

In April and June 2013, I fixed gill tissue for histological processing. To detect *L. callinectes* on decalcified gill that I embedded in paraffin and stained with hematoxylin and eosin in addition to detection on the frozen gills. The goal was to create a permanent, preserved record of infested gills. However, these histological processes led to loss of the loricae from gill tissue so all samples appeared negative. Consequently, I only assessed frozen gills for *L. callinectes* and abandoned histology on gills.

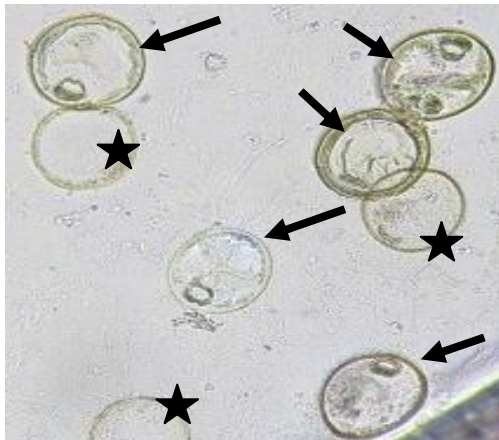


Figure 3.6: Occupied *Lagenophrys callinectes* loricae (arrows) co-occur with empty loricae (stars) on blue crab gills.

### 3.2.9 Detection of *Hematodinium perezii*

*H. perezii* can be detected on stained, fresh hemolymph smears and by PCR. Due to unfamiliarity and inexperience with this parasite, I was unable to identify *H. perezii* by staining hemolymph with neutral red stain, and therefore, only used PCR to detect the dinoflagellate (Shields 2012). Hemolymph was preserved in 95% ethanol for PCR (Section 3.2.3).



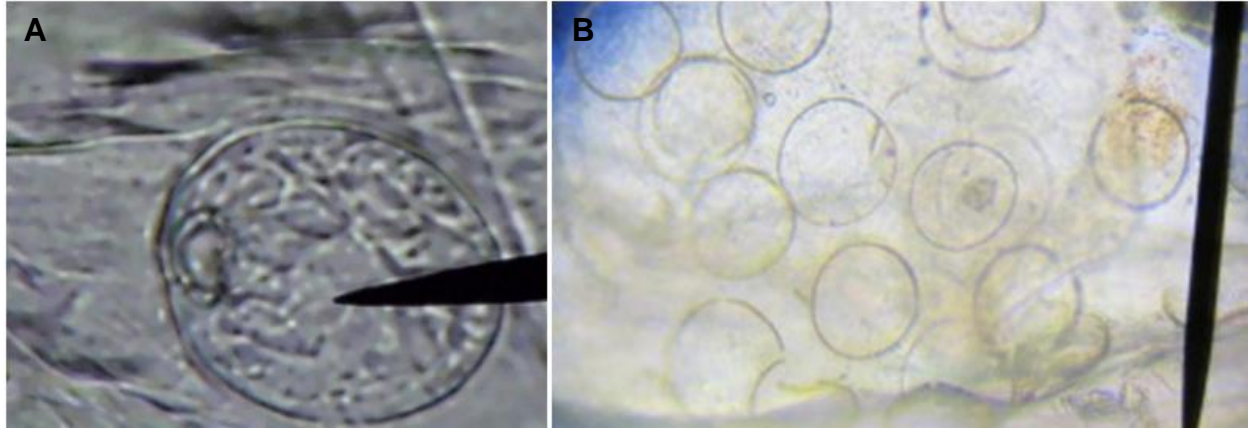


Figure 3.7: A: An occupied *Lagenophrys callinectes* lorica (arrow) present on gill tissue collected in April 2013. The lips of the lorica aperture are visible (left of arrow). This gill had the ectocommensalistic symbiont. B: Unoccupied *Lagenophrys callinectes* circular loricae present on gill tissue collected in April 2013.

Prior to DNA extraction, I centrifuged approximately 200  $\mu$ l of ethanol-preserved hemolymph at 1500  $g$  for 1 min and removed the excess ethanol (Lohan et al. 2012). Samples dried for at least 30 min to allow residual ethanol to evaporate. Samples were lysed overnight with a few rare exceptions when lysis was limited to approximately 4 hr (Lohan et al. 2012). To extract DNA from the lysed hemolymph, I used Qiagen's DNeasy Blood and Tissue kits per the manufacturer recommendations with two 5 min elution incubations and two 100  $\mu$ l elutions. For PCR, I used the general metazoan primers nSSU A (5'-AACCTGGRTTGATCCTGATCCTGCCAGT-3') and nSSU B (5'-GATCCTTCCGCAGGTTACCTAC-3') to verify that each sample contained amplifiable DNA (Lohan et al. 2012). This PCR reaction contained 1X standard *Taq* (Mg-free) reaction buffer (New England Biolabs, Ipswich, MA), 1.5 mM  $MgCl_2$ , 0.2 mM dNTPs, 1  $\mu$ M nSSU A, 1  $\mu$ M nSSU B, 1 unit of *Taq* polymerase, and 0.4 mg/mL BSA or RSA (Lohan et al. 2012). The reaction was brought to a volume of 10  $\mu$ l with Nanopure water. Thermocycling conditions were: 95.0  $^{\circ}C$  for 4 min; 45 cycles of 94.0  $^{\circ}C$  for 30 s, 45.0  $^{\circ}C$  for 30 s, 72.0  $^{\circ}C$  for 2 min; and a final extension at 72.0  $^{\circ}C$  for 5 min (modified from Lohan et al. 2012). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller.

To detect *Hematodinium* sp., I used the *Hematodinium* sp. primers HITS1F (5'-CATTCACCGTGAACCTTAGCC-3') and HITS1R (5'-CTAGTCATACGTTTGAAGAAAGCC-3') (Lohan et al. 2012). This PCR reaction contained 1X standard *Taq* (Mg-free) reaction buffer, 1.5 mM  $MgCl_2$ , 0.1 mM dNTPs, 0.5  $\mu$ M HITS1F, 0.5  $\mu$ M HITS1R, and 1 unit of *Taq* polymerase (Lohan et al. 2012). The reaction was brought to a volume of 10  $\mu$ l with Nanopure water. After November 1, 2013, PCR reactions contained 1 mM  $MgCl_2$  instead of 1.5 mM to optimize the reaction (Lohan et al. 2012). Thermocycling conditions were: 95.0  $^{\circ}C$  for 5 min; 40 cycles of 94.0  $^{\circ}C$  for 30 s, 58.0  $^{\circ}C$  for 30 s, 72.0  $^{\circ}C$  for 90 s; and a final extension at 72.0  $^{\circ}C$  for 5 min (modified from Lohan et al. 2012). Thermocycling was performed in an Eppendorf Mastercycler proS thermocycler.

To detect *Hematodinium* spp., I used a second set of primers that target the SSU rRNA gene that is more conserved than the ITS1 region targeted by HITS1 primers (Lohan et al. 2012). This second *Hematodinium* spp. primer pair was Hemat-F-1487 (5'-CCTGGCTCGATAGAGTTG-3') and Hemat-R-1654 (5'-GGCTGCCGTCCGAATTATTCAC-3') (Lohan et al. 2012). This PCR reaction contained 1X standard *Taq* (Mg-free) reaction buffer, 1.5 mM MgCl<sub>2</sub>, 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 BSA or RSA (Lohan et al. 2012). The reaction was brought to a volume of 10 µl with Nanopure water. Thermocycling conditions were: 95.0 °C for 10 min; 40 cycles of 94.0 °C for 30 s, 56.0 °C for 30 s, 72.0 °C for 1 min; and a final extension at 72.0 °C for 10 min (modified from Lohan et al. 2012). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller.

Amplified products were separated by 2% agarose gel electrophoresis after staining with EZ-Vision DNA Dye (Amresco, Solon, OH; diluted from 6X stock concentration to approximately 1.2X). I stained 2 µl of PCR product with 2 µl diluted EZ-Vision and then ran the electrophoresis at 150 volts in sodium borate (SB) buffer. Gels were visualized with a FisherBiotech 312 nm UV transilluminator and photographed with a Kodak Gel Logic 112. Each PCR run included a negative control and a positive control of DNA extracted from ethanol-preserved hemolymph infected with *H. perezii* that was obtained from Dr. Shields at Virginia Institute of Marine Science. Extraction of the positive control was consistent with extraction methods for the samples. Amplifications were only considered successful when the metazoan primers amplified DNA from each sample

(~ 1700 bp) and all three primer pairs amplified the positive control but none amplified the negative control (Fig. 3.8). If a sample did not amplify with the metazoan primers, I extracted a new sample of hemolymph and amplified it again. Presence of *H. perezii* was recorded whenever a 302 bp band was present on the gel for the HITS1 primers and a 187 bp band was present for the Hemat primers (Fig. 3.8; Lohan et al. 2012).

### 3.2.10 Detection of *Loxothylacus texanus*

Because the chance for infection by *L. texanus* was hypothesized to be low due to low salinity and active molting, I only performed PCR on 25 crabs collected from each shedding facility. The randomly selected 25 crabs were all pre-molt crabs with a CW of 15 cm or less. The maximum CW of 15 cm was chosen because few crabs larger than this have ever been recorded as infected by *L. texanus* (Adkins 1972, Ragan and Matherne 1974, Hochberg et al. 1992). Prevalence of this parasite peaks in the summer and fall so I analyzed fewer crabs collected in April (Adkins 1972, Ragan and Matherne 1974, Hochberg et al. 1992). At LU, SM, and HA, I analyzed 3 crabs collected from each facility in April, 7 from June, 8 from August, and 7 from October. At KM in Hopedale, I did not collect crabs in April or October so I analyzed 12 from June and 13 from August.

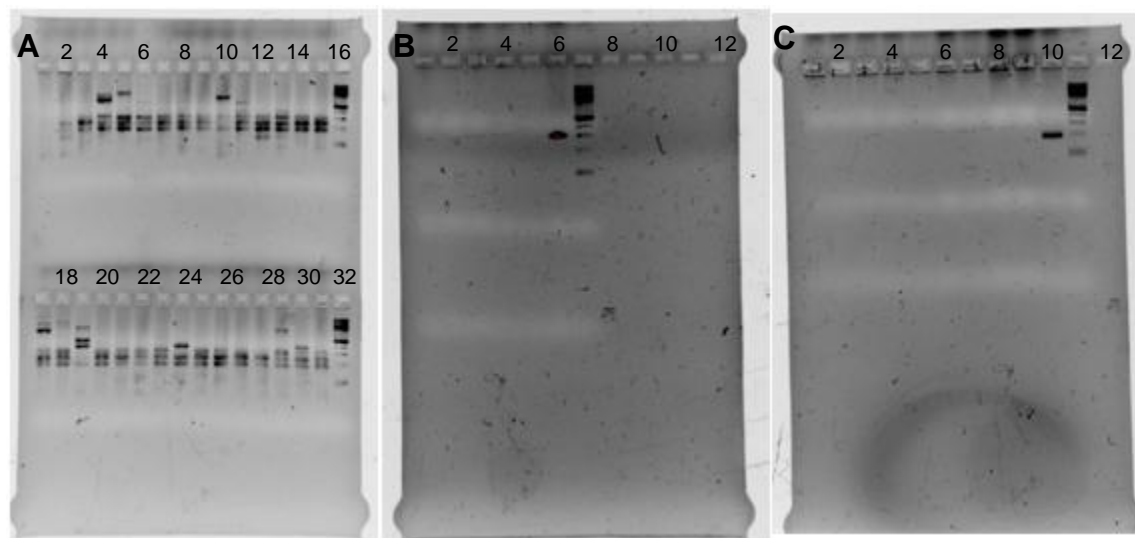


Figure 3.8: Agarose gel results from *Hematodinium perezii* PCR. These samples were collected at shedding facilities throughout 2013. A: Gel for the metazoan primers (nSSU A, nSSU B). Each lane (lanes 1-15, 17-31) with distinguishable bands indicates amplifiable DNA in the sample. The particular band of interest was the largest, ~1700 bp. Lanes 16 and 32 were 100 bp ladders. B: Gel for *H. perezii* specific primers (HITS1F, HITS1R). Only the positive control (lane 6) has a band, indicating the samples (lanes 1-5) were not infected with *H. perezii*. Lane 7 is a 100 bp ladder and lanes 8-12 are empty. C: Gel for *H. perezii* specific primers (Hemat-F-1487, Hemat-R-1654). Only the positive control (lane 10) has a band, indicating the samples (lanes 1-9) were not infected with *H. perezii*. Lane 11 is a 100 bp ladder and lane 12 is empty.

*L. texanus* parasitization was detected by the appearance of any externa during the initial dissection (Section 3.2.3). Additionally, to detect infections that had not yet developed externa, I did PCR on hepatopancreas and midgut from each crab (Section 3.2.3, Appendix A). All tissue samples were lysed overnight prior to extraction. To extract DNA from the tissues, I used Qiagen's DNeasy Blood and Tissue kits per the manufacturer recommendations with a 5 minute elution incubation and one 200  $\mu$ l elution. For PCR, I used the universal crustacean primers HI (5'-GTGCATGGCCGTTCTTAGTTG-3') and 329 (5'-TAATGATCCTTCCGCAGGTTACCTACG-3') to verify that each sample contained amplifiable DNA (Sherman et al. 2008). To detect *L. texanus* infections, I used the same forward HI primer with reverse Loxo3 primer (5'-ACGTTTGATTGCGCGCGCACTGTCTGC-3') (Sherman et al. 2008). Both PCR reactions contained 1X standard *Taq* (Mg-free) reaction buffer (New England Biolabs, Ipswich, MA), 1.75 mM  $MgCl_2$ , 0.2 mM dNTPs, 0.4  $\mu$ M HI primer, 0.4  $\mu$ M 329 or Loxo3 reverse primer, 1 unit of *Taq* polymerase, and 0.2 mg/mL BSA or RSA (Sherman et al. 2008). All reactions were brought to a volume of 10  $\mu$ l with Nanopure water. Thermocycling conditions were: 95.0  $^{\circ}C$  for 5 min; 30 cycles of 95.0  $^{\circ}C$  for 40 s, 66.8  $^{\circ}C$  for 25 s, 72.0  $^{\circ}C$  for 3 min; and a final extension at 72.0  $^{\circ}C$  for 10 min (Sherman et al. 2008). Thermocycling was performed in either an Eppendorf Mastercycler proS thermocycler or MJ Research PTC-100 Programmable Thermal Controller.

Amplified products were separated by 1% agarose gel electrophoresis and stained with EZ-Vision DNA Dye (Amresco, Solon, OH; diluted from 6X stock concentration to approximately 1.2X). I stained 2  $\mu$ l of PCR product with 2  $\mu$ l diluted EZ-Vision and then ran the electrophoresis at 150 volts in SB buffer. Gels were visualized with a FisherBiotech 312 nm UV transilluminator and photographed with a Kodak Gel Logic 112. All PCR runs included a negative control and a positive control of DNA extracted from a *L. texanus* externa provided by Dr. Sherman from University of South Alabama. Extraction of the positive control was consistent with extraction methods for samples. Amplifications were only considered successful when the crustacean primers amplified DNA from each sample (~ 500 bp), and both primer pairs amplified the positive control but neither amplified the negative control (Fig. 3.9). If a crab's hepatopancreas and midgut both did not amplify with the crustacean primers, I extracted new hepatopancreas and midgut samples and amplified them again. Presence of *L. texanus* was recorded whenever a 237 bp band was present on the gel for the primer pair HI and Loxo3 (Fig. 3.9; Sherman et al. 2008).

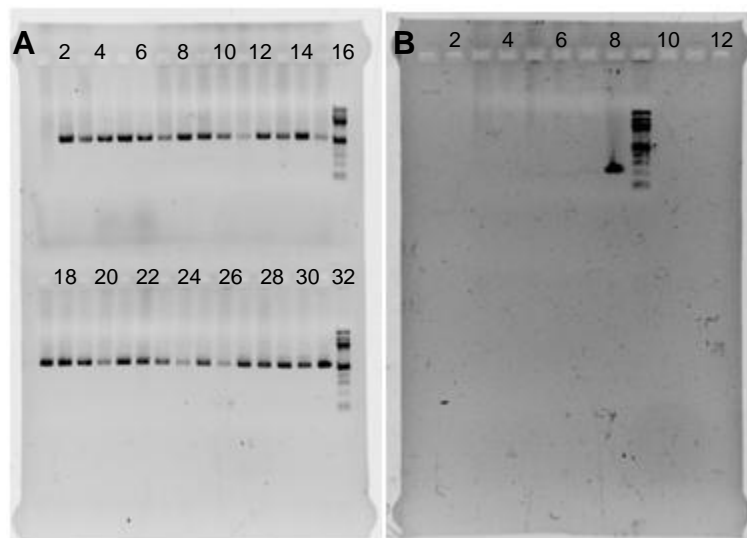


Figure 3.9: Agarose gel results from *Loxothylacus texanus* PCR. These samples were collected from shedding facilities in 2013. A: Gel for the crustacean primers (HI, 329). Each lane (lanes 1-15, 17-31) with distinguishable bands indicates amplifiable DNA in the sample. Lanes 16 and 32 are 100 bp ladders. B: Gel for *L. texanus* specific primers (HI, Loxo3). Only the positive control (lane 8) has a band, indicating that the samples (lanes 1-7) were not infected with *L. texanus*. Lane 9 is a 100 bp ladder and lanes 10-12 are empty.

### 3.2.11 Detection of *Urosporidium crescens*

I detected *U. crescens* by visual gross detection during the initial dissection (Section 3.2.3, Appendix A). Presence of *U. crescens* is very noticeable as black spots in the hepatopancreas or muscle (Fig. 3.10). In addition to visual detection, I planned to use histology to detect *U. crescens*. Like *L. callinectes*, the goal of including histology was to include a permanent slide record of the infection. However, due to histology's small field of inspection, this method was not particularly useful for *U. crescens*.

Therefore, I abandoned histology as a method of assessment for *U. crescens* infections after April and only detected this hyperparasite visually.



Figure 3.10: *Urosporidium crescens* hyperparasitizes a trematode found in blue crab hepatopancreas and muscle. A: Large, black, *U. crescens*-infected, encysted trematode larvae found in a crab collected at Grand Isle in October 2013 (red circle). B: Smaller, black *U. crescens* spots in the hepatopancreas of a crab collected at Grand Isle in June 2013 (red circles).

### 3.2.12 Statistical Analysis

I performed all statistical analyses in RStudio (R Core Team 2013). For the analysis of the effect of a predictor variable on the presence of a particular disease, parasite, or symbiont, I did a logistic regression as a generalized linear model (binomial distribution with a logit link) in the package *stats* in RStudio. Predictors variables included shedding facility, collection month, the facility\*month interaction, water temperature, crab sex, and crab size. I also performed an analysis of deviance Chi-square test in the *stats* package to evaluate model fit.

To generate a cumulative mult categorical logit model for shell rot intensity, I used package *vgam* in RStudio in which the categories were scored from 0 to 2 (Table 3.3; Agresti 2007, Yee 2010, Yee 2013). For cumulative logit models, likelihood ratio tests were performed in package *vgam* to analyze model fit (Yee 2010, Yee 2013). For all statistical tests, alpha was set at 0.05 for statistical significance.

## 3.3 Results

### 3.3.1 Prevalence and Intensity of Shell Rot

The aggregate prevalence of shell rot on pre-molt crabs at all four facilities was 64.3% (Fig. 3.11). At KM, shell rot was present on 95% of crabs in June and 65% in August (Fig. 3.12). At LU, this disease's prevalence ranged from 45 to 84.2% (Fig. 3.13). At SM, the lowest shell rot prevalence at any of the facilities was recorded at 30% and SM's highest prevalence was 80% (Fig. 3.14). At HA, prevalence of shell rot ranged from 40% in April to 80% in August (Fig. 3.15).



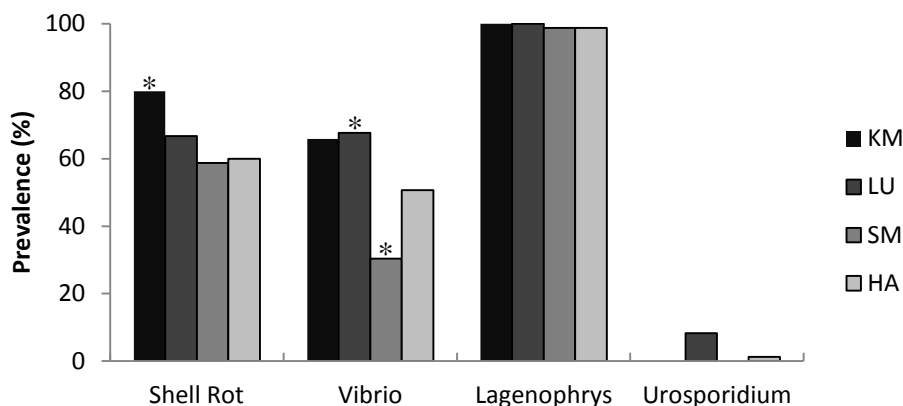


Figure 3.11: Aggregate prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, and *Urosporidium crescens* in crabs collected from four shedding facilities: Hopedale, KM; Dulac, LU; St. Mary's, SM; Erath, HA (\* denotes statistical significance compared to HA).

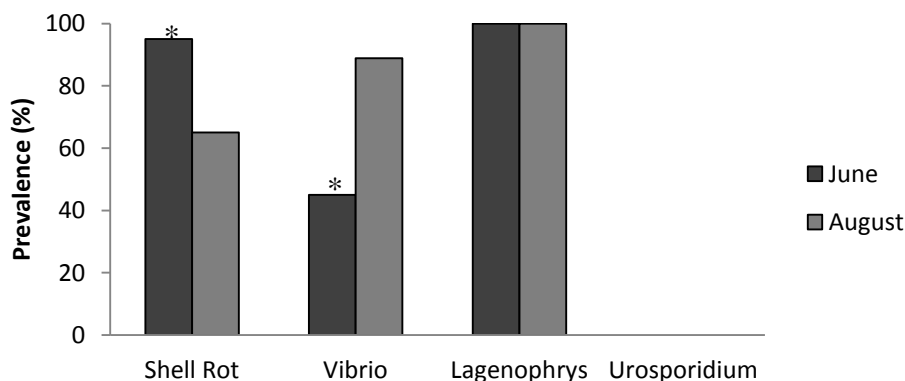


Figure 3.12: Prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, and *Urosporidium crescens* in June and August collections at the Hopedale (KM) facility (\* denotes statistical significance compared to prevalence in August).

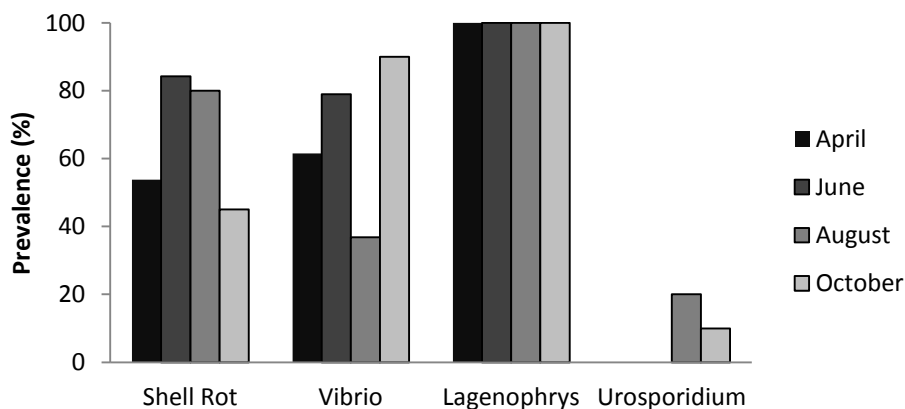


Figure 3.13: Prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, and *Urosporidium crescens* in April, June, August, and October collections at the Dulac (LU) facility.

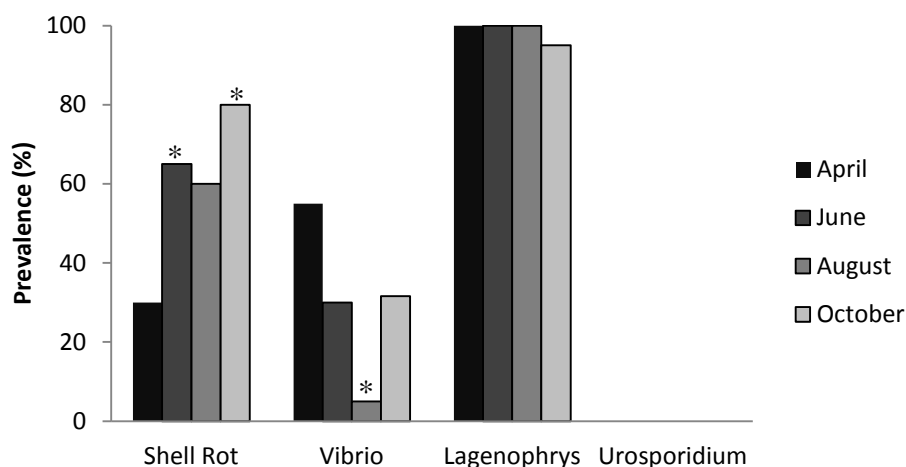


Figure 3.14: Prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, and *Urosporidium crescens* in April, June, August, and October collections at the St. Mary's (SM) facility (\* denotes statistical significance compared to prevalence in April).

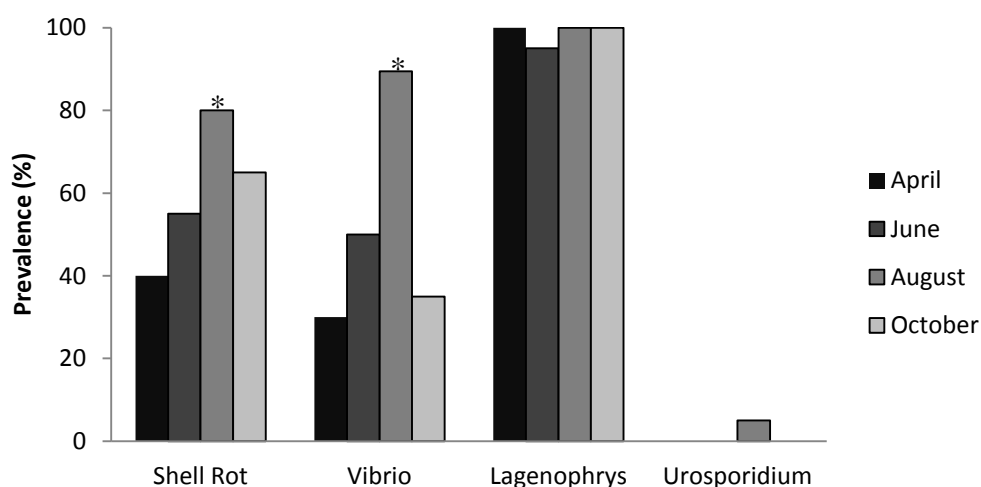


Figure 3.15: Prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, and *Urosporidium crescens* for April, June, August, and October collections at the Erath (HA) facility (\* denotes statistical significance compared to prevalence in April).

Shell rot varied significantly by collection month at three of the four facilities. At KM, probability of shell rot was significantly higher in June than in August (Table 3.4). Also, the model with month as a predictor variable was a better fit than the model without month ( $\chi^2 = 6.1937$ , d.f. = 1,  $p = 0.0128$ ). At SM, shell rot's prevalence varied significantly across the collection months with it highest in October and lowest in April (Table 3.4). The model with month as a predictor variable was a better fit than one without ( $\chi^2 = 11.172$ , d.f. = 3,  $p = 0.0108$ ). At HA, shell rot in August was significantly more prevalent than in April (Table 3.4). Although there was significant variation at HA across the months, the models with and without month as a predictor variable were not significantly different ( $\chi^2 = 7.3219$ , d.f. = 3,  $p = 0.0623$ ).

The probability of shell rot infections increased as water temperature at the shedding facility increased with the logistic regression equation:  $\log\text{-odds}(\text{shell rot}) = -3.6809 + 0.1583(\text{temperature})$  (Fig. 3.16, Table 3.4). This logistic regression without facility as an additional predictor variable was a better model than the model with facility and water temperature (AIC: 328.78 vs. 332.27,  $\chi^2 = 2.5058$ , d.f.= 3,  $p = 0.4742$ ).

Shell rot prevalence also varied significantly between the shedding facilities. When a model including facility, month, and the facility\*month interaction was analyzed, KM was significantly different than HA (Table 3.4). When each month of collection was analyzed separately, there was significant difference in the prevalence of shell rot between the four facilities in June as KM had a significantly higher prevalence (Table 3.4).

Unlike month and facility, the crab's sex was not a significant predictor for the prevalence variation of shell rot (Table 3.4) and a model including sex fit the data no better than one without sex ( $\chi^2 = 1.0297$ , d.f.= 1,  $p = 0.3102$ ). The odds of shell rot increased by 48.07% for every 1 cm increase in CW (Table 3.4). The average CW throughout the study was 13.17 cm. At KM, the average size was the largest at 14.32 cm. Models for shell rot should include size as a predictor variable ( $\chi^2 = 7.1907$ , d.f.= 1,  $p < 0.01$ ). The overall logistic regression for the prevalence of shell rot included collection month (or water temperature), shedding facility, and size (Table 3.5).

Table 3.4: Parameter estimates and p-values for predictor variables for prevalence of *Lagenophrys callinectes*, *Urosporidium crescens*, shell rot, and *Vibrio* spp. infections. Site, month, and site\*month parameter estimates for specific categories not listed were not significant. Category cells are blacked out for continuous variables.

Disease, Parasite, Symbiont	Predictor	Category	Parameter Estimate	p-value
Shell Rot	Site	KM	2.7438	0.0143
	Month	June (KM)	2.3254	0.0392
		June (SM)	1.4663	0.0302
		October (SM)	2.2336	0.0026
		August (HA)	1.7918	0.013
	Site*Month	KM*August	-3.5110	<0.01
	Water Temperature		0.1583	<0.01
	Size		0.3925	<0.01
<i>Vibrio</i> spp. Hemolymph and Organ Infections	Sex	Male	0.3748	0.3112
	Site	LU (August)	-2.603	<0.01
		LU (October)	2.816	<0.01
		SM (August)	-5.142	<0.0001
	Month	June (KM)	-2.3979	<0.01
		August (SM)	-3.1451	<0.01
		August (HA)	3.0445	<0.01
	Site*Month	LU*August	-3.92	<0.001
		SM*August	-6.1896	<0.0001
	Water Temperature		-0.0739	0.1938
<i>Vibrio</i> spp. Hemolymph Infections	Size		-0.4155	<0.01
	Sex	Male	-0.1299	0.7330
	Lab Assistant	Assistant 1	0.4895	<0.01
	Site	LU	1.3521	0.0305
	Month	August	-2.3783	<0.001

(Table 3.4 continued)

Disease, Parasite, Symbiont	Predictor	Category	Parameter Estimate	p-value
<i>Vibrio</i> spp. Hemolymph Infections	Site*Month	All possible	n/a	>0.05
	Water Temperature		0.3809	0.1524
	Size		-0.4953	<0.01
	Sex	Male	0.1840	0.6966
<i>Lagenophrys callinectes</i>	Site	All sampled	n/a	All >0.99
	Month	All sampled	n/a	All >0.99
	Site*Month	All possible	n/a	All >0.99
	Size		-0.4448	0.62
<i>Urosporidium crescens</i>	Site	All sampled	n/a	All > 0.99
	Month	All sampled	n/a	All > 0.99
	Site*Month	All possible	n/a	All > 0.99
	Size		-1.045	0.101

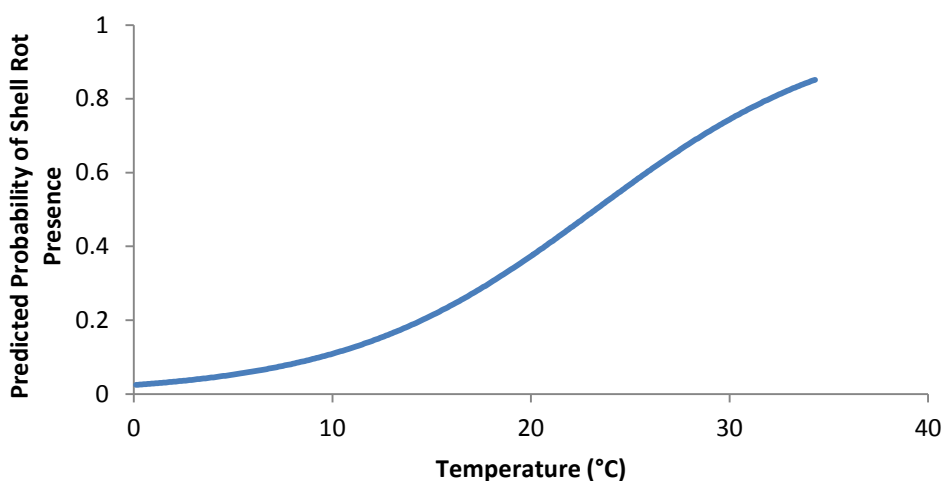


Figure 3.16: Logistic regression of the predicted probability of shell rot infections depending on water temperature at commercial soft shell shedding facilities in Louisiana.

Table 3.5: Predictor variables for the prevalence of shell rot, *Vibrio* spp., *Lagenophrys callinectes*, and *Urosporidium crescens* in pre-molt crabs collected in 2013 from commercial shedding facilities (\* denotes statistically significant predictor).

Disease, Parasite, Symbiont	Facility	Month	Facility-Month Interact.	Water Temp.	Size	Sex	Lab Assistant
Shell Rot	*	*	*	*	*		n/a
<i>Vibrio</i> spp. total	*	*	*		*		*
<i>Vibrio</i> spp. hemolymph	*	*		*	*		n/a
<i>L. callinectes</i>							n/a
<i>U. crescens</i>							n/a

In addition to the prevalence of shell rot at 64.3%, the average shell rot intensity was 0.89. Severe shell rot with necrotic lesions was present on 25% of the pre-molt crabs and was 63.6% of all shell rot cases diagnosed. The cumulative multcategory logit model for shell rot intensity included size and month of collection as predictors for shell rot intensity score (Table 3.3). Likelihood ratio tests indicated that intensity score was independent of crab sex and shedding facility but dependent on crab size and collection month (sex:  $\chi^2 = 3.0731$ , d.f. = 1,  $p = 0.0796$ ; facility:  $\chi^2 = 5.3696$ , d.f. = 3,  $p = 0.1467$ ; size:  $\chi^2 = 3.8721$ , d.f. = 1,  $p = 0.0491$ ; month:  $\chi^2 = 14.4$ , d.f. = 3,  $p < 0.01$ ).

The logit for shell rot intensity score 0 was (Agresti 2007):

$$3.23049 - 1.56593(\text{August}) - 1.16777(\text{June}) - 1.19777(\text{October}) - 0.21067(\text{Size}).$$

The logit for intensity score 1 was (Agresti 2007):

$$5.02851 - 1.56593(\text{August}) - 1.16777(\text{June}) - 1.19777(\text{October}) - 0.21067(\text{Size}).$$

Based on these logit equations, the cumulative probability for a crab having no shell rot (intensity 0) was (Agresti 2007):

$$P(Y=0) = \frac{e^{3.23049 - 1.56593(\text{August}) - 1.16777(\text{June}) - 1.19777(\text{October}) - 0.21067(\text{Size})}}{1 + e^{3.23049 - 1.56593(\text{August}) - 1.16777(\text{June}) - 1.19777(\text{October}) - 0.21067(\text{Size})}}.$$

The cumulative probability for a crab having no shell rot (intensity 0) or non-necrotic shell rot (intensity 1) was (Agresti 2007):

$$P(Y \leq 1) = \frac{e^{5.02851 - 1.56593(\text{August}) - 1.16777(\text{June}) - 1.19777(\text{October}) - 0.21067(\text{Size})}}{1 + e^{5.02851 - 1.56593(\text{August}) - 1.16777(\text{June}) - 1.19777(\text{October}) - 0.21067(\text{Size})}}.$$

Cumulative probability for a crab with shell rot intensity 0, 1, or 2 was 1.0 (100%) (Fig. 3.17).

Based on the individual category probabilities, the probability of no shell rot decreased with increasing size (Fig. 3.18 and 3.19). Conversely, the probability of shell rot (score 1 or 2) increased with crab size, with the exception of non-necrotic shell rot in June, August, and October (Fig. 3.18 and 3.19). In these three months, the probabilities leveled off and decreased slightly after 13 cm CW. Regardless of crab size, the probability of necrotic shell rot (score 2) was highest in August when the water temperatures were highest (Fig. 3.18 and 3.19). Crabs were most likely to not have shell rot in April (Fig. 3.18 and 3.19).

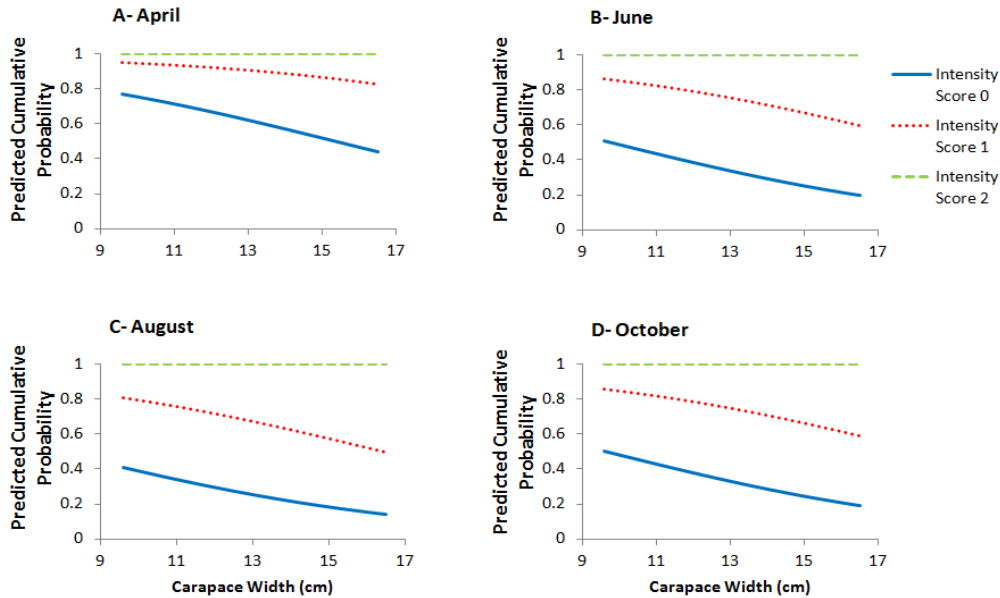


Figure 3.17: Cumulative probabilities from cumulative logit models for shell rot intensity scored as 0 (no shell rot), 1 (non-necrotic spots), or 2 (necrotic lesions) in April (A), June (B), August (C), and October (D) 2013. The cumulative probability of a crab having shell rot intensity 2 or any of the lower intensities is 1.0.

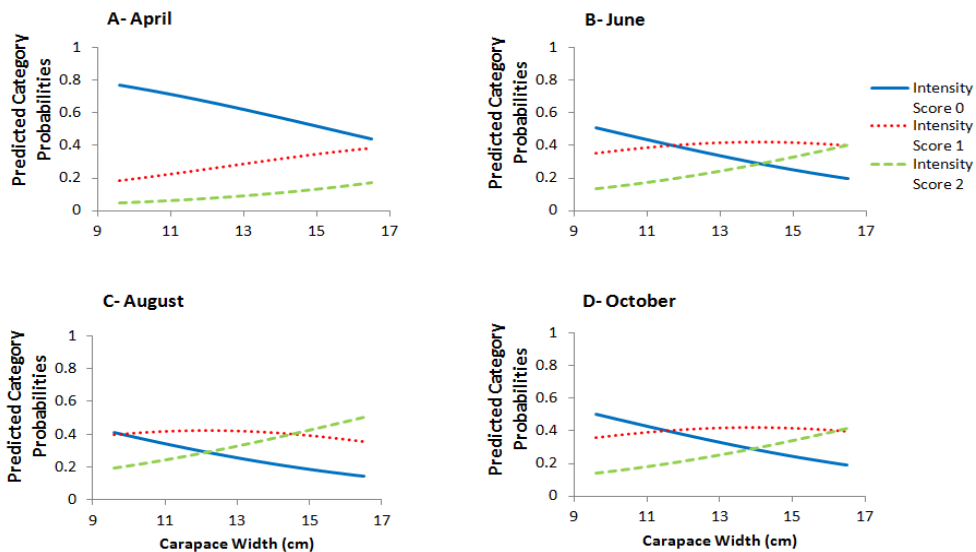


Figure 3.18: Category probabilities for crabs collected from shedding facilities in April (A), June (B), August (C), and October (D) 2013 having shell rot intensities scored as 0, 1, or 2. Score 0 represented a lack of shell rot while 1 was non-necrotic and 2 was necrotic shell rot.

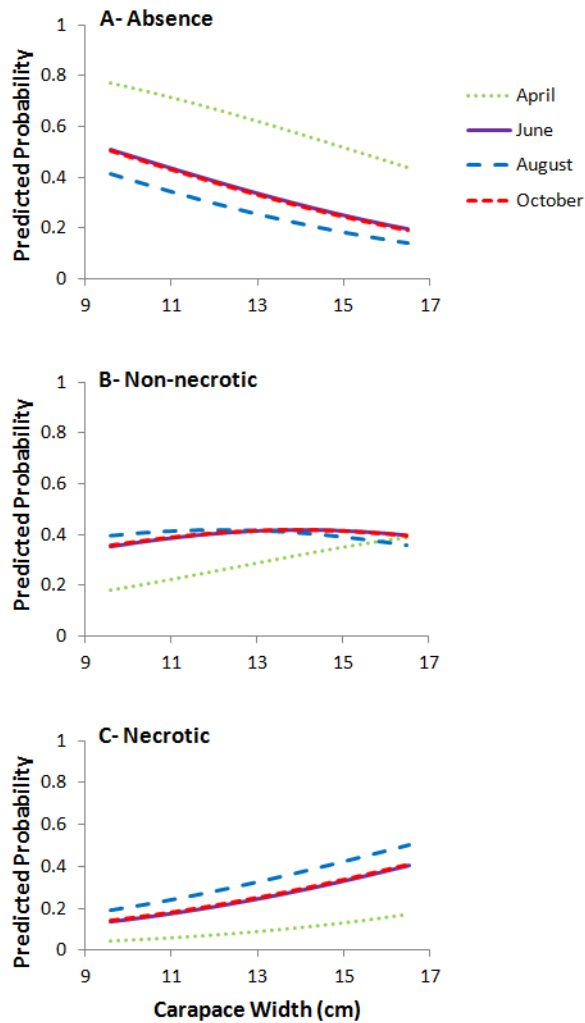


Figure 3.19: Predicted probabilities for crabs collected from shedding facilities in April, June, August, and October 2013 having no shell rot (A), non-necrotic shell rot (B), and severe, necrotic shell rot (C).

### 3.3.2 Prevalence of *Vibrio* spp.

Prevalence of *Vibrio* spp. infections in the crab hemolymph and internal organs ranged from 5 to 90% at the four facilities. The aggregate prevalence of *Vibrio* spp. in pre-molt crabs was 51.3% (Fig. 3.11). At KM, *Vibrio* spp. infections were observed in 45% of crabs in June and 89% in August (Fig. 3.12). At LU, *Vibrio* spp. prevalence ranged from 37% in August to 90% in October (Fig. 3.13). Prevalence at SM ranged from 5% in August to 55% in April (Fig. 3.14). *Vibrio* spp. infections varied at HA from 30 to 89% (Fig. 3.15).

The prevalence of *Vibrio* spp. in crabs from KM, SM, and HA varied significantly across the sample months. At KM and HA, prevalence rates of approximately 89% were significantly higher in August (Table 3.4). At SM, prevalence was significantly different in April and August (Table 3.4). For each facility, the logistic regression with month

included as a predictor variable fit the data better than a logistic regression without month included (all  $p < 0.01$ ). Water temperature was not significant predictor for prevalence of *Vibrio* spp. (Table 3.4). Inclusion of water temperature in the model did not significantly improve model fit ( $\chi^2 = 1.7452$ , d.f. = 1,  $p = 0.1865$ ).

In addition to monthly variation, *Vibrio* spp. prevalence also varied significantly between facilities with LU overall having the highest prevalence and SM having the lowest (Fig. 3.11). In the model for prevalence of *Vibrio* spp. with site, month, and the site\*month interaction, LU and SM in August were significantly different from HA (Table 3.4). When each month was analyzed individually for variation between facilities, LU was significantly different from HA in August and October, and SM's low prevalence in August of 5% was significantly lower than prevalence at HA (Table 3.4).

Sex was not a significant predictor variable for the prevalence of *Vibrio* spp. and its inclusion in the model did not improve model fit (Table 3.4;  $\chi^2 = 0.1167$ , d.f. = 1,  $p = 0.7326$ ). Crab size was a significant predictor for *Vibrio* spp. prevalence where the odds of *Vibrio* spp. infection decrease by 34% for every 1 cm increase in CW (Table 3.4). A model without size had poor fit ( $\chi^2 = 9.3646$ , d.f. = 1,  $p < 0.01$ ).

Prevalence of *Vibrio* spp. was significantly affected by the individual who pulled the hemolymph from each crab. Percent prevalence of *Vibrio* spp. ranged from 39% to 81% for the four people who pulled hemolymph. In a logistic regression with the person who pulled the hemolymph as the only predictor variable for prevalence of *Vibrio* spp., there was a significant difference in the probability of *Vibrio* spp. presence (Table 3.4). The regression without this variable did not fit the data as well as the regression with it ( $\chi^2 = 13.739$ , d.f. = 1,  $p < 0.01$ ).

Overall, prevalence of *Vibrio* spp. infections in the hemolymph and organs was moderate and the important predictor variables were shedding facility, collection month, the facility\*month interaction, crab size, and the lab assistant (Table 3.5).

When results erred by the lab assistant variation were removed from the dataset and only 179 of the results were analyzed (Section 3.2.5), *Vibrio* spp. prevalence in the hemolymph alone ranged from 5 to 61.5%, with an aggregate prevalence of 36.8% (Fig. 3.20). In the best model, hemolymph infection rates were affected by facility, month (or water temperature), the facility\*month interaction, and crab size (Table 3.5). Sex did not significantly affect *Vibrio* spp. hemolymph prevalence (Table 3.4). Month was retained in the model instead of water temperature because 20 samples from KM in June had no corresponding water temperature (Table 3.1). Size had a negative effect on prevalence of hemolymph infections (Table 3.4) and prevalence was significantly lower in August than in April based on only these 179 samples (Table 3.4). The only significant site difference was between LU and HA (Table 3.4).

### 3.3.3 Co-occurrence of Shell Rot and *Vibrio* spp.

Shell rot and *Vibrio* spp. hemolymph infections co-occurred in crabs at prevalence rates between 5% and 40%, with an aggregate co-occurrence rate of 24% (Fig. 3.21). However, the presence of a *Vibrio* spp. hemolymph infection had a minimal positive effect on the prevalence of shell rot ( $\beta_{\text{Vibrio}} = 0.6218$ ,  $p = 0.1229$ ). The shell rot prevalence model with *Vibrio* spp. included as a predictor variable was not a better model than one without *Vibrio* spp. (AIC: 215.42 vs. 215.87;  $\chi^2 = 2.4525$ , d.f. = 1,  $p =$



0.1173). Shell rot intensity was also independent of *Vibrio* spp. prevalence ( $\chi^2 = 1.9965$ , d.f. = 1,  $p = 0.1557$ ). The presence of shell rot had an insignificant positive effect on the prevalence of *Vibrio* spp. infections ( $\beta_{\text{Shell Rot}} = 0.5578$ ,  $p = 0.1489$ ) and the prevalence model without shell rot as a predictor variable fit the data comparably (AIC: 201.62 vs. 202.25;  $\chi^2 = 2.6329$ , d.f. = 1,  $p = 0.1047$ ).

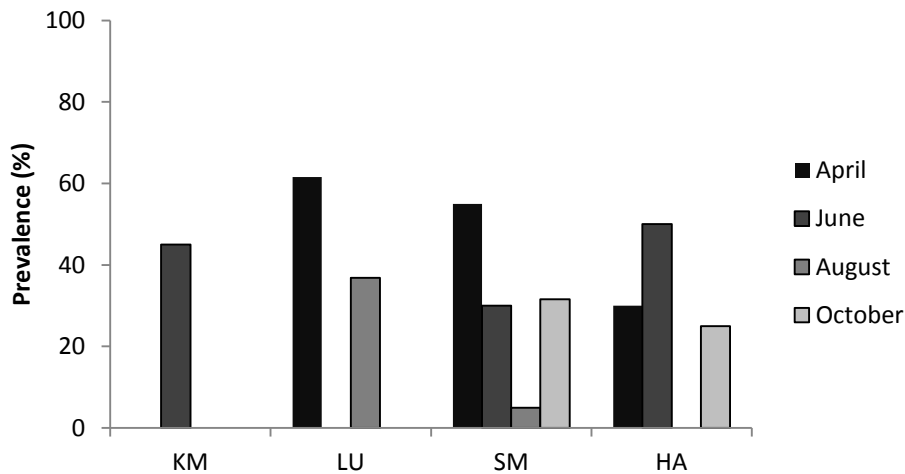


Figure 3.20: *Vibrio* spp. hemolymph infection rates in crabs collected from four shedding facilities (Hopedale, KM; Dulac, LU; Port of W. St. Mary's, SM; Erath, HA) in April, June, August, and October 2013. Missing bars indicate that results are missing due to error or unsuccessful collection.

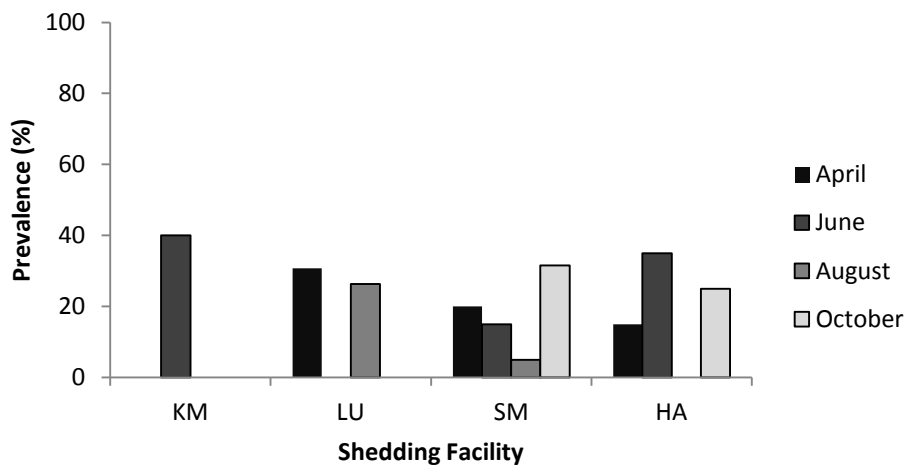


Figure 3.21: Shell rot and *Vibrio* spp. hemolymph infection co-occurrence rates in crabs collected from four shedding facilities (Hopedale, KM; Dulac, LU; Port of W. St. Mary's, SM; Erath, HA) in April, June, August, and October 2013. Missing bars indicate that results are missing due to error or unsuccessful collection.

### 3.3.4 Other Bacterial Infections

From API results, I also detected infections with: *Aeromonas* spp. (*A. hydrophila*, *A. sobria*, *A. caviae*), *Morganella morganii*, *Photobacterium damsela*, and *Shewanella putrefaciens*. These bacteria were isolated from the hemolymph and internal organs.

### 3.3.5 *Ameson michaelis* Results

One crab collected from SM in August was infected with *A. michaelis* according to electron micrographs. One sample from LU in October was tentatively assessed as infected but genetic sequencing is pending.

### 3.3.6 Reo-like Virus Results

One sample from HA collected in June was infected with a medium intensity reo-like (RLV) infection. Eleven samples from HA were analyzed resulting in a prevalence of 9.1%. For LU in June, nine samples were analyzed for RLV and two were positive resulting in 22.2% prevalence. At LU, both infections were low intensity. Aggregate prevalence of RLV at these two shedding facilities in the summer was 15%.

### 3.3.7 Prevalence of *Lagenophrys callinectes*

The aggregate prevalence of *L. callinectes* at the four facilities was 99.3% (Fig. 3.11). In the 27 post-molt samples collected from LU in April, the prevalence was 22.2%. At KM and LU, prevalence in pre-molt crabs was 100% (Fig. 3.11-3.13). Prevalence was 98.75% with one uninfested crab in October at SM and one in June at HA (Fig. 3.11, 3.14, 3.15).

There was no significant monthly variation and no significant site variation between the four facilities (Table 3.4). During April and August, there was 100% prevalence at all facilities. In June and October, there were unequal prevalence rates but the differences were not significant (Table 3.4). Additionally, crab size was not a significant indicator of *L. callinectes* prevalence (Table 3.4) and a model without size fit the data equally well ( $\chi^2 = 0.2544$ , d.f. = 1,  $p = 0.614$ ).

### 3.3.8 Prevalence of *Hematodinium perezii*

All 272 pre-molt and 27 post-molt samples (KM N=40, LU pre-molt N=72, LU post-molt N=27, SM N=80, HA N=80) were negative for *H. perezii* according to PCR amplifications with HITS1 and Hemat primers.

### 3.3.9 Prevalence of *Loxothylacus texanus*

None of the crabs collected had externa. All 100 samples (25 from each facility, Section 3.2.10) were negative for *L. texanus* according to PCR amplifications with the primer pair HI and Loxo3.

### 3.3.10 Prevalence of *Urosporidium crescens*

*U. crescens* was rarely observed in crabs from shedding facilities. Four crabs in August and two in October at LU were infected with this hyperparasite (20% and 10% prevalence, respectively; Fig. 3.13). One crab at HA was infected in August, resulting in 5% prevalence (Fig. 3.15). Overall, the prevalence of *U. crescens* at the four facilities was 2.6% (Fig. 3.11).

There was no significant monthly variation and no significant variation between the facilities for *U. crescens* (Table 3.4). Crab size was also not a significant indicator of the prevalence of *U. crescens* (Table 3.4).

## 3.4 Discussion

### 3.4.1 Prevalence and Intensity of Shell Rot

Shell rot prevalence varies greatly in wild blue crab populations. Prevalence rates have peaked to as high as 53.1% along South Carolina but prevalence rates along the East Coast are typically less than 10% (Shields and Overstreet 2007). Pathogens hypothesized to cause shell rot inhabit a wide range of salinities so the disease is often not correlated with salinity (Shields and Overstreet 2007). Conversely, shell rot is dependent on ecdysis, which depends on water temperature and crab size (Sandifer and Eldridge 1974). Shell rot is generally more prevalent and intense in older, larger crabs that molt less frequently and during periods of infrequent molting, especially in the winter. This moderately high prevalence of 64.3% in the shedding facilities where the salinity was consistently less than 8 ppt confirms that shell rot can be observed at low salinities.

In this study, shell rot prevalence varied significantly between the four collection months. Given that shedding facility samples were not collected in the winter when shell rot is expected to be highest due to infrequent molting (Chapter 2), I hypothesized that shell rot was most prevalent and intense in the warmest months sampled because pathogens that cause shell rot, such as *Vibrio* spp. and other bacteria, are more prevalent at warmer temperatures (Huq et al. 1984, Welsh and Sizemore 1985).

The probability of shell rot infections varied significantly between shedding facilities, particularly in June. In the model including month, facility, and the month\*facility interaction, KM had a significantly higher prevalence, whereas LU and SM did not differ from HA. This higher prevalence at KM in Hopedale may be a geographic difference if crabs that are brought to this facility are from an area of very high shell rot prevalence. The water quality at KM is likely not the explanation for higher prevalence 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 systems for a couple of days. A hypothesized explanation for the higher incidence of shell rot at KM is that crabs at KM, on average, were larger than at the other facilities, which is important because molt frequency decreases with increasing size (Smith and Chang 2007). Results indicated that shell rot prevalence is dependent on crab size and that the odds ratio of shell rot infections increases by 1.4807 as size increases by 1 cm. Therefore, at the facility with the largest crabs, shell rot should be more prevalent.

In a healthy system, it is expected that shell rot is predominantly absent but when it is present, it is at low intensities. Shell rot infections were most probable in the warmest month, August, and least probable in the coolest month, April (Fig. 3.18 and 3.19). Crabs molt more frequently in the early spring than during the hottest summer months so crabs collected in April would have newer shells than those collected in August (Guillory et al. 2001). Additionally, the probabilities for all three intensity scores (no, light intensity, high intensity shell rot) were nearly identical for June and October. This is most likely related to similar moderate water temperatures and similar lengths of the intermolt period following the two peak molting times in spring and early fall (Guillory et al. 2001).

Overall, the most significant predictors for shell rot prevalence and intensity are water temperature and crab size. The monthly variation in shell rot is likely due to water temperature changes. The moderate to high prevalence rates found at these four shedding facilities could be a cause of concern for commercial blue crab shedders. Shell rot 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 rot (Sandifer and Eldridge 1974). Also, high incidence of shell rot can be important in shedding systems because the causative pathogens are contagious (Sandifer and Eldridge 1974, Sindermann 1989). Necrotic lesions can expose crabs to bacteria that are often in high densities in shedding tanks (Sandifer and Eldridge 1974).

Fortunately for shedding facilities, if a crab successfully molts, it will shed its shell rot and will typically have a clean, soft shell that is appetizing and marketable (Vogan et al. 1999). However, shell rot discoloration was observed on one of the post-molt crabs from LU. The effect of this minor, non-necrotic shell rot discoloration on the soft shell crab is minimal to the industry.

### 3.4.2 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 water months, *Vibrio* spp. prevalence is hypothesized to be high and potentially detrimental to shedding crabs (Overstreet and Cook 1972, Huq et al. 1984, Welsh and Sizemore 1985, Shields 1997). High densities of *Vibrio* spp. occur because of proliferation and rapid reproduction of the bacteria at higher temperatures.

In this study, only 179 of the 267 plates were valid results due to lab error that included inconsistent amounts of hemolymph plated and/or puncture of internal organs that contain more bacteria than the hemolymph. In this reduced dataset, I detected monthly variation that was contrary to my hypothesis as prevalence was lowest in the warmest month, August. However, this result is influenced by the low sample sizes retained for August (N=39) and the low prevalence at SM in August (Fig. 3.20).

There was significant variation in prevalence rates between the four shedding facilities with LU having the highest overall prevalence rate and SM having the lowest aggregate prevalence rate (Fig. 3.20). Interestingly, SM is the only facility of the four to have a closed, recirculating system. In a closed system, I expected *Vibrio* spp. loads to be the highest, especially if there was improper water filtration (Messick and Kennedy

1990). However, a closed system could have better water quality than open systems if the nearby water source is polluted or has poor water quality (Guillory et al. 2001). Monitoring *Vibrio* spp. load in the tank water may have explained the variation in *Vibrio* spp. infections between the facilities.

Another potential explanation for the variation between facilities is crab handling as *Vibrio* spp. infections are more prevalent and intense in injured and roughly handled pre-molt crabs (Welsh and Sizemore 1985, Givens et al. 2013). Therefore, a potential explanation for the site variation is that crabs going to LU were not as carefully handled as those going to SM.

*Vibrio* spp. infections were not more prevalent in males or females, which is consistent with previous studies (Welsh and Sizemore 1985). Unlike sex, size was a factor for prevalence in the shedding facilities and the odds of a *Vibrio* spp. infection decreased by 39% for every 1 cm increase in CW. Size may be important because smaller crabs molt more frequently, which can increase exposure to *Vibrio* spp. (Davis and 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 and Overstreet 2007). In future studies, injuries, especially autotomized legs, should be noted to test a correlation between injury and *Vibrio* spp. infections. Additionally, determination of infection intensity, not just prevalence of *Vibrio* spp., is important to differentiate between potentially fatal and mild, harmless infections.

The overall moderate prevalence of 36.8% at the shedding facilities may represent a threat to the facilities' profitability as *Vibrio* spp. infections have been linked to high mortality rates at shedding facilities (Overstreet and 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 prevention of intense infections should focus less on bacterial loads in tank water and more on minimization of stress and injury.

### 3.4.3 Co-occurrence of Shell Rot and *Vibrio* spp.

*Vibrio* spp. are the main chitinoclastic bacteria that cause shell rot (Shields and Overstreet 2007). Shell rot lesions can expose crabs to *Vibrio* spp. present in the water, which can lead to internal infections (Millikin and Williams 1984). Co-occurrence of shell rot and *Vibrio* spp. infections has been recorded in many studies because of this inherent connection (Sandifer and Eldridge 1974, Iverson and Beardsley 1976, Malloy 1978, Shields and Overstreet 2007). I expected that these two infections would frequently co-occur but the rate of co-occurrence was only 24% in the pre-molt crabs (Fig. 3.21). Additionally, shell rot did not significantly depend on the presence of *Vibrio* spp. infections and *Vibrio* spp. infections did not depend on the presence shell rot.

Identification of bacteria from shell rot lesions may result in a correlation between shell rot and *Vibrio* spp. colonizing the crab's shell. However, other bacteria genera, fungi, and other pathogens may have be the etiological agents for the majority of the shell rot. For example, at low salinities like those at shedding facilities, *Aeromonas* spp. and *Pseudomonas* spp. have been found to be more prevalent and associated with shell rot than *Vibrio* spp. (Noga et al. 1994).

#### 3.4.4 Other Bacterial Infections

The non-*Vibrio* spp. bacteria isolated from crab hemolymph and organs were *Aeromonas* spp. (*A. hydrophila*, *A. sobria*, *A. caviae*), *Morganella morganii*, *Photobacterium damsela*, and *Shewanella putrefaciens*. *Aeromonas* spp. are common bacteria in blue crab hemolymph and can reach moderate prevalence rates (Babinchak et al. 1982). The presence of *Aeromonas* spp. infections may be a possible explanation for the lack of co-occurrence of *Vibrio* spp. and shell rot because *Aeromonas* spp. are also known to cause shell rot (Noga et al. 1994). *Photobacterium damsela* is known to be pathogenic and opportunistic in shellfishes and finfishes, and it may cause blue crab mortalities (Givens et al. 2013).

In future studies, identification of bacteria infecting crab hemolymph and organs and measurement of infection intensity should be studied in order to determine their effects on shedding facility mortalities.

#### 3.4.5 *Ameson michaelis*

*A. michaelis* can tolerate very low salinities like those often observed at shedding facilities (Overstreet and Whatley 1975, Findley et al. 1981). It is a rare parasite but can cause localized mass mortalities, especially in crowded shedding facilities (Messick and Sindermann 1992). In this study, I found two crabs infected with *A. michaelis*. This low prevalence rate does not currently represent a threat to the soft shell industry; however, if a mass mortality occurs in the future, deceased crabs should be visually inspected for lysed, discolored muscle.

#### 3.4.6 Reo-like Virus (RLV)

Reo-like virus may be a significant cause of death at shedding facilities, especially because pre-molt crabs are often stressed and crowded in shedding tanks (Shields 2003, Shields and Overstreet 2007, Bowers et al. 2010). Unlike *Hematodinium perezii*, this virus can withstand low salinities often observed at shedding facilities (Shields and Overstreet 2007). Also unlike shell rot and *Vibrio* spp. infections, mortality of infected crabs is hypothesized to be extremely high (Bowers et al. 2010). In the future, more samples should be analyzed because this parasite may pose a more serious threat to shedding facilities than any of the other diseases, parasites, or symbionts studied here and knowledge of its prevalence rates and transmission prevention may greatly decrease deaths in these facilities.

#### 3.4.7 Prevalence of *Lagenophrys callinectes*

*L. callinectes* is an ectocommensalistic ciliate that infests blue crab gills (Couch and Martin 1979). Blue crabs are rarely harmed by infestations but asphyxiation can occur when the crab is already stressed and the infestation is intense (Couch and Martin 1979, Scott and Thune 1986). *L. callinectes* does not directly vary with water temperature in the Gulf, but it does vary according to the crab's molt cycle because the

ciliate is shed with the old molt and reinfestation does not necessarily occur immediately (Shields and Overstreet 2007).

At the four shedding facilities, *L. callinectes* prevalence was very high at an overall rate of 99.3%. The much 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 instantaneous. The lack of monthly, site, and crab size variation was expected because this ciliate seems to be ubiquitous in Gulf blue crabs.

Even though this ciliate is present at very high rates in shedding facilities, it does not pose any harm to pre-molt blue crabs as it is commensalistic. To determine whether stressed pre-molt crabs may die of asphyxiation from this ciliate, future studies should study the intensity of infestations and at what level they may be fatal.

#### 3.4.8 Prevalence of *Hematodinium perezii*

*H. perezii* is a parasitic dinoflagellate that has been correlated with decreased blue crab commercial landings on the East Coast (Mancinelli et al. 2013). Eventually, all infected crabs will die prematurely as the dinoflagellate destroys hemocytes and the oxygen transporter protein, hemocyanin (Meyers et al. 1996, Lee and Frischer 2004). This dinoflagellate is dependent on salinities above 20 ppt, where the dinospore stage is active (Coffey et al. 2012). *H. perezii* has never been documented below 11 ppt (Newman and Johnson 1975, Messick and Shields 2000).

Because salinities at shedding facilities in Louisiana are usually below 10 ppt, the probability of finding pre-molt crabs infected with *H. perezii* is low. My results of 0% prevalence are consistent with this hypothesis. In 2011, the positive detections of *H. perezii* in pre-molt crabs in low salinity shedding tanks were likely false positives due to the unreliability of histology. Histology and hemolymph smears are not dependable methods of detection without genetic confirmation. PCR and new methodology with dinoflagellate viral nucleoproteins (DNVPs) should be used to assess crabs (Gornik et al. 2012, Gornik et al. 2013). DNVPs are used in immunofluorescence assays and Western blot methods to sensitively detect *Hematodinium* sp. infections (Gornik et al. 2013).

*H. perezii* does not directly affect the profitability of shedding facilities. However, the dinoflagellate's prevalence in the wild could indirectly affect the facilities by decreasing the number of crabs caught and transported to shedding facilities.

#### 3.4.9 Prevalence of *Loxothylacus texanus*

*L. texanus* is an internal barnacle that infects freshly molted crabs. After the infected crab molts five to nine times, the barnacle develops an externa which causes castration of the crab and suppression of its molt cycle (Ragan and Matherne 1974, Tindle et al. 2004, Shields and Overstreet 2007). Infected crabs are typically smaller than their uninfected counterparts with an average CW of 5.8 cm (Adkins 1972, Overstreet et al. 1983). Additionally, *L. texanus* is limited to salinities above 12 ppt for viable larvae (Shields and Overstreet 2007). The barnacle is most often observed at salinities above 25 ppt in the summer and fall (Adkins 1972, Hochberg et al. 1992, Shields and Overstreet 2007).

Three factors at shedding facilities greatly reduce the possibility of having a crab infected with *L. texanus* present in the tanks. First, similar to *H. perezii*, the low salinities at shedding facilities are intolerable for *L. texanus*. Also, the crabs in shedding facilities are typically larger than 9 cm CW so they are less likely to be infected with the barnacle. Lastly, the crabs in the facilities are actively molting, so no crabs bearing an externa would be at the facility. It is possible that pre-externa crabs with internal infections could be brought to the shedding facilities because they are still molting, but the typical pre-molt size restricts this possibility.

The absence of *L. texanus* at the shedding facilities confirms that this parasite has little direct effect on the soft shell industry. However, like *H. perezii*, *L. texanus* can indirectly harm the livelihood of the industry if prevalence in wild populations is high because fewer crabs would be reproducing and be of legal size to be landed, which could consequently decrease the number of crabs brought to shedding facilities.

#### 3.4.10 Prevalence of *Urosporidium crescens*

*U. crescens* is a hyperparasite of the blue crab that infects a trematode commonly found in blue crab muscle, hepatopancreas, and ventral ganglia (Newman et al. 1976, Messick and Sindermann 1992, Messick 1998). The parasitized trematode turns black and is visually unappetizing, which while harmless to the infected crab, it can negatively affect the commercial industry because of its appearance (Perkins 1971, Couch and Martin 1979). *U. crescens* may be intolerant to low salinities, as its prevalence at low salinities has never been published (Chapter 2; Messick 1998).

I observed very few infections with *U. crescens*, and I hypothesize this is due to the very low salinities at Louisiana shedding facilities. Because of the unappetizing appearance and taste of infected meat, the lack of many infections in pre-molt crabs is positive for the shedding facilities. I hypothesize that the few infections in from crabs caught in higher salinity areas than the average crabs in the systems. Continuing to fish low salinity areas for pre-molt crabs may protect the facilities from the economic losses caused by this harmless hyperparasite.

#### 3.4.11 Conclusions and Future Investigations

From the results of this study, I cannot definitively state the cause of most shedding facility mortalities. It is unlikely that high death rates are caused by *L. callinectes* unless the crabs are highly stressed from low dissolved oxygen and poor water quality. Due to salinity tolerance restrictions, deaths are also not attributable to *H. perezii*. Water quality is not a likely explanation for high loss rates because at all four facilities it was within the acceptable range for nitrites, ammonia, and pH and previous studies have not linked poor water quality and mortalities (Manthe et al. 1983, Chaves and Eggleston 2003).

High death rates may be caused by a combination of harmful parasites and diseases such as shell rot, *Vibrio* spp., *A. michaelis*, and RLV but more research needs to be conducted. Several factors need further investigation including: RLV, *Vibrio* spp. loads in the tank water, rough handling, injury, and stress from abrupt salinity changes.



As this was a one year study, more long-term monitoring is necessary, especially given the low hard crab landings of 17575 metric tons in 2013 as compared to average landings of 21052 metric tons (Appendix C). Future studies are important to understand the context of 2013's prevalence rates and the long-term health of the soft shell fishery.

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## CHAPTER 4: COMPARISON OF PREVALENCE RATES AT FIELD SITES AND SOFT SHELL SHEDDING FACILITIES

### 4.1 Introduction

Prevalence of diseases, parasites, and symbionts can vary between crabs held at commercial shedding facilities and wild crabs. Often because of crowding and stress, prevalence rates are hypothesized to be higher in shedding tanks than in nearby wild populations. In particular, shell rot, bacterial infections, reo-like virus, and *Ameson michaelis* may be more prevalent in shedding tanks because of the crowded, stressful conditions that increase susceptibility and facilitate transmission (Iverson and Beardsley 1976, Messick and Sindermann 1992, Shields and Overstreet 2007, Bowers et al. 2010). Conversely due to low salinities at shedding facilities in Louisiana, *Hematodinium perezii* and *Loxothylacus texanus* should be more prevalent in wild populations and nearly absent in shedding systems (Newman and Johnson 1975, Shields and Overstreet 2007). Also potentially due to the low salinities at shedding facilities, transmission of *Urosporidium crescens* spores in the tanks may be limited so infection rates should be higher in wild populations (Messick 1998). *Lagenophrys callinectes* has been hypothesized to be very abundant and detrimental to shedding facilities (Couch 1966, Couch and Martin 1979). However, *L. callinectes* has also been noted to be very abundant in wild populations so there may be little difference in prevalence rates between wild and captive populations (Shields and Overstreet 2007). In general, when salinity was not a limiting factor, I expected prevalence of diseases, parasites, and symbionts to be higher in the shedding facilities than in the sampled wild populations because of the stressful, crowded conditions and poorer water quality often observed at these facilities.

### 4.2 Comparison of Results

#### 4.2.1 Shell Rot

The overall prevalence for shell rot in crabs collected at the field sites was 54.8%, and at the shedding facilities, prevalence was 64.3% (Fig. 4.1). The odds of infections were significantly more probable in the captive populations than in the wild populations ( $\beta_{\text{Shedder}} = 0.3978$ ,  $p < 0.01$ ).

For shell rot intensity, the average score at the shedding facilities was 0.89 and at the field sites was 0.76 (Tables 2.6 and 3.3). Shell rot intensity score was dependent on the site predictor variable ( $\chi^2 = 6.4945$ , d.f. = 1,  $p = 0.0108$ ). The logit for no shell rot (intensity score 0) was (Agresti 2007):

$$-0.20791 - 0.33161 * \text{Shedder}.$$

The logit for non-necrotic shell rot (intensity score 1) was (Agresti 2007):

$$1.35886 - 0.33161 * \text{Shedder}.$$

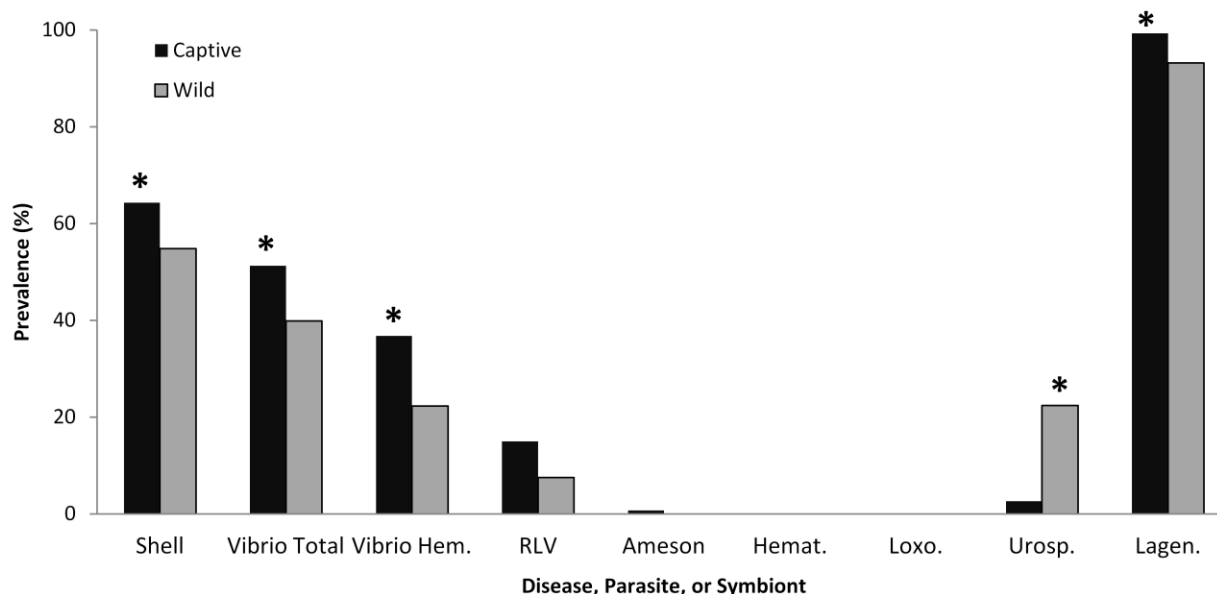


Figure 4.1: Prevalence rates of shell rot (Shell), total *Vibrio* spp. infections in hemolymph and internal organ (*Vibrio* Total), *Vibrio* spp. hemolymph only infections (*Vibrio* Hem.), reo-like virus (RLV), *Ameson michaelis* (Ameson), *Hematodinium perezii* (Hemat.), *Loxothylacus texanus* (Loxo.), *Urosporidium crescens* (Urosp.), and *Lagenophrys callinectes* (Lagen.) in captive blue crabs (black bars) at commercial shedding facilities and in wild blue crabs (grey bars) collected from four coastal locations in 2013 and the beginning of 2014 (\* denotes statistically significant difference between wild and captive rates).

Based on these logit equations, the cumulative probability for a crab having shell rot intensity 0 was (Agresti 2007):

$$P(Y=0) = \frac{e^{-0.20791 - 0.33161(\text{Shedder})}}{1 + e^{-0.20791 - 0.33161(\text{Shedder})}} \text{ (Agresti 2007).}$$

The cumulative probability for a crab having shell rot intensity 0 or 1 was (Agresti 2007):

$$P(Y \leq 1) = \frac{e^{1.35886 - 0.33161(\text{Shedder})}}{1 + e^{1.35886 - 0.33161(\text{Shedder})}}.$$

Cumulative probability for a crab with shell rot intensity 0, 1, or 2 was 1.0 (100%) (Fig. 4.2).

This result of higher prevalence of shell rot and more intense infections at the shedding facilities was expected. Iverson and Beardsley (1976) hypothesized that crabs held in captivity would be infected more often than wild crabs. This increased prevalence is likely due to the crowded conditions of shedding systems, in which this contagious disease can be transmitted easily between crabs (Malloy 1978, Sindermann 1989). Also, crabs are more likely to be injured when they are kept in small, crowded



tanks, and these injuries can expose the shell to pathogens causing shell rot (Sindermann 1989).

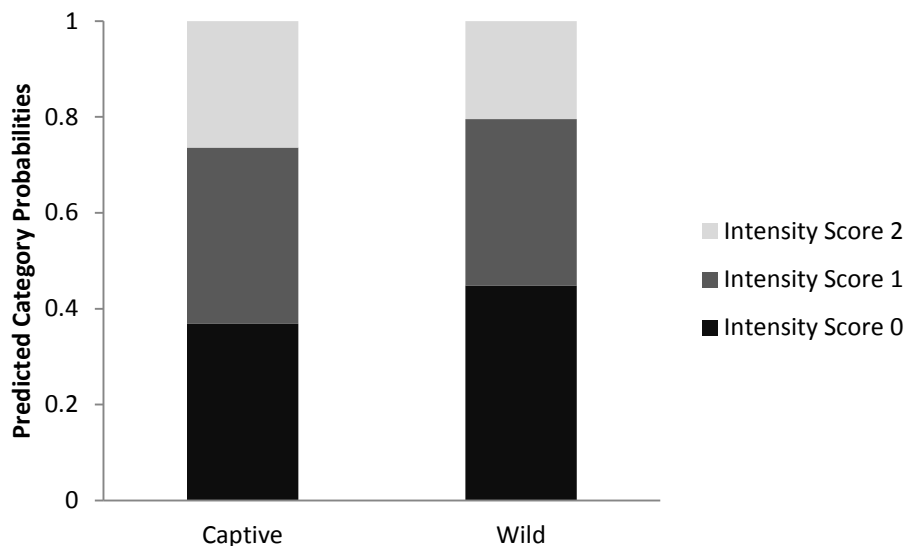


Figure 4.2: Predicted probabilities of wild and captive crabs in Louisiana having shell rot intensities scored as 0, 1, or 2. Score 0 represented the absence of shell rot while 1 was non-necrotic shell rot spots and 2 was the presence of shell rot lesions.

#### 4.2.2 Bacterial Infections in Hemolymph and Organs

The aggregate prevalence of *Vibrio* spp. in the wild populations was 39.9% (Fig. 4.1). Conversely, these bacteria were significantly more prevalent in the shedding facility crabs with an overall prevalence of 51.3% (Fig. 4.1;  $\beta_{\text{Shedder}} = 0.4613$ ,  $p < 0.01$ ). The odds of *Vibrio* spp. infections were 58.6% higher in the shedding facilities than in the wild populations. For infections only in the hemolymph, there was a significant difference between the prevalence rates at the shedding facilities and in the wild populations with rates of 36.8% and 22.3%, respectively (Fig. 4.1;  $\beta_{\text{Shedder}} = 0.7311$ ,  $p < 0.001$ ).

Like the results for shell rot, the higher prevalence rate in shedding systems was expected because crabs are stressed and kept in crowded conditions. Crabs that are commercially trapped and transported to shedding systems are often stressed, which can lead to increased bacterial loads in their hemolymph (Welsh and Sizemore 1985, Noga et al. 1998, Givens et al. 2013). The crabs collected from wild populations in this study were not commercially caught and may have been handled more carefully than if they had been caught by a fisherman. Also, I soaked all traps 48 hours or less to minimize stress that can cause increased bacterial loads in hemolymph (Shields and Overstreet 2007), and this may be inconsistent with commercial practices during different seasons.

Crabs collected from both the field sites and shedding facilities were infected with *Aeromonas* spp. (*A. sobria*, *A. hydrophila*, *A. caviae*), *Photobacterium damsela*, and *Shewanella putrefaciens*. *Aeromonas* spp. are correlated with shell rot infections so their presence in both captive and wild crabs may explain some of the shell rot

infections (Noga et al. 1994, Noga et al. 1998). *Photobacterium damsela* is pathogenic to blue crabs and may cause some mortalities (Givens et al. 2013). In the future, effects of *Shewanella putrefaciens* and other bacteria identified in this study and their potentially divergent prevalence and intensity rates in captive and wild crabs should be studied to determine if one of these non-*Vibrio* spp. bacteria could cause high mortality rates in shedding systems.

#### 4.2.3 Reo-like Virus (RLV)

Both wild and captive crabs were infected with RLV. Of the 39 analyzed from the field sites, three were infected for a prevalence rate of 7.7% (Fig. 4.1). Of the 20 analyzed from two shedding facilities, three were infected for a prevalence rate of 15% (Fig. 4.1). The higher rate at the shedding facilities was consistent with predictions that this virus is more prevalent in captivity (Bowers et al. 2010). This higher prevalence in shedding systems is hypothesized to be due to crowding and stress associated with the tanks (Bowers et al. 2010). Additionally, captivity allows more frequent cannibalism, especially on fresh molt crabs, and ingestion of infected tissue can spread the virus (Johnson 1977, Shields 2003, Bowers et al. 2010).

#### 4.2.4 *Ameson michaelis*

*A. michaelis* was only found at the shedding facilities (Fig. 4.1). One crab from Grand Isle was hypothesized to be infected as indicated by its white muscle, but it was uninfected according to electron microscopy. Although *A. michaelis* has been linked to mass mortalities in shedding systems, it is hypothesized to be rare both in the wild and in shedding systems (Overstreet and Whatley 1975, Messick and Sindermann 1992, Shields and Overstreet 2007). Epizootics may occur in shedding facilities because of cannibalism that can be common in the crowded tanks (Millikin and Williams 1984). It has also been hypothesized that this parasite thrives in waters with low dissolved oxygen, low salinity, and high temperatures (Findley et al. 1981). The low salinity and high temperature conditions are typical of Louisiana shedding systems in the summer and occasionally, low dissolved oxygen can also occur in shedding tanks (Manthe et al. 1983).

#### 4.2.5 *Hematodinium perezii*

No crabs in this study were infected by *H. perezii* (Fig. 4.1). Because of the low salinities sampled, I expected very low prevalence at the field sites and 0% prevalence in the shedding systems. The average salinity at the highest salinity field site, Grand Isle, was only 20.5 (Table 2.2), and this parasite is known to thrive at salinities above 25 ppt (Messick and Shields 2000). All salinities at the shedding facilities were below 8 ppt (Table 3.1), and no diseased crab has ever been recorded at salinities below 11 ppt (Newman and Johnson 1975, Messick and Shields 2000). In the future, higher salinity field sites with restricted water flow (i.e. canals, bayous, embayments) should be sampled.

#### 4.2.6 *Loxothylacus texanus*

No crabs in this study were infected with *L. texanus* (Fig. 4.1). Consistent with the *H. perezii* results, this was expected partially due to the salinities sampled (Tables 2.2 and 3.1) as infections are most common at salinities above 20 ppt (Boone et al. 2004, Tindle et al. 2004, Shields and Overstreet 2007). This prevalence rate was also biased by the sizes sampled. Infected crabs are typically smaller than 8 cm CW because this parasite preferentially infects frequently-molting juvenile crabs (Adkins 1972, Overstreet et al. 1983).

The average size collected at the shedding facilities was 13.2 cm CW. Also, pre-molt crabs brought to shedding facilities are often redliners that are within three days of molting so they would not have a suppressed molt cycle indicative of infection (Oesterling 1982). Therefore, I would not expect infected crabs at any shedding facility. At the field sites, I sampled mostly large juveniles and adults, so again I would not expect to see many infections. In the future, smaller juveniles from high salinity areas should be sampled, and then wild populations' prevalence rates may be above 0% and consistent with previous rates determined for Louisiana waters (Adkins 1972, Ragan and Matherne 1974).

#### 4.2.7 *Urosporidium crescens*

The overall prevalence of *U. crescens* in crabs collected at the four field sites was 22.4% (Fig. 4.1). At the shedding facilities, the prevalence was significantly lower at 2.6% (Fig. 4.1;  $\beta_{\text{Shedder}} = -1.5447$ ,  $p < 0.001$ ). This result may be an effect of salinity because shedding facility salinities were consistently less than 8 ppt while the field site salinities averaged 11.5 ppt (Tables 2.2 and 3.1). The odds of infection by this hyperparasite increase by 10% as salinity increases by 1 ppt ( $\beta_{\text{Salinity}} = 0.0961$ ,  $p < 0.0001$ ). *U. crescens* infections have been detected in crabs from waters with moderate salinities across a wide temperature range; however, no known data exist on infections at low salinities (Messick 1998). The lack of infected crabs in Lake Pontchartrain, the lowest salinity field site, supports my hypothesis that if the majority of pre-molt crabs are caught in lower salinity areas before being transported to the facilities, then few crabs should be infected before captivity. Additionally, low salinities in shedding systems may prevent transmission of infections.

#### 4.2.8 *Lagenophrys callinectes*

In the wild and captive crabs, prevalence rates of *L. callinectes* were 93.2% and 99.3%, respectively (Fig. 4.1). Prevalence was significantly higher at the shedding facilities than at the field sites ( $\beta_{\text{Shedder}} = 2.2828$ ,  $p < 0.01$ ). This result is consistent with results from Chesapeake Bay and nearby shedding facilities (Couch 1966, Couch and Martin 1979). The higher prevalence in shedding systems could be correlated with crowding that may facilitate transmission between crabs. Although there was a statistically significant difference in my results, the high prevalence rates in both wild and captive crabs shows that this ciliate is ubiquitous in Gulf blue crabs and has a wide salinity tolerance.

### 4.3 Summary and Implications of Comparison

Shell rot, *Vibrio* spp., *U. crescens*, and *L. callinectes* prevalence rates varied significantly between the shedding facilities and field sites (Fig. 4.1). Minimization of stress and injury is important for prevention of mortalities from both shell rot and bacterial infections because both increase with commercial handling and placement in shedding tanks (Overstreet and Cook 1972, Sandifer and Eldridge 1974, Shields and Overstreet 2007). Results for *U. crescens* suggest that the hyperparasite is intolerant of low salinities and is not prevalent at shedding facilities, which is economically beneficial for these facilities as soft shell meat should be largely uninfected and appetizing to consumers (Couch and Martin 1979). Reo-like virus was detected in wild and captive crabs. If more samples are analyzed in the future, I expect that prevalence will be higher in the shedding systems due to the crowded conditions and that dead crabs collected from shedding tanks will be infected with RLV (Bowers et al. 2010, Bowers et al. 2011). *A. michaelis* was only found at the low salinity shedding facilities, but it also may cause mortalities at low salinity coastal sites, especially during high temperature months (Findley et al. 1981). *H. perezii* and *L. texanus* parasitization should only be of concern at high salinity areas that are commercially fished because both have salinity tolerances above 11 ppt (Shields and Overstreet 2007). I do not expect either to cause mortalities in shedding systems but both could be detrimental to wild populations.

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## APPENDIX A: DISSECTION OF BLUE CRAB METHODOLOGY

With a Dremel 3000 or Dremel 7700 fitted with a cut-off wheel, I cut the dorsal carapace from the anterior margin of the head just outside the eye orbitals to the articulation of swimming leg (Fig. A1 black lines) (Johnson 1980). Then I cut transversely across the anterior and posterior regions of the the dorsal carapace (Fig. A1 red lines) (Johnson 1980). After cutting the crab carapace, I removed the two lateral spines and removed samples of the hepatopancreas found in the removed shell (Fig. A2). One sample of hepatopancreas was preserved in 95% ethanol and the other sample was frozen at -20 °C in the event that the ethanol-preserved sample degraded.

Next I removed two samples of gill and froze both at -20 °C (Fig. A2). The middle part of the cut shell was then removed to reveal the heart (Fig. A3). The heart was removed and frozen at -20 °C. After removing the heart, the midgut was visible underneath where the heart had been located. The fragile midgut was carefully removed and preserved in 95% ethanol.

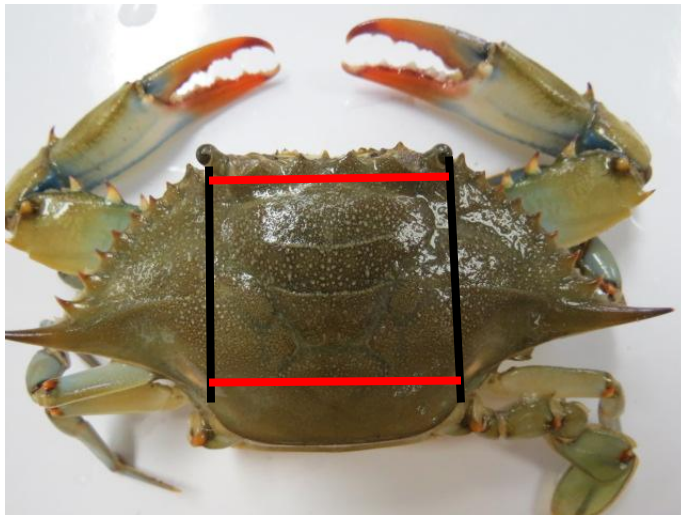


Figure A1: Black lines are the first two cuts in the dorsal carapace from the anterior margin of the head just outside the eye orbitals to the articulation of the swimming leg. Red lines are the transverse cuts across the anterior and posterior areas of the dorsal carapace.



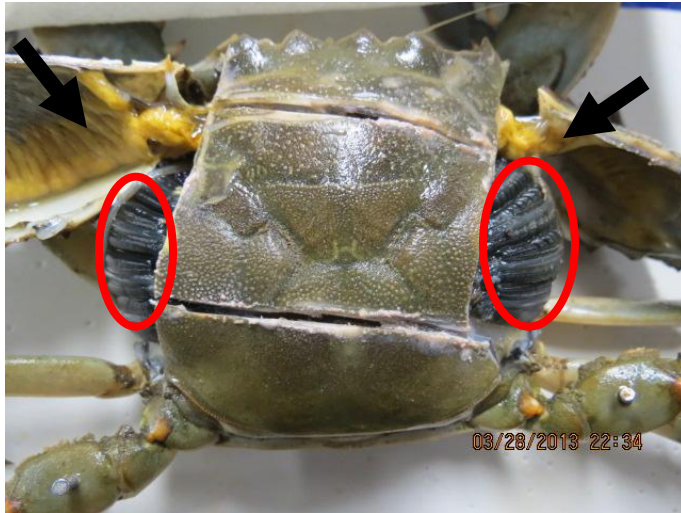


Figure A2: Dissected crab after the four cuts. The lateral spine portions of the shell were removed and contain the yellow hepatopancreas (black arrows). After the hepatopancreas samples were removed, two gills were also removed (red circles).



Figure A3: Dissected crab after the four cuts. The red circle denotes the centrally located, semitransparent heart and underneath the heart is the midgut.

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## APPENDIX B: FIELD SAMPLING METHODOLOGY

Table B1: Sampling methods used at the four field sites (Lake Pontchartrain, Cocodrie, Rockefeller Wildlife Refuge, and Grand Isle) in 2013 and the beginning of 2014. Blank cells indicate that this method was not attempted. Cells with a “0” indicate that the method was attempted but no crabs were caught.

Site	Season	Seining	Trawling	Bait Trawling	Baited Line	Baited Trap	Baited Capture (Line & Traps)	Unbaited Trap
Pontchartrain	Winter 2013	0						
	Spring	24						
	Summer	3				57		
	Fall	1			6	53		
	Winter 2013-14					0		
Cocodrie	Winter 2013	0						
	Spring	0						
	Summer	1	6	41		12		
	Fall			14		46		
	Winter 2013-14			29	0	31		
Rockefeller	Winter 2013	4	13					
	Spring	40	3					
	Summer	1	3			56		
	Fall	0	4			60		
	Winter 2013-14	0	8		0	52		
Grand Isle	Winter 2013	7						
	Spring	13						
	Summer	4			5	26		25
	Fall	1				19	40	
	Winter 2013-14					19	41	

### APPENDIX C: BLUE CRAB LANDINGS DATA

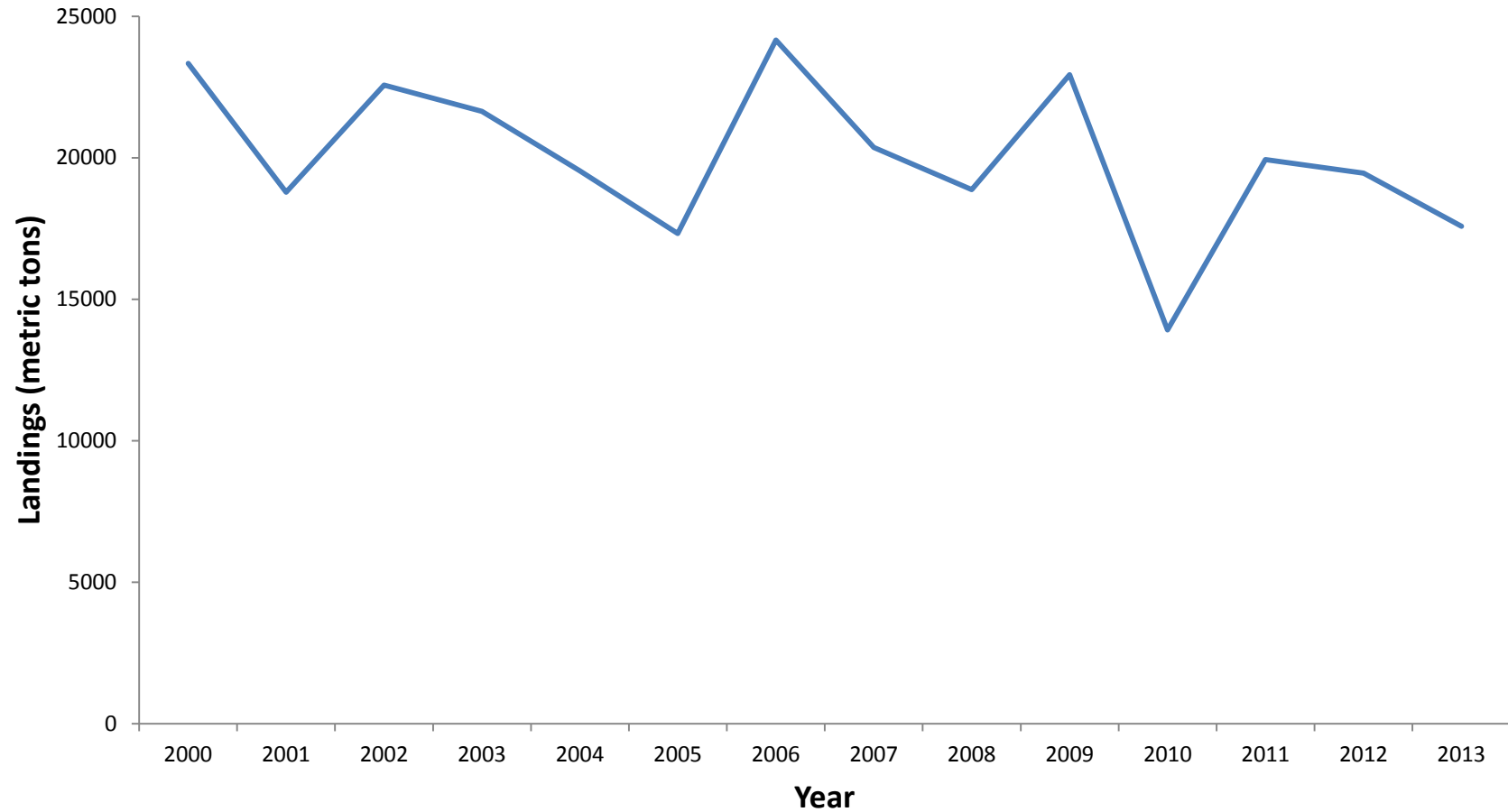


Figure C1: Louisiana blue crab landings from 2000 to 2013. Landings in 2013 were the third lowest in 14 years after low landings in 2005 following Hurricane Katrina and in 2010 following the Deepwater Horizon oil spill. Average landings, excluding these three years, were 21052 metric tons. Data source: Louisiana Department of Wildlife and Fisheries preliminary trip ticket data, 2014.

## **VITA**

Holly Anne Rogers was born in Cincinnati, Ohio. She graduated from Walnut Hills High School in 2008 and from the University of Cincinnati with a Bachelor's of Science in 2011 as an Environmental Studies major. Holly worked for the US EPA at the Experimental Stream Facility for 14 months before enrolling at Louisiana State University for her Master's of Science under the direction of Dr. Julie Anderson. She studied blue crab diseases, parasites, and symbionts during which she spent many hours unsuccessfully "seining" in the soft sediments of south Louisiana and extracting and amplifying DNA only to find no positive results.