ABSTRACT

Title of Document: ECOLOGY OF A FATAL BLUE CRAB VIRUS: DETECTION, RANGE, AND PREVALENCE OF *CALLINECTES SAPIDUS* REO-LIKE VIRUS

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Callinectes sapidus reo-like virus (RLV) is a fatal to blue crabs and consistently found within the Chesapeake Bay. Development of a sensitive and reliable RT-qPCR assay permitted a precise assessment of RLV prevalence in crabs captured from locations near and far from soft-shell crab production sites. Viral prevalence was temporally and spatially highly variable, but outbreaks of RLV appeared to be localized to an area of 1 - 2 km^2. When significantly different between sites, higher prevalence was observed near soft-shell crab production. RLV prevalence was not correlated with crab characteristics, with the exception of larger mean carapace width for infected crabs. Sequences of RLV PCR products were used to compare genotypes of outbreak and non-outbreak infections. Identical genotypes were found in outbreaks from the middle Chesapeake Bay and Long Island, NY. Together, the prevalence and genetic data are consistent with RLV outbreaks being caused by focal spread of the virus through a local population.

ECOLOGY OF A FATAL BLUE CRAB VIRUS: DETECTION, RANGE, AND PREVALENCE OF *CALLINECTES SAPIDUS* REO-LIKE VIRUS

by

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Chapter 1: Introduction

In the past decades pathogens in marine species have been recognized as an important area for research. This is driven in part by the need for sustainable fisheries and aquaculture, as part of global food security and economic security. Research that allows sustainable exploitation of commercial species aids preservation of those species. Collection of baseline data on pathogens, such as prevalence, range, seasonality, or correlation with host characteristics, is a necessary starting point as we move toward management that more effectively accounts for marine pathogens. This baseline information can directly contribute to management and lead to disease models, which in turn raise hypotheses about epizootic requirements and conditions.

The research in this thesis addresses fundamental questions about prevalence and genetic diversity of *Callinectes sapidus* reo-like virus (RLV). RLV is fatal to blue crabs, and as is true for many crab viruses, little ecological data are available for this pathogen. Here we develop a sensitive assay to quantitatively detect RLV, make a preliminary assessment of viral genetic diversity, and perform a multi-year assessment of RLV prevalence in the Chesapeake Bay. These data provide the foundation to move toward RLV epidemiological modeling and broader assessments of the virus' genetic diversity, evolution, and impact on the blue crab population.

1.1 Marine Disease Ecology

Challenges exist for quantifying pathogens in marine systems, in particular: high taxonomic diversity, complex or unknown life histories, open populations, anthropogenic impacts, and impracticality of terrestrial disease control strategies (McCallum et al.

2004). These same challenges exist when seeking to predict the spread of a pathogen or frequency and effects of epizootics. Despite these complexities, there is the opportunity to build on the wealth of techniques from terrestrial examples. Terrestrial disease models point to the importance of host density, transmission rates, basic reproduction number of the pathogen, and rates of death or recovery for the host. Therefore, a brief review of some examples in marine pathogen modeling is useful in thinking about current and future work on RLV.

The examples of sea lice (*Lepeophtheirus salmonis*) parasitizing Atlantic salmon (Frazer et al. 2012), and white-spot syndrome virus (WSSV) in penaeid aquaculture (Lotz and Soto 2002, Soto and Lotz 2001) demonstrate the variety of concerns for understanding marine host-parasite systems. Sea lice are macroparasites that have a direct lifecycle, with a free-living stage but no intermediate hosts (Frazer et al. 2011). In Atlantic salmon, sea lice remain at a low prevalence and intensity in the absence of the necessary host density and environmental conditions that facilitate epidemics, while epidemics feature increased prevalence and intensities associated with host mortality (Morton et al. 2004, Morton et al. 2005, Frazer et al. 2011). A key feature of this system is the impact of sea-cage aquaculture for salmon. There is a recognized potential for wild populations to seed pathogens in aquaculture and for aquaculture populations to amplify and disseminate pathogens and to be a source of cross-species infection (e.g. reviewed Kautsky et al. 2000, Morton 2004, Morton 2005). Similar to macroparasites, transmission of viral and bacterial pathogens may be enhanced by high densities in aquaculture or wild populations.

In addition to density, mode of exposure and species specificity are important considerations. Species-specific susceptibility, transmission coefficients, and density dependence of white spot syndrome virus (WSSV) have been addressed by Lotz and Soto (2002). WSSV has at least two natural modes of transmission, but it is clear that they are not equal in importane, and ingestion is more ecologically relevant than water exposure (Lotz and Soto 2002). *Litopenaeus setiferus* is more susceptible to WSSV than *Litopenaeus vannamei* (Soto and Lotz 2001), demonstrating the importance of looking at specific species of interest, even in cases where the hosts are closely related. Species-specific transmission rates may also inform management of aquaculture, depending on the relative value of the cultured species. For *L. vannamei*, WSSV transmission is minimal or absent as a result of cohabitation, but over an order of magnitude higher following ingestion (Soto and Lotz 2001). Low or absent transmission as a result of cohabitation may simply be the result of a low encounter rate as the virus is diluted in the water. The transmission rates observed may also reflect susceptibility of the cell type (cuticle or gill versus alimentary) exposed to the virus.

The examples above highlight important considerations in marine disease ecology. The type of pathogen in part dictates routes of exposure, and the route of exposure may greatly alter transmission rate. Density is a key feature in transmission and epizootics of some pathogens, in particular parasitoid pathogens. Aquaculture creates artificially high densities that elevate the risk of pathogen invasion. If pathogens are present, these high densities can cause large-scale mortality either because of increased infection rate or as an additional stress, as is seen in the classic boom-and-bust pattern of shrimp pond farming (Kautsky et al. 2000). Morton (2004) demonstrated that the high densities in

aquaculture can threaten wild populations if parasites in aquaculture are not adequately managed or sequestered. In species not subject to aquaculture, life history or behavior can lead to high densities (e.g. recruitment, spawning, or schooling), though these may not be as high as the densities achieved in farming. Fisheries are managed based on target density for maximum sustainable yield. At this time, pathogens in most fishery species have not been studied in a way that allows comparison between impacts of density on disease transmission and the densities created by life history or aquaculture.

1.1.1 Disease Detection in Marine Metazoans

The ability to reliably identify a pathogen is necessary to determine the ecological impacts of that pathogen. Detection methods for marine pathogens vary widely in terms of sensitivity and specificity. The above example of sea lice on salmon is a relatively tractable system for determining both prevalence and intensity of the parasite: sea lice can be counted under a dissecting microscope, or without magnification (Morton et al. 2005), and can be collected from the water column, as well as from the externae of infected fish. Microparasites are more challenging to quantify, and this is especially true in the case of viruses. Marine viruses are often identified in response to a large-scale die-offs in wild or cultured animals (e.g. Johnson and Bodammer 1975, Bowers et al. 2010, Lovell and Drake 2009). Pathogen identification has also historically been limited by the techniques available. Sensitive detection methods are critical for precise assessment of prevalence or identification of latent infections. This discussion of detection methods will focus on detection of viruses but address techniques applicable to many types of pathogens.

Crustacean viruses were first identified in the 1960s and 1970s, using histology and electron microscopy (review Johnson 1983). These techniques are useful for determining etiology and effects of a disease. For described pathogens, *in situ* hybridization and immunohistochemistry allow specific detection and localization of pathogens, and both can be used semi-quantitatively. Alternately, PCR may be used for qualitative or quantitative pathogen detection. PCR detection has been used to detect finfish and shellfish pathogens and address ecological questions and in some instances fishery management questions (e.g. Bain 2010, Garver 2014). Quantitative PCR (qPCR) has the advantage of rapid and sensitive quantification of pathogens. In some assays, it can reliably detect a single copy of the target nucleotide sequence and appropriate primer design can yield high specificity for a species of interest. In addition to specific primers, confirmation of the desired amplicon can be verified using the product melting temperature (reviewed Smith and Osborn 2009, Zipper et al. 2004, Wittwer et al. 1997). The length and nucleotide composition of the target will yield a characteristic melting temperature (Tm) under given salt conditions. Alternately, a fluorescent probe that anneals to a unique sequence between the primers can be used for quantification that reduces uncertainty related to primer dimers or other non-target amplification (Smith and Osborn 2009).

Quantification in PCR can be either relative or absolute. Both require a standard against which to compare unknown samples. In relative quantification, the amount of product is expressed relative to a gene of known copy/target number or a housekeeping gene with constant expression (Freeman et al. 1999, Wong and Medrano 2005). Absolute quantification requires a standard dilution of known copy number, and the template may

additionally be standardized against total nucleic acids in the sample or sample mass (Smith and Osborn 2009). These sensitive detection methods have the clear advantage of providing both a precise measure of prevalence and severity of infection. qPCR has been essential in identifying latent infections, transmission risks, and providing information for responsible aquaculture management (e.g. Durand et al. 2002, Bain 2010, Garver 2013)

1.1.2 Environmental Impacts & Crustacean Immune Function

Crustaceans are host to many bacterial, viral, protistan, and metazoan parasites. Although crustaceams lack the adaptive immune system found in jawed vertebrates, they mount a range of innate immune responses to kill or sequester pathogens. These innate immune responses are cellular, humoral, and organ based. They include hemolymph coagulation, the prophenoloxidase (PPO) cascade and melanization, antibacterial and antifungal peptides, stimulation of hemocyte-producing tissue, phagocytosis by hemocytes, and phagocytosis in the hepatopancreas (review Le Moullac and Haffner 2000, Shields and Overstreet 2004). The robustness of immune function can be altered by the same conditions that alter metabolism and hemopoiesis. In particular, hemopoiesis and hemocyte-based responses may vary naturally or in response to external or physiological stress.

Environmental conditions can affect immune function. However, in crustaceans the understanding of how the environment influences immune function is complicated by a lack of clear baseline information or trends that can be generalized across species. For example, hemocyte counts can fluctuate based on diel or tidal cycles, as well as seasonally. Within the portunid crabs, aspects of immune function have been explored in *Carcinus maenas* both at baseline levels, and in response to stressors (Hauton et al. 1995,

Chisholm and Smith 1994, Truscott and White 1990). *C. maenas* has circatidal variations in hemocyte counts and PPO activity (Truscott and White 1990, Hauton et al. 1995). PPO activity was highest during low tide for a typical tidal cycle, but this pattern was disrupted by acclimation to an extended low tide (Hauton et al. 1995). Crustacean species that live in fresh water, neritic, or oceanic habitats, are unlikely to show the same tidal oscillation. For example, crayfish hematopoiesis and PPO activity have circadian rhythmicity (Lin and Soderhall 2011, Noonin et al. 2013). Temperature or seasonality may also impact portunid immune function. In *C. maenas* the antibacterial response in hemocyte lysate supernatants was robust except for the months where temperatures were at their highest and lowest (Chisholm and Smith 1994). Research on *C. maenas* cannot be generalized to other portunid species without more information. However, *C. maenas* demonstrates the challenges of assessing overall immune function and indicates some factors that may impact blue crab immune responses. In blue crabs it is not known if immune responses have seasonal, circadian, or molt-based rhythms. However, the blue crab inhabits climates from temperate to tropical, and salinities from almost freshwater in the upper portions of estuaries to the oceanic salinity on the coastal shelf. Consequently, seasonality and acclimation should be considered in any future research on *C. sapidus* immune function.

In addition to natural variability, crustacean immune functions may increase or decrease in response to stress. For example, in marine penaeid shrimp, typical PPO activity is disrupted at high temperatures (32 – 33 °C), but may not be substantially altered across a range of lower temperatures (Pascual et al. 2003, Vargas-Albores et al. 1998). Conversely, PPO displayed a steady increase, rather than a threshold response, in

increasing salinity (28 – 44 ppt) (Vargas-Albores et al. 1998). Other species likely have different thresholds or patterns of altered immune function in response to physiological stress (e.g. Sung et al. 1996). In addition to abiotic stressors, pathogen exposure can illicit increased immunocompetance. Bacterial challenges initially decrease and then augment PPO activity in *C. maenas* (Hauton et al. 1996). Overall, crustacean immune function is frequently impaired at physiological extremes with decreased function correlating with the increased metabolic demands due to salinity or temperature.

Notably, when considering transmission of a pathogen, density functions separately from the stresses related to high density or poor environment conditions (Kermack and McKendrick 1927, Krkosek 2010). This should not minimize considerations of environmental impact on immune function when looking at disease ecology. Instead it is an example of the complex interactions that drive epizootics. Historically, aquaculture is a primary example of these principles, demonstrating high density coinciding with, or causing, poor water quality (reviewed Kautsky et al. 2000). However high densities and poor environmental conditions are not exclusive to aquaculture. High densities may be a feature of life-history stages and isolated events and climate change may create physiologically stressful conditions. Under such circumstances, epizootics become increasingly likely.

1.1.3 Crustaceans and Viral Infection: Portunidae and Reoviruses

More than 30 viruses, representing a range of families, have been identified in crustaceans: dsRNA viruses (Reoviridae, Birnaviridae), ssRNA viruses (Bunyaviridae, Picronaviridae, Roniviridae, Rhabdoviridae), dsDNA viruses (enveloped bacilliform viruses, Herpesviridase, Iridoviridae) and ssDNA viruses (Parvoviridae). Viruses

infecting crabs were among the first crustacean viruses identified, however many of these have not been biochemically characterized and were identified based on morphology and cellular localization (reviewed Bonami and Zhang 2011, Johnson 1983). While some crustacean viruses are cosmopolitan and will infect many hosts (e.g. WSSV, Rajendran et al. 1999), others have only been found in a single host (e.g. W2 viruses; Mari and Bonami 1988). In addition to the innate immune responses discussed above, crustaceans have non-specific defenses against viral infection. Crustacean response to viral infection may include cytokine mediated antiviral response, antiviral proteins, apoptosis, and RNA interference (RNAi). Portions of these pathways have been identified in penaeid shrimp, but the extent of their role in viral infections is not known (review Liu et al. 2009).

Many reoviruses have been identified infecting crabs. These include *C. sapidus* reo-like virus (RLV), *Macropipus depurator* P-virus, *Carcinus mediterraneus* W2 virus, two *Eriocheir sinensis* reoviruses (EsRV 905 and 806), and *Scylla serrata* reovirus (MCRV / SsRV) (reviewed Bonami and Zhang 2011). Reoviruses are double stranded RNA (dsRNA) viruses with a multi-layer capsid generally comprised of an outer capsid layer, intermediate capsid proteins, and an inner or core capsid (Huang et al. 2012, Roy 2006). The reovirus genome is linear and segmented with nine to twelve segments (Joklik 1981, Attoui et al. 2005). Generally speaking, each segment encodes a single gene product (review Roy 2006). The majority of crab reoviruses have been identified and taxonomically categorized by host range, electron microscopy, and histology, but not by genome sequence. Crab viruses were initially grouped as a single genus: "Cardoreoviruses" for Carcinus dodeca reoviruses. This genus was based on the common features of a crab hosts, 12-segment virus genome, similar electrophoretic pattern for

some members, and a putative non-turreted inner capsid structure (Mari and Bonami 1988, Zhang et al. 2004, Huang et al. 2012). However, recent genome sequencing indicates that crab reoviruses may not be members of a single genus, and offers a more nuanced view of their relatedness.

Recent reovirus phylogenies are typically constructed based on the RNA-dependent RNA-polymerase (RdRP) gene, as RNA-based viruses have a high mutation rate and the RdRP has essential, conserved function (Deng et al. 2012, Domingo and Holland 1997). To date, sequencing efforts include the RdRP of EsRV905, the complete genome of SsRV/MCRV (Deng et al. 2012, Chen et al. 2012, Chen et al. 2011), and all coding regions of the RLV genome (Bowers et al. 2010, Flowers, Warg, and Schott unpublished). SsRV and RLV share relatively high nucleotide identity, on the order of >70% across all sequence in GenBank (Bowers et al. 2010, Tang et al 2011). However SsRV and EsRV905 RdRP genome segments share only 55% identity (Deng et al. 2012). This suggests that SsRV and RLV may share a genus based on sequence similarity, morphology, genome structure, and portunid hosts. However it is unknown if all morphologically grouped Cardoreoviruses are members of this genus. Further complete genome sequencing is needed to resolve the phylogenetic relationships of crab reoviruses.

1.2 Blue Crab Life History & Pathogens

The blue crab, *Callinectes sapidus*, has a broad geographic range and is found along the U.S. East Coast from New England into the Gulf of Mexico. In South America, blue crabs are found in abundance near the equator in Venezuela, and as far south as the coast of southern Brazil and Uruguay, to northern Argentina (Williams 1974). They are therefore tolerant of a wide range of temperatures/climates. In the temperate regions,

crabs overwinter when water is below approximately 10˚C (Brylawski and Miller 2003). Blue crabs are a coastal and estuarine species and adults can tolerate salinities from hypersaline lagoons to freshwater (Mangum and Amende 1972). Oxygen demand increases at lower salinities (Towle and Burnette 2004), likely reflecting the increased metabolic demands of maintaining hyperosmotic hemolymph. The lifecycle of the blue crab includes a long pelagic larval stage. In coastal bays, recruiting juveniles disperse up the bay while mature crabs may migrate down bay to spawn or overwinter in deeper waters. Life history characteristics are important factors influencing the spread of pathogens in crab populations.

1.2.1 Life History

Unlike mature crabs, larval blue crabs require high salinities and live along the continental shelf. Larvae are released at the mouths of estuaries or along the coast, undergo eight zoeal stages, metamorphose into megalopa, and metamorphose again into juvenile crabs (Fig. 1.1). Recently hatched larvae display positive phototaxis, negative geotaxis and increased swimming speed with increased salinity (review Epifanio 2004). Swimming behavior, currents and tides disperse larvae along the coast. In the mid-Atlantic region, southward wind events drive larvae and megalopa inshore toward coastline and estuaries, allowing for settlement. In coastal or estuarine water, megalopa swimming patterns result in transport landward, or up estuaries (Forward et al. 1997, review Epifanio 2004). Megalopae settle and metamorphose in response to salinity and vegetation cues from estuaries (Welch et al. 1997, Brumbaugh and McConaugha 1995). Blue crab megalopae do not change settlement behavior in the presence of mature conspecifics (Welch et al. 1997). After settlement and metamorphosis, juvenile and

mature crabs undergo a series of dispersals. The first juvenile stages shelter in grass beds or other structured habitat, but individuals will disperse if intra-cohort densities are high (>10 crabs per m^2) (Reyns and Eggleston 2004). This emigration may be a means to avoid inter-cohort and intra-cohort predation. At this size, intra-cohort cannibalism is minimal but increases at high density, while inter-cohort cannibalism is potentially high and both size- and habitat-dependent (Moksnes et al. 1997). Subsequently, large juveniles (>20mm CW) have a size-dependent secondary dispersal from nursery habitat and mature in less-structured shoreline habitat (review Lipcius et. al. 2004).

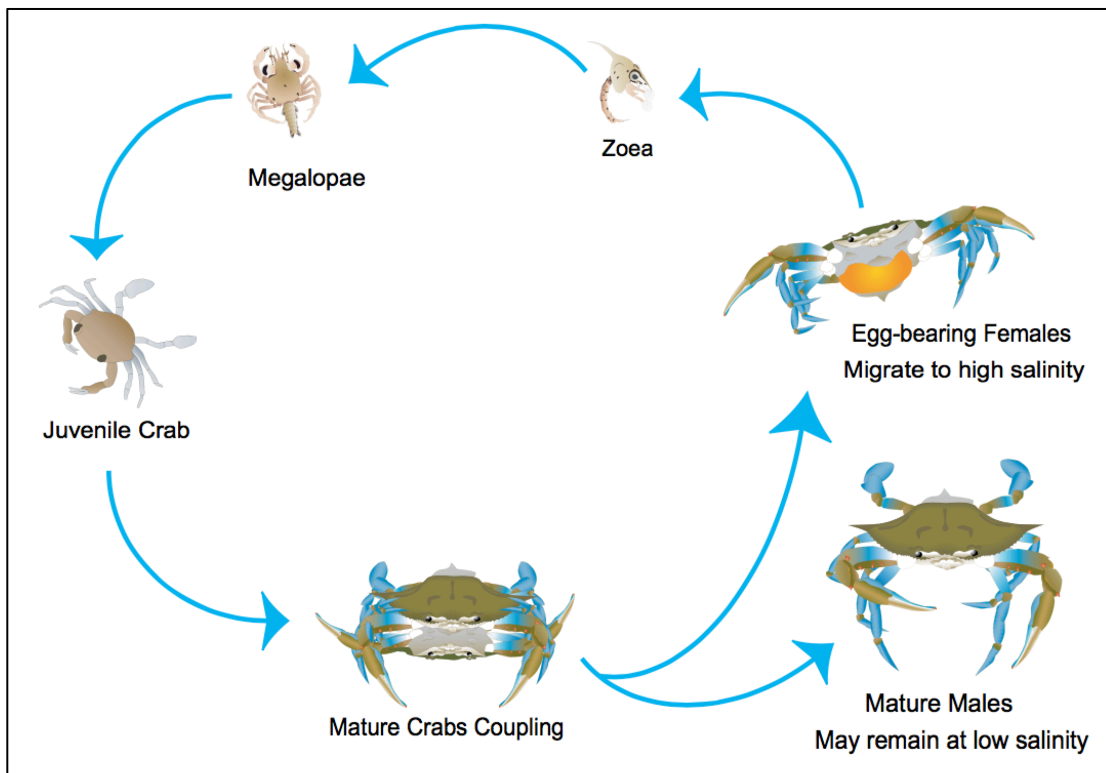

Figure 1.1. Blue crab life cycle. Major stages of the life cycle are shown. Courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

Once mature, both male and female crabs exhibit large-scale movements, in addition to meandering or foraging within habitats. Mature female crabs in the Chesapeake Bay, and other estuaries, undergo two phases of migration. In the first, they move to the higher-salinity lower estuary prior to setting sponges. Then during brood incubation, females again migrate to the mouth of the estuary, or off-shore, for the brood to hatch (Tankersley et al. 1998). In the Chesapeake Bay, most mature females stay in the lower bay throughout the summer without migrating out of the bay or up into lower salinities (Hines 2004). Mature male crabs do not undergo the same migration to high salinity, however in estuaries males migrate to deeper channels to overwinter. Apart from these large-scale movements, mature crabs will meander with periodic longer, directional movement (Hines and Ruiz 1995, Turner et al. 2003, Hines 2004). This type of movement is exemplified in Figure 1.2, which shows movement of a mature male in Rhode River off the Chesapeake Bay. In coastal waters, blue crabs occasionally travel large distances along the coast (> 1,200 km), however tagging studies show that mature crabs in estuaries generally stay within that estuary (review Hines 2004).

Once juvenile crabs exceed 40 mm CW, they begin preying on conspecifics more frequently. Cannibalism is significantly more common in adults than juveniles (Lipcius et al. 2004). Blue crabs grow by cycles of molting, which creates a period of vulnerability to predation in the time after ecdysis. The carapace fully hardens by the late postmolt stage, and crabs will spend approximately 70% of their life as postmolt or intermolt hardshells (Smith and Chang 2004). Females have a terminal molt to maturity during which they mate, however males continue to grow and may reach sizes exceeding 200 mm CW. Blue crab maturation and growth is in part temperature dependent and the time between molts

can be described by a degree-day function (Smith and Chang 2004). The intermolt period is dependent on the amount of time spent above a minimum temperature for growth, which was calculated to be 10.8 °C (Brylawski and Miller 2006). Consequently patterns of blue crab growth differ by latitude. Crabs at warmer latitudes may grow and molt year-round. Crabs in temperate latitudes, including the Chesapeake Bay, have seasonal recruitment and growth. Megalopa that recruit in fall will disperse as >20 mm juveniles before winter, but mature in the following year. In the mid-Chesapeake Bay, this creates mixed population and a bimodal size distribution with a cohort of large juveniles and a cohort of mature crabs (Hines et al. 1990).

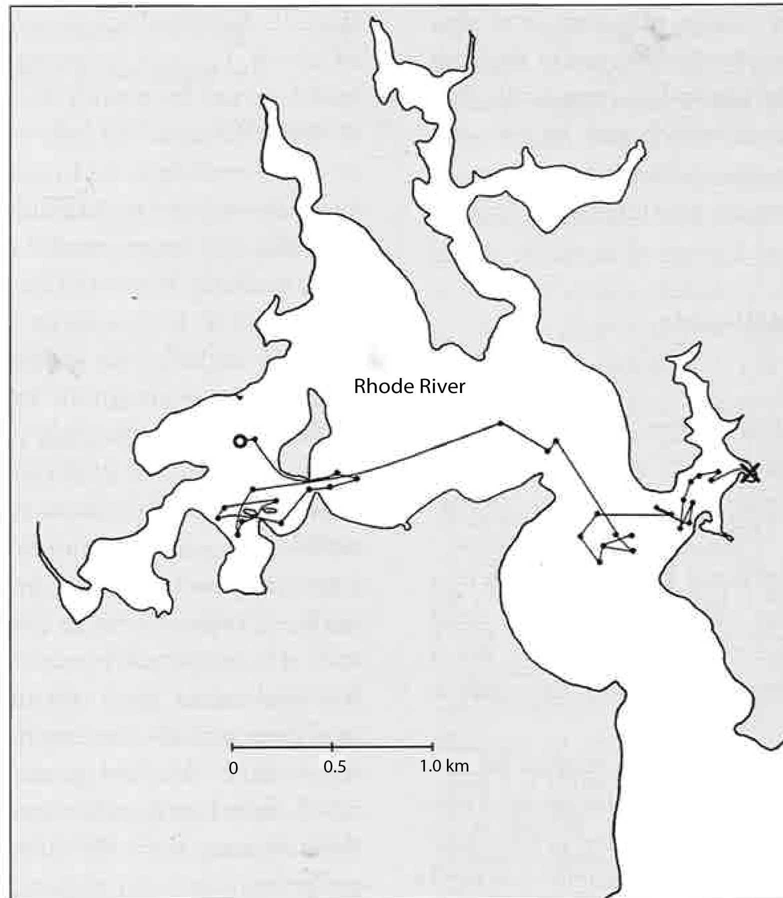

Figure 1.2. Small-scale blue crab movement; from Hines (2004). Example movement of a large male blue crab tracked by ultrasonic telemetry in the Rhode River, MD over the course of approximately two weeks. The open circle indicates the start point for tracking, and the X indicates the end point. Each dot represents approximately a 12-hour interval.

1.2.2 Blue Crab Stock Assessment and Fishery

In the US, blue crab supports a fishery with average annual landings worth more than $150 million. The largest fisheries are the Chesapeake Bay, North Carolina, and the Gulf of Mexico (predominantly Louisiana), with mean annual landing of 66, 29, and 55 million pounds respectively (2003 – 2012) (NMFS Commercial Fisheries Statistics). In the Chesapeake Bay, blue crab is caught commercially caught principally by pots/traps, scrapes, and trotline (MD DNR, VA MRC). In addition to the hard crab market, soft crabs can be sold as a higher-value product. To produce soft crabs, pre-molt crabs are held in flow through shedding systems until they molt. This is a labor-intensive, often artisanal process that involves holding crabs in recirculating or flow-through tanks and removing soft crabs within hours of molting (Oesterling 1995).

The ecological and economic importance of the blue crab has motivated onging efforts to maintain the fishery and population. Notably, the Chesapeake Bay crab population has substantial inter-annual variability and considerable effort has gone into managing this stock. Since 1989/1990 the Chesapeake Bay blue crab population has been monitored by a stratified random winter dredge survey (Miller et al. 2011). Stock maintenance is in part complicated by variability in natural mortality. Current stock management is based on maintaining an adequate spawning population of mature females through target (25.5%) and threshold (34%) exploitation rates (MD DNR). Harvest of females has been at or below target exploitation since 2008 when the management strategy was implemented (CBSAC 2014). Despite this, the Chesapeake Bay blue crab population was estimated to be at some of the lowest recorded densities in 2013 and again in 2014. The drop in population followed extremely high density in 2012 (MDNR). It is unclear what has

caused this. Possible contributing factors include cold winters; predation; and variable recruitment. In conjunction with the winter dredge survey, the importance of estimating mortality has been recognized (Hewitt et al. 2007, Hewitt & Hoenig 2005). In blue crabs, natural mortality estimates vary depending on the method of estimation (Hewitt et al. 2007, Bunnell and Miller 2005), and the contribution of disease to natural mortality and population fluctuation is unclear.

1.2.3 Pathogens of the Blue Crab

The population-level impact of blue crab diseases are unclear, and this has been identified as a critical data gap (CBSAC 2014). While population-level impacts require further research, many pathogens have been described in blue crab. Microbial pathogens, including viruses, bacteria, and protozoans, have been described in the blue crab, but fatal viral pathogens will be emphasized here. In addition to microbial pathogens, blue crabs may be infected by fungi (e.g. *Lagenidium callinectes*), and metazoan parasites, including helminths, nemerteans, and the parasitic barnacle, *Loxothylacus texanus* (review Shields and Overstreet 2004). A number of these parasites are fatal, reduce fecundity, or reduce marketability of the whole crab or meat.

Notable fatal diseases in the blue crab include bacterial infections, diverse protozoan infections, and at least three diseases of viral etiology. Systemic bacterial infections can cause hemocytic aggregation; internal clotting, which results in reduced clotting ability in withdrawn hemolymph; and death (Messick and Kennedy 1990). *Vibrio parahaemolyticus* is commonly seen in systemic infections. Shell disease is also associated with bacteria, though the etiology may be a combination of chitinoblastic bacteria and poor water quality. *Vibrio* and *Pseudomonas* species are commonly see in

shell lesions (Shields and Overstreet 2004). Shell disease is not invariably fatal and crabs may escape or slow the disease by molting. Protozoan pathogens fatal to blue crabs include *Mesanophyrus chesapeakensis* and *Orchitophrya stellarum* (Orchitophryid ciliates), *Hematodinium perezi* (dinoflagellate), *Ameson michaelis* (microsporidian), and *Paramoeba perniciosa* (amoeba) (review Shields and Overstreet 2004, Milliken and Williams 1984, Small et al. 2013). *A. michaelis* is notable for causing both mortality and reduced marketability. Microsporidosis is referred to as "cotton crab" for the muscle destruction that makes the meat of infected crab unpalatable. For many of these pathogens, patterns of prevalence have not been adequately characterized. *H. perezi* is the best studied, with infections throughout the temperate Atlantic coast and south to Florida. Throughout this range, infections are typically found at salinities above 18 ppt (Messick & Shields 2000, Stentiford & Shields 2005). In temperate regions, *H. perezi* infections fluctuate seasonally with high prevalence, particularly in juveniles, in late summer (August – November) (Messick 1994, Newman and Johnson 1975).

Relatively little information is available for viral diseases in the blue crab. At present three viruses are known to be fatal: bi-facies virus (BFV); Chesapeake Bay virus (CBV); and *Callinectes sapidus* reo-like virus (RLV or CsRV). Bi-facies virus is an enveloped, nuclear, dsDNA virus, originally thought to be a herpes-like virus (Johnson 1983, Bonami and Zhang 2011). BFV infects many tissue types, but has primarily been observed in hemocytes, with infection resulting in milky-white hemolymph that has reduced clotting ability (Johnson 1983). BFV has been identified in Chincoteague Bay, Virginia, and in Assawoman Bay, Delaware (Johnson 1983). In contrast to BFV, Chesapeake Bay Virus (CBV) is a cytoplasmic, RNA virus that appears picornavirus-

like; it was first identified in Tangier Sound, Chesapeake Bay during the summer (Johnson 1978). This virus infects tissue of ectodermal origin, and heavy infection in gill epethelia and/or neurosecretory cells likely contribute to death (Johnson 1983). Ommatidia may be infected and, as a result, crabs with CBV may be blind for as long as a month prior to death (Johnson 1983). Time to death was variable with CBV but some crabs survived as long as two months with this virus under laboratory conditions (Johnson 1978). RLV will be discussed in greater detail below. Briefly, RLV is a cytoplasmic virus that infects hemocytes and causes necrosis in the hemopoetic tissue, circulatory system, neuroglia, and nerve cells. RLV infection result in reduced hemolymph clotting, muscle weakness, and trembling pereopods, followed by death (Johnson 1977, Bowers et al. 2010). All three fatal blue crab viruses have been found in or near the Chesapeake Bay, and it is possible that all three could regularly be present in the Bay. Prior to the research undertaken here, information on range and prevalence of all three has been very limited. Here, RLV was selected for study due to repeated identification in soft-shell system mortalities (Bowers et al. 2010, Schott personal communication), suggesting a persistent prevalence in the wild population of the Chesapeake Bay.

The life history of the blue crab suggests opportunities for pathogens to spread throughout the Chesapeake Bay population. This is particularly important for pathogens, such as viruses, that are not free-living or have the potential to be diluted below infectious levels outside the host. Newly recruited juveniles remain in highly-structured nursery habitat, while large juveniles (>20 mm) move to shallow, unstructured sub-tidal habitat, and subsequently disperse up the Chesapeake Bay (Lipcius et al. 2004). Adult

crabs have small-sale movements but also migrate over long distances, and these adults display substantial levels of cannibalism (Lipcius et al. 2004). Dispersing juveniles are likely to carry pathogens up-Bay, while mature crabs may similarly carry pathogens over long distances, generally in the opposite direction as juveniles (toward the mouth of the Bay). The high-density of juveniles prior to dispersal has the potential to facilitate density-dependent disease transmission, while increased cannibalism by larger crabs presents a likely mode of disease transmission to mature crabs. Dispersal/migration in all life stages may carry pathogens across the Bay. Ecologically important modes of transmission have not been quantified for blue crab pathogens, however cannibalism is a likely mode of transmission. Similarly, the crab densities required for epizootics of any given pathogen are not known. Consequently for viral pathogens there is little understanding of when or if segments of the crab population are particularly vulnerable.

1.2.4 *Callinectes sapidus* Reo-like Virus (RLV)

RLV was initially described by Johnson and Bodammer (1975) in juvenile crabs experiencing high mortality under laboratory conditions. This virus has been repeatedly identified when crabs are held in captivity, particularly if they are housed at high density or for a period of weeks (Johnson and Bodammer 1975, Bowers et al. 2010). In particular Bowers et al. (2010) identified RLV in dead or dying crabs from a shedding system, while the virus was not detected in any outwardly healthy animals from the same facility. RLV infects juvenile and adult crabs; it is unclear if it can infect larval stages though preliminary evidence suggests it may not (Schott personal communication). Prior to the research here, RLV had been identified in Chincoteague Bay, Virginia, and the Chesapeake Bay (Johnson 1983). Other studies have identified RLV by histology and

electron microscopy, gel electrophoresis on total RNA, RT-PCR, and *in situ* hybridization (Johnson 1977, Johnson 1983, Bowers et al. 2010, Tang et al. 2011).

After the initial identification of RLV, it was subsequently classified as a reovirus based on the icosahedral, non-enveloped capsid of 55 – 60 nm in diameter, and RNA genome (Johnson 1977). The RLV genome consists of 12 segments of, totaling approximately 24 kb in length (Bowers et al. 2010). RLV creates cytoplasmic inclusions, typical of reovirus replication, with paracrystaline arrays of virions seen in advanced infections. Crabs infected with RLV become anorexic, lethargic, lose muscle tone, and have trembling periopods, however these symptoms only appear when the crab is nearing death (Johnson 1977, Bowers et al. 2010, personal observation). Lethargy and limited feeding may begin days before death, however weakness and trembling occurs when crabs are moribund. None of these symptoms are reliable diagnostics for RLV. RLV may be transmitted by injection, feeding infected tissue, and cohabitation (Johnson 1983, Bowers et al. 2010, Schott and Diamante personal communication). Time to death after injection is reliably two to four weeks (Bowers et al. 2010). Injection commonly results in a 100% transmission rate, and PCR-confirmed infections invariably result in death for both laboratory transmission and infections in wild-caught crabs (personal observation).

A closely related virus is *Scylla serrata* reovirus (SsRV or MCRV). Unlike the blue crab, the mud crab *Scylla serrata* is intensively cultured in China. SsRV was identified as the cause of “sleeping disease” in cultured crabs (Weng et al. 2007). Similar to RLV, SsRV causes lethargy, loss of appetite, and death, and infects connective tissues causing necrosis (Weng et al. 2007). RNA viruses have relatively high mutation rates on the order of 10^{-3} to 10^{-5} per nucleotide per replication, which results in 0.1 to 10 mutations per 10

kb (reviewed Domingo and Holland 1997). Despite the high variability in reovirus genomes, SsRV has >70% nucleotide identity with the portions of the RLV genome sequenced by Bowers et al. (2010) and Tang et al. (2011). Genome sequence indicates that SsRV is not closely related to *Eriocheir sinensis* reovirus, the type member of the Cardoreoviruses (Deng et al. 2012). Nor is SsRV closely related to Aquareoviruses based on genome sequence and smooth inner capsid morphology (Deng et al. 2012, Huang et al. 2012). A new genus has been proposed for SsRV (Deng et al. 2012). In total, published genome sequence and capsid morphology suggest RLV and SsRV both belong in a previously unrecognized genus of the Sedoreovirinae.

SsRV has primarily been described in mud crab aquaculture. Blue crabs are not currently cultured in North America, but soft shell shedding approximates many aspects of aquaculture on a shorter time scale. Shedding systems and aquaculture also have the potential to concentrate pathogens and return them to wild populations (e.g. Morton 2004). RLV has been studied in terms of shedding mortalities (Bowers et al. 2010), but not in terms of wild prevalence or pathogen range. For these reasons, the research here seeks to characterize the range of RLV within the northern US range of the blue crab, and to assess prevalence of the virus in relation to flow-through shedding systems in the Chesapeake Bay. This information is part of the groundwork for understanding how RLV interacts with the blue crab population. The Chesapeake Bay is an important system to evaluate as it is the site of the largest blue crab fishery in the US, and a region that generally has upwards of 70 million spawning females (MD DNR Winter Dredge data). Precise measures of prevalence can be challenging to obtain for wild populations but are needed in order to collect precise background data.

The following chapters address optimizing a quantitative detection method for RLV and studies of RLV range and prevalence. A sensitive, quantitative assay for RLV was desirable before beginning environmental studies of range or prevalence. Consequently, an RT-qPCR assay for RLV was developed. This assay was used to test for RLV in crabs from multiple locations along the Atlantic coast of the Americas. RLV from multiple locations was used for a preliminary assessment of genetic diversity in this virus. Within the Chesapeake Bay, the RT-qPCR assay was used to assess RLV prevalence in relation to shedding systems. This was also the first study to test for correlations between RLV infection and crab characteristics.

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Chapter 2: Development and validation of an RT-qPCR Assay for RLV

2.1 Importance of Quantitative RLV Detection

Prior to this research, RLV has been detected by histology paired with electron microscopy, RNA extraction and gel electrophoresis, and end-point RT-PCR (Johnson 1977, Messick et al. 1998, Bowers et al 2010). The end-point PCR assay for RLV is more sensitive than histology and gel electrophoresis on RNA extracts (Bowers et al. 2010). RLV prevalence as assessed by histology was low, to the point that the pathogen might have been considered rare. When examined by dsRNA methods, RLV prevalence was recognized to be substantially higher, particularly in soft crab shedding systems and when measured by RT-PCR the prevalence was higher still (Bowers et al. 2010, Schott and Messick unpublished). The greater sensitivity and precise quantification of viral loads are needed to address prevalence, tracking disease progression in individuals, and move toward an understanding of RLV epidemiology. Quantitative PCR (qPCR) is well established as a means of detecting and quantifying pathogens. It has repeatedly been used for sensitive detection of pathogens in water (Girones et al. 2010). In economically valuable marine invertebrates, qPCR has been used to detect and exclude viruses from farmed stock (e.g. Withyachumnarnkul 1999), as well as for environmental assessment of host range and prevalence (Bain et al. 2010).

Here we develop and validate a one-step, reverse-transcription, quantitative PCR (RT-qPCR) method for RLV detection. Sensitivity, reproducibility, and potential for contamination prior to PCR are all considered. Notably as sensitivity increases, the need to consider sources of contamination also increases, and the potential for contamination is

sometimes underappreciated. We evaluated contamination, and the potential for contamination informed the treatment of data in the subsequent chapter of this thesis. The sensitivity of the RT-qPCR assay is valuable for generating a more precise assessment of RLV prevalence than was previously possible. In addition, viral quantification allows the first testing for correlations between RLV viral load and host characteristics. This assay has been used in prevalence and range studies, as well as for tracking disease progression in laboratory studies. It also makes possible the crucial task of biosecurity screening of broodstock crabs destined for hatcheries and aquaculture.

Quantitative detection of RLV allowed identification of virus outbreaks and isolated infections across a wide geographic range from Massachusetts to Maryland, as well as off the east coast of Florida and southern coast of Brazil. Outbreaks were identified in a coastal bay of Long Island, NY, and the middle Chesapeake Bay. A portion of the RLV genome was sequenced from infected crabs representing these areas. Pairwise sequence comparisons permitted a preliminary assessment of the robustness of the RT-qPCR primers used, and an intriguing glimpse of the genetic diversity of RLV across its geographic range.

2.2. RLV RT-qPCR Assay Development

2.2.1 Considerations for qPCR

Quantitative PCR assays may follow the classic three-step cycle of primer annealing, extension, and melting, or adopt a combined annealing and extension step at a single temperature. qPCR requires a shorter amplicon than end-point PCR, approximately 75 – 150 bp in length. Both the primers and total amplicon should ideally have 50 – 60% GC

content (Taylor et al. 2010). qPCR assays can be characterized based on specificity, sensitivity, and reproducibility. Sensitivity and reproducibility are both quantifiable using data from the assay itself. Conducting qPCR on a dilution series of the target sequence can be used to calculate amplification efficiency and determine the lower limit of detection. Efficiency and detection limit can then be compared over multiple PCR runs to assess reproducibility.

Specificity of the assay pertains to two issues: false positives due to non-specific primers, and false negatives due to failure of the assay or variability in the target primer sites. Both were considered in the development of this assay. False positives should be considered during primer design. Amplification of the desired target can be verified by the product melting temperature or use of a specific probe (reviewed Smith and Osborn 2009, Zipper et al. 2004, Wittwer et al. 1997). Melting temperature depends on amplicon length, GC content, and salt concentration in the qPCR reaction mixture. Consequently melting temperature can identify secondary products (primer dimers or additional products due to non-specific priming), once the melting temperature for a desired product has been identified. False negatives may result from failure to amplify, which can occur for multiple reasons. Ethanol, isopropanol, and phenol can inhibit PCR, as well as endogenous inhibitory compounds can co-purify with RNA can inhibit PCR (Gallup 2011, Schultz et al. 2006). Another notable concern is variability of the primer sites. In well understood genomes and highly conserved genes this concern can be minimized. The RLV genome has not been published and viral RNA genomes have the potential to be highly variable (Domingo and Holland 1997). Therefore, RLV prevalence determined by PCR should be regarded as potentially conservative.

Quantifying pathogens with qPCR is accomplished by comparing an unknown sample to a standard. The standard may be nucleic acid directly from the pathogen of interest, or a cloned portion of that genome with the target sequence present. During amplification cycles, DNA product quantity is measured by a reporter dye that fluoresces in proportion to the amount of product present. A dilution series of known concentrations of the target DNA sequence can then be used as a reference for quantifying unknowns and determining assay efficiency by setting a detection threshold. The PCR cycle at which a sample reaches the detection threshold is termed the threshold cycle (Ct), and samples with a higher number of target sequences at the start will have a lower Ct. In addition, under ideal conditions the quantity of the amplicon will double each cycle, and increase by a factor of ten every 3.32 cycles. The reliability and reproducibility of the RT-qPCR assay was evaluated using a ten-fold dilution series. Efficiency can be calculated based on a graph of the standard dilution series. Graphing $\log_{10}$ copy number versus Ct gives a slope that corresponds to the number of cycles between ten-fold dilutions. Thus under ideal conditions the slope will be -3.322. The actual slope reflects the proportion of PCR product that is doubled during each replication cycle, and can be converted to efficiency. In general, efficiency of 95% to 105% is desirable for qPCR.

2.2.2 Primer Selection

Multiple primer sets were developed and tested for the qPCR assay. The ninth genome segment of RLV had been cloned, sequenced, and used in an end-point RT-PCR assay (Bowers et al. 2010). Consequently this segment was targeted for the qPCR assay using the cloned DNA target. The primer sets that were tested (Table 2.1) targeted amplicons of

125 – 200 bp in length. In addition primer set 002f and 1211r was designed to amplify the full length of the known sequence for RLV segment nine.

Primers that produced an amplicon via end-point PCR were then tested for qPCR performance on a DNA target, including background fluorescence in negative controls, and the presence of secondary products. Primer set 002f and 1211r failed to amplify. Primer sets 138f and 262r, as well as Set3 Fwd and Set1 Rev, produced amplicons but had higher background fluorescence than primer Set1 Fwd and Set1 Rev. Set 1 primers were ultimately selected for optimization in the qPCR assay. This primer set was tested at four concentrations: 500 nM, 200 nM, 100 nM, 50 nM. The 500 nM primer concentration was most sensitive and sensitivity was progressively lost as primer concentration decreased. Reactions with 200 nM primers amplified on average 1.5 cycles later than those with 500 nM primers. Reactions with 100 nM primers amplified on average 4.5 cycles later than reactions with 500 nM primers. At 50 nM primer concentrations amplification was not detected for multiple dilutions in the standard, and no comparison of efficiency was calculated.

Table 2.1. Potential qPCR primers for RLV detection. Primer sequence, salt adjusted melting temperature (Tm), GC content, and success producing an amplicon shown. Primer pairs are grouped within the table; primer Set3 Fwd was designed for use with Set1 Rev.

Name	Primer Sequence	Tm (°C)	GC%	Amplification
Set 1 Fwd	TGCGTTGGATGCGAAGTGACAAAG	65.2	50.0	Yes
Set1 Rev	GCGCCATACCGAGCAAGTTCAAAT	65.2	50.0	Yes
Set3 Fwd	AAGATGTCTCGCTCCTTGTCAGCA	65.2	50.0	Yes
138f	TAGCTACGGTGGGAGGAATG	60.5	55.0	Yes
262r	CTGAATGGCAAAGCACAGAA	56.4	45.0	Yes
002f	TCTTGAGGCCTAGATTCGTAG	59.5	47.6	No
1211r	TTACCTCATCTGTACCGTCTG	59.5	47.6	No

2.2.3 Standard Development

Absolute quantification with PCR requires a standard against which unknown samples can be compared; this standard is also used for developing and validating the assay. Here both dsRNA and plasmid DNA standards were used for assay development. Plasmid DNA with an insert from the ninth RLV segment was used to optimize qPCR cycling conditions independently of the reverse transcription step. Purified RLV genomic dsRNA was used for characterizing efficiency and reproducibility, as well as for routine use as a positive control for quantification in the assay. The DNA standard was prepared using the portion of the RLV genome cloned by Bowers et al. (2010). Plasmid was purified with a commercial kit (Zymo Plasmid MiniPrep Kit). The plasmid was then restricted with EagI which cut on either side of the insert to produce two linear segments, one consisting primarily of the RLV insert, and the other consisting of plasmid DNA. Restriction digest completion was confirmed by gel electrophoresis. NanoDrop quantification was used to

calculate copy number based on plasmid and insert size. To prepare the dsRNA standard, total RNA was extracted from a crab preserved at -80 °C and known to have a high viral load. From the total RNA, dsRNA was enriched using CF11 column chromatography (Castillo et al. 2011), followed by ethanol precipitation. Gel electrophoresis was used to confirm purity and integrity of the dsRNA genomic segments. NanoDrop quantification of dsRNA was used to calculate genome copy number based on an estimated genome size of 23.7 Kb (Bowers et al. 2010). For both the plasmid DNA and dsRNA standards, a dilution series ranging from 10^6 to 10^1 copies/µL was used. The dsRNA standard was diluted in 25 ng/µL carrier yeast tRNA.

2.2.4 RLV RT-qPCR Optimization

qPCR cycling conditions were optimized for efficiency and low background. qPCR optimization and RT-qPCR was conducted with TaqMan® Fast Virus 1-Step Master Mix with SYBR Green detector using a 7500 Real-Time PCR System (Life Technologies). Forward and reverse primers were at a final concentration of 0.5 µM. For this assay amplification is accomplished with a two-step cycle consisting of a melt step at 95 °C, followed by a single primer annealing and elongation temperature. Shorter cycling times and higher annealing temperature were found to decrease background without a measureable effect on efficiency. When using dsRNA we found that a heating and annealing step for template and primers, prior to reverse transcription, improved the sensitivity of the assay and decreased background fluorescence. After concluding the optimization above with standard 20 µL reaction, the assay was attempted with a 10 µL reaction volume. The assay was able to reliably detect 10 copies of template in both reaction volumes, and the smaller reaction volume was subsequently used.

Ultimately, the following procedure was adopted for the RLV RT-qPCR assay: dsRNA standard and RNA samples are prepared for heating by combining equal volumes of the template and a primer mixture, which contained the forward and reverse primers each at 5 µM. RNA with primers is heated to 95 °C for 5 minutes before addition to the qPCR mixture. 2 µL of the primer and template is added to create a 10 µL reaction with 1X TaqMan® Fast Virus 1-Step Master Mix and 1X SYBR Green. This commercial master mix allows reverse transcription and PCR cycling in the same tube. Reverse transcription is accomplished with a 5 minute 50 °C incubation. This is followed by 5 minutes at 95 °C to deactivate the reverse transcriptase and melt all secondary structures and double-stranded oligonucleotides. Amplification is achieved with a 10 second melt step at 95 °C followed by a 20 second annealing and elongation step at 61 °C, repeated a total of 35 times.

2.2.5 Characterization of the RLV RT-qPCR Assay

For the RLV assay, efficiency under ideal conditions was 100.8% for the dsRNA standard and 96.4% for the plasmid standard, calculated from the slopes in Figure 2.1. The difference in efficiency between the two standards may reflect bias in the Nanodrop quantification or purity of the dsRNA standard. Efficiency and sensitivity were also evaluated under typical use. For ten qPCR runs conducted between July and September of 2013, the mean slope was -3.44 with a standard deviation of 0.11. This is an average efficiency of 95.2% under typical use with the dsRNA standard.

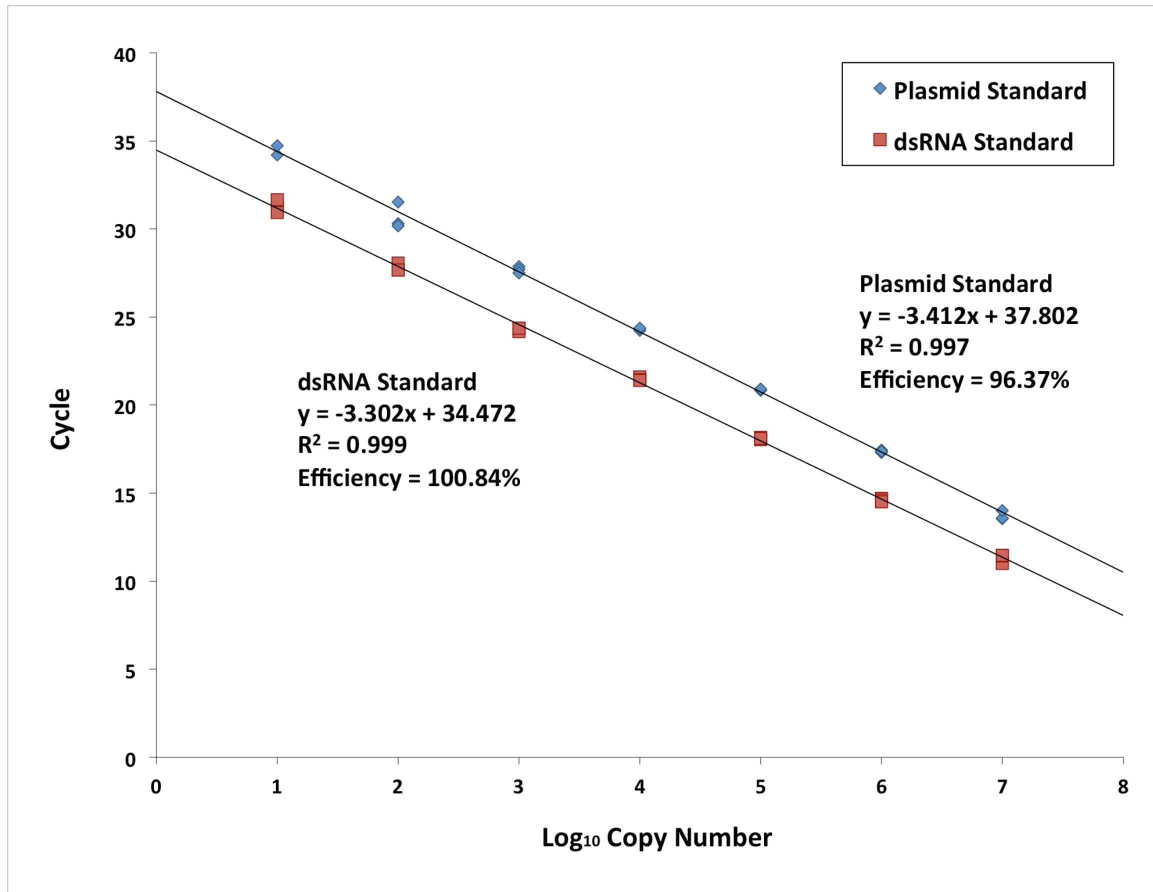

Figure 2.1. RLV qPCR efficiency. Comparison of efficiency for the RLV assay with a dsRNA standard and DNA standard. The threshold cycles for a $\log_{10}$ dilution series is used to assess efficiency relative to 100% efficiency at a slope of -3.32. Standards run in triplicate.

This RT-qPCR assay can reliably detect 10 target sequence or less. Assuming each target represents a complete RLV genome, this is less than a femtogram of nucleic acid. Given the sensitivity of this assay, contamination was a concern, particularly for samples that were dissected prior to routine detection by RT-qPCR. Dissections were performed with single-use autoclaved wooden instruments and razor blades. Before each dissection the

bench and the crab's carapace were cleaned with ELIMINase™. In the hands of two researchers experienced with these techniques, dissection of a crab containing 10^9 viral copies per mg of tissue yielded contamination on the order of 10^2 copies per mg in the subsequent dissection, and no contamination in the second dissection following the highly infected animal. Consequently for dissected samples, a conservative threshold of 10^3 copies per mg was used for assigning which animals were RLV positive. The need to account for dissection-related contamination does not negate the utility of a highly-sensitive technique, such as this assay. Although a relatively high threshold was set for RNA extracted from dissected leg muscle, RNA extracted from hemolymph withdrawn by single-use syringes will allow detection with a far lower threshold as there is far less potential for samples to come in contact with fluids or surfaces that the prior sample contacted.

2.3 RT-PCR Product Sequencing

2.3.1 Sample Selection and Analysis

The RT-qPCR assay was used in two studies that allowed a preliminary look at RLV genetic diversity. The study outline in the Chapter 3 analyzed crabs collected from the Chesapeake Bay during the summers of 2012 and 2013. Concurrently, a study of the RLV range in the northeast US, and opportunistic sampling in the southern US and Brazil allowed sequencing of RLV infections from a broad geographic range (Table 2.2). When sequencing candidates were identified, amplification was performed with the primers described by Bowers et al. (2010), which includes the portion of the genome amplified by the RT-qPCR primers. Two outbreaks (prevalence greater than 13.5%) were identified in the summer of 2012, one off Crisfield in the Maryland portion of the Chesapeake Bay,

and the other in Georgica Pond, a coastal inlet on Long Island, New York. A total of 22 RLV infections were chosen for sequencing.

Sequences were assessed by a pairwise comparison of nucleotide and amino acid percent identity and dN/dS ratio. The dN/dS ratio assigns each nucleotide position in the alignment to two bins. The first, dN, is the rate of non-synonymous changes, or nucleotide changes that alter the amino acid produced, relative to the number of possible non-synonymous substitutions in the alignment. The second bin, dS rate, is similarly the rate of synonymous changes relative to synonymous sites. A multiple sequence alignment was generated, trimmed, and used to assess number of segregating sites, total polymorphisms, and percent identities (CLC Genomics Workbench 7.0.4). Here, pairwise dN and dS values were estimated with the Yang and Nielson (2000) method in PAMLx (Xu and Yang 2013). A dN/dS ratio of one indicates changes are neutral, while ratios greater than one and less than one indicate selection against amino acid changes and for amino acid changes, respectively. Consequently, a dN/dS ratio less than one can be interpreted as selection pressure maintaining the amino acid sequence.

Table 2.2. Samples used in PCR product sequencing. Sampling year, sampling region, and presence of an outbreak is given for each sample. For samples collected in an outbreak, the RLV prevalence from that sample is included.

ID	Sampling Year	Sampling Location	Outbreak Site
MA 1	2012	Agawam River, MA	No
MA 2	2013	Agawam River, MA	No
NY 3	2011	Georgica Pond, NY	No
NY 4	2012	Georgica Pond, NY	Yes (22% RLV)
NY 5	2012	Georgica Pond, NY	Yes (22% RLV)
NY 6	2012	Georgica Pond, NY	Yes (22% RLV)
NY 7	2012	Georgica Pond, NY	Yes (22% RLV)
NY 8	2012	Georgica Pond, NY	Yes (22% RLV)
NY 9	2012	Georgica Pond, NY	Yes (22% RLV)
MD 10	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 11	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 12	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 13	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 14	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 15	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 16	2012	Middle Chesapeake Bay, MD	Yes (61% RLV)
MD 17	2012	Middle Chesapeake Bay, MD	No
MD 18	2012	Middle Chesapeake Bay, MD	No
VA 19	2012	Wachapreague Bay, VA	No
VA 20	2009	Lower Chesapeake Bay, VA	No
FL 21	2012	East Coast of Florida	No
Br 22	2013	Southern Brazil	No

2.3.2 RLV Sequence Results

The 22 sequences were compared over a 387 bp region, including the binding sites for the Set1 primers used in the RT-qPCR assay. Seventeen segregating sites were identified; each had one unique SNP. Pairwise comparisons showed that all nucleotide sequences were more than 95% identical. Ten of the genotypes sequenced were identical to the sequence deposited in GenBank (GI: 327179100). These ten include five of the sequences from the outbreak in the middle Chesapeake Bay and four of the sequences from the outbreak in New York. The total number of polymorphisms came to an average of 2.9×10^{-3} changes per base.

Pairwise comparisons within and between outbreaks showed that nucleotide sequences were greater than 99% identical (Fig 2.2). Outside of the outbreaks three of the sequenced genotypes stood out: MD 17, VA 19, and Br 22. These had less than 99% identity with all other genotypes sequenced and were identified from the middle Chesapeake Bay (non-outbreak), Wachapreague Bay, and southern Brazil respectively. Looking at putative amino acid sequence, all genotypes sequenced were more than 95% identical (Fig 2.3). MD 17 and Br22 were still relatively dissimilar from other genotypes and each other at the amino acid level.

The mean dN and dS were calculated with all 22 sequences, and this gave a dN/dS ratio of 0.34. However, selecting for outbreak samples may artificially weight results with those genotypes. To reduce bias, within each outbreak identical sequences were reduced to one representative of that genotype. This resulted in 13 sequences with a dN/dS of 0.33. The genotypes that stood out in pairwise comparisons were also considered. VA 19 had a relatively lower dN/dS of 0.18, reflecting the conservation of amino acid sequence

relative to other North American strains that is visible in the pairwise comparison. MD 17 and Br 22 had dN/dS ratios of 0.51 and 0.63 respectively.

One segregating site was identified in each primer sequence; the primers were robust despite these single nucleotide polymorphisms (SNPs). At total of three SNPs were found in the primer regions over all 22 sequences. This is an average of 3.49×10^{-3} changes per base. Overall, the primer functioned despite individual SNPs in the binding site. It is probable that a SNP in the 3' base would not be tolerated. The number of changes in the primer regions was comparable to the overall proportion of bases with SNPs.

2.4 Discussion

The RT-qPCR assay developed is highly sensitive and reliable. To date this assay has been used to assess RLV range in the US, and prevalence within the Chesapeake Bay, as well as testing more than 200 broodstock brought to the UMCES-IMET blue crab hatchery (Schott and Zmora unpublished). In the future, RLV quantification may be used to understand the prevalence of the virus in key life stages or to characterize disease progression or transmission during laboratory studies. Use of this methodology has the potential to dramatically change our perception of how prevalent, or important, RLV is to the ecology of blue crabs. The method of sampling and ample processing must be considered when picking a qPCR threshold to identify RLV-positive animals. Sampling by hemolymph draw is a convenient and non-fatal method that has minimal potential for contamination and may make full use of the RT-qPCR assay's sensitivity.

A preliminary assessment of the genetic diversity of RLV segment 9 demonstrated that the RT-qPCR primers are robust even with some polymorphisms in the primer annealing site. In other viruses degenerate primers have been used to detect diverse environmental

genotypes, and that approach may be useful in future studies of RLV (e.g. Rojas et al. 1993). Almost half the genotypes sequenced were identical to the sequence deposited in GenBank, and these included multiple samples from the two identified outbreaks used in sampling. These samples may be representative of a particularly successful and persistent North American genotype. The sequences here suggest that genetic differences will be greater across larger geographic distances, with the potential for different genotypes, and phenotypes (amino acids sequences), to be present in North American versus South American RLV. dN/dS ratios less than one reflected the nucleotide and amino acid conservation visible in the pairwise comparisons of percent identity. This analysis considers only one segment of the RLV genome. Other gene products, or non-coding regions in the RLV genome may be subject to different selection pressure resulting in more or less sequence conservation. Consequently, the whole genome should be considered for any future studies that seek to identify viral population boundaries or differences in regional selection pressure on this virus.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
MA	1																					
	2	99.48																				
NY	3	99.48	99.48																			
	4	99.74	99.74	99.74																		
	5	99.74	99.74	99.74	100.00																	
	6	99.74	99.74	99.74	100.00	100.00																
	7	99.48	99.48	100.00	99.74	99.74	99.74															
	8	99.74	99.74	99.74	100.00	100.00	100.00	99.74														
	9	99.48	99.48	100.00	99.74	99.74	99.74	100.00	99.74													
MD	10	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74												
	11	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74	100.00											
	12	99.48	99.48	99.48	99.74	99.74	99.74	99.48	99.74	99.48	99.74	99.74										
	13	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74	100.00	100.00	99.74									
	14	99.48	99.48	99.48	99.74	99.74	99.74	99.48	99.74	99.48	99.74	99.74	100.00	99.74								
	15	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74	100.00	100.00	99.74	100.00	99.74							
	16	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74	100.00	100.00	99.74	100.00	99.74	100.00						
	17	97.93	97.93	97.93	98.19	98.19	98.19	97.93	98.19	97.93	98.19	98.19	98.45	98.19	98.45	98.19	98.19					
	18	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74	100.00	100.00	99.74	100.00	99.74	100.00	100.00	98.19				
VA	19	98.71	98.71	98.71	98.97	98.97	98.97	98.71	98.97	98.71	98.97	98.97	99.22	98.97	99.22	98.97	98.97	98.71	98.97			
	20	99.74	99.74	99.74	100.00	100.00	100.00	99.74	100.00	99.74	100.00	100.00	99.74	100.00	99.74	100.00	100.00	98.19	100.00	98.97		
FL	21	99.22	99.22	99.22	99.48	99.48	99.48	99.22	99.48	99.22	99.48	99.48	99.74	99.48	99.74	99.48	99.48	98.19	99.48	98.97	99.48	
Br	22	98.19	98.19	98.19	98.45	98.45	98.45	98.19	98.45	98.19	98.45	98.45	98.19	98.45	98.19	98.45	98.45	97.16	98.45	97.42	98.45	97.93

Figure 2.2. Nucleotide pairwise comparison. Percent identity along a 384 bp portion of the ninth segment of the RLV genome. Pairwise percent identities comparing genotypes within outbreaks are indicated by the bold black boxes. Pairwise percent identities comparing genotypes between the two outbreaks are highlighted by the orange box.

Region		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
MA	1																					
	2	100.00																				
NY	3	99.19	99.19																			
	4	100.00	100.00	99.19																		
	5	100.00	100.00	99.19	100.00																	
	6	100.00	100.00	99.19	100.00	100.00																
	7	99.19	99.19	100.00	99.19	99.19	99.19															
	8	100.00	100.00	99.19	100.00	100.00	100.00	99.19														
	9	99.19	99.19	100.00	99.19	99.19	99.19	100.00	99.19													
MD	10	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19												
	11	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00											
	12	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00										
	13	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00									
	14	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00	100.00								
	15	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00	100.00	100.00							
	16	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00	100.00	100.00	100.00						
	17	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75	96.75					
	18	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00	100.00	100.00	100.00	100.00	96.75				
VA	19	99.19	99.19	98.37	99.19	99.19	99.19	98.37	99.19	98.37	99.19	99.19	99.19	99.19	99.19	99.19	99.19	97.56	99.19			
	20	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00	100.00	100.00	100.00	100.00	96.75	100.00	99.19		
FL	21	100.00	100.00	99.19	100.00	100.00	100.00	99.19	100.00	99.19	100.00	100.00	100.00	100.00	100.00	100.00	100.00	96.75	100.00	99.19	100.00	
Br	22	96.75	96.75	95.93	96.75	96.75	96.75	95.93	96.75	95.93	96.75	96.75	96.75	96.75	96.75	96.75	96.75	95.12	96.75	95.93	96.75	96.75

Figure 2.3. Amino acid pairwise comparison. Percent identity along a 128 amino acid sequence inferred from the sequenced portion of the ninth segment of the RLV genome.

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Chapter 3: Prevalence of *Callinectes sapidus* reo-like virus (RLV) in relation to flow-through shedding in the Chesapeake Bay

3.1 Introduction

Understanding the ecology of marine crustacean diseases is important to understanding variability in abundance, and therefore management, of commercially exploited crustacean species (Stentiford et al. 2012). Marine diseases typically receive attention when mass mortalities or fishery impacts are observable, as in the case of white spot syndrome virus (WSSV) in shrimp, and bitter crab disease in both crabs and lobsters (Sánchez-Martínez 2007, Meyers et al. 1987, Field et al. 1992). However, even in the case of a visually observable disease, pathogen prevalence and disease-related mortality are challenging to quantify (Shields 2005). For marine species there may be the added challenge of infected individuals dying and thus disappearing from the population after death, resulting in unobserved outbreaks or seasonal trends in disease-related mortality. In stock assessments, the contribution of disease is not specifically measured, but is aggregated into "natural mortality", which describes all mortality that is not fishing mortality. The spread of an infectious disease partly depends on rate of contact and rate of transmission between infected and susceptible individuals (Reno 2011). This mean species with substantial fluctuations in population density are unlikely to sustain an equilibrium with a pathogen, and may be subject to epizootics or escape from that pathogen. Similarly, a pathogen may disrupt otherwise predictable population variation, as in *Homarus americanus* where shell disease can disrupt the spawner-to-recruit relationship (Wahle et al. 2009). Diseases are often recognized as a factor in the decline

of populations (Ward and Lafferty 2004), but as Wahle et al. (2009) demonstrated, pathogens also play a role in the dynamics of exploited species, as a mortality source other than fishing.

Callinectes sapidus, the blue crab, is an ecologically important species in estuarine and coastal habitats along the Western Atlantic. The largest North American blue crab fishery is in the Chesapeake Bay, and this population experiences substantial inter-annual variation in abundance (Miller et al. 2011, NOAA NMFS, MD DNR). Disease ecology has been recognized as a research priority for this population (Chesapeake Bay Stock Assessment Committee 2014). For many blue crab diseases, such basic information as seasonality or factors influencing outbreaks may be lacking. At least three viruses that infect blue crabs are fatal: reo-like virus (RLV), Chesapeake Bay virus (CBV), and bifacies virus (BFV) (Bowers et al. 2010, Johnson 1977, Johnson 1983). However, there is limited information on the impact of these and other viral diseases on the crab population and fisheries (Johnson 1978, Shields 2003, Shields and Overstreet 2007). The Chesapeake Bay crab population undergoes considerable inter-annual variation, with varying estimates of natural mortality (Hewitt et al. 2007). It has been recognized there is a need for more research on the contribution of disease-related mortality to fluctuations in abundance of Chesapeake Bay blue crabs (CBSAC 2014).

The *C. sapidus* reo-like virus (hereafter RLV) is found throughout the North American range of the blue crab, including the largest North American blue crab fisheries along the Chesapeake Bay and Louisiana coast (Flowers and Schott unpublished, Rogers 2014). RLV is most closely related to the *Scylla serrata* reovirus (SsRV/MCRV) (Bowers et al. 2010). *S. serrata* is an important aquaculture species in Asia, and SsRV has received

attention for causing mass mortalities in aquaculture (Weng et al 2007). Unlike *S. serrata*, *C. sapidus* hard crabs are not produced in commercial aquaculture. However soft-shell production is a form of short-term aquaculture that is practiced throughout the blue crab North American range. RLV is found in more than 50% of blue crabs that die in soft-shell shedding systems, where high densities and additional stressors may render crabs more susceptible to disease (Bowers et al. 2010, Ary and Poirrier 1989). Similarly, in experimental aquaculture conditions RLV-infected crabs died in a matter of weeks (Bowers et al. 2010). Shedding systems may amplify virus and return it to wild populations via effluent and discarded mortalities. As in other crustacean viruses (e.g., shrimp white spot syndrome virus, Soto and Lotz 2001), RLV may be transmitted by scavenging and cannibalism, and waterborne transmission is probable. Transmission during cohabitation, without cannibalism, appears possible (Johnson 1977, Schott unpublished). The potential for shedding systems to affect the prevalence of RLV in nearby wild blue crabs motivated the current study.

This study provides a critical baseline for RLV prevalence in the Chesapeake Bay. Prior surveys indicated that RLV prevalence may be highly variable on both large and small spatial scales (Flowers and Schott unpublished, Messick 1998). Given the apparent variability of RLV prevalence, here we evaluate flow-through shedding as a specific activity of concern. We also consider seasonal trends in RLV prevalence during the fishing season, and the potential for RLV to disproportionately impact a particular sex or life stage. Crabs were collected by fishery-independent sampling at field sites near active shedding operations, and collaboration with soft crab producers allowed us to confirm the presence of RLV in flow-through systems adjacent to sampling locations.

3.2 Methods

3.2.1 Crab Sampling and Environmental Data

Blue crabs were sampled from the Maryland portion of the Chesapeake Bay between June and August of 2012 and 2013. The waterways off Deal Island and Crisfield, Maryland, were selected because they were impacted both by large flow-through shedding operations, and were adjacent to and a nearby pristine watersheds. The Rhode River was selected as a site with a historical dataset for crab abundance and environmental studies (e.g. Hines et al. 1987) but no active flow-through crab shedding. At both Crisfield and Deal Island, crabs were sampled at locations identified as near to and far from shedding sites. Near sites were immediately adjacent (less than 200 m) to shedding sites and effluent. Far sites were approximately 2 km away and adjacent to undeveloped land without crab shedding operations (Fig. 3.1). Sampling areas are as follows: Deal Island near (38.169 N, -75.947 W); Deal Island far (38.146 N, -75.910 W); Crisfield near (37.981 N, -75.861 W); Crisfield far (37.973 N, -75.875 W); Rhode River (38.871 N, -76.514 W). Crisfield and Deal Island were sampled on June 13 and August 28 in 2012 (n = 40); as well as June 21, July 9, July 23, and August 6 in 2013 (n = 25). Rhode River was sampled on June 27 and August 1 in 2012 (n = 40); as well as June 26, July 15, July 24, July 30, and August 9 in 2013 (n < 25). In the Rhode River during summer 2013 crab abundance was low and it was not possible to collect 25 crabs per sampling event.

Crabs were sampled by otter trawl with 5 - 10 minute tow duration. Tow times, catch per unit effort, and water quality parameters were recorded for each trawl site. For each crab, carapace width measured spine-to-spine, sex, maturity status for females, and prior injury

status were recorded. Because crabs are sometimes injured by the trawl or during sorting, presence of a prior injury was categorized as regrowth or absence of an open wound for injuries or lost pereopods. On sampling trips after June 2012, molt stage was also recorded. For crabs greater than 90 mm carapace width, tissue samples were collected by non-fatal leg autotomy. Smaller crabs were collected whole. Crab samples were individually bagged and immediately placed on ice for transport. Samples were stored at -20 °C pending RNA extraction and RT-qPCR.

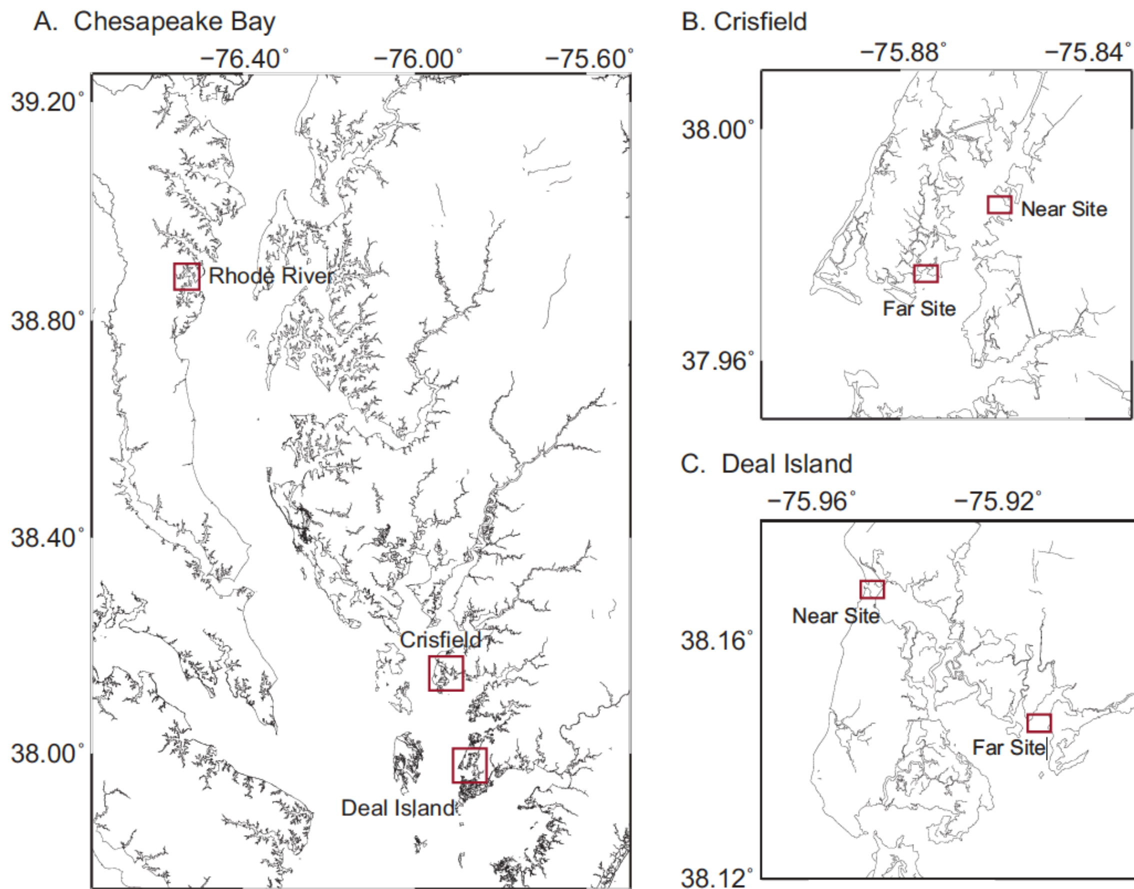

Figure 3.1. Maps of sampling locations. A. The Chesapeake Bay with the three sampling regions, Rhode River, Crisfield, and Deal Island. B. Close views of Crisfield and C. Deal Island with the near and far sampling sites.

3.2.2 Virus Detection

RNA extraction and RT-qPCR were conducted as described in the preceding chapter. Briefly, dissections were conducted with single-use, sterile implements and the dissection area and crab carapace were cleaned with ELIMINase™ prior to each dissection. Tissue was homogenized in 1.0 mL of cold TRIzol, using a Savant FastPrep™ FP120 homogenizer. RNA extraction was performed on approximately 50 mg of leg muscle, per TRIzol® specification. Resulting RNA pellets were dissolved in 50 µL of nuclease-free water and stored at -80 °C.

Prior to RT-qPCR, template and primers were annealed by heating at 95 °C for 5 minutes followed by cooling on ice. RT-qPCR was conducted using TaqMan® Fast Virus 1-Step Master Mix in 10 µL reactions. Primers described in the previous chapter were used at a final reaction concentration of 0.5 µM. Standard curves were constructued using purified dsRNA virus genome from an RLV infected crab, serially diluted in 25 ng/µL carrier yeast tRNA (six ten-fold dilutions, 10^6 to 10^1 RLV genome copies per µL). Amplicon quality was assessed by melting point analysis. As per the discussion in chapter 2, RLV quantification was achieved by comparison to a standard curve run with each plate (10^2 to 10^6 copies).

3.2.3 Statistical Analysis

All statistical tests were performed with R (R Core Team 2013). Differences in prevalence were assessed by Chi-square tests for independence or Fisher's exact test for small samples. A sampling site was characterized as experiencing an outbreak if RLV prevalence for that date was significantly higher than the mean prevalence for all crabs

combined (Chi-square, $p < 0.05$). Continuous variables were tested for normality using an Anderson-Darling test. Carapace width was not normally distributed ($p < 2.2e-16$). Viral load was corrected by subtracting 1000 and setting all negative values to zero. This gives a conservative assessment of prevalence and removes potential positives due to crab-to-crab contamination. Viral load was then $\log_{10}$ transformed, but was not normally distributed after transformation ($p < 0.0001$). Consequently, the nonparametric Wilcoxon rank sum test with continuity correction was used for analysis of continuous variables.

3.3 Results

During the summers of 2012 and 2013, a total of 898 crabs were sampled from five sampling sites (Fig. 3.1) and assessed for RLV viral load. 406 crabs were sampled in 2012 (n = 40 per sample), and 492 crabs were sampled in 2013 (n = 25 per sample when possible). A summary of crab characteristics is provided in Table 3.1. Carapace width ranged from 10 to 182 mm, with a mean size of 80.9 mm. There was no significant difference in mean size for 2012 and 2013 (Table 3.2., $p = 0.17$). Male crabs comprised 60.9% of the total, and the ratio of male to female crabs was not different between the two sampling years ($p = 0.98$).

Overall RLV prevalence was 13.5%, but there was significant inter-annual variation in prevalence. In 2012, 22% of crabs were infected with RLV (n = 406); in 2013, 5.9% of crabs were infected (n = 492). After correcting for possible contamination, viral load in infected crabs ranged from $10^{1.3}$ to $10^{9.8}$ target sequences per mg of muscle tissue, with a mean viral load of $10^{4.7}$ mg^{-1}.

Table 3.1. Summary of crab metrics. Percent of total crabs and percent RLV infected by category. There were no significant differences in prevalence.

	Category	Number	Percent of Total	Percent RLV+ by Category
Sex	Male	544	60.9	14.7
	Female	350	39.1	11.7
Molt Stage	Intermolt	184	20.6	7.6
	Premolt	388	43.4	9.3
	Postmolt	102	11.4	4.9
	Not Categorized	221	24.7	29.9
Injury Status	Uninjured	734	81.7	13.1
	Prior Injury	164	18.3	15.2

Table 3.2. Crabs grouped by infection status and year. Mean and range of carapace width (CW) given. * indicates mean CW was significantly different between RLV infected and non-infected crabs.

	Number	Mean CW (mm)	CW Range (mm)
All Crabs	898	80.9	10 – 182
All RLV +	121	88.1 *	15 – 151
All RLV-	777	79.8 *	10 – 182
2012	406	82.2	15 – 182
2013	492	79.7	10 – 172

3.3.1 Proximity to Shedding Locations

Overall, RLV prevalence was significantly higher in crabs sampled adjacent to shedding systems (Chi-square, $p < 0.001$). 18.8% of crabs were RLV positive at near sites ($n = 362$), while 7.6% were RLV positive at far sites ($n = 367$). Total Rhode River prevance was 14.5% ($n = 169$), which was significantly lower than near sites and higher than far sites (Chi-square, $p < 0.05$). On all sampling dates where there was a significant difference between near and far sites, the near sites had higher RLV prevalence (four comparisons, Fisher's exact test $p < 0.05$). For the purposes of this study, an outbreak was defined as prevalence significantly above the overall mean prevalence of 13.5%. Three of the four instances in which there was a significant difference between near and far sites coincided with outbreaks at the near site. This occurred at Deal Island and Crisfield in June of 2012 and at Deal Island during August of 2013. Outbreaks were never observed at far sites (1 – 2 km from shedding activity), but were seen in both of the 2012 Rhode River samples.

In 2012 Crisfield and Deal Island near sites experienced outbreaks which subsided to lower levels of RLV eight weeks later. June 2012 RLV prevalence was high overall, with the greatest prevalence adjacent to flow-through shedding tanks (Fig. 3.2). A notable change was seen at the Crisfield near site, where prevalence dropped from 61% to 0% in the 8 weeks between sampling events (Fig. 3.2). Prevalence at the Deal Island near site was similarly high in June (45%) and significantly lower in August (20%). In 2012 prevalence at far sites did not change significantly. Consequently, in contrast to June, August did not have significant differences between near and far prevalence. The highest

prevalence recorded at a far site during this study (24%) occurred at Crisfield in June 2012; this was concurrent with the 61% prevalence observed at the Crisfield near site.

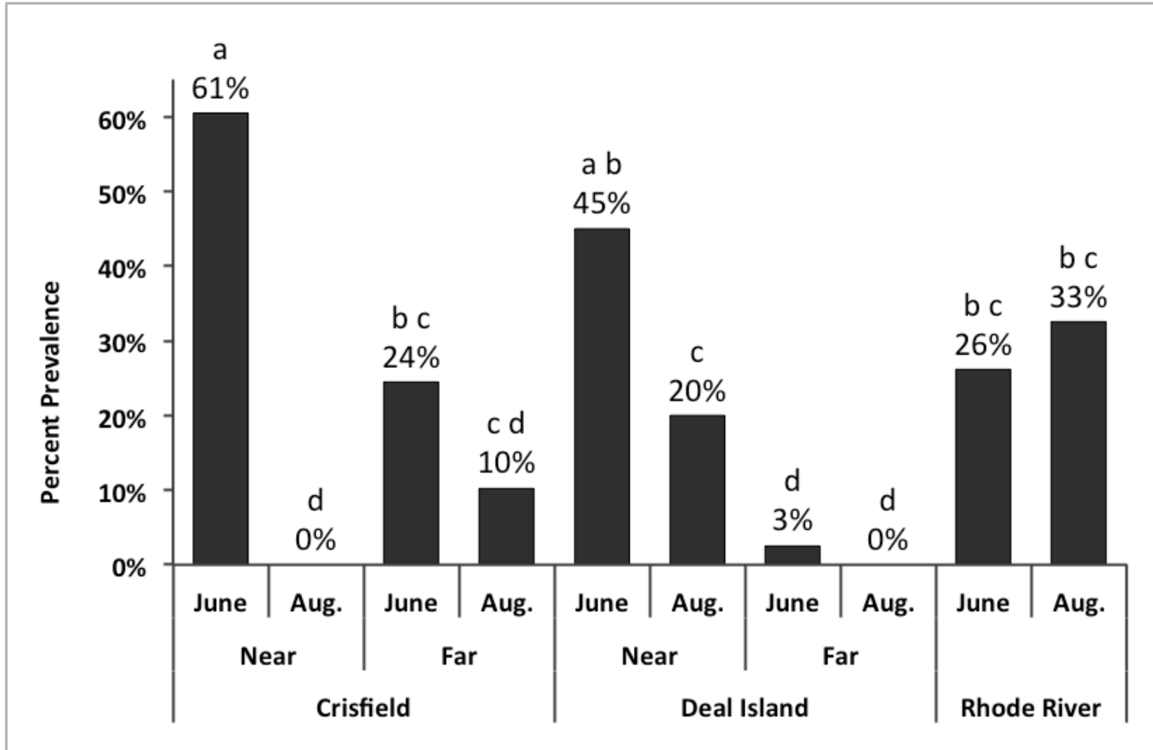

Figure 3.2. Reo-like virus prevalence in summer 2012. Crabs were sampled from three regions: Rhode River, and the waters off Crisfield and Deal Island, MD. Crisfield and Deal Island sites are designate as "near" (within 200 m of flow-through crab shedding), and "far" (1 – 2 km from shedding operations). Letters a - d refer to samples that are not significantly different by Fisher's exact test ($p > 0.05$).

In 2013 overall prevalence was lower, with smaller fluctuations in RLV between different sites and dates. At Crisfield, the near and far samples were not significantly different from each other on any sampling date. Similarly, at the Deal Island far site there was no significant difference between any of the sampling dates. Deal Island prevalence at the near site increased from early July to August. The August sample at the Deal Island near site did qualify as an outbreak with 32% prevalence, which emerged over the course of four weeks (Fig. 3.3). This was the sole instance in 2013 when a pair of near and far sites were significantly different, as well as the sole outbreak. In 2013 none of the crabs sampled from the Rhode River were RLV positive (n = 84), consequently Rhode River data are not shown in Fig. 3.3.

Confirmation the RLV was circulating in adjacent shedding facilities was obtained by testing mortalities provided by shedding operators. In 2012 shedding mortalities were provided from Crisfield. In 2013 mortalities were provided by a Deal Island watermen as well. Shedding mortalities from all dates were RLV positive, with prevalence ranging from 94% to 100% (n = 56 total shedding mortalities). RLV positive shedding mortalities had viral loads ranging from $10^{3.3}$ to $10^{9.4}$ mg^{-1} of leg muscle, with a mean of $10^{7.0}$ mg^{-1}.

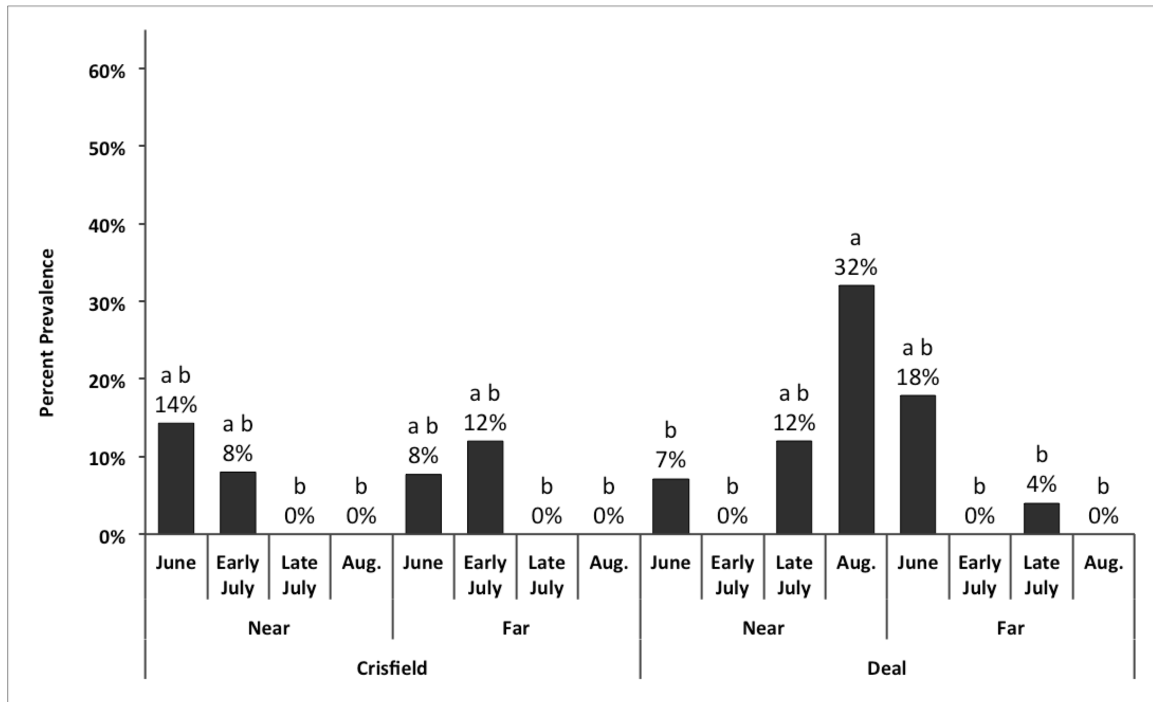

Figure 3.3. Reo-like virus prevalence in summer 2013. Crabs were sampled from three regions: Rhode River, and the waters off Crisfield and Deal Island, MD. Crisfield and Deal Island sites are designate as "near" (immediately adjacent to flow-through crab shedding), and "far" (1 – 2 km from shedding operations). Letters a and b refer to samples that are not significantly different by Fisher's exact test ($p > 0.05$).

3.3.2 Seasonality

In 2012, all three regions (Crisfield, Deal Island, and Rhode River) had prevalence categorized as an outbreak in at least one sample. Namely, the June samples at near sites, and both Rhode River samples experienced outbreaks (Fig. 3.2). Crabs from June 2012 ranged in size from 15 to 180 mm carapace width, and RLV was seen throughout the size range for both outbreak and non-outbreak sites (data not shown). Outbreaks at near sites

in June of 2012 had subsided to lower prevalence by August. Conversely, Rhode River had outbreak prevalence on both sampling dates in 2012, but it is unknown if this high prevalence was sustained between June and August. In 2013 only one outbreak, out of a total of twenty samples (5 sites, sampled on 4 dates) was observed, this was at the Deal Island near site in August. This outbreak appeared over the course of two to four weeks (Fig. 3.3). There was no significant difference in mean viral load ($p > 0.05$) for outbreak versus non-outbreak samples.

Prevalence was significantly higher in early summer for the total set ($p < 0.001$) (Fig. 3.3). However, when assessing seasonal prevalence by year, 2013 did not have significantly different prevalence between early and late summer ($p = 0.28$).

3.3.3 Crab Characteristics

Crab characteristics/metrics were considered for the entire data set. No significant difference in RLV status was found based on sex ($p = 0.2$); premolt, intermolt, or postmolt status ($p > 0.2$); or prior injury ($p = 0.5$). A total of 350 female crabs were sampled. Of these, 40 were mature, 39 were prepubertal, and the remaining 271 were immature; in females, RLV prevalence was not significantly different by maturity ($p > 0.1$). For all crabs there was a correlation between size (carapace width) and RLV prevalence. Crabs ranged in size from 10 mm to over 180 mm, and RLV positive individuals were found across this size range (Fig. 3.4). RLV positive crabs had a mean carapace with of 88.1 mm, while uninfected crabs had a mean size of 79.8 mm (Table 2). Carapace width was not normally distributed, so the difference in mean size was assessed by Wilcoxon rank sum test ($p = 0.009$). There was no significant difference in carapace

width for outbreak versus non-outbreak samples ($p = 0.051$). There was no significant difference in mean viral load based on injury status, or molt stage ($p > 0.05$).

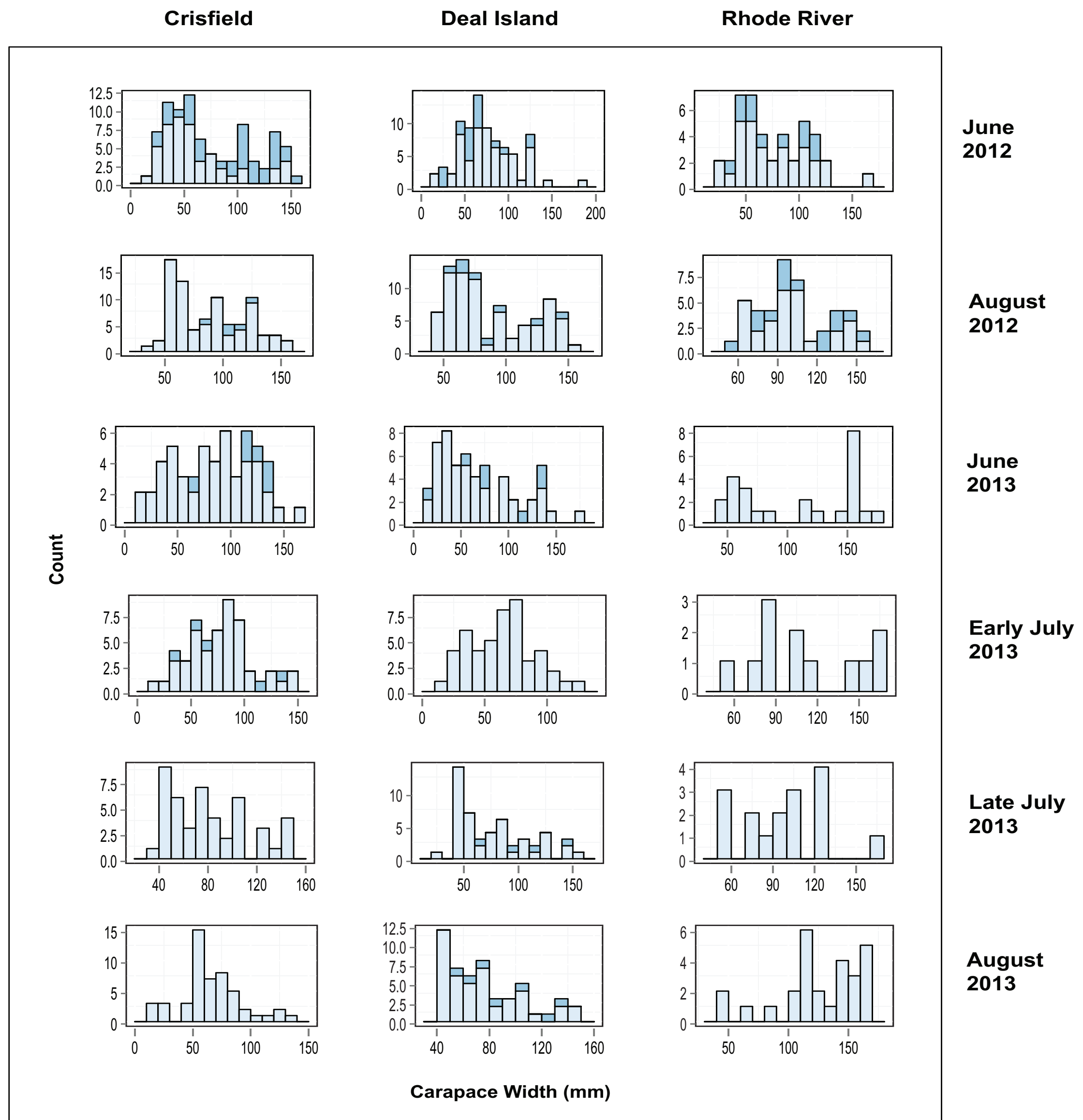

Figure 3.4. Histograms with infection status by region and date. RLV infected crabs represented by dark blue, uninfected crabs represented by light blue. Crabs are grouped by 10 mm increments of carapace width. Columns are regions from left to right: Crisfield, Deal Island, and Rhode River. Rows are sampling dates in chronological order from top to bottom.

3.4 Discussion

This study is the first to look at RLV prevalence at the same sites over a time series using scientific sampling methods. The results confirm initial indications that RLV prevalence is highly variable both spatially and temporally. This scale of outbreaks matches the described movement patterns of mature crabs in the Rhode River (Hines 2004). Previous assessments of RLV have recorded prevalence as high as 75% but have not included repeated sampling at the same location, sampling multiple times within the crab growing season, or assessment of possible fishery impacts on RLV prevalence (Flowers and Schott unpublished, Tang et al. 2011, Bowers et al. 2010, Messick 1998, Johnson 1977). In this study, RLV prevalence ranged from 0% to 61% with both extremes observed at the same site, eight weeks apart. High prevalence was associated with soft-crab shedding systems, but was not exclusive to shedding regions. Inter-annual variation in prevalence was substantial.

At regions with soft-shell shedding, Crisfield and Deal Island, all outbreaks observed were adjacent to flow-through shedding systems. In every case where there was a significant difference between near and far sites, the near site had higher prevalence. However, adjacent to shedding systems, prevalence was not consistently above the mean total or annual prevalence. Instead, near sites had disproportionately high prevalence, as compared to far sites, that was localized and temporally variable. Fluctuations in RLV prevalence cannot be explained by changes in the viral load in nearby shedding system, because at all dates 94 – 100% of dead peelers analyzed had high virus loads (on average 10^7 copies/mg tissue). The outbreaks in Rhode River during 2012, temporal variation in RLV prevalence, and continual presence of RLV in shedding systems, makes it unlikely

that effluent and water-born transmission are the primary drivers of RLV prevalence. Anecdotal information from fishermen and crab shedders reveals that oftentimes dead crabs from shedding tanks are discarded adjacent to docks or taken back to fishing grounds. Scavenging on RLV-infected mortalities is a possible mode of transmission and could contribute to the appearance of stochastic outbreaks, in keeping with the variability observed in this study.

Crab characteristics, including sex, maturity, molt stage, and prior injury, did not correlate with RLV prevalence. The exception was carapace width, which was on average larger for RLV infected crabs. This may reflect mixed populations and the inter-cohort cannibalism observed in blue crabs (Bunnell and Miller 2005, Moksnes et al. 1997). Size-dependent cannibalism is common in blue crabs (Mansour 1992, Moody 1994, reviewed Hines 1997). In a mixed population where larger adults have the opportunity to prey on juveniles, that predation presents an opportunity for transmission and removal of infected juveniles that could create higher prevalence in larger crabs. Alternately, prolonged infections with the crab surviving multiple molt cycles could contribute to higher prevalence in large crabs. This possibility is supported by the lack of significant difference in prevalence between premolt, intermolt, or postmolt crabs. Crabs in shedding systems with RLV infection are unlikely to survive a molt (Bowers et al. 2010), but this may be a result of stress from harvesting and captivity.

In this study, outbreaks occurred on the scale of only 1 – 2 kilometers and emerged over the course of a month or less. Outbreaks were found in all three regions sampled, in particular adjacent to shedding systems and in the Rhode River. Based on this, shedding practices may contribute to RLV prevalence, but flow-through shedding does not sustain

continuous high prevalence in adjacent crab populations. Environmental conditions or other stochastic events may influence the susceptibility of an individual or groups of crabs. Crab populations adjacent to shedding are likely at higher risk of an RLV outbreak than other populations with similar densities. Discarding dead crabs from shedding tanks could provide both a source of RLV and an incentive (scavenging) for crabs to stay at high density adjacent to shedding systems.

In addition to small-scale spatial and temporal variations in prevalence, inter-annual variation in RLV was substantial with a 16% (over three fold) difference between 2012 and 2013. The Chesapeake Bay blue crab population saw substantial inter-annual variation over the course of this study. In 2012 the MDNR Winter Dredge Survey found high densities of young of the year contributing to high overall crab densities. The 2012 dredge survey measured crab densities of 79.2 crabs per 1000 m^2, while 2013 had far lower crab densities of 31.8 crabs per 1000 m^2 (MD DNR). Based on the high crab density and high prevalence in 2012, followed by lower density and lower virus prevalence in 2013, density-dependent transmission should be considered as a factor in in Bay-wide RLV prevalence. Additional years of sampling are needed to test possible correlations between virus prevalence and Bay-wide crab abundance. It is similarly important sample over time series on additional years to evaluate seasonal trends in prevalence. While this data appears to support higher prevalence in early summer, this may be an artifact of the outbreaks at multiple sites in June 2012. In 2013 the sole outbreak was observed in August, highlighting the need for additional data to assess seasonality of RLV prevalence.

Targeted assessment of specific life stages should be considered in future assessments of RLV prevalence. The mean size of RLV infected crabs was larger, suggesting that RLV may disproportionately impact broodstock. Previous research (prior to molecular methods for RLV detection) found low RLV prevalence in overwintering Chesapeake Bay crabs, with RLV infection in just 0.3% of crabs (Messick 1998). However, the detection method used to assay overwintering crabs was far less sensitive than the methods in this study. Prevalence of RLV in overwintering crabs, and survival during overwintering with RLV should be considered. Smaller size classes should also be considered studied for possible density-dependent transmission in nursery habitat, which could subsequently transmit RLV to crabs to mature crabs. If crabs survive multiple molts while RLV infected, transmission between juveniles may disseminate RLV as they disperse throughout the Chesapeake Bay.

This and prior studies demonstrate that RLV can reliably be found in the Chesapeake Bay. Factors driving prevalence are not well understood, but may include crab densities, density and associated behavior of a particular year class, environmental stress altering susceptibility or duration of infection, and amplification or dissemination of RLV due to fishery activities. RLV and other pathogens may be a substantial and highly variable cause of natural mortality, which is not accounted for in current management strategies. RLV may have a profound effect on the blue crab population and fishery. It is therefore important to identify with greater precision the time from transmission to death with natural modes of transmission to better understand how quickly infected crabs are lost from the population and the fishery. It is similarly prudent to consider RLV transmission to newly recruiting year classes in the fall, as well as overwintering crabs or coastal crabs

as a source of RLV in the spring. While developing a more complete understanding of RLV transmission, some management changes may be implemented based on current knowledge. Land-based disposal of shedding mortalities is advisable in all instances.

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Chapter 4: Conclusions and Future Directions

4.1 Conclusions

The research here developed and applied new methods that substantially expand knowledge about *Callinectes sapidus* reolike virus (RLV). RLV has been identified in and around the Chesapeake Bay from the 1970s into the present (Johnson and Bodammer 1975, Johnson 1977, Messick 1998, Bowers et al. 2010). In this work a sensitive and quantitative detection method was validated, and that method was applied to a synoptic study in the Chesapeake Bay. This is the first time RLV prevalence has been assessed over a short time course and is a first step toward understanding the epizootic dynamics of this virus. Prior researched had indicated that RLV prevalence is variable over seasons and across regions of the Chesapeake Bay. Here for the first time it is possible to see the substantial changes in RLV prevalence occurring on a small scale of 1 - 2 km^2 and less than 20 days.

This assay has been used to verify that RLV extends throughout the northern range of the blue crab (Flowers and Schott unpublished), and is present in blue crabs off Florida and southern Brazil. In addition Rogers et al. (2014) detected RLV in the Gulf of Mexico, off Louisiana. RLV appears to be present throughout the North American range of the blue crab, and has been identified in two of the three largest US fisheries (Chesapeake Bay, and the Gulf of Mexico, the third largest fishery in North Carolina has not been extensively studied). Application of the qPCR method to other regions of the blue crab range revealed similar variability in RLV prevalence. Genetic characterization and comparison of genotypes of RLV “strains” from these different regions raises interesting

possibilities as to a mid-Atlanic or North America dominant strain as well as large-scale geographical variation.

Throughout the northeast range of blue crabs and in the Chesapeake Bay, RLV prevalence is highly variable, but outbreaks have been observed in multiple locations and during multiple years. Mortality rates and duration of RLV infections are unknown for wild blue crabs (and would be difficult to assess in the wild). However in laboratory studies, crabs determined to have RLV infections (experimentally or naturally acquired) die in two to eight weeks, depending on environmental conditions (Bowers et al. 2010, and unpublished data). Blue crabs do not have any identified adaptive immune system that would protect recovered individuals from reinfection and heavily infected crabs have never been observed to recover from RLV infections. Thus the current understanding of *C. sapidus* immune function and laboratory progression of RLV infection is in keeping with the observation from environmental data that outbreaks may be self-limited without substantial emigration. In fact, prevalence was also highly variable on a scale of one to two kilometers. RLV prevalence in the mid-Chesapeake Bay sites evaluated may correspond to patchy areas of foraging or meandering movement, with individual crabs occasionally moving between regions in a tributary. Apparently stochastic outbreaks may reflect the movement of one or a few infected individuals into an area with sufficient crab density to result in crab-crab transmission or environmental conditions that promote transmission or virus replication. Similarly, introduction of a dead, RLV infected crab could lead to an outbreak. Based on the correlation between shedding sites and RLV prevalence observed, particularly during the summer of 2013, disposal of shedding mortalities on land is advisable.

Within the Chesapeake Bay, and other coastal bays, there is no data available on patterns of RLV prevalence and spread. This research is the first to evaluate prevalence repeatedly at the same site within one season. Seasonal summer patterns were not readily apparent, but deserve further investigation. No correlation was found between RLV infection and the majority of crab metrics (sex, maturity, prior injury, molt stage). Future sampling should consider specific life stages that were not targeted here, especially early juveniles and overwintering females. The life history of the blue crab, particularly in the Chesapeake Bay, raises interesting questions about the effect of density or migration on pathogen transmission. The recruitment of megalopa/juveniles to nursery habitat suggests the possibility of semi-closed communities in which these new recruits are at high densities and could rapidly transmit a pathogen. This is a key life stage because these juveniles are both future breeding and fishery stock, and potential vectors for disease as they grow and migrate up the bay. On the other hand, overwintering mature crab, especially females, should be assessed for RLV. If mature females die due to pathogens during or shortly after overwintering, this is a potentially important loss of broodstock. Alternately, if crabs overwinter with RLV, at least in small numbers, this would explain how the virus reliably enters the Chesapeake Bay population each year.

In the future it will be valuable to understand how much natural mortality in blue crabs is attributable to pathogens, and to RLV specifically. It will also be valuable to understand how RLV behaves in different environments. Disease modeling can lead to a better understanding of the emergence and progression of outbreaks. At the same time complete genome sequencing, assessment of genetic diversity, and identification of different ecotypes will allow us to assess both viral population boundaries and whether rates and

models based on northeastern strains of this virus and it's host can be generalized to RLV dynamics in the Gulf of Mexico or South America.

4.2 Considering a Model of RLV Outbreaks

Development of mathematical models for the propagation of RLV from crab to crab would be a powerful and useful way to foster development of hypotheses about the effects of density, environmental factors, and the number of crabs lost to RLV. In any host-pathogen system there are factors that determine whether an infected individual leads to an outbreak or not. Development of a model for RLV epidemics may allow construction of hypotheses about the effect of density on virus spread and about situations in which there would either be an epizootic or extinction of the pathogen from a localized population. This would also also include hypotheses about, or estimates of, the number of individuals that die due to an outbreak in a given population. Terrestrial disease models, because they are the most advanced, can inform the investigation and modeling of marine diseases, but require careful consideration and adaptation to conditions that are particular to an aquatic environment.

4.2.1 Susceptible/Infected/Removed Models and RLV

A classic method for modeling communicable disease is the compartmental Susceptible / Infected / Recovered (SIR) model adapted by Kermack and McKendrick (1972). Discounting immigration and emigration (including recruitment of new generations) produces a model with a limited number of terms, which can be used as a starting point. In the simplest version of this model, individuals transition between susceptible (S), infected (I), and recovered (R) categories based on transmission rate as well as recovery

rate and/or death rate (duration of infection). Although the "R" class typically represents recovered and immune individuals, the model can instead denote "removed" individuals, meaning those that die from the pathogen. The system would likely be modeled with the "R" representing "removed," or the animals that die due to infection (Krkosek 2010). AN SI (Succeptible/Infected) model would similarly be useable for initial stages of modeling. However in future work it may be important to consider the number "removed" as a source of transmission via scavenging. We are then left with the highly simplified model in Figure 4.1 as a starting point. Individuals move between the SIR categories based on two rates: transmission rate and death rate once infected. This simplified set of variables is a valuable starting point for considering RLV epidemiology.

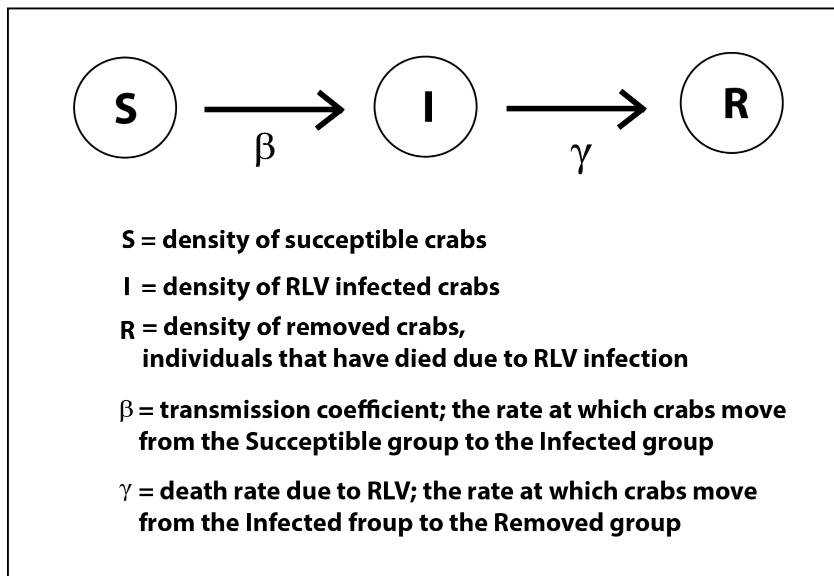

Figure 4.1. Potential RLV disease model adapted from Lloyd-Smith et al. (2005).

The model in Figure 4.1 is highly simplified both to make it useful as a starting point for RLV epidemiology and based on the known biology of this system. It assumes no births (recruitment), deaths unrelated to RLV, or population fluctuation due to immigration. This reflects a version of localized outbreaks in which time scale is short enough to allow us to disregard recruitment. This model also disregards other causes of natural mortality and fishing mortality. Ultimately a more complex approach may be needed to model localized outbreaks. Moreover, a spatially explicit model that accounts for immigration/emigration would likely be needed to depict RLV throughout the Chesapeake Bay, similar to modeling of *Hematodinium sp.* in South Carolina (Childress 2010). However, the SIR model above is useful in framing key considerations: the transmission coefficient(s) for RLV, and time to death for infected crabs.

4.2.2 RLV Transmission Coefficients

The transmission coefficient (β) depends on both the rate of contact and the probability of transmitting the pathogen. It may be tempting and seem intuitive to relate transmission and host density, however transmission may be modeled with many types of functions that depend on assumptions and the ecology of the system (McCallum 2001). Here I will briefly discuss the implications of the two simplest and most common ways to model transmission.

The SIR model is not spatially explicit and addresses density by increasing or decreasing number of individuals. In addition, the model assumes mass action, meaning that the hosts encounter each other in a random manner with roughly equivalent chance of any two individuals meeting. More recently a frequency-dependent SIR model has been proposed as a better fit for the assumption of contact described by mass action. This is

important because the density-dependent SIR model predicts thresholds for epidemics or removal of a pathogen from the population (McCallum et al. 2001, McCallum et al. 2004). These thresholds can be derived, and have been used to manage pathogens through culling or vaccination programs (McCallum 2001). Density thresholds are not implied by a frequency-dependent model in which the basic reproduction number of the pathogen depends on the transmission coefficient and rate of recovery, but not the total population density (McCallum 2001, Nelson et al. 2001). Any attempt to model RLV would need to consider both density- and frequency-dependent transmission and may ultimately need to consider more complex functions for transmission. In addition the assumption of mass action means SIR modeling would be appropriate primarily for smaller regions in which localized RLV outbreaks occur.

Practically speaking there are additional considerations for estimating transmission rate. As noted above, the transmission coefficient depends on both the rate of contact and the probability of transmitting the pathogen via a particular type of contact (e.g. cannibalism). Finkenstadt and Grenfell (2000) outline a method for determining the transmission coefficient from environmental prevalence data, however this method is extremely data intensive. For RLV, it would be more appropriate to estimate transmission through methods similar to those used by Soto and Lotz (2001) who employed a timed exposure to one infected individual or infected tissue to approximate the transmission. This method of ascertaining transmission rates allows controlled testing, but must be informed by information or contact rates or cannibalism in a complex environment. In a laboratory setting, transmission rates can either be assessed in studies specifically dedicated to this question, or derived from studies incorporating other aspects of disease

progression. The RT-qPCR assay for RLV would therefore be a powerful tool to investigate the effect of infectious dose on transmission rate.

4.2.3 Death Rate due to RLV

The second rate needed for a preliminary SIR model is the rate at which individuals leave the infected state. For blue crabs this is the rate of death due to infection. In the case of RLV, crabs are not known to survive infection, and decapods have no identified adaptive immunity. Duration of infection will be challenging to measure for wild crabs, and multiple approaches may be needed. A tag and recapture study would allow a direct measure of survival of naturally infected crabs in the wild. Tag and recapture studies have been used in blue crab to assess movement (e.g. Aguilar et al. 2004), and in other decapods to assess disease progression (e.g. *Homarus americanus* shell disease, reviewed Castro et al. 2006). Laboratory studies present the opportunity to test the impact of environmental conditions on duration of survival. Preliminary results indicate that increased temperature may lead to faster mortality. Salinity does not appear to affect time to death except at the low extreme of the blue crab's salinity range (Schott unpublished). A factorial study, for example, could be used to assess time to death with RLV infection with regard to initial viral dose and temperature.

4.3 The RLV Genome and Genetic Diversity

RLV has been identified in blue crabs throughout the US, and the southern tip of Brazil. RNA viruses, including reoviruses, are known to have high mutation rates (Domingo and Holland 1997). Within a single virus the high error rate during replication may give rise to a highly heterogeneous mix of genotypes in a viral population, often termed a viral

quasispecies (Domingo et al. 1998). Reovirus phylogenies may be challenging to construct and have a “starburst” pattern with polytomies (Nibert and Duncan 2013). Here we made a preliminary assessment of genetic diversity in Northeaster US RLV infections. Sequence identity was >99% within and between RLV outbreaks in Maryland and New York. High identity within outbreaks is consistent with clonal expansion of an infection, but the similarity between outbreaks indicated the potential for a highly successful genotype or ecotype that emerges when conditions are favorable. Both outbreaks were sampled in the summer of 2012. However, both share a comparable level of sequence identity with a sample collected in Virginia in 2009 (GI:327179100). This supports the possibility of a persistent dominant genotype or ecotype in the northeast. At the same time, virus samples with lower percent identities were identified in Virginia, Florida, and Brazil. This diversity may ultimately prove a more accurate reflection of the population diversity seen in the absence of an outbreak.

A study of RLV genetic diversity could address questions of viral population boundaries and ecotypes in the marine environment. However, a complete RLV genome is first needed as a reference for future genome assembly. The RLV genome has been estimated to be less than 24 Kb based on the electrophoretic pattern (Bowers et al. 2010), meaning that the genome is relatively small for modern assembly methods. The twelve segments of the RLV genome may either simplify or complicate genome assembly. The short segment lengths (< 5 Kb) mean contigs covering complete genome segments are likely to result from de novo assembly. RLV is most closely related to the *Scylla serrata* reovirus (SsRV) (Bowers et al. 2010). This may serve as a scaffold for RLV assembly, and be used to assess the length of gaps in any de novo assembly. Comparison between RLV and

SsRV, or other reoviruses, can give clues to genome segment function and allows hypotheses about conservation and selection pressure on specific segments.

Ultimately a completed RLV genome, and studies of RLV phylogeography can be used to address questions of reovirus evolution in the oceans and population boundaries of pathogen and host. The sequence analyzed here indicates that there may be a dominant genotype in the mid-Atlantic. There is not enough data at this point to indicate whether this genotype is present in the gulf and along South America. A broader assessment of RLV genetic diversity could begin to address whether the changes are due to genetic drift, or if true ecotypes can be identified for this virus.

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