ABSTRACT

Title of Thesis: MORTALITY AND REOVIRUS INFECTION IN SOFT-SHELL BLUE CRAB (CALLINECTES SAPIDUS) AQUACULTURE

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Soft-shell blue crab production the United States is an undervalued aquaculture practice experiencing high crab mortality rates from a series of stressors, including disease. The impact of one disease, the reovirus CsRV1, remains unquantified in major soft-shell crab production regions, despite the virus' known ubiquity and lethality. My research examined the mortality and CsRV1 infection rates of pre- and post-production crabs in Maryland, Virginia, and Louisiana soft-shell crab production facilities in 2016-2017, attempting to link these rates to water quality and aquaculture practice variables. I found that recirculating aquaculture systems lost half the proportion of crabs (16%) that flow-through systems did (33%). CsRV1 infection was the primary predictor of crab death in Chesapeake aquaculture, presenting in 75% of dead crabs compared to 22% of dead crabs in Louisiana aquaculture. Multi-state data suggests crab losses worth over $2 million are attributable to CsRV1, indicating a need for aquaculture effluent and discard control.

MORTALITY AND REOVIRUS INFECTION IN SOFT-SHELL BLUE CRAB (CALLINECTES SAPIDUS) AQUACULTURE

By

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Chapter 1: Introduction

Global Crustacean Fisheries Overview

Global crustacean fisheries and aquaculture make increasingly valuable contributions to the global economy and constitute 20 kg per capita annually in the diet of the world's growing human population as a major protein source (FAO 2018). While total capture from wild fisheries has not significantly increased since 1990, many crustacean fisheries have increased production during that time, including those for crabs (FAO 2018). Meanwhile, global aquaculture production has increased since 1990 to the point of overtaking total fisheries capture production, with crustacean species such as shrimp and crabs constituting important component fisheries (FAO 2016). Crab harvests reached record levels totaling >1.7 million metric tonnes (mt) in 2016 with a steadily increasing trend since the 1980's. Among many other species, the Asian blue crab species (*Portunus trituberculatus* and *P. pelagicus*, Wang et al. 2010), mud crab (*Scylla spp.*), and mitten crab (*Eriochier sinensis*, Wang et al. 2016) all represent regionally and globally important aquaculture species for human consumption (FAO 2016). Simultaneously, there is concern about the sustainability of crustacean stocks and fisheries given increasing fishing pressure (Worm et al. 2009), habitat degradation (Lotze et al. 2006), and disease-related mortality (Lafferty et al. 2004, Stentiford et al. 2012) in both wild capture fisheries and aquaculture.

Atlantic Blue Crabs

Geographical and Ecological Range

The Atlantic blue crab *Callinectes sapidus* is the target of one of top five largest wild capture crab fisheries in the world by volume (FAO 2016, 2018). Blue crabs prey and scavenge in western Atlantic, Gulf of Mexico, and Caribbean Sea coastal and estuarine environments from New England to Uruguay (Williams 1974, Millikin & Williams 1984). Increasingly frequent observations of *C. sapidus* in the Gulf of Maine and Nova Scotia have been attributed to climate shifts (Johnson 2015). Outside the American supercontinent, *C. sapidus* has become established as an invasive species supporting minor fisheries in the Mediterranean, Black, North, and Baltic Seas (Perdikaris et al. 2015). Across its life history, *C. sapidus* plays important food web roles as prey, predator, and scavenger, crossing and coupling the benthic and pelagic ecosystems where it exists and influencing populations of other commercially important marine species such as striped bass, red drum, and Atlantic croaker (Baird & Ulanowicz 1989, Miller 2001, Hines 2007).

Life History

Maturation

Blue crab life history stages are often defined by the 25-29 molting (ecdysal) stages of growing crabs. After eggs hatch in waters >22 ppt and 19-29°C (Sandoz & Rogers 1944, Kennedy & Cronin 2007), larval crabs (zoea) molt through seven pelagic planktonic stages, filter feeding in open ocean. Surviving zoea are transported by oceanic currents towards shallower, fresher coastal waters prior to the eighth molting stage, when they become megalopae and gain chelipeds that enable capture of larger prey (Kennedy

& Cronin 2007). Once in estuarine water, megalopae metamorphose into the first of 18-20 juvenile crab stages that are morphologically similar to adult crabs.

Sexual Dimorphism

Juvenile female crabs, colloquially known as "sallies," are morphologically distinguished from adults by their triangular abdominal apron. Adult female crabs, known as "sooks," possess a rounded dome-shaped apron that allows them to houses up to 2 million eggs in the sponge mass between this mature abdominal apron and rest of the body (Kennedy & Cronin 2007). By comparison, male crabs, known as "jimmies," possess an abdominal apron in the shape of a rounded "T" that cover the adult male gonopods used to deliver sperm to the newly molted females (Williams 1974, Kennedy & Cronin 2007).

There is also dimorphism in adult habitat distribution depending on time of year. Males typically reside in shallow, less saline waters except in the summer mating season, while females migrate toward estuary mouths to release hatched larvae from their egg mass. The maximum observed length between lateral spines is 9.1 inches (23 cm) for both males and females (Kennedy & Cronin 2007). Sexual maturity in both male and female crabs occurs at 12-18 months of age, when females generally reach 5-6 inches of carapace width and males 4.5-5.5 inches carapace width (Kennedy & Cronin 2007). Both sexes generally live for 2-3 years, although older crabs are sporadically observed and 8 years has been used as a maximum age in modelling (Rugolo et al. 1998, Bunnell & Miller 2005).

Molting

Ecdysis is essential for growth of crabs and the process plays a significant role in their ecology. Once crabs grow enough to require a new exoskeleton, the pre-ecdysal phase begins with the release of ecdysteroids from the Y-organ located behind crabs' eyestalk (Techa & Chung 2015). This triggers separation of the hypodermis from the old exoskeleton, release of enzymes to dissolve old exoskeletal components, and formation of the new exoskeleton from resorbed and absorbed inorganic salts (Kennedy & Cronin 2007). Crabs caught during this one-to-two week period are known colloquially as "peelers," which are identified by green hues on the dorsal carapace and white or red lines near dactyl edges of the swimmer leg (Oesterling 1995). After the new dermal layer has partly formed, crabs enter the ecdysal phase, where water is rapidly absorbed. Turgor pressure causes the old exoskeleton to split ventrally between the lateral spines, lending to the colloquial term for these crabs, "busters." Molting crabs then back out of the old shell and continue to absorb water, normally gaining 33% body mass per molt within 6 hours. Crab shells remain soft for 1-2 hours maximum after emergence from old shells, progressing to full hardness 96 hours after molting (Kennedy et al. 2007).

Throughout this entire process, crabs are in a physiologically stressed and highly vulnerable state (deFur et al. 1988; deFur 1990). Particularly as busters and soft crabs, crabs stop eating and moving, undergo dramatic changes in body chemistry, and cannot effectively defend themselves without functional, hard chelae (Ryer et al. 1997, Kennedy & Cronin 2007, Techa & Chung 2015). In nature, these crabs bury themselves in mud, sand, and other refuge found in shallow creeks with seagrass and marshland at low tide, where they are able to hide from predators and ensure a higher survival rate (Hines et al.

1987, Shirley et al. 1990, Ryer et al. 1997). Even so, mortality is elevated during this stage, although study of natural molting survival rates has been limited to tethering experiments (e.g. Ryer et al. 1997).

Blue Crab Fishery

History

Chesapeake Bay blue crab fishery records date back to European settlement in the 1600s, although harvest of the species throughout American regions likely predates recorded history (Kennedy & Cronin 2007). With the advent of crab traps in the 1920s and a shift of effort away from declining oyster fisheries in the late 20th century, blue crab fisheries were overexploited to the point that managers deemed closures necessary (Miller 2001, Lipcius & Stockhausen 2002, Aguilar et al. 2008, CBSAC 2011), but a series of successful management strategies are credited with boosting the fishery's health, sustainability, and profitability (Bunnell et al. 2010, CBSAC 2018). Crabbing plays a critical role in the livelihood of thousands of watermen, workers in the industrial distribution network, and their families throughout the eastern United States.

American wild capture fisheries annually harvested over 60,000 mt of blue crabs worth over USD$150 million each year from FY2014 to FY2016 (NOAA NMFS 2016). The three most productive regions from the fishery's inception to the present day are the Chesapeake Bay, Louisiana, and North Carolina, with either a Chesapeake state or Louisiana being the leading harvest state every year for over a decade (NOAA NMFS 2016, CBSAC 2018). Blue crab catch data and crab abundance estimates from fishery-independent sources such as the Chesapeake Bay Winter Dredge Survey (WDS) peaked in the 1990s, with declines and fluctuations from approximately 52,000 mt in the

Chesapeake Bay in 1993 down to less than 22,000 mt in the Chesapeake Bay in 2013 (CBSAC 2018). As of 2016, NOAA NMFS reports the combined Maryland and Virginia commercial catch at 29,548 mt and a value of $99.7 million (NOAA NMFS 2016). Recreational fishing pressure also remains non-trivial, but with uncertain magnitude. 1,600-2,000 mt of recreational harvest has been estimated by the Chesapeake Bay Stock Assessment Committee (CBSAC) in recent years (2011, 2018), based on a yield of 8% of the total commercial catch recommended by mark-recapture studies (Semmler, 2018). Senior managers consider those estimates to have limitations, however, based on annual variation of mark-recapture percentage data (T. Miller, pers. comm.). Declines in the abundance of blue crabs in the 1990s and 2000s led to the eventual closure of winter dredge fisheries, banning of orange sponged female crab catch, institution of the WDS in 2006, and tightly regulated seasonal closures based on harvest reporting and stock assessment (Aguilar et al. 2008, CBSAC 2018). Although Chesapeake blue crab abundance and catch continue to fluctuate, regional management measures are credited with the recovery of the regional fishery to annual catch, abundance, and juvenile and female stock proportion estimates above their 2008 low (CBSAC 2018).

Gear and Regulations

Although the methods of *C. sapidus* harvest are generally the same from state to state, regulations such as minimum size and take limits vary between states. Maryland has a 5-5.25” minimum size of hard crabs and 3.25-3.5” minimum size on peeler and soft crabs for commercial and recreational fishermen alike, plus a 1 bushel hard crab plus 2 dozen soft crab per person recreational limit (MD DNR 2018). By comparison, Louisiana has a 5” hard crab commercial and recreational minimum size, no minimum size whatsoever

on commercial and recreational soft crabs, and a 12 dozen (~2 bushel) all crab recreational limit (LA Wildlife and Fisheries 2018a,b). Currently, the predominant gear used by commercial and recreational crabbers alike is the crab trap (66% in the Chesapeake Bay, 99% in Louisiana; Bourgeois et al 2014), followed by trotlines (31% in the Chesapeake Bay; CapLog Reports 2011). Trotlines are heavy fishing lines, typically several hundred meters long, with regularly spaced branch lines that have baited sacks at the end. Crabs will cling to these bait sacks even as they are brought to the surface, whereupon they are net-harvested by crabbers. Traps are now generally required to have cull rings that minimize bycatch by letting juvenile crabs have an escape route (LA Wildlife and Fisheries 2018a,b, MD DNR 2018). Regardless, traps still have higher handling stress, derelict fishing potential, bycatch potential, and chance of harmful or disease-transmitting interaction than hand trotlines (Guillory and Prejean 1997, Sturdivant & Clark 2011). This is principally due to traps' confined environment, metal frame, and inherent rough and high-speed handling (McKenna & Camp 1992, Guillory 1993, Guillory & Prejean 1997, Barber & Cobb 2007, Havens et al. 2008).

Soft-Shell Blue Crab Aquaculture

Overview

An important practice dependent on the wild blue crab fishery is soft-shell crab production, a practice over 150 years old that yields fresh soft crabs from peelers (Roberts 1905, Oesterling 1995). Between 5% and 10% of harvested crabs are peelers, which are separated from the rest of catch by watermen and transported to enclosures known as shedding systems (Oesterling 1995). This controlled enclosure leads to the practice being defined as aquaculture by USDA and NOAA (NOAA NMFS 2016).

Peelers are held until they die or molt within 1-14 days, and resulting soft crabs are sold at 4-8 times the value of a hard crab by weight (NOAA NMFS 2016). Single shedding operations may hold only a couple tanks in parallel flow or dozens to hundreds of systems with different flow and water quality inputs (Oesterling 1995, Flowers et al. 2018). While crab aquaculture is an international practice that also applies to *P. trituberculatus, P. pelagicus, S. serrata, E. sinensis*, and other *Callinectes* species in Asia, Africa, Europe, and South America, many of these other practices are centered around hard crab maturation and feature longer holding times (Shelly & Lovatelli 2011, Azra & Ikhwanuddin 2015, Mirera & Moksnes 2015, Tavares et al. 2017). Tank enclosures are usually shallow (≤1 ft water depth) with easy overhead access by watermen. The molting of crabs in tanks is usually checked every 4-6 hours to avoid excessive shell hardening of soft crabs. Water quality monitoring varies widely; some or all of salinity, temperature, dissolved oxygen, and nitrogenous wastes may be checked daily, weekly, or less frequently depending on system operators. Crab abundances per tank range from a few dozen to hundreds depending on catch and season. Crabs may be fished by watermen who operate their shedding system or purchased from other watermen, who may fish locally or travel from out of state (Oesterling 1995, Tavares et al. 2017, Schott pers. comm.).

Water Flow in Aquaculture

While soft crab shedding systems outside the United States often use low-cost open systems that immerse sealed crab cages in open water, crab shedders in the United States predominantly use two types of land-based tank enclosure systems: flow-through (also referred to as semi-closed) and recirculating (Ogle et al. 1982, Oesterling 1995,

Tavares et al. 2017). The defining difference between flow-through and recirculating shedding systems is the way that water quality and level is maintained (Fig. 1.1). Nearby estuaries are used to supply flow-through system water, while recirculating system water must either be transported in from local estuaries or piped in from household or municipal freshwater supplies with salt (Oesterling 1995, Tavares et al. 2017). The other critical difference between these systems is that recirculating systems must have effective biofiltration to function, whereas flow-through systems do not (Tavares et al. 2017). Closed systems that handle waste-excreting organisms such as crabs must be able to recycle or transform waste into less toxic chemicals. In soft-shell shedding systems, nitrifying bacteria are used to reduce ammonia and nitrite into nitrate. This nitrate is less toxic and easier to manage by dilution out through regular partial water changes. Toxic proteinaceous waste not removed by this process may be eliminated by bubbling air through biofilter water. This acts to adsorb dissolved waste, including protein, into a surface foam that is skimmed from the top of filter. By comparison, flow-through systems regularly cycle water in and out, simply removing waste by direct replacement. The downside of this aspect of flow-through operations is that the water quality of the system is dependent on water quality of the environmental water source; if the surrounding environment is intolerable to crabs, the water in a flow-through system will likely be so as well.

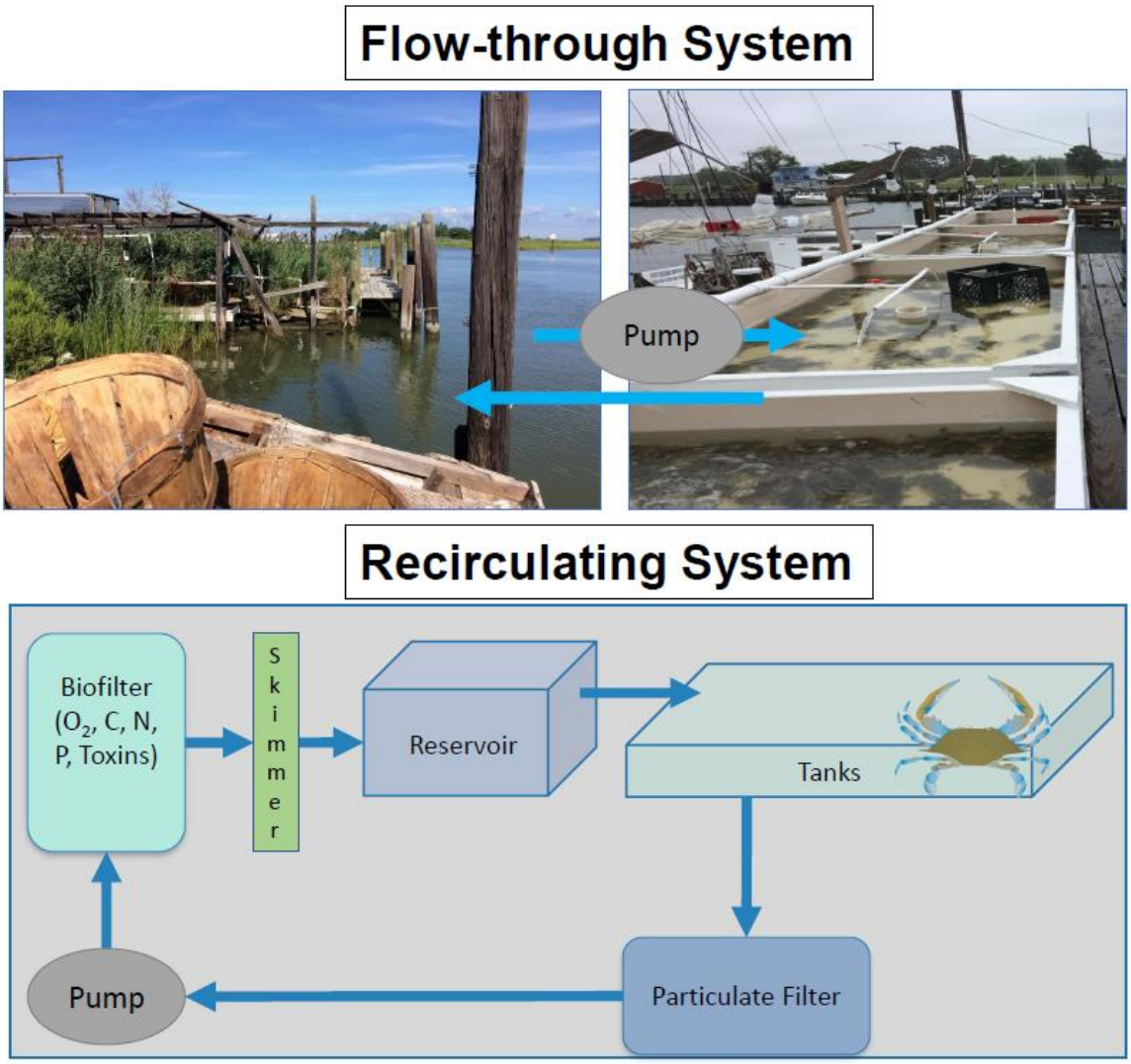


Fig. 1.1. Illustration of the central differences between flow-through and recirculating soft-shell crab shedding systems. Flow-through systems pump water directly from surrounding natural waters into aquaculture tanks, and deposit waste water directly back to those waters. Recirculating systems filter imported water through a biochemical filter and protein skimmer to remove or transform toxins such as nitrogenous waste, control the temperature and salinity of crab tank water via central reservoirs, and eliminate direct effluent to the environment by maintaining a closed water loop that only requires periodic water changes to remove nitrate.

Soft-Shell Production Records

In 2016, NOAA NMFS reported a national peeler catch of 640.3 mt worth $4.6 million (NOAA 2016). Virginia, North Carolina, and Louisiana reported the three highest yield states by peeler volume, with 333.4, 202.2, and 65.8 mt of catch, respectively. A discrepancy was found between the 2016 NMFS Maryland data, reporting 5.3 mt of peelers caught, compared to data from MD DNR, which recorded a peeler catch of 1222.8 mt based on direct count records collected from watermen (MD DNR, pers. comm.). The NMFS-reported MD dockside value for peelers of $102,442 for FY2016 rises to $23.7 million with the MD DNR correction, or 44% of the value of the MD hard crab catch. Even with a more conservative correction based on the FY2016 national value per crab from NMFS, the industry value would be much greater than reported at $8.8 million. This peeler catch record discrepancy dates back to at least 2005, when NMFS recorded a composite Maryland soft and peeler crab catch of 570.7 mt. While exact origins of this disparity are uncertain, privacy rules require the NMFS and the Atlantic Coastal Cooperative Statistics Program (ACCSP) to filter catch data from seafood dealers that employ three people or fewer. Since crabbers are not considered employees and most shedding systems are tended by small partnerships or families, most shedding may be excluded by this this privacy measure. While these are coarse estimates, the implication is that the soft crab aquaculture industry is a highly significant part of blue crab fishery as a whole. In addition, it is possible that similar underreporting of soft crabs and peelers may exist for other states.

Stresses and Mortality in Aquaculture

In an aquaculture setting, crabs experience molting stress caused by large osmotic, hormonal, and metabolic swings, and stress from being in the unsheltered environment of a crowded tank, resulting in some degree of mortality (Mangum et al. 1985, Ryer et al. 1997, Kennedy & Cronin 2007, Techa & Chung 2015). Flow-through systems in particular are prone to water quality fluctuations from their environmental water source, including algal toxins or hypoxia that would stress crabs even before factoring molting stress and increased susceptibility to disease (deFur et al. 1990, Tanner et al. 2006). Even if water conditions are ideal, aggression between crabs, including cannibalism, potentially increases mortality rates in soft-shell shedding tanks. (Oesterling et al. 1995, Sturdivant & Clark 2011). Additionally, predators such gulls, herons, and raccoons prey on crabs in uncovered systems. All these factors are believed to contribute to the death of approximately 25% of peelers before they successfully molt (Oesterling 1995, Chaves & Eggleston 2003). Other studies report mortality from <10% (Rose 2002) to >50% (Bowers et al. 2010). By comparison, prior study in controlled research aquaculture suggests that juvenile crabs experience 10-15% mortality each time they molt (Zmora et al. 2005).

Data Gaps Regarding Aquaculture Mortality

Few peer reviewed studies have explored the factors that contribute to soft-shell crab aquaculture mortality. Chaves & Eggleston (2003) found no significant difference in mortality between flow-through and recirculating systems mortality rates in North Carolina soft-shell crab aquaculture systems. That study, however, was limited to one state and one season; therefore, inferring that recirculating and flow-through systems in

other states and years do not differ in their mortality rates is unresolved. Similarly, while various manuals and studies indicate optimum temperature, salinity, and other water quality ranges (Ogle et al. 1982, Oesterling 1995, Tavares et al. 2017), these are often given with ranges rather than precise optima, opening the door for further research to refine the best culture conditions. Mortality analysis based on crab variables such as crab size, sex, whether peelers are purchased or fished, initial molt stage, and population per tank was studied by Chaves and Eggleston (2003), but their results often used relatively low sample sizes and yielded marginal statistical results, again leaving room for further investigation.

Diseases of Blue Crab

Overview of Pathogens

Given the stress and interactions present in crab traps and soft-shell aquaculture systems, blue crab disease is a concern for both fishery and aquaculture (Johnson 1983, Stentiford & Shields 2005, Shields & Overstreet 2007, Bonami & Zhang 2011, Stentiford et al. 2012, CBSAC 2018). Pathogens of importance include protozoans such as *Hematodinium perezi* (Stentiford & Shields 2005), bacteria such as *Vibrio* spp. (Krantz et al. 1969, Thibodeaux et al. 2009), fungi such as *Ameson michaelis* (Findley et al. 1981, Shields and Overstreet 2007) and a suite of viruses.

Callinectes sapidus Reovirus 1

Callinectes sapidus reovirus 1 (CsRV1) is one of four crab viruses associated with mortality of North Atlantic blue crabs, the others being the Chesapeake Bay picornavirus (CBV), Bi-Facies herpes-like virus (BFV or HLV; Johnson 1977, 1988, Bowers et al. 2010), and a rhabdo-like virus (Jahromi 1977) that was also reported to be associated

with CsRV1 (RLV) infections (Johnson 1984). CsRV1 was found in 21% of wild crabs sampled in the Northwest Atlantic during 2011-2012 (Flowers et al. 2016a). Mortality rates of >95% have been observed in infected crabs in laboratory settings (Bowers et al. 2010, Schott et al. unpub. data). Although found at lower prevalence in Gulf of Mexico, Caribbean Sea, and Southwest Atlantic crabs, it is still known to cause mortality in these regions (Rogers et al. 2015a, b, Schott et al. unpub. data).

CsRV1 is a 55-60 nm icosahedral reovirus possessing 12 segments of double stranded RNA (dsRNA). It was originally described by Phyllis Johnson as Reo-like Virus (RLV) (Johnson 1977; Bowers et al. 2010), and later as *C. sapidus* reovirus (CsRV; Tang et al. 2011). The nomenclature change to CsRV1 distinguishes the virus from a second reovirus discovered in *Callinectes* from Brazil with a distinct genome signature (Schott et al. unpub.). CsRV1 is genetically and morphologically similar to a series of 12 genome segmented viruses tentatively classified as Crabreovirus (Deng et al. 2012), including the P virus of *Macropipus depurator* (Vago 1966, Montanie et al. 1993), MCRV of *Scylla serrata* (Weng et al. 2007), and EsRV strains of *Eriocheir sinensis* (Zhang et al. 2004). In prior laboratory trials, homogenized and filtered preparations of crab tissue infected with CsRV1 caused 100% mortality after 9-16 days when injected into PCR-negative crabs at doses of $>10^6$ CsRV1 genome copies (Bowers et al. 2010). The virus infects hemocytes and hemopoetic tissue, leading to cellular inclusions and nodule formation in nervous, gill, and epidermal tissues (Johnson 1977, Bowers et al. 2010). CsRV1 does not present with external physical symptoms, instead manifesting as gradual lethargy, paralysis, tremors, and eventual death of crabs in the final days of infection (Johnson 1977, Bowers et al. 2010).

CsRV1 in Soft-shell Aquaculture

Johnson (1977, 1983) speculated that CsRV1 is likely transmitted by cannibalism of, and cohabitation with, infected crabs, but limitations to detecting CsRV1 by histopathology and continuing consideration of a reovirus-rhabdovirus complex in these early experiments leave natural transmission routes uncertain. While found at lower prevalence and load in wild blue crab population surveys, it has been found in >50% of soft shell crab production mortalities in Chesapeake Bay facilities (Bowers et al. 2010, Flowers et al. 2016a). Recent study also indicated that incidence of CsRV1 increases in populations of wild crabs sampled near soft-shell production facilities and that male crabs and larger crabs are more likely to be infected, although prevalence varied widely by time and locations in all cohorts (Flowers et al. 2018).

Data Gaps Regarding CsRV1

Understanding of the total fisheries impact of pathogenic crab viruses remains incomplete, even for CsRV1. No attempt has yet been made at quantifying the economic impact of CsRV1 on soft shell crab aquaculture, let alone the impact of the virus on wild populations overall. Questions remain regarding the cause of CsRV1 prevalence variability by geography, aquaculture practice, or water quality in areas of harvest. Difficulty of CsRV1 detection, extent of sampling replication and repetition, and exclusive focus on live hard crabs and dead peelers to date have limited our understanding of CsRV1 impact on natural mortality (e.g. Bowers et al. 2010, Flowers et al. 2015, 2018). Given the direct potential importance of CsRV1 as a challenge to soft-shell crab production in the United States, these knowledge gaps define the principle aims of this thesis in studying mortality and CsRV1 in aquaculture.

Central Thesis Objectives

1. This study was conducted to identify stressors related to peeler mortality in blue crab aquaculture while including more states, years, and crabs than prior studies. In order to determine the overall impact of aquaculture mortality on the industry, we surveyed crab mortality, water quality, and operating conditions in Maryland, Virginia, and Louisiana soft-shell crab aquaculture facilities in 2016 and 2017. If our findings were consistent with the results of Chaves & Eggleston, then we expected to observe 20-25% mortality that would be elevated in shedding systems with low crab per tank densities, as well as for crabs that were bought rather fished and female rather than male (2003). We expected that recirculating systems would experience less mortality than flow-through systems, likely associated with less hypoxia and lower nitrite in well-controlled recirculating systems (Manthe et al. 1994).

2. Investigating the role that CsRV1 in aquaculture peeler mortality was central to this study. Expanding on the mortality survey, we sampled live and dead crabs from soft-shell facilities for CsRV1 using a reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay for the virus genome and compared CsRV1 prevalence and intensity to mortality. This highly sensitive assay had not existed at the time of prior crab shedding studies, nor had the potential importance of the CsRV1 pathogen to crab shedding been realized. Based on earlier work by Bowers et al. (2010), we expected intense CsRV1 infection to be found in >50% of dead peeler crabs regardless of region. The expectation for live peelers and soft crabs was that Chesapeake Bay CsRV1 prevalence would be ~25% at lower intensity, based on the wild crab survey of Flowers et al. (2016a). Louisiana live crabs were expected to have ~15% CsRV1 prevalence at

low intensity, based on the studies of Rogers et al. (2015a,b). Given the differences expected in CsRV1 prevalence and intensity in dead and live crabs, we hypothesized that CsRV1 would be a strong factor predicting peeler death.

3. The last central objective of this thesis was to compare environmental stress and crab condition to CsRV1 prevalence and intensity in aquaculture, thus detecting any disease risk factors. Water quality, operating conditions, and crab characteristics were input into a stepwise process that selected the best generalized linear mixed model (GLMMs) predicting CsRV1 occurrence and intensity. Based on Flowers et al. (2018), we proposed that larger crabs and male crabs might be more prevalently and intensely infected with CsRV1, but that this relationship might be limited due to the narrow size range selected by this fishery-dependent practice. We did not expect a significant association of water quality parameters with CsRV1 infection levels, as no correlates have been observed in prior study (Bowers et al. 2010, Flowers et al. 2018).

Additional Thesis-Motivated Questions

While work on the primary thesis objectives is described in Chapter 2, a series of other research questions identified below were studied and detailed in Chapter 3 and Appendix 1.

1. This study sought to understand the elusive natural transmission routes of CsRV1 between crabs. These were of particular interest in supporting any conclusion about whether CsRV1 might be transmitted during fishery activities or within shedding systems. We exposed virus-naïve crabs to infected dead crabs via cohabitation for different periods of time, and fed others infected crab tissue to test for ingestion

transmission. These experiments were inconclusive with some still in progress, and will be discussed in Appendix 1.

2. The need to have accurate data on peeler crab fishing practices and catch increasingly became a concern of this study. As evidenced by the discrepancy between Maryland DNR and NOAA NMFS Maryland catch numbers, soft-shell production, trends, and value have not always been accurately reflected in official records. This limits effective management priority and decision making by both industry and regulatory agencies. Here, we pooled available data from managers and surveyed watermen to update the complete picture of soft-shell blue crab production in the United States.

Chapter 2: Investigating risk factors for mortality and reovirus infection in aquaculture production of soft-shell blue crabs (*Callinectes sapidus*)

Abstract

Crustaceans in aquaculture are prone to mortality from the combined effects of disease agents and the stresses associated with crowded, closed conditions. The culture practice of producing soft-shell blue crabs is no exception, suffering from mortality of about 25%. The virus, *Callinectes sapidus* reovirus 1 (CsRV1), has been reported at high viral loads in crabs dying in soft-shell shedding facilities. We investigated the relationship between crab mortality and CsRV1 prevalence and load in soft-shell crab production and whether death and virus infection correlated with identifiable aquaculture practices, environmental stresses, crab characteristics, or geographic regions. The patterns of CsRV1 prevalence, infection intensity, and mortality in blue crab aquaculture were studied in the Chesapeake Bay and Gulf of Mexico, USA. Using a genome-targeted assay, we compared virus loads in live and dead aquaculture crabs by individual sex and injury state from recirculating and flow-through systems of variable salinity, temperature, and crabs per aquaculture tank. Mortality was two-fold higher in flow-through aquaculture systems (33%) than in recirculating aquaculture systems (16%). Flow-through aquaculture systems had higher variability in daily water temperature than recirculating aquaculture, and hypoxic events were observed only in flow-through systems during this study. CsRV1 intensity above 10^6 virus per mg crab tissue was found in 62% of all pre-molt mortalities in production compared with 7% of successfully molted soft-shell crabs. The CsRV1 virus load in dead crabs was elevated in salinity above 7-8 psu. In a mixed-effect model analysis, the random effects of location and time

were more important than salinity in predicting CsRV1 load in all crabs and dead crabs. Our results support previous research showing that recirculating aquaculture has lower mortality in soft-shell production, and confirms the association of high viral loads of CsRV1 with crab mortality in these production systems. Moreover, the findings indicate that although CsRV1 is ubiquitous in these systems, management of culture conditions such as salinity and temperature may limit virus-associated mortality.

Introduction

Crustacean aquaculture supports extensive seafood industries worldwide, yet disease agents remain a major factor limiting production (Shields and Overstreet, 2007; Stentiford et al., 2012; FAO, 2018). Many factors may exacerbate both disease susceptibility and mortality in aquaculture, such as high population density, stress of confinement, water quality, temperature fluxes, or hypoxia (Le Moullac and Haffner, 2000; Mohanty et al., 2018). Certain aquaculture practices, including disposal of dead aquaculture animals into neighboring marine waters, use of dead animals as bait, or untreated effluent release, can facilitate the spread of disease, yet these practices remain common in the industry (Lafferty et al., 2015; Shields, 2017; Flowers et al., 2018).

The target of one of the world's four largest crab fisheries, the blue crab, *Callinectes sapidus*, is at the center of a multi-million dollar aquaculture practice in the eastern United States (Maryland, 2018; NOAA NMFS, 2016; FAO, 2018). This fishery-dependent practice involves holding pre-molt blue crabs, known colloquially as peelers, in shallow tanks until they molt into soft-shell crabs, which are a value added product consumed regionally and frozen for international trade (Oesterling, 1984; Chaves and

Eggleston, 2003; Tavares et al., 2017). The molting process is inherently stressful for crabs (Defur et al., 1988; Defur, 1990). Combined with external stressors associated with harvest and aquaculture, molting stress may contribute to the reported 25% - 50% mortality in soft-shell crab production (Chaves and Eggleston, 2003; Oesterling, 1984). Despite numerous methods and manuals designed to help optimize culture conditions (e.g., Ogle and Perry, 1982; Oesterling, 1984), peeler crab mortality remains unpredictable and costly to individual producers. Recent studies have investigated the potential for disease to contribute to crab mortality in the shedding systems used by the industry in soft-shell production (Bowers et al., 2010; Rogers et al., 2015).

Across their US range and in Brazil, blue crabs are infected by the pathogenic virus *Callinectes sapidus* reovirus 1 (CsRV1, Bowers et al., 2010; Flowers et al., 2015). CsRV1 (previously identified as Reo-Like Virus, RLV) was identified as a cause of crab mortality in captive crabs in the 1970s (Johnson, 1977; Johnson, 1978), and subsequently in soft crab aquaculture production and a scientific blue crab hatchery (Bowers et al., 2010). The virus infects hemocytes and hemopoietic tissue (Johnson, 1977; Tang et al., 2011). Injection of viral filtrate leads to paralysis and death of crabs in days or weeks and is associated with infiltration of hemocytes into neural tissues (Johnson, 1983; Bowers et al., 2010).

Application of sensitive quantitative molecular assays for CsRV1 has shown a mean prevalence of 20% in wild crabs surveyed from the northeast United States, with most infected animals harboring $<10^4$ virus genomes per mg crab muscle tissue (Flowers et al., 2015). In contrast, an earlier study of soft-shell crab aquaculture in Maryland and

Florida, USA, found CsRV1 in 71% of dead peeler crabs using an RNA electrophoresis assay that has an estimated detection limit of 10^5–10^6 genome copies per mg muscle tissue (Bowers et al., 2010). This association of CsRV1 with peeler crab mortality suggests that CsRV1 may be an important contributor to mortality during blue crab molting and a source of considerable economic loss to the soft-shell crab industry (Johnson, 1983; Flowers et al., 2018).

Few studies have investigated how disease may interact with aquaculture practices to cause crab mortality during soft-shell production. It is not known how crab mortality and CsRV1 prevalence are affected by culture practices, environmental holding conditions, individual crab characteristics, or specific geographical location of facilities. To determine whether specific biological or environmental risk factors exist in soft-shell crab production, we partnered with soft crab producers in Maryland, Virginia, and Louisiana to measure different parameters used in shedding systems and correlate these with virus prevalence and loads using a quantitative, real time polymerase chain reaction (RT-qPCR) assay (Flowers et al., 2015). Potential relationships between crab mortality, CsRV1 infection, aquaculture system type (flow-through vs. recirculation), salinity and water temperature, and the individual size, sex, and molting state of crabs were investigated using generalized linear mixed effect modelling (GLMM). Our findings may be useful for identifying management practices that reduce CsRV1-associated mortality and increase successful soft-shell crab production.

Materials and Methods

Crab collection and handling

Peeler crabs, culture system water, and culture practices were surveyed at soft-shell production facilities in Maryland, Virginia, and Louisiana from May to September, in 2016 and 2017. Freshly harvested live peelers that had not been placed in aquaculture, peelers that died in aquaculture, and successfully molted soft crabs were collected by participating watermen during one-week periods in each month, with 7–25 crabs of each type sampled. Live and dead crabs were transported on ice to the Virginia Institute of Marine Science (VIMS), Louisiana State University AgCenter (LSU AgCenter), or the Institute of Marine and Environmental Technology (IMET) depending on the location of the production facility. Crabs were either measured and dissected immediately or stored at −20 °C for later analysis. All crabs were measured (carapace width) and assessed for obvious limb loss or puncture injuries prior to dissection. A 1–4 cm section of walking leg was removed from each crab and frozen, and those sourced from VIMS were preserved in 95% ethanol. In addition, crab sex, sample date, molt stage, type of shedding system, and location were recorded for all crabs on accompanying data sheets. Molt stage was assessed by the color along the margin of the propodus of the 5th walking leg and progressive splitting of the carapace. The red color along the margin typically indicates molting will occur within 3 days, pink with molting in 1–2 days, and splitting of the carapace epimeral suture a sign that molting is imminent, and full molt indicating the full emergence of the new instar. All leg samples were transported to IMET for CsRV1 quantification.

Soft crab producers participating in the study were located in Pasadena, Lusby, Tilghman Island, and Rock Hall, Maryland, West Point, Sarah's Creek, and Chuckatuck Creek, Virginia, and Dulac and Franklin, Louisiana, USA (Fig. 2.1). Two additional sites at Bear Creek, MD and Violet, LA were initially surveyed, but were excluded from statistical analysis as high-mortality outliers that were affected by hypoxia and toxic nitrite levels beyond the limits required for sustainable aquaculture operation. Sites were categorized by open (flow-through) or closed (recirculation) water circulation type. Both systems at Tilghman Island were flow-through sites located within 1 km of each other, but were independent businesses and operations (Table A.1.). Daily water samples (10 mL) of all systems were collected at varying times of day by watermen and were assessed for salinity by refractometer, while nitrate, nitrite, general hardness, carbonate hardness, and pH were measured by aquarium test kit (5 in 1 Aquarium Test Strips, API®) for Maryland sites. Water temperatures in culture systems were measured hourly by automated HOBO™ dataloggers and used to derive weekly mean temperatures and temperature ranges (Onset Corporation®). Water temperature range was defined as the maximum difference between any hourly point and the weekly period average. Weekly mean salinity of each sampling period was averaged from daily water samples for site comparison. Participating watermen recorded whether they fished peeler crabs themselves or purchased peelers from other fishermen, the number of aquaculture tanks they used, average number of crabs per tank, and the number of crabs dying or molting to soft shell on each day of the survey. Mortality level was calculated by dividing the number of dead peelers reported by the sum of dead peelers and live soft crabs reported as output during a 7 day survey period.

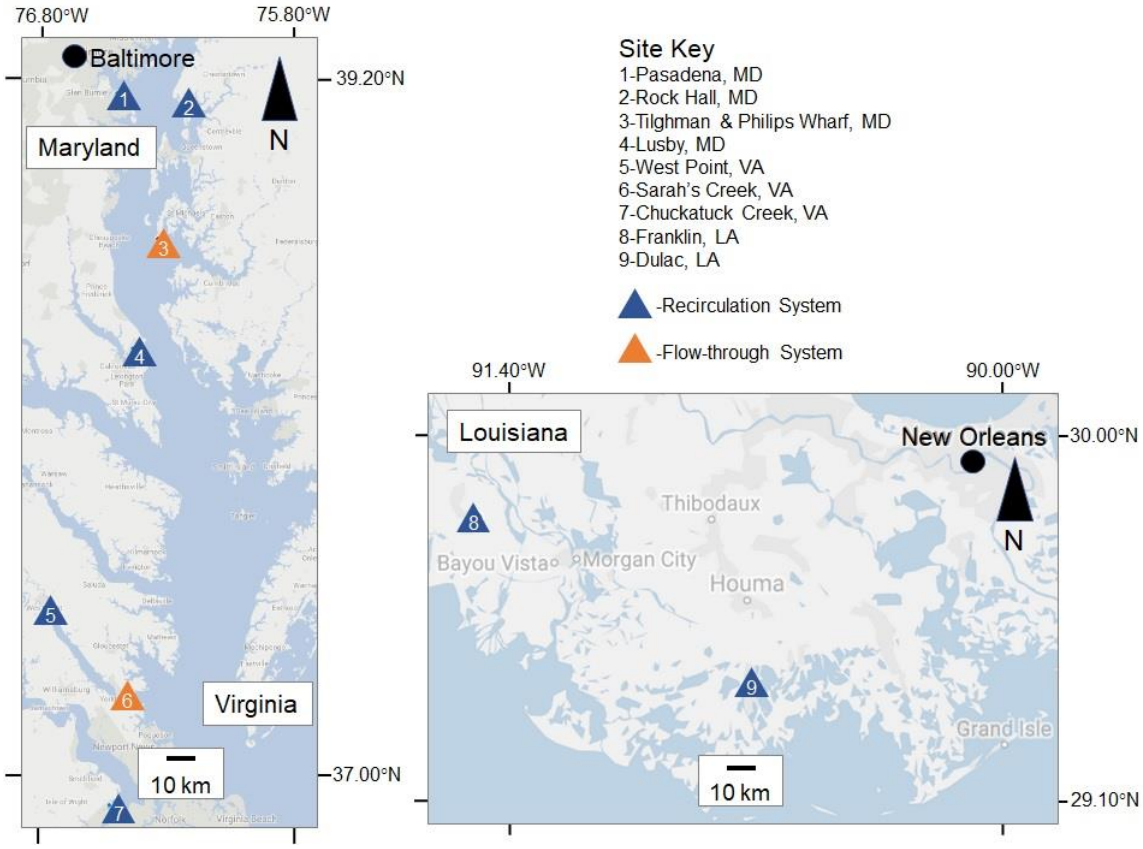


Fig. 2.1. Map of soft-shell blue crab aquaculture facilities surveyed in 2016–2017.

Crab dissection and RNA extraction

Crabs were dissected with sterile wooden rods and razor blades, and all handling and crab surfaces were cleaned with ELIMINase™. Samples (25–100 mg) of crab muscle and epidermis were excised from a walking leg of each crab and homogenized in 1.0 mL RiboZol® (VWR Scientific) using ceramic beads in a MP® FastPrep24 homogenizer. RNA extraction methods were similar to those used by Flowers et al. (2015), and followed manufacturer's instructions. RNA pellets were washed twice in 75% ethanol to ensure removal of traces of phenol. Resulting RNA pellets were dissolved in 1 mM

EDTA, and RNA purity and concentration were evaluated by NanoDrop™ spectrophotometry.

Quantification of CsRV1

PCR primer selection and dsRNA standard preparation were adapted from Flowers et al. (2015). The primer pair 5′-TGCGTTGGATGCGAAGTGACAAAG-3′ (RLVset1F) and 5′-GCGCCATACCGAGCAAGTTCAAAT-3′ (RLVset1R) are designed to detect an amplicon from the ninth genome segment of CsRV1 (GenBank entry KU311716) (Flowers et al., 2016). Standard curves of the CsRV1 genome were produced by purifying viral dsRNA from crabs infected with greater than 10e8 copies per mg muscle. Enrichment of dsRNA and verification of purity and quality followed the protocol of Bowers et al. (2010). Standard curves were consturcted using dsRNA at calculated concentrations ranging from 3.4×10^7 down to 10 genomes per µL were dissolved in 1 mM EDTA with 25 ng per µL yeast tRNA.

The qPCR reagents, thermocycler parameters, and process for annealing primers to crab RNA were modified from Flowers et al. (2015). The qPCR reaction components included 1 x One-Step Master Mix, Low ROX (qScript™ One-Step qRT-PCR Kit, Low ROX, Quanta Bio), SYBR® Green (Quanta) and 500 nM of each primer. Primers were dissolved in 1 mM EDTA. Amplification was conducted by using 40 cycles of 5 s at 95 °C (melting) followed by 30 s at 61 °C (annealing and extension), followed by melting point analysis from 61 °C to 95 °C for verification of the correct amplification product.

Statistical analysis

Statistical tests were conducted using JMP® Pro 13 (SAS, 2018) and the R 3.4.4 statistical package (R Development Core Team, 2018). Initial statistical correlations between mortality, CsRV1 prevalence and intensity, and specific aquaculture and crab variables were tested using Pearson correlation matrices and probability tests. Matrices and tests were produced using the corr.test function in R and the Multivariate Correlation tool in JMP®. Significant correlations were defined as those where $p \leq .05$. A $\log_{10}(x + 1)$ transformation was applied to CsRV1 load prior to running ANOVA or GLM models to account for the exponential nature of the qPCR assay measurements and to enhance visualization. The Shapiro-Wilk test for normality and Levene's test for homoscedasticity were applied when appropriate. When analyzing individual factor comparisons, parametric pairwise comparisons were tested via *t*-test, whereas one-way ANOVA with Tukey's Honest Significant Difference test was used for multiple comparisons to reduce Type I error. Non-parametric comparisons used Wilcoxon rank-sum testing with Dunn pairwise comparison to determine significant differences. In cases where continuous data was being compared, a series of linear regressions were undertaken comparing appropriate data transformations with a regression *t*-test and sum-of-squares lack of fit analysis. Stepwise selection of best GLMM with forward elimination was conducted using Akaike's information criterion (AIC) in R (stepAIC function, MASS package, lmer, lme4 package, R Development Core Team, 2018) was used to determine the factors that best model crab mortality and CsRV1 prevalence and intensity of infection. In individual crab data models, date was used as a random effect to account for uneven sample sizes from different dates and locations, and because crabs were typically harvested and

introduced to aquaculture in groups, which can create pseudoreplication effects (i.e., crabs that die on the same day may have been caught in the same time frame from the same location) (Thorson and Minto, 2015). Due to geographic separation from the Chesapeake region and the disparate number of sites, the crab data from Louisiana was not included in the statistical models. GLM models that include all potential analyzed risk factors for mortality or CsRV1 infection, prior to stepwise AIC, are listed in Supplementary Table A.3.

Results

Aquaculture mortality by site and date

Eight soft-shell crab aquaculture facilities were sampled at a total of twenty timepoints in 2016-2017, with each timepoint lasting one week. In total, 12,172 crabs were recorded as having passed through those facilities during those times, with 897 of those crabs sampled for CsRV1.

The mean crab mortality level from Chesapeake aquaculture facilities sampled was 21.7 ± 2.8% (n = 12,172 crabs) (Supplementary Table A.1). The salinity range was 3–20 psu in Virginia and 6–18 psu in Maryland. Mean temperature ranged from 23.8 to 29.4 °C with a maximum peak of 37°C in Virginia and 27.8 to 29.4 °C with a maximum peak of 32.2°C in Maryland. Crab mortality was significantly greater in flow-through aquaculture systems (32.9 ± 4.3%; n_{site} = 3, n_{time} = 7) than in recirculating systems (16.4 ± 3.1%; n_{site} = 5, n_{time} = 13) (Fig. 2.2, p .006). When we compared culture conditions between system types, salinity was significantly lower in recirculating systems (Supplementary Table A.1; Student's t; d.f. = 18, t = −2.55, p = .02). All systems surveyed

with average salinities below 8 psu had mortality of 15.0% or less; however, there was no significant difference in mortality based on salinity alone (Pearson's correlation, ρ = .3483, p = .1324).

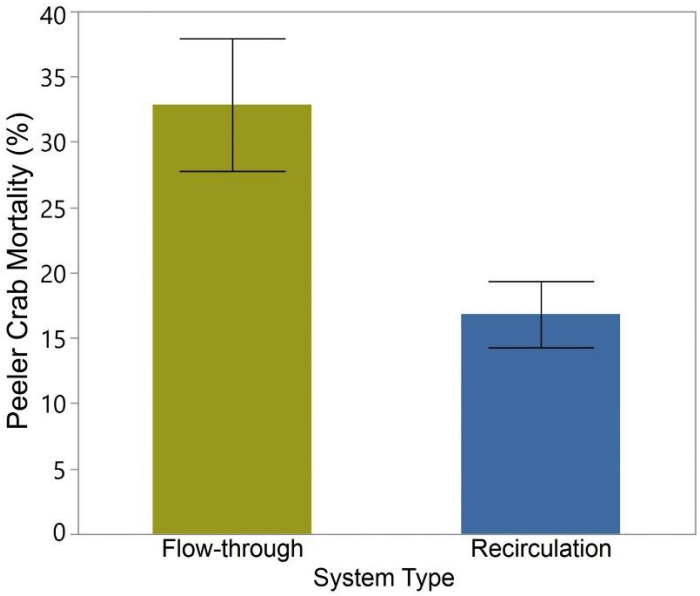


Fig. 2.2. Blue crab (C. sapidus) mortality (mean ± s.e.) observed in flow-through and recirculating aquaculture systems in the Chesapeake Bay soft shell crab industry. Means are significantly different between flow-through and recirculating systems (Student's t, d.f. = 18, t=-3.11, p = .0060).

The final model for peeler crab mortality (Table 2.1, Model A) had significant system type ($slope_{recirculation} - 21.8$, $p < .0001$) and temperature range fixed effects (slope = −1.20, $p = .03$). Maximum fluctuation in water temperature did not differ significantly between flow-through and recirculating systems (Fig. 2.3, Student's t, d.f. = 19, $t = -1.52$, $p = .09$). However, maximum temperature fluctuation measured at the flow through facility at Sarah's Creek, VA, was more than twice as high as at recirculating

sites. The Sarah's Creek facility experienced 21.3% mortality on average: lower than the flow-through system average, but comparable to the overall shedding mortality in this study. No other significant relationships between crab mortality and other variables were found.

Table 2.1. Final reduced generalized linear mixed models (GLMM) predicting significant effects on crab mortality and CsRV1 infection intensity.

Model	Fixed Effects					
	Predictor Variable	Slope Estimate	Standard Error	p-value	Variance	Standard Deviation
A. Crab Mortality (%) ~ System Type + Temperature Range (°C) d.f. = 17, AIC = 154.17 Non-normal Homoscedastic	System Type	-21.85	5.3	7.06E-04		
	Temperature Range (°C)	-2.16	0.94	0.0341		
	Intercept	44.32	6.28	1.93E-06		
B. Log(Crab CsRV1 Load) (genomes/mg) ~ Successful Molting + (1|Site) + (1|Date) d.f. = 518, AIC = 2561.85 Non-normal	Successful Molting	-3.16	0.31	<2E-16		
	Intercept	4.77	0.56	9.43E-05		
	Location				1.28	1.13
	Date				1.88	1.37
C. Log(Dead Crab CsRV1 Load) ~ Salinity + (1|Site) + (1|Date) d.f. = 320, AIC = 1601.30 Non-normal	Salinity (psu)	-0.13	0.07	0.0498		
	Intercept	6.25	1.13	8.42E-05		
	Location				4.94	2.22
	Date				1.05	1.03

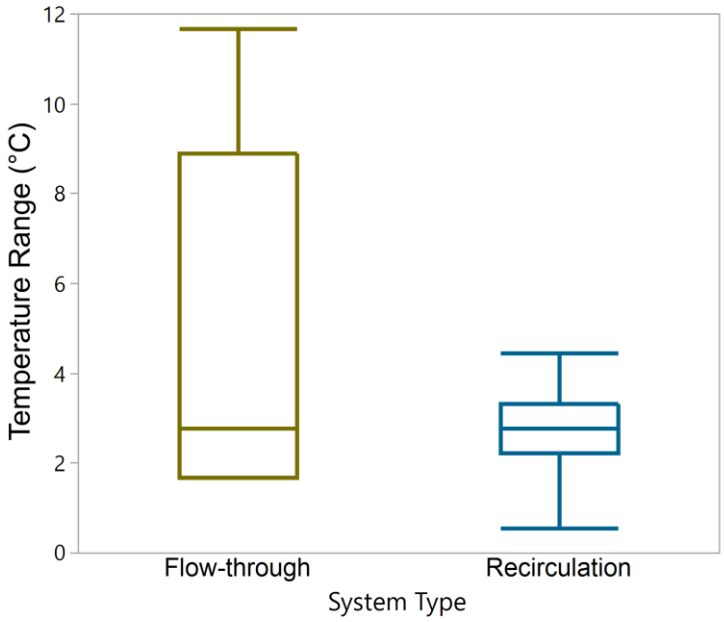


Fig. 2.3. Daily fluctuation from mean water temperature in flow-through and recirculating crab aquaculture systems (Student's t, d.f. = 19, t = −1.52, p = .09).

CsRV1 infection in aquaculture analyzed by individual crab

Throughout the aquaculture facilities surveyed in MD and VA, 75.4% of dead peelers ($n = 305$) were infected with CsRV1, compared with 23.9% of live soft-shell crabs ($n = 184$) and 33.3% of freshly harvested live peelers ($n = 60$; Fig. 4a-c). The difference between live and dead peelers was even more pronounced when considering infections of $>10^6$ CsRV1 genome copies per mg muscle tissue, with 62.3% of dead peeler crabs surveyed exceeding this infection intensity compared with 7.1% of live soft shell crabs ($n = 288$; Fig. 2.4a–c, Table 1, Model B). This 10^6 CsRV1 copy threshold is important because such loading is what is found in mortalities caused by CsRV1 transmission induced in laboratory experiments (Bowers et al. 2010; Schott et al. unpub. data). Successful molting was the only significant fixed effect (slope − 3.16, $p < .0001$)

with location (variation 1.28) and date (variation 1.88) as random effects associated with CsRV1 genome copy number (Table 1, Model B). No other factors, including salinity, system type, temperature, injury, or crab sex were significant factors influencing CsRV1 loads.

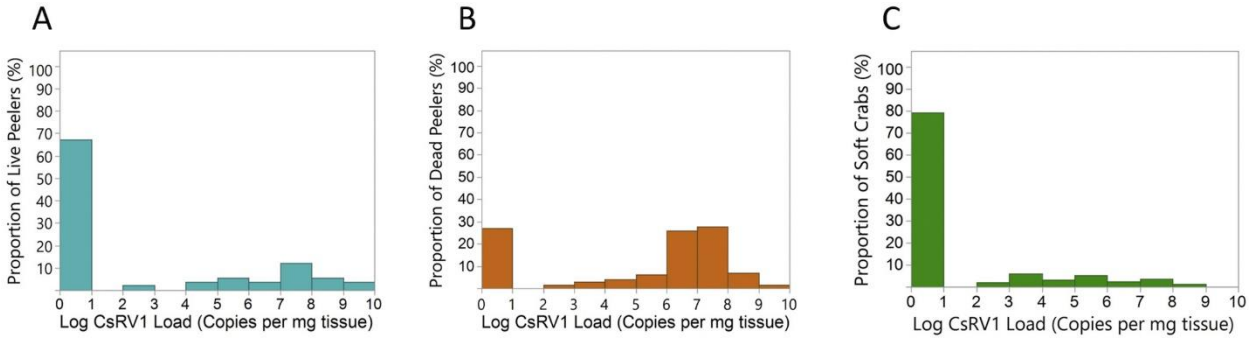


Fig. 2.4. Frequency histograms of log CsRV1 loads observed in Chesapeake Bay. a) live peelers, b) shedding mortalities, and c) soft shell blue crabs from aquaculture. Percent prevalence of CsRV1 in each group is defined as the proportion of crabs with a log CsRV1 Load greater than zero.

Considering specific environmental variables on a shedding system basis, higher salinity aquaculture sites experienced higher prevalence levels of the virus in dead peeler crabs (Fig. 2.5). Prevalence in dead peelers best fit a reciprocal relationship to salinity (CsRV1 Prevalence (%) = 98.0–251.3/Salinity (psu); $R^2 = 0.4374$, ANOVA d.f. = 19, $F = 13.99$, $p = .0015$). Salinity was the only fixed effect retained in the reduced mixed models of CsRV1 infection intensity (Table 2.1, Model C). The salinity effect was only marginally significant ($p = .0498$) with a low magnitude negative relationship (slope = −0.13) once other variables were accounted for in the total model. The random effects of date (variance = 1.05) and location (variance = 4.94) were both of higher

magnitude per unit change than salinity. No other effects were significant, including injury to crabs.

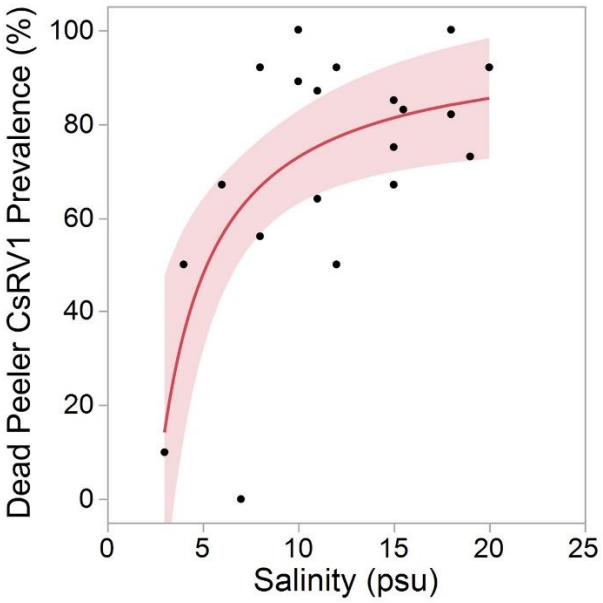


Fig. 2.5. Prevalence of CsRV1 infection in dead peeler crabs fit to a reciprocal regression. As salinity increased, CsRV1 prevalence in dead peelers increased towards an expected maximum of 85.4% at 20 psu. A major drop in CsRV1 prevalence was observed below 8 psu. (CsRV1 Prevalence (%) = 98.0–251.3/Salinity (psu); $R^2 = 0.4374$, ANOVA d.f. = 19, F = 13.99, p = .0015).

Louisiana mortality and prevalence data

Two Louisiana soft-shell crab production facilities were sampled during four week-long time points during 2016-2017. A total of 652 crabs were processed in these facilities during this time, of which 139 were sampled for CsRV1. This Louisiana data was not integrated in GLMMs with Chesapeake data, nor analyzed in separate GLMMs due to the low site sample size.

In Louisiana shedding facilities, the percentage of dead crabs was 14.5 ± 5.5% (n = 652 crabs) (Supplementary Table A.2). In 2016 and 2017, the prevalence of CsRV1 in dead crabs was 21.9% (n = 82). Only one dead crab from the Franklin facility had a detectable CsRV1 infection. Sampling of live crabs in November 2017 found that both soft shell crabs (n = 20) and live peelers (n = 37) had a prevalence of CsRV1 of 5%, with only one heavily infected crab detected. Due to the low site and crab sample sizes from this distinct geographical region, Louisiana data was not included in the GLMM analyses (Supplementary Table A.3). Aquaculture salinity was measured at 0–3 psu at Louisiana sites, while the mean temperature range was 21–29 °C.

Discussion

By collaborating with soft crab producers in three states, this study reconfirmed that soft crab production has variable and sometimes high mortality and that well-controlled recirculating aquaculture systems are crucial to minimize peeler crab mortality. Average peeler mortality was similar to that seen in a prior study in North Carolina, U.S.A., that reported 23% mortality in blue crab shedding systems (Chaves and Eggleston, 2003). In contrast to the North Carolina blue crab study, flow-through systems examined in the current study had twice the mortality of recirculating systems, where one in three peelers died on average. Despite the global importance of mortality and disease in crab aquaculture systems (Zhang et al., 2004; Deng et al., 2012; Oesterling, 1984), information on mortality in crab aquaculture is scarce (FAO, 2018). While reviews of soft-shell blue crab aquaculture practice exist (Oesterling, 1984; Tavares et al. 2018),

none have produced specific numbers on regional value and volume productivity, disease incidence, or losses due to disease.

The lower mortality of crabs in recirculating systems was likely associated with better control of environmental variables compared to flow-through systems. For example, temperature range was less in the recirculating systems, albeit the trend was not significantly different ($p = .08$) between system types. Other environmental parameters may also contribute to crab mortality in poorly-controlled or flow-through systems. For example, we excluded one flow-through system in the Baltimore area from the study because of separate events of hypoxia (< 2 mg/L) and high nitrite (>10 mg/L) which were both associated with crab mortality over 50%. In these latter examples, mortality was not likely a result of environmental variability per se, but the fact that one water quality parameter exceeded a biological threshold for crab survival.

Economically, peeler mortality represents a loss of time, effort and money to watermen. This amount of mortality appears to be a long-accepted cost of doing business for individual crab shedders, who may lose several thousand dollars' worth of peeler crabs per week, depending on the size of their shedding operation. Estimates of peeler crab mortality have been identified as a critical need by the Chesapeake Bay Stock Assessment Committee (Chesapeake Bay Stock Assessment Committee (CBSAC), 2018). Based on 2016 data for peeler harvests, Maryland harvested 1225 metric tons of peeler crab (Maryland, 2018, pers. comm.), while Virginia harvested 333 metric tons, and Louisiana 65 metric tons (NOAA NMFS Commercial Fisheries Statistics, 2016). Based on the 22% mortality observed in this study, an estimated 356.2 metric tons of peelers

(worth $2.58 million) died prematurely in these three states in FY 2016. Applied to the entire United States peeler harvest, this mortality represents a loss of 408.2 metric tons of blue crab. At 250 g estimated average weight per peeler, this represents 1.63 million peeler crabs lost in FY2016.

This study revealed additional information about the association of CsRV1 infection with peeler crab mortality. When analyzed on the basis of individual crabs, CsRV1 infection was the most significant predictor of peeler crab death regardless of shedding system type. Heavy infections were found in almost two thirds of the dead peeler crabs, and was nine times higher than the prevalence of heavy infections in successfully molted soft-shell crabs. Estimating the economic consequences of the 62% of peelers that died with heavy CsRV1 infections in MD and VA suggests that the virus is associated with the loss of at least 212 metric tons of peeler crabs worth $1.53 million. Furthermore, an association in the range of 62% may help explain the difference between research-controlled (13%) and Chesapeake soft-shell industry (22%) molting mortality rates. In value terms, it is also important to recognize that CsRV1 is not the only reovirus to kill crabs in aquaculture: both *Eriochir sinensis* (Zhang et al., 2004) and *Scylla serrata* (Deng et al., 2012) are reported to suffer from mortality associated with EsRV and MCRV, respectively, adding to global crab aquaculture losses associated with reoviruses.

Crabs with high virus loads likely did not acquire CsRV1 within the shedding systems. The prevalence of CsRV1 infections in live peeler crabs entering aquaculture (33%) was nearly the same as the estimated prevalence of CsRV1 from the combined numbers of dead and live crabs processed in these systems (35%). That is, the overall

prevalence of CsRV1 in peelers did not increase during the average of 5 days in short-term culture. This indicates that although virus replication within infected crabs may be accelerated during aquaculture, CsRV1 transmission between crabs in soft-shell crab production is minimal over the brief culture periods involved. We speculate that a certain fraction of crabs enter soft crab aquaculture with naturally-acquired CsRV1 infections which rapidly progress due to the additional stress of molting and sub-optimal conditions, and eventually contribute to mortality of peelers at the levels observed in this study.

Soft crab aquaculture conducted at lower salinities appeared to experience lower overall CsRV1 infection prevalence and intensity within the Chesapeake Bay than high salinity sites, but the difference was not significant. The current study documented that prevalence of CsRV1 was much lower in dead peelers from Louisiana shedding systems compared with dead peelers from Chesapeake systems. All Louisiana shedding facilities were at low salinity (0.6–6.5 psu), and also had lower dead loss than Chesapeake shedding facilities. While Louisiana sampling data were too sparse to permit powerful statistical comparison, the low mortality and CsRV1 prevalence found in Louisiana point to a need to better understand the effects of salinity on crab survival and CsRV1 infection in aquaculture. A 2002 North Carolina Fisheries Grant research report describes an intriguing study that shows very low peeler mortality in low salinity (2 psu) shedding systems (NC Fishery Resource Grant Program, 2002). Together, our results and the referenced studies provide motivation to study whether low salinity in harvest water or aquaculture systems reduces CsRV1 prevalence and/or peeler mortality.

It is apparent that infection trends were affected by factors that we did not measure or control. First, strong random effects of site and date on CsRV1 prevalence were identified by GLMM. Second, CsRV1 prevalence levels at middle salinity sites did not fit well with the salinity regression, indicating that other factors influence overall infection rates in mesohaline conditions. The site and time factors in final modelling of CsRV1 prevalence suggest that the actual location of crab harvest, position in the estuary, or related factors may influence disease prevalence even more directly than salinity. This site-by-site variation agrees with prior studies of CsRV1 prevalence in wild crabs, which showed wide variation by site, year, or month (Flowers et al., 2018; Flowers et al., 2015). As such, further study of these geographic and behavioral variables is recommended to increase the utility of these findings for regional best industry practices.

The association of high CsRV1 loads with crab mortality in aquaculture has implications for release of virus into the environment from aquaculture, particularly from flow-through systems. The 212 metric tons of heavily infected dead peelers estimated as discards from this study represents over 800,000 diseased crabs, which are potentially discarded into the Chesapeake Bay annually. This concern is supported by a prior study that documented elevated CsRV1 prevalence in blue crabs close to flow-through shedding facilities (Flowers et al., 2018). Although the transmission route of the virus remains unknown, many viruses in decapod crustaceans remain infective in carcasses (Oidtmann et al., 2018). Replacing flow-through systems with recirculating aquaculture and/or conscientious land-based disposal of dead crabs would interrupt the flow of CsRV1 to uninfected wild crabs, to the benefit of the fishery, watermen, and the environment.

Chapter 3: Discussion & Synthesis

Hypotheses Evaluation Summary

Soft Shell Crab Aquaculture Mortality

The levels of mortality observed in soft shell crab shedding systems in Maryland, Virginia, and Louisiana examined in this thesis are consistent with levels observed in prior studies in other locations and years. The 22% average mortality observed in Chesapeake state facilities is almost identical to the 23% level observed in North Carolina by Chaves & Eggleston (2003). Lower mortality at Louisiana sites surveyed (9 and 20%) still remains within the range of 10-50% derived from cursory studies from Chesapeake and North Carolina facilities, although the statistical power of those studies is limited (Rose 2002; Bowers et al. 2010; Chapter 2/Spitznagel et al. 2019). With USDA and NOAA defining soft-shell crab shedding as aquaculture industry, it is thus important to understand the efficiency of such industry on the national level. Given a national peeler catch of approximately 1800 mt, this would suggest that as much as 400 mt of peelers die before molting successfully in soft shell crab shedding facilities. The millions of dollars of value thereby lost to watermen and the industry as a whole highlights the need to minimize crab aquaculture mortality rates.

The findings of this study support the hypothesis that recirculating aquaculture systems experience lower mortality than flow-through systems, as mortality in recirculating systems was half that of flow-through systems. A possible reason may be that well-controlled recirculation and water quality increases the stability and regulation of aquaculture environment parameters, particularly dissolved oxygen and nitrite (Ogle et al. 1982, Manthe et al. 1994, Oesterling 1995, Tavares et al. 2017). This is supported by

my observations that the only mass mortalities during this study were associated with hypoxic events and toxic nitrite levels at flow-through shedding facilities or those with poorly controlled biofilters. Increased temperature range in certain flow-through sites, while not associated with crab mortality, is a further indicator of the better environmental control provided by recirculation, although shading and thermally regulating water supplies can be used to stabilize temperature in either system type. Overall, the gain in value from peelers molting in recirculating rather than flow-through systems should offset the cost of building and operating systems with recirculation and enhanced biofiltration (Tavares et al. 2017). There are no specific economic analyses of the economic trade-off between shedding system types and crab shedding mortality in publication, however, and should be pursued in future research, outreach, and demonstration projects. Preferential adoption of well-monitored recirculating systems in soft shell crab aquaculture is clearly indicated by this study.

Published observations of relationships between peeler mortality and crabs per tank, source of peelers, and crab sex (Chaves & Eggleston 2003) were not statistically supported nor contradicted by this study. The sampling scheme for mortality in this study was not conducted in such a way that sex could be analyzed as a mortality factor. Accomplishing this would require studies in which watermen sorted every crab in representative tanks by sex. While not specifically detailed in Chapter 2, no significant relationships were found between peeler mortality, mean number of crabs per shedding tank, and stocking of facilities with crabs caught or bought by shedders, unlike the findings of Chaves & Eggleston (2003).

A potential threshold relationship between mortality and salinity was indicated by two findings. The salinity-mortality relationship described in Chapter 2 indicates a threshold near 7 ppt below which mortality drops to <20%, based on data from both Chesapeake and Louisiana sites. This is similar to a 2002 North Carolina shedding study (Rose 2002), but potentially contradictory to a limited study on survival of stage 7-16 juvenile *Callinectes sapidus* (1-110 mm carapace width) that observed lower survival and molting at 5 ppt than 25 ppt (Chazaro-Olvera & Peterson 2004). It should be kept in mind that many unmeasured factors may correlate with salinity in this study, including geography or hydrology of peeler harvest regions. It is possible that these factors played a greater direct role than salinity in driving mortality patterns. In order to improve understanding of the optimum harvest and aquaculture conditions for soft-shell crab production, future wild and peeler crab surveys should seek to incorporate enough replication and power for geospatial and hydrological analyses to be applied.

CsRV1 in Soft Shell Aquaculture

Prior observations that a high proportion of dead peelers from the Chesapeake Bay are intensely infected with CsRV1 (Bowers et al. 2010, Flowers et al. 2016a) were supported with high statistical power by evidence from this study. In Maryland and Virginia, overall prevalence in dead peelers (75%) was three times that in live soft crabs, while heavy infections were nine times more common in dead peelers (62%) than in live soft crabs. Simultaneously, findings within this study shed light on how CsRV1 may get into aquaculture crabs. The 33% CsRV1 prevalence found in freshly harvested peelers from this study is within the prevalence range observed in wild hard crabs by Flowers et al. (2016a). This CsRV1 prevalence in live peelers is also equivalent to the 35%

aggregate CsRV1 prevalence in live soft crabs and dead peelers, suggesting that all CsRV1 infections in aquaculture crabs pre-existed in harvested crabs. This would mean that harvest location controls initial CsRV1 prevalence, while aquaculture conditions influence progression of infections to higher intensity and death (Fig. 3.1).

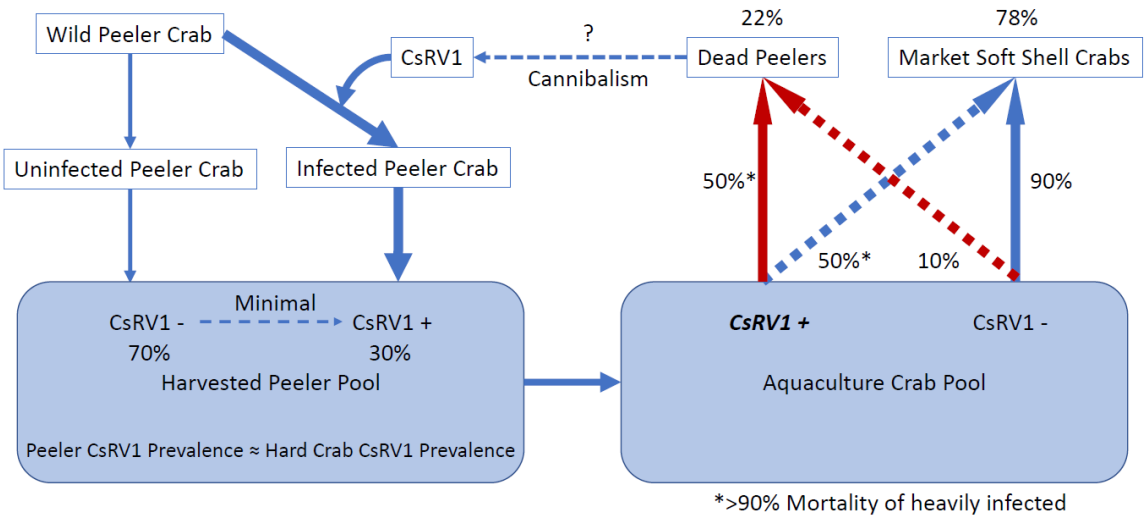


Fig. 3. 1. Model for how peeler crabs and CsRV1 travel and partition through soft-shell crab harvest and aquaculture. Crabs **g**enerally acquire CsRV1 prior to harvest from the wild, although some may acquire it during harvest and transport phases. Once harvested, peelers brought into soft-shell aquaculture, where CsRV1 infections amplify until peelers die or molt. Output crabs either exit as marketable soft crabs (78%) or are cycled back into the environment as dead peelers (22%) typically discarded into estuaries or used as bait, both of which may promote CsRV1 transmission. While 50% of all CsRV1-infected peelers die in aquaculture; among peelers with heavy infections, mortality increased towards 90-95%.

Natural osmotic, hormonal, metabolic, and starvation stresses of molting might all plausibly increase susceptibility of peelers to CsRV1 infection compared to hard crabs (Mangum et al. 1985, Kennedy & Cronin 2007, Techa & Chung 2015), as could aggressive interactions or injury during molting (Ryer et al. 1997) and in or around crab traps (Sturdivant & Clark 2011). Studies comparing physiological parameters in molting and hard crabs with or without CsRV1 infection in identical environments would help to determine which explanation is most likely. Regardless, this study confirms that CsRV1 is tightly associated with aquaculture peeler death, highlighting the need for further study of a disease linked to the loss of millions of crabs and industry dollars annually.

Rogers et al. (2015a,b) found a lower prevalence and intensity of CsRV1 infection in Louisiana than in the Chesapeake Bay, with 5% of all crabs and 15% of dead peelers infected with CsRV1. CsRV1 survey results from this study largely confirm their findings, and also show high variability as previously observed (Flowers et al. 2018). The Dulac shedding facility had 39% heavy CsRV1 infection prevalence in dead peelers sampled in 2016, eight times higher than in any other recorded sample from Louisiana aquaculture. CsRV1 infection in dead peelers at Dulac subsequently declined to 6% in 2017, indicating that temporal and geographic variability observed in infection of Chesapeake Bay aquaculture crabs may also apply to Louisiana aquaculture crabs. As such, loss of peelers attributable to CsRV1 in Louisiana may be less trivial than previously described by Rogers et al. (2015a,b).

Risk Factors for CsRV1 Peeler Infection

In contrast to the fishery-independent survey results of Flowers et al. (2018), this fishery-based study did not find any crab characteristics to correlate with CsRV1 infection intensity. This may be expected because peeler and hard crab catch in the fishery is prone to fishery-dependent bias and clustering, and thus is not representative of the total crab population. While prevalence of CsRV1 infections was significantly higher among intact dead peelers (85%) compared to those that had busted (70%), no difference in CsRV1 virus load was detected between infected crabs in these two groups. No apparent physical or behavioral differences attributable to CsRV1 infection were observed in infected crabs, although more subtle differences may exist. Instead, these findings support the working model that interactions during or prior to capture are how CsRV1 is introduced to aquaculture crabs, with aquaculture stress acting to modify the intensity of infections.

Salinity appears to be a potential CsRV1 prevalence risk based on *post hoc* analysis, but the complex relationship between salinity and CsRV1 intensity seen in mixed modelling requires further specific study. Random date and location carried much greater statistical weight than salinity in the final model of CsRV1 intensity. Furthermore, CsRV1 intensity in dead crabs decreased with higher salinity once time and site were factored in GLM, unlike the prevalence pattern. This suggests that specific unstudied factors related to time and site exert influence on CsRV1 infection patterns, whether or not these factors covary with salinity. Future studies on the effect of salinity on the lethal dose and time course of CsRV1 infection in laboratory settings should be conducted to clarify these apparent data conflicts. As part of these studies, the relationship between CsRV1 infection and how much time crabs spent outside of acceptable water quality

thresholds should be examined. The prevalence of other blue crab diseases has been linked to crab time spent in extreme salinity and temperature conditions (Sullivan & Neigel 2018), and thus it would be logical to examine whether this effect applies to CsRV1 infection.

A pivotal indication of this study is that CsRV1 infection prevalence in soft-shell aquaculture can be explained solely by CsRV1 prevalence in wild crab harvest, with no significant transmission evident during the commercial shedding process. The fastest progression from initial infection to mortality observed in prior CsRV1 injection trials was nine days (Bowers et al. 2010), while trials and surveys conducted during this thesis study indicate that <1% of peelers spend this long in aquaculture. Therefore, it may be that CsRV1 time to mortality is too long for the disease to kill in aquaculture. Peeler aquaculture crabs are a subset of wild harvest crabs, which in turn are expected to be represented within the crab samples found by independent surveys. Stressors that change the infection risk and survival of all wild crabs should therefore change the infection risk and survival of peelers.

There is a need to understand whether crab distribution and movement or other factors underlie the results of CsRV1 infection risk modelling. In such modeling, the strongest variables that predicted CsRV1 infection were random date and location effects, concurring with published fishery-independent study results (Flowers et al. 2016a; 2018). It seems highly unlikely, however, that elevation of CsRV1 infection in crabs from certain times and places is truly random. At least three non-random CsRV1 prevalence drivers can be envisioned. First, behavior and movement of crab populations alters likelihood of crabs interacting with each other or infection vectors (Lafferty et al. 2004,

Daversa et al. 2017). Second, hydrodynamics might control infection spread as well, since distribution of host, pathogen, and potential environmental reservoirs over time are more accurately predicted when flow of water and what is in it are accounted for (Viljugrein et al. 2009). Finally, the distribution of where infected bycatch or dead peelers are discarded may be a driving factor behind the observations of Flowers et al. (2018), since the elevated prevalence of CsRV1 in crabs near shedding sites is plausibly linked to infectious discards or effluents. Future study should work to integrate these factors into modelling CsRV1 infection throughout the blue crab population.

Future Directions

CsRV1 in Aquaculture Recommendation Summary

The relevance of CsRV1 to the entire United States soft-shell blue crab industry indicated by this study leads to recommendations to minimize spread of CsRV1 and avoid crab aquaculture stresses that amplify CsRV1. Recirculating aquaculture with good biofiltration with monitoring of water quality and crab mortality should be adopted as the industry standard. While the time and place of peeler harvest factored more in CsRV1 infection rates than water quality, control of dissolved oxygen, nitrite, salinity, and other factors should not be neglected as ways to improve harvest and eliminate mass mortalities. Disposal of peeler mortalities from aquaculture should be restricted to land-based end points to reduce potential CsRV1 transmission to wild crabs, particularly in the Chesapeake Bay where two-thirds of dead peelers were heavily infected. Finally, more intricate studies of factors that correlate with wild CsRV1 infection prevalence such as hydrology, fisheries practice, and movement of crab population should be conducted,

since it appears that infection in the wild peeler harvest is a major factor in CsRV1-associated death in the soft-shell blue crab industry.

Peeler Catch Reporting

A more accurate estimate of the economic impact of peeler mortality will require a rewrite of catch and aquaculture data and consistent measurement of peeler mortality. Major discrepancies between peeler catch numbers reported by state and federal agencies were discovered during this study. Data available through NOAA NMFS underestimates annual Maryland peeler and soft shell catch by ~600 fold as compared to MD DNR records. Depending on whether state or national estimates of the value per pound are used, the revenue from Maryland peelers is between $25 million and $60 million annually, or 45% to 110% of the value of NMFS reported hard crab landings in the state. Correction for this would raise national peeler catch estimates by three-fold, indicating that NOAA records greatly underestimate the significance of the soft-shell crab industry. Any similar national underreporting of other state records should be determined in future inquiry. One of the major causes of this discrepancy is that most soft crab aquaculture businesses, including those participating in this study, consist of three direct employees or fewer. Catch from such business is not counted in ACCSP and NOAA data records to protect small business privacy, even when these businesses may be collecting catch from dozens of crabbers (MD DNR, pers. comm.). Reformulating these filters should be evaluated as a solution to the need for accurate reporting of this or any other small business-driven industry at the national level. Furthermore, these and other errors continue to be propagated up through global recording and documentation. For example, the FAO blue crab aquaculture statistics indicate that there was no soft crab production

from 1950-1995 and 2005-2014, while the 1997-2004 records showing <500 mt total are low in light of the findings from MD DNR (FAO 2018). If national or international decisions are going to be made based on the FAO records, then immediate updating and correcting of these data is important.

Impact of CsRV1 on Wild Blue Crab Population and Fishery

While this study provided a wealth of new data on the impact of CsRV1 in blue crab aquaculture, the total impact of CsRV1 on wild blue crab populations remains totally unknown. CsRV1 infection of captive crabs is shown to cause mortality, but whether it significantly contributes to or increases natural mortality in wild crabs is a crucial unknown. Furthermore, if CsRV1 prevalence in shedding is dependent on prevalence in the wild as hypothesized, then it is desirable to know how lethal CsRV1 is in the wild and how that lethality varies. CsRV1 prevalence estimates exist for crab populations in the northeastern seaboard of the United States, Louisiana, and scattered locations elsewhere (Rogers et al. 2014a; 2014b; Flowers et al. 2016a; 2018).

This knowledge gap can only be filled by two types of studies, both involving tag-recapture approaches. The first involves tagging thousands of crabs that are live sampled for CsRV1 and released into the wild. Once those crabs are recovered by researchers or fishermen alive, they can be resampled for CsRV1 to compare change in virus titer to survival and growth or simply recorded as surviving crabs, accounting for additional stressors and uncertainties in the process. Some of these uncertainties will be studied in the second series of experiments, where large random samples of CsRV1-screened crabs from different regions would be kept in mesocosms that simulate their source habitats and traps. These closed, controlled mesocosms consist of large enclosures that allow non-

quarantine interaction with other crabs as per Sturdivant & Clark (2011). Proportions of peeler and hard crabs will or can be subsequently infected with CsRV1 as necessary to simulate different viral prevalence levels. Subsequent tissue sampling and mortality monitoring of CsRV1-naïve crabs will be used to establish transmission, prevalence, and mortality estimates. Drawing from existing blue crab stock models (e.g. CBSAC 2018), these CsRV1 parameter estimates could be used to determine the relevance of the disease in the wild. Both open or semi-contained mark-recapture study and mesocosm experiments should be pursued to boost our understanding of CsRV1 dynamics in the wild.

Conclusion

Studies in this thesis confirm the high prevalence of CsRV1 in soft shell crab aquaculture and the significant association between death and CsRV1 infection. Particularly in the Chesapeake Bay, it is reasonable to assert that CsRV1 contributes to millions of dollars of soft crab value lost. As such, measures such as discard control and use of recirculating systems should be adopted to limit both the spread of disease and crab mortality in general. An array of data gaps hindering our understanding of how valuable the soft crab industry is and how much value is thus lost due to CsRV1 must be addressed in the future. In particular, rectifying national and international production records, investigating wild CsRV1 infection and mortality patterns, and linking more specific environmental factors to CsRV1 dynamics in wild and aquaculture populations are all necessary to improve our comprehension of the soft crab industry and how it is affected by disease.

Appendix I. Transmission Trials

Three trials were conducted to try to determine how CsRV1 is naturally passed from crab to crab. Experiments 1 and 2 were done at IMET by Matthew Spitznagel to investigate cohabitation and single-dose cannibalism, respectively. Experiment 1 (cohabitation) was run in May 2016, while Experiment 2 (cannibalism) was run in August 2016. Experiment 3 was a repeated dose cannibalism trial conducted at LSU AgCenter by Dr. Julie Lively and lab members, initially conducted between June and September 2017.

CsRV1 infected and PCR-negative donor crabs were identified by the RT-qPCR assay described in Chapter 2. For Experiment 1, CsRV1 negative crabs were collected from Dundalk on May 24, 2016, while CsRV1-positive crabs with >2 x 10^6 genome copies per mg muscle were sourced from Deal Island, MD on May 22, 2016. CsRV1-negative and positive crabs were collected from Tilghman, MD for Experiment 2; positive donors collected on August 10, 2016 and negative donors on August 4, 2016. Experiment 3 CsRV1-negative crabs were identified from Violet, LA crabs sampled on June 23, 2016, with CsRV1-positive crabs were sourced from Dulac, LA collected June 1, 2016.

In Experiment 3, muscle from donor crabs or CsRV1-negative squid was dissected out, homogenized, solidified with alginate, and distributed into 5 g aliquots according to protocols in the Lively lab and described in a master's thesis (Clowes 2016). Positive injection control trials were conducted using filtrates of homogenized muscle in sterile saltwater with and without alginate to confirm that no materials decreased viral potency. Negative injection control trials were conducted using alginate in saline to

confirm that no materials aside from CsRV1 were toxic. All crabs and prepared exposure tissue were stored at -20°C when not being prepared or actively used.

Live crabs for all trials were large juvenile and adult hard crabs collected by trotline, individually wrapped in wet fabric during transport to minimize interactions, and pre-screened for CsRV1 using RT-qPCR on hemolymph samples before division into experimental groups (Figure A.1.). All crabs were maintained in 25°C and 15 ppt artificial seawater under constant aeration. Daily squid or shrimp feeding and a 12h:12h day:night cycle were maintained for experimental durations. Full water changes were conducted daily to limit nitrogenous waste and turbidity.

In Experiment 1, wild fishery-caught crabs were obtained from Florida and held in individual aquaria as "recipients". A whole dead crab (either carrying CsRV1 or not) was placed into the individual tank of each recipient live crab for 90 minutes. No recipient crabs were observed feeding during exposures in this trial, although some crabs manipulated dead donor material. After the transient exposure, recipient crabs were kept for 15 days in individual tanks with daily water changes. After this time, hemolymph was withdrawn and tested for the presence and quantity of CsRV1 using the qPCR assay methods of Flowers et al. (2016a) to detect change in CsRV1 titers. At 30 days, all crabs were sacrificed and muscle tissue was sampled. Muscle was also sampled from any crabs that died during the 30 duration of the experiment. No change in CsRV1 over time was detected by RT-qPCR on samples from PCR-negative live crabs in the CsRV1-exposed trial group (n = 20), nor was any significant difference in titer change noted between exposed and unexposed (n = 20) groups (Figure A.2.). Mortality was trivial and not

statistically different between exposure groups. Positive controls for this experiment consisted of recipient crabs injected with homogenized muscle from donor crabs that had >10e6 CsRV1 genomes per mg tissue filtered into saline. Experiment 1 CsRV1-positive dead crabs all died within 30 days and contained >10e7 CsRV1 per mg.

In Experiment 2, infected crab material was fed to recipients. Ingestion doses were prepared from 1 g of crab muscle and fed to PCR-negative crabs. Hemolymph was sampled just prior to feeding (t_0) and at 7, 13, and 21 days after experiment tissue feeding, with all remaining crabs sacrificed at 28 days for a final muscle tissue analysis. No difference in CsRV1 infection status was observed between crabs fed virus-laden muscle (n=19) and those fed negative control muscle (n = 23; Student's t, d.f. = 16.97, t = -0.6044, p = 0.2768), with no apparent virus acquisition in either group. Positive control crabs injected with the 0.2 micron-filtrate of homogenized CsRV1-positive muscle died within 21 days with heavy CsRV1 infections. Crabs injected with similarly prepared filtrate of CsRV1-negative muscle did not develop infections or die.

The seven-day CsRV1 feeding experiment (Experiment 3) analyzed hemolymph and muscle tissue at t_0, time of mortality, and 28 days after completion of the exposure period, at which time crabs were sacrificed. The infectivity of viral preparations was verified by directly injecting filtrates of the homogenized muscle (n = 19) and filtrates of alginate-mixed homogenate (n = 18) into control crabs. Positive control crabs experienced >80% mortality by 25 days, while negative control crabs experienced only one mortality on day 27 (Figure A.3.). Only two mortalities were observed in crabs fed virus-laden food (n = 30), which was not significantly different from mortality in the non-virus control group (n = 26). No significant difference was observed in CsRV1 titer

change between CsRV1-crab and negative squid-fed groups, with significant difference in titer change of both compared to injection control groups (ANOVA with Tukey's HSD, d.f. = 3, total F = 34.18, pairwise $p_{crab\text{-}squid} = 0.6374$). However, four previously CsRV1 PCR-negative crabs fed CsRV1-infected muscle did acquire CsRV1 infection with intensities higher than 10^7 genome copies per mg muscle. Of these, one crab subsequently died before completion of the trial, the first reported instance of CsRV1-linked mortality during an infection trial that used a natural mode of infection.

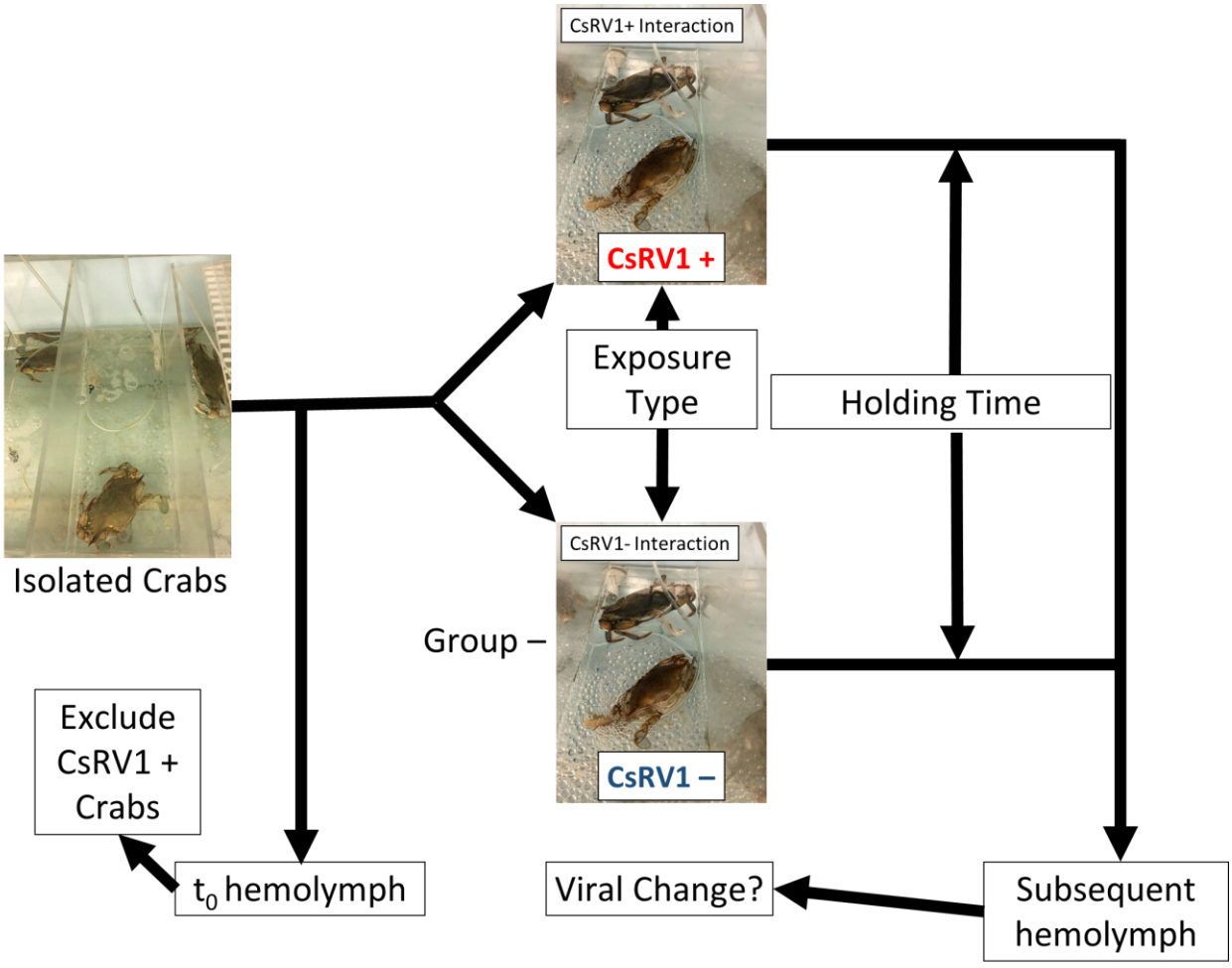


Fig. A1. 1. Design flowchart for CsRV1 transmission experiments in blue crabs. Wild crabs were acclimated to isolation enclosures and screened for pre-existing CsRV1 using a RT-qPCR assay prior to start of experiments. Crabs were then divided into CsRV1 negative and positive experimental groups and exposed to infected crabs or tissues. Holding periods of up to 35 days followed, during which time mortality was recorded and CsRV1 sampling repeated from hemolymph or muscle tissue to detect change in viral titers. Proximity interaction experiments are depicted in photographs.

In the short-term proximity exposure trial, no crabs were observed feeding during exposures in this trial. Non-lethal hemolymph samples were taken 15 days after exposure

to detect change in CsRV1 titers, followed by sacrifice and post-mortem muscle sampling of all crabs at 30 days. No change in CsRV1 over time was detected by RT-qPCR on samples from PCR-negative live crabs in the CsRV1-exposed trial group (n = 20), nor was any significant difference in titer change noted between positive and negative (n = 20) exposure groups (Figure A.2.). Mortality was trivial and not statistically different between exposure groups.

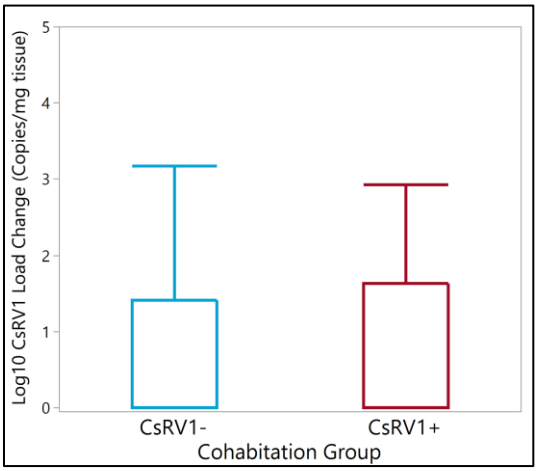
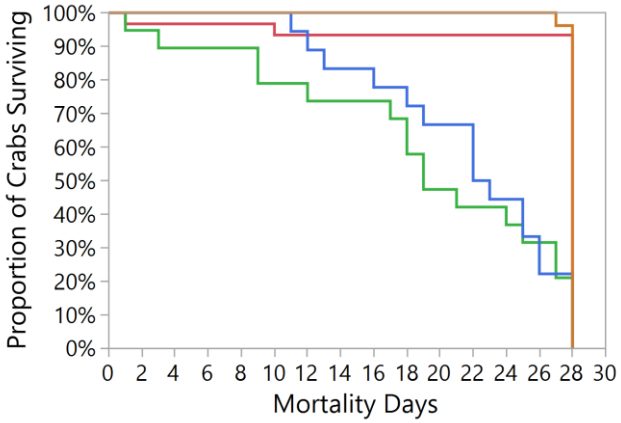
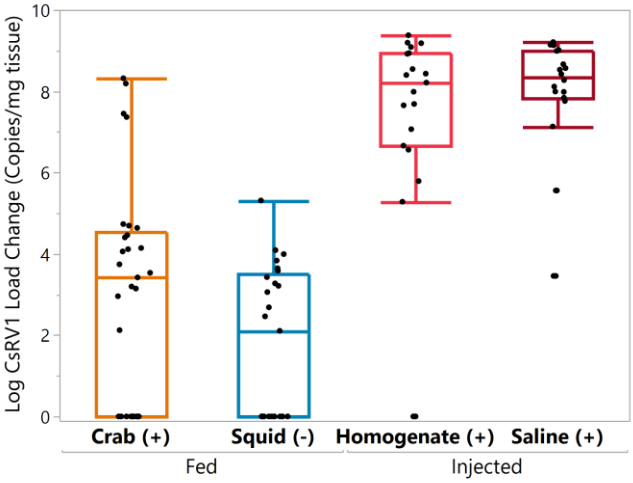


Fig. A1.2 Change in CsRV1 load of crabs exposed (virus+) or not exposed (control) to CsRV1-infected dead peelers. Crabs were exposed to donors for 90 minutes, then maintained in aquaculture for an additional 7 days. No increase in CsRV1 load was observed in crabs exposed to virus-laden dead peelers, nor was a difference between virus and control donor group titers observed (n= 20 each).



A)



B)

Fig. A1.3. Results of multiple-dose ingestion transmission trial. A) Multiple-dose ingestion trial mortality curve. No significant difference in mortality between CsRV1-negative squid (n = 26) and CsRV1-positive crab (n = 30) fed groups was detected. Crabs injected with virus filtered from homogenized pre- (n = 19) or post-alginate (n = 18) treatment crab material experienced mortality of >80%. B) Changes in CsRV1 load observed from t_0 to mortality or sacrifice on t_{28} in each experimental group. All but five injection control crabs developed a similarly intense level of CsRV1 infection (>10^6 genomes per mg tissue on RT-qPCR). There was no significant difference in CsRV1 load

change between crabs fed squid and crabs fed infected crab (Dunn, $p = 0.5179$), but four CsRV1-exposed crabs did develop infections of $>10^6$ genomes per mg tissue.

Appendix 2. Supplemental Figures to Aquaculture Field Survey

Table A.1. Record of sites, dates, water quality, system type, crab mortality, and CsRV1 prevalence data from surveyed Chesapeake soft shell crab aquaculture sites. Live soft and peeler crab sampling was progressively introduced to the experiment, and were not sampled at all sites.

State	Site	Starting Week of Sampling	System Type	Mortality (%)	Crabs per Float	Peak Temperature (°C)	Salinity (psu)	Mean Temperature (°C)	Dead CsRV1 Infection (%)	Live Crab CsRV1 Infection (%)	Peeler Crab CsRV1 Infection (%)	Temperature Range (°C)
VA	West Point	5/6/2016	Recirculation	15	88	22.2	7	19.4	0	0		2.8
VA	West Point	7/18/2016	Recirculation	9	15	31.1	4	28.9	50	25		2.2
VA	West Point	9/3/2016	Recirculation	12	10	29.4	3	26.1	10	0		3.3
VA	Sarah's Creek	5/9/2016	Flow-through	15	53	31.1	18	19.4	100			11.7
VA	Sarah's Creek	7/17/2016	Flow-through	21	35	37.2	12	28.3	92			8.9
VA	Sarah's Creek	9/9/2016	Flow-through	28	44	35	19	26.7	73			8.3
VA	Chuckatuck Creek	5/9/2016	Recirculation	31	125	25	15	20.5	85	8		4.4
VA	Chuckatuck Creek	7/15/2016	Recirculation	12	19	33.3	15.5	30	83	25		3.3
MD	Pasadena	6/28/2016	Recirculation	35	16	27.2	11	25	87			2.2
MD	Pasadena	8/18/2016	Recirculation	15	33	28.3	12	25.6	50	46		2.8
MD	Patuxent	6/4/2016	Recirculation	10	28	26.1	10	22.8	100			3.3
MD	Patuxent	8/17/2016	Recirculation	10	67	31.1	6	26.7	67	8		4.4
MD	Tilghman	6/15/2016	Flow-through	28	141	26.7	15	24.4	75			2.2
MD	Tilghman	8/2/2016	Flow-through	40	100	30	15	28.3	67	20		1.7
MD	Tilghman	9/14/2016	Flow-through	48	50	25.6	18	23.9	82	25	52	1.7
MD	Rock Hall	6/20/2016	Recirculation	6	35	24.4	8	23.9	56			0.6
MD	Rock Hall	8/21/2016	Recirculation	9	50	27.8	11	25.6	64	57		2.2
MD	Pasadena	8/19/2017	Recirculation	34	20	27.2	8	24.4	92	42	25	2.8
MD	Philips Wharf	7/11/2017	Flow-through	50	40	32.2	10	29.4	89	31		2.8
VA	Chuckatuck Creek	7/26/2017	Recirculation	15	38	31.7	20	29.4	92	17	0	2.2

Table A.2. Record of sites, dates, water quality, system type, crab mortality, and CsRV1 prevalence data from surveyed Louisiana soft shell crab aquaculture sites. Live soft and peeler crab sampling was progressively introduced to the experiment, and were not sampled at all sites. Mortality was not sampled in 2017.

Site	Week of Sampling	System Type	Mortality (%)	Peak Temperature (°C)	Salinity (psu)	Mean Temperature (°C)	Temperature Range (°C)	Crabs Sampled (Dead/Live)	Dead Crab CsRV1 Infection (%)	Dead Severe Infection (%)
Dulac	6/1/16	Recirculation	9	30	3	26.1	1.9	28/0	57	39
Franklin	8/31/16	Recirculation	20	30.6	0	29.4	1.1	22/0	5	5
Dulac	6/8/17	Recirculation	N/A	32.8	2	27.8	5	16/22	6	0
Franklin	11/19/17	Recirculation	N/A	23.3	1	21.1	2.2	16/31	0	0

Table A.3. Full generalized linear mixed model (GLMM) with potential effects on crab mortality and CsRV1 infection intensity.

Model	Predictor Variable	Estimate	Standard Error	p-value	Variance	Standard Deviation
	Fixed Effects				Random Effects	
A. Crab Mortality (%) ~ System Type + Crabs per Float + Salinity (psu) + Month + Year + Mean Temperature (°C) + Dead Crab CsRV1 Prevalence (%) + Temperature Range (°C) + (1|Site) d.f. = 9, AIC = 148.25	System Type (Recirculation)	-24.46	9.942	0.0695		
	Crabs per Float	0.0490	0.0808	0.5583		
	Salinity (psu)	-0.5443	0.7074	0.4579		
	Month	3.478	2.097	0.1340		
	Year	2.003	6.617	0.7690		
	Mean Temperature (°C)	-1.6875	0.8741	0.0877		
	Dead Crab CsRV1 Prevalence (%)	0.1963	0.1368	0.1791		
	Temperature Range (°C)	-2.8836	1.5379	0.1122		
	Location				93.64	9.68
	Intercept	-3951	13340	0.7738		
B. Log(Crab CsRV1 Load) (genomes/mg) ~ System Type + Mean Temperature (°C) + Salinity (psu) + Sex + Successful Molting + Injury + (1|Site) + (1|Date) d.f. = 513, AIC = 2573.41	System Type (Recirculation)	-0.5724	1.007	0.5739		
	Mean Temperature (°C)	-0.0338	0.0599	0.5747		
	Salinity (psu)	-0.1085	0.0695	0.1207		
	Sex (Male)	-0.5331	0.3064	0.0824		
	Successful Molting	-3.2752	0.3244	<2E-16		
	Injury	0.4574	0.2502	0.0681		
	Location				2.99	1.73
	Date				1.40	1.18
	Intercept	7.893	3.197	0.0156		

C. Log(Dead Crab CsRV1 Load) ~ System Type + Mean Temperature (°C) + Salinity (psu) + Sex + Injury (1|Site) + (1|Date) d.f. = 316, AIC = 1604.33	System Type (Recirculation)	-2.566				
	Mean Temperature (°C)	-0.1046				
	Salinity (psu)	-0.2606				
	Sex (Male)	-0.6106				
	Injury	0.2734				
	Location				6.63	2.58
	Date				1.35	1.16
	Intercept	14.05	3.947	7.04E-04		

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