Investigating conspecific CsRV1 transmission in *Callinectes sapidus*

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Highlights

- Single per os exposure with infected muscle did not result in detectable transmission.
- Repeated per os exposure resulted in transmission in 11% of individuals.
- The use of an alginate enabled a single source of virus to be over time.

Abstract

A reo-like virus, CsRV1, is found in blue crabs, *Callinectes sapidus*, from every North American location assessed, including Chesapeake Bay and the Atlantic and Gulf coasts, USA and associated with blue crabs in softshell production. CsRV1-associated crab mortality is prevalent in captive crabs, but it is still unknown how CsRV1 is transmitted. The purpose of this study was to examine the role that conspecific predation or scavenging may play in per os transmission in single exposure and repeated exposure experiments. For viruses without cell culture propagation, repeated exposure experiments have the challenge of presenting the virus consistently during the experiment and across time replicates. In a single-exposure experiment, none of the crabs fed muscle tissue of crabs carrying intense infections of CsRV1 developed CsRV1 infections. In a repeated-exposure trial, using infected muscle tissue prepared in alginate blocks, CsRV1 was detected in 11% of the crabs fed infected tissue but was not significantly different from the control group fed alginate lacking CsRV1. For repeated per os exposure experiments, the study demonstrated the utility of using alginate to present the same homogenous sample of virus, both injected and per os, over time for oral challenge experiments. Conspecific predation and scavenging could be a transmission route, but future work into this and other possible routes of transmission for CsRV1 is important to better understand the role this virus plays in wild crab populations and the soft-shell crab industry.

Keywords: CsRV1, reo-like virus, transmission, blue crabs, conspecific spread

1. Introduction

One of the most ecologically and economically important crustaceans in the United States is the blue crab, *Callinectes sapidus* (Rathburn, 1896). It is distributed from New England to the Gulf of Mexico and is fished both commercially and recreationally (Guillory and Perret, 1998; Millikin, 1978). A blue crab's epibenthic lifestyle and voracious feeding habits help regulate the environments in which they live along with the populations of organisms on which they feed.

Blue crabs commonly consume bivalves, fish, snails, live plants, crustaceans, dead animal matter and are also cannibalistic in nature (Hines, 2007; Hines and Ruiz, 1995). In the United States, the blue crab fishery has an annual dockside value of $200 million (National Marine Fisheries Service, 2020). In the Gulf of Mexico and Chesapeake Bay regions, commercial fisheries employ thousands of fishermen targeting the species (Louisiana Department of Wildlife and Fisheries, 2020; Virginia Marine Resources Commission, Personal Comm.). Many more are employed in nearshore activities supporting crab harvesters and retailers. Blue crab populations fluctuate due to a number of potential influences including fishing pressure, recruitment success and natural mortality (Hines and Ruiz, 1995; Rome et al., 2005). The role of pathogens in blue crab natural mortality is often ignored yet blue crabs are a host for a wide variety of pathogens, some of which are known to cause significant mortality (Bonami and Zhang, 2011; Lee and Frischer, 2004; Messick and Shields, 2000; Rogers et al., 2015; Shields and Overstreet, 2007; Spitznagel et al., 2019).

In the 1970s and 1980s, there were reports of a "reo-like virus" (then termed RLV, now *Callinectes sapidus* reovirus 1, CsRV1) associated with mortalities of captive blue crabs (Johnson, 1984; Johnson, 1977). Bowers et al. (2010) demonstrated that injection of CsRV1 is fatal to blue crabs in two to three weeks under laboratory conditions, and subsequent studies revealed that wild crabs carrying natural infections of CsRV1 also die within two to five weeks in captivity (Bowers et al., 2010; Johnson, 1977). CsRV1 is found in hard (intermoult) crabs from every location assessed in the United States, including Chesapeake Bay and the Atlantic and Gulf coasts (Bowers et al., 2011; Flowers et al., 2013; Flowers et al., 2016b; Rogers et al., 2014; Spitznagel et al., 2019). The virus causes destruction of the hemopoietic tissue and young hemocytes, with neurological damage most likely being the cause of death (Johnson, 1977; Tang et al., 2011). The impact of CsRV1 on wild crab populations is unknown, however its presence in over 50% of pre-molt mortalities in crabs held under aquaculture conditions suggests it is responsible for a considerable fraction of the overall mortality observed (Bowers et al., 2010; Spitznagel

et al., 2019). The captive (artificial) environment is stressful to blue crabs, especially during ecdysis, and in combination with high CsRV1 loads this likely contributes to mortality (Ary Jr and Poirrier, 1989; Spitznagel et al., 2019). However, the molting process is inherently stressful for crabs, and the natural level of background mortality of wild blue crabs during the molt remains a largely unknown proportion of natural mortality (Hewitt et al. 2007).

Few studies have been done on the factors linked to CsRV1- associated crab mortality (Flowers et al., 2016b; Spitznagel et al., 2019), and it is still unknown how CsRV1 is transmitted. Previous studies analysing wild blue crabs sampled near (<200 m) and far (>2 km) from flow-through shedding facilities in Chesapeake Bay documented the probability of detecting CsRV1 in crabs close to shedding facilities to be up to 78% higher than in crabs sampled from far sites (Flowers et al., 2018). In these flow-through systems, mortalities are often discarded into the adjacent waterway, which could introduce CsRV1 by two routes: via discarded crabs and free virus particles present in flow-through effluent. With wild prevalence values varying from 0 – 60% in hard crabs and 33.3.% in live freshly harvested pre-molt crabs (Spitznagel et al., 2019), determining the routes of transmission would increase understanding the role this virus plays in natural blue crab populations and the potential for effluents or discards to affect virus prevalence.

A better understanding of CsRV1 transmission routes can lead to improvements in blue crab shedding and wild capture practices, as both activities dispose of undersize, sick or dead animals back into the estuary. The purpose of this study was to examine the role that conspecific predation or scavenging may play in transmission. The collection of crabs in Louisiana for use in the bioassay also provided an opportunity to investigate the prevalence of CsRV1 in the natural population at several time points. Furthermore, this study provided an opportunity to examine the utility of using alginate to address the problem of using one batch of virus for an experiment over time for viruses without cell culture options for multiple transmission routes (injected and *per os*).

2. Materials and Methods

2.1 Single Exposure to CsRV1

2.1.1 Collection and prescreening of single exposure crabs

Wild mature molt blue crabs (carapace width 103-179 mm) were harvested by commercial traps in August 2016 from the Choptank River within 2 km of Tilghman Island, MD, USA. Ambient collection site surface water conditions were 31°C and 14 ppt salinity. These crabs were placed in coolers with cloth-covered ice packs and transported to the Institute of Marine and Environmental Technology (IMET) in Baltimore, MD, where they were housed in individual 16 liter acrylic tanks and acclimated for 7 days in 15 ppt artificial sea water (ASW, de-chlorinated municipal water with the addition of commercial grade food salt produced in the IMET Aquaculture Research Center) at 25°C. Crabs were held under constant aeration and a 12h:12h day:night light cycle prior to pre-trial screening for CsRV1. Full water changes were conducted daily to maintain water quality. Each day, crabs were offered a mixed diet of shrimp and squid for 1 h and uneaten food was removed.

All crabs used in the single exposure experiment (described below) were sampled for existing CsRV1 infections (T0) two days prior to their exposure to CsRV1-infected donor muscle tissue. From each crab, 100 µl hemolymph was withdrawn from directly beneath the arthrodial cuticle connecting the merus of the swimming leg to the carapace, after cleaning with 75% isopropanol. The hemolymph sample was drawn directly into 300 µl anticoagulant (0.3 M sodium chloride, 0.1 M glucose, 26 mM citric acid, 2.5 mM EDTA in sterile water at pH 6.3). Hemocytes were pelleted at 2,000 g and saved for later RNA extraction and qPCR screening as described in Sections 2.3 and 2.4.

2.1.2. Single exposure per os

CsRV1-free and CsRV1-infected crabs with a CsRV1 load approximately 10^8 CsRV1 genome copies per mg muscle tissue were identified by RT-qPCR among recently-dead shedding system mortalities.

Mortalities had been dead less than 6 h before being chilled on ice and were maintained on ice no more than 24 h until feeding trials. Infectiousness of the fed tissue was verified by preparation of tissue filtrates (Flowers et al. 2016b) and injection into an additional 4 naïve crabs housed under similar conditions; negative control injections (n=5) were performed with filtrates prepared from CsRV1-negative crabs. Filtrate was prepared using infected crab tissue mixed with sterile ASW (15 ppt) at a ratio of 100 mg tissue to 2 ml ASW and vortexed for 2 min. The resulting suspension was centrifuged at 500 g for 5 min to sediment debris. The remaining supernatant was filtered through a 0.2 µm filter. The injection area was sterilized with a 95% ethanol solution. Using a tuberculin syringe (1cc, 27-gauge needle), 20 µl of freshly prepared virus suspension representing a challenge dose of 2×10^7 CsRV-1 genome copies was injected into the hemal sinus at the joint between the thorax and the 5th (swimmer) leg.

In order to determine if ingesting a single meal of CsRV1-infected crab muscle could lead to transmission of the virus, 1 g of CsRV1-infected or uninfected crab muscle (infected same muscle tissue as prepared or filtrate above and representing an oral challenge dose of at least 10^{10} CsRV-1 genome copies) was placed in the 16 L aquaria of individual crabs (n=29 fed uninfected and n=29 fed infected muscle tissue) for 1 h. The full or partial consumption of the donor tissue by each naïve crab (all mature males) was recorded, and crabs (n=10 CsRV1 treatment; n=6 uninfected treatment) that did not eat the tissue after 1 h were excluded from further analyses. After 1 h, naïve crabs were removed from their enclosure and a full water change performed. Crabs were then maintained and monitored for health changes for 28 days post exposure. Crabs still living after 28 days were sacrificed by ice immersion. On the day that crabs died or were sacrificed, a single walking leg was removed and preserved at -20°C for subsequent CsRV1 analysis.

2.2 Repeated exposure to CsRV1

2.2.1 Collection and prescreening of crabs for repeated exposure

Wild blue crabs were caught from 2 locations within Louisiana, USA. In June and August 2017, crabs (carapace width 86-155 mm) were caught at Island Road, Point Aux Chene (2-3 ppt; 29 °C). In September 2018, crabs (carapace width 80-114 mm) were caught at Lake Rd, Lacombe (2 ppt; 29 °C). Crabs were caught using dip nets and placed into coolers with ice covered in burlap for transport to Louisiana State University AgCenter. The crabs were held in individual 76 l polyethylene treatment tanks and allowed to acclimate 7 days to 15 ppt ASW (de-chlorinated municipal water with the addition of Instant Ocean® Sea Salt) at 22 °C.

Before the start of the bioassay (within the 7-day acclimation period), each crab was prescreened for CsRV1 as described above (2.1.1) with one walking leg removed, placed in a plastic bag, and preserved at -20°C.

2.2.2. Repeated exposure treatments

The challenge in repeated per os experiments is to provide dosing over time as virus can currently only be obtained from infected blue crab tissue, during the course of a treatment and across replicates. In order to provide a uniform dosing across time, we used alginate to create a solid block of homogenized tissue for the crabs. This allowed the CsRV1 homogeneous tissue to be frozen as a uniform mixture with fresh blocks prepared daily across replicates, and the same sample available to prepare injected filtrate across replicates. As previous work has not been done with alginate and virus delivery, we soaked the CsRV1 alginate block in saline and injected the saline to verify that the alginate process did not interfere with the virus. The four treatments were 1) Fed CsRV1 alginate block, 2) Fed Squid alginate block (negative alginate control), 3) Injected virus filtrate (positive control for viability of virus), and 4) Injected CsRV1 alginate-soaked saline solution (positive control for alginate process). For the infected crab alginate and virus filtrate preparations, infected muscle tissues from crabs previously identified to have a CsRV1 loads of 10^7 and 10^8 per mg fresh weight (Spitznagel et al., 2019) were homogenized with a food processor for 5 min and then frozen until use.

To prepare the CsRV1 alginate block, we used a process similar to that developed for producing homogenous portions of shrimp processing waste into crab bait (Clowes, 2016). The homogenized infected blue crab tissue was mixed at a ratio of 3:2 alginate:crab, and the mixture hardened in a 1% CaCl2 solution for 1 h. The resulting crab alginate block was cut into 5 g portions (representing a total challenge dose of approximately 2 x 10^{10} CsRV1 genome copies) for subsequent use in feeding experiments. Fresh crab alginate blocks were prepared each day for 7 days. Materials and supplies, including food processors, were labeled and kept separate for the CsRV1 alginate and squid (control) alginate to prevent viral contamination.

The negative control squid alginate block was prepared as above with a ratio of 3:2 alginate:squid (commercial-grade frozen squid, *Ommastrephes sloani pacificus*, homogenized). Fresh squid alginate blocks were prepared each day.

For the virus filtrate preparation, we followed the procedure outlined by Bowers et al. (2011) and described in 2.1.2. Using a tuberculin syringe (1cc, 27 gauge needle), 20 µl of freshly prepared virus suspension representing a total challenge dose of approximately 2 x 10^{7} CsRV-1 genome copies was injected into the hemal sinus at the joint between the thorax and the 5^{th} (swimmer) leg. Aliquots of virus filtrate were prepared and frozen (-20°C) for each trial. Mortalities in the first 7 days post injection were not included in further analyses because of the likelihood that these crabs had a cryptic pre-existing infection with CsRV1, another pathogen, or succumbed to unnoticed injuries.

To verify that preparation of the alginate-crab muscle blocks did not eliminate CsRV1 infectivity, 5 g of crab alginate block was soaked in 25 ml of sterile ASW (15 ppt) for 16 h at 22°C. The solution was then centrifuged at 500 g for 5 min at room temperature to sediment the alginate and filtered through a 0.2 µm filter. Aliquots of virus-saline solution were frozen (-20°C) for each trial. The virus-saline was injected

as described above. Mortalities in the first 7 days post injection were not included in further analyses. Due to shipping issues, the viral load of the saline treatment was not available.

2.2.3 Repeated exposure *per os*

Three separate transmission bioassays were conducted using a total of 106 crabs (trial 1: 30 crabs, June 2017; trial 2: 40 crabs, July 2017; and trial 3: 36 crabs, September 2018). Conditions were maintained for all three trials at 15 ppt ASW at 22 °C.

To examine whether repeated doses of CsRV1 via feeding would result in CsRV1 transmission, a crab alginate block was prepared each day to ensure the same amount of virus was presented to each crab each day. Naïve crabs were randomly assigned into one of four treatment groups: 1) CsRV1 alginate block (n=32), 2) Squid alginate block (negative control, n=32), 3) Injected virus filtrate (positive control for virus, n=21), and 4) Injected alginate saline solution (positive control for homogenate, n=20). The first two treatment groups were fed 5 g of alginate block (squid or crab), each day for 7 days. Crabs were observed to ensure they consumed the alginate, and after 2 h, any remaining alginate was removed. The injected crabs were injected on Day 0 and fed a control diet of squid (*Ommastrephes sloani pacificus)* or shrimp (*Litopenaeus setiferus*) each day for 7 days.

Crabs from all four treatments were then maintained an additional 21 days following the end of the 7-day alginate feeding treatment to allow the virus time to replicate. During the 21 days following the treatment, the crabs were fed a regular diet of squid (*Ommastrephes sloani pacificus)* or shrimp (*Litopenaeus setiferus*) every 3 days. Crabs were monitored daily for mortality and molting. Water temperature and water quality (ammonia, nitrite, nitrate) were checked daily, and water changes were carried out as needed to maintain water quality. Water temperature ranged from 22-24°C. When a crab died or at the end of the 21 days, a walking leg was removed and stored at -20°C. All samples were shipped frozen to IMET for subsequent RNA extraction and qPCR analyses.

226

227 **2.3 Crab Dissection and RNA Extraction**

228 Hemolymph samples (prescreening for single exposure (2.1.1)) were extracted with Trizol as described
229 in Flowers et al. (2016b) and muscle tissues (prescreening for repeated exposure (2.2.1) and final
230 analysis for both) from preserved crab legs was dissected following Spitznagel et al. (2019). RNA
231 extraction methods followed Flowers et al. (2016b). Those pellets were then dissolved in 1mM EDTA and
232 evaluated (absorbance at 260 and 280 nm) on a NanoDrop spectrophotometer for RNA quantity and
233 purity (Spitznagel et al., 2019).

234

235 **2.4 Quantification of CsRV1**

236 Quantification of CsRV1 genome numbers was based on a standard curve of purified dsRNA from
237 infected crabs, following the methods of Spitznagel et al. (2019), amplified with the primer
238 pair 5'- TGCGTTGGATGCGAAGTGACAAAG- 3' (RLVset1F) and 5'- GCGCCATACCGAGCAAGTTCAAAT- 3'
239 (RLVset1R) used to detect a 158 bp amplicon from the ninth genome segment of CsRV1 (GenBank entry
240 KU311716) (Flowers et al. 2016b). Standard curves of the CsRV1 genome were prepared using dsRNA
241 from purified CsRV1. Standard ten-fold dilutions yielded genome concentrations of 3.4×107 down to 34
242 genomes per µL. All dilutions were prepared in 1 mM EDTA with 25 ng per µL yeast tRNA and stored at -
243 80 °C.

244

245 The qPCR reagents, parameters, and process selection followed Flowers et al. (2016b). The qPCR
246 reaction components included 1× qScript™ One-Step qPCR Kit Low ROX Master Mix (Quanta Bio), SYBR®
247 Green (Quanta) and 500 nM of each primer. Primers were dissolved in 1mM EDTA to prevent RNA
248 degradation. The product was amplified for 40 cycles of 5 s at 95 °C (melting) followed by 30 s at 61 °C
249 (annealing and extending).

250

Based on prior experience and the inclusion of negative control samples processed in parallel with crab samples (Bowers et al., 2011; Flowers et al., 2016a), the threshold for assessing a crab to be infected with CsRV1 was set at 1000 CsRV1 genome copies per mg muscle or per µl hemolymph as determined by RT-qPCR. Crabs with fewer than 1000 copies were considered negative, and the possible consequence of cross contamination.

2.5 Statistical Analysis

For the repeated exposure experiments, an ANOVA was used to compare the Log viral load amongst treatments. Tukey's HSD was used to determine which treatments were different when the ANOVA was significant. Chi-square compared the presence/absence of virus amongst the treatment groups, and a Fisher exact test was used to determine if the treatment groups of crab and squid were different. Statistical analyses were done in JMP (Pro 15.1) and Sigma Plot 14 (Systat Software). The p value was set at 0.05 for all analysis.

3. Results

3.1. Single exposure per os

No crabs fed infected tissue developed CsRV1 infections. None of the crabs fed infected or uninfected tissue died within 28 days. All crabs injected with the virus filtrate (n=4, prepared from CsRV1-positive muscle) died within 21 days with heavy CsRV1 infections. None of the crabs injected with control filtrate (n=5, prepared from CsRV1-negative muscle) developed infections or died within 28 days. The injection studies indicated that the virus fed to the crabs was competent to cause infection and disease.

3.2 Repeated exposure to CsRV1

3.2.1 Pre-existing prevalence

Screening for virus-free crabs produced data on natural prevalence values of the virus. Of the crabs that were caught in Point Aux Chenes in June 2017, 38.5% (10/26) were positive for CsRV1. At the same

277 location in August 2017, only 2.7% (1/37) crabs were positive for CsRV1. In the following year in
278 September, CsRV1 was detected in 5.6% (2/36) crabs from Lacombe. All crabs with detectable CsRV1
279 were excluded from the data analysis.
280
281 **3.2.2. Post-exposure prevalence and virus load**
282 For reasons outlined below, 21 crabs were excluded from this study. At To, 13 crabs were determined to
283 be positive for CsRV1 and excluded (see above). Any crab that died in the first week was excluded (n=5),
284 2 of those 5 were also CsRV1 positive at To. Low RNA yields from crab tissue extractions (n=4) and
285 possible contamination (n=1) were also excluded. Of the 84 crabs analyzed, 16 were injected with CsRV1
286 filtrate, 18 were injected with CsRV1 saline solution, 23 were fed squid alginate blocks and 27 were fed
287 CsRV1 alginate blocks. The viral genome number in the CsRV1 filtrate was 12 million copies per
288 microliter representing a challenge dose of 24 million CsRV1 genome copies per crab from the 20 µl
289 inoculum. The CsRV1 alginate represented a challenge dose of over 10 billion CsRV1 genome copies per
290 block. There were a total of 30 female and 75 male blue crabs in the study. Carapace width ranged from
291 80 to 175 mm with an average width of 112.7 mm.
292
293 CsRV1 was detected in 100% (16/16) of the crabs injected with the CsRV1 filtrate (Fig. 1), with an
294 average of 6.3 x 10^8 CsRV1 genome copies per mg muscle tissue (Fig. 2). CsRV1 was detected in 94%
295 (17/18) of crabs injected with the CsRV1 saline solution (Fig. 1), with an average viral genome load of 4.4
296 x 10^8 CsRV1 genome copies per mg muscle tissue (Fig. 2). A one-way ANOVA revealed there was a
297 significant difference between at least two of the groups ($F=113.15$, $p<0.001$). The final virus loads in the
298 two injected treatments were not significantly different (Tukey's HSD, $p=0.990$; Fig. 2). CsRV1 was not
299 detected in any of the crabs fed the squid alginate blocks (Fig. 1 & 2). CsRV1 was detected in 11% (3/28)
300 of the crabs fed the CsRV1 alginate blocks (Fig. 1) with an average viral genome load of 7.3 x 10^8 CsRV1
301 genome copies per mg muscle tissue for the three infected crabs (range 1.5 x 10^8 to 1.7 x 10^9 CsRV1

genome copies per mg muscle tissue) (Fig. 2). The injected groups were both significantly different from the per os groups (Tukey's HSD, $p<0.001$) for all four combinations.

While three cases of CsRV1 transmission occurred in the CsRV1 alginate block treatment group, the virus load in the two alginate block fed groups (CsRV1 and squid) were not statistically different from each other (Tukey's HSD, $p=0.700$; Fig. 2). The Chi square indicated significant different in presence and absence between at least two groups ($X2=69.26$, $df=3$, $p<0.001$) There was no significant difference in the presence/absence of virus between the CsRV1 alginate and squid fed groups (Fisher Exact, $p=0.240$; Fig. 1). When comparing the final presence/absence of CsRV1 in recipient crabs, fed and injected treatment groups were significantly different (Fisher Exact, $p<0.001$; Fig. 1), and final viral load was also significantly different between the fed and injected treatment groups (Fisher Exact, $p<0.001$; Fig. 2).

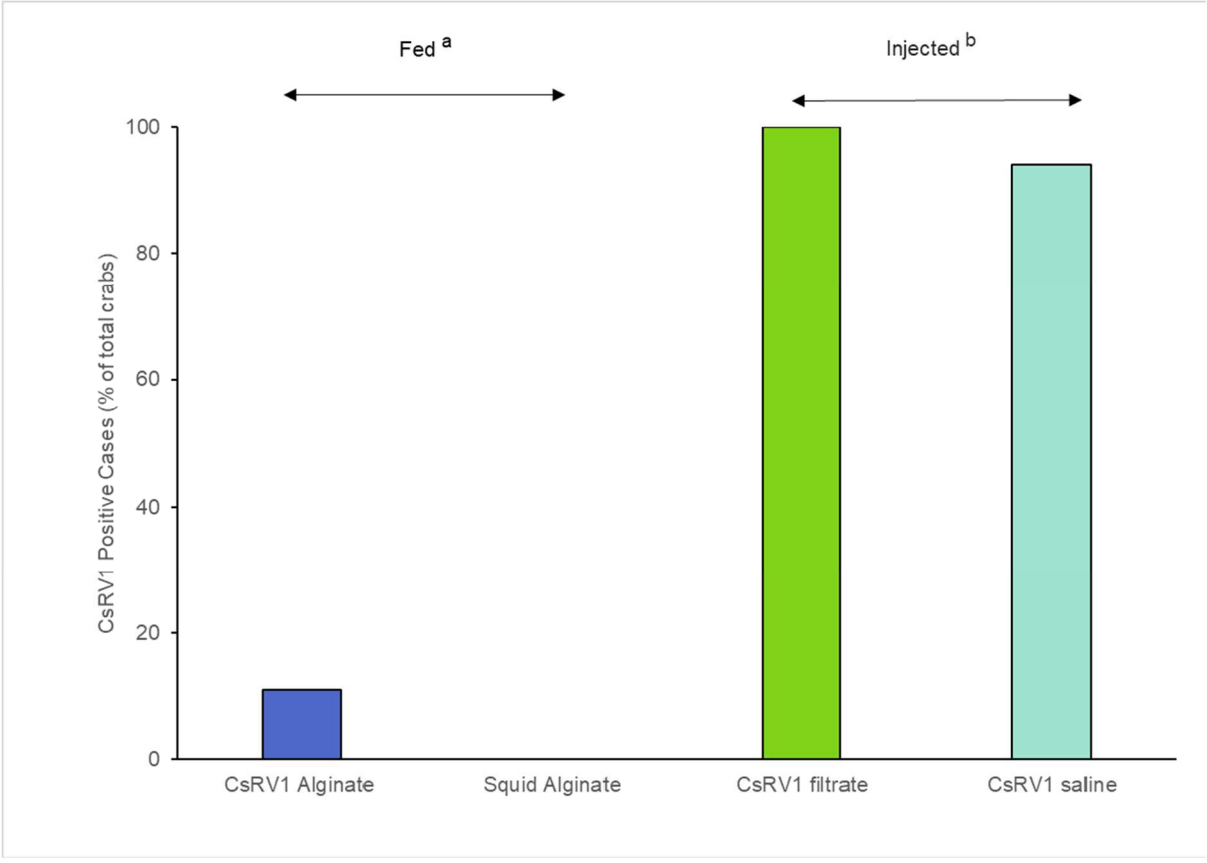

Figure 1. The positive cases of CsRV1 for each treatment group (% of total crabs). Different letters

indicate a significant difference in the fed (blue; CsRV1 alginate and squid alginate) and injected treatments (green; CsRV1 filtrate and CsRV1 alginate saline) ($p<0.001$). There were no significant differences between the fed or within the injected treatment groups.

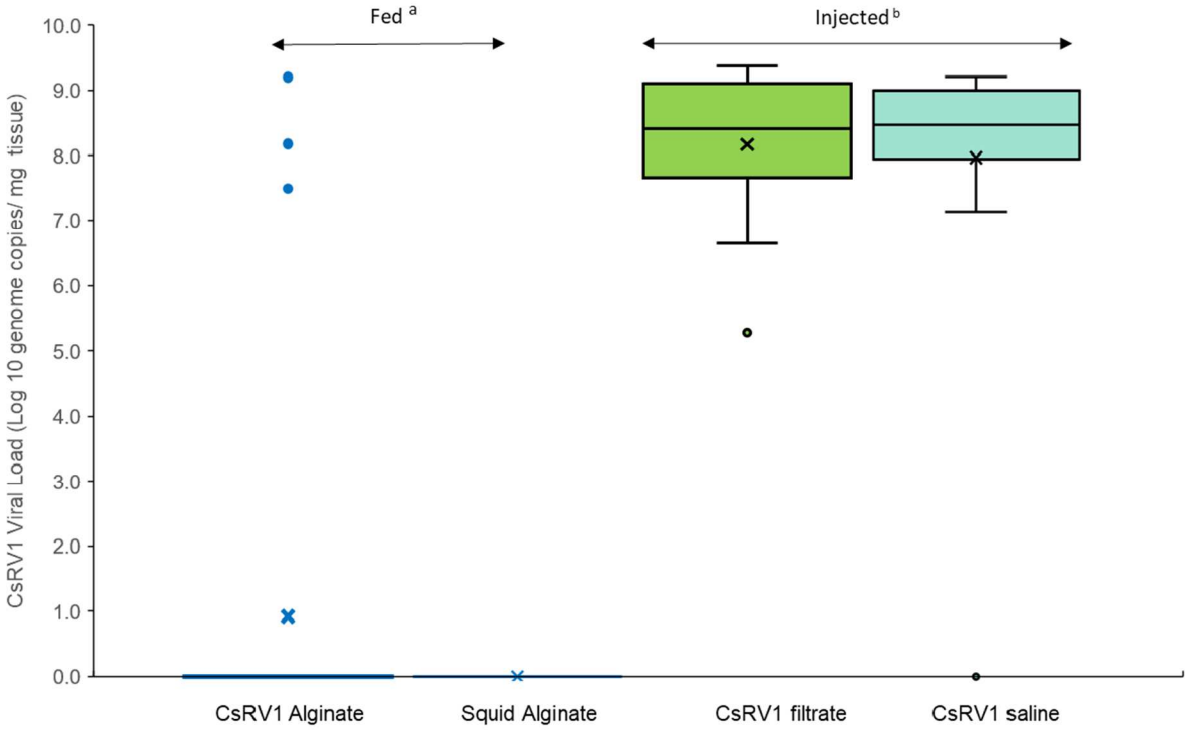

Figure 2. Box plot of the viral load of CsRV1 for each treatment group. X represents the mean with the box representing the upper and lower quartiles with a line at the median. The whiskers represent variability outside of the quartiles. Individual dots are outliers. For CSRV1 Alginate and Squid Alginate, the upper and lower quartiles are at zero. Different letters indicate a significant difference in the fed (blues; CsRV1 alginate and squid alginate) and injected treatments (greens; CsRV1 filtrate and CsRV1 alginate saline) ($p<0.001$). There was no significant difference within the fed or within the injected treatment groups.

3.2.2 Crab survival

CsRV1 was present in 100% of the crabs that died during the course of the experiment (27 crabs: 1 crab fed CsRV1 alginate blocks, 12 crabs injected with CsRV1 filtrate, and 14 injected with CsRV1 alginate

saline solution). Of the crabs that died (n=27), the range was 1.7 x 10^8 to 5.3 x 10^9 CsRV1 genome copies per mg muscle tissue with an average of 1.6 x 10^9 CsRV1 genome copies per mg muscle tissue.

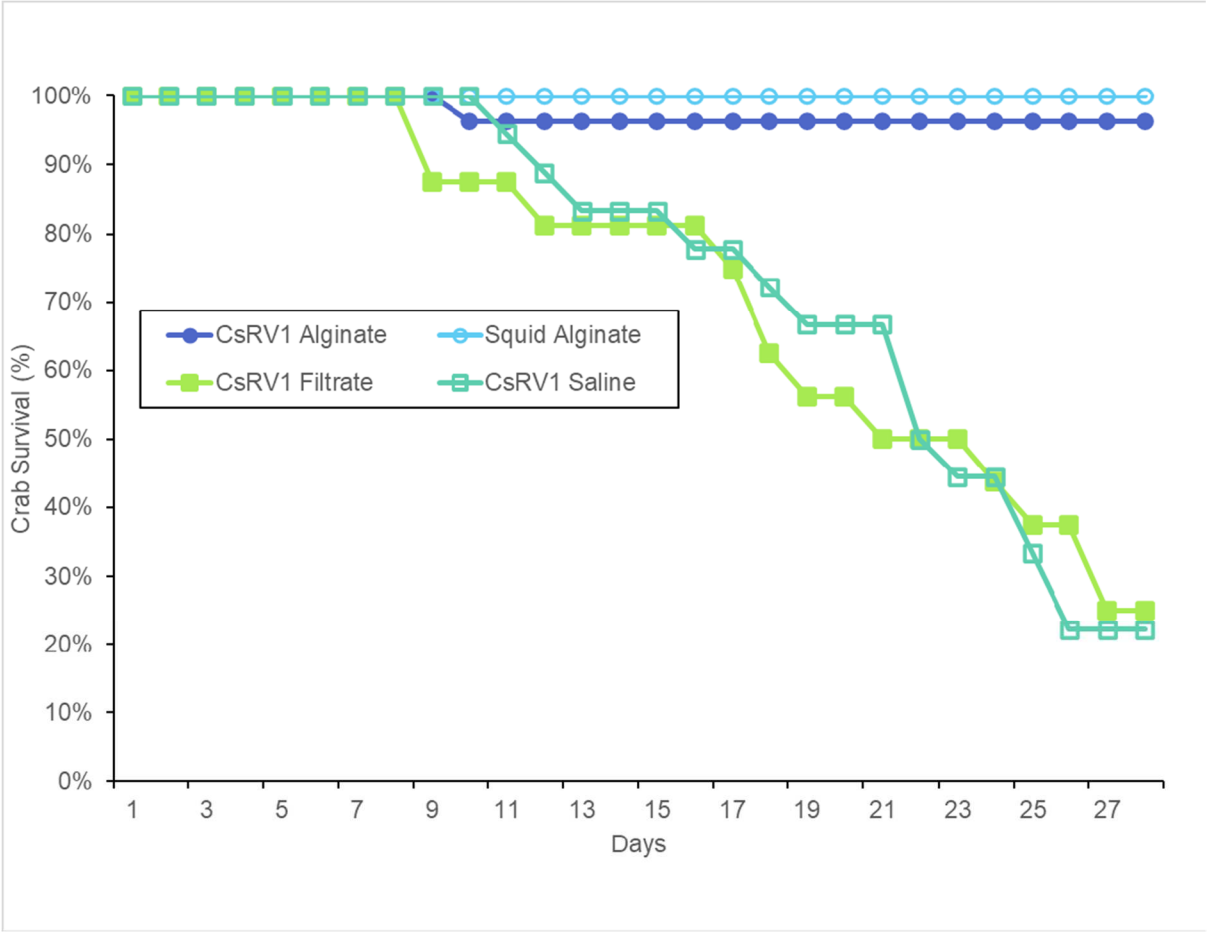

Figure 3. 28-day survival (%) of crabs in each treatment group. Injected treatments (Squares) are CsRV1 Filtrate (solid) and CsRV1 Saline (open); per os exposure (Circles) are CsRV1 Alginate (solid) and Squid Alginate (open).

Fifty-seven crabs were alive at the end of the 28-day bioassay (26 crab fed CsRV1 alginate blocks, 23 crabs fed squid alginate blocks, 4 crabs injected with CsRV1 filtrate, and 4 injected with the CsRV1 alginate saline solution; Figure 3). Of the 57 crabs that were alive at day 28, 16% had detectable CsRV1 levels (2 crab fed CsRV1 alginate blocks, 4 crabs injected with CsRV1 filtrate and 3 injected with the

CsRV1 alginate saline solution) ranging from 1.7 x 10^7 to 5.3 x 10^9 CsRV1 genome copies per mg muscle tissue with an average of 2.5 x 10^9 CsRV1 genome copies per mg muscle tissue.

4. Discussion

Despite the fact that the blue crab virus CsRV1 is associated with the majority of softshell crab mortalities and has a natural prevalence of up to 60% in hard shell blue crabs, little is known about natural transmission of the virus between blue crabs. Determining the possible routes of transmission for CsRV1 is important to better understand the role this virus plays in wild crab populations and the soft-shell crab industry. Conducting transmission studies with reproducible doses of CsRV1 is challenging because in vitro culture methods do not exist for crustacean viruses. The single exposure bioassay using infected muscle (representing a challenge dose of 10^{10} CsRV-1 genome copies per crab) did not result in detectable CsRV1 transmission. In contrast, the repeated exposure bioassay using alginate gel blocks formulated from muscle tissue homogenates containing CsRV1 (representing a challenge dose of 10^{10} CsRV-1 genome copies per crab) indicated that transmission occurred in 3 of 28 crabs offered this treatment. The use of an alginate gel proved to be an effective method for allowing virus from a single homogenized sample to de delivered to many crabs; virus infectivity was retained for at least 24 h after immersion in saline.

The exact mode of transmission to the three crabs in the CsRV1 alginate block treatment group is unclear. Since the injected CsRV1 alginate saline solution treatment confirmed that the virus remained infectious after submersion of the alginate block in saline for 24 h it is possible that unknown quantities of CsRV1 viral particles leached out the crab alginate blocks into the surrounding water, and crabs were exposed by this route, rather than by direct consumption of the CsRV1 alginate. In addition, the crabs were handling the CsRV1 alginate as they ate. Regardless of the exact mode, these data suggest that

370 blue crabs interacting with infected tissue from a dead conspecific could increase risk of conspecific
371 infection either from direct per os ingestion or another transmission route. Repeated exposure also is
372 likely to increase to risk of infection as the single exposure bioassay did not result in detectable CsRV1
373 transmission even though viral load was comparable (10^{10}). The apparent lack of infection through per
374 os exposure can be rationalized with the fact that the digestive system of a crab may not be a hospitable
375 place for virus particles. Digestive enzymes that liquefy prey items typically include proteases and
376 nucleases (Veróonica and Gimenez, 2013). A logical follow up study would be the exposure of virus
377 suspensions to crab digestive enzymes, to see if this abrogates infectivity.
378
379 A lot of information is still needed for this virus. Numerous (unpublished, Schott and Lively labs) trials
380 have shown that injection of blue crabs with approximately 100,000 virus genome equivalents leads to
381 50-100% infection and disease. The dose response curves are unknown for CsRV1, although work is
382 ongoing. In the repeated exposure experiment, crabs were injected with 10^7 while the crabs were
383 exposed to 10^{10} per os. Even with the threefold increase in magnitude, the per os route resulted in
384 significantly fewer infections than injection. This could be due to the transmission route, but there are
385 many other factors that are unknown with the virus.
386
387 The CsRV1 alginate block preparation method appeared to be an effective method for delivery of a
388 known concentration of CsRV1 (10^{10} CsRV-1 genome copies per crab). The associated CsRV1 alginate
389 saline solution studies indicated that after 24 h immersion, CsRV1 released into the saline solution
390 retained its infectiousness when inoculated into naïve crabs, and that resulting CsRV1 loads were
391 comparable to those produced by inoculation of CsRV1 homogenate prepared directly from infected
392 muscle tissues. Large-scale feeding transmission trials in crustaceans can be challenging to carry out. It is
393 difficult to ensure that all naïve animals are exposed to equal amounts of the challenge pathogen. The
394 method developed here would allow for exposure from one virus preparation for every individual every

day, and can easily be scaled down, or up, and could be used to investigate the transmission pathways of other viral pathogens of crustaceans.

There does not seem to be a specific viral load level at which blue crabs die from CsRV1 infection. Flowers et al. (2016b) suggested a level of heavy infection at 10^6 CsRV1 genome copies per mg muscle tissue. Of the 84 individuals analyzed in the current study, all the crabs that died had quantifiable CsRV1 viral loads that ranged from 1.7×10^8 to 5.3×10^9 CsRV1 genome copies per mg muscle tissue. Not all crabs that had detectable CsRV1 died. The surviving crabs had a CsRV1 loads that ranged from 1.7×10^7 to 5.3×10^9 CsRV1 genome copies per mg muscle tissue. There does not seem to be a definitive correlation between virus loads and host mortality. If the holding time had continued past 28 d, then more of these crabs might have died, but this and previous work (e.g. crabs surviving through molt while infected) suggests that individual fitness (ex. immunocompetence), susceptibility to stress, or other biological factors are involved in mortality (Spitznagel et al., 2019). Further studies are needed to better understand the role of the virus in crab mortality, especially in wild populations.

The three natural prevalence values calculated from the crabs collected for this study, 38.5% (PAC, June 2017), 2.7% (PAC, Aug 2017) and 5.6% (Lacombe, Sept 2018) contribute to the very limited CsRV1 prevalence data previously reported in Louisiana (Rogers et al., 2015). The mean natural prevalence of CsRV1 for wild caught crabs in this study was just below 13%. Rogers et al. (2015) collected crabs in the summer of 2013 at Grand Isle and Rockefeller Wildlife Management area, both these locations are farther south than where crabs were collected in this study, and the natural prevalence values were 7.7% (5% from Grand Isle and 10.5% from Rockefeller). In contrast, Flowers et al. (2016b) measured natural prevalence in Maryland waters at 22 and 5.9%, over two summers of sampling (2012, 2013). The prevalence values obtained in the current study support the existing literature that CsRV1 prevalence has high variability both spatially and temporally. A recent paper by Zhao et al. (2020) reveals a climate/

latitudinal gradient of prevalence, with highest CsRV1 incidence in temperate areas, and nearly absent in the lower latitudes.

Although our data does not conclusively identify an oral exposure route for CsRV1, it does suggest that naïve crabs can acquire infections when in the proximity of CsRV1, at least with repeated exposure either by consumption of diseased host tissues, or through the water. Our findings, therefore, have direct industry relevance, especially to the soft-shell industry as dead pre-molt crabs are often discarded in nearby waterways or sold/used as fishing bait. Our findings are consistent with Flowers et al. (2018) who reported an increased prevalence of CsRV1 in the vicinity of softshell shedding effluent sources. Returning dead crabs to the water may spread CsRV1 if the crabs are infected, and this may re-infect live wild crabs, whether through consumption or other exposure. Recommendations should be made to the industry to dispose of dead crabs from shedding systems on shore.

5. Conclusion

This is the first study to investigate a natural transmission route of CsRV1. While repeated exposure to a CsRV1 alginate preparation did lead to transmission in a limited number of naïve crabs, the exact transmission route is unclear. The alginate/CsRV1 infected muscle preparation was an effective method for delivery of a defined concentration of CsRV1 and allowed us to perform repeated exposures to CsRV1. Additional studies are needed to better understand transmission of this virus.

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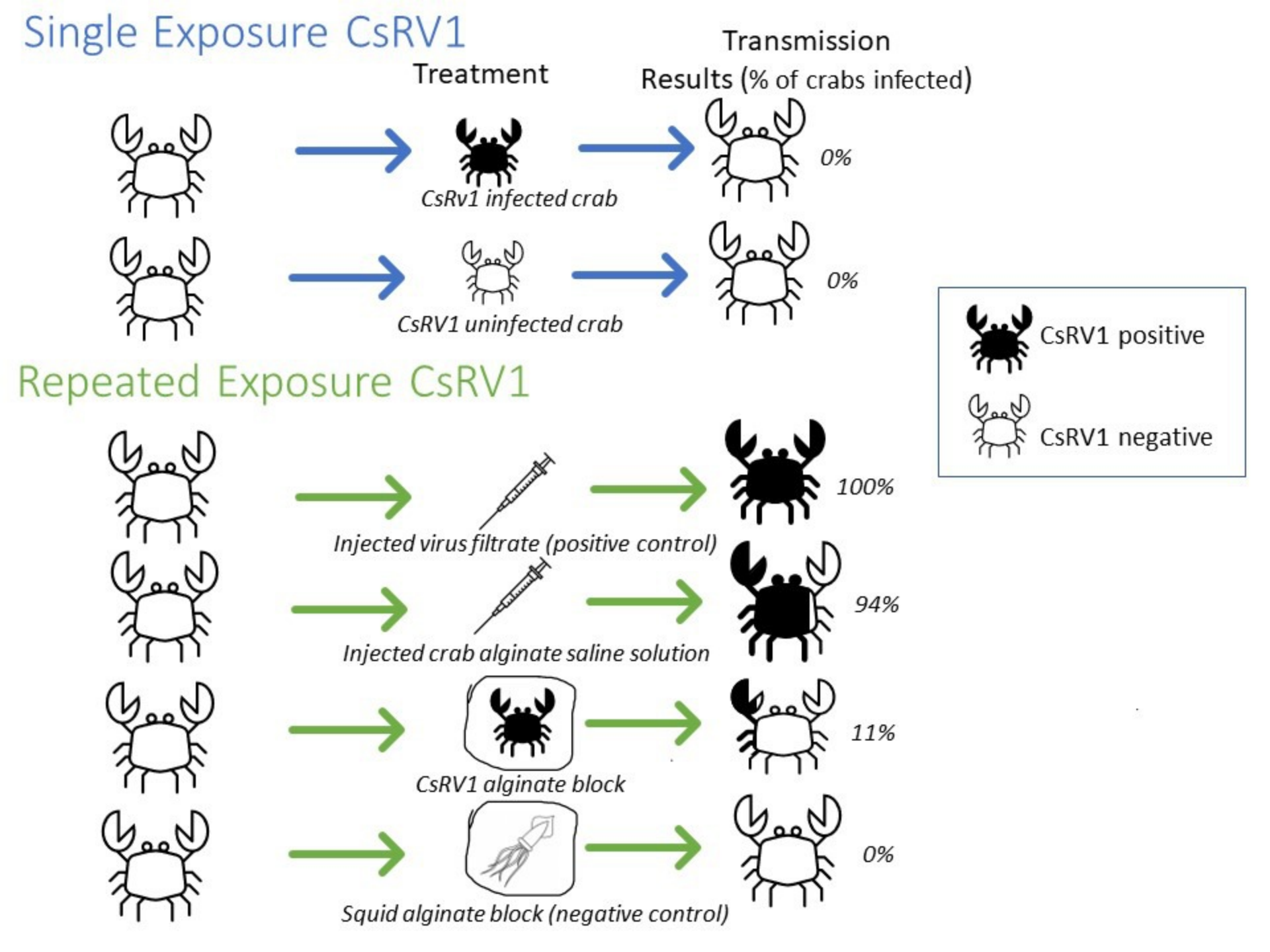
Single Exposure CsRV1
Treatment
Transmission
Results (% of crabs infected)
CsRv1 infected crab
0%
CsRV1 uninfected crab
0%
Repeated Exposure CsRV1
CsRV1 positive
CsRV1 negative
Injected virus filtrate (positive control)
100%
Injected crab alginate saline solution
94%
CsRV1 alginate block
11%
Squid alginate block (negative control)
0%