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

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27 **Abbreviations**
28 CsRV1: *Callinectes sapidus* reovirus 1
29 RT-qPCR: reverse transcriptase quantitative polymerase chain reaction
30

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31 **Abstract:**

32 Crustacean aquaculture is prone to mortality from the combined effects of disease agents and the stresses
33 associated with crowded, closed conditions. The culture practice of producing soft-shell blue crabs is no
34 exception, suffering from mortality of about 25%. The virus, *Callinectes sapidus* reovirus 1 (CsRV1), has
35 been reported at high viral loads in crabs dying in soft-shell shedding facilities. We investigated the
36 relationship between crab mortality and CsRV1 prevalence and load in soft-shell crab production and
37 whether death and virus infection correlated with identifiable aquaculture practices, environmental
38 stresses, crab characteristics, or geographic regions. The patterns of CsRV1 prevalence, infection
39 intensity, and mortality in blue crab aquaculture were studied in the Chesapeake Bay and Gulf of Mexico,
40 USA. Using a genome-targeted assay, we compared virus loads in live and dead aquaculture crabs by
41 individual sex and injury state from recirculating and flow-through systems of variable salinity,
42 temperature, and crabs per aquaculture tank. Mortality was two-fold higher in flow-through aquaculture
43 systems (33%) than in recirculating aquaculture systems (16%). Flow-through aquaculture systems had
44 higher daily water temperature variability than recirculating aquaculture, and hypoxic events were
45 observed only in flow-through systems during this study. Heavy CsRV1 intensity was found in 62% of all
46 pre-molt mortalities in production compared with 7% of successfully molted soft-shell crabs. The CsRV1
47 virus load in dead crabs was elevated in higher salinity conditions. Using a mixed-effect model, the
48 random effects of location and time were more significant than salinity in predicting CsRV1 load in all
49 crabs and dead crabs. Our results support previous research showing that recirculating aquaculture has
50 lower mortality in soft-shell production, and confirms the association of high viral loads of CsRV1 with
51 crab mortality in these production systems. Moreover, the findings indicate that although CsRV1 is
52 ubiquitous in these systems, management of culture conditions such as salinity and temperature may limit
53 virus-associated mortality.

54 **Key words**: *Callinectes sapidus,* disease, CsRV1, molting, sustainability

55

56 **Highlights**

57 • A collaborative study with US soft crab producers revealed that 22% of all pre-molt crabs die before
58 molting, with 16% mortality in recirculating systems compared to 33% mortality in flow through
59 systems.
60 • The pathogenic virus, CsRV1, was present at high levels in the majority of crabs that died in soft-shell
61 production but only in 7% of successfully molted crabs.
62 • Virus prevalence in dead crabs was associated with lower salinity, but random effects of location and
63 date were also associated with prevalence and load.
64 • Although CsRV1 is ubiquitous, management of culture conditions such as salinity and temperature
65 variation may limit virus-associated mortality.
66

67 **1. Introduction:**

68 Crustacean aquaculture supports extensive seafood industries worldwide, yet disease agents remain a
69 major factor limiting production (Shields & Overstreet 2007; Stentiford et al., 2012; FAO, 2017). Many
70 factors may exacerbate both disease susceptibility and mortality in aquaculture, such as high population
71 density, stress of confinement, water quality, temperature fluxes, or hypoxia (Le Moullac & Haffner,
72 2000; Mohanty et al., 2018). Certain aquaculture practices, including disposal of dead aquaculture
73 animals into neighboring marine waters, use of dead animals as bait, or untreated effluent release, can
74 facilitate the spread of disease, yet these practices remain common in the industry (Lafferty et al., 2015;
75 Shields 2017; Flowers et al., 2018).

76 The target of one of the world's four largest crab fisheries, the blue crab, *Callinectes sapidus*, is at the
77 center of a multi-million dollar aquaculture practice in the eastern United States (MD DNR, 2018; NOAA
78 NMFS, 2016; FAO, 2018). This fishery-dependent practice involves holding pre-molt blue crabs, known
79 colloquially as peelers, in shallow tanks until they molt into soft-shell crabs, which are a value added
80 product consumed regionally and frozen for international trade (Oesterling, 1995; Chaves & Eggleston,
81 2003; Tavares et al., 2017). The molting process is inherently stressful for crabs (deFur et al., 1988;
82 deFur, 1990). Combined with external stressors associated with harvest and aquaculture, molting stress
83 may contribute to the reported 25% - 50% mortality in soft-shell crab production (Chaves & Eggleston,
84 2003; Oesterling, 1995). Despite numerous methods and manuals designed to help optimize culture
85 conditions (e.g., Ogle et al., 1982; Oesterling, 1995), peeler crab mortality remains unpredictable and
86 high. Recent studies have investigated the potential for disease to contribute to crab mortality in the
87 shedding systems used by the industry in soft-shell production (Bowers et al., 2010; Rogers et al., 2015).

88 Across their US range and in Brazil, blue crabs are infected by the pathogenic virus *Callinectes*
89 *sapidus* reovirus 1 (CsRV1, Bowers et al., 2010; Flowers et al., 2015). CsRV1 (previously identified as
90 Reo-Like Virus, RLV) was identified as a cause of crab mortality in captive crabs in the 1970s (Johnson,
91 1977; Johnson, 1978), and subsequently in soft crab aquaculture production and a scientific blue crab
92 hatchery (Bowers et al., 2010). The virus infects hemocytes and hemopoietic tissue (Johnson, 1977; Tang

93 et al., 2011). Injection of viral filtrate leads to paralysis and death of crabs in days or weeks and is
94 associated with infiltration of hemocytes into neural tissues (Johnson, 1983; Bowers, et al., 2010).
95 Application of sensitive quantitative molecular assays for CsRV1 has shown a mean prevalence of
96 20% in wild crabs surveyed from the northeast United States, with most infected animals harboring $<10^4$
97 virus genomes per mg crab muscle tissue (Flowers et al., 2015). In contrast, an earlier study of soft-shell
98 crab aquaculture in Maryland and Florida, USA, found CsRV1 in 71% of dead peeler crabs using an RNA
99 electrophoresis assay that has an estimated detection limit of 10^5-10^6 genome copies per mg muscle tissue
100 (Bowers et al., 2010). This association of CsRV1 with peeler crab mortality suggests that CsRV1 may be
101 an important contributor to mortality during blue crab molting and a source of considerable economic loss
102 to the soft-shell crab industry (Johnson, 1983; Flowers et al., 2018).
103 Few studies have investigated how disease may interact with aquaculture practices to cause crab
104 mortality during soft-shell production. It is not known how crab mortality and CsRV1 prevalence are
105 affected by culture practices, environmental holding conditions, individual crab characteristics, or specific
106 geographical location of facilities. To determine whether specific biological or environmental risk factors
107 exist in soft-shell crab production, we partnered with soft crab producers in Maryland, Virginia, and
108 Louisiana to measure different parameters used in shedding systems and correlate these with virus
109 prevalence and loads using a quantitative real time polymerase chain reaction (RT-qPCR) assay (Flowers
110 et al., 2015). Potential relationships between crab mortality, CsRV1 infection, aquaculture system type
111 (flow-through vs. recirculation), salinity and water temperature, and the individual size, sex, and molting
112 state of crabs were investigated using generalized linear mixed effect modelling (GLMM). Our findings
113 may be useful for identifying management practices that reduce CsRV1-associated mortality and increase
114 successful soft-shell crab production.
115

116 **2. Materials and methods:**

117 2.1. Crab collection and handling

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

135 Soft crab producers participating in the study were located in Pasadena, Patuxent, Tilghman Island,
136 and Rock Hall, Maryland, West Point, Sarah's Creek, and Chuckatuck Creek, Virginia, and Dulac and
137 Franklin, Louisiana, USA (Figure 1). Two additional sites at Bear Creek, MD and Violet, LA were
138 initially surveyed, but were excluded from statistical analysis as high-mortality outliers that were affected
139 by hypoxia and toxic nitrite levels beyond the limits required for sustainable aquaculture operation. Sites
140 were categorized by open (flow-through) or closed (recirculation) water circulation type. Both systems at
141 Tilghman Island were flow-through sites located within 1 km of each other, but were independent

142 businesses and operations (Table A.1.) Water samples (10 ml) of all systems were collected daily by
143 watermen, were assessed for salinity by refractometer, while nitrate, nitrite, general hardness, carbonate
144 hardness, and pH were measured by aquarium test kit (5 in 1 Aquarium Test Strips, API®) for Maryland
145 sites. Water temperatures in culture systems were measured hourly by automated HOBO™ dataloggers
146 (Onset Corporation®). Mean water temperatures and salinities of each sampling period were computed
147 from daily water samples for site comparison. Water temperature variability was defined as the maximum
148 difference between any hourly point and the weekly period average. Participating watermen recorded
149 whether they fished peeler crabs themselves or purchased peelers from other fishermen, the number of
150 aquaculture tanks they used, average number of crabs per tank, and the number of crabs dying or molting
151 to soft shell on each day of the survey. Mortality level was calculated by dividing the total number of
152 reported crabs molting successfully by the sum of crabs dying or molting during a 7 day survey period.

153 2.2. Crab dissection and RNA extraction:

154 Crabs were dissected with sterile wooden rods and razor blades, and all handling and crab surfaces
155 were cleaned with ELIMINase™. Aliquots of 25-100 mg of crab muscle and connective tissue were
156 excised from a walking leg of each crab and homogenized in 1.0 mL RiboZol® (VWR Scientific) using
157 ceramic beads in a MP® FastPrep24 homogenizer. RNA extraction methods were modified from those
158 used by Flowers et al (2015). After RiboZol®-chloroform separation of RNA into isopropanol, two
159 12,000 g centrifuge washes with 500 µL 75% ethanol were carried out to increase RNA purity from levels
160 obtained with earlier sampling methods. Resulting RNA pellets were then dissolved in 1 mM EDTA to
161 decrease RNA degradation, and RNA purity and concentration were evaluated by NanoDrop™
162 spectrophotometry.

163 2.3. Quantification of CsRV1:

164 PCR primer selection and dsRNA standard preparation were adapted from Flowers et al (2015). The
165 primer pair 5′□TGCGTTGGATGCGAAGTGACAAAG□3′ (RLVset1F) and 5′□
166 GCGCCATACCGAGCAAGTTCAAAT□3′ (RLVset1R) are designed to detect an amplicon from the

167 ninth genome segment of CsRV1 (GenBank entry KU311716) (Flowers et al., 2016). Standard curves of
168 the CsRV1 genome were produced by purifying viral dsRNA from crabs infected with greater than 10e8
169 copies per mg muscle. Enrichment of dsRNA and verification of purity and quality followed the protocol
170 of Bowers et al (2010). Standard concentrations ranging from $3.4x10^7$ to 10 genomes per µL were
171 dissolved in 25 ng per µL yeast tRNA and used for sample comparison.

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

179 2.4. Statistical analysis:

180 Statistical tests were conducted using JMP® Pro 13 (SAS, 2018) and the R 3.4.4 statistical package
181 (R Core Team, 2018). Initial statistical correlations between mortality, CsRV1 prevalence and intensity,
182 and specific aquaculture and crab variables were tested using correlation matrices. Significant correlations
183 were defined as those where $p \leq 0.05$. In the case where any significant correlation was identified or more
184 complex relationship noted, $\log_{10}(x+1)$ transformations were done prior to running ANOVA or GLM
185 models. The Shapiro-Wilk test for normality and Levene's test for homoscedasticity were applied when
186 appropriate. When analyzing individual factor comparisons, parametric pairwise comparisons were tested
187 via t-test, whereas one-way ANOVA with Tukey's Honest Significant Difference test was used for
188 multiple comparisons. Non-parametric comparisons used Wilcoxon rank-sum testing with Steel-Dwass
189 pairwise comparison to determine significant differences. In cases where continuous data was being
190 compared, a series of linear regressions were undertaken comparing appropriate data transformations with
191 a regression t-test and sum-of-squares lack of fit analysis. Stepwise selection of best GLMM was

192 conducted using Akaike's information criterion (AIC) in R (stepAIC function, MASS package, lmer,
193 lme4 package, R Core Team, 2018) was used to determine the factors that best model crab mortality and
194 CsRV1 prevalence and intensity of infection. In individual crab data models, date was used as a random
195 effect in modelling because crab data was recorded daily rather than weekly, introducing potential sample
196 clustering effects if uneven sample sizes and replication at different locations and times were not
197 accounted for (Thorson & Minto, 2015). Due to geographic separation from the Chesapeake region and
198 the disparate number of sites, the crab data from Louisiana was not included in the statistical models.

199

200 **3. Results**

201 3.1. Aquaculture mortality by site and date

202 The mean proportion of dead crabs among the eight culture facilities sampled in the Chesapeake Bay
203 region was 21.7 ± 2.8% (n=12,172 crabs) (Table A.1). The mean salinity range was 3-20 psu in Virginia
204 and 6-18 psu in Maryland. Mean temperature ranged from 67 to 86°F with a maximum peak of 99°F in
205 Virginia and 73 to 85°F with a maximum peak of 90°F in Maryland. Mortality was significantly greater in
206 flow-through water aquaculture systems (32.9 ± 4.3%; n_{site} = 3, n_{time} = 7) than in recirculating water
207 systems (16.4 ± 3.1%; n_{site} = 5, n_{time} = 13) (Figure 2, Student's t, d.f. = 18, t = -3.11, p = 0.0060). When
208 we compared culture conditions between system types, salinity was significantly lower in recirculating
209 systems (Table A.1; Student's t; d.f. = 18, t = -2.55, p = 0.02). All systems surveyed with average
210 salinities below 8 psu had mortality of 15.0% or less; however, there was no significant difference in
211 mortality based on salinity alone (Regression ANOVA, F = 2.48, p = 0.13).

212 The final model for peeler crab mortality had significant system type ($slope_{recirculation}$ -21.8, p <
213 0.0001) and temperature variation fixed effects (slope = -1.20, p = 0.03; Model A, Table 1). Daily
214 fluctuation in water temperature did not differ significantly between flow-through and recirculating
215 systems (Figure 3, Student's t, d.f. = 1, t = -1.52, p = 0.09). However, maximum temperature fluctuation
216 measured at the flow through facility at Sarah's Creek, VA, was more than twice as high as those at

217 recirculating sites. The Sarah's Creek facility experienced 21.3% mortality on average: lower than the
218 flow-through system average, but comparable to the overall shedding mortality in this study. No other
219 significant relationships or variables correlated with crab mortality.

220 3.2. CsRV1 infection in aquaculture analyzed by individual crab

221 Throughout the aquaculture facilities surveyed in MD and VA, 75.4% of dead peelers (n = 305) were
222 infected with CsRV1, compared with 23.9% of live soft-shell crabs (n = 184) and 33.3% of freshly
223 harvested live peelers (n = 60; Figure 4a-c). The difference in survival was even more pronounced when
224 considering infections of $>10^6$ CsRV1 genome copies per mg muscle tissue, with 62.3% of dead peeler
225 crabs surveyed exceeding this infection intensity compared with 7.1% of live soft shell crabs (n = 288;
226 Figure 4a-c, Table 1, Model B). Successful molting was the only significant fixed effect (slope -3.16, $p <$
227 0.0001) with location (variation 1.28, $p < 0.0001$) and date (variation 1.88, $p < 0.0001$) as significant
228 random effects associated with CsRV1 genome copy number (Table 1, Model B). No other factors,
229 including salinity, system type, temperature, injury, or crab sex were significant factors influencing
230 CsRV1 loads.

231 Considering specific environmental variables on a shedding system basis, higher salinity aquaculture
232 sites experienced higher prevalence levels of the virus in dead peeler crabs (Figure 5). Prevalence in dead
233 peelers best fit a reciprocal relationship to salinity (CsRV1 Prevalence (%) = 98.0 – 251.3/Salinity (psu);
234 $R^2 = 0.4374$, ANOVA d.f. = 19, F = 13.99, p = 0.0015).

235 Salinity was the only fixed effect retained in the reduced mixed models of CsRV1 infection intensity
236 (Table 1, Model C). The salinity effect was only marginally significant (p = 0.0498) with a low
237 magnitude negative relationship (slope = -0.13) once other variables were accounted for in the total
238 model. The random effects of date (variance = 1.05, $p < 0.0001$) and location (variance = 4.94, $p <$
239 0.0001) were more significant and higher magnitude. No other effects were significant, including injury
240 to crabs.

241 3.3. Louisiana mortality and prevalence data

242 At two Louisiana shedding facilities surveyed in 2016, the percentage mortality of dead crabs was 14.5 ±
243 5.5% (n= 652 crabs) (Table A.2). In 2016 and 2017, the prevalence of CsRV1 in dead crabs was 21.9% (n
244 = 82). Only one dead crab from the Franklin facility had a detectable CsRV1 infection. Sampling of live
245 crabs in November 2017 found that both soft shell crabs (n=20) and live peelers (n=37) had a prevalence
246 of CsRV1 of 5%, with only one heavily infected crab detected. Due to the low site and crab sample sizes
247 from this distinct geographical region, Louisiana data was not included in the GLMM analyses (Table
248 A.3). Aquaculture salinity was measured at 0-3 psu at Louisiana sites, while the mean temperature range
249 was 70-85°F**.**

250 **4. Discussion:**

251 By collaborating with soft crab producers in three states, this study reconfirmed that soft crab
252 production has variable and sometimes high mortality and that well-controlled recirculating aquaculture
253 systems are crucial to minimize peeler crab mortality. Average peeler mortality was similar to that seen in
254 a prior study in North Carolina, U.S.A., that reported 23% mortality in blue crab shedding systems
255 (Chaves & Eggleston, 2003). In contrast to the North Carolina blue crab study, flow-through systems
256 examined in the current study had twice the mortality of recirculating systems, where one in three peelers
257 died on average. Despite the global importance of mortality and disease in crab aquaculture systems
258 (Zhang et al., 2004, Deng et al., 2012; Oesterling 1995), information on mortality in crab aquaculture is
259 scarce (FAO, 2018). Reports on aquaculture practices of *Scylla* spp. in southeast Asia and Africa indicate
260 mortality levels similar to or higher than that observed in the short-term production of soft-shell blue
261 crabs (Keenan, 1999; Mirera & Moksnes, 2015).

262 The lower mortality of crabs in recirculating systems was likely associated with better control of
263 environmental variables compared to flow-through systems. For example, temperature variation was less
264 in the recirculating systems, albeit the trend was not significantly different (p=0.08) between system
265 types. Other environmental parameters may also contribute to crab mortality in poorly-controlled or flow-
266 through systems. For example, we excluded one flow-through system in the Baltimore area from the

267 study because of separate events of hypoxia (< 2 mg/L) and high nitrite (>10 mg/L) which were both
268 associated with crab mortality of over 50%. In these latter examples, mortality was not likely a result of
269 environmental variability *per se*, but the fact that one water quality parameter exceeded a biological
270 threshold for crab survival.

271 Economically, peeler mortality represents a loss of time, effort and money to watermen. This amount
272 of mortality appears to be a long-accepted cost of doing business for individual crab shedders, who may
273 lose several thousand dollars' worth of peeler crabs per week, depending on the size of their shedding
274 operation. Estimates of peeler crab mortality have been identified as a critical need by the Chesapeake
275 Bay Stock Assessment Committee (CBSAC, 2018). Based on 2016 data for peeler harvests, Maryland
276 harvested 1225 metric tons of peeler crab (MD DNR, 2018, pers. comm.), while Virginia harvested 333
277 metric tons, and Louisiana 65 metric tons (NOAA NMFS, 2016). Based on the 22% mortality observed in
278 this study, an estimated 356.2 metric tons of peelers (worth $2.58 million) died prematurely in these three
279 states in FY 2016. Applied to the entire United States peeler harvest, this mortality represents a loss of
280 408.2 metric tons of blue crab. At 250g estimated average weight per peeler, this represents 1.63 million
281 peeler crabs lost in FY2016.

282 This study revealed additional information about the association of CsRV1 infection with peeler crab
283 mortality. When analyzed on the basis of individual crabs, CsRV1 infection was the most significant
284 predictor of peeler crab death regardless of shedding system type. Heavy infections were found in almost
285 two thirds of the dead peeler crabs, and was nine times higher than the prevalence of heavy infections in
286 successfully molted soft-shell crabs. Estimating the economic consequences of the 62% of peelers that
287 died with heavy CsRV1 infections in MD and VA suggests that the virus is associated with the loss of
288 212 metric tons of peeler crabs worth $1.53 million. CsRV1 is not the only reovirus to kill crabs in
289 aquaculture: both *Eriochir sinensis* (Zhang et al., 2004) and *Scylla serrata* (Deng et al., 2012) are reported
290 to suffer from mortality associated with reoviruses.

291 Crabs with high virus loads likely did not acquire CsRV1 within the shedding systems. The
292 prevalence of CsRV1 infections in live peeler crabs entering aquaculture (33%) was nearly the same as

293 the estimated prevalence of CsRV1 from the combined numbers of dead and live crabs processed in these
294 systems (35%). That is, the overall prevalence of CsRV1 in peelers did not increase during the average of
295 5 days in short-term culture. This indicates that although virus replication within infected crabs may be
296 accelerated during aquaculture, CsRV1 transmission between crabs in soft-shell crab production is
297 minimal over the brief culture periods involved. We speculate that a certain fraction of crabs enter soft
298 crab aquaculture with naturally-acquired CsRV1 infections which rapidly progress due to the additional
299 stress of molting and sub-optimal conditions, and eventually contribute to mortality of peelers at the
300 levels observed in this study.

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

313 It is apparent that infection trends were affected by factors that we did not measure or control. First,
314 strong random effects of site and date on CsRV1 prevalence were identified by GLMM. Second, CsRV1
315 prevalence levels at middle salinity sites did not fit well with the salinity regression, indicating that other
316 factors influence overall infection rates in mesohaline conditions. The site and time factors in final
317 modelling of CsRV1 prevalence suggest that the actual location of crab harvest, position in the estuary, or
318 related factors may influence disease prevalence even more directly than salinity. This site-by-site

319 variation agrees with prior studies of CsRV1 prevalence in wild crabs, which showed wide variation by
320 site, year, or month (Flowers et al., 2018; Flowers et al. 2015).

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

331

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333 None.

334

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351

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443 **Table and Figure captions:**

444

445 **Table 1.** Final reduced generalized linear mixed models (GLMM) predicting significant effects on crab

446 mortality and CsRV1 infection intensity.

447

448 **Figure 1.** Map of soft-shell blue crab aquaculture facilities surveyed in 2016-2017.

449

450 **Figure 2.** Blue crab (*C. sapidus*) mortality (mean±s.e.) observed in flow-through and recirculating water

451 aquaculture systems in the Chesapeake Bay soft shell crab industry.

452

453 **Figure 3.** Daily fluctuation from mean water temperature in flow-through and recirculating crab

454 aquaculture systems (Student's t, d.f. = 1, t = -1.52, p = 0.09).

455

456 **Figure 4.** Frequency histograms of log CsRV1 loads observed in Chesapeake a) live peelers, b) dead, and

457 c) soft shell blue crabs from aquaculture.

458

459 **Figure 5**. Prevalence of CsRV1 infection in dead peeler crabs fit to a reciprocal regression (CsRV1

460 Prevalence (%) = 98.0 – 251.3/Salinity (psu); R^2 = 0.4374, ANOVA d.f. = 19, F = 13.99, p = 0.0015).

461

462 **Appendices:**

463 **Table A.1.** Record of sites, dates, water quality, system type, crab mortality, and CsRV1 prevalence data

464 from surveyed Chesapeake soft shell crab aquaculture sites. Live soft and peeler crab sampling was

465 progressively introduced to the experiment, and were not sampled at all sites.

466

467

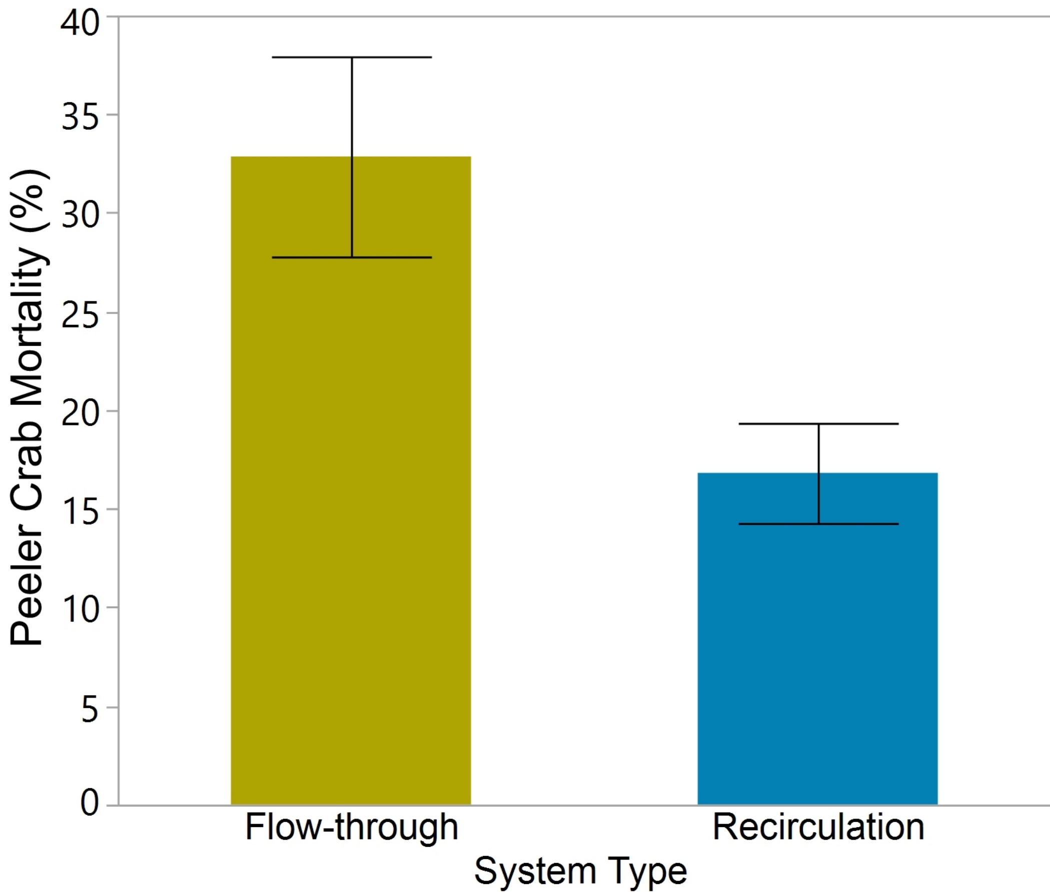
468 **Table A.2.** Record of sites, dates, water quality, system type, crab mortality, and CsRV1 prevalence data
469 from surveyed Louisiana soft shell crab aquaculture sites. Live soft and peeler crab sampling was
470 progressively introduced to the experiment, and were not sampled at all sites. Mortality was not sampled
471 in 2017.
472
473 **Table A.3.** Full generalized linear mixed model (GLMM) with potential effects on crab mortality and
474 CsRV1 infection intensity.
475

Site Key
1-Pasadena, MD
2-Rock Hall, MD
3-Tilghman & Philips Wharf, MD
4-Patuxent, MD
5-West Point, VA
6-Sarah's Creek, VA
7-Chuckatuck Creek, VA
8-Franklin, LA
9-Dulac, LA

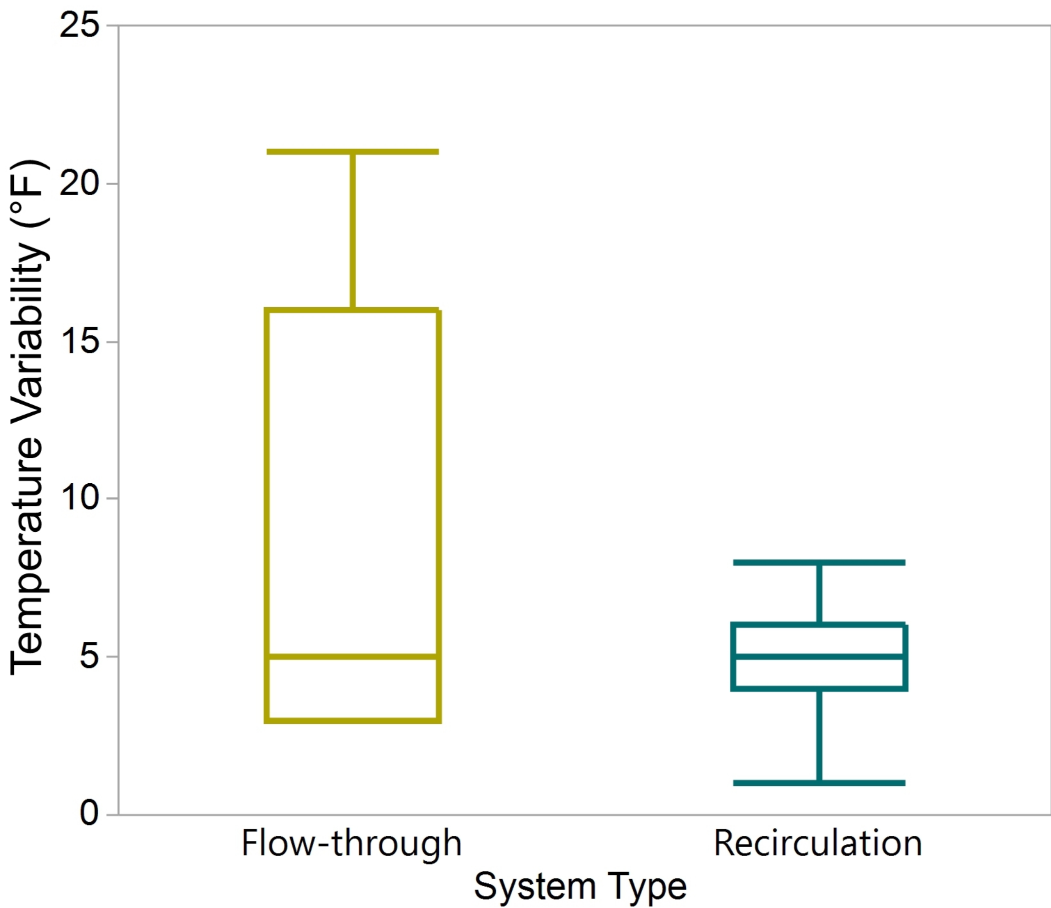
-Recirculation System

-Flow-through System

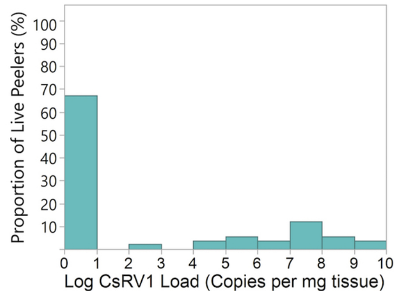
91.40°W
90.00°W
Louisiana
New Orleans
30.00°N
Thibodaux
N
8
Bayou Vista
Morgan City
Houma
9
Grand Isle
10 km
29.10°N

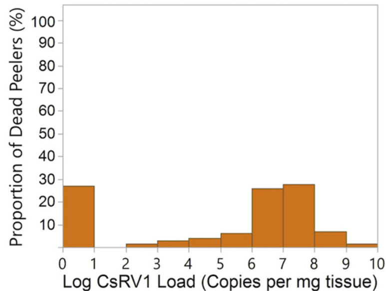


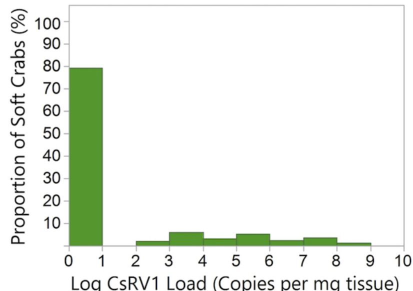
Peeler Crab Mortality (%)
40
35
30
25
20
15
10
5
0
Flow-through
Recirculation
System Type



Temperature Variability (°F)
25
20
15
10
5
0
Flow-through
Recirculation
System Type


A
Proportion of Live Peelers (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Log CsRV1 Load (Copies per mg tissue)


B
Proportion of Dead Peelers (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Log CsRV1 Load (Copies per mg tissue)


C
Proportion of Soft Crabs (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Log CsRV1 Load (Copies per mg tissue)

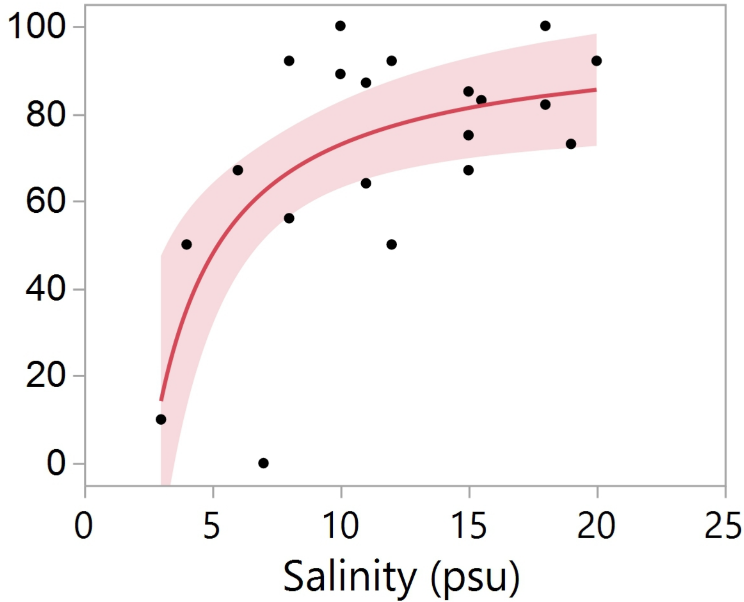

Dead Peeler Crab CsRV1
Infection Prevalence (%)
100
80
60
40
20
0
0
5
10
15
20
25
Salinity (psu)

Table 1. Final reduced generalized linear mixed models (GLMM) predicting significant effects on crab mortality and CsRV1 infection intensity.

Model	Predictor Variable	Slope Estimate	Standard Error	p-value	Variance	Standard Deviation	p-value
	Fixed Effects				Random Effects		
A. Crab Mortality (%) ~ System Type + Temperature Variation (°F) d.f. = 17, AIC = 154.17 Non-normal Homoscedastic	System Type (Recirculation)	-21.85	5.30	7.06E-04			
	Temperature Variation (°F)	-1.20	0.52	0.0341			
	Intercept	44.32	6.28	1.93E-06			
B. Log(Crab CsRV1 Load) (genomes/mg) ~ Successful Molting + (1|Site) + (1|Date)	Successful Molting	-3.16	0.31	<2E-16			
d.f. = 518, AIC = 2561.85	Location				1.28	1.13	8.15E-08
Non-normal	Date				1.88	1.37	7.20E-07
	Intercept	4.77	0.56	9.43E-05			
C. Log(Dead Crab CsRV1 Load) ~ Salinity + (1|Site) + (1|Date)	Salinity (psu)	-0.13	0.07	0.0498			
d.f. = 320, AIC = 1601.30	Location				4.94	2.22	7.41E-10
Non-normal	Date				1.05	1.03	7.14E-04
	Intercept	6.25	1.13	8.42E-05			