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White spot syndrome virus (WSSV) in cultured juvenile blue crabs *Callinectes sapidus*: oral versus injection exposure, and feeding frequency effects

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ABSTRACT: The efficacy of oral versus injection exposure and the effect of feeding frequency on the transmission of white spot syndrome virus (WSSV) in cultured juvenile blue crabs *Callinectes sapidus* were investigated. Crabs in Group 1 (G-1, n = 48) were exposed once orally to 100 mg of WSSV-infected shrimp tissue mg^{-1} of body weight (BW). The oral inoculum contained 2.6×10^9 WSSV genome copies mg^{-1} tissue. Group 2 (G-2, n = 46) received the same dosage once weekly for 5 wk. Group 3 (G-3, n = 12) was injected with 0.01 ml (2.6×10^7 genome copies 0.01 ml^{-1}) WSSV inoculum g^{-1} BW. Group 4 (G-4, n = 12) was injected with 0.01 ml WSSV-negative shrimp serum and saline mixture g^{-1} BW. Dead and moribund animals were frozen at −80°C. After 37 d, all remaining crabs were frozen. Genomic DNA from gill tissue was evaluated for the presence and quantity of WSSV using TaqMan real-time PCR. All G-3 animals died and tested positive. No G-4 animals died or tested positive. In the fed groups, WSSV prevalence was approximately 16%, but viral load was higher and survival was lower in G-2 compared to G-1. Injected animals carried a higher viral load than fed animals, and dead animals had higher viral loads than live animals. Blue crab juveniles are susceptible to WSSV, but oral exposure does not efficiently transmit WSSV in juvenile blue crabs. Some animals can die from WSSV if repeatedly exposed.

KEY WORDS: WSSV · Blue crabs · Oral exposure · Susceptibility

1. INTRODUCTION

White spot syndrome virus (WSSV) is the epizootic agent of white spot disease, a deadly, untreatable disease primarily of cultured penaeid shrimp. Since the first recorded outbreak of white spot disease in an aquaculture facility in Taiwan in 1992 (Chou et al. 1995), the virus has been found virtually everywhere shrimp aquaculture occurs (Flegel 1997). The virus has resulted in billions of dollars in economic damage to the aquaculture industry (Stentiford et al. 2012). White spot disease spreads rapidly among shrimp and causes nearly 100% mortality in crowded populations of penaeids within 2 to 7 d (Chou et al. 1995). Disease transmission occurs through cohabitation in confined space and particularly through consumption of infective carcasses (necrophagy) by uninfected shrimp (Chou et al. 1995, Lightner et al. 1998, Soto & Lotz 2001, Soto et al. 2002, Walker & Mahon 2009).

In North American coastal waters, penaeid shrimps, including 3 commercially harvested species in the northwestern Atlantic Ocean, are potentially most severely impacted by white spot disease (see Soto & Lotz 2001, Soto et al. 2002, Chapman et al. 2004), but other crustaceans, including several commercial species of interest to aquaculture such as blue crabs, crayfish, and prawns, are susceptible (i.e. can be

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infected) to WSSV to varying degrees (Flegel 1997, Richman et al. 1997, Sahul-Hameed et al. 2000, Escobedo-Bonilla et al. 2006, Sánchez-Martínez et al. 2007, Baumgartner et al. 2009). Our own opportunistic surveys for WSSV in decapods in coastal Mississippi (USA) during 2015–2018 revealed WSSV infections in 10 species of decapods (Muhammad 2016, our unpublished data), including the blue crab *Callinectes sapidus*, in which we observed WSSV in 36% (15 of 42) of the mature females examined.

C. sapidus occurs in warmer coastal habitats of the western Atlantic Ocean from Maine (USA) to Argentina. The species is especially abundant in the Chesapeake Bay and in the northern Gulf of Mexico, both of which support commercial fisheries for blue crabs. In northern Gulf marshes, blue crabs function ecologically as important prey items for many fish species. Blue crabs are also voracious predators that prey on a variety of fauna including many other decapod species that are susceptible to WSSV. Moreover, because habitat and life history of the blue crab overlap with those of other crustaceans such as penaeid shrimp, blue crabs may serve as reservoirs for infection of penaeid shrimp (Chang et al. 2001, Powell et al. 2015).

Few published studies have investigated the susceptibility of blue crabs to WSSV using molecular techniques. Chang et al. (2001) surveyed 206 living wild adult crabs of harvestable size from American waters, and found WSSV in 29, 24, and 25% of crabs from New York, New Jersey, and Texas, respectively. Powell et al. (2015) surveyed 176 adult blue crabs from South Carolina and 124 adult blue crabs from Georgia and found only a single crab from South Carolina (0.3%) infected with WSSV. Two other studies that surveyed blue crabs from the Gulf of Mexico for WSSV using molecular techniques did not detect the virus (Dorf et al. 2005, Rogers et al. 2015). Despite the fact that WSSV was not detected in those 2 studies and some variability in the methods among WSSV surveys in blue crabs, the collective evidence, including our own (unpublished), indicates that adult blue crabs are infected with WSSV in the northwest Atlantic Ocean.

The manner in which WSSV infects crustaceans other than penaeid shrimps is not well understood. Previous experiments in our laboratory using a Chinese isolate of WSSV confirmed that WSSV is capable of causing white spot disease and mortalities in adult blue crabs by injection and oral infection (Flowers 2002). Likewise, in other studies (Supamattaya et al. 1998, Yun et al. 2014, Yin et al. 2017), prevalence and mortality varied with the method of exposure, regardless of arthropod species studied. Additionally, differential susceptibility to WSSV between life history stages within the same decapod species has been demonstrated (for examples, see Venegas et al. 1999, Sahul-Hameed et al. 2000, Pérez et al. 2005), albeit not in blue crabs. The degree to which WSSV affects juvenile blue crabs and how juvenile blue crabs become infected remain ambiguous. Understanding whether juvenile and adult blue crabs differ in susceptibility to WSSV will help elucidate the potential for disease outbreaks in wild and cultured crab populations.

We investigated the efficacy of oral versus injection exposure and the effect of frequency of oral exposure on transmission of WSSV in cultured juvenile blue crabs. We incorporated the same Chinese isolate of WSSV that Flowers (2002) used to challenge adult blue crabs, thus maximizing the comparative value of the present results with those reported by Flowers (2002).

2. MATERIALS AND METHODS

2.1. Source and preparation of viral inoculum and infective tissue for oral exposures

The WSSV isolate used in the experiment came from gill tissue of cultivated penaeid shrimp that died from WSSV infection in the People's Republic of China. The tissue was collected and frozen (–20°C) in 1996 and shipped from China to the Gulf Coast Research Laboratory, Ocean Springs, Mississippi, where it was maintained frozen at –80°C as a source for infection experiments. Concentrated WSSV inoculum was prepared following protocols by Prior et al. (2003) and Escobedo-Bonilla et al. (2006). Gills from shrimp killed by WSSV infection were removed, minced using a single-edged razor blade, and mixed with 0.9% sterile saline solution (1:10 w/v dilution of gill tissue to saline solution). The mixture was then homogenized using a vortex mixer and centrifuged at 3000 × *g* (4°C for 20 min). The supernatant was collected and centrifuged again at 13 000 × *g* (4°C for 20 min). The final supernatant liquid containing the virus was filtered (0.4 µm filter syringe), divided into 1 ml aliquots, and stored at –80°C. These aliquots were used to inoculate juvenile blue crabs for this experiment and to prepare infective shrimp tissue for the present oral exposure experiment with juvenile crabs. The same procedure was followed using gill tissue from uninfected shrimp to prepare aliquots for inoculating negative control juvenile blue crabs.

Adult specimens of *Litopenaeus vannamei* (24–45 g body weight [BW]), obtained as postlarvae from Sea Products Development (Rockport, TX), were used for providing infective shrimp tissue for the oral exposure experiment. Inoculum prepared as described above was thawed and mixed with 0.9% sterile saline solution (1:10 w/v dilution of inoculum to saline solution) and filtered (0.4 µm filter syringe). Genomic DNA extracted from thawed diluted inoculum (50 µl) was evaluated using TaqMan real-time PCR, and 1 µl of extracted DNA was found to contain between 10^7 and 10^8 genome copies of WSSV. The thawed diluted inoculum was then injected (0.02 ml of WSSV inoculum g^{-1} BW; 0.02 ml contains 5.2×10^7 genome copies of WSSV) between the second and third tail segment (Lightner et al. 1998, Prior et al. 2003). Shrimp were maintained in 500 l circular fiberglass tanks with aerated artificial seawater (15 ppt) at 27–29°C and monitored after 48 h. Moribund and freshly dead shrimp were collected and frozen at –80°C. Genomic DNA was extracted from subsamples of muscle tissue (100 mg) for evaluation for WSSV infection using TaqMan real-time PCR. Flesh from the 5 infected shrimps was minced using a single-edged razor blade and mixed using a vortex mixer. Genomic DNA from the resulting mixed flesh (100 mg samples) was assessed using TaqMan real-time PCR and contained a mean of 2.6×10^6 genome copies of WSSV μg^{-1} of tissue. Genomic DNA samples from individual shrimp had from 1.1×10^5 to 6.2×10^6 genome copies of WSSV μg^{-1} of tissue. The flesh mixture was used for initial feeding on the first experimental day (Day 1) as described below. The remaining flesh was portioned and frozen at –80°C for subsequent thawing and feeding as described below during the remainder of the 5 wk experiment.

2.2. Source of experimental blue crabs

Juvenile crabs were spawned and reared at the Thad Cochran Marine Aquaculture Center (Ocean Springs, MS) using methods similar to those of Zmora et al. (2005). After crabs underwent the final molt from megalopa to juvenile stage, the early juvenile stage crabs were maintained in five 2500 l grow-out raceways for 6 wk at 26°C and fed frozen fish twice daily. After 6 wk, juvenile crabs (n = 118) measuring 15–20 mm in carapace width and weighing from 1–2 g were randomly selected and removed from the grow out raceways and isolated in 1 l tanks with aerated artificial sea water (20–22 ppt) in a temperature-controlled room (27–29°C). The isolated juvenile crabs were acclimated under these conditions for 4 d prior to experimentation. On the first acclimation day, each juvenile crab was allowed to feed on commercially prepared dry pellet food (Zeigler Intensive Grow Out, Zeigler Bros.), but food was withheld for the final 3 d of acclimation.

2.3. Experimental conditions

At the start of the experiment (Day 1), the 118 isolated, acclimated juvenile crabs were divided into 4 treatment groups: G-1: single oral exposure (n = 48); G-2: multiple oral exposure (n = 46); G-3: WSSV injection group (n = 12); and G-4: negative control injection group (n = 12). On Day 1, each member of both oral study groups (G-1 and G-2) was fed 100 mg of infected shrimp tissue ad libitum. The oral dose per feeding was 2.6×10^{11} WSSV genome copies (infected tissue contained approximately 2.6×10^9 genome copies WSSV mg^{-1} tissue). This quantity of infected shrimp tissue was approximately 10% of the overall BW of each crab. Members of G-1 were subsequently fed only commercial feed for the remainder of the 5 wk study. In contrast, members of G-2 were each fed 100 mg of WSSV-infected shrimp tissue (tissue contained 2.6×10^9 genome copies WSSV mg^{-1}) each week during the study supplemented by commercial feed once per week between exposures to infected shrimp tissue. Members of G-3 were injected with WSSV inoculum at a dose of 0.01 ml g^{-1} BW (0.01 ml of inoculum contains 2.6×10^7 genome copies of WSSV). The inoculum was injected between the trochanter and coxa of the rear (swimming) leg using a 1 ml syringe with a 30 gauge needle. Members of G-4 were injected with a comparable volume of negative control inoculum at a dose of 0.01 ml g^{-1} crab body weight. Both injection study groups were fed 100 mg of uninfected shrimp tissue ad libitum. Members of both injection groups were subsequently fed commercial feed for the remainder of the 5 wk study. Water in each of the 118 tanks was changed weekly throughout the experimental period with sea water prepared and maintained in the same temperature controlled room. All animals were monitored daily. Molting was not specifically monitored, but carapaces were removed as shed. Dead and moribund crabs were immediately isolated, frozen (–80°C), and the date and time of collection were recorded. The experiment was terminated after 37 d and all remaining live crabs were frozen (–80°C). Genomic DNA was extracted from gill tissue (100 mg samples) from the frozen carcasses, and evaluated for

presence and relative quantity of WSSV using TaqMan real-time PCR analysis.

2.4. DNA extraction and real-time PCR analysis

Shrimp and crab DNA was extracted from animals using High Pure Viral Nucleic Acid Kits (Roche Applied Science). Package instructions were modified by inclusion of a 48 h incubation at room temperature prior to the suggested 10 min heated incubation period, and tissues were crushed with a micropestle. Resulting DNA samples were frozen at −20°C. Total DNA was estimated in thawed samples using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific). Sequencing was accomplished using the forward primer WSS1011F (5′-TGG TCC CGT CCT CAT CTC AG-3′), reverse primer WSS1079R (5′-GCT GCC TTG CCG GAA ATT A-3′), and TaqMan probe WSSV1032-1050 (5′-AGC CAT GAA GAA TGC CGT CTA TCA CAC A-3′) labeled by the fluorescent dyes 5-carboxyfluoroscein (FAM) at the 5′ end and *N,N,N,N*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end (Durand et al. 2003). DNA Samples (5 µl) were analyzed in triplicate using a TaqMan assay, consisting of 12.5 µl of TaqMan Universal PCR Master Mix (ThermoFisher Scientific), 2.5 µl of both 0.3 µM primers, and 2.5 µl of 0.15 µM probe for a total sample volume of 25 µl (Durand et al. 2003). Mean viral load for each crab sample was calculated from the 3 replicate samples. A 10-fold serially diluted 65 bp WSSV standard oligonucleotide (5′-AAT GGT CCC GTC CTA GAA GCC ATG AAG AAT GCC GTC TAT CAC ACA CTA ATT TCC GGC AAA GCT CG-3′) (Trilink Biotechnologies) was amplified to provide standard WS curves for quantifying sample copy number for each plate run in real time (28 to 2.782 × 10^7 copies μl^{-1}), also in triplicate. The standard dilution concentrations were calculated based on the length of the oligonucleotide and the assumption that average weight of a base pair is 650 Da, coupled with conversions from weight to volume. A negative control sample consisting of 5 µl of purified water in place of DNA plus Master Mix, primers, and probe was included in triplicate in each reaction plate. Amplification of sample reactions was done at 50°C for 120 s for AmpErase uracil-N-glycosylase reaction, followed by AmpliTaq activation for 600 s at 95°C, then 40 cycles of 15 s each at 95°C and 60 s at 60°C (Durand & Lightner 2002, Durand et al. 2003). Reactions were run in an Applied Biosystems 7500 Fast Real-Time PCR System (ThermoFisher Scientific) and analyzed using 7500 Software V2.0.4 (ThermoFisher Scientific).

2.5. Data analysis

Survival in the 4 treatment groups was monitored daily and compared by Kaplan-Meier survival analysis using Mantel-Haenszel tests (Therneau 2018). Viral loads from animals that tested positive for WSSV from various treatments were compared using Kruskal-Wallis tests to compare more than 2 treatments and Mann-Whitney *U*-tests to compare 2 treatments (R Development Core Team 2018). Differences among groups were considered statistically significant at an α level of 0.05. Throughout the manuscript, values are given as means ± SD unless otherwise noted.

3. RESULTS

3.1. Prevalence of WSSV infection

The prevalence of WSSV in juvenile blue crabs fed infected tissue and detected from gill tissue at the end of the 37 d study was similar between G-1 and G-2 treatments. Eight out of 48 crabs (16.7%) fed infected shrimp tissue once only, on Day 1 (G-1), were infected with WSSV (Table 1). Seven out of 46 crabs (15.2%) fed infected shrimp tissue once per week (G-2) were infected with WSSV (Table 2). Twelve out of 12 crabs (100%) injected with WSSV inoculant on Day 1 (G-3) were infected with WSSV (Table 3). None of the 12 crabs (0%) in the injected negative control group (G-4) was infected with WSSV.

Table 1. Viral load for white spot syndrome virus (WSSV)-positive juvenile blue crabs from the single oral exposure treatment (G-1). Mortality day is the day on which the animal died, with Day 1 being the day of feeding of infected WSSV tissue and Day 37 being the experiment termination date. Seven of the 8 infected crabs survived and were frozen on Day 37

Crab ID no.	Mortality day	Viral quantity (genome copies) Mean	SD	Range
26	35	4[a]	1.8	1.8–5
2	37	46	11	34–56
8	37	1048	339	699.4–1378
9	37	645	98	534–719
31	37	29	1.2	28–30
34	37	26	9.6	17–36
39	37	1048	245	818–1305
42	37	188	79.1	112–270

[a]Below threshold of detection (~20 genome copies) using present real-time PCR methods

Table 2. Viral load for white spot syndrome virus (WSSV)-positive juvenile blue crabs from the multiple oral exposure treatment (G-2). Mortality day is the day on which the animal died, with Day 1 being the day of the first feeding of infected WSSV tissue and Day 37 being the experiment termination date. Four of the 7 infected crabs survived and were frozen on Day 37

Crab ID no.	Mortality day	Viral quantity (genome copies) Mean	SD	Range
43	10	8.86×10^5	1.39×10^5	7.3×10^5–9.6×10^5
7	22	20.3	1.9	18.9–22.4
26	35	5.05×10^5	3.4×10^4	4.7×10^5–5.35×10^5
37	37	19	22	3.6–44
24	37	15	20.2	0.5–38
42	37	11	6.8	6.6–18.8
48	37	53	36	31–95

Table 3. Viral load for white spot syndrome virus (WSSV)-positive juvenile blue crabs injected with WSSV inoculum (treatment G-3). Mortality day is the day on which the animal died, with Day 1 being the injection day

Crab ID no.	Mortality day	Viral quantity (genome copies) Mean	SD	Range
1	2	1.4×10^7	4.4×10^6	1.1×10^7–1.9×10^7
2	2	1.6×10^7	2.0×10^6	1.4×10^7–1.8×10^7
3	2	5.8×10^5	5.2×10^3	5.8×10^5–5.9×10^5
4	2	9.2×10^7	4.9×10^6	8.9×10^7–9.8×10^7
5	2	3.3×10^7	2.6×10^7	3.0×10^6–5.0×10^7
6	2	3.8×10^5	0	3.8×10^5
7	2	1.8×10^5	5.5×10^4	1.2×10^5–2.3×10^5
8	3	2.1×10^7	3.0×10^6	1.9×10^7–2.5×10^7
9	3	1.1×10^6	61 737	1.0×10^6–1.2×10^6
10	3	1.4×10^5	1.8×10^5	3.0×10^4–3.4×10^4
11	3	4.0×10^4	2.5×10^4	3.0×10^4–7.0×10^4
12	3	4.1×10^4	2.5×10^4	2.4×10^4–7.0×10^4

3.2. Viral load

The viral load from the 8 WSSV-infected crabs in G-1 ranged from 4 to 1048 genome copies μl^{-1} genomic DNA, and the mean viral load for all infected G-1 crabs was 379 ± 461 genome copies μl^{-1} genomic DNA (Table 1, Fig. 1). The load detected in the crab with the lowest measured load (4 ± 1.8 genome copies of WSSV μl^{-1} genomic DNA) was below the threshold of accurate detection (~20 genome copies) by the real-time PCR methods. This crab was found dead on Day 35 of the study. The other 7 infected G-1 crabs were still alive on Day 37. The viral load from the 7 WSSV-infected crabs in G-2 ranged from 11 to 8.9×10^5, and the mean viral load for all infected G-2 crabs was $2 \times 10^6 \pm 3.6 \times 10^5$ viral

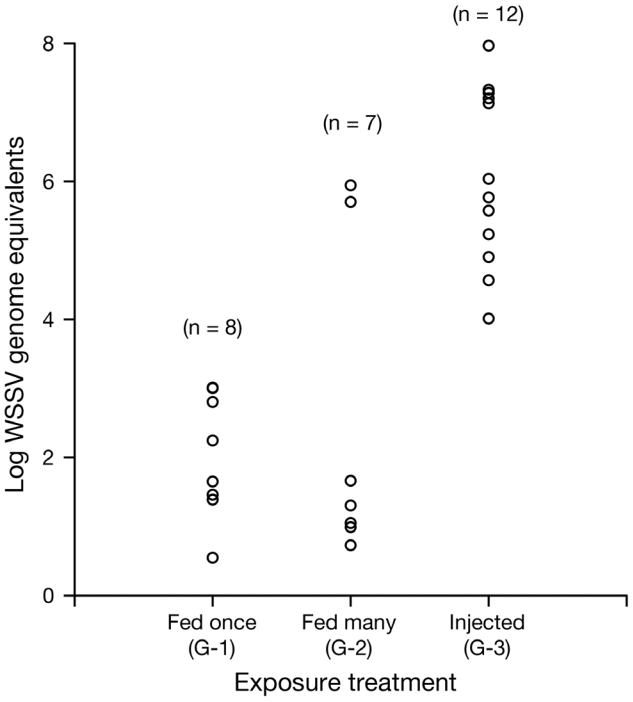

Fig. 1. Observed white spot syndrome virus (WSSV) loads from WSSV-positive juvenile blue crabs either injected (G-3), orally exposed once (G-1), or orally exposed 5 times (G-2). LogLoads differ among exposure groups (Kruskal-Wallis test, $p < 0.0006$). The LogLoad is the viral genome copy number in 1 µl of extracted DNA from 100 mg of gill sample expressed as $\log_{10}$

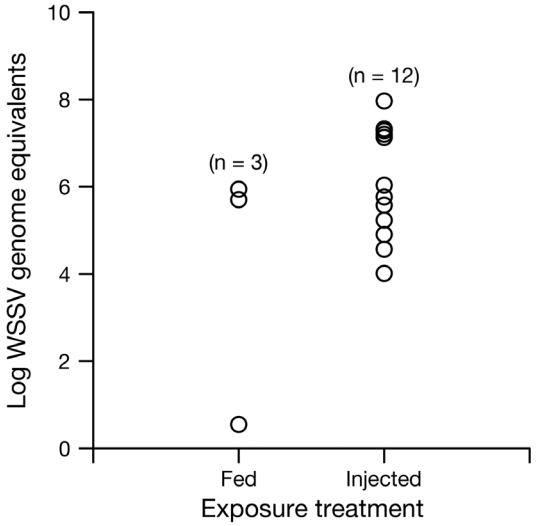

Fig. 2. Observed white spot syndrome virus (WSSV) quantities in orally exposed (G-2) and injected crabs that died from WSSV infection during the study. There was no difference in viral load between groups (Mann-Whitney *U*-test, $p > 0.3$). LogLoad is the viral genome copy number in 1 µl of extracted DNA from 100 mg gill sample expressed as $\log_{10}$. The fed crab with low load (0.5) died on Day 35 of the experiment

genome copies μl^{-1} genomic DNA (Table 2, Fig. 1). Only 2 of the 7 infected crabs (29%), both of which died early (Days 10 and 35), had high viral loads. The mean viral load for those 2 crabs was $7 \times 10^5 \pm 2.3 \times 10^5$ viral genome copies μl^{-1} genomic DNA. The viral load for the 12 G-3 crabs killed by injection with WSSV inoculant ranged from 4×10^4 to 9.2×10^7 viral genome copies μl^{-1} genomic DNA, and the mean viral load for all WSSV injected (G-3) crabs was $2 \times 10^6 \pm 3.6 \times 10^5$ viral genome copies μl^{-1} genomic DNA (Table 3, Fig. 1).

Viral load differed among the 3 exposure treatments (Kruskal-Wallis test; $p < 0.0006$; Fig. 1). The mean viral load in G-3 crabs killed by intramuscular injection with potent WSSV inoculant containing $1.5 \times 10^7 \pm 2.7 \times 10^7$ genome copies μl^{-1} of extracted DNA was not different than the mean viral load ($6.96 \times 10^5 \pm 2.3 \times 10^5$ genome copies μl^{-1} of extracted DNA) in 3 G-2 crabs that died early after multiple feeding exposures (Mann-Whitney *U*-test; $p > 0.3$; Fig. 2). The mean viral load in all crabs that died while infected with WSSV during the study ($1.3 \times 10^7 \pm 2.5 \times 10^7$ genome copies per μl^{-1} of extracted DNA) was significantly higher (Mann-Whitney *U*-test; $p < 0.00004$) than the mean viral load in crabs that survived with WSSV infections to the end of the 37 d study (262.3 ± 408 genome copies per μl^{-1} of extracted DNA) (Fig. 3).

3.3. Survivorship

Survival curves for the 4 groups are significantly different from one another ($p < 0.05$, log-rank/Mantel-Haenszel test) (Fig. 4). Mortalities were observed in treatments G-1, G-2, and G-3, but not in G-4. Forty-one of the 48 G-1 crabs (85.4%) survived to Day 37 and only 1 of these 7 crabs tested positive for WSSV (the same crab with a WSSV load below the detection limits for real-time PCR); consequently, none of the G-1 crabs experienced mortality that can be attributed to WSSV. Twenty-nine of the 46 G-2 crabs (63%) survived to Day 37 (Fig. 4). Four of the 7 G-2 crabs (57%) infected with WSSV survived to Day 37, whereas 3 of the infected crabs (43%) died before Day 37 (Table 2). Of the 3 crabs that died while infected with WSSV, 2 had an extremely high viral load (see Table 2), and 1 had a low viral load (20.3 ± 1.9 viral genome copies μl^{-1} of genomic DNA) (Table 2); therefore, WSSV was considered the likely cause of death for 2 of the 3 infected G-2 crabs. Thus, repeated oral exposure to WSSV over 37 d resulted in 2 G-2 crab mortalities or 4.3% mortality, and these mortalities occurred on Days 10 and 35 of the study. Mean time to death was 540 ± 424.3 h. Juvenile blue crabs injected with WSSV inoculant on Day 1 (G-3) began dying on Day 2, and all 12 injected crabs were

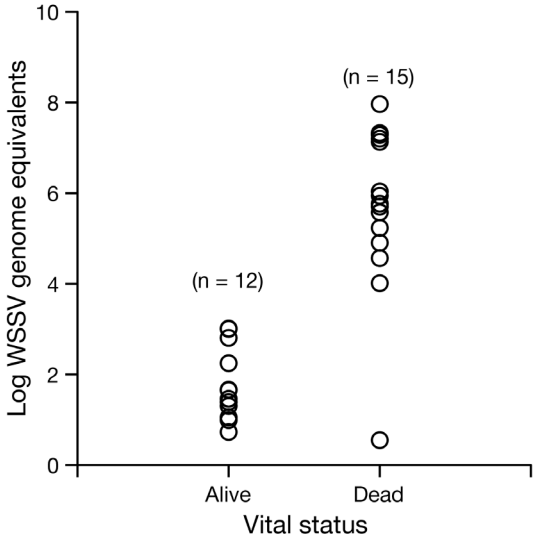

Fig. 3. Observed white spot syndrome virus (WSSV) quantities from all juvenile crabs that died from WSSV during the study and those that survived with WSSV infections at the end of the study. There was a significant difference in viral load between groups (Mann-Whitney *U*-test, $p < 0.00004$). The LogLoad is the viral genome copy number in 1 µl of extracted DNA from 100 mg gill sample expressed as $\log_{10}$

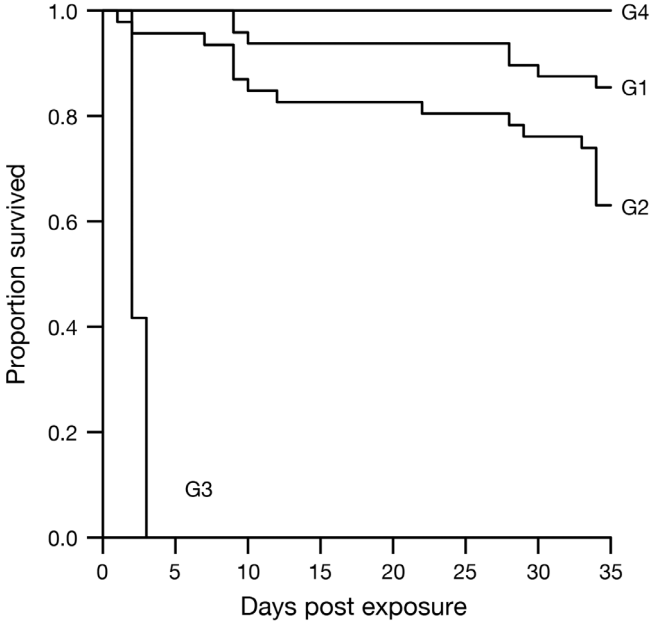

Fig. 4. Kaplan-Meier estimation of survivorship. Proportion (probability) survival versus time in days from exposure to white spot syndrome virus (WSSV) for 4 groups of juvenile blue crabs (G-1: orally exposed once; G-2: orally exposed 5 times; G-3: WSSV injection; G-4: negative injection control). Each curve is different at $p < 0.05$ (Mantel-Haenszel test)

dead by the end of Day 3 (Table 3). Mean time to death among G-3 crabs was 59.8 ± 7.77 h.

4. DISCUSSION

Factors contributing to susceptibility and virulence include, among others, mode of transmission, dose, viral isolate, temperature, and host species, as well as their complex interactions. Our results indicate that the oral exposure route produced lower prevalence and virulence than the injection route despite the higher dose used in the oral exposure. The oral exposure dose was 2.6×10^{11} WSSV genome copies crab^{-1} whereas the injection dose was $2.6–5.2 \times 10^{7}$ WSSV genome copies crab^{-1}. One explanation for why oral exposure is less virulent than injection exposure is that passage through the digestive system exposes viruses to various pHs and digestive enzymes that may denature some virions. Moreover, the gut wall provides a physical and chemical barrier that may also reduce the number of virions that gain access to the interior of the host (Jeswin et al. 2015). Conversely, exposure by injection avoids those obstacles, allowing a greater number of virions to reach internal cells of the host.

Infection by injection provides a mechanism for standardizing viral dosing for comparative purposes (Prior et al. 2003, Escobedo-Bonilla et al. 2006, Laramore et al. 2009), but represents an unnatural mode of transmission. Transmission among decapods is thought to occur by ingestion of WSSV-infected cadavers, horizontally by cohabitation with infected hosts, and possibly vertically from broodstock to offspring (Soto & Lotz 2001, Soto et al. 2002, Escobedo-Bonilla et al. 2006, Corteel et al. 2009, Jeswin et al. 2015). We focused on the injection and oral pathways of WSSV infection in the present study because both pathways have been studied previously in decapods, including in adult blue crabs (Flowers 2002). In the present study, survivorship was different among the 4 groups. All of the virus-injected crabs (G-3) died as a result of WSSV (i.e. had high viral loads) and none of the placebo crabs (G-4) died or tested positive for WSSV. No crabs fed infected tissue only once (G-1) died as a result of WSSV (i.e. none had a high viral load), but 2 crabs fed infected tissue multiple times died with a high viral load prior to the end of the experiment. Cumulative survivorship (Fig. 4) in the G-2 treatment (63%) was lower than that in G-1 treatment (85.4%). While factors other than WSSV may have influenced mortality in the oral exposure treatments, the difference in survivorship curves (Fig. 4) among the groups suggests that WSSV kills juvenile blue crabs and that WSSV has a greater effect on mortality in those crabs fed the virus multiple times. The nearly identical WSSV prevalence values in both oral treatment groups (G-1: 16.7%, G-2: 15.2%) is consistent with similar studies investigating influence of exposure time on WSSV susceptibility. Tuyen et al. (2014) similarly reported nearly identical prevalence of WSSV infection in shrimp fed WSSV-infected carcasses followed by either removal or retention of uneaten tissue. Our results and those of Tuyen et al. (2014) suggest that exposure time does not determine the prevalence of WSSV. Our study, however, suggests that repeated exposure results in increased mortality through an increased amount of virus in some infected animals.

In the present study, sample size prevented us from using median lethal dose (LD_{50}) for assessing virulence of WSSV in juvenile blue crabs, so we used mean viral load in dead and moribund shrimp (mean lethal load) as an indicator of WSSV virulence. We used gill tissue to assess viral load because Jeswin et al. (2015) showed that although WSSV load varied considerably among tissues and stages of infection in *Penaeus monodon*, gill tissue retained the highest viral load in moribund animals. The lethal viral load for WSSV varies among decapods, but penaeid shrimp dying from white spot disease generally exhibit the highest viral loads measured using real-time PCR. Durand & Lightner (2002) reported a mean lethal load of 1.2×10^{9} WSSV genome copies (from gill tissue) in moribund *Litopenaeus vannamei*, *L. stylirostris*, and *P. monodon*. Jamisolamin dela Calzada (2008), following procedures similar to those in the present study, inoculated the same Chinese isolate of WSSV into *L. vannamei* (n = 83) and *Farfantepenaeus duorarum* (n = 68) and reported mean lethal loads of 1.3×10^{9} and 5.9×10^{10} for moribund and fresh dead shrimp of those species, respectively. Jeswin et al. (2015) inoculated *P. monodon* (n = 5) with an unidentified isolate of WSSV (50 µl inoculant, containing 6×10^{7} WSSV genomic copies) and reported a mean lethal load derived from gill tissue of 6.45×10^{8} WSSV genomic copies. Additionally, Muhammad (2016), following procedures similar to those in the present study, inoculated the same Chinese isolate of WSSV into *Palaemonetes pugio* (n = 74) and *Uca panacea* (n = 71) and reported mean lethal loads of 9.21×10^{8} (SE = 8.69×10^{8}) and 1.53×10^{8} (SE = 2.12×10^{7}) for these species, respectively. Because the blue crabs sampled in the present study were not sampled until morbidity or death, it is not known whether or not surviving crabs were sampled

after an earlier peak of viral load or if the viral load in those crabs with low viral load would have increased over time. However, the mean lethal load that we found for the Chinese WSSV isolate in injected juvenile blue crabs (G-3) (1.5×10^7 genome copies of WSSV) appears to be 2 orders of magnitude lower than that in penaeid shrimp and slightly lower than that in other decapod species thus far similarly assessed for viral load. This suggests that while blue crab juveniles may be only somewhat susceptible to WSSV (i.e. capable of being infected) in comparison to other decapod species, they may be less tolerant of the virus (i.e. die with lower loads) than other decapods.

Laramore et al. (2009) showed that 7 geographic isolates of WSSV (including 1 from China) experimentally inoculated into *L. vannamei* produced different LD_{50} values and noted that the higher viral loads in dead shrimp did not necessarily correlate with higher LD_{50} values, suggesting that other factors related to genetic diversity play a role in WSSV virulence. The Chinese isolate used in the present study has not been compared genetically with other WSSV isolates; however, the present study and those by Jamisolamin dela Calzada (2008) and Soto et al. (2002) indicate that the isolate is highly virulent. Soto et al. (2002) assessed susceptibility by oral transmission in *L. vannamei* using this Chinese isolate and demonstrated that shrimp allowed to feed on infected shrimp cadavers for 16 h (cadavers prepared identically to those in the present study) exhibited 81% mean mortality after 5 d.

Temperature influences WSSV virulence, presumably through a combination of effects on the virus and host. While the optimal temperature for each species varies, the temperature used in the present experiment (27–29°C) falls both within the range of normal summer temperatures in the northern Gulf of Mexico and the optimal range for WSSV replication (Guan et al. 2003, Moser et al. 2012). The temperature used in the present experiment (27–29°C) was slightly higher than that (22–23°C) used by Flowers (2002). Nevertheless, all adult blue crabs challenged by injection with the Chinese WSSV isolate (volumes proportioned to crab weight) died rapidly, with mean ± SE time to mortality of 52 ± 1.18 h (Flowers 2002), compared with 59.8 ± 7.77 h (mean ± SD) in the G-3 juvenile crabs in the present study. In contrast, adults in the study by Flowers (2002) fed multiple doses of WSSV (prepared the same as in the present study) experienced 67% mortality (mean ± SE time to mortality of 375 ± 39.5 h), whereas juvenile crabs fed multiple infected doses in the present study experienced only 4.3% mortality (on Days 10 and 35, with mean ± SD time to mortality of 540 ± 424.3 h). Flowers (2002) did not assess viral load, but based on the present results and those of Flowers (2002), both adult and juvenile blue crabs are equally susceptible to WSSV by injection but have reduced susceptibility to WSSV infection through oral exposure as juveniles. The higher temperature used in the present experiment is closer to the high tolerance limit for WSSV (as demonstrated in shrimp) than that in the study by Flowers (2002); therefore, potential physiological differences between juvenile and adult crabs and any differences in the virus at the higher temperature may have contributed to the differences in oral transmission observed between the 2 studies.

Several studies have investigated variation in susceptibility to WSSV among decapods and even among life history stages of a single decapod species. Prior et al. (2003) demonstrated approximately 83.5% cumulative mortality for adult *L. vannamei* injected with a WSSV inoculant identically prepared but administered at twice the concentration (0.02 ml g^{-1} of shrimp body weight) as in the present study. Similarly, Yun et al. (2014) demonstrated 100% mortality for adult *Macrobrachium nipponense* injected with 2 relatively lower viral doses (2.5×10^4 and 900 genome copies g^{-1} prawn body weight), compared with dose used in the present study (2.6×10^7 genome copies g^{-1} crab body weight) after 2 and 10 d, respectively. Jeswin et al. (2015) injected the equivalent of 6×10^7 genome copies g^{-1} shrimp body weight into adult specimens of *P. monodon* and observed cumulative mortality of 100% within 96 h compared with approximately 60 h in this study. Venegas et al. (1999) demonstrated that susceptibility to WSSV is higher in older postlarval stages of *Marsupenaeus japonicus*. Sahul-Hameed et al. (2000) demonstrated that adults of cultured *Macrobrachium rosenbergii* are more susceptible to white spot disease than postlarvae. Similarly, Pérez et al. (2005) demonstrated that early postlarvae of *L. vannamei* were more resistant to WSSV infection than later stages. The present study determined that high WSSV susceptibility in injected juvenile blue crabs matched results in previous injection experiments, and susceptibility by oral transmission is higher in juvenile than adult blue crabs (see Flowers 2002), which is also consistent with the earlier life history challenge experiments involving other decapods.

In conclusion, the relatively constant prevalence of WSSV in juvenile blue crabs orally exposed in the present study, regardless of the number of times exposed, suggests that blue crabs could be the

source of infection for other decapods as postulated by Chang et al. (2001) and Powell et al. (2015). That most of the positive crabs carry a low viral load further suggests that WSSV is of little threat to individuals or the population of juveniles that might recruit to the adult population. The likelihood of a disease outbreak among juvenile crabs in aquaculture appears to be low considering the low infection rate and low incidence of white spot-induced mortality observed. Further research is required to establish possible effects on recruitment success or subsequent effects on adult crabs in the wild.

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