Vol. 120: 143–150, 2016
doi: 10.3354/dao03018

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 7

# Experimental inoculation of Louisiana red swamp crayfish *Procambarus clarkii* with white spot syndrome virus (WSSV)

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ABSTRACT: The red swamp crayfish *Procambarus clarkii* represents an important aquaculture species responsible for over half of all commercial aquaculture profits in Louisiana, USA. White spot syndrome virus (WSSV) is highly pathogenic in crustacean species and induces mass mortality in aquaculture operations worldwide. Natural outbreaks of WSSV occur yearly in cultured populations of crayfish in Louisiana. The goal of this study was to better understand the infectivity of WSSV in *P. clarkii*, by determining the minimum lethal dose necessary to initiate infection and to measure the resulting cumulative mortality following infection with different doses. A real time quantitative PCR (qPCR) method was used to detect WSSV in DNA extracted from gill tissue to ensure *P. clarkii* study populations were WSSV-free before the start of trials. Viable viral particles were isolated from naturally infected *P. clarkii* gill tissue and quantified using a novel digital PCR approach. Three infectivity trials were performed, and WSSV inocula were created by serial dilution, generating 5 treatments per trial. Five crayfish (weighing ~25 g) per dilution per trial received viral inoculations. Mortality was monitored daily for the duration of the trial in order to construct a median lethal dose ($LD_{50}$) curve, and probit regression analysis was used to determine $LD_{50}$ concentrations of viral particles. Knowledge of the infectivity of WSSV in native crayfish populations is of critical importance to the management of the commercial crayfish aquaculture industry in Louisiana. This is the first study to investigate the infectivity and to determine the $LD_{50}$ of the Louisiana strain of WSSV in native crayfish.

KEY WORDS: *Nimaviridae* · *Whispovirus* · Crustacean · Aquaculture · $LD_{50}$



## INTRODUCTION

White spot syndrome virus (WSSV) is a recently described, highly virulent virus and is the sole member of the family *Nimaviridae*, genus *Whispovirus* (Mayo 2002). It is a highly infective shrimp pathogen and has caused billions of dollars in economic losses in commercial shrimp farming operations worldwide (OIE 2006, Lightner 2011). WSSV can infect more than 90 aquatic crustacean species (Escobedo-Bonilla et al. 2008) and can spread to new areas through mechanisms such as importation of infected frozen shrimp commodities, transportation of live shrimp for aquaculture, seabirds, and release of untreated wastewater from shrimp packing plants (Lightner et al. 1997). It was first identified in penaeid shrimp in 1992 in Taiwan (Chen 1995) and was detected in the US by 1995 (Lightner 1996). In Louisiana, WSSV was first identified in wild and farmed crayfish populations in 2007 (Baumgartner et al. 2009).

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Crayfish are economically and culturally important to the people of Louisiana, with commercial profits from crayfish aquaculture valued at over $172 million, representing over half of all commercial aquaculture profits in the state (Richardson 2014). Louisiana is the largest producer of crayfish in the US, and the red swamp crayfish *Procambarus clarkii* constitutes 70 to 80% of the harvest annually (McClain et al. 2007). Crayfish are extensively farmed in large, shallow ponds, and production yields rely on natural reproduction rates from year to year. WSSV is transmitted horizontally, via both cannibalism and waterborne transmission (Chou et al. 1998). Although *P. clarkii* is generally resistant to diseases in the natural environment (Meng et al. 2013), the pond design of crayfish aquaculture provides an opportunity for the virus to spread rapidly and potentially decimate harvest yields. Moreover, some studies have shown that WSSV can remain latent in organisms without causing mortality, thereby preserving the virus in the environment or host until conditions are suitable for an outbreak (Tsai et al. 1999, Sanchez-Martinez et al. 2007).

Previous laboratory studies have confirmed experimental infections of WSSV in multiple species of penaeid shrimps, crabs, freshwater prawns, lobsters, copepods, insect larvae, and crayfish (Lo et al. 1996, Chen et al. 2000, Shi et al. 2000, Jiravanichpaisal et al. 2001, Sahul Hameed et al. 2001, Syed Musthaq et al. 2006), including *P. clarkii* (Maeda et al. 2000, Zhu & Quan 2012). Although these previous studies have shown that many taxa are susceptible to WSSV, mortality does not always occur from experimental infection. Freshwater prawns, lobsters, and crabs can exhibit histopathological signs of infection without mortality and could serve as reservoirs to facilitate the spread of WSSV to other species (Rajendran et al. 1999). In penaeid shrimp aquaculture operations, the presence of WSSV can significantly reduce commercial profits, as viral infection can induce mortality rates of up to 100% within 3 to 10 d (Lightner 1996, Zhan et al. 1998). The results of WSSV infectivity studies in *P. clarkii*, however, have produced various rates of mortality, and to date no infectivity study has been conducted on the Louisiana strain of WSSV in this species.

Crayfish harvests constitute the bulk of the commercial aquaculture profits in Louisiana, and the presence of WSSV poses a potential threat to the success of the industry. Although WSSV has not yet had a dramatic effect on commercial pond crayfish operations locally, the virus has negatively impacted soft shell crayfish production in closed recirculating aquaculture systems. Ultimately, WSSV is new to this region, and the long-term effects are unknown. The aim of the current study was to increase understanding of the detailed interactions between *P. clarkii* and WSSV by isolating and quantifying viable WSSV particles from infected *P. clarkii*, and performing infectivity trials in this species in order to determine a median lethal dose concentration of viral particles. This is the first study to determine the infectivity of the Louisiana strain of WSSV in native crayfish, and the results could be used to facilitate future investigations into ecosystem management initiatives and potential control strategies.

## MATERIALS AND METHODS

### Crayfish

*Procambarus clarkii* were collected using baited traps from crayfish culture ponds at the Louisiana State University Agricultural Center's Aquaculture Research Station in 2014 (Baton Rouge, Louisiana). Crayfish weighing 20 to 25 g were held in separate containers in individually recirculating 90 l tanks at a density of 5 crayfish tank$^{-1}$. Temperature was maintained at 24 ± 1°C, and crayfish were given a 1 wk acclimation period to these conditions prior to viral challenge. In total, 3 trials were performed, during which individuals received a commercial pelleted diet (50% protein, 14% fat; Cargill) at 1.5% of body weight every 3 d. A previously described quantitative TaqMan PCR (qPCR) method by Durand & Lightner (2002) and Baumgartner et al. (2009) was used on gill tissue from 30 individuals from each study population to ensure that crayfish were WSSV-free before the initiation of trials. Water quality parameters including alkalinity, hardness, total ammonia nitrogen, nitrite, and pH were measured weekly to ensure optimal water conditions.

### WSSV isolation and purification

The strain of WSSV used in the trials, LADL14-014, originated from infected *P. clarkii* collected from a soft-shell production facility (Livingston Parish, Louisiana) that were originally wild-caught from the Atchafalaya River Basin in 2014 (J. Hawke unpubl. case reports). Gill tissue was harvested from several individuals and used to isolate the virus via a modified technique described by Du et al. (2007). In summary, 6 g of WSSV-infected gill tissue were homoge-

nized in 35 ml of TNE buffer (0.05M Tris-HCl, 0.1M NaCl, 0.001M EDTA, pH 7.4). Samples were centrifuged at 8000 × *g* (20 min at 4°C), and the supernatant was passed through a 0.22 µm filter using a vacuum filter apparatus. The filtrate was layered onto a 30% sucrose solution and centrifuged at 30 000 × *g* (2 h at 4°C). The viral pellet was then suspended in 100 µl of TNE buffer. This procedure was performed on 3 separate occasions before the start of each of the 3 trials using gill tissue from previously experimentally infected and deceased crayfish, which provided a fresh source of viral particles from the previous trial. This process circumvented the damage related to freezing and thawing of samples kept at −80°C and changes in numbers of viable virions from one trial to the next. The same isolation protocol was also conducted on uninfected gill tissue to serve as a negative inoculation control for Trials 2 and 3.

### Viral quantification

Quantification of viral particles was carried out using the Open Array on Quantstudio 12K Flex Real Time PCR system (Applied Biosystems) before the start of each trial. Briefly, DNA was extracted from purified virions using the DNeasy blood and tissue kit as per the manufacturer's instructions (Qiagen). The DNA yield was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Molar concentration of DNA was calculated using the formula:

$$\mu g\,\mathrm{DNA} \times \frac{pmol}{660\,pg} \times \frac{10^6\,pg}{1\,\mu g} \times \frac{1}{N} = pmol\,\mathrm{DNA} \qquad (1)$$

The measured weight in grams was divided by molecular weight (number of nucleotide base pairs × 660 − the approximate double-strand DNA base weight of 660 Daltons). The circular DNA genome of WSSV contains 292 967 nucleotides (van Hulten et al. 2001); therefore, the WSSV molecular weight is 292 967 × 660 = 193 358 220 Da. If the DNA concentration measured via nano drop is 1 µg µl$^{-1}$, the molar concentration is $1 \times 10^{-6}/193\,358\,220 = 5.17 \times 10^{-15}$ M or 0.00517 picomol. Since 1 mol has Avogadro number of molecules ($6.023 \times 10^{23}$ mol) and a picomole = $6.023 \times 10^{11}$ mol, 0.00517 picomol will have $3.11 \times 10^{9}$ mol. This DNA is serially diluted to achieve a volume containing approximately $2 \times 10^{3}$ mol to be used for absolute quantification of DNA using the Quantstudio Flex.

The DNA samples were then diluted to a concentration of $1 \times 10^{3}$ particles µl$^{-1}$ and used as template for digital PCR. Reaction mix was prepared using Taqman Open Array Digital PCR master mix. Mastermix was prepared as per instructions with approximately 1800 to 2000 copies of viral DNA as a template. The Taqman primer–probe combinations used for the all reactions originated from Durand & Lightner (2002) and are listed in Table 1. The reaction mix was loaded onto open arrays using the Accufill ™ automated sample loader. Digital PCR was carried out on the QuantstudioFlex system, and the resulting data were analyzed using DigitalSuite™ Software. The software analyzes real-time PCR data by fitting it to a Poisson statistical model and reports digital results in copies µl$^{-1}$ with a statistically calculated confidence interval.

### Dilutions, inoculation, and WSSV infectivity

After quantification, the viral stock solution was serially diluted in 300 mOsm kg$^{-1}$ Hanks' balanced salt solution (HBSS) to create 5 inoculation dilutions. The dilutions ranged from $5.02 \times 10^{5}$ to $5.02 \times 10^{1}$ particles µl$^{-1}$, $2.05 \times 10^{1}$ to $2.05 \times 10^{-3}$ particles µl$^{-1}$, and $1.2 \times 10^{1}$ to $1.2 \times 10^{-3}$ particles µl$^{-1}$ for Trials 1, 2, and 3, respectively. For each trial, 10 crayfish dilution$^{-1}$ were intramuscularly injected using a 27-gauge needle with 100 µl between the carapace and abdomen. In Trial 1, an additional 10 crayfish received an injection of 300 mOsm kg$^{-1}$ HBSS solution to serve as a control. To better control for any differences resulting from the viral isolation protocol, the same procedure was performed on uninfected gill tissue, diluted to $10^{1}$ in 300 mOsm kg$^{-1}$ HBSS, and injected into 10

Table 1. Primer and probe sequences (GenBank accession number U50923) used for white spot syndrome virus quantitative PCR diagnosis and for absolute viral quantification using digital PCR

| Primer | Sequence |
|---|---|
| Forward (WSS1011F) | 5′-TTG TCC CGT CCT CAT CTC AG-3′ |
| Reverse (WSS1079R) | 5′-GCT GCC TTG CCG GAA ATT A-3′ |
| TaqMan probe | 5′(6FAM) AGC CAT GAA (ZEN) GAA TGC CGT CTA TCA CAC A (IBFQ)-3′ |

crayfish trial$^{-1}$ for Trials 2 and 3. Mortality of crayfish was monitored daily for 21 d in order to construct a median lethal dose ($LD_{50}$) curve. A probit regression analysis of the mortality data was conducted using *PoloPlus 2.0* software (LeOra Software Company®) in order to estimate $LD_{50}$ concentrations for each trial.

## RESULTS

### Viral isolation

Electron microscopy confirmed that intact viral particles can be isolated from WSSV-infected crayfish gill tissue using the described viral isolation protocol (Fig. 1). Preliminary testing of the inoculation process demonstrated that the viral particles retrieved from the isolation procedure successfully infected and induced mortality in *Procambarus clarkii.*

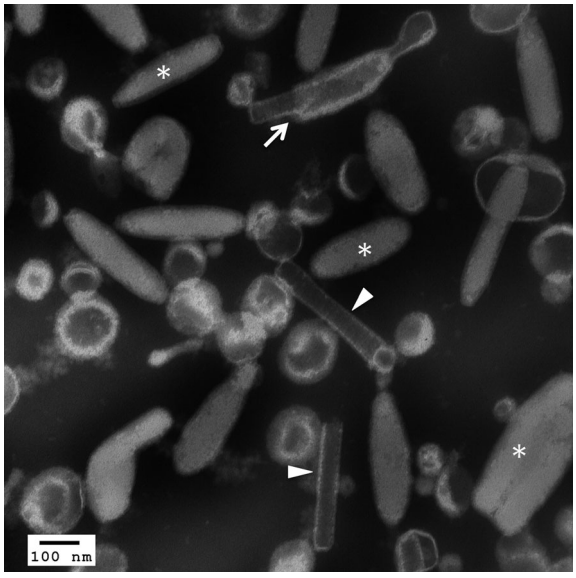


Fig. 1. Transmission electron microscopy image of white spot syndrome virus particles isolated from *Procambarus clarkii* gill tissue using a viral isolation protocol (negative staining with 2% phosphotungstic acid). Suspension of isolated viral particles diluted to 1:100000 containing mature virions (asterisks), ruptured virion with the exposed nucleocapsid (arrow), and naked nucleocapsids prior to being enveloped (arrowheads)

### Trial 1

In Trial 1, 100% mortality was reached by Day 5 in all viral dilutions ranging from $5.02 \times 10^5$ to $5.02 \times 10^1$ particles µl$^{-1}$, while all control crayfish remained alive (Fig. 2). Mortality began within 48 h post-inoculation, and nearly all crayfish were lethargic within 72 h. No WSSV-infected crayfish survived; therefore, an $LD_{50}$ value could not be calculated. As a result of the extremely rapid mortality rate, the WSSV dilution regime was further diluted for Trials 2 and 3.

### Trial 2

Further diluting the viral concentration regime for Trial 2 generated greater differences in cumulative mortality compared to Trial 1. Only the highest viral dilution group receiving $2.5 \times 10^1$ WSSV particles µl$^{-1}$ reached 100% mortality within 4 d, while no mortality was observed at the 2 lowest dilutions of $2.5 \times 10^{-2}$ and $2.5 \times 10^{-3}$ particles µl$^{-1}$, or in control crayfish (Fig. 3). An $LD_{50}$ value of 1.96 WSSV particles µl$^{-1}$ was estimated with a 95% confidence interval (1.362–2.929 WSSV particles µl$^{-1}$), which equates to a total exposure of 196 WSSV particles per individual crayfish.

### Trial 3

Crayfish in Trial 3 were exposed to approximately the same dilution regime of WSSV particles as in Trial 2; however, greater mortality was observed in Trial 3 (Fig. 4). The 3 highest viral dilution groups ranging

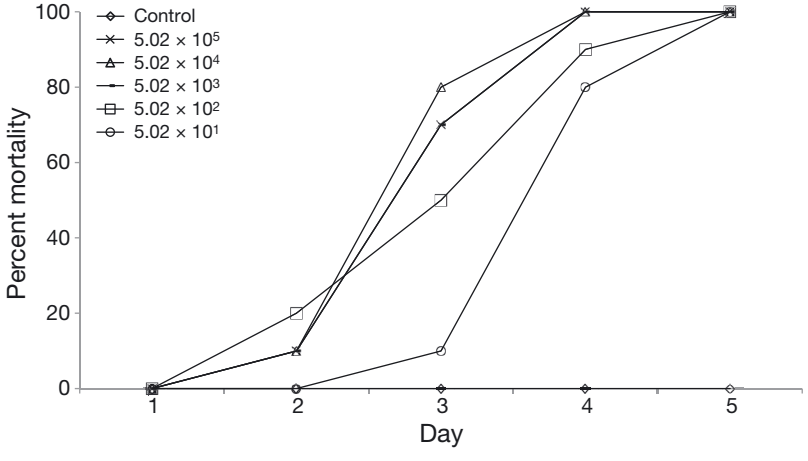


Fig. 2. Cumulative mortality for the 5 d of Trial 1. Crayfish *Procambarus clarkii* received inoculations ranging from $5.02 \times 10^5$ to $5.02 \times 10^1$ white spot syndrome virus particles µl$^{-1}$, with 10 crayfish inoculated in each dilution and control group

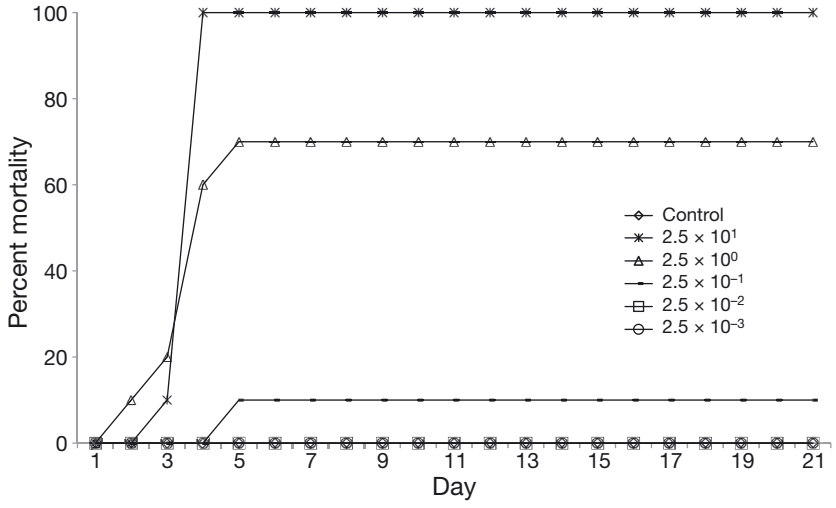


Fig. 3. Cumulative mortality chart for 21 d post-inoculation in Trial 2. A range of $2.5 \times 10^{1}$ to $2.5 \times 10^{-3}$ white spot syndrome virus particles $\mu l^{-1}$ was administered to 10 crayfish *Procambarus clarkii* in each dilution group and controls

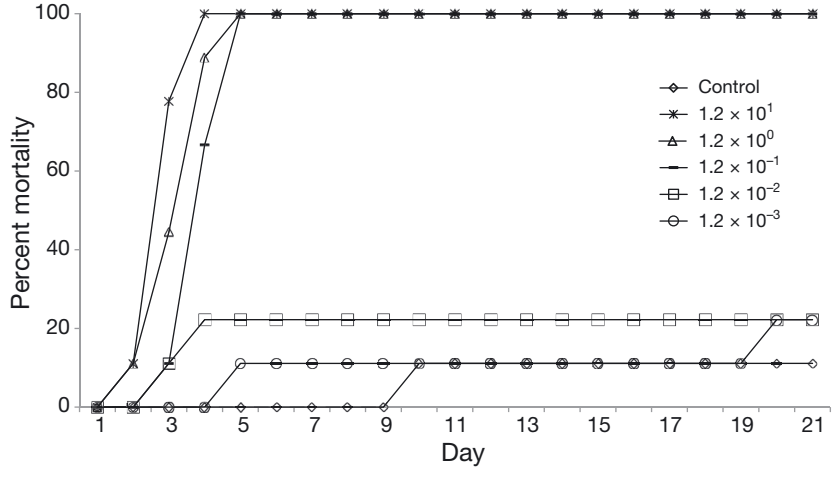


Fig. 4. Cumulative mortality chart for Trial 3 lasting 21 d. Viral dilution regime ranged from $1.2 \times 10^{1}$ to $1.2 \times 10^{-3}$ white spot syndrome virus particles $\mu l^{-1}$, and 9 crayfish *Procambarus clarkii* were inoculated in each dilution group

from $1.2 \times 10^{1}$ to $1.2 \times 10^{-1}$ WSSV particles $\mu l^{-1}$ reached 100% cumulative mortality within 5 d. Additionally, partial mortality was observed at all lower dilutions, including 1 control individual. Excluding the death of 1 control crayfish, the $LD_{50}$ value for this trial was estimated at 0.021 WSSV particles $\mu l^{-1}$ with a 95% confidence interval (0.0028–0.106 WSSV particles $\mu l^{-1}$), for a total exposure of 2.1 viral particles.

## DISCUSSION

The results of the current study demonstrate that WSSV is highly pathogenic in native Louisiana red swamp crayfish *Procambarus clarkii*. Few WSSV viral particles were needed to elicit 100% cumulative mortality within 5 d, and the calculated $LD_{50}$ values ranged from a total exposure of 2.1 to 196 viral particles. The greater mortality rate observed in Trial 3 compared to Trial 2 likely led to an underestimation of the true $LD_{50}$ value, and the $LD_{50}$ value of 196 viral particles is probably more accurate. The aim of Trial 3 was to produce similar results to Trial 2; however, it is possible that imperfect abiotic conditions contributed to the observed higher mortality. No deaths occurred in control crayfish with the exception of 1 individual in Trial 3, suggesting that differences in water quality or condition of the crayfish prior to experimentation could be responsible for the higher overall cumulative mortality in Trial 3. Water quality was monitored to ensure parameters were within the natural range for this species during each trial (Huner & Brown 1985); thus, health condition prior to viral challenge could potentially explain the differences in observed mortality rate.

Disease occurrence is affected by the interplay between environmental conditions, host, and the pathogen (Lightner & Redman 1998). The potential differences in health or abiotic conditions that likely led to higher mortality in Trial 3 can provide insight into how WSSV outbreaks fluctuate from year to year. In aquaculture ponds, water conditions can vary dramatically throughout the year. Farmed crayfish are frequently exposed to a wide range of environmental conditions including temperature fluctuations, low dissolved oxygen levels (Avault et al. 1975), and more recently, saltwater intrusion (Green et al. 2011). Drastic changes in temperature or salinity (Newsom & Davis 1994), as well as prolonged exposure to low dissolved oxygen (McClain 1999, Bonvillain et al. 2012), can induce stress in *P. clarkii,* and could potentially make crayfish more vulnerable to WSSV infection. Understanding how adverse environmental conditions affect the likelihood of WSSV infection will be critical to the development of disease prevention strategies.

In natural environments, crayfish are exposed to WSSV either via infected crayfish shedding viral particles into the water, or through the ingestion of infected tissue material (Lotz & Soto 2002). WSSV can persist in the environment and remain latent in organisms without inducing mortality or disease (Sanchez-Martinez et al. 2007). Consumption of infected tissue is a more effective route of transmission compared to waterborne transmission (Lotz & Soto 2002); therefore, crayfish in the current study were held in separate containers during experimentation. Previous work further demonstrates that higher viral loads are necessary to induce mortality in immersion experiments compared to inoculation in shrimp, and that the time course to mortality is much slower (Durand & Lightner 2002). Inoculation does not represent a potential exposure route in aquaculture ponds; however, it was selected over immersion in order to increase certainty in the number of particles each crayfish received. Although the quantification technique employed in the current study was also selected to increase confidence in the inoculation doses, digital PCR measures genome equivalents, and not necessarily intact, infectious virions. The inability to differentiate between infectious and noninfectious particles during quantification could explain some of the variation in observed mortality throughout the trials. Unfortunately, no crustacean cell lines are currently available for propagation of WSSV *in vitro*, so the number of infectious particles cannot be absolutely determined. For this preliminary investigation on WSSV infectivity in *P. clarkii,* the use of inoculation as an exposure route provides critical information on lethal and sublethal concentrations of viral particles that will be useful for work further investigating the interactions between the host and virus.

One novel aspect of the current study is the use of digital PCR to quantify WSSV viral particles. Earlier studies conducted on quantification of WSSV utilized competitive PCR techniques involving co-amplification of WSSV primers with an internal standard of known concentration to determine genome copy numbers (Tang & Lightner 2000, Du et al. 2008). More recently, real-time qPCR protocols were developed that rely on the creation of a standard curve by cloning WSSV-containing plasmids and comparing threshold cycle ($C_T$) values of unknown samples to determine number of viral particles (Durand & Lightner 2002, Jang et al. 2009, Zhu & Quan 2012). To date, our study is the first to quantify WSSV using a digital PCR approach. The use of digital PCR allows for easier, faster quantification of viral particles by eliminating the need for a plasmid vector required in the use of qPCR for WSSV quantification. Moreover, digital PCR increases measurement sensitivity by partitioning out the sample into thousands of smaller reactions, and reduces subjectivity by eliminating the use of a standard curve (Sedlak & Jerome 2013). Digital PCR technology has also proved useful in other virological studies. Compared to real-time qPCR, White et al. (2012) found that digital PCR had a higher detection limit and an on average 10% lower coefficient of variance for quantification of GB Virus Type-C, an RNA virus potentially important in HIV-1 infected patients. Digital PCR technology provides powerful insight into host–virus relationships and can determine which viruses infect individual bacterial cells (Tadmor et al. 2011).

The reported lethal dose of experimentally WSSV-infected *P. clarkii* varies significantly throughout the literature. Compared to the findings of other WSSV infectivity trials conducted in *P. clarkii*, the virus strain used in the current experiment appears to be more virulent. Using similar inoculation procedures, Zhu & Quan (2012) estimated the $LD_{50}$ value of WSSV to be $1.524 \times 10^5$ particles $\mu l^{-1}$, while Du et al. (2006) observed only a 75% mortality rate with an exposure of $1 \times 10^6$ total white spot viral particles. Moreover, both studies experienced an overall slower time course to mortality in all dilution groups compared to the mortality rates observed in the current study. In all trials of the current research, complete mortality was observed in all dilution groups above 1.2 particles $\mu l^{-1}$, and the majority of crayfish died within 5 d post-inoculation. Although these infectivity studies vary in viral quantification techniques and isolation procedures, the observations suggest that the Louisiana strain of WSSV used in this experiment is extremely virulent and could pose a threat to native crayfish. Several previous studies have confirmed differences in virulence between strains of WSSV worldwide in a variety of species (Marks et al. 2005, Pradeep et al. 2009, Gao et al. 2014). Marks et al. (2005) observed a higher cumulative mortality rate in *Penaeus monodon* when shrimp were infected with a smaller genome WSSV strain compared with a larger, ancestral genotype. When serially passaged in shrimp, smaller WSSV genomes exhibited greater fitness and were able to outcompete larger genome strains (Pradeep et al. 2009). In freshwater prawns, Corteel et al. (2012) observed virulence differences in WSSV strains from Thailand and Vietnam in *Macrobrachium rosenbergii* and concluded that strain ori-

gin affected mortality and infection rates. The WSSV genome is very similar among different geographic isolates and exhibits the most variation at 2 variable regions (Zwart et al. 2010). The variable regions of the WSSV strain used in the current trials have been sequenced (W.A. Baumgartner pers. comm.), although a virulence assessment has not yet been conducted. Complete sequence information and experiments to assess the virulence of the Louisiana strain of WSSV represent important next steps in predicting the spread and potential commercial impacts of WSSV on crayfish aquaculture.

This study successfully isolated, quantified, and determined lethal and sub-lethal concentrations of WSSV particles in Louisiana *P. clarkii*. The results of the infectivity trials suggest that the strain isolated and used in the current study may be more virulent in Louisiana *P. clarkii* compared to other WSSV strains in this species in other parts of the world. The strain of WSSV used in this experiment originated from the Atchafalaya River Basin, an area responsible for the majority of wild harvested crayfish in the US. The presence of the virus in the Atchafalaya River Basin poses a threat to the success of crayfish harvesting in Louisiana and has the potential to spread and impact other commercial crustacean fisheries that exist within connected waterbodies. Knowledge concerning the virulence and infectivity of the Louisiana strain of WSSV in native crayfish and other crustacean species will be of use for investigations into disease prevention and potential management strategies. The infectivity results can also be used to facilitate future investigations on the pathogenesis of WSSV and innate immune responses resulting from WSSV infection in Louisiana crayfish. Understanding how and when innate immune systems respond throughout a time course of WSSV infection could help explain differences in mortality rates observed between crayfish.

*Acknowledgements.* This work was supported by the Louisiana Board of Regents Research Competitiveness Subprogram Grant number LEQSF(2012-15)-RD-A-02. Facilities at the Louisiana State University (LSU) Agricultural Center Aquaculture Research Station and the LSU School of Veterinary Medicine were instrumental in the completion of this research. Molecular work was conducted at the LSU School of Veterinary Medicine in the Division of Biotechnology & Molecular Medicine and GeneLab Core supported by NIH-NIGMS 5P30GM110760. We are grateful for the support and assistance provided by Tanner Jones, Heidi Olivier, and Judy Wiles. This manuscript has been approved by the LSU AgCenter 2015-241-23829. Electron microscopy images were taken by Dr. Yulia Sokolova and the Microscopy center at the Department of Comparative Biomedical Sciences at the LSU School of Veterinary Medicine.

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*Editorial responsibility: Jeffrey Shields, Gloucester Point, Virginia, USA*

*Submitted: December 14, 2015; Accepted: May 9, 2016*
*Proofs received from author(s): June 20, 2016*