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Infectivity and Physiological Effects of White Spot Syndrome Virus (WSSV) in Farmed Louisiana Red Swamp Crayfish (Procambarus clarkii)

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INFECTIVITY AND PHYSIOLOGICAL EFFECTS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN FARMED LOUISIANA RED SWAMP CRAYFISH (*PROCAMBARUS CLARKII)*

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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. White spot syndrome virus (WSSV) is highly pathogenic and induces mass mortality in crustacean aquaculture operations worldwide. Crayfish lack the adaptive ability of the vertebrate immune system, and must depend on primitive, innate immune responses to combat viral infections. This study aims to investigate the dose-response of WSSV in *P. clarkii* and to examine viral-host interactions by examining the biochemical and immunological changes induced by WSSV infection in this species.

Viable viral particles were isolated from naturally infected *P. clarkii* gill tissue, quantified using a novel digital PCR approach, and inoculated into *P. clarkii* to determine a median lethal dose (LD_{50}) value of WSSV particles. After estimating an LD_{50} value, crayfish were inoculated at this nominal concentration of viral particles, and biological tissues were sampled across time to observe physiological and immunological changes throughout the course of WSSV infection. Antioxidant activity increased over time, while immunological gene expression was downregulated in the gill tissue of WSSV-infected crayfish.

Knowledge of the infectivity of WSSV in native crayfish is of critical importance to the management of the commercial aquaculture industry in Louisiana. Examination of the viral-host interactions in crayfish can be used to facilitate future investigations towards WSSV prevention and management, and serve to develop the use of *P. clarkii* to model innate immune responses to WSSV infection in other decapod crustaceans. This is the first study to investigate dose-response and immunological changes induced by the Louisiana strain of WSSV in native crayfish.

CHAPTER 1: INTRODUCTION

More than 640 species of crayfish have been described, with 5-10 new species identified each year (Crandall and Buhay 2008). Crayfish are native to every continent except Africa and Antarctica (Hobbs 1988), and the greatest species diversity occurs in North America and Australia (Taylor 2002). Crayfish are ecologically important, hardy decapod crustaceans capable of survival in a variety of environmental conditions. They are opportunistic omnivores and can play a keystone role in their ecosystem by facilitating energy flow through the environment (Momot 1995) and controlling community structure (Hobbs et al. 1989; Nystrom et al. 1996). Crayfish inhabit a variety of freshwater environments including lakes, rivers, streams, swamps, and estuaries (Nystrom 2002). To avoid predation and seek refuge during molting, reproduction, and extreme environmental changes, many species of crayfish construct burrows (Gherardi 2002). Burrowing crayfish are classified into three categories: primary, secondary, and tertiary burrowers. Natural crayfish abundance and distribution depends on factors such as water quality, temperature, habitat structure, competition, and predation (Lodge and Hill 1994). Changes in these abiotic and biotic factors can alter crayfish diversity and range, however, anthropogenic activities have much larger impacts on crayfish populations. Pollution, habitat destruction, and introduction of non-native crayfish into new areas have all contributed to the decline of several crayfish species (Wilcove et al. 1998). Estimations suggest that between one-third and one-half of all crayfish are threatened or endangered (Taylor et al. 2007).

While crayfish are ecologically important and can affect food web diversity and structure (Nystrom 2002), several species are also commercially valuable. The red swamp crayfish, *Procambarus clarkii*, is the most economically important crayfish species worldwide. The natural distribution of *P. clarkii* spans from the south-central United States northward along the

Mississippi River to Illinois, and southward to north-eastern Mexico (Hobbs et al. 1989). Due to the commercial importance of this species for aquaculture, it has been widely introduced to over twenty countries including China, Kenya, Ecuador, France, and Spain (Holdich et al. 1999). Introductions of *P. clarkii* outside of its native range have been successful due to several life history characteristics that make this species an effective invader. *Procambarus clarkii* is an extremely hardy crayfish, and can survive in waters with less than 3 mg/ml dissolved oxygen, live within a range of brackish to freshwater (Huner 2002), and tolerate polluted environments (Gherardi et al. 2000). As a secondary burrower, *P. clarkii* constructs simple burrows during dry periods (Gherardi 2002), enabling the species to withstand extreme environmental changes. *P. clarkii* exhibits early maturity, rapid growth rates, large numbers of offspring, and a short life-span (Gherardi 2006). Additionally, *P. clarkii* can outcompete native species (Gherardi and Cioni 2004), and decrease indigenous crayfish populations by spreading crayfish plague, a fungal disease to which it is resistant (Gherardi 2006). Together these characteristics enable *P. clarkii* to survive and adapt to changing conditions within its natural range, and also facilitate the ability of the species to invade and successfully colonize novel areas.

On a regional level, crayfish are important economically and culturally to the people of Louisiana. Louisiana is the largest producer of crayfish in the United States, and both *P. clarkii* and *P. zonangulus* are harvested from wild-caught populations and cultured pond operations (McClain et al. 2007). *Procambarus clarkii* occurs naturally statewide, with concentrated populations in the southern part of the state (Walls 2009), and represents 70-80% of the commercial harvest annually (McClain et al. 2007). In Louisiana, crayfish are extensively farmed in earthen ponds either in monoculture or with seasonally planted rice or soybeans to serve as suitable forage. Crayfish ponds are flooded and drained annually to mimic the seasonal

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fluctuations in water levels that occur in their natural habitat within swamps and rivers (McClain et al. 2007). Commercial harvest yields rely on natural reproduction within the pond, and during dry periods crayfish seek refuge in burrows to safely reproduce. Crayfish aquaculture is the most profitable aquaculture endeavor in Louisiana, with 91,373 hectares of ponds yielding $172,070,595 in commercial profits in 2014 (Louisiana Summary Agriculture and Natural Resources 2014). Although *P. clarkii* represents the most important aquaculture species, 39 species and sub-species of crayfish have been identified in Louisiana (Walls 2009). Many of these species exhibit limited natural ranges and are classified as threatened or endangered.

One potential threat to the success of the crayfish aquaculture industry is the worldwide spread of white spot syndrome virus (WSSV). WSSV is highly virulent, enveloped, rod-shaped, and has a tail-like appendage extending from the end of some virions (Wang et al. 1995). WSSV replicates in the nucleus, typically in ectodermal and mesodermal tissues (Lightner 1996). WSSV possesses double-stranded, circular DNA, and the genome is approximately 300kb (van Hulten et al. 2001; Yang et al. 2001). It is the sole member of the novel viral family *Nimaviridae,* genus *Whispovirus* (Mayo 2002). WSSV was first described in penaeid shrimp in 1992 in Taiwan (Chen 1995). Since its discovery, commercial shrimp farms have suffered billions of dollars in economic losses due to WSSV infections (OIE 2006; Lightner 2011). WSSV has a broad host range and can infect more than 90 aquatic crustacean species (Escobedo-Bonilla et al. 2008), including crabs (Hameed et al. 2003), aquatic insect larvae (Lo et al. 1996), and crayfish (Jiravanichpaisal et al. 2001). Penaeid shrimps develop white spots on the carapace, and other crustacean species exhibit lethargy and reduce feeding behavior when infected (Mohankumar and Ramasamy 2006). Spread of WSSV worldwide has been facilitated by the importation of frozen shrimp commodities, the transportation of live shrimp for aquaculture, seabirds, and the

release of untreated wastewater from shrimp packing plants (Lightner et al. 1997; Hasson et al. 2006). The virus reached the United States by 1995 (Lightner 1996), and was first identified in natural and farmed crayfish populations in 2007 in Louisiana (Baumgartner et al. 2009).

Previous laboratory studies have demonstrated experimental infections of WSSV in *P. clarkii* (Du et al. 2006; Zhu et al. 2009; Zhu and Quan 2012)*,* however, crayfish mortality rates are highly variable across studies. Reductions in mortality and viral replication rates have been observed when crayfish are maintained in hyperthermic (Du et al. 2006) or low temperature conditions (Du et al. 2008). Although WSSV outbreaks are infrequent in crayfish ponds in Louisiana, disease is the most significant factor contributing to economic losses in all aquaculture operations (Meyer 1991). In Louisiana, farmers can experience greater than 90% mortality in traps when infections occur (Baumgartner et al. 2009), and soft-shell producers suffer excessive mortality if the virus enters their recirculation systems (John Hawke, personal communication). While the relationship between WSSV and penaeid shrimps is well documented in the literature, there is a lack of information on the interactions between WSSV and *P. clarkii* in Louisiana. Several studies have demonstrated differences in virulence in crustaceans between WSSV strains worldwide (Wang et al. 1999; Marks et al. 2005; Gao et al. 2014), however, no experimental dose-response study has been performed utilizing the Louisiana strain of WSSV in native crayfish. Prior to the development of potential management strategies for crayfish farmers, the infectivity and pathogenesis of WSSV in *P. clarkii* needs to be assessed. In order to further develop preventative measures against WSSV infection, the interactions between the virus and critical components of the innate immune system must also be investigated. Finally, the current study provides an unique opportunity to conduct experimental research on a long-standing, farmed *P. clarkii* population. The crayfish used in this work were

harvested from active research ponds at the Louisiana State University Agricultural Center's

Aquaculture Research Station that are managed following prevalent industry practices. The

results of the current study are therefore directly applicable to the crayfish industry in Louisiana

and provide insight into the potential impacts of WSSV on crayfish farms. The overall aim of

this research was to increase knowledge of the detailed interactions between WSSV and *P.*

clarkii by determining the experimental infectivity of the virus via inoculation and to evaluate

changes in immune activity across time in response to infection.

CHAPTER 2: INFECTIVITY PATTERN OF WHITE SPOT SYNDROME VIRUS (WSSV) IN THE LOUISIANA RED SWAMP CRAYFISH *PROCAMBARUS CLARKII*

2.1 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 imported 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 detected in the United States by 1995 (Lightner 1996). In Louisiana, WSSV was first identified in natural and farmed crayfish populations in 2007 (Baumgartner et al. 2009).

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 (Louisiana Summary Agriculture and Natural Resources 2014). Louisiana is the largest producer of crayfish in the United States, and the red swamp crayfish *Procambarus clarkii* constitutes 70-80% 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 transmits horizontally, via both cannibalistic activity and waterborne transmission (Chou et al. 1998), as well as vertically from parent to offspring (Lo et al. 1997). While *P. clarkii* is generally resistant to diseases in the natural environment (Meng et al. 2013), the typical pond culture systems used in crayfish aquaculture provide 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, aquatic insect larvae, and crayfish (Lo et al. 1996; Chen et al. 2000; Shi et al. 2000; Hameed et al. 2001; Jiravanichpaisal et al. 2001; Musthaq et al. 2006), including *P. clarkii* (Maeda et al. 2000; Zhu and 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 (Ranjendren 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 days (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. While WSSV has not yet had a dramatic effect on commercial crayfish operations locally, the virus 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 Louisiana 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 white spot syndrome virus in native crayfish, and the results could be used to facilitate future investigations into ecosystem management initiatives and potential control strategies.

2.2 Materials and Methods

2.2.1 Crayfish

Procambarus clarkii were collected using baited traps from crayfish culture ponds at the Louisiana State University Agricultural Center's Aquaculture Research Station (Baton Rouge, Louisiana). Crayfish averaging 22 ± 1.5g and 45 ± 1.6mm (mean ± standard deviation) were held in separate, plastic containers in individually recirculating 90-liter tanks at a density of 5 crayfish/tank. Temperature was maintained at $24 \pm 1°C$ and crayfish were given a one-week acclimation period to these conditions prior to viral challenge. A total of 3 trials were performed, during which individuals received a commercial pelleted diet (50% protein, 14% fat, Cargill Minneapolis, Minnesota) at 1.5% of body weight every 3 days. Trials began on July 22, 2014, August 21, 2014, and January 16, 2015 for trials 1, 2, and 3, respectively. A previously described quantitative PCR (qPCR) method by Durand and Lightner (2002) and modified by Baumgartner et al. (2009) was conducted on gill tissue from 30 individuals from each study population to ensure 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. Water quality was maintained within the following ranges of mean and standard deviation: total ammonia nitrogen 0.02 ± 0.04 mg/l, nitrite 0.11 ± 0.13 mg/l, pH 8.49 ± 0.11, alkalinity 176 ± 13 mgCaCO$_3$/l, and hardness 208 ± 51 mgCaCO$_3$/l.

2.2.2 White Spot Syndrome Virus

WSSV-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 served as the viral source used in the challenges (Louisiana Animal Disease Diagnostic Laboratory Accession Number L1402972, Louisiana State University School of Veterinary Medicine, Baton Rouge, Louisiana). 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, 6g of WSSV-infected gill tissue were homogenized in 35ml of TNE buffer (0.05M Tris-HCl, 0.1M NaCl, 0.001M EDTA, pH=7.4). Samples were centrifuged at $8000 \times g$ for 20 min at 4°C, and the supernatant was passed through a 0.22µm filter using a vacuum filter apparatus. The filtrate was layered onto a 30% sucrose solution and centrifuged at $30,000 \times g$ for 2 hours at 4°C. The viral pellet was then suspended in 100µl of TNE buffer. This procedure was performed on three separate occasions before the start of each of the three trials such that gill tissue from experimentally infected and deceased crayfish was harvested and used to isolate fresh viral particles from the previous trial. The same isolation protocol was also conducted on uninfected gill tissue to serve as a negative inoculation control for trials 2 and 3.

2.2.3 Viral Quantification

Absolute quantification of viral particles was carried out using the Open Array on Quantstudio 12K Flex Real Time PCR system (Applied Biosystems, Grand Island, New York) before the start of each trial. Briefly, DNA was extracted from purified virions using the DNeasy blood and tissue kit as per manufacturer's instructions (Qiagen, Valencia, California). The DNA yield was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Molar concentration of DNA was calculated using the formula

$$\mu gDNA \times \frac{pmol}{660\ pg} \times \frac{10^6 pg}{1\ \mu g} \times \frac{1}{N} = pmolDNA$$

The DNA samples were then diluted to a concentration of 1×10^3 particles/µl 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 1800-2000 copies of viral DNA as a template. The Taqman primer - probe combinations used for all of the reactions were according to the protocol of Durand and Lightner (2002) and are listed in Table 2.1. The reaction mix was loaded on to open arrays using the Accufill [TM] automated sample loader. Digital PCR was carried out on the QuantstudioFlex system and the resulting data was analyzed using DigitalSuite™ Software.

Table 2.1: 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'-TTGTCCCGTCCTCATCTCAG-3'
Reverse (WSS1079R)	5'-GCTGCCTTGCCGGAAATTA-3'
TaqMan Probe	5'(6FAM)AGCCATGAA(ZEN)GAATGCCGTCTATCACACA(IBFQ)-3'

2.2.4 Dilutions, Inoculation, and WSSV Infectivity

After quantification, the viral stock solution was serially diluted in 300 mOsm/kg Hank's Balanced Salt Solution (HBSS) to create 5 dilutions for inoculation. The dilutions ranged from 5.02×10^5 to 5.02×10^1 particles/µl, 2.05×10^1 to 2.05×10^{-3} particles/µl, and 1.2×10^1 to 1.2×10^{-3} particles/µl for trials 1, 2, and 3, respectively. For each trial, 10 crayfish/dilution were injected intramuscularly using a 27-gauge needle with 100µl between the carapace and abdomen. In trial 1, an additional 10 crayfish received an injection of 300 mOsm/kg HBSS solution to

serve as a control. For additional controls to detect differences resulting from the viral isolation protocol, the same procedure was performed on uninfected gill tissue, diluted to 10^1 in 300 mOsm/kg HBSS, and injected into 10 crayfish/trial for trials 2 and 3. Mortality of crayfish was monitored daily for 21 days 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[®], Petaluma, California) in order to estimate LD_{50} concentrations for each trial.

2.3 Results

2.3.1 Viral Isolation

Electron microscopy confirmed that intact viral particles can be isolated from WSSV-infected crayfish gill tissue using the described viral isolation protocol (Figure 2.1), however, the percentage of intact virions was not estimated. Preliminary testing of the inoculation process demonstrated that the viral particles retrieved from the isolation procedure successfully infect and induce mortality in *P. clarkii*.

2.3.2 Trial 1

In trial 1, 100% mortality was reached by day 5 in all viral dilutions ranging from 5.02×10^5 to 5.02×10^1 particles/μl, while all control crayfish remained alive (Figure 2.2). Mortality began within 48 hours post-inoculation and nearly all crayfish were lethargic within 72 hours. No WSSV-infected crayfish survived, therefore an LD_{50} value could not be calculated. As a result of the extremely rapid mortality rate, the purified WSSV preparations were further diluted for trials 2 and 3.
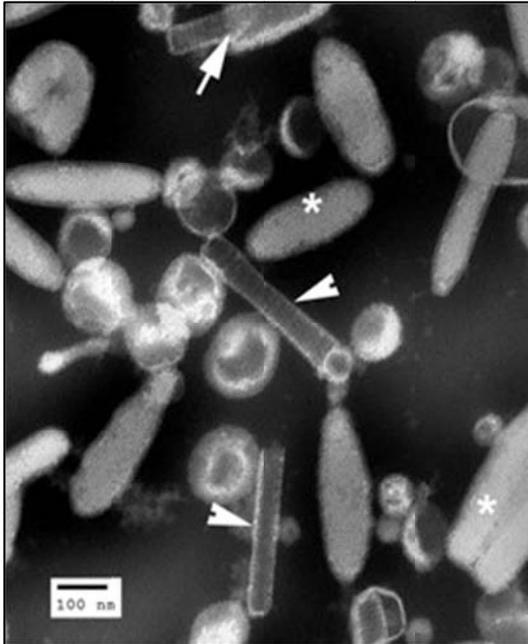
Figure 2.1: Transmission electron micrograph of white spot syndrome virus particles isolated from *Procambarus clarkii* gill tissue using viral isolation protocol *(negative staining with 2% phosphotungstic acid)*. Suspension of isolated viral particles diluted to 1:100,000 containing mature virions (asterisks), ruptured virion with the exposed nucleocapsid (arrow), and naked nucleocapsids prior to being enveloped (arrowheads).

2.3.3 Trial 2

Further diluting the viral concentrations in trial 2 generated greater differences in cumulative mortality compared to trial 1. Only the highest viral dilution group receiving 2.5×10^1 WSSV particles/µl reached 100% mortality within 4 days, while no mortality was observed in the two lowest dilutions of 2.5×10^{-2} and 2.5×10^{-3} particles/µl, or in control crayfish (Figure 2.3). An LD_{50} value of 1.96 WSSV particles/µl was estimated with 95% confidence interval (1.362, 2.929 WSSV particles/µl), which equates to a total exposure of 196 WSSV particles per individual crayfish.
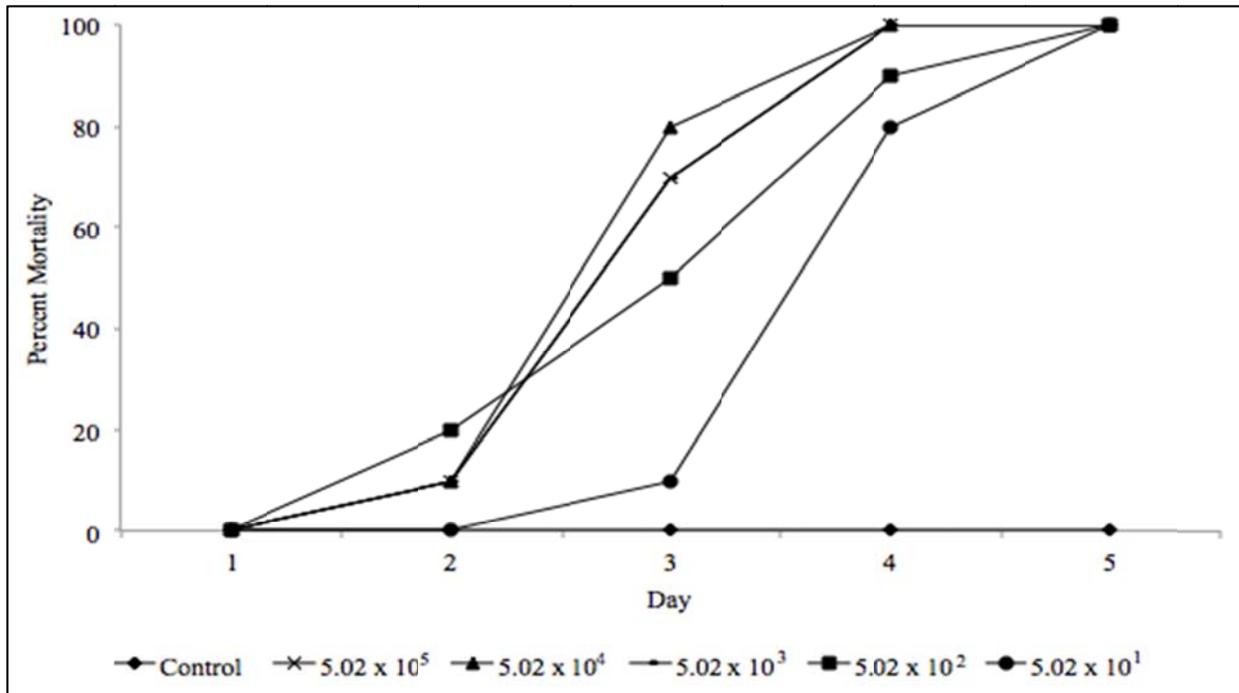
Figure 2.2: Cumulative mortality for 5 days post-inoculation in trial 1. Crayfish received inoculations ranging from 5.02×10^5 to 5.02×10^1 WSSV particles/μl, with 10 crayfish inoculated/dilution group.
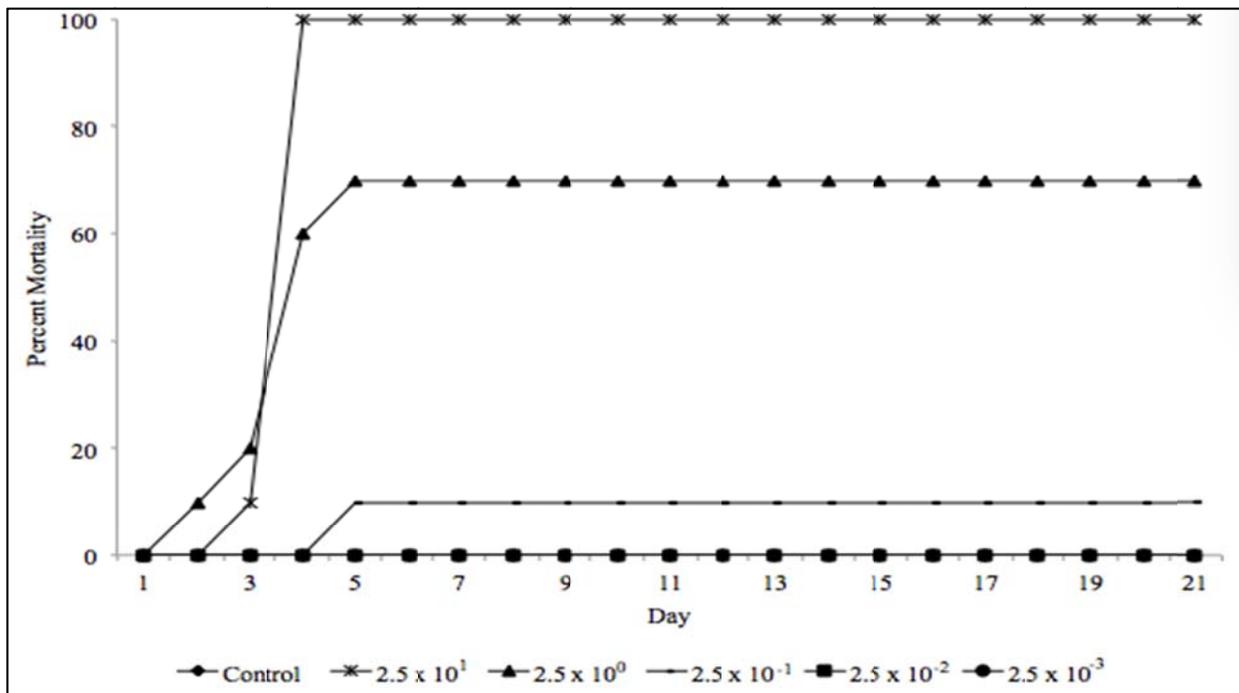
Figure 2.3: Cumulative mortality for 21 days post-inoculation in trial 2. Crayfish received inoculations ranging from 2.5×10^1 to 2.5×10^{-3} WSSV particles/μl, with 10 crayfish inoculated/dilution group.

2.3.4 Trial 3

Crayfish in trial 3 were exposed to approximately the same dilution regime of WSSV particles as trial 2, however, greater mortality was observed in trial 3. The three highest viral dilution groups ranging from 1.2×10^1 to 1.2×10^{-1} WSSV particles/µl reached 100% cumulative mortality within 5 days. Additionally, partial mortality was observed in all lower dilutions, including one control individual. Excluding the death of one control crayfish, the LD_{50} value for this trial was estimated at 0.021 WSSV particles/µl with a 95% confidence interval (0.0028, 0.106 WSSV particles/µl), for a total exposure of 2.1 viral particles.
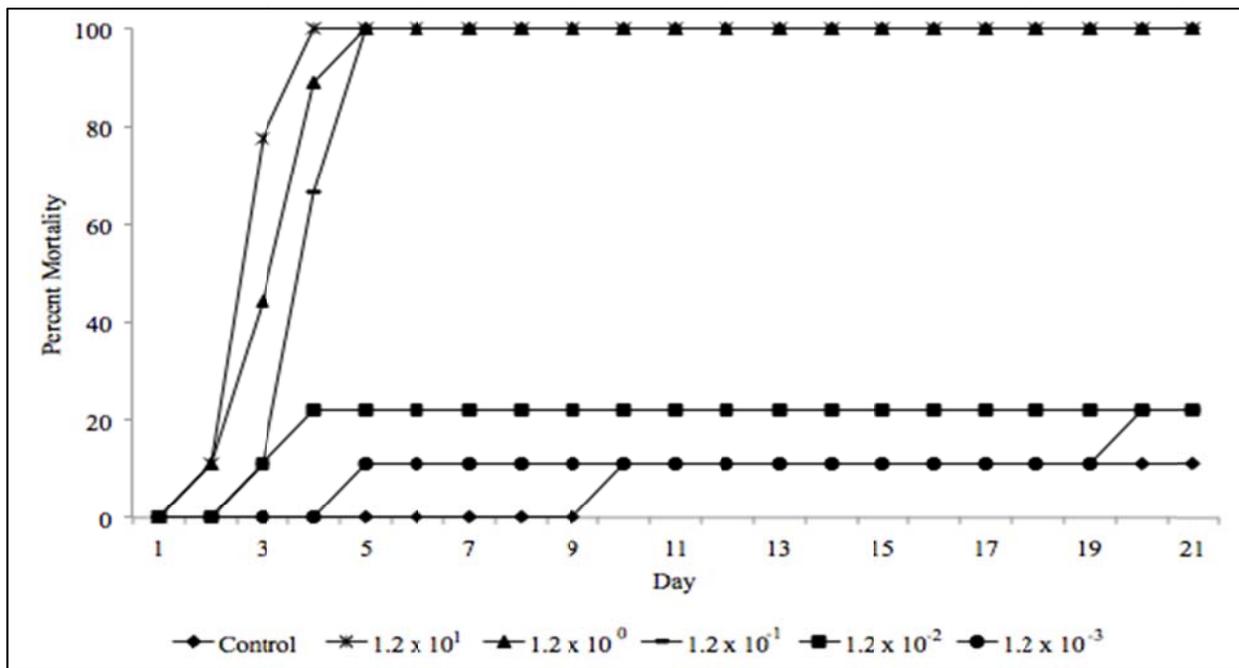
Figure 2.4: Cumulative mortality for 21 days post-inoculation in trial 3. Crayfish received inoculations ranging from 1.2×10^1 to 1.2×10^{-3} WSSV particles/µl, with 10 crayfish inoculated/dilution group.

2.4 Discussion

The results of the current study demonstrate that WSSV is highly pathogenic in native Louisiana red swamp crayfish. Very few WSSV viral particles are needed to elicit 100% cumulative mortality within 5 days, and the calculated LD_{50} values range from a total exposure

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of 2.1 to 196 viral particles/crayfish. 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 or viral estimations contributed to the observed higher mortality. No deaths occurred in control crayfish with the exception of one 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. Water quality was monitored to ensure parameters were within the natural range for this species during each trial (Huner and Brown 1985), therefore 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 and 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 and 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

and 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 and 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 of shrimp, and that the time course to mortality is much slower (Durand and Lightner 2002). Although inoculation does not represent a potential exposure route in aquaculture ponds, it was selected over immersion in order to increase certainty in the number of particles each crayfish received. For this preliminary investigation on WSSV infectivity in *P. clarkii,* the use of inoculation as an exposure route provides critical information on lethal and sub-lethal 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 (Tang and Lightner 2000; Du et al. 2008) or real-time quantitative PCR techniques (Durand and Lightner 2002; Jang et al. 2009; Zhu and Quan 2012) to determine genome copy numbers. To date, the current 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 and 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

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average 10% lower coefficient of variance for quantification of GB Virus Type-C, an RNA virus potentially important in HIV-1 infected patients.

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 and Quan (2012) estimated the LD_{50} value of WSSV to be 1.524×10^5 particles/µl, while Du et al. (2006) observed only a 75% mortality rate with an exposure of 1×10^6 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 1.2×10^0 particles/µl, and the majority of crayfish died within 5 days 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 (Marks et al. 2005; Pradeep et al. 2009; Gao et al. 2014). The variable regions of the WSSV strain used in the current trials have been sequenced (Baumgartner, personal communication) and represent an important step in predicting the spread and potential commercial impacts of the Louisiana strain of WSSV on crayfish aquaculture.

This study successfully isolated, quantified, and determined lethal and sub-lethal concentrations of WSSV particles in farmed Louisiana *P. clarkii*. The use of farmed *P. clarkii* in the experimental dose-response infections provides insight into the potential impacts of WSSV outbreaks on the crayfish aquaculture industry. Moreover, 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 United States. The presence of the virus in the Atchafalaya River Basin poses a threat to the success of wild 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.

CHAPTER 3: ANTIOXANT ACTIVITY AND IMMUNOLOGICAL GENE EXPRESSION INDUCED BY WHITE SPOT SYNDROME VIRUS (WSSV) INFECTION IN *PROCAMBARUS CLARKII*

3.1 Introduction

White spot syndrome virus (WSSV) is highly pathogenic and induces mass mortality in crustacean aquaculture operations worldwide. WSSV exhibits a broad host range in decapod crustaceans, including several species of penaeid shrimps, freshwater prawns, lobsters, and crayfish (Escobedo-Bonilla et al. 2008). Although the majority of economic loss associated with WSSV infection occurs in penaied shrimp farms, crayfish producers in Louisiana occasionally experience high mortality rates attributed to WSSV. The red swamp crayfish, *Procambarus clarkii*, represents an economically important species responsible for over half of all commercial aquaculture profits in Louisiana (Louisiana Summary Agriculture and Natural Resources 2014). WSSV has persisted in wild and farmed populations of *P. clarkii* in Louisiana since 2007 (Baumgartner et al. 2009), and diagnostic testing of aquaculture ponds suspected of infection occurs every year (John Hawke, personal communication).

To combat viral infection, crayfish possess a suite of simple defense mechanisms. Crayfish lack the adaptive ability of the vertebrate immune system, and must depend on primitive, innate immune responses (Söderhäll and Söderhäll 2002). One of the most important, innate immune factors of crayfish is the prophenoloxidase (proPO) system. This defensive system circulates in the hemolymph in inactive forms and exits the hemocytes when foreign pathogens are detected (Söderhäll and Söderhäll 2002). The proPO system can serve as a pattern-recognition system by activating upon detection of polysaccharides from microorganisms, such as bacteria (Aspán et al. 1995). Activation occurs in a stepwise manner involving serine proteases, and excess production is prohibited by specific protease inhibitors (Zeng and Lu

2009). Once activated, this system plays a role in the immune pathways that regulate

phagocytosis, nodule formation and encapsulation, and melanization of pathogens (Cerenius and

Söderhäll 2004). An increase in the gene expression of proPO in various tissues has been shown

to occur when *P. clarkii* individuals are challenged with WSSV (Li et al. 2012), indicating the

importance of this system as a viral defense.

Another defense mechanism crayfish utilize is the release of reactive oxygen species

(ROS) via respiratory burst in response to invasion by bacteria, fungi, or viruses (Liu et al.

2010). Viruses disrupt the pro/antioxidant balance in organisms by increasing the production of

pro-oxidants and reducing the production of antioxidants used to scavenge ROS (Schwarz 1996).

Previous research conducted on WSSV-infected *Penaeus monodon* and *Fenneropenaeus indicus*

indicates that WSSV induces excess release of ROS (Rameshthangam and Ramasamy 2006;

Mohankumar and Ramasamy 2006), leading to oxidative stress. Antioxidant enzymes such as

superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) protect cells by

neutralizing the harmful effects of ROS (Liu et al. 2007; Liu et al. 2010; Taylor et al. 2013), and

concentrations of these and other antioxidants can serve as indicators of stress in organisms

(Downs et al. 2001). These three enzymes work in concert to reduce cellular damage resulting

from ROS production. SOD converts the superoxide anion O_2^- into water and hydrogen peroxide,

which is then neutralized to water and oxygen by CAT and GPx (Liu et al. 2013). Mohankumar

and Ramasamy (2006) observed that activities of SOD, CAT, and GPx decreased in the

hemolymph, hepatopancreas, gills, and muscle tissue of *F. indicus* when challenged with WSSV.

Previous work investigating antioxidant enzyme activity in *P. clarkii* found that activity levels of

SOD and CAT significantly decreased in hepatopancreas tissue in response to exposure to the

toxic herbicide prometryne (Stará et al. 2014).

The current study aims to investigate critical viral-host interactions by examining the biochemical and immunological changes induced by sub-lethal WSSV infection in *P. clarkii*. Gene expression of proPO and SOD was quantified, and antioxidant activity levels of SOD, CAT, and GPx were assessed throughout a time course of WSSV infection. The results of the antioxidant assays and gene expression analysis can provide insight into the ability of the crayfish immune system to cope with the physiological changes stimulated by WSSV infection. Investigating innate immune responses could enable future research on potential control initiatives for WSSV, and afford beneficial information to the management of commercial crayfish aquaculture in Louisiana.

3.2 Materials and Methods

3.2.1 Crayfish

Using baited traps, *P. clarkii* were harvested from crayfish aquaculture ponds at the Louisiana State University Agricultural Center's Aquaculture Research Station (Baton Rouge, Louisiana). Crayfish averaging 22.5 ± 2g and 46 ± 2mm (mean ± standard deviation) were selected for trials and held in separate containers in individually recirculating 90-liter tanks at a density of 6 crayfish/tank. Tank temperature was maintained at 24±1°C and crayfish were acclimated to these conditions for one week before experimentation. A total of three trials were conducted, with trials 1, 2, and 3 beginning on April 8, 2015, May 17, 2015, and June 12, 2015, respectively. To ensure the source population was WSSV-free, a quantitative PCR (qPCR) procedure described by Durand and Lightner (2002) and Baumgartner et al. (2009) was conducted on gill tissue from 30 individuals prior to trials. Water quality parameters including alkalinity, hardness, total ammonia nitrogen, nitrite, and pH were measured before the initiation of trials to ensure optimal water conditions (Chapter 2, page 8).

3.2.2 WSSV Isolation and Quantification

Experimentally WSSV-infected *P. clarkii* from previous research conducted at the Louisiana State University School of Veterinary Medicine were utilized as the viral source to inoculate crayfish in the current study. WSSV particles were isolated from infected gill tissue following a modified procedure previously published by Du et al. (2007) and described previously in Chapter 2 (page 9). The isolation procedure was also conducted on uninfected gill tissue to serve as a negative inoculation control. Absolute quantification of viral particles was carried out using the Open Array on Quantstudio 12K Flex Real Time PCR system (Applied Biosystems, Grand Island, New York) before the start of trials, as previously described in the Chapter 2 methods section (page 9).

3.2.3 WSSV Inoculation and Tissue Sampling

Previous research conducted on the experimental dose-response of WSSV in *P. clarkii* estimated a median lethal dose (LD_{50}) value of approximately 1.96 WSSV particles/μl within 21 days post-inoculation (Chapter 2, page 12). This nominal dose of viral particles was targeted to produce a sub-lethal response in crayfish within a series of three separate trials. Following quantification of WSSV particles, the virus was diluted to concentrations of 1.38×10^0, 1.96×10^0, and 1.01×10^0 WSSV particles/μl for trials 1, 2, and 3, respectively. Each crayfish received an inoculation of 100μl of either WSSV viral particles or negative control solution intramuscularly between the abdomen and carapace. Biological tissues were harvested from 6 WSSV-inoculated crayfish and 6 control crayfish at 0, 12, 24, 72, and 120 hours post-inoculation for trials 1 and 2. For trial 3 the sampling time points were 0, 24, 72, 120, and 168 hours post-inoculation. At each sampling point, gill and hepatopancreas tissues were harvested from each individual. Gill and hepatopancreas tissues were collected via dissection and washed briefly in

phosphate buffered saline (PBS, pH=7.4) to remove any contaminants. Approximately 50 mg of

gill and hepatopancreas tissues were placed into 1ml of homogenizing buffer (50mM Tris-HCL,

5mM EDTA, PMSF 10μM). Tissues were manually homogenized and then centrifuged at

10,000g for 10 minutes at 4°C. The supernatant was removed, frozen in liquid nitrogen, and

stored at -80°C. For qPCR analysis, roughly 100 mg of gill and hepatopancreas tissue were

immersed in 1ml RNAlater solution and stored at -20°C.

3.2.4 Biochemical Assays

Assays to examine antioxidant activity were conducted on gill and hepatopancreas tissues

from all trials. Tissue samples were assayed for SOD activity, GPx activity, and protein

concentration. Only gill tissue samples were analyzed for CAT activity. All individual assay

samples were conducted in triplicate wells. The commercial assay kits Coomassie (Bradford)

Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), Amplex® Red Catalase Assay Kit

A22180 (Life Technologies Corporation, Grand Island, NY), SOD Assay Kit WST 19160

(Sigma-Aldrich, St. Louis, MO), and Glutathione Peroxidase Assay Kit 703102 (Cayman

Chemical Company, Ann Arbor, MI) were used to measure protein concentration, CAT, SOD,

and GPx activity, respectively. Assays were conducted as per manufacturer instructions and

absorbance values were measured using a BioTek Synergy 2 Microplate Reader (BioTek,

Winooski, Vermont).

3.2.5 Primer Design and Evaluation

Primers designed for gene expression analysis of elongation factor 1-α (EF1-α), SOD and

proPO were evaluated using gel electrophoresis prior to conducting qPCR. The EF1-α sequence

was acquired from previously published research conducted in *P. clarkii* on GenBank (Accession

Number KR135166), while SOD and proPO primers were designed using Integrated DNA

Technologies software (Table 3.1). For each primer pair of proPO and SOD, one positive control

containing sample cDNA and one negative control of molecular grade water were analyzed.

Master mixes for each primer pair consisted of 13.72μl molecular water, 5μl 5x GoTaq reaction

buffer, 0.5μl 10mM dNTP, 0.25μl forward primer, 0.25μl reverse primer, and 0.25μl GoTaq

DNA polymerase. Samples were run at 50°C for 2 minutes, 94°C for 2 minutes, and then 40

cycles were completed at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

Following PCR, 1μl SYBR Green, 2μl 4X gel loading dye, and 5μl of each sample were loaded

into a polyacrylamide gel (30:0.8 acrylamide:bisacrylamide, 0.1% Ammonium persulfate, 0.1%

Tetramethylethylenediamine) and run at a constant voltage of 150 volts for 45 minutes. Bands

present on the gel were detected using an E-Gel Imager with UV Light base (Life Technologies

Corporation, Grand Island, New York).

Table 3.1: Primer sequences and GenBank accession numbers for elongation factor 1-α (EF1-α), superoxide dismutase (SOD), and prophenoloxidase (proPO) utilized in quantitative PCR for gene expression analysis of white spot syndrome virus-infected *Procambarus clarkii*

Primer		Sequence
Elongation Factor 1-α	Forward	CCACAAAGGCAGGTGAAAAGG
(KR135166)	Reverse	ATTGGGTGAACCAAGCAGGG
Superoxide Dismutase	Forward	GTCTTCGTAGGTACGGTGTATG
(KC333177)	Reverse	CTTGCTACGCCTTGGTAGTT
Prophenoloxidase	Forward	TCGACCAGGCCTCCTAAATA
(EF595973)	Reverse	GGAAGGAGGAGGAGGATAGATT

3.2.6 Quantitative PCR Analysis

To prepare samples for qPCR, RNA was isolated from gill and hepatopancreas samples

using TRIzol reagent. Briefly, samples in RNAlater were centrifuged, washed in PBS, and

manually homogenized in TRIzol. Chloroform was added to facilitate separation of RNA, the

samples were incubated at room temperature for 5 minutes, and then centrifuged to isolate the

supernatant. The supernatant was removed and an equal volume of isopropanol was added before

samples were held overnight at -20°C to permit RNA precipitation. Samples were centrifuged to

pellet the RNA, washed with ethanol, and then allowed to air dry to remove excess liquid from

the pellet. A 50μl aliquot of a master mix containing 5μl 10x TURBO DNase buffer, 1μl

TURBO DNase, and 44μl nuclease-free water (Ambion, ThermoFisher Scientific, Waltham,

Massachusetts) was added to each sample to dissolve DNA. Samples were then placed in a 37°C

water bath for 30 minutes. A 5μl suspension of DNase inactivation reagent was added and

samples were incubated at room temperature for 5 minutes. Finally, samples were centrifuged

and the supernatant containing isolated RNA was removed. To determine RNA concentrations, a

2μl aliquot of each sample was diluted into 98μl molecular grade water and measured using a

GeneQuant pro Spectrophotometer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania).

Aliquots of samples containing RNA concentrations greater than 500ng/μl were diluted by 50%

in molecular grade water prior to the next step of complementary DNA (cDNA) synthesis.

Isolated RNA was then synthesized to cDNA using SuperScript IV Reverse Transcriptase

(Life Technologies Corporation, Grand Island, NY). The cDNA reaction was completed as per

manufacturers instructions and PCR was conducted using a Bio-Rad MyCycler Thermal Cycler

(Bio-Rad, Hercules, California). The samples were run at 65°C for 5 minutes, 4°C for 10

minutes, 55°C for 15 minutes, and then 80°C for 10 minutes.

Following cDNA synthesis, samples were prepared for qPCR analysis. A master mix for

each primer pair consisted of 5μl 5X GoTaq Reaction buffer, 0.5μl 10mM dNTP, 0.25μl forward

primer, 0.25μl reverse primer, 1.25μl 10X SYBR Green, 0.5μl Rox reference dye, 12μl

molecular grade water, and 0.25µl GoTaq DNA Polymerase. A 96-well plate was used to thoroughly mix 32µl of the master mix with 8µl of each cDNA sample prior to transfer to a 384-well plate with a final reaction volume of 10µl per well. A total of 6 plates were run, with one gene and tissue type per plate. Samples were run in triplicate for each primer pair, and qPCR was conducted at 50°C for 2 minutes, 94°C for 2 minutes, and then 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

3.2.7 Statistical Analysis

Biochemical assay results were initially analyzed using Gen5 Data Analysis Software (BioTek, Winooski, Vermont). Based on recommendations from assay research previously conducted in this laboratory group, if the coefficient of variation (CV) between standard well triplicates was greater than 12%, wells were removed and standard curves generated such that the R^2 values were greater than 0.97. Similarly, samples containing a CV greater than 12% among triplicate wells were modified to remove outlying values for SOD and CAT assays. As a kinetic assay, GPx did not calculate a CV value for triplicates, therefore values were eliminated if the value resided outside of the first and third interquartile range of the mean. All calculated assay values were averaged, and the mean for each sample was standardized by the corresponding protein concentration prior to statistical analysis.

Similar to the GPx analysis, qPCR C_t values for each sample were evaluated and outlying values removed based on the interquartile range of the mean. C_t values from each sample were then normalized to their corresponding EF1-α reference gene C_t value. Normalized values were analyzed using the $\Delta\Delta C_t$ method, and outlying $\Delta\Delta C_t$ values were removed if they resided outside of a 99% confidence interval of the mean.

The overall statistical analysis strategy was to separately test antioxidant activity in gill and hepatopancreas tissues by trial, time, and by treatment, either control of WSSV-infected crayfish, such that a total of 18 statistical tests were conducted. For proPO expression, gene expression in gill tissue was also analyzed by treatment and time, and only one statistical test was conducted. Each test was performed by a general linear model (PROC GLM, SAS version 9.4, Cary, NC) assuming a linear relationship between the model predicted values and observed values and normally distributed residuals. These assumptions were evaluated for each model and, where violated, a $\log_{10}$ transformation was applied. For each model, the response was antioxidant activity or gene expression, and the explanatory variables were treatment and hours post inoculation (hpi), which were 12, 24, 72, and 120 hpi for trials 1 and 2, and 24, 72, 120, and 168 hpi for trial 3. Because the relationship between antioxidant activity or gene expression over time may not have been linear (e.g., an initial decrease followed by a decrease), intrinsically linear polynomials were evaluated, including the quadratic and cubic forms. In models with fewer observations because of mortality, time passed was modeled as a continuous variable to conserve model degrees of freedom. Otherwise, time passed was treated categorically because it represented a designed experimental feature. Given that multiple comparisons were made on the same data, post-hoc tests were adjusted by the Tukey-Kramer adjustment. Statistical significance was evaluated at $\alpha=0.05$.

3.3 Results

3.3.1 Primer analysis

Primer pairs of SOD and proPO that were designed using IDT software were analyzed using PCR and gel electrophoresis on a polyacrylamide gel (30:0.8 acrylamide:bisacrylamide, 0.1% Ammonium persulfate, 0.1% Tetramethylethylenediamine). Both proPO and SOD positive

controls produced single products on the gel, and no products were observed in either negative

control (Figure 3.1).

1   2   3   4   5
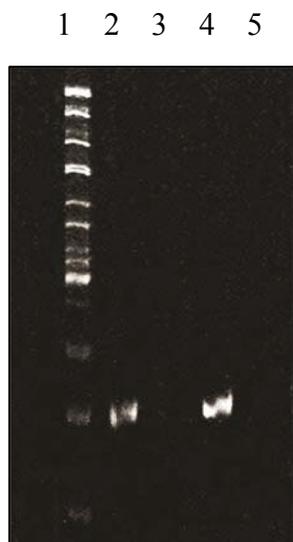
Figure 3.1: Polyacrylamide gel (30:0.8 acrylamide:bisacrylamide, 0.1% Ammonium persulfate, 0.1% Tetramethylethylenediamine) to analyze designed prophenoloxidase (proPO) and superoxide dismutase (SOD) primer pairs designed using IDT software. Lanes are numbered above 1-5 from left to right on the gel. Lane 1 represents a molecular ladder, Lane 2 contains the proPO positive control, Lane 3 has the proPO negative control, Lane 4 represents the SOD positive control, and Lane 5 contains the SOD negative control.

3.3.2 Trial 1

Crayfish in trial 1 received an inoculation of 1.38×10^0 WSSV particles/µl, for a nominal

dose of 138 WSSV particles/crayfish. Mortality occurred in three WSSV-infected crayfish prior

to the 120-hour sampling point. Antioxidant activity levels varied across time in gill and

hepatopancreas tissues for both control and WSSV-infected crayfish, however, no significant

differences were observed.

3.3.3 Trial 2

Crayfish in trial 2 received 1.96×10^0 WSSV particles/µl, for a nominal dose of 196

WSSV particles/crayfish. Mortality occurred in all WSSV-infected crayfish between 72 and 96

hpi, while all control crayfish survived to 120 hpi. Antioxidant activity varied across time in

control and WSSV-inoculated crayfish for both tissue types, however, significant differences were only observed in gill tissue. GPx concentration in gill tissue was significantly higher across all time points in WSSV-inoculated crayfish compared to control (Figure 3.2, p=0.0252). At 72 hpi, GPx concentration was significantly higher in the gills of WSSV-infected crayfish compared to control by 0.027 U/ml/μg protein (p=0.0003). Across all sampling time points, CAT was significantly higher in the gill tissue of WSSV-inoculated crayfish compared to control (Figure 3.3, p=0.0079). At 72 hpi, CAT concentration in the gills was significantly higher in WSSV-infected crayfish compared to control by 0.205 U/ml/μg protein (p=0.0128). SOD activity was not significantly different in gill or hepatopancreas tissue between control and WSSV-infected crayfish.

3.3.4 Trial 3

WSSV-infected crayfish in trial 3 were inoculated with 1.01×10^0 WSSV particles/μl, for a nominal dose of 101 viral particles. Antioxidant activity varied across time in both tissues in control and WSSV-infected crayfish. Mortality was observed in 3 WSSV-infected crayfish at 168 hpi, but no mortality occurred in control crayfish at any time point. The three dead crayfish were replaced with WSSV-infected crayfish from an extra 168 hpi tank in order to compensate for differences in sample size at that time point. No significant differences were observed in gill or hepatopancreas antioxidant activity between control and WSSV-inoculated crayfish. Gene expression analysis was performed only on tissues from trial 3 because this trial represented the most complete dataset. Mean relative proPO gene expression in gill tissue was significantly different across time (Figure 3.4, p=0.0045). At 120hpi, proPO expression was significantly downregulated in the gills by 2.7 units in WSSV-inoculated crayfish compared to control

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(p=0.0004). Gene expression of proPO in hepatopancreas and SOD expression in both tissues were not analyzed due to insufficient data.
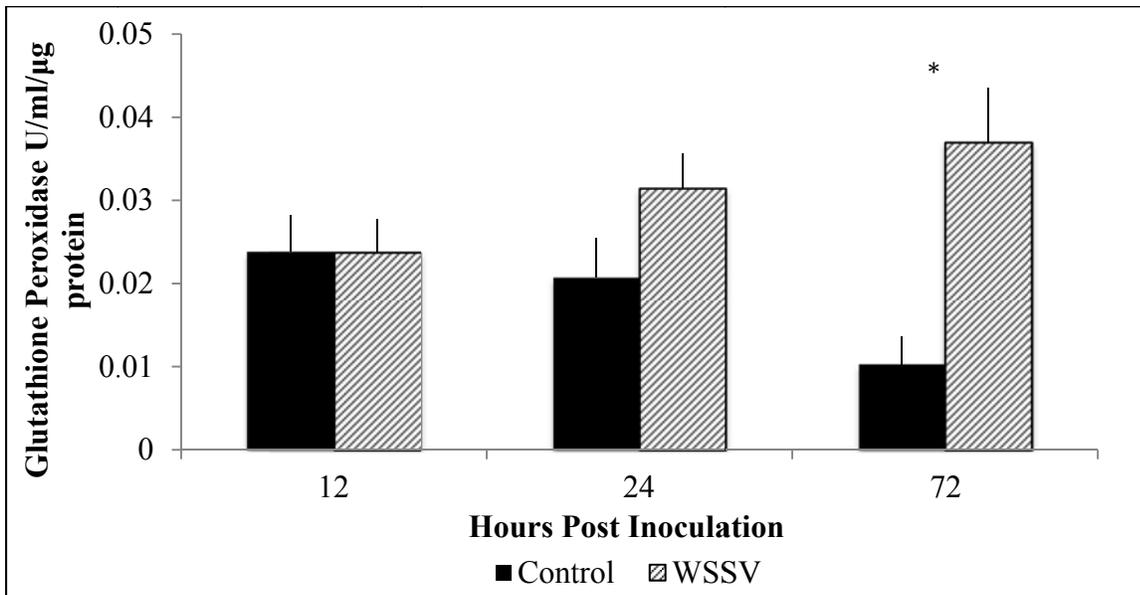
Figure 3.2: Glutathione peroxidase (GPx) activity in gill tissue from trial 2. Overall, GPx concentration was significantly higher across all time points in WSSV-inoculated crayfish compared to control (p=0.0252). At 72 hours post inoculation, GPx concentration was significantly higher in WSSV-infected crayfish compared to control (p=0.0003).
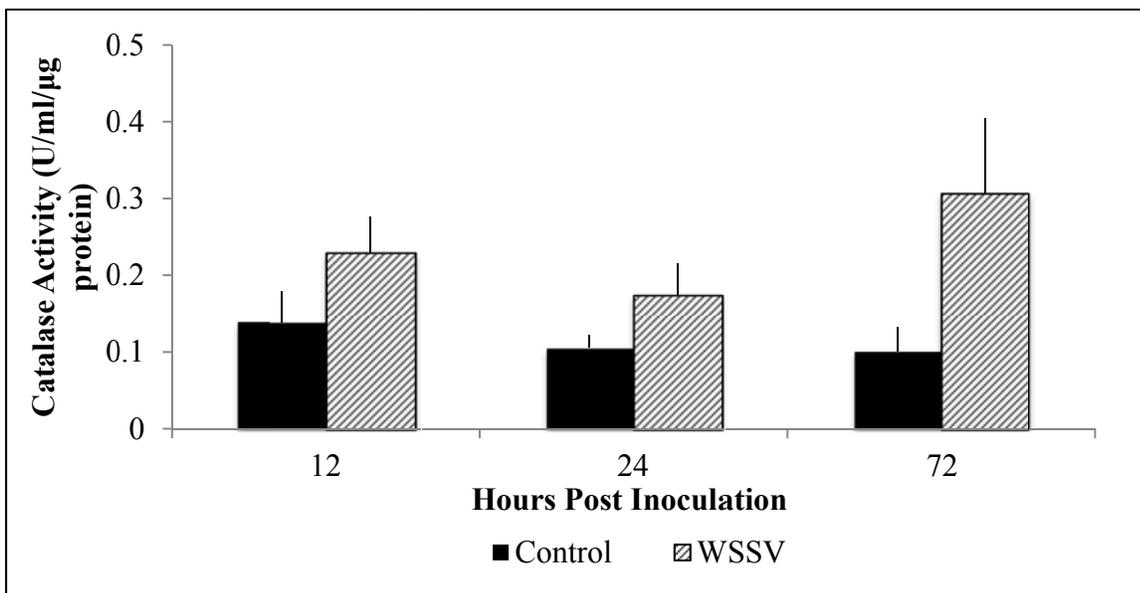
Figure 3.3: Catalase (CAT) activity of gill tissue in trial 2. Overall, CAT was significantly higher across all time points in WSSV-inoculated crayfish compared to control (p=0.0079). At 72 hours post inoculation, CAT concentration was significantly higher in WSSV-infected crayfish compared to control (p=0.0128).
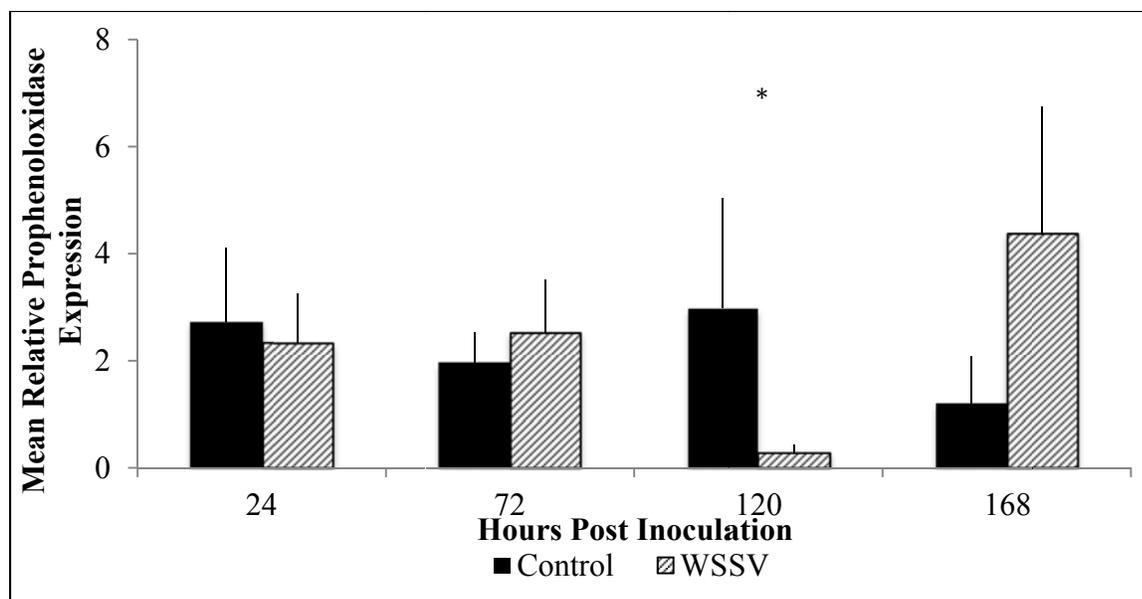
Figure 3.4: Mean relative prophenoloxidase expression in gill tissue of crayfish in trial 3. Gene expression was significantly different across time (p=0.0045), and was significantly downregulated at 120 hours post inoculation in WSSV-infected crayfish compared to control (p=0.0004).

3.4 Discussion

The results of the current study demonstrate an innate immune response in *P. clarkii* to WSSV infection over time. Gill and hepatopancreas tissues were examined based on the results of previous studies that have shown gills and hepatopancreas to be the most important tissues for immune reactions in crustaceans (Gross et al. 2001). Histological analysis has confirmed that WSSV targets multiple tissue types in crayfish, including the gills, cuticular epithelium, hematopoietic tissue, and hemocytes (Jiravanichpaisal et al. 2001). Although both gill and hepatopancreatic tissues are targeted by WSSV and respond immunologically to foreign pathogens, differences in gene expression and antioxidant activity were only observed in the gill tissue of WSSV-inoculated crayfish over time. No differences in antioxidant activity were observed in hepatopancreas samples, indicating that the gills may be more reactive to WSSV infection in this species.

Similar studies conducted in shrimp found that antioxidant activity decreases in multiple tissues in several species of WSSV-infected shrimp (Mohankumar and Ramasamy 2006; Rameshthangam and Ramasamy 2006; Yeh et al.2009; Taylor et al. 2013). While the results of the current study conflict with the shrimp literature in that a statistically significant increase in antioxidant activity was observed, there is evidence to suggest that crayfish may respond differently to infection than penaeid shrimps (Gao et al. 2014). In *P. clarkii*, SOD expression and total SOD activity increased when crayfish were challenged with *Spiroplasma eriocheiris* or *Aeromonas hydrophila* (Meng et al. 2013). Antioxidants such as SOD are activated and consumed in response to invasion by foreign pathogens, and activity of SOD increases to promote self-protection (Lin et al. 2008). When crayfish experience oxidative stress, SOD production may increase in an attempt to compensate for the consumption of the enzyme as the organism responds to the pathogen (Meng et al. 2013). Antioxidant enzymes are interdependent, and SOD is the first enzyme to combat oxidative stress via the dismutation of oxyradicals. In *Antheraea mylitta,* activities of SOD, GPx, and CAT increased when silkworms experienced oxidative stress due to increased temperature (Jena et al. 2013). While SOD activity was not significantly different in any tissue in any trial, the elevated activities of GPx and CAT in the gills suggest that *P. clarkii* is responding biochemically to WSSV infection.

In order to reduce variability among results, the current study quantified the nominal dose of WSSV particles prior to inoculation and sampled control crayfish at every time point throughout trials. Previously published studies also vary with regards to dose of WSSV particles administered to shrimp, experimental time sampling regime, and time passed before an observed immune response was demonstrated. Based on previous research conducted on WSSV infectivity in *P. clarkii*, the nominal dose of WSSV particles administered to crayfish affects the observed

mortality rate (Chapter 2, page 12). Although crayfish were inoculated at approximately the estimated LD_{50} value of WSSV particles, mortality rates varied throughout trials. In trials 1 and 2, mortality was observed in WSSV-infected crayfish before 120 hpi, while in trial 3, mortality did not occur until 168 hpi. The variability in mortality rate and antioxidant response among trials could be due to differences in number of WSSV particles inoculated per crayfish in each trial, the unpredictability of viral replication rate, or unknown physiological differences in individual crayfish condition prior to experimentation. While the LD_{50} value serves as an estimation of expected mortality, crayfish response depends on the biotic and abiotic factors affecting the virus, which are difficult to control. Another important consideration is the necessity of sampling control crayfish at every time point throughout trials. Several previous studies have compared antioxidant activity of WSSV-infected tissues to a single control time point (Mohankumar and Ramasamy 2006; Taylor et al. 2013) however, this approach does not account for differences in control antioxidant activity over time. In the current study, differences in both antioxidant activity and gene expression were observed in control crayfish over time. Antioxidant enzymes can be regulated by multiple factors other than pathogens, such as age, diet, seasonality and reproductive cycle, and temperature (Borkovic et al. 2008). Although crayfish were acclimated prior to trials, holding crayfish in laboratory conditions could induce a mild stress response and produce variation in innate immune activities.

In the current study, the gene expression of proPO in gill tissue varied significantly across time and was significantly downregulated at 120 hpi. Results of previous studies investigating both proPO gene expression and enzymatic activity levels in response to WSSV infection vary considerably throughout the literature. Li et al. (2012) found that gene expression of proPO increased over time in the intestine, hepatopancreas, muscle, ovary, cuticular

epidermis, branchia, stomach, hemocytes, and spermary of WSSV-infected *P. clarkii*. Similarly,

Sarathi et al. (2008) observed an increase in proPO enzymatic activity at 10 days post-

inoculation in the hemolymph of WSSV-infected *Macrobrachium rosenbergii.* Mortality was not

observed in either study, and proPO enzymatic activity returned to control levels in *M.*

rosenbergii after confirmed clearance of WSSV. The lack of mortality in these studies may

indicate that the increase in proPO gene expression was sufficient to combat viral infection, or

that the experimental dose administered was not concentrated enough to induce mortality. At

lower doses of WSSV particles that do not induce mortality, proPO gene expression may be

upregulated as organisms attempt to clear the viral infection. Conversely, several previous

studies have observed significant downregulation in proPO gene expression in experimentally

WSSV-infected animals. Liu et al. (2013) found that relative proPO gene expression was

significantly downregulated until 72 hpi in the hemocytes of the freshwater crayfish *Cherax*

quadricarinatus. In shrimp, gene expression of proPO was significantly downregulated in

WSSV-infected *Litopenaeus vannamei* compared to control (Yeh et al. 2009; Ji et al. 2011), and

proPO enzymatic activity was significantly lower in WSSV-inoculated *Penaeus monodon*

(Mathew et al. 2007). The current study as well as all three of these previous studies observed

mortality in experimental animals. At high doses of WSSV particles that experimentally induce

mortality, the innate immune system may be overwhelmed, thereby reducing proPO expression

and increasing cumulative mortality. The variability in upregulation or downregulation of proPO

gene expression and enzymatic activity observed throughout the literature could be attributed to

differences in the nominal dose of WSSV particles inoculated into experimental animals or the

response of upstream immune system genes that regulate proPO transcript levels. Activation of

the proPO system occurs in a step-wise cascade, and is regulated by several serine proteinases

including proPO activating enzymes (PPAEs) and proPO activating factors (PPAFs) (Cerenius and Söderhäll 2004). PPAFs and PPAEs convert proPO into its active form, phenoloxidase (PO), which is responsible for the production of melanin (Lee and Söderhäll 2002; Buda and Shafer 2005). When heavily infected with two pathogens, WSSV and *Vibrio anguillarum*, gene expression of both proPO and PPAFs in *L. vannamei* were significantly downregulated (Qiao et al. 2015). A decrease in proPO has been linked to a decrease in the activity of PO and a corresponding increase in susceptibility of shrimp to pathogens (Tassanakajon et al. 2013). While PPFAs gene expression and PO enzymatic activity were not investigated in the current study, the significant downregulation in gene expression of proPO at 120 hpi in trial 3 could explain the observed mortality by 168 hpi. Although not statistically significant, there was an increase in proPO gene expression in surviving crayfish at 168 hpi. This may indicate that the crayfish that survived to 168 hpi were able to recover from WSSV infection.

Although mortalities occurred at a low concentration of WSSV particles within 120 hours, previous studies indicate that shrimp exhibit a faster time course to mortality than crayfish and are more negatively impacted by WSSV-infection in commercial aquaculture operations. In shrimp farming, WSSV can produce up to 100% mortality within 3 to 10 days (Chou et al. 1995; Jiravanichpaisal et al. 2001). Comparatively in *P. clarkii*, experimental challenge dose studies infectivity studies have shown that WSSV-induced mortality rates are highly variable. Crayfish inoculated with WSSV particles may reach 100% mortality within 5 days, or may survive a 21-day challenge experiment (Chapter 2, page 12; Du et al. 2006; Du et al. 2007; Zhu and Quan 2012). In Louisiana, crayfish are extensively farmed in earthen ponds at relatively low densities compared to intensive shrimp aquaculture. In pond environments, crayfish become infected with WSSV via consumption of infected tissue material or the shedding of viral particles into the

water by infected crayfish (Lotz and Soto 2002). Although inoculation of purified WSSV particles is not an exposure route in nature, the current study utilized intramuscular injection in order to initiate an immune response. While crayfish do respond biochemically to experimental WSSV infection, severe disease outbreaks experienced by crayfish farmers are infrequent in Louisiana. Intensive shrimp farming at high densities creates a stressful environment, which could weaken the innate immune response and make shrimp more susceptible to disease. Moreover, higher densities provide more opportunity for WSSV to spread between animals via shedding of particles or cannibalistic activities. Conversely, the extensive nature and lower density of crayfish aquaculture may decrease the opportunity for crayfish to come into contact with WSSV particles. Lower densities provide a less stressful environment for crayfish, which may increase their innate ability to respond and combat WSSV infection, thereby decreasing the frequency of WSSV outbreaks in ponds.

Innate immune responses and mortality rates may also vary due to differences in virulence between WSSV strains. Sequencing of WSSV strains from different geographic regions have shown genotypic variation (Marks et al. 2004; Zwart et al. 2010). Marks et al. (2005) observed a higher cumulative mortality rate in *P. 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 exhibit greater fitness and are able to outcompete larger genome strains (Pradeep et al. 2009). Strains with smaller genomes may provide a replication advantage for WSSV, thereby increasing observed mortality rates (Marks et al. 2005). Differences in WSSV virulence have been experimentally observed in isolates from different species in various geographic locations. Wang et al. (1999) found that WSSV strains isolated from shrimp induced greater mortality in *L. vannamei* postlarvae and *Farfantepenaeus duorarum*

juveniles compared with strains isolated from crayfish. Different WSSV strains have also induced distinct innate immune responses in *C. quadricarinatus* (Gao et al. 2014). Virulence of three WSSV strains was assessed, and the innate immune components total hemocyte count, hemocyanin concentration, and the activities of proPO and PO varied in crayfish infected with different strains. The Louisiana strain of WSSV has yet to be entirely sequenced, however, the differences in the variable regions are most closely related to the WSSV isolates from China (Baumgartner, personal communication). The difference in virulence between WSSV strains may explain part of the variability in observed mortality and characterization of innate immune responses published in the literature.

In the current study, *P. clarkii* individuals responded physiologically and immunologically to WSSV-infection over time. Although responses were only observed in 2 out of 3 trials, increases in GPx and CAT activity as well as a decrease in proPO gene expression were observed. Compared to penaeid shrimps, *P. clarkii* appears to be more resistant to WSSV-induced mortality. Although only one crayfish species was investigated in this study, at least 39 species and sub-species of crayfish have been described in Louisiana (Walls 2009). WSSV has a broad host range and can infect more than 90 species of aquatic crustaceans (Lo et al. 1996). Moreover, WSSV has been found in wild crayfish populations in the Atchafalaya River Basin (ARB) (Baumgartner et al. 2009), which connects to multiple waterways throughout southwest Louisiana. Of particular concern is the Gulf Intracoastal Waterway (GIWW), which connects the ARB to the Gulf of Mexico. As a slow velocity waterway, the GIWW could be conducive for crayfish movement and the subsequent spread of WSSV into the Gulf. Because penaeid shrimps typically suffer greater cumulative mortality due to WSSV, the potential impacts of WSSV on the Gulf shrimping industry need to be assessed. Furthermore, several native crayfish in

Louisiana are listed as threatened or endangered due to habitat destruction and pollution. The potential spread of WSSV from the GIWW or the ARB into smaller streams could further decimate these smaller crayfish populations. Alternatively, if crayfish are naturally more resistant to WSSV infection compared to penaeid shrimps, threatened and endangered species may be less likely to suffer further population decline due to WSSV. The results of the current study provide a first look into the innate immune response of Louisiana crayfish to WSSV infection, and provide a foundation for future research into management initiatives and disease prevention.

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VITA

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