Epidemiology of White Spot Syndrome Virus in the Daggerblade Grass Shrimp (*Palaemonetes pugio*) and the Gulf Sand Fiddler Crab (*Uca panacea*)

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EPIDEMIOLOGY OF WHITE SPOT SYNDROME VIRUS IN THE DAGGERBLADE
GRASS SHRIMP (PALAEMONETES PUGIO) AND THE GULF
SAND FIDDLER CRAB (UCA PANACEA)

by

Muhammad

A Dissertation
Submitted to the Graduate School
and the School of Ocean Science and Technology
at The University of Southern Mississippi
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for the Degree of Doctor of Philosophy

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ABSTRACT

EPIDEMIOLOGY OF WHITE SPOT SYNDROME VIRUS IN THE DAGGERBLADE GRASS SHRIMP (*Palaemonetes pugio*) AND THE GULF SAND FIDDLER CRAB (*Uca panacea*)

by Muhammad

December 2016

Ever since the first outbreaks of *White spot syndrome virus* (WSSV), which causes White Spot Disease (WSD), in Asia in the early 1990s, the pathogen has been a major constraint to the profitability of the shrimp aquaculture industry across the globe. WSSV has a broad host range and is routinely detected in wild decapod crustaceans. In the present study, two common species in the tidal salt marsh along the northern coast of the Gulf of Mexico, the daggerblade grass shrimp (*Palaemonetes pugio*) and the Gulf sand fiddler crab (*Uca panacea*), were investigated for their role as reservoirs of WSSV and for the possible consequences of WSSV on their population dynamics. From 2013 to 2015, 1884 individuals of *P. pugio* and 1280 individuals of *U. panacea* were collected and screened for WSSV. The overall prevalence of WSSV in *P. pugio* and *U. panacea* was 7.27% and 12.97%, respectively. From experimental bioassays, the LD$_{50}$ of WSSV for *P. pugio* at 5 dpi was $8.24 \times 10^7$ WSSV genome copies g$^{-1}$ of body weight or $2.45 \times 10^7$ WSSV genome copies/grass shrimp, whereas the LD$_{50}$ of WSSV for *U. panacea* at 14 dpi was $1.67 \times 10^8$ WSSV genome copies g$^{-1}$ of body weight or $2.55 \times 10^8$ WSSV genome copies/fiddler crab. Experimental transmission of WSSV-China isolate to *P. pugio* occurred when exposed to infected living *P. pugio* ($\beta_{1,1} = 0.03$ d$^{-1}$), infected living *U. panacea* ($\beta_{1,2} = 0.02$ d$^{-1}$), infected *P. pugio* carcasses ($\chi_{1,1} = 0.08$ d$^{-1}$), and infected *U. panacea*.
*panacea* carcasses ($\chi_{1.2} = 0.03 \text{ d}^{-1}$). However, no WSSV transmission was observed when *U. panacea* was exposed to infected living or infected carcasses of either of the two species. Virulence of WSSV was higher in *P. pugio* ($\alpha_1 = 0.014 \text{ d}^{-1}$) than in *U. panacea* ($\alpha_2 = 0.00$). The decomposition of WSSV infectivity in carcasses of the two species was rapid ($\delta = 1 \text{ d}^{-1}$). The basic reproduction number ($R_0$) as calculated from single-host WSSV epidemic population models was 2.22 for *P. pugio* and 6.71 for *U. panacea*. $R_0$ from a two-host WSSV epidemic community model, including both *P. pugio* and *U. panacea*, increased to 17.09.
ACKNOWLEDGMENTS

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CHAPTER I – INTRODUCTION

General Background

Shrimp aquaculture is one of the most rapidly expanding aquaculture industries in the world. The expansion of shrimp aquaculture, which began in the mid-1980s, is driven by a huge demand for high-quality shrimp in the global seafood market. High profitability along with advances in shrimp aquaculture technologies (i.e., shrimp seed production and artificial feed) has led shrimp aquaculture to dominate the aquaculture industry in many Asian and South American countries. From 1980 to 2013, the world’s shrimp and prawns aquaculture production increased from 71.9 thousand metric tons to 4.45 million metric tons with an estimated average production growth of 14% annually (FAO-FIGIS 2015). Shrimp was the single most valuable seafood commodity in the world, contributing about 15% to the total value of traded seafood products in 2012. Although production is concentrated primarily in emerging and developing countries, the greater part of that production ended up in the global seafood market and developed countries (FAO 2014a). In 2012, 4.34 million metric tons of shrimp valued at US$ 19.4 billion were produced. The Pacific white leg shrimp (Litopenaeus vannamei) and the giant tiger shrimp (Penaeus monodon) are the predominant species in the market. China was the largest producer of farm-raised shrimp in 2012 with total production exceeding 1.5 million metric tons followed by Thailand, Vietnam, Indonesia and Ecuador (FAO 2014b). The United States is the single largest shrimp importer in the global seafood market. In 2014, the US total shrimp imports were 568,650 metric tons followed by Japan with total imports were 223,423 metric tons (FAO-GLOBEFISH 2015).
The shrimp aquaculture industry contributes significantly to national economies, especially in Asia, Central, and South America (Walker and Mohan 2009) where it is the leading earner of foreign exchange. In these countries, shrimp aquaculture serves as the primary source of income for small-scale farmers, specifically those in poor and underdeveloped coastal villages, and plays an essential role in poverty alleviation in developing countries. Although a success story, many factors have hindered the penaeid shrimp aquaculture industry. A primary constraint is infectious diseases.

Like any other organism, shrimp are vulnerable to a vast range of infectious agents such as protistans, fungi, metazoans, bacteria, and viruses. Epidemics of these infectious diseases cause significant production losses and threaten the viability of the shrimp aquaculture industry worldwide. Production losses of farm-raised shrimp due to infectious diseases have been estimated at 40% of total shrimp production annually, of which 60% have been attributed to viral diseases (Lundin 1996; Flegel 2006; Flegel 2012).

More than 20 penaeid shrimp viruses have been identified (Lightner 1999, Flegel 2012). White spot syndrome virus (WSSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV), Yellow head virus (YHV), and Infectious myonecrosis virus (IMNV) are considered the five most threatening viruses in penaeid shrimp aquaculture and are listed (Table 1) as World Organization for Animal Health (OIE – Office International des Epizooties) notifiable diseases (Lightner et al. 2012; OIE 2016). Among these viruses, WSSV is responsible for the largest share of economic losses in shrimp farming. According to Lightner (2012), financial losses due to WSSV since its emergence in the 1990s were close to US$ 15 billion, significantly higher
than the losses caused by the other penaeid viruses combined which was about US$ 5.7 billion. Approximately 300,000 metric tons of global shrimp production, worth about US$ 1 billion, is lost to WSSV annually (Stentiford et al. 2012).

Table 1

*OIE listed penaeid shrimp viruses (as of 2016)*

<table>
<thead>
<tr>
<th>Family</th>
<th>Type</th>
<th>Virus name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nimaviridae</td>
<td>dsDNA</td>
<td><em>White spot syndrome virus</em> (WSSV)</td>
</tr>
<tr>
<td>Parvoviridae</td>
<td>ssDNA</td>
<td><em>Infectious hypodermal and hematopoietic necrosis virus</em> (IHHNV)</td>
</tr>
<tr>
<td>Dicistroviridae</td>
<td>ssRNA+</td>
<td><em>Taura syndrome virus</em> (TSV)</td>
</tr>
<tr>
<td>Roniviridae</td>
<td>ssRNA+</td>
<td><em>Yellow head virus</em> (YHV)</td>
</tr>
<tr>
<td>Totiviridae</td>
<td>dsRNA+</td>
<td><em>Infectious myonecrosis virus</em> (IMNV)</td>
</tr>
</tbody>
</table>

WSSV has a remarkably wide host range; therefore, not only is the virus posing a major threat to cultivated shrimp species, but it also potentially endangers many species of decapod crustaceans in the natural environment. Our knowledge of the dynamics and consequences of WSSV in wild decapod populations is limited. According to Stentiford et al. (2012), understanding infectious agents in wild decapod crustacean populations is crucial to advancing our knowledge of emerging pathogens that threaten aquaculture. Further, study of wild decapod crustacean populations that maintain very high prevalences of infectious agents may provide compelling insights into fundamental theories of zoonoses and the mechanisms of epidemics (Stentiford et al. 2012).
Research Objectives

The primary goals of my research were to: 1) investigate the prevalence of WSSV in two species of wild decapod crustaceans collected from the northern Gulf of Mexico, the daggerblade grass shrimp (*Palaemonetes pugio*) and the Gulf sand fiddler crab (*Uca panacea*), 2) estimate the median lethal doses (LD$_{50}$s) of WSSV in *P. pugio* and *U. panacea*, 3) estimate epidemic parameters of WSSV infection both within-species and between-species of *P. pugio* and *U. panacea*, and 4) develop population epidemic models of WSSV within *P. pugio* and *U. panacea* populations as well as a 2-species community model.

Dissertation Organization

This dissertation is divided into six chapters. The first chapter provides a brief introduction to the research objectives followed by literature reviews of WSSV and mathematical modeling of infectious diseases. The second chapter addresses the prevalence and viral load of WSSV in the daggerblade grass shrimp *P. pugio* and the Gulf sand fiddler crab *U. panacea* collected from the tidal salt marshes along the northern coast of the Gulf of Mexico. The third chapter reports the median lethal doses (LD$_{50}$s) of WSSV in *P. pugio* and *U. panacea* after intramuscular challenge with WSSV. The estimated LD$_{50}$s for these two species of wild decapod crustaceans were compared to LD$_{50}$s of WSSV reported in penaeid shrimp. The fourth chapter describes the experimental protocols designed to estimate the epidemic parameters needed for the mathematical models of WSSV epidemics developed in the fifth chapter. The fifth chapter describes two epidemic models of WSSV: WSSV in single species host
populations and WSSV in a two-species host community. The sixth chapter summarizes all the results obtained from the research.

White Spot Syndrome Virus Review

WSSV is the most important penaeid shrimp virus and is the cause of White Spot Disease (WSD); the virus has been a major constraint to the shrimp aquaculture industry for more than two decades. Epidemics of WSD were detected for the first time in 1992 in kuruma shrimp (*Marsupenaeus japonicus*) farms in Fujian Province of China and the northern region of Taiwan (Chou et al. 1995; Zhan et al. 1998). WSD epidemics were also reported in Japan in 1993 causing up to 80% mortality of cultured kuruma shrimp (Nakano et al. 1994). Subsequently, the disease spread rapidly across the globe resulting in high mortality industry-wide. In many shrimp grow-out ponds complete mortality occurs within 3 – 10 days (Lightner 1996). The disease has been responsible for financial losses amounting to US$ 15 billion since its first emergence two decades ago (OIE 2006; Chou et al. 1995; Lightner et al. 2012). Dissemination of the virus into different geographic regions is believed to have occurred through importation of frozen product, live shrimp seed, and broodstock that harbored WSSV (Nunan et al. 1998; Durand et al. 2000; Reville et al. 2005; Lotz et al. 2005; Stentiford et al. 2012).

In the preliminary reports following its emergence, WSSV was classified as a nonoccluded baculovirus and was referred to by various names, such as white spot baculovirus (WSBV), hypodermal and hematopoietic necrosis baculovirus (HHNBV), rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ), penaeid rod-shaped DNA virus (PRDV), *Penaeus monodon* nonoccluded baculovirus III (PmNOB III), or systemic ectodermal and mesodermal baculovirus (SEMBV) (Inouye et al. 1994; Chou et al. 1995;
Wang et al. 1995; Durand et al. 1996; Venegas et al. 2000; Wongteerasupaya et al. 2003). Phylogenetic and genomic analyses of three major structural WSSV virion genes (thymidine-thymidylate kinase, ribonucleotide reductase, and protein kinase) provided evidence that WSSV was a new virus unrelated to baculoviruses or other viral families (van Hulten and Vlak 2001; van Hulten et al. 2000a; van Hulten et al. 2000b). Therefore, WSSV is now placed by the International Committee on Taxonomy of Viruses (ICTV) into the genus Whispovirus of the family Nimaviridae (“nima” is the Latin term for “thread”) due to its prominent tail-like appendage (Mayo 2002).

The WSSV virion is non-occluded, enveloped, with an olive to bacilliform shape, and containing a distinct tail-like projection at one extremity which is frequently visible on negatively stained WSSV virion preparations (Wang et al. 1995; Wongteerasupaya et al. 1995; Huang et al. 2001). The intact viral envelope ranges between 210 - 380 nm × 70 - 167 nm (Chang et al. 1996; Park et al. 1998; Rajendran et al. 1999). From transmission electron micrograph preparations, the viral envelope is 6 – 7 nm wide and has a trilaminar unit membrane complex containing two electron-translucent membranes divided by an electron-nontranslucent membrane (Wongteerasupaya et al. 1995; Durand et al. 1997). The WSSV nucleocapsid is about 200 × 65 nm in size, and surrounded by a 6 nm thick exterior wall which is divided into 15 – 16 segments. Each segment consists of dual layers of 14 globular subunits with a diameter of 8 nm (Wang et al. 1995; Durand et al. 1997; Huang et al. 2001).

WSSV contains a circular double-stranded DNA (dsDNA) genome of 292 to 307 kilobase pairs comprising 181 to 184 putative open reading frames (ORFs) (van Hulten et al. 2001a; Yang et al. 2001). To date, five WSSV isolates from different countries
(China, Korea, Taiwan, Thailand, and Mexico) have been comprehensively sequenced
(Wang et al. 1995; Yang et al. 2001; van Hulten et al. 2001a; Chai et al. 2013;
Rodriguez-Anaya et al. 2016). By comparing the complete sequences of three isolates
(Thailand, Taiwan and China), Marks et al. (2004) found that the isolates have a
nucleotide similarity of 99.32%. Their study also noted some genetic variation among
these three isolates, in particular: 1) a substantial deleted region in the genome of the
Thailand isolate, 2) a transposase sequence only in the genome of the Taiwan isolate, 3) a
variable region sensitive to recombination, 4) single nucleotide mutations and
polymorphisms, and 5) variation in the number of repeat units within homologous direct
repeats. These differences have been used extensively as genetic markers in molecular
epidemiological investigations to determine the virus’s spread history as well as to
pinpoint the potential reservoirs of disease (Wongteerasupaya et al., 2003; Dieu et
al.,2004; Musthaq et al., 2006).

Proteomic approaches have identified approximately 50 structural proteins in the
virus. Of these, 50% of the proteins are classified as envelope proteins which include
VP15, VP19, VP24, VP26, VP28, VP68, VP281 and VP466 (Tsai et al. 2004; Wu et al.
2005; Xie and Yang 2006; Zhou et al. 2009). VP28 is the most abundant of the envelope
proteins and likely has a critical role in WSSV infection (e.g., cell receptor recognition,
attachment, and entry) (van Hulten et. al 2001b; Yi et al. 2004; Wu et al. 2005).

Based on their temporal expression, Liu et al. (2005) classified the WSSV genes
into three different stages: immediate-early, early, and late genes. The immediate-early
genes of WSSV can be expressed independently of other viral genes if compatible host
proteins are present in the nucleus; however, expression of the early and late genes
requires transcription factors which result from expression of WSSV immediate-early
genes (Liu et al. 2005; Li et al. 2009). Currently, at least three immediate-early genes of
WSSV have been determined: $ie1$, $ie2$, and $ie3$. The transcripts of these genes are
observed approximately 2 hours post infection (Liu et al. 2005). WSSV targets the host’s
antiviral STAT (signal transducer and activator of transcription) protein and NF-κB
(nuclear factor- kappa B) protein and exploits these proteins to activate the promoter $ie1$
gene resulting in an increase in expression of this gene (Liu et al. 2007; Huang et al.
2010).

At the population level, three modes of WSSV transmission in penaeid shrimp
have been recognized: transmission by ingestion of infectious host cadavers, transmission
by cohabitation with infected hosts, and vertical transmission from broodstock to
offspring (Corteel et al. 2009). Factors associated with the host (e.g., species and age) as
well as factors associated with WSSV (e.g., strains, virulence) affect transmission of the
virus (Wang et al. 1998; Lightner et al. 1998; Soto et al. 2001). For successful
transmission to occur, the virus must overcome a number of barriers. The virus is
required to pass the first defense of the host, the exoskeleton. The exoskeleton is
impervious to viral transit except where broken by injury, thinned by molting, or
naturally thin (around the gills) (Corteel et al. 2009). In the course of horizontal
transmission by ingestion, the virus has to cross the gastrointestinal tract of the host
where the virus may be inactivated by digestive enzyme activities and pH fluctuation
(Jeswin et al. 2015). Once the virus passed these two barriers, the virus must able to find
compatible receptors on the surface of the host’s cells, such as $P. monodon$ Rab7 protein
(PmRab7), β-integrin, and $P. monodon$ chitin-binding protein (PmCB), for viral
attachment and entry (Sritunyalucksana et al. 2006; Li et al. 2007; Chen et al. 2009). Another important step for WSSV infection in the host is that the virus must overcome the shrimp’s immune response. Even though shrimp lack a true adaptive immune response, they have an effective innate immune response against a wide array of pathogens invasion including WSSV. Infection by the virus triggers numerous major innate immune response signaling pathways in shrimp such as Toll, immune deficiency (IMD), and Janus kinase/Signal transducers and activators of transcription (JAK/STAT) pathways (Chen et al. 2008; Li and Xiang 2013; Wen et al. 2014). In addition, the virus must challenge the shrimp’s cell-mediated antiviral responses such as phagocytosis, apoptosis and the prophenoloxidase (proPO) activating system (Sritunyalucksana and Söderhäll 2000; Wang and Zhang 2008; Wang et al. 2014).

WSSV has a diverse host range including many, if not all, cultivated penaeid shrimp species, caridean shrimp, lobsters, crayfish, crabs, krill, branchiopods, rotifers, copepods, polychaete worms, microalgae, and some aquatic insects (Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010; Liu et al. 2007). The gross clinical signs of WSD which may but not always be displayed by infected penaeid shrimp include anorexia, lethargy, loose cuticle, and pinkish to reddish body coloration (Chou et al. 1995; Otta et al. 1999). WSSV-infected shrimp often display white spots (diameter from 0.5 – 2.0 mm) on the underside of the cuticle on the carapace and appendages (Chou et al. 1995; Lightner 1996; Leu et al. 2009). However, this distinctive feature of WSSV infection is not a conclusive diagnostic feature because high alkalinity, bacterial infection, and other environmental stressors also cause white spots (Leu et al. 2009; Wang et al. 2000).
The virus targets several tissues, including ectodermal and mesodermal tissues, striated muscle, hematopoietic nodules, hemocytes, cuticular epidermis, foregut, nervous system, gills, and connective and epithelial tissues (Momoyama et al. 1994; Wongteerasupaya et al. 1995; Chang et al. 1996; Durand et al. 1996). Escobedo-Bonilla et al. (2008) reported that gills and stomach are the primary target organs for WSSV replication and those organs may be severely impaired during the course of infection. WSSV replication occurs in the nucleus causing nuclear hypertrophy, nuclear disintegration, and chromatin accumulation; and its replication is easily triggered by stressful conditions (Lo et al. 2003; Leu et al. 2009). Jeswin et al. (2015) analyzed the viral copy number in different tissues of the giant tiger shrimp _P. monodon_ following intramuscular exposure to WSSV inoculum and demonstrated that gills had the highest viral load followed by pleopods, muscles, and hemocytes.

Infectious Disease Modeling

Mathematical epidemic models have been used to investigate the dynamics of numerous infectious diseases impacting human and wildlife populations. Models have the potential to advance our knowledge of disease population processes which may lead to accurate forecasts and guidance for reducing the incidence and severity of infectious disease outbreaks (Heffernan 2011). The models are developed based on tractable assumptions to incorporate the processes of pathogen transmission from infected hosts to susceptible hosts at the population level. The application of mathematical models in the field of epidemiology has a long and extensive history. It began when Daniel Bernoulli published his seminal paper of a smallpox model in 1766 (Dietz and Heesterbeek 2002). Since then, the use of mathematical models in epidemiology has increased and become
more popular among theoretical epidemiologists particularly in the middle of the 20th century. Numerous mathematical population models have been synthesized, analyzed and applied to various infectious diseases (Hethcote 2000). Additionally, mathematical models of infectious diseases have evolved from models that focus on one-pathogen in one host to more complicated models involving multiple-hosts and multiple-pathogens (Holt and Pickering 1985; Anderson and May 1991; Grenfell and Dobson 1995; Dobson 2004). Advances in computational power and statistical methods have contributed to the increase in the robustness of infectious disease models.

Epidemic population models share much conceptually with population models of free-living species, particularly the concepts of births and deaths. Transmission is the parallel of births for epidemic models, i.e., transmission is the force that increases the number of infected hosts and therefore is one of the important factors that regulates the dynamics of infectious disease (McCallum et al. 2001). On the other hand, the loss of infectivity or removal rate of a pathogen can be translated as deaths for epidemic models, i.e., an infected dead host may lose its infectiousness due to decomposition or scavenging (Soto and Lotz 2001; Lotz et al. 2003; Lotz 2010). In most infectious disease models, the transmission rate of a disease in a population is highly dependent on the number of susceptible hosts and the force of infection (likelihood that an individual becomes infected). According to Begon et al. (2002), the force of infection is the product of the probability of contacts among the host in a population, the probability of a susceptible host making contact with the infective, and the probability of a susceptible host becoming infected after contact with the infected host.
The spread of a pathogen depends on the type of environment occupied by both the pathogen and the host, and therefore the underlying biological processes of disease transmission in aquatic environments may be different from those in terrestrial environments (McCallum et al. 2004). Aquatic pathogens and hosts inhabit three-dimensional environments that may quantitatively change the dispersal compared to that which occurs in terrestrial environments which are more-or-less two-dimensional (Murray 2009). Even though the dynamics of infectious diseases have rarely been studied in aquatic populations compared to those in terrestrial populations, the principles that regulate the disease dynamics in terrestrial animals should be applicable to studying the disease dynamics in aquatic organisms (Reno 1988). For example, Powell et al. (1996) identified some biological and environmental factors that are important in regulating epidemics of Perkinsus marinus, the causative agent of Dermo disease, in eastern oyster (Crassostrea virginica) populations such as food supply, recruitment rate, disease resistance, salinity, and temperature.

In shrimp aquaculture, various infectious agents, particularly viruses such as WSSV, TSV, IHHNV, YHV, and IMNV have been reported to cause severe production losses over the last several decades. Understanding of the population dynamics of the diseases caused by these viruses may be advanced through formulation of epidemic models that can provide essential information for controlling and forecasting disease outbreaks and consequences on shrimp farms. Dr. Jeffrey M. Lotz and his research group (USM, Gulf Coast Research Laboratory) have synthesized epidemic models of two of the most important viruses in shrimp aquaculture, WSSV and TSV (Soto and Lotz 2001; Soto et al. 2001; Lotz and Soto 2002; Soto and Lotz 2003; Lotz et al. 2003). In the
epidemic model of WSSV in *L. vannamei*, Lotz and Soto (2002) identified 6 host states: 1) susceptible, 2) latent infected, 3) acute infected, 4) dead infected, 5) chronically infected, and 6) dead infected that is no longer infectious because of decomposition or scavenging. This will be the starting point for the models in this dissertation.
CHAPTER II - PREVALENCE OF WHITE SPOT SYNDROME VIRUS IN THE
DAGGERBLADE GRASS SHRIMP (*Palaemonetes pugio*) AND
THE GULF SAND FIDDLER CRAB (*Uca panacea*)

Introduction

First detected in the early 1990s, the infectious disease caused by *White spot syndrome virus* (WSSV) remains one of the greatest threats to shrimp aquaculture. WSSV is a double-stranded DNA virus with a circular genome belonging to the genus *Whispovirus* of the family *Nimaviridae* (Mayo 2002; Vlak et al. 2004). The virus can cause up to 100% mortality in 3 to 10 days in commercial shrimp farms (Lightner 1996). The first epidemic of WSSV was reported in farm-raised kuruma shrimp (*Marsupenaeus japonicus*) in Fujian province of China and the northern region of Taiwan in 1992 (Chou et al. 1995; Zhan et al. 1998). Within a decade following these first observations, the virus disseminated rapidly throughout all shrimp farming regions of the world. The estimated economic loss associated with the disease since its first appearance in the early 1990s is approximately US$ 15 billion (Lightner et al. 2012).

WSSV has a remarkably wide host range which includes many, if not all, cultivated penaeid shrimp species, copepods, crabs, lobsters, crayfish, freshwater crabs, prawns, and rotifers as well (Zhang et al. 2006; Liu et al. 2007; Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010). Some species of wild crustaceans that are considered pest species and natural inhabitants of shrimp farms such as crabs and prawns likely serve as asymptomatic carriers of WSSV (Lo et al. 1996). Several studies have investigated the susceptibility of a variety of hosts and the prevalence of WSSV in the natural environment. Lo et al. (1996) reported that 40% of wild arthropods, including some
decapods and copepods, collected from different locations in Taiwan were infected by WSSV. Hossain et al. (2001) reported that 49% of wild penaeid shrimp from coastal waters of Bangladesh were infected by the virus. Moreover, a study on WSSV prevalence in the blue crab (*Callinectes sapidus*) from the North-American Atlantic Coast reported a prevalence of 27% (Chang et al. 2001). In addition, Chapman et al. (2004) reported prevalences of 4.78% in white shrimp (*Litopenaeus setiferus*), 0.84% in brown shrimp (*Farfantepenaeus aztecus*), and 0% in pink shrimp (*F. duodarum*). The presence of an exotic pathogen like WSSV in wild crustaceans is of concern to both aquaculture where these wild crustaceans may serve as reservoirs of infection and natural resources where the WSSV may threaten wild commercially important stocks and species that are critical components of the ecosystem.

The daggerblade grass shrimp (*P. pugio*) is a common inhabitant of tidal salt marsh habitats along the Atlantic and the Gulf of Mexico coasts of North America from Nova Scotia, Canada to Veracruz, Mexico (William and Wigley 1977; Anderson 1985; Glas et al. 1997). This shrimp not only serves as prey for many economically important fish and crustaceans that use the estuaries as nursery grounds but also as a predator that plays an essential role in energy transfer in the salt marsh ecosystem (Welsh 1975; Lund et al. 2000; Davis et al. 2003). The Gulf sand fiddler crab (*U. panacea*) also is a common inhabitant of tidal salt marsh habitats. It is one of the burrowing crustaceans inhabiting sandy intertidal habitats such as bordering bays, coastal marshes, tidal creeks, and lagoons along the coast of the Gulf of Mexico from north-west Florida to Tabasco, Mexico (Powers 1975; Barnwell and Thurman 1984). The burrowing associated with such crustaceans serves to facilitate marsh sediments oxygenation, substrate drainage,
and nutrient transfer in the coastal wetland ecosystem and thus greatly contributes to the fundamental vitality of salt marsh habitats (Montague 1980; Hoffman et al. 1984; Zeil et al. 2006; Chatterjee et al. 2014). Fiddler crabs also serve as important food items for many species including predatory birds, blue crabs, mud crabs, channel bass, and raccoons (Montague 1980; Nomann and Pennings 1998; Zeil et al. 2006).

To better understand the role of WSSV in natural communities of decapods, I undertook a survey of WSSV in Mississippi Sound. The objectives of the survey were to: 1) estimate WSSV prevalence in the daggerblade grass shrimp (*P. pugio*) and the Gulf sand fiddler crab (*U. panacea*) collected from tidal salt marshes of Mississippi Sound in the northern Gulf of Mexico (GoM), and 2) compare the WSSV viral load in these two species.

Materials and Methods

*Sample Collection*

The daggerblade grass shrimp (*P. pugio*) and the Gulf sand fiddler crab (*U. panacea*) were collected from tidal salt marshes of Mississippi Sound as part of an effort to obtain uninfected animals for laboratory studies. Specimens of *P. pugio* were collected from Davis Bayou (n = 1884), Ocean Springs, Mississippi (30°23′31″ N, 88°47′54″ W) by dip-netting during the period of November 2013 to October 2015. Specimens of *U. panacea* were hand-collected from Marsh Point (n = 613), Ocean Springs, Mississippi (30°22′58″ N, 88°48′20″ W) and from Deer Island (n = 667), Harrison County, Mississippi (30°22′08″ N, 88°49′30″ W) during September 2014 through August 2015. In total, 1884 specimens of *P. pugio* and 1280 specimens of *U. panacea* were collected. Captured animals were transported immediately to the Gulf
Coast Research Laboratory (GCRL) in Ocean Springs. Upon arrival, all animals were placed individually in 3-l isolation tanks containing 15 ppt aerated artificial seawater (Bio-Sea Marine Mix™, Aquacraft, CA, USA). The water depth in containers with *P. pugio* was 10 cm whereas that in containers with *U. panacea* was 1 cm. Animals were acclimated for 48 h at room temperature (26 ± 1 °C). After acclimation, a DNA sample was collected from each wild-captured animal and evaluated for WSSV infection using TaqMan real-time PCR.

**Genomic DNA Extraction**

Pleopods and hemolymph were used as sources of DNA for *P. pugio* and *U. panacea*, respectively. Total genomic DNA was extracted from 30 – 50 mg of tissue sample or approximately 10 μl of hemolymph using High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) in accordance with the company’s instructions. Total genomic DNA extract concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at – 20 °C until used for subsequent real-time PCR.

**Standard Curve**

A set of 10-fold standard serial dilutions ranging from 2.69×10¹ to 2.69×10⁶ WSSV genome copies 5-μl⁻¹ was generated from synthesized oligonucleotides of 75 bp corresponding to nucleotides 1008 – 1082 of SalI fragment of WSSV genomic sequence in GenBank U50923. The oligonucleotide sequence used to generate the standard curves was 5’– CAA TGG TCC CGT CCT CAT CTC AGA AGC CAT GAA TGC CGT CTA TCA CAC ACT AAT TTC CGG CAA GGC AGC TCG –3’ (Invitrogen, Carlsbad, CA, USA). The absolute WSSV copy numbers in all samples were quantified by
extrapolating the cycle threshold (C_T) values from the standard curve. The viral copy number was normalized to μg of total DNA extract.

Detection and Quantification of WSSV using TaqMan Real-Time PCR

WSSV detection and quantification was carried out according to the method described by Durand and Lightner (2002). The sequences of forward primer, reverse primer, and TaqMan probe were 5′–TGG TCC CGT CCT CAT CTC AG–3′ (WSS1011F), 5′–GCT GCC TTG CCG GAA ATT A–3′ (WSS1079R), and 5′–AGC CAT GAA GAA TGC CGT CTA TCA CAC A–3′ (WSSV1032-1050) and are listed in Table 2. These primers produced a 69 bp amplicon. The TaqMan probe was labeled with two fluorogenic dyes: 5–carboxyfluorescein (FAM) on the 5′ end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end.

Table 2

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Name</th>
<th>Sequence (5′ to 3′)</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>WSS1011F</td>
<td>TGG TCC CGT CCT CAT CTC AG</td>
<td>20</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>WSS1079R</td>
<td>GCT GCC TTG CCG GAA ATT A</td>
<td>19</td>
</tr>
<tr>
<td>Probe</td>
<td>WSSV1032-1050</td>
<td>AGC CAT GAA GAA TGC CGT CTA TCA CAC A</td>
<td>28</td>
</tr>
</tbody>
</table>

Real-time PCR was performed by using a TaqMan Universal PCR Master Mix (Life Technologies, Warrington, UK). Five μl of DNA template containing 20 – 30 ng μl⁻¹ of total DNA extract was added to a PCR mixture consisting of 0.3 μM of each
primer and 0.15 μM of TaqMan probe in a final reaction volume of 25 μl. Amplification was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: 2 minutes reaction for AmpErase uracil-N-glycosylase (UNG) at 50 °C, followed by the AmpliTaq Gold DNA polymerase activation for 10 minutes at 95 °C, 40 cycles of 15 seconds denaturation at 95 °C and 60 seconds annealing at 60 °C, and 60 seconds extension at 60 °C. All genomic DNA samples were run in duplicate with two non-template controls (NTC) as negative controls.

Data Analysis

Data obtained from the survey were prepared and analyzed using Microsoft Excel 2013 and SPSS version 24. The null hypotheses for this study were: 1) there is no difference in overall prevalence of WSSV infection between P. pugio and U. panacea, 2) there is no difference in prevalence of WSSV between years, and 3) there is no difference in mean viral load of WSSV between the two species. Pearson’s Chi-Square test was performed to determine differences in prevalence of WSSV infection in both species. Differences in mean log viral load of infected grass shrimp and fiddler crab were evaluated using an independent-samples t-test. The significance level (α) used to accept or reject the null hypothesis was 0.05.

Results

One-thousand eight-hundred eighty-four (1884) P. pugio and 1280 U. panacea were collected and evaluated for WSSV using TaqMan real-time PCR. The overall prevalence of WSSV infection in the two species collected during the period of 2013 to 2015 is presented in Table 3. The estimated mean prevalence of WSSV in wild P. pugio
and *U. panacea* collected during this surveillance study were 7.27% and 12.97%, respectively. The prevalence of WSSV in *U. panacea* was significantly higher than its prevalence in *P. pugio* ($\chi^2 = 28.567, P < 0.001$).

**Table 3**

*Prevalence of WSSV in Palaemonetes pugio and Uca panacea collected from the northern coast of the Gulf of Mexico*

<table>
<thead>
<tr>
<th>Species</th>
<th>Year</th>
<th>Total tested</th>
<th>Total positive</th>
<th>Prevalence (%)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. pugio</strong></td>
<td>2013</td>
<td>270</td>
<td>22</td>
<td>8.15</td>
<td>4.89 - 11.41</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>710</td>
<td>94</td>
<td>13.24</td>
<td>10.75 - 15.73</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>904</td>
<td>21</td>
<td>2.32</td>
<td>1.34 - 3.30</td>
</tr>
<tr>
<td>Group total</td>
<td></td>
<td>1884</td>
<td>137</td>
<td>7.27</td>
<td>6.1 - 8.44</td>
</tr>
<tr>
<td><strong>U. panacea</strong></td>
<td>2014</td>
<td>841</td>
<td>163</td>
<td>19.38</td>
<td>16.71 - 22.05</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>439</td>
<td>3</td>
<td>0.68</td>
<td>-0.09 - 1.45</td>
</tr>
<tr>
<td>Group total</td>
<td></td>
<td>1280</td>
<td>166</td>
<td>12.97</td>
<td>11.13 - 14.81</td>
</tr>
</tbody>
</table>

The prevalence of WSSV reached its highest in 2014 at 13.24% and 19.38% for *P. pugio* and *U. panacea*, respectively. The prevalence of WSSV in *P. pugio* collected in 2014 was significantly higher than the prevalence of WSSV in *P. pugio* collected in 2013 ($\chi^2 = 4.859, P = 0.028$) and in 2015 ($\chi^2 = 71.613, P < 0.001$). Similarly, the prevalence of WSSV in *U. panacea* collected in 2014 was significantly higher than that in *U. panacea*
in 2015 ($\chi^2 = 89.347, P < 0.001$). Moreover, the prevalence of WSSV in both species decreased to 2.32% for *P. pugio* and 0.68% for *U. panacea* in 2015.

By using the TaqMan real-time PCR, I was able to calculate the WSSV genome copy number in each infected *P. pugio* and *U. panacea* collected in this study (Table 4). The overall means of WSSV viral load in WSSV-positive *P. pugio* and *U. panacea* were $7.16 \times 10^2$ (SE $= 1.44 \times 10^2$) WSSV genome copies $\mu$g$^{-1}$ of total DNA and $8.17 \times 10^4$ (SE $= 7.83 \times 10^4$) WSSV genome copies $\mu$g$^{-1}$ of total DNA, respectively. The viral loads in WSSV-positive *P. pugio* and *U. panacea* were from $1.57 \times 10^2$ – $1.25 \times 10^4$ WSSV genome copies $\mu$g$^{-1}$ of total DNA and $2.80 \times 10^2$ – $1.30 \times 10^7$ WSSV genome copies $\mu$g$^{-1}$ of total DNA, respectively. During the period of this survey, I found only one Gulf sand fiddler crab (*U. panacea*) that was heavily infected by WSSV with a viral load of $1.30 \times 10^7$ WSSV genome copies $\mu$g$^{-1}$ of total DNA. An independent sample t-test detected a significant difference between the mean log viral load in WSSV-infected *P. pugio* and *U. panacea*, and based on this finding I rejected the null hypothesis ($t_{(301)} = 10.916, P < 0.001$).

This survey confirmed the presence of WSSV in wild populations of *P. pugio* and *U. panacea* in the northern coast of the Gulf of Mexico. Although the virus was detected in both *P. pugio* and *U. panacea*, no animals displayed clinical signs associated with WSSV infections in penaeid shrimp.
Table 4

*Mean viral load (WSSV genome copies µg\(^{-1}\) of total DNA) in WSSV-positive specimens of Palaemonetes pugio and Uca panacea collected from the northern coast of the Gulf of Mexico*

<table>
<thead>
<tr>
<th>Species</th>
<th>Year</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pugio</em></td>
<td>2013</td>
<td>22</td>
<td>3.16×10(^2)</td>
<td>5.87×10(^1)</td>
<td>1.57×10(^2) – 1.51×10(^3)</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>94</td>
<td>8.91×10(^2)</td>
<td>2.07×10(^2)</td>
<td>1.57×10(^2) – 1.25×10(^4)</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>21</td>
<td>3.51×10(^2)</td>
<td>5.10×10(^1)</td>
<td>1.62×10(^2) – 1.27×10(^3)</td>
</tr>
<tr>
<td>Group total</td>
<td></td>
<td>137</td>
<td>7.16×10(^2)</td>
<td>1.44×10(^2)</td>
<td>1.57×10(^2) – 1.23×10(^4)</td>
</tr>
<tr>
<td><em>U. panacea</em></td>
<td>2014</td>
<td>163</td>
<td>3.09×10(^3)</td>
<td>5.34×10(^3)</td>
<td>2.80×10(^2) – 4.48×10(^4)</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>3</td>
<td>4.35×10(^6)</td>
<td>4.32×10(^6)</td>
<td>2.49×10(^3) – 1.30×10(^7)</td>
</tr>
<tr>
<td>Group total</td>
<td></td>
<td>166</td>
<td>8.17×10(^4)</td>
<td>7.83×10(^4)</td>
<td>2.80×10(^2) – 1.30×10(^7)</td>
</tr>
</tbody>
</table>
Figure 1. Viral loads (log_{10} WSSV genome copies µg^{-1} of total DNA) in WSSV-positive *Palaemonetes pugio* (□) and *Uca panacea* (■) collected from the tidal salt marshes of the northern coast of the Gulf of Mexico. No *U. panacea* were collected and tested for WSSV in 2013.

Discussion

White Spot Disease caused by WSSV is one of the most important diseases in farmed penaeid shrimp species. Further, WSSV commonly infects wild populations of decapods as well as other crustacean species. However, our understanding of WSSV in wild crustacean populations is meager. *White spot syndrome virus* is virulent in many species, and the introduction of WSSV could pose a serious threat to critical components of salt marsh habitats as well as the sustainability of crustacean fisheries and aquaculture industries in the Gulf region. When the virus is successfully established in the wild, a decline in populations of shrimp and other economically important crustacean species may be expected (Nunan et al. 2001).

Several studies have suggested that the virus may be introduced into the coastal waters of the USA through imports of frozen shrimp that are infected by WSSV (Nunan
et al. 1998; Lightner 1999; Durand et al. 2000; Lotz et al. 2005). Moreover, as hundreds of shrimp-related industries such as shrimp processing plants and grocery stores are located along the Gulf of Mexico, it is possible for the virus to be introduced into the Gulf coastal waters if processing plant or household waste contacts the environment and susceptible wild host populations (Nunan et al. 1998; Hasson et al. 2006).

Even though the effect of WSSV on wild decapod crustacean populations is poorly understood, this study demonstrates the presence of WSSV infection in two of the local species of decapod crustaceans of the Gulf of Mexico. In this study, the prevalence of WSSV in *P. pugio* and *U. panacea* was 7.27% and 12.97%, respectively. In comparison to WSSV prevalence in three species of penaeid shrimps from coastal waters of the USA reported by Chapman et al. (2004), the prevalence of the virus in *P. pugio* and *U. panacea* was higher than that in penaeid shrimp. Despite the fact that the two species under study were infected by WSSV, no clinical signs of White Spot Disease (i.e., white spots on the cuticle, pinkish to reddish body discoloration, etc.) were observed. This finding suggests that *P. pugio* and *U. panacea* could act as asymptomatic carriers and thus serve as reservoirs for transmission of the virus to commercially important decapods.

This study also found that the prevalence of WSSV in *P. pugio* and *U. panacea* were significantly reduced in 2015. The reasons for the lower prevalence of WSSV in the two species screened during this period were not determined in this study. It is likely that some biological and environmental factors that are important for WSSV transmission in the wild were altered. Such factors might include, among others, reduced viral load in
the water (dose), salinity and temperature fluctuations, and changes in water current pattern.

Previous reports of the prevalence of WSSV in wild crustaceans collected from different geographic areas vary, except for a study conducted by Chapman et al. (2004). For example, Otta et al. (1999) reported a WSSV prevalence of 75% in wild captured tiger shrimp (*Penaeus monodon*) from the West coast of India, whereas Dutta et al. (2015) reported a prevalence of 56.20% in wild *P. monodon* captured along the East coast of India. Jang et al. (2009) also reported a prevalence of 75.5% in wild captured fleshy shrimp (*Fenneropenaeus chinensis*) broodstock from the coast of the Korean Peninsula. Moreover, Cavalli et al. (2013) found a prevalence of 20% in wild captured crabs (*Neohelice granulate*) from Brazil. Such variation in the prevalence of WSSV may be explained by variation in the number of samples collected, the geographic locations of the studies, the susceptibility of the hosts, or the diagnostic methods used for WSSV detection.

The mean viral load in WSSV-infected *P. pugio* and *U. panacea* is variable and often low (from 10^2 to 10^6 WSSV genome copies µg⁻¹ of total DNA). The highest viral load was found in only one individual of *U. panacea* collected in 2015 with an estimated viral load of 1.30×10^7 WSSV genome copies µg⁻¹ of total DNA. This was an exceptional case during the three years of this study. Even though the prevalence of WSSV was higher in *U. panacea*, there was no significant difference in the mean log WSSV load between *P. pugio* and *U. panacea*. It is of note that most of the infected wild *P. pugio* and *U. panacea* screened in this study managed to survive capture and holding for several days to weeks. Perhaps the low viral load in animals from the wild contributes to the lack
of disease or pathology. One plausible explanation as to why most animals screened for WSSV in this study contained a low viral load is that hosts with a high viral load succumb to WSSV; thus, leaving the low load survivors to be observed in this study. Another possible explanation is that severely infected hosts may have their health and physical movement affected due to heavy infection of WSSV; consequently, they would have a greater chance to be preyed upon. Additionally, the widespread prevalence of WSSV suggests that the virus may not be very pathogenic for both *P. pugio* and *U. panacea*. 
CHAPTER III - PATHOGENICITY OF WHITE SPOT SYNDROME VIRUS IN THE DAGGERBLADE GRASS SHRIMP (PALAEMONETES PUGIO) AND THE GULF SAND FIDDLER CRAB (UCA PANACEA)

Introduction

White spot syndrome virus (WSSV) is the most pathogenic of the penaeid shrimp viruses and has been a major constraint to the shrimp farming industry world-wide for more than two decades. The first epidemics of the disease attributable to this virus, namely White Spot Disease (WSD), were reported in kuruma shrimp (Marsupenaeus japonicus) farms in Fujian Province of China and the northern region of Taiwan in 1992 (Chou et al. 1995; Zhan et al. 1998). In 1993, WSD outbreaks were reported in Japan where it caused up to 80% mortality of cultured kuruma shrimp (Nakano et al. 1994). Following these first appearances in Asia, the disease spread rapidly worldwide causing up to 100% mortality in commercial shrimp farms over a period of 3 - 10 days (Lightner 1996). The disease has been responsible for estimated financial losses of US$15 billion (Lightner et al. 2012). The first epidemic of WSSV in the western hemisphere occurred in the white shrimp (Litopenaeus setiferus) on farms in south Texas USA in 1995 (Lightner 1996; Rosenberry 1996). Transmission of WSSV across international boundaries is assumed to occur through trade of frozen farmed shrimp products and infected live broodstock and seed (Nunan et al. 1998; Durand et al. 2000; Stentiford et al. 2012).

In the preliminary reports published following its emergence, the virus was classified as a nonoccluded baculovirus and given various names such as white spot baculovirus (WSBV), hypodermal and hematopoietic necrosis baculovirus (HHNBV),
rod-shaped nuclear virus of Penaeus japonicus (RV-PJ), penaeid rod-shaped DNA virus (PRDV), Penaeus monodon nonoccluded baculovirus (PmNOB III), shrimp explosive epidemic disease (SEED) or systemic ectodermal and mesodermal baculovirus (SEMBV) (Inouye et al. 1994; Chou et al. 1995; Jie et al. 1995; Wang et al. 1995; Durand et al. 1996; Venegas et al. 2000; Wongteerasupaya et al. 2003). Phylogenetic and genomic analyses of three major structural protein genes (thymidine-thymidylic kinase, ribonucleotide reductase, and protein kinase) provided evidence that WSSV is not related to baculoviruses or other known families (van Hulten and Vlak 2001; van Hulten et al. 2000a; van Hulten et al. 2000b). Based on this evidence, WSSV has been designated by the International Committee on Taxonomy of Viruses (ICTV) as belonging to the genus Whispovirus in the family Nimaviridae (“nima” is the Latin term for “thread”) in reference to the prominent tail-like appendage on the envelope (Mayo 2002).

At the population level, three modes of WSSV transmission in shrimp have been recognized: 1) transmission by ingestion of infectious host cadavers, 2) transmission by cohabitation with infected hosts, and 3) transmission from broodstock to offspring (Corteel et al. 2009). Factors associated with the host (e.g., species and age) as well as factors associated with the virus (e.g., strains and virulence) affect transmission of the virus within a host population (Wang et al. 1998; Lightner et al. 1998; Soto et al. 2001).

Variation in virulence among WSSV isolates of different geographic origins have been reported by some investigators (Laramore et al. 2009; Rahman et al. 2008). Rahman et al. (2008) examined virulence of three isolates of WSSV, two isolates from Thailand and one isolate from Vietnam in L. vannamei and demonstrated that Thailand isolates are more virulent compared to Vietnam isolate. Their study found that all
isolates resulted in a total mortality of shrimp within 72 – 108 hpi for Thailand isolates and 204 – 348 hpi for Vietnam isolate. Laramore et al. (2009) compared the virulence of seven geographic WSSV isolates from China, Ecuador, Honduras, Mexico, and Nicaragua in *L. vannamei*. The results of their study showed that WSSV isolates from Ecuador, Honduras and Mexico are the three most virulent isolates and suggested that the difference in virulence among isolates corresponded to geographical origin.

In the previous chapter I found that both *P. pugio* and *U. panacea* carried a low load of WSSV at a high prevalence. Those observations suggested that: 1) the two species are either resistant to WSSV and are able to control loads and perhaps mortality or 2) they are not resistant and many infected individuals are dying and not being observed. To address these alternatives, we experimentally exposed individuals to WSSV to determine if the species could control the load and mortality. The objectives of this study were to: 1) determine the median lethal dose (LD$_{50}$) of WSSV in two species of wild decapods, *P. pugio* and *U. panacea*, 2) compare the survival curves of these two species after challenge with various doses of WSSV, 3) compare the mean WSSV lethal load between *P. pugio* and *U. panacea*, and 4) determine the mean WSSV load in individuals that survived challenge.

**Materials and Methods**

*Experimental Animals*

Individuals of the daggerblade grass shrimp (*P. pugio*) and the Gulf sand fiddler crab (*U. panacea*) used throughout this study were collected from the tidal salt marshes along the northern coast of the Gulf of Mexico. Specimens of *P. pugio* were collected from Davis Bayou (n = 1884), Ocean Springs, Mississippi (30°23’31” N, 88°47’54” W)
by dip-netting during the period of November 2013 to October 2015. Specimens of *U. panacea* were hand-collected from Marsh Point (n = 613), Ocean Springs, Mississippi (30°22'58" N, 88°48'20" W) and from Deer Island (n = 667), Harrison County, Mississippi (30°22'08" N, 88°49'30" W) during September 2014 through August 2015. All animals were evaluated for WSSV infection using TaqMan based real-time PCR. WSSV-negative animals were maintained under laboratory conditions until used for bioassay. All negative animals were placed in 1-m² bottom-area cylindrical tanks containing artificial seawater (Bio-Sea Marine Mix™, Aquacraft, CA, USA) at a salinity of 15 ppt. Seawater depth was 30 cm for *P. pugio* and 1 cm for *U. panacea*. Animals were fed once daily with commercial shrimp pelleted feed (Zeigler Bros, Inc., Gardners, PA, USA). Water temperature was maintained at 26 ± 1 °C.

**WSSV Stock Preparation and Production**

The isolate of WSSV used in these studies was obtained from China in 1995 and has been passaged through *Litopenaeus vannamei* several times. The viral inoculum used for experiments was amplified as follows. Twenty (20) healthy specific pathogen free (SPF) *L. vannamei* (mean body weight = 17.65 ± 2.01 g) were placed in a 1-m² cylindrical tank containing 15 cm of aerated artificial seawater at a salinity of 15 ppt and a water temperature of 26 ± 1 °C and allowed to acclimate for 24 h. Shrimp were then injected intramuscularly with undiluted inoculum at a dose of 20 µl g⁻¹ of shrimp body weight using a 30 G Ultra-Fine™ insulin syringe (Becton, Dickinson & Co., NJ, USA). Moribund and freshly dead shrimp were collected and stored at – 80 °C until TaqMan real-time PCR was performed to confirm that these shrimp were infected with WSSV.
The gill and pleopod tissues of the moribund shrimp were harvested, homogenized using a tissue homogenizer, and then suspended in 0.9% saline solution at a ratio of 1:10 (w/v). This suspension was vortexed thoroughly and then centrifuged at 3000 × g for 20 minutes at 4 °C. The supernatant fluid was collected and re-centrifuged at 13,000 × g for 20 minutes at 4 °C (Escobedo-Bonilla et al. 2005). The final supernatant was filtered through a 0.45 µm filter (Whatman, GE Health Care, Buckinghamshire, UK) and then the filtrate was divided into 2-ml aliquots and stored at -80 °C. Virus-free/cell-free shrimp homogenate also was prepared from gill tissues of SPF *L. vannamei* and used as inoculum for the negative control groups. This homogenate was prepared following the protocol described above, except that the shrimp were not infected with WSSV and the final supernatant was filtered through a 0.2 µm filter (Whatman, GE Health Care, Buckinghamshire, UK). TaqMan real-time PCR was performed to quantify the viral load in the stock solution and to confirm that the shrimp homogenate was virus-free.

**WSSV Experimental Challenge Protocol**

Prior to injection with WSSV, all animals were placed in a 1-m² bottom-area cylindrical tank containing 15 ppt artificial seawater and allowed to acclimate for 48 h. Water temperature was maintained at 26 ± 1 °C. In total, 120 individuals of *P. pugio* (mean body weight = 0.30 ± 0.06 g) and 120 of *U. panacea* (mean body weight = 1.52 ± 0.34 g) were used in this experiment. Two-fold serial dilutions of WSSV inoculum were prepared from a known WSSV genome copy number stock solution: 1:2 – 1:32 dilutions *P. pugio*, and 1:4 – 1:64 dilutions for *U. panacea*, respectively. Viral inocula for *P. 
*pugio* and *U. panacea* were prepared from two viral stock solutions containing $7.25 \times 10^7$ and $4.58 \times 10^8$ WSSV genome copies μl$^{-1}$, respectively.

The experimental animals of each species was divided into five treatment groups and one negative control group, each group consisted of 20 susceptible animals. After 48 h acclimation, the susceptible animals from each treatment groups were injected with WSSV inoculum of each dilution at a dose of 20 μl g$^{-1}$ of body weight. Susceptible *P. pugio* were intramuscularly injected at the junction between the 3rd and 4th abdominal segments using 34 G Hamilton syringe (Hamilton, Reno, NV, USA), whereas susceptible *U. panacea* were injected with the inoculum using 30 G syringe (Becton, Dickinson & Co, NJ, USA) at the joint between the coxa and the base of the 4th (posterior) walking leg. For the negative control group, 20 susceptible animals were injected with virus-free and cell-free shrimp homogenate. Injected animals were placed individually in 3-l isolation tanks to avoid cross-infection between animals and observed every 4 h. Moribund and dead shrimp were recorded, collected, and stored at – 80 °C for subsequent real-time PCR analysis. At the end of the experiment, all surviving animals were collected and stored at – 80 °C.

**Genomic DNA Extraction and Quantification**

Pleopod tissue of *P. pugio* and periopod tissue of *U. panacea* were used as sources of DNA. Genomic DNA was extracted from approximately 30 – 50 mg of tissue using High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) in accordance with the company’s instructions. Total DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at – 20 °C.
**WSSV Copy Number Quantification by TaqMan Real-Time PCR**

The WSSV copy number quantification method as well as all of the primers sequences and TaqMan probe was carried out as described by Durand and Lightner (2002). The sequences of forward primer, reverse primer, and TaqMan probe were 5′–TGG TCC CGT CCT CAT CTC AG–3′ (WSS1011F), 5′–GCT GCC TTG CCG GAA ATT A–3′ (WSS1079R), and 5′–AGC CAT GAA GAA TGC CGT CTA TCA CAC A–3′ (WSSV1032-1050). These primers produced a 69 bp amplicon. The TaqMan probe was dual-labeled with fluorogenic dyes, 5–carboxyfluorescein (FAM) on the 5′ end and \(N,N,N',N'-\text{tetramethyl}-6\)-carboxyrhodamine (TAMRA) on the 3′ end.

Real-time PCR was performed using a TaqMan Universal PCR Master Mix (Life Technologies, Warrington, UK). Five µl of DNA template containing 20 – 30 ng µl\(^{-1}\) of total DNA was added to a PCR mixture consisting of 0.3 µM of each primer and 0.15 µM of TaqMan probe in a final reaction volume of 25 µl. Amplification was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 2 minute reaction for AmpErase uracil-N-glycosylase (UNG) at 50 °C, followed by the AmpliTaq Gold DNA polymerase activation for 10 minutes at 95 °C, 40 cycles of 15 seconds of denaturation at 95 °C and 60 seconds of annealing at 60 °C, and 60 seconds of extension at 60 °C. All genomic DNA samples were run in duplicate with two non-template controls (NTC) as negative controls.

The standard curve to quantify the viral copy number was prepared from a set of 10-fold serial dilutions of synthesized oligonucleotides. Standards ranged from 2.69×10^1 to 2.69×10^6 WSSV genome copies 5-µl\(^{-1}\). Synthesized oligonucleotides were 75 bp and corresponded to nucleotides 1008 – 1082 of SalI fragment of WSSV genomic sequence.
in GenBank U50923. The oligonucleotide sequence used for the standard was 5’– CAA TGG TCC CGT CCT CAT CTC AGA AGC CAT GAA GAA TGC CGT CTA TCA CAC ACT AAT TTC CGG CAA GGC AGC TCG –3’ (Invitrogen, Carlsbad, CA, USA). The absolute WSSV copy numbers in all samples were quantified by extrapolating the cycle threshold (C_T) values from the standard curve. The viral copy number was normalized per μg of total DNA.

Data Analysis

The LD_{50} was calculated using Logistic Regression of SYSTAT software version 13. Kaplan-Meier analysis was performed using SPSS version 24 to determine the median survival time of the animals after challenge with different viral doses. An independent-samples t-test was performed to test the null hypothesis that there is no difference in the mean lethal load between P. pugio and U. panacea at a significance level (α) of 0.05.

Results

Median Lethal Dose Estimates

Palaemonetes pugio was challenged with various doses of WSSV inoculum containing 7.14×10^8, 5.58×10^8, 2.58×10^8, 1.12×10^8 and 4.80×10^7 WSSV genome copies g\(^{-1}\) of P. pugio body weight (Table 5). The estimated median lethal dose (LD_{50}) for P. pugio at 5 days post injection (dpi) was 8.24×10^7 WSSV genome copies g\(^{-1}\) (CI: 4.97×10^7 – 1.17×10^8) of grass shrimp body weight or 2.45×10^7 WSSV genome copies/grass shrimp (Table 6). Uca panacea was challenged with various doses of WSSV inoculum containing 2.40×10^9, 1.09×10^9, 5.06×10^8, 2.10×10^8 and 1.01×10^8 WSSV genome copies g\(^{-1}\) of U. panacea body weight (Table 5). The estimated LD_{50} of WSSV for U. panacea
at 14 dpi was $1.67 \times 10^8$ WSSV genome copies g$^{-1}$ (CI: $7.93 \times 10^7$ – $2.59 \times 10^8$) of fiddler crab body weight or $2.55 \times 10^8$ WSSV genome copies/fiddler crab (Table 6). The results demonstrated that the estimated LD$_{50}$ of WSSV for $U. panacea$ was substantially higher than that for $P. pugio$. The association between mortality and the log dose of WSSV used for the two species is illustrated in Figure 2.

Table 5

*Logistic regression analysis summary for LD$_{50}$s of WSSV in Palaemonetes pugio and Uca panacea after challenge with various doses of WSSV inoculum.*

<table>
<thead>
<tr>
<th>Species</th>
<th>LD$_{50}$ (WSSV genome copies g$^{-1}$)</th>
<th>Log LD$_{50}$</th>
<th>95% Confidence Interval</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P. pugio$</td>
<td>$8.24 \times 10^7$</td>
<td>7.916</td>
<td>$4.97 \times 10^7$ – $1.17 \times 10^8$</td>
<td>7.697 – 8.066</td>
</tr>
<tr>
<td>$U. panacea$</td>
<td>$1.67 \times 10^8$</td>
<td>8.222</td>
<td>$7.93 \times 10^7$ – $2.59 \times 10^8$</td>
<td>7.899 – 8.413</td>
</tr>
</tbody>
</table>
Figure 2. Logistic regression of LD$_{50}$s of WSSV in *Palaemonetes pugio* (▲) and *Uca panacea* (□).
Table 6

Percent survival of *Palaemonetes pugio* and *Uca panacea* after challenge with serial dilutions of WSSV inoculum at the end of experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>Viral doses (WSSV genome copies g(^{-1}) of body weight)</th>
<th>Survival</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pugio</em></td>
<td>7.14×10(^8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5.58×10(^8)</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2.58×10(^8)</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1.12×10(^8)</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>4.80×10(^7)</td>
<td>13</td>
<td>65</td>
</tr>
<tr>
<td><em>U. panacea</em></td>
<td>2.40×10(^9)</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.09×10(^9)</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5.06×10(^8)</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2.10×10(^8)</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>1.01×10(^8)</td>
<td>12</td>
<td>60</td>
</tr>
</tbody>
</table>

Survival Curves

The experimental challenges of *P. pugio* and *U. panacea* were terminated at 15 dpi and 21 dpi, respectively. At the end of experiments, the cumulative survival of *P. pugio* after challenge with various doses of WSSV inoculum containing 7.14×10\(^8\), 5.58×10\(^8\), 2.58×10\(^8\), 1.12×10\(^8\) and 4.80×10\(^7\) WSSV genome copies g\(^{-1}\) of grass shrimp body weight were 0%, 5%, 15%, 45%, and 65%, while the cumulative survival of *U. panacea* after challenge with various doses of WSSV inoculum containing 2.40×10\(^9\), 1.09×10\(^9\), 5.06×10\(^8\), 2.10×10\(^8\) and 1.01×10\(^8\) WSSV genome copies g\(^{-1}\) of grass shrimp body weight were 0%, 5%, 15%, 45%, and 65%, respectively.
1.09×10⁹, 5.06×10⁸, 2.10×10⁸ and 1.01×10⁸ WSSV genome copies g⁻¹ of fiddler crab body weight were 5%, 10%, 20%, 50%, and 60%, respectively. The first mortality of *P. pugio* and *U. panacea* after challenge with the virus was recorded at 32 hpi and 44 hpi, correspondingly. No mortality was observed in the negative control groups of either species. All animals that died during the bioassays were heavily infected by WSSV. The gross signs observed on the two WSSV-infected species included anorexia, lethargy, and weakness. In some moribund WSSV-infected *U. panacea*, walking leg detachment was also observed. In addition, pinkish to reddish body discoloration was observed in *P. pugio*. No white spots were observed on the cuticle in either species.

The survival probabilities for both *P. pugio* and *U. panacea* were analyzed using Kaplan-Meier survival analysis. The median survival times of *P. pugio* after exposure to WSSV inoculum were 40 hours at 7.14×10⁸ WSSV genome copies g⁻¹, 44 hours at 5.58×10⁸ WSSV genome copies g⁻¹, 48 hours at 2.58×10⁸ WSSV genome copies g⁻¹, and 64 hours at 1.12×10⁸ WSSV genome copies g⁻¹. The median survival times of *U. panacea* following exposure to WSSV inoculum were 172 hours at 2.40×10⁹ WSSV genome copies g⁻¹, 188 hours at 1.09×10⁹ WSSV genome copies g⁻¹, 192 hours at 5.06×10⁸ WSSV genome copies g⁻¹, and 468 hours at 2.10×10⁸ WSSV genome copies g⁻¹. The median survival time at a dose of 4.80×10⁷ WSSV genome copies g⁻¹ for *P. pugio* and at a dose of 1.01×10⁸ WSSV genome copies g⁻¹ for *U. panacea* cannot be determined as the survival rates of the two species were higher than 50%.
Figure 3. Cumulative survival of daggerblade grass shrimp (*Palaemonetes pugio*) after challenged with various doses of WSSV inoculum.

- Negative Control, ○ $7.14 \times 10^8$ WSSV genome copies g$^{-1}$, ■ $5.58 \times 10^8$ WSSV genome copies g$^{-1}$, × $2.58 \times 10^8$ WSSV genome copies g$^{-1}$, ♦ $1.12 \times 10^8$ WSSV genome copies g$^{-1}$, ▲ $4.80 \times 10^7$ WSSV genome copies g$^{-1}$. 
Figure 4. Cumulative survival of the Gulf sand fiddler crab (*Uca panacea*) after challenged with various dilutions of WSSV inoculum.

- Negative Control, ○ $2.40 \times 10^9$ WSSV genome copies g$^{-1}$, ■ $1.09 \times 10^9$ WSSV genome copies g$^{-1}$, ▲ $5.06 \times 10^8$ WSSV genome copies g$^{-1}$, ▲ $2.10 \times 10^8$ WSSV genome copies g$^{-1}$, ▲ $1.01 \times 10^8$ WSSV genome copies g$^{-1}$. 

Cumulative survival vs Time (day post injection)
WSSV Lethal Loads

The WSSV lethal load in moribund and dead *P. pugio* and *U. panacea* was determined by using TaqMan real-time PCR. Regardless of dose used in the experiments, the mean WSSV lethal load for *P. pugio* was $9.21 \times 10^8$ (SE = $8.69 \times 10^8$) WSSV genome copies $\mu$g$^{-1}$ of total DNA, whereas the mean WSSV lethal load for *U. panacea* was $1.53 \times 10^8$ (SE = $2.12 \times 10^7$) WSSV genome copies $\mu$g$^{-1}$ of total DNA (Table 7). The log mean lethal loads in *P. pugio* and *U. panacea* were compared using an independent-samples t-test. The test detected the log mean lethal loads of the virus was statistically significantly higher in *P. pugio* than in *U. panacea* ($t_{(143)} = 2.592$, $P = 0.029$). The median lethal load in *P. pugio* was $7.10 \times 10^8$ WSSV genome copies $\mu$g$^{-1}$ of total DNA, while the median lethal load in *U. panacea* was $7.84 \times 10^7$ WSSV genome copies $\mu$g$^{-1}$ of total DNA. The majority of *P. pugio* and *U. panacea* that succumbed to WSSV infection in this study was heavily infected with the estimated lethal load ranging from $10^6$ – $10^9$ WSSV genome copies $\mu$g$^{-1}$ of total DNA (Figure 5).

The WSSV loads in surviving infected animals at the respective doses were quantified and those values are in Table 8. The mean viral load in surviving infected *P. pugio* was $1.49 \times 10^3$ WSSV genome copies $\mu$g$^{-1}$ of total DNA and ranged from $4.85 \times 10^2$ - $4.97 \times 10^3$ WSSV genome copies $\mu$g$^{-1}$ of total DNA. On the other hand, the mean viral load in surviving infected *U. panacea* was $6.61 \times 10^5$ WSSV genome copies $\mu$g$^{-1}$ of total DNA and ranged from $4.16 \times 10^2$ - $3.43 \times 10^6$ WSSV genome copies $\mu$g$^{-1}$ of total DNA (Table 8). The results indicate that the mean viral load in surviving infected *U. panacea* was higher by two logs than in surviving infected *P. pugio*. An independent-samples t-
test detected that the log mean viral load in surviving *U. panacea* was significantly higher compared to *P. pugio* ($t_{(32)} = 3.013, P = 0.005$).

*Figure 5.* Distribution of log lethal viral load in dead and moribund *Palaemonetes pugio* (□) and *Uca panacea* (■) after challenge with various doses of WSSV inoculum.
Table 7

Mean lethal viral load (WSSV genome copies $\mu$g$^{-1}$ of total DNA) in Palaemonetes pugio and Uca panacea after challenge with various doses of WSSV

<table>
<thead>
<tr>
<th>Species</th>
<th>Infectious Dose (WSSV genome copies g$^{-1}$)</th>
<th>n</th>
<th>Viral load (WSSV genome copies $\mu$g$^{-1}$ of total DNA)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pugio</td>
<td>7.14×10$^8$</td>
<td>20</td>
<td>7.57×10$^8$</td>
<td>4.14×10$^8$</td>
<td>1.98×10$^8$ – 1.75×10$^9$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.58×10$^8$</td>
<td>19</td>
<td>1.53×10$^9$</td>
<td>1.41×10$^9$</td>
<td>2.07×10$^8$ – 4.93×10$^9$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.58×10$^8$</td>
<td>17</td>
<td>5.20×10$^8$</td>
<td>4.47×10$^8$</td>
<td>9.00×10$^7$ – 1.65×10$^9$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.12×10$^8$</td>
<td>11</td>
<td>7.52×10$^8$</td>
<td>5.69×10$^8$</td>
<td>1.59×10$^8$ – 1.72×10$^9$</td>
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</tr>
<tr>
<td></td>
<td>4.80×10$^7$</td>
<td>7</td>
<td>7.59×10$^8$</td>
<td>1.23×10$^8$</td>
<td>6.13×10$^8$ – 9.67×10$^8$</td>
<td></td>
</tr>
<tr>
<td>Group Total</td>
<td></td>
<td>74</td>
<td>9.21×10$^8$</td>
<td>8.69×10$^8$</td>
<td>9.00×10$^7$ – 4.93×10$^9$</td>
<td></td>
</tr>
<tr>
<td>U. panacea</td>
<td>2.40×10$^9$</td>
<td>19</td>
<td>2.09×10$^8$</td>
<td>5.22×10$^7$</td>
<td>1.34×10$^7$ – 8.60×10$^8$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.09×10$^9$</td>
<td>18</td>
<td>1.85×10$^8$</td>
<td>5.15×10$^7$</td>
<td>1.78×10$^7$ – 7.17×10$^8$</td>
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<tr>
<td>Group Total</td>
<td></td>
<td>71</td>
<td>1.53×10$^8$</td>
<td>2.12×10$^7$</td>
<td>4.46×10$^6$ – 8.60×10$^8$</td>
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</tbody>
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Table 8

Mean viral load (WSSV genome copies µg⁻¹ of total DNA) in surviving infected Palaemonetes pugio and Uca panacea after challenge with various doses of WSSV

<table>
<thead>
<tr>
<th>Species</th>
<th>Infectious Dose (WSSV genome copies g⁻¹)</th>
<th>n</th>
<th>Viral load (WSSV genome copies µg⁻¹ of total DNA)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
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<tbody>
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<tr>
<td></td>
<td>7.14×10⁸</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>5.58×10⁸</td>
<td>1</td>
<td>5.81×10²</td>
<td>0</td>
<td>5.81×10²</td>
<td>5.81×10²</td>
</tr>
<tr>
<td>P. pugio</td>
<td>2.58×10⁸</td>
<td>3</td>
<td>2.01×10³</td>
<td>5.40×10²</td>
<td>1.08×10³</td>
<td>1.08×10³ - 2.95×10³</td>
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<tr>
<td></td>
<td>1.12×10⁸</td>
<td>9</td>
<td>1.67×10³</td>
<td>3.74×10²</td>
<td>4.85×10²</td>
<td>4.85×10² - 3.49×10³</td>
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<td></td>
<td>4.80×10⁷</td>
<td>12</td>
<td>4.31×10²</td>
<td>3.46×10²</td>
<td>6.33×10²</td>
<td>6.33×10² - 4.9×10³</td>
</tr>
<tr>
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<td>25</td>
<td>1.49×10³</td>
<td>2.19×10²</td>
<td>4.85×10³</td>
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<td>1</td>
<td>4.16×10²</td>
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<td>U. panacea</td>
<td>5.06×10⁸</td>
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<td>1.25×10⁶</td>
<td>1.22×10⁶</td>
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<td>5</td>
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<td></td>
</tr>
<tr>
<td>Group Total</td>
<td></td>
<td>9</td>
<td>6.61×10⁵</td>
<td>4.40×10⁵</td>
<td>4.16×10²</td>
<td>3.43×10⁶</td>
</tr>
</tbody>
</table>
Discussion

The primary objective of this study was to estimate the median lethal dose (LD$_{50}$) of WSSV in the daggerblade grass shrimp ($P. pugio$) and the Gulf sand fiddler crab ($U. panacea$) collected from the wild. This study uses TaqMan real-time PCR to quantify the viral concentration in each infectious dose used in the experimental bioassay to determine the LD$_{50}$ of WSSV in $P. pugio$ and $U. panacea$. Prior to the development of this method by Durand and Lightner (2002), most WSSV challenge experiments relied on standardized dilutions, e.g., Escobedo-Bonilla et al. (2005). TaqMan real-time PCR is a robust method that is fast, sensitive and specific, and has been used by many diagnostic laboratories for WSSV detection in shrimp.

My study estimated the LD$_{50}$ of WSSV in $P. pugio$ at 5 dpi to be $8.24\times10^7$ WSSV genome copies g$^{-1}$ of shrimp body weight or $2.45\times10^7$ WSSV genome copies/grass shrimp, whereas the estimated LD$_{50}$ of the virus in $U. panacea$ at 14 dpi was $1.67\times10^8$ WSSV genome copies g$^{-1}$ of crab body weight or $2.55\times10^8$ WSSV genome copies/fiddler crab. The result from these experimental bioassays revealed that the LD$_{50}$ of WSSV in $U. panacea$ was an order of magnitude higher that it was in $P. pugio$. In addition, the time required for the virus to kill 50% of the challenged $U. panacea$ was substantially longer compared to that for $P. pugio$. Various studies that estimate LD$_{50}$s of WSSV in penaeid shrimp as well as other crustaceans suggest that the LD$_{50}$s vary from one study to another (Durand and Lightner 2002; de la Calzada 2008; Laramore et al. 2009; Liu et al. 2011; Zhu and Quan 2012; Corteel et al. 2012). The differences in reported LD$_{50}$s of WSSV may be explained by differences in the exposure method, susceptibility of the host, or the isolate of virus used for the experimental bioassays. An investigation
conducted by Laramore et al. (2009), for example, showed differences in the virulence of WSSV among isolates from different geographic regions that resulted in variation of the LD$_{50}$s in *L. vannamei*.

Even though many studies have estimated the LD$_{50}$ of WSSV in some crustaceans including cultivated penaeid shrimp; only a few of these studies reported the viral quantity in the inoculum used for the experimental bioassay as in this current study (Laramore et al. 2009; Liu et al. 2011; Zhu and Quan 2012). Using TaqMan real-time PCR, Zhu and Quan (2012) assessed the viral load in the inoculum used in their study and reported an LD$_{50}$ of $1.52 \times 10^7$ WSSV genome copies g$^{-1}$ of red swamp crayfish (*Procambarus clarkii*). Another study conducted by Liu et al. (2011) reported an LD$_{50}$ of $7.34 \times 10^3$ WSSV genome copies g$^{-1}$ of mud crab (*Scylla serrata*) following exposure to serial dilutions of WSSV stock of $2.19 \times 10^7$ WSSV genome copies µl$^{-1}$, whereas the study of de la Calzada (2008) reported an LD$_{50}$ of $1.57 \times 10^4$ and $2.07 \times 10^5$ of WSSV genome copies µg$^{-1}$ of total DNA extract in *L. vannamei* and pink shrimp (*Farfantepenaeus duodarum*), respectively. Moreover, previous work of Durand and Lightner (2002) demonstrated that intramuscular injection of WSSV inoculum containing $10^4$ and $10^5$ WSSV genome copies ml$^{-1}$ resulted in 50% mortality of *L. vannamei* juveniles at 49 hpi and 52 hpi, respectively. By using viral titer to calculate WSSV load in the inoculum, Corteel et al. (2012) reported the median lethal doses of WSSV-Thailand and WSSV-Vietnam isolates in the giant freshwater prawn *Macrobrachium rosenbergii* juveniles were at $10^{5.4 \pm 0.4}$ LD$_{50}$ ml$^{-1}$ and $10^{2.3 \pm 0.3}$ LD$_{50}$ ml$^{-1}$, respectively. However, because of the differences in reporting the units and LD$_{50}$ calculations used by these investigators as well as limited information provided in their reports (i.e., average weight of animal), it is
difficult to make a comparison between the LD\textsubscript{50s} of WSSV in \textit{P. pugio} and \textit{U. panacea} obtained in this study with the LD\textsubscript{50} estimates reported in the studies above. However, a comparison can be made with the LD\textsubscript{50} estimates of WSSV in red swamp crayfish (Zhu and Quan 2012) and mud crab (Liu et al. 2011). The result from this study demonstrated that the estimated LD\textsubscript{50s} of WSSV in \textit{P. pugio} and \textit{U. panacea} were higher than the LD\textsubscript{50} of the virus in red swamp crayfish and mud crab.

TaqMan real-time PCR allowed for the quantification of WSSV lethal load in dead and moribund animals following intramuscular exposure to the virus. This study found that the WSSV lethal load in \textit{P. pugio} was higher than the lethal load in \textit{U. panacea}. One plausible explanation for the higher viral load in moribund and dead \textit{P. pugio} is that the host’s cells in target organs are more compatible with WSSV replication than in \textit{U. panacea}, which in turn results in an increased level of virus from lysed dead cells. Moreover, based on the survivorship curve, the progression of WSSV infection in \textit{P. pugio} was faster than that in \textit{U. panacea}. These findings provide evidence that WSSV-China isolate used in this study is more virulent to \textit{P. pugio} than to \textit{U. panacea} and also indicate that WSSV infection is species specific. Most of the animals that succumbed to WSSV infection contained a high viral copy number which ranged from \(10^6 – 10^9\) WSSV genome copies \(\mu g^{-1}\) of total DNA. The highest WSSV lethal load was observed in \textit{P. pugio} with a viral copy number of \(4.93 \times 10^9\) WSSV genome copies \(\mu g^{-1}\) of total DNA. Additionally, the viral load in WSSV-infected animals that survived was assessed using TaqMan real-time PCR. The viral load in these survivors ranged from \(10^2 – 10^3\) and \(10^2 – 10^6\) WSSV genome copies \(\mu g^{-1}\) of total DNA for both \textit{P. pugio} and \textit{U. panacea}, respectively. In comparison to \textit{P. pugio}, however, \textit{U. panacea} appears more
capable of tolerating a high viral load for a relatively long period without showing any signs of WSSV infection. The different responses of *P. pugio* and *U. panacea* following exposure to WSSV indicate that WSSV infection is species-specific.

In this study, the viral loads in animals that survived from WSSV experimental infection also were quantified. The result showed that all of the survivors contained a low viral load which ranged from $10^2$ – $10^6$ WSSV genome copies µg$^{-1}$ of total DNA. In comparison to the viral loads obtained in the WSSV prevalence study in previous chapter, it shows that the viral loads in survivors from this experiment are distributed within the same range as that of infected shrimp collected from the wild. This finding could suggest that the infected animals from the prevalence study contained a low viral load because the heavily infected animals might have died from the infection. Moreover, my study also found that the virus required a longer time to kill *U. panacea* than *P. pugio*. It is very likely that the replication rate of the virus in *U. panacea* to attain a sufficient killing dose was slower than in *P. pugio*.

In this study, the challenged animals were contained individually in isolation tanks following injection with various doses of WSSV. This protocol prevented inadvertent secondary infections from other infected animals, particularly through contact with virions shed into the water by other infected animals. Moreover, it can prevent viral dose magnification in challenge animals through cannibalism of moribund and dead animals (Prior et al. 2003).
CHAPTER IV - ESTIMATES OF PARAMETERS IMPORTANT FOR THE 
DYNAMICS OF WHITE SPOT SYNDROME VIRUS IN POPULATIONS 
OF THE DAGGERBLADE GRASS SHRIMP (PALAEMONETES PUGIO) 
AND THE GULF SAND FIDDLER CRAB (UCA PANACEA) 

Introduction 
Viral infectious diseases have been a major obstacle in the development of the shrimp aquaculture industry in many countries across the globe. White Spot Disease (WSD), a disease associated with White spot syndrome virus (WSSV), has negatively affected the sustainable growth of this industry by reducing profits and production over the last several decades. Since its first emergence in Asia in 1992, WSD has caused shrimp production losses worth about US$ 15 billion (Zhan et al. 1998; Lightner et al. 2012). WSSV infects almost all cultivated penaeid shrimp species and is highly pathogenic causing total mortality in farm-raised shrimp ponds within 3 to 10 days (Lightner 1996). The virus is a double stranded DNA virus with ovoid to bacilliform morphology assigned to the genus Whis povirus as the only member of the family Nimaviridae (Mayo 2002). Due to its economic importance, the virus has been listed as an OIE notifiable crustacean pathogen (OIE 2016).

WSSV is notoriously euryxenous, having a wide host range, including all cultivated penaeid shrimp species, planktonic copepods, crabs, lobsters, crayfish, freshwater crabs, prawns, and rotifers (Lo et al. 1996; Chen et al. 2000; Sahul-Hameed et al. 2003; Zhang et al. 2006; Waikhom et al. 2006; Liu et al. 2007; Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010). Lo et al. (1996) reported that some wild decapods commonly found in the vicinity of shrimp farms likely serve as asymptomatic carriers of WSSV.
The introduction of WSSV into wild decapod populations is believed to have occurred through the following pathways: 1) importation of shrimp seed and broodstock with latent infection, 2) escapes of WSSV-infected penaeid shrimp from aquaculture ponds, 3) discharge of improperly treated solids and liquid waste from shrimp processing plants, restaurants, and shrimp ponds experiencing WSSV outbreaks, 4) contamination from WSSV-infected shrimp used for bait, and 5) contamination from infected fouling organisms and/or ship ballast water (Lightner et al. 2002; Flegel and Fegan 2002; Hasson et al. 2006; de la Peña et al. 2007; Martorelli et al. 2012).

Pathogens use numerous modes of transmission to spread from infected to susceptible hosts. In the case of WSSV, its transmission can occur three ways: 1) ingestion of an infected host cadaver (horizontal), 2) cohabitation with infected hosts (horizontal), and 3) transfer from broodstock to offspring (vertical) (Corteel et al. 2009). Factors associated with the host (e.g., species and age) as well as factors associated with the virus (e.g., strains and virulence) affect transmission of the virus (Wang et al. 1998; Lightner et al. 1998; Soto et al. 2001).

Several studies have reported differences in virulence among WSSV isolates (Wang et al. 1999; Marks et al. 2005; Rahman et al. 2008; Laramore et al. 2009; Pradeep et al. 2009). Marks et al. (2005) reported that the virulence of WSSV is inversely related to the virus’s genome size. Isolates with smallest genomes were the most virulent. A similar result was reported by Pradeep et al. (2009). In other studies, the difference in virulence among WSSV isolates was associated with the number of repeat units (RU) in ORF94 in which isolates with fewer than 9 RU were more virulent (Wongteerasupaya et al. 2003; Musthaq et al. 2006; Pradeep et al. 2008). Rahman et al. (2008) reported that
the increased virulence of WSSV-Thailand compared to WSSV-Vietnam in juvenile *L. vannamei* was associated with the degree of the virus replication in gills. Additionally, Waikhom et al. (2006) demonstrated that passaging WSSV isolates through different hosts, such as *Macrobrachium rosenbergii* and *Portunus pelagicus*, before exposure to *Penaeus monodon* reduced the virulence and significantly decreased shrimp mortality.

While the dynamics of WSSV in many economically important penaeid shrimp species are documented, similar information for its dynamics in wild decapod populations is lacking. Understanding the dynamics of infectious agents in wild decapod populations is crucial to advancing our knowledge of emerging pathogens that threaten aquaculture (Stentiford et al. 2012) and may provide insights for control and eradication of WSSV from aquaculture as well as from wild populations.

The main objectives of this research was to estimate and to compare epidemiologically important parameters for WSSV dynamics within-species and between-species of the daggerblade grass shrimp (*P. pugio*) and the Gulf sand fiddler crab (*U. panacea*). In particular, I estimate transmission by cohabitation (\( \beta \)), transmission by ingestion (\( \chi \)), virulence (\( \alpha \)) and decomposition (\( \delta \)) coefficients of WSSV. The coefficient \( \beta \) is the probability that a susceptible host becomes infected after contact with an infected living host, whereas the coefficient \( \chi \) is the probability that a susceptible host becomes infected after contact with an infected host carcass. The coefficient \( \alpha \) is the pathogen-induced mortality or the virulence coefficient (probability that an infected individual dies in a unit of time), and the coefficient \( \delta \) is the likelihood that an infected host carcass loses its infectivity due to decomposition or consumption by scavengers.
Materials and Methods

Experimental Animals

The daggerblade grass shrimp (*P. pugio*) and the Gulf sand fiddler crab (*U. panacea*) used in this study were collected from the tidal salt marshes along the coast of Mississippi Sound in the northern Gulf of Mexico. Specimens of *P. pugio* were collected from Davis Bayou (n = 1884), Ocean Springs, Mississippi (30°23'31" N, 88°47'54" W) by dip-netting during the period of November 2013 to October 2015. Specimens of *U. panacea* were hand-collected from Marsh Point (n = 613), Ocean Springs, Mississippi (30°22'58" N, 88°48'20" W) and from Deer Island (n = 667), Harrison County, Mississippi (30°22'08" N, 88°49'30" W) during September 2014 through August 2015. All animals were screened for WSSV using TaqMan real-time PCR. WSSV-negative animals were maintained in 1-m² bottom-area cylindrical tanks filled with artificial seawater (Bio-Sea Marine Mix™, Aquacraft, CA, USA) at a salinity of 15 ppt (30-cm depth for *P. pugio* and 1-cm depth for *U. panacea*) and a temperature of 26 ± 1 °C until used for studies. Animals were fed once daily with commercial shrimp pelleted feed (Zeigler Bros, Inc., Gardners, PA, USA).

WSSV Stock Preparation

The WSSV isolate used in this study came from China in the mid-1990s and has been passaged through SPF *L. vannamei* several times at the Gulf Coast Research Laboratory. The viral isolate was amplified following a method developed by Escobedo-Bonilla et al. (2005) with slight modifications as described in Chapter III. The WSSV load used in this experiment was quantified using TaqMan real-time PCR.
Preparation of Infected Animals

To prepare the initial infected hosts \( (I_0, \text{infected at time } = 0) \) for transmission, I exposed 20 animals of each species, \( P. pugio \) (mean body weight = 0.37 ± 0.06 g) and \( U. panacea \) (mean body weight = 1.19 ± 0.21 g), with a WSSV dose of \( 1.45 \times 10^9 \) WSSV genome copies \( g^{-1} \) of animal body weight. After injection, all animals were placed individually in 3-l isolation tanks containing 15 ppt artificial seawater at a depth of 5-cm for \( P. pugio \) and 1-cm for \( U. panacea \). Freshly dead and moribund animals were collected and stored at –80°C and used as the source of infection for the transmission by ingestion estimate. Six (6)-hour-old and 3-day-old infections of \( P. pugio \) and \( U. panacea \), respectively, were used as the source of infection for transmission by cohabitation estimates. Viral load in each \( I_0 \) was quantified using TaqMan real-time PCR.

Experiments 1 and 2 - Estimation of Transmission Coefficients (\( \beta \) and \( \chi \)) within-Species and between-Species

For experiment 1, the within-species transmission coefficient estimation (e.g., from \( P. pugio \) to \( P. pugio \) and from \( U. panacea \) to \( U. panacea \)), was performed by exposing susceptible hosts to an infected living host or a freshly dead infected carcass of the same species. Briefly, 30 susceptible hosts of each species were placed in 1-m\(^2\) bottom-area cylindrical tank containing 15-cm depth of artificial seawater for \( P. pugio \) and 1-cm depth for \( U. panacea \) at a salinity of 15 ppt. Subsequently, they were exposed to one living infected host or one infected carcass of their respective species for 24 h. This experiment was performed in five replicates with one negative control. At 24 h, the initial infected host \( (I_0) \) was removed from the tank, weighed and stored at –80 °C. All
exposed susceptible animals were transferred individually to 3-l isolation tanks and observed every 4 h for 10 d. Moribund and freshly dead animals were collected, time of death was recorded and the carcasses were stored at –80 °C. At the end of the experiment, all surviving animals were collected and stored. TaqMan real-time PCR was performed to validate the status of infection.

For experiment 2, the between-species transmission coefficient (e.g., from *P. pugio* to *U. panacea* and vice versa) was estimated by exposing susceptible hosts to one infected living individual or one infected carcass of the other species as described above. Each estimate of transmission was conducted in five replicates with one negative control tank. At the end of the experiment, all animals were killed and stored at –80°C. TaqMan real-time PCR was performed to determine the infection status of exposed animals.

The transmission coefficient from infected hosts to susceptible hosts (β and χ) is estimated using a formula suggested by Soto and Lotz (2001) as follows,

$$\beta, \chi = 1 - \exp \left( \frac{\ln \left( \frac{S_t+1}{S_t} \right)}{I_t} \right),$$

(1)

where $S_t$ is the initial number of susceptible, $I_t$ is the initial number of living infected host, and $S_{t+1}$ is the number of susceptible animals remaining at the end of the time of interest. When $I_t = 1$, as in my case, β or χ, reduces to the proportion of susceptible animals that become infected.

**Experiment 3: Estimation of Decomposition Coefficient (δ)**

The decomposition rate, δ, was estimated by exposing susceptible hosts to infected carcasses aged for 0, 24, and 48 h prior to exposure. Thirty susceptible
individuals of each species, *P. pugio* and *U. panacea*, were injected intramuscularly with a viral dose of $1.51 \times 10^9$ WSSV genome copies g$^{-1}$ of host body weight and then placed individually in 3-l isolation tanks. Freshly dead animals along with their tissue sample were collected and stored at $-80^\circ$C until used as a source of infection. For negative control groups, ten susceptible individuals of each species were injected with cell-free and virus-free shrimp homogenate. The viral load in each infected host was quantified using TaqMan real-time PCR. Prior to exposures to susceptible animals, infected carcasses of the respective species were aged in 3-l isolation tanks containing 5 cm of aerated artificial seawater at a salinity of 15 ppt and a room temperature of $26 \pm 1^\circ$C. After 48 h acclimation, thirty susceptible individuals were exposed to one infected carcass that had been aged a particular length of time as described above in a 1-m$^2$ bottom-area cylindrical tank containing 15 ppt aerated artificial seawater. The seawater depth was 15 cm for *P. pugio* and 1 cm for *U. panacea*. The room temperature was maintained at $26 \pm 1^\circ$C. Each decomposition time was replicated five times with one negative control group. After 24 h of exposure, all animals were transferred individually to 3-l isolation tanks and observed every 4 h for 10 d. Moribund and dead animals were collected and stored at $-80^\circ$C. At the end of the experiment, all animals were collected and tested for WSSV using TaqMan real-time PCR.

*Estimation of Virulence Coefficient ($\alpha$)*

The probability that an infected host will die from the infection during one time-unit is the pathogen-induced mortality (virulence) and is represented by the symbol $\alpha$. An estimate of $\alpha$, can be obtained by fitting an exponential decay curve, $y = e^{-\omega t}$, to the observed survival data of infected animals. The probability of survival during one time-
unit \((t = 1)\) is \(e^{-\omega}\). Therefore, the probability of dying in one time-unit is \(1 - e^{-\omega}\) or \(\alpha\). The curve fitting was performed using the statistical software package SYSTAT version 13.

**Total DNA Extraction and Quantification**

Pleopod tissue (grass shrimp) and periopod tissue (fiddler crabs) was used as the source of DNA. Total genomic DNA was extracted from 30 – 50 mg of tissue using the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) in accordance with the company’s protocol. Total DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at –20 °C until used for subsequent TaqMan real-time PCR.

**Diagnosis of Infection Status by TaqMan Real-Time PCR**

TaqMan real-time PCR was performed to determine the remaining number of uninfected hosts after exposure to a WSSV-infected host. The diagnostic method, primers, and TaqMan probe, was carried out as described in Durand and Lightner (2002). The sequences of the TaqMan probe and the forward primer and reverse primers were: 5′-AGC CAT GAA GAA TGC CGT CTA TCA CAC A-3′ (WSSV1032-1050), 5′-TGG TCC CGT CCT CAT CTC AG-3′ (WSS1011F), and 5′-GCT GCC TTG CCG GAA ATT A-3′ (WSS1079R). The sequences of the primers were obtained from the WSSV genomic sequence in GenBank U50923; the product of these primers was 69 bp. A fluorogenic TaqMan probe was labeled with 5-carboxyfluorescein (FAM) on the 5′ end and \(N,N,N',N'\)-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end.

Real-time PCR was performed using a TaqMan Universal PCR Master Mix (Life Technologies, Warrington, UK). Five µl of DNA template containing 20 – 30 ng µl⁻¹ of
total DNA extract was added to the PCR mixture consisting of 0.3 μM of each primer and 0.15 μM TaqMan probe. The final reaction volume was 25 μl. Amplification was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: 2 minutes reaction time for AmpErase uracil-N-glycosylase (UNG) at 50 °C, followed by AmpliTaq Gold DNA polymerase activation for 10 minutes at 95 °C, 40 cycles of 15 seconds denaturation at 95 °C and 60 seconds annealing at 60°C, and finally 60 seconds extension at 60 °C.

Standards were prepared from 75 bp oligonucleotides corresponding to nucleotides 1008 – 1082 of the SalI fragment of WSSV genomic sequence. A set of 10-fold serial dilutions ranging from 2.69×10¹ to 2.69×10⁶ WSSV genome copies 5-μl⁻¹ was used to prepare the standard curve. The oligonucleotide used for the standard was 5’-CAA TGG TCC CGT CCT CAT CTC AGA AGC CAT GAA GAA TGC CGT CTA TCA CAC ACT AAT TTC CGG CAA GGC AGC TCG–3’ (Invitrogen, Carlsbad, CA, USA). The absolute WSSV genome copy number in all samples was quantified by extrapolating the cycle threshold (Cₜ) values from the standard curve. The viral copy number was normalized per μg of total DNA.

Data Analysis

Curve fitting was performed using SYSTAT 13 to estimate the virulence coefficient. Statistical analyses were carried out using the statistical software package SPSS version 24. Prior to statistical analyses, the coefficient data obtained from this study were subjected to an arcsine-root transformation. The null hypotheses of this study were: 1) there is no significant different between transmission coefficients within-species or between-species, 2) there is no significant difference between the mean viral load in
the initial infected host and 3) no significant different between the mean viral load in the initial infected living host at 0 h and 24 h of exposure time. An independent-samples t-test compared the mean coefficients as well as the mean log viral load in the initial infected host ($I_0$) obtained from the experimental transmission estimation bioassay. In addition, a paired-samples t-test compared the mean log load in the infected living hosts at 0 h and 24 h of exposure time. The significance level ($\alpha$) used to accept or reject the null hypothesis was 0.05.

Results

Experiment 1: Within Species Transmission Coefficient Estimates

The cohabitation transmission coefficient ($\beta$) estimates for $P. pugio$ and $U. panacea$ after exposure to an infected host of the same species were 0.03 and 0, respectively; whereas the ingestion transmission coefficient estimates ($\chi$) were 0.08 for $P. pugio$ and 0.00 for $U. panacea$ (Table 9). The cohabitation transmission coefficient and ingestion transmission coefficient estimates for $P. pugio$ ranged from 0.00 to 0.17 for $\beta$ and from 0.03 to 0.20 for $\chi$. In this experiment, none of the susceptible $U. panacea$ were positive after exposure to an infected host of the same species either by cohabitation or ingestion transmission. An independent-samples t-test detected no significant differences between the ingestion and cohabitation transmission coefficient estimates of $P. pugio$; and the null hypothesis failed to be rejected ($t_{(8)} = 1.734, P = 0.126$). In comparison, the mean value of transmission by ingestion was not significantly higher than the mean value of transmission by cohabitation.
Table 9

Summary of the within-species cohabitation transmission (β) and ingestion transmission (χ) coefficient estimates of WSSV

<table>
<thead>
<tr>
<th>Host</th>
<th>β estimate (range)</th>
<th>χ estimate (range)</th>
</tr>
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<tbody>
<tr>
<td>P. pugio</td>
<td>0.03 (0.00 – 0.17)</td>
<td>0.08 (0.03 – 0.20)</td>
</tr>
<tr>
<td>U. panacea</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Mortality of P. pugio occurred between 36 and 48 h post-exposure to infected carcass. The proportion dying was 0.02. No animals died in the negative control groups nor were any WSSV-positive during the experimental bioassays. For transmission by cohabitation of P. pugio, 5 animals were WSSV-positive with an estimated viral load of 4.13×10^2 - 1.40×10^3 WSSV genome copies µg^-1 of total DNA. On the other hand, 12 P. pugio were WSSV-positive in the transmission by ingestion experiment with an estimated viral load of 5.05×10^2 - 4.00×10^8 WSSV genome copies µg^-1 of total DNA. The summary of WSSV loads in infected susceptible hosts exposed to infected living individuals and infected carcasses of the same species are presented in Table 11.

Experiment 2: Between-Species Transmission Coefficient Estimates

The cohabitation transmission coefficient estimates (β) from an infected living U. panacea to susceptible P. pugio was 0.02, while the ingestion transmission coefficient from an infected carcass of U. panacea to susceptible P. pugio was 0.03 (Table 10). Both β and χ estimates from an infected U. panacea to susceptible P. pugio were from 0.00 to 0.03 and from 0.00 to 0.07, respectively. In this experiment; however, I found no transmission from an infected P. pugio to a susceptible U. panacea, either by
cohabitation or ingestion; thus, the $\beta$ and $\chi$ estimates from an infected *P. pugio* to susceptible *U. panacea* were 0.00. There was no significant difference between the mean between-species cohabitation transmission and ingestion transmission coefficient estimates from an infected *U. panacea* to a susceptible *P. pugio*. Consequently, the null hypothesis failed to be rejected ($t_{(8)} = 0.036$, $P = 0.972$).

**Table 10**

*Summary of the between-species cohabitation transmission ($\beta$) and ingestion transmission ($\chi$) estimates*

<table>
<thead>
<tr>
<th>Infected host</th>
<th>Susceptible host</th>
<th>$\beta$ estimate (range)</th>
<th>$\chi$ estimate (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>U. panacea</em></td>
<td><em>P. pugio</em></td>
<td>0.02 (0.00 – 0.03)</td>
<td>0.03 (0.00 – 0.07)</td>
</tr>
<tr>
<td><em>P. pugio</em></td>
<td><em>U. panacea</em></td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Similar to experiment 1, mortalities only occurred in *P. pugio* after exposure to infected carcasses of *U. panacea* (between 36 and 48 h post exposure). The proportion dying was 0.03. No *U. panacea* died following exposure to an infected living or carcass of *P. pugio*. No animals died in the negative control groups nor were any found to be WSSV positive. For cohabitation transmission of WSSV from infected living *U. panacea* to susceptible *P. pugio*, 3 animals were positive for WSSV. The estimated viral load was $2.73 \times 10^2$ - $2.71 \times 10^7$ WSSV genome copies $\mu g^{-1}$ of total DNA with a mean of $9.05 \times 10^6$ (SE = $9.05 \times 10^6$) WSSV genome copies $\mu g^{-1}$ of total DNA.

Four susceptible *P. pugio* were positive following exposure to infected carcasses of *U. panacea*. They carried viral loads from $1.11 \times 10^8$ - $3.56 \times 10^8$ WSSV genome copies $\mu g^{-1}$ of total DNA with a mean of $1.97 \times 10^8$ (SE = $5.63 \times 10^8$) WSSV genome copies $\mu g^{-1}$.
of total DNA. The summary of WSSV loads in newly infected susceptible hosts post exposure to infected living and infected carcasses of the same species are displayed in Table 11.
Table 11

Mean viral load (WSSV genome copies µg⁻¹ of total DNA) in infected Palaemonetes pugio and Uca panacea after exposure to infected live individuals or carcasses in experiments 1 and 2

<table>
<thead>
<tr>
<th>Mode of transmission</th>
<th>Experiment</th>
<th>I₀</th>
<th>S₀</th>
<th>Number infected</th>
<th>Viral load (WSSV genome copies µg⁻¹ of total DNA)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohabitation</td>
<td>1</td>
<td>P. pugio</td>
<td>P. pugio</td>
<td>5</td>
<td>6.65 × 10²</td>
<td>1.88 × 10²</td>
<td>4.13 × 10² - 1.40 × 10³</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>U. panacea</td>
<td>U. panacea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>P. pugio</td>
<td>U. panacea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>U. panacea</td>
<td>P. pugio</td>
<td>3</td>
<td>9.05×10⁶</td>
<td>9.05×10⁶</td>
<td>2.73×10² - 2.71×10⁷</td>
<td></td>
</tr>
<tr>
<td>Ingestion</td>
<td>1</td>
<td>P. pugio</td>
<td>P. pugio</td>
<td>12</td>
<td>3.63×10⁷</td>
<td>3.31×10⁷</td>
<td>5.05×10² - 4.00×10⁸</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>U. panacea</td>
<td>U. panacea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>P. pugio</td>
<td>U. panacea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>U. panacea</td>
<td>P. pugio</td>
<td>4</td>
<td>1.97×10⁸</td>
<td>5.63×10⁷</td>
<td>1.11×10⁸ - 3.56×10⁸</td>
<td></td>
</tr>
</tbody>
</table>
Comparison between Transmission Coefficients in Experiments 1 and 2

For *P. pugio*, an independent-samples t-test detected no significant difference between transmission by cohabitation within-species and between-species ($t_{(8)} = 0.214, P = 0.838$). Additionally, there was no difference detected between transmission by ingestion coefficients within-species and between-species coefficients ($t_{(8)} = 1.761, P = 0.116$). In the case of *U. panacea*, both transmission by cohabitation and transmission by ingestion coefficients were 0.00 from all experiments as no susceptible *U. panacea* became infected after exposure to infected hosts.

Experiment 3: Decomposition ($\delta$) Coefficient Estimates

The decomposition coefficient estimates were 1.0 for both *P. pugio* and *U. panacea* (Table 12). The ingestion transmission coefficient ($\chi$) for *P. pugio* was 0.14 following exposure to fresh (0-h decomposed) infected *P. pugio* carcasses (Table 13). In contrast, no *U. panacea* became infected after exposure to infected carcasses at any decomposition time. Moreover, no individuals of either species became infected after exposure to 24 h or 48 h decomposed infected carcasses. This finding indicates that the carcasses lose infectiousness over time due to decomposition. However, the virus was still detected in these carcasses even after decomposition for 48 h (Table 14). No mortalities were recorded in the negative control groups nor were any diagnosed with WSSV following exposure to decomposed carcasses.
Virulence (α) Estimates

To generate the observed cumulative survivorship curve, survival data from experiments 1 and 2 for the two species were pooled (Figure 6). The estimated virulence coefficient of WSSV for *P. pugio* was 0.014. In contrast, the virulence coefficient estimate for *U. panacea* was 0.00 since no mortality occurred following exposure to either infected living hosts or infected carcasses (Table 12). Moreover, 20 survivors of *P. pugio* were found to be infected, whereas none of the *U. panacea* survivors were WSSV positive. For *P. pugio*, the overall mortality was 2.3% of which only 7 grass shrimp died after infection. The first mortality was recorded at 36 h post exposure, and mortality stopped at day 3. No mortalities occurred during the remainder of the experiment. The only deaths of *P. pugio* were after exposure to carcasses. Additionally, neither mortality nor a positive WSSV test by TaqMan real-time PCR was recorded in the any of the negative control groups or individuals.

Table 12

Summary of the virulence (α) and the decomposition (δ) coefficient estimates of WSSV for *Palaemonetes pugio* and *Uca panacea*

<table>
<thead>
<tr>
<th>Host</th>
<th>α estimate</th>
<th>δ estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pugio</em></td>
<td>0.014</td>
<td>1.00</td>
</tr>
<tr>
<td><em>U. panacea</em></td>
<td>0.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Figure 6. Observed (▲) and best fit (—) cumulative survival curve of *Palaemonetes pugio* following exposure to WSSV infected host. Data were combined from experiments 1 and 2.

Table 13

*Summary of ingestion transmission coefficient ($\chi$) estimates of WSSV in Palaemonetes pugio and Uca panacea fed decomposed infected carcasses*

<table>
<thead>
<tr>
<th>Species</th>
<th>Ingestion transmission coefficient estimates ($\chi$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td><em>P. pugio</em></td>
<td>0.14 (0 – 0.7)</td>
</tr>
<tr>
<td><em>U. panacea</em></td>
<td>0.00</td>
</tr>
</tbody>
</table>
**Viral Loads in I₀ Used for Transmission of WSSV within-Species and between-Species Experiments**

The WSSV load in each infected host used as a source of infection (I₀) for cohabitation and ingestion within- and between-species experiments are shown in Table 15 and Table 16. The infected living hosts used in these experiments had infections that were 6 hours old and 3 days old for *P. pugio* and *U. panacea*, respectively. The overall mean viral loads in living infected *P. pugio* and *U. panacea* used for transmission by cohabitation experiments within- and between-species at 0 h of exposure time were 1.22×10⁴ (SE = 3.44×10³) and 3.48×10⁶ (SE = 1.53×10⁶) WSSV genome copies µg⁻¹ of total DNA, respectively. At 24 h of exposure time, viral loads in living infected *P. pugio* and *U. panacea* increased to 4.07×10⁷ (SE = 1.49×10⁷) and 5.83×10⁷ (SE = 2.57×10⁷) WSSV genome copies µg⁻¹ of total DNA, respectively. A paired-samples t-test showed a significant difference between the mean log load at 0 h and 24 h for *P. pugio* ($t_{(9)} = 15.244, P < 0.001$) and *U. panacea* ($t_{(9)} = 4.849, P = 0.001$).

The mean viral loads in infected living hosts increased by approximately 3 logs for *P. pugio* and 1 log for *U. panacea* during the 24 h of exposure time. This finding indicates that the virus replicates faster in *P. pugio* than in *U. panacea*. Moreover, an independent-samples t-test indicated no significant difference between the mean log viral loads in infected living hosts of *P. pugio* used for within-species and between-species experiments ($t_{(8)} = 0.040, P = 0.969$). In contrast, the t-test detected a significant difference in the mean log viral loads between infected living hosts of *U. panacea* used for within-species and between-species experiments ($t_{(8)} = 3.431, P = 0.009$).
The overall mean viral loads of infected host carcasses used for transmission by ingestion within-species and between-species for *P. pugio* and *U. panacea* were 9.03×10⁸ (SE = 2.42×10⁸) and 1.20×10⁸ (SE = 2.05×10⁷) WSSV genome copies µg⁻¹ of total DNA, respectively. An independent-samples t-test showed that the mean log viral load of *P. pugio* infected carcasses used for transmission by ingestion between-species experiment was statistically significantly higher than the mean log viral load of *P. pugio* infected carcasses used for transmission by ingestion within-species experiment (*t*(8) = 2.595, *P* = 0.032). Additionally, there was no difference between the mean log viral load of *U. panacea* infected carcasses used for transmission by ingestion within-species and between-species experiments (*t*(8) = 1.514, *P* = 0.169).

Table 14

*Summary of mean viral loads (WSSV genome copies µg⁻¹ of total DNA) in decomposed infected carcasses of Palaemonetes pugio and Uca panacea used in Experiment 3*

<table>
<thead>
<tr>
<th>Species</th>
<th>Decomposition time (h)</th>
<th>Viral load (WSSV genome copies µg⁻¹ of total DNA)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pugio</em></td>
<td>0</td>
<td>3.32×10⁹</td>
<td>4.66×10⁸</td>
<td>2.40×10⁹ - 3.91×10⁹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.04×10⁹</td>
<td>4.83×10⁷</td>
<td>9.50×10⁸ - 1.12×10⁹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>7.48×10⁸</td>
<td>2.64×10⁸</td>
<td>2.22×10⁸ - 1.05×10⁹</td>
<td></td>
</tr>
<tr>
<td><em>U. panacea</em></td>
<td>0</td>
<td>8.45×10⁷</td>
<td>6.04×10⁶</td>
<td>7.29×10⁷ - 9.32×10⁷</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>8.42×10⁷</td>
<td>1.60×10⁷</td>
<td>5.80×10⁷ - 1.13×10⁸</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3.76×10⁷</td>
<td>6.44×10⁶</td>
<td>3.06×10⁷ - 5.04×10⁷</td>
<td></td>
</tr>
</tbody>
</table>
Table 15

Summary of WSSV loads (WSSV genome copies µg\(^{-1}\) of total DNA) in infected living hosts used in cohabitation transmission of *Palaemonetes pugio* and *Uca panacea*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>I(_0)</th>
<th>S(_0)</th>
<th>Time (h)</th>
<th>Viral load (WSSV genome copies µg(^{-1}) of total DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>P. pugio</td>
<td>P. pugio</td>
<td>0</td>
<td>1.00×10(^4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>6.54×10(^7)</td>
</tr>
<tr>
<td></td>
<td>U. panacea</td>
<td>U. panacea</td>
<td>0</td>
<td>7.56×10(^5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>3.20×10(^7)</td>
</tr>
<tr>
<td>2</td>
<td>P. pugio</td>
<td>U. panacea</td>
<td>0</td>
<td>1.44×10(^4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>1.54×10(^7)</td>
</tr>
<tr>
<td></td>
<td>U. panacea</td>
<td>P. pugio</td>
<td>0</td>
<td>6.20×10(^6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>8.45×10(^7)</td>
</tr>
</tbody>
</table>
Table 16

Summary of WSSV load (WSSV genome copies µg⁻¹ of total DNA) in infected host carcasses used in ingestion transmission of *Palaemonetes pugio* and *Uca panacea*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>I₀</th>
<th>S₀</th>
<th>Viral load (WSSV genome copies µg⁻¹ of total DNA)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>P. pugio</em></td>
<td><em>P. pugio</em></td>
<td>1.42×10⁹</td>
<td>3.36×10⁸</td>
<td>2.71×10⁸ - 2.30×10⁹</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>U. panacea</em></td>
<td><em>U. panacea</em></td>
<td>1.47×10⁸</td>
<td>3.15×10⁷</td>
<td>7.28×10⁷ - 2.51×10⁸</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>P. pugio</em></td>
<td><em>U. panacea</em></td>
<td>3.86×10⁸</td>
<td>1.23×10⁷</td>
<td>1.14×10⁸ - 8.42×10⁸</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>U. panacea</em></td>
<td><em>P. pugio</em></td>
<td>9.21×10⁷</td>
<td>2.28×10⁷</td>
<td>4.56×10⁷ - 1.62×10⁸</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

In the present study, I compared the transmission by cohabitation (\(\beta\)) and transmission by ingestion (\(\chi\)) within- and between-species, virulence (\(\alpha\)), and decomposition (\(\delta\)) coefficients of the daggerblade grass shrimp \(P. pugio\) and the Gulf sand fiddler crab \(U. panacea\) following exposure to WSSV-infected individuals. Two modes of transmission, cohabitation and ingestion, were used to study WSSV transmission within-species and between-species of \(P. pugio\) and \(U. panacea\). Transmission by cohabitation of WSSV was performed by exposing susceptible animals with an infected living host, whereas the transmission by ingestion was performed by exposing susceptible animals with an infected host carcass.

The experimental challenge protocol of WSSV used in this study assumed that the susceptible host population of a fixed size was homogenously mixed. It was assumed that each susceptible individual had the same probability of making contact with the infected host during the 24 h of exposure time. In Experiment 1, I found that transmission by ingestion was greater than transmission by cohabitation in \(P. pugio\). However, there was no significant difference between transmission by cohabitation and transmission by ingestion in \(P. pugio\). A similar result was obtained from Experiment 2, in which WSSV transmission was detected only in \(P. pugio\) after exposure to infected living and infected carcasses of \(U. panacea\). The results from Experiment 1 and 2 showed that both transmission by cohabitation and transmission by ingestion coefficients in \(P. pugio\) are low and lie within a relatively narrow range. In comparison to transmission through contact with an infected living host that is actively shedding the virus, transmission through ingestion of infected host carcasses is the more effective way.
to transmit WSSV to individuals of *P. pugio*. The results obtained from this study agree with the results reported by Soto et al. (2001) and Soto and Lotz (2001) that WSSV transmission by ingestion of infected carcasses is over an order of magnitude higher than transmission by cohabitation. Interestingly, none of the susceptible *U. panacea* (from Experiment 1 and 2) were found to be WSSV-positive following exposure to the infected host, regardless of the mode of transmission used in this study. Moreover, my study also demonstrated that the two species of wild decapods showed a different response to the virus; *P. pugio* seemed to be more susceptible to WSSV infection than *U. panacea*. 

Quantification of viral load in the initial living infected host at 0 h and 24 h post exposure also revealed that the replication rate of WSSV in *P. pugio* was faster by 2-log than it was in *U. panacea*. The difference in susceptibility to WSSV infection may be due to several possible factors: 1) differences in feeding behavior between the two species where *P. pugio* is more aggressive than *U. panacea*, 2) differences in cell receptors that bind WSSV envelope proteins where *P. pugio* has either more receptors or receptors with higher affinity, 3) differences in intracellular machinery that support viral replication, or 4) differences in immune recognition and response to WSSV. Additionally, using the same WSSV isolate used in this study, Soto et al. (2000) and Soto and Lotz (2001) obtained higher WSSV transmission by ingestion rates in *L. vannamei* and *L. setiferus*. It is likely that WSSV susceptibility varies among decapods and is species-specific.

In Experiment 3, susceptible hosts were exposed to infected host carcasses that had been aged for 0, 24, and 48 hours to investigate the effect of carcass decomposition on infectivity of WSSV in the two species. The overall estimated decomposition coefficients (δ) of WSSV for both *P. pugio* and *U. panacea* are 1.0 because none were
infectious after 24 h. However, infection was observed when *P. pugio* were exposed to infected carcasses that had been decomposed for 0 h. This result implies that infected host carcasses lose infectiousness rapidly due to decomposition and contrasts with unpublished data from our lab that found decomposition in *L. vannamei* to be much lower (0.48 d⁻¹). It is very likely that the size of host, particularly host’s body weight, affects the decomposition rate and viral dose of infected host carcass.

The quantification of viral load in the initial infected host carcasses showed that the loads were high (10⁸ – 10⁹ WSSV genome copies µg⁻¹ of total DNA for *P. pugio* and 10⁷ WSSV genome copies µg⁻¹ of total DNA for *U. panacea*) even after the carcasses have been decomposed for 48 h. This finding provides more evidence that the infectivity of WSSV in decomposed host carcasses will be reduced due to decomposition. In addition, although my study was able to quantify the WSSV load in each decomposed host carcass using TaqMan real-time PCR, this technique cannot identify whether the virus in infected carcasses is still infectious.

My study found that the overall virulence rate (pathogen-induced mortality) of WSSV in the daggerblade grass shrimp (*P. pugio*) was very low (α₁ = 0.014), whereas it was zero for the Gulf sand fiddler crab (*U. panacea*) following exposure to a WSSV-infected host (α₂ = 0.00). These results indicate that WSSV infection in wild populations of *P. pugio* and *U. panacea* may not play a significant role in the survival of the two species in the natural environment. However, based on results obtained from the experimental transmission and prevalence study in Chapter II, WSSV can cause a latent infection in the two species. Despite the fact that the virus produced only a very low mortality in *P. pugio* and no mortality in *U. panacea*, the experimental WSSV infections
as described in Chapter III showed a different result. The virus could kill the two species following intramuscular injection of the virus; however, the injection method is not a natural way for WSSV transmission in the wild. The two modes of WSSV transmission used in this study, transmission by cohabitation and transmission by ingestion, are possibly the most natural courses of WSSV transmission in the wild environment. Low virulence implies that these species may serve as reservoir carriers of WSSV and may pose a threat to the survival of other decapods in the wild that are more susceptible to WSSV infection.
CHAPTER V – EPIDEMIC MODEL OF WHITE SPOT SYNDROME VIRUS IN THE DAGGERBLADE GRASS SHRIMP (*Palaemonetes pugio*) AND THE GULF SAND FIDDLER CRAB (*Uca panacea*)

Introduction

Over the last three decades, the profitability of shrimp aquaculture has been reduced by numerous infectious diseases, particularly those of viral etiology. The first virus of penaeid shrimp, *Baculovirus penaei* (BP), was reported in 1974. Since then approximately 20 shrimp viruses have been recognized; only a few of them pose a threat to penaeid shrimp aquaculture (Couch, 1974; Lightner 1999; Walker and Winton, 2010; Flegel 2012). Presently, five viruses are considered the most threatening to penaeid shrimp and are listed as notifiable infectious disease agents by the World Organization for Animal Health (OIE), *White spot syndrome virus* (WSSV), *Infectious hypodermal and hematopoietic necrosis virus* (IHNV), *Taura syndrome virus* (TSV), *Yellow head virus* (YHV), and *Infectious myonecrosis virus* (IMNV) (OIE 2016). WSSV is the most pathogenic and causes enormous production losses (Lightner et al. 2012).

WSSV, the infectious agent of White Spot Disease (WSD), is non-occluded with a genome of circular double-stranded DNA. It is the sole member of the genus *Whispovirus* in the family of *Nimaviridae* (Wang et al. 1995; van Hulten et al. 2001a; Yang et al. 2001; Mayo 2002). WSD epidemics were first observed in Fujian Province of China and the northern region of Taiwan in 1992 causing high mortalities in farmed kuruma shrimp (*Marsupenaeus japonicus*). Within a decade it dispersed throughout all shrimp producing countries worldwide (Chou et al. 1995; Zhan et al. 1998). The disease can result in 100% mortality in commercial shrimp farms within 3 -10 days and has been
responsible for estimated economic losses of US$15 billion since its emergence in 1992 (Chou et al. 1995; Lightner 1996; OIE 2006; Lightner et al. 2012). Approximately 300,000 tons of production is lost to WSSV every year at a cost of about US$ 1 billion (Stentiford et al. 2012). There is no effective treatment for the disease. White Spot Disease not only causes massive economic losses but it also causes great social impacts. WSSV outbreaks on commercial shrimp farms have caused lost employment and income for many communities that depend on shrimp aquaculture and other related industries (Alday de Graindorge and Griffith 2001).

Much work has been done on transmission of WSSV in penaeid shrimp and a few other decapod crustacean species. Three modes of transmission have been identified: 1) ingestion of infected hosts (horizontal); 2) waterborne transmission or cohabitation with infected hosts (horizontal); and 3) transmission from broodstock to offspring (vertical) (Supamattaya et al. 1998; Chang et al. 1998; Rajendran et al. 1999; Soto et al. 2001; Corteel et al. 2009; Vazquez-Boucard et al. 2010). Biological determinants such as cannibalism, predation, age of shrimp and variability in virulence among WSSV isolates affect horizontal transmission of WSSV within a host population (Soto et al. 2001; Lightner et al. 1998; Wang et al. 1998).

Investigations of the dynamics of white spot disease in some penaeid shrimp species have combined experimental study and mathematical modeling. For example, Lotz and Soto (2002) formulated a compartmental epidemic model of WSSV in *Litopenaeus vannamei* based on Reed-Frost disease transmission process. Their model revealed that WSSV epidemics in *L. vannamei* are governed by the number (density) of susceptible hosts, transmission, virulence, and removal coefficients, and the initial dose
of the virus (Lotz and Soto 2002). Additionally, Tuyen et al. (2014) formulated a stochastic Susceptible-Infected-Recovered (SIR) model of WSSV in juvenile Penaeus monodon and L. vannamei that employed a Kermack-McKendrick transmission term. Their study found that the transmission coefficient of WSSV was higher in P. monodon than in L. vannamei. Moreover, they found that the basic reproductive number ($R_0$) of WSSV infection was lower in P. monodon than in L. vannamei and that the disease has a longer infectious period in P. monodon (Tuyen et al. 2014). Modeling the dynamics of WSSV transmission in wild populations or communities of decapod crustaceans has not been done.

Although poorly understood in natural habitats, WSSV could negatively impact wild shrimp and other crustaceans if it is successfully introduced and established. Therefore, it is important to understand the dynamics and consequences of WSD in wild host populations and communities (de la Peña et al. 2007; Escobedo-Bonilla et al. 2008; Villarreal 2009; Dang et al. 2010). In this contribution, I develop a simple mathematical model of WSSV dynamics in two local decapod crustacean species of the northern Gulf of Mexico, the daggerblade grass shrimp (Palaemonetes pugio) and the Gulf sand fiddler crab (Uca panacea), in an attempt to further our understanding of the role that WSSV might play in wild populations and communities. Firstly, I develop a mathematical model of WSSV dynamics in single host populations which is modified from previously developed models (Soto and Lotz 2001; Lotz and Soto 2002; Lotz et al. 2003). Secondly, I extend those models to formulate a model of WSSV dynamics in a two-host community. Thirdly, I incorporate model parameter estimates from Chapter IV into the
model to investigate the consequences of WSSV epidemics. Finally, I explore how variation in those parameters affect WSSV dynamics.

**Modeling Procedure**

*Dynamic Models of WSSV in a Host Population*

My mathematical model of WSSV dynamics in a host population of wild decapods was modified from a previously developed compartment model (Lotz and Soto, 2002). Three compartments or states of the host have been identified: susceptible hosts (S), infected hosts (I) and infected carcasses (D) which have died from infection but remain infectious. Figure 7 illustrates the hypothetical course of WSSV infection in a wild crustacean host population and is a standard compartmental SID epidemic model with a Reed – Frost method of transmission (Abbey 1952; Black and Singer 1987).

![Diagram](image)

*Figure 7.* Diagram of WSSV life cycle in a host population
The blunted arrows on the diagram identify the initial sources of infection and link the infected compartments (I, D) to the susceptible compartments (S). The coefficients on the transition arrows represent the likelihoods of transition from one compartment to another during one unit of time. The coefficient \( \beta \) represents the likelihood that a susceptible host becomes infected after contact with an infected living host, whereas the coefficient \( \chi \) represents the likelihood that a susceptible host becomes infected after contact with an infected host carcass. The coefficient \( \alpha \) is the likelihood that an infected living host dies from infection in one unit of time (pathogen-induced mortality, or virulence), and the coefficient \( \delta \) is the likelihood that an infected host carcass loses its infectivity due to decomposition or consumption by scavengers. The diagram can be translated into a set of mathematical equations as follow:

\[
S_{t+1} = S_t - S_t \cdot \left( \left( 1 - (1 - \beta)^I \cdot (1 - \chi)^D \right) \right) \\
I_{t+1} = I_t + S_t \cdot \left( \left( 1 - (1 - \beta)^I \cdot (1 - \chi)^D \right) - I_t \cdot \alpha \right) \\
D_{t+1} = D_t + I_t \cdot \alpha - D_t \cdot \delta
\]

Eq. (2) describes the dynamics of WSSV in the susceptible host compartment (S), where \( \beta \) is the transmission by cohabitation coefficient or the likelihood that a susceptible host becomes infected after exposure to an infected living host per unit of time, \( \chi \) is the transmission coefficient by ingestion or the likelihood that a susceptible host becomes infected after exposed to an infected host carcass per unit of time. The likelihood that a susceptible host manages to escape an infection after contact with all infected living individuals and all infected carcasses is equal to \( (1 - \beta)^I \cdot (1 - \chi)^D \), where \( I_t \) and \( D_t \) represent the number of infected living host and the number of infected carcass at time \( t \), respectively.
Eq. (3) describes the dynamics of WSSV in the infected host compartment (I), where the number of infected hosts is increased by the number of susceptible hosts that become infected and decreased by the number of infected hosts that succumb from infection per unit of time.

Eq. (4) describes the dynamics of WSSV infection in the dead infected host compartment (D), where the number of infected carcasses is increased by the number of infected hosts that die from infection and decreased by the number of infected carcasses that lose infectiousness due to decomposition or consumption by scavengers per unit of time. The coefficient $\delta$ is the likelihood that an infected carcass loses its infectivity per unit of time.

This model reasonably assumes that the epidemic occurs in a closed population with no births or natural deaths because the known generation time of WSSV epidemics in aquaculture settings is much shorter than the generation time of the hosts (days vs months). I further assume a constant transmission rate during the infectious period and a homogeneously mixed population.

One of the most important parameters for an infectious disease that can be determined from the model is the basic reproduction number, $R_0$. $R_0$ is the average number of new infections generated by one infected host during its time of infectivity in a completely susceptible host population (Diekmann et al. 1990). When $R_0 < 1$, the disease will fade from the host population, and when $R_0 > 1$, an epidemic will ensue (Heffernan et al. 2005). From the set of equations described above, $R_0$ of WSSV infection in a host population can be determined (Lotz and Soto 2002) following Eq. (2-4) as follows:
where $\beta/\alpha$ is the number of new infections that arise from a living infected during its expected life time $1/\alpha$ and $\chi/\delta$ is the number of new infections that arise from an infected carcass during it’s expected life time $1/\delta$.

**Dynamic Model of WSSV in a Host Community**

The SID model discussed above is modified into a matrix population model which allows incorporating species heterogeneity in the model (Diekmann et al. 2010; Caswell 2001). Matrix models are easily extended to more complex host-parasite systems, particularly multi-species communities. Figure 8 represents a life cycle diagram for WSSV in a two-host community of wild crustacean hosts.

A matrix representation of the two-host community for this study is a block diagonal matrix $M$ (Eq. 5), where $H_i$ is the $3 \times 3$ sub-matrix of the dynamics of WSSV infection in host species $i$.

$$M = \begin{bmatrix} H_1 & 0 \\ 0 & H_2 \end{bmatrix}$$  \hspace{1cm} (6)

The off-diagonal submatrices are filled with zeros because species do not change individuals among themselves. However, the transmission terms now include transmission of infection from species $j$ to species $i$ ($\beta_{ij}$ and $\chi_{ij}$) as well as transmission within species ($\beta_{ii}$ and $\chi_{ii}$). The transition matrix for $H_i$ is formulated in Eq.7 as presented below:
\[ H_1 = \begin{bmatrix} \left( \prod_{i=1}^{2} \left( (1 - \beta_{1i})^{I_i} \cdot (1 - \chi_{1i})^{D_i} \right) \right) & 0 & 0 \\ 1 - \left( \prod_{i=1}^{2} ((1 - \beta_{1i})^{I_i} \cdot (1 - \chi_{1i})^{D_i}) \right) & (1 - \alpha_1) & 0 \\ 0 & 1 - (1 - \alpha_1) & (1 - \delta_1) \end{bmatrix} \] (7)

\textit{Figure 8.} Diagram of WSSV life cycle in two-host species community. Dashed lines ending in circles indicate transmission from one species to another and solid lines ending circles indicate within species transmission.
The life cycle diagram of WSSV in a two-host community (Figure 8) can be transformed into a life table of infection (Table 17). The $R_0$ for WSSV transmission dynamics in the two-host species community can be easily calculated from a life table of infection as presented in Eq. (8) below:

$$R_0 = \left( \frac{\beta_{1,1} + \beta_{2,1}}{\alpha_1} + \frac{\chi_{1,1} + \chi_{2,1}}{\delta_1} + \frac{\beta_{2,2} + \beta_{1,2}}{\alpha_2} + \frac{\chi_{2,2} + \chi_{1,2}}{\delta_2} \right)$$

(8)

Table 17

*Life table of WSSV infection.*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Duration</th>
<th>Transmission</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>I$_1$</td>
<td>$1/\alpha_1$</td>
<td>$\beta_{1,1} + \beta_{2,1}$</td>
<td>$(\beta_{1,1} + \beta_{2,1})/\alpha_1$</td>
</tr>
<tr>
<td>D$_1$</td>
<td>$1/\delta_1$</td>
<td>$\chi_{1,1} + \chi_{2,1}$</td>
<td>$(\chi_{1,1} + \chi_{2,1})/\delta_1$</td>
</tr>
<tr>
<td>I$_2$</td>
<td>$1/\alpha_2$</td>
<td>$\beta_{2,2} + \beta_{1,2}$</td>
<td>$(\beta_{2,2} + \beta_{1,2})/\alpha_2$</td>
</tr>
<tr>
<td>D$_2$</td>
<td>$1/\delta_2$</td>
<td>$\chi_{2,2} + \chi_{1,2}$</td>
<td>$(\chi_{2,2} + \chi_{1,2})/\delta_2$</td>
</tr>
</tbody>
</table>

Model Simulation

*Epidemic Parameters for Model Simulation*

The epidemic parameters used to simulate the model of WSSV dynamics within single-species host populations and a two-species host community of *P. pugio* and *U. panacea* was obtained from the experimental bioassays described in Chapter IV. The summary of epidemic parameters of WSSV dynamics in the two species is presented in Table 17 and 18. No transmission of WSSV was detected to *U. panacea*, either from *U. panacea* or from *P. pugio*; however, the design of the experiment precluded detection of
transmission smaller than 1/150 = 0.0067. Therefore, the values of these coefficients were adjusted for the purposes of simulation. I adjusted the value of $\beta_{2,2}$ ($U. panacea$ to $U. panacea$) and $\beta_{2,1}$ ($P. pugio$ to $U. panacea$) and $\chi_{2,2}$ and $\chi_{2,1}$ to 0.0067. This value was the smallest possible number than could be obtained from my transmission experiments following an assumption that only one out of 150 susceptible hosts became infected after contact with infected living host or infected host carcass. The true value of these two coefficients may be less than 0.0067. Also, because the virulence ($\alpha_2$) of WSSV in $U. panacea$ obtained from the experimental transmission study was 0.0, I set $\alpha_2$ for model simulation to a daily mortality of 0.001. This is likely close to the natural mortality rate and results in a life expectancy of infected $U. panacea$ of 3 years. This was done to avoid division by zero in the model simulation, particularly in $R_0$ calculation.

Table 18

Summary of WSSV transmission coefficients in Palaemonetes pugio and Uca panacea

<table>
<thead>
<tr>
<th>Susceptible host</th>
<th>Infected host</th>
<th>$\beta$ (range)</th>
<th>$\chi$ (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-host</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P. pugio$</td>
<td>$P. pugio$</td>
<td>0.03 (0.001 – 0.10)</td>
<td>0.08 (0.001- 0.10)</td>
</tr>
<tr>
<td>$U. panacea$</td>
<td>$U. panacea$</td>
<td>0.0067 (0.001 – 0.10)</td>
<td>0.0067 (0.001 – 0.10)</td>
</tr>
<tr>
<td><strong>Between-host</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P. pugio$</td>
<td>$U. panacea$</td>
<td>0.02 (0.001 – 0.10)</td>
<td>0.03 (0.001 – 0.10)</td>
</tr>
<tr>
<td>$U. panacea$</td>
<td>$P. pugio$</td>
<td>0.0067 (0.001 – 0.10)</td>
<td>0.0067 (0.001 – 0.10)</td>
</tr>
</tbody>
</table>
Table 19

Summary of WSSV virulence and decomposition coefficients in Palaemonetes pugio and Uca panacea

<table>
<thead>
<tr>
<th>Host</th>
<th>$\alpha$</th>
<th>$\delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pugio</td>
<td>0.014 (0.001 – 0.1)</td>
<td>1 (0.01 – 1.0)</td>
</tr>
<tr>
<td>U. panacea</td>
<td>0.001 (0.001 – 0.1)</td>
<td>1 (0.01 – 1.0)</td>
</tr>
</tbody>
</table>

Characteristics of WSSV Epidemic Model within P. pugio and U. panacea Populations.

Simulation of WSSV epidemic models was performed using Python software version 3.4. Simulations generate important features of WSSV epidemics in P. pugio and U. panacea, including the epidemic curve, the epidemic peak, the duration of the epidemic, and the size of the epidemic. The epidemic curve visualizes the number of infected hosts in a population as a function of time (Bailey 1975; Wallinga and Teunis 2004; Jiang et al. 2009). The epidemic peak is defined as the maximum point of an epidemic curve and is the point where an epidemic begins to decline (Bailey 1975; Soto and Lotz 2003). The speed of an epidemic is the time required for an epidemic to reach its peak, whereas the epidemic duration is the width of the epidemic curve. The size of an epidemic describes the number of susceptibles that become infected during the course of an epidemic (Bailey 1975; Soto and Lotz 2003). I simulated epidemics of WSSV using the estimated parameter values from Tables 18 and 19. Results are displayed in Figures 9 and 10 for P. pugio and U. panacea populations, respectively. The number of susceptible P. pugio and U. panacea that become infected after exposure to a WSSV-infected host increases over time. For P. pugio, the epidemic reaches its peak at day-8.
post exposure with a final epidemic size of 92%. The epidemic peak in *U. panacea* occurs at day-43 post exposure with a final epidemic size of 97%. These results indicate that the WSSV epidemic in *P. pugio* progresses faster than in *U. panacea*; however, the epidemic size in *U. panacea* is higher than in *P. pugio*.

The estimated basic reproduction number (*R*_0) for WSSV in *P. pugio* and *U. panacea* populations is 2.22 and 6.71, respectively. The estimated values of *R*_0 for WSSV in the two species are greater than one suggesting that an epidemic of WSSV can establish within *P. pugio* and *U. panacea* if the virus is introduced into their populations.
Figure 9. Model epidemic illustrating characteristics of WSSV epidemic in *Palaemonetes pugio* population. (1) epidemic curve, (2) epidemic peak at Time 8, (3) size of epidemic was 92%. Dead (●) are also infected by WSSV.

Figure 10. Model epidemic illustrating characteristics of WSSV epidemic in *Uca panacea* population. (1) epidemic curve, (2) epidemic peak at Time 43, (3) size of epidemic was 97%. Dead (●) are also infected by WSSV.

A series of simulations were performed to study the consequences of variation in model parameters on the characteristics of WSSV epidemics. In particular, I changed the number (density) of susceptible animals ($S_0$), the number (density) of infected living animals ($I_0$), the number (density) of infected carcass ($D_0$) and the value of the coefficients ($\beta, \chi, \alpha, \text{and} \delta$). Simulations were executed by changing one parameter at a time while holding the other parameters constant as in Tables 18 and 19.

Transmission by Cohabitation ($\beta$) and Transmission by Ingestion Coefficients ($\chi$).

Generally, increasing the transmission parameters increased the speed, size, and $R_0$ of the epidemics. For $P. pugio$, increasing $\beta$ coefficients from 0.005 to 0.1 with an increment of 0.005 while keeping the other model parameter values constant increased the speed of the epidemic and the final size of the epidemic. Similarly, increasing $\beta$ for $U. panacea$ epidemics increased both the speed and the size of the epidemic. The final epidemic sizes with the increased $\beta$ in $P. pugio$ and $U. panacea$ were 97% and 100%, respectively (Figure 11 and Figure 12). Increasing the $\chi$ coefficients from 0.005 to 0.1 with an increment of 0.005 while holding the other parameters constant increased the speed of the WSSV epidemic in both species, but not as much as increasing $\beta$. No changes in the size of epidemic were observed as a result of increasing the $\chi$ coefficients in either $P. pugio$ or $U. panacea$. The estimated final size of WSSV epidemic in $P. pugio$ and $U. panacea$ were 92% and 97%, accordingly (Figures 13 and 14). These findings suggest that transmission by cohabitation is an important factor that governs the spread of the virus in the two species.
Figure 11. Effect of transmission by cohabitation coefficient (β) on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 12. Effect of transmission by cohabitation coefficient (β) on characteristics of model WSSV epidemics in *Uca panacea*. 
Figure 13. Effect of transmission by ingestion coefficient ($\chi$) on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 14. Effect of transmission by ingestion coefficient ($\chi$) on characteristics of model WSSV epidemics in *Uca panacea*. 
Virulence Coefficient ($\alpha$). An increase in $\alpha$ from 0.001 to 0.1 with an increment of 0.005 while holding the other parameters of the model constant resulted in an increase in the speed of the epidemic in the two species; however, increasing this coefficient significantly reduced the size of epidemic in both species. By increasing $\alpha$, the estimated final sizes of the WSSV epidemics in $P. pugio$ and $U. panacea$ were reduced to 69% and 19%, respectively (Figures 15 and 16).

Decomposition Coefficient ($\delta$). An increase in the decomposition coefficient from 0.1 to 1.0 with an increment of 0.05 while holding the other parameters constant did not affect the size of the WSSV epidemic in either $P. pugio$ or $U. panacea$ (Figures 17 and 18). However, a minor change was observed in the speed of the epidemic in the two species. Increasing the $\delta$ coefficients reduced the speed of the epidemic to reach its peak for both $P. pugio$ and $U. panacea$. 
Figure 15. Effect of virulence coefficient (α) on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 16. Effect of virulence coefficient (α) on characteristics of model WSSV epidemics in *Uca panacea*.
Figure 17. Effect of decomposition coefficient (δ) on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 18. Effect of decomposition coefficient (δ) on characteristics of model WSSV epidemics in *Uca panacea.*
Initial Number of Susceptible Animals ($S_0$). An increase in the initial number or density of susceptible animals from 1 to 100 while the other parameters in the model remained constant increased the speed and the size of the WSSV epidemic for both species. The final sizes of the WSSV epidemics in $P. pugio$ and $U. panacea$ were 97% and 99%, respectively. Figures 19 and 20 summarize the effect of changing the initial number of susceptible host on the WSSV epidemic model in $P. pugio$ and $U. panacea$, respectively.

Initial Number of Infected Animals ($I_0$, $D_0$). An increase in the initial number of infected hosts ($I_0$) and infected host carcasses ($D_0$) from 1 to 10 while the other parameters in the model were held constant increased the speed of the epidemics in both $P. pugio$ and $U. panacea$; however, there was no change in the size of the epidemics in the two species. The final size of the epidemic in $P. pugio$ and $U. panacea$ was 92% and 97%, respectively. The final size was constant regardless of the initial number of infected hosts used at the beginning of exposure (Figures 21 and 22). On the other hand, increasing the initial number of infected host carcasses slightly increased the size of an epidemic in $P. pugio$ from 92% to 93%; but, no change was detected in the size of the WSSV epidemic in $U. panacea$. Moreover, a significant increase in the speed of the epidemic was detected in $P. pugio$ as the number of infected host carcasses increased. Only a minor change in the speed of the epidemic was observed in $U. panacea$. Figures 23 and 24 summarize the effect of increasing $D_0$ in both species.
Figure 19. Effect of susceptible host on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 20. Effect of susceptible host number on characteristics of model WSSV epidemics in *Uca panacea*.
Figure 21. Effect of initial living infected host number on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 22. Effect of initial infected living host number on characteristics of model WSSV epidemics in *Uca panacea*.
Figure 23. Effect of initial infected host carcasses number on characteristics of model WSSV epidemics in *Palaemonetes pugio*.

Figure 24. Effect of initial infected host carcasses number on characteristics of model WSSV epidemics in *Uca panacea*. 
Effects of Model Conditions on the Basic Reproduction Number ($R_0$) of within Species WSSV Epidemics in *P. pugio* and *U. panacea*

Further inspection of Eq. (5) of the one-host WSSV epidemic model indicates that the values of $R_0$ for WSSV in *P. pugio* and *U. panacea* is governed by the values of $\beta$, $\chi$, $\alpha$, and $\delta$. The simulations to investigate the effects of changes of these coefficients on $R_0$ demonstrated that increasing the values of $\beta$ or $\chi$ while holding other parameters constant, increased $R_0$ of WSSV in both species. Increasing the transmission by cohabitation coefficient caused a higher change in $R_0$ in *P. pugio* and *U. panacea* compared to changes in transmission by ingestion for the two species. In contrast, increasing the virulence and decomposition coefficients reduced $R_0$ in both *P. pugio* and *U. panacea*. Figures 25 and 26 illustrate the effects of changing epidemic model parameters on $R_0$ of WSSV in *P. pugio* and *U. panacea*, respectively.
Figure 25. Effects of increasing epidemic model parameters on basic reproduction number ($R_0$) of WSSV in *Palaemonetes pugio*. 
Figure 26. Effects of increasing epidemic model parameters on basic reproduction number ($R_0$) of WSSV in *Uca panacea*.
Characteristics of a Two-Host Community Epidemic Model

Simulations of the two-host community epidemic in *P. pugio* and *U. panacea* were performed by using model parameters obtained from the experimental transmission studies as presented in Tables 18 and 19. The simulation of the two-host community epidemic in *P. pugio* and *U. panacea* incorporates transmission within-species and between-species. The characteristics of the WSSV epidemic model in the two species community are illustrated in Figure 27 and Figure 28.

In the two-host community WSSV epidemic, the presence of infected *P. pugio* and *U. panacea* increased the speed of the epidemic in both species. The time required for the epidemic in *P. pugio* to reach its peak was reduced from 8 days to 7 days when infected *U. panacea* was present. The presence of infected *P. pugio* significantly reduced the duration of the epidemic in *U. panacea* from 43 days to 20 days. Moreover, the final sizes of WSSV epidemics in *P. pugio* increased from 92% to 94% in the presence of infected *U. panacea* in the two-host community. Similarly, the final size of the epidemics in *U. panacea* increased from 97% to 98% in the presence of infected *P. pugio* in the two-host community. These findings provide evidence that the presence of alternative susceptible species increases the size and speed of a WSSV epidemic in the two hosts. \( R_0 \) in a two-host community of *P. pugio* and *U. panacea* is 17.09 and is higher than the \( R_0 \) obtained from a one-host WSSV epidemic model in either *P. pugio* (\( R_0 = 2.22 \)) or *U. panacea* (\( R_0 = 6.71 \)). This finding indicates that the virus is capable of causing an epidemic when infected *P. pugio* and *U. panacea* are present in the two hosts community.
Figure 27. Model epidemic illustrating a WSSV epidemic in *Palaemonetes pugio* in the two-species community. (1) epidemic curve, (2) epidemic peak at Time 7, (3) size of epidemic was 94%. Dead (●) are also infected by WSSV.

Figure 28. Model epidemic illustrating a WSSV epidemic of *Uca panacea* in the two-species community. (1) epidemic curve, (2) epidemic peak at Time 20, (3) size of epidemic was 98%. Dead (●) are also infected by WSSV.
Sensitivity Analysis of a Two-Host WSSV Epidemic Model in *P. pugio* and *U. panacea* Community

Simulations were performed to investigate the effects of changing only one parameter in the two-host WSSV epidemic model on infection dynamics in the *P. pugio* and *U. panacea* community while holding the other parameters at the values given in Tables 18 and 19.

*Transmission by Cohabitation (β) and Transmission by Ingestion (χ) Coefficients.*

Increasing the value of within-species transmission by cohabitation from 0.001 to 0.1 with an increment of 0.005 for *P. pugio* (β₁₁) or between-species transmission by cohabitation from *U. panacea* to *P. pugio* (β₁₂) increased the size and speed of the epidemic in *P. pugio*. Increasing these two coefficients in *U. panacea* only increased the speed of the epidemic. On the other hand, increasing the value of between-species transmission by cohabitation from 0.001 to 0.1 with an increment of 0.005 for *P. pugio* to *U. panacea* (β₂₁) or within-species transmission by cohabitation of *U. panacea* (β₂₂) slightly increased the speed and the size of the WSSV epidemic in *P. pugio*. Increasing the β₂₁ and β₂₂ coefficients also increased the size of the epidemic in *U. panacea* to 100%. Moreover, increasing these two coefficients substantially increased in the speed of the WSSV epidemic in *U. panacea* (Figure 29).

Interestingly, increasing the value of within-species transmission by ingestion coefficients from 0.001 to 0.1 with an increment of 0.005 for *P. pugio* (χ₁₁) and *U. panacea* (χ₂₂) and between-species transmission by ingestion coefficients from *U. panacea* to *P. pugio* (χ₁₂) and from *P. pugio* to *U. panacea* (χ₂₁) did not increase the size of the epidemic in either species. The final size of the epidemic remained constant at
94% for *P. pugio* and 98% for *U. panacea*. On the other hand, increasing $\chi_{1,1}$ coefficient resulted in a minor change in the speed of the epidemic in *P. pugio*. Moreover, increasing the values of $\chi_{1,2}$, $\chi_{2,1}$, and $\chi_{2,2}$ only increased the speed of the WSSV epidemic in *U. panacea* (Figure 30). These findings indicate that the transmission by ingestion coefficient does not play an important role in the dynamics of WSSV in the two species.
Figure 29. Effects of increasing transmission by cohabitation coefficients on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.
Figure 30. Effects of increasing transmission by ingestion coefficients on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.
**Virulence Coefficient** ($\alpha$). Simulations were performed by increasing the virulence coefficient in a two-host community epidemic model from 0.001 to 0.1 with an increment of 0.005 while holding the other parameters of the model at the same values as given in Tables 18 and 19. Increasing the virulence in *P. pugio* ($\alpha_1$) resulted in an increase in the speed of epidemic in *P. pugio*; however, it concomitantly reduced the size of the epidemic in this species. In contrast, increasing this coefficient did not change the size of epidemic in *U. panacea* and, interestingly, it reduced the speed of the epidemic in *U. panacea*. Increasing virulence ($\alpha_2$) in *U. panacea* drastically increased the speed of epidemic and reduced the size of epidemic in *U. panacea*. However, manipulating this coefficient did not affect the size or the speed of the epidemic in *P. pugio* (Figure 31).

**Decomposition Coefficient** ($\delta$). Simulations were performed to investigate the effects of increasing the decomposition rate ($\delta$) in the two species on characteristics of the two-host community epidemic from 0.001 to 1.0 with an increment of 0.05 while holding the other model parameters as listed in Tables 18 and 19. The simulations revealed that increasing the decomposition coefficient did not affect the size of the epidemic in either species. In contrast, increasing this coefficient reduces the speed of the WSSV epidemic in the two species. This finding provides evidence that the decomposition coefficient does not play an important role in regulating the WSSV epidemic in the community of *P. pugio* and *U. panacea* (Figure 32).
Figure 31. Effects of increasing virulence coefficients on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.

Figure 32. Effects of increasing decomposition coefficients on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.
**Initial number of Susceptible Host ($S_0$).** Simulations were performed to study the effects of changing the initial number (density) of susceptible hosts on the dynamics of WSSV epidemics in a *P. pugio* and *U. panacea* community. Simulations were executed by increasing $S_0$ from 1 to 100 while the other model parameters were held at the values listed in Tables 18 and 19. As the initial number of susceptible *P. pugio* increased, the size and the speed of the epidemic in *P. pugio*, as well as the speed of epidemic in *U. panacea*, were increased. However, increasing this number resulted in only a minor increase in the size of the epidemic in *U. panacea*. Further, increasing the initial number of susceptible *U. panacea* resulted in an increase in the speed of the epidemic in both species. Nonetheless, increasing this value resulted in a small change in the size of epidemic in both species populations (Figure 33).

**Initial Number of Infected Hosts ($I_0$, $D_0$).** The dynamics of the two-host community epidemic model in *P. pugio* and *U. panacea* were studied by performing a series of simulations in which the number of initially acutely infected hosts ($I_0$) and infected dead hosts ($D_0$) was increased from 1 to 10 while the other parameters were held at the values listed in Tables 18 and 19. In this simulation, increasing the initial number of infected hosts resulted in an increase of the viral dose given to susceptible hosts. An increase in the number of infected hosts ($I_0$ and $D_0$) of *P. pugio* or *U. panacea* concurrently increased the speed of the epidemic in each of the two species populations. On the contrary, increasing the initial number of infected hosts caused an inconsequential change in the size of the WSSV epidemic in both species populations (Figures 34 and 35).
Figure 33. Effects of increasing the initial number of susceptible host \( (S_0) \) on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.

Figure 34. Effects of increasing the initial number of living infected host \( (I_0) \) on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.
Figure 35. Effects of increasing the initial number of infected host carcass ($D_0$) on WSSV epidemic in *Palaemonetes pugio* and *Uca panacea* community.

**Effects of Model Conditions on Basic Reproduction Number ($R_0$) of WSSV within a Two-Species Community**

Similar to a one-host WSSV epidemic model, further inspection of the life table for WSSV infection (Table 17) and Eq. (7) of the two-host model, the values of $R_0$ for WSSV in *P. pugio* and *U. panacea* are reliant of the values of $\beta$, $\chi$, $\alpha$, and $\delta$ coefficients. The simulations to investigate the effects of changes of these coefficients on $R_0$ of WSSV demonstrated that increasing within-species transmission by cohabitation of *U. panacea* ($\beta_{2,2}$) or the between-species transmission by cohabitation from *P. pugio* to *U. panacea* ($\beta_{2,1}$) while holding other parameters in the model constant, increased $R_0$ in the two species community (Figure 36). On the other hand, increasing the value of within-species transmission by ingestion of *P. pugio* and *U. panacea* ($\beta_{1,1}$ and $\beta_{2,2}$, respectively) resulted in a minor increase in $R_0$ in the community. This result also was observed when
the value of within-species and between-species transmission by ingestion of *P. pugio* and *U. panacea* were varied while the other parameters were held constant. These findings provide more evidence that transmission by ingestion is less important than transmission by cohabitation in regulating WSSV epidemics in the two-species communities (Figure 37).

Further, increasing the virulence of WSSV in the two species from 0.001 to 0.1 significantly reduced $R_0$ in the community of *P. pugio* and *U. panacea* (Figure 38). This simulation also reveals that increasing virulence in *U. panacea* reduces $R_0$ in the two-host species community more than increasing it in *P. pugio*. Similarly, increasing the decomposition rate in the two species reduced $R_0$ in the two-host community (Figure 39).
Figure 36. Effects of increasing transmission by cohabitation coefficients on basic reproduction number ($R_0$) of WSSV in *Palaemonetes pugio* and *Uca panacea* community.

Figure 37. Effects of increasing transmission by ingestion coefficients on basic reproduction number ($R_0$) of WSSV in *Palaemonetes pugio* and *Uca panacea* community.
Figure 38. Effects of increasing virulence coefficient on basic reproduction number (R₀) of WSSV in *Palaemonetes pugio* and *Uca panacea* community.

Figure 39. Effects of increasing decomposition coefficient on basic reproduction number (R₀) of WSSV in *Palaemonetes pugio* and *Uca panacea* community.
Discussion

This study is the first to investigate the dynamics of WSSV epidemics in two-species decapod communities. I used laboratory parameter estimates from the daggerblade grass shrimp \( (P. \text{pugio}) \) and the Gulf sand fiddler crab \( (U. \text{panacea}) \) to produce mathematical models of the progress of WSSV epidemics in one- and two-host communities. The one-host epidemic and the two-host epidemic models used in this study were based on an epidemic model previously formulated by Soto and Lotz (2001), Lotz and Soto (2002), and Lotz et al. (2003). These models are developed to further our understanding of factors that modulate the dynamics of WSSV outbreaks in the natural communities.

The transmission by cohabitation coefficient \( (\beta) \), transmission by ingestion coefficient \( (\chi) \), virulence coefficient \( (\alpha) \), decomposition coefficient \( (\delta) \), the initial number of susceptible host \( (S_0) \), and the initial number of infected hosts \( (I_0) \) were identified as important for governing WSSV outbreaks in \( P. \text{pugio} \) and \( U. \text{panacea} \) populations and communities. Increasing the value of only one parameter in the models while holding the other parameters constant resulted in changes in the size and speed of WSSV epidemics in the two species under study.

The peak of the WSSV epidemic in the one-host model was predicted to occur in \( P. \text{pugio} \) at day-8 post-exposure with a final epidemic size of 92%. The WSSV epidemic peak was predicted to occur in \( U. \text{panacea} \) at day-43 post-exposure with a final size of 97%. It is interesting to note that the time required for the virus to cause an outbreak in \( U. \text{panacea} \) is longer than in \( P. \text{pugio} \). One possible explanation for this finding is that \( U. \text{panacea} \) may be less susceptible to WSSV infection than \( P. \text{pugio} \) resulting in a slow
progression of the disease in populations of this species. However, because the time required to reach the peak of the epidemic is longer, the virus has a greater probability of making more contacts with susceptibles, which in turn results in all of the susceptibles becoming infected.

In comparison to the time required to reach the peak of the WSSV epidemic in the one-host epidemic model, the time to reach the peak in the two-host community was reduced from 8 days to 7 days in \textit{P. pugio} and from 43 days to 20 days in \textit{U. panacea}. Correspondingly, the presence of infected \textit{P. pugio} and \textit{U. panacea} in the two-host community also increased the size of WSSV epidemic in both populations. In this case, the size of the epidemic in \textit{P. pugio} increased from 92\% to 94\% while the size of the epidemic in \textit{U. panacea} increased from 97\% to 98\%. These findings may provide evidence that the presence of alternative susceptible species in the host community shortens the time required to reach the peak of an epidemic and simultaneously increases the size of WSSV epidemic in the host population. It is unsurprising considering that the presence of alternative or substitutive hosts in the community may not only increase the frequency of contact between susceptible hosts and infected hosts as the density of hosts increases, but also increases the dose of the virus by increasing the number of initial infectives which provides an additional source of infection in the host population. Further, if a WSSV infection has already occurred within each species, then spillover of the virus between species will result in a more severe WSSV epidemic in the two-host species community.

Simulations of the two models demonstrated that transmission by cohabitation with WSSV-infected animals is over an order of magnitude higher than transmission by
ingestion of infected carcasses. Further, simulations of the two models revealed that transmission by ingestion does not play an important role in the dynamics of WSSV in the two species. This is not unreasonable since the decomposition rate of the virus in infected carcasses of the two species is high. Therefore, it is likely that infected carcass has already lost its infectiousness due to decomposition when ingested by susceptible hosts. Moreover, because the number of infected animals that die from WSSV infection is low, the disease has a negligible impact on the survival of *P. pugio* and *U. panacea* populations. However, these two species likely carry the virus for long periods and therefore continuously shed the virus into the environment. Therefore, these species, although not themselves affected, could threaten the survival of other species that occupy the same space and are more susceptible to the infection. In addition, simulations of one-host and two-host of WSSV epidemic models revealed that increasing the virulence results in a significant decrease in the size of WSSV epidemic in the two species. Because the virulence in both *P. pugio* and *U. panacea* is low, infection by WSSV does not affect survival of these two species in the natural environment. However, low virulence is beneficial to the virus as it increases the period over which the virus is infective in the host population.

Even though the models predict that WSSV epidemics can occur in the two species, a different result might be observed in the real world. Factors such as habitat overlap and competition for food resources can influence the dynamics of WSSV in wild populations of *P. pugio* and *U. panacea*. For example, in the wild, the species in this study occupy completely different habitats, decreasing the probability of contact between the two species. The only possible way for *P. pugio* and *U. panacea* to make contact is
when they overlap one another during high tides. Their contact will be limited by the duration of high tides.

The results obtained from my model depend on the assumptions used in developing the model. For example, in both the one-host and two-host models reported here, it is assumed that host populations are homogenously mixed and of fixed sizes. These assumptions imply that all susceptible hosts have an equal chance of making contact with each one of the susceptible and infected hosts. Extending the one-host model into a two-host model consequently increases the density of hosts and therefore significantly increases the frequency of contact between hosts. This is not unreasonable since the space occupied by the two hosts remains the same. As the frequency of contact between hosts increases, the size of the epidemic and the speed at which the peak is attained are increased.

The expected size of an epidemic in host populations is greatly influenced by the basic reproduction number (R$_0$). Further inspection of Eq. (4) and Eq. (7) make it clear that R$_0$ depends on the values of $\beta$, $\chi$, $\alpha$ and $\delta$. In particular, increasing $\beta$ and $\chi$ will increase the value of R$_0$, and increasing $\alpha$ and $\delta$ will reduce the value of R$_0$. My study found that R$_0$ from a two-host WSSV epidemic model (R$_0$ = 17.09) is greater than those from one-host epidemic models (R$_0$ = 2.22 for P. pugio and R$_0$ = 6.71 for U. panacea).

To further analyze R$_0$s of WSSV in P. pugio and U. panacea, I compared the results obtained from this study with R$_0$ of the virus in L. vannamei as reported by Lotz and Soto (2002). Even though Lotz and Soto (2002) did not estimate R$_0$ in their report, they provided all the important parameter estimates to calculate R$_0$. By solving the equations from their WSSV epidemic model, R$_0$ of WSSV in L. vannamei is equal to 60. A high
value of $R_0$ obtained from their study can be expected as the formula used to calculate $R_0$ incorporates the initial number of susceptible hosts ($S_0$). Lotz et al. (2003) observed that $R_0$ is reliant on $S_0$ and its value decreased as the density of susceptible hosts decreased due to infection. When $S_0$ is employed in calculation of $R_0$, it will significantly increase $R_0$ of WSSV in the two species under current study ($R_0 = 66.69$ for $P. pugio$ and $R_0 = 201.2$ for $U. panacea$). By comparing $R_0$ of WSSV from these two species, it is very likely that WSSV has greater potential to disperse and establish in $P. pugio$ and $U. panacea$ populations.
CHAPTER VI – SUMMARY

White spot syndrome virus, the causative agent of White Spot Disease, is the most pathogenic penaeid shrimp virus and has been responsible for enormous production losses in shrimp aquaculture for more than two decades. WSSV is a large dsDNA virus that was originally observed to cause massive mortality in kuruma shrimp *Marsupenaeus japonicus* in Fujian Province of China and the northern region of Taiwan in 1992. Since 1992, the virus has spread throughout the world, particularly in shrimp producing countries. Moreover, there is a growing body of evidence that many species of crustaceans are susceptible to WSSV infection. Much research has been focused on the roles and impacts of WSSV in some economically important cultivated penaeid shrimp species. Numerous findings have advanced our understanding of the dynamics of WSSV outbreaks in shrimp aquaculture. However, little is known about the dynamics of WSSV outbreaks in wild populations of decapod crustaceans. There is increasing concern about adverse impacts of WSSV infection on native estuarine crustacean species, particularly when this virus is introduced into new geographic regions.

The primary objectives of my research were to: 1) investigate the prevalence of WSSV in two species of wild decapod crustaceans collected from the northern Gulf of Mexico, the daggerblade grass shrimp *Palaemonetes pugio* and the Gulf sand fiddler crab *Uca panacea*, 2) estimate the median lethal doses (LD₅₀) of WSSV in *P. pugio* and *U. panacea*, 3) estimate the epidemic parameters of WSSV infection within- and between-species, and 4) develop mathematical models of WSSV transmission in *P. pugio* and *U. panacea*. 

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The result from the three-year survey conducted in this research showed that the two local species of crustaceans, the daggerblade grass shrimp *P. pugio* and the Gulf sand fiddler crab *U. panacea*, are susceptible to WSSV infection. The prevalence of WSSV in these two species during the three years of study varied. The overall prevalence of WSSV over the three years of study in *P. pugio* and *U. panacea* were 7.27% and 12.97%, respectively. The prevalence of WSSV in *U. panacea* was significantly higher than that in *P. pugio*. Interestingly, none of the animals that were WSSV-positive showed symptoms that often reported in infected penaeid shrimp. This suggests that these two species can serve as asymptomatic reservoirs for the virus. Additionally, I presented evidence that the virus was consistently detected during the three years of the study, but the viral loads were low. This supports the hypothesis that the virus has been introduced and established in wild populations of crustaceans in the coastal estuaries of the Gulf of Mexico.

The experimental challenges of WSSV were conducted to determine the median lethal dose (LD$_{50}$) of the virus in the two species. The animals were challenged with various doses of WSSV inoculum via intramuscular injection under controlled laboratory conditions. The viral loads in each dose used in this study were quantified using TaqMan real-time PCR. After injection, challenged animals were placed individually in isolation containers to avoid secondary infection. The LD$_{50}$ of WSSV in *U. panacea* was substantially higher than that in *P. pugio*. However, the time required for the virus to cause 50% mortality of the population was much longer in *U. panacea* than in *P. pugio*. Moreover, the median lethal viral loads of dead and moribund infected animals showed that the load was higher in *P. pugio* than in *U. panacea*. Another interesting finding from
my research was that the infected survivors were able to maintain the virus for long periods of time. In this study, the virus was detectable in infected survivors for 15 dpi in *P. pugio* and 21 dpi in *U. panacea*.

Following intramuscular injection of the virus, infected animals started to display signs of WSSV infection after 24 hpi for *P. pugio* and 32 hpi for *U. panacea*; the signs included anorexia, lethargy, and weakness. In some moribund WSSV-infected *U. panacea*, walking leg detachments were observed. Pinkish to reddish body discoloration was observed in *P. pugio*; however, no white spots were observed on the cuticle of either species. Mortality in WSSV-infected animals was observed at 32 hpi and 44 hpi for *P. pugio* and *U. panacea*, correspondingly.

The survival probabilities following exposure to WSSV in both *P. pugio* and *U. panacea* were analyzed using Kaplan-Meier survival analysis. The median survival time of the two species was inversely related to the viral dose. My study demonstrated that the median survival time of *U. panacea* was considerably higher than that of *P. pugio*. The highest viral doses administered to *P. pugio* and *U. panacea* were $7.14 \times 10^8$ and $2.40 \times 10^9$ WSSV genome copies g$^{-1}$ of animal body weight. These doses resulted in 100% and 95% mortalities in *P. pugio* and *U. panacea*, respectively. The results obtained from the experimental challenges with WSSV provide strong evidence that: 1) *P. pugio* and *U. panacea* are susceptible to and can succumb to WSSV infection; 2) both species may play roles as reservoirs for WSSV in wild populations; and 3) *U. panacea* is more resistant to WSSV infection than *P. pugio* and is able to maintain high viral loads without displaying any signs of infection.
Transmission by cohabitation and transmission by ingestion were used to study the dynamics of WSSV transmission within-species and between-species. Transmission by cohabitation was performed by exposing susceptible animals to infected living hosts, whereas transmission by ingestion was performed by exposing susceptible animals to infected host carcasses. The estimated transmission by cohabitation ($\beta_{1,1}$) and transmission by ingestion ($\chi_{1,1}$) coefficients within-species for *P. pugio* were 0.03 and 0.08, respectively. In contrast, both coefficients ($\beta_{2,2}$ and $\chi_{2,2}$) were found to be 0 in *U. panacea* as there was no WSSV transmission detected. The estimated transmission by cohabitation from infected living ($\beta_{1,2}$) and transmission by ingestion from an infected carcass ($\chi_{1,2}$) for *U. panacea* to *P. pugio* were 0.02 and 0.03, respectively. Conversely, the transmission from infected living and infected carcass from *P. pugio* to *U. panacea* ($\beta_{2,1}$ and $\chi_{2,1}$) were found to be 0 in *U. panacea*. The overall estimated virulence coefficient ($\alpha_1$) was 0.014, and the overall estimated decomposition coefficient ($\delta_1$) was 1 for *P. pugio*. The overall estimated virulence coefficient ($\alpha_2$) was 0.0, and the overall estimated decomposition coefficient ($\delta_2$) was 1 for *U. panacea*. These results indicated that the probability of susceptible animals becoming infected after exposure to either infected living or infectious carcass of either species is low.

To study the dynamics of WSSV epidemics in one-host species and two-host species communities, two epidemic WSSV models were formulated based on the previously developed WSSV epidemic model for *Litopenaeus vannamei*. The epidemic model is governed by three states of the hosts: susceptible, infected, and dead hosts; the model is a compartment model that uses a Reed-Frost transmission term. A series of simulations was performed to investigate the characteristics of WSSV epidemics within
P. pugio and U. panacea populations as well as in the two species community using the epidemic parameters obtained from WSSV transmission experiments. To investigate the effects of the parameters on the dynamics the values of $\beta$, $\chi$, and $\alpha$ coefficients in U. panacea were modified. We also studied the effects of initial values of susceptible ($S_0$), infected living ($I_0$) and infected dead animals ($D_0$) on epidemic dynamics.

The epidemic model showed that the epidemic peaked in P. pugio at day-8 following exposure to an infected host with a final epidemic size of 92%, whereas the peak epidemic occurred in U. panacea at day-43 following exposure to an infected host with a final epidemic size of 97%. My study found that the basic reproduction number ($R_0$) of WSSV in both P. pugio and U. panacea is greater than 1 indicating that the virus is able to cause an epidemic in populations of both species. The estimated $R_0$s for P. pugio and U. panacea were 2.22 and 6.71, respectively. Further, simulations of the two-host WSSV epidemic model in this study demonstrated that the speed of an epidemic in P. pugio increases when infected U. panacea are present in the host community. The presence of infected P. pugio in the community drastically increases the speed of the epidemic in U. panacea. Additionally, the final size of WSSV epidemics in the two species increases from 92% to 94% for P. pugio and from 97% to 98% for U. panacea. These findings provide evidence that there is WSSV spill-over between the two species, which in turn regulates the dynamics of WSSV epidemics in the community. The estimated $R_0$ obtained from the two-host WSSV epidemic model was 17.09 and substantially larger than the estimated $R_0$ obtained from either one-host WSSV epidemic model.
The characteristics of WSSV epidemics in a one-host population and in the two-host community of *P. pugio* and *U. panacea* was studied by manipulating one model parameter while holding the other parameters constant. An important finding obtained from the simulation is that transmission by cohabitation plays a more significant role than transmission by ingestions in governing the WSSV epidemic in both species. Moreover, the simulations revealed that increasing the initial number of susceptible and the initial number of infected animals resulted in an increase in the size of the epidemic as well as in the speed of the epidemic. Finally, based on the results obtained from simulations of the two epidemic models, I provided evidence that neither ingestion nor decomposition of infected host carcass plays an important role in regulating the epidemics in *P. pugio* and *U. panacea* populations.


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