FINAL REPORT

I. Title : Evaluation of ecological and commercial impact of white spot syndrome virus (WSSV) infections in the white shrimp, *Litopenaeus setiferus*, and the blue crab, *Callinectes sapidus*, in Southeastern United States using an immunoasssay technique

Craig L. Browdy, Eleanor F. Shepard, and James W.B. Powell

South Carolina Department of Natural Resources – Marine Resources Research Institute
217 Fort Johnson Road, Charleston, SC 29412

This study was funded by the Saltonstall-Kennedy program of NMFS/NOAA, Grant No. **NA03NMF4270090**

II. Abstract

White spot syndrome virus (WSSV) was discovered in shrimp aquaculture facilities in South Carolina in 1997. The outbreaks were thought to be related to collections of wild shrimp for reproduction in captivity. As the disease is known to cause devastating mortality in cultured shrimp in Southeast Asia, its discovery in SC prompted concern for the health of wild crustacean populations. An initial study conducted in 1999 found detectable virus in wild stocks of the Atlantic white shrimp, *Litopenaeus setiferus* and the brown shrimp, *Farfantepenaeus aztecus* with incidence and severity levels lower in the latter species. One *L. setiferus* individual, collected offshore, displayed a significant level of acute disease. The present study was designed to evaluate the current status of WSSV infection and to explore interactions between ovarian development, spawning and incidence of WSSV in wild populations of *L. setiferus* and the blue crab, *Callinectes sapidus*, using an existing immunoassay technique. A molecular diagnostic tool (i.e. real-time PCR), and a standardized *in vivo* bioassay were used to confirm viral infection and viability. A total of 1,808 *L. setiferus* and 300 *C. sapidus* specimens at various reproductive stages were collected and examined for WSSV infection using a commercially available immunoassay diagnostic test kit (Shrimple®). Shrimple® detects viral infection by utilizing a specific antibody that recognizes vp28, a viral structural protein. Specimens that tested positive for WSSV were further examined using real-time PCR and *in vivo* bioassays. Although 87 shrimp and 11 crabs tested positive with the Shrimple® kits, none of the shrimp and only one crab was found to carry viable virus at levels significant enough to cause infection in injection bioassays. Thus, although continuing to be present, current incidence of WSSV in wild shrimp and crab populations along the Southeastern Atlantic coast is minimal even during the physiologically rigorous reproductive cycle. An education/outreach program component of this project afforded researchers the opportunity to share ideas with members of the commercial fisheries industry and develop awareness regarding the commercial and environmental importance of diseases in the shrimp industry. Participants included members of the commercial fisheries in South Carolina, Georgia, and Florida, as well as members of educational institutions and government agencies. Dissemination of research results to cooperators, other researchers and key constituencies is ongoing.

III. Executive Summary

White spot syndrome virus (WSSV) was first reported in the U.S. in Texas in 1995. This was followed by its discovery in shrimp aquaculture facilities in South Carolina in 1997 and was thought to be related to collections of wild shrimp for reproduction in captivity (Browdy and Holland 1998). As the disease is known to cause devastating losses in cultured shrimp (Lundin 1997, Lightner 1999), its discovery in SC prompted concern for the health of wild crustacean populations and the estuarine ecosystem. WSSV is known to be an extremely virulent pathogen in penaeid shrimp with a wide host range (Chang, et al. 1998, Lo et al. 1996). In an initial survey of shrimp collected from south Atlantic US waters in 1999, investigators found detectable virus in wild stocks of the Atlantic white shrimp, *Litopenaeus setiferus* and the brown shrimp, *Farfantepenaeus aztecus* with incidence levels lower in the latter species (Chapman et al, 2004). One *L. setiferus* individual, collected offshore, displayed a significant level of acute disease. The present study was designed to assess the current status of WSSV infection in the wild by providing a scientifically sound and statistically rigorous analysis of WSSV incidence, and to analyze if there is a correlation between disease incidence, ovarian development and spawning of wild populations of *L. setiferus* and *Callinectes sapidus*. This was accomplished by using a new antibody-based detection kit (Shrimple®), sensitive molecular diagnostic tools (real-time PCR and one-step PCR), and *in vivo* bioassays. These diagnostic techniques were selected to provide for rapid screening of large numbers of individuals, to allow for the most sensitive detection of viral DNA in the samples (Durand and Lightner 2002) and to assess the viability and virulence of the virus to determine if it is severe enough to pass on to susceptible cohorts in injection bioassays (Prior et al 2003).

A total of 1,808 *L. setiferus* and 300 *C. sapidus* specimens were collected during this study. Samples comprised 1,368 female *L. setiferus*, approximately 65% of which were in the advanced stages of reproductive development, and 440 specimens were males. The distribution of the shrimp specimens collected across South Carolina, Georgia, and Florida was 991, 566, and 251, respectively. For the blue crabs, 263 were females, 82% of which were reproductively mature, and 37 were males. A total of 179 crabs were collected in South Carolina and 121 were collected in Georgia. Frozen samples were tracked using an Access database (Microsoft), prepared for analyses and archived for further research.

A commercially available immunochromatographic detection kit (Shrimple®) was used for rapid screening of samples in the field and in the laboratory. Prior to use in the present research, a series of bioassays were carried out to determine the sensitivity of Shrimple® relative to real time PCR (Powell, et al. In Press). Specific Pathogen Free (SPF) *Litopenaeus vannamei* were injected with a WSSV inoculum and sampled from 1 to 32 hours post infection (p.i.). By analyzing corresponding samples per time interval, the Shrimple® test results were correlated with estimated viral copy numbers from quantitative PCR. All of the negative controls were confirmed virus negative by both diagnostic assays. No false positives were found. Real-time PCR detected infections in 100% of inoculated shrimp, while the Shrimple® test kits detected infection in only

57.26% of the specimens. Results of this study indicate that the Shrimple® test kits failed to detect WSSV infection prior to 12 hours p.i. and demonstrated a significantly low detection efficiency during the early onset of infection—i.e., 4.35% compared to 100% with real-time PCR from 1 to 8 hours post-infection (p.i). False negative results from the Shrimple® kits were observed for specimens containing 4-1061 viral copies/ng genomic DNA. Faint positives were observed for specimens containing 36-1784 viral copies/ng genomic DNA. Although considerably less sensitive than real-time PCR, the preliminary studies suggested that the kits provide a useful tool for detection of viral infections prior to development of gross signs of acute disease.

In the first screening using the Shrimple® test kits, reactions which should indicate WSSV infections were detected in both *L. setiferus* and *C. sapidus*. Positive reactions were detected in 4.8% and 3.7% of *L. setiferus* and *C. sapidus*, respectively. The next phase of the research focused on confirmation of infection and further screening using the more sensitive real-time PCR viral quantification.

A significant amount of time and effort was invested in validation of real time PCR methodologies for the screening of wild *L. setiferus* and *C. sapidus*. DNA isolated from wild *L. setiferus* was determined to inhibit real-time PCR reactions. A series of trials were conducted using multiple primer sets applying both Qiagen QuantiTect Probe PCR Kits (Durand and Lightner, 2002) and SYBR-Green (Dhar, et al., 2001) PCR Kits (Qiagen, Inc.; Valencia, CA). Dilution concentrations were tested to optimize PCR conditions and reduce the inhibition to the extent possible in the PCR reaction. By applying the SYBR-Green technologies and diluting the genomic DNA to one-tenth its initial concentration, the inhibition was reduced significantly while still allowing the reaction to proceed. Optimized protocols were used to screen all Shrimple® positive samples and a sub-sample of the Shrimple® negative shrimp and crabs. One step PCR protocols (Chapman, et al., 2004) were used to validate real time PCR results.

A total of 207 shrimp and 21 crabs were tested by PCR comprising all specimens that gave positive test results using the Shrimple® assay. No WSSV amplification was obtained for any of the *L. setiferus* tested using either real-time or one-step PCR. A further effort was dedicated to subsequent rigorous screening of shrimp sub-samples that tested positive with the Shrimple® kits. This included referral of samples to outside diagnostic laboratories for further verification. No WSSV amplification could be confirmed from any of these specimens following repeated screening against five established PCR primers. The results of the present study have led us to conclude that the positive reactions observed from wild shrimp tested with the Shrimple® detection kit are most likely false positives. Further studies are planned in collaboration with the manufacturers of the kits to better understand the nature of this non-specific binding. Similar results were found for the *C. sapidus* samples. Of the 11 crabs that tested positive using the Shrimple® test kits, only one specimen was confirmed positive using real-time PCR.

A standard injection bioassay protocol (Prior, et al., 2003) was used to provide further validation of the Shrimple® results and real-time PCR diagnostic results. Each of the 87

shrimp and 11 crabs suspected to be infected with WSSV according to the Shrimple®, as well as 17 shrimp and 3 crabs presumed to be virus free were further analyzed by bioassay. An inoculum was prepared from course filtered, centrifuged tissue homogenates according to the methods detailed by Prior, et al. (2003). Positive, negative and test inocula were injected into susceptible SPF *L. vannamei*. Mortality was recorded daily and moribund shrimp or shrimp surviving at the termination of the 10-day assays were collected and tested for WSSV infection by PCR. All positive controls reached 100% mortality as expected and WSSV infection was confirmed among moribund shrimp. Mortality in negative controls was not significantly different than that recorded for shrimp injected with test inocula with one exception. Inoculum prepared from the *C. sapidus* specimen found to be WSSV positive by PCR caused acute disease, i.e., 100% mortality in the bioassay. Moribund shrimp from this bioassay were confirmed WSSV positive by PCR. PCR products were sequenced and found to match the primers' target fragment of the WSSV genome. Further studies are being carried out to characterize more variable regions of the genome of the blue crab's WSSV genome. Additional research beyond the scope of the present studies will focus on better understanding of the pathogenicity of WSSV in the blue crab.

The results of the present study indicate that although WSSV persists in the region, levels of infection in shrimp and crab populations are low, even during the physiologically rigorous reproductive cycles; and currently, its occurrence does not pose a threat to the shrimp industry. The education/outreach program established and implemented through the course of this project provided educational opportunities to members of the commercial fisheries industry. The program shared the objectives and methodology of the study and built cooperative relationships that will afford collaborative researchers opportunities in the future. Scientific and technical reporting of research results is ongoing and follow-up efforts to disseminate research findings among cooperators and key stakeholders are planned.

IV. Purpose

A. Description of the problem.

Major viruses of concern relative to South Atlantic native stocks include Taura Syndrome Virus (TSV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), and the Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV)(Lightner 1999). WSSV was first reported from Southeast Asia in 1992 and spread throughout the region during the 1990's causing devastating declines in farmed shrimp production (Flegel 1996). WSSV was identified in captive shrimp in South Carolina in 1997 and in native shrimp in the southeast Atlantic coast in 1999 (Chapman, et al. 2004). The available evidence suggested that WSSV infections in South Carolina were derived from the wild (Browdy and Holland 1998). WSSV is unique among shrimp viruses in that it infects a variety of crustaceans. WSSV-like genetic material has been found in samples from white shrimp, grass shrimp, fiddler crabs, blue crabs, stone crabs and other crustaceans. These crustaceans potentially serve as reservoirs for WSSV with the possibility of re-

infecting wild as well as farmed shrimp populations. Archived DNA samples suggest a WSSV-like virus may have existed in the southeastern U.S. as early as 1988 (Browdy and Holland 1998). WSSV infections identified in captive Pacific white shrimp in South Carolina could have resulted from an introduction from Asia or from indigenous carriers.

Risks of shrimp virus introduction are not limited to transfer of live animals for shrimp culture. Other potentially important sources of shrimp viruses include carrier organisms in ship ballast water, frozen seafood product, and avian dispersal (Durand, et al. 2000; Lightner, et al. 1992; VanPatten, et al. 2004). In many countries, farmed shrimp are harvested during the acute phase of virus outbreaks to salvage part of the crop and minimize losses. The infected shrimp are frozen and sold in the U.S. for consumption or for use as fishing bait. Viable virus particles have been found in imported frozen commodity shrimp in the US (Durand, et al. 2000). If introduced into local waters as bait or processing waste, these tissues could represent a vector for infection of indigenous crustaceans.

General population surveys for WSSV presence in *L. setiferus* and *C. sapidus* have previously been performed. A study in 1999 was conducted (Chapman, et al. 2004) targeting penaeid shrimp collected along the South Atlantic Bight, extending from Cape Hatteras, North Carolina, to Cape Canaveral, Florida. A total of 586 *L. setiferus* specimens were tested with 1-step PCR, 28 (or 4.7%) of which were determined positive for WSSV. The current study expanded the previous population survey to include evaluating *C. sapidus* specimens as potential biological reservoirs for the virus. Rather than sampling multiple penaeid shrimp species, the current study focused specifically on *L. setiferus*, the most abundant commercial fisheries shrimp in the southeastern U.S., as well as the species which resulted in the highest percentage of WSSV infections in the 1999 study. Efforts were made to collect samples spanning developmental stages of both male and female specimens to allow researchers to target physiological (reproductive development) and any biotic or abiotic factors which may favor increase in the incidence of WSSV infections.

B. Objectives

The present study has been designed to provide a scientifically sound and statistically rigorous analysis of WSSV incidence in commercially significant crustacean populations in the waters of the Southeastern United States. The study aimed to:

1. develop, test, and assess the reliability of immuno-based tools (using polyclonal and/or monoclonal antibodies) as diagnostic tools for detecting WSSV in the laboratory and in the field;
2. evaluate the impact of WSSV on the *L. setiferus* and *C. sapidus* populations and their general health in the waters of the Southeastern United States with particular reference to reproductive populations to assess amplification of the disease and potential for vertical transmission;
3. promote awareness by working with commercial fishermen about the commercial implications and ecological significance of shrimp and blue crab diseases; and

4. generate a database for the evaluation of the impacts of WSSV and interrelationships between infection levels and environmental parameters.

V. Approach

A. Methodology

1. Site selection and research collaboration

In an effort to expand the sample locations throughout the commercial southeastern Atlantic U.S. *L. setiferus* fisheries areas, research collaborators were enlisted to assist with sample collection. The survey area was divided into the three states—South Carolina, Georgia, and Florida. Within each state, a central location was selected to direct sampling. The location was decided upon based on relative distance from central locations in bordering states, potential research collaborators, and being heavily involved in commercial shrimp fisheries. Charleston, South Carolina; Brunswick, Georgia; and Jacksonville, Florida, served as the three central locations in the study area.

The South Carolina Department of Natural Resources (SC-DNR), University of Georgia—Marine Extension Service (UG-MES), Georgia Department of Natural Resources (GA-DNR), and Florida Marine Resources Research Institute (FL-MRRI) were the primary research collaborators who facilitated the overall sample collection throughout the study. Additionally, commercial fishermen in each state were recruited to regularly collect samples once the commercial seasons were opened in each state. Shrimple® test kits were used by SC-DNR biologists when screening shrimp onboard commercial shrimp boats.

In addition to collections made in each of the three central locations, samples were collected along the coasts of South Carolina and Georgia during trawl surveys conducted by each state agency. The trawl surveys were regular trips north and south of the central locations providing some additional samples from more remote locations.

2. Specimen collection and storage

Specimens were collected in sites frequented by local commercial fishing industries as well as being readily accessible by each research collaborator in South Carolina, Georgia, and Florida (Figure 1). All specimens were stored in -20°C freezers at each research collaborator's facility until transfer of the specimens to Hollings Marine Laboratory (HML) in Charleston, South Carolina, could be arranged. The specimens were shipped on dry ice to HML and then stored in a -40°C freezer until tissue samples of interest were collected from each specimen. Following sample preparation, tissue samples were either stored in a -80°C freezer or in sarcosyl-urea (1% sarcosyl, 8M urea, 20mM sodium phosphate, 1mM EDTA, pH 6.8) to ensure the viability of any potential viral materials.

Weekly sample collections were attempted at each collection site throughout the commercial shrimp season. Additionally, samples were collected through state agency surveys prior to the commercial season. Inclement weather and conflicting schedules limited sampling periodically throughout the project but were kept to a minimum.

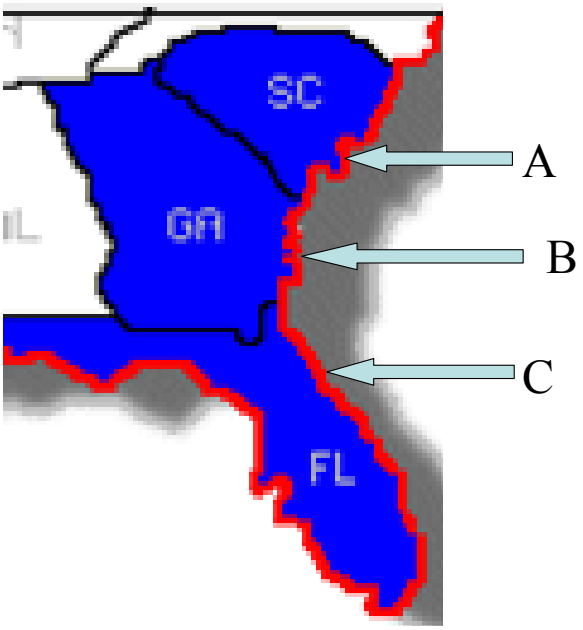


Figure 1. *L. setiferus* and *C. sapidus* collection sites. (A = South Carolina – Charleston, Key commercial areas, Bull's Bay to Port Royal, Coastal Surveys N & S; B = Georgia – Brunswick and Coastal Survey N & S; C = Florida – Jacksonville).

Each sample collection was tagged with a specific identification number that correlated to the date and location of collection. All pertinent data for each specimen within each collection was recorded and stored in a database. Each tissue sample collected during dissection was assigned a unique identification number. A database was created to contain information regarding each collection of shrimp and crabs as well as each individual specimen collected. All samples collected have been entered in the database and archived for future analysis or related studies.

Three tissue samples were prepared per specimen, in addition to the sample removed for diagnosis with the Shrimple® test kit. All dissections were carried out in a biosecure laboratory and aseptic techniques were applied to prevent any potential cross contamination among specimens. From each shrimp specimen the following samples were prepared: 2 pleopoda stored in 1000 ul of sarcosyl-urea in a microcentrifuge tube, tail fan, or uropod, stored in 1000 ul of sarcosyl-urea in a microcentrifuge tube, and the cephalothorax stored at -40°C in a sealable plastic bag. Pleopoda were selected as the primary tissue for analysis based on previous studies confirming that peeled shells, pleopoda and tail fan, contain nearly 10-fold more WSSV on a per weight basis than do whole heads or whole tails (Lightner, et al. 2001). From each crab specimen the following samples were prepared: 2 gills stored in 1000 ul of sarcosyl-urea in a microcentrifuge tube, 2 swimming legs stored at -80°C in a sealable plastic bag, and a

composite sample consisting of the hepatopancreas, heart, gills, eyestalks, stomach, muscle, and reproductive organs stored in a 50-ml sample jar at -80°C. Specific crab tissues were selected based on previously conducted research that demonstrated detectable WSSV in gills, hemolymph, stomach, eyestalks, testis, heart, ovary, muscle, and hepatopancreas (Kou, et al. 1998, Lightner, et al., 2001).

DNA isolations and real-time PCR were performed on the shrimp pleopoda and crab gills stored in sarcosyl-urea (1% sarcosyl, 8M urea, 20mM sodium phosphate, 1mM EDTA, pH 6.8). Samples were lysed via 72-hour incubation at 37ºC. Shrimp cephalothoraxes and composite samples from the crabs were used for performing bioassays and for confirmatory testing. The shrimp uropoda and crab swimming legs were collected as an additional sample from each specimen in the event a replicate or confirmatory sample was needed for any aspect of the study.

3. Shrimple® test kit validation

Real-time PCR was used to determine the efficiency and sensitivity of Shrimple® diagnostic test kits (Powell, et al. In Press). Detection of tagged oligonucleotide probes by gold nanoparticles, such as those in Shrimple® test kits, has been determined to be a fast, sensitive, and economical method for pathogen detection (Li and Rothberg, 2004).

All Shrimple® tests were performed according to the manufacturer's protocol (EnBioTec Laboratories; Tokyo, Japan). Kit components consist of Shrimple® test strip, disposable eye dropper, tissue grinder, and a 1.5 mL microcentrifuge tube filled with grinding buffer. Pleopoda were removed from the shrimp, placed in the microcentrifuge tube, and ground using the tissue grinder. Three drops, or approximately 75 ul, of the supernatant were applied to the test strip.

The immunochromatographic assay (Shrimple®) developed by EnBioTec Laboratories utilizes a sandwich immunoassay technique. A monoclonal rat anti-WSSV antibody-colloid gold conjugate pad is positioned next to the sample pad region on the membrane test strip. The membrane test strip is pre-coated with anti-rat IgG on the control (C) zone, where a pink band will appear if the test kit is valid and has performed properly, and monoclonal rat anti-WSSV on the test (T) zone, where a pink band will appear if the animal being tested is positive for WSSV. A test that results in pink bands both at the C-zone and at the T-zone is positive for white spot syndrome virus (Figure 2).

L. vannamei total genomic DNA and associated viral DNA were extracted from 3 pleopoda per animal using Sprint Prep kit (Agencourt Bioscience; Beverly, MA) in accordance with the manufacturer's protocol. To ensure that all DNA isolations were successful and yielded intact gDNA, subsamples from the isolations were electrophoresed on a 0.8% agarose gel with ethidium bromide, and quantified on an ultraviolet spectrophotometer with a 96-well plate reader (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA) at 260/280nm. The average concentration of isolated DNA per sample was 3.93 ng/µl +/- 2.13 ng/µl.

Thermal cycling was performed on the Applied Biosystems 7500 Sequence Detection System with preliminary data analysis by the 7500 Sequence Detection Software v. 1.2.2. (Applied Biosystems; Foster City, CA). Quantitative real-time PCR was performed using the Qiagen QuantiTect Probe PCR Kit (Qiagen, Inc.; Valencia, CA) according to the recommended procedure. Briefly, 2 ul samples (5-10 ng gDNA) from each DNA isolation were added to a PCR mixture containing 0.3 uM of each primer and 0.15 uM TaqMan probe into a final reaction volume of 25 ul. The amplification program consisted of 10 m at 95°C to activate the hot-start AmpliTaq Gold polymerase, followed by 40 cycles of 15 s denaturation and 60 s annealing/extension at 60°C. Plasmid DNA samples specific to the WSSV template of known concentration were included as absolute standards. The instrument determined fluorescence at the end of each annealing/extension cycle. Background fluorescence was monitored using ROX dye as part of the reaction mixture, and all samples were normalized to the background level.

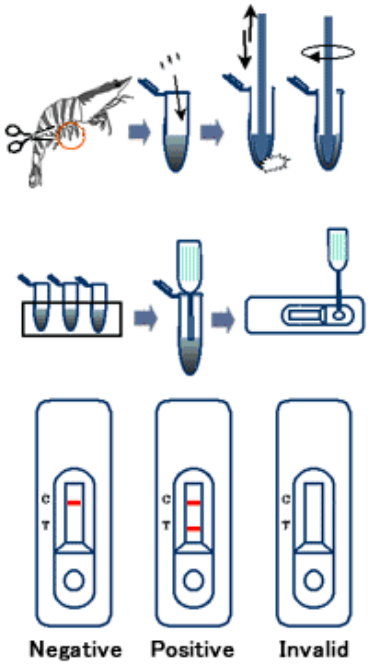


Figure 2. Shrimple® diagnostic test kit procedural diagram and potential test results. Reprinted with permission from EnBioTec Laboratories Co., Ltd. Shrimple® Product Guide.

Sequences for PCR primers and TaqMan probe used for the detection of WSSV were obtained from a previously published study (Durand and Lightner, 2002). The primers (WSS1011F and WSS1079R) generated a 69-bp amplicon and were selected from a region of WSSV genomic sequence in GenBank U50923. The TaqMan probe was dual-labeled with fluorescent dyes, 5'-5-carboxyfluoroscein (FAM) and 3'-N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) (Table1).

Following real-time PCR amplification, a baseline and threshold were defined using the Applied Biosystems 7500 Sequence Detection Software (ABI 7500 SDS v. 1.2.2), resulting in a fractional cycle number (C_T value) assigned to each individual sample. A set of standard dilutions (from 1 x 10^7 to 1 x 10^{-1} viral copies/µl) was created from a WSSV plasmid prep DNA of 4.0 x 10^7 viral copies/ ul and ran simultaneously with the samples from the time-course experiment. Regression of the log of viral copy number

and C_T value was used as a standard curve for determining viral load. Viral copy number was normalized per ng genomic DNA.

Table 1. WSSV Primers used for real-time PCR

Virus	Upstream primer	Downstream primers	Probe
WSSV	5'-TGGTCCCGTCCTCATCTCAG-3' WSS1011F (TaqMan)	5'-GCTGCCTTGCCGGAAATTA-3' WSS1079R (TaqMan)	5'-AGCCATGAAGAATGC CGTCTATCACACA-3'
WSSV	5'-GATAAGAGAGGTAGACACT AGTAGTGTTATTGCT-3' 101-110F (SYBR-Green)	5'-CCACTGTGCCAGCTATTGCA 101-165R (SYBR-Green)	No probe used for SYBR-Green real-time PCR

4. Shrimple® testing of specimens

All 1,808 *L. setiferus* specimens were tested for WSSV using the Shrimple® test kits. Pleopoda from each individual were removed and tested in accordance with the manufacturer's protocol. Similarly, each of the 300 *C. sapidus* specimens were tested for WSSV using the kits. A gill from each individual was removed and tested.

5. DNA isolation

Various DNA extraction and isolation techniques were tested and compared to determine the optimal method for high throughput and for optimization of both genomic DNA quantity and quality. Total genomic DNA and associated viral DNA were extracted from 2 pleopoda per shrimp and 2 gills per crab using the Sprint Prep kit (Agencourt Bioscience; Beverly, MA) in accordance with the manufacturer's protocol. Tissue lysate (50 µl) was mixed with 80 µl isopropanol and 10 µl magnetic beads. The samples were placed on a 96-well magnetic plate where the DNA-bound beads were drawn out of suspension to the bottom of each well. The samples were then washed five times with 100 µl of cold 70% ethyl alcohol and allowed to air dry on the magnetic plate before being eluted in 50 µl TE. To ensure all DNA isolations were successful and yielded intact genomic DNA, sub-samples from the isolations were electrophoresed on a 0.8% agarose gel with ethidium bromide, and quantified on an ultraviolet spectrophotometer with a 96-well plate reader (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA) at A = 260/280nm . The mean concentration of isolated DNA per sample was 3.93 ng/µL +/- 2.13 ng/µl. When the real-time PCR protocol was changed from TaqMan chemistry to SYBR-Green real-chemistry, amplification of the endogenous gene, β-actin at 20+/- 2 CT was added to the reaction to confirm presence of intact genomic DNA.

6. Real time PCR validation and optimization

Durand and Lightner (2002) published a quantitative real-time PCR method for the measurement of WSSV in penaeid shrimp. In order to verify and optimize the established real-time PCR protocol, a series of reaction experiments were performed. A serial dilution of a WSSV plasmid of known concentration yielded a set of WSSV standards ranging from 1.0×10^{7} to 1.0×10^{-1}.

In addition to the established TaqMan chemistry based real-time PCR, a quantitative real-time PCR protocol based on SYBR-Green chemistry (Dhar, et al., 2001) was also used. The SYBR-Green quantitative PCR incorporates the use of an endogenous gene amplification rather than standards for quantification. DNA isolate from each specimen is amplified for both the WSSV target and for ß-actin, a general crustacean gene. Quantification of viral load in an individual specimen is possible by calculating the ratio of detection values for WSSV versus ß -actin.

To validate the PCR methods, a bioassay was performed on 25 wild caught *L. setiferus* to determine if experimental WSSV infections could be detected with real-time PCR and 1-step PCR using the DNA isolation method and PCR protocols adopted for this study. 5 *L. setiferus* were held in each of 5 recirculating aquaria. Specimens 1 – 5 were injected with 0.6mL 1xTN buffered saline and specimens 6 – 25 were injected with 0.6mL WSSV inoculum derived from the infected crab specimen SK040167-1 from the field survey portion of this study. Specimens spanning an early infection, post injection time course were collected, tested with Shrimple®, and pleopoda were removed for DNA isolation and subsequent PCR analysis. Throughout the experiment, dead and moribund shrimp were collected, tested, and analyzed, as well. Additionally, positive controls were established for *L. vannamei* and negative controls were derived from genomic DNA isolated from SPF *L. vannamei* reared in the biosecure husbandry facility.

7. PCR testing of specimens

Preliminary real-time PCR analysis was performed using the 7500 Sequence Detection Software v. 1.2.2. (Applied Biosystems; Foster City, CA). However, an upgraded model Applied Biosystems' 7500 Sequence Detection System was used for the rest of the trials. Taqman quantitative real-time PCR was performed using the Qiagen QuantiTect Probe PCR Kit (Qiagen, Inc.; Valencia, CA) according to the recommended procedure. Briefly, 2 ul samples (5-10 ng gDNA) from each DNA isolation were added to a PCR mixture containing 0.3 uM of each primer and 0.15 uM TaqMan probe into a final reaction volume of 25 ul. The amplification program consisted of 10 m at 95°C to activate the hot-start AmpliTaq Gold polymerase, followed by 40 cycles of 15 s denaturation and 60 s annealing/extension at 60°C. Plasmid DNA preps from specific WSSV template of known concentration were utilized as absolute standards. The instrument determined fluorescence at the end of each annealing/extension cycle. Background fluorescence was monitored using ROX dye as part of the reaction mixture, and all samples were normalized to the background level.

Sequences for PCR primers and TaqMan probe used for the detection of WSSV were obtained from a previously published study (Durand and Lightner, 2002). The primers (WSS1011F and WSS1079R) generated a 69-bp amplicon and were selected from a region of WSSV genomic sequence in GenBank U50923. The TaqMan probe was dual-labeled with fluorescent dyes, 5'-5-carboxyfluoroscein (FAM) and 3'-N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA). In each 96-well plate, a dilution series of the plasmid standard for WSSV was run along with the unknown samples.

Additionally, real-time PCR was performed using Sybr-Green chemistry and Qiagen QuantiTect Sybr-Green PCR Kits (Qiagen, Inc.; Valencia, CA) according to the recommended procedures. Sequences for Sybr-Green real-time PCR primers were obtained from a previously published study (Dhar, et al. 2001). The primers (101-110F and 101-165R) generated a 306-bp amplicon. Primers for an endogenous gene (178F and 228R) were designed based on a shrimp (*Penaeus monodon*) ß-actin sequence in GenBank AF100987. 2 ul samples (5-10 ng gDNA) from each DNA isolation were added to a PCR mixture containing 0.24 uM of each primer and 7.1 ul 2x QuantiTect SYBR- Green Master Mix into a final reaction volume of 25 ul. The amplification program consisted of 2m at 50°C and 10m at 95°C, followed by 40 cycles of 10 s at 95°C and 1m at 60°C. Each sample was replicated two times and was run with both WSSV and ß -actin primers. Any samples determined positive for WSSV were re-confirmed through subsequent real-time PCR analysis.

8. Further analysis and verification of screening results by real time and one step PCR analysis

To verify real-time PCR testing results, two sets of samples were retested to address verification and validation issues. The first sample set included (1) wild *L. setiferus* samples that tested negative, weak positive, and strong positive with Shrimple® and (2) laboratory infected *L. vannamei* collected at three time points after infection with WSSV, such that Shrimple® negative, weak positive, and strong positive reactions were included. The second sample set included (1) wild *L. setiferus* samples that tested negative, weak positive, and strong positive with Shrimple®, (2) samples from WSSV amplification in *L. setiferus* specimens, (3) laboratory infected *L. vannamei* collected at three time points after infection with WSSV, such that Shrimple® negative, weak positive, and strong positive reactions were included, (4) negative control SPF *L. vannamei*, and (5) positive control *L. vannamei*. The second of the two retested sample sets included an additional set of samples from laboratory infected *L. setiferus* collected at three time points after infection with WSSV, similar to the methodology applied to the collection of experimentally infected *L. vannamei* samples. All positive and negative controls amplified appropriately with all diagnostic methods. The sample sets were tested using real-time PCR—the first sample set with TaqMan chemistry and the second sample set with SYBR-Green chemistry. —and 1-step PCR analysis, using 3 different primer sets for WSSV.

In addition, one-step PCR (Chapman, et al., 2004) was used to further confirm and validate the findings obtained from the real-time PCR protocols. Samples were screened for genomic DNA using primer pairs - 143F/145R (Lo et al., 1996). WSSV detection was conducted using three primer sets previously developed by Lo et al. (1996), Kimura et al. (1996), and Wang & Lightner (NCBI accession number AF178573). A 25-ul PCR reaction volume contained 2 ul of template DNA, 1 ul buffer, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 uM of each primer, and 2.5 U Taq DNA polymerase. Thermocycle parameters used included an initial cycle of 94ºC for 4 min, 52ºC for 45s and 72°C for 3 min. This was followed by 34 cycles of 94, 52, and 72°C at 45s each, respectively. A 72ºC, 30 min extension phase concluded the thermocycle. Amplification products were

separated in a 0.8% analytical agarose gel with ethidium bromide. The one-step PCR protocols were the same as those used in a previous SK-funded WSSV survey (Chapman, et al. 2004).

The first sample set was screened across three dilutions of genomic DNA isolate to determine if there was a dilution effect, either with too little or too much DNA. All reactions in the SYBR Green sample set and 1-step PCR sets were run with a 1:10 dilution of genomic DNA.

9. Bioassay testing

Field collected shrimp and crab samples suspected to be WSSV-infected were further analyzed by performing bioassays on SPF *L. vannamei* with inoculum prepared from the samples following the protocol developed by Prior, et al. (2003). SPF *L. vannamei* were obtained from the Oceanic Institute (U.S. Marine Shrimp Farming Program; Kailua-Kona, HI) as post-larvae and reared in an indoor, environmentally-controlled, biosecure husbandry facility at the SCDNR campus in Charleston, SC. Water quality parameters were monitored regularly and adjusted to maintain optimal conditions.

Experimental infections were carried out in a biosecure, environmentally-controlled challenge laboratory (27°C, 12L:12D photoperiod) in a recirculating water system with 25 19-liter polypropylene aquaria, filled with 3 liters of polished seawater. The system is equipped with a sump, a series of biofiltration units, and an ultraviolet disinfection unit. A total of 15 shrimp (1.0 – 1.5 g) were stocked at a density of 15 animals per aquarium and injected with viral or control inoculum at a volume of 0.02mL g^{-1}. Test animals were fed with one pellet of a commercial shrimp grower diet per shrimp per day.

10. External laboratory testing and verification

In order to verify results obtained from internal real-time PCR and one-step PCR testing, specimens were sent to two separate external laboratories: Texas A&M Veterinary Medical Diagnostic Laboratory System and University of Arizona Veterinary Science and Microbiology Laboratory. Twelve *L. setiferus* specimens, which had been previously tested and determined negative for WSSV using both real-time and one-step PCR but determined positive for WSSV using Shrimple® test kits were submitted to each laboratory.

11. Molecular sequencing

One-step PCR was performed on genomic DNA isolated from the crab specimen confirmed to be infected by WSSV by Shrimple®, PCR, and *in vivo* bioassay. Established WSSV primers and thermocycling parameters were used (Lo, et al. 1996). The PCR product was electrophoresed and bands were cut from the agarose gel, purified (QIAquick Gel Extraction Kit, Qiagen Inc.), and submitted to an external laboratory for molecular sequencing. The results from the molecular sequencing were compared to WSSV DNA sequence stored in the National Center for Biotechnology Information

(NCBI) BLAST search website. In addition, the sequences were aligned against a known positive control for WSSV using the European Bioinformatics Institute ClustalW Sequence Analysis program.

12. Educational outreach

One of the main objectives of this project is to promote awareness by working with commercial shrimpers, about the commercial implications and ecological significance of shrimp and blue crab diseases. Involvement of commercial fishermen in this project aimed to help develop their awareness with respect to the importance of viral diseases and their relationship to shrimp fishery and environmental quality.

Pamphlets detailing the objectives and methodology of the project, implications of WSSV, and the importance of shrimpers' participation in research were developed and distributed to members of the commercial shrimping industry in South Carolina, Georgia, and Florida. On-board and dockside training in disease diagnosis using the Shrimple test kits was made.

B. Project management and oversight

Overall project oversight was provided by Dr. Craig L. Browdy. Supervision of project methodology and molecular laboratory components was provided by Dr. Eleanor F. Shepard. Coordination of research collaborators, collection of specimens, sample preparation and analyses, and bioassays were performed by James Powell with assistance from Caroline Payne, Delilah Arrington, Michelle Pate, Tanya Polon, Selena Kirby, and Justin Yost. Dr. Erin J. Burge, Dr. Robert Chapman, and Dr. Arun Dhar provided training, supervision, and oversight of the PCR components of the study.

This research was made possible by a collaborative effort among the following state agencies and members of the commercial fishing industry: SCDNR's Crustacean Management Survey team (Larry Delancey, Jimmy Jenkins, and Mark Maddox), FL MRRI - Fisheries Independent Study (Russ Brodie), GA-DNR (Dwayne Roberson), UGA-MES (Carolyn Belcher), and commercial fishermen (Mr. Fred Dockery and Mr. Tommy Edwards).

VI. Findings

1. Site selection and research collaboration

The 1,808 *L. setiferus* specimens obtained during the course of this study were collected by research collaborators in South Carolina, Georgia, and Florida (Figure 3) and the 300 *C. sapidus* specimens from Charleston, South Carolina and Brunswick, Georgia (Figure 4). The majority of the crab and shrimp specimens were collected from South Carolina, where our research collaborators were both more numerous and available for more periodic sample collection. South Carolina shrimp samples were primarily collected

from the Charleston Harbor, Georgia shrimp from numerous rivers and creeks in Brunswick, Georgia, and Florida samples from the Jacksonville area. In South Carolina, other collection sites included: Bull's Bay, Port Royal Sound, Edisto River, and St. Helena Sound.

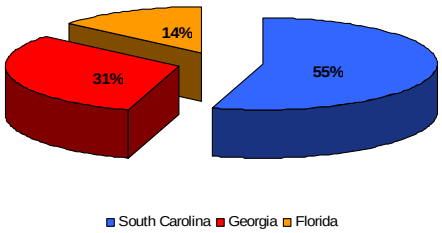


Figure 3. Distribution of shrimp specimens among collection states (n=1,808)

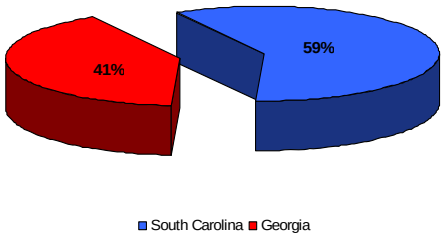


Figure 4. Distribution of crab specimens between collection states (n=300)

Because a big part of the current study is to direct particular reference to reproductive populations and its possible influence on WSSV infection in the wild, a significant percentage of the shrimp and crabs collected targeted reproductively active females (Figures 5 and 6).

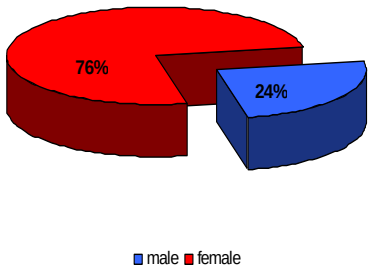


Figure 5. Sex distribution of shrimp specimens (n=1,808)

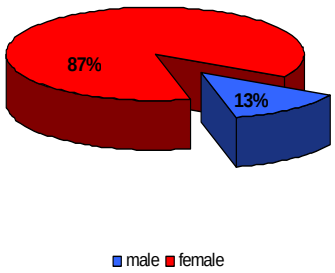


Figure 6. Sex distribution of crab specimens (n=300)

2. Specimen collection and storage

Samples were collected with specific regard to developmental stage (Figures 7, 8 9, and 10), targeting females undergoing increasing reproductive stress to determine the potential for WSSV amplification during the rigorous ovarian developmental stages.

Surveys of shrimp populations follow an established categorization of reproductive development that allows researchers to delineate the distribution of reproductive stages

observed in any given sample set. Stages 1 through 5 characterize increasing ovarian development from Stage 1, undeveloped, through Stage 5, spent female. A Stage 4 female is in the most progressive stage of reproductive development and often exhibits a deep, brownish green color in her ovaries. A Stage 3 female is vitellogenic exhibiting a bright yellow or orange colored ovary. A stage 2 female demonstrates the earliest visible signs of ovarian development. Male reproductive development stages begin with a Stage 6 male, which is undeveloped and exhibits separate, unfused patasmids, and progresses to a Stage 9 where spermatophores are fully developed. Stage 0 describes a fully developed male who has just released its developed spermatophore and has yet to develop a new set. A Stage 7 male is characterized by fused patasmids but no appearance of spermatophore development. At Stage 8, male shrimp have fully fused patasmids and spermatophores are becoming visible on the anterior-ventral area of the body.

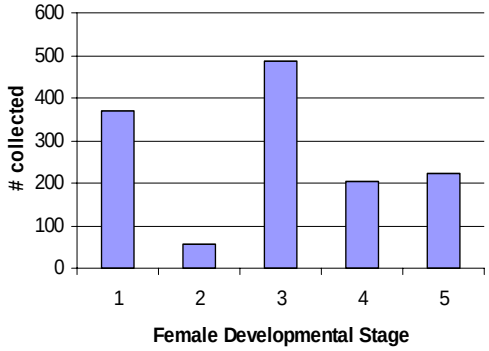


Figure 7. Distribution of female *L. setiferus* collected across developmental stages (n=1,368)

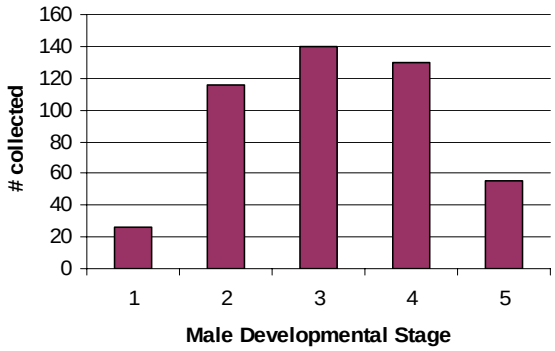


Figure 8. Distribution of male *L. setiferus* collected across developmental stages (n=440)

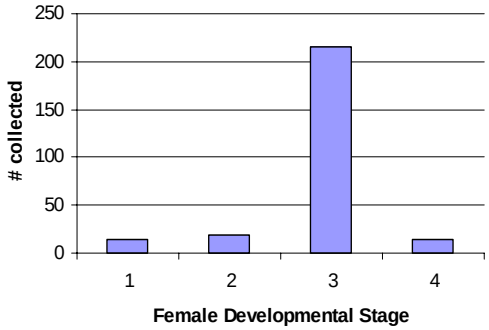


Figure 9. Distribution of female *C. sapidus* collected across developmental stages (n=263)

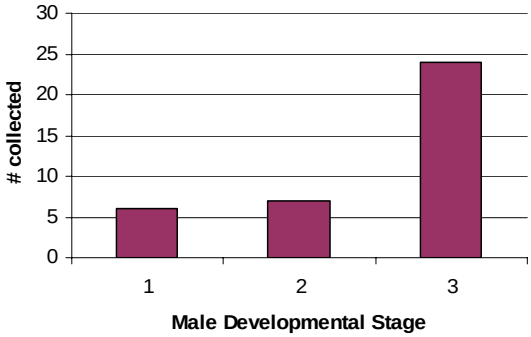
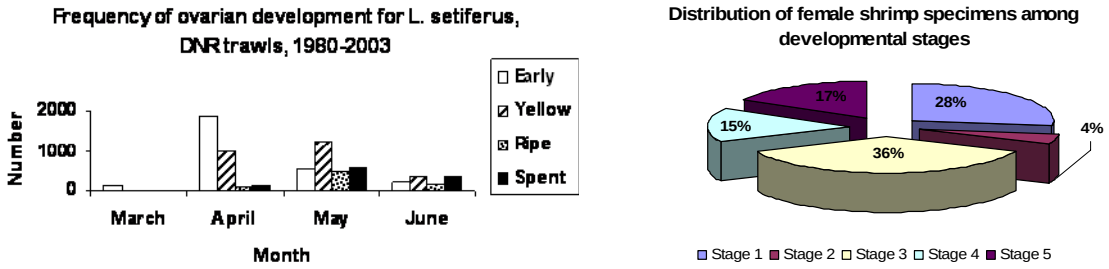


Figure 10. Distribution of male *C. sapidus* collected across developmental stages (n=37).

Targeting specific individuals at ovarian specific developmental stages is constrained by the natural distribution of females within a population at any given time, and the distribution of males and females during collections. Efforts were made by collaborators to collect more females than males. Collection was intensified during the most active part of the reproductive season (Figure 11). Historical data has demonstrated that mature

crabs are available throughout the year, with peaks from March through December and collections for this study were carried out during these months.



(A) (B)

Figure 11. (A) Frequency of ovarian development observed in *L. setiferus* collected during monthly trawl surveys performed by the South Carolina Department of Natural Resources – Crustacean Management Unit from 1980 through 2003. (B) Frequency of ovarian development observed in *L. setiferus* collected during the course of this study.

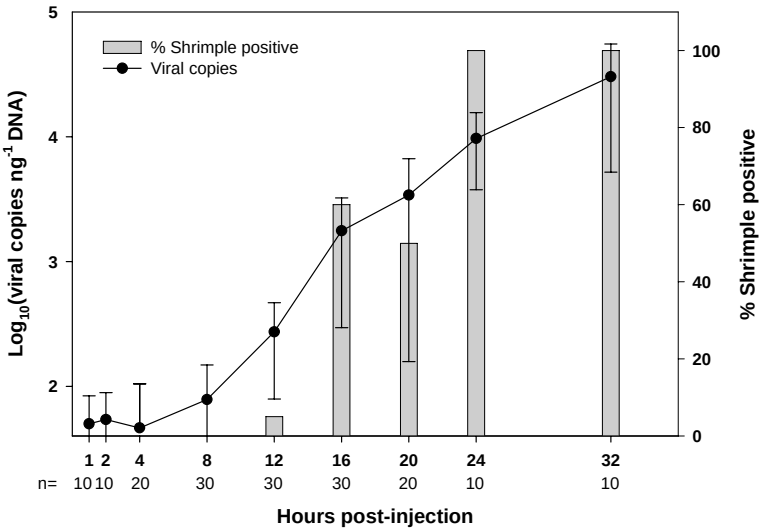


Figure 12. Quantification of WSSV copy number, measured by real-time PCR, at various time points over the course of the bioassay compared to the percentage of positive Shrimple® test results for WSSV. All specimens were positive for WSSV as determined by real-time PCR. No Shrimple® positive test results were obtained from specimens prior to 12h p.i., but all specimens were positive by 24h p.i. Sample sizes are indicated below the x-axis.

3. Shrimple® test kit validation

A time-course experiment was conducted to test the reliability and precision of the Shrimple® test kit in detecting viral infection. From one hour after injection of viral inoculum to 32 hours p.i., 100% (122 of 122) of the specimens tested positive for WSSV by real-time PCR, despite displaying no gross signs of the disease. Negative control specimens (20 of 20) tested negative for WSSV with both real-time PCR and Shrimple®

test kits. From 1 to 8 hours p.i., none of the specimens tested positive with Shrimple®, however, at 12 hours p.i. 5.26% (1 of 30) tested positive, increasing over time to 100% positive by 24 hours p.i. (Figure 12). At 8 hours p.i., 1 of 19 Shrimple® tests resulted in an ambiguous, faint band in the T-zone. This faint band was qualitatively different from the negative controls but was not consistent with a true positive test as it did not result in a discernable colored band. By 12 hours p.i., these faint bands comprised the majority of the test results (15 of 19); by 16 hours p.i., 60% of the Shrimple® tests were unambiguously positive (18 of 30).

Samples that tested negative with Shrimple® (43 of 122), but positive with real-time PCR, yielded 4 to 1061 viral copies/ng genomic DNA with an average of 126.16 +/- 220.80 viral copies/ng genomic DNA (Figure 13). Shrimple® test results classified as faint were determined by real-time PCR to contain 36 to 1784 viral copies/ng genomic DNA, or an average of 613.65 +/- 551.42. Higher infection levels gave a strong positive result. A one-way ANOVA test indicated that the three groups (Shrimple® negative, faint, and positive) were significantly different from one another (at $P < 0.001$).

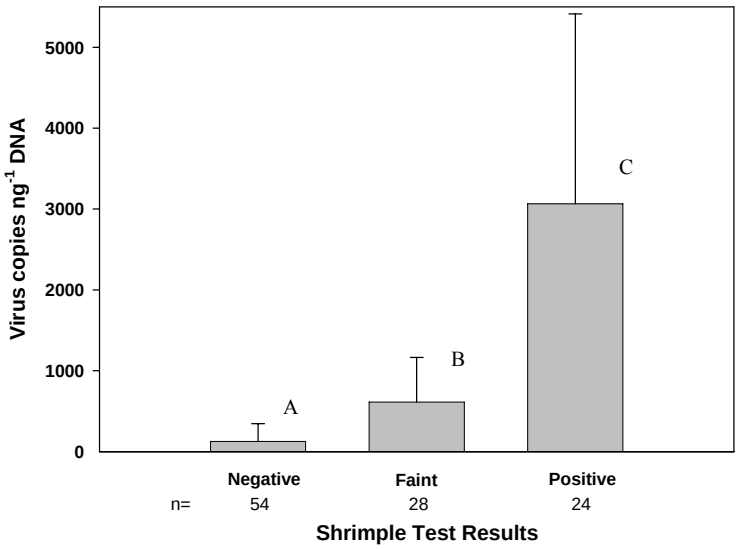


Figure 13. Average viral copies/ng genomic DNA for specimens with negative, faint, and positive Shrimple® test results (1-24 hours post injection). Test results reported as faint were characterized by an apparent, light-grey colored band not present with negative test results. However, the band was not as distinct as that for positive test results. Statistical analysis showed a significant difference between treatments ($P<0.001$).

EnBioTec Laboratories designed the Shrimple® diagnostic test kit to detect WSSV in aquaculture-reared shrimp. The primary concern with such a tool is the potential for erroneous results; i.e., false negative results, allowing the virus the opportunity to manifest into devastating mass mortalities, whereas false positive results may lead to shrimp farmers performing unnecessary rapid harvests resulting in tremendous economic losses.

All of the negative controls were confirmed virus negative by both diagnostic assays. No false positives were found. During the early onset of infection (from 1 to 8 hours p.i.),

Shrimple® test kits failed to detect WSSV infections, whereas 100% of the same specimens were determined positive for WSSV with real-time PCR. Throughout the entire study 34.7% of the specimens tested were determined positive for WSSV using Shrimple® test kits compared to 100% with real-time PCR. Real-time PCR is more sensitive, and will detect WSSV infection earlier, than the diagnostic test kits; however, Shrimple® provides detection of viral presence prior to gross anatomical signs of infection and prior to mortality, which in reality, gives ample time to apply loss preventing measures.

The present research showed that Shrimple® test kits are sensitive enough to detect a relatively low-level infection in *L. vannamei*, prior to gross anatomical evidence of disease. The level at which the Shrimple® test kit is capable of detecting viral infection varies. While a faint Shrimple® band measured as low as 36 viral copies/ng genomic DNA, the lowest true chromatographic positive result was observed at 356 viral copies/ng genomic DNA. Disparity in the sensitivity of the test kit exists in that some specimens that tested negative with Shrimple® were determined to have infection levels as high as 1,098 viral copies/ng genomic DNA.

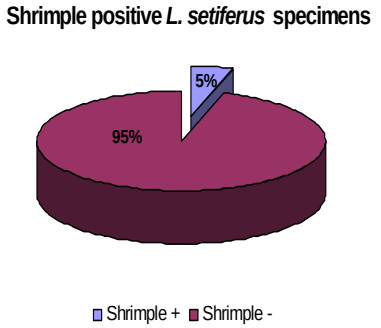


Figure 14. Frequency of Shrimple® positive reactions in *white shrimp L. setiferus*

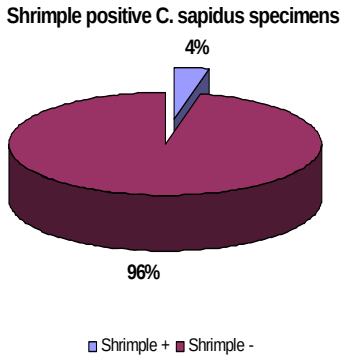


Figure 15. Frequency of Shrimple® positive reactions in blue crab *C. sapidus*

4. Shrimple® testing of specimens

A significant number of white shrimp and blue crabs were found to react positively with the Shrimple® test kits. The positive bands ranged in intensity from weak to strong. Figures 14 and 15 show the breakdown of Shrimple® positive samples for both *L. setiferus* and *C. sapidus*. Note that only one of these samples was subsequently confirmed WSSV-positive by PCR. Similarly, only the PCR positive crab sample was confirmed positive by *in vivo* bioassay. Nevertheless, an analysis of the distribution of the Shrimple® positive shrimp and crabs are reported here to provide some insight into the nature of these apparently non-specific reactions. Approximately 4.8% of *L. setiferus* (Figure 14) and 3.8% of *C. sapidus* specimens (Figure 15) were determined to be Shrimple® positive. Statistically, there is no significant difference in the percentage of Shrimple® positive reactions between male and female *L. setiferus* (Figure 16); however, there were significantly more Shrimple® positive reactions (at $P<0.05$) among female crabs *C. sapidus* (Figure 17).

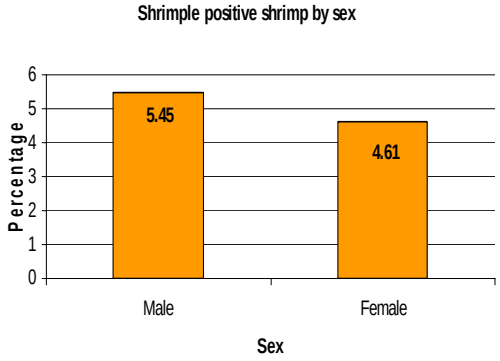


Figure 16. Percentages of male and female shrimp determined Shrimple® positive

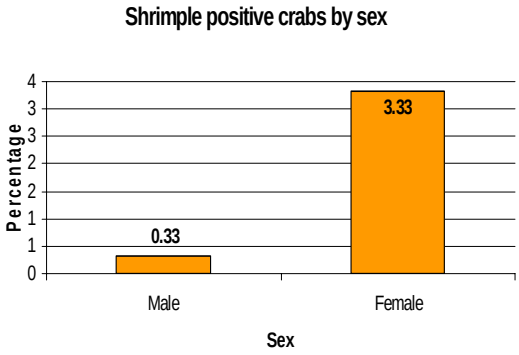


Figure 17. Percentages of male and female crabs determined Shrimple® positive

Complete summaries of specimens that were determined Shrimple® positive are available in the Tables 2, 3, and 4 below. Each table summarizes the individual specimens, including the sex and developmental stage of the specimen, as well as the state in which the animal was collected.

Table 2. Summary of Shrimple® positive female *L. setiferus* specimens collected.

Specimen	Sex	Development Stage	State		Specimen	Sex	Development Stage	State
040025-2-1	F	1	SC		040155-8-1	F	1	GA
040036-6-1	F	1	GA		040155-83-1	F	1	GA
040044-20-1	F	1	GA		040042-19-1	F	3	GA
040044-2-1	F	1	GA		040042-24-1	F	3	GA
040044-27-1	F	1	GA		040042-4-1	F	3	GA
040044-29-1	F	1	GA		040072-15-1	F	3	GA
040046-7-1	F	1	SC		040072-21-1	F	3	GA
040048-1-1	F	1	SC		040084-6-1	F	3	SC
040056-10-1	F	1	SC		040114-1-1	F	3	SC
040056-1-1	F	1	SC		040144-11-1	F	3	FL
040056-13-1	F	1	SC		040152-12-1	F	3	FL
040056-2-1	F	1	SC		040164-8-1	F	3	GA
040056-3-1	F	1	SC		040079-22-1	F	4	SC
040056-6-1	F	1	SC		040145-22-1	F	4	FL
040067-16-1	F	1	SC		040152-50-1	F	4	FL
040067-17-1	F	1	SC		040163-2-1	F	4	SC
040067-20-1	F	1	SC		040050-55-1	F	5	SC
040067-9-1	F	1	SC		040050-67-1	F	5	SC
040068-11-1	F	1	SC		040050-71-1	F	5	SC
040068-6-1	F	1	SC		040051-45-1	F	5	SC
040069-9-1	F	1	GA		040077-1-1	F	5	SC

040072-24-1	F	1	GA		040077-14-1	F	5	SC
040077-13-1	F	1	SC		040077-17-1	F	5	SC
040110-20-1	F	1	SC		040077-19-1	F	5	SC
040112-7-1	F	1	SC		040077-2-1	F	5	SC
040148-29-1	F	1	GA		040077-23-1	F	5	SC
040149-40-1	F	1	FL		040077-24-1	F	5	SC
040155-6-1	F	1	GA		040077-25-1	F	5	SC
040155-65-1	F	1	GA		040077-4-1	F	5	SC
040148-47-1	F	5	GA		040088-10-1	F	5	SC
040159-2-1	F	5	SC					

Table 3. Summary of Shrimple® positive male *L. setiferus* specimens collected.

Specimen	Sex	Stage	State		Specimen	Sex	Stage	State
040076-13-1	M	6	SC		040155-71-1	M	7	GA
040077-20-1	M	6	SC		040155-76-1	M	7	GA
040163-10-1	M	6	SC		040043-12-1	M	8	SC
040036-2-1	M	7	GA		040043-5-1	M	8	SC
040056-15-1	M	7	SC		040043-7-1	M	8	SC
040072-28-1	M	7	GA		040048-16-1	M	8	SC
040074-12-1	M	7	GA		040050-82-1	M	8	SC
040112-5-1	M	7	SC		040082-2-1	M	8	SC
040148-39-1	M	7	GA		040051-50-1	M	9	SC
040155-42-1	M	7	GA		040051-51-1	M	9	SC
040155-43-1	M	7	GA		040079-13-1	M	9	SC
040049-17-1	M	0	SC		040049-14-1	M	0	SC
040049-3-1	M	0	SC		040049-15-1	M	0	SC

Table 4. Summary of Shrimple® positive *C. sapidus* specimens collected.

Specimen	Sex	Stage	State
040032-1-6	F	4	GA
040032-7-6	F	2	GA
040058-3-6	F	3	GA
040059-3-6	M	8	GA
040065-15-6	F	3	GA
040065-1-6	F	3	GA
040065-5-6	F	3	GA
040065-6-6	F	3	GA
040095-4-6	F	3	SC
040100-13-6	F	3	SC
040167-1-6	F	3	SC

Frequency of Shrimple® positive samples for *L. setiferus* decreased as water temperature increased into June and July, with the highest rates of infection detected in March, April, September, and October (Figures 18 and 19). These findings agree with expectations for WSSV infection in that previous studies that have demonstrated that hyperthermia can actually induce protection from WSSV in *L. vannamei* (Vidal, et al., 2001). Shrimp reared in temperatures greater than 31ºC demonstrated a significantly reduced susceptibility to WSSV than those reared in cooler temperatures. Thus the results obtained from the Shrimple test kits fits the expected viral infection prevalence trends. Applying previous findings to the current study, one would expect to see a decrease in WSSV infection rates in the months from March to August when the bottom water temperature is increasing, with lowest incidence in June and July when bottom water temperature is at its highest. No temporal relationship was apparent for the crab samples (Figure 19).

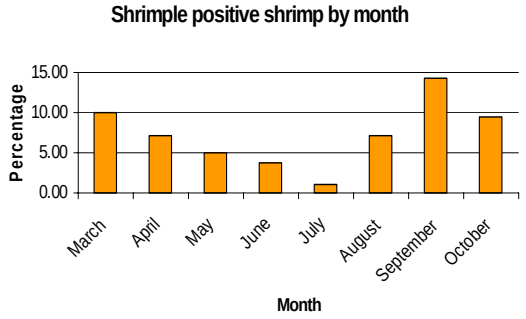


Figure 18. *L. setiferus* Shrimple® positive test results across months

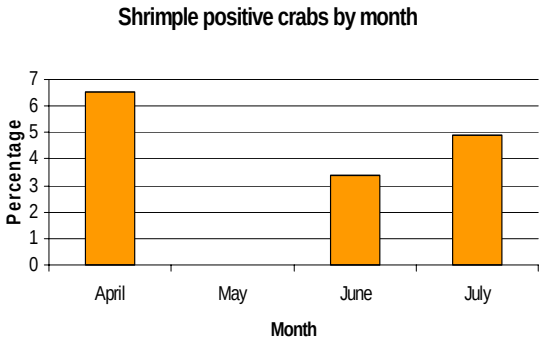


Figure 19. *C. sapidus* Shrimple® positive test results across months

Geographical variation in Shrimple® positive samples for *L. setiferus* were not significantly different between South Carolina and Georgia but were significant between South Carolina and Florida, as well as Georgia and Florida (Figure 20). Although sample sizes were small, results suggest a possible negative correlation between temperature and frequency of shrimp that test positive with the Shrimple® kits. Geographical variation in Shrimple® positive samples for *C. sapidus* was observed when comparing the specimens from Charleston, South Carolina, versus those from Brunswick, Georgia (Figure 21).

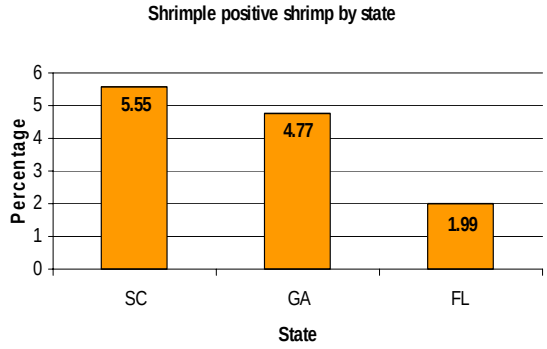


Figure 20. Variation in Shrimple® positive rates observed in shrimp, among states.

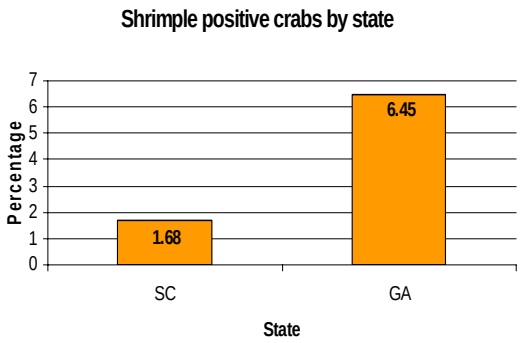


Figure 21.Variation in Shrimple® positive rates observed in crabs, between states.

5. DNA isolation

A subset of *L. setiferus* pleopoda were randomly selected to optimize the efficiency DNA extraction methods. High throughput capabilities as well as improved quality and quantity of DNA was obtained via the magnetic bead protocol compared to other DNA extraction methods tested. Phenol-chloroform isolation (PCI) and DNAzol® were selected as comparative methods. The quantity of DNA obtained via magnetic bead isolation exceeded the quantity of DNA obtained from PCI and DNAzol®. Additionally, the methodology of DNA isolations via magnetic beads is significantly more time efficient and does not involve using hazardous chemicals as required in PCI. Isolation with sarcosyl-urea affords researchers the ability to lyse tissues using incubation (at 70ºC) rather than manual homogenization, thereby increasing efficiency in high throughput applications. The quality of DNA isolated across the three methods was comparable.

6. Real time PCR validation and optimization

Established techniques using TaqMan real-time PCR (Durand and Lightner, 2002) and SYBR-green real-time PCR (Dhar, et al, 2001) to detect WSSV in penaeid shrimp were utilized to ensure that the protocols described would be compatible with the genomic DNA isolation methods used for this study. A WSSV plasmid of known viral concentration, 4.0×10^{7}, was obtained and diluted to establish a series of known concentrations to develop a standard curve (Figure 22) of viral concentration.

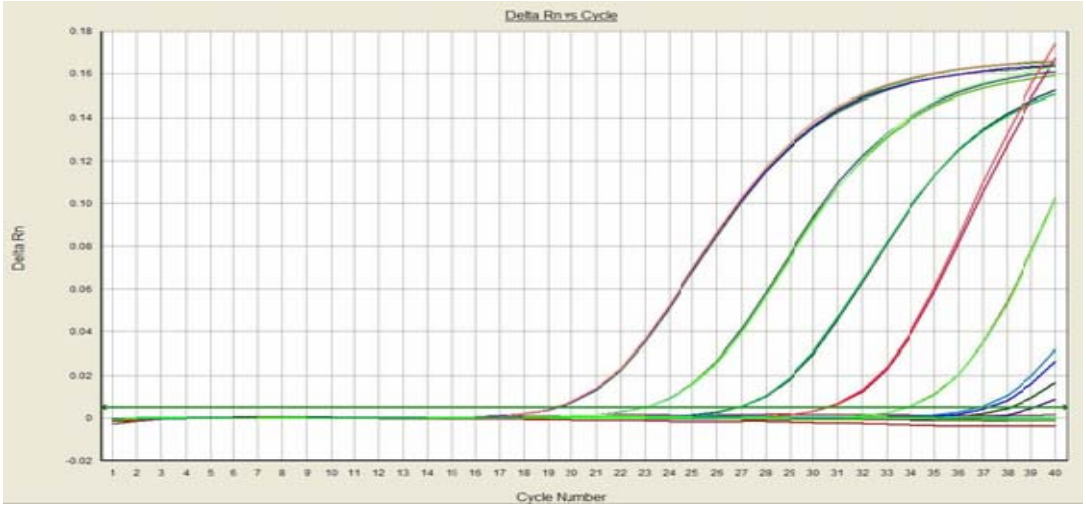


Figure 22. WSSV Standards from 1×10^{7} to 1×10^{1}. Standard curves were generated by correlating individual standard template concentration to a given CT-value (the point where the curve crosses the horizontal threshold line). Unknown sample concentrations can then be estimated comparing its CT-value to the standards curve.

Results of the bioassay performed on wild caught *L. setiferus* confirmed that injection of the WSSV inoculum caused mortality, while injection of the same volume 1xTN buffered

saline caused no mortality. Samples were taken at early time course points: 4 hours, 8 hours, and 16 hours post injection and from all dead and moribund shrimp throughout the experiment. Table 5 shows a summary of details the specimens tested and PCR results obtained for the preliminary bioassay experiment.

Real-time PCR, 1-step PCR, and Shrimple® failed to detect early WSSV infections (4 hours p.i.). From 8 hours p.i. onward, detection by the various methods is similar, even when comparing the signal from both Shrimple® and 1-step PCR. WSSV is capable of causing mortality in experimentally infected *L. setiferus* specimens and the DNA isolation techniques as well as the PCR protocols adopted for this project are both capable of detecting viral infections as well as adequate for such applications.

Table 5. Diagnostic test results for experimentally WSSV infected *L. setiferus*.

Shrimp #	Treatment	Sample collected	Shrimple®	1-step PCR	Real-time PCR
1	Saline inj.	4 days p.i.	-	-	-
2	Saline inj.	4 days p.i.	-	-	-
3	Saline inj.	4 days p.i.	-	NA	NA
4	Saline inj.	4 days p.i.	-	NA	NA
5	Saline inj.	4 days p.i.	-	NA	NA
6	WSSV inj.	4 hours p.i.	-	-	-
7	WSSV inj.	4 hours p.i.	-	-	-
8	WSSV inj.	8 hours p.i.	Faint	Weak +	+
9	WSSV inj.	8 hours p.i.	+	+	+
10	WSSV inj.	16 hours p.i.	+	+	+
11	WSSV inj.	16 hours p.i.	+	+	+
12	WSSV inj.	2 days p.i.	+	NA	NA
13	WSSV inj.	2 days p.i.	+	NA	NA
14	WSSV inj.	2 days p.i.	+	NA	NA
15	WSSV inj.	2 days p.i.	+	NA	NA
16	WSSV inj.	3 days p.i.	+	NA	NA
17	WSSV inj.	3 days p.i.	+	+	+
18	WSSV inj.	3 days p.i.	+	NA	NA
19	WSSV inj.	4 days p.i.	+	NA	NA
20	WSSV inj.	4 days p.i.	+	NA	NA
21	WSSV inj.	4 days p.i.	+	NA	NA

To further validate the PCR methods and test for non-specific inhibition, a series of experiments were run with DNA isolated from known infected and control populations as follows. First, standard curves were generated by correlating individual standard template concentration to a given CT-value (the point where the curve crosses the horizontal threshold line). Unknown sample concentrations could then be estimated comparing its CT-value to the standard curves. Next, amplification for laboratory infected WSSV positive control L. vannamei were compared with similar samples which had been spiked with DNA from several sources: 1. from negative control L. vannamei, 2. from L. setiferus, and 3. from common mummichog, *Fundulus heteroclitus*.

When WSSV positive controls were spiked with DNA from SPF *L. vannamei* (Fig. 24) and from the common mummichog, *Fundulus heteroclitus* (Fig. 25), no significant inhibitions were observed. However, different levels of inhibition were observed when WSSV-positive controls were spiked with DNA from wild caught *L. setiferus* (Fig. 23*)*.

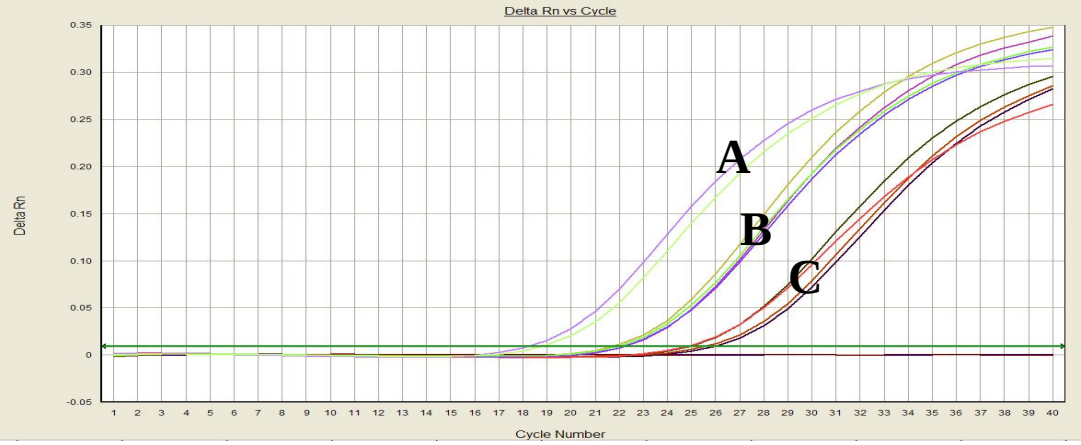


Figure 23. Real-time PCR inhibition exhibited by spiking WSSV positive control template with DNA from wild-caught *L. setiferus* specimens. A = WSSV positive control, B = WSSV positive control spiked with DNA from 2 different *L. setiferus* specimens, C = WSSV positive control spiked with DNA from 2 different *L. setiferus* specimens.

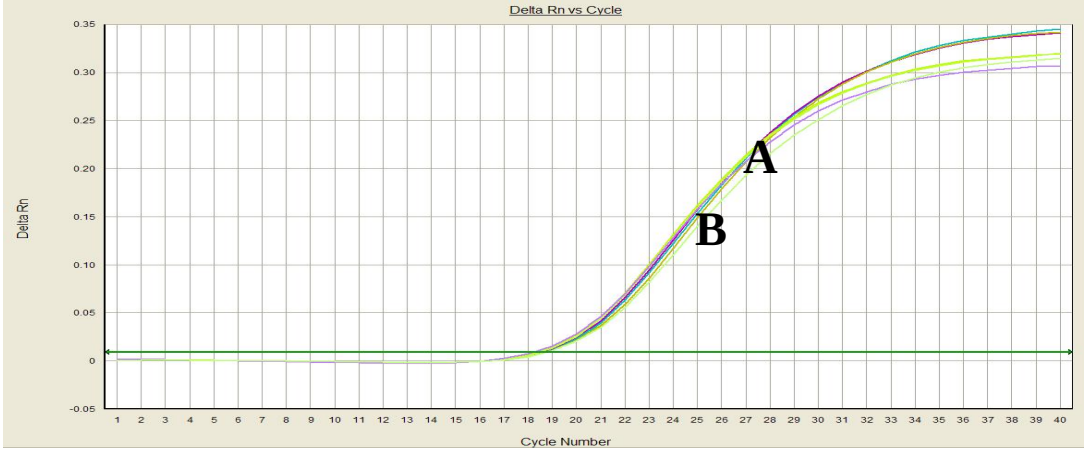


Figure 24. Real-time PCR reactions of spiked WSSV positive control template with DNA from specific pathogen free *L. vannamei* specimens. A = WSSV positive control, B = WSSV positive control spiked with *L. vannamei* DNA. Note that there is no difference between the two reactions. All amplifications have the same CT value (~18.5).

It is possible that the wild *L. setiferus* genomic DNA contain impurities or unacceptable levels of protein:nucleic acid ratio that hinder the real-time PCR amplification. Further research is necessary to fully investigate this potential inhibition factor and its effects on the ability to accurately screen wild specimens by PCR-based detection. To overcome this potential problem, isolated genomic DNA was diluted to one-tenth its initial concentration. The inhibition was reduced significantly while still allowing the reaction

to proceed (Figure 26). In a separate study, a similar, inexplicable real-time PCR inhibition was exhibited while detecting hepatitis A virus in seawater (Brooks and Dhar, 2005).

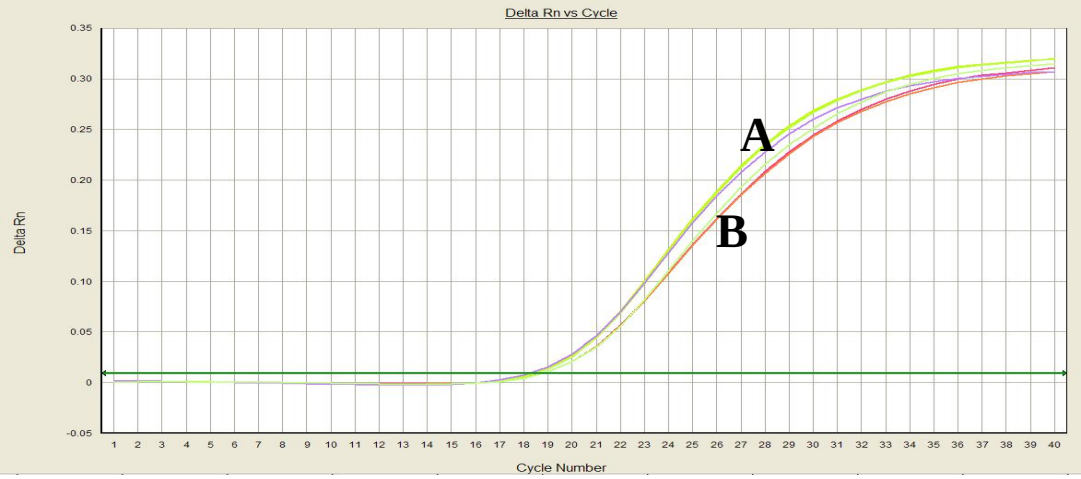


Figure 25. Real-time PCR reactions of spiked WSSV positive control template with DNA from the common mummichog, *Fundulus heteroclitus*. A = WSSV positive control, B = WSSV positive control spiked with *F. heteroclitus* DNA. Note that there is no difference in the two reactions, all amplifications have the same CT value (~18.5).

The dilution approach was adopted for analysis of the unknown samples as described below. To assure the quality of these analyses, endogenous gene reactions were included assuring proper amplification of DNA from each sample.

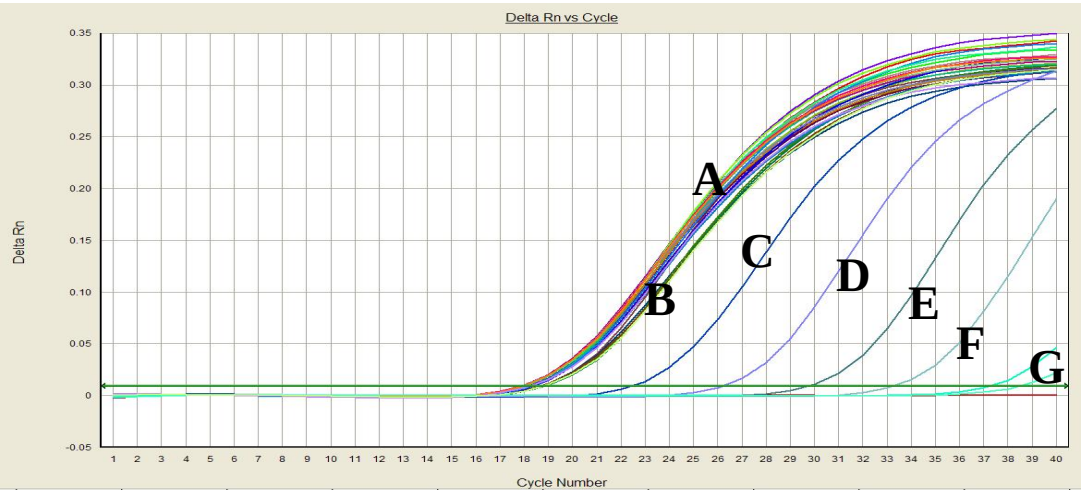


Figure 26. Real-time PCR inhibition exhibited by spiking WSSV positive control template with a 1:10 dilution of DNA from wild-caught *L. setiferus* specimens. A = WSSV positive control, B = WSSV positive control spiked with DNA from 4 different *L. setiferus* specimens diluted 1:10; C → G = standards 10^6 through 10^ 2.

7. PCR testing of specimens

Real-time PCR was performed on DNA isolated from 228 specimens. Specimens selected for real-time PCR testing included all shrimp and crab specimens that tested positive using Shrimple®. In addition, a representative sample of specimens that tested negative for WSSV was included as negative controls. All real-time PCR reactions were run in duplicates with primers for the target WSSV sequence and an endogenous gene, β-actin. Of all specimens tested, only one crab sample (SK040167-1) was determined positive for WSSV using real-time PCR. For confirmation, DNA was re-isolated from specimen SK040167-1 and subsequent real-time PCR reactions were performed, yielding supporting results.

Specimen SK040167-1 was a mature, female *C. sapidus* collected in Charleston, South Carolina. The sample tested positive for WSSV using Shrimple® diagnostic test kits, real-time PCR, and one-step PCR.

8. Further analysis and verification of screening results by real time and one step PCR analysis

None of the Shrimple® positive wild caught *L. setiferus* specimens were determined PCR positive by any of the methods or DNA concentrations tested. Using a 1:10 dilution of the genomic DNA, confirmatory amplification was observed in positive and negative controls as well as experimentally infected *L. vannamei* and *L. setiferus* that span the possible Shrimple® outcomes (Table 6). The DNA isolation protocol used for extracting genomic DNA from the selected specimens and the selected real-time and 1-step PCR protocols are adequate and acceptable for use in screening all specimens collected for this study.

Table 6. Summary of verification testing results with real time and one step PCR analysis.

	Species	DNA concentration	# Tested	# qPCR TaqMan +	#qPCR SYBR-Green +	# 1-step PCR +
Wild caught						
Shrimple® –	*L. setiferus*	Full strength	5	0	0	0
Shrimple® faint	*L. setiferus*	Full strength	5	0	0	0
Shrimple® +	*L. setiferus*	Full strength	30	0	0	0
Shrimple® –	*L. setiferus*	1:100	5	0	0	0
Shrimple® faint	*L. setiferus*	1:100	5	0	0	0
Shrimple® +	*L. setiferus*	1:100	30	0	0	0
Shrimple® –	*L. setiferus*	1:10	5	0	0	0
Shrimple® faint	*L. setiferus*	1:10	5	0	0	0
Shrimple® +	*L. setiferus*	1:10	30	0	0	0
Lab infected						
Shrimple® –	*L. vannamei*	1:10	2	2	2	2
Shrimple® faint	*L. vannamei*	1:10	1	1	1	1
Shrimple® +	*L. vannamei*	1:10	2	2	2	2
Shrimple® –	*L. setiferus*	1:10	4	NA	0	0
Shrimple® faint	*L. setiferus*	1:10	1	NA	1	1
Shrimple® +	*L. setiferus*	1:10	4	NA	4	4

9. Bioassay testing

Bioassays were performed using all the Shrimple® positive samples i.e., 87 shrimp and 11 blue crab specimens or a total of 98 samples. Bioassays were performed in a recirculating water system, each consisting of 25 aquaria. Each bioassay included replicated 10 treatments (each treatment representing 1 Shrimple® positive specimen), 2 positive control tanks, 2 negative control tanks, and 1 tank with uninjected animals held for water quality control. A total of 15 one-gram *L. vannamei* were used per replicate where 1 aquarium represents a replicate. In addition to the 98 Shrimple® positive specimens tested, bioassays on randomly selected Shrimple® negative specimens were also performed giving a total of 140 samples tested. Of these, only one resulted in a cumulative mortality similar to the positive control (SK040167-1). It was the same blue crab specimen that tested positive for WSSV with Shrimple® and real-time PCR. DNA was isolated from pleopod samples collected from moribund individuals in the bioassay and were determined positive for WSSV using real-time PCR. Furthermore, Shrimple® tests were performed on each moribund or freshly dead individual collected during from the bioassay. Each specimen tested positive for WSSV (Table 7) with an increasing chromatographic response over time. Figure 27 below depicts the cumulative mortality of SK040167-1 (black) in comparison to the positive control (red).

10. External laboratory testing and verification

Dr. Ken Hasson's research group at Texas A&M Veterinary Diagnostic Laboratory and Dr. Donald Lightner's laboratory at the University of Arizona Microbiology Department

Table 7. Specimens collected and tested for WSSV during the bioassay using inocula from SK040167-1.

Sample number	Hours post injection	Shrimple® result	Real-time PCR	Real-time PCR CT
1	12	Faint	Positive	29.99
2	24	Faint	Positive	31.01
3	24	Faint	Positive	32
4	48	Faint	Positive	30.24
5	48	Positive	Positive	29.35
6	48	Positive	Positive	29.22
7	72	Positive	Positive	28.59
8	72	Positive	Positive	28.81
9	72	Positive	Positive	29.9
10	96	Positive	Positive	28.99
11	96	Positive	Positive	26.49
12	96	Positive	Positive	27.48

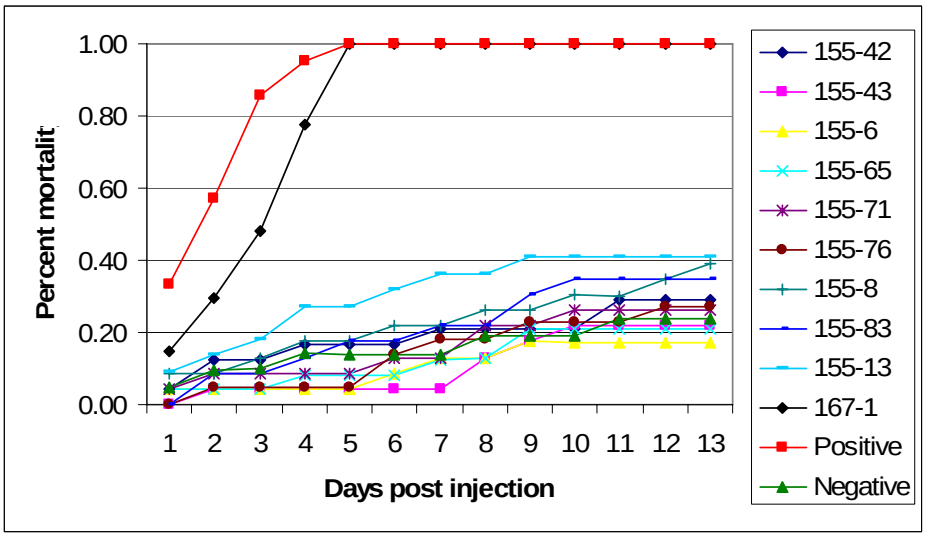


Figure 27. Cumulative mortality of bioassay BA78-14. Specimen 167-1 was the only specimen to demonstrate cumulative mortality markedly similar to the positive control.

were both contracted as external verification sources for the results of this study. Each lab was given a sample set including a series of shrimp determined Shrimple® positive, real-time PCR negative as well as a sample from the real-time PCR positive, Shrimple® positive crab specimen (SK040167-1). Both laboratories determined that the shrimp specimens were negative for WSSV using real-time PCR, 1-step PCR, and nested PCR, all of which were based on the use of established WSSV primers. Both laboratories confirmed that the crab specimen (SK040167-1) was positive for WSSV. The findings of these independent laboratories coincided with the results of the real-time PCR and one-step PCR analyses performed during the course of this study. All samples that tested positive for WSSV with Shrimple® were determined to be negative by PCR except for one crab.

11. Molecular sequencing

In order to obtain a cleaner PCR product for genetic sequencing, 1-step PCR was performed on SK040167-1 DNA as well as DNA isolate obtained from *L. vannamei* showing signs of acute WSSV infection following injection challenge with a composite inoculum prepared from the potentially infected crab. WSSV detection was performed using a previously developed primer set (Lo, et al. 1996). To further confirm the WSSV infection from the blue crab specimen SK040167-1, the amplicon from the PCR reaction was sequenced and compared to the expected published viral sequences. It was determined that the DNA from the crab specimen, and from the bioassay shrimp specimens have sequence similar to the published WSSV sequence. Using the web-based software on the NCBI website, a BLAST search of the sequenced PCR product from the infected crab resulted in a score of 876. The sequenced products were aligned against our

standard positive control (from a 1998 disease outbreak in an aquaculture facility in South Carolina) using the European Bioinformatics Institutes web-based software (Figure 28).

Figure 28. Alignment of sequenced PCR products against sequenced PCR product obtained from a known WSSV infected shrimp. Shrimp 2 = bioassay shrimp infected with inoculum from Shrimp1, + control = positive control sample from *L. vannamei* infected with stock WSSV inoculum, Crab*** = SK040167-1, Shrimp 1 = bioassay shrimp infected with inoculum from Crab***(SK040167-1).

12. Education outreach

Information pamphlets detailing the current study and WSSV background information were developed and distributed to members of the commercial shrimp and crab fisheries industry in South Carolina, Georgia, and Florida. Dockside and on-board training was conducted in each state to demonstrate detection of WSSV using Shrimple® diagnostic test kits. Selected members of the commercial shrimp industry performed a set of tests on shrimp collected during their normal operations and reported the results. A total of 100 tests were performed by five commercial shrimpers. All samples were determined Shrimple® negative. The educational outreach portion of this study provided a platform for opening channels of communication between the fisheries industry and research scientists. Numerous relationships were established that should provide for future collaboration on various studies. Commercial shrimpers and crabbers were made aware

of diseases that researchers, such as those involved in this project are investigating with the goal of providing better management of economically significant fisheries stocks.

B. Problems

Two significant problems were encountered during the course of this research. The first was the unexpected numbers of false positives found when applying the Shrimple® test kits to wild shrimp and crabs. A significant effort was devoted to validation of the kits in laboratory based exposures in SPF *L. vannamei* as published by Powell et al. (2006). The kit was found to be relatively sensitive with no false positives. The nature of the false positive results found in the screening of wild samples remains to be determined. It is possible that a reactive viral protein from a related virus was present in the samples. This would explain the apparent correlations with temperature expected for WSSV infections (reduced infections in summer months when temperatures were higher) found for the shrimp positive samples. On the other hand, non-specific binding from another unrelated protein can not be ruled out.

A second problem involved the inhibitory effect of the DNA extracted from wild *L. setiferus* on real time PCR amplification of known positive samples from laboratory infections of SPF *L. vannamei*. The inhibitory effect was significant as compared to control DNA from non infected laboratory populations of *L. vannamei* and of the mummichog, *Fundulus heteroclitus*. The problem was overcome through dilution of DNA from wild shrimp. Amplification of an endogenous gene from the wild shrimp assured proper extraction and amplification. Results were verified by one step PCR and submission to outside laboratories.

C. Need for additional work

The results of this study suggest that at the present time, WSSV is still present in wild populations of blue crab *C. sapidus*. Little is known about the effect of WSSV in blue crabs. Additional work on the pathology of infection in blue crabs is warranted. This could entail bioassay studies to look at tissue distributions and pathogenicity in crabs. Further study on the virus itself could yield information on viral strain and potential source. This would require amplification and sequencing of variable regions of the viral genome and comparison with known strains of WSSV.

The lack of infection in white shrimp *L. setiferus* and low infection rate in the crabs suggest that at the time of sampling for this study, the virus was not likely affecting commercial fisheries and was unlikely to be a major threat to aquaculture operations as long as adequate biosecurity precautions were in place. Nevertheless, the continued presence of WSSV in local waters would justify continued vigilance particularly in case of mass mortality events or times of extreme stress on wild populations.

Further research on the nature of the false positives encountered with the Shrimple® tests could be carried out to improve application of these kits and to explore the possibility of

presence of a potentially related viral pathogen in local stocks. Similarly, the nature of the PCR inhibition encountered should be the topic of additional research to improve the sensitivity of real time PCR based diagnostic tools for *L. setiferus.*

VII. Evaluation

A. Describe the extent to which project goals and objectives were attained.

1. Were goals and objectives attained?

1. Develop, test, and assess the reliability of immuno-based tools (using polyclonal and/or monoclonal antibodies) as diagnostic tools for detecting WSSV in the laboratory and in the field. This goal was attained in that a commercially available immuno-based tool was tested and applied with a complete assessment of reliability in detecting WSSV in the laboratory and in the field. See section B. below for a discussion of the modifications made to this objective.

2. Evaluate the impact of WSSV on the *L. setiferus* and *C. sapidus* populations and their general health in the waters of the Southeastern United States with particular reference to reproductive populations to assess amplification of the disease and potential for vertical transmission. This goal was achieved in that substantial collections of shrimp and crabs from SC GA and FL were made including individuals in all stages of the reproductive cycle. Fortunately, very low levels of infection were encountered with none of the shrimp and only one crab indicating diagnosable and transferable infection with WSSV. The results of this study are relevant to assessment of the ability of various detection methods to diagnose viral presence and assessment of the risks to shrimp fisheries and aquaculture in the southeastern United States, particularly in South Carolina, Georgia, and Florida. The lack of infection in white shrimp *L. setiferus* and low infection rate in the crabs suggest that at the time of sampling for this study, the virus was not likely affecting commercial fisheries and was unlikely to be a major threat to aquaculture operations as long as adequate biosecurity precautions were in place. Nevertheless, the continued presence of WSSV in local waters would justify continued vigilance particularly in case of mass mortality events or times of extreme stress on wild populations.

3. Promote awareness by working with commercial fishermen about the commercial implications and ecological significance of shrimp and blue crab diseases. This goal was achieved in that an education/outreach effort was established and implemented through the course of this project providing educational opportunities to members of the commercial fisheries industry. Information pamphlets were developed and distributed and dockside and on-board training was conducted in each state. The program shared the objectives and methodology of the study and built cooperative relationships that will afford collaborative researchers opportunities in the future. Scientific and technical reporting of research results is ongoing and follow-up efforts to disseminate research findings among cooperators and key stakeholders are planned.

4. Generate a database for the evaluation of the impacts of WSSV and interrelationships between infection levels and environmental parameters. This goal was achieved in that a complete database was built with sampling and diagnostic information. Due to the lack of detection of WSSV infection in wild specimens, the proposed application of the database on sample distributions to the evaluation of relationships between biological and environmental parameters and infection levels was modified as discussed in section B. below.

2. Were modifications made to the goals and objectives?

The parameters of the current study were altered by changing 'Objective 1,' removing the condition that the immuno-based tools for detecting WSSV be developed. EnBioTec Laboratories, Inc. in Tokyo, Japan, developed a commercially available monoclonal antibody-based immunoassay for WSSV (Shrimple®) prior to our development of a similar tool. We had the opportunity to develop and optimize conditions for a sandwich ELISA-based diagnostic technique for detection of WSSV called antigen capture ELISA (Ac_ELISA) (Shepard, et al. unpublished results). However, with the commercial availability of the Shrimple® diagnostic kit, objectives were realigned (as discussed in progress reports) to allow for increased efficiency in use of funds and expansion of sample sizes. Resources were used to evaluate the sensitivity of the kit which resulted in the drafting and publication of a manuscript (Powell, et al. 2006), fulfilling the essential goal of objective 1. Due to the lack of WSSV infection detected in wild specimens, the proposed application of the database on sample distributions to the evaluation of relationships between biological and environmental parameters and infection levels was not generated as per objective 4. Some interesting results were found when a similar evaluation of the Shrimple® positives was made (as described in section VI:5 above). Further research will be needed to better understand the cause of the false positive results observed for the Shrimple® diagnostic test kits.

B. Dissemination of project results

A manuscript covering the efficiency and sensitivity of Shrimple® has been published (Powell, et al. 2006) and accepted for publication in Aquaculture. A short communication covering real-time PCR inhibition induced by genomic DNA from wild-caught *L. setiferus* is being written and, once complete, will be submitted for publication in a peer-reviewed journal. Experiments are currently being conducted to determine the susceptibility and pathogenecity of WSSV in *C. sapidus* as well as the distribution of WSSV in experimentally infected blue crabs. Once the bioassay challenge experiments are concluded and the samples collected during the experiments are analyzed, a manuscript will be written and submitted for publication in a peer-reviewed journal. In addition, a manuscript is also in preparation to report the overall results of this project.

A summation of the results for specimens collected in Georgia and Florida will be sent to Georgia Department of Natural Resources and Florida Marine Resources Research Institute, respectively. Presentations have been made at internal meetings held within South Carolina Department of Natural Resources—Marine Resources Division and at a

joint meeting with a research laboratory within the College of Charleston. Dr. Eleanor Shepard presented the findings from this study at the 31st Annual Eastern Fish Health Workshop in Mt. Pleasant, South Carolina on April 28, 2006. James Powell presented the findings of this study at the Joint Meeting of the South Carolina Chapter of the American Fisheries Society and the South Carolina Fisheries Workers Association on February 17, 2006.

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