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## Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV

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### Abstract

There is currently concern and controversy in the shrimp industries of the Americas about the risk posed by the importation and reprocessing of shrimp infected with white spot syndrome virus (WSSV) and yellow head virus (YHV). To further understand the risk, more knowledge concerning the quantitative virus load of infected shrimp is needed. The present study was carried out to better define, using qualitative and quantitative methods, the relative virus load of shrimp heads and tails. For these studies, specific pathogen-free (SPF) *Penaeus vannamei* were infected with WSSV. Emergency harvest of these shrimp was simulated by collecting the infected shrimp at the onset of postinfection mortalities and determining the relative virus loads of the head and tails by quantitative real-time PCR and histology methods. Routine histology and in situ hybridization assay with a WSSV-specific DNA probe demonstrated qualitatively similar levels of WSSV infection in the heads and tails of experimental infected shrimp. The novel real-time PCR method demonstrated quantitatively that the head had a slightly higher WSSV load than did the tail. However, since the tail represents 58% of the total body weight, the total virus load on a per weight basis turns out to be similar in the head (49%) and tail (51%) of the same shrimp with acute phase WSSV infections. In proportion to the total tail weight, the virus load of the peeled shell represents 55% of the total viral load in tail.

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*Keywords:* WSSV; Shrimp; Quantitative PCR; In situ hybridization

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## 1. Introduction

In the wake of the introduction, establishment, and severe impact of white spot syndrome virus (WSSV) on many of the shrimp farming regions of the Americas, there is now considerable concern that yellow head virus (YHV), or other potentially serious significant pathogens from Asia, Africa, Australia, or elsewhere, might follow the same path of WSSV to add another harmful blow to the industry. Recent risk assessments (RA) carried out in the United States and in Australia have addressed questions relating to the risk of introduction and establishment of exotic shrimp pathogens (such as WSSV, YHV, and others) that are posed by various routes (Van der Schalie and Austin, 1998; AQIS, 2000). The conclusions reached from the U.S. RA were severely limited by the paucity of available data and controversy over the data that was available. Hence, the U.S. RA identified several areas where research was needed before a more thorough or quantitative RA could be carried out. Among the topics identified, and relevant to the present work, was improved diagnostic methods. In situ hybridization of fixed sections of shrimp tissues with specific DNA probes has become a common and widely accepted method for detection of penaeid shrimp/prawn viruses and for diagnosis of the diseases that they cause (Lightner, 1996; OIE, 2000). For the latter application, in situ hybridization has been applied to determine qualitatively such things as tissue distribution of WSSV in infected hosts (Durand et al., 1996; Wongteerasupaya et al., 1996; Lo et al., 1997; Nunan and Lightner, 1997; Kou et al., 1998; Chang et al., 1998).

PCR-based methods, for which there are both qualitative and quantitative methods, have been developed for use with shrimp tissues. These methods, such as competitive PCR and the newly developed technique of real-time PCR, permit the determination of the number of copies of a target segment of DNA in a particular tissue sample. These methods have been applied to penaeid shrimp virology and to develop assays for WSSV and IHNV (Tang and Lightner, 2000, 2001; Durand and Lightner, in press). In this study, small juvenile *Penaeus vannamei* that had been experimentally exposed to WSSV in the laboratory were subjected to a simulated emergency harvest when mortalities due to the disease were first detected. The relative virus load of the heads (cephalothoracies) and tails (abdomens) of these shrimp was determined qualitatively using routine histological methods and in situ hybridization with a WSSV-specific DNA probe. The relative virus load of the heads, tails, peeled tail muscle, and abdominal shells was determined quantitatively by real-time PCR.

## 2. Materials and methods

Juvenile (average weight: 3 g), specific pathogen-free (SPF) *P. vannamei* (following the taxonomic scheme of Holthuis, 1980) were stocked (18 and 16 shrimp, respectively), into two laboratory aquaria (40 l) filled with artificial seawater. Experimental shrimp were fed with minced WSSV-infected tissue (1994 isolate from Thailand) at 5% (tank A) and 2.5% (tank B) of body weight for 2 days. Following the feeding of WSSV-infected tissue, the experimental shrimp were maintained on a commercial pelleted ration. After the first mortalities were observed in the WSSV-exposed tanks, the remaining shrimp were

collected to simulate an emergency harvest. Representative shrimp taken on day 0 of the study served as the negative control samples, while the harvested shrimp taken at day 3 were either fixed in Davidson's AFA fixative for histological and in situ hybridization analysis or frozen at  $-70^{\circ}\text{C}$  for real-time PCR analysis.

### 2.1. Histology and in situ hybridization

Six shrimp were randomly selected and fixed in Davidson's AFA fixative and processed for routine histology using standard methods (Bell and Lightner, 1988; Lightner, 1996). Two specimens were day-0 negative controls and four were from day-3 post-WSSV exposure. The experimental shrimp were dissected and embedded so that lateral and midsagittal sections of the cephalothoraxes and abdomen would be obtained. From the resultant paraffin blocks, consecutive 4- $\mu\text{m}$ -thick sections were prepared. From each pair of consecutive sections, one was stained with H&E and the other was reacted with a WSSV-specific DIG-labeled DNA probe using in situ hybridization.

### 2.2. Real-time PCR quantification of WSSV

For real-time PCR assay, 16 of the remaining shrimp from tanks A and B were collected to simulate an emergency harvest. With eight of these shrimp, the cephalothorax (head) was separated from the abdomen. From the remaining eight shrimp, only tails were used. The shell, tail fan (telson and uropods), and the pleopods of the abdomen (tail) were removed by peeling, leaving the peeled tail meat (tail muscle, hindgut, midgut, nerves, bits of cuticular epidermis).

### 2.3. DNA extraction

Total shrimp DNA was extracted from the experimental shrimp heads, tails, and shells using the High Pure Genomic DNA kit (Roche Molecular Biochemical, Indianapolis, IN) in accordance with the manufacturer's protocol. Tissues were crushed with a micropestle and cells are lysed during a 1-h incubation with proteinase K in the presence of a chaotropic salt (guanidine HCl). Cellular nucleic acids bind selectively to glass fiber fleece in a special centrifuge tube. The nucleic acids remain bound while a series of rapid "wash-and-spin" steps removes contaminating small molecules. Finally, low salt elution removes the nucleic acids from the glass fiber fleece. The concentration of total DNA in the sample was estimated using a BioPhotometer (Eppendorf Scientific, Hamburg, Germany) spectrophotometer.

### 2.4. Primers and probes

The sequences of PCR primers and TaqMan probe used for the detection of WSSV were selected from a region of WSSV genomic sequence in GenBank U50923. The primers (WSS1011F/WSS1079R) generate a 69-bp amplicon (Table 1). The TaqMan probe, starting from nucleotide 1032 to nucleotide 1050, was synthesized and labeled with fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and *N,N,N,N*-tetramethyl-6-

Table 1  
The sequences of WSSV and IHNV primers and TaqMan probes

Virus	Upstream primers	Downstream primers	TaqMan probes
WSSV	5'-TGGTCCCGTCCTCAT- CTCAG-3' WSS1011F	5'-GCTGCCTTGCCGGAA- ATTA-3' WSS1079R	5'-AGCCATGAAGAATGCCGTC- TATCACACA-3'

carboxyrhodamine (TAMRA) on the 3' end. Specificity tests showed that there is no cross-reaction with shrimp DNA, other baculoviruses DNA such as BP, MBV, and parvoviruses DNA such as IHNV and HPV. The selected primers recognized representative WSSV isolates from China, South Carolina, Texas, India and WSSV from crayfish. As for the sensitivity of the test, this method was found to detect from  $4 \times 10^7$  to 4 copies (Durand and Lightner, in press).

### 2.5. PCR mixture and cycling

The TaqMan assay was carried out with the TaqMan Universal PCR Master Mix containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, and optimized buffer components (PE Applied Biosystems, Foster City, CA). A sample of 10–100 ng of DNA was added to a PCR mixture containing 0.3  $\mu\text{M}$  of each primer and 0.15  $\mu\text{M}$  of TaqMan probe in a final volume of 25  $\mu\text{l}$ . Amplification was performed with the following conditions: 2-min reaction for AmpErase uracil-*N*-glycosylase (UNG) at 50 °C and activation of the AmpliTaq for 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The quantity of each sample was determined by GeneAmp 5700 Sequence Detection System software (SDS 1.0). Each sample was tested in duplicate. A series of dilutions from a WSSV recombinant plasmid (copy number known) were made to be used as standards for quantification.

A *t*-test (paired-samples *t*-test) was used to compare statistically the means between the WSSV copy number values from different parts of the shrimp.

## 3. Results

Experimental shrimp began to die 3 days after per os exposure to WSSV-infected shrimp tissues. Negative control shrimp from day-0 and the remaining WSSV-exposed shrimp collected on day-3 post-WSSV exposure.

### 3.1. Histological and in situ hybridization results

The results from histological sections processed using routine H&E staining and in situ hybridization with a WSSV probe showed that three of the four WSSV-exposed shrimp sampled presented advanced systemic WSSV infections. The fourth shrimp was negative for signs of WSSV infection by H&E histology and by in situ hybridization assay with the WSSV-specific DNA probe (Table 2). Negative control shrimp taken on day 0 were likewise negative for WSSV infection by H&E histology and in situ hybridization. Using

Table 2

Comparisons of qualitative WSSV infection grade of the heads and tails of juvenile *P. vannamei* assayed by routine H&E and in situ hybridization with a WSSV-specific DNA probe

Sample number	Treatment	H&E		In situ DNA probe	
		Head	Tail	Head	Tail
1 and 2	Day-0 control	0	0	0	0
3	Day-3 harvest	3	3	3	3
4	Day-3 harvest	0	0	0	0
5	Day-3 harvest	4	4	4	4
6	Day-3 harvest	3	3	3	3

The values given are infection severity grades: 0 = not detected; 1 = low; 2 = moderate; 3 = heavy; and 4 = severe.

the grading scheme for infection severity from Lightner (1996), the infection severity grades were determined for the control and the experimental shrimp. Grade 0 scores were given to the two unexposed negative control shrimp and to one of four of the WSSV-exposed shrimp because they presented no signs of WSSV by routine H&E histology and in situ hybridization. However, three of the four WSSV-exposed shrimp presented grades 3–4 (i.e. severe) WSSV infections as determined by both H&E histology and by in situ hybridization with a WSSV-specific probe (Table 2; Fig. 1). In these shrimp, characteristic WSSV basophilic intranuclear inclusion bodies were abundant in the cuticular epithelium and connective tissues of the general body cuticle and appendages (i.e. the carapace, abdominal tergal and pleural plates, gills, antenna, maxillipeds, pereopods, pleopods, telson, and uropods), in the foregut (esophagus and anterior and posterior chambers of the stomach), and in hindgut and rectum. With direct comparisons of the head and tail sections from the same shrimp, no difference in severity of WSSV infection was apparent in the cuticular epithelium present in the head (i.e. stomach, head appendages) or tails (i.e. pleopods, uropods, and telson). WSSV inclusions were also apparent, but generally in lower numbers, in the circulating hemocytes, fixed phagocytes, antennal gland tubule epithelial cells, in lymphoid organ sheath cells, in the parenchymal tissues of the hematopoietic tissues, and in connective tissues throughout the shrimps' bodies.

### 3.2. WSSV quantification by real-time PCR

The DNA yield, after extraction with the High Pure Genomic DNA kit, was within the expected range of 5–20 µg of DNA as specified by the manufacturer (Table 3). However, it was found that DNA yield (and DNA yield on a per weight basis) was different between the tissues tested. The DNA yield from head tissues was slightly greater than from tail tissues, and DNA yield from muscle tissues and shells were lower than that from either tails or heads.

For each run, a standard curve was generated from samples of purified cloned-WSSV plasmid ranging from  $4.00 \times 10^7$  to 400 copies (Fig. 2). Copy numbers were calculated by interpolation of the experimentally determined threshold cycle ( $C_T$ ). From these copy numbers, the final results were expressed as the mean copy number of WSSV per microgram of total DNA and mean copy number of WSSV per gram of tissue (Table 4).

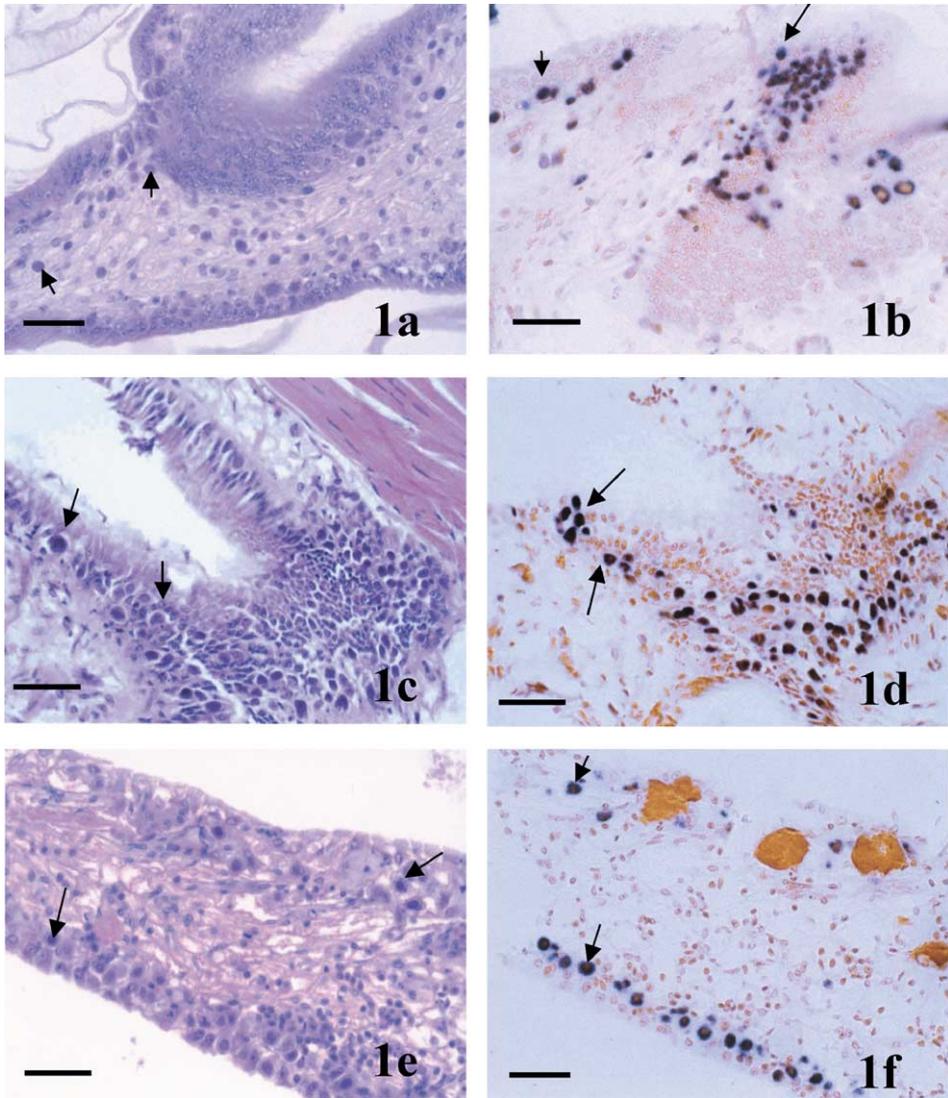


Fig. 1. Matched pairs of consecutive sections from the head and tail of a WSSV-infected *P. vannamei* stained with H&E or reacted by in situ hybridization using WSSV-specific gene probes. a (bar=155  $\mu$ m) and b (bar=145  $\mu$ m) are sections of the stomach cuticular epithelium and subcuticular connective tissues that are located in the cephalothorax (“head”); c (bar=90  $\mu$ m) and d (bar=145  $\mu$ m) are sections of the cuticular epithelium and subcuticular connective tissues at the junction of a uropod and the telson (tail fan); and e (bar=148  $\mu$ m) and f (bar=125  $\mu$ m) are sections of the cuticular epithelium and subcuticular connective tissues of a uropod (tail). Sections illustrated on the left are stained with H&E, while the consecutive sections of each on the right have been reacted with a DIG-labeled DNA probes to WSSV and counterstained with Bismarck Brown. Arrows indicate examples of characteristic WSSV intranuclear inclusion bodies that are basophilic with H&E staining and marked with a blue-black stain where they have reacted with the gene probe.

Table 3  
Mean values of total DNA after extraction from shrimp tissues

Tissues	Extracted total DNA (ng of DNA/mg of tissues)	Total DNA yield ( $\mu$ g of DNA)
Head	342.2	19.1
Tail	300.7	15.7
Muscle	241.1	11.3
Shell	150.7	8.9

The mean WSSV copy number (Table 4) in whole heads was found to be slightly greater than the mean in whole tails (significant at 90% level of confidence). Two times more WSSV copies were found in the head than in the tail when the WSSV copy numbers

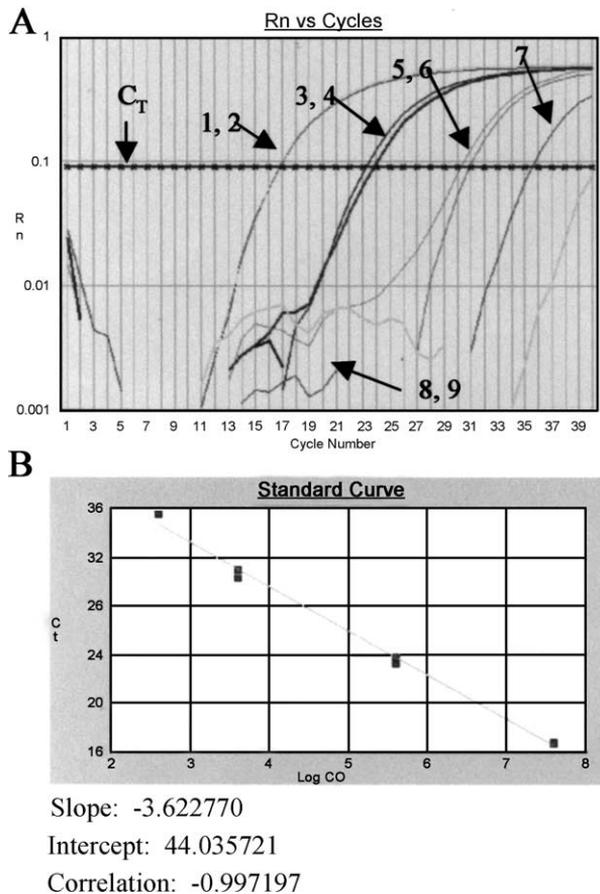


Fig. 2. Standard curve of the WSSV copy number. (A) Amplification curve of the standard (supercoiled plasmid containing 69-bp insert) samples;  $4.00 \times 10^7$  (1, 2),  $4.00 \times 10^5$  (3, 4),  $4.00 \times 10^3$  (5, 6),  $4.00 \times 10^2$  (7), no template control (8, 9). Rn: fluorescence signal,  $C_T$ =Cycle number. (B) Standard curve made with two replicates of each of four dilutions and the value of the slope, intercept and correlation.

Table 4

Mean value comparison of copy number of WSSV DNA from whole head, whole tail, tail muscle, and tail shell samples of juvenile *P. vannamei* with acute WSSV infections and assayed by real-time PCR

Sample	N	Copy number per microgram of total DNA extracted			Copy number per gram of tissue		
		Mean	Confidence (%) <sup>a</sup>	Range	Mean	Confidence (%)	Range
Whole heads	8	$2.5 \times 10^7$	90	$1.10 \times 10^6$ – $8.20 \times 10^7$	$2.00 \times 10^{10}$	90	$2.09 \times 10^5$ – $8.56 \times 10^{10}$
Whole tails	8	$1.2 \times 10^7$		$4.00 \times 10^5$ – $4.80 \times 10^7$	$1.53 \times 10^{10}$		$9.60 \times 10^7$ – $9.57 \times 10^{10}$
Tail muscle	8	$3.4 \times 10^7$	95	$2.70 \times 10^5$ – $1.80 \times 10^8$	$1.86 \times 10^{10}$	95	$6.30 \times 10^7$ – $7.68 \times 10^{10}$
Tail shells	8	$4.8 \times 10^8$		$5.50 \times 10^5$ – $1.47 \times 10^9$	$7.32 \times 10^{10}$		$1.28 \times 10^8$ – $1.55 \times 10^{11}$

The copy number per gram of tissue was calculated with the following formula:

$$\text{Copy number/gram} = \frac{(\text{Copy number per microliter of extracted total DNA})(\text{total volume of total extracted DNA})}{\text{Grams of tissues used to extract the total DNA}}$$

<sup>a</sup> The difference in means between head/tail and shell/muscle demonstrated with the *t*-test is displayed with the confidence level of 90% or 95% as indicated.

are expressed per microgram of total DNA (significant at 90% level of confidence). As for the WSSV content of shells and tail muscle, the WSSV copy number mean was between 4 and 10 times greater in the abdominal shells than in the tail muscle (significant at 95% level of confidence) (Table 4). No tank effect was noted as no difference was found between WSSV copy number between tanks A and B.

The relative ratio of the total body weight of the head versus tail in most penaeids is about 40:60 (AQIS, 2000). In *P. vannamei*, it is 42:58. Hence, the shrimp's tail makes up about 58% of a shrimp's total weight, while the head makes up about 42%. When the WSSV copy number values, expressed as number per gram of tissue, are compared and extrapolated to this 42:58 body weight ratio, we find that 51% of WSSV load is in the tail and 49% is present in the head. Within the tail portion of the shrimp, 55% of the virus content is in the shell, tail fan (uropods and telson), and pleopods, while 45% is present in the muscle, epidermis, and connective tissues associated with the hindgut and midgut.

#### 4. Discussion

Routine histology and in situ hybridization assay with a WSSV-specific DNA probe demonstrated qualitatively similar levels of WSSV infection in the heads and tails of experimental shrimp from a simulated emergency harvest. The novel real-time PCR method demonstrated quantitatively that the head had a slightly higher WSSV load than did the tail. However, the total virus load on a per weight basis turns out to be similar in the head and tail of the same shrimp with acute phase WSSV infections. Peeled shells from the tails (with pleopods and the tail fan) contain almost 10-fold more WSSV on a per

weight basis than do whole heads or whole tails. When the shrimp are unshelled, 45% of the total virus load of the tail remains in the edible tail meat, while 55% of the virus content of the tail is present in the peeled shell.

The shrimp used in this study were exposed to WSSV per os by feeding them WSSV-infected shrimp carcasses. Of the four postharvest specimens examined for severity of WSSV infection, one presented no signs of WSSV infection while three presented moderate to severe infections. The same proportion of low grade to advanced WSSV infections would be expected in the 16 postharvest shrimp assayed for WSSV by real-time PCR. This inherent variability in viral load in the experimental shrimp was apparent in the PCR findings. However, since the data were paired (i.e. the viral content of heads and tails from the same shrimp), the effect of stage of infection on viral content was minimized.

The relatively lower yield of DNA from shells and muscle tissue compared to whole heads and whole tails was due to the nature of these tissues (Table 3). The shells contain a very high proportion of acellular cuticle relative to the amount of cellular cuticular epithelium present. Likewise, muscle cell nuclei make up a relatively low proportion of the total cell volume relative to their cytoplasm with its high content of actin, myosin, and other subcellular structures. However, the effect of the difference of DNA yield was minimized since a known quantity DNA sample (10–100 ng of DNA) was added to the PCR mixture and the final DNA quantities were expressed on  $\mu\text{g}$  total DNA basis.

Small count size frozen tails of *Penaeus monodon* with gross signs of acute phase WSSV infections (and likely to be from emergency harvests) have been common in retail outlets in the United States as early as 1994. In laboratory studies, such products have been found to transmit WSSV and YHV to SPF indicator shrimp in laboratory studies (Nunan et al., 1998; Durand et al., 2000). The findings of the present study confirm these earlier studies by demonstrating that small size “green” frozen shrimp tails, resulting from an emergency harvest due to the onset white spot disease, do contain large amounts of the virus and that the amount of virus present in such tails is equivalent in magnitude to that found in the heads from the same shrimp. The results of the present paper also confirm the findings of Soto et al. (2001), who compared mortality rates of challenged indicator shrimp (*P. vannamei*) exposed to a WSSV-infected cephalothoraxes, abdomens, or whole shrimp cadavers. In that study, it was found that there was no apparent difference in infectivity (and, hence, the presumed viral load) of WSSV-infected cephalothoracies (heads) or abdomens (tails) (Soto et al., 2001).

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