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Hongwei Ma
University of Southern Mississippi and Medical College of Wisconsin

Robin M. Overstreet
Gulf Coast Research Laboratory, robin.overstreet@usm.edu

Jean A. Jovonovich
University of Southern Mississippi, Jean.JovonovichAlvillar@usm.edu

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Daggerblade Grass Shrimp (*Palaemonetes pugio*): A Reservoir Host for Yellow-Head Virus (YHV)

Hongwei Ma,[1,2] Robin M. Overstreet,[1] and Jean A. Jovonovich[1]

1. Department of Coastal Sciences, University of Southern Mississippi, Ocean Springs, Mississippi, USA
2. Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin, USA

Corresponding author – Robin M. Overstreet, email robin.overstreet@usm.edu

Abstract

Yellow-head virus (YHV) is a major pathogen in penaeid shrimps. We surveyed 13 crustacean species in eight families from two orders that are commonly found in the Mississippi coastal area and freshwater environments as potential reservoir or carrier hosts of YHV. Using semi-nested polymerase chain reaction (PCR) on relatively small sample sizes, we did not detect any natural infection. However, when the daggerblade grass shrimp, *Palaemonetes pugio*, and the blue crab, *Callinectes sapidus*, were exposed to YHV by injection and per os, YHV was detected in the tissue of *P. pugio* and in the hemolymph of *C. sapidus* when tested by semi-nested reverse transcription PCR (RT-PCR) and real time quantitative reverse transcription PCR (qRT-PCR). YHV replicated in *P. pugio*, causing 8% mortality (9/112) after injection, with the viral titer reaching a peak at 14 days post-inoculation (dpi) and remaining detectable at 36 dpi. The number of infected animals and viral load, however, were relatively low, but the virus still remained infectious to penaeids when administered by feeding. When YHV was injected into *P. pugio*, in situ hybridization detected a positive response to it at 7 dpi in connective tissue of hepatopancreas, muscle, and midgut. Viral RNA in injected *C. sapidus* remained at a low level for 3 days, and it was not detected from 7 dpi onward. In fed *C. sapidus*, the viral RNA reached a peak at 3 dpi and still detectable at 7 dpi, but it became undetectable at 14 and 21 dpi. These data suggest that *P. pugio* under some conditions could act as a reservoir host for YHV but that the blue crab could serve as a poor, short-term carrier host only.

Keywords: *Callinectes sapidus*, *Palaemonetes pugio*, qRT-PCR, reservoir host, vector, yellow-head virus

1. Introduction

Yellow-head virus (YHV) was first described from an epizootic infection in Thai penaeid shrimp farms (Limsuwan, 1991). Subsequent outbreaks of YHV have been reported from cultivated penaeid shrimp throughout Asia (Walker, 2006). Infections have also been reported from frozen imported commodity shrimp in the United States (Durand et al., 2000; Ma and Overstreet, unpublished) and from two cultured penaeid shrimps, *Litopenaeus vannamei* and *Litopenaeus stylirostris*, on the west coast of Mexico (de la Rosa-Vélez et al., 2006; Sánchez-Barajas et al., 2008). As the cause of an important emerging commercial shrimp disease, YHV has caused an estimated economic loss of US$500 million from its discovery in 1991 until 2006 (Lightner, 2007). The YHV with two related viruses from Australia, gill-associated virus (GAV) and lymphoid organ virus (LOV), have been placed in the family Roniviridae of the order Nidovirales (Cowley and Walker, 2002; Gorbalenya et al., 2006).

Populations of grass shrimps (*Palaemonetes* spp., Palaemonidae, Decapoda) are important consumers and are the key prey for many crustacean and fish species and "potential vectors of YHV." Among these grass shrimp species, the daggerblade grass shrimp, *Palaemonetes pugio*, living in habitats of shallow water in or around tidal marshes, submerged vegetation, and oyster reefs (Anderson, 1985), is distributed in Atlantic and Gulf coasts from Massachusetts to Texas. It survives in salinity less than 1‰ to over 30‰ (Heard, 1982) and constitutes more than 95% of all grass shrimp from estuarine tidal areas in many locations along the coastlines of the Gulf of Mexico and the South Atlantic.

Other crustacean species found in the area that might have access to YHV include the blue crab, *Callinectes sapidus*, which occurs commonly along the Gulf of Mexico; its natural distribution includes most coasts of the western Atlantic Ocean from Nova Scotia to Argentina. It comprises one of the most valuable commercial fisheries in the US (Kennedy et al., 2007). Recent research has shown that the blue crab can serve as a vector for the white spot syndrome virus (WSSV) (Chapman et al., 2004; Matthews and Overstreet, unpublished).

A vector can be defined as an organism that directly or indirectly transfers an infectious disease agent to another organism. It may be a "reservoir," in which the agent replicates but is typically not harmed, that transfers the agent to an organism of economic or medical importance that typically is harmed. In contrast, it may be a "carrier," or "mechanical vector," which is never harmed by the nonreplicating agent that it transfers to the "important" organism.

Studies by Thai researchers on YHV carrier or reservoir hosts reported a limited number of potential hosts such as the sergestid *Acetes* sp. (Flegel et al., 1995a,b) and the palaemonids *Palaemon serrifer*, *Palaemon styliferus*, *Macrobrachium sintangense*, and *Macrobrachium lanchesteri*. In contrast, 16 species of crabs belonging to six families (Longyant et al., 2005, 2006) did not support virus replication. The purpose of our study was: (1) to increase the knowledge of potential reservoir or carrier species, (2) to study the dynamics of YHV upon intramuscular inoculation or per os feeding in tissue and hemolymph of *P. pugio* and *C. sapidus*, respectively, by both qRT-PCR and semi-nested RT-PCR.

2. Materials and methods

2.1. YHV isolate

The YHV isolate (YHV92) used in this study was originally collected in 1992 from *Penaeus monodon* in Thailand. We sequenced the whole genome of this isolate and deposited it in GenBank as Acc. No. FJ848673. The YHV92 inoculum was prepared as follows: 10 g cephalothorax tissue was homogenized with 90 ml TN buffer and centrifuged at 1800g and 4°C for 5 min; the supernatant was centrifuged at 1800*g* and 4°C for 5 min and again at 18,000*g* and 4°C for 20 min. This supernatant was then aliquoted into 1.5 ml vials and stored at –80°C until required. For injection experiments, an aliquot was diluted with 2% saline and filtered through a 0.45 μm membrane. A 100-μl inoculum (diluted 1:1000) was injected into the third abdominal segment of specific pathogen free *L. vannamei* (Shrimp Improvement System, Florida). At 3 days post inoculation (dpi) shrimp became moribund. At that time, hemolymph was drawn using an EDTA-coated 1-ml syringe and samples were pooled as a reference virus stock. The pooled hemolymph and tissues were stored at –80°C until required.

2.2. Experimental animals

During May–September 2007, 13 crustacean species (belonging to eight families in two orders) were collected in the coastal areas and a freshwater environment in Mississippi (Table 1). *P. pugio*, four species of *Uca*, *Armases cinereum*, and *Sesarma recticulatum* were collected from the Gulf Coast Research Laboratory campus, Ocean Springs, Mississippi (30°23′31.07″N, 88°47′56.67″W); *C. sapidus*, *Clibanarius vittatus*, *Menippe adina*, and *Squilla empusa* were captured near Deer Island, Biloxi, Mississippi (30°22′6.50″N, 88°50′11.95″W); *Emerita talpoida* was collected from the west end of Horn Island, Mississippi (30°14′20.41″N, 88°45′52.86″W); and the freshwater *Palaemonetes kadiakensis* was collected from Tuxachanie Creek, Harrison County, Mississippi (30°37′56.86″N, 88°59′27.8″W). After collection, we surveyed some individuals from each species for natural YHV-infection using semi-nested PCR. Blue crab (*C. sapidus*) hemolymph was drawn and the nucleic acid was extracted and tested for YHV as well as for white spot syndrome virus (WSSV) and the dinoflagellate disease agent *Hematodinium* sp. Afterward, animals were acclimatized in 19-l tanks containing artificial sea water (hw-Marinemix professional, Houston) at 20 g/l salinity (the same as at the collection sites) for 7 days before YHV inoculation, the exception being the species *P. kadiakensis*, which was acclimatized in fresh water for 7 days prior to YHV inoculation.

2.3. Maintenance of experimental animals

Five Pacific white shrimp (*L. vannamei*) (averaging 12 g) were maintained individually in 19-l aerated tanks for 7 days. The blue crabs, all negative for YHV, WSSV, and *Hematodinium* sp., were maintained individually in 19-l aerated tanks containing 20 g/l artificial sea water mixture for 7 days before being administered YHV. About 200 daggerblade grass shrimp (*P. pugio*) were distributed equally among ten 19-l aerated tanks containing 20 g/l artificial sea water for 7 days before being administered YHV. Sixty Mississippi grass shrimp (*P. kadiakensis*) were maintained among six 19-l aerated tanks containing fresh wa-

ter for 7 days before being administered YHV. Crabs (*Uca* spp., *A. cinereum*, and *S. recticulatum*) were maintained in tanks containing sand minimally covered by water. For other crustaceans, 1–10 individuals of each species, depending on their size, were maintained in 19-l aerated tanks containing 20 g/l artificial sea water for 7 days before being administered YHV. These animals were then fed once a day with commercial pelleted feed (size #3, Rangen, Buhl, Idaho); half of the sea water was changed every other day, and the temperature was controlled by circulating water heated at 26.0 ± 0.5°C around the tanks maintained in a single water bath.

2.4. Virus inoculation

2.4.1. Intramuscular route

A 100-μl inoculum of 1:1000 diluted homogenate of YHV92 with a total virus copy number of 2.36×10^4 (as determined by qRT-PCR) was injected into the third abdominal segment of five *L. vannamei*, a 20-μl inoculum of 1:100 diluted YHV92 homogenate with a 4.72×10^4 virus copy number was injected into 120 *P. pugio* and 60 *P. kadiakensis*, a 200-μl inoculum of 1:100 diluted homogenate of YHV92 with a total viral copy number of 4.72×10^5 was injected intra-muscularly (im) into the propodus of the cheliped through the flexible arthrodial membrane joining the dactyl in five *C. sapidus*, and the same amount of viral particles was also injected into the body cavity through the right rear coxa of five additional crabs. Other experimental animals were injected im with a total virus copy number of 4.72×10^4.

2.4.2. Per os route

In the feeding experiments, five *C. sapidus* were each fed 1 g of YHV-positive tissue from *L. vannamei* during each of three consecutive days, and 50–70 μl of hemolymph was drawn at 6 h after the third feeding, which was recorded as 6 h post inoculation (hpi). Thereafter, individuals of *C. sapidus* were given pelleted feed. Individuals of *P. pugio* (n = 80) were equally distributed among four tanks, and 1 g minced YHV-positive tissue was introduced each day into each tank for three consecutive days of feeding after which the shrimp were switched to pelleted food. The same feeding protocol was applied to all other animals.

2.5. Tissue and hemolymph sampling

At 1, 3, 5, 7, 14, 21, and 36 dpi, two *P. pugio* were randomly picked from each tank and stored in RNAlater (Ambion Inc.) at 4°C overnight and then stored at –20°C until RNA extraction. In addition, two *P. pugio* were fixed in Davidson's fixative for tissue sections on 3, 7, 14, and 21 dpi. About 50–70 μl of hemolymph from *L. vannamei* and *C. sapidus* were drawn by EDTA-coated 1-ml syringes at 6 hpi and at 1, 3, 7, and 14 dpi, and stored at –80°C. For all other species, 3–10 individuals were sampled on 3, 7, and 14 dpi and stored as indicated above.

2.6. Total RNA extraction

Total RNA from tissue was extracted using the protocol from the High Pure Tissue RNA Kit (Roche). Briefly, the animals were rinsed with autoclaved distilled water, and 9–20 mg of tissue from the cephalothorax was sliced and homogenized in 400 μl of lysis/binding

buffer using a pestle in a 1.5-ml tube. After being digested in DNase I and washed, the RNA was eluted into 100 μl of autoclaved RNase-free water and stored at –80°C. Hemolymph RNA was extracted following the protocol from the High Pure Viral Nucleic Acid Kit (Roche). Briefly, 150 μl of autoclaved nuclease-free water was added to 50 μl of hemolymph and mixed with 250 μl of binding buffer containing poly A and proteinase K. The extracted RNA was eluted into 100 μl of autoclaved RNase-free water and stored at –80°C.

2.7. Conventional one primer pair set RT-PCR (1-set PCR)

Conventional one primer pair set RT-PCR (1-set PCR) of the sample total RNA was processed following the protocol of SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) in which the following primers were added: primer 273F: 5′-CAA GAT CTC ACG GCA ACT CA-3′ and 273R: 5′-CCG ACG AGA GTG TTA GGA GG-3′ (Tang and Lightner, 1999). The reaction mixture was placed in an MJ Thermocycler with a 30-min incubation at 50°C to synthesize the cDNA. Inactivation of the reverse transcriptase at 94°C for 2 min was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 30 s. A final extension step at 68°C for 5 min completed the reaction after which it was held at 4°C. This 1-set PCR reaction yields a 273 bp amplification product which can be visualized by gel electrophoresis with the addition of ethidium bromide to the 2% agarose gel and analyzed with a FluorS MultiImager (BioRad).

2.8. Semi-nested RT-PCR

The nonstop, semi-nested RT-PCR also was conducted using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity in a 25-μl reaction mixture containing 1.1 μM P64A1, 0.1 μM P64S1, 0.2 μM P64S2, and 0.8 μM P64S3 can yield three amplicons (1003, 553, and 247 bp) (Kiatpathomchai et al., 2004). The reaction was initiated by reverse transcription at 50°C for 30 min followed by transcriptase inactivation at 94°C for 2 min. This procedure was followed by the sequential cycling protocols of (1) 5 cycles of 94°C for 15 s, 60°C for 45 s, and 72°C for 1 min; (2) 15 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s; (3) 35 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s; and (4) 1 cycle of 72°C for 5 min after which the reaction was placed on hold at 4°C. After PCR amplification, we resolved (10 μl) products by electrophoresis in a 1.2% (w/v) agarose gel and then visualized them by ethidium bromide staining and the FluorS MultiImage analyzer.

2.9. qRT-PCR

The iScript One-Step RT-PCR Kit for Probes (BioRad) was used to perform qRT-PCR with primers YHV141F: 5′-CGT CCC GGC AAT TGT GAT C-3′, YHV206R: 5′-CCA GTG ACG TTC GAT GCA ATA-3′ (Dhar et al., 2002), and YHV TaqMan: 5′-/FAM/CCA TCA AAG CTC TCA ACG CCG TCA/TAMRA-Sp-3′ (Integrated DNA Technologies, Inc.). The standard was a 72-bp segment from a sequence (GenBank AF148846) that contained 66-bp amplicon with an extra 3 bp on both ends.

The qRT-PCR amplifications were undertaken in an iCycler Thermocycler (BioRad). The qRT-PCR was conducted in a 25-μl reaction volume containing 2 μl RNA, 12.5 μl 2 × RT-PCR reaction mix for probe, 300 nM of each of the forward and reverse primers, 100 nM probe,

and 0.5 μl iScript Reverse Transcriptase Mix for One-Step RT-PCR. The thermal profile of qRT-PCR was 10 min at 50°C for cDNA synthesis, 5 min at 95°C for iScript reverse transcriptase inactivation, and 40 cycles of 15 s at 95°C and 30 s at 56°C (data collection step) for amplification and then held at 4°C (Ma et al., 2008).

2.10. In situ hybridization

After being inoculated with YHV, four specimens of *P. pugio* were collected at 7 dpi for quantification of the viral load by qRT-PCR, and an additional four for tissue processing were fixed with Davidson's solution for 20 h and then transferred into 70% ethanol (molecular grade). In situ hybridization (ISH) analysis followed the method of Tang et al. (2002). Four-μm sections prepared for ISH were then overlaid with the YHV probe (GenBank accession no. AF148846) labeled with digoxigenin-11-dUTP (20–40 ng/ml) following the procedures detailed by Tang and Lightner (1999).

2.11. Statistical analysis

The log viral titers per μg RNA were expressed as mean ± standard error (SE). Statistical comparisons of log viral titers for the three groups: (1) 6–24 hpi, (2) 3–14 dpi, and (3) 36 dpi, were performed with one-way ANOVA based on SPSS 15.0 for Windows. Values of p less than 0.05 were accepted as statistically significant.

3. Results

3.1. Natural infections

No natural YHV infection was detected in any of the 13 species of crustaceans examined by semi-nested PCR (Table 1). To assure no YHV or confounding agent occurred in animals used for experiments, we also examined for YHV using semi-nested RT-PCR an additional 20 specimens of *P. pugio* from the Davis Bayou, Ocean Springs, Mississippi, to represent a couple hundred collected to be used; they were also examined for WSSV. Also, 30 specimens of *C. sapidus* from Deer Island off Biloxi, Mississippi, were examined for YHV as well as for WSSV and *Hematodinum* sp., and they were used as experimental animals. No individual of either decapod expressed a positive reaction for any of the agents.

3.2. Experimental infections

Based on 1-set PCR, two species of palaemonids and the blue crab were positive after YHV inoculation while all other crustacean species were YHV-negative. Although some animals either fed or injected with YHV demonstrated a YHV-positive reaction by semi-nested PCR at 5 and 7 dpi, all individuals returned to YHV-negative status by 14 dpi (Figs. 2 and 3, arrowheads; Table 1).

Table 1. Experimental infection of yellow-head virus in decapod and stomatopod crustaceans from coastal Mississippi determined by RT-PCR

				Natural infection*	Injection (days PI)*							Feeding (days PI)*		
				Semi-nested	1-set PCR			Semi-nested PCR				Semi-nested PCR		
Superfamily	Family	Species	Common name	PCR	3	7	14	3	7	14	36	3	7	14
Palaemonoidea	Palaemonidae	*Palaemonetes pugio*	Daggerblade grass shrimp	0 (20)	6 (10)	6 (10)	6 (10)	10 (10)	10 (10)	10 (10)	10 (10)	4 (10)	4 (10)	5 (10)
Palaemonoidea	Palaemonidae	*Palaemonetes kadiakensis*	Mississippi grass shrimp	0 (10)	3 (5)	3 (5)	2 (5)	5 (5)	5 (5)	5 (5)	nd	2 (5)	2 (5)	nd
Portunoidea	Portunidae	*Callinectes sapidus*	Blue crab	0 (20)	6 (10)	1 (10)	0 (10)	10 (10)	2 (10)	0 (10)	nd	8 (10)	5 (10)	0 (10)
Paguroidea	Diogenidae	*Clibanarius vittatus*	Thinstripe hermit	0 (10)	0 (5)	0 (5)	0 (5)	4 (10)	2 (10)	0 (5)	nd	2 (10)	0 (10)	nd
Ocypodoidea	Ocypodidae	*Uca spinicarpa*	Spined fiddler	0 (10)	0 (5)	0 (5)	0 (5)	4 (10)	4 (10)	0 (5)	nd	0 (5)	nd	nd
Ocypodoidea	Ocypodidae	*Uca longisignalis*	Gulf marsh fiddler	0 (10)	0 (5)	0 (5)	0 (5)	2 (10)	2 (10)	0 (5)	nd	0 (5)	nd	nd
Ocypodoidea	Ocypodidae	*Uca virens*		0 (6)	0 (5)	0 (5)	0 (5)	2 (10)	2 (10)	0 (5)	nd	0 (5)	nd	nd
Ocypodoidea	Ocypodidae	*Uca panacea*	Gulf sand fiddler	0 (5)	0 (5)	0 (5)	0 (5)	0 (10)	0 (5)	0 (5)	nd	0 (5)	nd	nd
Grapsoidea	Sesarmidae	*Armases cinereum*	Squareback crab	0 (5)	0 (5)	0 (5)	0 (5)	4 (10)	2 (10)	0 (5)	nd	0 (5)	nd	nd
Grapsoidea	Sesarmidae	*Sesarma recticulatum*	Purple marsh crab	0 (5)	0 (5)	0 (5)	0 (5)	4 (10)	2 (10)	0 (5)	nd	0 (5)	nd	nd
Xanthoidea	Menippidae	*Menippe adina*	Gulf stone crab	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	nd	nd	nd	nd
Hippoidea	Hippidae	*Emerita talpoida*	Atlantic sand crab	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	nd	nd	nd	nd
Squilloidea	Squillidae	*Squilla empusa*	Mantis shrimp	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	nd	nd	nd	nd

nd – not detected; PI – post-inoculation

* Positive (examined)

3.2.1. Viral dynamics in P. pugio

Specimens of P. pugio injected with YHV showed a gradual increase of viral copy number per μg RNA during the 36-d period. The mean log viral copy number per μg RNA showed a peak between 72 and 336 hpi. This combined peak is significantly higher ($p < 0.05$, one-way ANOVA) than the values recorded at 6–24 hpi or at 864 hpi (Fig. 1). Semi-nested RT-PCR also exhibited slightly brighter bands for individuals sampled from 3 to 14 dpi (Fig. 2, lower panel). The cumulative mortality of im injected individuals was 8% (9/112) during the 36-d period.

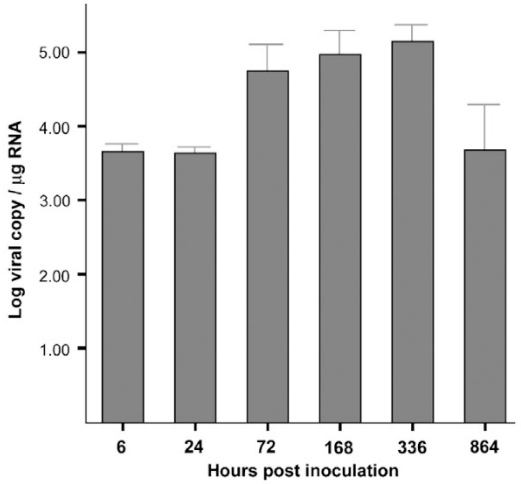

Figure 1. qRT-PCR quantification of YHV RNA levels in the daggerblade grass shrimp, *Palaemonetes pugio*, after injection at different time periods. The log viral copy per μg RNA of combined data from 72–336 h post inoculation (hpi) is significantly higher than those of 6–24 hpi and 864 hpi ($p < 0.05$, one-way ANOVA).

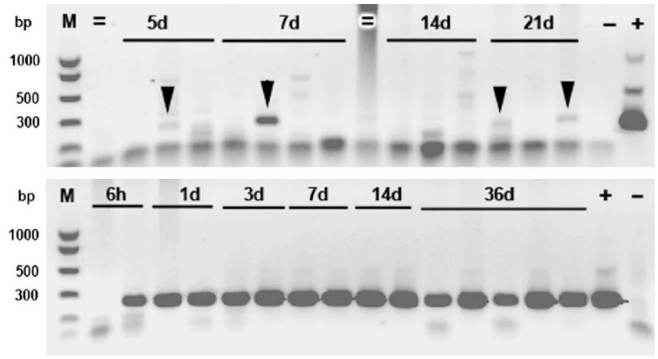

Figure 2. Semi-nested RT-PCR amplification of RNA from tissue of *Palaemonetes pugio* that was fed (upper panel) and injected (lower panel) virus from YHV-positive *Litopenaeus vannamei*. Lane M, 100 bp ladder DNA marker; +, positive control from *L. vannamei* administered YHV; –, RNase-free water; =, *P. pugio* injected with autoclaved saline for 5 and 14 days. Arrows show the faint band indicating YHV-positive.

P. pugio inoculated per os did not show mortality (0/80). The semi-nested RT-PCR demonstrated that the viral load in those individuals was lower than that in injected ones (Fig. 2). A few shrimp showed a faint positive result by semi-nested RT-PCR (Fig. 2, arrowheads) and others showed no band.

3.2.2. Viral dynamics in C. sapidus *and* L. vannamei

After *C. sapidus* was exposed to YHV-positive tissue per os, semi-nested RT-PCR demonstrated positive results at 6 hpi, and reached the highest signal 3 days after the last feeding, with the signal becoming weaker at 7 dpi and undetectable at 14 dpi (Fig. 3). The positive control from YHV-infected *L. vannamei* showed a strong signal of three bands; crab hemolymph showed one positive band, indicating that the viral load was low. The crab experienced different infection patterns when fed or injected. The viral load in crab hemolymph reached its peak at 3 dpi after the 3 days of feeding but was still detectable at 7 dpi. Afterward, the viral number was undetectable. In contrast, when YHV was injected into either the muscle or body cavity, the viral load was not significantly different at 6, 24, and 72 hpi, and the viral number did not increase to a peak as seen in crabs fed the virus. The viral load was undetectable for injected groups by 7 dpi onward.

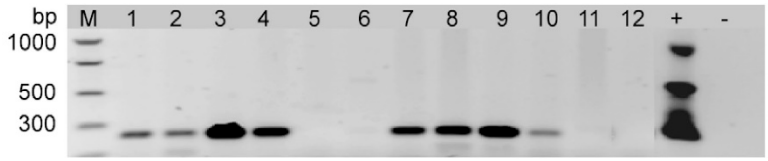

Figure 3. Semi-nested RT-PCR 247 bp amplification of YHV in hemolymph of *Callinectes sapidus* administered by feeding tissue from YHV-positive *Litopenaeus vannamei*. M, 100 bp ladder DNA marker; 1–6, crab A; 7–12, crab B; +, positive control from *L. vannamei* administered YHV and showing three positive bands; –, negative control. 1, 7: 6 hpi; 2, 8: 1 dpi; 3, 9: 3 dpi; 4, 10: 7 dpi; 5, 11: 14 dpi; and 6, 12: 21 dpi.

The mean log value of the viral copy number per μg blue crab hemolymph RNA during the first 72 h is shown in Table 2 and Fig. 4. No statistically significant difference was found in mean viral copy number between injection into the body cavity and muscle. Also, no statistically significant difference was found in the mean viral copy number between 6 and 72 hpi.

Table 2. Comparison of YHV log viral copy/μg hemolymph RNA administered YHV
SE = standard error

Host	Time (h, PI)	Injection site	*N*	Mean of log viral copy/μg RNA	SE
Callinectes sapidus	6	Body cavity	5	2.702	0.318
	6	Muscle	5	3.057	0.026
	6	Total	10	2.879	0.162
	24	Body cavity	5	2.918	0.047
	24	Muscle	5	2.38	0.036
	24	Total	10	2.628	0.101
	72	Body cavity	5	2.972	0.495
	72	Muscle	5	3.276	0.254
	72	Total	10	3.124	0.267
Litopenaeus vannamei	6	Muscle	5	3.086	0.163
	24	Muscle	5	4.738	0.418
	72	Muscle	4	8.028	0.217

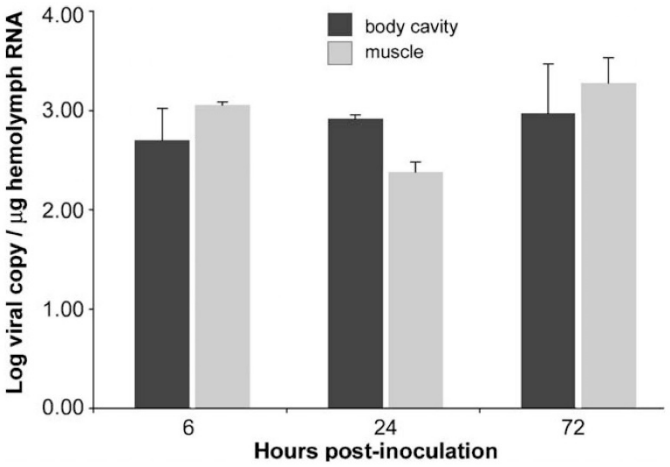

Figure 4. The qRT-PCR quantification of YHV RNA levels in hemolymph of *Callinectes sapidus* at different hours after being injected with 4.72×10^5 YHV copies into the body cavity and muscle.

3.4. In situ hybridization for P. pugio

Of the four tested individuals of *P. pugio* analyzed by ISH, one expressed a strong positive reaction and two showed weak positive reactions in connective tissue within skeletal muscle, and the fourth was negative. Positive shrimp demonstrated dark blue precipitation in the cytoplasm of connective tissue of hepatopancreas tubules, skeletal muscle, and midgut (Fig. 6). The average viral load for these shrimp at 7 dpi was 1.5×10^4/μg RNA.

4. Discussion

The term "reservoir host" as used here refers to a host in which the agent replicates and the host serves as a reservoir of the virus able to infect a host of interest. In our study, the virus replicated in *P. pugio*, indicating it could serve as a reservoir host for YHV to infect economically important commercial penaeid shrimps. The positive ISH results confirm the infection in the palaemonid and support the likelihood of the viral titer being high enough to be infectious to the penaeid. The results from the crab showed that YHV did not replicate in it, but the copy number could be sustained for 72 h after which the virus was not detectable. In contrast, YHV replicated rapidly after being injected into the commercial penaeid *L. vannamei*, and the initial mortality of that penaeid appeared before 72 hpi, with mortalities progressing to 100% before 120 hpi (Table 2, Fig. 5). Since YHV rapidly kills most penaeid shrimps, these decapods may not serve as normal hosts. The chronic infection in *P. pugio* can serve as a long-term source of virus that can infect penaeids, especially in areas or during seasons when no other source would be available. Free virus remains infective for over 6 days at 4°C and 25°C (Ma et al., 2008) and even longer when in tissue. "Carrier hosts" are those vectors in which the virus does not replicate, but it can remain active for a short time.

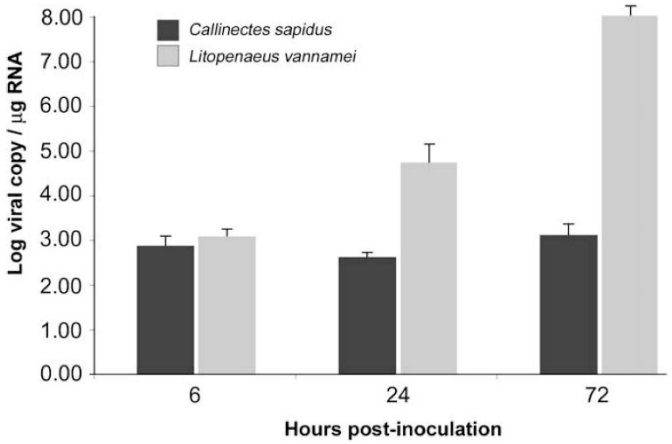

Figure 5. The qRT-PCR quantification of RNA levels in hemolymph of *Callinectes sapidus* and *Litopenaeus vannamei* at different hours after being injected with 4.72×10^5 and 2.36×10^4 copies of YHV, respectively.

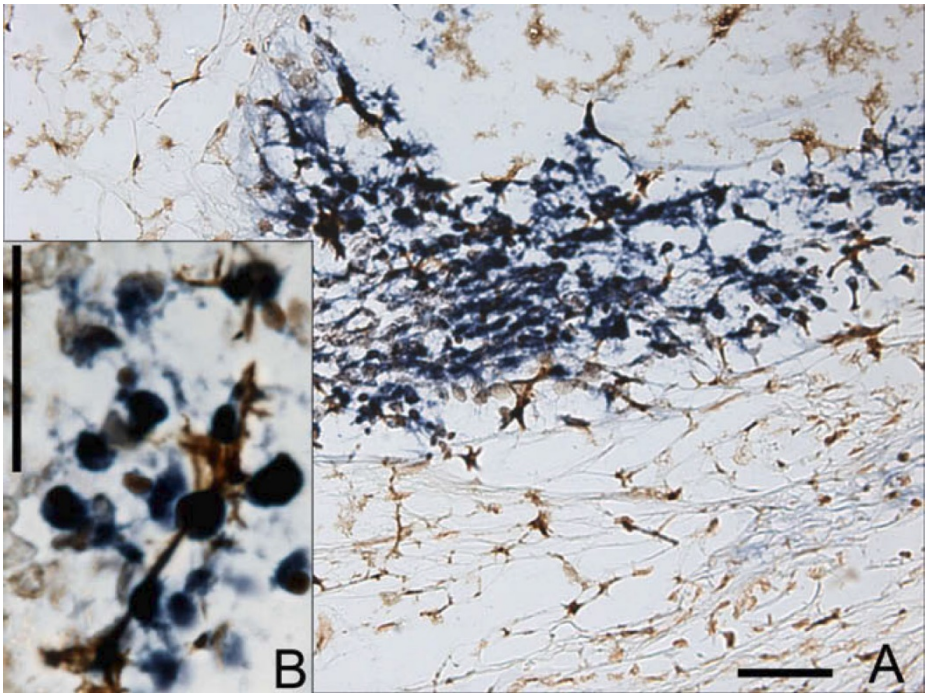

Figure 6. In situ hybridization analysis detecting YHV in experimentally exposed *Palaemonetes pugio*. The positive reaction is depicted by the dark blue precipitation in the cytoplasm of connective tissue. (A) Low magnification and (B) High magnification. Scale bars = 30 μm.

Most tested penaeid shrimps are susceptible to disease from YHV, such as *Farfantepenaeus aztecus*, *Farfantepenaeus duorarum*, *Fenneropenaeus indicus*, *Fenneropenaeus merguiensis*, *Litopenaeus setiferus*, *L. stylirostris*, *L. vannamei*, *Metapenaeus affinis*, *Metapenaeus brevicornis*, *Metapenaeus ensis*, *Metapenaeus japonicus*, and *P. monodon* (Flegel et al., 1995a,b; Wang et al., 1996; Lightner et al., 1997; Lu et al., 1994, 1997; Longyant et al., 2006). However, differentiation of reservoir from carrier hosts of non-penaeid species was rarely documented. *Acetes* sp. and *Palaemon styliferus* may serve as reservoir hosts (Flegel et al., 1997). Longyant et al. (2005) screened five species of palaemonid shrimp, *Macrobrachium rosenbergii*, *M. lanchesteri*, *M. sintangense*, *P. styliferus*, and *P. serrifer*, collected near farms containing *P. monodon* in Thailand using RT-PCR and monoclonal antibodies specific to structural proteins of YHV, but they did not detect a natural YHV infection in any individual examined. We expect that a more extensive survey of local crustaceans would reveal infection in at least a few wild species. After injection with YHV, a small proportion of *M. lanchesteri* showed mild YHV infections at day 3 but no infection at 10 and 30 dpi; *M. sintangense*, *P. styliferus*, and *P. serrifer* were susceptible to YHV (Longyant et al., 2005).

If some local Mississippi crustaceans harbor the exotic YHV, we examined too few individuals to detect it, but we corroborated the studies by Longyant et al. (2005, 2006) showing that the virus could be established temporarily in many decapod crustaceans when injected with YHV. Feeding the virus to those crustaceans, however, produced few that

exhibited a positive PCR reaction, especially after 3 days. *P. pugio* and *C. sapidus* appeared the most likely vector hosts, so we placed emphasis on them to determine if they could serve as a reservoir or carrier host. The 1-set PCR method was not as effective in detecting YHV because the YHV load was one to several log values lower than those in highly susceptible hosts (e.g., *P. monodon* and *L. vannamei*). This low viral load may be the reason why Longyant et al. (2006) did not detect YHV after 3 days in 16 species of crabs after injection. The more sensitive semi-nested RT-PCR can grade infections into three levels depending on bands present on a gel (Kiatpathomchai et al., 2004) and serves as a more useful method than the 1-set PCR method for detecting vector hosts. From our observations, the semi-nested RT-PCR method was consistent with that of qRT-PCR; however, conventional RT-PCR could produce negative results when just one primer pair set was used to evaluate YHV in both *P. pugio* and *C. sapidus* when fed the virus or even in the early stages when injected im. But the disadvantage to using the semi-nested RT-PCR method is that it may produce a false positive result because of high sensitivity. In an attempt to make our results comparable with those obtained by Kiatpathomchai et al. (2004) using their original semi-nested RT-PCR method for YHV, we decreased the amplification cycles in consideration of both sensitivity and specificity values.

When *P. pugio* was fed YHV, the semi-nested RT-PCR amplification detected the virus in the tissue on 5, 7, and 21 dpi in some individuals, even though the viral concentration was low. There are several possibilities that can produce this situation (1) the PCR detection limitation, (2) some individuals may consume more viral particles than others, and (3) the species may have a different feeding behavior from *C. sapidus*, consuming only a small amount of YHV-positive tissue. After im injection into *P. pugio*, it still showed positive results at 36 dpi in five random samples with a viral load at about 10^4 copies μg^{-1} RNA by the semi-nested RT-PCR method. If *P. pugio* is preyed upon by susceptible hosts, that dose is high enough to infect penaeid shrimps and perhaps other hosts.

Palaemonid shrimps, penaeid shrimps, and the blue crab constitute the primary crustacean food source for fishes, crustaceans, and other animals in most estuarine habitats from Texas to northeast Florida as well as along the Atlantic coast (e.g., Christmas and Langley, 1973; Kneib and Wagner, 1994; Rozas and Minello, 2001; Granados-Dieseldorff, 2006). As hypothesized by Odum et al. (1982), those crustaceans appear to represent a major link between detritus production in wetlands and coastal food webs. *P. pugio* is the primary dietary component for many fishes. For example, it dominates the diet of juvenile red drum (*Sciaenops ocellatus*). In Alabama, it occurs in 3-cm standard length (SL) fish and becomes the most important component in 4- to 7-cm SL fish (Morales and Dardeau, 1987), and it also contributes heavily to the diet of larger red drum in Mississippi (Overstreet and Heard, 1978). Overstreet and Heard (1982) also report other inshore fishes in Mississippi as consumers of *P. pugio* and *Palaemonetes vulgaris*. Predation of infected grass shrimps by fishes results in infected feces, another possible source for infections in penaeids, palaemonids, and other vectors.

P. pugio is a permanent resident of the inshore estuaries. Commercial penaeids and the *C. sapidus* are not, but they use the estuaries as nurseries; the penaeids spawn offshore and *C. sapidus* spawns near barrier islands. The grass shrimp feeds on postlarval *C. sapidus* (Olmi and Lipcius, 1991). Its behavior shows that it is not a "search and capture" predator

but rather it depends on chance encounters and state of hunger (Morgan, 1980); it also feeds on epiphytes, detritus, and algae (Quiñones-Rivera and Fleeger, 2005). As trophic generalists with mixed diets, the grass shrimps as well as the penaeids and *C. sapidus* all feed on each other, depending on body size, habitat type, time of day and tide, and season. Penaeids have been shown to eat grass shrimps (e.g., Leber, 1985; Kneib, 1987), and the blue crab feeds on grass shrimps and other caridean shrimps as well as on penaeids, other *C. sapidus* individuals, and fishes (Laughlin, 1979). Seasonal differences and overlapping of these different crustaceans in the northern Gulf of Mexico (e.g., Livingston, 1984) allow continual feeding on each other and potential transmission of YHV and other agents.

The Mississippi grass shrimp, *P. kadiakensis*, which is commonly found in fresh water environments in the central and southern United States (Pennak, 1978; Anderson, 1985), also showed a YHV-positive reaction by 1-set and semi-nested RT-PCR after being either injected or fed. As a congener of *P. pugio*, *P. kadiakensis* is also a potential reservoir host for YHV. Hence, both fresh water and brackish palaemonids from disjunct localities in the USA and abroad seem to serve as reservoir hosts for YHV.

Reasons for different infection patterns by feeding and injection of YHV in *C. sapidus* are not clear. The individuals may have consumed YHV-positive tissue at different times. Even if they all consumed tissue at the same time, the viral number in the tissue and that consumed may have differed. Hence, the qRT-PCR may not be an exact method for evaluating virus in fed animals. One reason that YHV was lost more quickly from the hemolymph of *C. sapidus* injected rather than fed might be that the relatively large amount of virus injected all at once induced a rapid host response eliminating the virus. However, the fed virus may have taken longer to pass from the digestive tract to the hemolymph or it may have remained free in the hemolymph longer and not been sequestered as rapidly by host hemocytes. Probably, digestive activity on the fed virus influences replication. In any event, feeding on the virus by *P. pugio*, *C. sapidus*, or other hosts is how select crustaceans in nature can become vectors.

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