Parasitic crustaceans as vectors of viruses, with an emphasis on three penaeid viruses

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

Department of Coastal Sciences, The University of Southern Mississippi, Ocean Springs, MS 39564, USA

Synopsis Parasitic crustaceans serve as both hosts and vectors of viruses as well as of parasites and other microbial pathogenic agents. Few of the presumably numerous associations are known, but many can be anticipated. Recently, branchiurans and gnathiid isopods have been documented to host helminths and blood parasites. Because the agents can be observed readily with a microscope, these are better recognized than are the smaller viral, bacterial, and fungal agents. Some agents are harmful to the host of the crustacean parasite and others are not. Viruses probably fit both these categories, since viruses that do not appear pathogenic are often seen in ultrastructural images from a range of invertebrate hosts, including crustaceans. Some viruses have been implicated in causing disease in the host, at least under appropriate conditions. For example, lymphocystis virus may possibly be transmitted to the dermis of its fish hosts by copepods and to the visceral organs by a cymothoid isopod. Similarly, argulid branchiurans seem to transmit the viral agent of spring viremia of carp as well as carp pox, and copepods have been implicated in transmitting infectious hematopoietic necrosis, infectious salmon anemia, and infectious pancreatic necrosis to salmon. Other viruses can be vectored to their hosts through an additional animal. We exposed three viruses, Taura syndrome virus (TSV), white spot syndrome virus (WSSV), and yellowhead virus (YHV), all of which cause mortalities in wild and cultured penaeid shrimps, to crustacean parasites on fish and crabs. Using real-time polymerase chain reaction analysis, we show that TSV in the cyclopoid copepod *Ergasilus manicatus* on the gill filaments of the Gulf killifish, *Fundulus grandis*, the acorn barnacle *Chelonibia patula* on the carapace of the blue crab, *Callinectes sapidus*, and gooseneck barnacle *Octolasmis muelleri* on the gills of *C. sapidus*, can replicate for at least 2 weeks and establish what should be an infective dose. This result was additionally supported by positive *in situ* hybridization reactions. All three parasites are the first known non-penaeid hosts in which replication occurs. The mean log copy number of WSSV also suggested that replication occurred in *E. manicatus*. The mean log copy number of YHV gradually decreased in all three parasites and both hosts over the 2-week period. The vector relationships indicate an additional potential means of transmitting and disseminating the disease-causing agents to the highly susceptible and economically valuable penaeid shrimp hosts.

Introduction

A quotation extracted from ''On poetry: a rhapsody'' by Swift (1733) reads ''The Vermin only teaze and pinch Their Foes superior by an Inch. So Nat'ralists observe, a Flea Hath smaller Fleas that on him prey, And these have smaller Fleas still to bite 'em, And so proceed ad infinitum.'' The verse, which has been repeated in various forms, evolved into the nursery rhyme entitled ''The Siphonaptera,'' sometimes referred to as ''Fleas,'' and constitutes the theme of this presentation. The simple rhyme states ''Big fleas have little fleas, Upon their backs to bite 'em, And little fleas have lesser fleas, and so, ad infinitum.''

Crustacean parasites (representing the fleas) belonging in several, if not most, orders probably harbor a large array of viruses (some of the littler fleas), just as their free-living crustacean counterparts. On the other hand, few cases have been documented, let alone well studied (e.g., Cusack and Cone 1986). Viruses in free-living crustaceans are becoming well known because they act as limiting factors to the economic success of penaeid shrimp aquaculture and result in billions of US dollars in lost harvest (Lightner 2007). Viral infections also influence other economically important crustaceans such as the blue crab, crayfishes, and spiny lobster (e.g., Edgerton et al. 2002; Shields and Overstreet 2007; Li et al. 2008). Parasitic crustaceans appear to vector viruses to fishes in aquaculture, further increasing the monetary loss to the industry resulting from viral infections. Losses to animals in the wild may also be significant.

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[1]E-mail: robin.overstreet@usm.edu

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Perhaps the great majority of viruses from crustaceans exhibit little virulence, but most that are recognized presently are pathogenic.

Numerous parasites already are known to vector infectious agents. Helminth and protozoan parasites have been proven or suggested to transmit viruses to mammalian, fish, or unknown suspected hosts with harmful effects. For example, metastrongyle nematode species (lungworms) from pigs have long been known to harbor the virus responsible for swine influenza and maintain it in their eggs or in larvae within the earthworm intermediate host for over a year (Shope 1943). The virus apparently remains ''masked'' in a noninfective form in a metastrongyle within the pig until the pig is further stressed, such as by migrating larvae of *Ascaris suum* or multiple injections of extracts of that ascarid nematode (Sen et al. 1961). Another nematode, *Trichinella spiralis*, experimentally transmitted the virus responsible for lymphocytic choriomeningitis (Syverton et al. 1947). Viral-like particles have been shown by TEM to occur naturally in cecal epithelium of the mammalian lung-infecting trematode platyhelminth *Paragonimus kellicotti* (transmitted to cats from a second intermediate crayfish host and possibly the agent of feline influenza) (Byram et al. 1975). Other viral particles have been reported from various cells of the trematode *Cotylogaster occidentalis* infecting freshwater molluscs (Ip and Desser 1984), the monogenean platyhelminth *Diplectanum aequans* on gills of fishes (Mokhtar-Maamouri et al. 1976), and the nematode *Trichosomoides crassicauda* in the gall bladder of wild rats (Foor 1972). Virus-like particles are even described from the cytoplasm of the coccidian protozoan *Goussia aculeati* from the intestinal epithelium of a fish (Stienhagen et al. 1993) and from within food vacuoles of the apostome ciliate parasite *Hyalophysa chattoni* on the daggerblade grass shrimp, *Palaemonetes pugio* (see Kucera 1992). Further investigations of those and other such infections and of their relationships with parasite health and host disease remain a fruitful field.

Crustacean parasites are not well known for transmitting viruses, but that deficiency probably reflects the little attention directed to the field. Recently, branchiurans (Moravec et al. 1999) and gnathiid isopods (Davies et al. 2004; Smit et al. 2006) have been documented to host parasites, similar to their terrestrial-counterpart arthropod hosts, and some may even serve as hosts in addition to recognized leech hosts, once thought the sole vector transmitting most or all aquatic blood parasites. Because parasitic agents can be observed readily under a microscope, these agents have been recognized more easily than have the smaller viral, bacterial, and fungal agents. The parasites also visually portray how ''Big fleas have little fleas. . . .''

The purpose of this report is to indicate the actual or presumed role of crustacean parasites in association with viruses as provided in the literature and to document original data on experimental infections in three such crustacean parasites with known viruses that readily kill their commercially important penaeid (shrimp) hosts. The term ''parasite'' is used in a broad sense to include attached, potentially harmful, ''commensal'' barnacles on the carapace and gills of the blue crab, *Callinectes sapidus*. Because of the relatively large barnacle weight and the loss of the crab's respiratory surface area, the harmful barnacles can reduce the longevity of the crab and increase its vulnerability to predators.

Materials and methods

Animals

For purposes of our original experiments, we exposed three viruses to the cyclopoid copepod *Ergasilus manicatus* on the gill filaments of the Gulf killifish, *Fundulus grandis*, and diamond killifish, *Adinia xenica*, and the potentially parasitic acorn barnacle *Cheloniba patula* on the carapace and gooseneck barnacle *Octolasmis muelleri* on the gills of *C. sapidus* (blue crab). The copepod was identified according to the generic revision of Roberts (1970), and the taxonomically important basal inflation and lateral process of the second segment of the prehensile antenna are indeed thin walled and inflatable as Roberts suggested. Young (1990) considered *O. muelleri* distinct from *Octolasmis lowei*, a position we follow but which requires confirmation. The biology of the barnacles has been summarized by Overstreet (1983) and Shields and Overstreet (2007).

The fish came from Simmons Bayou, Ocean Springs, Jackson County, off Mississippi Sound (30°22′22.80″N; 88°45′10.13″W) on July 29–31, 2008 when the salinity was 17–20 ppt. *F. grandis* measured 46–95 mm total length (TL) and *A. xenica* measured 26–38 mm TL. They were acclimated to 15 ppt artificial sea water (Fritz Super Salt Concentrate, synthetic sea salt, Dallas Texas) in an aerated 26.0 ± 0.5°C water bath for 7 days before being administered the viruses. The specimens of *C. sapidus*, measuring 137–180 mm in carapace width (point to point), came from the eastern end of West Ship Island (Gulf Island National Seashore, 30°13′9.10″N; 88°56′19.56″W) off Mississippi when the salinity was 30 ppt. They were acclimated for 7 days in aerated synthetic sea

water (hW Marinemix® professional, Wiegandt, Germany) to 20 ppt and maintained at 26.0 ± 0.5°C.

Viruses

The three viruses comprising the mixture were white spot syndrome virus (WSSV), Taura syndrome virus (TSV), and yellowhead virus (YHV). All are highly pathogenic to penaeid shrimps, capable of killing them within a few days after initial exposure.

WSSV remains the sole member of Nimaviridae in the genus *Whispovirus* (see Vlak et al. 2004) and consists of a double-stranded DNA virus with a large circular genome of about 300 kb, representing one of the largest genomes of animal viruses known. It is rod-shaped, nonoccluded, enveloped, and as large as 350 by 130 nm, with a cylindrical nucleocapsid up to 260 by 96 nm (Durand et al. 1997; Lo 2006). There is some variation among sequenced gene fragments of several WSSV isolates (Wang et al. 2000). Also, Galavíz-Silva et al. (2004) examined many isolates and considered two or three profiles, Kiatpathomchai et al. (2005) defined 12 types, and Laramore et al. (2009) studied the virulence of seven isolates, some of which we used to conduct research on decapods. Mass mortalities and infections in the wild are known from both the eastern and western hemispheres (Lo 2006).

The WSSV isolate used in this study was originally obtained by Shiao Wang, The University of Southern Mississippi, from infected *Penaeus monodon* in an aquaculture pond in Guangzhou, China, in 1997. This highly virulent isolate had undergone two passages through *Litopenaeus vannamei* before used in this study. Our studies show that an infective dose of this isolate typically kills its penaeid host in 2–4 days, depending on the dose and on both species and genetic stock of penaeid host. All shrimp used as a reference source consisted of a specific-pathogen-free TSV-sensitive strain of *L. vannamei* originating from The Oceanic Institute in Hawaii (e.g., Xu et al. 2007). As few as 3000 genome copies per μg of total DNA (host and virus) can be fatal in that host. Chronic infections can result in deposits of calcium salts in the internal cuticular surface, producing the "white spot" condition.

TSV is a small icosahedral positive-sense, nonenveloped, single-stranded RNA virus in the genus *Cripavirus*, recently transferred from the Picornaviridae to the Discistroviridae; it is related to the cricket paralysis-like viruses (CrPV-like) (Mari et al. 2002). The virus has been recognized as causing mass mortalities in penaeids from South America as well as Central America and the United States since the early 1990s. It later spread to Southeast Asia in 1999 with the culture of *L. vannamei*. The virus has been divided into three distinct groups, with the most virulent represented by an isolate from Belize, Central America, that caused 50% mortality in 3 days compared with 4–6 days for the other virulent isolates (Erickson et al. 2005; Tang and Lightner 2005). Wild *Litopenaeus setiferus* (white shrimp), but not *Farfantepennaeus aztecus* (brown shrimp) and *F. duorarum* (pink shrimp), from the Gulf of Mexico died when fed a Texas isolate. Tissues from the two exposed resistant species obtained at Day 79 postexposure could kill susceptible bioassay *L. vannamei* when fed to it (Overstreet et al. 1997). A few specific stocks of *L. vannamei*, the primary cultured penaeid in the world, developed through breeding programs are resistant even to highly infective TSV (Argue et al. 2002; Moss et al. 2005; Overstreet personal observations).

The TSV isolate for this study originated from a 1995 aquaculture disease outbreak in Texas, where it killed most exposed *L. vannamei*. An homogenate of this virulent Texas isolate of TSV maintained in our laboratory since the 1995 outbreak was mixed with additional material of the same isolate from two chronically infected shrimp. An infective dose typically kills most of its penaeid hosts in 3–4 days.

YHV belongs in the genus *Okavirus*, in the ronivirid order Nidovirales (Lightner 1999). Virions are rod-shaped, enveloped particles, 150–200 nm long by 40–60 nm wide with rounded ends and 8–11 nm projections extending from the surface. The virus occurs in the cytoplasm of cells of ectodermal and mesodermal origin as well as in intracellular vesicles. It is presently common in Southeast Asia, including India (e.g., Walker 2006). There is one known pathogenic genotype, with known isolates that exhibit genetic diversity (Walker 2006; Ma and Overstreet personal observations), as well as five other genotypes that do not cause mortality of infected penaeids.

We used the YHV92 isolate obtained from Donald Lightner, the University of Arizona, which has been sequenced in its entirely by Ma et al. (manuscript in preparation). It was originally collected in 1992 from *Penaeus monodon* in Thailand. An infective dose typically kills its penaeid host in 1–2 weeks.

Viral administration and animal sampling—fish

Material of all three viruses, consisting of 9.5 g of WSSV-infected tissue from about 10 individuals, 18 g of TSV-infected homogenate, and 20 g of YHV-infected gill tissue were blended together.

This homogenate was separated into 1.5-g aliquots, with each group of fish fed one aliquot on Day 0 and a second on Day 1 for a total of a 3-g feeding. The administered viruses were quantified as 5.73 log WSSV copy number/μg of total DNA, 7.68 log TSV copy number/μg of total RNA, and 6.62 log YHV copy number/μg of total RNA, and all represented what has been shown in our laboratory to be above that necessary to produce lethal infections to *L. vannamei*.

These two aliquots of homogenate of all three viruses were introduced into the water so that the test animals could feed on them. There were two groups of infested fish, one with 43 *F. grandis* and one with 32 *A. xenica*; each was exposed in 4 l of water in a 19-l aquarium, with seven and five individuals, respectively, kept as nonexposed controls in 76-1 and 57-l aquaria, respectively. The exposed groups were netted on Day 3 and washed in a series of three 37-l aquaria to eliminate any remaining unfed infected material. They were then transferred to 114-1 and 76-l aquaria and fed commercially pelleted feed (Size no. 3, Rangen, Buhl, Idaho) once a day. All aquaria had aeration through sponge filters (AZOO® sponge filter, Aquatic Eco-systems, Inc., Apopka, Florida) and were maintained at 26.0 ± 0.5°C. A 50% seawater exchange was performed every day. Feces were removed from the bottom daily. A representative sample of five fish per time period was killed by decapitation and the copepods on the gill as well as the liver and intestine of each fish were collected for real-time PCR (both qPCR [WSSV] and qRT-PCR [TSV and YHV]) and stored at –80°C. Samples were collected at 0 h postfeeding, which was 48 h after being initially exposed to the viruses and immediately after being removed from the viral material and washed, as well as at 6, 24, 72, 168, and 336 h postfeeding.

Viral administration and animal sampling—crabs

Crabs were treated similarly as the fish. However, some differences were necessary. They were originally placed in individual plastic cages within an aerated 300-l raceway for 3 days while their eggs were released and all food obtained from the wild was voided. They were then moved to individual aquaria in a single temperature-controlled waterbath at 26.0 ± 0.5°C. Initially, 16 barnacle-infested crabs were exposed to the virus individually in 4 l of 20 ppt sea water in 9-l aquaria, and three controls were maintained without viral exposure. Each crab was then washed in a series of three 19-l aquaria before being transferred to its own 37-l aquarium aerated with a sponge filter; a 50% water exchange was conducted every other day after viral exposure.

Because the crab hosts had to be killed to sample *O. muelleri* on the gills, these were sampled serially at the indicated time periods (0, 6, 24, 72, 168, and 336 h postfeeding), the same as for *E. manicatus* on the gills of fish. Samples of soft tissues from *C. patula* on the carapace of the host, however, were removed at the same intervals indicated above; at those times a hemolymph sample was also drawn from each crab using a 25-ga, EDTA-coated, 1-ml syringe.

RNA (TSV, YHV) and DNA (WSSV) nucleic acid extraction

Tissue RNA from the whole barnacles and copepod and from fish liver and fish intestine were extracted using the protocol from the High Pure Tissue RNA Kit (Roche, Cat no. 12033674001). Briefly, the tissues were rinsed with autoclaved distilled water, and 9–20 mg of tissue was aliquotted 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 50–100 μl of sterile DEPC-treated, RNase-free, distilled water and stored at –80°C. Blue crab hemolymph nucleic acid (NA) was extracted following the protocol from the High Pure Viral Nucleic Acid Kit (Roche, Cat no. 11858874001). Briefly, 150 μl of autoclaved nuclease-free water was added to 50 μl of hemolymph and mixed with 250 μl binding buffer containing poly A and proteinase K. The extracted NA was eluted into 100 μl of sterile, DEPC-treated, RNase-free, distilled water and stored at –80°C.

Tissue DNA was extracted using the protocol from the High Pure Template Preparation Kits (Roche, Cat no. 11796828001). Briefly, the tissue (from both barnacle species, fish intestine, fish liver, and copepod) was rinsed with sterile distilled water, and 10–20 mg of tissue were aliquotted, sliced and homogenized in 200 μl Tissue Lysis Buffer and 40 μl Proteinase K, incubated for 1–3 h at 55°C and extracted according to the instructions. The DNA was eluted into 50–100 μl of sterile DNase- and RNase-free water and stored at –80°C. A Nanodrop® ND-1000 UV-Vis spectrophotometer was used to determine the NA concentration of all extracted samples.

Real time PCR

The iScript™ One-Step RT-PCR Kit for Probes (BioRad, Cat no. 170-8895) was used to

Table 1 Primers and conditions for real-time PCR

	Primer sequence and cycle parameters	References
WSSV		Durand and Lightner (2002)
1011F	5′-TGGTCCCGTCCTCATCTCAG-3′	
1079R	5′-GCTGCCTTGCCGGAAATTA-3′	
TaqMan	5′-AGCCATGAAGAATGCCGTCTATCACACA-3′	
Parameters	95°C 3′, (95°C 15″, 60°C 30″) × 45	
YHV		Dhar et al. (2002)
141F	5′-CGTCCCGGCAATTGTGATC-3′	
206R	5′-CCAGTGACGTTCGATGCAATA-3′	
TaqMan	5′-CCATCAAAGCTCTCAACGCCGTCA3′	
Parameters	50°C 10′, 95°C 5′, (95°C 15″, 56°C 30″) × 40	
TSV		Tang et al. (2004)
1004F	5′-TTGGGCACCAAACGACATT-3′	
1075R	5′-GGGAGCTTAAACTGGACACACTGT-3′	
TaqMan	5′-CAGCACTGACGCACAATATTCGAGCATC-3′	
Parameters	50°C 10′, 95°C 5′ (95°C 15″, 60°C 30″) × 40	

perform qRT-PCR for YHV and TSV (Table 1). Three HPLC-purified (Invitrogen) synthesized standards containing the amplicon with three extra bp on both sides of the amplicon were as follows: YHV standard was a 72-bp segment from the sequence (GenBank AF148846), TSV standard was a 78-bp segment from the sequence (GenBank AF277675), and WSSV standard contained a 75-bp segment from the sequence (GenBank U50923). The TaqMan probes for the three viruses were synthesized and labeled with fluorescent dyes, 6-carboxyfluoroscein (FAM) on the 5′ end and *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end (Integrated DNA Technologies).

The qRT-PCR amplifications were undertaken in an iCycler Thermocycler (BioRad). The qRT-PCR was conducted in a 25 µl reaction volume containing 5 µl RNA, 12.5 µl 2 × RT-PCR reaction mix for probe, 300 nM of primers, 100 nM probe, and 0.5 µl iScript Reverse Transcriptase Mix for One-Step RT-PCR. IQ^{TM} SuperMix (BioRad, Cat no. 170-8862) was used for detection of WSSV in a 25 µl reaction volume, containing 5 µl DNA, 12.5 µl 2 × SuperMix, 300 nM of primers, and 100 nM probe.

Analysis of data

Following real-time PCR amplification, a baseline and threshold were defined using the BioRad iCycler iQ PCR detection system, resulting in a fractional cycle number (C_T value) assigned to each individual sample. A set of standard dilutions was run simultaneously with all 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 µg total genomic DNA or total RNA. One-way ANOVA analysis (SPSS 15.0 for Windows) was used to compare viral titer at the different sampling times. Comparisons between the titer in parasite versus host as well as parasite versus parasite at those individual periods of time were analyzed using the Kolmogorov-Smirnov two-sample test (K-ST) (Hollander and Wolfe 1999).

In situ hybridization

Because log copy numbers of WSSV and TSV were detected at 3.5–4.5/µg total DNA and RNA, respectively, we sent 2-week old infected material to Donald Lightner at Aquaculture Pathology, University of Arizona, for analysis using the methods described by Lightner (2006a, b). The material, however, had been transferred from –80°C to cold Davidson's fixative rather than fixed directly.

Viruses harmful to parasitic crustaceans

The entoniscid isopod *Portunion conformis* of the yellow shore crab, *Hemigrapsus oregonensis*, represents the best known system of a pathogenic viral infection in a parasitic crustacean. The cytoplasm of various cell types in the parasitic isopod appears to be infected by two viruses. One is relatively large (58 nm in diameter) and unidentified, and the other is a small (25 nm in diameter), tentatively-identified

RNA picorna-like virus (Kuris et al. 1979). In spite of the great abundance of this small virus throughout the isopod as well as in the host crab, the authors originally found no evidence at the initial geographic locality for the virus killing the parasitic crustacean, other than the presence of an occasional dead female parasite. However, high prevalence of viral infections in the isopod from other Californian locations seemed to incriminate the virus with female mortality on the basis of high viral titers in dead individuals (Kuris et al. 1979, 1980). Apparently, mortality of these isopod individuals diminishes the typical cellular hemocytic response of the crab that encapsulates the healthy isopod, completing the marsupium and forming an opening to the gill chamber. That opening allows release of the isopod epicaridean larvae. Consequently, the diminished response of the previous parasitically castrated crab now deposits a melanin layer around the dead isopod, and the crab regains its reproductive capacity. The virus in the crab's host tissue does not seem to harm the crab, but the virus benefits the crab by killing the parasite, resulting in the ability of the crab to encapsulate the parasite as a foreign body and once again produce its own eggs.

Even though not as specific to *C. sapidus* (blue crab) as *C. patula*, the facultative symbiotic ivory barnacle, *Balanus eburneus*, also infests *C. sapidus* in addition to fouling many hard substrata (Shields and Overstreet 2007). Disease from a large 222 by 175 nm enveloped icosahedral DNA iridovirid-like virus in laboratory-reared *B. eburneus* was the first viral infection reported from any barnacle (Leibovitz and Koulish 1989). Based on their TEM study, the virus replicated in parenchymal cells, producing hypertrophy and necrosis, and hemocytes, resulting in pathological alterations somewhat similar to those reported from terrestrial isopods (Federici 1980) and arthropods (e.g., Carey et al. 1978).

The relationship of most viruses with their hosts (as seen by TEM or detected by modern means) is certainly not clear, but the finding of such viruses suggests that disease is common. However, being able to obtain small, moribund animals in the natural environment, or even in culture, remains a difficult task. Infestations of parasitic crustaceans both on vertebrates and on invertebrates often decrease rapidly with time, and perhaps some of that loss could be attributed to viral infections of the parasites. Based on low survival of many crustacean parasites on their hosts or upon reaching their hosts, the possibility of a pathogenic virus seems real and should be investigated.

Viruses in crustaceans not recognized as pathogenic

Based on TEM involving miscellaneous studies on and surveys of viruses from insects, plants, and miscellaneous organisms (e.g., Munn 2006), there are probably many nonpathogenic viruses in parasitic crustaceans (e.g., Adams and Bonami 1991). This assumption, however, is probably rather subjective because under specific conditions some of the viruses probably harm their hosts. For example, there are over 20 known pathogenic viruses not including strains or complexes that produce mortality of commercial penaeid shrimps, and these have been characterized and investigated in considerable detail (e.g., Lightner 1999, 2006a). In addition, TEM sections made to evaluate biological features of penaeids have detected other viruses. Foster et al. (1981) found virus-like particles in the cytoplasm of fixed phagocytic cells in the heart of *Farfantepenaeus aztecus*, and Krol et al. (1990) discovered a reo-like virus in the cytoplasm of epithelial cells of the anterior midgut and specific hepatopancreatic cells of *Litopenaeus vannamei*. The latter was being studied to determine the pathogenic effect of the endemic virus *Baculovirus penaei*. In some cases, reoviruses of a variety of crabs and shrimps can be pathogenic by themselves or when co-occurring with other specific viruses (e.g., Shields and Overstreet 2007). The blue crab along the Atlantic coast harbors seven or eight different viruses described by Johnson (1986), at least two of which are also known to occur in the Gulf of Mexico. Not all are known to be harmful, and only three are lethal to the crab; two co-occur with other viruses apparently producing synergistic pathogenic effects. There are also multiple strains of some viruses, especially, but not consistently, RNA viruses such as YHV (Walker 2006) and TSV (e.g., Tang and Lightner 2005). Some of our unpublished studies with WSSV have involved isolates that do not kill their penaeid host. We suggest that species of the parasitic branchiuran genus *Argulus* may harbor a variety of both harmful and harmless viruses.

An attempt was made to determine whether aquatic viruses might affect copepods. Drake and Dobbs (2005) experimentally exposed the cultured copepod *Acartia tonsa* to elevated concentrations of natural viruses in sea water but did not detect any negative effect on larval or adult survival or on fecundity.

Nonpathogenic viruses also may provide cross-immunity to pathogenic strains, benefitting hosts by inhibiting or eliminating infection and disease.

Nonpathogenic as well as pathogenic viruses also may be transferred to a susceptible host by a mechanical vector, a ''host'' in which the virus does not replicate.

In some cases, a virus can accumulate within a crustacean parasite and replicate. That vector may serve as an alternative host for the virus. That situation can best be demonstrated using real-time PCR and experimental infections.

Vector of pathogenic viruses in fishes

Viral infection in fishes is promoted by the ability of species of *Argulus*, parasitic copepods (*Caligus*, *Lepeophtheirus*, and others), and isopods (cymothoid, exocorallanid, tridentellid, and gnathiid) to either switch hosts (translocate from one individual fish to other individuals of the same species or to other species) or to obtain multiple blood meals from fishes. Species of *Argulus* and of various copepods already have been demonstrated to serve as mechanical vectors of viruses that produce serious disease in fishes.

Salmonid viral diseases are good examples of diseases transmittable by crustacean copepod vectors. Using cumulative mortality as the end point, Nylund et al. (1993) demonstrated that the copepod sea louse *Lepeophtheirus salmonis* in the laboratory could passively transfer the infectious salmon anemia virus (ISAV) to farmed Atlantic salmon (*Salmo salar*). Not all salmonids exhibit clinical disease. Replication of ISAV can occur in the brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) as well as in non-salmonid herring (*Clupea harengus*) without causing disease, perhaps because they become neutralized (e.g., Devold et al. 2000; Kristiansen et al. 2002). However, Nylund et al. (1993, 1994) suggested, based on transmission studies, that the copepods *Caligus elongatus* and *L. salmonis* may transfer the agent from those life-long salmonid carrier hosts to *S. salar*. The host-virus relationship differs among fish species. No replication or persistence of ISAV occurs in saithe (*Pollachius virens*), whether injected with virus or cohabiting space with diseased *S. salar*; however, those copepods that switch hosts may pose a threat to *S. salar* after feeding on the resistant saithe, a gadid that occurs naturally with *S. salar* (see Snow et al. 2002).

Additional viruses from copepods parasitic on salmonids have also been reported. High levels (up to 8.7×10^5 *Pfu*/g) of infectious hematopoietic necrosis virus (IHNV) were detected in *Salmincola* sp. as well as in the leech *Piscicola salmositica* from the gills of spawning sockeye salmon (*Oncorhynchus nerka*) (Mulcahy et al. 1990). Also, infectious pancreatic necrosis virus (IPNV) has been detected in *Lepeophtheirus salmonis*, and, even without evidence of copepods serving as vectors for any virus from field studies, Johnson et al. (2004) promoted copepod control. Criteria have been adopted in Atlantic Canada and Scotland as an integral part of management strategies for control of ISA (Johnson et al. 2004).

Carp viral diseases also have been transmitted by crustacean parasites. Spring viremia of carp (SVC), or infectious dropsy, has been demonstrated by Ahne (1985) to be vectored to *Cyprinus carpio* by *Argulus foliaceus* as well as by the leech *Piscicola geometra*. This transmission is most notable when temperatures ranged from 5°C to 20°C. Lack of replication of this rhabdovirus in the argulid was determined by comparing virus titers (plaque forming units, *Pfu*) in blood of both carp and argulid using titration in an established fish cell line derived from tissue posterior to the anus of the fathead minnow (*Pimephales promelas*) (FHM-cells) 1 week after introducing the argulid to infected carp with 10^4–10^5 *Pfu*/ml of blood, and 2 weeks after introducing the argulid with that blood meal to noninfected carp. At 2 weeks, the *Pfu*/g of tissue in the argulid had decreased to 0 while that from the internal organs of the carp averaged 10^3–10^4. However, Bussereau et al. (1975) were able to detect replication of SVCV in the fruit fly *Drosophila melanogaster*. In carp aquacultural facilities in Israel, a decline in *Argulus japonicus* coincided with complete disappearance of the papillomas of carp pox, a herpesvirus infection, which previously had been abundant (Landsberg 1989).

Viral diseases of fishes other than salmonids and carps are also suspected of being acquired from crustacean parasites. Cymothoid isopods and ergasilid copepods may play a role in transmission of ''spontaneous'' lymphocystis virus. The viral infections are characterized by hypertrophied cells surrounded by a thick hyaline capsule typically found in connective tissue cells of the fins and skin.

Circumstantial evidence indicates that individuals of the cymothoid isopod *Livoneca redmanii* (senior synonym of *Lironeca ovalis*) transmit infection of the lymphocystis virus to internal tissues of the silver perch (*Bairdiella chrysoura*) while devouring the fish's gills. Of 38 fish with infections in Ocean Springs, Mississippi, most had lesions observable on fins, gills, skin, and operculum. Of 21 fish with the lymphocystis lesions on the gills, 20 had one or more isopods present. Of those, 14 had

infections internally. Internal infections in infected fish involved the spleen, coelomic mesentery, nostrils, lateral line pits, liver, area behind the eye, heart, kidneys, ovaries, eye, testes, and gall bladder, with prevalence in that order (Lawler et al. 1974). Either the isopod irritated host tissue, allowing the virus to enter the host or the isopod to transmit the virus. An isolate of this strain of virus can infect both *B. chrysoura* and the sympatric banded drum, *Larimus fasciatus*, but not other tested sciaenids or members of other families in Mississippi. This was determined by unpublished (RMO) studies in which attempts to cross-infect specific fish lymphocystis hosts with injections of the silver perch isolate and infect the silver perch with isolates of lymphocystis discovered from the corresponding non-silver perch fishes failed. Moreover, data from transmission electron microscopy observations indicated that some of those isolates differed from each other morphologically. Internal infections other than those involving the eye have not been seen by us in other local fishes. Dukes and Lawler (1975) described eye infections in *Cynoscion arenarius* from Texas and Nigrelli and Smith (1939) reported single hypertrophied cells in some visceral organs in a fish from New Jersey. Superficial infections were common, although internal infections were not, in fishes from the New York Aquarium where host to host contact was likely (Nigrelli and Ruggieri 1965). With modern molecular tools, it is possible to determine whether the virus from Mississippi occurred in or replicated in the isopod, but lymphocystis infections are not as common now in *B. chrysoura* nor in other local Mississippi fishes as they were in the 1970s.

Nigrelli (1950) noted a positive correlation between a lymphocystis infection of the skin and presence of ergasilids, and he suggested that that group of copepods, as well as argulids may serve as vectors (Nigrelli and Ruggieri 1965). Jones and Hine (1983) noted a similar potential vector-relationship between lymphocystis in the marine orange-spotted spinefoot (*Siganus guttatus*, Siganidae) and *Ergasilus rotundicorpis* from the Philippines in an aquaculture pond.

Vectors of fatal penaeid shrimp viral diseases

Because of the economic importance of wild and cultured penaeid shrimps, we examined the potential role of parasitic crustaceans in vectoring three viruses known to cause mortality to penaeid shrimps. All three of those viruses are recognized by the World Animal Health Organization (OIE) as agents of OIE-listed diseases. The economic losses from the three are dramatic, reaching approximately US$8 billion for WSSV, US$3 billion for TSV, and US$0.5 billion for YHV from their discoveries in the 1990s until 2006 (Lightner 2007). We simultaneously fed WSSV, TSV, and YHV to the parasitic gooseneck barnacle *O. muelleri* on the gills and the acorn barnacle *C. patula* on the carapace of *C. sapidus* (blue crab) and *E. manicatus* on the gills of *F. grandis* (Gulf killifish) and *A. xenica* (diamond killifish). All the parasites and hosts exposed to the viral mixture accumulated virus and some replicated as reported below. Values for viruses in *E. manicatus* all refer to the copepod on *F. grandis*. None of the control crabs or fish exhibited any virus.

Results of experiments on TSV viral exposure show that the fish copepod *E. manicatus*, the crab carapace barnacle *C. patula*, and the crab gill barnacle *O. muelleri* all contained between10^3 and 10^4 mean copy numbers of TSV/μg total RNA at Days 7 and 14 (Figs. 1 and 2) except for *C. patula* at Day 7 (Fig. 2). When copy numbers were obtained from the hemolymph of a single crab and a series of *C. patula* from the crab, they showed similar values at 1 week, with an especially high value at 2 weeks for *C. patula* of 10^5 copies per μg when compared with 10^4 copies/μg for *O. muelleri* (Fig. 3). Those Log copy numbers of TSV/μg total RNA were higher in *E. manicatus* (Fig. 1) and in *C. patula* and *O. muelleri* (Fig. 2) than in their respective

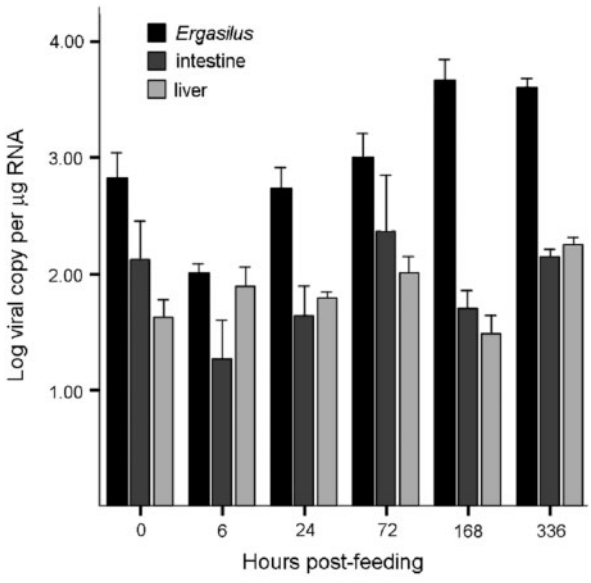

Fig. 1 TSV log titer in intestine and liver tissues from *F. grandis* with associated copepod, *E. manicatus*, attached to the gills. Bar = mean, line = 1 standard error.

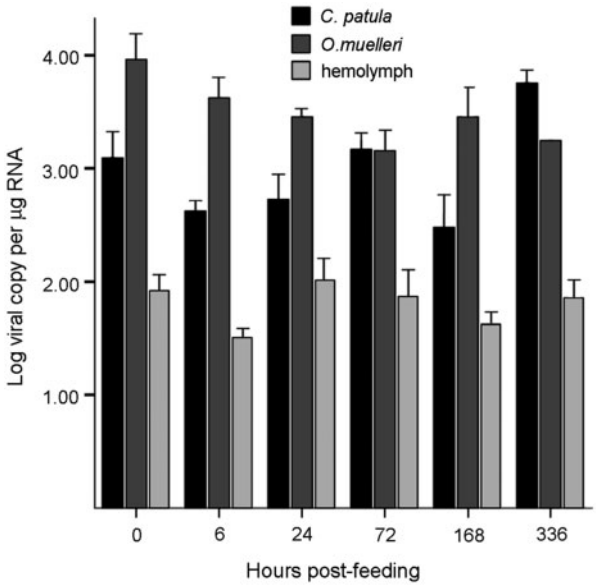

Fig. 2 TSV log titer in hemolymph from *C. sapidus* with associated barnacles *C. patula* on the carapace and *O. muelleri* on the gills. Bar = mean, line = 1 standard error.

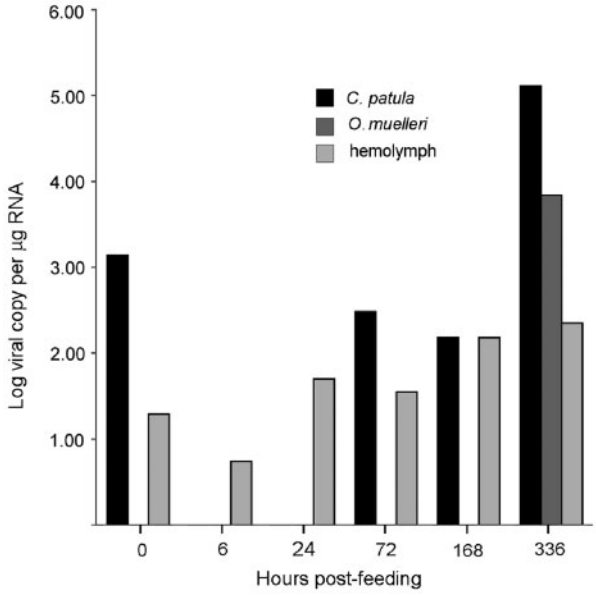

Fig. 3 TSV log titer of hemolymph from a single specimen of *C. sapidus* and from the tissues of its associated barnacle, *C. patula*, sampled across the time period and the gill-associated barnacle, *O. muelleri*, sampled at end of experiment.

hosts (ANOVA, $P<0.001$). Differences between copy numbers in *E. manicatus* and in both killifish liver and killifish intestine at 3, 7, and 14 days but not at 24 h, when viral replication was initiating, were significant (K-ST, $P<0.001$) (Fig. 1). The copy number in the copepod at 2 weeks was significantly

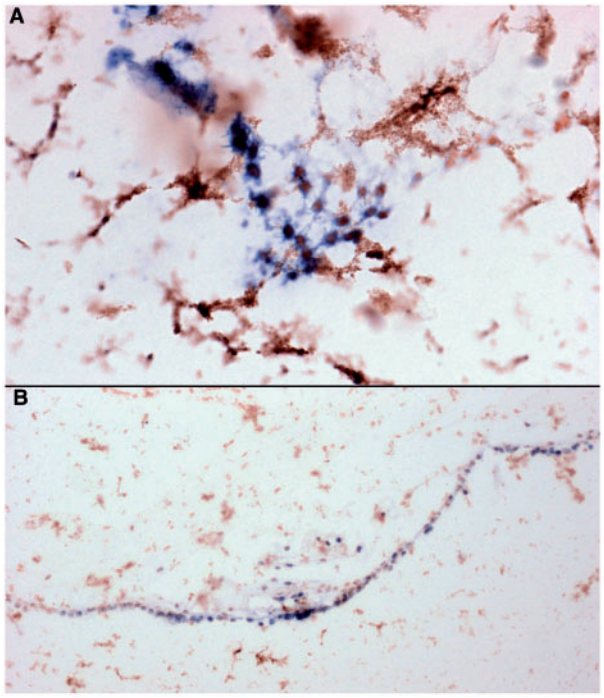

Fig. 4 *In situ* hybridization assay demonstrating a positive reaction to the TSV-specific probe visualized by the dark blue-black precipitate in the epithelial cells. (**A**) TSV-positive reaction in the gill-associated gooseneck barnacle, *O. muelleri*, from the blue crab, *C. sapidus*. (**B**) TSV-positive reaction in the surface acorn barnacle *C. patula* from the blue crab, *C. sapidus*.

more than in the copepod at Day 3 (K-ST, $P<0.005$), demonstrating that the virus appeared to replicate (Fig. 1). Values for differences between viral copy number in each of the two barnacles and that in crab hemolymph were also significant (ANOVA, $P<0.001$), and copy number values at 3, 7, and 14 days also differed between each parasite and the crab (K-ST, $P<0.03$) (Fig. 2). Sample sizes of the copepod at Days 3–14 were 10–14, and those for the crab and barnacles were 6–10. The copy numbers in both the fish and crab hosts remained constant throughout 2 weeks; however, the values were relatively low. *In situ* hybridization (ISH) assays from 2-week old infections for the barnacles produced positive signals in the cytoplasmic and nuclear areas of the cuticular epithelium (Fig. 4).

Log WSSV copy number increased after Day 3 in *E. manicatus*, with the number of copies of WSSV/µg total DNA averaging about 10^4 on Days 7 and 14 (Fig. 5). Differences between viral copy numbers in *E. manicatus* and in both killifish liver and killifish intestine at 3, 7, and 14 days but not at 24 h were significant (K-ST, $P<0.001$). Significant differences (K-ST, $P<0.04$) occurred between the copy number in the copepod at Day 3 and the

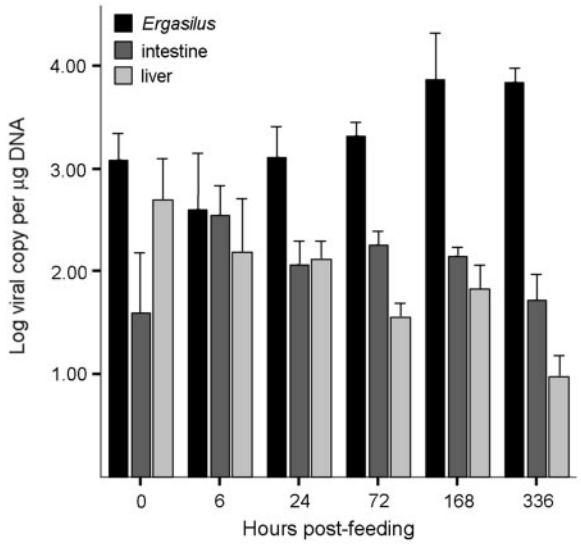

Fig. 5 WSSV log titer in intestine and liver tissues from *F. grandis* with associated copepod *E. manicatus* attached to the gills. Bar = mean, line = 1 standard error.

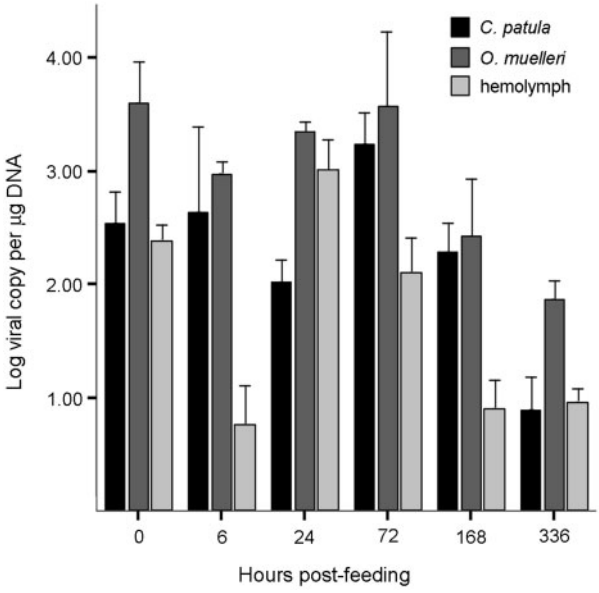

Fig. 6 WSSV log titer in hemolymph from *C. sapidus* with associated barnacles *C. patula* on the carapace and *O. muelleri* on the gills. Bar = mean, line = 1 standard error.

corresponding values both at Days 7 and 14 (Fig. 5). The copy numbers of the virus in fish liver, intestine, and feces (data not shown) showed a trend to gradually decrease throughout the 2 weeks (Fig. 5). There was no indication based on copy number that WSSV replicated in either barnacle or the crab host (Fig. 6), even though the virus was still detectable at a relatively low level at 2 weeks. The barnacles also did not produce positive-WSSV ISH signals but copepod

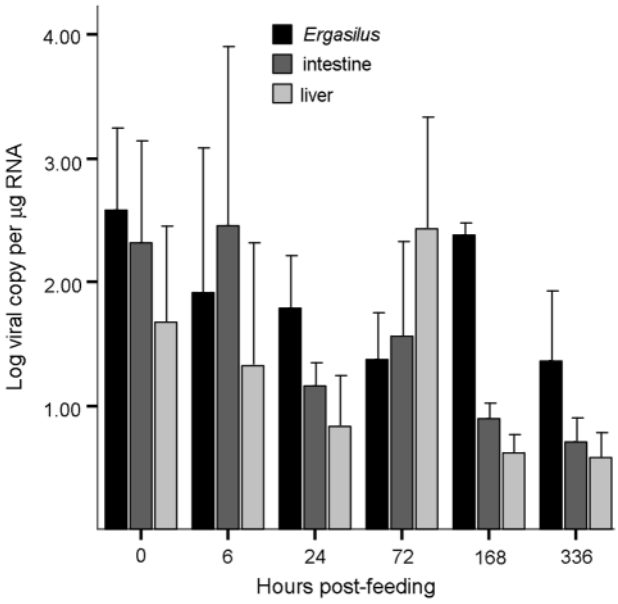

Fig. 7 YHV log titer in intestine and liver tissues from *F. grandis* with associated copepod, *E. manicatus*, attached to the gills. Bar = mean, line = 1 standard error.

tissue was not available for analysis. Values for copy number of WSSV in the copepod only suggested replication.

The general trend for mean log YHV copy number in the copepod, fish, both barnacles, and crab showed a gradual decrease over the 2-week period, resulting in hardly detectable levels (< 100 [usually < 10] copy numbers of YHV/µg total RNA) (Figs. 7 and 8). The decreasing trend for copy number was especially notable for the crab and barnacles in which the values dropped rapidly after Day 1 (Fig. 8).

Potential problems can exist when tissue samples are small, especially like that of the copepod (about 1-mm long) when tested using the Nanodrop instrument. However, the values seem accurate based on the consistent values from numerous samples fitted within the standard curve (data not shown), the relatively small standard errors, and the confirmation of the presence of a positive reaction for ISH. According to Lightner (University of Arizona, personal communication), viral copy numbers usually need to reach 10^3–10^4 copy numbers of a virus/µg total RNA or DNA to detect the ISH signal as long as the infection is not highly focused.

Even though we administered a dose high enough to be infective to penaeids, we note that in our experience, more agent may be necessary when feeding it rather than when injecting it into the experimental animals. For WSSV-infections, which

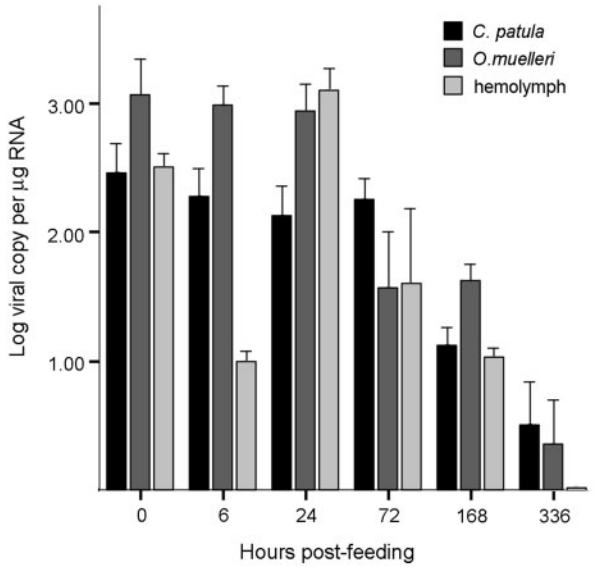

Fig. 8 YHV log titer in hemolymph from *C. sapidus* with associated barnacles *C. patula* on the carapace and *O. muelleri* on the gills. Bar = mean, line = 1 standard error.

typically produce 100% mortality, mortality is delayed when the dose is administered by immersion rather than by injection, and the copy number in moribund individuals of different species reaches different levels (Durand and Lightner 2002). Also, the copy number of a virus may not replicate as dramatically in fed non-penaeid animals. Consequently, the dose administered as well as the source of the virus and the method of exposure seems adequate and realistic. The study was conducted during a period when parasites were not at a high abundance, even though for purposes of establishing their potential as vectors, the need for large samples was not critical. The intensity of *E. manicatus* was about 1–5 per infected host, which seems typical in Ocean Springs, Mississippi, but it can reach 30 elsewhere (Barse 1998). Other species of *Ergasilus* occur locally in numbers surpassing 100 per fish, and the species are larger and their hosts more migratory, making them better prospects as vectors.

The importance of this study is to evaluate the potential of virally exposed crustacean parasites to transmit the viruses to penaeids in the wild or in aquaculture. We presume that none of the commercial penaeid species that die within a few days of exposure to the three test viruses represent a natural host of the agent but rather serve as an accidental host that exhibits a severe pathological response. The establishment of large-scale penaeid aquaculture somehow exposed the viruses to cultured susceptible shrimp, which then became infected and spread those infections rapidly through the industry. Consequently, invertebrates that could tolerate the replicating viruses probably included the initial natural hosts.

The relatively high copy number and presumed replication of TSV in the copepod and barnacles show them to be potential reservoir hosts. Neither the copepods on either killifish nor the two barnacles on the crab died during the experiment from TSV, another virus, or other reason. These crustacean parasites represent the first known potential reservoirs for TSV in which viral replication occurs. Previously, there was no known non-penaeid carrier other than an hemipteran insect known as the salt marsh water boatman (*Trichocorixa reticulata*, Corixidae) and birds that feed on shrimp carcasses in farm ponds; they were shown in the laboratory to be mechanical vectors only (Lightner 2006a). Because the viral load in our experimentally infected crustacean parasites is probably enough to produce infections in penaeids does not necessarily mean that the crustaceans represent the only such hosts in nature. Neither does it mean that any copepod or even any poecilostomatoid copepod or any barnacle could host the virus.

The crustacean parasites might act like a partially TSV-resistant (TSR) penaeid to a virulent TSV exposure. Nunan et al. (2004) and Tang et al. (2004) developed real-time methods to assess copy number. Srisuvan et al. (2006) fed four characterized TSV isolates with widely divergent virulence to both especially bred TSR and TSV-susceptible (TSS) stocks of *Litopenaeus vannamei* (Pacific white shrimp). Their results exhibited primarily suspected survival, log copy number of TSV/µl RNA, histopathological response in shrimp, and ISH response to the eight corresponding combinations over a 14-day period; 100–80% of the TSR stock survived and none to 20% of the TSS stock survived, reflecting different rates of mortality. Living TSS shrimp exposed to three of the four isolates maintained at least 10^6 copy numbers of TSV/µl RNA, but, by Day 14, living TSR shrimp had only 10^2–10^3 copies, with positive signals present in shrimp given the most virulent isolate only. Our test copepods and barnacles tolerated the virus, and the values of copy numbers in them were somewhat higher than those encountered by Srisuvan et al. (2006) 2 weeks after their exposures of highly virulent Belize and Thailand isolates in TSR shrimp. The values measured much higher than those resulting from exposures to the less virulent Hawaiian and

Venezuelan isolates and to the least virulent Venezuelan isolate fed to the TSS shrimp stock.

Numerous animals, mostly decapod crustaceans, have been reported to be infected with WSSV, but replication in few of those animals has been reported. A copepod and an ephydrid insect larva from shrimp farms in epizootic areas of WSSV infection in Taiwan, Republic of China, were examined by both 1-step and 2-step PCR for WSSV (Lo et al. 1996). Two of six pooled samples of the free-living calanoid copepod *Schmackeria dubia* tested positive with 1-step PCR, and that number increased to four of six with the more sensitive 2-step procedure. The insect larva also increased from four of ten to eight of ten when the 2-step procedure was used. Both were reported as reservoirs, but Lo et al. (1996) stated that it was not clear if the virus replicated or caused disease in copepod, insect, or "sea slater" (Isopoda, probably *Ligia* sp.), also associated with shrimp ponds (Lo 2006). We do not know if the infection in *E. manicatus* was typical for copepods in general, but we doubt it since the presence of virus could be detected in the distantly related barnacles at 2 weeks, but replication did not occur. A non-crustacean, the eunicid polychaete, *Marphysa gravelyi*, in southern India was shown to have between 17% and 75% prevalence of WSSV in various locations receiving effluent from WSSV-infected ponds but none in areas without shrimp farms (Vijayan et al. 2005). Analyses were based on 2-step PCR; few samples demonstrated a positive response with the 1-step procedure. Half the samples of worms sold as food for broodstock in shrimp hatcheries were infected, demonstrating the importance that mechanical vectors of WSSV may have on aquaculture.

The near loss of YHV from all three crustacean parasites as well as their hosts over 2 weeks probably demonstrates well what happens to the virus in most non-penaeids. Perhaps the parasites could serve as mechanical, or passive, vectors for a very short period unless being continually exposed, but they are likely not reservoirs. Reservoir hosts other than penaeids for YHV are not well understood. Ma et al. (2009) demonstrated that the injected virus could replicate in the daggerblade grass shrimp, *Palaemonetes pugio*, reaching a peak at 14 days and still detectable at 36 days, but the copy number could be sustained in the blue crab (*C. sapidus*) for 3 days and not detectable by Day 7. It remained detectable at Day 21 in crabs fed the virus.

A good question concerns whether the crustacean parasites could serve to infect penaeids.

The viruses have been detected in waters associated with aquaculture and other sources (e.g., Lightner 1999), and the parasites could acquire them. The copepod *E. manicatus* on species of *Fundulus* and related fishes is a reasonable reservoir or vector for any of the three tested viruses. First, the male copepod is free-living and can be infected by the virus in suspension and disperse far from where it was originally infected. The female, which could become infected when free-living or attached, can detach from a fish host by environmental stress (such as an increase or decrease in salinity), by predation of the fish, or by other situations resulting in host death. Especially since the copepod is small, it provides a good source of food and relative viral genome concentration for larval and young juvenile penaeid shrimps as well as for older individuals. In other words, the roles of the copepod would be to (1) disperse the viral agents to habitats where the virus would not necessarily be expected to come in contact with shrimp larvae and young postlarvae or (2) to have infected individuals present at a time when the virus was not otherwise available to the migratory penaeid stocks. Presumably other species of *Ergasilus* and possibly even members of other copepod genera can serve a similar role, and, as indicated above, they can be abundant on highly migratory fishes such as mullets.

The blue crab and a few other species, such as the horseshoe crab (*Limulus polyphemus*), get infested initially with the barnacles by their cyprids in high salinity water. The female blue crab usually undergoes anecdysis (a terminal molt at maturity) and becomes infested when the mature individual spawns in the high salinity water near barrier island passes. It routinely dies after its first through fourth spawn. Consequently, those dead and dying crabs and their barnacles can contribute to a feeding frenzy by birds and fishes, leaving an abundance of loose infected tissue available to penaeids and other organisms. Also, the male blue crab, which does molt throughout its life, makes its molt with attached barnacles available to shrimp. The occasional male and mature female blue crabs that leave the high salinity spawning grounds and venture into sounds, bays, and bayous can make viruses available to shrimp. The young postlarval penaeids would be especially available to feed on infested molts and infected barnacle fragments.

We have shown that the three tested parasites could theoretically transmit viruses to vulnerable penaeid shrimps in culture or the wild. More important, there is a possibility that crustacean parasites may play an important role in bridging gaps in the

food web for spreading a variety of viral diseases. Crustacean parasites have previously been overlooked as vectors of disease agents, with the few exceptions of those hosting economically important infectious agents to salmonids and carp. As more attention is directed toward crustacean parasites as hosts for viruses, many more viruses will probably be found. These may include those that cause disease, those that do not harm the host unless there is some synergistic relation to environmental or anthropogenic stress, and those that can be transmitted to commercially important or other animals. Such transmission can play an important role in either aquaculture or in affecting animals and biodiversity in the natural environment.

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