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Feeding hermit crabs to shrimp broodstock increases their risk of WSSV infection

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ABSTRACT: White spot syndrome virus (WSSV) is a serious shrimp pathogen that has spread globally to all major shrimp farming areas, causing enormous economic losses. Here we investigate the role of hermit crabs in transmitting WSSV to *Penaeus monodon* brooders used in hatcheries in Vietnam. WSSV-free brooders became PCR-positive for WSSV within 2 to 14 d, and the source of infection was traced to hermit crabs being used as live feed. Challenging hermit crabs with WSSV confirmed their susceptibility to infection, but they remained tolerant to disease even at virus loads equivalent to those causing acute disease in shrimp. As PCR screening also suggests that WSSV infection occurs commonly in hermit crab populations in both Vietnam and Taiwan, their use as live feed for shrimp brooders is not recommended.

KEY WORDS: White spot syndrome virus · Hermit crab · Feed organism · *Penaeus monodon* · Shrimp brooder

INTRODUCTION

White spot syndrome virus (WSSV) is a major shrimp pathogen responsible for mass mortalities that have caused huge economic losses to the global shrimp farming industry over the past 2 decades (Lo et al. 1996, 2005, Escobedo-Bonilla et al. 2008). The WSSV infection state of shrimp postlarvae produced in hatcheries is critically important to avoid disease occurring during culture (Lo et al. 1998, Hsu et al. 1999, Satoh et al. 1999, Peng et al. 2001, Seok et al. 2007). WSSV can be transmitted vertically from spawners to their offspring (Lo et al. 1997, Hsu et al. 1999, Tsai et al. 1999, Peng et al. 2001). In shrimp hatcheries, therefore, broodstock surveillance for WSSV is critical for producing virus-free postlarvae for stocking ponds. As WSSV has the widest host range of all known crustacean viruses (Sánchez-Paz 2010) and is distributed widely, there are many means by which it can bypass disease-prevention control measures used at shrimp farms. Susceptible species include polychaetes, which are used commonly in hatchery diets for shrimp broodstock to promote maturation and spawning. Additionally, some hatcheries use species available locally with high nutritive value such as crabs and clams. However, these pose risks for transmitting the WSSV infection to shrimp (Kanchanaphum et al. 1998, Sahul Hameed et al. 2003, Vijayan et al. 2005, Chang et al. 2011).

Here we report an example of how WSSV infections were established in *Penaeus monodon* brooders at a hatchery in Vietnam through the use of hermit

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crabs as live feed. The susceptibility of hermit crabs to WSSV infection and their potential for transmitting the virus are reported.

MATERIALS AND METHODS

Shrimp hatchery sampling

Farming practices and the WSSV infection status of *Penaeus monodon* brooders, hermit crabs and polychaetes being used as feed were investigated at a hatchery at Ca Na Beach, Ninh Thuan province, Vietnam, in 2004. Pleopods were sampled at random from a batch of *P. monodon* brooders immediately after their arrival at the hatchery, and then again 2 d and 2 wk later. Cephalothorax tissue of hermit crabs and pieces of polychaetes were also collected for PCR screening for WSSV and other viruses. Wild-caught hermit crabs captured in the vicinity of the hatchery were also sampled.

WSSV and hepatopancreatic parvovirus (HPV) PCR

WSSV detection was performed using a commercial OIE (World Organisation for Animal Health)-certified nested PCR WSSV-detection kit (IQ2000 WSSV Detection and Prevention System; GeneReach Biotechnology). HPV was detected using the IQ2000^{TM} HPV-nested PCR Detection System (GeneReach Biotechnology). Using the samples described above, DNA extraction (DTAB/CTAB) and PCR were performed following the manufacturer's instructions. Briefly, each nested PCR employs a competitive PCR system in which the reaction produces larger-sized amplicons as the amount of viral DNA increases. Three patterns for 'severe', 'medium', and 'light' infection levels are recognized according to patterns produced using 2×10^3, 2×10^2 and 2×10^1 WSSV DNA copies, or 2×10^4, 2×10^3 and 2×10^2 HPV DNA copies, respectively.

RT-PCR for RNA viruses

Hermit crabs captured outside the hatchery were screened by RT-PCR for the presence of the RNA viruses Taura syndrome virus (TSV), yellow head virus/gill-associated virus (YHV/GAV), and Mourilyan virus (MoV) using the corresponding IQ2000^{TM} RT-PCR kits (GeneReach Biotechnology) according to the manufacturer's protocols.

WSSV inoculum preparation

The inoculum was prepared using the WSSV-TW strain (GenBank No. AF440570) originating from *Penaeus monodon* collected in Taiwan in 1994 (Wang et al. 1995). Briefly, 0.5 g muscle tissue from several *P. monodon* was minced and then homogenized in 4.5 ml sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) pre-chilled to 4°C. After centrifugation at 400 × *g* for 10 min at 4°C, the supernatant was filtered through a 0.45 µm membrane filter, and 50 µl of the filtrate was injected into muscle tissue of the fourth abdominal segment of adult-specific pathogen-free (SPF) *Litopenaeus vannamei* (High Health Aquaculture) (45 g mean body weight). About 3 d after injection, hemolymph was collected using a syringe and a 26-gauge hypodermic needle from the moribund *L. vannamei*, and hemocytes were removed by centrifugation at 1000 × *g* for 10 min at 4°C. The supernatant was then diluted 4-fold with PBS and stored at −80°C as virus stock.

WSSV infectivity for hermit crabs

To assess WSSV infectivity, 3 hermit crab species, *Diogenes* aff. *nitidimanus*, *Pagurus minutus* and *P. angustus* (mean weight 3.12, 2.8 and 2.87 g, respectively), were collected from the middle of the West Coast of Taiwan. Crabs were kept in tanks filled with aerated, filtered 28‰ salinity seawater at 25°C, and prior to challenge, the IQ2000 PCR was used to determine the WSSV infection status of ~10 randomly selected crabs using the cephalothorax as the sample tissue. The WSSV stock was diluted 50-fold with cold PBS and filtered through a 0.45 µm filter. The filtrate was then injected into individuals of each of the 3 crab species at the coxae of the second pereopod at a dosage of 20 µl g^{-1} body weight. At 72 h post-challenge (hpc), pereopods were collected from crabs and checked for WSSV infection using the IQ2000 PCR.

Electron microscopy

Since the stomach is a major target organ of WSSV, slices of the stomach of a WSSV-challenged *Pagurus minutus* in which high WSSV DNA amounts were detected by PCR were pre-fixed in 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) for 24 h at 4°C and then postfixed in 1% osmium

tetraoxide in the same buffer for 2 h at 4°C. The fixed samples were then dehydrated through a graded series of increasing alcohol concentrations and embedded in Spurr epon resin. Ultrathin sections were cut using a Reichert OMU3 ultramicrotome and stained with uranyl acetate and lead citrate. Observations were made using a Hitachi H-600 electron microscope set at 100 kV.

Fig. 1. (A) A polychaete worm and (B) hermit crabs (undetermined species) from the shrimp hatchery where they were being used in a maturation diet for *Penaeus monodon* brooders

Bioassay of WSSV-challenged hermit crabs and shrimp

A bioassay was performed to evaluate the sensitivity of hermit crabs to WSSV infection. Three replicate groups of 10 *Pagurus minutus* hermit crabs from a batch in which no evidence of WSSV infection was detected by IQ2000 PCR were challenged with WSSV by injection at the coxae of the second pereopod as described above. Three groups of 10 hermit crabs were also injected with PBS alone as negative controls. For comparison, groups of 10 WSSV-free *Litopenaeus vannamei* (provided by Tungkang Biotechnology Research Center, Fisheries Research Institute Taiwan) were also challenged with WSSV by intramuscular injection at the same dosage (i.e. 20 µl g^{-1} body weight). Mortalities were recorded twice daily. Additional batches of hermit crabs and shrimp injected similarly were sampled for a time course analysis to track increases in WSSV infection levels over time using the IQ2000^{TM} WSSV PCR. Two individuals were sampled from each group at 0 (i.e. before challenge), 24, 48, and 96 hpc. Hermit crabs were also sampled at 144 hpc.

RESULTS

Hatchery practices

At the *Penaeus monodon* brooder hatchery at Ca Na Beach, wild-captured brooders were kept in indoor tanks at a density of ~10 shrimp per tank. The brooders were matured on diets consisting of live commercial polychaete worms (Fig. 1A) and wild-captured hermit crabs of undetermined species (Fig. 1B). After removing the shells, the crab flesh was homogenized and mixed with the worms before being fed to the brooders. Other live shell-less hermit crabs were placed directly into the brooder tanks. In the year following this 2004 study, we learned that the hatchery had suffered a serious WSSV disease outbreak and was no longer in operation.

Detection of WSSV in *Penaeus monodon* brooders and their feed organisms

The IQ2000 WSSV PCR kit failed to detect WSSV in any of the 4 *Penaeus monodon* brooders sampled immediately after the batch arrived at the hatchery (Fig. 2A). Two days later, 2 out of 3 of these brooders were scored by PCR as having low to medium WSSV infection (Fig. 2B). After 2 wk in the hatchery, all 3 brooders were scored by PCR as having a severe WSSV infection (Fig. 2C). No WSSV was detected in either of the 2 polychaetes tested (Fig. 2D), but in the 5 tested hermit crabs, 4 were infected and 3 of these were scored as having severe WSSV infection (Fig. 2E). We note that the WSSV positive controls in Fig. 2A–C did not produce the expected patterns at lower WSSV DNA template amounts. While the reason for this is unknown, the tests still clearly differentiated between the presence and absence of WSSV, and we have used the expected control patterns to classify the WSSV infected samples as either medium or severe.

PCR screening of wild-captured hermit crabs for viruses

Hermit crabs captured from outside the shrimp hatchery at Ca Na Beach were tested for several viruses using IQ2000 PCR tests. PCR detected WSSV in all 4 crabs tested (Fig. 3A), but TSV, YHV/GAV, MoV and HPV were not detected (Fig. 3B–E).

Hermit crab challenge with WSSV

WSSV-negative groups (n = 3) of *Diogenes* aff. *nitidimanus*, *Pagurus minutus* and *P. angustus* (Fig. 4A–C) were injected with WSSV, and 72 h later crabs were re-tested using the IQ2000 WSSV PCR. High WSSV DNA amounts were detected in all 3 samples of all 3 species of hermit crab after the WSSV challenge (Fig. 4D).

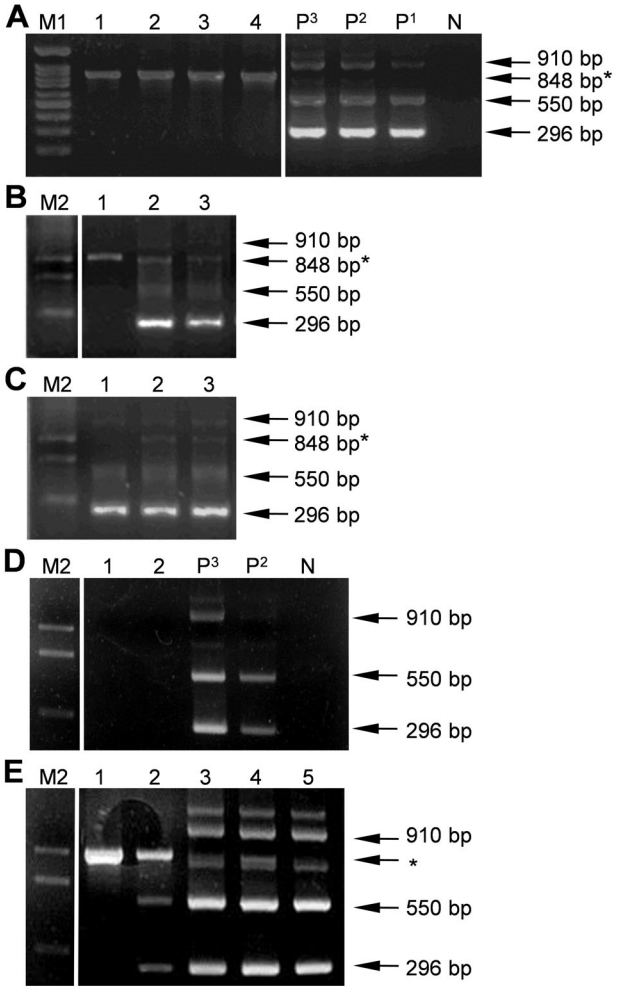

Fig. 2. White spot syndrome virus (WSSV) infection status in *Penaeus monodon* brooders, polychaetes and hermit crabs (undetermined species) used as feed determined using the IQ2000 WSSV PCR. (A) Four brooders sampled immediately upon arrival at the hatchery (Lanes 1 to 4), (B) Three other brooders tested 2 d later (Lanes 1 to 3), (C) Two weeks after arrival, all 3 of the brooders sampled had severe WSSV infections (Lanes 1 to 3), (D) Polychaete worms (Lanes 1 to 2), (E) Hermit crabs (Lanes 1 to 5). P^3, P^2 and P^1 are PCR test positive controls containing 2×10^3, 2×10^2 and 2×10^1 WSSV DNA equivalents (plasmid DNA), respectively. N: PCR negative control yeast tRNA (40 ng μl^{-1}) as template. DNA bands at 910, 550 and 296 bp are WSSV-specific. Asterisk indicates the crustacean-specific DNA PCR product (848 bp). M1: 100 bp DNA ladder; M2: DNA size markers of 848, 630 and 333 bp provided with the kit

Electron microscopy confirmation of WSSV infection in hermit crabs

To confirm WSSV infection in the injected hermit crabs, transmission electron microscopy (TEM) was used to examine the stomach of a WSSV-challenged *Pagurus minutus* in which the PCR banding pattern predicted severe WSSV infection. Rod-shaped virus particles indicative of WSSV (Wang et al. 1995, Lo et al. 1997) were readily seen in the nuclei of the stomach cells (Fig. 5).

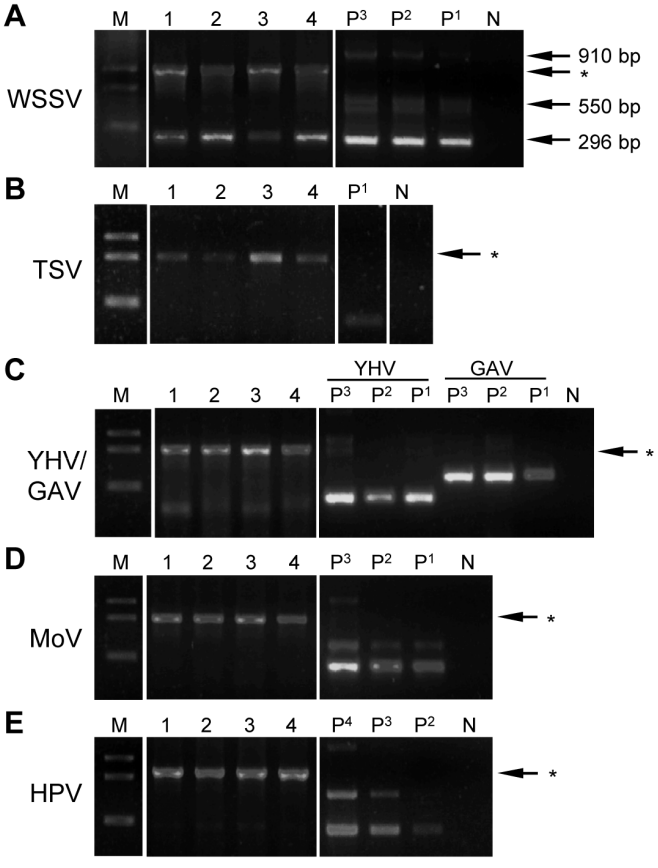

Fig. 3. Four wild-caught hermit crabs (undetermined species) (Lanes 1 to 4) from Ca Na Beach, Vietnam, screened for the presence of (A) white spot syndrome virus (WSSV), (B) Taura syndrome virus (TSV), (C) yellow head virus/gill-associated virus (YHV/GAV), (D) Mourilyan virus (MoV) and (E) hepatopancreatic parvovirus (HPV) using the respective IQ2000 PCR kits. P^4, P^3, P^2 and P^1 are positive controls containing 2×10^4, 2×10^3, 2×10^2 and 2×10^1 viral RNA/DNA equivalents, respectively, used in each PCR. N: PCR negative control yeast tRNA (40 ng μl^{-1}). Asterisks indicate a crustacean (hermit crab)-specific PCR product. M: DNA size markers of 848, 630 and 333 bp provided with the kits

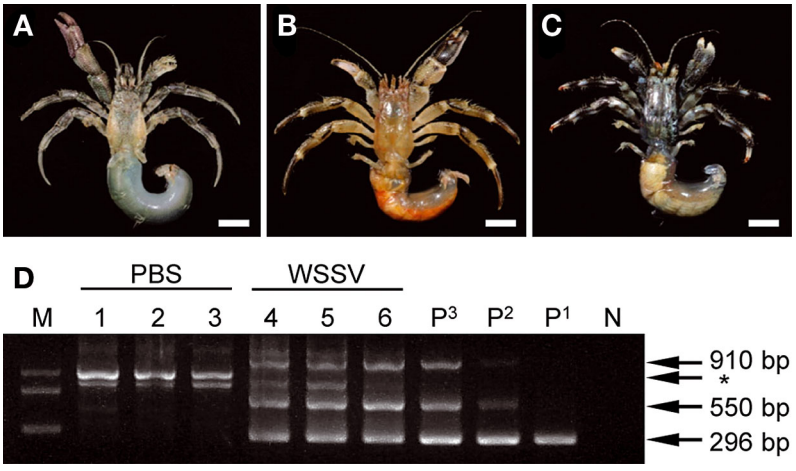

Fig. 4. (A) *Diogenes* aff. *nitidimanus*, (B) *Pagurus minutus*, and (C) *P. angustus* collected from the west coast of Taiwan. White spot syndrome (WSSV) detection in 3 hermit crab species challenged experimentally with WSSV as detected by IQ2000 WSSV PCR. Scale bars = 1 cm. (D) IQ2000 WSSV PCR amplification results with phosphate-buffered saline (PBS)-injected *D.* aff. *nitidimanus*, *P. minutus*, and *P. angustus*, respectively (Lanes 1 to 3); same species challenged with WSSV (Lanes 4 to 6). PCR-positive controls (P^3, P^2 and P^1), and the negative control (Lane N) are as described in Fig. 2. Asterisk indicates PCR products amplified from crustacean (hermit crab) genomic DNA. M: DNA size markers of 848, 630 and 333 bp

Resistance of hermit crabs to WSSV

To evaluate the resistance of hermit crabs to WSSV disease, a bioassay was performed. *Pagurus minutus* were used due to their ready availability compared to other 2 species. Pre-screening with the WSSV IQ2000 PCR detected WSSV in ~43% of the wild-caught *P. minutus*. Only individual crabs that tested WSSV-negative in the PCR were used in the bioassay. For comparison, WSSV-negative *Litopenaeus vannamei* shrimp were challenged similarly. Mortalities accumulated steadily among the WSSV-challenged shrimp from 48 hpc, and all shrimp had died at 108 hpc. No deaths occurred among the WSSV-challenged hermit crabs or among the PBS-injected crabs over the 144 h bioassay period (Fig. 6). Tracking WSSV infection levels over time using the IQ2000 WSSV PCR detected WSSV at 24 hpc and at high infection loads in the WSSV-challenged shrimp and hermit crabs at 96 and 48 hpc, respectively (Fig. 7). The high WSSV loads were sustained in the challenged hermit crabs sampled at 144 hpc.

DISCUSSION

In 2004, after observing that WSSV-free *Penaeus monodon* brooders introduced to a Vietnamese hatchery soon became WSSV-positive, the likely

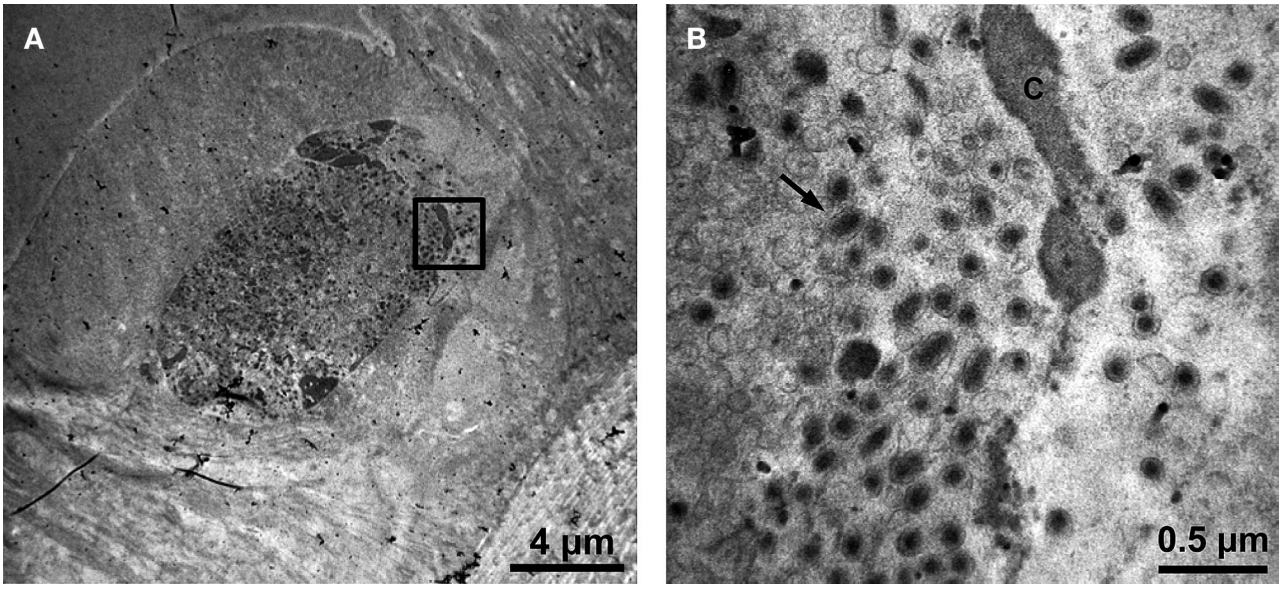

Fig. 5. *Pagurus minutus.* Electron micrographs of an ultrathin section from the stomach tissue of a challenged hermit crab with white spot syndrome virus (WSSV). (A) Infected cells and (B) the cell nucleus (box in A) at higher magnification showing (arrow) rod-shaped WSSV particles. C: chromatin

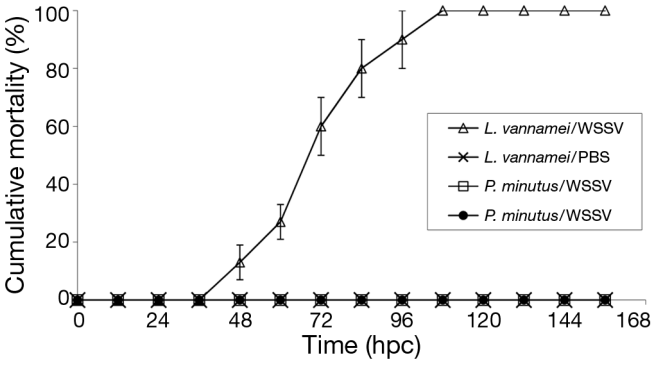

Fig. 6. *Pagurus minutus* and *Litopenaeus vannamei*. Mean (±SD) cumulative mortality among groups of 2 species of hermit crab injected with white spot syndrome virus (WSSV) or with phosphate-buffered saline (PBS) alone for 3 replicate groups of 10 individuals maintained in separate tanks. hpc: hours post-challenge

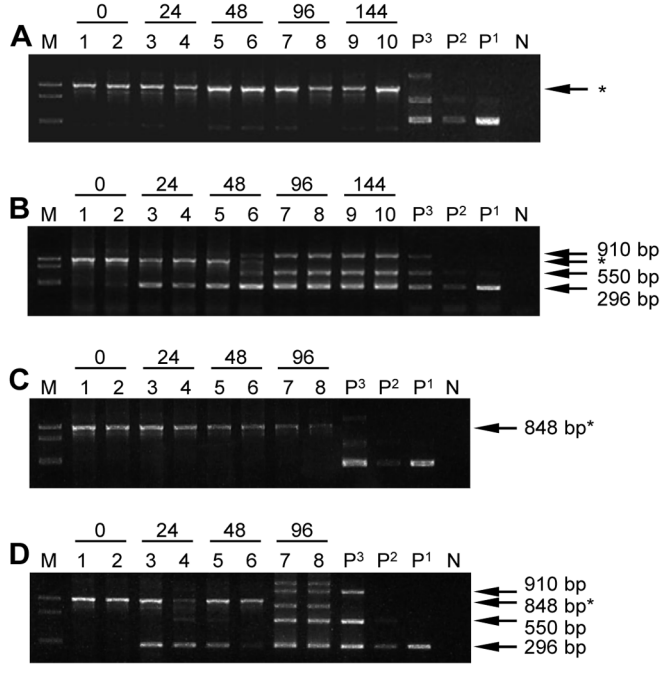

Fig. 7. *Pagurus minutus* and *Litopenaeus vannamei*. Progress of white spot syndrome virus (WSSV) infection levels in experimentally challenged hermit crabs. The IQ2000 WSSV PCR was used to determine WSSV DNA amounts at 24 h intervals in (A) mock-infected hermit crabs, (B) WSSV-challenged hermit crabs, (C) mock-infected shrimp and (D) WSSV-challenged shrimp. Lane headings show times post-challenge (h). The positive controls (P^3, P^2 and P^1), negative control (Lane N), and (asterisk) crustacean DNA bands are as described in Fig. 2. M: DNA size markers of 848, 630 and 333 bp

source of infection was traced to either polychaete worms or hermit crabs being used as feed. Live polychaete warms are commonly used as feed in shrimp hatcheries, and as WSSV can accumulate in their digestive tracts, shrimp can become infected by ingesting polychaetes carrying WSSV (Vijayan et al. 2005). PCR testing showed that the polychaetes being used in the hatchery were free of WSSV, but many of the hermit crabs were WSSV-positive. PCR testing of the hermit crabs for several other important viruses of shrimp found no evidence of TSV, YHV/GAV, MoV or HPV. In 2010, 6 yr after the Vietnamese hatchery experiment, small numbers of hermit crabs from Taiwan tested by PCR for WSSV showed evidence of light WSSV infection in 43% of 240 *P. minutus* examined (data not shown). WSSV was not detected in *Diogenes* aff. *nitidimanus* or *P. angustus* species of hermit crabs, but these negative results may be due to the small sample size (n = 4 for each species). When initially WSSV-negative *P. minutus* were retested after a 1 wk interval, some of them became WSSV-positive as evidenced by faint WSSV-specific DNA bands being amplified by PCR. These crabs seemed to be carrying WSSV loads initially below the level detectable by the IQ2000 WSSV PCR. During the 1 wk interval, stress may have triggered elevated WSSV replication and if so, its prevalence in *P. minutus* may be higher than the ~43% estimated here.

Although over 93 species of arthropods have been reported to be hosts or carriers of WSSV (Sánchez-Paz 2010), hermit crabs, have so far been overlooked, and their roles in WSSV disease has not been investigated. TEM observation confirmed that WSSV can replicate in hermit crabs, and PCR detected high levels of virus in both challenged and wild hermit crabs in Vietnam. However, no gross signs of disease were observed in challenged crabs even when high WSSV loads were detected by PCR, indicating that they tolerate infection well.

Taken together, the data reported here indicate that hermit crabs are a common natural host of WSSV and that WSSV is easily transmitted to *Penaeus monodon* brooders that ingest infected hermit crabs used as feed in hatcheries. Hermit crabs thus present a risk factor for WSSV disease through vertical virus transmission to seedstock entering culture systems.

In health surveillance programs implemented in shrimp culture systems, all species susceptible to WSSV infection should be considered. This includes live feed organisms such as hermit crabs, which are often used in hatcheries as they are an important dietary source for brooder maturation. Our data indicate that shrimp hatcheries should not use hermit crabs as a feed.

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