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Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab *Scylla serrata*

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ABSTRACT: White spot syndrome virus (WSSV), the causative agent of white spot syndrome in shrimp, has a wide host range which extends to crabs, copepods and other arthropods. In this study, benthic larvae of the mud crab *Scylla serrata* were captured from Taiwan's coastal waters and screened for the presence of WSSV by polymerase chain reaction (PCR) and *in situ* hybridization. WSSV was detected in around 60% of the larvae, and this prevalence rate remained fairly constant when the captured larvae were subsequently maintained in an aerated system in the laboratory. WSSV-free larvae obtained from a hatchery were challenged by immersion in a WSSV inoculum. Fifteen days after challenge, cumulative mortality in the experimental group reached 43% compared to 20% in the control group. PCR detection of WSSV in both moribund and surviving specimens clearly implicated the virus as the cause of death in most cases. Histological and *in situ* hybridization data confirmed that WSSV tissue tropism in *Scylla serrata* crab larvae is similar to that found in shrimp.

KEY WORDS: Natural infection · WSSV · Benthic larvae
Crab seeds · *Scylla serrata* · Tissue tropism · PCR

White spot syndrome virus (WSSV) is the causative agent of a disease which has led to mass mortalities of cultured shrimps in Taiwan and many other countries. WSSV has a wide host range and it has been observed not only in shrimps but also in crabs and other arthropods such as copepods, insects and pest prawns (Lo et al. 1996a).

Natural WSSV infections have been found in captured and cultured specimens of the mud crab *Scylla serrata* both in Taiwan (Lo et al. 1996a) and elsewhere (Flegel 1997, Kanchanaphum et al. 1998). It is also known that mortality can result when adults of this

commercially valuable species are infected with WSSV by injection (Supamattaya et al. 1998). To date, however, although *S. serrata* crab seeds (benthic and megalopa crab larvae are commonly referred to by farmers as crab 'seeds') used for stocking culture ponds are almost invariably caught from the wild, there have been no investigations of the prevalence of WSSV in these wild populations. In the present study we therefore use PCR to screen for the presence of WSSV in *S. serrata* larvae captured from the coastal waters of south Taiwan. We also subject WSSV-free larvae to experimental challenge by immersion in a WSSV inoculum.

Materials and methods. Experimental infection: Benthic *Scylla serrata* larvae with a carapace width of 1 cm were supplied by a crab hatchery in Tung Kang, south Taiwan. These larvae were hatched from eggs derived from wild-caught WSSV-free breeder crabs. Randomly selected specimens were checked using WSSV diagnostic 2-step PCR (Lo et al. 1998) and were all found to be PCR negative.

WSSV inoculum was prepared from seriously infected (i.e. 1-step PCR positive; Lo et al. 1996b) black tiger shrimp *Penaeus monodon*. Carapace and integument tissue (5 g) was minced and then homogenized in 40 ml sterile seawater (12.5% w/v). After centrifugation (1000 × *g* for 10 min at 4°C), the supernatant was filtered through a 0.45 µm membrane and used immediately. A blank (control) inoculum was similarly prepared from a 2-step WSSV-negative shrimp.

The experimental group (n = 180) was challenged by immersion for 2 h in an aerated tank containing viral stock solution diluted 1:150 with sterile seawater (total volume = 6 l). The control group (n = 260) was similarly treated using the blank inoculum. After immersion, the larvae were removed from the tank and washed twice with sterile seawater. Each group was then randomly

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separated into 3 subgroups ($n = 60$), and each subgroup was placed in an aerated 30 l plastic tank. The larvae were maintained at $27 \pm 2^\circ\text{C}$ and fed with artificial feed once a day for the duration of the 14 d experiment. Mortalities were recorded daily for each group, and dead specimens were removed from the tanks each day. All dead individuals were checked for the presence of WSSV by using the DNA extracted from the whole body in 2-step PCR (Lo et al. 1996a,b).

In situ hybridization and H&E staining: At 6 h, 12 h, 24 h, 2 d, 4 d, 6 d, 8 d, 10 d and 12 d after challenge, several randomly selected moribund individuals from the above groups were also fixed for histology and *in situ* hybridization analysis under a light microscope. The DNA probe for *in situ* hybridization was prepared from a pms146 PCR product (Lo et al. 1996b) and non-radioactively labeled with digoxigenin-dNTP (Boehringer Mannheim Biochemical) using a random priming method. Specimens were fixed in Davidson's fixative. Tissue sectioning and the hybridization procedure was carried out as previously described (Chang et al. 1996, Lo et al. 1997). For H&E staining, tissues were treated with Davidson's fixative for 48 h, then sectioned and stained with hematoxylin and eosin.

Wild-captured benthic larvae: In the course of 4 field trips to the same collecting area during August and September 1998, benthic larvae of *Scylla serrata* (1.5 to 2 cm carapace width) were captured from their natural environment in the coastal waters around southern Taiwan and immediately transported to the nearby Tung Kang Marine Laboratory of the Taiwan Fisheries Research Institute in Ping Tung Prefecture. From each batch of collected larvae, DNA was extracted (Lo et al. 1998) from randomly chosen specimens ($n = 10$ or 15). The DNA was not extracted from any specific organ, but from a mixture of many tissues excluding only the hepatopancreas. The extracted DNA was then tested for the presence of WSSV using a commercial kit (AcuGen Asia Co., Ltd, Taipei, Taiwan) based on a more recently developed 1-step nested WSSV PCR protocol (Lo et al. 1998) that classifies a WSSV infection into 1 of 4 levels. The interpretation of these 4 levels of infection in terms of the diagnoses made by the 2-step nested PCR protocol used in the first part of this study is described in Table 1.

The crabs collected on the fourth field trip (September 17) were also used in a follow-up study. Specimens from this group that were not used for the initial WSSV screening ($n = 104$) were subsequently cultured for 14 d in the laboratory. To prevent cannibalism, each larva was maintained and fed separately in its own cell in a wire-mesh grid

Table 1 Interpretation of the diagnoses made by the PCR protocols used in this study

No. of copies of DNA template in initial PCR reaction mixture	Diagnosis by 1-step nested PCR ^a	Diagnosis by 2-step nested PCR ^b
$> 2 \times 10^3$	Level 1	1-step
2×10^3	Level 2	1- or 2-step
2×10^2	Level 3	2-step
2×10^1	Level 4	2-step
$< 2 \times 10^1$	Negative	Negative

^aBased on Lo et al. (1998); ^bbased on Lo et al. (1996b)

apparatus equipped with a seawater flow-through system and aeration. To minimize the chance of WSSV infection from food, the larvae were fed only with an artificial diet. Carcasses and molted shells were collected several times a day, and tissue samples (100 mg) were extracted using a commercially available kit (AcuGen Asia Co., Ltd, Taipei, Taiwan) developed from a newer and more convenient DNA extraction protocol (Lo et al. 1996a, 1998). Another DNA extraction method, based on Lo et al. (1996b), was then used to test the extracted samples for the presence of WSSV DNA. Because the primer sets compete for the DNA templates of virus and shrimp that co-exist in the extraction mixture, the PCR results were divided into 4 levels. Levels 1 and 2 indicated a higher quantity of virus in the specimen, and Levels 3 and 4 indicated a lower quantity (Lo et al. 1998). At the end of the 14 d culture period, 15 of the surviving larvae were chosen at random and also subjected to the same AcuGen DNA extraction and testing products.

Results. Experimental infection: Cumulative *Scylla serrata* mortalities are shown in Fig. 1. Almost 70%

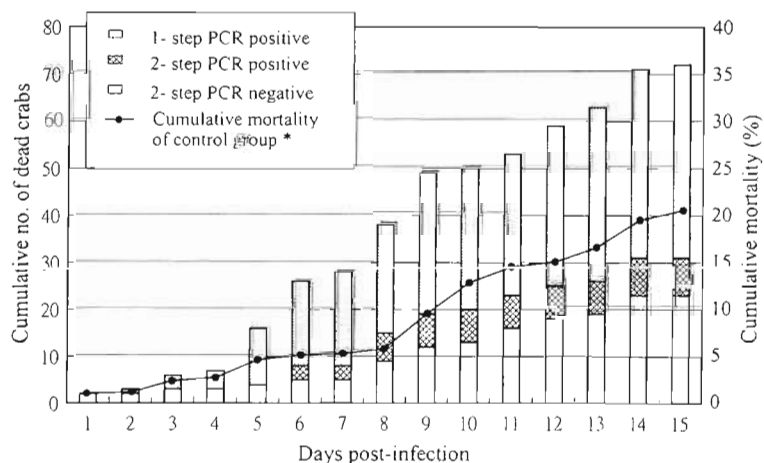


Fig. 1. *Scylla serrata*. Time-course mortality analysis for mud crabs experimentally challenged with WSSV infection. *WSSV was not detected in any of the crabs from the control group

(49/71) of the carcasses in the experimental group were PCR positive (8 were 2-step positive only; 41 were both 1- and 2-step positive), while none of the carcasses or survivors were positive in the control group. At Day 15 post-challenge, WSSV was also detected in 33% (24/72) of the tested survivors. H&E staining and *in situ* hybridization detected no obvious

histopathological change after 24 h infection (data not shown) but at 2 d post infection, cell degeneration and positive hybridization signals were frequently observed, especially in the external organs (gill and integument) (Fig. 2A–D). Subsequently almost all the organs, both internal and external, were infected by WSSV (Fig. 2).

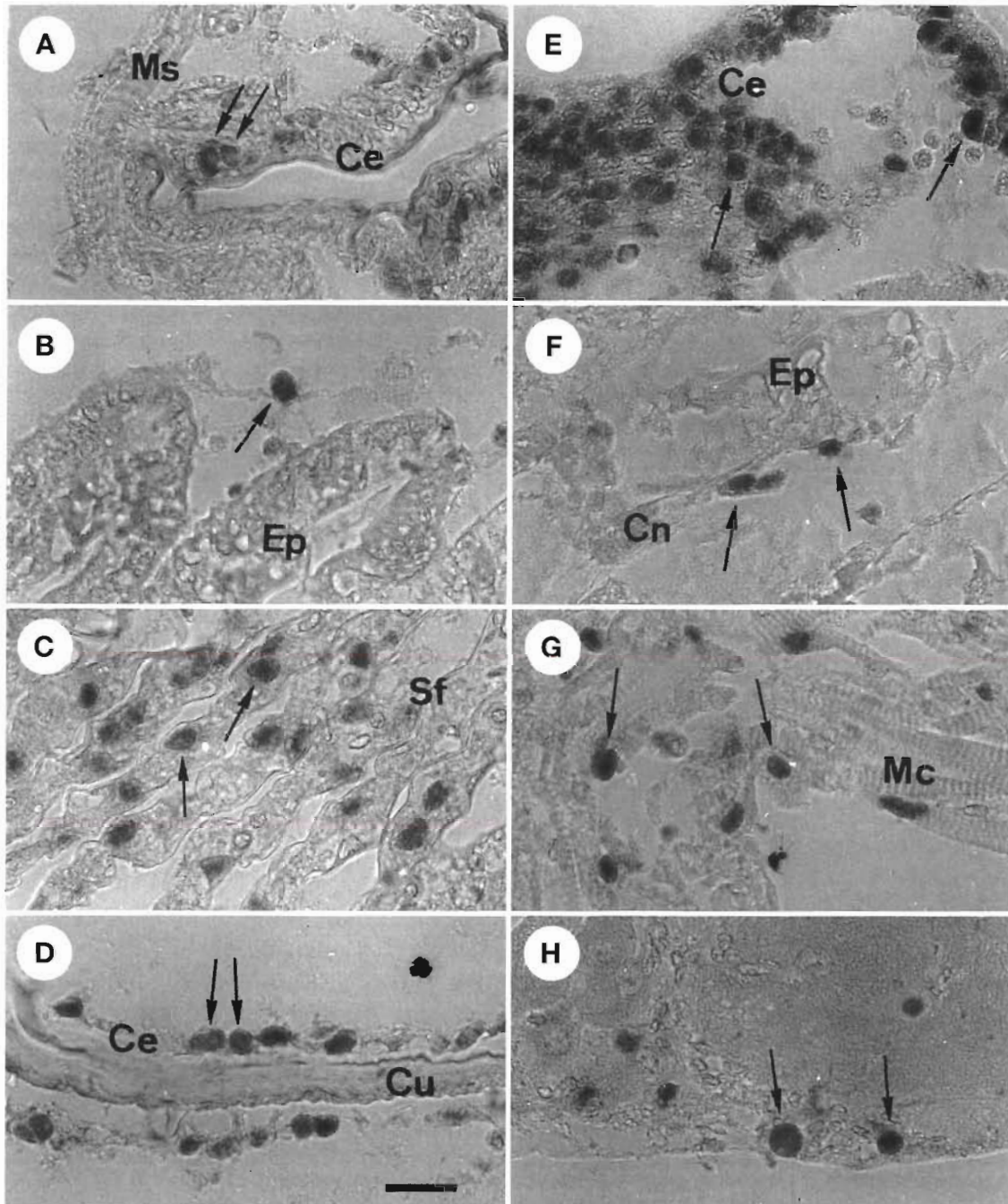


Fig. 2. *Scylla serrata*. Detection of WSSV DNA in tissue of experimentally infected monband mud crabs by H&E stain and *in situ* hybridization (A–D) 2 d and (E–H) 6 d after challenge. Positive signals were detected in: (A, E) stomach, (B, F) hepatopancreas, (C) gill; (D) integument; (G) heart; and (H) nervous tissue. Arrows indicate WSSV-infected cells. Ce: cuticular epithelium; Cn: connective tissue; Cu: cuticle; Ep: epithelium cell; Mc: myocardium; Ms: muscle; Sf: secondary filament. Scale bar = 20 μ m.

WSSV prevalence in wild-captured benthic larvae infections: Based on the small number of tested specimens taken from each group, the WSSV infection rates on Day 1 ranged from 50 to 73% (Table 2). Mortality rates for the next 14 d for the 104 benthic larvae that were cultured in the laboratory are shown in Fig. 3. WSSV infection level data for the cast shells and 15 of the surviving crabs in the group are shown in Table 2.

Discussion. The results of the challenge experiment suggest that the increased numbers of mortalities in the experimental groups were caused by a severe (1-step PCR-positive) WSSV infection (Fig. 1). This in turn suggests that *Scylla serrata* larvae are susceptible to WSSV via a waterborne infection pathway. It should be noted that the crab mortality data in Fig. 1 in fact underreport the true number of dead larvae. In addition to the totals shown in this figure, a further 93 carcasses (57 from the experimental and 36 from the control groups) were not tested because these crab larvae were cannibalized before they could be collected. However, since relatively few larvae were lost to cannibalism and since the numbers of cannibalized larvae were roughly the same in both the experimental and control groups, there is no prima facie reason to expect that these lost data would have substantially altered the results shown in Fig. 1. Nevertheless, for the second part of this study, the wire-mesh grid apparatus was used to ensure that cannibalism did not occur.

Fig. 2 shows that in external organs and tissues such as the gill and integument, positive signals were de-

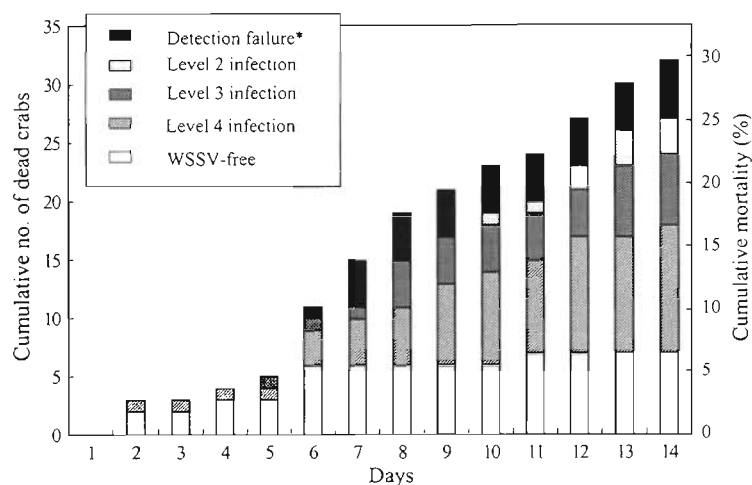


Fig. 3. *Scylla serrata*. Time-course mortality analysis for 104 wild-captured benthic seeds during 2 wk of cultivation in the laboratory. *Neither WSSV nor shrimp DNA-specific PCR products were obtained

tected sooner and more frequently than in the internal organs (heart and nervous system). Thus, as well as confirming that *Scylla serrata* larvae are susceptible to a WSSV challenge via immersion, the H&E stain and *in situ* hybridization data (Fig. 2) further suggest that for the waterborne infection pathway, WSSV first infects the external organs and then the internal ones. The virus is also evidently able to replicate over time, especially in the cuticular epithelial lining of the stomach (Fig. 2A,B,E,F). We conclude that the tissue tropism in *S. serrata* crab larvae is similar to that found in many species of shrimp (cf. Lo et al. 1996a,b, 1997).

There was a relatively high number of detection failures (5 out of 33) when the single-step nested PCR protocol was used to screen crab carcasses in the second part of this study (Fig. 3). We attribute these detection failures to the fact that the crab seed carcasses decay very readily in water; several of the tested carcasses in the second part of the study had already started to smell bad and showed signs of muscle decay. By contrast, none of the carcasses tested in the first part of this study had reached such an advanced state of decay; since they were not segregated within individual cages, those crabs that died in the experimental infection trials would have been cannibalized well before decay could have advanced to such an extent. This account of the detection failures is also consistent with the data shown in Table 2, where in 73 1-step nested PCR tests of live specimens and cast shells only 1 detection failure occurred.

Table 2. Detection of WSSV by WSSV diagnostic PCR in benthic larvae of *Scylla serrata* captured from coastal waters. -: no WSSV detected; DF: detection failure. Neither WSSV nor shrimp DNA-specific PCR products were obtained

Collection date	Day tested	PCR level					Tissue source
		1	2	3	4	-	
15 Aug 1998	1 ^b			5/10	5/10		Live specimens
24 Aug 1998	1 ^b			5/10	4/10	1/10	Live specimens
16 Sep 1998	1 ^b			11/15	4/15		Live specimens
17 Sep 1998 ^a	1 ^b			9/15	6/15		Live specimens
	8		1/1				Cast shell
	10			1/2	1/2		Cast shells
	12			1/2	1/2		Cast shells
	13		1/2		1/2		Cast shells
	14			9/15	6/15		Live specimens
	14				1/1		Cast shell

^a119 specimens collected on this date. After the initial 1-step PCR testing of 15 larvae, the remaining 104 crab seeds were cultured in the laboratory for 14 d

^bSpecimens were tested within 5 h of being collected

A comparison of Figs. 1 & 3 shows that while more than half of the carcasses in Fig. 1 were 1-step PCR positive, none of the wild caught crab carcasses were diagnosed with anything more severe than a Level 2 infection (Fig. 3). The relatively high levels of WSSV infection shown in Fig. 1 are probably due to the high dosage of viral inoculum used in the first part of this study. It is worth noting, however, that the WSSV prevalence in the carcasses in both parts of the study was approximately the same (about 60%).

A fairly consistent prevalence of around 60% was observed in the benthic larvae captured in August and September (Table 2). It is possible that this apparently stable and rather severe natural infection rate of wild mud crab seeds in the coastal waters around southern Taiwan might be persist throughout the year; there is also some evidence that mud crab population in shrimp culture ponds have a persistent WSSV infection rate of around 60% (G.-H.K. unpubl. data). If so, this is potentially very worrying for farmers because many of the crab seeds they use for stocking earth ponds are caught from the wild. Table 2 also shows that despite the deaths that may be attributed to WSSV during the 2 wk observation period, the prevalence in the live specimens at the beginning was the same as the prevalence at the end. This provides yet more evidence for the persistent and stable dispersal of WSSV in the mud crab population.

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