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NOTE

Experimental infection of white spot syndrome virus in freshwater crayfish *Pacifastacus leniusculus*

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ABSTRACT: The signal freshwater crayfish *Pacifastacus leniusculus* was found to be susceptible to infection with white spot syndrome virus (WSSV). Histopathological observations of various tissues of virus-injected crayfish showed similar symptoms to those from WSSV-infected penaeid shrimp, but no appearance of white spots on the cuticle or reddish body colour were observed, although these are the prominent gross signs of white spot disease in shrimp. A gene probe for detecting WSSV was developed in order to detect the virus in affected cells and tissues using *in situ* hybridisation. Strong signals were observed in cells of virus-injected crayfish, but not in control-injected crayfish. The number of granular haemocytes in virus-injected crayfish was significantly higher than in sham-injected and non-injected crayfish from Days 5 to 8 ($p \leq 0.05$) and Days 3 to 8 ($p < 0.01$) post-injection, respectively. The proportion of granular haemocytes in virus-injected crayfish was also significantly higher than in sham-injected controls from Days 3 to 8 ($p < 0.01$). These results indicate that WSSV has a significant effect on the proportion of different haemocyte types in the freshwater crayfish.

KEY WORDS: White spot syndrome virus · WSSV · Crayfish · *Pacifastacus leniusculus* · *In situ* hybridisation

White spot disease is one of the most disastrous in shrimp culture, having reduced shrimp production extensively worldwide. Mortality rates are usually very high and cumulative mortality can reach 100% within 3 to 10 d from the onset of visible gross signs (Chou et al. 1995, Wongteerasupaya et al. 1995). White spot disease has been reported from several Asian countries since the early 1990s (Inouye et al. 1994, Momoyama et al. 1994, Nakano et al. 1994, Chou et al. 1995, Flegel et al. 1995, Huang et al. 1995, Wongteerasupaya et al. 1995, Mohan et al. 1998, Park et al. 1998, Y. G. Wang et al. 1999), and more recently from South America and from the south-eastern coast of the USA (Lightner et al. 1997, Lo et al. 1999, Q. Wang et al. 1999, 2000).

White spot syndrome virus (WSSV) is a rod-shaped crustacean virus (Takahashi et al. 1994, Chou et al. 1995, C. H. Wang et al. 1995, Lo et al. 1996, Y. G. Wang et al. 1999) with a wide geographic distribution and host range. All the major species of cultivated penaeid shrimp can be naturally infected by this virus (Inouye et al. 1994, Nakano et al. 1994, Chou et al. 1995, Wongteerasupaya et al. 1995, Lo et al. 1996, Flegel 1997, Lightner et al. 1997, Mohan et al. 1998, Nunan et al. 1998, Park et al. 1998, Q. Wang et al. 1999). In addition to penaeid species, both natural and experimental infections have also been reported in caridean shrimp (*Exopalaemon orientalis* and *Macrobrachium rosenbergii*), crayfish (*Orconectes punctimanus*), wild crabs (*Calappa lophos*, *Portunus sanguinolentus*, *P. pelagicus*, *Charybdis* sp., *Helice tridens* and *Scylla serrata*), wild lobsters (*Panulirus* sp.), palaemonidian pest shrimp, krill (*Acetes* sp.), planktonic copepods and pupae of an ephydridian insect (Chang et al. 1998, Kanchanaphum et al. 1998, Peng et al. 1998, Supamattaya et al. 1998, Y. C. Wang et al. 1998, Q. Wang et al. 2000).

The virus can be detected by the use of polymerase chain reaction (PCR), *in situ* hybridisation or monoclonal antibody assays (Kanchanaphum et al. 1998, Peng et al. 1998, Poulos et al. 2001). Thus, in recent years molecular probes have been developed as diagnostic tools and these are already being used for pathogen detection in commercial shrimp farms.

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The aim of the present study was to ascertain whether the freshwater signal crayfish *Pacifastacus leniusculus* is susceptible to WSSV infection. This species has been introduced in many European waters to replace native crayfish species that have been eradicated by the crayfish plague fungus (Söderhäll & Cerenius 1999). Crayfish constitute a major part of the freshwater fauna and play an important ecological role. They are also useful laboratory models for the study of defence mechanisms among crustaceans. Therefore, a gene probe for detecting WSSV in crayfish was developed for further studies on virus-host interaction at the cellular level.

Materials and methods. ***Experimental animals:*** Freshwater crayfish *Pacifastacus leniusculus* were purchased from Berga Kräftodling, Södermanland, Sweden and maintained in tanks with running aerated water at 10°C. Only healthy male intermolt crayfish were used for the experiments. Prior to the initiation of the experiments all crayfish were acclimated for 24 h at 20°C where the experiments were to be performed.

WSSV propagation: WSSV was propagated *in vivo* by inoculation of clarified gill tissue homogenates from previously naturally infected *Penaeus monodon* with prominent white spots on the cuticle. The homogenates were maintained in M199 medium in 15% seawater at –80°C. WSSV from *P. monodon* was also propagated in crayfish. Gill tissues from 8 moribund crayfish (3 g) were collected, homogenised in 10 ml of Leibovitz medium (L15) at 4°C and clarified by centrifugation at 10 000 × *g* for 30 min at 4°C. The supernatant was then diluted 10-fold with L15 medium. The same amount of gills from healthy crayfish was treated similarly and used as a control. The supernatants were divided into aliquots and stored at –80°C until used. For infection experiments, an amount of 200 µl from each dilution was injected via the base of the fourth walking leg of crayfish. The lowest effective dosage of the dilution that was able to kill crayfish within 10 d was selected for further studies.

DNA isolation: DNA was isolated from gills of both control and virus-infected crayfish using a DNA Isolation Kit (PURE GENE®, Gentra Systems, Minneapolis, MN, USA) following the manufacturer's protocols. DNA concentration was determined by measuring absorbance at 260 nm using a visible UV spectrophotometer. DNA extracts were stored at –20°C.

PCR conditions: Specific WSSV primers were designed from a WSSV genomic sequence (GenBank accession No. AF178573). The primer sequences were 5′TTG ACG ACG ACT ATG ATG AC3′ as the upstream primer and 5′GCT TAC GGA TTT ACA CCT CT3′ as the downstream primer. PCR amplification was carried out in a 50 µl volume containing primers (0.5 µM each), deoxynucleotide triphosphates (200 µM each), Taq polymerase (2.5 U per 50 µl), $MgCl_2$ (1.5 mM) in PCR buffer (1×) and 100 ng of a DNA template. The PCR amplification was performed in an automatic thermocycler (Perkin-Elmer Gene Amp PCR System 2400). The amplification program was as follows: initiation denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 s and polymerisation at 72°C for 1 min, and final polymerisation at 72°C for 7 min. A negative control using sterile distilled water instead of DNA template was included to monitor for possible contamination. After amplification, PCR products were analysed in 1% agarose gel, stained with ethidium bromide and visualised under UV illumination.

DNA cloning and sequencing: The amplified products were purified using GFX™ PCR DNA and a Gel Band Purification kit (Amersham, Uppsala, Sweden) and cloned into PCR 2.1-TOPO vector (Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol. Several clones derived from PCR amplifications were sequenced using dye terminator reactions (Perkin-Elmer) by an automated sequencer (ABI Prism 377 DNA Sequencer, Applied Biosystems, Foster City, CA, USA). The sequences were analysed using the BLAST search program (National Centre for Biotechnology International, Bethesda, MD, USA).

Probe preparation: The 1537 base pair DNA fragment of WSSV obtained from PCR amplification was randomly labelled with digoxigenin-11-deoxyuridine triphosphate using the High Prime DNA labelling kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. Labelled DNA was visualised using alkaline phosphatase conjugated anti-digoxigenin antibody and the reagents nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Histopathological studies and in situ hybridisation: The samples of sham-injected and virus-injected crayfish at Days 3 and 8 post-injection were fixed in Davidson fixative (Bell & Lightner 1998) for histology and *in situ* hybridisation assay. All specimens were then dehydrated through a series of alcohol, cleared in xylene and finally embedded in paraffin wax. Tissue sections were prepared as previously described by Bell & Lightner (1998) and stained with haematoxylin and eosin (H&E) stain. Sections from the same tissues were used for *in situ* hybridisation assays. Briefly, after dewaxing and rehydration, the sections were incubated for 15 min at 37°C with 100 µl of Proteinase K (100 µg ml^{-1}) per slide. The slides were fixed in 0.4% formaldehyde for 5 min at 4°C and immersed in distilled water for 5 min. Then, the sections were hybridised with 500 ng ml^{-1} of digoxigenin-labelled probe in 50 µl of hybridisation buffer containing 1× Denhardt's solution, 10% dextran sulphate, 8× saline sodium citrate buffer and 50% formamide. After hy-

bridisation, hybrids were detected using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments according to Boehringer Mannheim's protocols. A positive hybridisation reaction produced a dark purple precipitate against the light green background of the counterstain (methyl green) and this appeared as black in black-and-white photographs.

Circulating haemocyte counts: The crayfish were divided into 3 groups of 8. The viral challenge group was injected with 200 µl of viral supernatant into the soft tissue at the base of the fourth walking leg using a sterile syringe with a needle (23 G1, 0.6 × 25 mm). The sham-injected control group received the same volume of non-viral supernatant. The third group of crayfish did not receive an injection. All crayfish were kept under the same conditions. Circulating haemocytes were counted from the virus-injected and sham-injected control group on Days 1, 3, 5 and 8 post-injection while those from the non-injected group were counted on Day 1 only. Haemolymph samples (100 µl) were taken with a sterile needle (18G1/2, 1.2 × 40 mm) from just under the soft ventral cuticle of the abdomen and immediately fixed with 10% formalin in 0.15 M NaCl. Haemocytes were immediately counted in a haemocytometer.

Statistical analysis: The data from haemocyte counts were examined for significance either by 1-way ANOVA when several groups were compared or by a *t*-test when only 2 groups were compared. Differences were considered statistically significant at $p \leq 0.05$.

Results. ***Gross pathology:*** Typical gross signs of WSSV infection in shrimp (i.e., appearance of white spots in the cuticle, overall reddish body coloration, or both) were not found in the crayfish injected with WSSV. Gross signs of WSSV infection in crayfish were (1) reddish haemolymph, (2) a significantly delayed clotting reaction (Hall et al. 1999, Sritunyalucksana & Söderhäll 2000), (3) a reduction in activity and locomotion, and (4) a weak response to stimulation. Crayfish usually started to die from Days 7 to 8 post-injection and all crayfish were dead at Day 10 post-injection.

Histopathological observations: Lesions caused by viral injection were observed in the gill lamellae (Fig. 1a), cuticular epidermis around the stomach (Fig. 1b), nuclei of adipose connective tissue (Fig. 1c), haematopoietic tissue (Fig. 1d), heart and haemocytes of all viral injected crayfish, but no affected cells were found in the hepatopancreatic tubules. The target tissues revealed foci of cells with enlarged nuclei that contained marginated chromatin separated from central, homogeneous, eosinophilic regions by an unstained ring at the early stage of infection (Fig. 1b,d, white arrowheads). These intranuclear, eosinophilic inclusions subsequently expanded to meet the marginated chromatin (Fig. 1a to d, black arrowheads). In moribund crayfish, these tissues displayed high numbers of affected nuclei.

Production of a probe by PCR and DNA sequence analysis: The DNA purified from gill tissue of virus-injected crayfish was used as a template for production of a probe by PCR. Electrophoresis of the PCR product showed a discrete band of approximately 1500 base pairs. These results indicated a successful amplification of the DNA fragment (Fig. 2) and the product was named cfWSSV. The cfWSSV fragment from the PCR amplification was sequenced and verified as a partial sequence of WSSV of penaeid shrimp (GenBank accession No. AF178573).

In situ hybridisation assay: The probe cfWSSV was shown to be specific for WSSV because it produced a positive reaction as indicated by a strong signal in affected cells of various target tissues after *in situ* hybridisation (Fig. 1e). No reactions were observed in tissues or cells of sham-injected or uninjected control crayfish (Fig. 1f) or in the tissue from virus-injected crayfish when the *in situ* hybridisation protocol was followed without the cfWSSV probe.

Circulating haemocyte counts upon viral infection: The numbers of both granular cells and total haemocytes in the haemolymph of sham-injected crayfish increased with a peak on Day 3, then declined near to the numbers of haemocytes of non-injected crayfish by Day 5. By contrast, granular cell numbers of WSSV-injected crayfish were slightly higher on Day 1 and continuously increased thereafter with a peak on Day 3. They subsequently stayed higher than the numbers of sham-injected control crayfish. The total haemocyte count (THC) of the virus-injected group was not significantly different from the THC of the non-injected crayfish ($p > 0.05$, Fig. 3), whereas the THC of the sham-injected control group was significantly higher than that of the non-injected group only on Day 3 ($p = 0.05$, Fig. 3). By contrast, the granular cell number in the virus-injected group was significantly higher than that of the non-injected group from Day 3 to Day 8 ($p < 0.01$, Fig. 3), whereas the granular cell number of the sham-injected group was significantly higher than that of the non-injected group only on Day 3 ($p < 0.01$, Fig. 3).

Comparison between the virus-injected group and the sham-injected group showed that the number of granular cells was significantly higher in the WSSV-injected group from Days 5 to 8 ($p \leq 0.05$, Fig. 3). However, the percentage of granular cells after injection with or without WSSV was significantly higher than that in the non-injected crayfish from Days 1 to 8 ($p < 0.001$, Fig. 4). Comparison between the virus-injected group and the sham-injected group showed that the granular cell proportion of the virus-injected group was significantly higher from Days 3 to 8 ($p < 0.01$, Fig. 4).

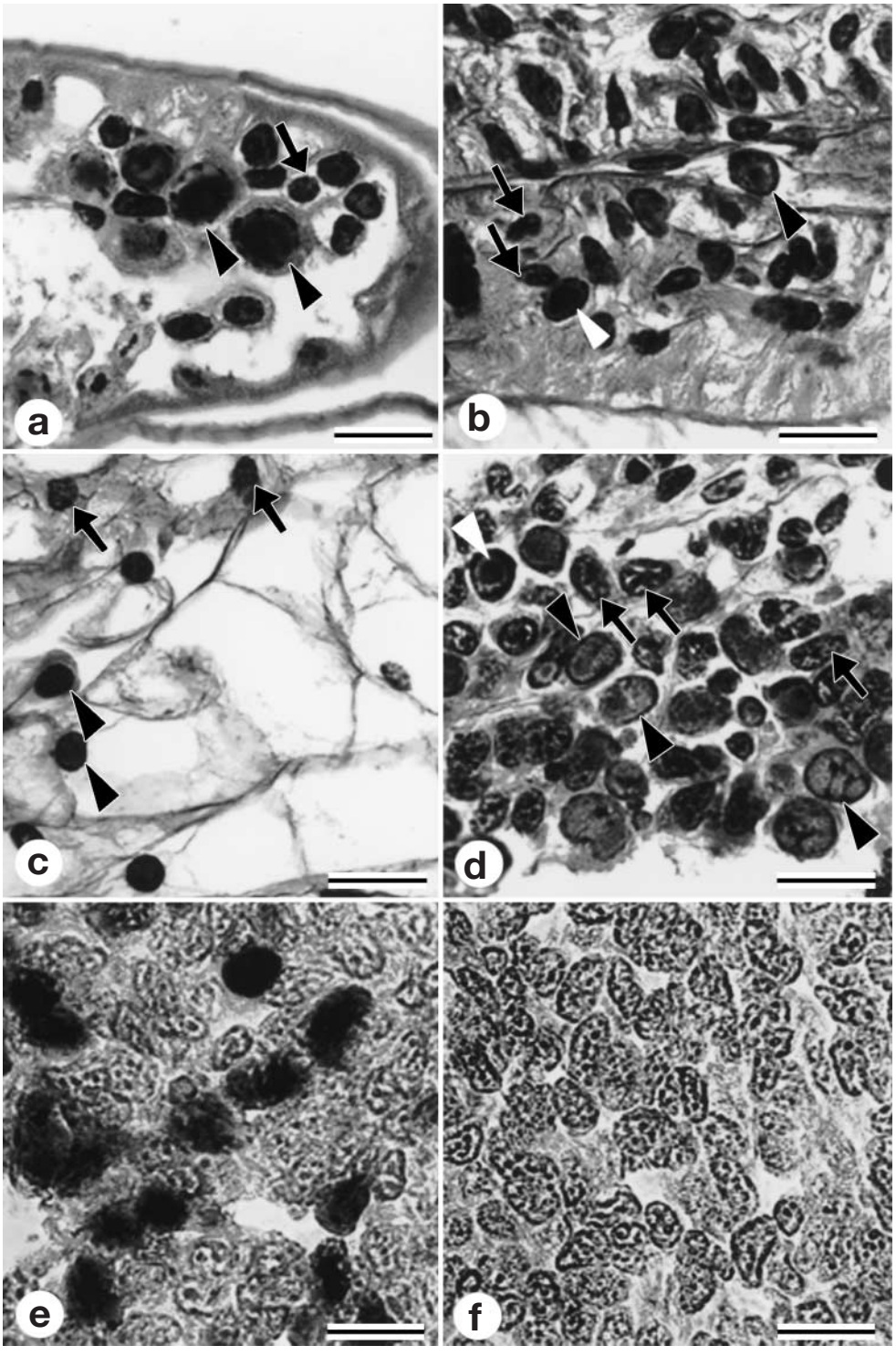

Fig. 1. Histopathology in target tissues from experimentally white spot syndrome virus (WSSV)-infected freshwater crayfish *Pacifastacus leniusculus*. Haematoxylin and eosin staining (a to d) revealed the characteristics of WSSV infection including enlarged nuclei with marginated chromatin, and central, homogeneous, eosinophilic inclusions (black arrowheads). Note an eosinophilic inclusion separated from the marginated chromatin by an unstained zone (white arrowheads). Gill lamellae (a), cuticular epithelium (b), adipose connective tissue (c) and haematopoietic tissue (d) show enlarged nuclei (black arrowheads) in most cells and normal nuclei (black arrows) in only a few. *In situ* hybridisation assay (e,f) of haematopoietic tissue from Day 8 shows a positive hybridisation reaction (dark nuclei) from virus-injected crayfish (f) and a negative reaction (no dark nuclei) from sham-injected crayfish (e). Scale bars = 10 µm

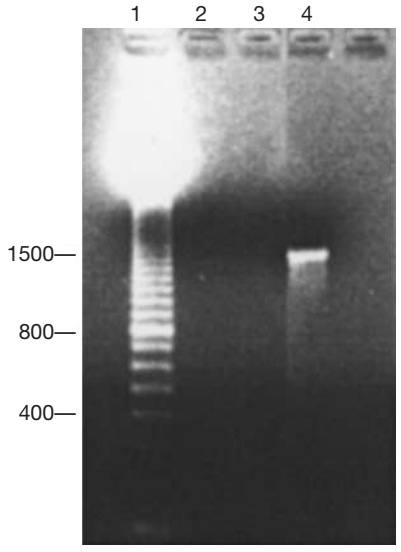

Fig. 2. Agarose gel showing a specific WSSV DNA fragment from polymerase chain reaction amplification using a DNA template from gills of virus-infected crayfish (lane 4). The negative controls (lanes 2 and 3) are distilled water and DNA from non-infected gills, respectively. Molecular weight markers (100 base pair ladder) are shown in lane 1

Discussion. The present study clearly shows that freshwater crayfish is one among many species of crustaceans susceptible to WSSV. Therefore, it is important to emphasise the risk of WSSV spread in European countries where it may have direct effects on native crayfish populations.

The histopathological study of organs and tissues in WSSV-injected crayfish revealed pathology similar to that of WSSV-infected penaeid shrimp (Chou et al. 1995, Huang et al. 1995, Durand et al. 1997, Y. G. Wang et al. 1999). In all moribund crayfish, target organs were heavily infected with WSSV, and the high incidence of affected cells throughout the organ probably led finally to death through loss of organ function. However, the epithelial cells of tubules in the hepatopancreas were not affected with WSSV even when the crayfish were moribund. This observation was consistent with a previous report of Y. G. Wang et al. (1999) that the hepatopancreatic epithelial cells of *Penaeus monodon* are not infected by WSSV. The prominent gross signs of WSSV infection in penaeid shrimp (white spots in the cuticle and reddish body colour) were not seen on crayfish. This may have been due to the thickness of the cuticle, and its dark colour due to melanin pigment.

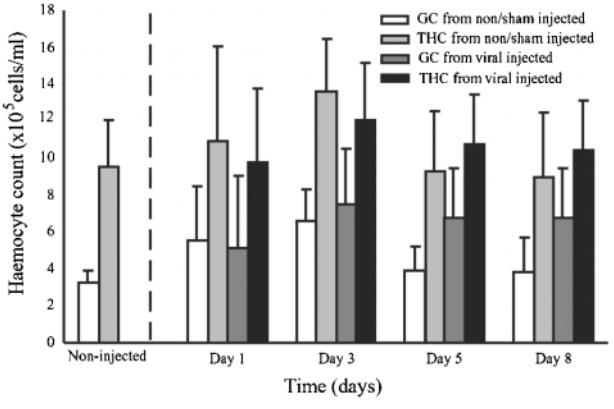

Fig. 3. Granular cell counts (GC) and total haemocyte counts (THC) in the haemolymph of injected and non-injected crayfish at different time intervals. The vertical bars represent the standard deviation of the means (n = 8)

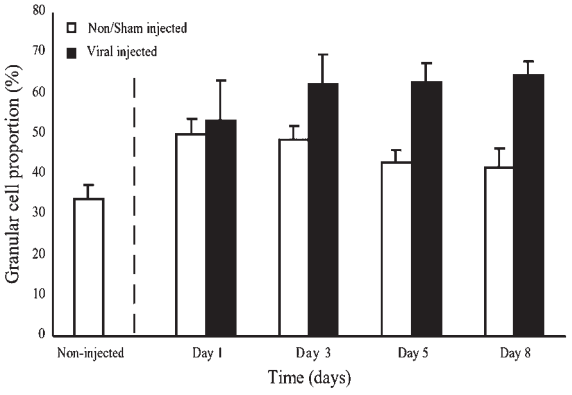

Fig. 4. Proportion of granular cells (%) in virus-infected crayfish in comparison with sham-injected and non-injected crayfish. Vertical bars represent the standard deviation of the means (n = 8)

The results from *in situ* hybridisation in various tissues supported the results for viral infection based on histopathological observations with H&E staining. The positive reaction in affected cells revealed that the cfWSSV used in this study was specific to WSSV and did not cross-react with uninfected cells or normal tissue. Furthermore, there were variations in signal intensity among the affected cells that probably depended on the stage of infection. Specifically, affected cells in the early stage of infection (i.e., with enlarged nuclei and intranuclear eosinophilic inclusions separated from marginated chromatin by an unstained space) gave a lightly positive reaction while later stage nuclei with full intranuclear eosinophilic inclusions or very dense inclusions gave stronger positive reactions.

It may be assumed that injection of gill tissue homogenates in L15 medium with or without WSSV would stimulate crayfish cellular defence mechanisms since the homogenates would contain foreign molecules. This might be one reason for higher granular cell num-

bers from Days 1 to 3 in the injected groups. However, the elevated granular cell numbers in sham-injected crayfish returned to the level of uninfected animals at 5 d post-injection, whereas those of the virus-injected crayfish remained elevated until death or the end of the test period. This clearly showed that normal haemocyte balance was rapidly restored after sham injection but not after virus injection. Since the THCs remained unchanged, the relative proportion of granular cells increased. There are several possible explanations for this. For example, it is possible that granular cells are more resistant to viral infection than non-granular cells and that a longer period is required for them to be destroyed by the virus. Indeed, C. H. Wang et al. (2000) have shown from transmission electron microscopy of WSSV-infected primary, lymphoid organ cell cultures that 18% of fibroblast-like cells were infected followed by 11% of adipose cells, 8% of reticular cells and 6% of granular cells. Another possible explanation arose from our histopathological studies, where we found that haematopoietic tissue was a heavily infected target for WSSV. Since haematopoietic tissue renews haemocytes by continuous release into the haemolymph (Söderhäll & Cerenius 1992), WSSV may affect haematopoietic tissue development and lead to changes in the proportions of different circulating blood cell types. Yet another possible explanation might be the presence of an antiapoptosis gene in WSSV, similar to those found in the DNA virus of the family *Baculoviridae* (Clem et al. 1991). A tentative product of such an antiapoptosis gene might be able to prevent apoptosis and allow viral replication to occur in some cells but not others. This latter possibility may be less likely now that WSSV is known to be only distantly related to the baculoviruses (Tsai et al. 2000, Liu et al. 2001), contrary to the conjecture in earlier publications that it is closely related (e.g., Wongteerasupaya et al. 1995). In any case, further studies are necessary to determine the susceptible level to WSSV among haemocyte types, even though haemocytes have been reported to be targets of WSSV in penaeid shrimp (Durand et al. 1996, Y. G. Wang et al. 1999, C. H. Wang et al. 2000) and crabs (Kanchanaphum et al. 1998).

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