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Molt stage and cuticle damage influence White Spot Syndrome Virus immersion infection in penaeid shrimp

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Abstract

Transmission of white spot syndrome virus (WSSV) in shrimp has been reported to occur by feeding and immersion. In the present study, the impact of the molt process and artificial lesions in the cuticle on shrimp susceptibility to WSSV was examined using intramuscular and immersion routes.

For the intramuscular route, *Penaeus (Litopenaeus) vannamei* shrimp (n=450) were injected with $10^{-2.3}$ up to $10^{2.7}$ shrimp infectious dose 50% end point (SID₅₀) of WSSV in early and late post-molt, inter-molt, early and late pre-molt; resp. A-, B-, C-, D1- and D2-stage. The resulting infection titers demonstrated that no difference ($p>0.05$) in susceptibility existed between different molt stages when virus was injected.

For the waterborne route, shrimp in different molt stages were immersed in sea water containing 10^4 SID₅₀ ml⁻¹ of WSSV. In a first study, *P. vannamei* (n=125) incubated in cell culture flasks, became infected with WSSV mostly in post-molt stages. In a second study, 2 groups of *P. vannamei* (n=100) and *P. monodon* (n=100) were transferred into plastic bags to prevent damage to the cuticle; and in 1 group a pleopod was cut off prior to incubation. Induction of damage increased infection significantly ($p<0.05$) in A-stage from 0-40% to 60-100%, in B-stage from 0-20% to 40-60%, in C-stage from 0-20 to 20-60%, while infection was 0% in D-stages with both immersion methods.

This study proved that shrimp are more susceptible to WSSV infection via immersion after molting than in the period before molting and wounding facilitates infection.

Keywords: White Spot Syndrome Virus; waterborne transmission; *Penaeus vannamei*; *Penaeus monodon*; molt stage.

1. Introduction

White spot syndrome virus (WSSV) is one of the most wide-spread viruses in penaeid shrimp aquaculture and is considered to be responsible for a large portion of crop failures (for reviews on WSSV, see: Sanchez-Martinez et al., 2007; Escobedo-Bonilla et al., 2008). Since the first reports on the virus, it has become generally accepted that transmission between shrimp and other Decapod Crustacea can occur via 3 routes: (1) oral uptake of tissues from infected hosts; (2) waterborne, when virus is transmitted via the water by immersion or cohabitation and (3) *per ovum* (vertical) and possibly *intra-ovum* from broodstock to offspring. When reviewing literature on WSSV, one finds a high number of experimental studies demonstrated that feeding of WSSV-infected shrimp tissues is an effective way to infect shrimp and other decapods. Especially the early reports on WSSV helped to build the image that the virus is highly contagious, even though many researchers had to administer WSSV-infected tissues more than one feeding, sometimes as long as 7 days. For the waterborne route, many studies reported that immersion and even cohabitation exposure readily allowed WSSV to cause infection, although older shrimp were reported to be less susceptible.

It is important to note, however, that most of the studies published so far were performed with non-specific pathogen-free (SPF) animals, without knowing the administered doses of WSSV and without screening the inoculum for the presence of other pathogens. Often, possible secondary transmissions after inoculation were not ruled out, temperature of the rearing water was not under control and most importantly, WSSV infections were rarely confirmed.

These facts make it difficult to reproduce those studies or make reliable conclusions. Probably the best-controlled experimental studies on WSSV transmission so far, were published by Soto and Lotz (Soto et al., 2001; Lotz and Soto, 2002; Soto and Lotz, 2003) and

Prior et al. (Prior et al., 2003). Soto and Lotz concluded that ingestion of infected tissues was a far more effective treatment than immersion in infected water. Remarkably however, even when *P. vannamei* were isolated to ensure they had equal chance to consume the infected tissues offered to them, not all shrimp became infected (50-60%). Prior et al. (2003) succeeded in determining the lethal intramuscular dose of a WSSV stock and also tried to develop a controlled bio-assay by immersion of *P. vannamei*. Although very large amounts of infectious virus were added to the water (as shown by the injection study), mortality rates stayed below 40%. Recently, another study clearly illustrated the difficulty to infect animals by WSSV immersion challenge (Gitterle et al., 2006), while a study on an ornamental shrimp's susceptibility to WSSV resulted in a discussion of the problems encountered with experimental feeding challenges (Laramore, 2007). Gitterle et al. (2006) showed that merely adding virus inoculum to the water was not sufficient to result in *P. vannamei* infection but needed to place the shrimp in tanks in which orally infected shrimp had previously died to finally obtain successful transmission. Finally, in the PhD thesis by Dr. Bonny Bayot (2006), less than 17% of *P. vannamei* shrimp became infected upon individual challenge with WSSV via oral route and none or merely 3% by immersion.

The overall conclusion from these publications is that there are restrictions on the ability of WSSV to gain entry into its host. With feeding of virus-infected tissues to shrimp, this is to be expected as the lack of control on the dose of virus actually reaching the site of entry, inherently creates irreproducible results. The fact that any portion of the animals might not be feeding (due to molting, stress, ...) for instance, can easily prevent an equal chance to become infected. Another factor which cannot be ignored is that all tissues known to be susceptible to WSSV replication are protected from the outside world by cuticle (Escobedo-Bonilla et al., 2007). This is also true for the gills and the epithelium of stomach and hindgut (Bell and Lightner, 1988).

Although little details are known about the structure and function of the cuticle of penaeid shrimp, it is well-known that it changes dramatically in time (Chan et al., 1988; Compère et al., 2004; Promwikorn et al., 2007). During the course of its life, a shrimp passes through consecutive molt cycles. Therefore, in a study examining transmission of pathogens in shrimp, it could be important to take the molt stage into account (Le Moullac et al., 1997; Mugnier et al., 2008).

Considering the inability to reproducibly cause infection in shrimp exposed to WSSV by immersion, the present study was set-up to investigate the factors determining WSSV infection by waterborne route. In a first hypothesis we tested whether the susceptibility of shrimp to WSSV infection changes during the course of their molt cycle. The virus was delivered intramuscularly, thus passing the cuticle in order to compare the internal susceptibility between the different molt stages. In a second approach, the barrier function of the cuticle against natural infection by waterborne virus was tested in a series of immersion inoculation experiments of shrimp in different molt stages. Groups of artificially damaged shrimp were compared with control shrimp to test the hypothesis that the cuticle presents a barrier against WSSV and that wounding can promote infection.

2. Materials and Methods

2.1. Experimental animals and conditions

The shrimp used in this study were *Penaeus (Litopenaeus) vannamei* from Molokai Sea Farms Int., Hawaii, USA and *P. monodon*, from Moana Technologies Nucleus Breeding Centre, Hawaii, USA. The batches of shrimp from Moana Technologies were certified to be SPF by Jim Brock, DVM. Those from Molokai Sea Farms

had SPF status according to inspection services by the Aquaculture Development Program, State of Hawaii. Batches of 10,000 PL-10 shrimp were shipped to Belgium and reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. They were fed with *Artemia* nauplii twice daily for a period of 3 weeks and were then weaned onto a commercial pelleted feed (A2 monodon high performance shrimp feed, INVE Aquaculture SA, Belgium), fed twice daily at a total rate of 5 % of their mean body weight (MBW). Water temperature was kept at $27 \pm 1^\circ\text{C}$ and salinity at $35 \pm 1 \text{ g l}^{-1}$. Regular water changes kept total ammonia-N below 0.5 mg l^{-1} and nitrite-N below 0.15 mg l^{-1} . The room was illuminated 12 hours per day by dimmed TL-light. For the viral challenge experiments, shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the experiments were performed under bio-safety conditions.

2.2. Molt stage determination

Molt stages were determined based on the descriptions by Robertson et al., 1987 and Chan et al., 1988. Briefly, shrimp were restrained for a few seconds and their uropods were examined by inverted microscope. At a magnification of 100 to 200X, the exopodites of uropods were analysed on the appearance of the cuticle, epidermis and molt processes such as apolysis and the formation of new cuticle. Shrimp were separated into 5 major molt stages: early and late post-molt (A and B), inter-molt (C) and early and late pre-molt (D1 and D2).

Post-molt stages are characterized by an epidermis in close contact with all of the still thin cuticle. The epidermis is present in the setae in A-stage and retracts in B-stage, while it constantly secretes additional layers to the cuticle. In the inter-molt stage the epidermis lies in a straight line at the bottom of the setae while the construction of the cuticle is finalized. The pre-

molt phase starts as the epidermis retracts from the cuticle in stage D1 and begins formation of a new cuticle. In the final stage before the molt, D2, the newly forming cuticle and setae become visible.

2.3 Virus

The WSSV Thai-1 isolate was used in the present study. This isolate has been studied before (Jiravanichpaisal et al., 2001; Escobedo-Bonilla et al., 2005; Escobedo-Bonilla et al., 2006; Escobedo-Bonilla et al., 2007; Rahman et al., 2008). It was collected from naturally infected *Penaeus monodon* in Thailand in 1996 and passaged in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001). Crayfish gill suspension containing WSSV Thai-1 was kindly provided by K. Söderhäll (Uppsala University, Sweden) and amplified in SPF *P. vannamei* juveniles to produce virus stocks. The median infectious titer of the stock used for all experiments in this study was determined to be $10^{6.0}$ shrimp infectious dose 50% end point (SID₅₀) per ml, following the *in vivo* intramuscular titration procedure in SPF *P. vannamei* described by Escobedo et al. (2005).

2.4 *In vivo* titration by intramuscular inoculation using shrimp in different molt stages

P. vannamei juveniles (MBW = 5.6 ± 2.7 g; n = 450) were taken from stock cultures maintained at ARC and screened for their molt stage as described in 2.2. Thirty shrimp were selected in each of the 5 major molt stages (A, B, C, D1 and D2) and inoculated intramuscularly with 50 µl of a 10-fold serial dilution of the WSSV stock (10^{-2} to 10^{-7}), with 5 shrimp per dilution. After the inoculation, shrimp were housed individually in covered 10 l aquaria, filled with artificial seawater at a salinity of 35 g l⁻¹, provided with constant aeration

and maintained at 27°C by air heaters. Approximately 2.5% of BW of a commercial shrimp diet was provided to each shrimp in 2 rations per day. Moribund and dead shrimp were recorded, removed from the aquaria and processed for detection of WSSV infection. The experiment was terminated at 120 hpi, when surviving shrimp were sacrificed and analyzed for WSSV infection. The experiment was performed in triplicate.

2.5 Study of WSSV infection by immersion route

2.5.1 Immersion inoculation inside cell culture flasks

The aim of this experiment was to develop a model for WSSV infection by immersion. A total of 125 SPF *P. vannamei* were used. As the batch of shrimp grew up, 5 groups of shrimp with a MBW of 1, 4, 6, 11 and 20 g were taken from the stock culture at ARC and screened for their molt stage as described in 2.2. For each size group, 5 shrimp per molt stage were immersed. The WSSV inoculum used to immerse the shrimp was a 1% dilution of the WSSV stock. It was prepared in a volume of 25 ml artificial seawater (35 g l⁻¹) per g bodyweight, resulting in a dose of 10⁴ SID₅₀ ml⁻¹. Shrimp of 1 g were put inside '25 cm²' cell culture flasks (Nunc A/S, Denmark) containing 25 ml of the inoculum. Animals of 4, 6 and 11 g were put inside '75 cm²' cell culture flasks containing respectively 100, 150 and 275 ml of the inoculum. Shrimp of 20 g were put inside '175 cm²' cell culture flasks containing 500 ml of the inoculum. Flasks were placed on a lateral side in order to allow the shrimp to stay in a physiological position. The duration of the immersion was 3 hours and water was aerated with an airstone.

After the inoculation, the procedures were identical to those in 2.4, but no food was given the first 12 h after the immersion to avoid additional oral up-take of virus via the food. Shrimp were monitored for clinical signs every 12 h and dead shrimp were removed and processed for detection of virus replication. The experiment was terminated 5 days post

immersion. At this time, all surviving shrimp were euthanized and processed for virus detection. Mortality and infection rates were compared between the molt stages and between the sizes.

2.5.2. Immersion inoculation inside plastic bags of shrimp with and without damaged cuticle.

In this experiment, damage was induced to 1 group of shrimp by cutting off a pleopod while shrimp of the control group were left undamaged. Both groups were put inside plastic bags to limit physical damage as much as possible. The aim was to evaluate whether mechanical damage would allow a higher incidence of WSSV infections in shrimp.

A total of 100 *P. vannamei* and 100 *P. monodon* were used in this experiment. For each species, 2 size groups of 50 shrimp were tested with a MBW of 2 and 15 g. Shrimp were taken from the stock culture at ARC and screened for their molt stage as described in 2.2. An attempt was made to minimize damage to the cuticle by carefully catching and handling the animals. Of each species and size, 10 shrimp of each molt stage were selected and placed individually in 4 l transparent polyethylene bags (220x330nm, 50my, Binpac) filled with sea water. These were placed inside buckets lined with shock-absorbing plastic for transport to the facilities of the Laboratory of Virology. At the start of the immersion, the water in the plastic bags containing the individual shrimp was replaced by 50 ml of the inoculum for 2 g shrimp and 375 ml for 15 g shrimp. The inoculum was prepared as described for 2.5.1. Per molt stage, 5 shrimp were then briefly recaptured and 1 pleopod of the first abdominal segment was cut off by bistouri blade at the level of the coxa. During the immersion, bags were hung in mid-air in order to allow the animals to stay in a physiological position in the layer of inoculum on the bottom and a tube with an aeration stone was inserted to allow aeration of the water. After 3 hours of incubation, the inoculum was drained from the bag and shrimp were placed straight into aquaria. The set-up of the remainder of the experiment was identical to that described for experiment 2.5.1.

Mortality and infection rates were compared between the molt stages, artificially damaged and intact shrimp and their respective sizes.

2.6 Detection of WSSV infection by indirect immunofluorescence(IIF)

The procedure to detect WSSV infection by IIF was described before (Escobedo-Bonilla et al., 2005). In brief, the cephalothoraxes of dead shrimp were dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at -20 °C. Cryosections (5 µm) were made and immediately fixed in 100% methanol at -20 °C for 20 min. Sections were washed three times for 5 min each in phosphate buffered saline (PBS) and incubated with 2 µg ml⁻¹ of the monoclonal antibody 8B7 (Diagxotics Inc. USA) directed against viral protein VP28 (Poulos et al., 2001) for 1 h at 37 °C. Then, sections were washed three times for 5 min each in PBS and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1 h at 37 °C. Sections were finally washed in PBS, rinsed in deionised water, dried and mounted with a solution of glycerine and 1, 4-diazabicyclo[2,2,2]-octane (DABCO) (ACROS organics, USA). Slides were analyzed by fluorescence microscopy (Leica DM RBE).

2.7 Statistical analysis

The virus titers of the intramuscular titration (2.2) were compared between molt stages using the Wilcoxon rank-sum non-parametric test (Zar, 1996).

Differences between molt stages within groups of 5 to 10 shrimp per group (2.3 and 2.4) were tested for significance using Fisher's exact test (Kirkwood and Sterne, 2003).

In 2.4, both species and ages were pooled into groups of 20 shrimp, and the difference in infection rates was tested between the molt stages and between the control and the pleopod cut groups by Pearson's Chi Square tests with Yates' correction.

All calculations were performed using S-plus version 6.1 (Lucent Technologies).

3. Results

3.1. *In vivo titration by intramuscular inoculation (Table 1)*

IIF analysis of dead and surviving shrimp revealed the following virus infection titers: 10^6 , $10^{6.5}$ and $10^{6.8}$ for A-stage ($10^{6.5 \pm 0.4}$); $10^{6.6}$, $10^{6.8}$ and $10^{7.5}$ for B-stage ($10^{7.1 \pm 0.4}$); $10^{6.5}$, $10^{6.7}$ and $10^{6.8}$ for C-stage ($10^{6.7 \pm 0.2}$); $10^{6.8}$, $10^{6.8}$ and $10^{7.1}$ for D1-stage ($10^{6.9 \pm 0.2}$) and $10^{6.3}$, $10^{6.7}$ and 10^7 for D2-stage ($10^{6.7 \pm 0.3}$) (Table 1). No significant differences in infection titers were observed between the stages ($p > 0.05$).

3.2. *Immersion inoculation inside cell culture flasks (Table 2)*

Of the 1 g shrimp, only 1 shrimp in the A-stage group started to become anorectic and lethargic at 36 hpi. This was the only shrimp to die due to WSSV before the end of the experiment. All other shrimp were euthanized at 120 hpi and were negative for WSSV on IIF. When the immersion was performed with 4 g shrimp, all survived the experiment uninfected. At a size of 6 g, 3 out of 5 A-stage shrimp started to show clinical signs at 36 hpi and died at 60-84 hpi. When the experiment was performed with 11 g, all A- and one B-stage shrimp showed clinical signs and died due to WSSV infection between 48 and 120 hpi. Two D2-stage animals molted during the immersion and one died before the end of the 3 hours procedure.

This was the only mortality during the course of the experiment which was not caused by WSSV infection. In the experiment performed on 20 g shrimp, all A-, 2 out of 5 B- and 1 C-stage shrimp showed clinical signs after 36 hpi. These shrimp died between 48 and 72 hpi and were confirmed to be infected with WSSV, while all other shrimp survived and were uninfected. The difference in infection rate was significantly higher in A-stage than in the other stages in 11 g shrimp, and between A- and C-, D1- and D2-stage in 20 g animals ($p < 0.05$).

During this experiment, it was noticed that the 11 and 20 g post-molt shrimp had suffered injuries to appendages during the immersion procedure. Because of this observation, an alternative immersion procedure using plastic bags was designed in an attempt to limit self-generated damage to the shrimp.

3.3. Immersion inoculation inside plastic bags (Table 3)

One pleopod of the first abdominal segment could be removed at the level of the coxa by bistouri blade without causing any clinical signs or mortality. Damaged sites showed melanization within 12-24 hours after injuries had occurred. Melanizations which were present on the animals after natural damage and prior to immersion were recorded. Thus, the physical damage occurring during the immersion procedure could be estimated.

Throughout the experiment, anorexia was recorded in D2-stage shrimp 24 to 48 h before molting and in A-stage shrimp. Uninfected animals started eating normally again by the end of A-stage. Infected animals displaying anorexia on the other hand also became lethargic between 48 to 72 hours post immersion (hpi), generally 24 hours before dying.

In 2 g juvenile *P. vannamei* immersed in plastic bags without cutting of pleopods, 2 shrimp in A-stage and 1 in C-stage died. Of the shrimp with cut off pleopods, 3 in A- and B-

and 1 in C-stage died between 48 and 72 hpi. All other shrimp of the various molt stages with pleopods left intact or cut survived until the end of the experiment at 120 hpi.

Of 15 g *P. vannamei* with no pleopod cut, only 1 out of five A- and B-stage animals died at 72 hpi. Cutting a pleopod increased the mortality to 5 in A-stage (48 to 120 hpi), 2 out of 5 in B-stage (96 hpi) and 1 in C-stage (120 hpi). All other shrimp survived until 120 hpi.

In 2 g juvenile *P. monodon* immersed with pleopods intact, only 1 shrimp in A-stage died. Of those with cut off pleopods, 3 in A- and B- and 2 in C-stage died between 48 and 120 hpi. All other shrimp survived until the end of the experiment.

Of 15 g *P. monodon* with pleopods left intact, 2 out of five A- and 1 B-stage shrimp died (48 or 72 hpi). Cutting a pleopod induced mortality in 3 shrimp in A-stage (48 to 72 hpi), 2 in B-stage (48 to 72 hpi) and 3 in C-stage (72 to 84 hpi). All other shrimp survived until the end of the experiment.

In all cases, dead shrimp were WSSV positive on IIF, and surviving shrimp were WSSV negative.

Only in 15 g *P. vannamei* with cut pleopods, significant differences were calculated between A-stage on one hand and C-, D1- and D2-stage on the other (Fisher's exact test; $p < 0.05$). When the infection rates of species and sizes were pooled (Table 3B), the Chi Square test on the results showed the following: 1) a significantly higher infection rate in A-stage than in D1- or D2-stage of the control groups ($p < 0.05$); 2) a highly significant difference between A- and D1- or D2-stage in the pleopod cut groups ($p < 0.001$); 3) no significant difference between A-, B- or C-stage in the pleopod cut groups ($p > 0.05$); 4) significantly more infected shrimp in B- and C-stage than in D1- and D2-stage of the pleopod cut groups ($p < 0.05$); 5) significantly more infected shrimp in A-, B and C-stages with cut pleopods than in the control group ($p < 0.05$).

4. Discussion

In preliminary WSSV immersion experiments leading up to this study, an influence of the molt cycle on the susceptibility to the virus had been observed. In the present study, an *in vivo* titration of the virus stock in shrimp in different molt stages was first performed by intramuscular route. This experiment showed that no significant intrinsic difference in susceptibility to WSSV existed between shrimp in the different molt stages. Hence, the underlying mechanism responsible for the difference in susceptibility to WSSV between molt stages had to be examined using trials mimicking natural transmission.

A new immersion inoculation procedure was set up to study the infection of WSSV by waterborne route. Studies on the waterborne route of WSSV transmission in literature all employed simply aquaria for inoculations of shrimp, except for Prior et al. (2003) who used cell culture flasks. At first sight, cell culture flasks seemed to be adequate tools to perform an immersion procedure as these containers are sterile, do not inactivate virus and allow observation of the animals. However, prevention of uncontrollable physical damage to the animals during transport in buckets and the immersion procedure in cell culture flasks proved to be difficult. All shrimp instinctively struggled by contracting their tail during catching and handling in an attempt to escape and jumped violently against the walls of the containers. Only post-molt (A- and B-stage) shrimp suffered visible damage. Most affected were appendages such as rostrum, telson, uropods, antennae, pleo- and pereopods. The damage was mainly comprised of fractures of the cuticle, noticed by deformities and hemolymph bleeding from the fractures. Sometimes this resulted in loss of appendages. Especially the larger 11 and 20 g shrimp were suffering injuries due to the relatively small access of the flasks.

As an alternative immersion recipient, polyethylene bags were tested in this study. When shrimp were carefully placed inside plastic bags before transport and the water replaced

by inoculum, the amount of resistance and jumping of the shrimp was reduced and much less obvious injuries could be observed while the shrimp hung suspended in mid-air. Even though the bags proved to be useful, it remained impossible to completely prevent the occurrence of damages in the soft post-molt shrimp.

Overall, the incidence of infection and mortality was clearly higher in shrimp immersed in WSSV inoculum during the post-molt stages than in pre-molt stages. It was postulated that immersion inoculation of shrimp in hard-walled containers could result in infection in larger shrimp in post-molt stages, because of damage to the cuticle which is softer and thinner in these stages. An inoculation procedure using plastic bags resulted in much less infection in post-molt stages as the animals were handled more carefully. A clear correlation between damage of the cuticle and infection was demonstrated by cutting a pleopod at the start of immersion. The incidence of infection was increased 2 to 8-fold between undamaged and artificially damaged groups. Similar results could also be obtained by cutting the rostrum in A-stage shrimp (data not shown). However, even with the infliction of a wound, no infection was ever recorded in shrimp which had been pre-molt at the time of exposure to waterborne virus. While differences were seen in infection rates between ages in shrimp immersed in cell culture flasks, no such differences were recorded between 2 or 15 g shrimp inoculated inside plastic bags.

The actual portal of entry of WSSV from the water into a host has never been described, but some assume that the gills are the best candidates (Chang et al., 1996; Witteveldt et al., 2004; Arts et al., 2007). The experimental findings of the present study demonstrate that an artificially induced wound in the cuticle increases the rate of WSSV infection upon immersion. Cutting off a pleopod creates an open wound which can allow either (1) infection of cells at the site of the wound or (2) entry of WSSV into the hemolymph followed by direct systemic spread or on the other hand (3) reduce the competence of shrimp to resist WSSV infection. In the first two scenarios, entry of the virus would occur through the opening in the cuticle itself. If one

considers the (ultra)structure of the cuticle of crustacea such as shrimp, it is not difficult to imagine that the cuticle constitutes an impregnable barrier against viruses from food or the environment (Compère, pers.comm.). Although damage to the cuticle appears to be the key to WSSV infection from the water, the situation is more complex. Even when an open wound is present in shrimp, this does not always lead to infection, especially in molt stages when the exoskeleton is well-developed (i.e. inter- and pre-molt). Factors which determine whether WSSV can ultimately invade a shrimp could be: (1) morphological and physiological (cuticle and epidermal cells) or (2) (a)specific defence-related (coagulation time, phagocytosis, phenoloxidase and reactive oxygen species activity etc.). All these factors are likely or are already known to vary between different stages of the molt cycle (Charmantier et al., 1994; Le Moullac et al., 1997; Liu et al., 2004; Chiou et al., 2007; Promwikorn et al., 2007; Mugnier et al., 2008). The third alternative explanation for the increased chance for WSSV infection in damaged shrimp, would be that wounding has a direct or indirect effect on the capacity of shrimp to resist to WSSV infection. Indeed, removal of a pleopod will induce stress, which could have an effect on the subsequent immune response of the shrimp. The inflicted damage and subsequent clotting, hemocyte migration and exocytosis at the site of the wound, and immune responses to other microorganisms which may enter, can all alter possible defence against WSSV infection.

Overall, the findings in the present paper give the impression that there are important restrictions on the ability of WSSV to gain entry to its host and question whether the water in which shrimp live is a natural medium for the spread of the virus, as long as the cuticle of shrimp is a firm barrier. This clearly differs from some reports on WSSV infections from water in literature (Kanchanaphum et al., 1998; Witteveldt et al., 2004; Arts et al., 2007), while it is supported by other (Prior et al., 2003; Bayot, 2006; Gitterle et al., 2006). Differences in

virulence or invasive ability of WSSV isolates, administered dose and methodology are the likely explanations for these variable results.

5. Conclusion

This study revealed that the molt stage of penaeid shrimp does not influence their susceptibility to WSSV infection when the virus is injected, but that on the other hand shrimp in post-molt stages of the molt cycle become more easily infected with WSSV from water than in pre-molt stages. The procedure by which shrimp were immersed in WSSV inoculum strongly affected the chances for infection. The rate of infection was significantly higher in animals with damages to the exoskeleton due to immersion in hard-walled containers or with a pleopod removed. From these findings we postulate that the cuticle is a barrier against WSSV infection and wounding can increase the susceptibility of shrimp.

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Table 1. Infection titers of White Spot Syndrome Virus stock by intramuscular inoculation in *P. vannamei* in different molt stages (3 repetitions of 5 shrimp per dilution). Average titers were not significantly different between molt stages ($p>0.05$).

Molt Stage	Dilution of WSSV	Mortality	Confirmed infected by IIF	Infection titer
A	10^{-2}	15/15	15/15	$10^{6.5\pm0.4}$ SID ₅₀ ml ⁻¹
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	10/15	10/15	
	10^{-6}	0/15	0/15	
	10^{-7}	0/15	0/15	
B	10^{-2}	15/15	15/15	$10^{7.1\pm0.4}$ SID ₅₀ ml ⁻¹
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	11/15	11/15	
	10^{-6}	6/15	6/15	
	10^{-7}	0/15	0/15	
C	10^{-2}	15/15	15/15	$10^{6.7\pm0.2}$ SID ₅₀ ml ⁻¹
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	10/15	10/15	
	10^{-6}	2/15	2/15	
	10^{-7}	0/15	0/15	
D1	10^{-2}	15/15	15/15	$10^{6.9\pm0.2}$ SID ₅₀ ml ⁻¹
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	13/15	13/15	
	10^{-6}	4/15	4/15	
	10^{-7}	0/15	0/15	
D2	10^{-2}	15/15	15/15	$10^{6.7\pm0.3}$ SID ₅₀ ml ⁻¹
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	10/15	10/15	
	10^{-6}	4/15	4/15	
	10^{-7}	0/15	0/15	

Table 2. Immersion of *P. vannamei* in different molt stages inside cell culture flasks containing inoculum with 10000 SID₅₀ ml⁻¹ of White Spot Syndrome Virus.

Weight	Molt stage	Mortality (hpi)	Confirmed infected by IIF
1 g	A	1/5 (60)	1/5
	B	0/5	0/5
	C	0/5	0/5
	D1	0/5	0/5
	D2	0/5	0/5
4 g	A	0/5	0/5
	B	0/5	0/5
	C	0/5	0/5
	D1	0/5	0/5
	D2	0/5	0/5
6 g	A	3/5 (60, 60, 84)	3/5
	B	0/5	0/5
	C	0/5	0/5
	D1	0/5	0/5
	D2	0/5	0/5
11 g	A	5/5 (48, 48, 48, 72, 72)	5/5
	B	1/5 (120)	1/5
	C	0/5	0/5
	D1	0/5	0/5
	D2	1/5†	0/5
20 g	A	5/5 (48, 60, 60, 60, 72)	5/5
	B	2/5 (60, 60)	2/5
	C	1/5 (60)	1/5
	D1	0/5	0/5
	D2	0/5	0/5

†: 1 shrimp died during immersion (<3 hpi)

Table 3. Immersion of *P. vannamei* or *P. monodon* in different molt stages inside plastic bags containing inoculum with 10000 SID₅₀ ml⁻¹ of White Spot Syndrome Virus, with or without removal of one appendage.

Species	Weight	Removal of appendage	Molt stage	Mortality (hpi)	Confirmed infected by IIF
<i>P. vannamei</i>	2 g	none (control)	A	2/5 (48, 72)	2/5
			B	0/5	0/5
			C	1/5 (72)	1/5
			D1	0/5	0/5
			D2	0/5	0/5
		1 pleopod	A	3/5 (48, 60, 72)	3/5
			B	3/5 (60, 60, 72)	3/5
			C	1/5 (60)	1/5
			D1	0/5	0/5
			D2	0/5	0/5
	15 g	none (control)	A	1/5 (72)	1/5
			B	1/5 (72)	1/5
			C	0/5	0/5
			D1	0/5	0/5
			D2	0/5	0/5
		1 pleopod	A	5/5 (48, 72, 84, 84, 120)	5/5
			B	2/5 (96, 96)	2/5
			C	1/5 (120)	1/5
			D1	0/5	0/5
			D2	0/5	0/5
<i>P. monodon</i>	2 g	none (control)	A	1/5 (72)	1/5
			B	0/5	0/5
			C	0/5	0/5
			D1	0/5	0/5
			D2	0/5	0/5
		1 pleopod	A	3/5 (48, 72, 72)	3/5
			B	3/5 (72, 72, 84)	3/5
			C	2/5 (72, 120)	2/5
			D1	0/5	0/5
			D2	0/5	0/5
	15 g	none (control)	A	2/5 (48, 72)	2/5
			B	1/5 (48)	1/5
			C	0/5	0/5
			D1	0/5	0/5
			D2	0/5	0/5
		1 pleopod	A	3/5 (48, 60, 72)	3/5
			B	2/5 (48, 72)	2/5
			C	3/5 (72, 84, 84)	3/5
			D1	0/5	0/5
			D2	0/5	0/5