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

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5 **Mathias Corteel¹, João J. Dantas-Lima¹, Mathieu Wille², Victoria Alday-Sanz³,**
6 **Maurice B. Pensaert¹, Patrick Sorgeloos² and Hans J. Nauwynck^{1*}**

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8
9
10
11 ¹Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133,
12 B-9820 Merelbeke, Belgium.

13 ²Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering,
14 Ghent University, Rozier 44, B-9000 Gent, Belgium.

15 ³Aquatic Animal Health, Portal del Angel, 3-5, 2, 08002 Barcelona, Spain.

16
17
18
19
20
21 *Corresponding author: Email: Hans.Nauwynck@UGent.be

22 Tel: +32 9 264 7373

23 Fax: +32 9 264 7495

24 **Abstract**

25

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

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

35 For the waterborne route, shrimp in different molt stages were immersed in sea water
36 containing 10^4 SID₅₀ ml⁻¹ of WSSV. In a first study, *P. vannamei* (n=125) incubated in cell
37 culture flasks, became infected with WSSV mostly in post-molt stages. In a second study, 2
38 groups of *P. vannamei* (n=100) and *P. monodon* (n=100) were transferred into plastic bags to
39 prevent damage to the cuticle; and in 1 group a pleopod was cut off prior to incubation.
40 Induction of damage increased infection significantly ($p<0.05$) in A-stage from 0-40% to 60-
41 100%, in B-stage from 0-20% to 40-60%, in C-stage from 0-20 to 20-60%, while infection was
42 0% in D-stages with both immersion methods.

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

45

46

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

49 1. Introduction

50

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

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

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

74 Prior et al. (Prior et al., 2003). Soto and Lotz concluded that ingestion of infected tissues was a
75 far more effective treatment than immersion in infected water. Remarkably however, even
76 when *P. vannamei* were isolated to ensure they had equal chance to consume the infected
77 tissues offered to them, not all shrimp became infected (50-60%). Prior et al. (2003) succeeded
78 in determining the lethal intramuscular dose of a WSSV stock and also tried to develop a
79 controlled bio-assay by immersion of *P. vannamei*. Although very large amounts of infectious
80 virus were added to the water (as shown by the injection study), mortality rates stayed below
81 40%. Recently, another study clearly illustrated the difficulty to infect animals by WSSV
82 immersion challenge (Gitterle et al., 2006), while a study on an ornamental shrimp's
83 susceptibility to WSSV resulted in a discussion of the problems encountered with experimental
84 feeding challenges (Laramore, 2007). Gitterle et al. (2006) showed that merely adding virus
85 inoculum to the water was not sufficient to result in *P. vannamei* infection but needed to place
86 the shrimp in tanks in which orally infected shrimp had previously died to finally obtain
87 successful transmission. Finally, in the PhD thesis by Dr. Bonny Bayot (2006), less than 17%
88 of *P. vannamei* shrimp became infected upon individual challenge with WSSV via oral route
89 and none or merely 3% by immersion.

90 The overall conclusion from these publications is that there are restrictions on the ability
91 of WSSV to gain entry into its host. With feeding of virus-infected tissues to shrimp, this is to
92 be expected as the lack of control on the dose of virus actually reaching the site of entry,
93 inherently creates irreproducible results. The fact that any portion of the animals might not be
94 feeding (due to molting, stress, ...) for instance, can easily prevent an equal chance to become
95 infected. Another factor which cannot be ignored is that all tissues known to be susceptible to
96 WSSV replication are protected from the outside world by cuticle (Escobedo-Bonilla et al.,
97 2007). This is also true for the gills and the epithelium of stomach and hindgut (Bell and
98 Lightner, 1988).

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

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

115

116 **2. Materials and Methods**

117

118 *2.1. Experimental animals and conditions*

119

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

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

136

137 2.2. Molt stage determination

138

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

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

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

152

153 2.3 Virus

154

155 The WSSV Thai-1 isolate was used in the present study. This isolate has been studied
156 before (Jiravanichpaisal et al., 2001; Escobedo-Bonilla et al., 2005; Escobedo-Bonilla et al.,
157 2006; Escobedo-Bonilla et al., 2007; Rahman et al., 2008). It was collected from naturally
158 infected *Penaeus monodon* in Thailand in 1996 and passaged in crayfish *Pacifastacus*
159 *leniusculus* (Jiravanichpaisal et al., 2001). Crayfish gill suspension containing WSSV Thai-1
160 was kindly provided by K. Söderhäll (Uppsala University, Sweden) and amplified in SPF *P.*
161 *vannamei* juveniles to produce virus stocks. The median infectious titer of the stock used for all
162 experiments in this study was determined to be $10^{6.0}$ shrimp infectious dose 50% end point
163 (SID₅₀) per ml, following the *in vivo* intramuscular titration procedure in SPF *P. vannamei*
164 described by Escobedo et al. (2005).

165

166 2.4 *In vivo* titration by intramuscular inoculation using shrimp in different molt stages

167

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

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

179

180 *2.5 Study of WSSV infection by immersion route*

181

182 *2.5.1 Immersion inoculation inside cell culture flasks*

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

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

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

202

203 2.5.2. Immersion inoculation inside plastic bags of shrimp with and without damaged cuticle.

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

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

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

226

227 *2.6 Detection of WSSV infection by indirect immunofluorescence(IIF)*

228

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

241

242 *2.7 Statistical analysis*

243

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

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

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

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

252

253 **3. Results**

254

255 *3.1. In vivo titration by intramuscular inoculation (Table 1)*

256

257 IIF analysis of dead and surviving shrimp revealed the following virus infection titers: 10^6 , $10^{6.5}$
258 and $10^{6.8}$ for A-stage ($10^{6.5\pm0.4}$); $10^{6.6}$, $10^{6.8}$ and $10^{7.5}$ for B-stage ($10^{7.1\pm0.4}$); $10^{6.5}$, $10^{6.7}$ and $10^{6.8}$
259 for C-stage ($10^{6.7\pm0.2}$); $10^{6.8}$, $10^{6.8}$ and $10^{7.1}$ for D1-stage ($10^{6.9\pm0.2}$) and $10^{6.3}$, $10^{6.7}$ and 10^7 for
260 D2-stage ($10^{6.7\pm0.3}$) (Table 1). No significant differences in infection titers were observed
261 between the stages ($p>0.05$).

262

263 *3.2. Immersion inoculation inside cell culture flasks (Table 2)*

264

265 Of the 1 g shrimp, only 1 shrimp in the A-stage group started to become anorectic and
266 lethargic at 36 hpi. This was the only shrimp to die due to WSSV before the end of the
267 experiment. All other shrimp were euthanized at 120 hpi and were negative for WSSV on IIF.
268 When the immersion was performed with 4 g shrimp, all survived the experiment uninfected.
269 At a size of 6 g, 3 out of 5 A-stage shrimp started to show clinical signs at 36 hpi and died at
270 60-84 hpi. When the experiment was performed with 11 g, all A- and one B-stage shrimp
271 showed clinical signs and died due to WSSV infection between 48 and 120 hpi. Two D2-stage
272 animals molted during the immersion and one died before the end of the 3 hours procedure.

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

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

283

284 3.3. Immersion inoculation inside plastic bags (Table 3)

285

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

291 Throughout the experiment, anorexia was recorded in D2-stage shrimp 24 to 48 h before
292 molting and in A-stage shrimp. Uninfected animals started eating normally again by the end of
293 A-stage. Infected animals displaying anorexia on the other hand also became lethargic between
294 48 to 72 hours post immersion (hpi), generally 24 hours before dying.

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

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

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

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

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

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

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

320

321 4. Discussion

322

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

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

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

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

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

363 The actual portal of entry of WSSV from the water into a host has never been described,
364 but some assume that the gills are the best candidates (Chang et al., 1996; Witteveldt et al.,
365 2004; Arts et al., 2007). The experimental findings of the present study demonstrate that an
366 artificially induced wound in the cuticle increases the rate of WSSV infection upon immersion.
367 Cutting off a pleopod creates an open wound which can allow either (1) infection of cells at the
368 site of the wound or (2) entry of WSSV into the hemolymph followed by direct systemic spread
369 or on the other hand (3) reduce the competence of shrimp to resist WSSV infection. In the first
370 two scenarios, entry of the virus would occur through the opening in the cuticle itself. If one

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

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

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

397

398 **5. Conclusion**

399

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

408

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410

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414

415 **7. References**

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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	$10^{6.5\pm 0.4}$ 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	$10^{7.1\pm 0.4}$ 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	$10^{6.7\pm 0.2}$ 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	$10^{6.9\pm 0.2}$ 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	$10^{6.7\pm 0.3}$ 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	