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Virus replication cycle of white spot syndrome virus in secondary cell cultures from the lymphoid organ of *Litopenaeus vannamei*

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The replication cycle of white spot syndrome virus (WSSV) was investigated in secondary cell cultures from the lymphoid organ of *Litopenaeus vannamei*. The secondary cells formed a confluent monolayer at 24 h post-reseeding, and this monolayer could be maintained for 10 days with a viability of 90 %. Binding of WSSV to cells reached a maximum (73 ± 3 % of cells and 4.84 ± 0.2 virus particles per virus-binding cell) at 120 min at 4 °C. WSSV entered cells by endocytosis. The co-localization of WSSV and early endosomes was observed starting from 30 min post-inoculation (p.i.). Double indirect immunofluorescence staining showed that all cell-bound WSSV particles entered these cells in the period between 0 and 60 min p.i. and that the uncoating of WSSV occurred in the same period. After 1 h inoculation at 27 °C, the WSSV nucleocapsid protein VP664 and envelope protein VP28 started to be synthesized in the cytoplasm from 1 and 3 h p.i., and were transported into nuclei from 3 and 6 h p.i., respectively. The percentage of cells that were VP664- and VP28-positive in their nuclei peaked (50 ± 4 %) at 12 h p.i. Quantitative PCR showed that WSSV DNA started to be synthesized from 6 h p.i. *In vivo* titration of the supernatants showed that the progeny WSSV were released from 12 h p.i. and peaked at 18 h p.i. In conclusion, the secondary cell cultures from the lymphoid organ were proven to be ideal for examination of the replication cycle of WSSV.

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INTRODUCTION

Of all cultured crustaceans worldwide, penaeid shrimp are considered to be economically one of the most important species. Diseases, especially viral diseases, have threatened this booming aqua-industry and caused huge economic losses (Flegel, 1997). White spot syndrome virus (WSSV) is lethal for shrimp and other crustaceans (Kasornchandra *et al.*, 1995; Wongteerasupaya *et al.*, 1995). Cell cultures are basic and useful tools for the study of replication cycles of viruses and the development of antivirals. Many trials have been performed on the development of cell cultures from different organs of various crustaceans (mainly shrimp) with increasing frequency over the last 30 years (Assavalapsakul *et al.*, 2003; Han *et al.*, 2013; Kasornchandra *et al.*, 1999; Li *et al.*, 2014; Wang *et al.*, 2000). In brief, cells derived from lymphoid, ovary and haematopoietic tissues were maintained for a certain period, and some of these cell cultures were reported to be susceptible to WSSV (Jiravanichpaisal *et al.*, 2006; Li *et al.*, 2014). However, to date, the WSSV replication cycle remains incompletely understood.

WSSV is now considered as one of the most prevalent and widespread viruses (Flegel, 1997). The size of WSSV ranges from 210 to 420 nm in length and from 70 to 167 nm in diameter (Lu *et al.*, 1997). The WSSV envelope consists of >35 different proteins (Lin *et al.*, 2002), of which VP28 is the most abundant. VP28 is the major envelope protein (van Hulten *et al.*, 2001) and was reported to play a crucial role in the infection process as an attachment protein, helping the virus to enter the cytoplasm (Yi *et al.*, 2004). The proteins constituting the WSSV nucleocapsid are still mostly unknown (Leu *et al.*, 2009). VP664, which consists of a long polypeptide of 6077 aa encoded by an intron-less giant ORF of 18 234 nt, is a major nucleocapsid protein (Sánchez-Paz, 2010). VP664 appears to form the stacked ring structures that are visible in the nucleocapsid

under transmission microscopy (Leu *et al.*, 2005). To date, the different steps of the replication cycle remain incompletely understood.

In the present study, the binding, entry, disassembly, protein and genome synthesis, assembly, production and release of WSSV were investigated in secondary lymphoid organ cells of *Litopenaeus vannamei*.

RESULTS

Behaviour of secondary cell cultures from the lymphoid organ of *L. vannamei*

A full cell monolayer was formed within 24 h post-seeding (Fig. 1a). The cell monolayer contained two cell types: fibroblast-like cells and epitheloid cells (Fig. 1b). In the first 7 days, the fibroblast-like cells formed networks. The epitheloid cells were present in these networks as cell islands (Fig. 1b). The epitheloid cells degenerated after 7 days (Fig. 1c), and fibroblast-like cells occupied all available spaces and became the dominant cell type in the cell monolayer (Fig. 1d). After 10 days, the fibroblast-like cells degenerated (Fig. 1e, f).

Viability of secondary cell cultures

The percentage of viable cells in the secondary cell cultures from the lymphoid organ of *L. vannamei* was determined at 1, 4, 7 and 10 days post-seeding by ethidium monoazide bromide (EMA) and Hoechst staining. The proportion of viable cells in the cell monolayer culture was 95 ± 3 % at 1 day, 92 ± 4 % at 4 days, 90 ± 2 % at 7 days and 92 ± 2 % at 10 days post-seeding. The high proportion of viable cells during the first 10 days post-seeding indicated that the secondary cell cultures from the lymphoid organ of *L. vannamei* survived well and were ready for investigation of the WSSV replication cycle.

Kinetics of WSSV binding

The percentage of virus-binding cells and the number of virus particles bound per virus-binding cell were determined. Both the percentage of virus-binding cells and the number of virus particles bound per virus-binding cell rose with increasing time at 4 °C. Especially during the first 60 min post-inoculation (p.i.) at 4 °C, WSSV attached quickly to cells. The percentage of virus-binding cells reached 56 ± 3 % (Fig. 2a) and the number of bound virus particles per virus-binding cell reached 3.66 ± 0.19 at 60 min (Fig. 2b). From 120 min p.i., the percentage of virus-binding cells (73 ± 3 %; Fig. 2a) and the number of virus particles bound per virus-binding cell (4.84 ± 0.2; Fig. 2b) reached maximal levels.

Co-localization of WSSV and early endosomes during entry

After the secondary cell cultures were incubated with WSSV at 4 °C until maximum virus binding was reached (120 min, as described above), the cells were incubated at 27 °C, subsequently fixed at different time points, labelled

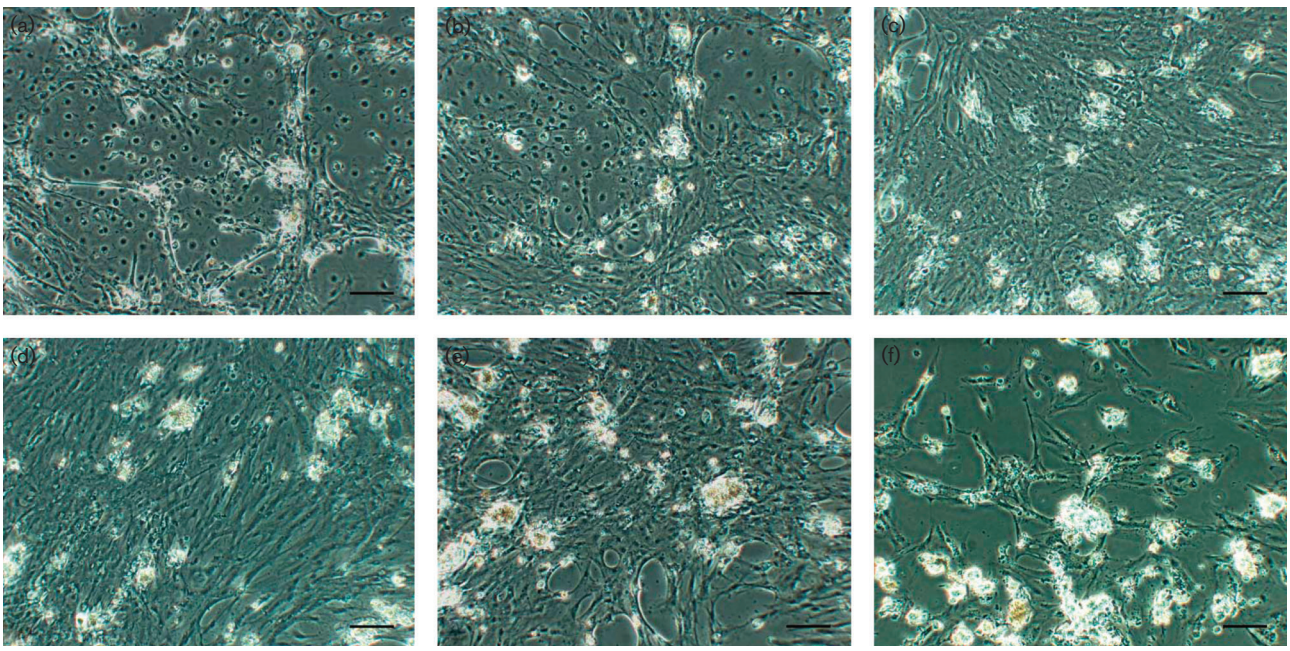

Fig. 1. Secondary cell cultures from the primary cell cultures at (a) 1, (b) 4, (c) 7, (d) 10, (e) 13 and (f) 16 days post-reseeding. Bar, 100 μm.

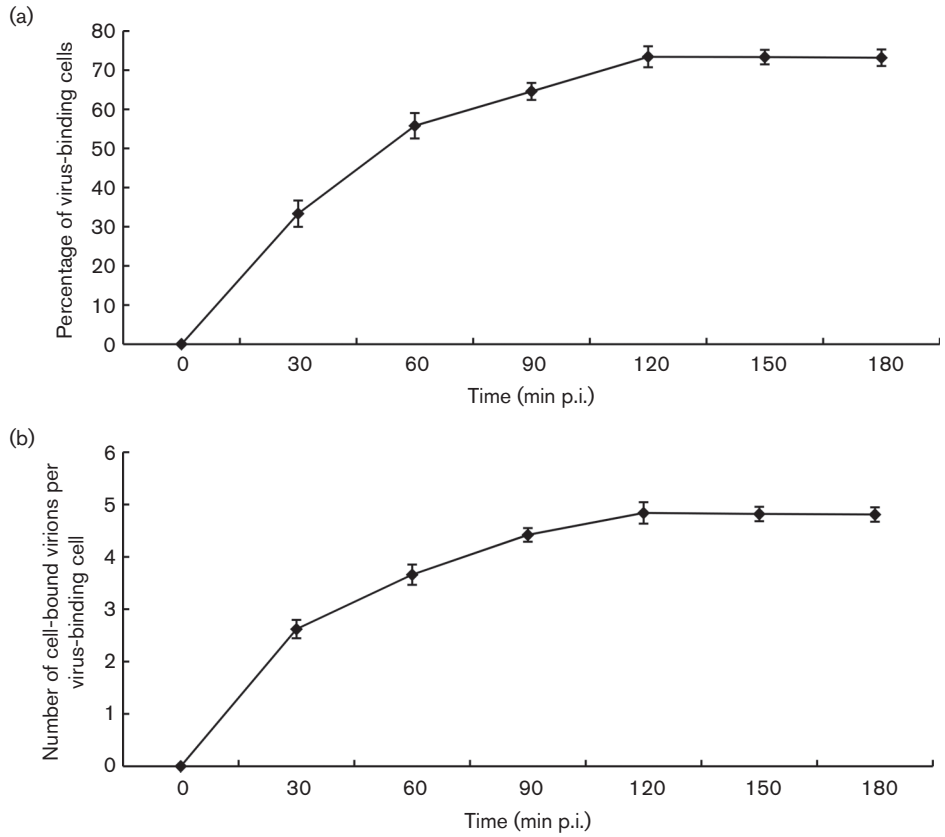

Fig. 2. (a) Percentage of virus-binding cells and (b) number of cell-bound virus particles per virus-binding cell in secondary cell cultures of the lymphoid organ of *L. vannamei* at different time points during WSSV incubation at 4 °C.

for VP28 and EEA1 and observed under a confocal microscope. Green fluorescent spots representing bound virus were observed on cell membranes (Fig. 3a). After further incubation at 27 °C, the percentage of cells with virus attached to the outside decreased and the number of cells with internalized virus increased (Fig. 3i). The virus particles were widely distributed inside the early endosomes (Fig. 3b, c). The percentage of cells with WSSV-containing endosomes reached a maximum (62 ± 3 %) at 60 min at 27 °C (Fig. 3i). At the same time (30–60 min p.i.), the number of virus-positive endosomes per virus-positive cell reached a maximum number of particles per positive cell (Fig. 3ii). From 60 to 180 min, the percentage of cells with WSSV-containing endosomes and the number of virus particles inside endosomes per virus-positive cell decreased gradually (Fig. 3i, ii).

Kinetics of uncoating and disassembly of WSSV in the cytoplasm

After 2 h inoculation at 4 °C, the number of WSSV particles bound to the cell surface reached a maximum. Once the cells were incubated at 27 °C, WSSV particles started to enter cells. From 0 to 150 min at 27 °C, the number of complete virions (co-localizing VP28 with VP664) decreased (Fig. 4), especially during the first 60 min at 27 °C (from 4.84 ± 0.20 to 0.55 ± 0.25 per virus-positive cell; Fig. 5). At the same time, the number of single VP28 and single VP664 signals began to rise. After 60 min at 27 °C, the number of single VP28 and single VP664 signals increased to 4.25 ± 0.86 and 4.95 ± 0.27 per virus-positive cell, respectively (Fig. 5). After 150 min at 27 °C, VP664 began to be expressed throughout the cytoplasm.

Kinetics of VP28 and VP664 expression and transport to the nucleus

To assess the kinetics of VP28 and VP664 expression, cells were inoculated with WSSV at 27 °C for 1 h, after which they were fixed at different time points (0, 1, 3, 6, 9, 12, 15, 18 and 24 h p.i.), and the percentage of VP28- and VP664-positive cells was determined (Fig. 6). New nucleocapsid protein VP664 began to be synthesized in the cytoplasm from 1 h p.i. (17 ± 3 %). The newly produced VP664 was found throughout the cytoplasm (Fig. 6a). From 3 h p.i., VP664 was transported into the nucleus (40 ± 4 % of cells; Fig. 6b). This expression and transport of VP664 was maintained during the entire WSSV virion assembly procedure. Starting from 3 h p.i., the envelope protein VP28 became expressed in the cytoplasm (5 ± 2 %; Fig. 6) in which VP664 was already present. The newly produced

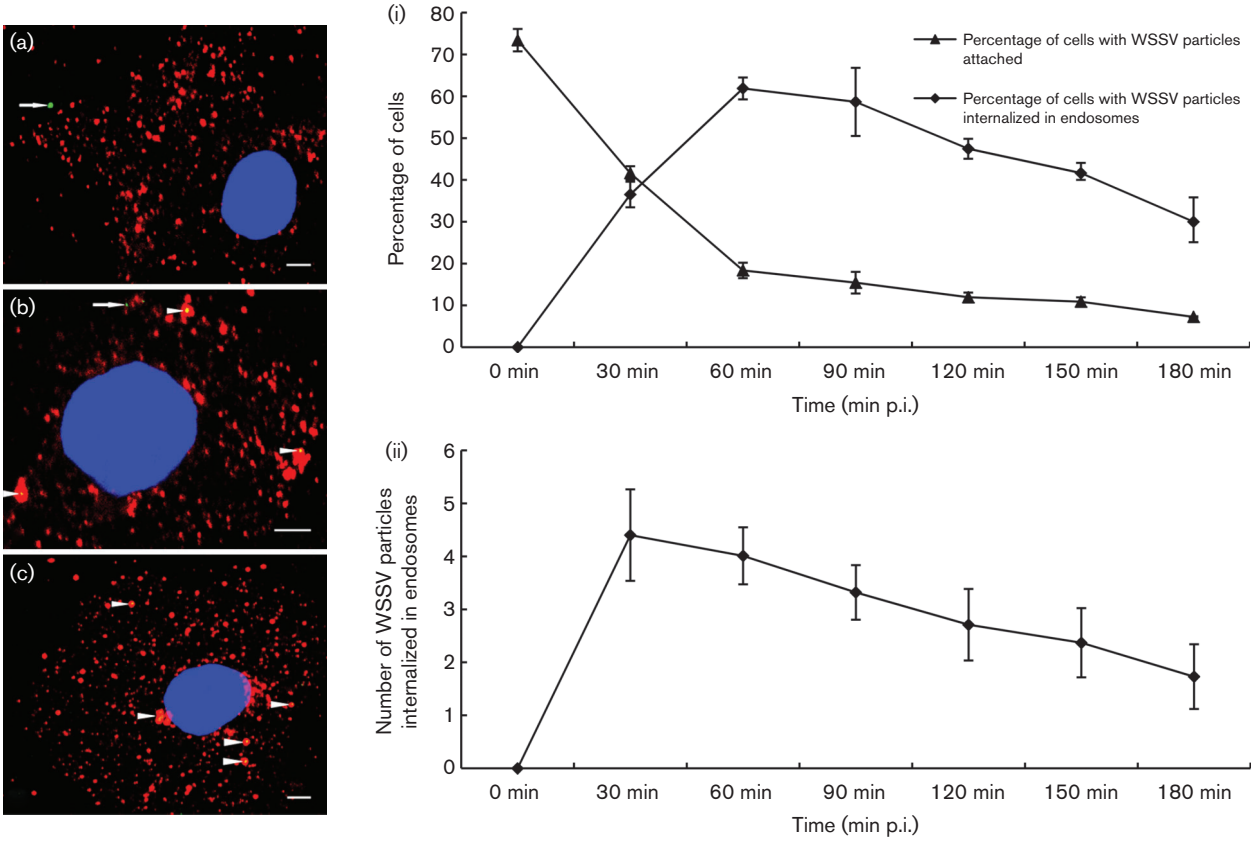

Fig. 3. (i) Percentages of WSSV-binding cells (white arrows: plasma membrane-bound WSSV particles) and cells with WSSV particle-internalized endosomes (white arrowheads: internalized WSSV particles) from 0 to 180 min p.i. at (a) 0, (b) 30 and (c) 60 min p.i. (ii) Number of WSSV particle-internalized endosomes per virus-infected cell from 0 to 180 min p.i. at (a) 0, (b) 30 and (c) 60 min p.i. Blue: Hoechst 33342, staining for nuclei; green: w29 and goat anti-mouse IgG-FITC, staining for WSSV particles; red, EEA1 (C-15) and rabbit anti-goat Alexa Fluor 594, staining for early endosomes; yellow, co-localization. Bar, 3 μm.

VP28 was highly concentrated in certain regions in the cytoplasm close to the nucleus and clearly separated from the areas where VP664 was synthesized (Fig. 6c). From 6 h p.i., VP28 was transported to the nucleus (10 ± 3 %) and from that time point VP664 and VP28 both became concentrated in the nucleus (Fig. 6d). The percentage of cells both VP664- and VP28-positive in the nucleus reached a maximum (50 ± 5 %) at 12 h p.i. and then dropped to 24 h p.i., at 13 ± 2 %.

Quantification of intra- and extracellular WSSV DNA load

Quantitative PCR (qPCR) demonstrated that the WSSV DNA began to be synthesized in the cells from 6 h p.i. (6.20 $\log_{10}$ ml^{-1}) and steadily increased up to 7.14 $\log_{10}$ ml^{-1} at 24 h p.i. WSSV DNA began to be released in the supernatant between 9 and 12 h p.i. (from 5.22 to 7.10 $\log_{10}$ ml^{-1}), and remained at that level to 18 h p.i. (7.37 $\log_{10}$ ml^{-1}). Between 18 and 24 h p.i., a marked increase in extracellular DNA load was observed, from 7.37 to 8.87 $\log_{10}$ ml^{-1} (Fig. 7a).

WSSV production and release

Titration of the supernatant collected from WSSV-inoculated secondary cell cultures from 0 to 24 h p.i. at 27 °C showed that the titre increased from 12 h p.i. [3.42 $\log_{10}$ SID_{50} (shrimp infectious dose 50% end point) ml^{-1}] and reached a maximum at 18 h p.i. (4.33 $\log_{10}$ SID_{50} ml^{-1}) (Fig. 7b).

DISCUSSION

In the present study, a secondary cell culture from the lymphoid organ of *L. vannamei* was established which allowed the study of the replication cycle of WSSV in shrimp cells.

Primary cell cultures derived from shrimp lymphoid organ have been reported in many other publications (Assavalapsakul *et al.*, 2003; Han *et al.*, 2013; Wang *et al.*, 2000) and their susceptibility to WSSV has already been demonstrated (Li *et al.*, 2014; Tapay *et al.*, 1995). However, little is known about the different steps of the replication cycle. Cell migration out of lymphoid organ explants is very

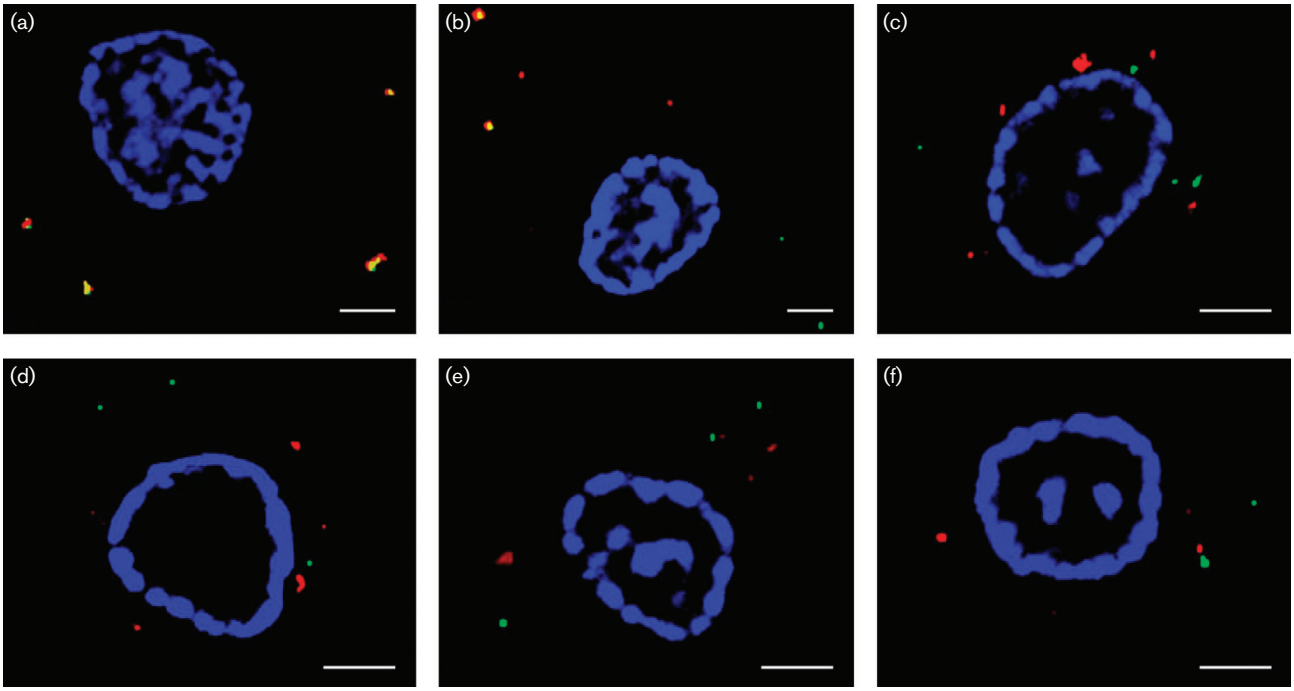

Fig. 4. Double-indirect immunofluorescence staining of the uncoating and disassembly of WSSV particles from 0 to 150 min p.i. at (a) 0, (b) 30, (c) 60, (d) 90, (e) 120 and (f) 150 min p.i. Blue: Hoechst 33342, staining for nuclei; green: w29 and goat anti-mouse IgG-FITC (F2761), staining for WSSV envelope; red: WSSV419 and goat anti-rabbit Alexa Fluor 594, staining for WSSV nucleocapsid; yellow: co-localization. Bar, 3 μm.

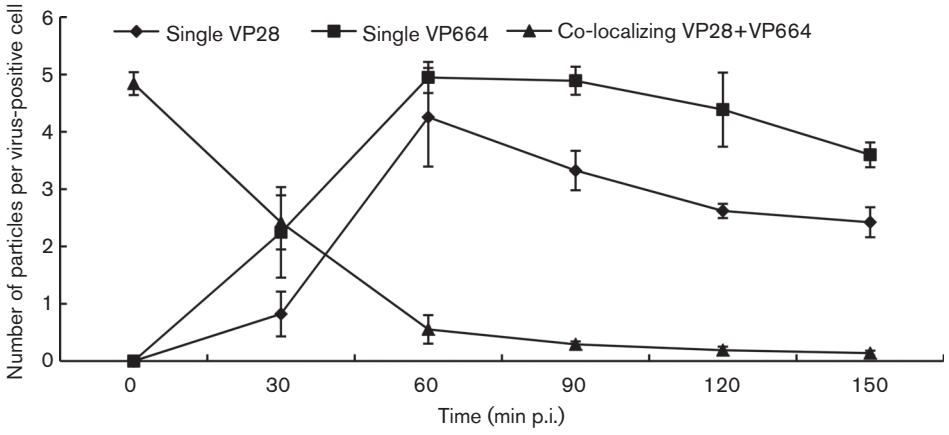

Fig. 5. Kinetics of uncoating of internalized WSSV particles from 0 to 150 min p.i.

variable in terms of numbers and proportions of cell types in the different wells of primary cell cultures. Therefore, it is impossible to obtain reproducible results. Furthermore, the cells remain at a short distance from the explants and form thick multi-layers. Bringing virus in contact with these cultures will not result in reliable results. In the present study, a solution was found to these problems by using secondary cell cultures. These secondary cell cultures formed a uniform monolayer within 24 h of post-reseeding and this monolayer could be maintained for >10 days with a viability of 90 %. Moreover, cell number and type in each well were fairly uniform, which enables study of the different steps of the WSSV replication cycle in a standardized, reproducible way.

The binding of WSSV to the cells gradually increased during 120 min of incubation at 4 °C. After incubation of the

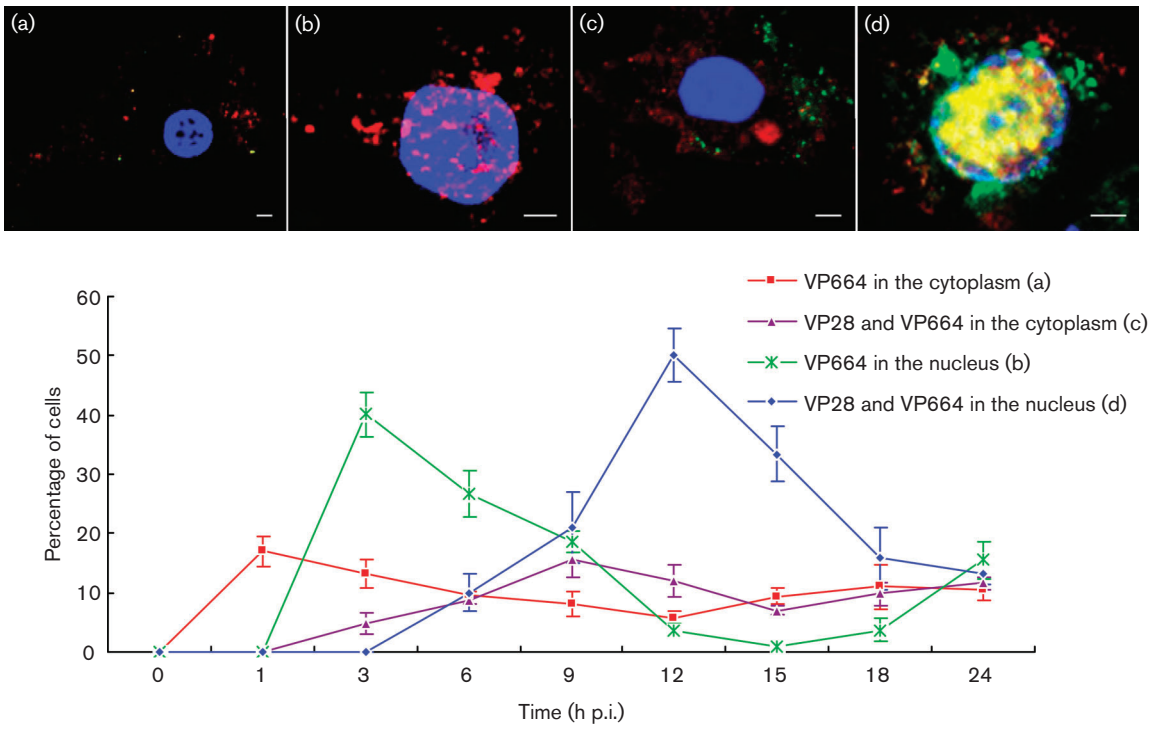

Fig. 6. Kinetics of WSSV infection in secondary cell cultures from 0 to 24 h p.i.: (a) WSSV nucleocapsid protein VP664 synthesized in cytoplasm. (b) WSSV nucleocapsid protein VP664 synthesized in cytoplasm and transported to the nucleus. (c) WSSV nucleocapsid protein VP664 and envelope protein VP28 synthesized in cytoplasm. (d) WSSV nucleocapsid protein VP664 and envelope protein VP28 synthesized in cytoplasm and transported to the nucleus. Blue: Hoechst 33342, staining for nuclei; green: w29 and goat anti-mouse IgG-FITC, staining for WSSV envelope; red: WSSV419 and goat anti-rabbit Alexa Fluor 594, staining for WSSV nucleocapsid; yellow: co-localization. Bar, 3 μm.

secondary cell cultures with WSSV, the number of viruses bound per cell was different among cells. In 27 % of cells, no virus was attached. In contrast, in 73 % of cells, 4.84 ± 0.20 virus particles were bound. This difference may be determined by the absence or presence of the receptors. The fact that the secondary cell culture consists of two cell types (fibroblast-like and epitheloid cells) may explain this finding. It is highly likely that only one of these two cell types is susceptible to WSSV infection. Furthermore, the virus particles were not equally distributed over the surface of each virus-positive cell, which indicated that the concentration of receptors was variable on different parts of the cell surface.

Following the binding to cell surface receptors, WSSV particles undergo endocytosis and are present as intact virions (nucleocapsid surrounded by the envelope) in endosomes. These endocytic events were clearly demonstrated by confocal microscopy. Sritunyalucksana *et al.* (2006) reported that a VP28-binding protein (PmRab7) was found in *Penaeus monodon*. PmRab7 has a high homology to the small GTP-binding protein Pab7, which represents a family of >30 proteins that are localized on the surfaces of distinct membrane-enclosed compartments of exocytic and endocytic pathways (Chavrier *et al.*, 1990; Feng *et al.*, 1995). Huang *et al.* (2013) previously reported that endocytosis was involved in the entry of WSSV into shrimp haemocytes. More work needs to be performed to determine the different viral and cellular proteins involved in this endocytic pathway.

Virus was found in small endosomes inside the cells from 30 min p.i. Subsequently, between 30 and 60 min p.i., large, intensely fluorescing spots were observed, suggesting that WSSV virions had accumulated in the cytoplasm over that time period. During virus entry, the virus envelope and nucleocapsid became separated from each other, which notably occurred during the first 60 min p.i. The nucleocapsid was released from the virus endosome and transported to a region close to the nucleus. Degradation of viral particles took place between 60 and 150 min p.i.

After 1 h p.i. at 27 °C the synthesis of the nucleocapsid protein VP664 was detected, followed by the synthesis of envelope protein VP28 at 3 h p.i. This is in agreement with previous studies. Leu *et al.* (2005) reported that VP664 and VP28 transcripts were detected in small amounts from 2 h post-intramuscular injection. The

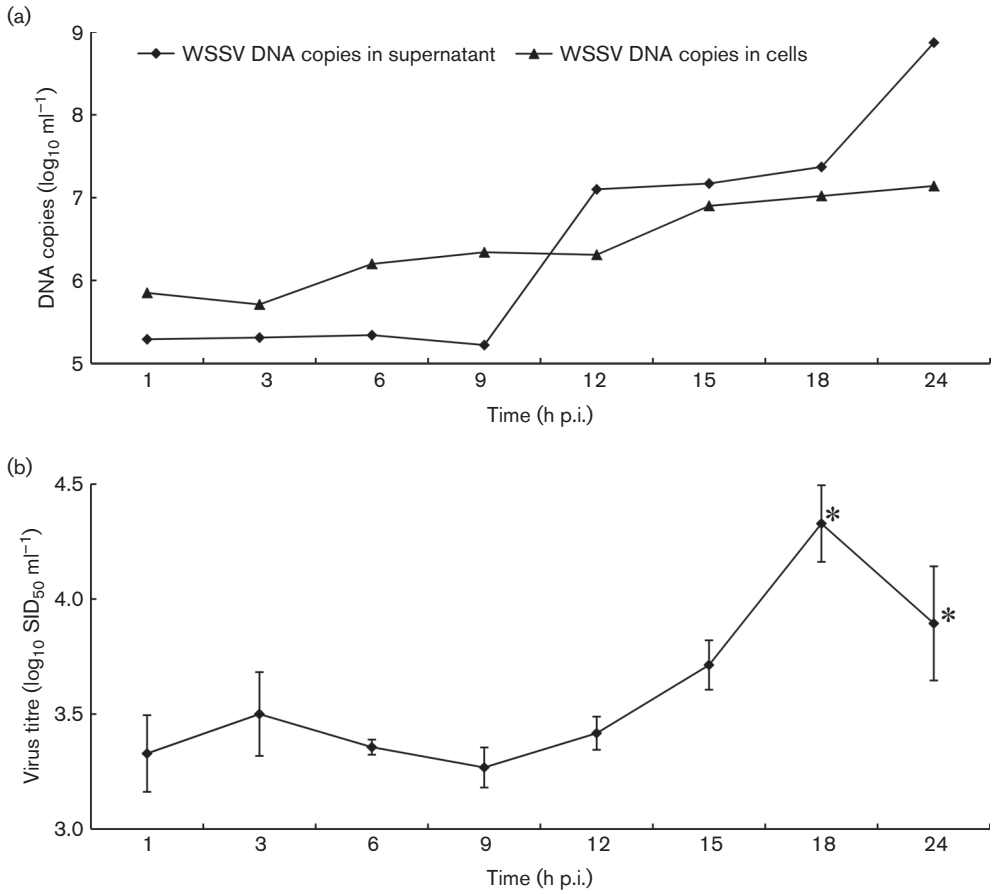

Fig. 7. (a) qPCR for the quantification of intra- and extracellular WSSV DNA load. (b) WSSV production in secondary cell cultures of the lymphoid organ of *L. vannamei*. Detection limit $\leq 10^{0.8}$ SID_{50} ml^{-1}; *significantly different ($P<0.05$) in comparison with the data from 1 h p.i.

signal of VP664 was detected in most parts of the cytoplasm, which indicates that the nucleocapsid protein VP664 was synthesized in the cytosol. VP664 was detected in the nucleus from 3 h p.i. The synthesis of VP664 continued over time. The signal could still be observed in the cytosol when the assembly of WSSV particles in the nucleus had already started. The envelope protein VP28 was first detected in the cytoplasm from 3 h p.i. and in the nucleus from 6 h p.i. Li *et al.* (2009) showed that the gene expression of VP28 was first detected at 4 h post-infection (samples were collected from primary crayfish haemocyte cultures). VP28 was not synthesized over the entire cytoplasm, unlike VP664, but was restricted to certain regions close to the nucleus. The lack of antibodies against organelles in shrimp cells made the identification of the synthesis site impossible. Based on the position of the concentrated signal of VP28, the rough endoplasmic reticulum (rER) might be a good candidate.

The qPCR results indicated that WSSV DNA synthesis began from 6 h p.i. in cells, which indicated that the capsid protein VP664 and envelope protein VP28 both began to be synthesized in the cytoplasm before the DNA genome had been synthesized in the nucleus. Consequently, VP664 and VP28 may be considered as immediate-early proteins. This might indicate that VP664 and VP28 have important functions in virus replication, such as regulation of cell metabolism and antiviral response, activation of the expression of late genes, genome replication and intranuclear assembly. However, further experiments should be performed to support this hypothesis. In the supernatant, the number of WSSV DNA copies increased between 9 and 12 h p.i. (from 5.22 to 7.10 $\log_{10}$ ml^{-1}), and remained at that level at both 15 and 18 h p.i. At 24 h p.i., a significant release of viral DNA was detected. As the infectious virus titre in the supernatant peaked earlier (at 18 h p.i.) and was much lower (4.33 $\log_{10}$ SID_{50} ml^{-1}) than the viral DNA copies, the large number of DNA copies at the end of the experiment is thought to have been non-assembled free viral DNA from disrupted cells. This is supported by the observation of cell lysis in the infected monolayer at that time point. Cell lysis is most probably also the reason why a drop was seen in numbers of cells carrying VP28 and VP664 in

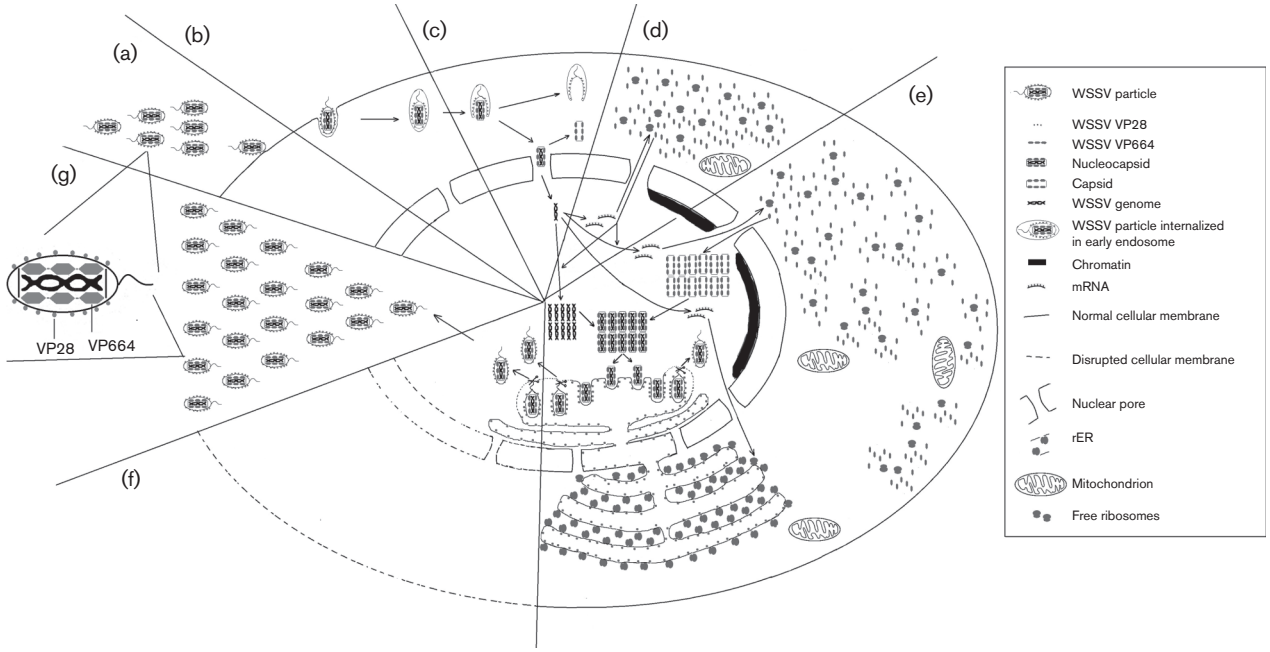

Fig. 8. A proposed model of the replication cycle of WSSV in secondary cell cultures from the lymphoid organ of *L. vannamei*. See text for description of stages in the process.

the nucleus. As only low infectious virus titres were obtained in the supernatant, cells from the lymphoid organ can be considered as low-productive cells. Another explanation could be the induction of an antiviral state resulting in the blocking of virus assembly.

Based on these results, a model of the replication cycle of WSSV in secondary cell cultures from the lymphoid organ of *L. vannamei* is hypothesized. The WSSV replication cycle begins with the attachment of a WSSV particle (Fig. 8a). After the virus particle is attached to the surface of the cell membrane, the WSSV particle enters the cell by endocytosis (Fig. 8b). In the early endosome, the disassembly of the virus particle begins. The WSSV envelope fuses with the endosome membrane and the nucleocapsid is released into the cytoplasm. The free nucleocapsid migrates close to the nucleus and injects the viral genome via a nuclear pore within the nucleus. Subsequently, both envelope and capsid proteins become degraded in the cytoplasm (Fig. 8c). Within the nucleus, transcription begins and the mRNAs of immediate-early genes are produced and subsequently migrate into the cytoplasm, where they become transformed into proteins by free ribosomes. The WSSV major capsid protein VP664 is expressed from this time point (1 h p.i.). The immediate-early proteins activate early and late proteins (Fig. 8d). From 3 h p.i., the mRNAs for envelope proteins (e.g. VP28) become transcribed. The WSSV major envelope protein VP28 is expressed in the rER and is transported to the inner nuclear membrane, which then proliferates within the nucleus. From 6 h p.i., the virus genome multiplies in the nucleus and the virus capsids become assembled around new viral genomes. Nucleocapsids are formed, then the nucleocapsids bud at the inner nuclear membrane with VP28 (Fig. 8e). The newly formed particles are present in the lumen of the expanded inner nuclear membrane (Fig. 8f). Finally, the new WSSV particles are released via cell lysis (Fig. 8g).

METHODS

Experimental animals. *Penaeus* (*Litopenaeus*) *vannamei* post-larvae were imported from Syaqua Siam. They were certified to be specific pathogen-free for white spot syndrome virus, taura syndrome virus, yellow head virus, infectious hypodermal and haematopoietic necrosis virus and infectious myonecrosis virus. After arrival, the post-larvae were stocked in a recirculating aquaculture system at 3.5 % salinity at the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium. After 4 months, shrimp in pre-moult stage, weighing 20 ± 2 g, were used for the collection of the lymphoid organ.

Primary and secondary cell cultures from the lymphoid organ of L. vannamei. The primary cell cultures were prepared as described previously (Li *et al.* 2014). The eye extract was prepared as described by Li *et al.* (2015). The cultures were incubated at 27 °C and one third of the culture medium was changed every 3 days. After 9 days of culture, cells were harvested by flushing the wells with a pipette. After the explants were removed, cells were pelleted by centrifugation at 400 *g* for 10 min and resuspended in 1 ml fresh culture medium. After counting with a Bürker–Türk counting chamber, cells were diluted and 1.1×10^5 cells were seeded in each well of 24-well plates (containing a glass coverslip coated with 0.1 % gelatin). At 24 h post-seeding, the secondary cell cultures (1×10^5 cells per well in 24-well plates) were ready for subsequent experiments.

Determination of the percentage of viable cells in the secondary cell cultures. The percentage of viable cells was determined in the secondary cell cultures every 3 days. One third of the culture medium was changed every 3 days. EMA and Hoechst staining were performed to count dead and total cells as described previously (Li *et al.* 2014).

WSSV purification and titre determination. A previously studied WSSV strain (WSSV-Thai-1; Escobedo-Bonilla *et al.*, 2005) was used in the purification experiments. The purified WSSV inoculum and its titration test were prepared as described previously by Dantas-Lima et al. (2013). The titre of WSSV inoculum was calculated according to Reed & Muench (1938), as $10^{7.7}$ SID_{50} ml^{-1}. The mock inoculum was prepared from healthy shrimp.

Analysis of WSSV binding to cells. To characterize the binding of WSSV to the secondary cells of the lymphoid organ, direct virus-binding studies were carried out. The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 0, 30, 60, 90, 120, 150 and 180 min at 4 °C. After three washes with cold culture medium, cells were fixed in cold 4 % paraformaldehyde for 10 min at room temperature. After one wash with PBS for 5 min, the cells were incubated in 200 µl mAb w29 (1 : 100 in PBS; Chaivisuthangkura *et al.*, 2004), which is directed against WSSV protein VP28, at 37 °C for 1 h. After three washes with PBS for 5 min, they were incubated in 200 µl goat anti-mouse IgG-FITC (1 : 100 in PBS; Sigma-Aldrich) at 37 °C for 1 h. After three washes with PBS for 5 min, the cells were incubated in 200 µl Phalloidin Texas red (1 : 40 in PBS; Life Technologies) at 37 °C for 1 h. After three washes with PBS for 5 min, the cultures were incubated in 200 µl Hoechst 33342 solution (10 µg ml^{-1} in PBS) for 10 min. After washes with PBS and ultra-pure water, the cells were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analysed with a confocal fluorescence microscope. The percentage of virus-binding cells (750 cells were chosen randomly) and the number of cell-bound virions on each virus-binding cell (150 cells were chosen randomly) at every time point were calculated.

Analysis of WSSV entry process by confocal microscopy. The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 2 h at 4 °C. After three washes with cold culture medium, 500 µl culture medium was added to each well and the cells were incubated at 27 °C. The cells were collected at 0, 30, 60, 90, 120, 150 and 180 min p.i., fixed in cold 4 % paraformaldehyde for 10 min, and permeabilized in 0.1 % Triton X-100 for 10 min at room temperature. After one wash with PBS for 5 min, the cells were incubated in 200 µl polyclonal antibody EEA1 (C-15) (1 : 100 in PBS; Santa Cruz), which is directed against early endosomes, at 37 °C for 1 h. After three washes with PBS for 5 min, the cells were incubated in 200 µl rabbit anti-goat Alexa Fluor 594 (1 : 100 in PBS; Sigma-Aldrich) at 37 °C for 1 h. Then, after three washes with PBS for 5 min, the cells were incubated in 200 µl w29 (1 : 100 in PBS) at 37 °C for 1 h. After three washes with PBS for 5 min, they were incubated in goat anti-mouse IgG-FITC (1 : 100 in PBS) at 37 °C for 1 h. After three washes with PBS for 5 min, the cell cultures were incubated in 200 µl Hoechst 33342 solution (10 µg ml^{-1} in PBS) for 10 min. After washes with PBS and ultrapure water, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analysed with a confocal fluorescence microscope. The percentage of virus-binding cells, percentage of cells containing WSSV particle-positive endosomes (750 cells were chosen randomly) and the number of WSSV particle-positive endosomes per virus-positive cell (150 cells were chosen randomly) were calculated.

Analysis of WSSV disassembly in cytoplasm. The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 2 h at 4 °C. After three washes with cold culture medium, 500 µl fresh culture medium was added to each well and the cells immediately incubated at 27 °C. The cells were collected at 0, 30, 60, 90, 120, 150 and 180 min, fixed in cold 4 % paraformaldehyde for 10 min and permeabilized in 0.1 % Triton X-100 for 10 min at room temperature. After one wash with PBS for 5 min, the cells were incubated in 200 µl polyclonal antibody WSSV419 (1 : 200 in PBS; Leu *et al.*, 2005), which is directed against WSSV nucleocapsid protein VP664, at 37 °C for 1 h. Then, after three washes with PBS for 5 min, the cells were incubated in 200 µl goat anti-rabbit Alexa Fluor 594 (1 : 100 in PBS, Sigma-Aldrich) at 37 °C for 1 h. After three washes with PBS for 5 min, the cells were stained with w29 (1 : 100 in PBS), goat anti-mouse IgG-FITC (1 : 100 in PBS) and Hoechst 33342 solution (10 µg ml^{-1} in PBS) as described above. After washes with PBS and ultra-pure water, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analysed with a confocal fluorescence microscope. The numbers of entire WSSV particles and nucleocapsids without envelope per virus-positive cell (150 cells were chosen randomly) were calculated.

Analysis of synthesis and transport of WSSV envelope VP28 and nucleocapsid VP664 proteins in cells. The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 1 h at 27 °C. After three washes with culture medium, 500 µl fresh culture medium was added to each well and the cells were further incubated at 27 °C. The cells were collected at 0, 1, 3, 6, 9, 12, 15, 18 and 24 h p.i. at 27 °C. At the same time, the supernatant was collected and stored at −70 °C after 2000 ***g*** centrifugation for 10 min. The cells were fixed in cold 4 % paraformaldehyde for 10 min and permeabilized in 0.1 % Triton X-100 for 10 min at room temperature. After one wash with PBS for 5 min, the cells were incubated in 200 µl of WSSV419 (1 : 200 in PBS) at 37 °C for 1 h. Then, after three washes with PBS for 5 min, the cells were incubated in 200 µl of goat anti-rabbit Alexa Fluor 594 (1 : 100 in PBS) at 37 °C for 1 h. After three washes with PBS for 5 min, the cells were stained with w29 (1 : 100 in PBS), goat anti-mouse IgG-FITC (1 : 100 in PBS) and Hoechst 33342 solution (10 µg ml^{-1} in PBS) as described above. After washes with PBS and ultra-pure water, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analysed with a confocal fluorescence microscope. The percentage of cells in which only VP664 or both VP664 and VP28 were detected in the cytoplasm and the percentage of cells in which only VP664 or both VP664 and VP28 were found in the nucleus were calculated (750 cells were chosen randomly).

qPCR for the quantification of intra- and extracellular WSSV DNA load. The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 1 h at 27 °C. After three washes with cold culture medium, 500 µl fresh culture medium was added to each well. The cells were further incubated at 27 °C and collected at 0, 1, 3, 6, 9, 12, 15, 18 and 24 h p.i. at 27 °C. Meantime, the supernatant was collected and stored at −70 °C after 2000 ***g*** centrifugation for 10 min. The cells were scraped and stored together with the cells from supernatant at −70 °C. Then DNA was extracted from the supernatant and the cell fraction using a QIAamp DNA Mini kit (Qiagen). Primers were designed in a conserved region of the VP19 coding sequence using the Primer3Plus website. A 20 µl PCR mixture was used per reaction, which contained 10 µl PrecisionPLUS 2× qPCR MasterMix with SYBR Green and ROX (PrimerDesign), 50 nM forward primer 5′-ATTGGTATCCTCGTCCTGGC-3′, 200 nM reverse primer 5′-GTTATCGTTGGCAGTGTCGTC-3′, 6.5 µl DNase/RNase-free H_2O and 3 µl sample DNA or diluted DNA standard (see below). An enzyme activation step at 95 °C for 2 min was followed by 40 cycles of 15 s at 95 °C and 60 s at 58 °C. A first-derivative melting curve analysis was performed by heating the mixture to 95 °C for 15 s, then cooling to 60 °C for 1 min and heating back to 95 °C at 0.3 °C increments. Amplification, monitoring and melting curve analysis were carried out in a StepOnePlus Real-Time PCR system (Applied Biosystems).

Synthetic DNA standards for absolute quantification. DNA was extracted from WSSV-Viet stock viruses using a QIAamp DNA Mini kit (Qiagen). The VP19 DNA fragment was amplified using the above-described primers in a 50 µl PCR mixture, composed of 10 µl OneTAQ Standard reaction buffer (New England Biolabs), 1 µl dNTPmix, 1 µl WSSV_VP19Fw primer, 1 µl WSSV_VP19Rv primer, 0.25 µl OneTAQ DNA Polymerase (New England Biolabs), 32.75 µl DNase/RNase-free H_2O and 4 µl DNA. After an enzyme activation step at 94 °C for 30 s, 35 cycles of amplification, each 20 s at 94 °C, 20 s at 55 °C and 60 s at 68 °C, were followed by a terminal elongation of 5 min at 68 °C. Fragment length was controlled by agarose gel electrophoresis, and fragments of the correct length were excised and purified from the gel using a Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel). Specificity of the fragments was controlled by assessment of the sequence (performed by GATC Biotech). The amount of DNA was determined using the NanoDrop 2000 system. Ten fold serial dilutions of the DNA were made over a range of 6 log units (10^6–10^1) for the generation of the standard curve (efficiency 97.28 %; R^2 0.99).

Titration of the supernatant from WSSV-inoculated secondary cell cultures from 0 to 24 h p.i. at 27 °C. The supernatant collected from the WSSV-inoculated secondary cell cultures from 1 to 24 h p.i. was diluted ten fold (10^{-1} to 10^{-4}); 50 µl of each dilution was injected into one shrimp (5.0 g) and five shrimp were used per dilution. All moribund shrimp were stored at −70 °C. After 7 days, all surviving shrimp were euthanized. The cephalothoraxes were dissected longitudinally, embedded in 2 % methylcellulose (Fluka), frozen and cryosectioned. Cryosections were stained with w29 (1 : 100 in PBS), goat anti-mouse IgG-FITC (1 : 100 in PBS) and Hoechst 33342 solution (10 µg ml^{-1} in PBS) as described above. The number of WSSV-positive shrimp in each dilution was counted and finally the titre of each supernatant from 0 to 24 h p.i. at 27 °C was evaluated as described previously by Escobedo-Bonilla *et al.* (2005).

Statistical analysis. All results are mean values from three independent replicates. The data were analysed statistically in SPSS 19.0. Differences were considered to be significant at $P<0.05$.

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