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Experimental studies on the portal of entry of White spot syndrome virus in *Penaeus vannamei*

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List of Abbreviations

AA	Antennal artery
AG	Antennal gland
AHPND	Acute hepatopancreatic necrosis disease
ALA	Anterior lateral artery
ALDH	Dehydrogenase
AM1	Adductor muscle of 1st antennal segment
AM4	Adductor muscle of 4th antennal segment
AN	Nervous cord
ARC	Artemia Reference Center
ASP	Antennal scale promotor muscle
ATM	Lateral anterior thoracic muscle 1
B	Bladder
BM	Basement membrane
C	Cuticle
CC	Cardiac chamber
CHH	crustacean hyperglycemic hormone
CL	Caudal lobe
CLH	Cuticle lined hindgut
CP	Compact part
Cp	Connecting point
CR	Comb row setae
CT	Connective tissue
CV	Cardiopyloric valve
DABCO	Diaza-bicyclo-octane
DL	Dorsal lobe
DOV	Dorsal ostial valve
E	Ecdysis
EC	Epithelial cell
EM	Esophageal muscles
En	Endocuticle

En	Endosomes
Ep	Epicuticle
ES	Epistomal stator muscles
Ex	Exopodite
FAO	Food and Agriculture Organization of the United Nations
FASW	Filtered, autoclaved seawater
FEC	Folded epithelial cells
FITC-WGA	Fluorescein isothiocyanate-Wheat germ agglutinin
GF	Gland filters
GIH	Gonad-inhibiting hormone
GMA	Anterior gastric mill attractor muscle
H	Hepatopancreas
H&E	Hematoxylin and eosin
HA	Hepatic artery
Hsp70	Heat shock protein 70
HV	Hemolymph vessel
IHHNV	Infectious hypodermal and hematopoietic necrosis virus
IIF	Indirect immunofluorescence
L	Labyrinth
LD_{50}	Lethal dose 50%
LL	Lateral lobe
LOV	Lateral ostial valve
LP	Lymphoid organ
LT	Lateral teeth
MB	Marine broth
MBW	Mean body weight
MIH	Molt-inhibiting hormone
ML	Medial lobe
MOETVIED	Vietnamese Ministry of Education and Training
MOIH	Mandibular organ-inhibiting hormone
MT	Median tooth
MV	Microvilli
N	Nephropore
NaCl	Sodium chlorua

PA	Posterior aorta
PA	Protocephalon attractor muscle
PBS	Phosphate-buffered saline
PC	Podocytes cell
PC	Pyloric chamber
PL	Post-larvae
PLM	Posterior protocephalon levator muscles
PM	Peritrophic membrane
PO	Phenoloxidase
proPO	Prophenoloxidase
RISC	RNA-induced silencing complex
RM	Rotator muscle of 3rd antennal segment
RNAi	RNA interference
Rpm	Rounds per minute
S	Stomach
SA	Scale adductor muscle
SA	Sternal artery
SG	Sinus gland
SID_{50}	Shrimp infectious dose 50% endpoint
SPF	Specific pathogen free
Sv	Small vesicles
TSV	Taura syndrome virus
UPO	Uropyloric ossicle
V	Vacuoles
V-CC	Ventral connecting channel
VL	Ventral lobe
VNC	Ventral nervous cord
VOV	Ventral ostial valve
WSSV	White spot syndrome virus
YHV	Yellow head virus
ZO	Zygocardiac ossicles

Chapter 1

Introduction

1.1 Aquaculture production

According to the latest statistic data collected globally by the Food and Agriculture Organization of the United Nations (FAO) in 2014, the global capture fishery and aquaculture production have grown rapidly in the last few decades with an average annual rate of 3.2%. Total capture fishery production was 93.7 million tonnes in 2011, 86.6 million tonnes in 2012 and 91.4 million tonnes in 2013. World food fish aquaculture production expanded at an average annual rate of 9.5% in the period from 1990 - 2000 and 6.2% in the period 2000 - 2012. In 2012, aquaculture production was about 90.4 million tonnes, including 66.6 million tonnes of fish, crustaceans, molluscs and other aquatic animals and 23.8 million tonnes of aquatic algae. In 2013, global aquaculture production attained about 70.5 million tonnes of food fish and 26.1 million tonnes of aquatic algae. Inland aquaculture contributed for about 63% of total farmed food fish production. China alone produced 43.5 million tonnes of food fish and 13.5 million tonnes of aquatic algae. The value of total aquaculture production was about 119.4 USD billion dollars in 2010 and 144.4 USD billion dollars in 2012. For shrimp aquaculture, total global production in 2012 was about 6.4 million tonnes, of which 60.8% came from marine-culture and 39.2% from inland aquaculture. The major production was dominated by white leg shrimp (*Penaeus vannamei*) and black tiger shrimp (*Penaeus monodon*), which are mainly produced in developing countries. Much of this production was exported. The trade in terms of value of shrimp aquaculture accounted for about 15% of the total value of internationally traded fishery products (FAO, 2014; FAO, 2012a, b, c).

1.1.1 Aquaculture of Penaeus vannamei

Over a few decades, global aquaculture of penaeid shrimp has largely expanded. Among species of farmed shrimp, namely *P. monodon, P. chinensis, P. merguiensis, P. japonicus and P. indicus, P. vannamei* has several advantages by its low protein requirement in the diet, specific pathogen resistance, high growth rate, high survival of larvae during rearing, high tolerance to a wide range of salinity, temperature and a high stocking density. Due to these features *P. vannamei* became the major species of shrimp aquaculture industry (Briggs *et al.*, 2004). The first spawning of *P. vannamei* was achieved in 1973 in Florida. From 1976 the commercial culture of *P. vannamei* began in South and Central America and subsequently expanded to Hawaii. In 1988, Chinese aquaculturists imported a batch of *P. vannamei* into

the mainland of China. In 1994, they produced their own seeds. Since 1996, commercial shrimp culture began in China, Taiwan and extended to the other coastal Asian countries. The global commercial production of *P. vannamei* increased from 0.002 million tonnes in 1976 to 0.071 million tonnes in 1987, and even to 0.181 million tonnes in 1997, but declined to 0.154 million tonnes in 2000 due to WSSV outbreaks. The production of *P. vannamei* reached approximately 2.352 million tonnes in 2007 and 3.314 million tonnes in 2013 (FAO) (Figure 1). At present, with the rapid expansion, this industry is facing several infectious diseases. The most dangerous infectious diseases are white spot syndrome (WSS) and acute hepatopancreatic necrosis disease (AHPND). White spot syndrome which is caused by a virus (WSSV) was first described in 1992. WSSV-infected shrimp are featured by redness of their body and white spots on their shell (Chou *et al.*, 1995). AHPND emerged in 2009 and is caused by *Vibrio parahaemolyticus*. AHPND is characterized by severe atrophy of the hepatopancreas with massive sloughing of epithelial cells (Lightner *et al.*, 2012). Until now, despite many attempts to eradicate these diseases, no effective control measures are available.

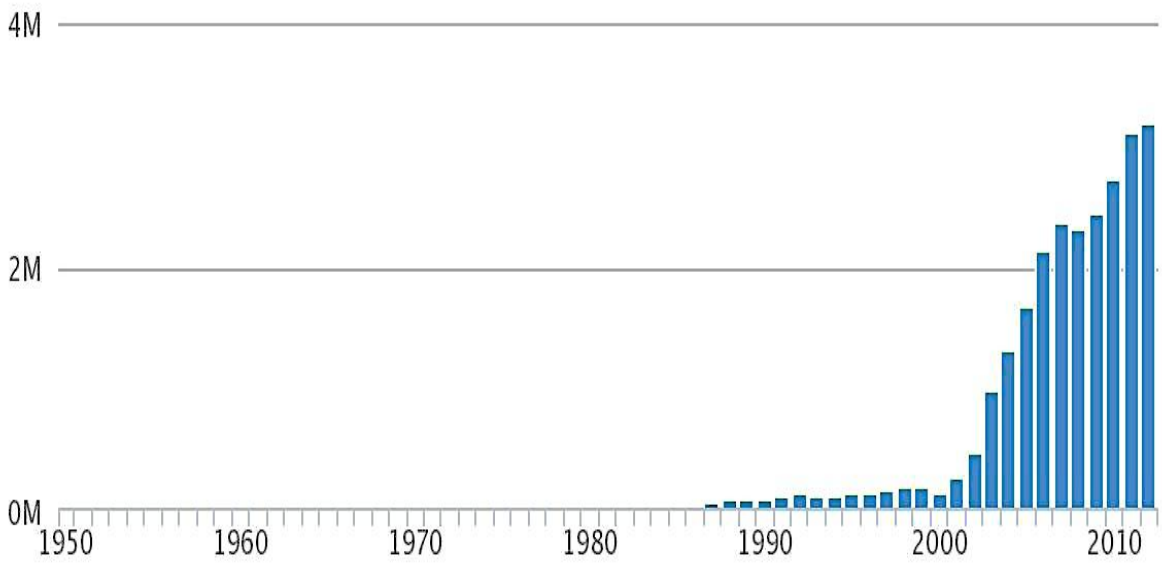

Figure 1. Global aquaculture production of *P. vannamei* from 1950 to 2013 (http://www.fao.org/fishery/culturedspecies/*Litopenaeus vannamei*/en)

1.2 Biology of *Penaeus vannamei*

1.2.1 Taxonomy and distribution of P. vannamei

Penaeus vannamei belongs to the decapod crustaceans, family *Penaeidae*, genus *Penaeus*. It is distinguished from other crustaceans by the presence of the spines on both the upper and lower margin of the rostrum. The taxonomic position of *Penaeus vannamei* is described as follows (Wyban and Sweeney, 1991; Martin and Davis, 2001):

Phylum: Arthropoda
Class: Cruscacea
Subclass: Malacostraca
Series: Eumalacostraca
Superorder: Eucarida
Order: Decapoda
Suborder: Dendrobrachiata
Family: Penaeidae
Genus: *Penaeus*
Species: *Penaeus vannamei*, Boone 1931

P. vannamei is native in the Western Pacific coast of Latin America from south Peru (5° S, 83° W) to north Mexico (28° N, 112° W). Shrimp live both inshore and offshore. Larvae and juveniles inhabit estuaries and coastal areas while adult shrimp move offshore for spawning (Wyban and Sweeney, 1991; Bailey-Brock, 1992).

1.2.2 Morphology

Similar to all penaeid shrimp, the body of white leg shrimp is divided into three regions: the cephalothorax, the abdomen and the tail. The cephalothorax contains appendages and internal organs. The appendages consist of a dorsal rostrum with 7-10 dorsal and 2-4 ventral spines, a pair of antennae, antennulae, mandibles, maxilules and maxillae, three pairs of maxillipeds, and five pairs of walking legs (pereiopods). The internal organs include heart, hepatopancreas, hematopoietic tissue, lymphoid organs, stomach, reproductive system and the gills. The abdomen has six segments, mainly composed of muscle and bearing five pairs of swimming legs (pleopods). The tail fan consists of the telson and two pairs of uropods (Wyban and Sweeney, 1991; Ruppert and Barnes, 1994; Budd, 2002) (Figure 2).

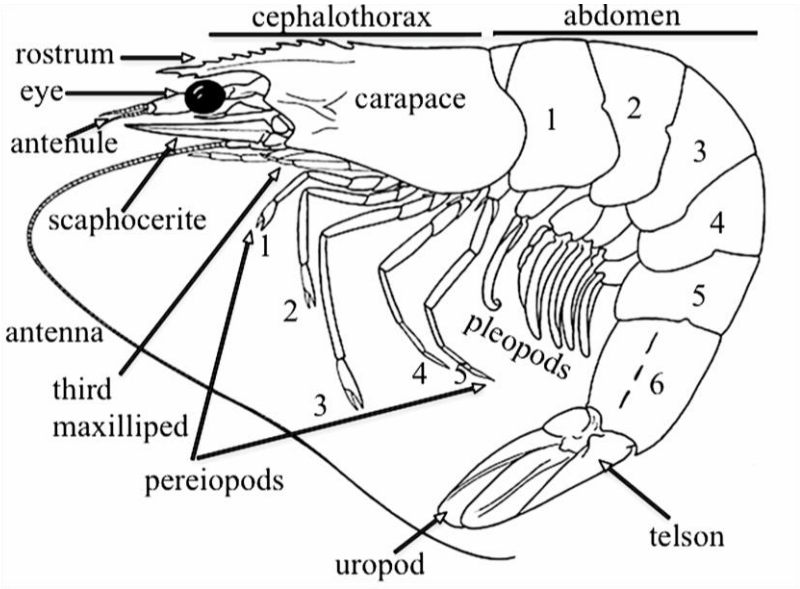

Figure 2. *P. vannamei* drawing.
(http://www.fao.org/fishery/culturedspecies/*Penaeus-vannamei*/en)

1.2.3 Life cycle

The lifespan of white leg shrimp is about 1.5 to 2 years. In its life cycle, shrimp undergo a series of complex metamorphoses from nauplius larvae into adults (Bailey-Brock, 1992). Adult shrimp mature, mate, and spawn in offshore water. Eggs are fertilized in water when females release simultaneously eggs and sperm (Bailey-Brock, 1992). Fertilized eggs drift in the water and undergo mitosis. The embryo changes about twenty-four hours before hatching into a nauplius. During the nauplius stage, the larvae nourish on the yolk reserve in the cytoplasm. After five moltings, the nauplius metamorphoses into protozoea. The protozoea have feeding activities on phytoplankton (microalgae). Next, they metamorphose into mysis and from that moment they live on zooplankton. After three instars stages, shrimp metamorphose and become post-larvae. Post-larvae are omnivorous feeders, morphologically similar to the adult. Post-larvae migrate into coastal areas where they nurse on epibenthic and develop into juveniles. Then, they return to the sea for maturing, mating and spawning (Bray WA, 1992; Sánchez, 1997) (Figure 3).

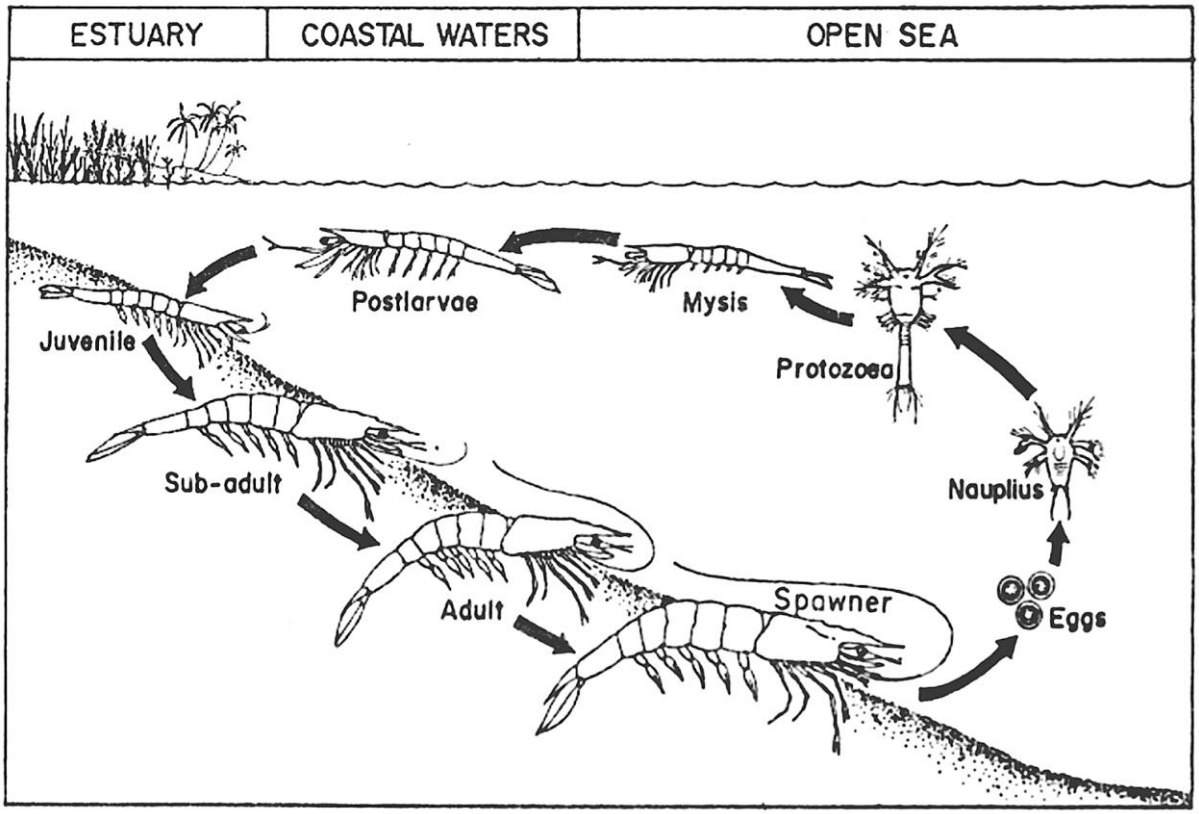

Figure 3. Life cycle of penaeid shrimp (Bailey-Brock, 1992).

1.2.4 Molting

Molting is essential for the growth of crustaceans. It is defined as a process of shedding the old cuticle and re-generating the new one. In penaeid shrimp, the degree of epidermal retraction from the setae and the old cuticle coupled with the development of new setae, the changes in morphology of the integument (layers of cuticle can be seen in Figure 7A & B), the presence of pigmentation, internal cones in the setal lumen are the criteria that are used to determine the molt stages of the animal. According to previous publications, the molt cycle of penaeid shrimp can be divided into some major stages (Longmuir, 1983; Robertson *et al.*, 1987; Chan *et al.*, 1988; Promwikorn *et al.*, 2004; De oliveira Cesar *et al.*, 2006; Promwikorn *et al.*, 2007; Corteel *et al.*, 2012).

Early post-molt stage “A”

Stage A begins immediately after molting. In this stage, the setae are soft. The setal lumen is filled with setal matrix. The epidermis is transparent with little pigmentation. The setal cone is not yet seen at the base of the setae. The new exoskeleton is soft and composed of two

layers: the epicuticle and exocuticle. Shrimp do not feed and lie most of the time at the bottom of the water. The duration of this stage lasts about one day, which depends on the environmental temperature and the size of animal.

Late post-molt stage “B”

The exoskeleton is hardened and the animal starts eating. This stage can be divided into sub-stages B1 and B2. Stage B1 is defined by the retraction of the epidermis in the setae to the region where cones will be formed. At stage B2, new setal cones are formed. The endocuticle layer is synthesized.

Inter-molt stage “C”

This stage can be sub-divided in C1, C2, C3 and C4. Stage C1 is defined by completed formation of internal cones. Cylindrical structures of setae become clearly visible in the C2 stage. Stages C1 and C2 are also defined by an increase of integument rigidity. The rigidity reaches its final stage in stage C3 and stage C4. The inter-molt stage is considered as a resting stage in the molt cycle.

Early pre-molt stage “D” (D0 and D1)

The early pre-molt stage is a period of biological preparation of organs and tissues all over the animal body for the next shedding. It is divided into sub-stages D0 and D1, which depends on the degree of retraction of the epidermis from the cuticle. When the epidermal tissue begins to retract from the cuticle, shrimp are identified as being at stage D0 of their molt cycle. An observed clear straight margin of the epidermal tissue underneath the setae determines the ending of stage D0. Stage D1 is defined by the increase in space between the setal base and the epidermis. The clear zone is the site for the formation of a new cuticle.

Late pre-molt stages “D2 and D3”

The stage of pre-molt can be subdivided into stages D2 and D3 according to the formation of the new setae. During stage D2, new setae form barbules and the setal spines extend into the base of the old setae. The epidermal retraction results in large empty spaces between the old cuticle and epidermis. In stage D3, new setae ere completely formed and folded under the old carapace. Shrimp stop eating by the end of this stage.

Ecdysis stage *"E"*

Prior to molting, shrimp absorb water to expand their body. When entering shedding, shrimp start a series of muscle contractions to accelerate a marked swelling at the softened point between the end of the cephalothorax carapace and the shell of the first abdominal segment. When this region breaks, shrimp withdraw their limbs and other appendages including the legs, gills, mouthparts, antennae, antennules, eyestalks and pleopods from the old skeleton. Then, they further kick their tail to escape from the old shell (Figure 4).

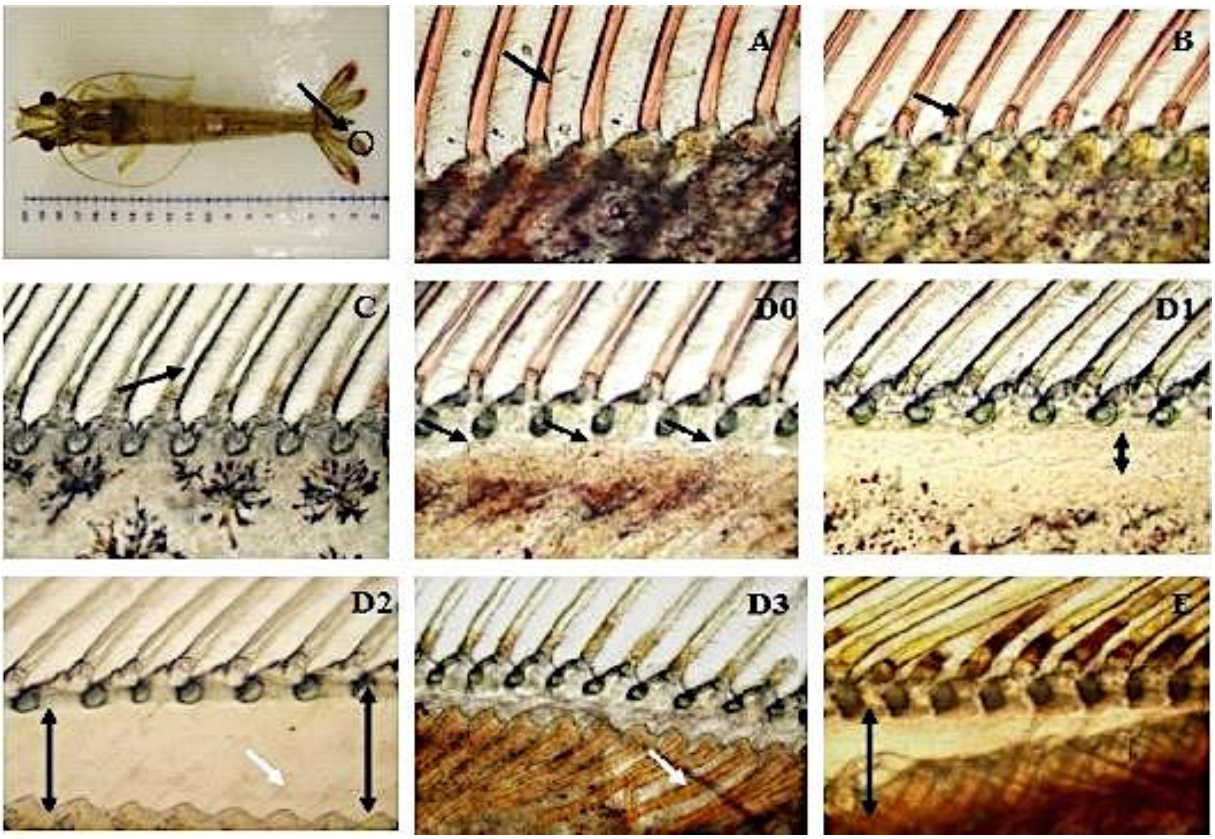

Figure 4. Photographs of the *P. vannamei* uropods during the major molt stage. A: early post-molt stage; presence of setal matrix (arrow) in the setal lumen, B: late post-molt stage; retraction of setal matrix and formation of internal cones (arrow), C: inter-molt stage; the setal lumen is empty, D0: early pre-molt stage; separation of the epidermis from the cuticle (arrows), D1: early pre-molt stage; increase in space between the cuticle and the epidermis (arrow), D2: late pre-molt stage; formation of new setae and larger space between the cuticle and the epidermis (arrow), D3: late pre-molt stage; new setae completely formed and folded under the old carapace, E: ecdysis; shedding of the old skin (De oliveira Cesar *et al.*, 2006; Corteel *et al.*, 2009; Corteel *et al.*, 2012).

1.2.4.1 Hormonal regulation of molting

In crustaceans, the eyestalk neurosecretory complex contains a group of neurosecretory perikarya in the medulla terminalis (X-organ, XO) which direct the sinus gland (SG) to

produce and secrete a wide range of neuropeptide hormones, which are involved in regulation of the carbohydrate metabolism (crustacean hyperglycemic hormone, CHH), molting (molt-inhibiting hormone, MIH), gonadal growth (gonad-inhibiting hormone, GIH) and activity of the mandibular organ (mandibular organ-inhibiting hormone, MOIH) (Chang, 1985; Skinner, 1985). CHH is originally categorised as the central hormone, which regulates the carbohydrate metabolism, inhibits ecdysteroid secretion and controls brachial ionic transport and osmoregulatory functions (Santos and Keller, 1993; Chung *et al.*, 1999; Spanings-Pierrot *et al.*, 2000). MIH has an inhibitory action on ecdysteroid synthesis in the Y-organ, which is located in the epithelium of the anterior brachial chambers (Bourguet *et al.*, 1977; Spindler *et al.*, 1980; Spaziani, 1990; Lachaise *et al.*, 1993; Blais *et al.*, 1994). When shrimp prepare themselves for ecdysis, the level of MIH in hemolymph drops which activates the release of major ecdysteroid hormone. Blais et al. (1994) reported that shrimp at the late pre-molt stage excreted major ecdysteroid compounds: 3-dehydroecdysone, ecdysone, and major 20-hydroxyecdysone. Y-organs cannot convert 3-dehydroecdysone or ecdysone to 20-hydroxyecdysone. This is conducted by peripheral tissues, such as gonad, hindgut, abdominal ganglia, eyestalk ganglia, hepatopancreas, antennal gland, and epidermis. Gonad-inhibiting hormone (GIH) is suggested to control vitellogenesis by inhibiting vitellogenin synthesis (Wilder *et al.*, 2010). The MOIH peptide hormone suppresses the production of methyl farnesoate (MF), which is a sesquiterpenoid compound synthesized in the mandibular organ. It was reported that high MF concentrations cause acceleration of the molting process and stimulate gonadal development in crustaceans (Wainwright *et al.*, 1996; Laufer *et al.*, 2005; Alnawafleh *et al.*, 2014) (Figure 5).

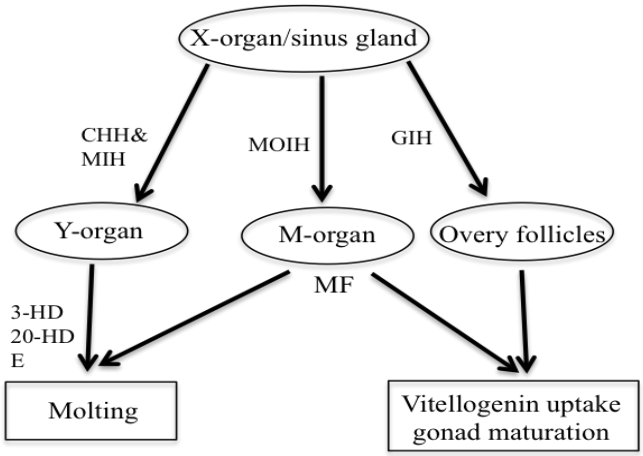

Figure 5. Hormone signaling pathways regulating molting in penaeid shrimp. Gonad inhibiting hormone (GIH), mandibular organ inhibiting hormone (MOIH), molt inhibiting hormone (MIH), crustacean hyperglycaemic hormone (CHH), mandibular organ (MO), 3-dehydroecdysone (3-HD), 20-hydroxyecdysone (20-HD), ecdysteroids (E), methyl farnesoate (MF).

1.2.4.2 Uptake of water during molting

In aquatic crustaceans, the molting process is generally accompanied by a rapid increase in wet weight as a result of water uptake (Dall and Smith, 1978; Mykles, 1980; Neufeld and Cameron, 1994). This process allows the swelling necessary for successful ecdysis and the subsequent increase in size of animals after molting (Defur et al. 1985; Cheng and Chang, 1991). It is generally accepted that the gills and midgut are pathways of water uptake during molting of crustaceans (Dall and Smith, 1978; Neufeld and Cameron, 1994). The uptake of NaCl by the gills results in an osmotic gradient and provides the force necessary for water influx (Travis, 1954; Capen, 1972; Jasmani *et al.*, 2010). Mechanisms related to water absorption via digestive tract of animals are not well understood. Chung et al. (1999) suggested that the surge of hyperglycemic hormone (CHH) in the foregut and hindgut may regulate ion uptake during molting crab *Carcinus maena*, thus allowing water uptake by drinking.

1.2.5 Digestive system

The digestive system of decapod crustaceans is an internal tube, which is localized in the dorsal region of shrimp and is divided into three regions: the foregut, the midgut and the hindgut. The foregut is composed of the mouth, the esophagus (E) and the stomach (cardiac and pyloric chambers) (Figure 6). The epithelial cells of the foregut have an ectodermal origin and are covered by cuticle (Felgenhauer and Abele, 1985; Lin, 1996; Ceccaldi, 1997). The mouth is covered by oral appendages of mandibles, labrum, maxillipeds and maxilla (Felgenhauer and Abele, 1985). The mandibles, a paired structure, function in grinding feed. Each mandible is composed of a molar and a flattened mandibular palp. The palp helps the labrum to collect particles. The maxilla 1 functions in holding and pushing food items between the mandibles. The maxilla 2 is involved in preening food items (Bell & Lightner 1988; Ceccaldi 1997; Bauer 1999). The maxillipeds function in feeding, grooming and possible pheromone reception (Alexander et al., 1980). The esophagus is a short, narrow J-shaped tube that ends into the anterior floor of the cardiac stomach. The esophagus is composed of simple columnar epithelial cells surrounded by muscles. The wall of the esophagus is slightly folded and lubricated by mucus secreted by tegumental glands, which facilitates closure and prevents feed from passing back into the mouth region (Felgenhauer and Abele, 1985; Minagawa and Takashima, 1994; Ceccaldi, 1997). The cardiac chamber

(CCH) is a gastric spacious sac (gastric mill) composing of a variety of internal structures of lateral teeth (LT), cardiopyloric valve (CV), comb row setae (CR), median tooth (MT). These structures facilitate mastication and sorting of food. A pair of lateral teeth are extensions of the zygocardiac ossicles (ZO) which are construct with the median teeth (MT). At the bottom of the cardiac stomach, the comb row setae are present formed by four rows of setae (Felgenhauer and Abele, 1985; Felgenhauer, 1992; King and Alexander, 1994). The pyloric chamber (PCH) is divided into dorsal and ventral sub-chambers. The dorsal pyloric sub-chamber is an extensive structure of a dorsal uropyloric ossicle (UPO). Ventral sub-chamber contains a pair of very complicated gland filters (GF). The gland filter is composed of upper and lower ampullary chambers that are armed with setae to form a screen. Food gets into the gland filter, enters the upper ampullary chamber and is squeezed into the lower ampullary chamber by extrinsic longitudinal muscles. Particles too large to pass through the dense setae of the lower chamber move posteriorly to the midgut. Fine particles and material in solution flow into the ampullary channels and pass directly into the hepatopancreas. The midgut starts at the junction with the pyloric stomach and ends in the posterior midgut caecum. Penaeid shrimp possess a pair of midgut ceca (one anterior caecum and one posterior midgut caecum). The anterior midgut caecum is located at the junction of the midgut with the phyloric stomach. The posterior midgut caecum is situated at the midgut-hindgut juncture. Midgut ceca are blind sacks with large, distinctive epithelial folds projecting into the cecal lumen. The ceca are part of the midgut, thus their lumens are lined by columnar epithelial cells with an apical microvillous brush border (Bell and Lightner, 1988). It is suggested that midgut ceca play a role in ion and water regulation, production of some enzymes and maintenance of pH balance (Holliday *et al.*, 1980; Lovett and Felder, 1990). Unlike the foregut and hindgut that are lined by cuticle, the wall of the midgut is lined by columnar epithelial cells (EC) with a basal lamina and apical microvilli secreting the peritrophic membrane. The peritrophic membrane is a non-cellular structure, composed of chitin fibrils and proteins, only permeable to inert particles smaller than 20 nm and functions as intestinal barrier to protect the midgut epithelium from mechanical damage of food particles and invasion of pathogens (Lehane, 1997; Martin *et al.*, 2006; Wang *et al.*, 2012). The hindgut begins behind the posterior midgut caecum and runs the length to the anus. The epithelial cells in the hindgut are of ectodermal origin and are thus covered with cuticle (Barker and Gibson, 1978). The hindgut is active in expelling the peritrophic membrane and its contents by rhythmic contractions along its length (Dall and Moriarty, 1983; Bell and Lightner, 1988) (Figure 6).

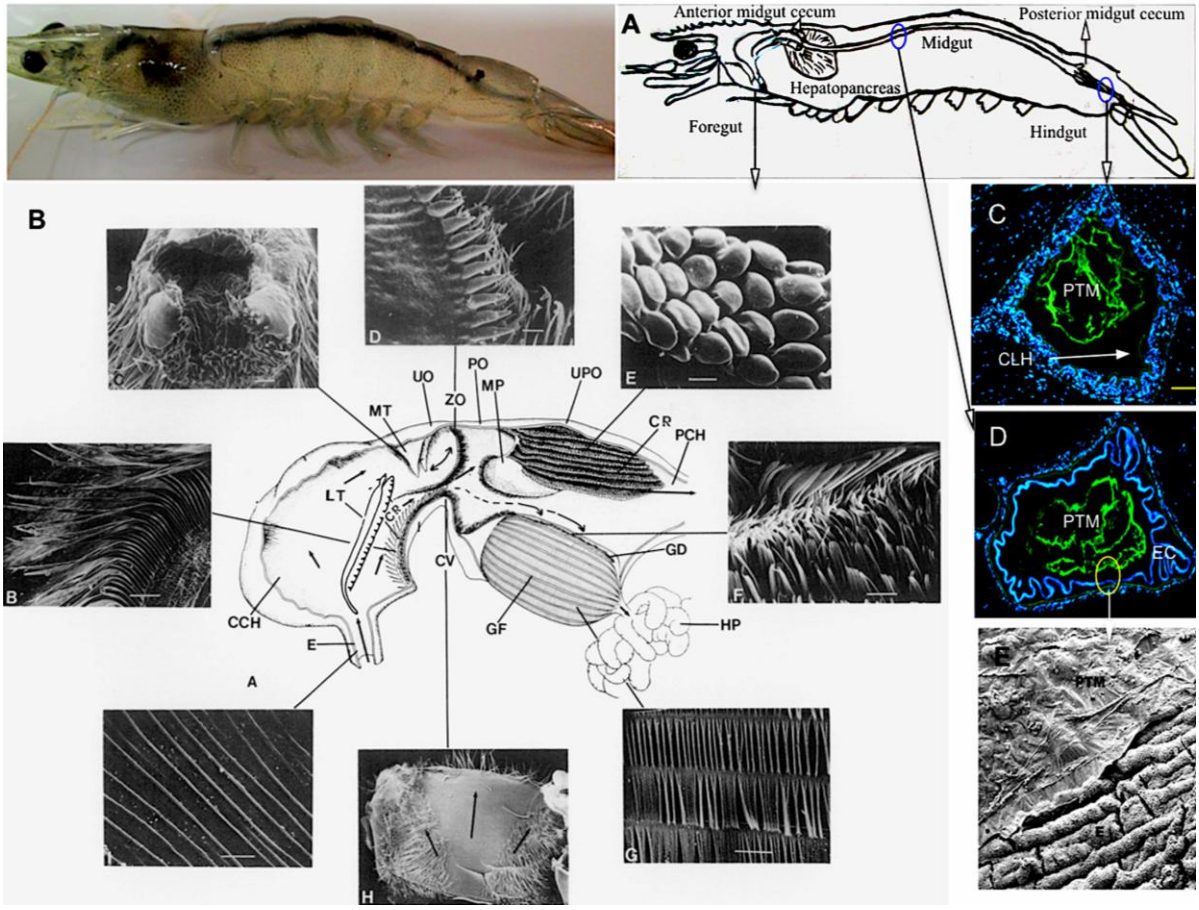

Figure 6. General anatomy of the digestive system of *Penaeid* shrimp, showing the foregut, midgut, and hindgut regions. A: Schematic drawing indicating the position of different component of the shrimp digestive system. B: Lateral view of the foregut and ultrastructure of different parts as by Scanning Electron Microscopy. Esophagus (E), cardiac chamber (CCH), lateral teeth (LT), comb row (CR) setae, cardiopyloric valve (CV), median tooth (MT), uropylorico ossicle (UO), pyloric chamber (PCH), uropyloric ossicle (UPO), zygocardiaco ossicle (ZO), gland filter (GF), guarding denticles (GD), hepatopancreas (HP). C & D: Cross-sections of the midgut and hindgut stained with FITC-linked succinylated (WGA) wheat germ agglutinin and analyzed by fluorescence microscopy: epithelial cells (EC), cuticle lining the hindgut (CLH), peritrophic membrane (PTM). E: Peritrophic membrane lining the midgut of shrimp revealed by Scanning Electron Microscopy (Felgenhauer and Abele, 1985; Martin *et al.*, 2006; Thuong *et al.*, 2016).

1.2.6 Gills and their functions

Penaied shrimp have dendrobranchiate gills located in the branchial chamber. Each gill has a central axis bearing a series of paired branches. The branches are primary filaments that are further divided into secondary filaments (Foster and Howse, 1978; Bell and Lightner, 1988). The surface of gills is covered by cuticle. The thickness of the cuticle varies from axis (16 μm) to branches (2.5 μm) and filaments (less than 1.0 μm). The pore canals (0.14 μm)

penetrate the exocuticle (Ex) and endocuticle (En) of the axis cuticle but do not enter the epicuticle (Ep) (Foster and Howse, 1978) (Figure 7A & B). Gill epithelium is composed of different cell types including nephrocytes, granular, flange and pillar cells (Bell and Lightner, 1988; Freire *et al.*, 2008). Nephrocytes are mainly located in the axial efferent vessels. Granular cells are abundant throughout the branchial tissue and are involved in the synthesis of cuticular material. Pillar cells are found in gill lamellae. They sustain the intralamellar septum and limit deformation of the hemolymph space during changes in hydrostatic pressure (Foster and Howse, 1978; Bell and Lightner, 1988). Crustacean gills are known to have multiple functions including gas exchange, ammonia excretion, balance the pH, osmotic and ionic homeostasis (Henry *et al.*, 2012). The exchange of oxygen and carbon dioxide is mediated by the countercurrent circulation of hemolymph and water on the surface of the filaments. Blood circulation in the gills occurs through a pair of infrabranchial sinuses that collect hemolymph from the tissue and drain into the afferent branchial vein. From the branchial vein, hemolymph is supplied to individual lamellae. Hemolymph, after oxygen uptake passes through the lamellae, flows into the efferent branchial veins and runs into the branchio-cardiac vein to the heart (Taylor and Taylor, 1992). Onken and Riestenpatt (1998) suggested that penaeid shrimp are strong hyperosmoregulators. Na^+ absorption is conducted via apical Na^+ channels and basolateral Na^+/K^+-ATPase. Cl^- absorption is carried out via apical Cl^-/HCO_3^- exchangers and basolateral Cl^- channels (a carbonic anhydrase (CA) provides the cellular substrates (H^+, HCO_3^-) for the apical transporters). Briefly, the basolateral Na^+/K^+ ATPase activates transbasolateral Na^+ absorption and generates a negative cellular potential by maintaining an outwardly directed K^+ concentration gradient across the plasmamembrane (i). An apical $V(H^+)$-ATPase generates increased extracellular HCO_3^-, supporting transapical Cl^- absorption (ii). Hyperpolarized cellular negativity supports the transapical entry of Na^+ and exit of Cl^- across the basolateral membrane (iii) (Figure 7C). It was suggested that NaCl uptake resulted in an osmotic gradient and provided the force necessary for intracellular passive absorption of water (Figure 7D) (Travis, 1954; Capen, 1972; Jasmani *et al.*, 2010; Larsen *et al.*, 2014). In seawater fish, the gills are also known as the place for active NaCl secretion (Evans et al., 1999). However, the mechanism of active NaCl secretion in the gills of crustaceans is not known. Most probably, in penaeid shrimp, the antennal gland is the major organ responsible for the excretion of sodium and chloride via urine (Lin *et al.*, 2000). Apart from these functions, the gills of shrimp are also an important place for ammonia/ammonium excretion, pH regulation, transbranchial absorption of Ca^{2+} (Flik *et al.*, 1994; Weihrauch *et al.*, 1998; Henry *et al.*, 2012)

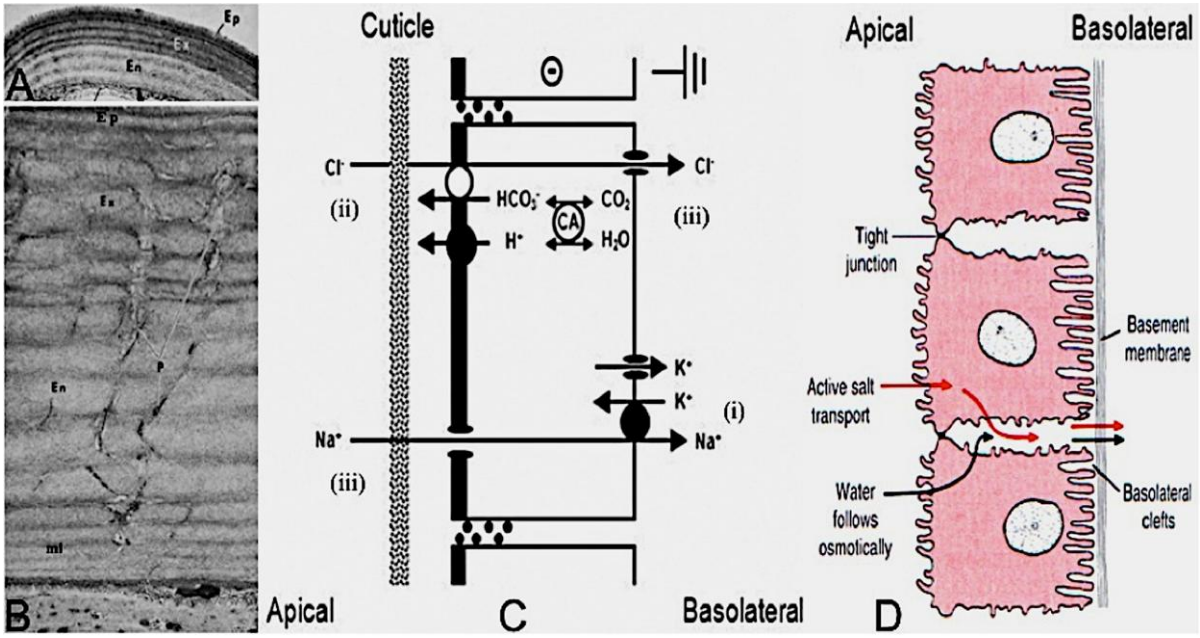

Figure 7. Morphology of the cuticle of the gill filament of *P. aztecus* and mechanism of NaCl and water transbranchial absorption across the gills. A & B: Cross-section of gill filament by transmission electron microscopy showing different layers of cuticle and penetration of pore channels through the exocuticle and endocuticle. Epicuticle (Ep), exocuticle (Ex), endocuticle (En), membranous layer (ml), pore canals (P). C: Mechanism of NaCl transbranchial absorption across the gills. Basolateral K^+ channel activates transbasolateral Na^+ absorption and generates a negative electrical potential in the cell (i). Apical V(H^+)-ATPase generates increased extracellular HCO_3^-, supporting transapical Cl^- absorption (ii). Hyperpolarized cellular negativity supports the transapical entry of Na^+ and exit of Cl^- across the basolateral membrane (iii). D: Active uptake of NaCl causes a deviation of apical osmotic pressure and results in passive entrance of water across the apical barriers (Foster and Howse, 1978; Onken and Riestenpatt, 1998; Larsen *et al.*, 2014).

1.2.7 Antennal glands and excretory function

Antennal glands are important organs of crustaceans, which are located at the base of the antennae and involve in excretion and osmoregulation functions of the hosts (Figure 8A, B). Studies on the antennal glands have shown that structure and function of the antennal glands are different among crustaceans depending on their habitats (Lin *et al.*, 2000; Khodabandeh *et al.*, 2005b). The antennal glands of species living in freshwater such as crayfishes are composed of four major different units: the coelomosac (end sac), the labyrinth, the proximal and distal tubules (nephridial canal) and the urinary bladder (Figure 8C). The antennal glands of freshwater crustaceans are taking care of the osmoregulation (Felgenhauer, 1992; Khodabandeh *et al.*, 2005c). The structure of the antennal glands of crustaceans living in seawater is composed of three major units: the coelomosac, the labyrinth, and the urinary

bladder (Figure 8D). The antennal glands in these species function in ion excretion (Bell and Lightner, 1988; Khodabandeh *et al.*, 2005b; Tsai and Lin, 2014). Generally, of both freshwater and marine crustaceans, the coelomosac locates in the center of the antennal gland. The coelomosac region represents regularly organized cavities limited by single layer of podocytes. The coelomosac is basally lined by a basement matrix with some parallel collagen-like fibrils. The podocyte cells are characterized by pedicels (p) on basal lamina. Cytoplasm of the podocytes contains numerous small vesicles (sv), vacuoles (v) and endosomes (en). The apical portions of the podocytes are devoid of microvilli (Figure 8E, H). These features of the podocytes indicate that the coelomosac cells perform an ultrafiltration and secretory activity (Walter and Wägele, 1990; Khodabandeh *et al.*, 2005a; Khodabandeh *et al.*, 2005b; Tsai and Lin, 2014). The coelomosac opens and leads to the underlying labyrinth. In this region, two sub-regions (labyrinth I and labyrinth II) are identified in the marine species. The labyrinth I immediately follows the coelomosac and forms the main part of the labyrinth. The labyrinth II is distal from the coelomosac and connects to the urinary bladder (Figure 8D). In freshwater crayfishes, the labyrinth follows the coelomosac and connects to the bladder through the nephridial canal (proximal and distal tubules) (Figure 8C). Two types of cells in the labyrinth have been observed, which are columnar cells and cuboidal cells. The common features of these cells are apical microvilli (mv), apical cytoplasmic extrusions, apical small vacuoles (sv), apical and sub-apical globular vesicles (v), basal membrane infoldings associated with mitochondria (m) (Figure 8F, I). It was suggested that the cells of the labyrinth have both reabsorption and secretion functions (Doughtie and Rao, 1983; Bell and Lightner, 1988; Khodabandeh et al., 2005a). In crayfishes, two sub-regions of proximal and distal tubules are observed in the structure of the antennal gland (Figure 8C). The cells in the tubule are cuboid and columnar cells with basal membrane infoldings associated with mitochondria and without apical microvilli. It was suggested that the nephridial canal appears as compulsory in fresh water species, which may support salt-reabsorbing function leading to the formation of dilute urine (Dall, 1970; Khodabandeh *et al.*, 2005a). In both marine and fresh water crustaceans, the bladder is lined by a single thin layer of epithelial cells on top of thin fibrous connective tissue layers with a network of hemolymph vessels in between them (Khodabandeh *et al.*, 2005a; Khodabandeh *et al.*, 2005b). The number of layers was variable according to its location. In crayfishes, the features of the bladder epithelial cells are devoid of apical microvilli. Apical cytoplasm bulges into the lumen and contains small vacuoles (sv). The basal region of the bladder cells is occupied by the large number of mitochondria associated with basal infoldings (Figure

8G). It was suggested a possible involvement in ion reabsorption (Khodabandeh *et al.*, 2005a). In lobster, the features of the epithelial cells of the bladder are apical microvilli, apical cytoplasmic extrusions, endosomes, cytoplasmic vacuoles, and basal plasma membrane infoldings associated with mitochondria. It was suggested an active ion secretion function for these cells, associated with reabsorption of biologically interesting molecules such as glucose, amino acids and small proteins (Khodabandeh *et al.*, 2005b). The urinary bladder is connected to the nephropore via a ureter (Bell and Lightner, 1988; Nakamura and Nishigaki, 1991; Khodabandeh *et al.*, 2005b). The nephropore is a cuticular valve-like split protruding at the base of the second antennae, which is controlled by a sphincter muscle system (Bushmann and Atema, 1996).

Ultrafiltration occurs across the basement membrane of the coelomosac when hemolymph passing the coelomosac artery that is located between the basement membrane of the coelomosac. The filtrate then flows through the different regions of the labyrinth, the proximal tubule, the distal tubules and the urinary bladder, while it is modified through absorption and secretion process. It is reported that freshwater crustaceans produce diluted urine, while marine species excrete hyperosmotic urine (Lin *et al.*, 2000; Khodabandeh *et al.*, 2005a).

Although little details are known about the structure and function of the antennal glands of euryhaline crustaceans habitat in both fresh and seawater such as penaeid shrimp, it is well known that structure of the antennal glands of these species is similar to that described in marine species, which composes of thee major unit: the coelomosac, the labyrinth, and the urinary bladder. It is also known that antennal glands of these species posses a complexity, by which the labyrinth diverges more complicatedly toward rostral direction, urinary bladder appears to be multi-lobed and connects between two side of the glands (Bell and Lightner, 1988; Nakamura and Nishigaki, 1991).

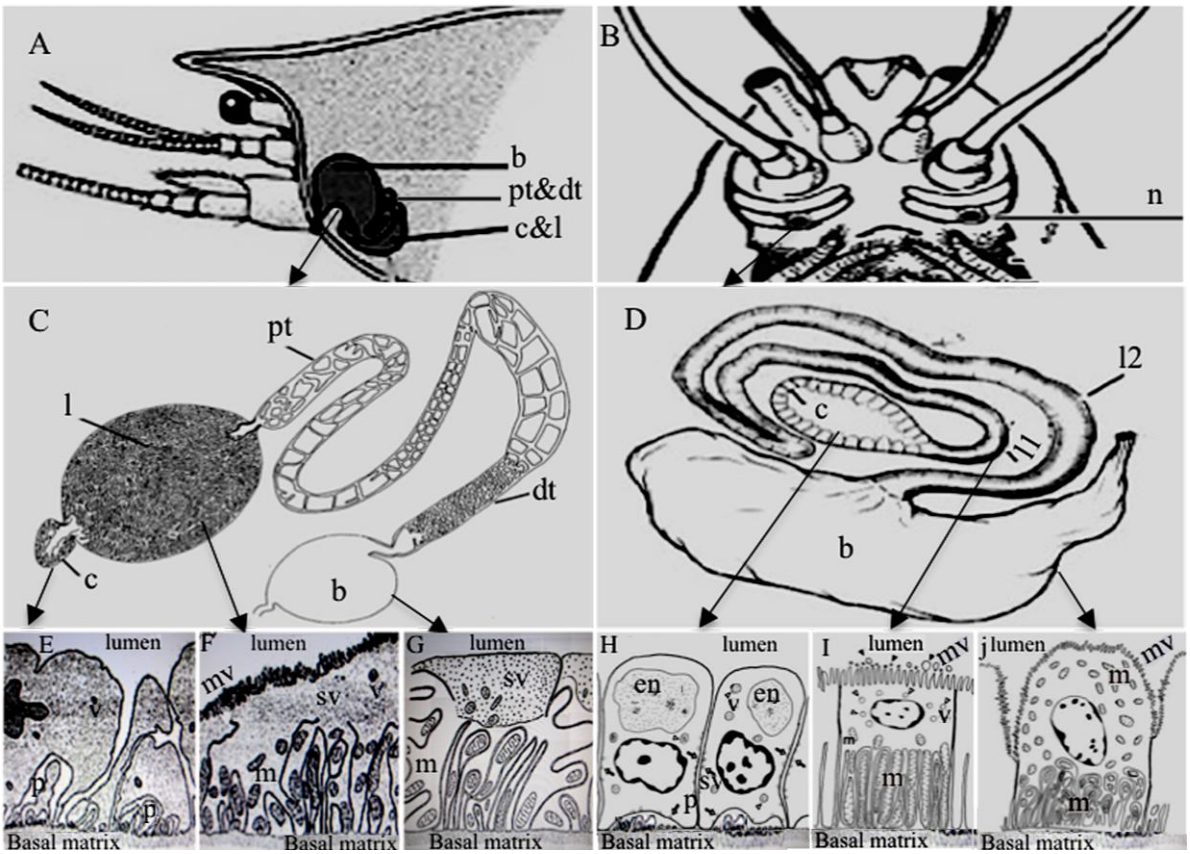

Figure 8. Schematic drawing of the structure of the antennal glands of freshwater and seawater crustaceans. A & B: The position of the antennal gland. C: Major regions of the antennal gland of fresh water crustaceans (crayfish *Procambarus leonensis).* Coelomosac (c), labyrinth (l), proximal tubule (pt), distal tubule (dt), bladder (b). D: Major regions of the antennal gland of seawater crustaceans (lobster *Homarus gammarus*, crab *Ocypode stimpsoni).* Coelomosac (c), labyrinth 1 (l1), labyrinth 2 (l2). E: Cell of the coelomosac of crayfish. F: Cell of the labyrinth of crayfish, microvilli (mv), small vacuoles (sv), vesicles (v), mitochondria (m). G: Cell of the bladder of crayfish. H: Cell of the coelomosac of lobster, pedicels (p), small vesicles (sv), vacuoles (v), endosomes (en). I: Cell of the labyrinth of lobster. J: Cell of the bladder of lobster (Felgenhauer and Abele, 1985; Khodabandeh *et al.*, 2005a; Khodabandeh *et al.*, 2005b; Tsai and Lin, 2014).

1.2.8 Circulatory system

The cardiovascular system of penaeid shrimp can be defined as a system consisting of four parts: the heart, hematopoietic tissues, lymphoid organ, and the associated arterial and venous hemolymph system. Hemolymph is the circulating body fluid, which is composed of haemocytes, proteins, lipoproteins, glycoproteins, antimicrobial peptides, free aminoacids, carbohydrates, fatty acids, electrolytes, metals, salts, water, oxygen and metabolic waste products (Shimizu et al., 2001). Hemocyanin is a major component of the hemolymph proteins. It is a large copper-containing protein originally recognized as oxygen transporter

(Figueroa-Soto *et al.*, 1997; McMahon, 2001). More recently, it has been demonstrated that hemocyanin is a multifunctional protein, also participating in immune defense and maintaining colloid-osmotic pressure (Zhang *et al.*, 2006; Zhang *et al.*, 2009). Unlike the typical vertebrate four-chambered heart, with one entrance and one exit per ventricle, crustaceans have multiple ostial and aortic valves. Hemolymph enters the heart through three pairs of muscular ostial valves namely: ventral ostial valve (VOV), dorsal ostial valve (DOV) and lateral ostial valve (LOV). Hemolymph leaves the heart via aortic valves that lead to five arterial systems: anterior aorta (AA), anterior lateral artery (ALA), hepatic artery (HA), posterior aorta (PA) and sternal artery (SA) (Figure 9). Pre-branchial hemolymph from active tissue is collected by paired infrabranchial sinuses and delivered to the gills for re-oxygenation. Post-branchial hemolymph enters the branchio-cardiac veins and delivers to the pericardial sinus surrounding the heart. Contraction of the ventricle then distributes the oxygenated hemolymph to the arterial systems that branch repeatedly to terminate and directly flows into the tissues (McMahon, 2001). Hematopoietic tissues are located in different areas of the cephalothorax, mainly at the dorsal side of the stomach, in the coxae of the maxillipeds and anterior esophagus towards the antennal gland. These tissues are arranged as spherical and elongated lobules which are surrounded by fibrous connective tissues (Bell and Lightner, 1988). Haematopoietic tissue is responsible for the production of haemocytes (Hyaline, semigranular or granular cells) (Hose *et al.*, 1992; Johansson *et al.*, 2000; Van de Braak *et al.*, 2002b). Lymphoid organs of penaeid shrimp consist of two lobes located ventro-anterior the hepatopancreas. Each lobe receives the haemolymph from the anterior artery, which is further branched into contorted tubules and haemal sinuses. The tubules consist of the central lumen lined by flattened endothelial cells. Next to the endothelial lining, two types of stromal cells have been identified: type one has more darkly stained cytoplasm and type two unstained cytoplasm (H&E stain) (Bell and Lightner, 1988). It was reported that haemocytes penetrate into the space between stromal cells, where they settle and later migrate into the haemal sinuses (Van de Braak *et al.*, 2002c). The lymphoid organ acts as a true filtering organ for the purposes of filtering and elimination of foreign materials and infectious agents from the haemolymph (Van de Braak *et al.*, 2002c). The lymphoid organ also plays an important role in the defense against infections especially viral infections (Hasson *et al.*, 1999; Khanobdee *et al.*, 2002).

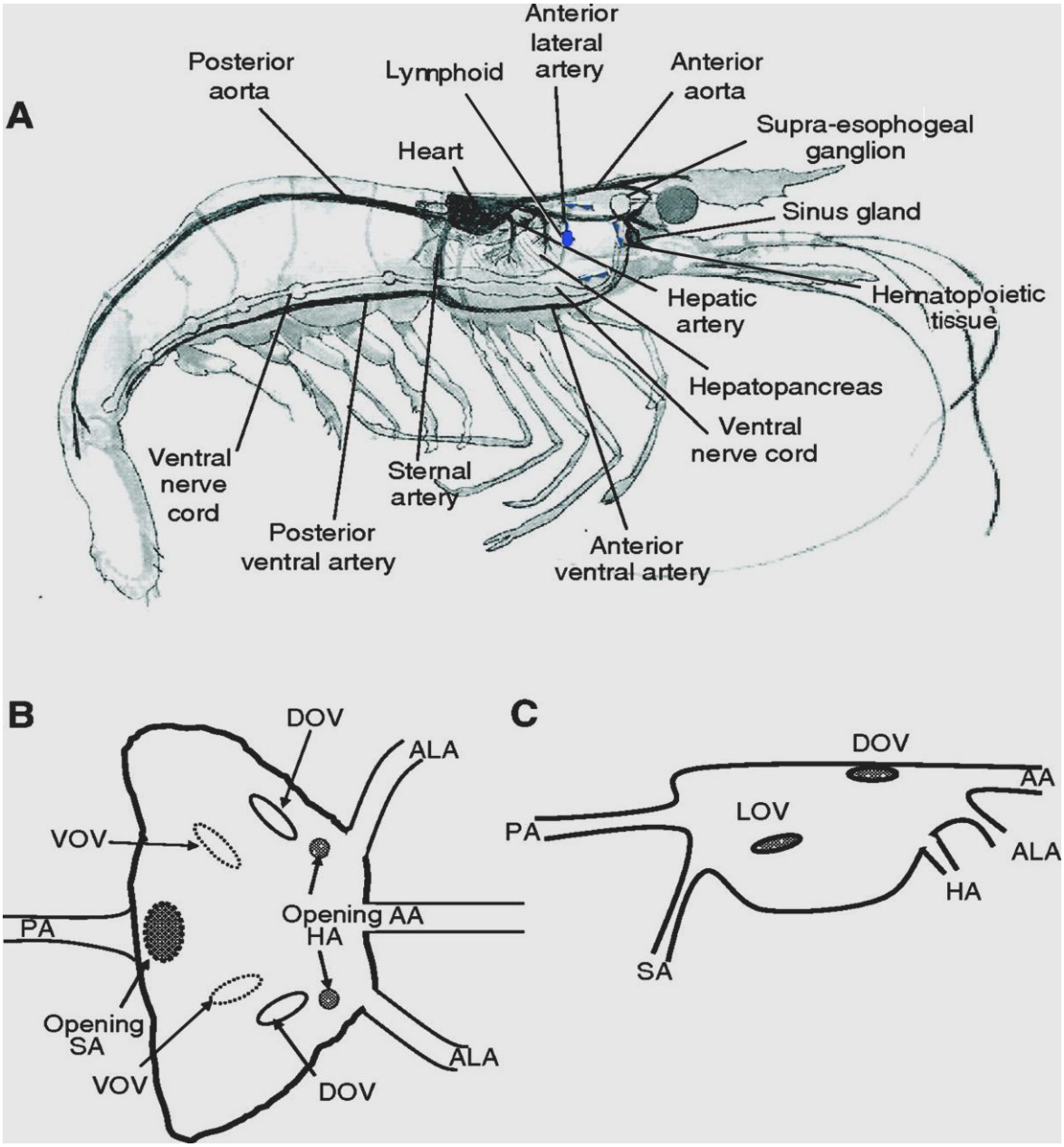

Figure 9. Schematic overview of the circulatory anatomy of shrimp. A: Position of circulatory system, B: Dorsal view of heart, C: Lateral of view of heart. Anterior aorta (AA), anterior lateral artery (ALA), hepatic artery (HA), posterior aorta (PA), sternal artery (SA), ventral ostial valve (VOV), dorsal ostial valve (DOV), lateral ostial valve (LOV) (Guadagnoli *et al.*, 2011).

1.2.9 Defense systems

In contrast to vertebrates, crustaceans do not have an adaptive immune defense and generally use a range of innate cellular and humoral defenses to protect themselves from pathogens that manage to gain access to their internal tissues. Haemocytes are the cells principally involved in the defense reactions against invading microorganisms. In the past, they were separated

into three subpopulations: hyaline cells, semi-granular cells and granular cells (Söderhäll and Smith, 1983; Van de Braak *et al.*, 1996). More recently, haemocytes of *P. vannamei* were divided into five subpopulations (Dantas-Lima *et al.*, 2013; Tuan *et al.*, 2015). These types of haemocytes have been reported to perform a number of special key actions including wound repair to prevent the penetration of pathogens into the main body cavity, phagocytosis, encapsulation, nodulation and killing of invaders such as protozoans, bacteria, fungi, and viruses (Söderhäll and Smith, 1983; Söderhäll *et al.*, 1985; Smith, 2010; Roulston and Smith, 2011; Zhi *et al.*, 2011; Tuan *et al.*, 2015). Antimicrobial peptides are mainly produced and released by haemocytes and are key factors in the elimination of microorganisms (Jiravanichpaisal *et al.*, 2006; Smith, 2010). In crustaceans, 15 distinct antimicrobial peptide families are currently recognized. The predominant families are penaeidins and crustins. They are small molecules containing normally 15 to 100 amino acids (Munoz *et al.*, 2002; Rosa and Barracco, 2010). Antimicrobial peptides are generally cationic molecules that have the ability of killing microbial pathogens by destabilization and disruption of their phospholipid bilayer membranes. For example, lysozyme is known to function primarily in bacterial lysis through cleaving the β-1,4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan of the pathogens (Hikima *et al.*, 2003; Kaizu *et al.*, 2011).

1.2.9.1 Recognition

The activation of the immune response starts when the presence of pathogen is recognized by molecular pattern receptors such as beta-glucan-binding-protein, lipopolysaccharide receptors, peptidoglycan binding protein, C-type lectin and toll-like receptors. These receptors detect the molecules like bacterial lipopolysaccharides, peptidoglycan and lipoteichoic acid, fungal beta-1,3-glucans and viral RNA (Cerenius *et al.*, 2010; Smith, 2010). Detection of a ligand activates signal pathways to induce a degranulation of haemocytes, which simulates the proPO system to release prophenoloxidase-activating enzyme into hemolymph. A cascade of serine proteinases cleaves the prophenoloxidase-activating enzyme into an active form, which further requires Ca^{2+} to activate phenoloxidase (PO). The active phenoloxidase catalyzes the hydroxylation of monophenols into o-diphenols and their oxidation into toxic quinones. Quinones are converted into melanin by a non-enzymatic process. Melanin is a dark brown pigment that is generated at sites of infection (Gollas-Galván *et al.*, 1999; Cerenius and Söderhäll, 2004).

1.2.9.2 Antiviral immunity

Recent studies have shown crustacean's response to viral infection via activation of RNA interference (RNAi), phagocytosis, apoptosis and antiviral peptides. RNAi is a biological process in which dsRNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. RNAi is initiated by the processing of long dsRNA into short 21-23 oligonucleotides, referred to as small interfering RNAs (siRNAs), which subsequently serve as guide sequences to direct the multi-component nuclease RISC (RNA-induced silencing complex) to destroy mRNAs bearing a complementary sequence. Integral to this process is the type III endoribonuclease Dicer, which is responsible for cleavage of long dsRNA into siRNAs. Numerous studies have revealed that the administration of artificial dsRNA/siRNA to shrimp can provide an effective protection against virus invasion (Bartel, 2004; Yodmuang *et al.,* 2006). Applications of RNAi technology against WSSV using dsRNA and siRNA specific to WSSV genes showed better survival than other antiviral aids in *Penaeus monodon, Penaeus chinensis* and *Litopenaeus vannamei*. A 100% protection against WSSV was reported using dsRNA-VP28 in *P. monodon* and *P. chinensis*. More than 95% survival was reported in *P. vannamei* and 75% survival in *P. japonicus* (Robalino *et al.,* 2005; Kim *et al.*, 2007; Tirasophon *et al.,* 2007; Xu *et al.,* 2007; Sarathi *et al.*, 2008). Most of the genetic material used to inhibit WSSV infection encodes structural proteins involved in virion architecture such as VP28, VP281, VP19, VP2 and VP15 (Robalino *et al.*, 2005; Westenberg *et al.,* 2005; Tirasophon *et al.*, 2007; Sarathi *et al.,* 2008). A few studies have assessed the antiviral efficacy of RNAi against non-structural WSSV proteins such as DNA polymerase, and protein kinase (Kim *et al.*, 2007). Although RNAi is a promising methodology for viral disease control in penaeid shrimp, it is difficult to be used in the field. Because dsRNA/siRNA can only be delivered to shrimp through intramuscular injection, this technology cannot be used at farm level.

Apoptosis has been identified to be important in the host antiviral response. In shrimp, several genes encode caspase family proteins, which are known to coodinate the regulation of apoptosis. dsRNA-mediated silencing of these genes results in the increase of WSSV copy numbers, indicating the involvement of shrimp caspases in apoptotic responses against viral infection (Wang et al., 2013). Ran GTPases are enzymes, which have been identified to play a role in the host antiviral responses via enhancement of phagocytic activity. Improvement of phagocytic activity through the activation of the Ran proteins could effectively inhibit WSSV infection in shrimp (Zhao et al., 2011). Rab family proteins, that have functions in endocytic

and exocytic membrane trafficking, have been shown to bind to the WSSV envelope protein VP28 and lead to a decrease in *P. monodon* mortality (Sritunyalucksana et al., 2006). It was reported that aldehyde dehydrogenase (ALDH) and heat shock protein 70 (Hsp70) play an important role in the inhibition of WSSV replication at high temperature. ALDH and Hsp70 markedly upregulated in WSSV-infected shrimps at 32°C. When ALDH and Hsp70 were knocked down, shrimps became severely infected, while shrimp without knockdown of ALDH and Hsp70 remained uninfected (Lin et al., 2011).

1.3 Penaeid shrimp diseases

Introduction

In the recent years, the rapid growth of shrimp aquaculture worldwide is accompanied by an increased incidence of infectious disease problems. Viruses, bacteria, protozoa and fungi have been identified as major agents of these diseases (Brock and Lightner, 1990; Lightner, 1996; Lightner *et al.*, 1998). More than 15 viruses have been reported to infect farmed shrimp, of which, 9 viruses namely white spot syndrome virus (WSSV), taura syndrome virus (TSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), gill-associated virus (GAV), infectious myonecrosis virus (IMNV), yellow-head virus (YHV) and mourilyan virus (MoV) are responsible for serious production losses (Bonami, 2008; Lightner, 2011; Ganjoor, 2015). Bacterial disease caused by Gram-negative, rod shaped, facultative anaerobic, motile bacteria in the family of *Vibrionaceae* are major pathogens responsible for high mortality in shrimp farming industry. Disease outbreaks caused by *Vibrio* species such as *V. harveyi, V. alginolyticus, V. damsela, V. parahaemolyticus, V. campbellii, V. vulnificus and V. penaeicida* have been observed in nursery and growout ponds of penaeid shrimp (Lightner *et al.*, 1990; Lavilla-Pitogo *et al.*, 1998). Vibriosis outbreaks in culture systems are known to occur in combination with stress factors (pH, salinity, ammonia, temperature) or following primary infections with other pathogens (Horowitz and Horowitz, 2001). Protozoan parasites are also important pathogens in shrimp aquaculture. They may infect the inside or outside of a host body. Those at the outside are found harmless when present at low numbers. Those at the inside can cause disease. Three major protozoan diseases of cultured shrimp are: cotton shrimp disease caused by microsporans, enteric cephaline gregarine infections infected by cephaline gregarines and protozoan fouling caused by ciliates (Johnson, 1989; Rajendran,

1997). Besides virusies, bacteria and protozoa, fungi are known as shrimp pathogens. Twenty genera and 46 fungal species are identified in shrimp, of which two common genera infect larval shrimp (*Lagcnidium* and *Sirolpidium).* One common genus (*Fusarium*) is found to attack juvenile and adult shrimp (Johnson, 1989; Silva *et al.*, 2011). At present, WSSV and *Vibrio parahaemolyticus* are major pathogens causing significant production losses in shrimp aquaculture worldwide.

1.3.1 White Spot Syndrome (WSS)

1.3.1.1 Classification

WSSV was first detected in Taiwan in 1992 (Chou *et al*., 1995), then it spread and caused serious mortality in penaeus shrimp farming in japan in 1993, United States of America in 1995, Central and South-America in 1999, Europe in 2002 (Inouye *et al*., 1994). During the first decade, the virus was given different names such as rod-shaped nuclear virus of *Penaeus japonicus* (Inouye *et al.,* 1994), hypodermal and haematopoietic necrosis baculovirus (Jie *et al.,* 1995), systemic ectodermal and mesodermal baculovirus (Wonteerasupaya *et al.*, 1995), white spot baculovirus (Wang *et al.,* 1995). Later, these viruses were recognized as one and the same virus and assigned by the International Committee on Virus Taxonomy to its own new genus, *Whispovirus*, in the family *Nimaviridae* (Mayo, 2002).

1.3.1.2 Morphology and structural proteins

WSSV is a dsDNA, which is rod-shaped, non-occluded and enveloped with tail-like appendages. Intact enveloped virions are 80-120 nm wide and 250-380 nm long (Inouye *et al.*, 1994; Chou *et al.*, 1995; Wang *et al.*, 1995; Durand *et al.*, 1996; Kanchanaphum *et al.*, 1998) (Figure 10). The viral envelope is 6 - 7 nm thick and is constructed by a lipid bilayer membrane. The space between the envelope and the nucleocapsid ranges from 2 to 7.5 nm. The nucleocapsid is a cylinder of 65 x 200 nm. The external wall of the nucleocapsid is 6 nm thick (Durand et al., 1997).

More than 40 structural proteins have been identified so far. Of these, 30 are located in the viral envelope, 5 in the tegument and 7 in the nucleocapsid (Leu *et al.,* 2009). Among the envelope proteins, VP28 was found to be the most dominant. It is synthesized after three hours post inoculation (van Hulten *et al.,* 2001; Li *et al.,* 2015). VP28 is reported to bind to

the tegument proteins VP24 and VP26 to anchor the envelope onto the underlying tegument layer (Leu *et al.*, 2009). VP28 was also known to bind to the Rab7 protein, which is localized on the surfaces of membrane-enclosed compartments of exocytic and endocytic pathways and regulates vesicle budding and fusion events (Sritunyalucksana *et al.*, 2006). The major tegument protein VP26 has been described as a matrix-linker protein between the viral envelope (VP28) and nucleocapsid (VP51A) to form a VP51A-VP26-VP28 structure (Chang *et al.*, 2008). VP26 interacts with β-actin and plays an important role in WSSV infection (Liu *et al.*, 2011). VP664 is a major capsid protein, which contributes primarily to the assembly and morphogenesis of the virion (Leu *et al.*, 2005)

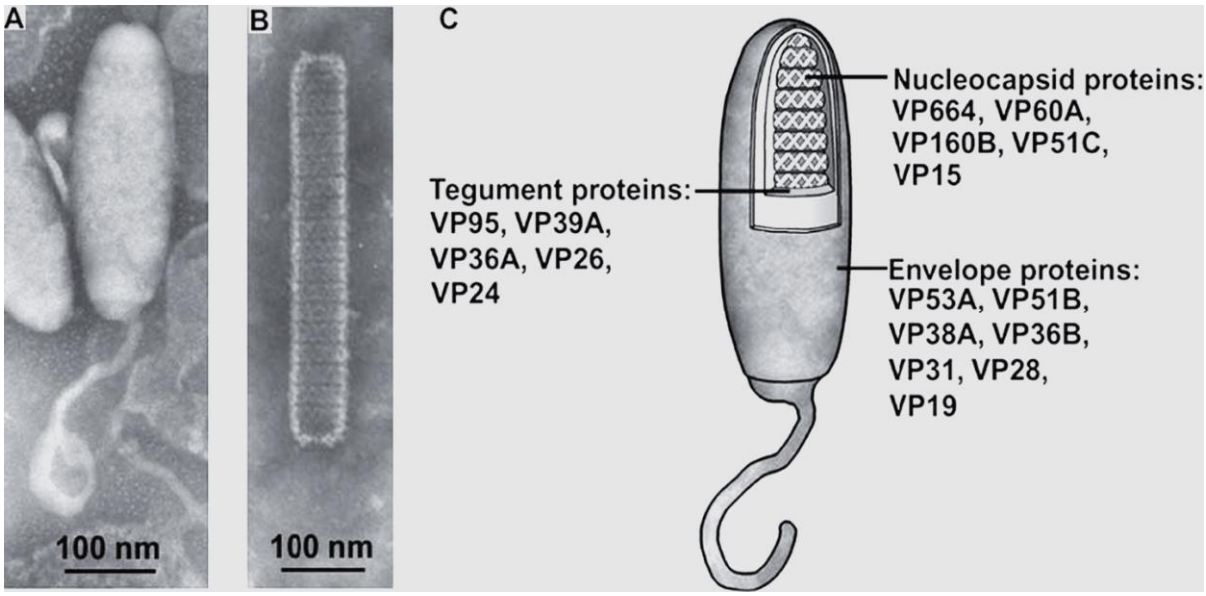

Figure 10. Morphology of WSSV. A & B: negative contrast electron micrographs. A & C: Intact WSSV virion with tail-like extension, B: WSSV nucleocapsid (Leu *et al.*, 2005).

1.3.1.3 Physical inactivation

Inactivation of WSSV by both chemical and physical treatments has been reported in literature. WSSV is inactivated within 90 min at 50°C and within less than 5 min at 70°C (Chang *et al.*, 1998; Nakano *et al.*, 1998). WSSV could be inactivated with U.V. irradiation of 3×10^4 μW.sec/cm^2 (Nakano *et al.*, 1998). The virus was completely inactivated by high acidity (pH 1 for 10 min, pH 3 for 1 h) and alkalinity (pH 12 for 10 min at 25^o C). WSSV was completely inactivated by 5 g l^{-1} formalin and 30% ethanol within 1 min (Nakano *et al.*, 1998). Sodium chloride at concentrations ranging from 0-10% gave no negative effect on the infectivity of WSSV (Chang *et al.*, 1998). Under laboratory conditions, WSSV was infective in seawater for at least 40 days at 30 °C. In shrimp pond sediment, the virus remained

infective for 19 days under sun-drying. When the water stays in the pond, WSSV remained viable for a period of 35 days (Kumar *et al.*, 2013).

1.3.1.4 Host range and WSSV transmission

WSSV has a wide range of hosts or carriers. To date, more than 98 species including all cultured marine shrimps, crabs, lobsters, crayfishes and some aquatic benthic organisms such polychaete worms, sea salters have been found as hosts or carriers of WSSV (Yan *et al.*, 2004; Vijayan *et al.*, 2005; Escobedo‐Bonilla *et al.*, 2008; Sánchez-Paz, 2010). In some species, WSSV results in a non-lethal or latent infection, making these species potential virus reservoirs and important sources of infection in shrimp ponds (Wainwright *et al.*, 1996; Zhang *et al.*, 2006; Laramore, 2007; Corteel *et al.*, 2012b; Raja *et al.*, 2015). Since its first report in Taiwan in 1992, transmission studies of WSSV among individuals have demonstrated that transmissions occur through horizontal (transmission by direct contact or ingestion of WSSV infected organisms) and vertical pathways (from an infected female parent to its offspring) (Lo *et al.*, 1997; Kanchanaphum *et al.*, 1998; Soto *et al.*, 2001; Yan *et al.*, 2004). Some studies have shown that WSSV is highly contagious in penaeid shrimp via feeding of WSSV-infected tissue, peroral inoculation or immersion (Lightner *et al.*, 1998; Escobedo-Bonilla *et al.*, 2005). However, other studies have reported difficulties to infect shrimp via immersion, cohabitation as well as peroral feeding (Gitterle et al., 2006; Laramore, 2007; Corteel et al., 2009; Tuyen et al., 2014). Studies on the factors determining WSSV transmission in shrimp have reported that an abrupt drop in temperature and salinity due to heavy rain is a hazard factor for a WSSV outbreak (Rodríguez *et al*., 2003; Peinado-Guevara and López-Meyer, 2006; Gunalan *et al*., 2010; Tendencia et al., 2010; Selvam *et al*., 2012; Kakoolaki *et al*., 2015). Larvae and early post-larvae of *P. monodon* were reported to resist to a WSSV infection by immersion and oral infection; further stages (late post-lavae and adults) were found more susceptible to WSSV challenge (Yoganandhan *et al.*, 2003). Further, shrimp are more susceptibly to WSSV infection via immersion shortly after molting than in the period before molting (Corteel *et al.*, 2009).

1.3.1.5 Pathogenesis

Understanding the way of entry, identifying target organs and unraveling spreading mechanisms of WSSV from the primary replication sites to other target organs provide important insights for early diagnosis and disease control. Numerous pathogenesis studies of WSSV infections in penaeid shrimp conducted so far have suggested that WSSV target cells can be found in organs of both ectodermal and mesodermal origin (epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eyestalk, heart, gonads, haematopoietic tissues) (PohShing *et al.*, 1996; Escobedo-bonilla *et al.*, 2007; Rahman *et al.*, 2007; Corteel *et al.*, 2012b). PohShing *et al.* (1996) reported that the primary sites of WSSV infection in early juvenile *P. monodon* were the subcuticular epithelial cells of stomach and integument, cells in gills, and connective tissue of the hepatopancreas. Arts *et al.* (2007) suggested that the gills were primary replication sites of WSSV. Another study of Escobedo-bonilla *et al.* (2007) determined foregut and cells in the gills as primary sites of WSSV replication. Haemocytes and epithelial cells of anterior and posterior midgut caeca and midgut trunk are refractory to WSSV infection (Hameed *et al.*, 1998; Van de Braak *et al.*, 2002a; Arts *et al.*, 2007). The pathogenesis of WSSV infection in penaeid shrimp was reported to depend on the viral strain, administered dose and route of inoculation. Rahman *et al.* (2007) showed that upon intramuscular infection WSSV-Thai-1 and WSSV-Thai 2 were more virulent than WSSV-Viet. Using WSSV-Thai-1 to infect shrimp via peroral inoculation, Escobedo-bonilla *et al.* (2007) found the first WSSV-infected cells in the foregut and gills at 18 hpi in shrimp inoculated with a low dose ($10^{1.5}$ SID_{50}) and at 12 hpi in shrimp inoculated with a high dose (10^{4} SID_{50}). In *P. monodon*, upon peroral feeding of WSSV infected tissues, infected cells were detected in gills at 16 hpi (PohShing *et al.*, 1996).

1.3.1.6 Clinical signs and diagnostic techniques

In the field, farmers often report WSS symptoms, which appear in ponds 30-60 days post-stocking. When disease occurs, WSSV-infected shrimp gather near the border of the pond. Cumulative mortality may reach up to 100% within 10 days after the first mortalities occur (Nakano *et al.*, 1994; Chou *et al.*, 1995; Lightner, 1996; Sudha *et al.*, 1998). Shrimp infected with WSSV often show white spots with a diameter of 0.5 to 3.0 mm in the exoskeleton of the carapace, appendages and body (Chou *et al.*, 1995; Lo *et al.*, 1996). However, these spots are not always present, and can also be detected in shrimp during bacterial infections, by the

use of probiotics or under certain water quality conditions (Lo *et al.*, 1996; Wang *et al.*, 2000). Therefore, identification of a WSSV infection cannot be based solely on the presence of these white spots. Several methods have been developed for the diagnosis of WSS in shrimp: histopathology, transmission electron microscope, DNA and immunostaining. Histopathology has a low sensitivity to detect WSSV-infected hosts at the early stage of infection. Rajendran *et al.* (2005) could detect WSSV-infected cells as early as 18 hpi, but not before 18 hpi. WSSV-infected cells are characterized by enlarged nuclei, containing Cowdry A-type basophilic inclusions (Lightner, 1996; Alday de Graindorge and Flegel, 1999). DNA based techniques including *in situ* hybridization, PCR, nested-PCR, *in situ* PCR and qPCR have been widely used and became powerful tools to detect the WSSV genome in WSSV-infected animals (Durand *et al.*, 1996; PohShing *et al.*, 1996; Lightner *et al.*, 1998; Li *et al.*, 2015). These DNA based techniques are much more sensitive than histology and have been used for the early detection of WSSV infection. Immunostainings using WSSV-specific antibodies have been developed for the detection of WSSV antigens. These assays include western blot, immuno-dot blot, ELISA, immunohistochemistry and immunofluorescence. Major advantage of immuno-based diagnostic methods is the ability to detect and grade the severity of viral infections in the tissue of the host (Nadala *et al.*, 1997; Poulos *et al.*, 2001; Zhang *et al.*, 2001; Escobedo-Bonilla *et al.*, 2005).

1.3.1.7 Environmental factors effecting WSSV outbreaks

Disease is the end result of a complex interaction between host, environment and pathogens (Lightner *et al*., 1998). Under some conditions, shrimp and its pathogens co-exist with little or no adverse effect. In recent years, more and more researchers have discovered that WSSV outbreaks were associated with the abrupt change in environmental factors such as temperature, salinity and pH (Rodríguez *et al*., 2003; Peinado-Guevara and López-Meyer, 2006; Gunalan *et al*., 2010; Tendencia *et al*., 2010; Selvam *et al*., 2012; Kakoolaki *et al*., 2015). Abrupt lower environmental temperature has been recognized as one of the main factors that trigger WSSV disease in shrimp cultures (Kautsky *et al.*, 2000; Esparza‐Leal *et al.*, 2010; Gunalan *et al.*, 2010). On the other hand, increasing the environmental temperature may be an important tool for WSSV disease control (Vidal *et al*., 2001; Rahman *et al*., 2006; Gao *et al*., 2011). It was suggested that WSSV-infected shrimp at high temperature (32^{o} C) upregulate the host proteins aldehyde dehydrogenase ALDH and Hsp70, which may be

involved in the inhibition of WSSV replication (Lin *et al*., 2011). A drop in salinity has also been found to increase the viral load in shrimp, resulting in 80% mortality on a farm in Mexico (Peinado-Guevara and López-Meyer, 2006). Acute drop in environmental salinity contributed to an increase of the susceptibility of *P. monodon* to WSSV infection (Joseph and Philip, 2007). Although the mechanism by which WSSV gets into shrimp during a drop of environmental salinity has not been found, it is known that the immune performances (prophenoloxidase activities-proPO) of penaeid shrimp change during acute temperature and salinity stress, which may result in increasing replication of pathogens in shrimp (Liu *et al*., 2006; Jia *et al*., 2014). Beside temperature and salinity, pH and ammonia are also found as risk factors, which may trigger WSSV infection in shrimp farms (Gunalan *et al*., 2010; Selvam *et al.*, 2012). Gunalan *et al*. (2010) reported that a pH between 6.8 and 8.7 was optimal for the growth and production of *P. monodon*. A high pH of 9.0 - 9.7 in the culture system might be the trigger for a WSSV outbreak. Generally, heavy rainfall could be an important stressor, because it causes a drop of water temperature, salinity, and pH (Peinado-Guevara and López-Meyer, 2006; Gunalan et al., 2010; Tendencia *et al*., 2010).

1.3.2 Vibrio diseases

Bacterial diseases, mainly due to *Vibrio*, are big problems in global aquaculture industry. Major *Vibrio spp,* including *V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbellii, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei,* have been reported to be associated with mass mortality in both hatcheries and grow-out ponds (Brock and Lightner, 1990). Among these species, *V. harveyi*, is one of the most virulent pathogens that has been associated with mass mortalities (80-100%) of *P. monodon* larval rearing systems (Liuxy *et al*., 1996; Lavilla-Pitogo *et al*., 1998; Uma *et al*., 2008). *V. harveyi* is luminescent and found to detach the epithelial cells from the basal lamina of the midgut of infected shrimp (Lightner *et al*., 1990; Martin *et al*., 2004). Recently, *Vibrio parahaemolyticus* has been identified as the causative agent of acute hepatopancreatic necrosis disease (AHPND) causing significant production losses in shrimp aquaculture worldwide (Tran *et al*., 2013; Zorriehzahra and Banaederakhshan, 2015). AHPND is known to be the cause of acute mortalities in both *P. monodon* and *P. vannamei* within the first 30-35 days of culture. AHPND-infected shrimp are characterized by brown-black cuticle lesions, black lymphoid organ and melanization of appendages. Histopathology reveals necrosis and inflammation of different organs (lymphoid

organ, gills, heart), severe atrophy of the hepatopancreas with massive sloughing of epithelial cells in diseased animals (Lightner *et al*., 2012). The mechanism by which *Vibrio* invades its host and results in disease has not been clearly elucidated. Some studies suggested that the digestive tract, particularly the stomach is the site for colonization and penetration of the *Vibrio* bacteria into shrimp (Lavilla-Pitogo *et al*., 1990; Martin *et al*., 2004; Tran *et al*., 2013; Soonthornchai *et al*., 2015). Other studies suggested that the gills are ideal places for bacterial invasion because they are covered by a thin exoskeleton (Aguirre‐Guzmán *et al.*, 2010). Alday-Sanz *et al*. (2002) were not successful in infecting shrimp with *V. vulnificus* via immersion and peroral inoculation. The authors suggested that *V. vulnificus* can only infect shrimp through a damaged cuticle. It is suggested that some *Vibrio* species cause mortality via excretion of extracellular virulent factors such as phospholipase, hemolysin, metalloprotease, cystein protease, chitinase and low molecular weight lipopolysaccharide. After release, these products pass through the internal barrier of the digestive tract (cuticle & PM), enter the blood stream and imbalance the shrimp's immune system (Aguirre‐Guzmán *et al.*, 2004; Martin *et al.*, 2004). *V. parahaemolyticus, V. alginolyticus* and several strains of *V. harveyi* release proteases, which cause mortality in *P. monodon* through destroying enzymatically the haemolymph clotting system in shrimp (Liuxy *et al*., 1996; Lee *et al*., 1999). Liu and Lee (1999) reported that a cysteine protease is the major toxin produced by *Vibrio harveyi* strain 820514, which can inhibit the clotting ability of shrimp haemolymph.

1.4 Control measures for shrimp infectious diseases

1.4.1 Traditional use of antibiotics

Antibiotics have been widely used in attempts to combat bacterial diseases in shrimp farms. They include chloramphenicol, gentamycin, trimethoprim, tiamulin, tetracyclines, quinolones and sulfonamides. However, in the recent years the use of these products has resulted in resistance of many *Vibrio* species (Hossain *et al.*, 2012; Yano *et al.*, 2014; Letchumanan *et al.*, 2015). Consequently, antibiotics are no longer effective in treating diseases caused by a wide range of *Vibrio* species. Karunasagar *et al.* (1994) reported mass mortality in black tiger shrimp (*Penaeus monodon*) larvae caused by *V. harveyi* strains, which were resistant to cotrimoxazole, chloramphenicol, erythromycin and streptomycin. The development of antibiotic resistance in numerous *Vibrio* bacteria increased the concern of transferring

resistant genes to pathogens humans and land animals (Cabello, 2006; Zhang *et al.*, 2009a; Albuquerque Costa *et al.*, 2015; Uddin *et al.*, 2015). Approaches to control diseases in shrimp aquaculture can be achieved by improved management and increased shrimp internal immune defense.

1.4.2 Vaccination

As an alternative strategy to antibiotic use in aquaculture, vaccines against shrimp pathogens received more attention during recent years. Vaccination requires primary immunization with antigens and relies on specific defense mechanisms, which is traditionally believed to exist only in vertebrates. However, some reports have described an increase in survival of shrimp in experimental "vaccination" trials (Namikoshi et al., 2004; Ha et al., 2008; Rowley and Pope, 2012). These studies have explored different anti-WSSV strategies to protect shrimp including inactivated virus, DNA, recombinant protein and dsRNA vaccines (Namikoshi *et al.*, 2004; Witteveldt *et al.*, 2004; Rout *et al.*, 2007; Fu *et al.*, 2010). It was demonstrated that the durations of protection and efficacy of vaccines varied with the viral protein genes used. Shrimp vaccinated with recombinant proteins showed a longer protection against WSS than shrimp vaccinated with inactivated virus (Namikoshi *et al*., 2004). Shrimp vaccinated with WSSV recombinant VP28 and VP281 envelope proteins resulted in a short-term protection for less than 3 weeks post-vaccination. Animals vaccinated with DNA plasmid vaccines encoding VP28 and VP281 showed a protection against viral disease up to 7 weeks (Rout et al., 2007). These experiments suggest that shrimp may achieve a short-term protection against a viral disease by vaccination with a whole virus inactivated vaccine and long-term antigen exposure is required to prolong the protection. Vaccination was mainly performed by injection, which restricts its use under field conditions.

1.4.3 Probiotics

Probiotics as 'biofriendly agents' such as lactic acid bacteria and *Bacillus spp* have recently attracted great attention in shrimp aquaculture. In literature, model actions of probiotics were described to include: production of inhibitory compounds, competition for nutrients, competition for adhesion sites in the gastrointestinal tract, enhancement of the immune response and production of essential nutrients and enzymatic contribution to digestion (Vine *et al.*, 2006). It was reported that the addition of *Bacillus* strain S11 to *P. monodon* culture

infected with a pathogenic *V. harveyi* strain increased survival of the shrimp from 26% to 100% after ten days (Rengpipat *et al.*, 1998). Zokaeifar et al. (2012) found that *B. subtilis* L10 and G1 strains showed a strong antibacterial activity against two pathogens *V. harveyi* at pH 7.3-8.0 and against *V. parahaemolyticus* at pH 6.0-8.0. A probiotic mixture of 5 *Bacillus* species (*B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. laterosporus* and *B. circulans*) was described to increase the conversion ratio, specific growth rate, and final production of shrimp receiving the probiotic compared to control shrimp (Ziaei-Nejad *et al.*, 2006). However, some important limitations to the use of probiotics are the need to be added regularly at high concentrations because they are not able to maintain themselves. Furthermore, *vibrios* may develop resistance, as has occurred for numerous antibiotics (Vine *et al.*, 2006)

1.4.4 Immunostimulation

Immunostimulants are substances that activate the immune system of animals to make them more resistant to microbial infections. The use of immunostimulants such as beta-glucans, vitamin C, seaweed extracts, peptidoglycans and lipopolysaccharides in shrimp has been reported to result in increased phenoloxidase activities and hemocyte counts, and significantly increased survival of shrimp after infection with virulent vibrios (Thanardkit et al., 2002; Marques et al., 2006). It was shown that immunostimulants like chitin, fucoidan, ergosan, glucan may induce resistance of shrimp to virus infection (Montero-Rocha *et al.*, 2006; Sajeevan *et al.*, 2009; Kumar *et al.*, 2015). Chotigeat *et al.* (2004) described that fucoidan, a sulfated polysaccharide extracted from brown algae, may help to prevent shrimp from WSSV infection. Zhao *et al*. (2011) found that the injection of lysophosphatidylcholine in shrimp enhanced the phagocytosis activity and effectively inhibited WSSV infection in shrimp. Although immunostimulation gives promising results, there are some limitations. The duration of response is likely to be short and immunostimulants do not always promote disease resistance (Sakai, 1999).

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Chapter 2

Aims of thesis

White spot syndrome is an infectious disease, caused by a DNA virus, which has become one of the most devastating viruses in shrimp aquaculture industry worldwide. Over a few decades, despite many attempts to prevent disease, no effective control measures have been found. Early studies on the transmission of WSSV reported that WSSV infected hosts can transmit the virus to native shrimp via horizontal and vertical routes and depicted the image that WSSV is highly contagious in penaeid shrimp. Recently, some experimental studies have reported the difficulty to infect shrimp via immersion, cohabitation as well as peroral feeding. In the field, a salinity drop in the water due to excessive rainfall and molting have been mentioned to be risk factors for WSSV outbreaks. In response to salinity stress and molting, shrimp absorb fluid and regulate their hemolymph osmolality by controlled exchange of ions in their antennal gland. It could make sense that during fluid absorption and urination shrimp may need to open their nephropore "nephropore (= slit in the cuticular disc at the base of the second antennae that forms an opening of the bladder to the outer world)" more frequently, which may allow WSSV to enter the bladder of the antennal gland and invade into shrimp. This working hypothesis will be exanimated in the present thesis.

The specific aims of this thesis are

(i) To examine the effect of the physical composition of the viral inoculum on the infectivity of WSSV in *P. vannamei* via peroral route and to study the internal barrier function of the peritrophic membrane of shrimp against WSSV infection

(ii) To evaluate the effect of an acute change in environmental salinity and shedding of the old cuticle shell on the susceptibility of *P. vannamei* to WSSV infection via waterborne route.

(iii) To investigate if the antennal gland is a main portal of entry for pathogens in shrimp. To this end, the three-dimensional structure of the antennal gland of *P. vannamei* was first investigated. The portal of entry of WSSV in shrimp was determined by comparing the shrimp infectious dose 50% endpoint of a WSSV stock and, the shrimp lethal dose 50% of *Vibrio campbellii* by different routes of inoculation: intramuscular injection, peroral and intra-antennal gland inoculation. In addition, the replication kinetics of WSSV in shrimp upon intra-antennal gland inoculation was examined.

Chapter 3

Per os infectivity of white spot syndrome virus (WSSV) in white-legged shrimp (*Litopenaeus vannamei*) and role of peritrophic membrane

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Abstract

As earlier observations on peroral infectivity of white spot syndrome virus (WSSV) in white-legged shrimp are conflicting, here, a standardized peroral intubation technique was used to examine (i) the role of the physical composition of the viral inoculum and (ii) the barrier function of the PM. In a first experiment, the infectivity of a WSSV stock was compared by determining the shrimp infectious dose with a fifty percent endpoint (SID_{50}) by intramuscular injection, peroral inoculation or via feeding. The following titers were obtained: $10^{8.77}$ SID_{50} g^{-1} by intramuscular injection, $10^{1.23}$ SID_{50} g^{-1} by peroral inoculation and $10^{0.73}$ SID_{50} g^{-1} by feeding. These results demonstrated that $10^{7.54}$ to $10^{8.03}$ infectious virus is needed to infect shrimp by peroral inoculation and via feeding. Next, it was examined if damage of the PM may increase the susceptibility for WSSV by peroral route. The infectivity of a virus stock was tested upon peroral inoculation of shrimp with and without removal of the PM and compared with the infectivity upon intramuscular inoculation. The virus titers obtained upon intramuscular injection and peroral inoculation of shrimp with and without PM were $10^{8.63}$ SID_{50} ml^{-1}, $10^{1.13}$ SID_{50} ml^{-1} and $10^{1.53}$ SID_{50} ml^{-1}, respectively. This experiment confirmed the need of $10^{7.1}$ to $10^{7.5}$ infectious virus to infect shrimp via peroral route and showed that the removal of the PM slightly but not significantly ($p > 0.05$) facilitated the infection of shrimp. This study indicated that WSSV contaminated feed is poorly infectious via peroral route, whereas it is highly infectious when injected into shrimp. The PM plays a minor role as internal barrier of shrimp against WSSV infection.

1. Introduction

Since its first description in 1992 (Chiang and Lo, 1995), WSSV is responsible for a large number of failures of shrimp culture worldwide (Escobedo-Bonilla *et al.*, 2008). WSSV is a rod-shaped, enveloped virus that infects a broad range of crustaceans (Durand *et al.*, 1996; Pradeep *et al.*, 2008); to date, more than 90 crustacean species have been found as hosts or carriers of WSSV (Sánchez-Paz, 2010). WSSV may be transmitted via vertical and horizontal routes (Fang *et al.*, 1997; Soto and Lotz, 2001; Prior *et al.*, 2003; Corteel *et al.*, 2009; Soowannayan and Phanthura, 2011; Tuyen *et al.*, 2014). Some environmental parameters such as temperature, salinity drop and pH are known as stressors influencing transmission and may influence the occurrence of WSSV outbreaks (Vidal *et al.*, 2001; Peinado-Guevara and López-Meyer, 2006; Rahman *et al.*, 2006; Gunalan *et al.*, 2010; Tendencia *et al.*, 2010). It is difficult to infect shrimp with WSSV via immersion or cohabitation with infected hosts (Prior *et al.*, 2003; Gitterle *et al.*, 2006; Corteel *et al.*, 2009; Tuyen *et al.*, 2014) and per os WSSV inoculation by intubation or via feed results in contradictory findings. Some researchers found it a powerful tool to induce WSSV infection in shrimp (Lightner *et al.*, 1998; Vidal *et al.*, 2001; Escobedo-Bonilla *et al.*, 2006) whereas others had difficulties to reproduce these results (Pérez *et al.*, 2005; Gitterle *et al.*, 2006; Hasson *et al.*, 2006; Laramore, 2007). Differences in virulence of the WSSV strain, virus dose, way of administration, and experimental conditions of the animals may be responsible for these controversial observations.

The peritrophic membrane (PM) is a non-cellular structure, composed of chitin fibrils and proteins, which are synthesized and secreted by epithelial midgut cells. It is lining the epithelial midgut and acts as a barrier preventing animals from physical damages and pathogen invasion (Hegedus *et al.*, 2009). In insects, it is well known that the inhibition of PM formation may increase the susceptibility of the host to virus infection (Wang and Granados, 2000; Rao *et al.*, 2004; Mitsuhashi *et al.*, 2007; Plymale *et al.*, 2008; Takemoto *et al.*, 2008). In order to establish an infection in the digestive tract of the host, pathogens may use their own chitinase to facilitate the penetration of PM (Huber *et al.*, 1991; Langer and Vinetz, 2001). In shrimp, it was already demonstrated that bacteria such as *Vibrio parahaemolyticus* may destroy the PM barrier to initiate colonization and replication in the midgut and invasion in the shrimp body (Martin *et al.*, 2004; Tran *et al.*, 2013). In addition, prior to molting, shrimp increase the expression of endogenous chitinases, which may help in the degradation process of the PM and facilitate the pathogen invasion (Peters *et al.*, 1999;

Tan *et al.*, 2000; Priya *et al.*, 2009; Proespraiwong *et al.*, 2010). At present, it is not clear if the PM forms a barrier to WSSV and if a removal of the PM may help WSSV to infect midgut epithelial cells and invade into the shrimp.

In the present study, it was examined if the physical composition of the viral inoculum and presence/absence of the PM changes the capacity of WSSV to infect its host.

2. Materials and methods

2.1 Experimental animals and growing conditions

The shrimp used in this study were *Penaeus* (*Litopenaeus*) *vannamei* from Piti Syaqua Farm, Syaqua Siam Co. Ltd., Thailand. The batch of 10000 PL_{8-12} was certified to be specific pathogen free (SPF) for the viruses WSSV, TSV, YHV and IHHNV by PCR and histopathology. The PL were transported to the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. At the ARC, shrimp were grown in a bio-filter recirculation system, fed with pelleted feed at a rate of 5% of mean body weight per day. Temperature was maintained at 27 ± 1 °C, salinity at 35 ± 1 g l^{-1}. Total ammonia and nitrite were controlled to be lower than 0.5 and 0.15 mg l^{-1}, respectively. For the inoculation experiments, shrimp were transported to the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University.

2.2 Determination of molt stage

Based on the descriptions of Robertson *et al.* (1987), Chan *et al.* (1988) and Corteel *et al.* (2012), the molt cycle of shrimp was determined and the shrimp were separated into 5 major stages. Briefly, shrimp were restrained and their exopodites of uropods were examined and analyzed on the appearance of setae, epidermis and cuticle under an inverted microscope at a magnification of 100x. In the early post-molt stage (A), the epidermis is present in the setae and retracts in later post-molt (B). In the inter-molt stage (C), the epidermis retracts under the setae and forms a straight line at the bottom of setae. In the early pre-molt (D1), the epidermis retracts from the old cuticle and starts forming a new cuticle. In the final stage, (before-molt, D2) new setae are formed under the old cuticle.

2.3 WSSV preparation

Preparation of WSSV stock

A WSSV Thai-1 used in this study was collected from infected *Penaeus monodon* in Thailand in 1996 and amplified in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal *et al.*, 2001). A homogenate of WSSV infected crayfish gills, kindly donated by P. Jiravanichpaisal and K. Soderhall (Uppsala University, Sweden), was inoculated in SPF *P. vannamei* juveniles to produce a starting WSSV stock. The median infectious titer of the stock was $10^{6.6}$ SID_{50} ml^{-1} as determined by in vivo intramuscular titration (Escobedo-Bonilla *et al.*, 2005).

Preparation of WSSV stocks (WSSV stock 1a & 1b and WSSV stock 2)

From the starting WSSV stock, a dilution of 10^{-2} was made in phosphate-buffered saline (PBS, pH 7.4) and injected intramuscularly into SPF *P. vannamei* juveniles to amplify the virus. Moribund shrimp were collected and confirmed to be WSSV positive by indirect immunoflourescence (IIF). Three inoculation stocks were prepared.

A. Stocks 1a & 1b: One hundred grams of moribund WSSV-infected shrimp were weighed and thawed. The shell, hepatopancreas and gut were removed and the remaining body was longitudinally cut into two parts. The first part was homogenized at 5000 rpm for 5 min using an IKA T 25 digital ultra-turrax. Then, the homogenate was further minced by serial syringe needles (1.2, 0.9 and 0.55 × 20 mm). Briefly, the homogenate was sucked up and blown out several times through the needle of 1.2 × 20 mm attached to a 20 ml syringe; this was repeated with needles of 0.9 × 20 mm and 0.55 × 20 mm, aliquoted and stored at - 70 °C for intramuscular injection and peroral inoculation experiments (stock 1a). The second part of WSSV infected tissue was cut into small pieces of 0.5-1 mm^2 and stored at - 70 °C for feeding (stock 1b).

B. Stock 2: For the preparation of WSSV stock 2, 50 g of thawed shrimp without shell, hepatopancreas and gut were chopped, suspended in PBS at a ratio of 1:3, homogenized at 5000 rpm for 1-1.5 min using IKA T 25 digital ultra-turrax and centrifuged at 3500 rpm for 10 min (4 °C). Then, supernatant was collected and stored at - 70 °C (WSSV stock 2).

2.4 Experimental design

2.4.1 Effect of physical composition of the viral inoculum on the oral infectivity of WSSV

The aim of this experiment was to compare the infectivity of a WSSV stock by determining the SID_{50} by intramuscular and peroral inoculation of a viral suspension and via feeding of infected tissue from the same shrimp. In the experiment, early pre-molt (D1) *P. vannamei* juveniles (MBW = 4.86 ± 0.37 g, *n* = 210) were collected and acclimated individually for 24 h in 10-liter tanks. Then, one group of twenty shrimp was injected with 50 mg of a 10-fold serial dilution (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, five animals per dilution) of WSSV stock 1a. Another group of twenty shrimp was inoculated perorally with 50 mg of a 10-fold serial dilution (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, five animals per dilution) of the same WSSV stock 1 a using a 0.74 × 19 mm - 24G Surflo-W catheter (a 10-fold serial dilution was prepared by mixing 1 portion of WSSV stock with 9 portions of pathogen-free shrimp minced tissue). Thirty shrimp of the third group were naturally fed per os with 0.5, 5, 50, 100, 250 and 500 mg of WSSV chopped tissue, with 5 shrimp per dose. Shrimp were fed one meal for the dose of 0.5, 5, 50 and 100 mg, 3 and 5 meals for the feeding of 250 and 500 mg of WSSV chopped tissue, respectively. The time interval between the two meals was 1 h. After inoculation, shrimp were housed individually and kept for 5 days. Cephalothoraxes and midguts of dead and moribund shrimp were collected every 12 h and terminated at 120 hpi. Samples of cephalothoraxes and midguts of dead, moribund and euthanized shrimp at 120 hpi were processed for detection of WSSV infection by IIF. The experiment was performed three times.

2.4.2 Role of PM as intestinal barrier

Removal of the PM

In this experiment, it was aimed to remove the PM by a peroral flushing of the midgut. A total of 24 *P. vannamei* juveniles (MBW = 4.62 ± 0.68 g) were screened for their molt stages (B, C, D_1, D_2). Six shrimp were selected in each of the four major molt stages and divided into two groups. Shrimp of both groups were fed with pathogen-free shrimp chopped tissue. The animals were starved then for 4 h. Shrimp in the first group were given a peroral flush using 1 mL of PBS. This was done with a 1 ml syringe attached to a 24G Surflo-W catheter. The catheter was gently inserted inside the shrimp mouth chamber. By a gentle press on the

plunger of the syringe, the PBS was forced through the shrimp digestive tract. Shrimp in the second group were not flushed.

Cryosection and staining of peritrophic membrane

After flushing, the fecal material that was expelled out of the anus was collected, fixed in 4% paraformaldehyde and incubated with 25 µg ml^{-1} fluorescein-linked succinylated WGA (wheat germ agglutinin lectin from *Triticum vulgarus*; Vector FL 1021S) for PM analysis. After flushing, shrimp of both groups were euthanized on ice and dissected (5 mm in length) at 3 sites (S_2, S_4, S_6, see Figure 1). Dissected tissues were fixed in 4% paraformaldehyde at room temperature (22 °C) for 15 min, washed with PBS for 15 min, embedded in 2% methylcellulose and frozen in liquid nitrogen liquid for 8 min. Cryosections (5 µm) were made and mounted on slides, washed with PBS for 5 min, incubated with fluorescein-linked succinylated WGA (25 µg ml^{-1}) for 30 min and Hoechst (10 µg ml^{-1}) for 15 min. Then, the slides were washed twice with PBS and once in deionised water (3 min each), dried and mounted with glycerin DABCO. The slides were analyzed by fluorescence microscopy (Leica DM RBE) and microphotographs were made at 100× magnification.

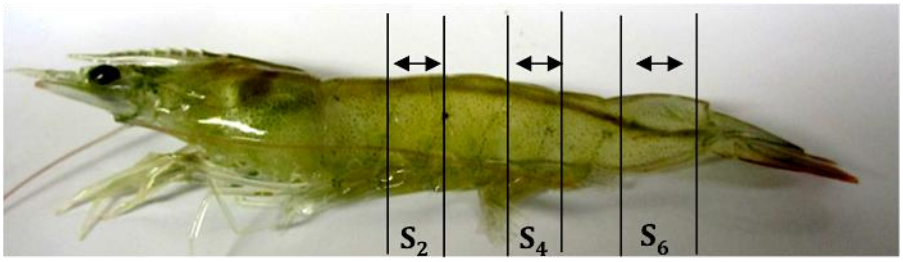


Figure 1. Three sampling sites for analysis of the peritrophic membrane. Segment S_2 and S_4 contain midgut, segment S_6 contains hindgut.

Effect of PM removal on WSSV infection upon peroral inoculation with WSSV

The aim of this experiment was to evaluate the barrier function of PM to WSSV infection via oral route. In this experiment, early pre-molt (D1) *P. vannamei* juveniles (MBW = 4.55 ± 1 g, *n* = 110) were screened, housed individually in 10 liter-aquaria and acclimated for 24 h. Prior to inoculation, shrimp were fed before a starvation period of 4 h. Then, fifteen shrimp were injected intramuscularly with 50 µl of a 10-fold serial dilution (10^{-6} to 10^{-8}, five animals per dilution) of WSSV stock 2. In twenty shrimp, the PM was removed by a peroral flush as described in sub-section "Removal of the PM" and twenty shrimp were kept intact. Afterwards, the animals were inoculated perorally with 50 µl of a 10-fold serial dilution (10^{0},

10^{-1}, 10^{-2}, 10^{-3}, five animals per dilution) of the same WSSV stock 2. After inoculation, shrimp were housed individually and fed with commercial shrimp diet at a rate of 5% of mean body weight per day. Moribund and dead shrimp were recorded and removed from the aquaria every 12 h until the end of the experiment at 120 hpi. Cephalothoraxes and midguts of dead, moribund and surviving shrimp were processed for detection of WSSV infection by IIF. The whole experiment was repeated twice. Five shrimp were used per dilution in the first repeat. In the second repeat, fifteen shrimp were used per dilution.

2.5 Detection of WSSV infection by indirect immunofluorescence (IIF)

WSSV infected shrimp were evaluated by indirect immunofluorescence (IIF) based on the description of Escobedo-Bonilla *et al.* (2005). Briefly, cephalothoraxes of dead, moribund and euthanized shrimp were collected, embedded in 2% methylcellulose and frozen at -20 °C. Cryosections (6 µm) were made and fixed for 10 min in 100% methanol at – 20 °C. The sections were washed three times in PBS (5 min each) and were incubated with 2 µg ml^{-1} of monoclonal antibody 8B7 (Diagxotics Inc.USA) directed against viral protein VP28 for 1 h at 37 °C. Then, samples were washed three times in PBS (5 min each), incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1h at 37 °C. The cell nuclei were stained by Hoechst for 15 min. Finally, the samples were washed twice with PBS, rinsed once in deionised water, dried and mounted with a solution of glycerine and 1,4-diaza-bicyclo-octane (DABCO) (ACROS organics, USA). Sections were analyzed by fluorescence microscopy (Leica DM RBE).

2.6 Statistical analysis

Virus infection titers (SID_{50}) (Sub-sections "Effect of physical composition of the viral inoculum on the oral infectivity of WSSV" and "Effect of PM removal on WSSV infection upon peroral inoculation with WSSV") were calculated based on the method of Reed and Muench (1938). Briefly, the numbers of infected and uninfected shrimp in each dilution were recorded. Accumulated values for the total number of animals that were infected or uninfected were obtained by adding values in the direction of the lowest to the highest values. The ratio and percentage of accumulated infected animals on the sum of the accumulated infected and uninfected animals were calculated for each dilution.

Two adjacent values, with one above (a) and one below (b) 50% were selected to calculate the proportional distance to the 50% endpoint by the following formula: (a-50%)/(a-b). The

proportional distance was added to the log10 of the dilution, that contained the percentage above 50% (a). The value of shrimp infectious dose 50% endpoint (SID_{50}) per ml was calculated taking into account to volume of the inoculum.

Shrimp of 3 replicates (Sub-section "Effect of PM removal on WSSV infection upon peroral inoculation with WSSV") were pooled into 2 groups (100 shrimp with and 100 shrimp without removal of the peritrophic membrane). The difference in infection rates between 2 groups was tested by Pearson's Chi-square test. All calculations were performed using R version 3.1.2.

3. Results

3.1 Effect of physical composition of the viral inoculum on the oral infectivity of WSSV

Shrimp inoculated intramuscularly with WSSV concentrations 10^{-6} to 10^{-8} of stock 1a (WSSV suspension finely minced with needles) had a mortality of 5 out of 5, 4 out of 5 and 1 out of 5, respectively. All shrimp inoculated with dilution 10^{-9} survived until 120 hpi. The same results were obtained in the second experiment. In the third repeat, 5 out of 5 shrimp in the dilution 10^{-6} and 4 in the dilution 10^{-7} died. Other shrimp survived until the end of the experiment. Upon oral inoculation with the dilutions 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} of the same WSSV stock 1a, only 2, 3, and 2 out of 5 shrimp inoculated with the dilution 10^{0} died in the three different repeats. When peroral feeding was performed with 0.5, 5, 50, 100, 250 and 500 mg of WSSV chopped tissue 1b, 0, 1, 0, 1, 2 and 3 animals out of 5 shrimp died, respectively, in the first experiment, 0, 0, 1, 2, 3 and 4 in the second experiment and 0, 0, 1, 2, 2 and 4 in the third repeat. IIF analysis of cephalothorax of dead, moribund and surviving shrimp revealed that all dead and moribund animals were WSSV positive, while all surviving shrimp were WSSV negative. Analysis of midgut of dead and moribund shrimp showed that connected tissue (CT) of the midgut of dead and moribund shrimp were WSSV positive, whereas epithelial cells (EC) were WSSV negative (Figure 2). The mean virus titers determined upon intramuscular injection, peroral inoculation and by feeding WSSV infected tissue were $10^{8.76 \pm 0.06}$, $10^{1.23 \pm 0.23}$ and $10^{0.73 \pm 0.12}$ SID_{50} g^{-1}, respectively (Table 1). Compared with the intramuscular route, $10^{7.53}$ times more virus was needed to infect a shrimp via oral inoculation, while $10^{8.03}$ times more virus was necessary to infect a shrimp via peroral feeding.

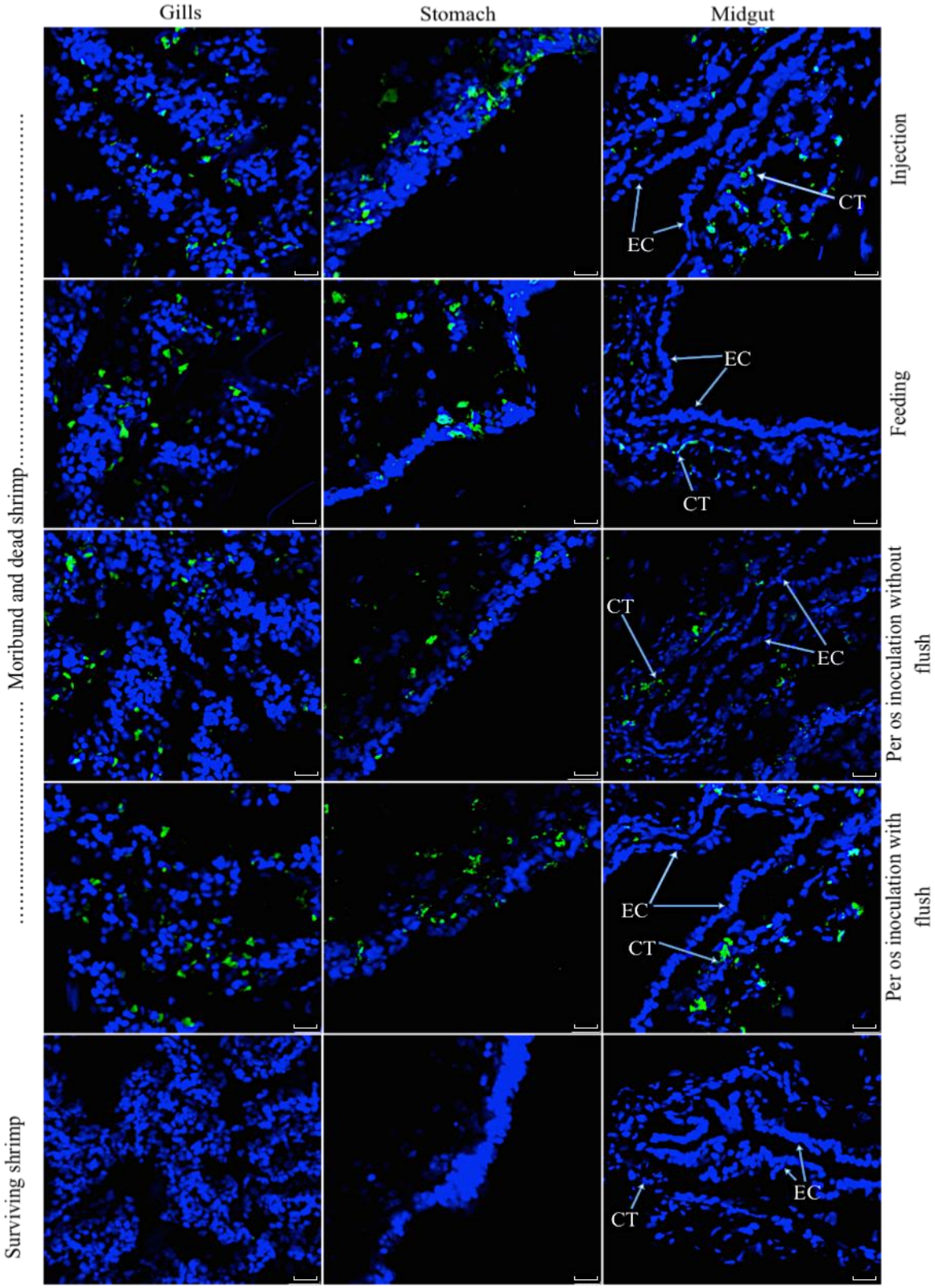


Figure 2. Representavive photomicrographs of viral antigen positive cells (green) in different tissues of moribund and dead shrimp. WSSV was detected by IIF using a VP28-specific mouse monoclonal antibody and an FITC-conjugated goat-anti mouse IgG. The cells of connective tissue (CT) of the midgut were WSSV positive, while the epithelial cells (EP) were WSSV negative. Bar = 50 µm.

Table 1. Determination of virus titers of a WSSV stock in *P. vannamei* by different inoculations. Intramuscular injection, peroral inoculation and feeding (feed).

Experiment	Inoculation route	Dilution of homogenate or amount of tissue (mg)	No. of shrimp	Mortality at different time points (hpi)									Infection by IIF	Virus titer
				24	36	48	60	72	84	96	120	Total		
1	intramuscular	10^{-7}	5		1	1	1		1			4/5	4/5	$10^{8.8}SID_{50}\ g^{-1}$
		10^{-8}	5				1					1/5	1/5	
		10^{-9}	5									0/5	0/5	
	peroral	10^{0}	5			1	1					2/5	2/5	$10^{1.1}SID_{50}\ g^{-1}$
		10^{-1}	5									0/5	0/5	
		10^{-2}	5									0/5	0/5	
		10^{-3}	5									0/5	0/5	
	feed	500	5					3				3/5	3/5	$10^{0.6}SID_{50}\ g^{-1}$
		250	5		1	1						2/5	2/5	
		100	5		1							1/5	1/5	
		50	5									0/5	0/5	
		5	5		1							1/5	1/5	
		0.5	5									0/5	0/5	
2	intramuscular	10^{-6}	5		1	3		1				5/5	5/5	$10^{8.8}SID_{50}\ g^{-1}$
		10^{-7}	5			1	2	1				4/5	4/5	
		10^{-8}	5				1					1/5	1/5	
		10^{-9}	5									0/5	0/5	
	peroral	10^{0}	5				2	1				3/5	3/5	$10^{1.5}SID_{50}\ g^{-1}$
		10^{-1}	5									0/5	0/5	
		10^{-2}	5									0/5	0/5	
		10^{-3}	5									0/5	0/5	
	feed	500	5		1		2	1				4/5	4/5	$10^{0.8}SID_{50}\ g^{-1}$
		250	5		2	1						3/5	3/5	
		100	5				2					2/5	2/5	
		50	5				1					1/5	1/5	
		5	5									0/5	0/5	
		0.5	5									0/5	0/5	
3	intramuscular	10^{-6}	5		2	3						5/5	5/5	$10^{8.7}SID_{50}\ g^{-1}$
		10^{-7}	5			2	2					4/5	4/5	
		10^{-8}	5									0/5	0/5	
		10^{-9}	5									0/5	0/5	
	peroral	10^{0}	5				1	1				2/5	2/5	$10^{1.1}SID_{50}\ g^{-1}$
		10^{-1}	5									0/5	0/5	
		10^{-2}	5									0/5	0/5	
		10^{-3}	5									0/5	0/5	
	feed	500	5				2	1	1			4/5	4/5	$10^{0.8}SID_{50}\ g^{-1}$
		250	5				1	1				2/5	2/5	
		100	5					2				2/5	2/5	
		50	5					1				1/5	1/5	
		5	5									0/5	0/5	
		0.5	5									0/5	0/5	

3.2 Role of PM as intestinal barrier

Removal of PM by a peroral flush

Microscopical observation of cryosections showed that three structures were stained with fluorescein-linked succinylated WGA: the PM, the basement membrane (BM) and the cuticle that lined hindgut lumen (CLH, only in the hindgut). The PM was absent in the midgut (sections S_2 and S_4) and hindgut (section S_6) in the shrimp that were flushed perorally, while the PM was clearly visible in the control samples at the 3 sampled segments (Figure 3, Additional file 1 and Table 2). The PM in the midgut of intact control shrimp stained with FITC-WGA consisted of multiple layers (Figure 3 and Additional file 1). The intensity of the FITC-WGA staining of the fecal material collected after flushing increased from the anterior to posterior position (Figure 4).

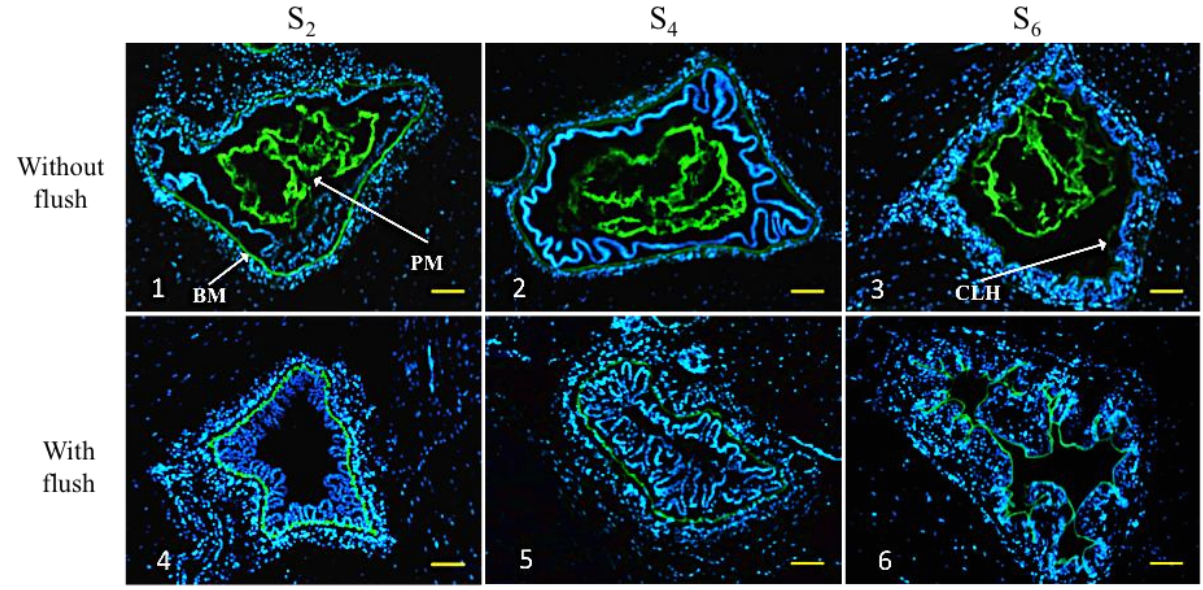
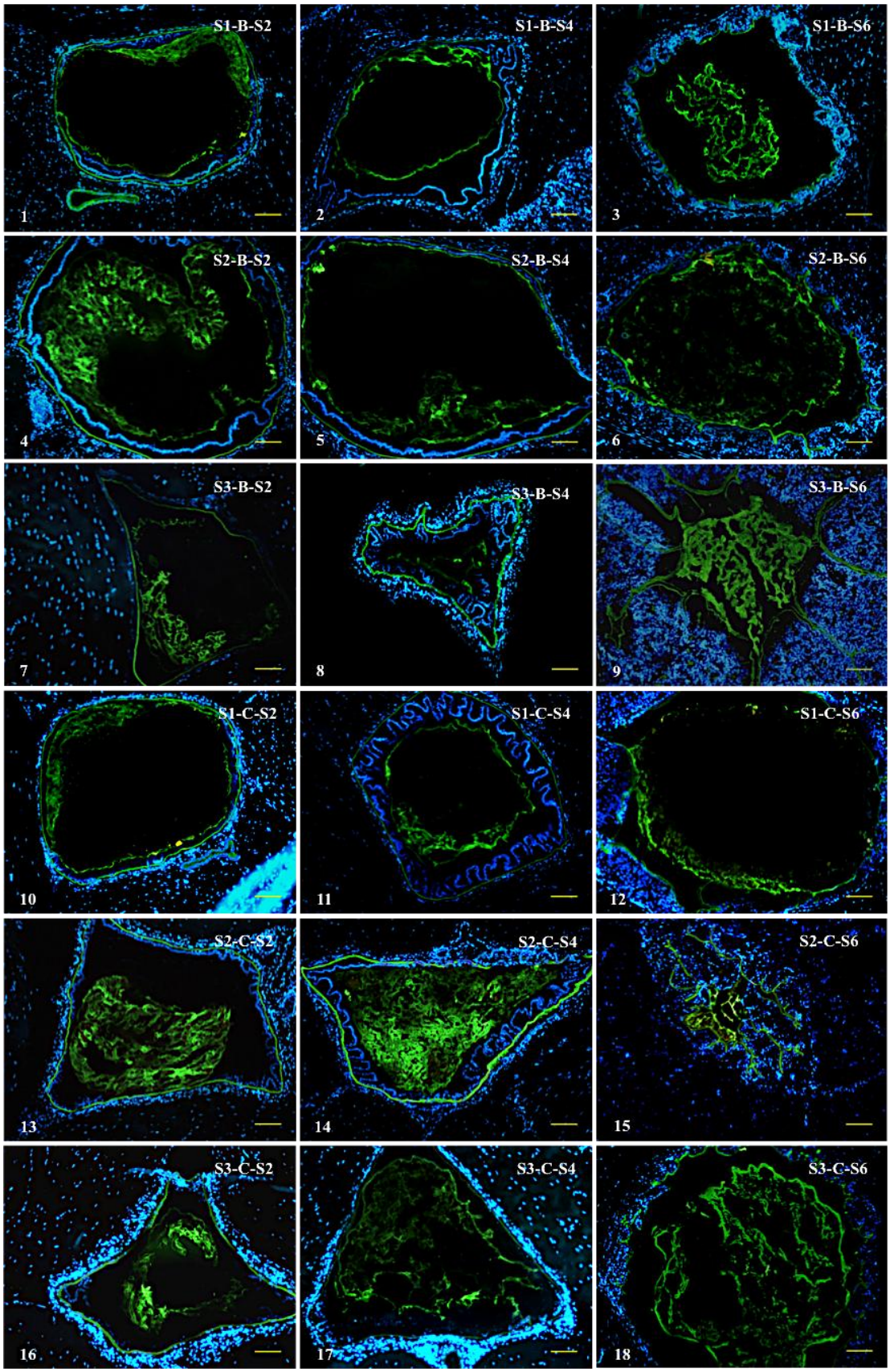


Figure 3. Presence/absence of peritrophic membrane in the gut of shrimp without and with a peroral flush. Peritrophic membrane (PM) was stained with FITC-linked succinylated (WGA) wheat germ agglutinin and analyzed by fluorescence microscopy. Bar = 100 µm. The cell nuclei were stained by Hoechst. Photomicrographs 1 to 3 show the presence of PM in the midgut (segment S_2 and S_4) and hindgut lumen (segment S_6) of control samples (without a peroral flush). Photomicrographs 4 to 6 show the absence of PM in the midgut and hindgut lumen of perorally flushed shrimp. FITC-linked succinylated WGA also labeled the basement membrane (BM) of the midgut (photomicrographs 1, 2, 4 and 5) and the cuticle that lined the hindgut lumen (CLH, photomicrographs 3 and 6).


S1-B-S2
1
S1-B-S4
2
S1-B-S6
3
S2-B-S2
4
S2-B-S4
5
S2-B-S6
6
S3-B-S2
7
S3-B-S4
8
S3-B-S6
9
S1-C-S2
10
S1-C-S4
11
S1-C-S6
12
S2-C-S2
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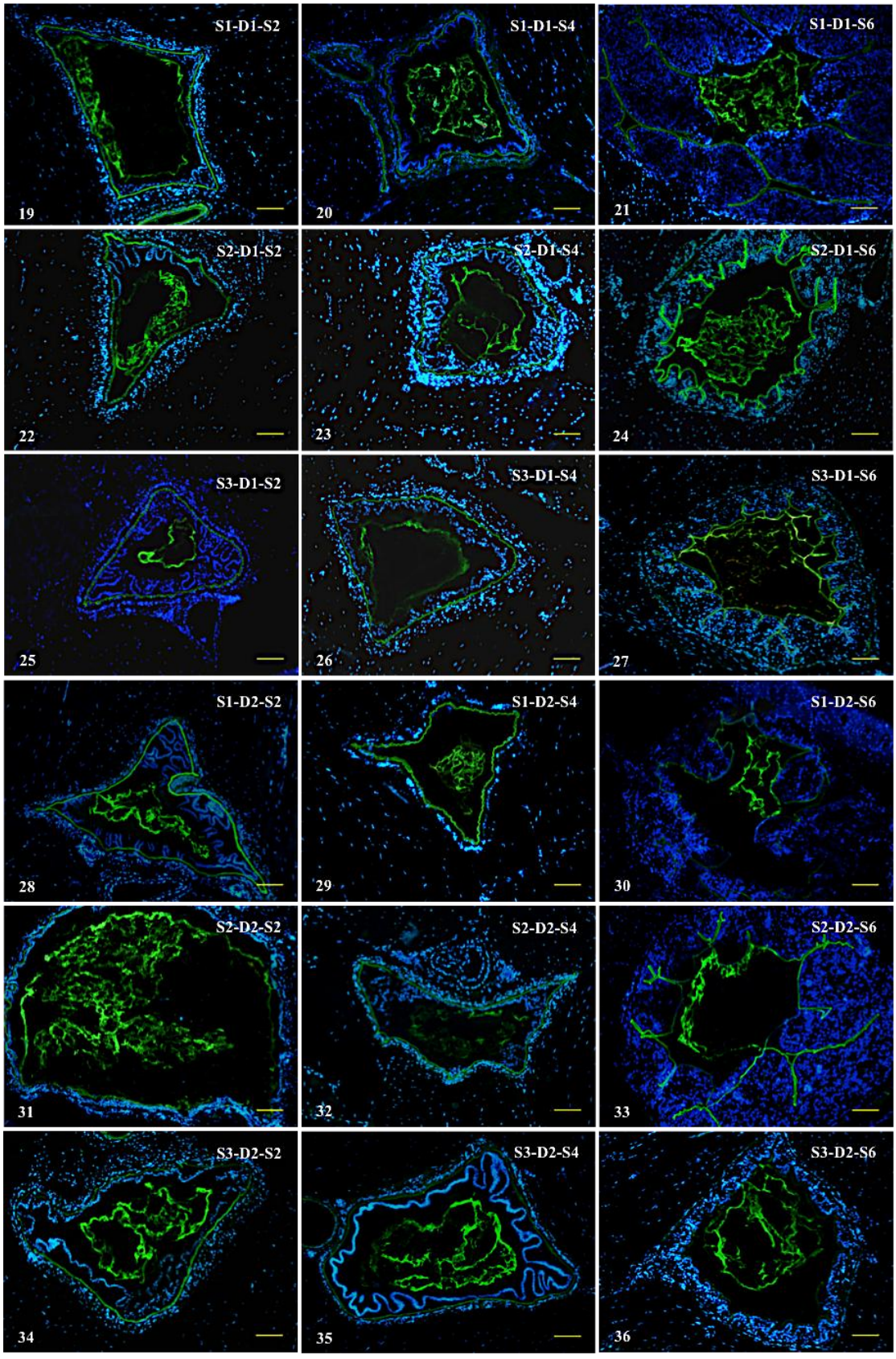
17
S3-C-S6
18


S1-D1-S2
19
S1-D1-S4
20
S1-D1-S6
21
S2-D1-S2
22
S2-D1-S4
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S2-D1-S6
24
S3-D1-S2
25
S3-D1-S4
26
S3-D1-S6
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S1-D2-S2
28
S1-D2-S4
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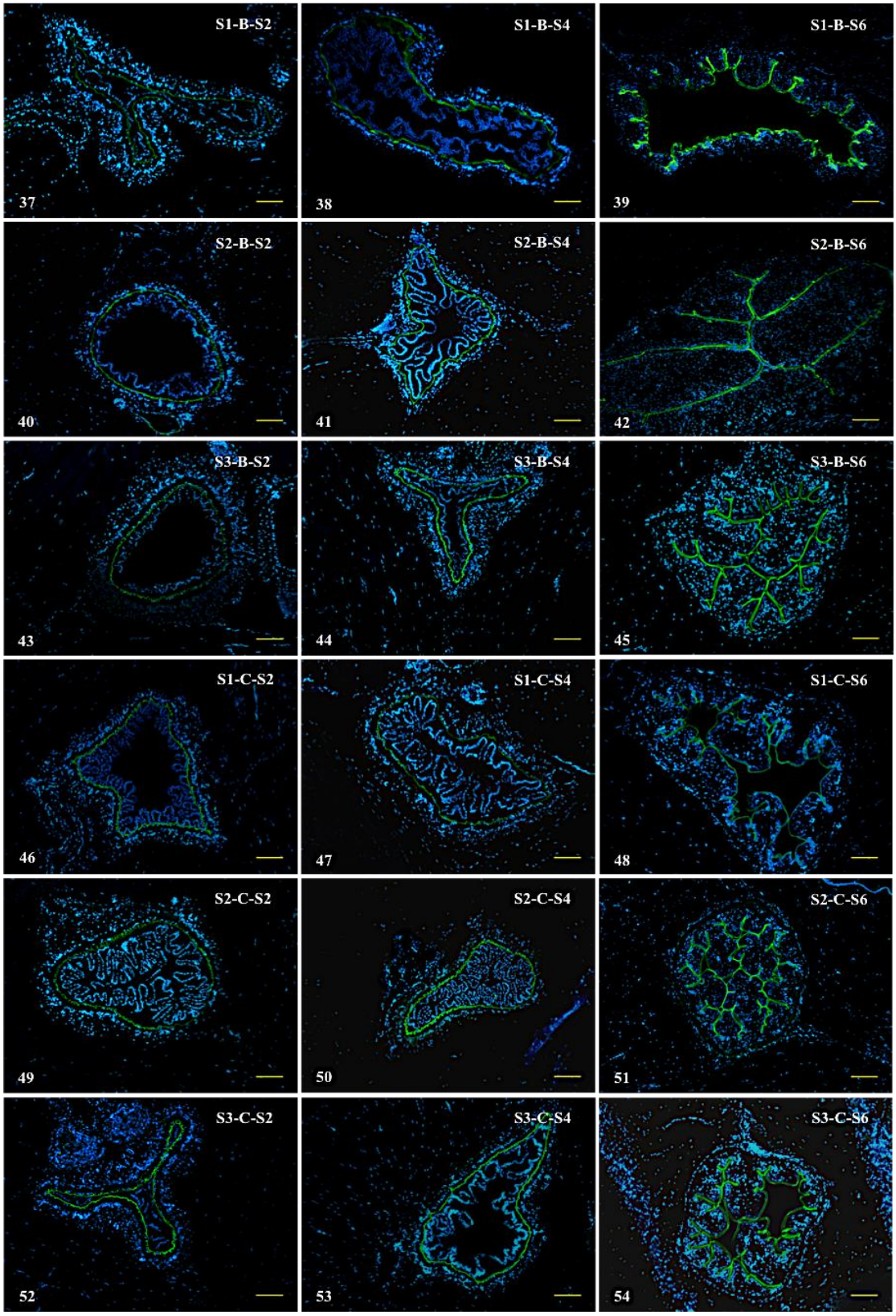
S3-D2-S6
36


S1-B-S2
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S1-B-S4
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S1-B-S6
39
S2-B-S2
40
S2-B-S4
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42
S3-B-S2
43
S3-B-S4
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S3-B-S6
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S1-C-S2
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S1-C-S4
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S1-C-S6
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S2-C-S2
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S3-C-S4
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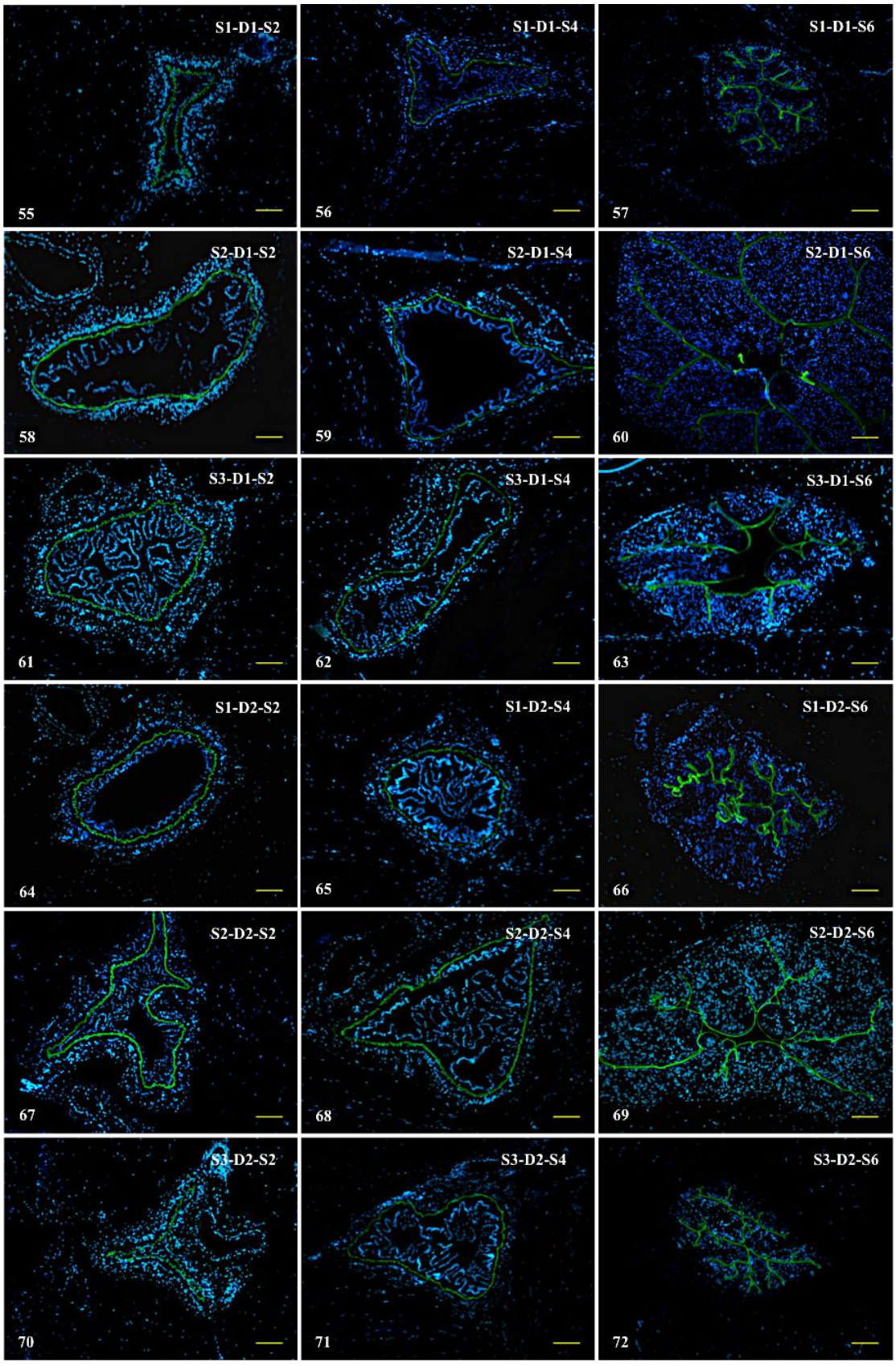
S3-C-S6
54


S1-D1-S2
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S1-D1-S4
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S1-D1-S6
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S2-D1-S2
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S2-D1-S4
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S2-D1-S6
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S3-D1-S2
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S3-D1-S4
62
S3-D1-S6
63
S1-D2-S2
64
S1-D2-S4
65
S1-D2-S6
66
S2-D2-S2
67
S2-D2-S4
68
S2-D2-S6
69
S3-D2-S2
70
S3-D2-S4
71
S3-D2-S6
72

Additional file 1. Detection of peritrophic membrane in the gut of shrimp without and with a peroral flush. Peritrophic membrane was stained with FITC-linked succinylated WGA wheat germ agglutinin, cell nuclei with Hoechst and analyzed by fluorescence microscopy. Bar = 100 µm. Photomicrographs 1 to 36 show the presence of PM in the midgut (segment S_2 and S_4) and hindgut (segment S_6) of control samples (without a peroral flush). Photomicrographs 37 to 72 show the absence of PM in the midgut and hindgut of perorally flushed shrimp. Code explanation: S1-B-S2; S1: shrimp number 1, B: shrimp in stage B of the molt cycle, S2: cross-section at segment 2.

Table 2. The presence of peritrophic membrane in *P. vannamei* shrimp without and with a peroral flush. Midgut sample sites (S_2, S_4), hindgut sample site (S_6).

Peroral flush	Molt stage	No. of shrimp	PM confirmed by FITC- WGA			No. of shrimp with the presence of PM
			S_2	S_4	S_6	
No (Control)	B	3	+ + +	+ + +	+ + +	3/3
	C	3	+ + +	+ + +	+ + +	3/3
	D1	3	+ + +	+ + +	+ + +	3/3
	D2	3	+ + +	+ + +	+ + +	3/3
Yes	B	3	− − −	− − −	− − −	0/3
	C	3	− − −	− − −	− − −	0/3
	D1	3	− − −	− − −	− − −	0/3
	D2	3	− − −	− − −	− − −	0/3

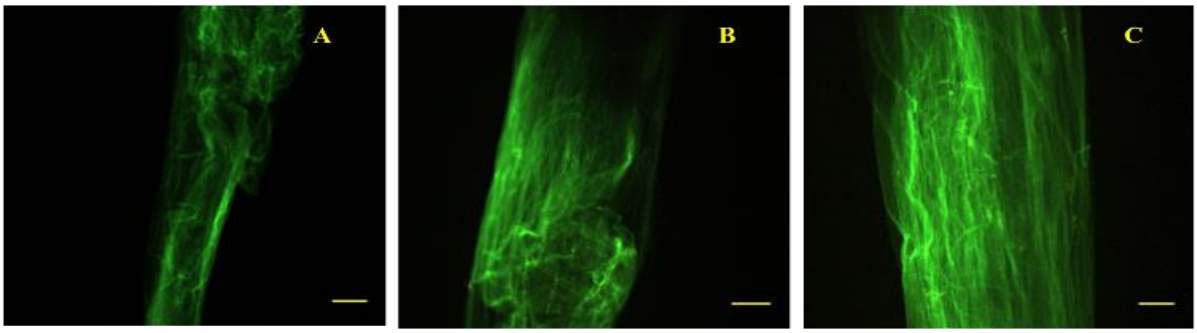


Figure 4. FITC-WGA labeled peritrophic membrane enclosing a feed bolus, collected upon peroral flushing. (A) Anterior region. (B) Middle region. (C) Posterior region. Bar = 100 µm.

Effect of PM removal on WSSV infection upon peroral inoculation with WSSV

In the first experiment, among the groups of shrimp injected with dilutions 10^{-6}, 10^{-7} and 10^{-8} of WSSV stock 2, all shrimp in dilution 10^{-6} and 3 out of 5 shrimp in dilution 10^{-7} died between 36 and 60 hpi. All other animals survived until the end of the experiment. When a peroral inoculation was performed in PM-intact shrimp with dilutions 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} of the same WSSV stock, only 2 out of 5 shrimp of dilution 10^{0} died. In PM-negative shrimp,

mortality was observed in 2 shrimp of dilution 10^0 and 1 shrimp of dilution of 10^{-1}. All other shrimp survived until the end of the experiment.

In the second experiment, among the groups of shrimp injected with 10^{-6}, 10^{-7} and 10^{-8} of WSSV stock 2, all shrimp in dilution 10^{-6} and 4 out of 5 shrimp in dilution 10^{-7} died between 36 and 60 hpi. All other shrimp survived until the end of the experiment. When a peroral inoculation was performed in PM-intact shrimp with 10^0, 10^{-1}, 10^{-2} and 10^{-3} of WSSV stock 2, 1 out of 5 shrimp in dilution 10^0, 1 in dilution 10^{-1}, 1 in dilution 10^{-2} died between 36 and 60 hpi. Peroral inoculation of PM-negative shrimp induced mortality in 3 shrimp with dilution 10^0 and 1 in dilution 10^{-1} between 48 and 60 hpi. Other shrimp survived until the end of the experiment.

In the third experiment, in the groups of shrimp injected with WSSV inoculum diluted 10^{-6} to 10^{-8}, all shrimp in the dilution 10^{-6} and 12 out of 15 shrimp in the dilution 10^{-7} died between 36 and 84 hpi. Other shrimp survived until the end of the experiment. When shrimp were inoculated perorally with 10^0, 10^{-1}, 10^{-2} and 10^{-3} of WSSV stock 2, 5 deaths out of 15 PM-intact shrimp in dilution 10^0 and 1 in dilution 10^{-1} were recorded between 36 and 60 hpi. Peroral inoculation in PM-negative shrimp caused mortality in 11 shrimp in dilution 10^0 and 2 shrimp in dilution 10^{-1}. All other shrimp survived until the end of the experiment at 120 hpi. IIF analysis of cephalothorax of dead, moribund and surviving shrimp revealed that all dead and moribund animals were WSSV positive, while all surviving shrimp were WSSV negative. Analysis of midgut of dead and moribund shrimp showed that connected tissue (CT) of the midgut of dead and moribund shrimp were WSSV positive, whereas the epithelial cells (EC) of the midgut were WSSV negative (Figure 2). The mean virus titers that were determined in the three experiments upon intramuscular injection and peroral inoculation of shrimp with and without PM were $10^{8.63 \pm 0.12}$, $10^{1.13 \pm 0.06}$ and $10^{1.53 \pm 0.21}$ SID_{50} ml^{-1}, respectively (Table 3). Compared with the intramuscular route, $10^{7.5}$ times more virus was needed to infect a PM-intact shrimp, while $10^{7.1}$ times more virus was necessary to infect a PM-negative shrimp via oral inoculation. The chi-square test on infection rates of shrimp showed that there was not a significant effect of removal of PM on the susceptibility of shrimp to WSSV infection ($p > 0.05$).

Table 3. Infection titers of a WSSV stock in *P. vannamei* without and with removal of the peritrophic membrane. Intramuscular injection, peroral inoculation.

Experiment	Inoculation route	Removal of PM	Dilution of tissue homogenate	No. of shrimp	Mortality at different time points (hpi)									Infection by IIF	Virus titer
					24	36	48	60	72	84	96	120	Total		
1	intramuscular	No	10^{-6}	5		2	2	1					5/5	5/5	$10^{8.5}$ SID_{50} ml^{-1}
		No	10^{-7}	5			1	2					3/5	3/5	
		No	10^{-8}	5									0/5	0/5	
	peroral	No	10^{0}	5			2						2/5	2/5	$10^{1.1}$ SID_{50} ml^{-1}
		No	10^{-1}	5									0/5	0/5	
		No	10^{-2}	5									0/5	0/5	
		No	10^{-3}	5									0/5	0/5	
		Yes	10^{0}	5		1	1						2/5	2/5	$10^{1.3}$ SID_{50} ml^{-1}
		Yes	10^{-1}	5						1			1/5	1/5	
		Yes	10^{-2}	5									0/5	0/5	
		Yes	10^{-3}	5									0/5	0/5	
2	intramuscular	No	10^{-6}	5		2	1	2					5/5	5/5	$10^{8.7}$ SID_{50} ml^{-1}
		No	10^{-7}	5			2	2					4/5	4/5	
		No	10^{-8}	5									0/5	0/5	
	peroral	No	10^{0}	5		1							1/5	1/5	$10^{1.2}$ SID_{50} ml^{-1}
		No	10^{-1}	5				1					1/5	1/5	
		No	10^{-2}	5				1					1/5	1/5	
		No	10^{-3}	5									0/5	0/5	
		Yes	10^{0}	5			1	2					3/5	3/5	$10^{1.6}$ SID_{50} ml^{-1}
		Yes	10^{-1}	5			1						1/5	1/5	
		Yes	10^{-2}	5									0/5	0/5	
		Yes	10^{-3}	5									0/5	0/5	
3	intramuscular	No	10^{-6}	15		2	4	4	4	1			15/15	15/15	$10^{8.7}$ SID_{50} ml^{-1}
		No	10^{-7}	15		1	2	4	4	1			12/15	12/15	
		No	10^{-8}	15									0/15	1/15	
	peroral	No	10^{0}	15		1	1	3					5/15	5/15	$10^{1.1}$ SID_{50} ml^{-1}
		No	10^{-1}	15		1							1/15	1/15	
		No	10^{-2}	15									0/15	0/15	
		No	10^{-3}	15									0/15	0/15	
		Yes	10^{0}	15		2	3	2	3	1			11/15	11/15	$10^{1.7}$ SID_{50} ml^{-1}
		Yes	10^{-1}	15			2						2/15	2/15	
		Yes	10^{-2}	15									0/15	0/15	
		Yes	10^{-3}	15									0/15	0/15	

4. Discussion

In vivo titration is generally used to define the infectivity of a WSSV stock (Escobedo-Bonilla *et al.*, 2005; Escobedo-Bonilla *et al.*, 2006; Corteel *et al.*, 2009). In the present study, the infectivity of WSSV was first determined in *P. vannamei* by intramuscular injection and

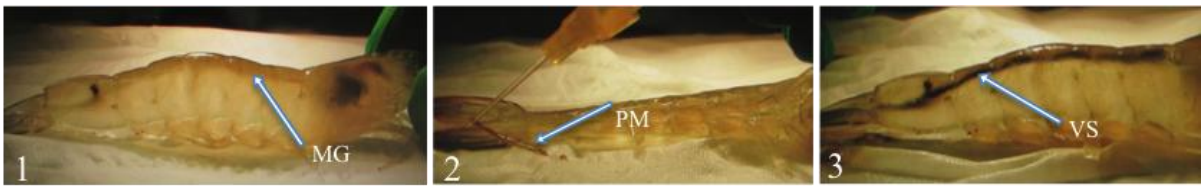
peroral inoculation. The results indicated that a homogenate of WSSV infected shrimp is highly infectious when directly injected into shrimp. In contrast, there are strong restrictions on the infection of shrimp via the digestive system even when the PM is removed. These findings were in conflict with earlier results from our group published by Escobedo-bonilla *et al.* (2006) In that paper, it was shown that shrimp could be easily infected upon peroral inoculation using a rigid plastic pipette tip (no. 790004 Biozym). However, by using dye, we could demonstrate that the fluid was crossing the gastrointestinal tract and was entering the hemocoel. Therefore, we have changed the inoculation device. In the present study, we have used a softer and flexible 24G Surflo-W catheter. By the use of dye, it was demonstrated that peroral inoculation of shrimp with this 24G Surflo-W catheter is not damaging the gastrointestinal tract. The findings in the present study were also in contrast with other researchers who detected a 100% mortality by feeding infected shrimp (Lightner *et al.*, 1998; Wang *et al.*, 1999). Different explanations can be forwarded. In the latter studies, animals were pooled in the same tank, fed with WSSV infected tissue shrimp for several days and terminated often after more than 5 days. It is very well possible that a larger amount of virus has been given with the feed and that cannibalism occurred when a few primarily infected shrimp became infected and died, resulting in large amounts of virus becoming homogeneously distributed in the water of the tanks. This may have triggered infection via waterborne route. However, our results are in agreement with the findings of others. Laramore (2007) reported that individual peroral feeding of *P. vannamei* with WSSV infected tissue at 10% of their body weight did not result in a 100% mortality. Another study on peroral infection of *P. vannamei* with 200 µl of a WSSV inoculum, containing 10^7 WSSV genome copies ml^{-1}, conducted by Gitterle *et al.* (2006) also showed that cumulative mortality of shrimp was less than 100%.

In order to understand the factors determining virus infectivity via oral route, it is very important to have a good knowledge on the anatomy of the host digestive system. In decapod crustaceans, the digestive system is divided into three major regions: foregut, midgut, and hindgut. The foregut is composed of the mouth, esophagus, cardiac and pyloric stomach chambers, which are covered with a cuticle layer. The food ingested via the mouth moves through the esophagus and ends into the cardiac stomach chamber. Through the process of cutting, crushing, mixing by the lateral teeth systems and filtering by a cardiac setal screen in the cardiac chamber, the processed food is drained into the pyloric chamber. In the pyloric region, the processed material is further sorted by the ampullary setal screen of gland filters into a liquid form for further digestion in the hepatopancreas and particles for subsequent

transport into the midgut region (Felgenhauer and Abele, 1985; Lin, 1996; McGaw and Curtis, 2013). The midgut region secretes the peritrophic membrane which wraps the material coming from the pyloric chamber (Martin *et al.*, 2006; Wang *et al.*, 2012). The hindgut is a simple cuticle lined tube, that functions in expelling the peritrophic membrane, containing the feces (Barker and Gibson, 1978; Dall and Moriarty, 1983). In several species of crustaceans, the mesh size of the ampullary setal screen is estimated to be smaller than 100 nm (Kunze and Anderson, 1979; Ngoc‐Ho, 1984). The pore size of midgut peritrophic membrane can be as small as 20 nm (Martin *et al.*, 2006). From these data and the size of WSSV of 70-380 nm (Wang *et al.*, 1995), it is likely that if the internal barriers of cuticle and peritrophic membrane are not ruptured, WSSV can not reach the epithelial cells. That is why, in the present study, it was examined if removal of the PM could facilitate the infection of the underlying epithelial cells. In the present study, N-acetyl-D-glucosamine of the PM was stained with FITC-WGA and staining of cryosections of midgut and feces revealed that the PM of *P. vannamei* is multilayered, which is similar to what has been described in penaeid shrimp by Wang *et al.* (2012) and Martin *et al.* (2006). The authors described that *P. vannamei* possesses a type I of PM, that is continuously formed on the surface of epithelial midgut cells and consists of three stages: PM in stage 1 and 2 is closely attached to the microvilli of epithelial midgut cells, and PM in stage 3 is detached. After the removal of the PM by a peroral flush and directly thereafter the peroral inoculation of WSSV suspension, it was observed that the inoculum was filling the complete gastrointestinal tract (Additional file 2). Therefore, the virus had direct access to the epithelial cells. Within this short time frame, the epithelial cells were not able to produce a new PM. However, this PM removal did not facilitate WSSV infection of the underlying epithelial cells. This observation was in agreement with that found by Arts (2007). The author reported that nuclei of epithelial midgut cells of WSSV-infected *Penaeus monodom* were negative with WSSV by VP28-immunoreactive and electron microscopy study. This is indicative for a state of resistance of epithelial cells to infection and the absence of receptors on the luminal surface of these cells. In addition, the infectivity of WSSV in shrimp via peroral infection may be largely decreased by digestive enzymes. This impact could be similar to the one described for insects (Brackney *et al.*, 2008). Another factor that may limit the penetration of WSSV through shrimp midgut is the basement membrane, a firm layer of connective tissue underneath the epithelial cells. In insects, the basement membrane is well known to prevent virus entry into the hemocoel (Mellon, 1992; Martin *et al.*, 2004; Passarelli, 2011). In the present study, the

basement membrane could be visualized by FITC-WGA which is in agreement with the finding of Martin *et al.* (2006). When one considers all barriers and viral unfriendly environmental factors that WSSV encounters in the digestive system of shrimp, it is easy to understand why WSSV is poorly infectious for shrimp via peroral route.

In conclusion, the present study revealed that a homogenate of WSSV infected shrimp is highly infectious when injected into shrimp, whereas it is poorly infectious via oral route. Removal of the PM slightly but not significantly facilitated the infection of shrimp. From these findings, it is highly questioned if per os uptake of WSSV is the major route of spread for the virus. More work needs to be done on finding more important portals of entry for WSSV in shrimp which will finally lead to the development of more effective WSSV control measures.



Additional file 2. Presence of virus inoculum in the midgut of shrimp after a peroral inoculation. (1) Before a per os flush to remove the PM, the midgut (MG) of shrimp was visible in the back of the animal. (2) After a per os flush, the PM was coming out of the anus. (3) Afterwards, the shrimp was inoculated with 50 µl of WSSV suspension (VS), directly the removal of the PM. The inoculum was present all over the gastrointestinal tract as can be seen in the figure.

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Chapter 4

Effects of acute change in salinity and molting on the infection of white leg shrimp *(Penaeus vannamei)* with white spot syndrome virus upon immersion challenge

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Abstract

In the field, molting and salinity drop of the water due to excessive rainfall have been mentioned to be risk factors for WSSV outbreaks. Therefore, in the present study, the effect of an acute change in environmental salinity and shedding of the old cuticle shell on the susceptibility of *Penaeus vannamei* to WSSV was evaluated by immersion challenge. For testing the effect of abrupt salinity stress, early pre-molt shrimp that were acclimated to 35 g l^{-1} were subjected to salinities of 50 g l^{-1}, 35 g l^{-1}, 20 g l^{-1}, 10 g l^{-1}, 7 g l^{-1} or 5 g l^{-1} and simultaneously exposed to $10^{5.5}$ SID_{50} ml^{-1} of WSSV for 5 hours, after which the salinity was brought back to 35 g l^{-1}. Shrimp that were transferred from 35 g l^{-1} to 50 g l^{-1}, 35 g l^{-1} and 20 g l^{-1} did not become infected with WSSV. Shrimp became infected with WSSV after an acute salinity drop from 35 g l^{-1} to 10 g l^{-1} and lower. The mortality in shrimp, subjected to a salinity change to 10 g l^{-1}, 7 g l^{-1} and 5 g l^{-1}, was 6.7%, 46.7% and 53.3%, respectively ($p < 0.05$). For testing the effect of molting, shrimp in early pre-molt, molting and post-molt were immersed in seawater containing $10^{5.5}$ SID_{50} ml^{-1} of WSSV. The resulting mortality due to WSSV infection in shrimp inoculated during early pre-molt (0%), ecdysis (53.3%), and post-molt (26.72%) demonstrated that a significant difference exists in susceptibility of shrimp during the short molting process ($p < 0.05$). The findings of this study indicate that during a drop of environmental salinity lower than 10 g l^{-1} and ecdysis, shrimp are at risk for a WSSV infection. These findings have important implications for WSSV control measures.

1. Introduction

Known as a euryhaline organism with strong salinity tolerance, *P. vannamei* has been widely cultured in low salinity water. Its culture contributed significantly to the increase of global shrimp aquaculture production (Bray *et al.*, 1994; Saoud *et al.*, 2003; Green, 2008). With the rapid expansion, more infectious diseases such as white spot syndrome (WSS) and acute hepatopancreatic necrosis disease (AHPND) have occurred. An approach to control infectious diseases in shrimp is to better understand the factors determining pathogen infection and to identify the portals of entry.

Since its first report in 1992, WSSV has become one of the most devastating viruses causing huge economic losses in shrimp aquaculture industry worldwide (Escobedo-Bonilla *et al.*, 2008; Lightner, 2011). Over a few decades, despite many attempts to prevent disease, no effective control measures have been found. Early studies on the transmission of WSSV reported that WSSV infected hosts can transmit the virus via horizontal and vertical routes (Lo *et al.*, 1997; Kanchanaphum *et al.*, 1998; Soto *et al.*, 2001), and depicted the image that WSSV is highly contagious in penaeid shrimp (Lightner *et al.*, 1998; Escobedo-Bonilla *et al.*, 2005). However, recently, some experimental studies have reported the difficulty to infect shrimp via immersion, cohabitation as well as peroral feeding (Gitterle *et al.*, 2006; Laramore, 2007; Corteel *et al.*, 2009; Tuyen *et al.*, 2014; Thuong *et al.*, 2016). Prior *et al.* (2003) used large amounts of infectious virus for immersion infection, but they failed to determine the lethal immersion dose of WSSV. Gitterle *et al.* (2006) indicated that only adding virus to the water was not sufficient to result in shrimp infection. Also more recently, Corteel *et al.* (2009) were not successful in infecting early pre-molt shrimp by immersion. Durand and Lightner (2002) concluded that a minimum of 10^5 WSSV copies ml^{-1} was necessary to induce WSSV infection via immersion.

When reviewing the occasional occurrences of WSSV outbreaks in traditional penaeid shrimp farms, higher incidences of WSSV outbreaks are reported during rainy season (Rodríguez *et al.*, 2003; Peinado-Guevara and López-Meyer, 2006; Gunalan *et al.*, 2010; Tendencia *et al.*, 2010). Generally, rainfall affects water temperature, salinity, pH and dissolved oxygen through various processes. An increase in rainfall dilutes the pond water and results in lower salinity and water temperature. These changes over a particular range may increase the susceptibility of shrimp to microbial infections (Liu *et al.*, 2006; Joseph and Philip, 2007; Ramos-Carreño *et al.*, 2014).

Ecdysis, a process of shedding the old cuticle and re-generating a new one, is an essential step for the growth of most species of crustacea during the course of their life. During molting, the animal shows a dramatic change in biology, behavior and structure of cuticle (Chan *et al.*, 1988; Promwikorn *et al.*, 2007). Especially, water absorption has been described as an essential phenomenon of molting in crustacea. In spiny lobster, *Panulirus argus,* the body-wet weight of the animal during ecdysis was reported to increase by approximately 10% (Travis, 1954). The major route of water uptake at ecdysis is most probably happening through the digestive tract (Neufeld and Cameron, 1994; Chung *et al.*, 1999). In addition, it is also believed to occur through the soft inter-segmental membranes and gills (Dall and Smith, 1978). At present, it is not clear if the abrupt change in environmental salinity conditions and the process of getting out of the old exoskeleton may facilitate the entry for WSSV into shrimp via waterborne route. Thus, the present study was set-up (i) to evaluate the effect of an acute salinity change on the susceptibility of *P. vannamei* to WSSV infection and (ii) to investigate the susceptibility of shrimp during the molting process to WSSV infection via waterborne route.

2. Materials and methods

2.1 Experimental animals

Specific pathogen-free (SPF) penaeid shrimp, *Litopenaeus vannamei*, with a mean body weight (MBW) of 2.8 g imported from Holland were reared in the laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. Shrimp were cultured in a bio-filter circulation system, fed with pelleted feed at a rate of 5% of their mean body weight per day. Temperature and salinity of the culture system were maintained at 27 ± 1 ^{o}C and 35 ± 1 g l^{-1}. Total ammonia and nitrite were controlled to be lower than 0.5 and 0.15 mg l^{-1}, respectively. For the experiments, shrimp were transported to the laboratory of Virology, Faculty of Veterinary Medicine, Ghent University.

2.2 Determination of molt stages

Based on the descriptions of Robertson *et al.* (1987), Chan *et al.* (1988), Corteel *et al.* (2012), the molt cycle of shrimp was determined and separated into 6 major stages. In brief, shrimp were restrained and their exopodites of uropods were examined and analyzed on the

appearance of setae, epidermis and cuticle under an inverted microscope at a magnification of 200x. In the early post-molt stage (A), the epidermis is present in the setae and retracts in later post-molt (B). In the inter-molt stage (C), the epidermis retracts under the setae and forms a base line at the bottom of setae. The internal cone is visual in the lumen of setae. In early pre-molt (D1), the epidermis retracts from the old cuticle and starts to form a new cuticle. In before-molt ($D2_a$), new setae are formed under the old cuticle. During the ready to molt stage ($D2_b$), new setae are interrupted by folds of the old setae (Figure 1).

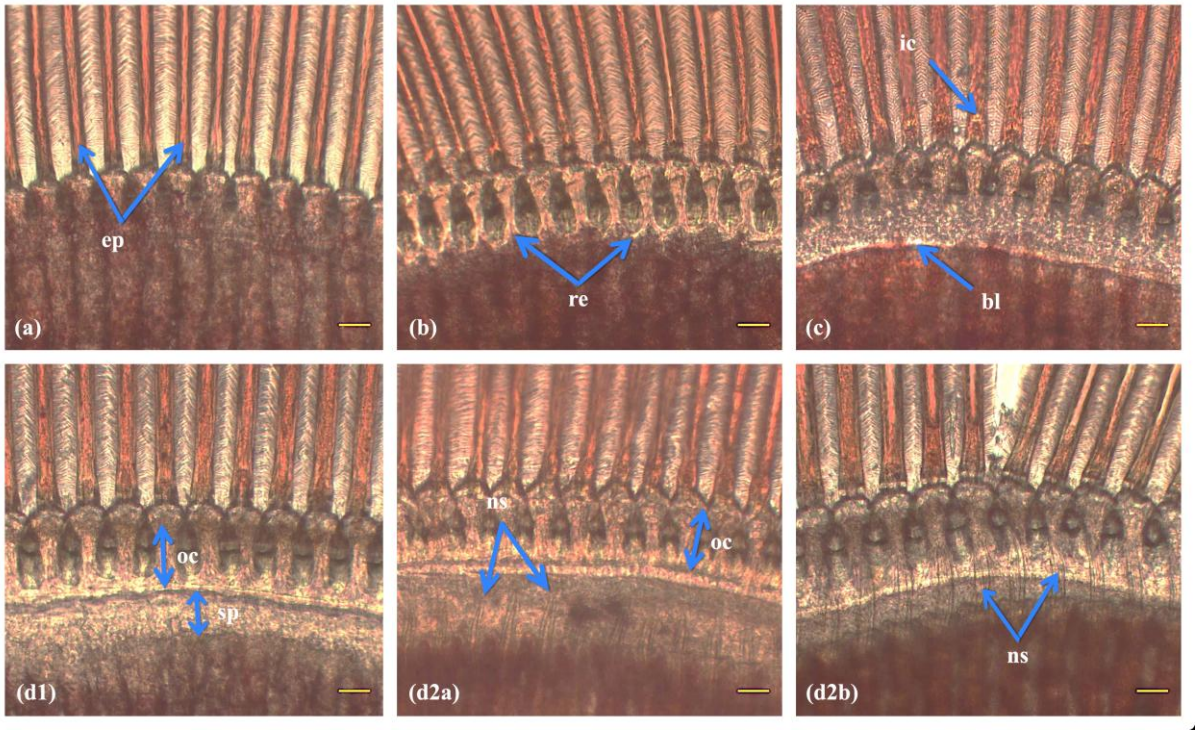

Figure 1. Determination of molt stages by changes of the exopodites of uropods. (A) During early post-molt, the epidermis is present in the setae (ep). (B) During late post-molt, the epidermis is retracted from the setae (re). (C) During the inter-molt, the epidermis is retracted underneath the setae and forms a base line at the bottom of the setae (bl) and the internal cones become visible (ic). (D1) During early pre-molt, the epidermis is fully separated from the old cuticle (oc) and a space is formed between the old cuticle and the epidemis (sp). ($D2_a$) Next before-molt, new setae are formed under the old cuticle (ns). ($D2_b$) When ready to molt, new setae (ns) are interrupted by folds of the old setae. Scale bar = 100 µm.

2.3 WSSV preparation

The WSSV Thai-1 used in the present study 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 donated by K. Soderhall (Uppsala University, Sweden). The virus was amplified in SPF *P.*

vannamei juveniles to produce virus stock. The median infectious titer of the stock was $10^{6.5}$ shrimp infectious dose 50% end point (SID_{50}) ml^{-1}, as determined by *in vivo* intramuscular titration in SPF *P. vannamei* (Escobedo-Bonilla *et al.*, 2005). A 10^{-2} dilution of this stock was made in phosphate-buffered saline (PBS), pH 7.4 and injected intramuscularly into SPF *P.vannamei* juveniles to amplify the virus. Then, moribund shrimp were collected and confirmed to be WSSV positive by indirect immunoflourescence (IIF). Thawed shrimp without shell, hepatopancreas and gut were chopped, suspended in PBS at a ratio of 1:3, homogenized at 5000 rpm for one min using an IKA T 25 digital ultra-turrax and centrifuged at 5000 g for 20 min (4 ^{o}C). Supernatant was collected, filtered (0.45 µm) and aliquoted for storage at -70 ^{o}C. All manipulations were done inside the laminar flow cabinet with sterile and pre-cooled conditions. The median infectious titer of the stock was determined to be $10^{8.5}$ SID_{50} ml^{-1} by *in vivo* intramuscular titration in SPF *P. vannamei*.

2.4 Immersion inoculation of shrimp with WSSV during an abrupt change in salinity

Thirty shrimp (MBW = 11.5 ± 2.8 g, n = 180) were divided into 6 groups. The animals were abruptly transferred individually from 35 g l^{-1} to six different tested salinities (5 g l^{-1}, 7 g l^{-1}, 10 g l^{-1}, 20 g l^{-1}, 35 g l^{-1} and 50 g l^{-1}) and simultaneously exposed to $10^{5.5}$ SID_{50} ml^{-1} of WSSV. In addition, 6 groups of 30 shrimp were transferred to the same 6 different salinities without virus and were used as controls. Because shrimp are easily wounded by manipulation during transfer, which could jeopardize the interpretation of the results, a new type of aquarium was constructed: the 10-liter two-compartment tank (Figure 2). This tank was divided in two compartments (compartment A & compartment B) by fixing a pyramid separation barrier in the middle of the tank. When one liter was added in each compartment, then the water was fully separated by the barrier. Larger volumes resulted in an overflow over the barrier. Several tanks were constructed; each individual experimental animal was housed in its own 10-liter two-compartment tank.

Inter-molt *P. vannamei* juveniles were housed individually in two-compartment tanks, filled with 4 liters of artificial seawater at a salinity of 35 ± 1 g l^{-1} (Figure 2). Shrimp were acclimated for 2 days (shrimp were then in D1 stage of their molt cycle), fed 2 times per day at a rate of 5% of their mean body weight. Each day, shrimp were gently flipped over the pyramid two times using a soft plastic patch. The purpose of using the two-compartment tank and gently flipping shrimp over the pyramid was to prevent stress and to avoid damage to the cuticle of the animals during manipulation. For WSSV exposure, animals were separated in

compartment A of the tank 3h after feeding. Compartment B was emptied and refilled with one liter of seawater with a certain salinity (5 g l^{-1}, 7 g l^{-1}, 10 g l^{-1}, 20 g l^{-1}, 35 g l^{-1} and 50 g l^{-1}). WSSV inoculum used to immerse the shrimp was prepared by adding 1 ml of WSSV stock ($10^{8.5}$ SID_{50} ml^{-1}) in the water of chamber B, resulting in $10^{5.5}$ SID_{50} ml^{-1} WSSV inoculum. Then, the animal was gently flipped from chamber A over the pyramid to chamber B and immersed for 5 h. After inoculation, the animal was flipped back to chamber A. The WSSV inoculum was siphoned out and the tank was refilled with 3 liters of artificial seawater (35 g l^{-1}) and maintained at 27 ^{o}C for 5 days. Moribund and dead shrimp were collected every 12 h and terminated at 120 hpi, when surviving shrimp were sacrificed. All moribund, dead and euthanized shrimp were processed for detection of WSSV infection by indirect immunofluorescence (IIF). The experiment was performed three times.

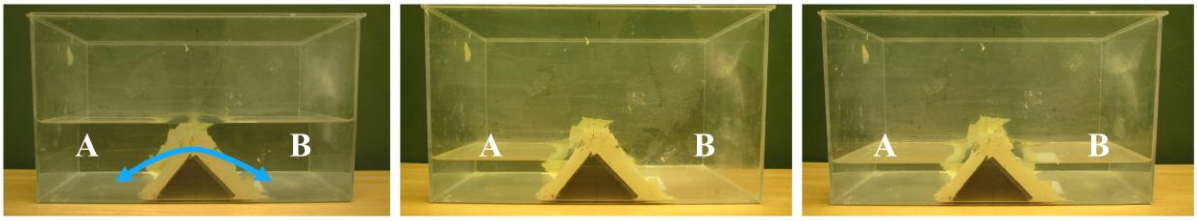

Figure 2. Two-compartment (A & B) tanks. (1) When filled with 4 liters, shrimp can freely migrate between compartment A & B (arrow). (2) When filled with 1-liter seawater in compartment A, the fluid does not overflow to compartment B and the shrimp stays in that compartment. (3) When filled with 1-liter seawater in each compartment, the fluid does not overflow in between the two compartments and shrimp can easily flipped from one compartment to the other.

2.5 Immersion inoculation of shrimp during early pre-molt, molting and post-molt

In this experiment, early pre-molt (D1) and before-molt ($D2_a$) *P. vannamei* juveniles (11.2 ± 2.5 g) were screened and housed individually in two-compartment tanks as described in 2.4 (Figure 2). The tank was filled with 4 liters of artificial seawater at a salinity of 35 g l^{-1}. Shrimp were maintained at 27 ^{o}C for 2 days, fed 2 times per day at the rate of 5% of their mean body weight. Then, shrimp in before-molt ($D2_a$) were re-examined for their molting process using an inverted microscope at a magnification of 200x. Animals with new setae interrupted by folds of the old setae ($D2_b$) were re-selected and rehoused individually in the same type of tank, filled with 4 liters of new artificial seawater at a salinity of 35 g l^{-1}. Because of the difficulty in predicting the precise timing of ecdysis, shrimp were frequently checked after rehousing (15 min intervals) for its molting process by looking at the junction

place between thorax and abdomen carapace. Animals with a wide dorsal junction between the thorax and abdomen carapace were separated in chamber A of the two-compartment tank. Then, 2 liters of water were siphoned out and 1 ml of WSSV stock ($10^{8.5}$ SID_{50} ml^{-1}) was added in the water of chamber A (1 liter) resulting in $10^{5.5}$ SID_{50} ml^{-1} WSSV inoculum. Shrimp that molted within 180 min of WSSV inoculation were taken into account and further immersed until finishing the inoculation period (5 h). For the inoculation of post-molt shrimp, 15 min after molting, shrimp were immersed with $10^{5.5}$ SID_{50} ml^{-1} of WSSV for 5 h. Early pre-molt (D1) shrimp were also inoculated with $10^{5.5}$ SID_{50} ml^{-1} of WSSV for 5 h. After inoculation, all shrimp were gently flipped from chamber A over the pyramid to chamber B. Afterwards, the WSSV inoculum was siphoned out from chamber A. The tank was refilled with 3 liters of artificial seawater, constant aeration was provided and the water was maintained at 27 ^{o}C for 5 days. Moribund and dead shrimp were collected every 12 h until the end of experiment at 120 hpi. Then, surviving shrimp were sacrificed. All moribund, dead and euthanized shrimp were analyzed for WSSV infection by IIF. The experiment was performed three times.

2.6 Detection of WSSV infection by indirect immunofluorescence (IIF)

WSSV infected shrimp were evaluated by indirect immunofluorescence (IIF) based on the description of Escobedo-Bonilla *et al*. (2005). Briefly, cephalothoraxes of dead, moribund and euthanized shrimp were collected, embedded in 2% methylcellulose and frozen at - 20 ^{o}C. Cryosections (6 µm) were made and fixed for 10 min in 100% methanol at - 20 ^{o}C. The sections were washed three times in PBS (5 min each) and incubated with 200 µl of monoclonal antibody w29 (1:100 in PBS), which is directed against viral protein VP28 for 1 h at 37 ^{o}C. Then, samples were washed three times in PBS (5min each), incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1 h at 37 ^{o}C. Finally, the samples were washed twice in PBS, rinsed once in deionised water, dried and mounted with a solution of glycerine and 1,4-diaza-bicyclo-octane (DABCO) (ACROS organics, USA). Sections were analyzed by fluorescence microscopy (Leica DM RBE).

2.7 Statistical analysis

In this study, shrimp of 3 replicates were pooled into groups of 15 animals, and the effect of treatments on the susceptibility of shrimp to WSSV infection was statistically analyzed by Pearson's Chi-square test. The difference in infection rates between two different salinity groups, and two molt stages were tested using Pearson's Chi-square tests with Yates' correction. Differences were considered significant at $p < 0.05$. All calculations were performed using R version 3.1.2.

3. Results

3.1 Immersion inoculation of shrimp with WSSV during an abrupt change in salinity

Shrimp transferred from a salinity of 35 g l^{-1} to 50 g l^{-1}, 35 g l^{-1}, 20 g l^{-1}, 10 g l^{-1}, 7 g l^{-1} and 5 g l^{-1} (mock inoculation) showed 100% survival at 120 hpi. Shrimp abruptly dropped into a salinity of 50 g l^{-1} to 35 g l^{-1} and 20 g l^{-1} also stayed alive until 120 hpi in the three experiments. Transfer of shrimp from 35 g l^{-1} to a lower environmental salinity resulted in mortality: 1 dead animal per 15 shrimp in 10 g l^{-1} (6.7%), 6 dead animal per 15 shrimp in 7 g l^{-1} (40%) and 8 dead animal per 15 shrimp in 5 g l^{-1} (53.3%) in three experiment. IIF analysis of dead, moribund and surviving shrimp revealed that all dead and moribund animals were WSSV positive. Only one surviving shrimp in the treatment of 7 g l^{-1} in the second replicate was WSSV positive. Other surviving shrimp were WSSV negative (Table 1). The chi-square test on infection rates of pooled shrimp gave the following results: (1) a significant effect of salinity change on the susceptibility of shrimp to WSSV infection ($p < 0.05$); (2) significantly higher infection rates of shrimp inoculated at a salinity of 7 g l^{-1} and 5 g l^{-1} than shrimp inoculated at a salinity of 10 g l^{-1}, 20 g l^{-1}, 35 g l^{-1} and 50 g l^{-1} ($p < 0.05$); (3) no significant differences in infection rates among the groups of shrimp challenged in 50 g l^{-1}, 35 g l^{-1}, 20 g l^{-1} and 10 g l^{-1} ($p > 0.05$); (4) no significant difference in infection rates between the two groups of shrimp inoculated at a salinity of 7 g l^{-1} and 5 g l^{-1} ($p > 0.05$) (Table 2).

Table 1. Immersion inoculation of *P. vannamei* in D1 stage of the molt cycle with $10^{5.5}$ SID_{50} ml^{-1} of white spot syndrome virus during an abrupt change in environmental salinity.

Experiment	Inoculation	Salinity switch ($g\ l^{-1}$)	Mortality (hpi)	Infection determined by IIF
1	WSSV	35 to 5	2/5 (60, 72)	2/5
		35 to 7	2/5 (60, 84)	2/5
		35 to 10	1/5 (84)	1/5
		35 to 20	0/5	0/5
		35 to 35	0/5	0/5
		35 to 50	0/5	0/5
	Mock	35 to 5	0/5	0/5
		35 to 7	0/5	0/5
		35 to 10	0/5	0/5
		35 to 20	0/5	0/5
		35 to 35	0/5	0/5
		35 to 50	0/5	0/5
2	WSSV	35 to 5	3/5 (84, 84, 96)	3/5
		35 to 7	2/5 (72, 84)	3/5
		35 to 10	0/5	0/5
		35 to 20	0/5	0/5
		35 to 35	0/5	0/5
		35 to 50	0/5	0/5
	Mock	35 to 5	0/5	0/5
		35 to 7	0/5	0/5
		35 to 10	0/5	0/5
		35 to 20	0/5	0/5
		35 to 35	0/5	0/5
		35 to 50	0/5	0/5
3	WSSV	35 to 5	3/5 (72, 84, 96)	3/5
		35 to 7	2/5 (84, 96)	2/5
		35 to 10	0/5	0/5
		35 to 20	0/5	0/5
		35 to 35	0/5	0/5
		35 to 50	0/5	0/5
	Mock	35 to 5	0/5	0/5
		35 to 7	0/5	0/5
		35 to 10	0/5	0/5
		35 to 20	0/5	0/5
		35 to 35	0/5	0/5
		35 to 50	0/5	0/5

Table 2. Effect of salinity change on mortality and infected rates in shrimp inoculated with $10^{5.5}$ SID_{50} ml^{-1} of WSSV by immersion (pooled from three replicates).

Acute change in salinity ($g\ l^{-1}$)	Mortality (dead/total)	Infection by IIF	% Infected
35 to 5	8/15	8/15	53.3^{a}
35 to 7	6/15	7/15	$46.7^{a,b}$
35 to 10	1/15	1/15	$6.7^{c,d}$
35 to 20	0/15	0/15	0^{d}
35 to 35	0/15	0/15	0^{d}
35 to 50	0/15	0/15	0^{d}

a, b, c, d: percentages indicated by different superscripts were significantly different by chi-square test ($p < 0.05$).

3.2 Immersion inoculation of shrimp during early pre-molt, molting and post-molt

In the three experiments, all early pre-molt shrimp survived until the end of the experiment. Immersion inoculation of shrimp during molting induced mortality in 2 animals in experiment 1, 3 animals in experiment 2 and 3 animals in experiment 3. Immersion inoculation of post-molt shrimp resulted in 1, 2 and 1 dead animals in the first, second and third experiment respectively. IIF analysis of dead, moribund and euthanized shrimp revealed that all dead and moribund animals were WSSV positive. Surviving shrimp that were euthanized were all confirmed to be WSSV negative (Table 3). The chi-square test on infection rates of pooled shrimp showed the following results: (1) there was a significant effect of molting stages on the susceptibility of shrimp to WSSV infection ($p < 0.05$); (2) significant differences existed in susceptibility of molting shrimp and pre-molt shrimp ($p < 0.05$); (3) infection rate of post-molt shrimp was not different from that of pre-molt and molting shrimp ($p > 0.05$) (Table 4).

Table 3. Inoculation of *P. vannamei* with $10^{5.5}$ SID_{50} ml^{-1} of WSSV by immersion in different molt stages: early pre-molt (D1), ecdysis (E), post ecdysis (A).

Experiment	Molt stage	Number of shrimp	Time of virus added before (-) and after (+) ecdysis (hours)	Mortality (hpi)	WSSV infection determined by IIF
1	Early pre-molt (D1)	5	- 168 h	0/5	0/5
	Ecdysis (E)	5	- 60 min - 40 min - 60 min - 30 min - 105 min	2/5 (72, 108)	2/5
	After ecdysis (A)	5	+ 15 min	1/5 (72)	1/5
2	Early pre-molt (D1)	5	- 168 h	0/5	0/5
	Ecdysis (E)	5	- 60 min - 60 min - 90 min - 120 min - 120 min	3/5 (60, 84, 96)	3/5
	After ecdysis (A)	5	+ 15 min	2/5 (48, 72)	2/5
3	Early pre-molt (D1)	5	- 168 h	0/5	0/5
	Ecdysis (E)	5	- 60 min - 40 min - 60 min - 30 min - 105 min	3/5 (72, 84, 84)	3/5
	After ecdysis (A)	5	+ 15 min	1/5 (72)	1/5

Table 4. Effect of ecdysis stages on infection rates in shrimp inoculated with $10^{5.5}$ SID_{50} ml^{-1} of WSSV by immersion (pooled from the three replicates).

Molt stage	Number of	Mortality	Infection by IIF	% Infected
Early pre-molt	15	0/15	0/15	0^{a}
Ecdysis (E)	15	8/15	8/15	53.3^{b}
After ecdysis (A)	15	4/15	4/15	$26.7^{b,a}$

a, b: percentages indicated by different superscripts was significantly difference by Chi-square test

4. Discussion

In this study, it has been shown that early pre-molt shrimp are highly susceptible to WSSV infection via immersion during acute salinity stress, particularly at low salinity stress conditions, and that during ecdysis and in early post-molt, shrimp are more susceptible to WSSV infection than in early pre-molt shrimp. These findings confirm the conclusions of some previous reports. Corteel *et al.* (2009) reported that *P. vannamei* are more susceptible to WSSV infection via immersion after molting than in the period before molting. However, in their study the authors did not evaluate the susceptibility of shrimp during ecdysis. Another study in *P. monodon* showed that the susceptibility of shrimp fed with infected tissues at 0 g l^{-1} salinity was higher than that of shrimp fed at 15 g l^{-1} and 35 g l^{-1} salinity (Joseph and Philip, 2007). It is known that the immune performance (prophenoloxidase activities (proPO)) of penaeid shrimp changes during acute salinity stress and molting, which may result in increasing replication of pathogens in shrimp. Le Moullac and Le Groumellec (1997) reported that variations of the proPO system of *P. stylirostris* in different molt stages are correlated with the sensitivity of shrimp to *Vibrio AM23* infection. Wang and Chen (2006) concluded that tiger shrimp *P. monodon* transferred from 25 g l^{-1} to low salinity levels 15 g l^{-1} and 5 g l^{-1} reduced its immune response and decreased its resistance against *Photobacterium damselae subsp* infection. Another study in *Fenneropenaeus chinensis* also revealed that salinity changes over a certain range resulted in a decreased phenoloxidase (PO) index and led to an acute outbreak of WSSV from a latent infection (Liu et al., 2006). In the present study, shrimp were at risk for a WSSV infection under stress conditions of lower environmental salinity and ecdysis, which suggests that lower salinity stress and ecdysis may facilitate the entry of WSSV into shrimp. The entry of the virus may be helped through the

influx of water during ecdysis and osmotic regulation of shrimp in low salinity water. Mykles (1980) reported that the midgut of American lobster, *Homarus americanus*, is the major surface for water absorption during ecdysis. Another study of Dall and Smith (1978) suggested that fluid absorption during ecdysis of rock lobster likely takes place at the gills and the whole body surface. *P. vannamei* are euryhaline organisms maintaining their hemolymph osmolarity in approximate equilibrium with the surrounding environment (Castille and Lawrence, 1981; Parado-Estepa *et al.*, 1987). Under sudden salinity stress, the osmotic concentration of hemolymph changes rapidly in order to establish a new equilibrium state between the animal and external environment. The animals dramatically extrude ions when exposed to a low salinity (Parado-Estepa *et al.*, 1987; Huong *et al.*, 2010). The transportation of ions is conducted actively by the use of ion pumps that are present in epithelial cells of gills, midgut and antennal glands. The osmotic difference between hemolymph and the external environment drives water passively through the water pores (Copeland, 1968; Capen, 1972; D'orazio and Holliday, 1985; Wheatly, 1987; Khodabandeh *et al.*, 2005b; Freire *et al.*, 2008; Henry *et al.*, 2012). In this scenario, the digestive system and the gills could be the best candidates of WSSV entry into shrimp as an assumption of some studies (Arts, 2007; Escobedo-bonilla *et al.*, 2007). During ecdysis, shrimp expand their head and contract their tail and other appendages to escape from the old skeleton. They kick and jump violently inside the tank, which may result in damages of the soft shell, allowing the virus to enter the shrimp's body (Travis, 1954; Clemens *et al.*, 1999). The finding in this study that early pre-molt shrimp resist to a WSSV infection in a stable environmental salinity is in agreement with Corteel *et al.* (2009). The authors reported that WSSV is poorly infectious in pre-molt shrimp even in shrimp with removed appendages.

Previous studies reported that shrimp respond to low salinity stress by regulating their hemolymph osmolality and urinary ion excretion through excretory antennal glands (Lin *et al.*, 2000; Buranajitpirom *et al.*, 2010). Lin *et al.* (2000) showed that the urine production of *P. monodon* rose dramatically from 0.17% to 2.4% of the body weight per hour after abrupt transferring the animal from 45 g l^{-1} to 15 g l^{-1} seawater. It could make sense that during urination WSSV may enter the bladder and infect the antennal glands. For shrimp in molting, a previous study has reported that urine production of shrimp increased rapidly as the molt ceased (Hagerman and Larsen, 1977). Therefore, after shedding and in diluted environmental salinity shrimp may frequently open their nephropore, which may allow WSSV to enter the bladder of the antennal glands and invade into the shrimp. In this scenario, the epithelial cells

of the bladder could be an interesting target of WSSV because there is no cuticle covering the epithelial cells of this organ (Khodabandeh *et al.*, 2005a).

In conclusion, this study revealed that there are restrictions on the ability of WSSV to enter into early pre-molt shrimp within a salinity range of 20 - 50 g l^{-1}, but that stressful conditions of an abrupt drop of salinity and shedding the old skeleton facilitate WSSV to enter shrimp via waterborne route. These findings give directions to WSSV control measures in penaeid shrimp farms. A fast drop of salinity in *P. vannamei* culture ponds should be minimized in order to prevent losses as a result from a WSSV infection. A low salinity exposure during WSSV immersion represents a new inoculation model that can be used for testing strategies to reduce the impact of a WSSV infection in shrimp.

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Chapter 5

The antennal gland is a major portal of entry for pathogens in shrimp

Abstract

Viral and bacterial diseases are major problems in shrimp aquaculture and pathogeneses have been intensively studied. However, the way how pathogens enter their host is still a matter of debate. Here, the role of the antennal gland (AG) as portal of entry was studied. First, the anatomy, histology and the three-dimensional structure of the *P. vannamei* AG were investigated. The AG of shrimp has a complex structure, which is composed of two symmetrical compact parts, each of which consists of a centrally located coelomosac surrounded by labyrinth I, located at the base of the second antennae, and rostrally extended by labyrinth II and a caudal multi-lobed urinary bladder ending in two vesicles and two pores protruding at the base of the second antennae. A fully standardized new technique was developed to infect shrimp via inoculating pathogens directly into the external pore of the AG. To identify the gastrointestinal tract or AG as portal of entry for WSSV and *Vibrio campbellii*, the shrimp infectious dose 50% endpoint of a WSSV stock and the shrimp lethal dose 50% of *V. campbellii* were assessed by intramuscular injection (reference) and upon peroral and intra-AG inoculation. 56 $SID_{50\text{-}im}$ (as determined by im route) was needed to infect 50% of shrimp via intra-AG inoculation and 29 x 10^6 $SID_{50\text{-}im}$ via peroral inoculation. Titration of *V. campbellii* showed the need of 62 $LD_{50\text{-}im}$ *V. campbellii* to kill 50% of shrimp upon intra-AG inoculation, while mortality did not occur in shrimp inoculated with $10^{4.3}$ $LD_{50\text{-}im}$ *V. campbellii* via peroral route. Next, the replication kinetics of WSSV in shrimp upon intra-AG inoculation was investigated. The first WSSV-infected cells were observed at 18 hpi in the epithelial cells of the bladder. From 24 hpi onwards, WSSV-infected cells were seen in all examined tissues. The highest numbers of WSSV-infected cells were observed in the coelomosac, gills and cuticular epithelium. Our findings show that the AG is an important portal of entry for pathogens in shrimp, which will stimulate and direct future research on how to control infectious shrimp diseases.

1. Introduction

Over the last three decades, global aquaculture production of shrimp has been booming. However, the rapid expansion of this industry has been accompanied by the emergence of very serious infectious diseases. Particularly, white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* have been associated with high mortality and a large proportion of crop failures in penaeid shrimp farming (Lightner, 2011). Despite many attempts to prevent diseases, no effective control measures have been found yet. The major obstacles behind the lack of control measures are the gaps in knowledge of the pathogenesis of viral and bacterial diseases, such as the portal of entry. Regarding WSSV, early studies gave the impression that WSSV is highly contagious and that the digestive system is most likely the major portal of entry in shrimp (Lightner *et al.*, 1998; Escobedo-Bonilla *et al.*, 2005). However, recently, several research groups had difficulties in infecting shrimp via immersion, peroral inoculation or per os feeding (Gitterle *et al.*, 2006; Laramore, 2007; Corteel *et al.*, 2009; Thuong *et al.*, 2016). Similarly, it is poorly understood how pathogenic *Vibrio* species manage to enter their host. Alday-Sanz *et al.* (2002) were not successful in infecting shrimp with *V. vulnificus* via immersion and peroral inoculation. The authors suggested that *V. vulnificus* could only infect shrimp via oral route through damaged cuticle. Aguirre‐Guzmán *et al.* (2010) suggested that gills and hepatopancreas may be possible entry portals of *V. parahaemolyticus* in shrimp. Other researchers were convinced that *V. parahaemolyticus* may invade the digestive system and may cause infection via waterborne route (Martin *et al.*, 2004; Tran *et al.*, 2013).

Studies on risk factors of WSSV outbreaks in traditional shrimp farms have reported that an abrupt drop in temperature and salinity due to heavy rain are hazard factors for a WSSV outbreak (Peinado-Guevara and López-Meyer, 2006; Tendencia *et al.*, 2010). Generally, an increase in rainfall results in lower environmental salinity. Shrimp respond to a lower environmental salinity stress by regulating their hemolymph osmolality and urinary ion excretion through the AG (Lin *et al.*, 2000; Buranajitpirom *et al.*, 2010). Lin *et al.* (2000) showed that the urine production of *P. monodon* rose dramatically from 0.17% to 2.4% of the body weight per hour after abruptly transferring the animal from 45 g l^{-1} to 15 g l^{-1} seawater. It could make sense that salinity stress may activate shrimp to open their external pores of the AG for urination and that during urination pathogens may enter the bladder and infect the AG. In lobster *(Homarus gammarus)*, crab (*Ocypode stimpsoni)* and crayfish (*Astacus leptodactylus)*, much is known on the morphology of the AG (Fuller *et al.*, 1989;

Khodabandeh *et al.*, 2005a), while it is poorly described in penaeid shrimp. The aims of the present study were (i) to examine the three-dimensional structure of the AG of *P. vannamei*, (ii) to identify the portal of entry by comparing the shrimp infectious dose 50% endpoint of a WSSV stock and the shrimp lethal dose 50% of *Vibrio campbellii* by different routes of inoculation: intramuscular (im) injection, peroral (po) and intra-antennal gland (intra-AG) inoculation and (iii) to investigate replication kinetics of WSSV in shrimp upon intra-AG inoculation.

2. Materials and methods

2.1 Experimental animals

Specific pathogen-free (SPF) penaeid shrimp, *Litopenaeus vannamei*, with a mean body weight (MBW) of 2.8 g imported from Holland were reared in the Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. Shrimp were cultured in a bio-filter circulation system, fed with pelleted feed at a rate of 5% of their mean body weight per day. Temperature and salinity of the culture system were maintained at 27 ± 1 ^{o}C and 35 ± 1 g l^{-1}. Total ammonia and nitrite were controlled to be lower than 0.5 and 0.15 mg l^{-1}, respectively. For the experiments, shrimp in early pre-molt were screened (Corteel *et al.*, 2009) and transported to the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University.

2.2 WSSV production

The WSSV Thai-1 used in the present study 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 donated by K. Soderhall (Uppsala University, Sweden). The virus was amplified in SPF *P. vannamei* juveniles to produce a virus stock. The median infectious titer of the stock was $10^{6.6}$ shrimp infectious dose 50% end point (SID_{50}) ml^{-1}, as determined by *in vivo* intramuscular titration in SPF *P. vannamei* (Escobedo-Bonilla *et al.*, 2005). A 10^{-2} dilution of this stock was made in phosphate-buffered saline (PBS), pH 7.4 and injected intramuscularly into SPF *P.vannamei* juveniles to amplify the virus. Then, moribund shrimp were collected and confirmed to be WSSV positive by indirect immunofluorescence (IIF). Thawed shrimp without shell, hepatopancreas and gut were chopped, suspended in PBS at a ratio of 1:3,

homogenized at 5000 rpm for one min using an IKA T 25 digital ultra-turrax and centrifuged at 5000 g for 20 min (4 ^{o}C). Supernatant was collected, filtered (0.45 µm) and aliquoted for storage at -70 ^{o}C. All manipulations were done inside a laminar flow cabinet under sterile and pre-cooled conditions.

2.3 Vibrio campbellii production

Rifampicin-resistant bacteria (*Vibrio campbellii*: LMG21363) that are pathogenic for penaeid shrimp were obtained from the ARC, Gent University, Belgium. From the original stock, 20 µl of bacterial suspension was inoculated in 20 ml Marine Broth (MB) 2216 (Difco Laboratories, USA) containing 100 mg l^{-1} of rifampicin for 12 h at 27 °C in a shaker at 90 rotations per minute (rpm). Then, the bacteria were sub-cultured under the same conditions for 14 h. Afterwards, the suspension was washed and centrifuged three times at 2000 g for 10 minutes. Finally, an estimated concentration of 10^{10} cfu ml^{-1} of bacterial suspension (stock) was made based on determining the optical density by spectrophotometry at an absorbance of 600 nm (OD_{600}). An optical density value (OD_{600}) of 1.0 corresponds to 1.2 x 10^{9} cells ml^{-1} (McFarland standard).

2.4 Three-dimensional structure of the antennal gland of P. vannamei

Early pre-molt *P. vannamei* shrimp (MBW 9.13 ± 0.92 g) from the ARC were screened and brought to the Laboratory of Morphology, Gent University, Belgium. In the Laboratory of Morphology, shrimp were euthanized on ice. Then, cephalothoraxes were dissected and fixed in a solution of paraformaldehyde and picric acid (85 ml of 2% paraformaldehyde and 15 ml of saturated picric acid, pH 7.4) at 37 ^{o}C for 12 h. The cephalothoraxes were embedded in paraffin. Cross-sections (10 µm) were made and stained with Harris' hematoxylin and counter-stained with eosin (H&E stain). The sections were analyzed and photographed under an Olympus BX61 microscope. The three-dimensional structure of the antennal gland was made using Amira software (version 5.6).

2.5 Infectivity of a WSSV stock in P. vannamei by different routes of inoculation

The aim of this study was to compare the infectivity of a WSSV stock in shrimp by different inoculation routes: im injection, po and intra-AG inoculation. In the experiment, early pre-

molt *P. vannamei* (25.4 ± 3.3 g) were collected and acclimated individually for 24 h in 10-liter tanks. Then, fifteen shrimp were injected intramuscularly with 5 µl of a 10-fold serial dilution (10^{-5}, 10^{-6} and 10^{-7}) of the WSSV stock, prepared in section 2.2, per animal (5 animals per dilution). Twenty shrimp were inoculated perorally with 50 µl of a ten-fold serial dilution (10^{0} to 10^{-3}) of the same WSSV stock per animal (5 animals per dilution). Twenty shrimp in the third group were inoculated into the bladder of the antennal gland with 5 µl of a ten-fold serial dilution (10^{-3} to 10^{-6}) of the same WSSV stock per animal (5 animals per dilution). The inoculation procedures were fully standardized. Briefly, im injection was performed with a 25-gauge needle (Terumo) mounted on an accurate syringe (Model 1710 LT SYR, 100 µl, Hamilton Bonaduz) filled with 5 µl of WSSV suspension. For oral inoculation, shrimp wrapped in tissue paper were placed ventral side up under a stereomicroscope. The tip (2mm) of a 0.64 x 19 mm-26G Surflo-W catheter (Terumo) mounted on a 100 µl Hamilton Bonaduz, filled with 50 µl of a WSSV suspension, was introduced into the oral cavity and the inoculum was delivered into the lumen of the foregut. For antennal gland inoculation, first, urine was removed from the bladder after gently introducing the tip (0.5 mm) of a 0.64 x 19 mm-26G Surflo-W catheter (Terumo) in the nephropores. The catheter was kept stable for a few seconds until the urine fully filled the catheter. Afterwards, the catheter was removed and replaced with a new 0.64 x 19 mm-26G Surflo-W catheter (Terumo) connected with a 100 µl Hamilton Bonaduz syringe, filled with 5 µl of a WSSV suspension. The WSSV suspension was inoculated by a gentle pressure on the plunger of the syringe. After inoculation, shrimp were housed individually and kept for 5 days. Cephalothoraxes of dead and moribund shrimp were collected every 12 h. The experiment was terminated at 120 hpi. At the latter time point all surviving shrimp were euthanized. Samples of dead, moribund and euthanized shrimp were processed for detection of WSSV infected cells by indirect immunofluorescence (IIF). The experiment was performed three times.

2.6 Lethality of Vibrio campbellii in P. vannamei by different inoculation routes

From a bacterial stock (10^{10} cfu ml^{-1}), serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were made in filtered, autoclaved seawater (FASW). Then, these dilutions were used to determine the lethal titers with 50% endpoint (LD_{50} ml^{-1}) in *P. vannamei* by im injection, po and intra-AG inoculations. Fifteen shrimp were injected with 100 µl of a 10-fold serial dilution (10^{-2}, 10^{-3} and 10^{-4}) of the *Vibrio* stock, produced in 2.3, per animal (5 animals per dilution). Fifteen

shrimp were inoculated perorally with 100 µl of a ten-fold serial dilution (10^0, 10^{-1} and 10^{-2}) of the same *Vibrio* stock per animal (5 animals per dilution). Fifteen shrimp in the third group were inoculated intra-AG with 10 µl of a ten-fold serial dilution (10^0, 10^{-1} and 10^{-2}) of the same *Vibrio* stock per animal (5 animals per dilution). After inoculation, shrimp were housed individually and kept for 5 days. Dead and moribund shrimp were collected every 6 h and the experiment was terminated at 120 hpi. At the latter time point all surviving shrimp were euthanized. For counting the bacterial density in dead, moribund and euthanized shrimp, shrimp were washed once with 70% alcohol and twice with FASW. Then, the whole shrimp were homogenated in FASW at a ratio of 1:5 (10^0) and diluted (10^{-1}, 10^{-2} and 10^{-3}) in FASW. 100 µl of each dilution was plated on marine agar containing 100 mg l^{-1} rifampicin (MAR). The plates were incubated at 28 ^oC for 24 h, then counting was conducted with the plate containing between 30 and 300 colonies.

2.7 Replication of WSSV in P. vannamei upon intramuscular and intra-antennal gland inoculation

Early pre-molt SPF-shrimp (25.2 ± 3.8 g), acclimated for 1 day at 27 ^oC, were divided into 3 groups. Animals were injected intramuscularly with 10 µl containing $10^{5.5}SID_{50}$ of WSSV at the junction between the 4^{th} and 5^{th} abdominal segments. Animals were inoculated intra-AG with 10 µl containing $10^{5.5}SID_{50}$ of WSSV. Animals were mock inoculated by intramuscular injection of 10 µl of PBS and used as controls. After inoculation, blood and cephalothoraxes of 5 shrimp were sampled at 0, 6, 12, 18, 24, 36 and 48 hpi. For the determination of WSSV-infected cells in hemolymph, 500 µl of hemolymph were collected with anticoagulant buffer at the ratio of 1:1. Hemolymph was further diluted 1:1 in PBS. Then, 100 µl of the diluted hemolymph was cytospinned. The cytospins were centrifuged at 300 g for 4 min, fixed for 10 min in methanol and WSSV viral antigens were detected by IIF. For the determination of WSSV-infected cells in tissues, the cephalothoraxes were cross-sectioned (20 µm) at the site of AG, hepatopancreas, gills, lymphoid organs, heart and hematopoietic tissues. Then, the sections were fixed in methanol for 10 min and WSSV viral antigens were detected by IIF. WSSV-infected cells in gills, hepatopancreas, lymphoid organ, coelomosac, heart and hematopoietic tissues were counted in 6 randomly selected fields and expressed as number of WSSV-infected cells per square millimeter (cells mm^{-2}). WSSV-infected and uninfected cells were counted randomly in 6 fields of hemocytes, bladder epithelial cells and cuticular

epithelial cells of the body, head and hindgut and expressed as percentage of infected cells (%).

2.8 Detection of WSSV viral antigens by IIF

Viral antigen positive cells in WSSV infected shrimp were detected by indirect immunofluorescence (IIF) as described by Escobedo-Bonilla *et al*. (2005). Briefly, cephalothoraxes of dead, moribund and euthanized shrimp were collected, embedded in 2% methylcellulose and frozen at - 20 ^{o}C. Cryosections (6 µm) were made and fixed for 10 min in 100% methanol at - 20 ^{o}C. The sections were washed three times in PBS (5 min each) and incubated in 200 µl of monoclonal antibody w29 (directed against WSSV viral protein VP28 1:100 in PBS) at 37 °C for 1 h. Then, samples were washed three times in PBS (5min each), incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1h at 37 ^{o}C. Finally, the samples were washed twice with PBS, rinsed once in deionised water and mounted with a solution of glycerine and 1,4-diaza-bicyclo-octane (DABCO) (ACROS organics, USA). Sections were analyzed with a confocal fluorescence microscope.

2.9 Statistical analysis

Determination of viral infectious and bacterial lethal titers was done based on the method of Reed and Muench (1938).

3. Results

3.1 Three-dimensional structure of the antennal gland of P. vannamei

- Anatomy of the antennal gland (Fig 1a, b, c)

The *P. vannamei* AG is composed of (i) two symmetrical compact parts (CP) located at the base of the second antennae, (ii) a rostral zone of the labyrinth (L) extending from the compact parts towards the base of the eyestalk and (iii) a multi-lobed urinary bladder ending in two vesicles and two pores (nephropore (N)) at the base of the second antennae. Each pore is equipped with an exopodite (Ex) bearing multiple tiny setae.

- Histological and three-dimensional structure of the antennal gland (Fig 1d-z)

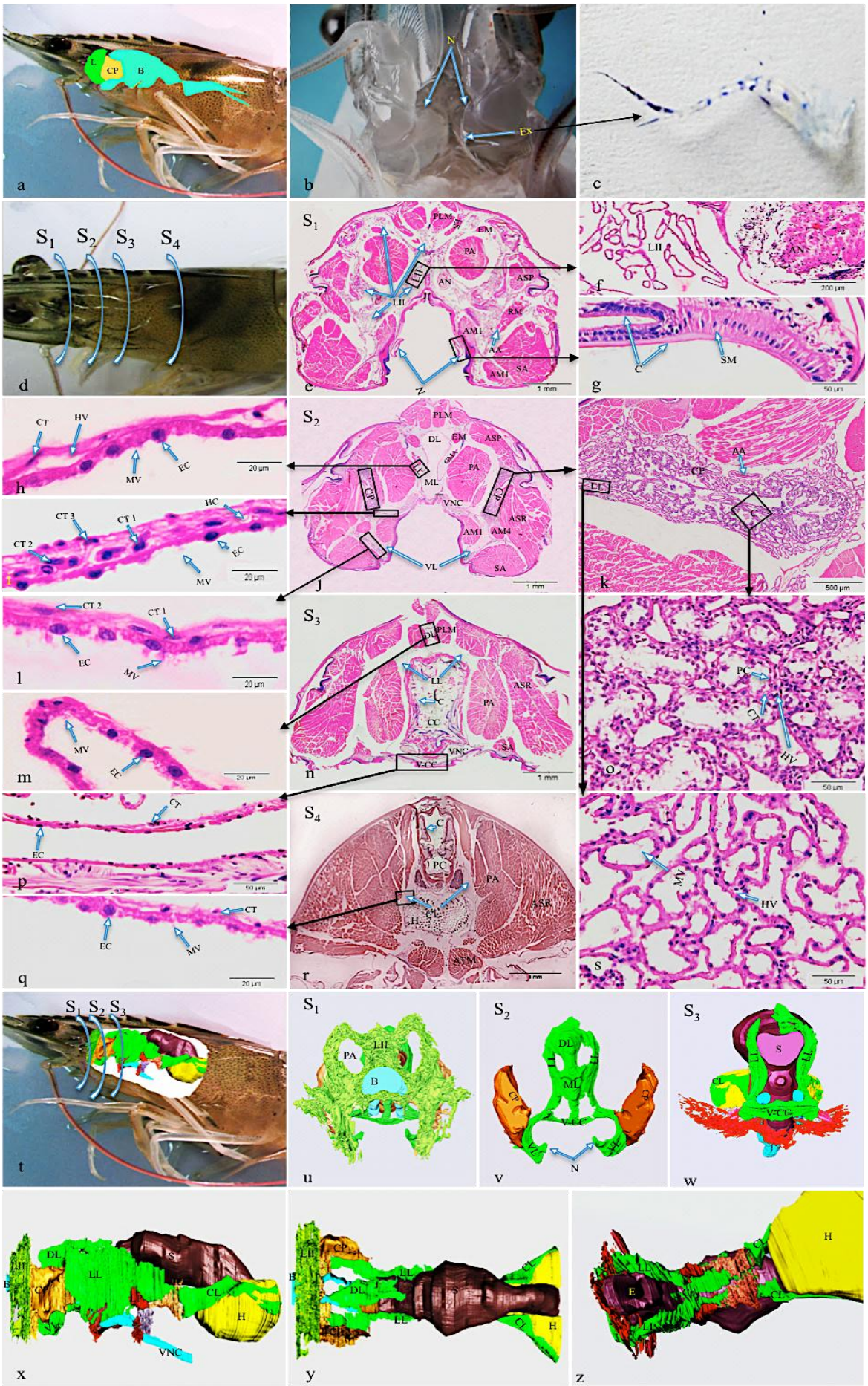
The AG is composed of three units: the coelomosac, the labyrinth, and the urinary bladder. The coelomosac (C) is located in the central region of the compact part (CP) (Fig 1j, k, v). The coelomosac consists of cyst-like structures, which are composed of a single layer of podocyte cells (PC) embedded in connective tissue (CT). The cyst-like structures are bathing in hemolymph, which is provided by hemolymph vessels (HV) entering the dorsal side of the cyst-like structures (Fig 1o).

The labyrinth displays crescent-like structures that are formed by a single layer of columnar cells with large central nuclei and apical microvilli (MV) (Fig 1f, s). The labyrinth was found in two regions. Region 1 (LI) is located in the compact part, where the crescent-like structures of the labyrinth and the coelomosac come into close contact with each other (Fig 1k, s). Region 2 (LII) is an extension part of LI of the compact parts in rostral direction surrounding antennal nervous cords (AN) and the protocephalon attractor muscle (PA) (Fig 1e, f, u, x, y). The labyrinth I structures are bathing in hemolymph, which is supplied from the hemolymph vessels (HV) entering the dorsal side of the crescent-like structures (Fig 1s).

The urinary bladder consists of multiple lobes, designated ventral, medial, lateral dorsal and caudal (Fig 1j, n, r, v, x). The ventral lobe (VL) is a voluminous vesicle occupying the ventral site of each compact part (CP) and linked with the nephropore (N) (Fig 1j, v). The nephropore is a cuticular valve-like split protruding at the base of the second antennae (Fig 1e, g). The two ventral lobes are connected with a ventral-connecting channel (V-CC) (Fig 1n, p, v, w). The medial lobe (ML) is a large reservoir starting at the ventral-connecting channel and branching in lateral and dorsal lobes (Fig 1j, v). The lateral lobe (LL) is a tubular sac enclosing the esophagus (Fig 1n, v, w, x, y). The dorsal lobe (DL) is a vesicle sac extending near the dorsal surface of the cephalothorax (Fig 1j, n, v, x, y). The caudal lobes (CL) are two tubular lobes, which are prolonging parts of lateral bladders running along the length of the hepatopancreas and terminating at the posterior part of the hepatopancreas (Fig 1r, x, y, z).

The bladder wall is lined by a single layer of epithelial cells (EC) with irregular apical microvilli (MV). The epithelial cell layer is supported by thin fibrous connective tissue layers (CT) and a system of hemolymph vessels presented in between the layers (HV) (Fig. 1h). The number of layers is variable according to their location. The wall of the ventral bladder is surrounded by two to three fibrous connective tissue layers (Fig 1i, l), whereas the wall of lateral, dorsal and caudal bladder is surrounded by one fibrous connective tissue layer (Fig 1h, m, q).

In conclusion, the AG of *P. vannamei* is a complex structure, which constitutes of two central compact parts (CP), a rostral extension of the labyrinth II and a multilobular urinary bladder (Fig 1u, v, w). These parts form an elongated structure running alongside the cepalothorax from the rostral side of the brain up to the posterior hepatopancreas (Fig. 1t, x, y, z).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p
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CT
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20 µm
r
S4
C
PC
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t
S1 S2 S3
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S2
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CP
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S3
S
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V-CC
x
DL
S
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CP
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VL
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y
LII
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Figure 1. Anatomy, histology and three-dimensional structure of the AG of *P. vannamei*. (a) Schematic drawing of different components of the AG in the cephalothorax, (b) position of the nephropores and exopodites, (c) dissected exopodite from shrimp, (d) selected cross-sections illustrate the major structure of the AG at different sites (S_1, S_2, S_3, S_4) through the cephalothorax, (e) section of the AG at S_1 with labyrinth II and nephropore, (f) labyrinth II, (g) nephropore histology, (h) bladder lateral wall, (i, l) bladder ventral wall, (j) section of the AG at S_2 contains two compact parts and four lobes of the urinary bladder (ventral, medial, lateral, dorsal lobes), (k) compact part, (m) bladder dorsal wall, (n) section of the AG at S_3 showing dorsal, lateral and connecting channel of medial bladder, (o) coelomosac, (p) connecting channel, (q) bladder caudal wall, (r) section of the AG at S_4 containing two lobes of the caudal bladder. (s) labyrinth I, (t) three-dimensional view with the location of three cross sections (S_1, S_2, S_3) through the cephalothorax, (u) cross section of the AG at S_1 shows the large network of the labyrinth II, (v) view of the AG at S_2 shows two compact parts and ventral, medial, dorsal, lateral bladders, (w) view of the AG at S_3 showing the ventral connection between the left and right lateral lobe, (x) lateral view of the AG shows an elongated structure of the labyrinth II, compact parts, ventral, dorsal, lateral and caudal bladder, (y) dorsal view of the AG shows the complex of labyrinth II, compact parts, dorsal, lateral and caudal bladder, (z) ventral view of the AG shows the connecting point of the bladder under the stomach.
AA: antennal artery, AM1: adductor muscle of 1st antennal segment, AM4: adductor muscle of 4th antennal segment, AN: antennal nerve, ASR: antennal scale remotor muscle, ATM: lateral anterior thoracic muscle 1, B: bladder, B: brain, C: coelomosac, C: cuticle, CC: cardiac chamber, CP: compact part, Cp: connecting point. CT: connective tissue, DL: dorsal lobe, E: esophagus, EC: epithelial cell, EM: esophageal muscles, ES: epistomal stator muscles, Ex: exopodites, GMA: anterior gastric mill attractor muscle, H: hepatopancreas, HV: hemolymph vessel, LII: labyrinth II, LL: lateral lobe, LP:Lymphoid organ, ML: medial lobe, MV: microvilli, N: nephropore, PA: protocephalon attractor muscle, PC: podocyte cells. PC: pyloric chamber, PLM: Posterior protocephalon levator muscles, RM: rotator muscle of 3rd antennal segment, S: stomach, SA: scale adductor muscle, SM: sphincter muscle, V-CC: ventral-connecting channel, VL: ventral loble, VNC: ventral nervous cord.

3.2 Infectivity of a WSSV stock in P. vannamei by different routes of inoculation (Table 1)

IIF analysis of dead, moribund and euthanized shrimp in the three experiments revealed that all moribund and dead shrimp were WSSV positive, while surviving shrimp were WSSV negative. In the three experiments, the virus titers determined upon im injection were $10^{8.67}$, $10^{8.67}$ and $10^{8.80}$ SID_{50} ml^{-1} ($\overline{x}$ = $10^{8.71}$ SID_{50} ml^{-1}); upon po inoculation: $10^{1.14}$, $10^{1.30}$ and $10^{1.30}$ SID_{50} ml^{-1} ($\overline{x}$ = $10^{1.25}$ SID_{50} ml^{-1}); upon intra-AG inoculation: $10^{6.97}$, $10^{6.60}$ and $10^{7.30}$ SID_{50} ml^{-1} ($\overline{x}$ = $10^{6.96}$ SID_{50} ml^{-1}). Taken together, this means that compared with the im route, 56.2 times more infectious virus is needed to infect 50% of shrimp via intra-AG inoculation, while 28.8 x 10^6 times more virus is necessary to infect 50% of shrimp via oral inoculation.

Table 1. Infection titer of a WSSV stock by intramuscular injection (im), peroral inoculation (po) and by intra-AG inoculation in *P. vannamei*

Experiment	Inoculation route	Dilution of WSSV stock	No of shrimp	Number of dead animals at….hpi									Number of infected animals/total (IIF)	Virus titer (SID_{50} ml^{-1})
				24	36	48	60	72	84	96	120	Total		
1	im	10^{-5}	5			3	1	1				5	5/5	$10^{8.67}$
		10^{-6}	5			2	2					4	4/5	
		10^{-7}	5									0	0/5	
	po	10^{0}	5			2						2	2/5	$10^{1.14}$
		10^{-1}	5									0	0/5	
		10^{-2}	5									0	0/5	
		10^{-3}	5									0	0/5	
	intra-AG	10^{-3}	5			2	2	1				5	5/5	$10^{6.97}$
		10^{-4}	5			1		2	1			4	4/5	
		10^{-5}	5				1	1				2	2/5	
		10^{-6}	5									0	0/5	
2	im	10^{-5}	5			3	2					5	5/5	$10^{8.67}$
		10^{-6}	5			1	2	1				4	4/5	
		10^{-7}	5									0	0/5	
	po	10^{0}	5			1	1					2	2/5	$10^{1.30}$
		10^{-1}	5					1				1	1/5	
		10^{-2}	5									0	0/5	
		10^{-3}	5									0	0/5	
	intra-AG	10^{-3}	5			3	2					5	5/5	$10^{6.60}$
		10^{-4}	5			1	1	1				3	3/5	
		10^{-5}	5				1					1	1/5	
		10^{-6}	5									0	0/5	
3	im	10^{-5}	5		3	1	1					5	5/5	$10^{8.80}$
		10^{-6}	5		2	1	1		1			5	5/5	
		10^{-7}	5									0	0/5	
	po	10^{0}	5				2					2	2/5	$10^{1.30}$
		10^{-1}	5					1				1	1/5	
		10^{-2}	5									0	0/5	
		10^{-3}	5									0	0/5	
	intra-AG	10^{-3}	5			2	2	1				5	5/5	$10^{7.30}$
		10^{-4}	5			2	1	1				4	4/5	
		10^{-5}	5				2		1			3	3/5	
		10^{-6}	5									0	0/5	

3.3 Lethality of Vibrio campbellii in P. vannamei by different inoculation routes (Table 2).

In the three experiments, mortality did not occur in shrimp injected intramuscularly with dilution 10^{-4} of the *V. campbellii* stock. Shrimp died within 12 hours after injection with dilutions 10^{-2} and 10^{-3} of the same *Vibrio* stock. Upon intra-AG inoculation, no mortality was recorded in the group of shrimp inoculated with dilution 10^{-2} of the *V. campbellii* stock. Mortality was observed in the group of shrimp inoculated with dilutions 10^{0} and 10^{-1} of the same *Vibrio* stock. Inoculation of shrimp with doses of *V. campbellii* via peroral route did not result in mortality. Determination of bacteria by plate counting indicated that all dead and moribund shrimp were *V. campbellii* positive and contained high densities of *V. campbellii*

($3.8 \pm 1.0 \times 10^5$ cfu ml^{-1} of homogenate), whereas surviving shrimp showed a clearing mechanism to eliminate bacteria from their body ($2.4 \pm 3.4 \times 10^2$ cfu ml^{-1} of homogenate). The lethal titers (LD_{50} ml^{-1}) of *Vibrio* upon im injection were $10^{4.16}$, $10^{4.37}$ and $10^{4.16}$ LD_{50} ml^{-1} ($\overline{x} = 10^{4.23}$ LD_{50} ml^{-1}) and upon intra-AG inoculation were $10^{2.50}$, $10^{2.50}$ and $10^{2.32}$ LD_{50} ml^{-1} ($\overline{x} = 10^{2.44}$ LD_{50} ml^{-1}). Taken all results together, these finding demonstrated that compared with the intramuscular route, 62 times more *V. campbellii* are required to kill 50% of shrimp via intra-AG inoculation and that it was not possible to kill shrimp by peroral route with up to $10^{9.0}$ *V. campbellii.*

Table 2. Lethality titers of *Vibrio campbellii* by intramuscular injection (im), peroral inoculation (po) and by intra-AG inoculation in *P. vannamei*

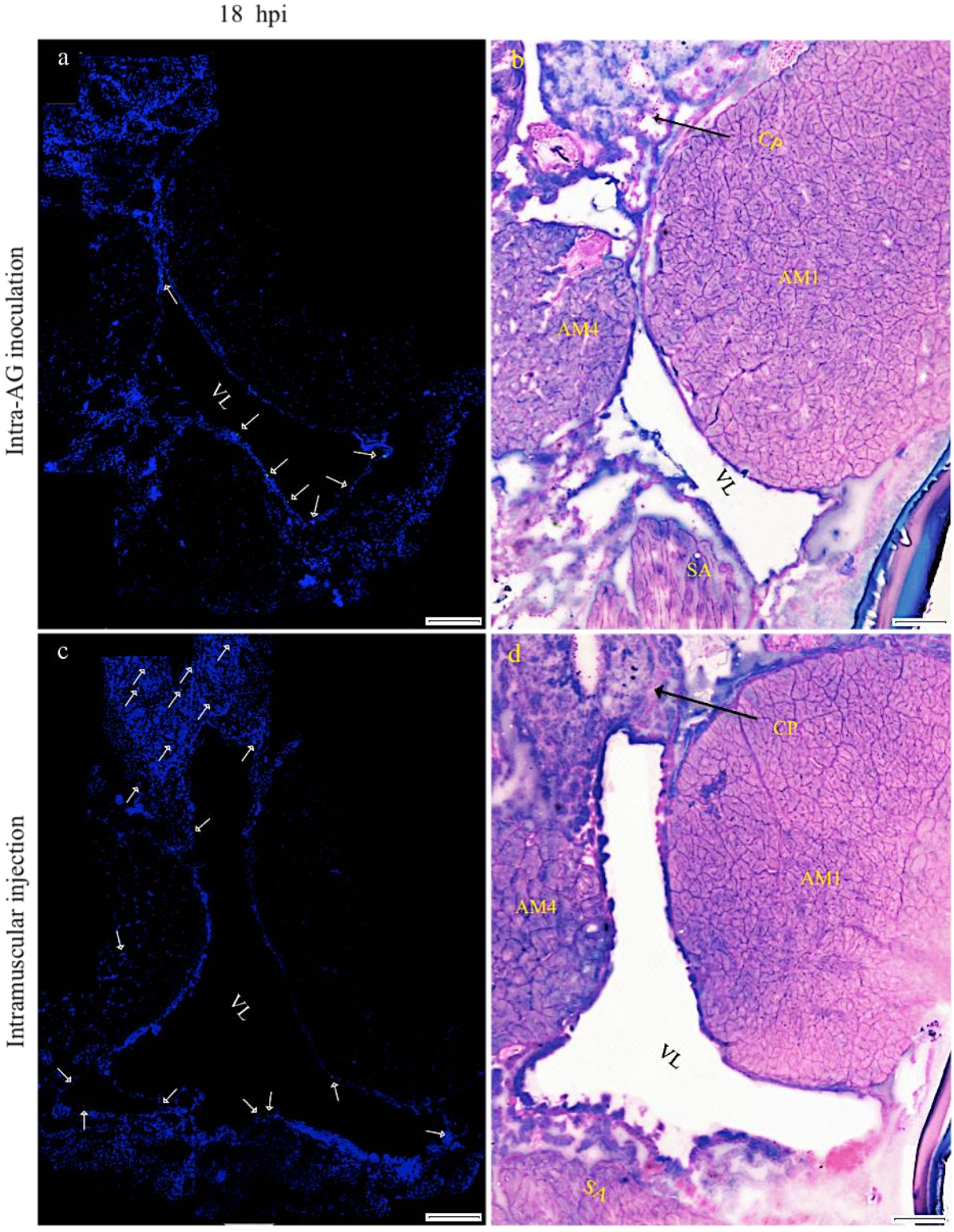
Experiment	Inoculation route	Dilution of *Vibrio* stock	No of shrimp	Number of dead animals at different time points									Mortality (%)	Lethal titers (LD_{50} ml^{-1})
				0	6	12	18	24	30	36	120	Total		
1	im	10^{-2}	5		5							5	100	$10^{4.16}$
		10^{-3}	5		1	2						3	60	
		10^{-4}	5									0	0	
	po	10^{0}	5									0	0	-
		10^{-1}	5									0	0	
		10^{-2}	5									0	0	
	inta-AG	10^{0}	5			2	1					3	60	$10^{2.49}$
		10^{-1}	5			2						2	40	
		10^{-2}	5									0	0	
2	im	10^{-2}	5		3	2						5	100	$10^{4.37}$
		10^{-3}	5		2	2						4	80	
		10^{-4}	5									0	0	
	po	10^{0}	5									0	0	-
		10^{-1}	5									0	0	
		10^{-2}	5									0	0	
	intra-AG	10^{0}	5		2	1						3	60	$10^{2.49}$
		10^{-1}	5			2						2	40	
		10^{-2}	5									0	0	
3	im	10^{-2}	5		5							5	100	$10^{4.16}$
		10^{-3}	5		2	1						3	60	
		10^{-4}	5									0	0	
	po	10^{0}	5									0	0	-
		10^{-1}	5									0	0	
		10^{-2}	5									0	0	
	intra-AG	10^{0}	5		2	1						3	60	$10^{2.32}$
		10^{-1}	5			1						1	20	
		10^{-2}	5									0	0	

3.4 Replication of WSSV in P. vannamei upon intramuscular and intra-antennal gland inoculation (Table 3).

In the intramuscular injection group, the first WSSV-infected cells were observed at 18 hpi in all investigated tissues: gills, coelomosac, heart, lymphoid organs, hepatopancreas, hematopoietic tissues and cuticular epithelium of head, body and hindgut. WSSV positive cells in hemolymph were detected at 24 hpi. Upon intra-AG inoculation, WSSV-infected cells were only detected in the epithelial cells of the bladder at 18 hpi. From 24 hpi onwards, WSSV positive cells were seen in other investigated tissues and some cells in the hemolymph (Fig 2). Of both inoculation routes, only a small proportion (< 2%) of cells in hemolymph was WSSV positive. WSSV-infected cell numbers of all investigated tissues were low at 24 hpi. The WSSV-infected cells increased rapidly and reached high levels after 36 hpi. The highest number of WSSV-infected cells was found in the coelomosac, gills and the cuticular epithelial cells. The number of WSSV-infected cells observed in im injected shrimp were higher than that observed in intra-AG inoculated animals in all investigated tissue.

Table 3. Quantification of infected cells in various organs of *P.vannamei* shrimp inoculated with WSSV via intramuscular injection (im) and intra-AG inoculation.

Inoculation route	Time (hpi)	Quantity of WSSV infected cells in											
		Antennal gland			Hematopoietic tissue (mm^{-2})	Hepatopancreas (mm^{-2})	Heart (mm^{-2})	Lymphoid organ (mm^{-2})	Gills (mm^{-2})	Cuticular epithelium			Hemolymph (%)
		Bladder Epithelium (%)	Labyrinth (mm^{-2})	Coelomosac (mm^{-2})						Head cuticular epithelium (%)	Body cuticular epithelium (%)	Hindgut cuticular epithelium (%)	
im	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0	0	0
	18	0.8±0.5	7±10	43±29	25±16	36±18	13±13	19±10	41±28	0.3±0.2	0.6±0.5	0.5±0.4	0
	24	2.0±1.2	66±24	93±34	81±27	157±50	68±37	62±20	249±75	1.8±0.6	2.7±1.5	3.5±2.4	0.4±0.3
	36	8.4±3.7	341±96	739±93	593±347	336±107	304±129	226±81	847±160	20.4±7.9	16.4±3.1	13.9±6.4	0.8±0.5
	48	13.3±4.5	914±301	2.057±274	1.186±354	795±203	504±270	981±221	2.037±207	22.9±7.6	35.9±7.1	32.9±6.3	1.5±0.4
intra-AG	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0	0	0
	18	0.8±0.4	0	0	0	0	0	0	0	0	0	0	0
	24	1.6±0.9	8±7	84±27	23±11	15±12	12±9	11±7	16±10	0.3±0.2	0.7±0.4	0.3±0.2	0.1±0.1
	36	5.9±1.9	138±35	561±114	187±121	233±48	205±72	179±39	407±179	11.6±4.0	6.0±2.8	5.6±1.9	0.6±0.4
	48	6.7±2.6	392±75	1.980±331	573±212	405±166	408±158	420±151	1.861±205	17.6±4.5	24.7±6.2	22.6±6.1	1.0±0.4


18 hpi
Intra-AG inoculation
Intramuscular injection
a
b
c
d
VL
CP
AM1
AM4
SA

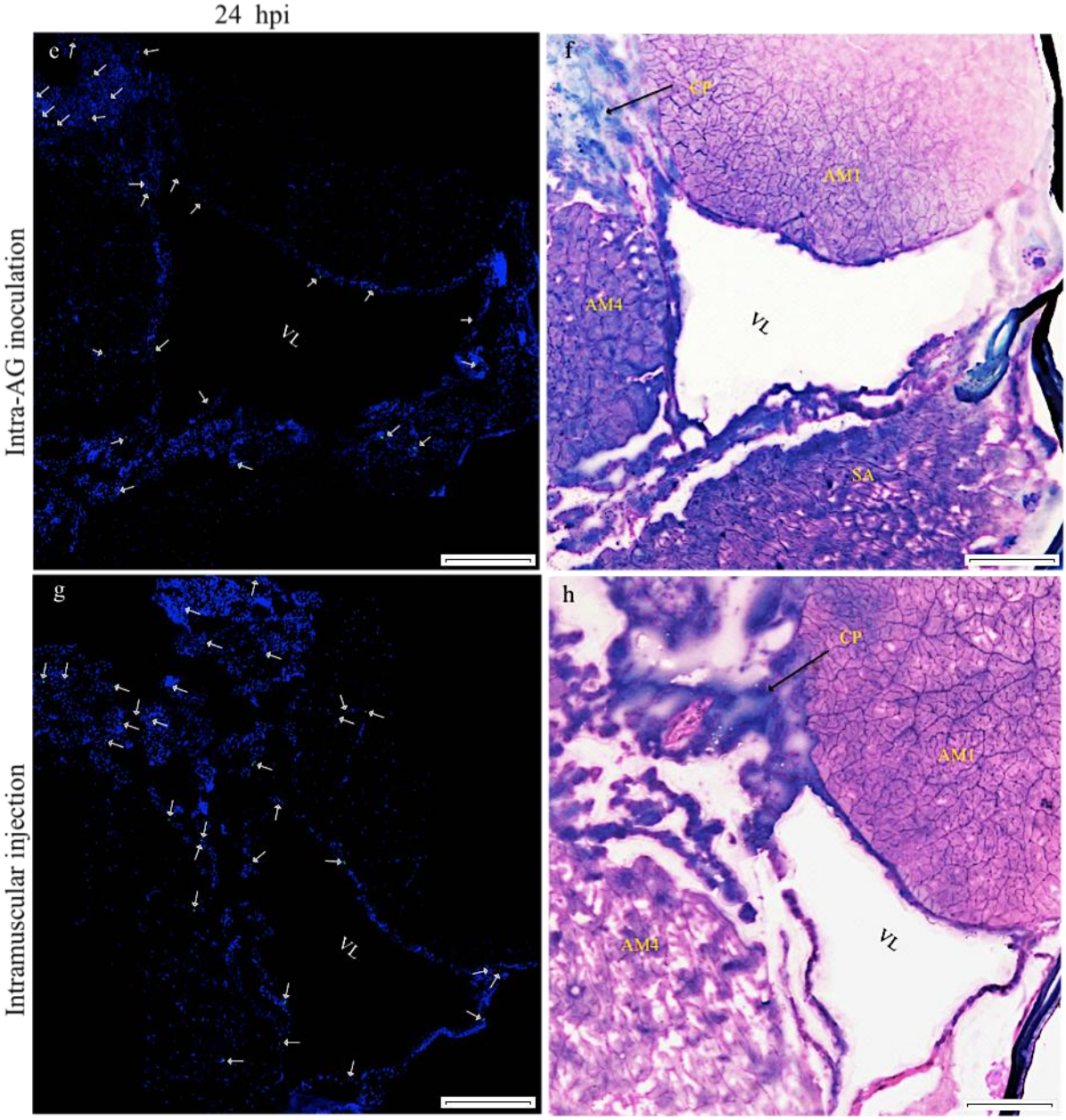


Figure 2. Photomicrographs of viral antigen positive cells (green) in the AG and surrounding areas of injected and intra-AG inoculated shrimp sampled at 18 hpi and 24 hpi. WSSV-infected cells were detected by IIF using a VP28-specific mouse monoclonal antibody and an FITC-conjugated goat-anti mouse IgG. Photomicrographs a & e show the first WSSV-infected epithelial cells in the ventral lobe (VL) of the bladder at 18 hpi and WSSV positive cells in the ventral lobe of the bladder, compact part (CP) and muscle surrounding the AG at 24 hpi of intra-AG inoculated shrimp (arrows). Photomicrographs c & g show WSSV-infected epithelial cells in the ventral bladder (VB), compact part (CP) and surrounding areas of the AG at 18 and 24 hpi in intramuscularly injected shrimp. Photomicrographs b, d, f & h show the histology of the AG and surrounding areas. The tissues were stained using Diff-Quick staining. VB: ventral bladder, CP: compact part, AM1: adductor muscle of 1st antennal segment, AM4: adductor muscle of 4rd antennal segment, SA: scale adductor muscle. Bar = 200 µm.

4. Discussion

A full knowledge of the anatomy of an animal is essential for the study of the portals of entry for pathogens. A detailed description of the *P. vannamei* AG is reported here, which allowed us to identify the AG as a very important portal of entry for viruses and bacteria. Indeed, upon intra-AG inoculation of two main shrimp pathogens, white spot syndrome virus (WSSV) and *Vibrio campbellii*, it was demonstrated that these pathogens could easily infect shrimp. This was in strong contrast with their infectivity when inoculated via peroral route. It was clearly demonstrated that WSSV first replicates in the epithelial cells of the bladder at 18 hpi and spreads afterwards all over the body.

Macroscopic, microscopic and ultrastructural studies of crustacean AG have been conducted mostly in lobster, crab, crayfish and some species of penaeid shrimp (*P. monodon, P. japonicus).* In these studies, crustacean AG were described to be composed of some basic units: the coelomosac, the labyrinth, the nephridial canal and the urinary bladder (Bell and Lightner, 1988; Khodabandeh *et al.*, 2005a; Khodabandeh *et al.*, 2005b; He *et al.*, 2012). In the current study, we found that *P.vannamei* AG constitutes of a coelomosac, a labyrinth and a urinary bladder, but lacks the nephridial canal. These observations were similar to those reported in some marine crustaceans such as lobster *Homarus gammarus* and penaeid shrimp *P. monodon, P. japonicus* (Bell and Lightner, 1988; Nakamura and Nishigaki, 1991; Khodabandeh *et al.*, 2005b). However, the morphological features of *P.vannamei* AG were different from that observed in fresh water crayfish *Astacus leptodactylus.* The AG of crayfish was described to contain a coelomosac, a labyrinth, a nephridial canal and a urinary bladder (Dall, 1970; Khodabandeh *et al.*, 2005a). The authors suggested that the nephridial canal functions in salt reabsorption in crayfish, which does not appear as compulsory in marine species. Our finding is also different from the description of He *et al.* (2012). The authors reported that the AG of *P. vannamei* is composed of a coelomosac, a labyrinth and a nephridial canal, but lacked a urinary bladder. It is important to note that for the morphological studies the latter authors dissected only minor parts of the AG at the base of the antennae but did not section the whole cephalothorax. Therefore, they lost the rostral part of the labyrinth (labyrinth II) and did not recognize the presence and the complexity of the urinary bladder. Interestingly, based on the three-dimensonal reconstruction of the AG, we found that *P. vannamei* possess a very complex AG, which consists of (i) two symmetrical compact parts located at the base of the antennae, (ii) an extension part of the labyrinth derived from the compact part in rostral direction, surrounding the rostral brain, (iii) a multi-

lobed urinary bladder, branched from the base of the antennae and stretching beyond the posterior margin of the hepatopancreas. The two sides of the AG are connected through a urinary channel in between the two lateral lobes and the medial lobe. These features were different from those observed in crayfish *Procambarus clarkii* and krill *Meganyctiphanes norvegica* (Herberholz *et al.*, 2004; Wirkner and Prendini, 2007). The AG of these crustaceans was reported to have two separated compact parts. Each part has an ovoid shape that contains all the components of an AG: the coelomosac, the labyrinth and the urinary bladder. To our knowledge, this is the first work describing the 3D-structure of a crustacean AG in detail that supports a fully comprehensive understanding of this organ.

In vivo titration is generally used to define the infectivity of pathogens in shrimp (Reed and Muench, 1938; Escobedo-Bonilla *et al.*, 2005; Joshi *et al.*, 2014). In this study, the SID_{50} of a WSSV stock and the LD_{50} of a *V. campbellii* stock was first determined in *P. vannamei* by intramuscular (im) injection, intra-AG and peroral (po) inoculation. The results obtained in the present study indicated that pathogens are poorly infectious to shrimp via the digestive system. This finding confirms an earlier study from our group (Thuong *et al.*, 2016). It strongly contrasts to the studies that detected a 100% mortality (Lightner *et al.*, 1998; Wang *et al.*, 1999). It is important to know that most of these studies were not conducted in a standardized controlled way. All animals were pooled in the same tank, fed with tissues from WSSV infected shrimp for several days and terminated often after more than 5 days. It is very well possible that a large amount of virus was homogeneously distributed in the water of the tank due to overfeeding and cannibalism, which may have triggered infection via waterborne route. Our results are in agreement with the findings of Laramore (2007) and Gitterle *et al.* (2006). The authors indicated that individual peroral inoculation of *P. vannamei* with WSSV may not result in a 100% mortality. Although through waterborne route pathogens can reach all potential sites of entry at external surfaces, stomach and gut of shrimp, it was found extremely difficult to infect shrimp with pathogens via that route. Corteel *et al.* (2009) did not succeed to infect early pre-molt shrimp with WSSV using a dose of 10^4 SID_{50} ml^{-1}. Phuoc *et al.* (2009) used a very high concentration (10^8 CFU ml^{-1}) of pathogenic *V. campbellii* to infect shrimp via immersion challenge, which also did not result in any mortality. Alday-Sanz *et al.* (2002) was also not successful to infect penaeid shrimp with pathogenic *V. vulnificus* isolated during a disease outbreak in a shrimp farm via both immersion and peroral inoculation. The authors suggested that bacteria could only enter the digestive tract of the host by oral intubation or immersion when the cuticula was disrupted. However, some recent studies found *V. parahaemolyticus* to be very highly infectious to *P. vannamei* via immersion (Tran

et al., 2013; Joshi *et al.*, 2014). The authors of these studies suggested that *V. parahaemolyticus* gets inside the gastrointestinal tract via waterborne route and releases their toxins, which afterwards become resorbed and cause tissue devastation and dysfunction of the hepatopancreas (Tran *et al.*, 2013). In crustaceans, digestive epithelial cells are covered by either a cuticle layer or a peritrophic membrane (Barker and Gibson, 1978; Dall and Moriarty, 1983; Felgenhauer and Abele, 1985; Lin, 1996; Martin *et al.*, 2006; McGaw and Curtis, 2013). Under intact conditions, these structures are believed to prevent entry of pathogens (Martin *et al.*, 2006; Corteel *et al.*, 2009). In insects, in order to establish an infection from the digestive tract, pathogens use their own chitinase or metalloproteinases to facilitate penetration through the peritrophic membrane (Huber *et al.*, 1991; Langer and Vinetz, 2001). In this study, histology of the bladder showed that there is no cuticle covering the epithelial cells of this organ, which could explain the high infectivity of pathogens to shrimp via intra-AG inoculation.

In vivo studies on the pathogenesis of WSSV in shrimp have established the concept that virulence of WSSV depends on the host, viral strains, administered dose and route of inoculation (Escobedo-bonilla *et al.*, 2007; Rahman *et al.*, 2007; Corteel *et al.*, 2012). Corteel *et al.* (2012) found that *M. rosenbergii* is more capable of resisting infection and disease in comparison to *P. vannamei*. Rahman *et al.* (2007) showed that WSSV-Thai-1 is more virulent than WSSV-Thai-2 and WSSV-Viet strains upon intramuscular injection. In our study, upon intramuscular injection, the first WSSV-infected cells were detected at 18 hpi in all investigated tissues, which is similar to the observation made in some previous publications (Escobedo-bonilla *et al.*, 2007; Rahman *et al.*, 2007). This suggested that upon intramuscular injection virus directly reaches the hemolymph, circulates through hemolymph all over the body and reaches all tissues. Recently, studies on the replication cycle of WSSV have found that a new viral progeny may be released from infected cells after 9 hpi (Li *et al.*, 2014; Li *et al.*, 2015). Therefore, it seems likely that WSSV-antigen positive cells detected at 18 hpi in WSSV-intramuscular injected shrimp were already in the second cycle of WSSV replication. In the present study, upon intra-AG inoculation, WSSV-positive cells were first observed in the epithelial bladder at 18 hpi and other investigated tissues at 24 hpi, which suggested that upon intra-AG inoculation WSSV needs first to replicate in the epithelial bladder cells in order to spread afterwards to other investigated tissues. In this study, only a small proportion of WSSV positive cells were observed in hemolymph of infected shrimp, which is in agreement with the findings in some previous publications (Van de Braak *et al.*, 2002a; Wang *et al.*, 2002; Shi *et al.*, 2005; Escobedo-bonilla *et al.*, 2007). Wang *et al.* (2002) reported that

WSSV infects 2 types of shrimp hemocytes as semigranular and granular cells, while hyaline cells were refractory to virus. In the present study, the high number of WSSV-infected cells observed in the gills and coelomosac were in agreement with the findings reported by Escobedo-bonilla *et al.* (2007) and Rahman *et al.* (2007). In shrimp, the gills and coelomosac are known to perform multiple functions such as respiration, excretion of ammonia, control of the acid-base balance and exchange of ions (Freire *et al.*, 2008). It is posible that virus replication causes lesions in these organs, leading to dysfunction and finally death of the animal.

In conclusion, the AG of *P. vannamei* described here is a complex structure, which constitutes of two compact parts, a rostral extension part of the labyrinth and a multi-lobed urinary bladder. Investigating the infectivity of pathogens in shrimp via different routes of inoculation indicated that pathogens are highly infectious in shrimp via intra-AG inoculation, whereas, they are poorly infectious via peroral route. Our pathogenesis study upon intra-AG inoculation demonstrated that epithelial cells of the bladder are very important targets for primary replication of WSSV, and that gills, coelomosac and epithelial cells are the main target tissues for WSSV replication. Our findings indicate that the external pores of the AG are important natural ports of entry for pathogens in shrimp. In the future, more work will be done to develop effective disease control measures, in view of these new findings.

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Chapter 6

General discussion

Over the last decades the rapid expansion of global shrimp aquaculture industry has created ideal circumstances for the emergence of infectious diseases with white spot syndrome (WSS) as the most important one. Despite many attempts to prevent WSS, effective control measures have not been found yet. The search for the portal of entry of WSSV will help in understanding the viral transmission in between shrimp. Together with the investigation of the factors determining viral transmission, it is an essential step for the development of WSSV control measures. In this thesis, the susceptibility of *P. vannamei* to WSSV infection via peroral inoculation and the barrier function of the peritrophic membrane (PM) to WSSV infection were fully elucidated. The findings indicated that WSSV is poorly infectious to shrimp via peroral route, demonstrating that the peroral route is not the most important natural route of infection of WSSV into shrimp. These findings stimulated us to perform a study to investigate the factors that facilitate WSSV infection and to find more important portals of entry into shrimp. An abrupt drop of environmental salinity and shedding of the old skeleton were found to be risk factors for infection of WSSV into shrimp via waterborne route. Shrimp respond to a low salinity stress by excreting ions through the excretory antennal gland (AG) in order to maintain their hemolymph osmolality. Multiple excretion activities may facilitate WSSV to enter into shrimp. A detailed structure of the AG of *P. vannamei* is described in the present thesis, which supports a fully comprehensive understanding of this organ. Via inoculating pathogens directly into the AG via the external pore of the AG, it was demonstrated that main viral and bacterial pathogens such as WSSV and *Vibrio campbelli* can easily enter shrimp via the AG. This finding directs the way for further research and the development of disease control measures.

Barriers preventing the entry of WSSV into shrimp via peroral route

In the first part of this thesis, the susceptibility of *P. vannamei* to WSSV infection via peroral inoculation and the barrier function of the PM to WSSV were investigated. Early experimental studies on transmission of WSSV in shrimp have raised the thought that WSSV is highly contagious to shrimp via peroral route (Lightner *et al.*, 1998; Escobedo-Bonilla *et al.*, 2006). However, it is important to note that most of these studies were performed under non-standardized conditions. Animals in different molt stages were kept together in the same tank, inoculated via feeding several meals without knowing the exact administered infectious dose of WSSV or used unsuitable tools for direct peroral inoculation causing lesions in the

cuticula and underlying cuticular epithelial cells (Lightner *et al.*, 1998; Vidal *et al.*, 2001; Escobedo-Bonilla *et al.*, 2006). As a consequence of lacking the proper controls, WSSV may be homogeneously distributed in the water and trigger infection via waterborne route. Because other researchers were not able to reproduce infection and disease via feeding or peroral inoculation, the oral route of infection remained a matter of debate (Gitterle *et al.*, 2006; Laramore, 2007). Therefore, in the present PhD thesis the peroral infectivity of WSSV in *P. vannamei* was evaluated under strictly standardized conditions. Shrimp at the same stage of molting were individually starved and maintained under the same conditions. They were inoculated by peroral inoculation, using a thin, flexible catheter, which mimics a natural pathway of WSSV infection *in vivo* and helps to deliver an exact amount of virus to the gastrointestinal tract within a short time frame. The results obtained in this study demonstrated that WSSV is poorly infectious to shrimp via peroral route. This finding is similar to that that has been reported in some earlier publications (Gitterle *et al.*, 2006; Laramore, 2007). However, in the latter studies, no progress has been made to explain the mechanism responsible for the observations. It is known that penaeid shrimp are fully protected from the environment by a thin layer of the exocuticle and the peritrophic membrane (PM) composed of chitin fibrin, chitin-binding proteins, calcium and mucins (Martin *et al.*, 2006; Wang *et al.*, 2012). When intact, these structures prevent pathogens to enter their host. An induced wound in the outer cuticle increases the rate of WSSV infection (Corteel *et al.*, 2009). In insects, some viruses such as occlusion baculoviruses and entomopoxviruses acquire an entry through the digestive tract of the host via chitinase, metalloproteinases, such as enhancins and fusolins, which are embedded into proteinaceous crystalline bodies, to digest chitin and mucin in the PM and thus enhance infection (Huber *et al.*, 1991; Langer and Vinetz, 2001; Mitsuhashi *et al.*, 2007; Hoover *et al.*, 2010). In some cases, non-occlusion baculoviruses became highly infectious to larvae by peroral inoculation when administered together with spindles (proteinaceous structures) of entomopoxviruses (Furuta *et al.*, 2001). It is well known that WSSV is a non-occluded rod-shaped viral particle. The viral particle lacks enzymes to facilitate its penetration through the PM. Therefore, it seems likely that WSSV cannot establish an infection in the digestive tract of the host. In the field, many bacteria such as *V. harveyi* and *V. parahaemolyticus* are present in the water of shrimp ponds and are pathogenic to them. Under certain condition, they can colonize the digestive tract and secrete virulent factors such as chitinase and proteases (toxins), which can facilitate the digestion of shrimp PM or cause the detachment of the epithelium from midgut and thus create openings in the PM through which the virions can pass (Martin *et al.*, 2004;

Tran *et al.*, 2013). Under controlled laboratory conditions, these interfering factors are absent which result in an intact PM. With this background, it was hypothesized that the PM plays a major role as internal barrier of shrimp against WSSV infection. However, surprisingly, the removal of the PM in the present thesis did not help to increase WSSV infection of the underlying epithelial cells. It has been shown before that epithelial cells of the midgut are not infected with WSSV (Wang *et al.*, 1999; Arts *et al.*, 2007; Escobedo-bonilla *et al.*, 2007). However, it was not known whether this was due to the protection by the PM or to the resistance of the underlying epithelial cells. Based on the results of the present thesis, a state of resistance of epithelial cells was demonstrated probably by the absence of receptors on the luminal surface of the epithelial midgut cells or presence of digestive enzymes. In some insects, it is well known that digestive enzymes may decrease the infectivity of virus via peroral infection (Brackney *et al.*, 2008). In addition, the basement membrane is also known to prevent virus entry into the hemocoel and may be responsible in part for the low infectivity of WSSV upon oral inoculation (Mellon, 1992; Martin *et al.*, 2004; Passarelli, 2011). From the findings in these experiments, it has become clear that there are restrictions for WSSV to gain entry into its host via the digestive system.

Physical responses of shrimp during molting and acute drop in environmental salinity increase the susceptibility of shrimp to WSSV infection and may help in finding the sites of WSSV entry into its host

The second part of this thesis focused on the effect of shedding of the old cuticle shell and an acute change in environmental salinity on the susceptibility of *P. vannamei* to WSSV infection by immersion. In the present study, we found that shrimp in ecdysis are more susceptible to WSSV infection than shrimp in post-molt and early pre-molt. Our finding is in agreement with the observation of Corteel *et al.* (2009). The mechanism responsible for the difference in susceptibility to WSSV is likely to be linked to the impact of the change of certain factors of shrimp during the molting process such as: (1) change in barrier structure of cuticle and PM and (2) immunological parameters. Concerning the first factor, it has been reported that through immersion WSSV can reach all potential sites of entry at the surface of the cuticle at the exoskeleton and gastrointestinal tract (esophagus, stomach and hindgut) and the PM. However, WSSV cannot penetrate the cuticle and the PM when these barriers are in an intact condition. An open wound in the cuticle increases the chance for a WSSV infection to become established (Martin *et al.*, 2006; Corteel *et al.*, 2009). In penaeid shrimp, the

cuticle and the PM of penaeid shrimp are known to be renewed during their molt cycle. The formation of new cuticle is finalized during early post-molt (Chan *et al.*,1988; Promwikorn et al., 2007). Therefore, during ecdysis and early post-molt, shrimp may have a reduced barrier to pathogens, which could leave shrimp more vulnerable to infections. In addition, during ecdysis shrimp kick and jump violently in order to get out of their old cuticle, which could result in damaging the new soft shell and increasing the chance for virus to reach susceptible cells. In the field, shrimp may also be damaged by cannibalistic attacks.

The hypothesis that a change in immunological parameters during molting cycle could determine the susceptibility of shrimp to a WSSV infection at the level of viral entry was raised before (Corteel *et al.*, 2009). The authors showed that artificial wounds of the cuticle by cutting appendages increased the susceptibility of post-molt shrimp, but did not change the resistance of early pre-molt shrimp to a WSSV infection upon an immersion challenge. Although the mechanism of WSSV resistance of early pre-molt shrimp after an artificially induced wound is not clear, the authors discussed that some specific immune factors related to coagulation time, phagocytosis, phenoloxidase and reactive oxygen activity may vary between different stages of the molt cycle and determine the susceptibility of shrimp to WSSV infection. Le Moullac and Le Groumellec (1997) indicated that the variation in total haemocyte count and the change in prophenoloxidase system during the molt cycle are correlated with the sensitivity of *P. stylirostris* to *Vibrio AM23* infection. In support with this idea, Liu et al. (2004) showed that the resistance of *P. vannamei* to *V. alginolyticus* infection varies with molt stages. *P. vannamei* in inter-molt displayed a higher resistance to a *V. alginolyticus* infection which correlated with increased haemocyte counts, phenoloxidase activity and respiratory burst. From these observations and the results obtained in the present study, it could be stated that disease occurred frequently more during ecdysis and early post-molt shrimp as the result of a complex interaction of the change in barrier structure of cuticle and PM and immunological parameters.

The field observation that WSSV outbreaks often occur simultaneously with a drop in environment salinity fits very well with our experimental findings. Overall, our findings clearly showed that a salinity drop increases the chance for a WSSV infection to become established. It is known that under sudden salinity stress, the osmotic concentration of hemolymph changes rapidly in order to establish a new equilibrium state between the shrimp and external environment. The animal takes up water and largely excretes ions in order to keep the osmolality constant (Parado-Estepa et al., 1987; Huong et al., 2010). It is generally accepted that absorption of water is conducted via gills and digestive tract (Travis, 1954;

Capen, 1972). Urinary ion excretion is implemented through excretory antennal glands (Lin et al., 2000; Buranajitpirom et al., 2010). Therefore, through water uptake more WSSV can reach potential sites of entry at the surface of the gills and the digestive tract. However, the surface of gills and midgut are fully covered with cuticle and peritrophic membrane (Foster and Howse, 1978; Martin *et al.*, 2006). As discussed above, WSSV cannot penetrate the cuticle of the gills and the PM when these barriers are intact (Corteel *et al.*, 2009).

Therefore, another hypothesis was needed. It was postulated that shrimp may be infected via the external pores of the antennal gland. The diameter of the nephropore is wide enough for virions to pass. In addition, there is no cuticle covering the epithelial cells of the urinary bladder (Bell and Lightner, 1988; Khodabandeh *et al.*, 2005). Therefore, it could make sense that during urination WSSV may enter through the external pores of the bladder and infect the antennal gland.

The findings in the present study give directions to WSSV control measures in penaeid shrimp farms. For instance, by minimizing a fast drop of salinity in *P. vannamei* culture ponds transmission of WSSV may be prevented.

Antennal glands: the sites of entry of pathogens into shrimp

In the present PhD thesis, it has been shown that there are restrictions for WSSV to enter shrimp via the digestive tract and that an acute drop of environmental salinity and ecdysis are risk factors for a WSSV infection in shrimp via immersion route. These findings suggested that the multiple excretion activity of the antennal gland may facilitate WSSV to enter shrimp. This stimulated us to perform a study to investigate if the antennal gland is a portal of entry for pathogens into shrimp. In the third part of this thesis, the anatomy, histology and the three-dimensional structure of the *P. vannamei* AG were first investigated. The histology of the AG of *P. vannamei* described here indicated that this organ has a complex structure, which is composed of two compact parts, each of which consists of a centrally located coelomosac surrounded by the labyrinth I, a rostral zone of the labyrinth II and a multi-lobed urinary bladder ending in two vesicles and two nephropores at the base of the second antennae. The features of the AG observed in the present study raise the question why the labyrinth and the bladder of *P. vannamei* have a complex structure. *P. vannamei* is a euryhaline species that has to migrate through different environmental salinities during their life cycle. Adult shrimp mature, mate and spawn in offshore water. During nauplius, zoea and mysis stages, the larvae inhabit seawater. Post-larvae migrate into coastal areas where freshwater runoff or heavy

rainfall can cause a sudden drop in environmental salinity. In low environment salinity, shrimp quickly regulate their hemolymph osmolality and produce large volumes of diluted urine. The specific structure of the labyrinth and the urinary bladder allows the production and secretion of large amounts of diluted urine. The histology of the AG also showed that there is no cuticle covering the epithelial cells of the urinary bladder and that the diameter of nephropores is wide enough for pathogens to pass, suggesting that pathogens may enter into shrimp via the external pores of the AG and is not hindered by a cuticle. However, one could argue that pathogens are not able to move against the outward flow of urine. Crucial elements that may help pathogens to enter the nephropores are exopodites. They bear multiple tiny setae, which brush frequently on the surface of the nephropores (around 2.5 times per second). It is very well possible that within a short time frame, when urination ceases, the movement of exopodites on the surface of the nephropores may insert pathogens inside the pores. Based on the knowledge obtained during the morphology study of the AG, a fully standardized new technique was developed to inoculate the AG via the nephropores. By using dye, we could demonstrate that the fluid inoculated via a soft catheter into the external pores of the AG entered the bladder and did not damage the epithelium of the bladder. We used this new intra-AG inoculation technique to study different aspects of infectivity and pathogenesis of pathogens in shrimp. The infectious dose 50% endpoint of a WSSV stock and the shrimp lethal dose 50% of a *V. campbellii* stock were determined by different routes of inoculation: intramuscular injection, peroral and intra-AG inoculation. The outcome of the experiments indicated that peroral inoculation resulted in a low WSSV infection level in shrimp, and that peroral inoculation of shrimp with *V. campbellii* resulted in no mortality at all. As discussed above, possible mechanisms responsible for a low infectivity of pathogens via peroral route could be the impact of the barrier function of the cuticula and peritrophic membrane and the presence of digestive enzymes. The infectivity of WSSV and *V. campbellii* in shrimp upon intra-AG inoculation was slightly lower than that upon intramuscular injection, probably due to the fact that a part of the pathogens inoculated inside the bladder was spilled during the removal of the catheter. In addition, the infectivity of WSSV and *V. campbellii* could be reduced by an antimicrobial activity of urine. In a preliminary study, WSSV and *V. campbellii* were incubated with shrimp urine for 4 h. Then, the infectivity of these stocks was determined in shrimp via intramuscular injection. We found that urine did not affect the infectivity of WSSV, whereas it could inactivate *V. campbellii*. The results obtained in the 3D-reconstruction study of the AG and the infectivities of pathogens in shrimp via AG inoculation route indicate that external pores of the AG are important natural ports of entry

for pathogens into shrimp. This idea fits very well with the field observation that WSSV outbreaks often occur rapidly and simultaneously over wide areas after a sudden heavy rainfall (Gunalan *et al.*, 2010; Tendencia *et al.*, 2010). It is known that urine production of shrimp is activated by a sudden drop in environmental salinity but also by feeding. Lobster (*Homarus americanus)* excretes three times more urine during the first hour of feeding (Breithaupt *et al.,* 1999). It is very well possible that dead WSSV-infected shrimp are preferred feed for non-infected shrimp. They can consume several meals of WSSV-infected tissues. During oral uptake, large amounts of virus may pass over the nephropores because the nephropores are located close to the mouth. Therefore, by feeding WSSV may enter into shrimp via external pores of the AG instead of via intestinal tract. This is likely the explanation for the difficulties in reproducing WSSV infection via peroral route. At present, further research in our laboratory is underway to examine if viral or bacterial particles do actually enter the antennal gland under certain circumstances.

Upon intra-AG inoculation, the early pathogenesis of WSSV in shrimp was studied in *P. vannamei*. WSSV-infected cells appeared first at 18 hpi in the epithelium of the bladder and at 24 hpi in all other investigated tissues. Previous studies using indirect immunofluorescence staining and quantitative PCR to investigate the replication cycle of WSSV have reported that different components of a virion as envelop proteins, capsid proteins, DNA were synthesized at different time points. Particularly, at 27°C, nucleocapsid protein VP664 was already detected at 1 hpi. The synthesis of envelope protein VP28 was observed from 3 hpi onwards. WSSV DNA synthesis started from 6 hpi (Leu *et al.*, 2005; Li *et al.*, 2015). Li *et al.* (2015) also showed that the cycle for a new virus generation is about 9 to 12 hpi. Therefore, it was postulated that VP28 should have been detected at 12 hpi and not at 18 hpi. Using immunohistochemistry for the detection of WSSV infection, Escobedo-bonilla *et al.* (2007) found the first WSSV-positive cells at 18 hpi in shrimp inoculated with a low dose ($10^{1.5}$ SID_{50}) of a WSSV stock. However, when shrimp were inoculated with a high dose (10^4 SID_{50}) of the same WSSV stock, the first WSSV-infected cells were observed at 12 hpi. The authors suggested that the signal of WSSV-antigen VP28 detected at 12 or 18 hpi is dependent on the viral inoculated dose. In the present study, shrimp were injected intramuscularly with 10^6 SID_{50} of a WSSV stock and sampled at 6, 12 hpi and processed for detection of WSSV-infected cells by indirect immunofluorescence. We found that these shrimp were WSSV positive at 12 hpi. From these observations, we could postulate that indirect immunofluorescence has a low sensitivity to detect the first infected cells. With a low dose, it is very well possible that two cycles of WSSV replication are required to detect VP28

antigen positive cells in shrimp. In the present study, WSSV-antigen positive cells detected at 18 hpi in the epithelial bladder cells are likely infected in the second wave of replication. After the first cycle of replication (9 to 12 hpi) in the epithelial bladder cells, new virions are released, which then enter the hemolymph, circulate all over the body and infect other tissues.

Based on the results of the present study, a general hypothetical model for WSSV replication kinetics in *P. vannamei* via antennal gland route can be proposed. Upon natural exposure to WSSV, nephropores serve as portal of entry for WSSV. Upon primary replication in epithelial cells of the bladder, the virus reaches the hemolymph, circulates through hemolymph all over the body and reaches all tissues.

In conclusion, the research described in this thesis has successfully led to some concepts. There are important restrictions for pathogens to get into *P. vannamei* via peroral route and peroral route may not be an important natural transmission pathway of WSSV in shrimp. Stress factors of an acute drop in environmental salinity and shedding of the old cuticle shell facilitate the transmission of WSSV into shrimp via waterborne route. *P. vannamei* possess complex structured antennal glands, which are important natural ports of entry for pathogens in shrimp.

Selective breeding for shrimp resistance to the infectious pathogens is an attractive option for disease control. A standardized intra-AG inoculation technique developed in this thesis is very useful to study aspects of viral infectivity, viral replication kinetics and to test, sort out resistant animals to the pathogen. This intra-AG inoculation protocol will be applied to identify resistant animals for the use in breeding programs.

The present thesis clearly showed that shrimp are at risk for a WSSV infection during a sudden drop of environmental salinity. This finding helps us to explore the potential of inducing protection in shrimp aquaculture. Culturing shrimp in dry season or in greenhouse-enclosed systems allows maintaining stable salinity and reducing the risk of a WSSV outbreak. In addition, greenhouse-enclosed culture systems help to keep water temperature as high as 33°C. This temperature has been proven to be effective in preventing WSSV-infected shrimp to die in previous studies from our lab. Based on these findings, an updated management practice for shrimp aquaculture through the use of greenhouse-enclosed culture systems associated with continuous monitoring water temperature and salinity will be a preferred way of working in tropical shrimp farming countries.

In this thesis, a sudden drop in environmental salinity is found as a risk factor that facilitates WSSV entry in shrimp. The mechanism by which shrimp regulate their hemolymph osmolality during a lower salinity stress through urinary ion excretion by the antennal glands

is suggested to help WSSV to enter its host. Besides a lower salinity stress, other factors such as different feeding regimes and aggressive behavior of shrimp are also known to affect the frequency of urinary excretion through antennal glands. Therefore, feeding regimes and aggressive behavior could be additional factors for a WSSV outbreak. Further studies will focus on these factors.

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Chapter 7

Summary

In chapter 1, an overview of shrimp aquaculture and infectious diseases is given. Further, the ecdysis process and response to a lower salinity stress are reviewed.

In chapter 2, the aims of the thesis are: (i) to better understand the susceptibility of shrimp to a WSSV infection via peroral route and the internal barrier function of the peritrophic membrane (PM) of shrimp against WSSV infection, (ii) to evaluate if an acute change in environmental salinity and shedding of the old cuticle shell could facilitate the entry of WSSV in shrimp, (iii) to investigate if the antennal gland is a main portal of entry for pathogens in shrimp.

In chapter 3, as earlier observations on peroral infectivity of WSSV in *P. vannamei* are conflicting, we evaluated the infectivity of WSSV-contaminated feed in penaeid shrimp via peroral route, and test whether the removal of the PM can help to increase the infectivity of WSSV via the digestive system of shrimp. To this end, the infectivity of a WSSV stock was compared by determining SID_{50} upon intramuscular injection, peroral inoculation or via feeding. The following virus titers were obtained: $10^{8.77}$ SID_{50} g^{-1} by intramuscular injection, $10^{1.23}$ SID_{50} g^{-1} by peroral inoculation and $10^{0.73}$ SID_{50} g^{-1} by feeding. Next, the infectivity of a virus stock was tested upon peroral inoculation of shrimp with and without removal of the PM and compared with the infectivity upon intramuscular inoculation. The following titers were obtained: $10^{8.63}$ SID_{50} ml^{-1} by intramuscular inoculation, $10^{1.13}$ SID_{50} ml^{-1} by peroral inoculation in shrimp with PM, $10^{1.53}$ SID_{50} ml^{-1} by peroral inoculation in shrimp without PM. This study indicated that WSSV contaminated feed is poorly infectious to shrimp via peroral route, which contrasts with its infectivity when injected directly into shrimp. The PM plays only a minor role as internal barrier of shrimp against WSSV infection.

Molting and rapid drop of environment salinity have been mentioned before to be risk factors for WSSV outbreaks. In chapter 4, we evaluated the effect of an abrupt drop in environmental salinity and shedding of the old cuticle shell on the susceptibility of *P. vannamei* to WSSV infection by waterborne route. For testing the effect of an abrupt salinity stress, early pre-molt shrimp acclimated to 35 g l^{-1} were subjected to salinities of 50 g l^{-1}, 35 g l^{-1}, 20 g l^{-1}, 10 g l^{-1}, 7 g l^{-1} or 5 g l^{-1} and simultaneously exposed to $10^{5.5}$ SID_{50} ml^{-1} of WSSV for 5 hours. Then, shrimp were brought back to 35 g l^{-1}. Shrimp that were transferred from 35 g l^{-1} to 50 g l^{-1}, 35 g l^{-1} and 20 g l^{-1} did not become infected with WSSV. They became infected with WSSV after an acute salinity drop from 35 g l^{-1} to 10 g l^{-1} and lower. For testing the effect of molting, shrimp in early pre-molt, molting and post-molt were immersed in seawater

containing $10^{5.5}$ SID_{50} ml^{-1} of WSSV. The resulting mortality due to WSSV infection in shrimp inoculated during early pre-molt, ecdysis and post-molt was 0%, 53.3% and 26.72%, respectively. The findings of this study indicated that shrimp are at risk for a WSSV infection during molting and a drop of environmental salinity lower than 10 g l^{-1}.

In chapter 5, the role of the antennal gland (AG) as portal of entry was studied. First, the anatomy, histology and the three-dimensional structure of the *P. vannamei* AG were investigated. The AG of shrimp has a complex structure, which is composed of two symmetrical compact parts, each of which consists of a centrally located coelomosac surrounded by labyrinth I, located at the base of the second antennae, and rostrally extended by labyrinth II and caudally by a multi-lobed urinary bladder ending in two vesicles and two pores protruding at the base of the second antennae. In order to identify if the gastrointestinal tract or the AG is a portal of entry for WSSV and *Vibrio campbellii* in shrimp, the shrimp infectious dose 50% endpoint of a WSSV stock and the shrimp lethal dose 50% endpoint of *V. campbellii* were assessed by intramuscular injection (reference) and upon peroral and intra-AG inoculation. This experiment showed the need of 56 $SID_{50\text{-im}}$ (as determined by im route) to infect 50% of shrimp via intra-AG inoculation and 29 x 10^6 $SID_{50\text{-im}}$ to infect 50% of shrimp via peroral inoculation. Titration of *V. campbellii* showed the need of 62 $LD_{50\text{-im}}$ *V. campbellii* to kill 50% of shrimp upon intra-AG inoculation, while mortality did not occur in shrimp inoculated with $10^{4.3}$ $LD_{50\text{-im}}$ *V. campbellii* via peroral route. Next, the replication kinetics of WSSV in shrimp upon intra-AG inoculation was investigated. The first WSSV-infected cells were observed at 18 hpi in the epithelial cells of the bladder. From 24 hpi onwards, WSSV-infected cells were seen in all examined tissues. The highest numbers of WSSV-infected cells were observed in the coelomosac, gills and cuticular epithelium. Our findings show that the AG is an important portal of entry for pathogens in shrimp.

In chapter 6, the main findings of this thesis are discussed. The infectivity of WSSV in *P.vannamei* via peroral route, the susceptibility of shrimp to WSSV infection during an abrupt drop in environmental salinity, the structure of *P.vannamei* antennal gland, the replication kinetics of WSSV in shrimp via intra-AG inoculation are valuable data for future studies on transmission of WSSV in shrimp and for development of infectious disease control measures.

Samenvatting

In hoofdstuk 1 wordt een overzicht gegeven van de garnaal aquacultuur en de betrokken infectieuze ziekten. Verder werden het vervellingsproces en de reactie van een garnaal op een een lager zoutgehalte eveneens besproken.

Hoofdstuk 2 vat de doelstellingen van het proefstuk samen. Het was ons doel om (i) een beter inzicht te krijgen in de gevoeligheid van garnalen aan een WSSV-infectie via perorale weg en in de interne barrièrefunctie van het peritrofisch membraan (PM) van garnalen tegen een WSSV infectie, (ii) na te gaan of een plotse verandering in het zoutgehalte en het vervangen van de oude cuticula de WSSV-infectie kan vergemakkelijken, (iii) om te onderzoeken of de antennale klier een belangrijke toegangspoort is voor ziekteverwekkers bij garnalen.

In hoofdstuk 3 werd de infectiviteit van WSSV-gecontamineerd voeder bij penaeid garnalen via perorale route nagegaan en werd het onderzocht of het verwijderen van het PM kan leiden tot een hogere infectiviteit van WSSV via het verteringssysteem bij garnalen. Dit werd uitgevoerd aangezien eerdere observaties met betrekking tot perorale infectiviteit van WSSV in *P. vannamei* tegenstrijdig waren. Hiervoor werd de infectiviteit van een WSSV-stock vergeleken door de SID_{50} te bepalen na intramusculaire injectie, perorale toediening en via het voeder. Volgende virus titers werden bekomen: $10^{8.77}$ SID_{50} g^{-1} na intramusculaire injectie, $10^{1.23}$ SID_{50} g^{-1} na perorale inoculatie en $10^{0.73}$ SID_{50} g^{-1} via het voeder. Vervolgens werd de infectiviteit van een virusstock getest na perorale inoculatie van garnalen, met en zonder verwijdering van het PM en vergeleken met de infectiviteit na intramusculaire injectie. Volgende titers werden bekomen: $10^{8.63}$ SID_{50} ml^{-1} na intramusculaire toediening, $10^{1.13}$ SID_{50} ml^{-1} na perorale inoculatie bij garnalen met PM en $10^{1.53}$ SID_{50} ml^{-1} na perorale inoculatie bij garnalen zonder PM. Deze studie toont aan dat WSSV-geïnfecteerd voeder weinig infectieus is voor garnalen via orale route, hetgeen in sterk contrast is met de infectiviteit na directe injectie bij garnalen. Het PM speelt een ondergeschikte rol als interne barrière tegen een WSSV-infectie.

Vervellen en een plotse daling in het zoutgehalte in de omgeving worden genoemd als risicofactoren voor WSSV-uitbraken. In hoofdstuk 4 werd het effect van een plotse daling van het zoutgehalte en het afwerpen van de oude cuticula op de vatbaarheid van *P. vannamei* na een WSSV-infectie via water nagegaan. Om het effect van de stress na een verandering in het zoutgehalte te testen, werden aan-35 g l^{-1}-geacclimatiseerde pre-molt (voor vervellen) garnalen gelijktijdig onderworpen aan zoutgehaltes van 50 g l^{-1}, 35 g l^{-1}, 20 g l^{-1}, 10 g l^{-1}, 7 g l^{-1} of 5 g l^{-1} én een $10^{5.5}$ SID_{50} ml^{-1} WSSV-infectie gedurende 5 uren. Nadien werden

de garnalen in een omgeving met een zoutgehalte van 35 g l^{-1} gebracht. Garnalen die van 35 g l^{-1} naar 50 g l^{-1}, 35 g l^{-1} en 20 g l^{-1} verplaatst werden, werden niet geïnfecteerd met WSSV. Garnalen werden echter wel geïnfecteerd na een plotse daling van het zoutgehalte van 35 g l^{-1} naar 10 g l^{-1} en lager. Om het effect van vervelling na te gaan, werden garnalen in pre-molt, molt en post-molt (voor, tijdens en na vervellen) fase ondergedompeld in zeewater met $10^{5.5}$ SID_{50} ml^{-1} WSSV. De mortaliteit na inoculatie met WSSV was 0% (pre-molt), 53.3% (molt) en 26.72% (post-molt), respectivelijk. De resultaten van deze studie geven aan dat garnalen risico lopen op een WSSV-infectie tijdens het vervellingsproces en in een milieu waarvan het zoutgehalte lager is dan 10 g l^{-1}.

In hoofdstuk 5 werd de rol van de antenale klier (antennal gland, AG) als mogelijke intredepoort bestudeerd. Eerst werd de anatomie, de histologie en de drie-dimensionale structuur van de AG van *P. vannamei* onderzocht. De AG van garnalen heeft een complexe structuur dat bestaat uit twee symmetrische compacte delen, waarvan elk deel bestaat uit een centraal gelokaliseerde coelomosac, omgeven door labyrinth I, gelegen ter hoogte van de secundaire antennen, dat rostraal verlengd is door labyrinth II, en een caudale meerlobbige urineblaas eindigend in twee blaasjes en twee poriën die uitsteken aan de basis van de secundaire antennen. Om te bepalen of het maagdarmkanaal of de AG een toegangspoort is voor WSSV en *Vibrio campbellii* in garnalen, werden de garnalen infectieuze dosis met 50% eindpunt van een WSSV stock én de garnalen dodelijke dosis met een 50% eindpunten van *V. campbellii* beoordeeld na een intramusculaire injectie (referentie), na perorale en intra-AG inoculatie. Dit experiment toonde aan dat van 56 $SID_{50\text{-im}}$ (zoals bepaald door intramusculaire route) nodig was om 50% van de garnalen te infecteren via intra-AG inoculatie en 29 x 10^6 $SID_{50\text{-im}}$ om 50% van de garnalen via perorale inoculatie te infecteren. Titratie van *V. campbellii* toonde aan dat 62 $LD_{50\text{-im}}$ *V. campbellii* nodig was om 50% van de garnalen na intra-AG inoculatie te doden, terwijl er geen mortaliteit werd geobserveerd bij garnalen geïnoculeerd met $10^{4.3}$ $LD_{50\text{-im}}$ *V. campbellii* via perorale route. Vervolgens werd de vermeerderingskinetiek van WSSV in garnalen na intra-AG inoculatie bestudeerd. De eerste WSSV-geïnfecteerde cellen werden teruggevonden 18 uur na infectie, in de epitheliale cellen van de blaas. Vanaf 24 uur na infectie en later werden WSSV-geïnfecteerde cellen gezien in alle onderzochte weefsels. Het hoogste aantal geïnfecteerde cellen werden teruggevonden in de coelomosac, de kieuwen en het cuticulair epitheel. Onze bevindingen tonen aan dat de AG een belangrijke toegangspoort is voor ziekteverwekkers bij garnalen.

De belangrijkste bevindingen van deze thesis worden besproken in hoofdstuk 6. De infectiviteit van WSSV in *P. vannamei* via perorale route, de gevoeligheid van garnalen aan WSSV-infectie tijdens een plotse daling in het zoutgehalte van de omgeving, de structuur van de AG van *P. vannamei*, de vermeerderingskinetiek via intra-AG inoculatie zijn belangrijke bevindingen voor verder onderzoek naar de overdracht van WSSV in garnalen en de ontwikkeling van controlemaatregelen tegen infectieuze ziekten.

Curriculum vitae

Khuong Van Thuong was born on 16th August, 1977 in Hungyen city, Vietnam. In 2001, he obtained a diploma of Aquaculture at Nha Trang University, Nha Trang city, Vietnam. From 2001 to 2008, he worked as a researcher at Biotechnology Center, Research Institute for Aquaculture No.1, Vietnam. In 2008, he obtained a scholarship by BTC (Belgian technical cooperation) and was enrolled in a two-year international course program "Master of Science in Aquaculture" at the Laboratory of Aquaculture and Artemia Reference Center (ARC), Ghent University. From 2010 to 2011, he continued working at the Research Institute for Aquaculture No.1 as a researcher. At the end of 2011, he started a PhD program under the supervision of Prof. Hans Nauwynck at the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University. His PhD program was funded by a scholarship of the Vietnamese government.

Khuong Van Thuong is author or co-author of 8 scientific publications.

Publications

Publications in Peer-reviewed international journals

1: Mathias Corteel, João J Dantas-Lima, Vo Van Tuan, **Khuong Van Thuong**, Mathieu Wille, Victoria Alday-Sanz, Maurice B Pensaert, Patrick Sorgeloos, Hans J Nauwynck (2012). Susceptibility of juvenile *Macrobrachium rosenbergii* to different doses of high and low virulence strains of white spot syndrome virus (WSSV). Diseases of Aquatic Organisms 100 (3): 211-218.

2: Wenfeng Li, Van Thao Nguyen, Mathias Corteel, João José Dantas-Lima, **Khuong Van Thuong**, Vo Van Tuan, Peter Bossier, Patrick Sorgeloos, Hans Nauwynck (2014). Characterization of a primary cell culture from lymphoid organ of Litopenaeus vannamei and use for studies on WSSV replication. Aquaculture 433:157–163.

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5: Li, W., L.M.B., De Cryse, G.M.A., Thuns, Tuan, V.V., **Thuong, K.V**., Bossier, P., Nauwynck, H (2015). Virus replication cycle of white spot syndrome virus (WSSV) in secondary cell cultures from the lymphoid organ of *Litopenaeus vannamei*. Journal of General Virology DOI: 10.1099/vir.0.000217.

6: V V Tuan, G. De Cryse, **K V Thuong**, P Bossier, H J Nauwynck (2016). Kinetic analysis of internalization of white spot syndrome virus (WSSV) by haemocyte subpopulations of penaeid shrimp, *Litopenaeus vannamei* (Boone), and the outcome for virus and cell. Journal of Fish Diseases DOI: 10.1111/jfd.12482

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8: **K V Thuong**, V V Tuan**,** Wenfeng Li, P Sorgeloos, P Bossier, H J Nauwynck (2016). Effects of acute change in salinity and molting on the infection of white leg shrimp *(Penaeus vannamei)* with white spot syndrome virus upon immersion challenge. Journal of Fish Diseases DOI: 10.1111/jfd.12471.

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