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White spot syndrome virus infection in *P. vannamei* and *M. rosenbergii*: experimental studies on susceptibility to infection and disease

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Cover image: *Caridina cantonensis* "crystal red" shrimp, winner of the 2nd place on the Holland Shrimp Show 2012, bred and photographed by the author. This ornamental shrimp species is susceptible to WSSV infection as well.

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List of Abbreviations

3DE	3-dehydroecdysone
ArF	Argon fluoride
BGBP	β-glucan-binding protein
CCAP	Crustacean cardioactive protein
CHH	Crustacean hyperglycaemic hormone
DNA	Deoxyribonucleic acid
FITC	Fluorescein isothiocyanate
GIH	Gonad inhibiting hormone
hpi	Hours post-injection/immersion/inoculation
IHC	Immunohistochemistry
IIF	Indirect immunofluorescence
IN	Integrase
kbp	Kilo base pair
LD_{50}	Lethal dose
LGBP	Glucan-binding protein
LOS	Lymphoid organ spheroids
LPS	Lipopolysaccharide
MBW	Mean body weight
MIH	Moult inhibiting hormone
MOIH	Mandibular organ inhibiting hormone
nm	Nanometre
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PID_{50}	Prawn infectious dose 50% endpoint
PL	Post-larva
PM	Peritrophic membrane
proPO	Prophenoloxidase
PRPs	Pattern recognition proteins
RNAi	RNA interference
RT	Reverse transcriptase
RV-PJ	Rod-shaped nuclear virus of *P. japonicus*
SID_{50}	Shrimp infectious dose 50% end-point
siRNA	Short interfering RNA
SPF	Specific pathogen-free
TEM	Transmission electron microscopy
TSV	Taura syndrome virus
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VNTR	Variable number tandem repeat
VP	Viral protein
WSSV	White Spot Syndrome virus
XO-SG	X-organ/sinus gland system
µl	Microlitre
µm	Micrometre

CHAPTER 1

Introduction and aims of the thesis

During the course of the three past decades, aquaculture of penaeid shrimp (commonly called “scampi” in Belgium) has expanded dramatically in many (sub)tropical countries around the world. Since 2010, production volume has passed 3 million metric tons with a value of more than 12 billion dollars. However, the intensification of the industry has come with a toll, and infectious diseases have severely affected the development and sustainability of the sector. One of the most prevalent and lethal infectious agents has been the white spot syndrome virus (WSSV). Since its discovery in the early 90’s, it is said to be responsible for more shrimp crops lost before harvest than any other disease agent in shrimp aquaculture. Typically, shrimp suddenly start to show disease symptoms, including white spots under their skin, and within a few days all shrimp in the pond die. Disease outbreaks with WSSV often occur in waves, when a vast number of shrimp farms are hit by the virus over wide geographical areas during the course of a few weeks. Up to date, despite many attempts by governmental research institutes and commercial companies, no effective control measures have been developed to control this virus in farms. One of the key problems behind the lack of preventive or curative treatments, is the still fragmentary knowledge on the factors determining the susceptibility of the host to infection and disease. Both the pathogenesis of WSSV and the anti-viral defense system of decapod Crustacea are only rudimentarily understood. Very little is understood about how WSSV manages to enter a host, and how WSSV appears to cause less infection and disease in some hosts. It was against this background that the two parts of the research project in this thesis were conceived.

Firstly, we investigated the factors involved in the process of WSSV to gain entry into shrimp from the environment. Results obtained with experimental inoculations of WSSV into the rearing water of shrimp are highly variable. Some published studies show a high percentage of infected shrimp after exposure to waterborne WSSV, others show that shrimp do not become infected, even when exposed to high virus doses as determined by intramuscular titrations. Overall, these contradictory results show that certain crucial variables are not clear and that the factors, which are responsible for the efficient entry of WSSV into its host, need to be determined.
We do know the cell types in which WSSV is able to replicate, and we know that all these cells are shielded from viruses in the outside world by the exoskeleton or cuticle of the shrimp. The chance of the virus gaining entry will thus depend on the

possibility to pass the external barriers of the shrimp. As the cuticle is a dynamic structure, changing both in composition and thickness during the moult cycle, we hypothesised that the barrier function of the cuticle would vary in time.
The first aim of this thesis was thus to compare the susceptibility of shrimp to waterborne WSSV and to identify the stages of the moult cycle in which shrimp are more susceptible or resistant to infection. We also went on to test whether wounds artificially induced in the cuticle could serve as entry points for the virus.

Secondly, we wanted to identify a host which was less susceptible to infection and disease caused by WSSV than penaeid shrimp. For this, we looked at the freshwater prawn *M. rosenbergii* (usually referred to as "reuze zoetwatergarnaal" in Belgium). Several published studies have indicated that this species has a significantly lower susceptibility to WSSV infection and disease than penaeid shrimp. If *M. rosenbergii* would indeed possess an anti-viral defense against WSSV, this would present a very interesting lead for research on control strategies. Unfortunately, unstandardised methodologies were used in the studies on WSSV in *M. rosenbergii*, and the published results were conflicting, with the prawns being totally refractory to infection or suffering severe infection and mortality due to WSSV.
The second aim of this thesis was thus to irrevocably establish how susceptible *M. rosenbergii* is to WSSV infection and disease by means of standardised methodology. For this, we used the methods which were previously set up for penaeid shrimp in our laboratory. *M. rosenbergii* were inoculated via intramuscular route and the obtained quantitative data on the pathogenesis, infectivity and pathogenicity of the virus in infected *M. rosenbergii* were compared with data previously obtained in penaeid shrimp.

CHAPTER 2

Literature review

2.1. Penaeid shrimp and palaemonid prawns

2.1.1 Taxonomy

Shrimp of the *Penaeidae* family and prawns of the *Palaemonidae* family both resort under the order of *Decapoda*. In this thesis, the species of interest were: *Penaeus vannamei* and *P. monodon*, and *Macrobrachium rosenbergii*. All three belong to the largest phylum in the Animal Kingdom, the Arthropoda, whose members are characterised by a chitinous exoskeleton that is periodically moulted, a segmented body and jointed, paired appendages. There are thousands of terrestrial species in this phylum, and a large, predominately aquatic subphylum, the Crustacea. The penaeid shrimp and the palaemonid prawns are both in the Order of Decapoda (with 10 walking legs) and are among the more highly evolved crustaceans of the Class Malacostraca (Bailey-Brock and Moss, 1992).

Phylum	Arthropoda	
Subphylum	Crustacea	
Class	Malacostraca	
Subclass	Eumalacostraca	
Superorder	Eucarida	
Order	Decapoda	
Suborder	Dendrobranchiata	Natantia
Superfamily	Penaeoidea	
Family	Penaeidae	Palaemonidae
Genus	Penaeus	Macrobrachium
Species	*P. vannamei* and *P. monodon*	*M. rosenbergii*

Considerable confusion exists in international literature on the use of the terms “shrimp” or “prawn” for naming Decapoda of the families Palaemonidae or Penaeidae, respectively. In the anglo-saxon world, “prawn” is the preferred name for penaeids, whereas in the rest of the world it refers to freshwater palaemonids. In the current thesis, “shrimp” will be used to denominate penaeids and “prawn” for palaemonids.

Another controversy exists about the genus names of penaeid shrimp. Pérez-Farfante and Kensley (1997) proposed to elevate the subgenus names in the family of Penaeidae to the genus level. Instead of classifying all penaeid shrimp in 1 genus *Penaeus*, the species would resort under the genera: *Farfantepenaeus*, *Fenneropenaeus*, *Litopenaeus* (including *L. vannamei*), *Marsupenaeus*, *Melicertus* and *Penaeus* (including *P. monodon*). This change was followed by a part of the authors publishing on penaeid shrimp, but not by another part, resulting in a confusing situation in which both systems existed in literature. Flegel (2007a) pointed out that no one is obliged by the rules of zoological nomenclature to accept the revisions in penaeid shrimp binomials proposed by Pérez Farfante and Kensley (1997). He suggested that the scientific community would accept the sub-genus names by including them in brackets between the genus name Penaeus and the relevant species names, as is recommended by the rules of zoological nomenclature [e.g., *Penaeus* (*Litopenaeus*) *vannamei*]. This idea was also supported by the editors of Aquaculture journal (Alderman *et al.*, 2007) and was supported by genetic analyses by Ma *et al.* (2011). In the present thesis, the suggestions of Flegel (2007a) will be followed.

All freshwater prawns cultured for consumption belong to the genus *Macrobrachium*, the largest genus of the family *Palaemonidae*. About 200 species have been described, of which 49 species are commercially exploited, mainly *M. nipponense* which is smaller than *M. rosenbergii* (Holthuis, 1980). As the genus name indicates, all members develop typical, over-sized chelipeds.

2.1.2. Penaeid shrimp

2.1.2.1. Morphology and physiology

As in all Malacostraca, the body of penaeid shrimp is composed of 19 segments (Figure 1). Five make up the head, 8 are located in the thorax and 6 in the abdomen. The head and thorax are fused into the cephalothorax, also known as pereon. Each segment of the cephalothorax bears a pair of bi- or triramous appendages, composed of an exo-, endo- and epipodite. The first 2 appendages of the head have a sensory function (antennae and antennulae), the following 6 are used in feeding (a set of mandibles and 5 pairs of maxillae). The last 5 limbs of the cephalothorax are the

walking legs (pereiopods), of which the first 3 are equipped with chelae for grabbing food. The exoskeleton of the cephalothorax (carapace) covers the gills with a protective gill chamber (branchiostegite) and forms a dorsal keel-shaped rostrum between the eyes. The abdomen (pleon) has six segments, mainly composed of muscle, and bears paired swimming legs (pleopods) on the first 5. The final segment is the tail fan, composed of 2 pairs of uropods and the telson, which the shrimp uses to quickly jump backwards in case of danger (Ruppert and Barnes, 1994; Budd, 2002).

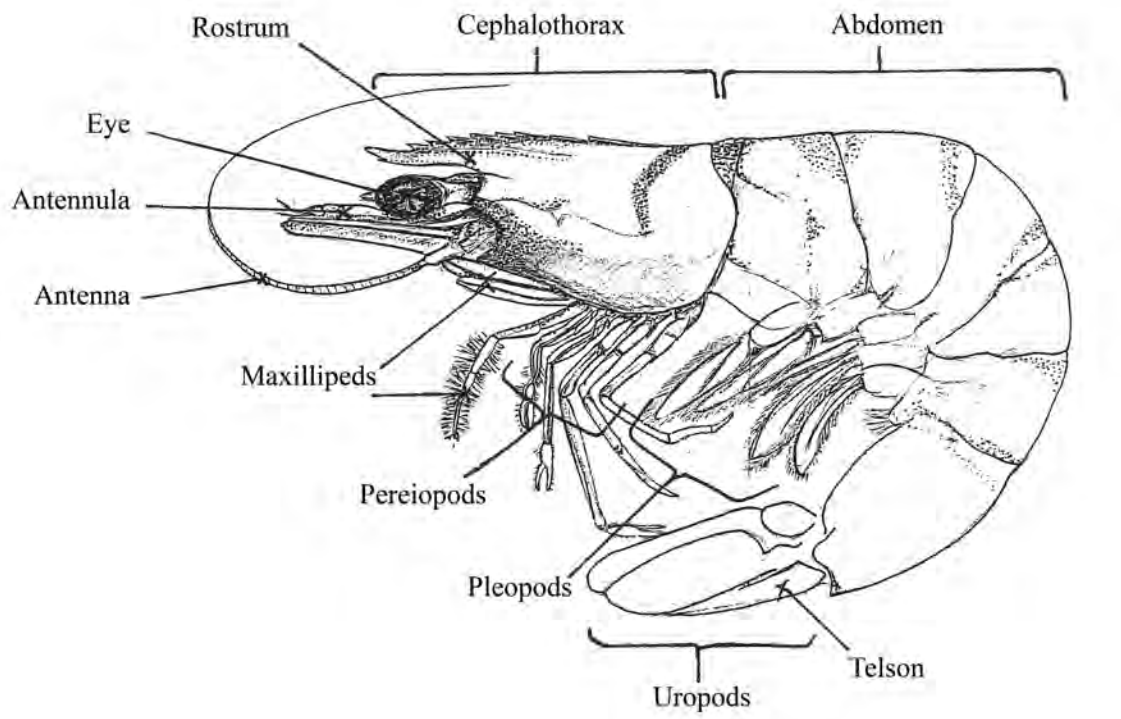

Figure 1. External morphology of a penaeid shrimp (Corteel, 2005).

2.1.2.1.1. Integument and moult

This section has been published as: Corteel M and Nauwynck HJ (2010) Chapter 4, The integument of shrimp: cuticle and its moult cycle. In: Alday-Sanz V (Ed) "The shrimp book", Nottingham University Press, United Kingdom.

INTRODUCTION

Because the integument of penaeid shrimp plays a central role in the research of this thesis, it will be discussed here in detail.

As all arthropods, shrimp possess an extremely efficient integument which serves a dual function as skin barrier and skeleton. This exoskeleton, which is formed by the epidermal cells of the integument, is usually called cuticle or cuticula. It is primordial to take into account that the integument of shrimp is a dynamic organ. Especially in growing animals, the integument is constantly involved in a cyclic process of moulting. To allow growth and regeneration, a new cuticle is deposited under the old one. Immediately after the old cuticle is shed, the new skin is stretched while it is still soft and the animal expands. Because of this, much of a shrimp's physiology is orchestrated in the tempo of the moult cycle, with periods of accumulation of reserves alternating with periods of rapid growth.

Much of the knowledge on decapod crustacean cuticle has been gathered through investigations in Astacidea and Brachyura. Extensive research on penaeid shrimp is lacking, and for instance no detailed data are available on the morphology and composition of shrimp cuticle. Hence, much of the information below applies to all Crustacea. Specific reference to penaeid shrimp will be given where possible.

a) Integument morphology

The exoskeleton of Crustacea is a complex biocomposite. It is composed of the polysaccharide chitin, proteins, minerals and some lipids. For an extensive review on the morphology and biochemistry of crustacean integument, see Compère *et al*. (2004). Recently, the organisation of arthropod cuticle was reviewed by Fabritius *et al*. (2008), using *Homarus americanus* as example (see also Raabe *et al*., 2006, 2007).

The bio-polymer chitin provides the supportive framework of the structure (Neville, 1975; Stevenson, 1985). Much like the iron bars in reinforced concrete used for the building of human constructions, the chitin supplies the cuticle with resistance against

tension. The elementary molecules of chitin are the monosaccharides N-acetyl-D-glucosamine and D-glucosamine. These building blocks are polymerised by β-1,4 bindings into long, linear chains. In arthropods, chitin is arranged in an anti-parallel manner: the α-crystalline form of chitin. Eighteen to 25 chitin polymer chains (19 according to Atkins in Neville, 1984) organise together in a crystalline core with a diameter of 2 to 5 nm. This chitin crystalline core constitutes a central axis, around which a sheath of protein subunits is deposited. Together, the chitin-protein complex forms a so-called microfibre of 7.25 nm wide and 0.3 μm long (Blackwell *et al.*, 1982). Considering the size of this smallest organisational unit of chitin and protein, the name nanofibril, as used by Raabe *et al*., is more suitable.

Proteins are deposited around the chitin strands and between the nanofibrils as the concrete around reinforcing bars in reinforced concrete. They render the composite impermeable and resistant against mechanical compression. The protein component and associated water molecules are determining for the mechanical properties of the cuticle (Skinner *et al.*, 1992; Andersen, 1999). Two categories of protein occur: those covalently bound to chitin or another component of the cuticle, and those non-covalently bound. Covalent bindings between proteins and between proteins and chitin solidify and stiffen the cuticle. This process is catalysed by phenoloxidase enzymes which convert phenol molecules into reactive quinones (Neville, 1975; Roer and Dillaman, 1993). The resulting bridges between the molecules give rise to the characteristic 'tanning' or sclerotising of the cuticle, rendering the proteins insoluble in water. Non-covalently bound proteins are 'free' proteins, only bound to other cuticular compounds by electrostatic and hydrogen bonds. As a result, these proteins can be extracted from the cuticle quite easily and are soluble in water-based buffers. In Crustacea, the external layers become sclerotised in the hours after moulting, during which the initially soft and pliant cuticle becomes tough and rigid (see below).

Inorganic minerals comprise 30-50% of the dry weight of shrimp cuticle (Welinder, 1974). This makes shrimp cuticle weakly mineralised compared to that of for instance the well-studied brachyurans. The mineral salts in cuticle are calcium-magnesium and strontium carbonates, which are deposited as crystalline calcite. Obviously, the mineralisation of the cuticle increases its hardness.

Transmission electron microscopy (TEM) revealed that the chitin-protein microfibres (also called nanofibrils) are combined by the dozens into macrofibres with a diameter up to 100 nm (Neville, 1975; Giraud-Guille, 1984). Depending on the location and

function of the cuticle, the macrofibres are arranged differently, thereby responsible to a large extent for the final mechanical properties of the cuticle. The macrofibres in most of the cuticle are organised in horizontal planes running parallel with the surface of the cuticle. In each plane, macrofibres are deposited with the long axis parallel to each other in horizontal sheets. Their direction changes from sheet to sheet by a few degrees. The overall structure of cuticle is thereby a helicoidal, twisted plywood-like construction with stacks of horizontal planes of macrofibres. Visually, this becomes evident as lamellae in vertical crosssections of the cuticle and as a parabolic pattern in oblique sections. Every lamella comprises the distance between two sheets of macrofibres orientated in the same direction. Between these two outer sheets of which the macrofibers are orientated in the same direction, the direction of other sheets gradually rotates 180°.

By light microscopic observation, four layers can be seen in fully formed, inter-moult cuticle of shrimp (Bell and Lightner, 1988; Roer and Dillaman, 1984; Promwikorn *et al*., 2007). From out- to inside, these are: epi-, exo-, and endocuticule and the membranous layer (Figure 2).

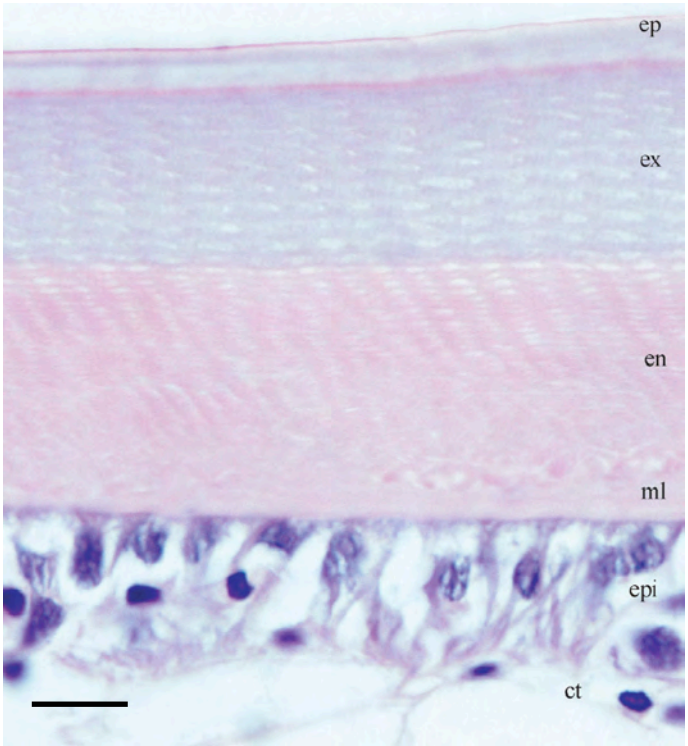

Figure 2. Light microscopic photograph of 15 g *P. vannamei* cuticle and underlying epithelium of the uropod (HE staining; scale bar = 10 µm)
(ep: epicuticle; ex: exocuticle; en: endocuticle; ml: membranous layer; epi: epidermal cells ; ct: connective tissue).

Lamellae of macrofibres are present and clearly visible in the exo- and endocuticle. The epicuticle is different from the other layers in composition and structure. It is very thin and contains lipids, proteins (as well as lipo- and glycoproteins) and minerals, but no chitin. The epicuticle is the first barrier against the outside world, and mainly regulates permeability. The exocuticle is present before the moult and becomes tanned and mineralised shortly after. This layer is the primary support of the exoskeleton. The endocuticle is clearly distinct from the exocuticle and contains much calcium. It supplements the exocuticle's supportive function. In shrimp, the organisation of the exocuticle appears more fibrillar than the endocuticle. The situation in crab and lobster, where the stacking height of the lamellae in the exocuticle is smaller than in the endocuticle, appears to be reversed in shrimp. The membranous layer lies just above the cuticular epithelia cells and is basically the last part of the endocuticle to be secreted. It is unmineralised and composed of thin lamellae. It becomes functionally important during the process of shedding the exuvium.

Underneath the cuticle lay the epidermal cells. This single layer of pseudostratified epithelium is responsible for the secretion of the entire exoskeleton, including its many elaborate structures. Close to the basal lamina, star-shaped chromatophores spread out (Noël, 1994). These cells can rearrange pigments in their cytoplasm and thereby influence the color of the shrimp. Other categories of cells can also be present: trichogenic cells which send out sensory bristles, tegumental glands which deposit their exocrine products via a duct through the cuticle, and "accessory cells" which have not been fully characterised yet, but appear to be the equivalent of the oenocytes known in insects, involved in synthesis of cuticular material (Locke, 1984).

b) The moult cycle: cyclic morphological and physiological integument changes

The moult process in Crustacea is most often described as a cycle, which repeats itself every time the cuticle is shed. Typically, this recurrent cycle is divided into 3 stages which occur between the pivotal moments of the shedding of the old cuticula (ecdysis). Chronologically, these are: post-moult (metecdysis), inter-moult (anecdysis) and pre-moult (proecdysis). One of the first researchers working on the moult of Crustacea, (Drach, 1939) applied a letter code to these stages: A and B for early and late post-moult respectively, C for inter-moult and D for post-moult. The

distinction between the stages was initially based on the hardness of the skeleton and histology (Drach and Tchernigovtzeff, 1967). In later studies, systems were developed to classify the stages in the moult cycle by microscopic observation of appendages, preferably areas with setae where morphological changes are more pronounced and can be observed more clearly (Stevenson, 1972; Aiken, 1973; Vranckx and Durliat, 1978; Lyle and Macdonald, 1983; Criel and Walgraeve, 1989; Musgrove, 2000; Gorokhova, 2002).

Studies on the moult process in penaeid shrimp which list selection criteria for the various moult stages have been published for *Penaeus (Farfantepenaeus) duorarum* (Schafer, 1968) *Penaeus (Farfantepenaeus) merguiensis* (Longmuir, 1983) *Penaeus (Litopenaeus) setiferus* and *Penaeus (Litopenaeus) stylirostris* (Robertson *et al*., 1987), *Penaeus (Litopenaeus) vannamei* (Chan *et al*., 1988; Cesar *et al*., 2006) and *Penaeus monodon* (Promwikorn *et al*., 2004; Promwikorn *et al*., 2007) (Table 1). The key criteria used to determine the moult stage are the appearance of the epidermis and the setae. This includes pigmentation, the formation of new setae (setogenesis), the presence of matrix or internal coni in the setal lumen and the formation of so-called setal organs (nodes) at the basis of the setae (Table 2).

Table 1. Published studies on the characterisation of moult stages in penaeid shrimp.

Species	Microscopic Observation	Age	Weight (g)	Length (cm)	Waterparameters		Author
					Temperature (°C)	Salinity (g l^{-1})	
P. setiferus *P. stylirostris*	10-70X Uropods	Adult	43-57	-	27-29	34-41	Robertson *et al.,* 1987
P. vannamei	100X Exised pleopods	Juvenile	-	11.5-13	20-22	28-30	Chan *et al.,* 1988
P. merguiensis	400X Exised pleopods	Juvenile	-	-	20.5-24.0	35	Longmuir 1983
P. monodon	100X Uropods	Juvenile	10-20	-	-	10-20	Promwikorn *et al.,* 2004

Table 2. Characteristics of the major moult stages in penaeid shrimp according to the publications of Table 1.

Author	Light microscopic criteria of moulting stages with duration in days (d)									
Robertson *et al.*, 1987	A	B	C1-C2-C3			D0-D1			D2-D3	
	Pigmented cellular matrix fills setal bases 1d	Matrix retracts from setal bases 2d	Matrix absent from setal bases, pigmented epidermal line at the basis of the setal nodes 2d			Pigmented epidermis retracts from basis of setal nodes 3d			Further retracting of epidermis + formation of new setae 4d	
Chan *et al.*, 1988	A	B	C			D0	D1		D2	D3
	Epidermis translucent Setae filled with translucent matrix 1d	Matrix granular and retracts from setae Start formation of internal cone in setae base	Epidermis granular Matrix retracted from setae Internal cones completed 1-2d	Setal organs clearly visible 6-8d	Setal bases more dense Setal organs more distinctive 4-7d	Separation between cuticle and epidermis New cuticle not present 3-6d	New cuticle present Formation new setae 8-10d		New setae form barbules + tips in bases old setae 2-3d	Setal organs disappear New setae fold 1-2d
Longmuir 1983	A	B	C		D0	D1	D1’	D1”	D1”’	D2	D3-D4
	Translucent cellular matrix fills setae and setal bases	Start formation internal cone Matrix retracts from setae	All setae contain internal cones		Epidermis retracts from setal bases	New setae start to form	Setal tips stick out of slightly irregular epidermis	Invagination epidermis around setae Setules visible	Invagination complete = half size of old seta	New cuticle thick as old Setal fold rounded	Not delineated by setogenesis
Promwikorn *et al.*, 2004	A	B	C		D0	D1	D2	D3	D4	
	Soft delicate setae No setal cones	Young setal cones	Mature setal cones Full-spread epidermis		Clear margin of epidermal tissue at base of setal cones	Clear narrow zone between setal cones and epidermis	Wider clear zone Wavy edge of epidermis	Highly wavy edge of epidermis White layer at edge of epidermis	Obvious wider clear zone Serrated edge epidermis Light-reflecting white layer at edge epidermis Paralleled-band fashion of epidermis	

Below we will define the stages of the moult cycle of penaeid shrimp according to literature and own research. In contrast with Astacideae and Brachyurae, the cycle of penaeid shrimp is rather short. Hence we divide the cycle in a limited number of major stages (Table 3 and Figure 3 and 4). Per stage, the morphological and physiological changes in the integument will be reviewed. In the subtitles, between brackets, letter codes used by other authors to define more elaborate (sub)stages are mentioned (Drach, 1939; Skinner, 1985; Compère *et al*., 2004).

Table 3. Characteristics of the major moult stages in *P. vannamei* and *P. monodon* according to Corteel *et al.* (2009).

Moult stage	Characteristics
A Early post-moult	- epidermis in contact with all of cuticula, runs up into setae - setal lumen filled with (granular) epidermal matrix - no internal cones in setae - setal nodes between setae vaguely visible
B Late post-moult	- retraction of epidermis from setae - epidermal matrix still in base of setae - small internal cones start to become visible in setae - setal nodes clearly visible
C Inter-moult	- epidermis lies in a straight line at bottom of the setal nodes - no epidermal matrix in setal lumen and base - internal cones clearly visible - setal nodes
D1 Early pre-moult	- epidermis retracts from cuticula leaving a translucent zone (apolysis) - epidermis begins formation of new cuticula, but still invisible - no epidermal matrix in setal lumen - internal cones - setal nodes
D2 Late pre-moult	- translucent zone - new cuticula visible - newly forming setae visible, folded into epidermis - no epidermal matrix in setal lumen - internal cones - setal nodes
E Moult	shedding of old cuticula

METECDYSIS

Early post-moult stage "A" (~ A1, A2)

Immediately after kicking off the exuvia, shrimp will take up water and expand their new cuticle. This stage is, therefore, the only moment in time for shrimp to grow, replace damaged cuticle and regenerate extremities. The new cuticle only comprises the epicuticle and exocuticle, which have been secreted prior to the moult. The latter layer is still unsclerotised and unmineralised at the start of this stage, leaving the exoskeleton of shrimp very soft, pliant and fragile. In this stage, the epidermal cells are in intimate contact with the cuticle and infiltrate it with cellular extensions through pore canals. The epidermis continues to secrete additional layers to the cuticle to form the endocuticle. Towards the end of this process, the epidermis will recede from the cuticle and withdraw from the lumen of setae.

The behaviour of shrimp is strongly affected by the state of their cuticle. Shrimp in A-stage are not able to use their walking legs initially and will spend their time swimming in the water column. They do not feed and are more vulnerable to cannibalistic attacks.

Late post-moult stage "B" (~ B1, B2, C1, C2, C3)

In the B-stage, the processes which started in A-stage are finalised. Especially the process of sclerotisation or tanning becomes evident as the epi- and exocuticle become stiff and darker. The final endocuticle layers are secreted (including the membranous layer as a last), and mineralisation of the exo- and endocuticle takes place. The epithelial cells start to decrease in size, even while they are still secreting the endocuticle. Meanwhile, the water which was taken up just after moulting is replaced by tissue, a process which will be finalised in the next moult stage.

Shrimp have a sufficiently hard cuticle to start walking and feeding in this stage. One of the first things they usually eat is (parts of) their exuvium.

ANECDYSIS

Inter-moult stage "C" (~ C4)

The inter-moult stage is considered a resting stage in the moult cycle. The cuticle is fully formed (pre- and post-ecdysal layers complete), and the underlying cuticular epithelial cells are relatively inactive, reducing in size to a cuboidal morphology. The physiology of the animal is concentrated on accumulation of reserves in this stage. Glycogen, lipids, calcium etc. are stored, mainly in the hepatopancreas and muscles.

PROECDYSIS

Early pre-moult stage "D1" (~ D0, D1', D1'', D1''')

Once the hormonal control triggers the formation of a new cuticle, the pre-moult phase commences. One of the first observable changes is the enlargement (especially elongation) of the epidermal cells. The cells metamorphosise into secretory cells with an extensive cellular production machinery and transport capacity. Also, an increase in mitotic activity can be noted in the epidermis, up to the moment of ecdysis. Proenzymes (chitinases and proteases) are secreted into the membranous layer which will partly digest the old cuticle before it is shed, and gelify it. In D1-stage, the first signs of the new cuticle formation can be seen with TEM at the apical membrane of the cells. Patches appear on the apical membrane and soon merge, forming the new epicuticle. At the same time, the process of apolysis starts with an ecdysal cleft opening between the epidermis and the old cuticle.

In this stage, the hepatopancreas and muscles will start the mobilisation of reserves needed for the cuticle construction.

Late pre-moult stage "D2" (~ D2, D3, D4)

As epidermal cells continue to increase in size and activity, they start the secretion of the exocuticle underneath the epicuticle by D2-stage. Short chitin oligomers are synthesised and secreted together with cuticular proteins as chitoproteins. Further polymerisation of chitin microfibers / nanofibrils is catalysed by chitin synthetase and the fibres are added to the exocuticle in the area around the cytoplasmic extensions of epidermal cells. This extensive building operation will result in depletion of reserves in hepatopancreas and muscle, although the spectacular atrophy of these organs as seen in crabs can not be noted in shrimp (Cesar *et al*., 2006).

By this stage, the degradation of the old cuticle will have reached its maximal point. The enzymes which had been secreted in the beginning of the pre-moult stage gelify the membranous layer and the lower regions of the endocuticle. The ecdysal cleft which started to form in the early pre-moult is filled with moulting fluid. From here, resorption of the basic molecules such as glucosamine, calcium and amino acids has to happen before the new cuticle becomes impermeable.

Thinning of the old cuticle results in preferential break lines, the ecdysial lines. In shrimp, these are located around the caudal and lateral edges of the cephalothorax and longitudinally on the legs. These allow easy exit for the animals during ecdysis. Probably because of the weakening of the old cuticle and the presence of two

superimposed cuticle layers also in the stomach, shrimp stop feeding by the end of the pre-moult stage. From here on, until they start eating again in the next B-stage, the metabolism of the shrimp will depend on reserves previously stored.

ECDYSIS

Stage "E"

Shrimp start a series of muscle contractions to loosen the old exoskeleton. A marked swelling can be seen on the end of the cephalothorax, in the arthrodisal membrane between the carapace and the first abdominal segment. It is from here that the old cuticle of the carapace opens like a hatch through which the shrimp will leave the exuvia.

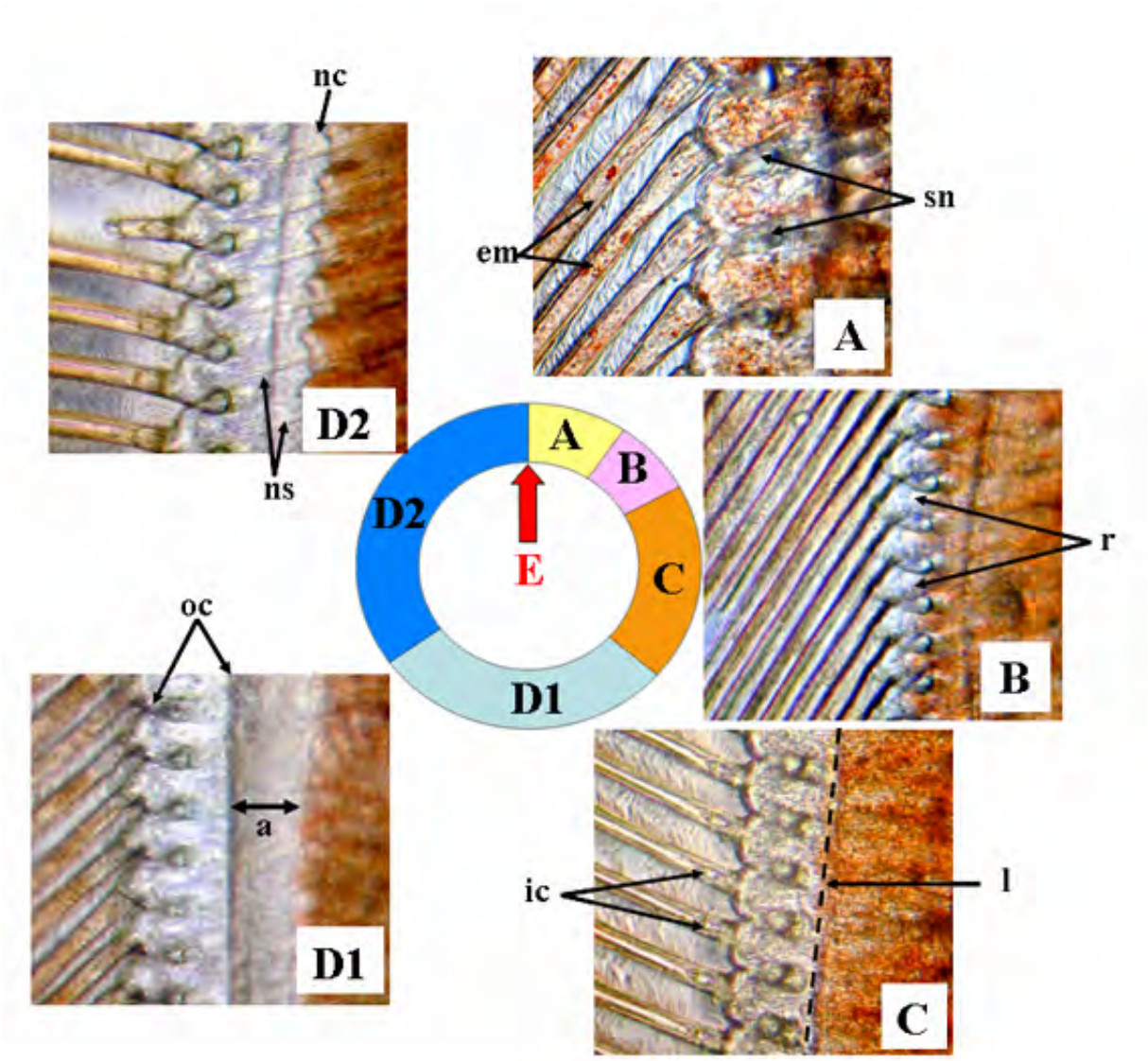

Figure 3. Photographs of the edge of 15 g *P. vannamei* uropods during the major moult stages, positioned chronologically around a representation of the moult cycle. A: early post-moult stage, setal nodes (sn) are forming, epidermal matrix (em) is present inside the setal lumen (magnification 200X); B: late post-moult stage, epidermis is retracting (r) from the setae (magnification 100X); C: inter-moult stage, epidermis lies on a line (l) just underneath the basis of the setal nodes, small internal cones (ic) fill the base of the setae (magnification 100X); D1: early pre-moult stage, apolysis (a) causes a space to form between the old cuticula (oc) and the epidermis (magnification 100X); D2: late pre-moult stage, epidermis forms the new, folded cuticula (nc) and the new setae (ns) (magnification 100X); E: ecdysis, the shedding of the old moult skin.

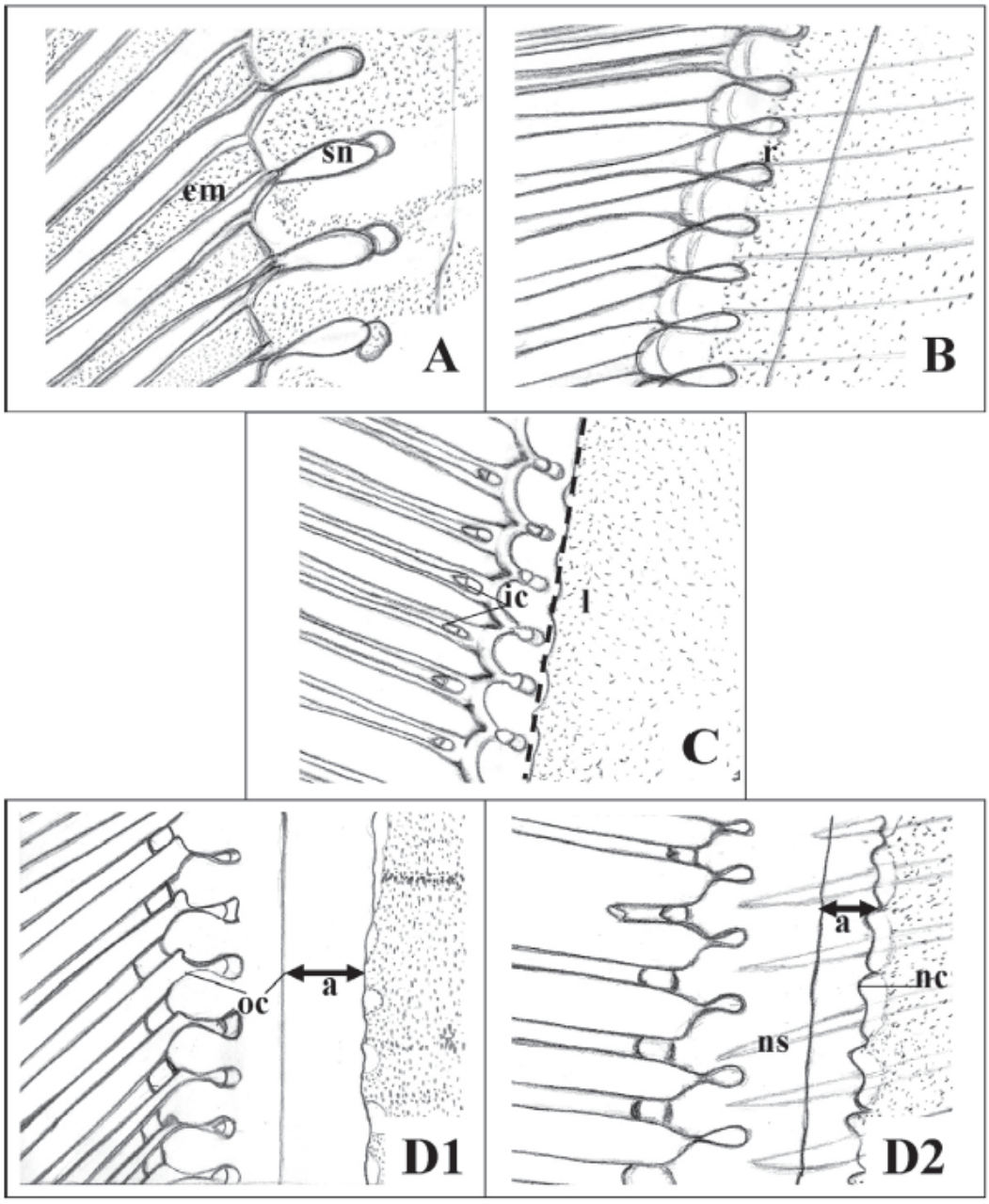

Figure 4. Schematic drawings of setae and epidermal tissue on the edge of uropods during the major moult stages. A: early post-moult stage, setal nodes (**sn**) are forming, epidermal matrix (**em**) is present inside the setae lumen; B: late post-moult stage, epidermis is retracting (**r**) from the setae; C: inter-moult stage, epidermis lies on a line (**l**) just underneath the basis of the setal nodes, internal cones (**ic**) fill the base of the setae; D1: early pre-moult stage, apolysis (**a**) causes a space to form between the old cuticula (**oc**) and the epidermis; D2: late pre-moult stage, epidermis starts to form the new cuticula (**nc**) layer and the new setae (**ns**).

c) Hormonal control of the moult cycle

The moult process in shrimp, as in all Crustacea, is primarily controlled by four types of endocrine substances: peptides, steroids, terpenoids and biogenic amines (Quackenbush, 1986; Van Herp and Payen, 1991; Keller, 1992; Chang *et al.*, 1993; Huberman, 2000; Hartnoll, 2001). The major role is played by two antagonistic hormones: the peptide moult inhibiting hormone and the steroid moulting hormone, ecdysone. The moult inhibiting hormone (MIH) is produced by neuroendocrine cells in the X-organ/sinus gland system (XO-SG), located in the eyestalks (Chang, 1985; Skinner, 1985; Yang *et al.*, 1996). The moulting hormone ecdysone is produced mainly by the pair of Y-organs, which are 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). As long as adequate levels of MIH are maintained in the hemolymph, the moult process is halted and shrimp remain in anecdysis. A reduction in MIH allows the release of more ecdysone in the haemolymph. Blais *et al.* (1994) showed that the major secreted moult hormone from the Y-organ in *Penaeus vannamei* was 3-dehydroecdysone (3DE). This is subsequently metabolised into 20-OH -ecdysone at the level of the epidermal cells (Devaraj and Natarajan, 2006), which stimulates the cells to make preparations for the ecdysis. A peak of ecdysteroids occurs around the end of early pre-moult, beginning of late pre-moult stage when the change of the epidermal cells into secretory mode is maximal. This peak in the ecdysone level is followed by a sharp decline by the end of the pre-moult stage. Throughout metecdysis, the levels remain low. Crustacean hyperglycaemic hormone (CHH) was originally categorised as the central hormone regulating the carbohydrate metabolism (Keller and Sedlmeier, 1998). In fact, CHH is a member of the same family of neuropeptides as MIH, gonad inhibiting hormone (GIH) and mandibular organ inhibiting hormone (MOIH) (Wainwright *et al.*, 1996; Webster, 1998). Together they orchestrate a variety of physiological processes in crustaceans which are interrelated, such as moulting, carbohydrate metabolism, reproduction and osmoregulation (Chung and Webster, 2003; Fanjul-Moles, 2006). CHH's are also secreted by the sinus gland complex and, as they are related to MIH, have an inhibitory action on ecdysteroids secretion. Another substance which is known to play a role in the control of the moult process in Crustacea is the sesquiterpene methyl farnesoate (Yudin *et al.*, 1980; Laufer *et al.*, 1987; Homola and

Chang, 1997). Mostly studied in crabs and crayfish (Rodriguez *et al.*, 2002), its involvement in the moult process and gonadal development of shrimp was recently further investigated (Nagaraju *et al.*, 2002; Hui *et al.*, 2008), although all the roles of this multifunctional signal molecule remain to be fully established (Nagaraju, 2007). This hormone, which is related to the better-known juvenile hormone in insects (Riddiford, 1994), is produced in the mandibular organ of shrimp. Next to MOIH it is also inhibited by MIH and mostly secreted in pre-moult stages, when it has a stimulatory influence on ecdysteroid levels and the moult process.

Finally, two other (neuro)hormones have to be mentioned here. Crustacean cardioactive protein (CCAP) has been studied for quite some time already (Stangier *et al.*, 1986), however, its involvement in the ecdysis of Crustacea is not clear (Chung *et al.*, 2006). Bursicon is known to mediate the sclerotisation process of the cuticle in insects (Dewey *et al.*, 2004), but indications of its presence in Crustacea have only recently been discovered (Wilcockson and Webster, 2008).

Although the major active substances involved in moult regulation are known, their control, functions and interactions are probably more complex than the current model shows (Figure 5). For instance the supposition that MIH levels drop at the onset of pre-moult has never been demonstrated (Nakatsuji and Sonobe, 2004), while evidence exists that MIH even increases in the last stage before moulting (Chung and Webster, 2005).

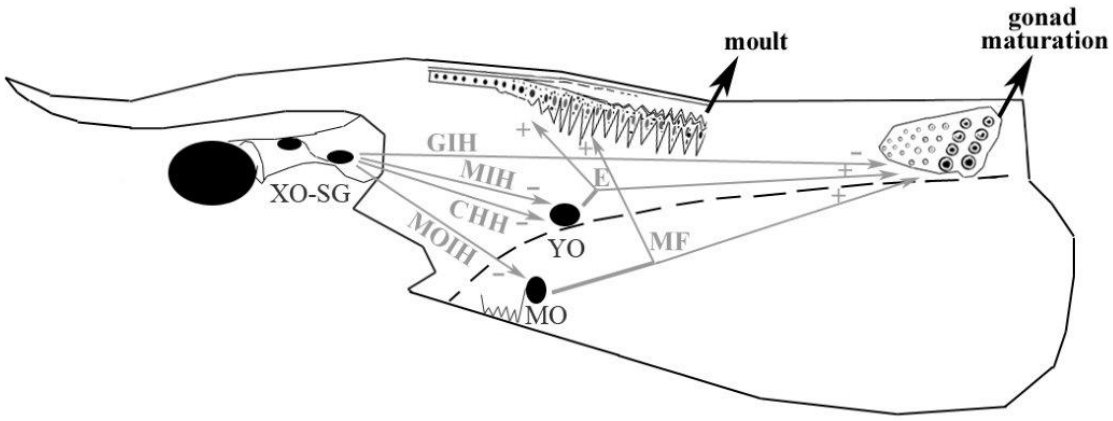

Figure 5. Schematic overview of the endocrine control of the moult process in penaeid shrimp. (XO-SG: X-organ/sinus gland complex; GIH: gonad inhibiting hormone; MIH: moult inhibiting hormone; CHH: crustacean hyperglycaemic hormone; MOIH: mandibular organ inhibiting hormone; YO : Y-organ; E: ecdysteroids; MO: mandibular organ; MF: methyl farnesoate).

The practical relevance of the moult process in penaeid shrimp farming is well-known due to the link between reproduction and moulting (Chang, 1997; Charmantier *et al.*, 1997). As a result, the mechanisms by which the peptide hormones GIH /MIH /CCH and ecdysone decide upon the moulting process have been studied (Fingerman, 1987; De Kleijn and Van Herp, 1998). Gonadal and somatic development occur simultaneously (Subramoniam, 2000) and crucial in the control of both is the XO-SG. By the practice of unilateral eyestalk ablation in female broodstock, farmers of penaeid shrimp remove the inhibitory influence of this endocrine gland (Bray and Lawrence, 1992). The inhibitory function of GIH and MIH is halved, the ovaria develop and precocious moulting occurs. Females spawn faster and at a higher frequency. However, the metabolic overdrive for vitellogenesis and production of moult skins which this procedure induces (Rosas *et al.*, 1993; Racotta *et al.*, 2003) ultimately leads to exhaustion of the brooders (Palacios *et al.*, 1999; Vazquez Boucard *et al.*, 2004). Although unilateral eyestalk ablation does allow for satisfactory reproduction of penaeid shrimp for some time (Marsden *et al.*, 2007), a more selective intervention with the gonad inhibiton, which does not interfere as much with moulting and the metabolism, could be attempted.

2.1.2.1.2. Digestive system

Overall, the digestive tract in Penaeidae is divided in three regions: fore-, mid- and hindgut (Figure 6). Embryologically, the epithelial cells in the fore- and hindgut are of ectodermal origin and thus covered with cuticle. The epithelial cells of the midgut are of entodermal origin, devoid of cuticle, but lined by a peritrophic membrane (Lovett and Felder, 1989; 1990a; 1990b).

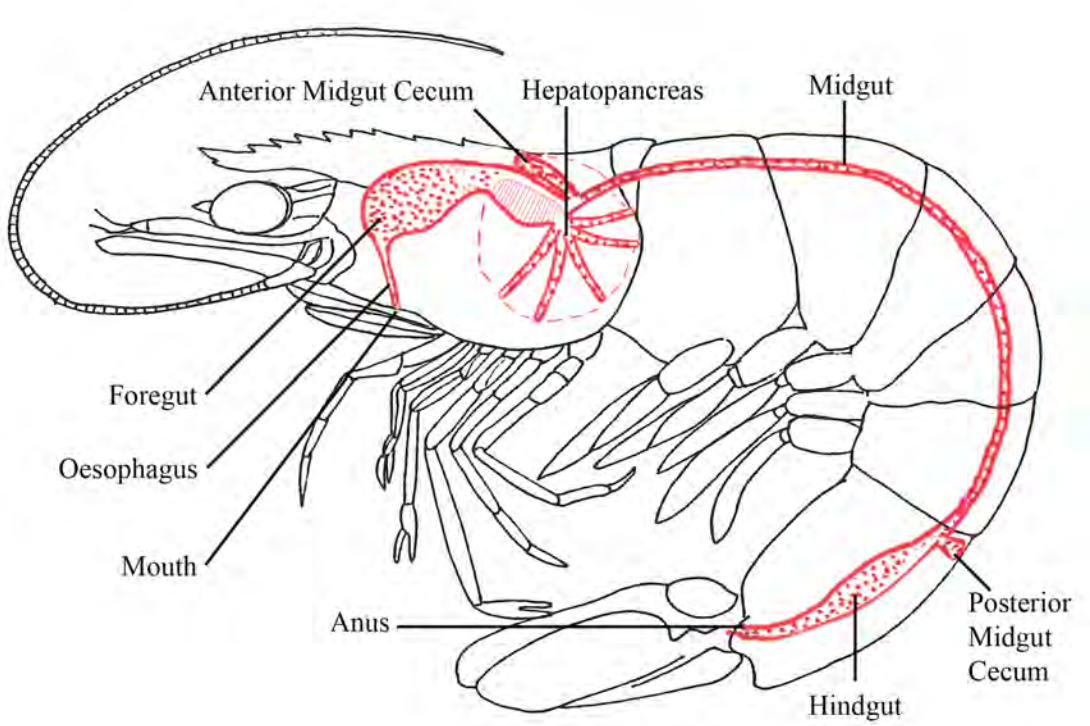

Figure 6. Schematic drawing of the digestive system of *P. vannamei*.

The foregut starts at the mouth, located rostro-ventrally in the cephalothorax, covered by the labrum and mouth parts. The appendages around the mouth are packed with mucus-secreting tegumental glands (Fingerman, 1992; Ceccaldi, 1998). From the mouth, the oesophagus leads dorsally to the stomach which is divided in an anterior and posterior chamber. The anterior chamber functions as a gastric mill, where muscles move the wall of the stomach and its cuticular tooth-like structures in order to grind the food. The second part of the stomach functions essentially as a ballows with a sieve in it. Food passes dorsally over a sieve composed of evenly spaced cuticular hairs, which is moved up and down. Liquid and solid particles which are

smaller than 1 μm pass in ventral direction through the sieve into the hepatopancreas. Larger particles pass on into the midgut (Icely and Nott, 1992; Ceccaldi, 1997).
At the exit of the stomach, the midgut starts and splits up in three directions. Dorsally, the anterior midgut cecum makes a sharp bend in rostral direction, where it lies against the stomach as a narrow pouch with a folded epithelium. Ventrally, the stomach sieve drains into the tubes of the hepatopancreas. This digestive gland is composed of hundreds of blind-ending tubes enveloping the posterior part of the stomach and most of the cecum. Its main functions are: chemical digestion, nutrient absorption, reserve storage and metabolism (Icely and Nott, 1992; Ceccaldi, 1997; 1998). In between the cecum and the hepatopancreas, the tubular midgut trunk leads the coarse solid food particles from the stomach and the digested liquids from the hepatopancreas out of the cephalothorax to the last segment of the tail (Icely and Nott, 1992; Martin and Chiu, 2003). There, the midgut trunk diverts dorsally in the posterior cecum, which is similar to the anterior cecum, and connects to the hindgut. The high columnar epithelial cells of the midgut are known to secrete a peritrophic membrane as a thin barrier around the passing food, but there is discussion in literature whether these cells are invovled in the absorption of nutrients and water (Lovett and Felder, 1990b; Martin *et al.*, 2006).
The hindgut, which is essentially the shrimp's rectum, has a folded, non-calcified epithelium and leads the fecal pellet to the anus below the telson (Dall *et al.*, 1990).

2.1.2.1.3. Respiratory system

Shrimp have 14 dendrobranchiate gills on both sides of their cephalothorax, protected by the cuticle cover of the branchial chamber or branchiostegite (Figure 7). The gills insert on the basis of the legs or body wall with their main axis and their basic plan is that of a tree, with paired filaments projecting at right angles along the length of the main trunk, and small lamellae increasing the surface of the branches, much like leaves do on a tree. The entire surface of the gills is covered by uncalcified cuticle, with a thickness of less than 1 μm on the lamellae (Bell and Lightner, 1988; Dall *et al.*, 1990; Taylor and Taylor, 1992).

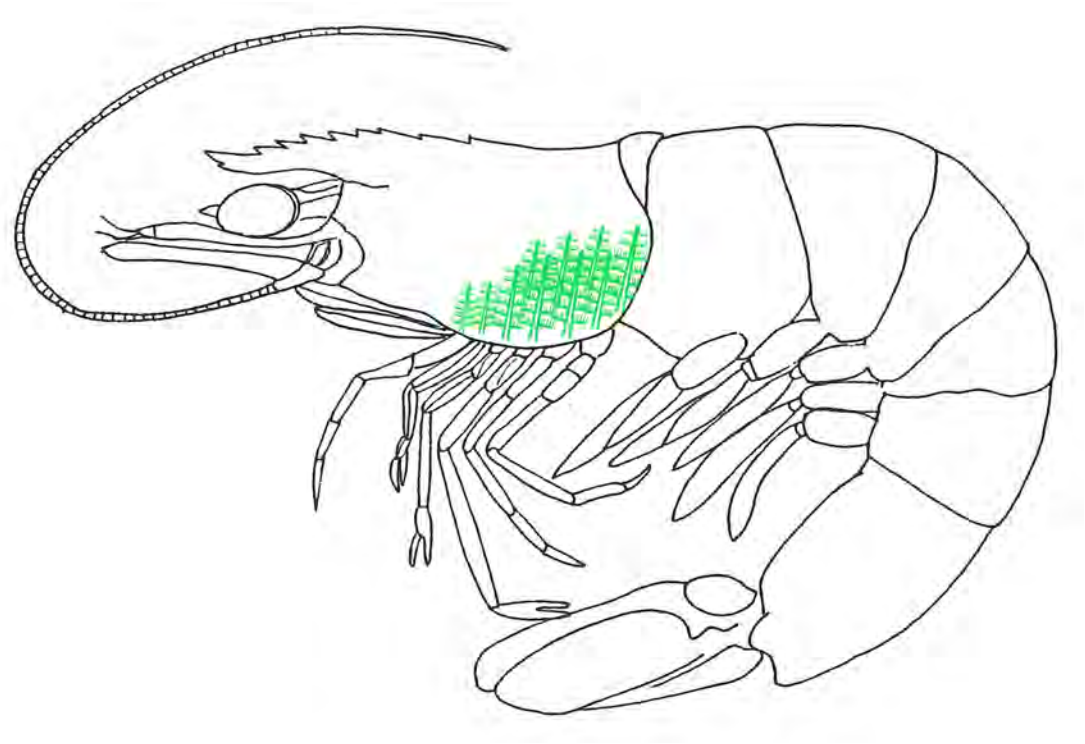

Figure 7. Schematic drawing of the gills of *P. vannamei*.

This is the location where the gas exchange takes place between, on the one hand, the water pumped by the scaphognatite appendage of the second maxilliped over the gills and, on the other hand, the hemolymph pumped by the heart via afferent and efferent vessels through the gills (Bauer, 1999; McGaw and Reiber, 2002).

Apart from this function, the gills of shrimp are also an important osmoregulatory organ. A specific cell type, the nephrocytes, which are large cells (20 - 50 μm) ressembling vertebrate glomerular nephrocytes, perform salt/water balance, acid/base regulation, ammonia excretion and calcium uptake (Foster and Howse, 1978; Taylor and Taylor, 1992; Ahearn *et al.*, 1999; Bauer, 1999).

Lastly, the gills have also been observed to be the site for expelling encapsulated foreign objects, bacteria and possibly lymphoid organ spheroids from the body of shrimp (Maina, 1998; Smith and Ratcliffe, 1980; 1981; Martin *et al*., 1993; 1996; 2000).

2.1.2.1.4. Excretory system

The antennal gland, also named ‘green gland’ in crayfish, is the main excretory organ of shrimp. From comparison with other Crustacea, it is known that it is composed of 3 parts: a bladder, the labyrinth and the coelomosac, but its anatomy is still poorly described in penaeid shrimp (Bell and Lightner, 1988; Fingerman, 1992; Felgenhauer 1992b) (Figure 8). From as far as the hepatopancreas, the tubules of the antennal gland can be found throughout the hemocoel. There is also an insertion into the lymphoid organ which implies a functional connection between these two organs (Duangsuwan et al., 2008; Rusaini and Owens, 2010).

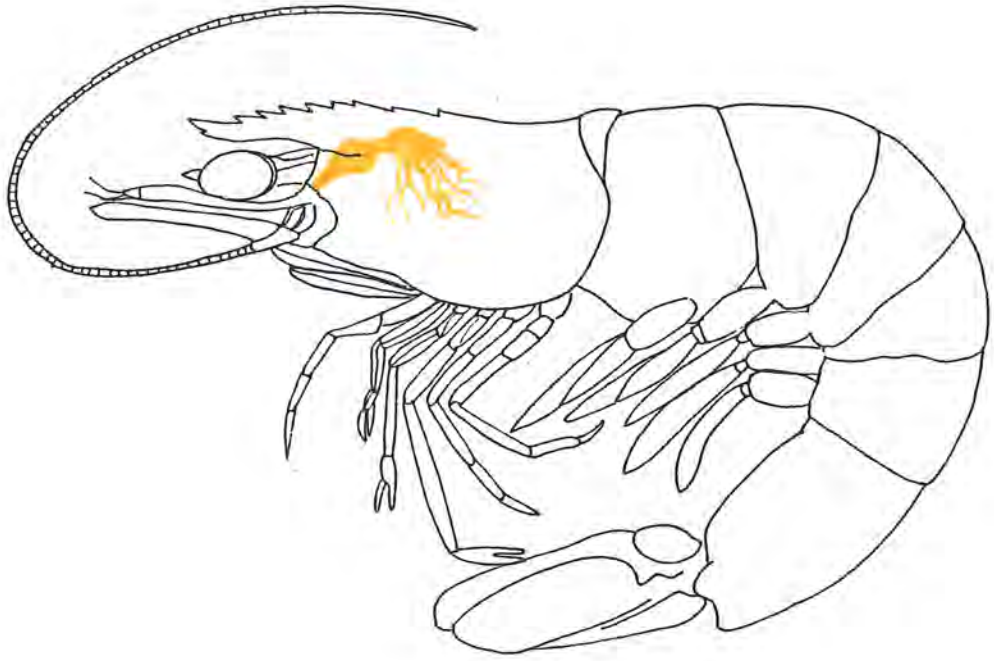

Figure 8. Schematic drawing of the antennal gland in *P. vannamei*.

The structure of the tubules of the antennal gland shares some similarity with vertebrate glomeruli and renal tubules, both by the histological aspect of the tubule cells, as by the presence of podocytes. Similar as for kidneys, the main functions of the antennal gland are osmo-regulation, acid/base homeostasis and detoxification (Potts and Parry, 1964; Ahearn *et al.*, 1999; Wheatly, 1999; Lin *et al.*, 2000). Finally, close to the base of the antennae, the bladder expels urine through a pore. Most of the nitrogenous waste of shrimp is under the form of ammonia, although some is converted to urea (Chen and Cheng, 1995).

2.1.2.1.5 Circulatory system

The heart of shrimp is located at the dorsal edge of the cephalothorax (Figure 9). Hemolymph collects in the spongy epicard, and is pumped from there by a series of subchambers with valves in three general directions around the body. The paired anterio-lateral, hepatic and subgastric arteries and the anterior aorta supply the cephalothorax, the sternal arteriy leads straight down to the ventral parts of the body, while the posterior aorta runs down the abdomen, next to the midgut (Martin *et al.*, 1989; Dall *et al.*, 1990).

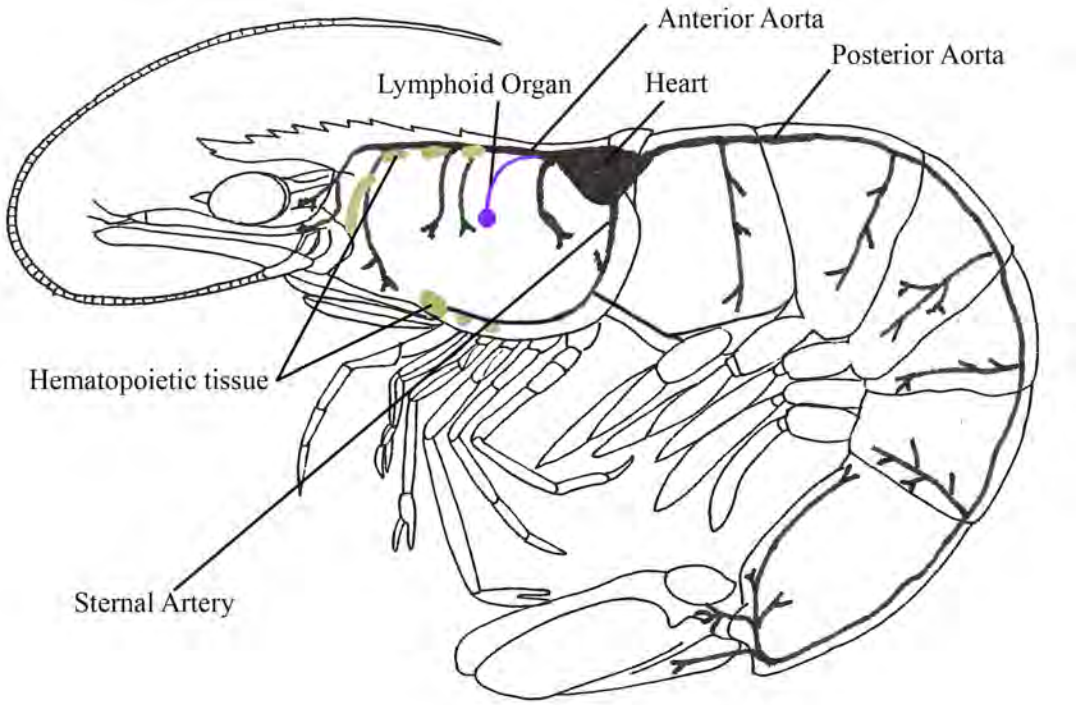

Figure 9. Schematic drawing of the circulatory system of *P. vannamei.*

Unlike vertebrate blood, shrimp hemolymph does not contain red blood cells or platelets. Instead, oxygen is transported by hemocyanin proteins and the clotting function of platelets is replaced by clotting protein. The only circulating cells are the hemocytes which are comparable to leucocytes. Other main plasma components are: electrolytes and proteins for osmoregulation, lipoproteins for fat and cholesterol transport, glucose as the main energy reserve molecule of shrimp, minerals for the calcification of the cuticle and waste, mostly in the form of ammonia (Shimizu *et al.*, 2001).

The hematopoietic tissue is the formation site of hemocytes (Dall, 1964; Oka, 1969; Martin *et al.*, 1987; van de Braak *et al.,* 2002a). These lobules of tissue lie dorsally on the stomach and in the coxae of the maxillipeds, closely associated to hemolymph vessels. They are constituted of dense packages of highly mitotic precursor cells of the hemocytes and maturing prohemocytes (Bell and Lightner, 1988; Martin and Hose, 1992).

The lymphoid organs of shrimp lie as a pair of lobes at the end of the subgastric arteries, ventrally of the stomach and just anterior of where the stomach enters the hepatopancreas (Martin *et al.,* 1987; Bell and Lightner, 1988). The artery branches many times into contorted tubules with a central hemolymph lumen surrounded by an endothelium and a manchette of cells. These stromal cells which lie around the hemolymph vessels show similarities to hemocytes and are observed to filter particles from the passing hemolymph as it drains from the incoming vessels to the hemal spaces between the tubes and out of the lymphoid organ. The functions of the organ in antibacterial and antiviral immunity have been studied, and a notable transformation of groups of cells into lymphoid organ spheroids (LOS) was observed in many viral infections. These basophilic clusters of hypertrophic cells appear to be a mass of phagocytic cells involved in the encapsulation of pathogens, which are thereby immobilised and eliminated (Martin *et al.,* 1996; van de Braak *et al.,* 2002b; Duangsuwan *et al.*, 2008; Rusaini and Owens, 2010).

2.1.2.1.6. Central nervous system

Penaeid shrimp have a ganglion in each segment of the body, with a single ventral nerve cord connecting them along the body (Figure 10). Larger ganglions lie in the anterior part of the cephalothorax, where the nerve cord makes a ring around the esophagus. This supra-esophagal ganglion is often considered the brain of the shrimp and is mainly involved in processing of the sensory functions of eyes, antennae and antennulae, as well as coordinating the rest of the body and the ingestion of food.

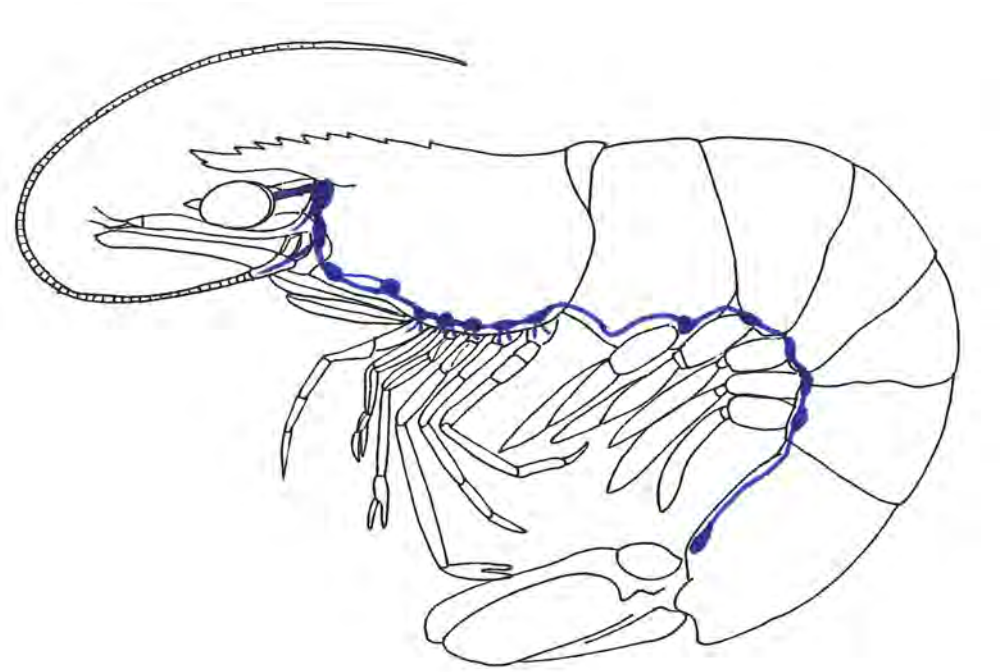

Figure 10. Schematic drawing of the central nervous system of *P. vannamei*.

Furthermore, the central nervous system is closely linked to neuroendocrine organs such as the X-organ/sinus gland complex in the eye stalk, the Y-organ and the mandibular organ. (Sandeman, 1982; Cooke and Sullivan, 1982; Skinner, 1985; Fingerman, 1992; Subramoniam, 2000; Diwan, 2005).

2.1.2.1.7. Reproductive system

Penaeid shrimp have separated sexes which can be easily distinguised by their genital organs. Males have petasma, a pair of extra appendages on the first abdominal segment which are used to deliver spermatophores. Internally, the male has two testes which deposit non-motile spermatozoa via the vas deferens into the terminal ampoules on the border between the cephalothorax and abdomen where the spermatophore packages are stored (Bailey-Brock and Moss, 1992; Krol *et al.*, 1992). For receiving the spermatophores, the female has a thelycum located between the bases of the 4th and 5th walking legs. In open thelycum species such as *P. vannamei*, the spermatophore is introduced into the female while her exoskeleton is hard in inter- or pre-moult stages. The female gonads are a pair of large ovaries, which can run up into the abdomen, and oviducts leading the eggs to the gonopores, opening towards

the thelycum, where they are fertilised with sperm from the spermatophores (Bailey-Brock and Moss, 1992).

2.1.2.1.8. Defense system

As they are invertebrates, the immune system of shrimp is essentially aspecific or innate, with only a few indications that they might possess specific or adaptive immune responses as well (Hauton and Smith, 2007). In general, shrimp recognise non-self molecules and mount rapid responses by means of their humoral and cellular defense systems (Beutler, 2004).

The activation of the defense response is initiated when the presence of pathogen-associated molecular patterns (PAMPs) inside the body is detected by pattern recognition proteins (PRPs) of the shrimp. PAMPs are typically conserved molecules of microbial origin, such as lipopolysaccharides (LPS) and Beta-1-3-glucans. PRPs are B-glucan-binding protein (BGBP), LPS- and glucan-binding protein (LGBP) and lectins such as C-type lectin. The opsonisation and detection of pathogens will start the activation of defense cascades, mainly that of the prophenoloxidase-activating (proPO) system (Söderhäll and Cerenius, 1998). Once the proPO-activating enzyme (a serine protease) has cleaved the inactive proPO into the active phenoloxidase (PO), a chain reaction occurs, involving both humoral and cellular responses (Cerenius and Söderhäll, 2004). PO is responsible for the well-known melanisation reaction, which is generally observed in wounded areas or during immune responses in invertebrates (Sritunyalucksana and Söderhäll, 2000). Besides, PO also triggers other defense mechanisms such as cell adhesion (Holmblad and Söderhäll, 1999), opsonisation (Söderhäll and Cerenius, 1998), phagocytosis (Roch, 1999), encapsulation (Lee and Söderhäll, 2002), antibacterial activity and bacterial clearance (Bachère *et al.*, 1995; Vargas-Albores *et al.*, 1996; Jimenez-Vega *et al.*, 2005; Lai *et al.*, 2005). Other humoral responses consist of reactive oxygen species (ROS), antimicrobial peptides, and lysozymes (Bachère *et al.*, 1995; Söderhäll and Cerenius, 1998; Roch, 1999; Sritunyalucksana and Söderhäll, 2000; Cerenius and Söderhäll, 2004; Lai *et al.*, 2005).

The cellular arm of the immune system consists essentially of the shrimp white blood cells, the hemocytes. These are categorised, based on the increasing amount of

granules present in the cytoplasm, in three groups: hyaline, semi-granular and granular hemocytes (Bauchau, 1981; Söderhäll and Cerenius, 1992).

The hyaline cells represent 5-20% of the hemocyte population, are ovoid to spindle-shaped and generally smaller that the other two hemocyte types. With 60-75% of the total, semi-granular hemocytes are the predominant subpopulation, with the granular cells taking up the remaining 10-25% (Martin and Graves, 1985; Hose *et al.*, 1987). These two categories are possibly different stages of maturation, originating from the same progenitor prohemocytes (Bauchau, 1981; Hose *et al.*, 1990; van de Braak *et al.*, 2002a; Söderhäll *et al.*, 2003: Zhang *et al.*, 2006). Up to date, little is known with certainty about the functions of the different subpopulations in shrimp. Hyalinocytes tend to lyse quickly *in vivo* and *in vitro*, releasing anti-microbial and pro-inflammatory compounds, and are in that sense reminiscent of neutrophil granulocytes of mammals (Dantas-Lima *et al.*, 2012). Semi-granulocytes are thought to be the main phagocytic cell type in shrimp and also degranulate rapidly when they detect non-self molecules (van de Braak *et al.*, 1996; Johansson *et al.*, 2000; Zhang *et al.*, 2006). These cells will take up and digest foreign particles within phagolysosomes by producing lysozyme and other hydrolytic enzymes and ROS. Granulocytes obviously serve their main function as storage cell for immunoactive compounds. They are the main source of proPO, which is released by degranulation during the processes of encapsulation and nodulation when combating fungi and bacteria, respectively (Hose and Martin, 1989).

As one will notice, the defense system described above mainly revolves around anti-fungal and anti-bacterial responses. Antiviral immunity in penaeid shrimp remains poorly understood (Liu *et al.*, 2009; Cerenius *et al.*, 2010; Smith *et al.*, 2010; Flegel and Sritunyalucksana, 2011). RNA interference (RNAi) is one of the few pathways know to play an important role in crustacean innate antiviral immunity, and has been studied in shrimp mainly in the context of anti-WSSV defense. In this thesis we discuss this subject in chapter 2.2.6.3. as part of the WSSV-host interactions.

2.1.2.2. Life cycle of penaeid shrimp

The life cycle of penaeid shrimp is quite complex (Bailey-Brock and Moss, 1992; Treece and Yates, 1988) (Figure 11). Twenty-four hours after a female lays her eggs, the shrimp emerges as a nauplius. In this stage, the larva does not feed but uses its yolk reserves to develop the body. After five moults (instars), the nauplius larva metamorphoses into the zoea stage, when it starts to feed on microalgae. After three zoea instars, the larva metamorphoses again into the mysis stage. From this stage on, the shrimp will eat zooplankton (such as *Artemia* nauplii in aquaculture facilities). After 3 instars as a mysis, the shrimp will go through a final metamorphosis and become a post-larva. The stages before post-larva are found in off-shore, pristine water. Post-larvae and juveniles migrate into estuaries and mangroves until mature, when they return to the sea to spawn.

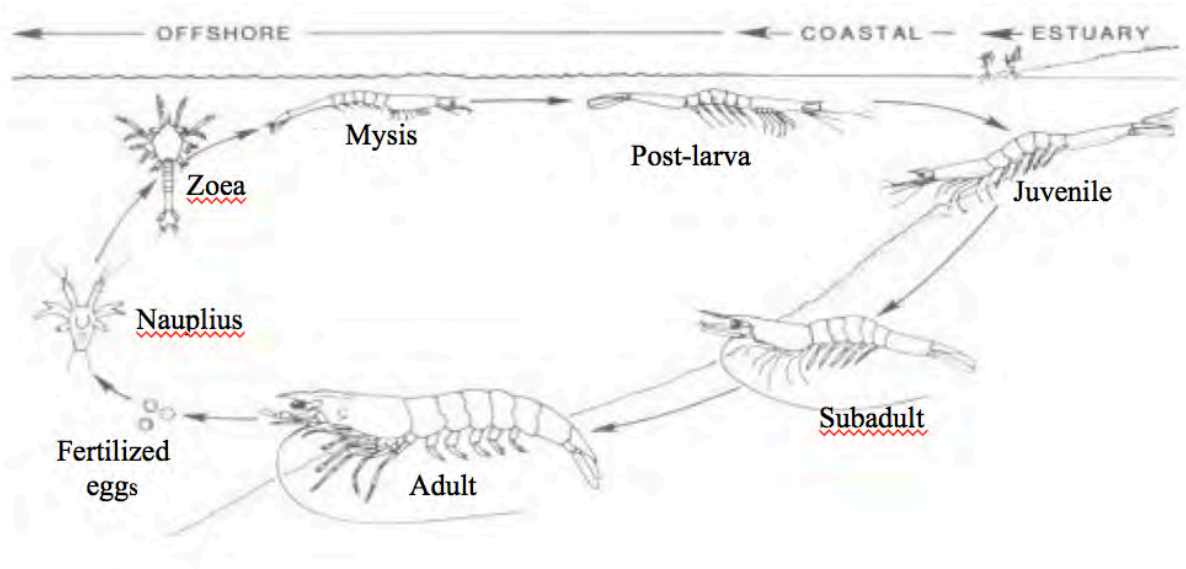

Figure 11. Life cycle of penaeid shrimp (after Bailey-Brock and Moss, 1992).

After spermatophore transfer from male to female, the eggs are released and fertilised externally. Embryogenesis subsequently occurs in the water during 12 to 14 hours. The eggs sink, but the phototropic nauplii quickly swim to the sea surface where they will develop in plankton-rich waters.

2.1.3 Palaemonid prawn biology

2.1.3.1. Morphology and physiology

The external anatomy of *M. rosenbergii* is similar to that of penaeid shrimp (Figure 12). We refer to the chapter on penaeid shrimp for the general information, and will only discuss the noteworthy differences here.

The cephalothorax of *M. rosenbergii* is relatively larger compared to their abdomen, and the rostrum is well-developed. This is more pronounced in males, which also have a more narrow abdomen.

One of the most spectacular characteristics of *M. rosenbergii* is their extremely large second pair of walking legs (chelipeds). Especially in adult, dominant males, these claws are very long and bright blue in colour. These claws mainly function in social hierarchy, for territorial competition and the protection of females. Next to the dominant "blue claw" males, a society of *M. rosenbergii* prawns also includes large "oranje claw" males and small "white claw" males or "runts".

The abdomen of female *M. rosenbergii* is quite different from that of penaeid shrimp, as the exoskeleton (pleura) forms a protective brood chamber and the pleopods are modified to hold eggs. The genital pores of the male are between the bases of the fifth walking legs, those of the female at the base of the third walking legs. Once a female is mature and her ovaries are carrying eggs, she will moult and seek the protection of a dominant male. The hard-shelled male will mate with her while she still has a soft shell. Within a few hours after mating, the female will lay her eggs and glue them onto her pleopods. She will hold, clean and aerate them for about 3 weeks, when they hatch. Females normally mature once they are 15 to 20g, but berried females have been observed as small as 6.5g (Daniels *et al.*, 2000). *M. rosenbergii* is the largest of all Macrobrachium species, adult males having been reported with a total body length of up to 33 cm, and adult females of up to 29 cm (FAO, 2002).

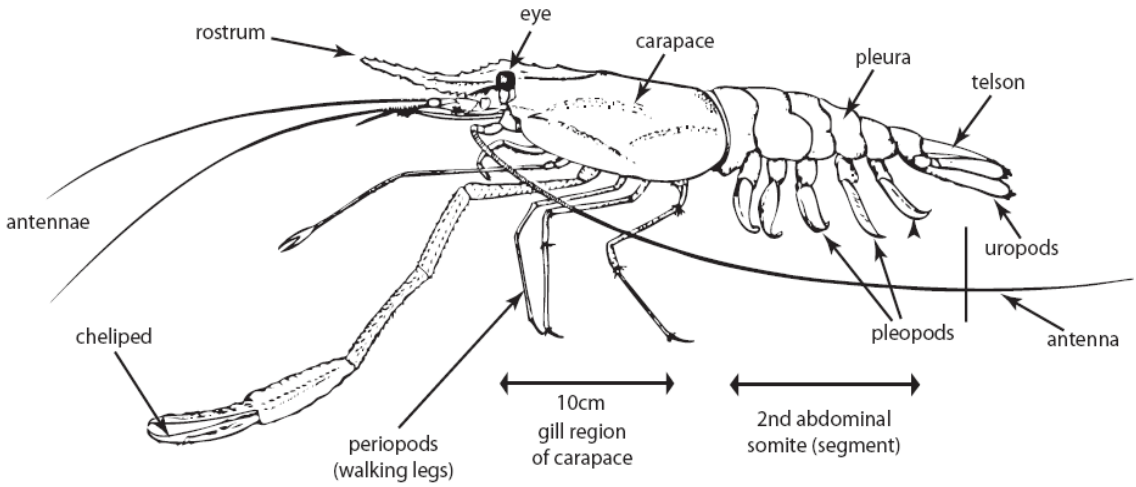

Figure 12. External morphology of *M. rosenbegii* prawns (Forster and Wickins, 1972).

The internal anatomy and physiology of the different organ systems in *M. rosenbergii* has not been extensively described in literature (Brown *et al.*, 2009). Here we will only discuss those aspects which are different from penaeid shrimp.

The gill architecture is not dendrobranchiate (tree-like) in *M. rosenbergii*, but phyllobranchiate. Each gill consists of a main axis, on which many lamellae are inserted perpendicularly, more comparable to a heating radiator.

The digestive system is grosso modo the same as in penaeids, although the fine structure of the stomach, stomach diverticulae and midgut cecae has not been investigated up to date. Although it has been mentioned that the gastric mill is absent in Caridea prawns such as *M. rosenbergii* (Dall and Moriarty, 1983), even though the foregut of this species can be clearly seen masticating food in a similar way as in penaeid shrimp species.

Other systems, such as the integument, the excretory, nervous and reproductive systems, are all comparable to those of penaeid shrimp. It is important to realise however, that many gaps still exist in the current knowledge. For instance, even basic information on the lymphoid organ or hematopoietic tissue and hematogenesis in *M. rosenbergii* is not available.

2.1.3.2. Life cycle of palaemonid prawns

The life cycle of *M. rosenbergii* is less complex than that of penaeid shrimp. Upon hatching from the eggs which are held by the mother on her pleopods, the planktonic (zoeae) larvae of *M. rosenbergii* go through 11 distinct stages over a period of 15 to 40 days (Uno and Kwon, 1969) (Figure 13). These larvae swim actively in brackish water, with their ventral side up and in the direction of their tail. Their diet consists of zooplankton (*Artemia* nauplii in aquaculture facilities). After the last moult as larva, the prawns metamorphose into postlarvae and start their return to freshwater where they will mature and mate. The cycle will be completed once a gravid female goes downstream and releases her larvae in brackish water.

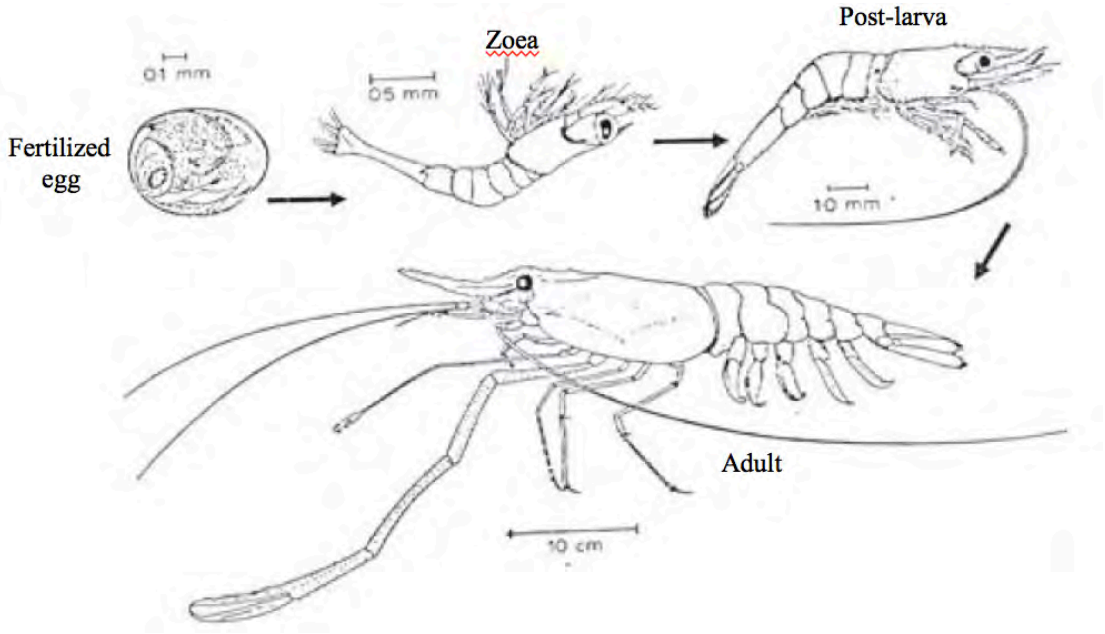

Figure 13. Life cycle of *M. rosenbergii* (Wickins and Lee, 2002).

2.2. White Spot Syndrome (WSS)

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White Spot Syndrome has been the most problematic infectious agent affecting the global shrimp farming industry since emerging in 1992 and is caused by White Spot Syndrome virus (WSSV). The disease was named after its primary clinical sign in affected *P. monodon*: formation of circular white calcium deposits on the underside of the cuticle of the cephalothorax. This denomination can be misleading as WSSV rarely induces white spots in infected American penaeids and similar spotting of the cuticle may result from other causes, such as bacterial infection (Wang *et al.,* 2000a).

2.2.1. The virus

2.2.1.1. Morphology and classification

White Spot Syndrome virus is an enveloped, bacilliform double-stranded DNA-virus (Figure 14). Both the size of the virion (up to 350 nm in length) and the size of the genome (30,000 kbp) are exceptionally large. The agent was assigned to a newly created virus family, the *Nimaviridae*, and placed in the genus, *Whispovirus*. WSSV stands alone in this family group and has only distant genomic resemblance to other DNA viruses such as pox, herpes and baculovirus (Vlak *et al.,* 2002). The virus was initially given a variety of names by researchers located in different countries who perceived the outbreaks to be caused by different viral agents. These early names included hypodermal and haemotopoietic necrosis baculovirus (HHNBV), rod-shaped nuclear virus of *P. Japonicus* (RV-PJ), systemic ectodermal and mesodermal baculovirus (SEMBV), white spot baculovirus (WSBV), and *P. monodon* non-occluded baculovirus (PMNOB) (Durand *et al.,* 1997; Karunasagar *et al.,* 1997; Chou *et al.,* 1998; Sahul-Hameed *et al.,* 1998). All of these agents are currently recognised as one virus, which is called WSSV.

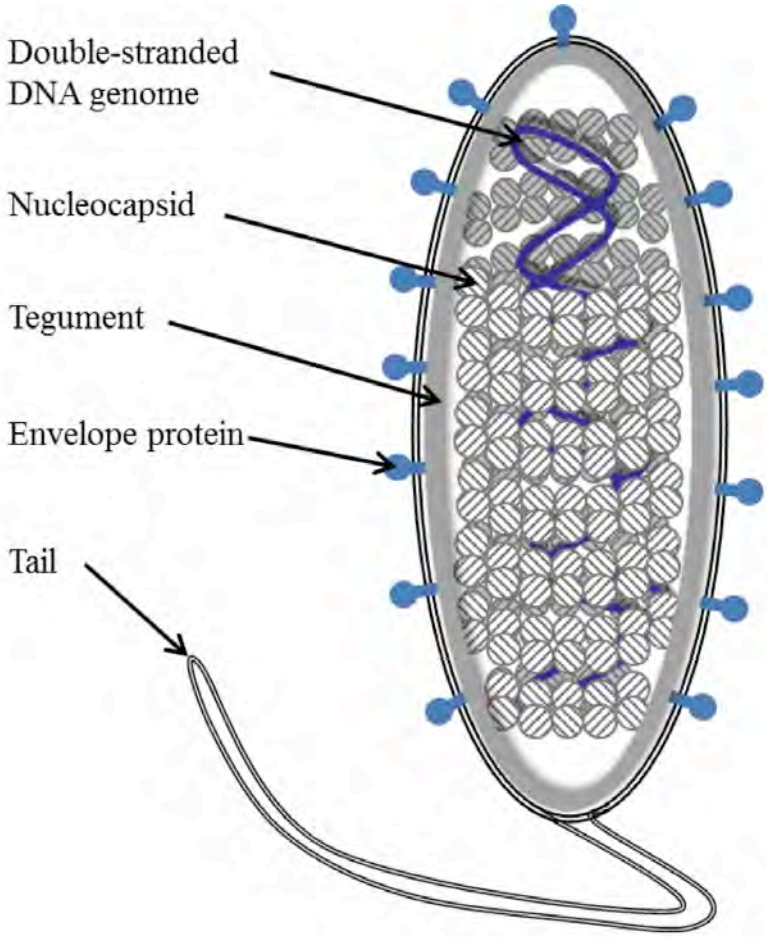

Figure 14. Schematic drawing of a WSSV virion.

2.2.1.2. Physical inactivation

There are reports describing that WSSV can be inactivated in less than 2h at 50°C (Chang *et al.*, 1998a; Nakano *et al.,* 1998) and in less than 5 minutes at 60°C; that it remains viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama *et al.,* 1998); and is viable in ponds for at least 3-4 days (Maeda *et al.,* 1998, Nakano *et al.,* 1998). Experiments on incubating a WSSV suspension in artificial sea water showed a 50% reduction of infectious titer after 3 hours at 27°C (Corteel *et al.,* 2009).

2.2.1.3. Variability in isolates

Few differences have been found among various geographical isolates of WSSV. Protein profiles and aminoacid sequences are similar (Lo *et al.,* 1999; Wang *et al.,*

2000b; Rajendran *et al.,* 2004) with little antigenic variability found among isolates using polyclonal or monoclonal antibodies in different immunoassays (Nadala and Loh, 2000; Shih *et al.,* 2001; Poulos *et al.,* 2001; Anil *et al.,* 2002; You *et al.,* 2002; Yoganandhan *et al.,* 2004). WSSV also appears to have a very stable genome compared to other viruses. In three of the isolates which have been fully sequenced, from Thailand, China and Taiwan (van Hulten *et al.,* 2001; Yang *et al.,* 2001; Chen *et al.,* 2002), only minor differences were found in a limited number of variable regions, mainly in ORF 14/15 and 23/24 (Marks *et al.,* 2004; Marks, 2005). These variable parts of the genome exhibit deletions, recombinations and a transposase region. The presence of a 13 kbp deletion in ORF23/24 has been correlated with an increased virulence of WSSV, accelerating the median lethal time from 14 to 3.5 days (Marks *et al.,* 2004; 2005). However, this is in conflict with the publication by Lan *et al.* (2002), which describes that a similar deletion mutant of WSSV was less virulent. None of the WSSV isolates analysed by Pradeep *et al.,* (2008) contained the sequence which is lost by this deletion in ORF23/24, confirming that the sequence for the coded nucleocapsid protein VP35 is not important for virulence in WSSV. Zwart *et al.* (2010) used the ORF14/15 and ORF23/24 variable regions as molecular markers to study the evolution of the WSSV genome as it spread through Asia. They saw a shrinkage of the genome over the years while WSSV spread, from 312 kbp to 293-298 kbp, at which it stabilised. By data analysis and bioassays, the authors further reinforce the hypothesis that deletions in the genome are correlated with improved WSSV fitness.

In the WSSV genome, there are also three other areas which vary between isolates, namely the variable number tandem repeat (VNTR) loci which are present in ORF75, ORF94 and ORF125 (Wongteerasupaya *et al.,* 2003; Marks *et al.,* 2004). These VNTR allow genotyping of WSSV and have been used to track the molecular epidemiology and evolution of WSSV (Dieu *et al.,* 2004; Pradeep *et al.,* 2008; Dieu *et al.*, 2010a; 2010b). The repeats appear to change in size remarkably fast between virus generations, might be influenced by the host species during passage and are suspected to affect virulence (Waikhom *et al.*, 2006).

Hoa *et al.* (2011) found a correlation between the occurrence of different genotypes of WSSV with different number of tandem repeats and the absence of disease outbreaks in shrimp ponds. Syed-Musthaq *et al.* (2006) had previously stated that the virulence of WSSV was not affected by the number of tandem repeats, but these authors did not

show the results of their experiments gauging the virulence of the different WSSV isolates. Walker *et al.* (2011) also observed a multitude of WSSV genotypes circulating at the same time in shrimp farms, but did not identify a correlation between particular (virulent) genotypes and disease outbreaks. Rather, these authors pointed out that pond management was primordial in determining whether disease outbreaks would occur in ponds with WSSV-infected shrimp.

A few laboratory studies have confirmed that certain WSSV isolates cause significant differences in clinical expression. The study by Wang *et al.,* (1999a) showed differences in virulence of six WSSV isolates in *P. vannamei* post-larvae and juvenile *P. duorarum* inoculated *per os*. An isolate obtained from US shrimp induced 100% mortality in *P. vannamei* faster than an isolate from crayfish, while neither of the isolates induced death among experimentally infected *P. duorarum*.

In another study by Rahman *et al.,* (2008), clear differences in virulence between three isolates (2 Thai and 1 Vietnamese) were shown with a reproducible intramuscular inoculation procedure and known doses of virus. The most virulent Thai isolate caused an onset of mortality at 36 hours post-injection (hpi), 100% cumulative mortality by 72-84 hpi and a median lethal time of 47 hpi. This represents one of the early isolates from the mid 90´s. In contrast, the manifestations of disease due to the least virulent, Vietnamese strain were timed at 36–60, 204–348 and 120 hpi, respectively. The Vietnamese isolate induced a more chronic disease and slower mortality rate than that observed with the Thai isolates, possibly because it replicated in significantly fewer cells in target organs. This difference was most pronounced in gills. This Vietnamese isolate was more recently isolated in the mid 2000's, and might represent an evolutionary adaptation of the virus.

2.2.2. Host range

WSSV has an exceptionally broad host range. Over 50 crustacean species have been found to be susceptible to WSSV. It is generally assumed that the virus can replicate in tissues of ecto- and mesodermal origin of all decapod crustaceans from marine and brackish or freshwater sources (Lo *et al.,* 1996a; Flegel, 1997; Chang *et al.,* 1998b, Flegel and Fegan, 2002; Sahul-Hameed *et al.,* 2003, OIE 2006; for review: see Escobedo *et al.,* 2008), including all commercially important penaeid species. In general, Natantia (notably shrimp) show more severe symptoms and mortality than

Reptantia (crabs and lobsters). One exception appears to be the giant freshwater prawn, *Macrobrachium rosenbergii*. On the one hand, some publications claim that natural and experimental WSSV infections of *M. rosenbergii* can occur. These reports mention that the infection causes disease and mortality mainly in young life stages with rather high viral loads, but that it can usually only be detected in adults by 2-step PCR. In some of these studies, a degree of mortality and higher viral loads were detected by bioassay and histology in older animals (Lo *et al.,* 1996b; Peng *et al.,* 1998; Rajendran *et al.,* 1999; Hossain *et al.,* 2001; Pramod Kiran *et al.,* 2002). In contrast, several other publications show no mortality among WSSV-inoculated *M. rosenbergii* juveniles and adults with induction of transient infection during the first few days after inoculation. These infections were found to be detectable by Western blot, 2-step or RT-PCR and, subsequently, became undetectable (Sahul-Hameed *et al.,* 2000; Yoganandhan *et al.,* 2006; Waikhom *et al.,* 2006). Although the variable experimental outcomes in the above studies are partly due to a lack of standardised and reproducible methodology, the fact that *M. rosenbergii* has some capacity to survive and even clear WSSV infections has now become clearly established and suggests that this species possesses an effective defense response against WSSV (Sarathi *et al.,* 2008). Based on a hemagglutination test of hemolymph from WSSV-injected *M. rosenbergii* and *P. monodon*, Pais *et al.,* (2007) suggested that hemagglutinins or lectins of the fresh water prawn may be involved in the antiviral response.

Due to its tremendously wide host range, together with the international movement of shrimp stocks of unknown health status, WSSV has become one of the most wide-spread viruses in the industry, occurring in all shrimp-farming countries except Australia (Flegel and Fegan, 2002). Once the virus becomes established in neighboring wild populations, exclusion of the virus from shrimp ponds becomes a difficult task.

All life stages are potentially susceptible to infection, from eggs to broodstock. To date, it is still not clear whether WSSV-infected shrimp eggs can undergo development (Lo *et al.,* 1997; Manjusha *et al.,* 2009). Very low, undetectable levels of infection might allow the development of the egg and *per ovum* and possibly *intra ovum* transmission of WSSV can occur from broodstock to offspring.

2.2.3 Geographical distribution and prevalence

First described in China and Taiwan in 1992, WSSV spread throughout East, South-East and South Asia causing a panzootic by 1994 (Escobedo-Bonilla *et al.,* 2008). There were some occasional reports of the virus in North America during the mid 90s until it created a second panzootic wave, which reached North, South and Central America in 1999 (Escobedo-Bonilla *et al.,* 2008). Generally speaking, we can say that WSSV is present in all shrimp producing countries except for Australia and some African countries.

The disease prevalence is highly variable and seasonal. During the cold and/or rainy seasons, the prevalence increases both in captive and wild populations.

2.2.4. Disease pattern

2.2.4.1 Clinical signs

In the field, WSS symptoms appear in farm ponds 14-40 days post-stocking. As mentioned, the characteristic white spots are not always present, particularly in *P. vannamei.* In addition, similar white spots have been reported due to the use of probiotics and under certain water quality conditions (Wang *et al.,* 2000a). Apart from the white spots, symptoms of WSS are aspecific (Figure 15). Farmers often report unusual gathering of shrimp at the edges of ponds and a cessation of feeding. After experimental inoculations, anorexia and lethargia appear within 1 to 2 days. Sometimes a change in colour can be noted, with legs and uropods becoming red or the whole body turning whitish. Mortalities typically reach 100% within 5 to 10 days of disease onset. In contrast, latent infections have been described in which the animals do not become diseased, but the latency has never been documented under laboratory conditions.

Figure 15. Clinical signs of WSSV infection in *P. vannamei*. Symptoms of WSS are variable and aspecific: white spots are not always present (arrow), red coloration of extremities (arrowhead) and opaqueness of muscle are variably present. Lethargy and anorexia can be noted by the empty stomach and midgut.

2.2.4.2. Pathology

The virus causes systemic infections that show characteristic lesions in tissues of ectodermal and mesodermal origin. It does not affect tissues of endodermal origin (e.g. midgut and hepatopancreatic epithelia) although it does infect cells in the interstitial tissue of the organ (mesodermal origin). In early stages of viral development, the hyperthrophied nuclei of infected cells show an acidophilic (reddish) central inclusion surrounded by a thin non-stained zone that is framed by a basophilic (blue) ring of marginated chromatin. In the later stages of infection, the central inclusion expands to fill the whole hyperthrophied nucleus and it becomes progressively more basophilic with age (Alday and Flegel, 1999). To confirm WSSV histologically, the best tissues to examine in *P. vannamei* and *P. monodon* is the epithelium of the stomach and gills. In the case of *P. stylirostris* subcuticular epithelium is a better option. In general, WSSV induced disease is rather easy to diagnose histologically because of the tremendous number and widespread distribution of infected cells present in a moribund penaeid shrimp.

2.2.5. Epidemiology

2.2.5.1. Transmission

Since the first reports on the virus, it has become generally accepted that transmission between shrimp and other Decapod crustacea can occur via 3 routes: (1) oral, by consumption of tissues from infected hosts (2) waterborne, when virus is transmitted via the water by immersion or cohabitation and possibly (3) *trans-ovum* or *per ovum* vertically from broodstock to offspring (Lo *et al.*, 1997).

A high number of experimental studies demonstrated that feeding of infected shrimp tissues is an effective way to transmit the virus to shrimp and other decapods (Chang *et al.*, 1996; Chang *et al.*, 1998b; Sahul-Hameed *et al.*, 1998; Supamattaya *et al.*, 1998; Wang *et al.*, 1998; Rajendran *et al.*, 1999; Rajan *et al.*, 2000; Tan *et al.*, 2001; Wu *et al.*, 2001). It was mainly these early reports which helped to build the image of WSSV being a highly contagious pathogen. However, many authors needed to administer infected tissues in several feedings, for periods sometimes as long as 7 days (Lightner *et al.*, 1998; Wang *et al.*, 1999b; Sahul-Hameed *et al.*, 2001; Kiran *et al.*, 2002; Jiravanichpaisal *et al.*, 2004; Bonnichon *et al.*, 2006; Jha *et al.*, 2007). Vidal *et al.* (2001) and Escobedo-Bonilla *et al.* (2006) published procedures for delivering WSSV inoculum straight into the stomach by intubation (Vidal *et al.*, 2001; Escobedo-Bonilla *et al.*, 2006). Both procedures resulted in infection in all inoculated shrimp. However, only in the latter study the viral stock had been titrated and a known dose was given to the animals (Escobedo-Bonilla *et al.*, 2005).

For the waterborne route, there are many studies which reported that immersion and even cohabitation readily allow the entry of WSSV into hosts (Wang *et al.*, 1997; Kanchanaphum *et al.*, 1998; Chen *et al.*, 2000; Witteveldt *et al.*, 2004; Witteveldt *et al.*, 2006), and older shrimp were reported to be less susceptible (Chou *et al.*, 1995; Yoganandhan *et al.*, 2003).

It is important to note, however, that most of the studies cited above were not performed under fully controlled experimental circumstances in terms of specific pathogen-free (SPF) status of experimental animals, administered dose, occurrence of secondary transmissions after the inoculation, presence of other pathogens in the inoculum, temperature of the rearing water and detection of actual WSSV replication. These features make it impossible to reproduce those studies and prevent reliable

conclusions. Probably the best-controlled experimental studies on WSSV transmission so far were published by Soto and Lotz (Soto *et al.*, 2001; Lotz and Soto, 2002; Soto and Lotz, 2003) and Prior *et al.* (2003). Soto and Lotz concluded that ingestion of infected tissues was far more effective in transmitting the virus between shrimp than immersion in infected water. Remarkably, however, even when shrimp were isolated to ensure they had equal chances to consume the infected tissues offered to them, not all shrimp became infected (50-60%). Prior *et al.* (2003) succeeded in determining the lethal intramuscular dose of a WSSV stock and also tried to develop a controlled bio-assay by immersion of shrimp. Although very large amounts of infectious virus were added to the water (as shown by the injection study), mortality rates stayed below 40%. Later, another study by Gitterle *et al.* (2006) showed the difficulty encountered when experimental infection by waterborne route is attempted. Merely adding virus inoculum to the water proved insufficient to result in infection and shrimp were placed in tanks in which orally infected shrimp had previously died.
The overall impression from these studies is that there are restrictions on the ability of WSSV to gain entry to its host. This does not have to seem illogical as specific behaviour such as active feeding has to be present in order to have a high exposure to the virus. Another factor which can not be neglected is that all the tissues known to be susceptible to WSSV replication are protected from the out-side world by cuticula (Wongteerasupaya *et al.*, 1995; Chang *et al.*, 1996; Durand *et al.*, 1996; Mohan *et al.*, 1998; Escobedo-Bonilla *et al.*, 2007). This is even true for the gills and the stomach epithelium (Bell and Lightner, 1988). Although little details are known about the structure and function of the cuticula of penaeid shrimp, it is well-known that they change dramatically in time (Chan *et al.*, 1988; Cariolou and Flytzanis, 1994; Promwikorn *et al.*, 2007). Therefore, to elucidate the transmission of WSSV in shrimp, it will be important to take the moult stage into account (Le Moullac *et al.*, 1997; Mugnier and Soyez, 2005).

2.2.5.2. Persistent / latent infection

Apart from typically causing mass mortality among shrimp populations, WSSV has also been observed in the field to persist inside its hosts in a latent state. In these cases, animals are asymptomatic carriers for extended periods and the virus is present in low amounts. Although these low levels of WSSV have been confirmed using

sensitive diagnostic methods (ie. 2-step PCR), the reproduction of latent infections under controlled laboratory conditions has not been reported (Tsai *et al.,* 1999; Chen *et al.,* 2000; Magbanua *et al.,* 2000; Thakur *et al.,* 2002). Further reports from the field indicate that WSSV can reactivate under stressing circumstances such as ablation, spawning (Lo and Kou, 1998), low temperature (Vidal *et al.,* 2001), etc.

The description of latency-related genes in the WSSV genome supports the possibility that this virus indeed can halt its lytic cycle and allow the host to survive (Hossain *et al.,* 2004). Usually these genes are identified based on their similarity to gene sequences of other viruses that are known to go into latency, such as *Herpesviridae* and *Baculoviridae* (Groves *et al.,* 2001; Hughes *et al.,* 1997; Leib *et al.,* 1991; Leight and Sugden, 2000).

One of the main problems in clarifying the issue on whether the infection is latent or persistent, both in the laboratory and field, is detection. All diagnostic tests have a detection limit and it is believed that WSSV can still be present in shrimp, even if 2-step PCR results are negative for the virus (Khadijah *et al.,* 2003). Additionally, appropriate target tissues have to be sampled for detection of latent infections. Pleopods are often collected for PCR analysis because the procedure is nonlethal and ideal for testing valuable broodstock. However, WSSV may be present in low concentrations in other tissues and missed by this testing method. For example, human Herpes simplex virus type 1 (HSV-1) is known to remain inside neural ganglia in a latent state during the life of the host, but is capable of reactivation (Roizman and Knipe 2001). As a result, sampling of tissues, other than neural ganglia for the presence of HSV-1, will result in a false negative result.

The possibility of WSSV going into latency creates the dangerous risk that animals labeled as ‘specific pathogen-free’ (SPF) might, in fact, be WSSV carriers that are capable of introducing the disease unknowingly. A recent publication supported this possibility by measuring viral latency-associated genes transcription in asymptomatic shrimp, which were SPF according to routine PCR testing (He and Kwang, 2008). It was suggested that the WSSV genome can be present in shrimp over extended periods of time while its lytic cycle is halted. These findings should also motivate shrimp growers to only purchase SPF stocks from reputable sources that have an established record of providing disease-free shrimp.

2.2.5.3. Vectors and source of contamination

Mechanical vectors include rotifers, non-decapod crustacea such as *Artemia sp.* and copepods, bivalves and polychaete worms, all common feeds for larvae and broodstock. In addition, non-crustacean aquatic arthropods such as sea slaters (*Isopoda*) and *Euphydradae* insect larvae have all been found to be PCR-positive for WSSV (Escobedo *et al.,* 2008). All of these species have been found capable of accumulating high concentrations of viable WSSV, although there is no evidence of virus replication (Lo *et al.,* 1996a; Chang *et al.,* 2002).

Infected frozen shrimp for human consumption or used as fishing bait may also act as a carrier of WSSV (Lightner *et al.,* 1997; Hasson *et al.,* 2006). Improper disposal of processing wastes (head, shells, etc.) and water may be a source of contamination if disposed near wild or farmed shrimp stocks.

2.2.6. WSSV - host interactions

During the last few years, various reports have been published indicating that some hosts are capable of stopping, eliminating or at least tolerating WSSV infections. Many researchers have studied the shrimp-WSSV interaction with the hope that a better understanding of the underlying mechanisms invoking virus elimination or persistence could lead to the development of strategies to control or prevent shrimp viral diseases in the future (Flegel, 2010; 2011).

Since the report of Venegas *et al.,* (2000) that shrimp possess some kind of defense against WSSV enabling them to survive infection, many observations have been published that penaeid shrimp can mount a defensive response against WSSV. As mentioned before, palaemonid shrimp appear to have an efficient mechanism to withstand and clear WSSV infection (Sarathi *et al.,* 2008).

2.2.6.1. WSSV-receptor and cellular ligand

A crucial interaction in viral infection is the receptor-ligand binding, which needs to occur between the viral particle and its host cell (Liu *et al.,* 2009). In both naturally occurring innate defense and vaccination attempts, preventing WSSV from

binding/fusing with target cells could be the basis for successful control of the infection.

At the cellular level, a shrimp protein called *Penaeus monodon* Rab7 (PmRab7), identified from the membrane of hemocytes, may function as one of the receptors for the virus (Sritunyalucksana *et al.,* 2006). It binds directly to the major viral envelope protein VP28 and is present in most shrimp tissues. *In vivo* neutralisation assays demonstrated that PmRab7 is essential for infection. Other researchers have concluded that the Rab-dependent signaling complex might act as a virus recognition protein that triggers a phagocytic defense against the virus, which aids in fighting infection (Wu *et al.,* 2007). They reported that the PjRab protein (found in *Marsupenaeus japonicus*) could regulate shrimp hemocytic phagocytosis through a protein complex consisting of the PjRab, beta-actin, tropomyosin, and enveloped protein VP466 of WSSV. Another molecule that may serve as a WSSV receptor is the beta-integrin molecule (Li *et al.,* 2007).

Two of the major WSSV envelope proteins known to be involved in the interaction with host cells are VP28 and VP19, but many others have been implicated in different studies, while more than 35 structural proteins have been characterised (Escobedo *et al.,* 2008). Interfering with several of these proteins directly or administering them in a recombinant form to shrimp has been demonstrated to hamper WSSV infection (van Hulten *et al.,* 2001; Yi *et al.,* 2004; Wu *et al.,* 2005; Li *et al.,* 2006; Xie and Yang, 2006; Ha *et al.,* 2008). As more becomes known about the structure of the WSSV virion, it is becoming clear that the many structural proteins are interacting with each other, forming protein complexes in the envelope (Chang *et al.*, 2010) and nucleocapsid (Tsai *et al.*, 2008). In the latter, VP664, the largest viral protein ever described, is also note-worthy (Leu *et al.*, 2005).

2.2.6.2. Apoptosis

The process of programmed cell-death or apoptosis is one of the main innate anti-viral defense responses known in animals (Everett and McFadden, 1999). This ‘scorched earth policy’ by the host in response to WSSV infection has been observed in WSSV-infected shrimp and implicated as an important reason for the death of shrimp by some authors (Wongprasert *et al.,* 2003; Flegel, 2007b). Anti-apoptotic genes, which support this hypothesis, have been recognised in the WSSV genome (Wang *et al.,*

2004). In addition, the apoptosis cascade that occurs in penaeid shrimp has been studied and one of the central enzymes necessary for initiating and executing apoptosis in animals, caspase, has been described. This enzyme was upregulated in survivors of WSSV challenge according to one study, suggesting that shrimp can increase their chance of survival by eliminating target cells before the virus can use them to replicate (Wang *et al.,* 2008a). Investigations into the regulation of host cell apoptosis by WSSV demonstrated that the virus can prevent programmed cell death through ubiquitination of a tumor suppressor-like protein (He *et al.,* 2006) and that the process of ubiquitination plays an important role in the regulation of WSSV latency (He and Kwang, 2008). It has now been established that WSSV has a gene coding for an anti-apoptosis protein, which serves as a direct caspase inhibitor (Leu *et al.,* 2008).

Despite this progress, the cellular pathways and interactions involved are still poorly understood in shrimp and in invertebrates in general. The role of apoptosis in shrimp death or survival following viral infection has not been established. In a lot of the research on WSSV pathogenesis and virus-host interactions, apoptosis is not discussed. If this process of cell death would be of major importance, it would be unlikely that it could be overlooked. In those studies which do focus on apoptosis in WSSV-infected shrimp, conflicting conclusions have been reached. Some researchers on the one hand, concluded that apoptosis could be considered as a host anti-viral defense response, as the down-regulation of an initiator caspase gene favored the replication of WSSV (Wang *et al.*, 2008b) or the administration of apoptosis inhibitors increased survival rates of WSSV challenged shrimp (Wang and Zhang, 2008), while on the other hand Rijiravanich *et al.* (2008) described that knock-out of caspase-3 gene resulted in improved protection against death due to WSSV infection. In conclusion, more work is needed to clarify the role of apoptosis during WSSV infections in shrimp.

Another interesting study looking into the complex host-virus interaction shows that WSSV can use a shrimp signal transducer and activator of transcription protein (STAT) to enhance expression of the immediate-early gene ie1, which is an important promotor in the early stages of WSSV infection. Thereby, WSSV is taking advantage of a mechanism which is normally supposed to be a defense against virus infection, as was seen in *Drosophila*, and using it to enhance viral replication (Dostert *et al.,* 2005; Liu *et al.,* 2007).

2.2.6.3. RNA interference

The technique of RNA interference (RNAi) has long been a potent research tool to down-regulate expression of target genes in a wide range of eukaryotes (Fire *et al.,* 1998; Friedman and Perrimon, 2004). The administration of sequence-specific RNA that was designed using WSSV-sequences was shown to be very successful in blocking the viral infection in shrimp, either with long dsRNA or short interfering RNA (siRNA) (Robalino *et al.,* 2004; 2005). Huang and Zhang (2012) and Haung *et al.* (2012) further confirmed that this mechanism is used by shrimp. These researchers showed that the central Argonaute effector proteins of the siRNA and miRNA pathways are upregulated during WSSV infection, leading to reduced WSSV loads.

The interesting role of RNAi in the interaction between WSSV and the shrimp host was prominently mentioned in a recent hypothesis forwarded by Flegel (2009). Upon observing persistent IHHNV, YHV and TSV infections in grossly healthy animals, it was proposed that a process of active accommodation of the viruses by the host was taking place in which the virus prevents an apoptotic response, which would otherwise kill the host (Flegel, 2007b). However, with the discovery of reverse transcriptase (RT), integrase (IN) and viral-like sequences in the genome of shrimp and insects, it is possible that they would use viral mRNA as a weapon against the viruses themselves and create a balance with the pathogen. The principle is that viral mRNA would be recognised by the host cell and by means of RT and IN, the mRNA sequence would be copied into the hosts' genome. This, in turn, would lead to the production of viral antisense immunospecific RNA and the induction of the host RNA interference (RNAi) mechanism, thus reducing viral mRNA transcription. Many important steps such as the recognition of foreign viral mRNA, viral sequences present in the genome of viral infection survivors and hereditary resistance via integration in gonad cells need to be confirmed, but it would provide an explanation for the observed reduction of viral load and persistent viral infections in shrimp. The finding by Huang *et al.* (2011) that over 20% of the *P. monodon* genome is made up of WSSV-like sequences, is already a strong support for the idea of Flegel (2009), and presents a fascinating insight in the origin of this virus and the co-evolution with its host.

2.2.6.4. Viral interference IHHNV – WSSV

Finally, a natural phenomenon of viral interference between IHHNV and WSSV has been described (Tang and Lightner, 2002; Bonnichon *et al.,* 2006). It was discovered by routine histology that *P. stylirostris* survivors from a WSSV infectivity study were also infected by IHHNV. Subsequent laboratory studies with IHHNV and WSSV in *P. styilirostris* showed that animals with an active IHHNV infection were clinically protected from WSSV, achieving survivals of 80% following a WSSV challenge lethal for control shrimp. The underlying mechanism remains unknown, but could be very relevant, as multiple viral pathogens are often present in shrimp under culture conditions.

2.2.6.5. Conclusion on WSSV - host interaction

While advanced studies of WSSV infection and the host response are undertaken, it does appear that an effort needs to be made to standardise the methodology used. It is still common practice to conduct experiments with poorly characterised viral inoculums and randomly purchased or collected shrimp of unknown health history. Other shrimp pathogens, especially the major viruses that are widespread, can easily interfere with the outcome of such studies. Additionally, factors such as temperature, moult stage, stocking density and possible transmission between experimental animals must be strictly recorded and controlled.

Even with this caution for the interpretation of published results on WSSV infection in shrimp, different opportunities to decrease the effects caused by this viral infection have been established. Particularly defense-modulation and improvement of host resistance appear promising directions of investigation to contain the losses caused by the disease.

2.2.7. Diagnosis

It is ill-advised to diagnose WSSV solely based on symptomatology, as the general signs of anorexia, lethargy, chromatophore expansion and rapid mortality can be the result of many types of both infectious and non-infectious diseases. Even white spots on the cuticle are not pathognomonic for WSSV and can occur during bacterial colonisation as well (Goarant *et al.,* 2000; Wang *et al.,* 2000a).

There is a full range of methods for the detection of WSSV. Each of them has advantages and disadvantages. The following table (Table 4) has been modified from the Manual of Diagnostic Tests for Aquatic Animals where the description of each method is available (www.oie.int).

Table 4. Description of each diagnostic method for WSSV according to OIE.

Method	Surveillance				Disease diagnosis	
	Larvae	PLs	Juvenile	Adults	Presumptive	Confirmatory
Gross signs	D	C	C	D	C	D
Bioassays	D	C	D	D	C	C
Whole mount light microscopy	D	C	C	D	C	C
Histopathology	D	C	C	C	B	B
TEM	D	D	D	D	D	A
Antibody based methods	D	C	D	D	B	B
DNA-probes	C	B	B	C	A	A
PCR	A	A	A	A	A	A
Sequence	D	D	D	A	D	A

From A: most suitable method to D: not recommended method

Commercial diagnostic kits available: Dot blot, *in situ* hybridization, PCR, immunodot, immunohistochemistry, immunosquash and immunochromatography.

2.2.8. Control and prevention

2.2.8.1. "Vaccination" or "immunisation"

Many reports have described an increased relative survival of shrimp in experimental "vaccination" trials (Johnson *et al.,* 2008; Rowley and Pope, 2012). The possibility of including recombinant viral proteins (mainly VP28) in either injectable or *per os* vaccines has shown promise for use in the field (Witteveldt *et al.,* 2004, Jha *et al.,* 2006, Fu *et al.,* 2008).

Ning *et al.,* (2009) reported a technique by which the oral administration of transfected bacteria could increase the survival among WSSV-challenged crayfish. Viral gene fragments encoding WSSV envelope protein VP28 were introduced in the attenuated *Salmonella* bacteria, which upon uptake via the food, were successfully expressed inside the tissues of the crayfish. This would then give rise to VP28 exposure of the host during about 7 days and confer protection by inducing an antiviral response.

A different strategy is to introduce a constructed DNA plasmid coding for the viral proteins via injection directly into the host where it induces the production of WSSV proteins by the host cells. By using this technique, Rout *et al.,* (2007) demonstrated that they could improve the relative survival of their *P. monodon* test shrimp to WSSV challenge and showed that expression of the DNA vaccine in the tissues of the experimental animals lasted for up to 2 months. It was proposed that an increase in prophenoloxidase, superoxide dismutase and superoxide anion levels occurred as an antiviral response mounted by the shrimp against the endogenously produced viral proteins (Rajesh Kumar *et al.,* 2008). Alternatively, the DNA vaccine could be delivered successfully to shrimp through chitosan nanoparticles (Rajesh Kumar *et al.,* 2009).

While "vaccination" of shrimp can result in clinical protection, it is uncertain whether it leads to prevention or elimination of WSSV infection. It is thus important to note that "vaccinated" shrimp could remain lifelong asymptomatic carriers, capable of spreading infectious virus to other shrimp populations.

Despite this progress in published work on protecting shrimp against WSSV, the underlying mechanism by which viral antigens activate the shrimp's defense system remains unknown. One of the major questions yet to be answered is whether the

shrimp's response to the presented pathogen, or subunit thereof, is specific. Indeed, the use of the word "vaccination" is not proper in the context of invertebrates where the existence of adaptive immunity is unclear. Little solid proof exists that adaptive immunity exists in shrimp or other invertebrates (Hauton and Smith, 2007; Johnson *et al.*, 2008). Evidence for an antibody response, involving B-cells, T-cells, etc. is lacking in invertebrates. As long as the underlying mechanisms remain unknown, the ability to design a successful WSSV vaccine will be hampered.

To date, only innate immunity has been demonstrated in shrimp and most of the knowledge obtained relates to defense against bacterial and fungal infections. For any hopes on an active creation of immune memory, this leaves us with the possibility for a form of "innate immunity training" in shrimp in response to exposure to pathogen associated molecular patterns (PAMPs). In fact, a number of studies have shown improved survival among WSSV-challenged shrimp following exposure to unrelated molecules originating from bacteria or yeast. Anti-lipopolysaccharide factors, a category of antimicrobial peptides known to stop bacterial and fungal infections in shrimp (de la Vega *et al.,* 2007), were also reported to interfere with WSSV replication in the crayfish *Pacifastacus leniusculus* (Liu *et al.,* 2006).

2.2.8.2. "Immunostimulation" or enhancement of anti-viral defense

Numerous papers have reported either variable levels of improved protection by using beta-glucans, vitamin C, seaweed extracts (fucoidan) and various other natural substances under experimental conditions (Cruz *et al.,* 2002, Soltanian *et al.,* 2009). However, the mode of action of these additives remains unknown and most descriptions in literature appear to point in the direction of an aspecific enhancement of the defense system (cfr. innate immunity training). Up to date, no data supporting full protection of shrimp against WSS in the field have been published (Chotigeat *et al.,* 2004; Rahman *et al.,* 2006a; Balasubramanian *et al.,* 2008; Rameshthangam and Ramasamy, 2007). No specific antiviral therapies have been described either.

2.2.8.3. Selective breeding for resistance

Resistant stocks against WSSV infection are not commercially available. Unlike for taura syndrome virus (TSV), the process of selective breeding of shrimp for WSSV

resistance appears to be particularly difficult (Gitterle *et al.*, 2006; Cock *et al.*, 2009). Some larvae producers have claimed a reduced susceptibility to WSS in past, but this had not been supported by scientific evidence, until recently by Cuéllar-Anjel *et al.* (2012). Selective breeding of *P. vannamei* over the course of 10 years in Panama had resulted in improved survival and lower infection rates than in unselected control lines.

2.2.8.4. Good husbandry and biosecurity

Improvement of biosecurity measures in and around shrimp farms reduces the risk of disease. Special attention should be given to water inlets and outlets, culture systems, possible carriers and movement of people and materials. Use of SPF broodstock and PL screened by nested PCR provides the best possibility of preventing WSSV entry into the farm or hatchery (Bondad-Reantaso *et al.*, 2005).
The construction of plastic greenhouses over ponds helps in isolating them from the surroundings, as well as its function to increase pond water temperature.

2.2.8.5. Temperature

For reasons not yet understood, it has been demonstrated that WSSV replication in shrimp is blocked when the water temperature is maintained at 32°C or higher (Vidal *et al.,* 2001; Guan *et al.,* 2003; Du *et al.,* 2006; Granja *et al.,* 2003; 2006; Rahman *et al.,* 2006b; 2007) and below 15°C (Jiravanichpaisal *et al.,* 2004). As a result, numerous farms have constructed greenhouses over nursery ponds and reported that this strategy has helped to prevent WSSV outbreaks during the nursery phase as well as reduce WSSV losses during growout. Recent discoveries by Lin *et al.* (2011) have shown the involvement of heat-shock proteins and aldehyde dehydrogenase in the suppression of WSSV by high temperatures.

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CHAPTER 3

Influence of moult stage and cuticle damage on inducing an experimental infection with waterborne WSSV in penaeid shrimp

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3.1. Study of the moult cycle in *Penaeus vannamei* and *P. monodon*

Corteel M, Dantas-Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2012) Moult cycle of laboratory-raised *Penaeus (Litopenaeus) vannamei* and *P. monodon*. Aquaculture International 20:13-18

Abstract

This study was carried out to gather quantitative data on the moult cycle and its different stages in laboratory-raised shrimp, kept at a constant temperature of 27°C. The stages of the moult cycle were differentiated and characterised by microscopic analysis of cuticle, epidermis and moulting processes in the uropods of *Penaeus vannamei* and *P. monodon*. Five major moult stages were defined: early and late post-moult (A and B), inter-moult (C) and early and late pre-moult (D1 and D2). Total moult cycle duration was around 5 and 6.5 days for 2 g *P. vannamei* and *P. monodon*, and 11 and 12 days for 15 g *P. vannamei* and *P. monodon*. Overall, the relative duration of the moult stages within the cycle was 5-10% for A, 9-16% for B, 12-20% for C, 28-36% for D1 and 30-38% for D2 stage. It was concluded from this study that the pre-moult stages comprised the dominant phase of the cycle and that *P. monodon* moulted at a significantly slower rate than *P. vannamei*, under the given conditions. Without the use of invasive techniques, the moult process was charted in laboratory-raised shrimp in Europe, providing a tool for taking into account this important physiological factor in further experiments.

Introduction

Like in all Crustacea, the body surface of penaeid shrimp is covered by an exoskeleton, called cuticula or cuticle. To allow growth and regeneration, this shell has to be shed periodically during a cyclic process called moulting. Most metabolic and endocrinological functions revolve around this cycle, making the moult a pivotal event in shrimp physiology (Skinner 1962; Skinner 1985; Chang 1995).

Typically, the moult cycle of Crustacea is divided into 4 recurrent stages: post-moult (metecdysis), inter-moult (anecdysis), pre-moult (proecdysis) and the moment of the shedding of the old cuticle (ecdysis). For a long time already, a letter-code is used to refer to these stages: A and B for early and late post-moult, C for inter-moult and D for pre-moult (Drach 1939). Studies on the moult process in penaeid shrimp which list selection criteria for the various moult stages have been published for *Penaeus (Farfantepenaeus) duorarum* (Schafer 1968) *Penaeus (Farfantepenaeus) merguiensis* (Longmuir 1983) *Penaeus (Litopenaeus) setiferus* and *Penaeus (Litopenaeus) stylirostris* (Robertson *et al*. 1987), *Penaeus (Litopenaeus) vannamei* (Chan *et al*.

1988; Cesar *et al*. 2006) and *Penaeus monodon* (Promwikorn *et al*. 2004). The key criteria for characterising the stages were the appearance of the epidermis, pigmentation, the formation of new setae (setogenesis) and the presence of matrix or internal coni in the setal lumen. Relative durations of the moult stages in shrimp have been provided so far by Chan *et al*. (1988) for 11.5-14 cm *P. vannamei* whose moult cycles took 34 days on average at a rearing temperature of 20-22°C. Since the retraction of Promwikorn *et al*. (2007), no data is available in literature for *P. monodon*.

Seeing the importance of the moult process in shrimp physiology, much more consideration should be given to it in animal experimentation. The aim of the present work was to record the duration of the stages of the moult cycle in *P. vannamei* and *P. monodon* under the rearing conditions at the laboratory of the authors.

Materials and Methods

Experimental animals and conditions

The shrimp used in this study were: *Penaeus vannamei* from Molokai Sea Farms Int. and *P. monodon* from Moana Technologies Nucleus Breeding Centre (both on Hawaii, USA). All batches of shrimp were certified to be SPF by Dr. James Brock of Moana Technologies. Batches of 10,000 shrimp arrived as post-larvae stage 10 and were reared in a recirculation system at the Laboratory of Aquaculture and Artemia Reference Center, Gent University, Belgium. They were fed with *Artemia* nauplii twice daily for 3 weeks and were then weaned onto a commercial pelleted feed (A2 monodon high performance shrimp feed, INVE aquaculture nv, Belgium), fed twice daily at a total rate of 5 % of their mean body weight (MBW). Water temperature was kept at 27 ± 1°C and salinity at 35 ± 1 g l^{-1}. Bio-filtration and regular water changes kept total ammonia-N below 0.5 mg l^{-1} and nitrite-N below 0.15 mg l^{-1}. The room was illuminated 12 hours per day by dimmed TL-light.

Moult stage determination

The stages of the moult cycle were differentiated and characterised based on the studies by Drach (1939), Robertson *et al*. (1987), Chan *et al*. (1988) and Compère *et*

al. (2004). By analyzing the aspect of cuticle, epidermis and moult processes of uropods, 5 major moult stages were defined: early and late post-moult (A and B), inter-moult (C) and early and late pre-moult (D1 and D2). Briefly, the main characteristics used to discern the stages were A: epidermal tissue is present inside the setal lumen; B: the epidermis is retreating from the setae but is still present in the base of the setae; C: the epidermis lies on a line just underneath the base of the setae; D1: apolysis causes a translucent space to form between the old cuticle and the epidermis; D2: the new, folded cuticle and the new setae have become visible; E: ecdysis, the shedding of the old moult skin. As E stage lasted only a few minutes, the moult was considered as the transition from D2 to A, and was not further included in the analysis.

Illustrations of the moult stages and the criteria to differentiate them can be found in chapter 2.1.2.1.1. of this thesis.

Study of the moult cycle of *P. vannamei* and *P. monodon*

The moult cycle of *P. vannamei* was followed when they had a size of 2.0 ± 0.3 g and 14.8 ± 0.9 g, at the age of 61 and 150 days, respectively. *P. monodon* were examined when they had reached a size of 2.1 ± 0.5 and 15.2 ± 1 g at the age of 55 and 158 days, respectively. During each of the observation periods, 12 shrimp were followed individually for the duration of one entire moult cycle. Shrimp had been previously tagged with visible implant elastomer (kindly provided by Dr. David Solomon of Northwest Marine Technology, USA) in different locations of the tail muscles to allow identification and housed inside the recirculation system. Feeding regime and environmental circumstances were maintained as described for the growing of the shrimp. All animals were taken from the system and examined by inverted microscope every 12 hours. Digital photographs (at a magnification of 100X and 200X) were made of the exopodites of uropods, consistently focusing on the central part of the caudal end. During this procedure, shrimp were immobilised for about 30 seconds by gently wrapping them inside a Styrofoam tube, with only the last tail segment remaining outside for placement on the microscope. Examination was stopped for each animal as soon as it had shed its moult twice, thereby passing at least one whole moult cycle while under observation.

Photographs were analysed on the appearance of the cuticle, epidermis and moult processes such as apolysis and setogenesis. The durations of the stages and the total cycle were measured. For *P. vannamei*, the study was repeated in 2 subsequent batches of shrimp. Differences between stages and between shrimp species were analysed by *t*-test.

Results

Moult stage determination

The characteristics by which the stages were defined, were found to be uniform for both ages studied and for both *P. vannamei* and *P. monodon* (except for the obvious differences in size and pigmentation). They could therefore readily be used to differentiate the different moult stages in both species.

Study of the moult cycle

Total moult cycle duration was 4.8 and 6.4 days for 2 g *P. vannamei* and *P. monodon*, and 10.9 and 12.3 days for 15 g *P. vannamei* and *P. monodon* (Table 1). Overall, the relative duration of the moult stages within the cycle was 5-10% for A, 9-16% for B, 12-20% for C, 28-36% for D1 and 30-38% for D2 stage. In all species and ages, the pre-moult stages were found to be significantly longer than the post- and inter-moult stages ($p<0.05$). Statistically significant differences were found between 2 and 15 g *P. vannamei* in all moult stages and in D stages for *P. monodon*. In *P. vannamei*, all moult stages increased proportionally in duration with age, while in *P. monodon*, the post- and inter-moult stages only increase slightly in duration but the elongation of the pre-moult phase was responsible for the longer moult cycle. When total moult cycle durations were compared between shrimp groups of the same size, *P. monodon* moulted at a significantly slower rate than *P. vannamei*. Obviously, 15 g shrimp moulted at a significantly slower pace than 2 g.

Table 1. Average durations of the major moult stages and total moult cycles of 2 and 15 g *P. vannamei* and *P. monodon*.

Species (weight; number of shrimp)	Average duration of moult stage in days ± SD (percentage of total cycle) A	B	C	D1	D2	Duration of total cycle
P. vannamei (2.0 ± 0.3 g; n = 36)	0.5 ± 0.1 (10%)	0.5 ± 0.1 (11%)	0.6 ± 0.2 (12%)	1.7 ± 0.4 (35%)	1.5 ± 0.3 (32%)	4.8 ± 0.5 [a*]
P. vannamei (14.8 ± 0.9 g; n = 36)	0.8 ± 0.3 (7.5%)	1.1 ± 0.5 (10%)	1.6 ± 0.5 (15%)	3.8 ± 0.8 (34.5%)	3.6 ± 0.7 (33%)	10.9 ± 1 [b]
P. monodon (2.1 ± 0.5 g; n = 12)	0.5 ± 0.2 (8%)	1 ± 0.5 (16%)	1.3 ± 0.5 (20%)	1.8 ± 0.5 (28%)	1.9 ± 0.4 (30%)	6.4 ± 0.9 [c]
P. monodon (15.7 ± 1.2 g; n = 12)	0.6 ± 0.1 (5%)	1.1 ± 0.3 (9%)	1.5 ± 0.4 (12%)	4.4 ± 0.7 (36%)	4.7 ± 0.6 (38%)	12.3 ± 0.6 [d]

*different subscripts indicate statistically significant differences

Discussion

In the present study, the moult process was assessed in individual shrimp at two stages of development by light microscopical analyses twice daily. This was done without the use of invasive techniques such as cutting off parts of appendages, as these manipulations are known to interfere with the moult rate of Crustacea (Skinner, 1985).

The relative durations of the stages in the moult cycle were found to be remarkably similar between species and ages. In absolute time, *P. vannamei* shrimp shed their

skin at a higher frequency than *P. monodon* of the same age and size. The pre-moult stages became lengthier with age, and this was more pronounced in *P. monodon*. Overall, the pre-moult phase was by far the longest, occupying as much as two thirds of the entire moult cycle.

Up to now, few publications have provided quantitative data on the moult cycle in *P. vannamei*. In a first study by Chan *et al.* (1988), the total cycle duration of 34 days was a lot longer compared to the findings of the present study, while a relatively long period was taken up by the inter-moult stage. As rearing temperature is known to have a major impact on the metabolism and the moult process of shrimp (Vijayan and Diwan, 1995; Verhoef *et al*., 1998), it is likely that the difference in temperature of 6°C between the studies is responsible for this acceleration, specifically speeding up the inter-moult stage which is essentially a resting phase in the cycle. In the study of Cesar *et al.* (2006), in which rearing temperature was about the same as that used in our study, 1 month-old and 3 month old *P. vannamei* shrimp moulted at the same rate as 2 month-old and 5 month-old shrimp respectively in our study. Since weights of the animals were not reported, a clear comparison could not be made, but this is an indication that laboratory-raised shrimp can have a delayed development compared to shrimp in pond culture. Cesar and Yang (2007) registered a 12-day long cycle in 3-month old *P. vannamei*, with again the inter-moult making up half of the cycle, but did not mention the rearing temperature. Since the retraction of Prowikorn *et al*. (2007), no quantitative data is available on the moult cycle in *P. monodon* except a description of the moult stages (Promwikorn *et al.,* 2004).

In our study, we limited the moult stages to those major phases which can be readily and practically indentified with light microscopic examination: early and late post-moult (A and B), inter-moult (C) and early and late pre-moult (D1 and D2). Robertson *et al*. (1987), Cesar *et al*. (2007), Liu *et al*. (2004; 2010) and Sanchez-Paz *et al*. (2003) all used a similar division of the moult cycle into 5 stages. As Robertson *et al*. (1987) also argued, a more refined separation of these major stages is only possible by TEM and thus not practical or usually not even relevant for research into the moult process and its impact on other factors.

For the present study, we were most interested in the moult cycle and the relative importance of the different moult stages of the experimental shrimp present at the Laboratory of Aquaculture in Belgium. From comparison with literature it becomes

clear that quantitative data on the moult cycle of shrimp can not be simply extrapolated to all shrimp and conditions. In Europe, research on tropical shrimp relies on the availability of laboratory-raised shrimp. Even though their growth might be slower than under farm conditions, the highly controlled environment does allow for reproducible reference values to be registered. The fact that three consecutive batches of *P. vannamei* had almost identical cycles at a similar weight, gave us confidence to trust the acquired data and to consider the selection system reliable to pick out shrimp in specific moult stages. This selection system will be used in experiments to investigate the impact of the moult stage on shrimp susceptibility to infections.

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3.2 Moult stage and cuticle damage determine WSSV immersion infection in penaeid shrimp

Corteel M, Dantas-Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2009) Molt stage and damage to the cuticula determine white spot syndrome virus infection by immersion in penaeid shrimp. Veterinary Microbiology 137:209-216

Abstract

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

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

For the waterborne route, shrimp in different moult stages were immersed in sea water containing 10^4 SID_{50} ml^{-1} of WSSV. In a first study, *P. vannamei* (n=125) incubated in cell culture flasks, became infected with WSSV mostly in post-moult stages. In a second study, 2 groups of *P. vannamei* (n=100) and *P. monodon* (n=100) were transferred into plastic bags to prevent damage to the cuticle; and in 1 group a pleopod was cut off prior to incubation. Induction of damage increased infection significantly ($p<0.05$) in A-stage from 0-40% to 60-100%, in B-stage from 0-20% to 40-60%, in C-stage from 0-20 to 20-60%, while infection was 0% in D-stages with both immersion methods.

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

Introduction

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

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

These facts make it difficult to reproduce those studies or make reliable conclusions. Probably the best-controlled experimental studies on WSSV transmission so far, were published by Soto and Lotz (Soto *et al.*, 2001; Lotz and Soto, 2002; Soto and Lotz, 2003) and Prior *et al.* (Prior *et al.*, 2003). Soto and Lotz concluded that ingestion of infected tissues was a far more effective treatment than immersion in infected water. Remarkably however, even when *P. vannamei* were isolated to ensure they had equal chance to consume the infected tissues offered to them, not all shrimp became infected (50-60%). Prior *et al.* (2003) succeeded in determining the lethal intramuscular dose of a WSSV stock and also tried to develop a controlled bio-assay by immersion of *P. vannamei*. Although very large amounts of infectious virus were added to the water (as shown by the injection study), mortality rates stayed below

40%. Recently, another study clearly illustrated the difficulty to infect animals by WSSV immersion challenge (Gitterle *et al.*, 2006), while a study on an ornamental shrimp's susceptibility to WSSV resulted in a discussion of the problems encountered with experimental feeding challenges (Laramore, 2007). Gitterle *et al.* (2006) showed that merely adding virus inoculum to the water was not sufficient to result in *P. vannamei* infection but needed to place the shrimp in tanks in which orally infected shrimp had previously died to finally obtain successful transmission. Finally, in the PhD thesis by Dr. Bonny Bayot (2006), less than 17% of *P. vannamei* shrimp became infected upon individual challenge with WSSV via oral route and none or merely 3% by immersion.

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

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

Considering the inability to reproducibly cause infection in shrimp exposed to WSSV by immersion, the present study was set-up to investigate the factors determining WSSV infection by waterborne route. In a first hypothesis we tested whether the susceptibility of shrimp to WSSV infection changes during the course of their moult cycle. The virus was delivered intramuscularly, thus passing the cuticle in order to compare the internal susceptibility between the different moult stages. In a second approach, the barrier function of the cuticle against natural infection by waterborne virus was tested in a series of immersion inoculation experiments of shrimp in

different moult stages. Groups of artificially damaged shrimp were compared with control shrimp to test the hypothesis that the cuticle presents a barrier against WSSV and that wounding can promote infection.

Materials and Methods

Experimental animals and conditions

The shrimp used in this study were *Penaeus (Litopenaeus) vannamei* from Molokai Sea Farms Int., Hawaii, USA and *P. monodon*, from Moana Technologies Nucleus Breeding Centre, Hawaii, USA. The batches of shrimp from Moana Technologies were certified to be SPF by Jim Brock, DVM. Those from Molokai Sea Farms had SPF status according to inspection services by the Aquaculture Development Program, State of Hawaii. Batches of 10,000 PL-10 shrimp were shipped to Belgium and reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. They were fed with *Artemia* nauplii twice daily for a period of 3 weeks and were then weaned onto a commercial pelleted feed (A2 monodon high performance shrimp feed, INVE Aquaculture SA, Belgium), fed twice daily at a total rate of 5 % of their mean body weight (MBW). Water temperature was kept at 27 ± 1°C and salinity at 35 ± 1 gl^{-1}. Regular water changes kept total ammonia-N below 0.5 mg l^{-1} and nitrite-N below 0.15 mg l^{-1}. The room was illuminated 12 hours per day by dimmed TL-light. For the viral challenge experiments, shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the experiments were performed under bio-safety conditions.

Molt stage determination

Molt stages were determined based on the descriptions by Robertson *et al.* (1987) and Chan *et al.* (1988). Briefly, shrimp were restrained for a few seconds and their uropods were examined by inverted microscope. At a magnification of 100 to 200X, the exopodites of uropods were analysed on the appearance of the cuticle, epidermis and moult processes such as apolysis and the formation of new cuticle. Shrimp were

separated into 5 major moult stages: early and late post-moult (A and B), inter-moult (C) and early and late pre-moult (D1 and D2).

Post-moult stages are characterised by an epidermis in close contact with all of the still thin cuticle. The epidermis is present in the setae in A-stage and retracts in B-stage, while it constantly secretes additional layers to the cuticle. In the inter-moult stage the epidermis lies in a straight line at the bottom of the setae while the construction of the cuticle is finalised. The pre-moult phase starts as the epidermis retracts from the cuticle in stage D1 and begins formation of a new cuticle. In the final stage before the moult, D2, the newly forming cuticle and setae become visible.

Virus

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

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

P. vannamei juveniles (MBW = 5.6 ± 2.7 g; n = 450) were taken from stock cultures maintained at ARC and screened for their moult stage. Thirty shrimp were selected in each of the 5 major moult stages (A, B, C, D1 and D2) and inoculated intramuscularly with 50 µl of a 10-fold serial dilution of the WSSV stock (10^{-2} to 10^{-7}), with 5 shrimp per dilution. After the inoculation, shrimp were housed individually in covered 10 l aquaria, filled with artificial seawater at a salinity of 35 g l^{-1}, provided with constant aeration and maintained at 27°C by air heaters. Approximately 2.5% of BW of a

commercial shrimp diet was provided to each shrimp in 2 rations per day. Moribund and dead shrimp were recorded, removed from the aquaria and processed for detection of WSSV infection. The experiment was terminated at 120 hpi, when surviving shrimp were sacrificed and analyzed for WSSV infection. The experiment was performed in triplicate.

Study of WSSV infection by immersion route

Immersion inoculation inside cell culture flasks

The aim of this experiment was to develop a model for WSSV infection by immersion. A total of 125 SPF *P. vannamei* were used. As the batch of shrimp grew up, 5 groups of shrimp with a MBW of 1, 4, 6, 11 and 20 g were taken from the stock culture at ARC and screened for their moult stage. For each size group, 5 shrimp per moult stage were immersed. The WSSV inoculum used to immerse the shrimp was a 1% dilution of the WSSV stock. It was prepared in a volume of 25 ml artificial seawater (35 g l^{-1}) per g bodyweight, resulting in a dose of 10^4 SID_{50} ml^{-1}. Shrimp of 1 g were put inside '25 cm^2' cell culture flasks (Nunc A/S, Denmark) containing 25 ml of the inoculum. Animals of 4, 6 and 11 g were put inside '75 cm^2' cell culture flasks containing respectively 100, 150 and 275 ml of the inoculum. Shrimp of 20 g were put inside '175 cm^2' cell culture flasks containing 500 ml of the inoculum. Flasks were placed on a lateral side in order to allow the shrimp to stay in a physiological position. The duration of the immersion was 3 hours and water was aerated with an airstone.

After the inoculation, the procedures were identical to those as described for the intramuscular route, except no food was given the first 12 h after the immersion to avoid additional oral up-take of virus via the food. Shrimp were monitored for clinical signs every 12 h and dead shrimp were removed and processed for detection of virus replication. The experiment was terminated 5 days post immersion. At this time, all surviving shrimp were euthanised and processed for virus detection. Mortality and infection rates were compared between the moult stages and between the sizes.

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

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

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

Detection of WSSV infection by indirect immunofluorescence (IIF)

The procedure to detect WSSV infection by IIF was described before (Escobedo-Bonilla *et al.*, 2005). In brief, the cephalothoraxes of dead shrimp were dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at -20 °C. Cryosections (5 µm) were made and immediately fixed in 100% methanol at -20 ºC

for 20 min. Sections were washed three times for 5 min each in phosphate buffered saline (PBS) and incubated with 2 µg ml^{-1} of the monoclonal antibody 8B7 (Diagxotics Inc. USA) directed against viral protein VP28 (Poulos *et al.*, 2001) for 1 h at 37 ºC. Then, sections were washed three times for 5 min each in PBS and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1 h at 37 ºC. Sections were finally washed in PBS, rinsed in deionised water, dried and mounted with a solution of glycerine and 1, 4-diaza-bicyclo[2,2,2]-octane (DABCO) (ACROS organics, USA). Slides were analyzed by fluorescence microscopy (Leica DM RBE).

Statistical analysis

The virus titers of the intramuscular titration were compared between moult stages using the Wilcoxon rank-sum non-parametric test (Zar, 1996).
Differences in WSSV infection after immersion between moult stages within groups of 5 to 10 shrimp per group were tested for significance using Fisher's exact test (Kirkwood and Sterne, 2003).
In the experiments with immersion and induction of damage, both species and ages were pooled into groups of 20 shrimp, and the difference in infection rates was tested between the moult stages and between the control and the pleopod cut groups by Pearson's Chi Square tests with Yates' correction.
All calculations were performed using S-plus version 6.1 (Lucent Technologies).

Results

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

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

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

Molt Stage	Dilution of WSSV	Mortality	Confirmed infected by IIF	Infection titer
A	10^{-2}	15/15	15/15	$10^{6.5 \pm 0.4}$ SID_{50} ml^{-1}
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	10/15	10/15	
	10^{-6}	0/15	0/15	
	10^{-7}	0/15	0/15	
B	10^{-2}	15/15	15/15	$10^{7.1 \pm 0.4}$ SID_{50} ml^{-1}
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	11/15	11/15	
	10^{-6}	6/15	6/15	
	10^{-7}	0/15	0/15	
C	10^{-2}	15/15	15/15	$10^{6.7 \pm 0.2}$ SID_{50} ml^{-1}
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	10/15	10/15	
	10^{-6}	2/15	2/15	
	10^{-7}	0/15	0/15	
D1	10^{-2}	15/15	15/15	$10^{6.9 \pm 0.2}$ SID_{50} ml^{-1}
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	13/15	13/15	
	10^{-6}	4/15	4/15	
	10^{-7}	0/15	0/15	
D2	10^{-2}	15/15	15/15	$10^{6.7 \pm 0.3}$ SID_{50} ml^{-1}
	10^{-3}	15/15	15/15	
	10^{-4}	15/15	15/15	
	10^{-5}	10/15	10/15	
	10^{-6}	4/15	4/15	
	10^{-7}	0/15	0/15	

Immersion inoculation inside cell culture flasks (Table 2)

Of the 1 g shrimp, only 1 shrimp in the A-stage group started to become anorectic and lethargic at 36 hpi. This was the only shrimp to die due to WSSV before the end of the experiment. All other shrimp were euthanised at 120 hpi and were negative for WSSV on IIF. When the immersion was performed with 4 g shrimp, all survived the experiment uninfected. At a size of 6 g, 3 out of 5 A-stage shrimp started to show clinical signs at 36 hpi and died at 60-84 hpi. When the experiment was performed with 11 g, all A- and one B-stage shrimp showed clinical signs and died due to WSSV infection between 48 and 120 hpi. Two D2-stage animals moulted during the immersion and one died before the end of the 3 hours procedure. This was the only mortality during the course of the experiment which was not caused by WSSV infection. In the experiment performed on 20 g shrimp, all A-, 2 out of 5 B- and 1 C-stage shrimp showed clinical signs after 36 hpi. These shrimp died between 48 and 72 hpi and were confirmed to be infected with WSSV, while all other shrimp survived and were uninfected. The difference in infection rate was significantly higher in A-stage than in the other stages in 11 g shrimp, and between A- and C-, D1- and D2-stage in 20 g animals ($p<0.05$).

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

Table 2. Immersion of *P. vannamei* in different moult stages in cell culture flasks containing WSSV inoculum with 10000 SID_{50} ml^{-1}.

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

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

Immersion inoculation inside plastic bags (Table 3)

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

recorded. Thus, the physical damage occurring during the immersion procedure could be estimated.

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

In 2 g juvenile *P. vannamei* immersed in plastic bags without cutting of pleopods, 2 shrimp in A-stage and 1 in C-stage died. Of the shrimp with cut off pleopods, 3 in A- and B- and 1 in C-stage died between 48 and 72 hpi. All other shrimp of the various moult stages with pleopods left intact or cut survived until the end of the experiment at 120 hpi. Of 15 g *P. vannamei* with no pleopod cut, only 1 out of five A- and B-stage animals died at 72 hpi. Cutting a pleopod increased the mortality to 5 in A-stage (48 to 120 hpi), 2 out of 5 in B-stage (96 hpi) and 1 in C-stage (120 hpi). All other shrimp survived until 120 hpi.

In 2 g juvenile *P. monodon* immersed with pleopods intact, only 1 shrimp in A-stage died. Of those with cut off pleopods, 3 in A- and B- and 2 in C-stage died between 48 and 120 hpi. All other shrimp survived until the end of the experiment. Of 15 g *P. monodon* with pleopods left intact, 2 out of five A- and 1 B-stage shrimp died (48 or 72 hpi). Cutting a pleopod induced mortality in 3 shrimp in A-stage (48 to 72 hpi), 2 in B-stage (48 to 72 hpi) and 3 in C-stage (72 to 84 hpi). All other shrimp survived until the end of the experiment. In all cases, dead shrimp were WSSV positive on IIF, and surviving shrimp were WSSV negative.

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

Table 3. Immersion of *P. vannamei* or *P. monodon* in different molt stages inside plastic bags containing inoculum with 10000 SID_{50} ml^{-1} of White Spot Syndrome Virus, with or without removal of one appendage.

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

B	Species	Removal of appendage	Molt stage	Mortality	Confirmed infected by IIF	% infected
	All shrimp	none (control)	A	6/20	6/20	30 [ab]
			B	2/20	2/20	10 [ac]
			C	1/20	1/20	5 [ac]
			D1	0/20	0/20	0 [c]
			D2	0/20	0/20	0 [c]
		1 pleopod	A	14/20	14/20	70 [d]
			B	12/20	12/20	60 [bd]
			C	7/20	7/20	35 [bd]
			D1	0/20	0/20	0 [c]
			D2	0/20	0/20	0 [c]

a,b,c: percentages indicated by different superscripts were significantly different by χ^2 analysis ($p<0.05$, except between A- and D-stages in pleopod cut group $p<0.001$.)

Discussion

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

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

As an alternative immersion recipient, polyethylene bags were tested in this study. When shrimp were carefully placed inside plastic bags before transport and the water replaced by inoculum, the amount of resistance and jumping of the shrimp was reduced and much less obvious injuries could be observed while the shrimp hung suspended in mid-air. Even though the bags proved to be useful, it remained impossible to completely prevent the occurrence of damages in the soft post-moult shrimp.

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

The actual portal of entry of WSSV from the water into a host has never been described, but some assume that the gills are the best candidates (Chang *et al.*, 1996; Witteveldt *et al.*, 2004; Arts *et al.*, 2007). The experimental findings of the present study demonstrate that an artificially induced wound in the cuticle increases the rate of WSSV infection upon immersion. Cutting off a pleopod creates an open wound which can allow either (1) infection of cells at the site of the wound or (2) entry of WSSV into the hemolymph followed by direct systemic spread or on the other hand (3) reduce the competence of shrimp to resist WSSV infection. In the first two scenarios, entry of the virus would occur through the opening in the cuticle itself. If one considers the (ultra)structure of the cuticle of crustacea such as shrimp, it is not difficult to imagine that the cuticle constitutes an impregnable barrier against viruses from food or the environment (Compère, pers.comm.). Although damage to the cuticle appears to be the key to WSSV infection from the water, the situation is more complex. Even when an open wound is present in shrimp, this does not always lead to infection, especially in moult stages when the exoskeleton is well-developed (i.e. inter- and pre-moult). Factors which determine whether WSSV can ultimately invade a shrimp could be: (1) morphological and physiological (cuticle and epidermal cells) or (2) (a)specific defence-related (coagulation time, phagocytosis, phenoloxidase and reactive oxygen species activity etc.). All these factors are likely or are already known

to vary between different stages of the moult cycle (Charmantier *et al.*, 1994; Le Moullac *et al.*, 1997; Liu *et al.*, 2004; Chiou *et al.*, 2007; Promwikorn *et al.*, 2007; Mugnier *et al.*, 2008). The third alternative explanation for the increased chance for WSSV infection in damaged shrimp, would be that wounding has a direct or indirect effect on the capacity of shrimp to resist to WSSV infection. Indeed, removal of a pleopod will induce stress, which could have an effect on the subsequent immune response of the shrimp. The inflicted damage and subsequent clotting, hemocyte migration and exocytosis at the site of the wound, and immune responses to other microorganisms which may enter, can all alter possible defence against WSSV infection.

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

Conclusion

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

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CHAPTER 4

Susceptibility of *Macrobrachium rosenbergii* to WSSV infections

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Corteel M, Dantas-Lima JJ, Tuan VV, Thuong KV, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (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 100:211-218

Abstract

As some literature on the susceptibility of different life stages of *Macrobrachium rosenbergii* to white spot syndrome virus (WSSV) is conflicting, the pathogenesis, infectivity and pathogenicity of 2 WSSV strains (Thai-1 and Viet) were investigated here in juveniles using conditions standardized for *Penaeus vannamei*. As with *P. vannamei*, juvenile *M. rosenbergii* (2 to 5 g) injected with a low dose of WSSV-Thai-1 or a high dose of WSSV-Viet developed comparable clinical pathology and numbers of infected cells within 1 to 2 d post-infection. In contrast, a low dose of WSSV-Viet capable of causing mortality in *P. vannamei* resulted in no detectable infection in *M. rosenbergii*. Mean prawn infectious dose 50% endpoints (PID_{50} ml^{-1}) determined in *M. rosenbergii* were in the order of 100-fold higher for WSSV-Thai-1 ($10^{5.3\pm0.4}$ PID_{50} ml^{-1}) than for WSSV-Viet ($10^{3.2\pm0.2}$ PID_{50} ml^{-1}), with each of these being about 20-fold and 400-fold lower, respectively, than found previously in *P. vannamei*. The median lethal dose (LD_{50} ml^{-1}) determined in *M. rosenbergii* was also far higher (~1000-fold) for WSSV-Thai-1 ($10^{5.4\pm0.4}$ LD_{50} ml^{-1}) than for WSSV-Viet ($10^{2.3\pm0.3}$ LD_{50} ml^{-1}). Based on these data, it is clear that juvenile *M. rosenbergii* are susceptible to WSSV infection, disease and mortality. In comparison to *P. vannamei*, however, juvenile *M. rosenbergii* appear more capable of "resisting" infection and disease, particularly in the case of a WSSV strain with lower apparent virulence.

Introduction

White spot syndrome virus (WSSV) infects a wide spectrum of crustaceans and is one of the most important pathogens of cultured penaeid shrimp. Over 80 species, including freshwater prawns, crayfish, lobsters and crabs, have been described to be hosts or carriers of WSSV (Escobedo-Bonilla *et al.*, 2008). Crustaceans that can carry WSSV pose a potential risk of transmitting infection and disease to cultured shrimp (Rajendran *et al.*, 1999; Flegel, 2007).

M. rosenbergii is the most widely cultured freshwater prawn species worldwide (New, 2002) with annual yields exceeding 30,000 t (FAO, 2009). Compared to penaeid shrimp, it is generally considered less prone to disease in culture (Bonami and Widada, 2011). With respect to WSSV, however, there have been some conflicting reports on the susceptibility of different *M. rosenbergii* life stages. For

example, some studies have reported larval and post-larval stages to be susceptible but older prawns to be quite refractive to acute infection and mortality (Lo *et al.*, 1996; Peng *et al.*, 1998; Pramod Kiran *et al.*, 2002). Indeed, in a comparative study including 2 other *Macrobrachium* sp. *(M. idella* and *M. lamerrae*) as well as *Penaeus monodon*, *M. rosenbergii* juveniles (1 to 2 g) and adults (5 to 7 g) were confirmed to be less susceptible to disease and mortality when challenged with WSSV by water-borne exposure, tissue ingestion and intramuscular injection (Sahul Hameed *et al.*, 2000). Follow-up studies showed WSSV infection to be transient, diminishing within a few days post-challenge (Waikhom *et al.*, 2006, Yoganandhan *et al.*, 2006). PCR tracking of WSSV loads in *M. rosenbergii* adults challenged by injection has also shown that the majority of WSSV is cleared within 5 d post-challenge, after which time low levels of virus remained detectable in some organs for 25 to 50 d (Sarathi *et al.*, 2008). Although not investigated in detail, there is some evidence to suggest hemagglutinins or lectins are involved in the process that protects *M. rosenbergii* against WSSV (Pais *et al.*, 2007).

In the present study, the pathogenicity of WSSV strains of high (Thai-1) and low (Viet) virulence for penaeid shrimp (Rahman *et al.*, 2008) was investigated in juvenile *M. rosenbergii* under standardized conditions used to determine their pathogenicity for *P. vannamei*. Tracking of numbers of infected cells in different organs over time in prawns injected with high and low doses of each strain and determinations of prawn infectious dose (PID_{50}) and lethal dose (LD_{50}) 50% end-points for the 2 WSSV strains confirmed the lower susceptibility of juvenile *M. rosenbergii* to infection and disease compared to *P. vannamei*, especially for the low virulence strain.

Materials and Methods

Prawns

M. rosenbergii were bred and reared using standard practices in the aquarium facilities at Ghent University, Belgium (New, 2002). Prawns used were 3rd generation offspring from broodstock imported from Thailand. Juvenile *M. rosenbergii* (2 to 5 g) were fed commercial penaeid shrimp feed pellets at a rate of 2.5% of their weight per day and maintained at 27 ± 0.5°C water temperature.

WSSV

The WSSV strains used to challenge *M. rosenbergii* originated from diseased *P. monodon* from either Thailand in 1996 (WSSV-Thai-1) or Vietnam in 2003 (WSSV-Viet) (Rahman *et al.*, 2008). WSSV-Thai-1 had been passaged once in *Pacifastacus leniusculus* (Jiravanichpaisal *et al.*, 2001) and WSSV-Viet had been passaged once in *Cherax quadricarinatus.* Crayfish gill homogenates containing WSSV-Thai-1 (from P. Jiravanichpaisal and K. Söderhäll, Uppsala University, Sweden) or WSSV-Viet (from Research Institute for Aquaculture no. 2, Ho Chi Minh City, Vietnam) were passaged in specific pathogen-free (SPF) *P. vannamei* to produce inocula and determine infectious titers as described previously (Escobedo-Bonilla *et al.*, 2005). Shrimp infectious dose 50% endpoint (SID_{50}) ml^{-1} titers were $10^{6.6}$ and $10^{5.8}$ for WSSV-Thai-1 and WSSV-Viet, respectively. Inocula were stored at –70°C and dilutions used to challenge *M. rosenbergii* were prepared in ice-cold phosphate-buffered saline (PBS).

Challenge protocols

In all bioassays, WSSV inoculum (50 ml) was injected into muscle at the junction between the 3rd and 4th abdominal segments. Methods to assess WSSV pathogenesis followed closely those described by Rahman *et al.* (2008). In brief, 140 *M. rosenbergii* juveniles (2 to 5 g) were stocked into 50 l aquaria (5 prawns per aquarium), each equipped with a water filter and heater. Based on SID_{50} ml^{-1} titers, each WSSV strain was injected into 30 prawns at either a low dose (LD, 30 SID_{50}) or a high dose (HD, 10000 SID_{50}). At 12, 24, 36, 48, 72 and 120 h post injection (hpi), prawns surviving in 1 tank were euthanized to collect and process cephalothorax tissue for immunohistochemistry (IHC). Prior to sampling, prawns were observed for gross disease signs and mortality was recorded. A group of 5 prawns was sampled at the beginning of the trial (0 hpi).

Bioassays to determine the PID_{50} were performed essentially as described previously (Escobedo-Bonilla et al. 2005, 2006), except that the WSSV infectivity titer was determined at 48 hpi instead of 120 hpi based on when most prawns were found to be infected by indirect immunofluorescence (IIF). In brief, 5 prawns (2 g) in each of 3 replicate 10 l aerated and covered plastic aquaria (15 prawns per dilution) were

injected with 10-fold serial dilutions of either WSSV-Thai-1 (10^{-1} to 10^{-6}) or WSSV-Viet (undiluted to 10^{-4}). Prawns were examined at 12 h intervals for gross disease signs and at 48 hpi, all prawns were euthanized and cephalothoraxes were processed for IIF.

The challenge procedure used to determine infectivity was used similarly to determine the LD_{50}, except that prawns (2 g) were maintained for longer (5 d). Prawns were examined at 12 h intervals for gross disease signs and to record deaths and moribund prawns (considered as dead). At 120 hpi, all surviving prawns were euthanized to process cephalothoraxes for IIF.

IHC

The cephalothoraxes of dead and euthanized prawns were fixed with Davidson's fixative for 48 h (Bell and Lightner, 1988; Lightner, 1996) sectioned longitudinally and embedded in paraffin. Sections of 5 μm were made and placed on silane-coated slides, and stained for IHC according to the procedure described by Escobedo-Bonilla *et al.*, (2007).

Sections were deparaffinized by heating at 55-60°C for 30 min and rehydrated by immersion in xylene and in gradual decreasing ethanol concentration (from 100% to 50%) and Tris buffer (pH 7.6). Endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in sodium azide (1%) and hydrogen peroxide (0.02%) in Tris buffer. Then sections were incubated with 2 μg ml^{-1} 8B7 for 1 h at 37°C. They were washed 3 times for 5 min each in Tris buffer and incubated for 1 h at 37°C with 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN 1001, Amersham Biosciences). Afterwards, they were washed 3 times in Tris buffer and incubated in streptavidine-biotinylated horseradish peroxidase complex (RPN 1051, Amersham Biosciences, UK) for 30 min at room temperature and washed 3 times again. Finally, they were incubated for 10-15 min in 0.01% of 3,3′ diaminobenzidine (D8001, Sigma Aldrich) for color development and counterstained with Gill's hemaluin, washed, dehydrated and mounted with Depex Polystyrene dissolved in xylene (DPX mountant for histology, Fluka, Biochemika, 44581, UK).

As in the study of *P. vannamei* (Rahman *et al.*, 2008), WSSV-infected cell numbers in gills, hematopoietic tissue and cuticular epithelium of stomach and body wall were quantified by light microscopy at 400× magnification. For gills and hematopoietic tissue, infected cells in 5 randomly selected fields were counted and expressed as cells mm^{-2}. For cuticular epithelium, both WSSV-infected and uninfected cells were counted in 5 fields selected at random and expressed as average percentage (%) infected cells. Differences in numbers of infected cells were tested for significance using *t*-tests.

IIF

Tissues of prawns were processed for IIF to detect WSSV using procedures described previously (Escobedo-Bonilla *et al.*, 2006). The cephalothoraxes of dead and euthanized prawns were dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at -20 °C. Cryosections of 5 µm were made and immediately fixed in absolute methanol at -20 ºC for 20 min. Sections were washed three times for 5 min each in phosphate buffered saline (PBS) and incubated for 1 h at 37°C with 2 µg ml^{-1} of monoclonal antibody 8B7 (Diagxotics, USA) against WSSV envelope protein VP28 (Poulos *et al.*, 2001). Then they were washed three times for 5 min each in PBS and incubated for 1 h at 37°C with 0.02 µg ml^{-1} of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG antibodies (F-2761 Molecular Probes, The Netherlands). Finally, they were washed in PBS, rinsed in deionised water, dried and mounted with a solution of glycerine and 1, 4-diaza-bicyclo[2,2,2]-octane (DABCO) (ACROS organics, USA). Slides were analyzed by fluorescence microscopy (Leica DM RBE). Tissues of moribund penaeid shrimp known to be infected with WSSV and uninfected shrimp were stained as positive and negative controls, respectively.

Results

WSSV pathogenesis in *M. rosenbergii*

When injected with a low dose of WSSV-Thai-1, the number of *M. rosenbergii* prawns displaying disease signs peaked at 48 hpi (all 5 prawns) and then declined, with none of the prawns displaying disease signs at 120 hpi (Table 1). Over this period, only 1 of 5 prawns became moribund at 48 hpi, and 2 of 5 prawns at 72 hpi. IHC analysis of gills, hematopoietic tissue and cuticular epithelium of stomach and body detected WSSV-infected cells in the majority of prawns sampled from 36 hpi onwards (Table 1). In the 3 prawns in which WSSV was detected at 120 hpi, infected cell numbers were lower than in prawns sampled at either 48 hpi or 72 hpi. Except for at 24 hpi ($p > 0.05$), infected cell numbers seen in organs of *M. rosenbergii* (Table 1) were not significantly different from numbers seen in comparable organs of *P. vannamei* challenged with the same dose of WSSV (Rahman *et al.*, 2008)

When injected with a high dose of WSSV-Thai-1, the number of prawns displaying disease signs peaked similarly at 48 hpi and declined thereafter very similarly to the low-dose challenge (Table 1). More moribund shrimp were evident at 36 hpi and at 48 hpi (3 of 5), 72 hpi (2 of 5) and 120 hpi (1 of 5) compared to the low dose challenge. IHC also detected WSSV-infected cells earlier (2 of 5 prawns at 24 hpi) and in all prawns sampled thereafter. Similarly to the low dose of WSSV-Thai-1, WSSV-infected cell numbers increased from 24 hpi to a maximum around 48 to 72 hpi before declining to very low levels at 120 hpi (Table 1, Fig 1A). Curiously, except for hematopoietic tissue at 48 hpi ($p < 0.05$), infected cell numbers did not differ significantly in any tissue type compared to those seen with the low dose WSSV-Thai-1 inoculum.

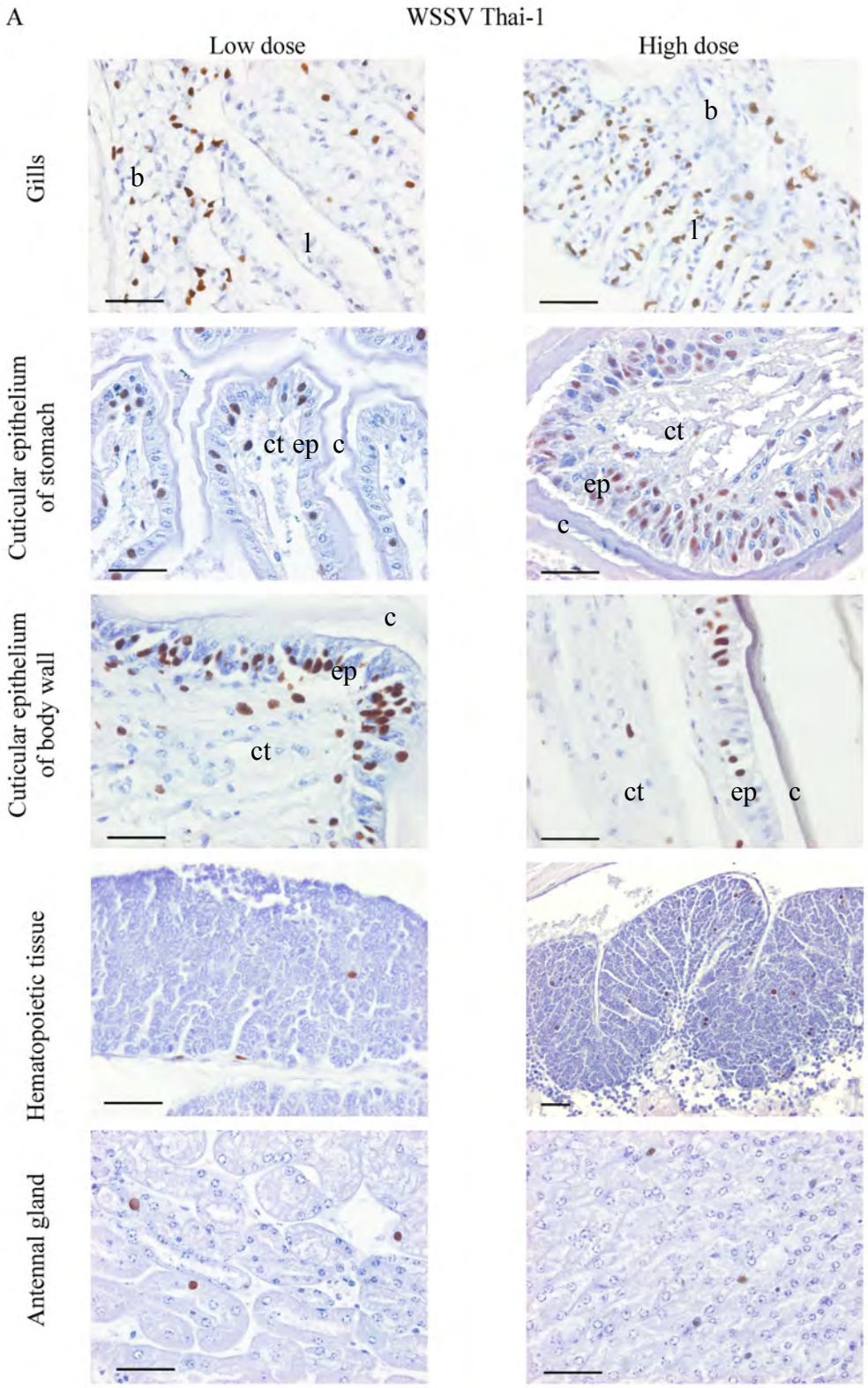
When injected with a low dose of WSSV-Viet, none of the prawns displayed gross disease signs, none died and no WSSV-infected cells were found by IHC analysis at any time point (Table 1, Fig. 1). At the high dose, however, 1 of 5 prawns showed disease signs at 24 hpi and this increased to a maximum of 4 of 5 prawns at 36 hpi and 48 hpi before declining to no prawns at 120 hpi (Table 1). Despite prawns showing disease signs, no deaths occurred prior to when prawns were sampled. WSSV-infected cells were first detected by IHC in low numbers at 36 hpi (12 hpi later than with WSSV-Thai-1) and numbers peaked at 48 hpi before declining (Table

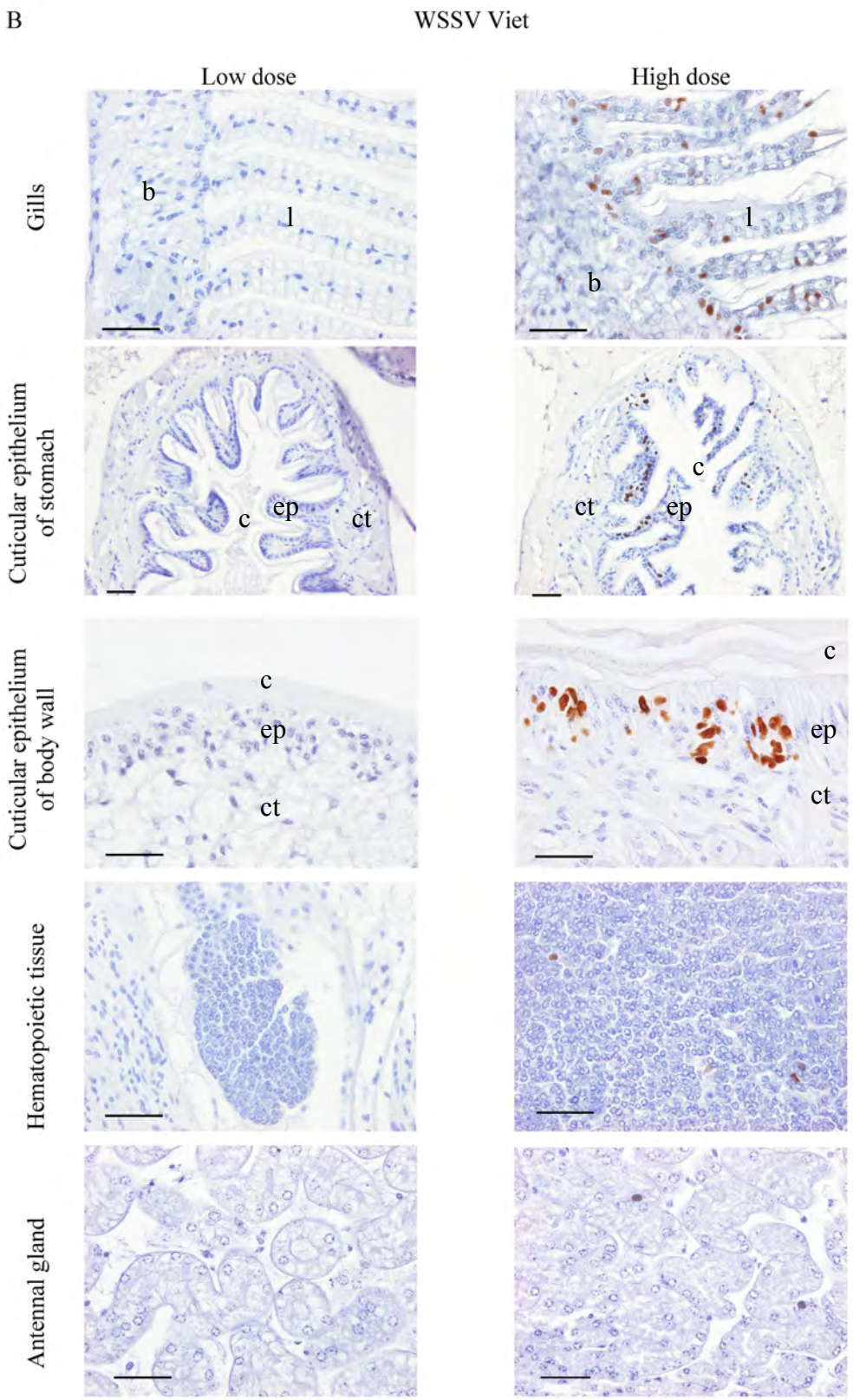
1, Fig. 1B). Infected cell numbers in gill tissues at 36 hpi (5 ± 9) and 72 hpi (18 ± 29) were significantly lower ($p < 0.05$) than those seen at these times with WSSV-Thai-1 (49 ± 32 and 157 ± 94, respectively), but at all other times there were no significant differences ($p > 0.05$) across the tissues examined.

Table 1. Immunohistochemistry quantification of infected cells in various organs of *M. rosenbergii* injected with either WSSV-Thai-1 or WSSV-Viet.

WSSV Strain	Dose	hpi	Number of prawns (Total *n* = 5)			Average number of infected cells in infected prawns			
			Disease signs	Mortality	Infected cells detected	Gills (mm^{-2})	Stomach epitheliu m (%)	Cuticular epithelium (%)	Hematopoietic tissue (mm^{-2})
Thai-1	Low	0	0	0	0	0	0	0	0
		12	0	0	0	0	0	0	0
		24	1	0	0	0	0	0	0
		36	3	0	4	39±42	2±4	12±9	23±15
		48	5	1	5	129±149	9±12	19±21	53±33
		72	3	2	5	239±203	29±13	28±14	15±16
		120	0	0	3	1±3	0.8±2	3±5	0
	High	0	0	0	0	0	0	0	0
		12	0	0	0	0	0	0	0
		24	2	0	2	7±7	0.8±0.7	0.6±0.4	2.5±0.7
		36	4	3	5	49±32	10±8	8±8	22±20
		48	5	3	5	199±270	13±11	14±13	109±23
		72	4	2	5	157±94	20±5	22±4	37±24
		120	2	1	5	3±3	5±12	6±2	8.6±12
Viet	Low	0	0	0	0	0	0	0	0
		12	0	0	0	0	0	0	0
		24	0	0	0	0	0	0	0
		36	0	0	0	0	0	0	0
		48	0	0	0	0	0	0	0
		72	0	0	0	0	0	0	0
		120	0	0	0	0	0	0	0
	High	0	0	0	0	0	0	0	0
		12	0	0	0	0	0	0	0
		24	1	0	0	0	0	0	0
		36	4	0	3	5±9	9±11	0.4±0.8	7.5±9
		48	4	0	5	53±33	21±15	30±20	43±21
		72	3	0	5	18±29	7±6.5	11±13	10±19
		120	0	0	2	15±17	9±7	2±4	21±39

Fig. 1. Photomicrographs of gills, cuticular epithelia of the stomach and body wall, hematopoietic tissues, and antennal glands of juvenile *M. rosenbergii* sampled at 48 h post-injection with either 30 SID_{50} (low dose) or 10,000 SID_{50} (high dose) of either (A) WSSV-Thai-1 or (B) WSSV-Viet. Infected cells were detected by immunohistochemistry using a VP28-specific monoclonal antibody, resulting in a brown-red colour; b = gill branch; l = gill lamella; c = cuticula; ep = epithelium; ct = connective tissue; scale bars = 50 µm.


A
WSSV Thai-1
Low dose
High dose
Gills
b
l
b
l
Cuticular epithelium of stomach
ct ep c
ct
ep
c
Cuticular epithelium of body wall
c
ep
ct
ct
ep
c
Hematopoietic tissue
Antennal gland


B
WSSV Viet
Low dose
High dose
Gills
b
l
l
b
Cuticular epithelium of stomach
c
ep
ct
c
ct
ep
Cuticular epithelium of body wall
c
ep
ct
c
ep
ct
Hematopoietic tissue
Antennal gland

Determination of the PID_{50} of the WSSV strains

Among groups of prawns injected with WSSV-Thai-1 inoculum diluted 10^{-1} to 10^{-6}, those injected with 10^{-1}, 10^{-2} and 10^{-3} dilutions began to display disease signs from 24 hpi. Based on IIF detection of WSSV infection across prawns injected with the various inoculum dilutions and sacrificed at expected peak viremia (48 hpi), the geometric mean infectious dose determined for the 3 replicate prawn groups ($10^{5.05}$, $10^{5.13}$ and $10^{5.80}$ PID_{50} ml^{-1}) was $10^{5.33\pm0.41}$ PID_{50} ml^{-1} (Table 2). Among groups of prawns injected with WSSV-Viet inoculum diluted up to 10^{-4}, all prawns injected with the undiluted and 10^{-1} diluted inoculum began to display disease signs from 24 hpi. Based on IIF detection of WSSV infection across prawns from all dilutions sacrificed at 48 hpi, the geometric mean infective titer determined from the 3 replicate groups ($10^{2.80}$, $10^{3.13}$ and $10^{3.67}$ PID_{50} ml^{-1}) was $10^{3.20\pm0.44}$ PID_{50} ml^{-1} (Table 2).

Table 2. Numbers of *M. rosenbergii* found to be infected at 48 h post-injection of 10-fold dilutions of either WSSV-Thai-1 or WSSV-Viet as determined by IIF staining.

Dilution	% prawns infected (n = 15/dilution)	
	WSSV-Thai-1	WSSV-Viet
undiluted	ND	100
10^{-1}	100	100
10^{-2}	100	40
10^{-3}	100	0
10^{-4}	53	0
10^{-5}	7	ND
10^{-6}	0	ND

ND = not done

Determination of the LD_{50} of the WSSV strains

All prawns injected with 10^{-1}, 10^{-2} and 10^{-3} dilutions of WSSV-Thai-1 began to shown gross disease signs from 24 hpi. Among prawns injected with dilutions of 10^{-4}, 10^{-5} and 10^{-6}, only those in which infected cells were evident when sampled at 120 hpi showed disease signs from 24 hpi. Except for the absence of white spot formation in cuticle, disease signs were comparable to those seen in penaeid shrimp and included anorexia, lethargy and whitening of the body. Deaths occurred from 48 hpi onwards and the LD_{50} determined when the bioassay was terminated (120 hpi) for the 3 replicate groups of prawns ($10^{5.51}$, $10^{5.14}$ and $10^{5.48}$ LD_{50} ml^{-1}) was $10^{5.38\pm0.21}$ LD_{50} ml^{-1}.

All prawns injected with undiluted and 10^{-1} diluted WSSV-Viet began to show gross disease signs from 24 hpi. Among prawns injected with the 10^{-2} dilution, only those in which infected cells were evident when sampled at 120 hpi showed disease signs from 24 hpi. Deaths occurred from 48 hpi onwards and the LD_{50} determined when the bioassay was terminated (120 hpi) for the 3 replicate groups of prawns ($10^{2.00}$, $10^{2.50}$ and $10^{2.30}$ LD_{50} ml^{-1}) was $10^{2.27\pm0.25}$ LD_{50} ml^{-1}. A reduced LD_{50} compared to PID_{50} for prawns injected with the WSSV-Viet strain was indicative of its lower relative virulence predicted from bioassays in penaeid shrimp.

Discussion

Some challenge experiments have reported juvenile and adult life stages of *M. rosenbergii* to be quite refractive to WSSV infection (Sahul Hameed et al. 2000, Waikhom et al. 2006, Yoganandhan et al. 2006). However, in the present study, with bioassays using high and low virulence strains of WSSV, juvenile (2 to 5 g) *M. rosenbergii* were found to readily support WSSV replication and succumb to disease and mortality. These data concur with alternative findings of higher infection levels and mortality occurring in earlier life stages (larvae and juveniles) than in adults (Lo et al. 1996, Peng et al. 1998, Rajendran et al. 1999, Pramod Kiran et al. 2002). While the differences in clinical outcomes with juvenile *M. rosenbergii* remain to be determined, possibilities include differences in *M. rosenbergii* age and origin, stress factors such as water temperature, and dose and virulence of the WSSV strain used. In examining WSSV strain virulence and dose factors in the bioassays reported here,

18.6-fold more WSSV-Thai-1 virus and 398-fold more WSSV-Viet virus was found to be required to establish infection in juvenile *M. rosenbergii* compared to *P. vannamei* shrimp (Escobedo-Bonilla *et al.*, 2005). These data indicate clearly that higher doses of WSSV are needed to establish infection in *M. rosenbergii* compared to shrimp, and that the WSSV strain origin can affect what dose is required for it to be capable of causing disease and mortality.

While both WSSV-Thai-1 and WSSV-Viet originated from diseased *P. monodon*, each had been passaged through different crayfish species before being passaged through SPF *P. vannamei* to prepare the inocula used to challenge juvenile *M. rosenbergii*. It is possible that passage through the different crayfish species had some role in determining the virulence of the inocula. However, as the double-stranded DNA genome of WSSV evolves quite slowly (Zwart *et al.*, 2010), virulence differences appear more likely to be inherent to each strain rather than a factor of their recent passage history.

Published bioassays with *M. rosenbergii* have used various, often poorly described conditions and water temperatures ranging between 18 and 32°C. It is quite possible that water temperature, which is known to affect WSSV replication (Rahman *et al.*, 2006), had a major impact on the clinical and virological outcome. Here the water temperature was standardized to 27°C, as this is optimal for replication of the WSSV-Thai-1 and WSSV-Viet strains in *P. vannamei* (Rahman *et al.*, 2006; 2007).

IHC detection of infected cells in cephalothorax tissues of *M. rosenbergii* showed WSSV to replicate in the same target organs as found in *P. vannamei* (Escobedo-Bonilla *et al.*, 2007; Rahman *et al.*, 2008), with the exception of the lymphoid organ for which no equivalent organ has been described in *M. rosenbergii* (P. Sithigorngul pers. comm.). Apart from the detection of infected cells being delayed from 24 to 36 hpi in *M. rosenbergii* compared to *P. vannamei* challenged with a low dose of WSSV-Thai-1, their numbers did not differ significantly across the organs examined. Indeed there were few significant differences between infected cell numbers seen in any organs at any times following challenge with either low or high doses of WSSV-Thai-1 and a high dose of WSSV-Viet. However, in contrast to this as well as observations in *P. vannamei*, no infected cells were detected in any *M. rosenbergii* challenged with a low dose of WSSV-Viet.

Similarities in infected cell numbers seen in juvenile *M. rosenbergii* challenged with high/low doses of WSSV-Thai-1 and a high dose of WSSV-Viet are confounding

considering the differences in clinical outcomes. However, fewer infected gill cells were apparent with WSSV-Viet than with WSSV-Thai-1, which supports the hypothesis that gill infection levels provide a good barometer of clinical outcomes in shrimp (Rahman *et al.*, 2008). Consistent with previous observations of a transitory viremic period in which disease signs and WSSV are readily detectable (Sahul Hameed *et al.*, 2000; Waikhom *et al.*, 2006; Yoganandhan *et al.*, 2006; Sarathi *et al.*, 2008), there was a general trend of falling numbers of infected cells in *M. rosenbergii* between 3 and 5 d post-challenge. More pronounced clearance effects appear to occur in challenged adult prawns (Sahul Hameed *et al.*, 2000, Sarathi *et al.*, 2008), and infection during the first couple of days following challenge has been tracked by immune-detection of the WSSV VP28 protein (Yoganandhan *et al.*, 2006).

The mechanism by which WSSV infection is cleared by *M. rosenbergii* remains a mystery that, if solved, could help devise strategies to protect cultured shrimp species. WSSV challenge affects levels of prophenoloxidase (proPO), superoxide anion, superoxide dismutase, total hemocyte count and clotting time, factors generally involved in antibacterial defense responses (Sarathi *et al.*, 2008). There is evidence to suggest some role for proPO in defending non-crustacean invertebrates against viruses (Shelby and Popham, 2006). However, the increases in proPO levels in hemolymph and melanized lesions of shrimp infected with Taura syndrome virus (Hasson *et al.*, 1999, Song et al. 2003) do not occur in *M. rosenbergii* infected with WSSV. No hemocytic infiltrations, encapsulations or ectopic spheroids typical of bacterial or viral infections in penaeid shrimp occur in WSSV-infected *M. rosenbergii* (Sarathi *et al.*, 2007), so direct hemocyte-mediated intervention appears unlikely.

Hemagglutinins or lectins in the hemolymph of *M. rosenbergii* might be the reason for their greater tolerance for WSSV infection compared to *P. monodon* (Pais *et al.*, 2007). However, if they are, their mode of action must be far more effective than the C-type lectins stimulated in response to WSSV infection in highly susceptible shrimp (Luo *et al.*, 2003; Ma *et al.*, 2007; 2008; Wang *et al.*, 2009; Zhao *et al.*, 2009). Moreover, while lectins may have roles in defending both vertebrates and invertebrates against viruses as well as bacteria and fungi (Wang *et al.*, 2009; Cerenius *et al.*, 2010), their function relies on their carbohydrate recognition domains (Cambi *et al.*, 2005). As none of the 5 major structural proteins of WSSV appear to be glycosylated (van Hulten *et al.*, 2002; Wei *et al.*, 2012), any direct interaction between lectins and WSSV seems unlikely.

M. rosenbergii defense against WSSV involves some mechanism that actively clears most infected cells within a few days of challenge. However, as *M. rosenbergii* that survive WSSV challenge appear to maintain low levels of virus detectable only by nested-PCR (Peng *et al.*, 1998), the clearance mechanism might be evaded or deactivated once infection loads reach levels which can persist indefinitely, in the absence of pathology.

In summary, data reported here confirm that juvenile *M. rosenbergii* have lower susceptibility to infection and more effective mechanisms for clearing infection and thus protecting themselves against disease than penaeid shrimp. These abilities were particularly evident here with a WSSV strain of lower apparent virulence. However, when challenged with a strain of higher virulence or with high doses of the low virulent strain, similar numbers of infected cells are established as in the more susceptible *P. vannamei* challenged using identical conditions. This finding clearly indicates that once some acute infection load threshold has been passed, whatever defense mechanisms are mounted by *M. rosenbergii* become swamped, and the clinical outcome of disease through to mortality progresses similarly to that in shrimp with acute infection. The dose and strain variables assessed in this study are likely to explain in part why differences in the susceptibility of juvenile *M. rosenbergii* have been reported, and highlight the importance of using well-characterized WSSV strains and standardized challenge conditions. *M. rosenbergii* and other palaemonid prawns can serve as useful model crustaceans for understanding anti-WSSV protection mechanisms and how these might be primed to protect these and cultured penaeid shrimp against disease caused by WSSV and other problematic viruses.

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CHAPTER 5

General Discussion

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1. THE CUTICLE AS BARRIER TO PREVENT ENTRY OF WSSV IN SHRIMP VIA THE WATER

The first part of this thesis focused on the barriers which can protect penaeid shrimp against WSSV entry. The limited work that has been done on the pathogenesis of WSSV showed that oral or immersion inoculations resulted in primary replication in stomach epithelium and gills (Wongteerasupaya *et al.*, 1995; Chang *et al.*, 1996; Arts *et al.*, 2007; Escobedo-Bonilla *et al.*, 2007). How the virus can reach these sites of primary replication has never been understood, as these target cells are all shielded from the outside world by their exoskeleton, the cuticle. In a lot of experimental studies, it was simply assumed that WSSV reaches these cells, and there was little to no attention to possible barriers. The fact that no studies have shown the entry site of WSSV with certainty should be seen in the broader frame of shrimp research. In fact, for all systemic shrimp viruses, neither the site of entry nor the sites of primary replication have been clearly defined (Lightner, 2011).

The cuticle of shrimp has the same function as skin in mammals and, when intact, will present a barrier against the entry of any invading virus. As in all crustaceans, the cuticle of shrimp is essentially composed of chitin, tightly-packed with chitin-binding proteins and calcium (Compère *et al.*, 2004). It forms an acellular layer covering all external surfaces of the shrimp body, and the stomach and hindgut as well. Even on the gills and in the stomach, where the cuticle is only a few micrometer thick, ultrastructural analyses have shown that there are very few openings to the surface through which virus particles could enter (Bell and Lightner, 1988; Andrews and Dillaman, 1993). The ultrastructure of the outermost thin epicuticle, on one hand, and the exo- and endocuticle, on the other, provide an impregnable shield which does not even allow water molecules to pass. The only openings in the cuticle with a diameter wide enough for virions to pass, are the antennal gland pore and excretory canals of tegumental glands. Although the detailed anatomy of the antennal and tegumental glands in shrimp have not been described (Bell and Lightner, 1988), it seems unlikely that any virus would be able to move against the outward stream of urine and tegumental secretion. Another weak spot in the armour of shrimp, where there is no cuticle, lies in the midgut. Here, the cells are only protected from the outside world by a much thinner and looser layer called the peritrophic membrane (PM). This layer is composed of chitin strands and chitin-binding proteins such as peritrophins and

intestinal mucins (Wang and Granados, 2001). Two studies have already considered the midgut as the site of entry of WSSV (Di Leonardo *et al.*, 2005; Arts *et al.*, 2007). However, as WSSV has never been described to replicate in cells of endoblastic origin, virus particles would have to pass the epithelial cells and the basement membrane. But before that, they would also have to cross the PM which shields the enterocytes from the passing gut contents (Wang and Granados, 2001; Martin *et al.*, 2006). Some insect viruses such as entomopoxviruses and baculoviruses are known to cross the PM (Mitsuhashi and Miyamoto, 2003; Hoover *et al.*, 2010). They use special enzymes for this, known as enhancins or fusolins, which digest the protein and chitin in the PM, thus creating holes through which the virions can pass (Peng *et al.*, 1999). Further research in our laboratory is under way to examine the possibility that WSSV uses a similar mechanism to enter into shrimp.

Again, in analogy with the situation in mammals, dermotropic viruses or viruses which enter the host via the skin do not cross the skin when it is intact but rather require wounds to be present (cfr. papilloma- and poxviruses) or depend on vectors to be deliverd transcutaneously (cfr. arboviruses). The involvement of a single macroscopic organism responsible for the transcuticular delivery of WSSV into shrimp is highly unlikely, as this would have been identified in shrimp farms by now (cfr. sea lice in fish). However, the role of microscopic organisms in facilitating entry of WSSV can not be ruled out. Many bacteria living on and around shrimp are facultatively pathogenic to them, and often possess the capacity to produce chitinase and proteinases capable of digesting shrimp cuticles (Hood and Meyers, 1977; Suginta *et al.*, 2000). This is a potential threat to the primary barriers of a shrimp and, under the right circumstances, could result in WSSV target cells becoming exposed.

Unlike the skin in mammals, the exoskeleton of shrimp is not renewed and repaired in a continuous manner. Instead, it is replaced periodically, and during this moulting, the new cuticle is very thin, fragile and the secreting cells send apical projections close to the surface. The pores left by the cellular extensions are not well-studied in shrimp and could potentially be large enough to allow certain virus particles to pass (Compère and Goffinet, 1987a; b), or at least they could greatly increase the chances for viruses to reach susceptible cells when superficial wounds in the thin cuticle are present. It is with this background that we hypothesised that the susceptibility of shrimp to WSSV infection changes during the moult cycle and that it can be increased by the presence of open wounds.

We started our investigations first with *per os* inoculations. Using a catheter, careful oral inoculation of a high dose of WSSV in shrimp in all stages of the moult cycle did not result in any infection. When the same dose was administered by intramuscular injection in shrimp in all moult stages, infection occurred in all subjects. This outcome showed that the digestive tract remained shielded from WSSV entry in all moult stages, both at the level of stomach cuticle and at the peritrophic membrane in the midgut.

We then went on to inoculate shrimp by immersing them in WSSV suspensions. This method of exposing the host should allow the virus to reach all external surfaces, as well as leaving a possibilitiy for the virus to be ingested, thus reaching the stomach and gut. After a first set of experiments, it became clear that despite bringing the virions in contact with all potential sites of entry, no infection was established. It was thus concluded that it was impossible for the virus to penetrate successfully through intact cuticle, either when it was fully formed in inter-moult, or still thin and weak in post-moult stages. However, when accidental wounds of the cuticle were observed, or when wounds were inflicted in a controlled way by cutting appendages, the infection did occur, but most consistently in those shrimp which had recently moulted. As the moult cycle progressed (D stages), shrimp became refractory to WSSV infection from water. It, therefore, appears that a period exists after moulting (A and B stage, less in C stage), during which WSSV has an increased chance to enter shrimp via waterborne contact route. When an *in vivo* titration of the virus stock was performed by the intramuscular injection route, no significant intrinsic differences in susceptibility to WSSV infection existed between shrimp in different moult stages. This allows to conclude that the underlying mechanism responsible for the difference in susceptibility to WSSV by waterborne route is likely to be linked to the impact of the moult process on the site of viral entry.

Possible underlying mechanisms of a moult-dependent change could be: (1) clotting time (i.e. leaving a longer window of opportunity for the virus to enter), (2) innate antiviral defense at the level of viral entry (i.e. an antiviral factor circulating in the hemolymph), (3) activated antiviral response (i.e. hemocytes involved in clotting), (4) cell structure, polarity or physiology. Concerning the first three hypotheses, it has been shown several times that the defense competence of shrimp varies during the moult cycle (Le Moullac et al., 1997; 1998; Cheng et al., 2003b; Liu et al., 2004). Although one would expect that during the critical period of moulting, evolution

would have taken care of protection of shrimp against pathogens, it is possible that some of these systems are undermined when rearing shrimp in captivity. In support of the fourth hypothesis, it is known that the epidermal cells exhibit a dramatic change in size and activity during the moult cycle (Skinner, 1985). The cytoskeleton changes and the cells metamorphose from a cubical, dormant epithelium into a high columnar, secretory epithelium with a highly increased transport through the cells, first to the bloodstream from the old cuticle when this is being resorbed (D1 stage), and subsequently towards the new cuticle in order to lay down chitin, proteins etc. (D2 stage to B stage). It is conceivable that the expanded apical surface during cuticle formation increases the chances for WSSV binding with its target cells. Moreover, the virus can (ab)use the cytoskeleton of the secretory epithelial cells to more easily start its replication, or can pass through in the direction of the basement membrane in order to reach the hemolymph circulation. The hypothesis that hemolymph coagulation could be determining in WSSV susceptibility was already supported by preliminary experiments in our laboratory (unpublished results). These tests showed that the time required for hemolymph to clot and a wound to be closed was 2 to 4 times shorter in post-moult than in pre-moult shrimp.

Overall, our findings clearly showed that open wounds in the cuticle increase the chance for a WSSV infection to become established. To our knowledge, this is the first description of a shrimp virus entering its host via wounds.

In a follow-up study (unpublished results, Tan 2008), we tried to aswer the question whether WSSV enters directly into the bloodstream of shrimp, or first needs to establish a primary replication at the site of the wound (tissue or hemocytes) prior to spreading systemically. For this, we exposed post-moult shrimp to WSSV via immersion after removal of a pleopod and screened the cells in and around the wound, the circulating hemocytes and the internal target organs of WSSV for presence of virus at different time points. The first WSSV-infected cells at the wound site were found at 24 hpi. By that time, the virus was also already detected in the gills and haematopoietic tissue. This indicates that entry of WSSV into the haemolymph occurs early and that the spread can be directly systemic. At 36 hpi, a very limited number of hemocytes was found positive for WSSV, supporting the idea that hemocytes do not play an important role in WSSV pathogenesis (Escobedo-Bonilla *et al.*, 2007).

During the course of this thesis, many attempts were made to further develop the inoculation model with WSSV entering through the cuticle. By using a dental drill

fitted with metal and diamond burrs, or an argon fluoride (ArF) ablation laser, attempts were made to remove the cuticle and expose the underlying cells (unpublished data). None of these experiments were successful in reproducibly inducing WSSV infection in all exposed animals, not even in those which had recently moulted. This shows that the virus cannot infect cuticular epithelial cells from the outside.

From the work in this thesis, it has become clear that the entry of WSSV into its host needs to be closely examined and that existing notions on inoculation models need to be revisited. For instance the role of open wounds in natural infections needs to be investigated. Shrimp are known to be cannibalistic, so it is not unlikely that some pathogens may be transmitted easily when shrimp are living in overcrowded and stressful conditions.

Sudden environmental changes, such as a drop in temperature, salinity or pH are known to induce a peak in moulting in a shrimp pond (Vijayan and Diwan, 1995). While synthesis of the new moult skin is not entirely finalized, it has a reduced barrier function, and precocious moulting could leave the shrimp more vulnerable to infections. This idea fits very well with the field observation that WSSV outbreaks often occur simultaneously over wide areas affected by sudden climatological changes (Lightner, 2011).

Focusing on maintaining a healthy, strong cuticle could be an effective strategy to reduce the transmission of WSSV and other shrimp viruses.

2. THE REDUCED SUSCEPTIBILITY OF *M. rosenbergii* TO WSSV INFECTION AND DISEASE COMPARED TO *P. vannamei*

In the second part of this thesis, the susceptibility of *M. rosenbergii* to WSSV infection and disease was investigated. WSS was first described in penaeid shrimp in 1993-1995 (Chou *et al.*, 1995; Wongteerasupaya *et al.*, 1995). Within a few years it was noticed that the situation of WSS in *M. rosenbergii* was different from that in penaeids. Several observational and experimental studies showed that the incidence of WSSV infection in *M. rosenbergii* was lower and that disease severity and mortality due to WSSV infections were lower (Chang *et al.*, 1998; Peng *et al.*, 1998; Sahul Hameed *et al.*, 2000; Bonami and Sri Widada, 2011). All these existing studies were

performed with poorly standardised and hardly reproducible methodologies and thus did not yield conclusive results. Moreover, little progress has been made to explain the mechanisms which are responsible for the observations. So far, only changes in some aspecific immune parameters (prophenoloxidase (proPO), superoxide anion, superoxide dismutase, total hemocyte count and clotting time) (Sarathi *et al.*, 2008) and an increase of C-type lectins in hemolymph have been reported (Pais *et al.*, 2007). How these parameters connect to the pathogenesis of the virus is not understood.

Our work on WSSV infections in *M. rosenbergii* showed that *M. rosenbergii* indeed possesses some degree of reduced susceptibility to WSSV infection and disease. However, the situation turned out to be more complex than what had been reported so far, as we found that, under certain conditions, the outcome of the infection was similar to that in *P. vannamei* i.e. wide-spread viral replication and acute mortality.

By using a standardised methodology, as previously used in our laboratory for penaeid shrimp (Rahman *et al.*, 2008) (the only difference was the use of fresh water instead of salt water), our study in *M. rosenbergii* confirmed that (1) 20-400x more virus was needed to establish a WSSV infection in *M. rosenbergii* than in *P. vannamei*, (2) *M. rosenbergii* showed clinical signs and mortality comparable to *P. vannamei* when the dose was high, (3) infected animals had the same WSSV target organs and similar numbers of WSSV-infected cells in time as *P. vannamei*. These last two observations indicate that if the initial defense of *M. rosenbergii* is overcome by inoculating sufficiently high virus quantity, a lethal infection with a replication rate comparable to that observed in *P. vannamei* follows.

The difference in virulence between the WSSV Thai-1 and WSSV Viet isolates in *M. rosenbergii* is similar as was observed in penaeid shrimp, with the former being more virulent than the latter. It could even be concluded that the WSSV Viet is even less virulent in *M. rosenbergii*, as relatively more virus was needed to establish infection and cause mortality. This underlines the importance of virus isolate characterisation and virus dose determination prior to performing experimental infections.

Our findings that in *M. rosenbergii* WSSV is able to replicate and to cause severe signs of disease are in accordance with most publications on the topic. However, the observation that the virus causes mortality is in sharp contrast with most published data. Since 2002, no publications have mentioned significant mortality caused by WSSV replication. Because we used quantified doses of WSSV, we can now

conclude that high doses of WSSV can overwhelm the prawn's defense system and lead to acute death.

These results shed new light on the published studies describing the clinical outcome of WSSV infections in *M. rosenbergii*, as well as on the underlying mechanism. The delayed detection of infected cells, and the reduction in infectious titer compared to *P. vannamei* both indicate that *M. rosenbergii* possesses an early defense. Additionally, the reduction in the number of infected cells 4-5 days after inoculation is a proof for a late(r) defense response.

In order to better understand the defense mechanisms, it will be necessary to differentiate the possibilities of a lack of susceptibility to the virus, or (a)specific antiviral defense. A first hypothesis is that *M. rosenbergii* has less susceptible cells or less receptors per cell than penaeid shrimp, which results in less vital organs being affected by WSSV. The receptor(s) for WSSV is currently still unknown (Li *et al.*, 2007), and only their discovery will allow to verify this concept. However, the counting of infected cells showed that, compared to those in penaeid shrimp, target organs are the same, and the number of infected cells are similar.

Another hypothesis is that *M. rosenbergii* better "tolerates" WSSV infection, allowing the virus to replicate without the development of pathology. This implies a different virus-host interaction where, for instance, the virus allows for better survival and function of infected cells than in penaeid shrimp (i.e. less cell lysis, less interference with cell metabolism). The better virus-host adaptation which apparently exists for WSSV in *M. rosenbergii* would be the result of a longer, more beneficial co-evolution of the virus and the prawns. This in the logic that over the course of evolution, parasites generally tend to allow better survival of their hosts, in the interest of their own fitness.

Closely related to this idea of tolerance is the theory that some hosts will accomodate WSSV replication, in a proces described as "viral accomodation" (Flegel, 2007). The viral accommodation theory attributes a central role to apoptosis. Either a viral-induced, massive and uncontrolled apoptosis (kakoapoptosis) will lead to the death of the host, or the viral replication is tolerated in the absence of kakoapoptosis and the host survives (Sahtout *et al.*, 2001; Flegel, 2009; Flegel and Sritunyalucksana, 2011). In these scenarios, viral-induced apoptosis is considered detrimental. This is in direct conflict with the more commonly accepted concept that apoptosis is an antiviral defense response, which is beneficial for the host. This has also been shown for

WSSV infections in shrimp where increased apoptosis levels were correlated with increased survival rates (Wang *et al.*, 2008; Wang and Zhang, 2008). In either case, no signs of increased apoptosis were noted by histopathological examination during our study. Specific detection techniques for apoptotic cells e.g. TUNEL and caspase stainings did not work and, therefore, other approaches are required to reach a conclusion in this matter.

A third hypothesis explaining the increased resistance against WSSV of *M. rosenbergii* compared to penaeid shrimp is that the freshwater prawn can mount an active defense response. This could be the inactivation of inoculated virus by a humoral factor or an immediate cellular response which is already present at the time of inoculation (innate defense). As already indicated in previous research, this could be a circulating lectin or antiviral protein (Pais *et al.*, 2007, Sarathi *et al.*, 2008). This could be complemented with specific antiviral defense which eliminates infected cells i.e. apoptosis, cytotoxic hemocytes etc. If freshwater prawns are capable of mounting an active antiviral response during WSSV infection, then evidence of classic inflammatory processes should be present. In our work, we did not encounter signs of hemocytic infiltrations, encapsulations and lymphoid organ spheroids. These hemocytic responses are typically found in chronic virus infections of shrimp (Lightner, 2011), where it is clear that the host is "fighting" against the virus. This indicates that the antiviral reponse of *M. rosenbergii* is more likely to be of humoral nature and less cellular, or it is subtle in terms of histopathological changes. The identification of the antiviral defense of *M. rosenbegii* is extra interesting, in the light of the accumulating evidence that invertebrates might possess some level of adaptive immunity. This has been described for instance in *Drosophila* (Pham *et al.*, 2007) and is referred to as "innate immunity training" (Netea *et al.*, 2011). WSSV infections in *M. rosenbegii* could be a good model to investigate this principle in a crustacean, which can open possibilities towards control strategies.

It is also important to point out that the apparent clearance of detectable WSSV replication in *M. rosenbergii* could in fact be the process of WSSV going into latency, rather than the shrimp's defense system eliminating the virus. Long-term studies with the appropriate sensitive detection methods will be necessary to differentiate these two scenarios.

In any case, it is clear that *M. rosenbegii* is less susceptible to WSSV infection and disease than penaeid shrimp, and the decreasing number of infected cells in time are

an indication that *M. rosenbegii* can mount an antiviral defense response against the virus. Seeing the low incidence of viral diseases in *M. rosenbergii* in general, the species' antiviral defense could have a broad activity against all viruses.

The importance of understanding the exact mechanism of WSSV infection in *M. rosenbergii* lies in two areas. Firstly, *M. rosenbergii* is often cultured and processed in the same areas as penaeid shrimp. It is therefore of great importance to know whether *M. rosenbergii* is shedding infectious virus during the course of infection and can present a reservoir for the virus. Secondly, *M. rosenbergii* is a promising model species for studying the antiviral defense apparently lacking or blocked by WSSV in penaeid shrimp.

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CHAPTER 6

Summary

Since its appearance in the early 90's, White Spot Syndrome Virus (WSSV) has continuously caused devastating outbreaks of penaeid shrimp mortality, from the shores of South-East Asia, all over Latin-America and more recently in all middle-eastern countries were shrimp culture had only started to expand. In all that time, only limited progress has been made in reducing the impact of the virus on shrimp production. Unlike with many of the other shrimp viruses circulating in the culture ponds, no signs of resistance against WSSV infection and disease in penaeid shrimp have been documented.

In chapter 1, shrimp aquaculture is introduced, the problem of WSSV infections is situated and the aims of this thesis are outlined. We aimed to lay the groundwork for two strategies which have the potential to stop WSSV entry. The first was to better understand the primary barrier of shrimp, their exoskeleton cuticle, and its potential role in the start of the infection. The second strategy was to look at the pathogenesis of WSSV in a non-penaeid species such as the freshwater prawn *Macrobrachium rosenbergii*, which reportedly, has a better capacity to survive WSSV challenges.

In chapter 2, an overview of the current knowledge is given, firstly on the main cultured shrimp species *Penaeus (Litopenaeus) vannamei* and *M. rosenbergii*, and secondly of WSSV.

In chapter 3, we focused on the route of infection of WSSV in penaeid shrimp, with special attention to the barrier function of the cuticle. In WSSV research so far, the entry of the virus and the protective barriers of the shrimp have been mostly overlooked. The virus is usually administered via infected tissues or water and simply assumed to have entered the host. In our work, we aimed to test whether the cuticle of the shrimp can function as a barrier, rendering shrimp non-susceptible to waterborne infection. However, as the cuticle of shrimp is periodically changing in composition and thickness during the animal's moult process, we hypothesized that the barrier function of the cuticle would vary between different stages in the moult cycle. The first aim of this study was thus to compare the susceptibility of shrimp in different moult stages to WSSV and test whether shrimp in certain stages were less susceptible to waterborne infection. The second aim was to investigate whether wounding the cuticle could increase the chances of waterborne WSSV infection.

Before we studied WSSV infection, we performed an extensive study of the moult process in our experimental animals in part 3.1. Both *P. vannamei* and *P. monodon* were microscopically examined for the aspect of their cuticle, epidermis and moulting

processes. This allowed the differentiation and characterisation of 5 major moult stages: early and late post-moult (A and B), inter-moult (C) and early and late pre-moult (D1 and D2). The total moult cycle duration for 2 g *P. vannamei* and *P. monodon* was around 5 and 6.5 days, respectively. For 15 g *P. vannamei* and *P. monodon*, this was 11 and 12 days, respectively. The relative duration of the moult stages within the cycle was A: 5-10%, B: 9-16%, C: 12-20%, D1: 28-36% and D2: 30-38%. One of the conclusions of this study was that the majority of the cycle was comprised of the pre-moult stages. In literature data, these stages had been relatively shorter in duration. Also we saw that *P. monodon* moulted less frequent than *P. vannamei*, under the given conditions. By avoiding the use of invasive techniques, we minimized the possible iatrogenic influences on the moult process. With the moult cycle in our experimental animals mapped, we possessed the necessary tools to take this important physiological factor into account during the following experiments.

In part 3.2, the impact of the moult cycle on the susceptibility of shrimp to WSSV, both by intramuscular and immersion route, was examined. The intramuscular route was investigated by performing a standard *in vivo* titration via injection in SPF *P. vannamei* in different moult stages. The resulting infection titers were similar for all moult stages, showing that no changes in internal susceptibility occur during the moult cycle. Next, to study the barrier function of the cuticle against WSSV in the water, the cuticle was damaged in some shrimp and the outcome of WSSV exposure was compared with undamaged shrimp. For this, SPF shrimp in different moult stages were immersed in sea water containing a high dose of WSSV. In a first study, juvenile *P. vannamei* of different sizes in different stages of the moult cycle were incubated in WSSV suspensions inside cell culture flasks. Five days after this exposure, it was noted that more shrimp in post-moult stage than shrimp in inter- or pre-moult stages had become infected with WSSV. The number of infected shrimp rose with age, and once shrimp reached 11 g, 100% of A-stage shrimp were infected. As accidental damage occurred inside the cell culture flasks, the study was repeated using plastic bags, both for *P. vannamei* and *P. monodon*. To confirm the role of wounds in the establishment of WSSV infection, a pleopod was cut off prior to incubation in 1 group. For both species, the cutting of a pleopod increased the infection rates in A-stage from 0-40% to 60-100%, in B-stage from 0-20% to 40-60% and in C-stage from 0-20 to 20-60%. In shrimp which had been in D1- and D2-stages at the time of inoculation inside the cell culture flasks or bags, no WSSV infection was observed. These

experiments lead us to conclude that shrimp have a higher chance to become infected with WSSV when they have recently moulted. This is the first evidence that the exoskeleton of shrimp protects against WSSV entry and that the virus may reach susceptible cells via open wounds.

In chapter 4, we examined the situation of WSSV in the freshwater prawn *M. rosenbergii*. Several findings have been published, showing that this prawn possesses a level of decreased susceptibility to WSSV infection and disease, compared to penaeid shrimp. However, some of the results in literature are conflicting and the use of unstandardized methodology renders a lot of the information inconclusive. Hence, we aimed to examine the susceptibility of *M. rosenbergii* to WSSV infection using conditions standardized for *P. vannamei*. We collected quantitative data on the infectivity, pathogenesis and pathogenicity of 2 WSSV strains (Thai-1 and Viet) in juvenile *M. rosenbergii* and compared these with data previously obtained in penaeid shrimp. *M. rosenbergii* injected with a low dose of WSSV-Thai-1 and a high dose of WSSV-Viet developed clinical pathology and numbers of infected cells within 1 to 2 days post-infection comparable to *P. vannamei*. On the other hand, a low dose of WSSV-Viet which was previously capable of causing mortality in *P. vannamei* did not result in infection in *M. rosenbergii*. About 100 times more infectious virus was needed to establish infections in *M. rosenbergii* with WSSV-Viet than with WSSV-Thai-1, and the mean prawn infectious dose 50% endpoints (PID_{50} ml^{-1}) for the respective strains were 20 to 400 times lower that the titers obtained previously in *P. vannamei*. The median lethal dose (LD_{50} ml^{-1}) determined in *M. rosenbergii* was also far higher (~1000-fold) for WSSV-Thai-1 ($10^{5.4\pm0.4}$ LD_{50} ml^{-1}) than for WSSV-Viet ($10^{2.3\pm0.3}$ LD_{50} ml^{-1}). These experiments clearly showed that juvenile *M. rosenbergii* can be infected with WSSV and that the virus can cause pathology and mortality. However, it was confirmed that the freshwater prawns are less susceptible to the infection and disease, in particular when challenged with the low virulent WSSV Viet strain.

In chapter 5, the general discussion elaborated on the main findings in this thesis and the conclusions were formulated. The findings presented in this thesis have opened the doors leading to two novel strategies to combat WSSV. The discovery that WSSV can enter shrimp via wounds urges shrimp farmers and researchers to pay more attention to the barrier function of the cuticle. The importance of environmental factors on WSSV outbreaks had already been recognised, but our findings can give new directions for improvement of cuticle quality by changes in management and nutrition. The confirmation that *M. rosenbergii* is indeed less susceptible to WSSV infection and disease than penaeid shrimp rises the hope that a successful anti-viral response can be mounted against WSSV. Once the underlying mechanism has been uncovered, this knowledge can be extrapolated to penaeid shrimp and the anti-viral defense can be improved by therapeutic measures or genetic selection.

CHAPTER 7

Nederlandse samenvatting

De laatste twee decennia worden de meeste penaeïde garnalen ("scampi") gekweekt in gevangenschap, voornamelijk in Azië en Latijns-Amerika. Vanaf het eerste verschijnen van het White Spot Syndrome Virus (WSSV) in de vroege jaren 90, heeft dit virus ieder jaar zware verliezen veroorzaakt in de garnalenkwekerijen. Sindsdien is er maar weinig vooruitgang geboekt in de bestrijding van dit virus. In tegenstelling tot vele andere garnalenvirussen, zijn er voor het WSSV geen aanwijzingen dat er zich resistentie ontwikkelt bij de penaeïde garnalen tegen infectie en ziekte. Ondanks uitgebreide inspanningen van onderzoekers en kwekers is er tot op heden nog geen afdoende bestrijdingsmethode ontwikkeld tegen het WSSV.

Hoofdstuk 1 van deze thesis introduceert de aquacultuur van garnalen, situeert de problemen met de WSSV-infecties en beschrijft de doelstellingen van deze thesis. De doelstellingen van deze thesis kaderden in de zoektocht naar twee nieuwe strategieën om het optreden van WSSV infecties te voorkomen.

De eerste strategie was gericht op een beter begrip van de primaire barrière van de garnaal, het exoskelet, en de mogelijke rol die het speelt bij de start van de infectie. Voor de tweede strategie gingen we na of bepaalde gastheren een betere capaciteit bezitten om WSSV-infecties te overleven.

In hoofdstuk 2 wordt een literatuuroverzicht gegeven van de meest gekweekte garnaalsoorten (de witpootgarnaal *Penaeus (Litopenaeus) vannamei* en de zoetwatergarnaal *Macrobrachium rosenbergii*) en van het White Spot Syndrome Virus.

In hoofdstuk 3 bestudeerden we de infectieroute van het WSSV in penaeïde garnalen met speciale aandacht voor de barrièrefunctie van het exoskelet, de cuticula. Tot dusver werd in het WSSV-onderzoek niet stilgestaan bij de mogelijk beschermende barrières bij de gastheer die het binnendringen van het virus kunnen verhinderen. Meestal wordt in experimenteel onderzoek het virus via geïnfecteerd weefsel of water toegediend aan de dieren en wordt er simpelweg aangenomen dat het virus de gastheer binnentreedt. Tijdens ons werk wilden we testen of de cuticula van de garnaal als barrière kan functioneren waardoor de garnaal niet gevoelig zou zijn voor virusinfectie via het water.

Omdat de cuticula van een garnaal echter periodiek verandert qua samenstelling en dikte gedurende de vervellingscyclus van het dier, formuleerden we de hypothese dat de barrièrefunctie van de cuticula varieert naargelang het vervellingsstadium.

De eerste doelstelling van deze studie was bijgevolg om de gevoeligheid van garnalen in verschillende vervellingsstadia te vergelijken, om te testen of bepaalde stadia minder gevoelig waren voor WSSV-infectie vanuit het water.
De tweede doelstelling was om na te gaan of wonden in de cuticula de kans op infectie met het WSSV vanuit het water konden vergroten.
Maar voor we konden overgaan tot de studie van de WSSV-infecties, waren we genoodzaakt om een uitgebreide studie van het vervellingsproces bij onze proefdieren uit te voeren.
In deel 3.1 werd bij zowel *P. vannamei* als bij *P. monodon* aan de hand van een microscopische studie het uitzicht van de cuticula, de epidermis en het vervellingsproces bekeken.
Dit liet ons toe om 5 vervellingsstadia te differentiëren en te karakteriseren: vroeg en laat post-vervelling (A en B), inter-vervelling (C) en vroeg en laat pre-vervelling (D1 en D2). De totale cyclus duurde 5 en 6,5 dagen bij respectievelijk *P. vannamei* en *P. monodon* van 2 gram en 11 en 12 dagen bij respectievelijk *P. vannamei* en *P. monodon* van 15 gram.
De verschillende stadia namen de volgende percentages van de vervellingscyclus in: A: 5-10%, B: 9-16%, C: 12-20%, D1: 28-36% en D2: 30-38%.
Een van de conclusies van deze studie was dat het grootste deel van de cyclus werd ingenomen door de pre-vervellingsstadia. Andere onderzoekers hadden deze stadia tot nu toe steeds als korter beschreven. Ook zagen wij in onze proefopstelling dat *P. monodon* minder frequent vervelde dan *P. vannamei*. Door geen invasieve technieken te gebruiken, beperkten we de risico's op iatrogene invloeden op het vervellingsproces.
In deel 3.2 werd de impact van de vervellingscyclus op de gevoeligheid van garnalen voor het WSSV geëvalueerd, zowel via intramusculaire weg als langs immersieroute. De intramusculaire route werd onderzocht aan de hand van een gestandaardiseerde *in vivo* titratie, waarbij het virus geïnjecteerd werd in SPF-*P. vannamei* tijdens de verschillende vervellingsstadia. De resulterende infectueuze titers waren gelijkaardig voor alle stadia, wat aantoont dat de interne gevoeligheid niet varieert gedurende het verloop van de vervellingscyclus. Om vervolgens de barrièrefunctie van de cuticula tegen WSSV-infecties vanuit het water te bestuderen, werd gekeken naar het resultaat na blootstelling van garnalen met een intacte cuticula tegenover dieren met een beschadigde cuticula. Hiervoor werden SPF-garnalen in verschillende vervellingsstadia ondergedompeld in zeewater dat een hoge dosis WSSV bevatte.

In een eerste studie werden jonge *P. vannamei* van verschillende groottes in de 5 vervellingsstadia blootgesteld aan het WSSV in celcultuurflessen. Vijf dagen later werd vastgesteld dat garnalen die zich op het moment van blootstelling in post-vervellingsstadia bevonden, meer kans hadden om geïnfecteerd te worden met het WSSV dan de proefdieren in de inter- en pre-vervellingsstadia. Het aantal geïnfecteerde garnalen nam toe met de leeftijd en eenmaal de garnalen 11 gram wogen, was 100% van de dieren in het A-stadium geïnfecteerd.

Vermits er accidentele schade aan de cuticula werd vastgesteld na het verblijf in de celcultuurflessen, werd de proef herhaald in plastic zakken. Daarbij werd de rol van wonden in het optreden van WSSV-infecties bevestigd door een zwempoot bij de garnalen af te snijden op het moment van blootstelling.

Voor beide garnaalsoorten noteerden we dat het afsnijden van een zwempoot het percentage geïnfecteerde garnalen in het A-stadium deed toenemen van 0-40% naar 60-100%, in het B-stadium van 0-20% naar 40-60% en in het C-stadium van 0-20% naar 20-60%. Bij de garnalen die zich in het D1- en D2-stadium bevonden op het moment van inoculatie in de celcultuurflessen of de plastic zakken, werd nooit WSSV- infectie vastgesteld.

Het besluit van deze experimenten was dat de kans op WSSV-infectie bij garnalen groter was wanneer de dieren recent verveld waren.

Deze studie is het eerste bewijs dat het exoskelet van garnalen een bescherming biedt tegen het binnendringen van het WSSV en dat het virus gevoelige cellen kan bereiken via open wonden.

In hoofdstuk 4 onderzochten we de situatie van het WSSV in de zoetwatergarnaal *M. rosenbergii*. Een aantal publicaties wijzen erop dat deze diersoort minder gevoelig is voor infectie en ziekte veroorzaakt door het WSSV in vergelijking met penaeïde garnalen.

De informatie in deze literatuur bevat echter tegenstrijdigheden en de methodes die gebruikt werden, laten niet toe om met zekerheid conclusies te trekken. Vandaar dat het ons doel was om de gevoeligheid van *M. rosenbergii* ten opzichte van het WSSV te bestuderen met dezelfde, gestandaardiseerde methodologie waarmee dit eerder in ons labo werd gedaan voor *P. vannamei*.

In deze studie verzamelden we kwantitatieve data betreffende de infectiviteit, pathogenese en pathogeniciteit van 2 WSSV-isolaten (Thai-1 and Viet) in juveniele *M. rosenbergii* en vergeleken we deze data met wat we eerder geobserveerd hadden in

penaeïde garnalen. Wanneer *M. rosenbergii* geïnjecteerd werden met een lage dosis WSSV-Thai-1 en een hoge dosis WSSV-Viet, ontwikkelde er zich klinische pathologie en waren de aantallen geïnfecteerde cellen gelijkaardig aan deze in *P. vannamei* binnen de eerste 2 dagen na de inoculatie. Dit stond in contrast met de resultaten na inoculatie met een lage dosis WSSV-Viet, want waar deze dosis eerder in *P. vannamei* sterfte had veroorzaakt, werd er geen infectie gedetecteerd in *M. rosenbergii*. Er was ongeveer 100 maal meer infectieus WSSV nodig om een infectie tot stand te brengen in *M. rosenbergii* met WSSV-Viet dan met WSSV-Thai-1 en de "mean prawn infectious dose 50% endpoints" (PID_{50} ml^{-1}) voor de respectievelijke isolaten waren 20 tot 400 maal lager dan de titers die voordien bekomen waren in *P. vannamei*. De "median lethal dose" (LD_{50} ml^{-1}) bekomen in *M. rosenbergii* was ook veel hoger (~1000 maal) voor WSSV-Thai-1 ($10^{5.4\pm0.4}$ LD_{50} ml^{-1}) dan voor WSSV-Viet ($10^{2.3\pm0.3}$ LD_{50} ml^{-1}).

Deze experimenten toonden duidelijk aan dat jonge *M. rosenbergii* geïnfecteerd kunnen worden door het WSSV en dat het virus wel degelijk pathologie en sterfte kan veroorzaken. Er werd echter ook duidelijk bevestigd dat de zoetwatergarnalen minder gevoelig zijn aan infectie en ziekte, iets wat vooral duidelijk was in het geval van WSSV-isolaten met lage virulentie.

In hoofdstuk 5 wordt dieper ingegaan op de belangrijkste bevindingen in deze thesis en worden de conclusies geformuleerd. De resultaten van deze thesis openen perspectieven op twee nieuwe strategieën om het WSSV te bestrijden. De ontdekking dat het WSSV een gastheer kan binnentreden via wonden is een aanzet voor kwekers en onderzoekers om meer aandacht te besteden aan de barrièrefunctie van de cuticula van garnalen. Het belang van omgevingsomstandigheden bij de WSSV-uitbraken was reeds erkend maar onze bevindingen kunnen een aanzet zijn voor verbeteringen in de cuticulakwaliteit door middel van specifieke ingrepen in het management en de voeding van de dieren. De definitieve bevestiging dat *M. rosenbergii* daadwerkelijk minder gevoelig is voor WSSV-infecties en ziekte doet de hoop rijzen dat een succesvolle antivirale afweerreactie tegen het WSSV mogelijk is.

Eenmaal de onderliggende mechanismen verantwoordelijk voor deze afweer gekend zullen zijn, kan deze kennis geëxtrapoleerd worden naar penaeïde garnalen om ook hun antivirale afweer te verbeteren door therapeutisch ingrijpen of door genetische selectie.

Curriculum vitae

Mathias Corteel was born on 20th of October 1981 in Borgerhout, Antwerp.
He obtained the degree of veterinarian with distinction in 2005 at Ghent University. During his senior year, he followed aquaculture-related subjects and wrote his msc thesis titled: "Pathogenesis of two White Spot Syndrome Virus (WSSV) strains after oral inoculation in the shrimp *Litopenaeus vannamei*."
Subsequently, he started his doctoral studies under the supervision of Prof Dr Hans Nauwynck at the Laboratory of Virology of the Faculty of Veterinary Medicine on the continuation of the research of his master thesis. In 2006 he obtained a 4-year scholarship from the Institute for the Promotion of Innovation through Science and Technology in Flanders (nr. 53534, IWT-Vlaanderen, Belgium), after which he was employed for 2 years as research assistant.
Since September 2012, he works for INVE Aquaculture in Dendermonde as Research and Development Engineer Health.

Mathias Corteel is author or co-author of 12 scientific publications and two book chapters. He supervised 11 master students and attended several international conferences and symposia.

Publications

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Proceedings

Abstracts

Escobedo-Bonilla CM, Rahman MM, **Corteel M**, Dantas Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2006) Effect of high water temperature on the virological and clinical outcome of experimental infections with white spot syndrome virus (WSSV) in specific pathogen free (SPF) *Litopenaeus vannamei*. Aqua 2006, May 9-13, Firenze, Italy

Rahman MM, Escobedo-Bonilla CM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2006) Evaluation of virulence of three white spot syndrome virus (WSSV) strains by intramuscular inoculation in specific pathogen free shrimp *Litopenaeus vannamei*. Aqua 2006, May 9-13, Firenze, Italy

Rahman MM, **Corteel M**, Dantas-Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2007) Daily fluctutuations of water temperature (27°C and 33°C) affect clinical and virological outcome of White Spot Syndrome Virus (WSSV) infection in *Penaeus vannamei* juveniles. Asian-Pacific Aquaculture 2007, August 5-8, Hanoi, Vietnam, p.47

Rahman MM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2007) Degree of replication in gills may be associated with virulence of white spot syndrome virus (WSSV) strains in infected *Penaeus vannamei* juveniles. Asian-Pacific Aquaculture, August 5-8, Hanoi, Vietnam, p. 242

Rahman MM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2007) Efficacy of high water temperature (33°C) to prevent disease and reduce mortality in *Penaeus vannamei* juveniles infected white spot syndrome virus (WSSV) depends on progression of infection. Asian-Pacific Aquaculture, August 5-8, Hanoi, Vietnam, p. 243

Scientific communications

Oral presentations (*presenting author)

Escobedo-Bonilla CM, Rahman* MM, **Corteel M**, Dantas-Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2006) Effect of high water temperature on the virological and clinical outcome of experimental infections with white spot syndrome virus (WSSV) in specific pathogen free (SPF) *Litopenaeus vannamei*. Aqua 2006, May 9-13, Firenze, Italy

Rahman MM, **Corteel* M**, Dantas-Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2007) Daily fluctutuations of water temperature (27°C and 33°C) affect clinical and virological outcome of White Spot Syndrome Virus (WSSV) infection in *Penaeus vannamei* juveniles. Asian-Pacific Aquaculture 2007, August 5-8, Hanoi, Vietnam

Rahman* MM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2007) Degree of replication in gills may be associated with virulence of white spot syndrome virus (WSSV) strains in infected *Penaeus vannamei* juveniles. Asian-Pacific Aquaculture, August 5-8, Hanoi, Vietnam

Rahman* MM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2007) Efficacy of high water temperature (33 °C) to prevent disease and reduce mortality in *Penaeus vannamei* juveniles infected white spot syndrome virus (WSSV) depends on progression of infection. Asian-Pacific Aquaculture, August 5-8, Hanoi, Vietnam

Poster presentations

Rahman MM, Escobedo-Bonilla CM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2006) Evaluation of virulence of three white spot syndrome virus (WSSV) strains by intramuscular inoculation in specific pathogen free shrimp *Litopenaeus vannamei*. Aqua 2006, May 9-13, Firenze, Italy

Rahman MM, Escobedo-Bonilla CM, **Corteel M**, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2006) Evaluation of virulence of three white spot syndrome virus (WSSV) strains by intramuscular inoculation in specific pathogen free shrimp *Litopenaeus vannamei*. Annual meeting of BSM, November 24, Brussel, Belgium

Corteel M, Dantas-Lima JJ, Wille M, Alday-Sanz V, Pensaert MB, Sorgeloos P, Nauwynck HJ (2008) Molt stage and cuticle damage influence White Spot Syndrome Virus infection in penaeid shrimp. Fish & Shellfish Immunology Workshop, June 22-27, Wageningen University, The Netherlands

Book chapters

Corteel M, Nauwynck HJ (2010) Chapter 4, The integument of shrimp: cuticle and its moult cycle. In: Alday-Sanz V (Ed) The shrimp book. Nottingham University Press, UK

Cuéllar-Anjel J, **Corteel M**, Galli L, Alday-Sanz V, Hasson KW (2010) Chapter 22, Principal shrimp infectious diseases, diagnosis and management. In: Alday-Sanz V (Ed) The shrimp book. Nottingham University Press, UK

Not peer-reviewed

Corteel M, Dantas-Lima JJ, Nauwynck HJ, Pensaert MB, Wille M, Sorgeloos P, Alday-Sanz V, Decamp O (2010) A standardised white spot disease challenge test to evaluate the efficacy of a biocide. Aquaculture AsiaPacific Magazine 6:31-33

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