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EXPERIMENTS ON THE EFFECT OF TEMPERATURE ON WHITE SPOT SYNDROME VIRUS INFECTION IN *LITOPENAEUS VANNAMEI* SHRIMP

EXPERIMENTEN OP HET EFFECT VAN TEMPERATUUR OP WHITE SPOT SYNDROME VIRUS INFECTIE IN *LITOPENAEUS VANNAMEI* GARNALEN

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ABSTRACT

White Spot Disease (WSD) is an aggressive and devastating viral disease caused by the White Spot Syndrome Virus (WSSV). This highly pathogenic and widespread disease, present throughout Asia and the Americas, can cause up to 100% mortality within 3-7 days after infection. It is annually responsible for huge ecological and economical losses in the main producing countries and forms as such one of the greatest threats for the further sustainable development of shrimp aquaculture.

Previous research showed that manipulation of physical factors gave promising results: manipulation of the environmental factors such as temperature produced the most interesting and promising results. For this thesis three experiments were performed, all in which pacific white shrimp (*Litopenaeus vannamei*) were intramuscularly inoculated with a well-defined viral dose (30 and/or 10000 SID_{50}) and exposed to high water temperature via standardised protocols. The first experiment looked at the efficacy of elevated temperature for protecting shrimp against WSSV. Practically, four temperature treatments in which an elevated temperature (33 °C) was either applied before virus inoculation, after the inoculation, both before and after inoculation, and in the fourth treatment a low temperature (27ºC) was used throughout the test. In the second series of experiments the protective value of high temperature after an initial period of viral replication was evaluated. Water temperature was raised from 27ºC to 33ºC at 0, 12 or 24 hours post WSSV inoculation. Maintaining and controlling such high water temperatures for longer periods of time is of course very unpractical in field conditions and probably economically unfeasible, so the third experiment evaluated the effectiveness of shorter cyclic exposure periods to high water temperature. Hence, the shrimp were exposed to daily temperature cycles (33ºC/27ºC) with 6, 12 and 18 hours of high water temperature, during five consecutive days.

Experiment 1 demonstrated a total blocking of disease progression when hyperthermia was applied immediately post inoculation. The protection was very effective even with a high viral dose (10000 SID_{50}). The second experiment, at a low viral dose (30 SID_{50}), showed that high temperature to some extent also worked therapeutic in that previously 24 hours of virus replication could be allowed. At a high infection dose (10000 SID_{50}) the level of protection was however not so effective. In Experiment 3, only a minimum of 18 hours at 33°C resulted in a significant lower

mortality with the infected shrimp. The results from all the experiments clearly show the potential of high water temperature for preventing mortality in WSSV infected shrimp.

ABSTRACT

White Spot Disease (WSD) is een uiterst agressieve en letale virale aandoening die veroorzaakt wordt door het White Spot Syndrome Virus (WSSV). Deze zeer infectueuze en wijdverspreide ziekte (Azië en Amerika), kan binnen 3 tot 7 dagen na infectie tot 100% mortaliteit leiden. Het is jaarlijks verantwoordelijk voor reusachtige ecologische en economische verliezen in de producerende landen en vormt dus één van de grootste bedreigingen voor de verdere duurzame ontwikkeling van garnalenkweek.

Vorige onderzoeken toonden reeds aan dat de manipulatie van fysische variabelen tot veelbelovende resultaten kon leiden: zo bleek temperatuur één van de meest veelbelovende te zijn. Voor deze thesis werden drie experimenten uitgevoerd, allen met *Litopenaeus vannamei,* die intramusculair geïnoculeerd werden met een welbepaalde virale dosis (30 en/of 10000 SID_{50}) en vervolgens blootgesteld werden aan een verhoogde watertemperatuur volgens gestandardiseerde protocols. Het eerste experiment bekeek het beschermend effect van de temperatuursverhoging tegen WSSV. De hoge temperatuur (33 °C) werd hierbij toegepast ofwel vóór de inenting met het virus, ofwel na de inenting, zowel vóór en na de inenting en in de vierde behandeling werd een lage temperatuur (27ºC) gebruikt gedurende geheel de test. In de tweede experimentenreeks kon het virus zich eerst gedurende een bepaalde periode vermenigvuldigen (0, 12 of 24 uur na inoculatie), vooraleer de temperatuursverhoging (van 27°C naar 33 °C) uitgevoerd werd. In de praktijk is het handhaven en controleren van dergelijke hoge watertemperaturen gedurende langere tijdspannes onpraktisch en waarschijnlijk economisch onhaalbaar, zodat het derde experiment opgezet werd om de doeltreffendheid van kortere cyclische periodes van blootstelling aan een verhoogde watertemperatuur uit te testen. Hiertoe werden de garnalen onderworpen aan dagelijkse temperatuurscycli (33ºC/27ºC) van 6, 12 en 18 uur blootstelling aan de verhoogde watertemperatuur, en dit gedurende vijf opeenvolgende dagen.

Experiment 1 toonde aan dat een continue verhoogde temperatuur onmiddellijk na de virale inoculatie voor een heel efficiënte bescherming zorgt, zelfs bij inentingen met een hoge virale dosis (10000 SID_{50}). Het tweede experiment toont aan dat de verhoogde temperatuur bij een lage virale dosis (30 SID_{50}) zelfs een zekere curatieve werking heeft, in die zin dat het virus zich initieel tot 24 uur mag vermenigvuldigen. Bij een hoge dosis (10000 SID_{50}) is de mate van bescherming echter niet zo efficiënt. In

Experiment 3, is er slechts één behandeling die resulteert in een significant lagere mortaliteit, namelijk de blootstelling aan 33°C gedurende 18 uur per dag. De resultaten van alle experimenten tonen duidelijk het potentieel van een verhoogde watertemperatuur ter bestrijding van WSSV besmetting.

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CHAPTER 1 – INTRODUCTION

White spot syndrome virus (WSSV) causes an aggressive and devastating disease (white spot disease, WSD) in shrimp farms throughout Asia, North and South America. Mortalities of 100% can occur within 3-10 days after the onset of disease in grow-out operations. White spot disease constitutes a huge ecological and economical threat for the development of shrimp fisheries and aquaculture. First recorded in Taiwan in 1992 (Chou *et al*., 1995), it has spread to several shrimp-farming countries in Asia and Latin America (Wang *et al*., 2000). The disease is characterised by the presence of white spots on the inner surface of the exoskeleton from which the disease name is derived (Lo *et al.,* 1996). Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Otta *et al*., 1999). WSSV is an enveloped, non-occluded bacilli-form-shaped virus containing a double-stranded DNA.

Since the outbreak of white spot disease, shrimp production has decreased significantly in many countries and farmers are facing serious difficulties in continuing production. The resulting economic losses and their impacts are now significantly affecting national economies and the livelihoods of shrimp farmers. Provision of assistance for combating this situation is considered highly appropriate and timely. Such assistance will help secure shrimp aquaculture development, national income through trade (both local and international), and livelihoods of farmers and other service providers (FAO, 2003).

In order to face this serious problem, the scientific community promptly answered to gather knowledge on this specific viral disease. Also a considerable number of measures to control WSSV were tested; however with little conclusive results and limited applicability. Of those, temperature manipulation for controlling this specific pathogen appears to be one of the most promissory and potentially applicable in the field.

Temperature is one of the most important environmental factors because it can affect an aquatic animals metabolism, oxygen consumption, growth rate, moult cycle, and survival rate directly. Temperature can also affect aquatic animals indirectly when combined with other environmental factors such as salinity and dissolved oxygen. Moreover temperature can have an impact on the development of pathogens and thus

disease in aquatic animals. Studies on the interaction between temperature and crustacean pathogens are however limited. So far there are only three reports on the effect of temperature on WSSV infection in crustaceans (Vidal *et al*., 2001 and Guan *et al*., 2003 in penaeid shrimp and Jiravanichpaisal *et al*., 2004 in freshwater crayfish).

The aim of this study was to evaluate the effect of high water temperature (33ºC) on survival of WSSV-infected shrimp (*Litopenaeus vannamei*), using a highly standardised challenge procedure, with a known infectious dose of white spot syndrome virus.

Three experiments were performed. The first experiment aimed to confirm the effectiveness of high water temperature for protecting shrimp against WSSV. For that, four temperature treatments were compared, in which high temperature was applied both before and after the virus inoculation, only before inoculation or only after the inoculation. In a last treatment low temperature (27ºC) was maintained all the time. In the second experiment the objective was to evaluate the protective value of high water temperature, after an initial period of viral replication. The water temperature was raised from 27ºC to 33ºC at different time points, at 12 and 24h after virus inoculation. Knowing that in field conditions, keeping high water temperature for long periods of time will probably be economically unfeasible, the third experiment evaluated the effectiveness of shorter periods of high water temperature exposure. The shrimp were submitted to daily temperature cycles (33ºC/27ºC) with 6, 12 and 18 hours of high water temperature exposure, during five consecutive days.

CHAPTER 2 – LITERATURE REVIEW

2.1 – Global aquaculture production

According to FAO statistics from 2004, the contribution of aquaculture to global aquatic production continues to grow, increasing from 3.9 percent of total aquatic production by weight in 1970 to 29.9 percent in 2002. Aquaculture continues to grow more rapidly than all other animal food-producing sectors. Worldwide, the sector has grown at an average rate of 8.9 percent per year since 1970, compared with only 1.2 percent for capture fisheries and 2.8 percent for terrestrial farmed meat-production systems over the same period. In 2002, total world aquaculture production (including aquatic plants) was reported to be 51.4 million tonnes (Fig. 1) by quantity and US$ 60.0 billion by value. This represents an annual increase of 6.1 and 2.9 percent in quantity and value respectively, over reported figures for 2000.

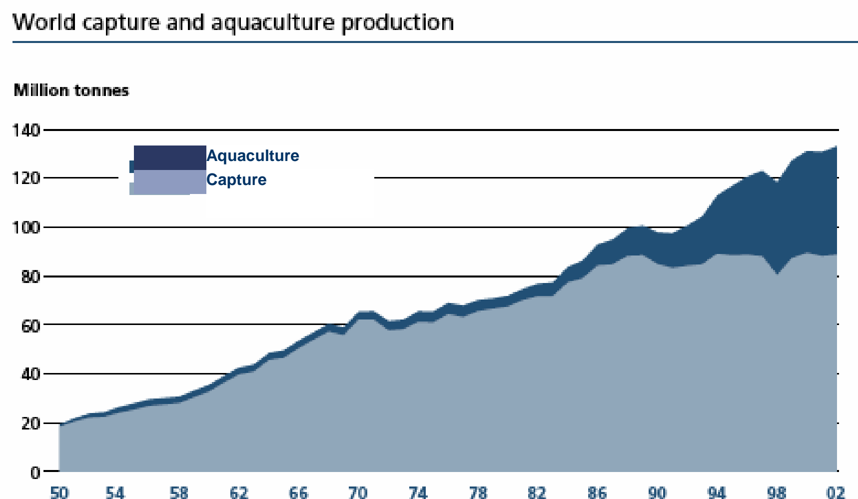

Fig. 1 - Global capture fisheries and aquaculture production data show the increasing importance of aquaculture in the annual global aquatic production (FAO, 2004).

According to data published by FAO (2002), increases in world aquaculture production will be driven by increases in Chinese production, with South Asia, Latin America and the Caribbean and Europe providing smaller increases. Freshwater species and molluscs will dominate aquaculture production. In order to meet growing projected consumption needs in Europe, total production increases in volume are estimated to result primarily from increases in aquaculture production. Indeed, the model estimates

that farmed production will likely double by 2030, exceeding 2.5 million tonnes in 2015 and reaching 4 million tonnes in 2030.

2.1.1 – Global shrimp production

Although cultured crustaceans represented only 5.4 percent of total aquaculture production by weight, they comprised 20.1 percent of total global aquaculture by value in 2002. One of the fastest growing aquaculture production sectors is that of penaeid shrimp. Within this family, the main cultivated species are the giant tiger prawn (*Penaeus monodon*), the fleshy prawn (*Fenneropenaeus chinensis*) and the whiteleg shrimp (*Litopenaeus vannamei*), these three species accounting for over 86% of total shrimp aquaculture production in 2000. Despite being affected by serious disease outbreaks in both Latin America and Asia, the annual rate of growth of the cultured shrimp sector was 6.8 percent (by weight) between 1999 and 2000. Although this had dropped to 0.9 percent during 2002, these growth rates are still relative high compared to other food producing sectors.

In recent years, *Litopenaeus vannamei* has become the leading farm-raised species, representing more than half of the total world production (Fig. 2) (FAO Fishstat database3, 2003). Since a few years China has shifted production towards *L. vannamei*, producing more than 270 000 metric tonnes in 2002 and an estimated 300 000 metric tonnes (71 percent of the country's total shrimp production) in 2003, which is higher than the current production of the whole of the Americas. Other Asian countries with developing industries for this species include Thailand (120 000 metric tonnes estimated production for 2003), Viet Nam and Indonesia (30 000 metric tonnes estimated for 2003 each). Total production of *L. vannamei* in Asia was approximately 316 000 metric tonnes in 2002, and it has been estimated that this has increased to nearly 500 000 metric tonnes in 2003, which is worth approximately US$ 4 billion in terms of export income (FAO, 2004).

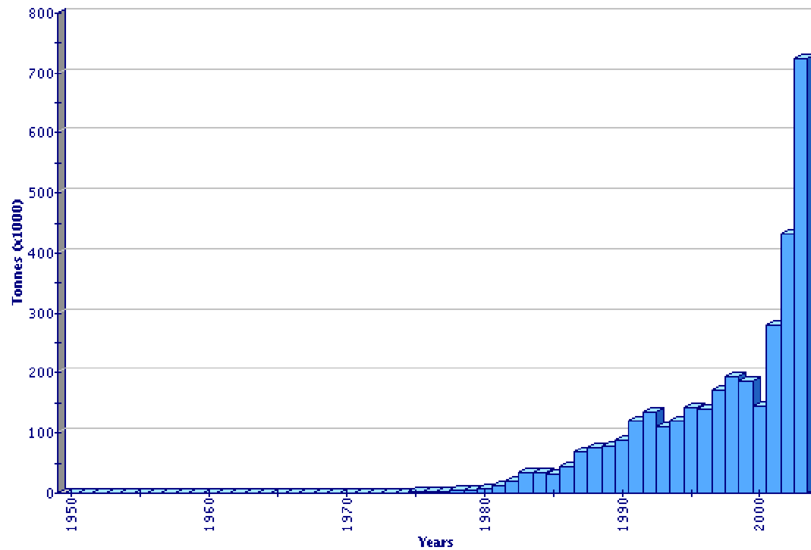

Fig. 2 – Global aquaculture production of *Litopenaeus vannamei* (FAO Fishstat database3, 2003).

2.2 – Penaeid shrimp biology

2.2.1 – Habitat and geographical distribution

Penaeid shrimp can be found in tropical and subtropical waters around the world, from about 40°N to 40°S latitude. Adult shrimp are rarely found below 180 m and typically inhabit off-shore waters, while juveniles generally occur in protected coastal habitats (Bailey-Brock & Moss, 1992).

Litopenaeus vannamei, is native from the pacific coast of America, from Mexico to Peru (Fig. 3), in areas where water temperatures are normally over 20ºC throughout the year (Rosenberry, 2004). This marine shrimp likes muddy bottoms at depths from the shoreline down to about 72 meters (Dore and Frimodt, 1987). It is not currently known whether there is one population or if isolated populations exist, although there appear to be differences between stocks from various areas under culture conditions.

Fig. 3 – Geographical distribution of *Litopenaeus vannamei*

2.2.2 –Taxonomy

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. This group of animals is characterised by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up of 42.000, predominantly aquatic species that belong to 10 classes. Within the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda. Within the suborder Dendrobranchiata, the penaeid shrimp, together with gamba prawns, gamba shrimps, benthesicymid shrimps, rock shrimps and solenocerid shrimps are included in the Superfamily Penaeoidae. The family of the Penaeidae (penaeid shrimp) contains apart from *Litopenaeus vannamei* many important farmed species such as *Penaeus monodon*, *Litopenaeus stylirostris*, *Marsupenaeus japonicus*, *Fenneropenaeus indicus* and *Fenneropenaeus chinensis*.

Domain = Eucarya
Kingdom = Animalia
Phylum = Anthropoda
Subphylum = Crustacea
Class = Malacostraca
Subclass = Eumalacostraca
Superorder = Eucarida
Order = Decapoda
Suborder = Dendrobranchiata
Super family = Penaeoidea
Family = Penaeidae
Genus = *Litopenaeus*
Species = *vannamei*

Fig. 4 – Taxonomic classification of *Litopenaeus vannamei*

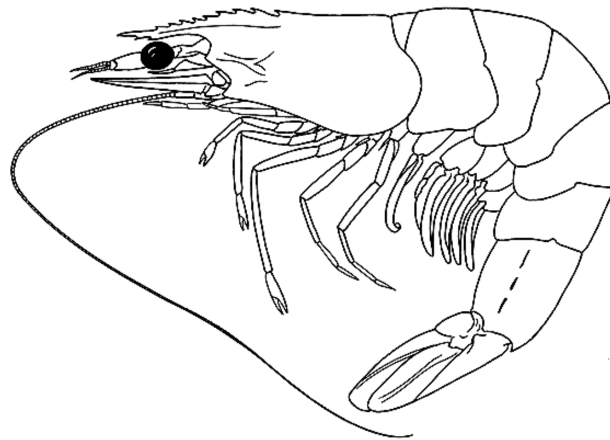

Fig. 5 – Drawing of *Litopenaeus vannamei*

Recently, Pérez Farfante and Kensley (1997) revised the taxonomic classification into genera within the family Penaeidae. The changes for the most important farmed species are shown in the table below.

Previous	Actual
Penaeus stylirostris	*Litopenaeus stylirostris*
Penaeus vannamei	*Litopenaeus vannamei*
Penaeus japonicus	*Marsupenaeus japonicus*
Penaeus indicus	*Fenneropenaeus indicus*
Penaeus penicillatus	*Fenneropenaeus penicillatus*
Penaeus chinensis	*Fenneropenaeus chinensis*
Penaeus monodon	*Penaeus monodon*

Fig. 6 – Taxonomic changes in important cultured shrimp species (Pérez Farfante & Kensley, 1997)

2.2.3 – Morphology

2.2.3.1 – External morphology

Like the other decapod crustaceans, shrimp are bilateral symmetric. The body is protected by an exoskeleton and divided into two regions: the cephalothorax (one unique piece) and the abdomen (several articulated pieces). They are covered by a chitin skeleton more or less calcified (calcium carbonate). This organ is flexible in the abdomen articulation for allowing movement (Morales, 1991). In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae form the jaw-like structures that are involved in food uptake (Solis, 1988). Appendages of the cephalothorax vary in appearance and function. The maxillipeds are the first three pairs of appendages, modified for food handling and the remaining five pairs are the walking legs (pereopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Bell and Lightener, 1988; Baily-Brock and Moss, 1992).

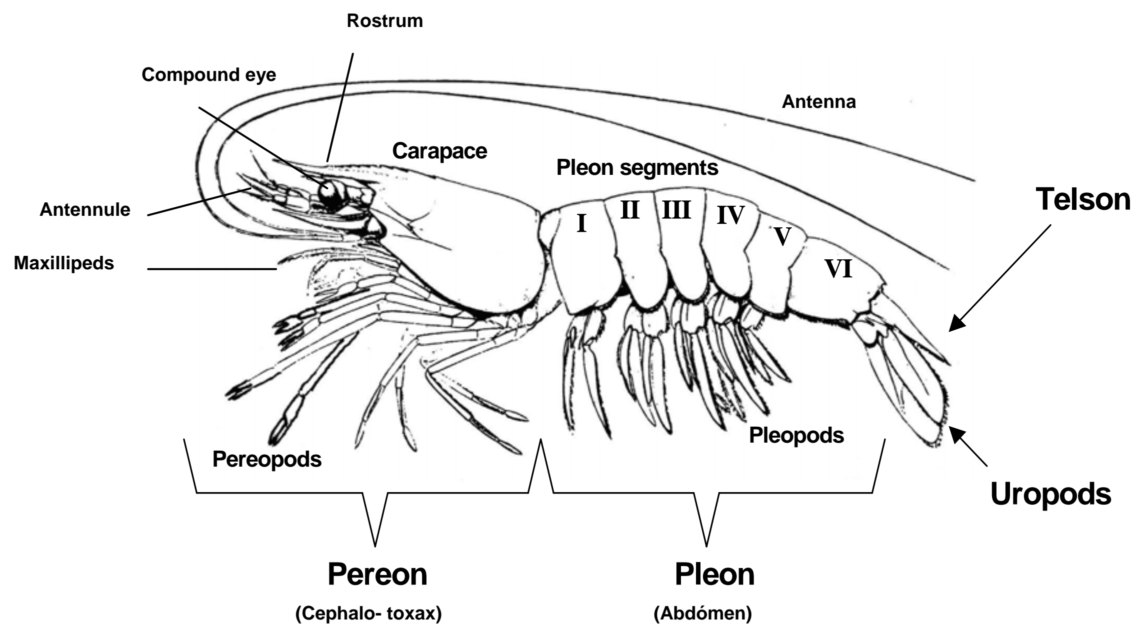

Fig. 7 - External morphology of shrimp. The body organization of decapod crustaceans is divided into tagmata or specialized regions. These are the pereon, (head and main internal organs), pleon (highly muscularized and specialized for swimming) and telson, or reminiscent tail-like structure. Each tagma possesses specialized appendages, either for feeding and crawling (pereopods) or for swimming and ventilation (pleopods). The uropods of the tail fan are used for escape propulsion.

2.2.3.2 – Internal morphology

The internal morphology of the penaeid shrimp is outlined in Figure 7. Penaeids and other arthropods have an open circulatory system and, therefore, the blood and the bloodcells are called haemolymph and haemocytes, respectively. The open spaces in the body are the haemocoel and contain haemolymph. Crustaceans have a muscular heart that is dorsally located in the posterior cephalothorax. It is short and wide, and tapering anteriorly and posteriorly. The blood is pumped by the heart through a complex array of arteries to the haemocoel. The valved haemolymph vessels leave the heart and branch several times before the haemolymph arrives at the sinuses that are scattered throughout the body, where exchange of substances takes place. After passing the gills, the haemolymph returns in the heart by means of three wide non-valved openings. The haemocytes are produced in the haematopoietic tissue. This organ is dispersed in the cephalothorax, but mainly present around the stomach and in the onset of the maxillipeds (Bauchau, 1981; Fox, 2001).

The digestive system is divided into a complex, cuticle-lined foregut region; a compact digestive (or midgut) gland at the beginning of the midgut region, followed by a long tubular, simple part; and a cuticle-lined hindgut region, consisting principally of the rectum (Dall, 1967). The stomach and oesophagus are part of the foregut. The stomach is, by divisions, composed of a cardiac and a pyloric region. In the cardiac stomach the cuticle is elaborated to form a complex and intricate gastric mill to grind food. Posterior to the cardiac stomach is located a smaller stomach region, the pyloric stomach which contains a sieve, or filter press, made of cuticular setae (Fox, 2001). In the midgut the hepatopancreas is located. This digestive gland consists of diverticula of the intestine. The spaces between these hepatopancreatic tubules are occupied by haemolymph sinuses. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980).

The reproductive system in crustaceans is the following. The male has two pairs of modified abdominal appendages on the first and second abdominal segments (the petasma) that deliver sperm to the female's external receptacle (the thelycum) located between the bases of the fifth walking legs.. The gonads (ovaries and testes) are paired tubular structures in the cephalothorax that connect to the exterior by the external sexual

appendages (thelycum and petasma) via paired gonoducts (oviducts and vasa deferentia). (Bailey-Brock & Moss, 1992).

The decapod excretory organs are a pair of antennal glands located at the base of the head leading by a duct to the nephridiopores on the second antenna. The antennal gland is a small white pad of tissue just anterior to and lateral to the oesophagus (Fox, 2001)

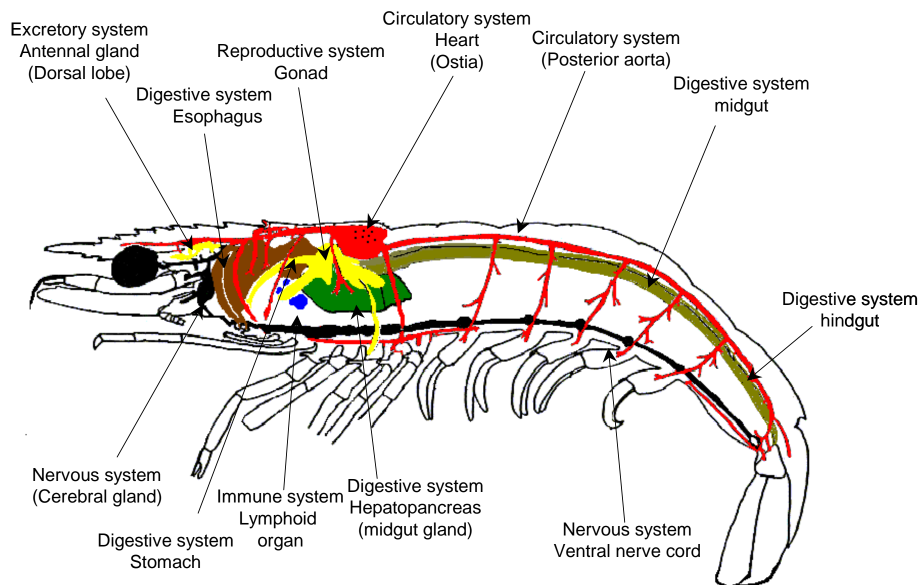

Fig. 8 - Diagram of the internal morphology of penaeid shrimp.

2.2.4 – Penaeid shrimp life cycle

Sexes are separated in most cultivated decapods, although occasionally individuals in an intersex hermaphroditic condition are found. In mature decapods mating generally occurs when the female is in a soft-shelled condition (i.e. newly moulted) and results in a deposition of one or more spermatophores in, or close to the genital openings of the female. The spawning occurs directly into the sea in the case of penaeid shrimp, or to the brood chamber beneath the abdomen in other groups. Penaeids eggs hatch a few hours after spawning and each larvae is left to fend for itself as it develops through the nauplius, protozoea and mysis stages before metamorphosing into a post larvae (Fig. 7) (Wickins and Lee, 2002). Their diet ranges from the hereditary yolk sack, during the early naupliar stage, to phytoplankton (microscopic plant organisms) and then to

zooplankton (microscopic animals). Finally, at mysis stage and beyond, the shrimp is able to eat a wide variety of organisms. During this period, the larvae drift with the currents. A small percent of them are swept into the bays and estuaries by the currents. Here, the postlarvae remain, through their juvenile stages, until they mature and seek the offshore spawning grounds. It has been estimated that only 1 percent of those spawned in nature actually reach the adult stage (Treece and Yates, 1988).

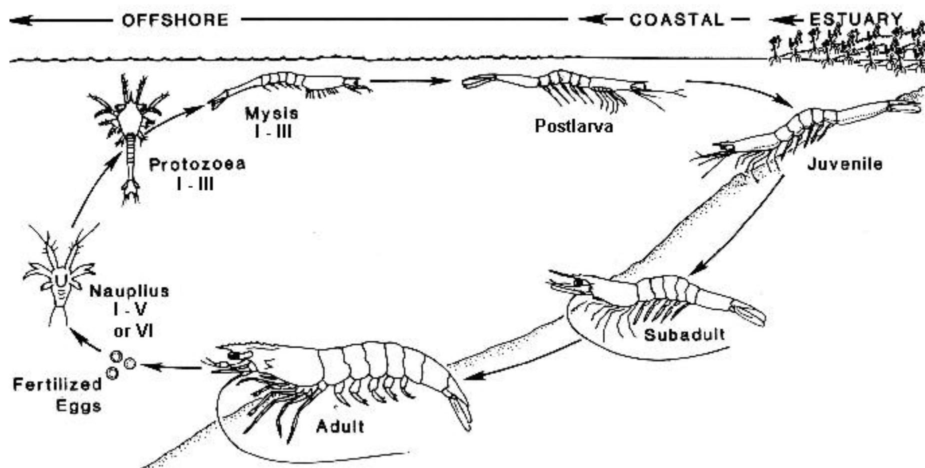

Fig. 9 – Penaeid shrimp life cycle (Baily-Brock and Moss, 1992).

2.2.5 – Physiology

2.2.5.1 – Immune system

The immune system is commonly divided into two major branches: innate and adaptative immunity. Since vertebrates lack an adaptive immune system in which memory is the hallmark, their defence mechanisms only rely on innate immune responses. Hence, crustaceans cannot readily be vaccinated against particular pathogens. Instead, their defence systems, while effective, tend to be more general and based on haemocytes that can mount phagocytic, cytotoxic and inflammatory responses to invading microbes (Wickins and Lee, 2002). Recently, however, cumulative experimental data from invertebrates provide some specificity and memory might exist in invertebrates (Kurtz, 2005).

Aquatic crustaceans are in intimate contact with their environment, particularly in intensive culture systems, which are enriched with bacteria and viruses. Some of these are pathogenic and many are saprophytic. However, under normal conditions animals maintain a healthy state by defending themselves against potential pathogens (Jiravanichpaisal, 2005).

The first line of defence against microbial invasion is the cuticle. It is a physical hard barrier with antimicrobial proprieties, for example it contains inhibitors against enzymatic attack. If it is penetrated, there is an immediate recognition of the non-self material by haemocytes and plasma proteins (Wickins and Lee, 2002).

The digestive tract, which is the main route of invasion, is partially lined with chitinous membranes and its hostile environment of acids and enzymes is able to inactivate and digest many virures and bacteria. In most cases the cuticular defences are sufficient to protect against even quite virulent pathogens, which often only produce disease when the integument has been physically damaged. Once pathogens gain entry into the hemocoel of the host, they encounter a complex system of innate defence mechanisms involving cellular and humoral responses (Jiravanichpaisal, 2005).

The cellular reactions involve three subpopulations of haemocytes which are responsible for a whole number of reactions: containment of the PO system, phagocytosis, degranulation and release of reactive oxygen intermediates (Song and Hsieh, 1994), and coagulation (Söderhäll and Smith, 1986).

The humoral components include the activity of soluble enzymes, either activated in circulating hemolymph, or released by cells that serve to detoxify toxic molecules or inhibit the physiology of invading pathogens (Cardenas and Dankert, 2000). Antimicrobial peptides, proteases and protease inhibitors, as well as lectin-like molecules exist in the white shrimp species *Litopenaeus vannamei* and *L. stylirostris* (Gross *et al.,* 2001; Cerenius and Söderhäll, 2004).

2.2.5.1.1- Haemocytes

In crustaceans, the circulating haemocytes play a crucial role in defence against infection, including recognition, phagocytosis, melanization, cytotoxicity and cell–cell communication (Johanson *et al.*, 2000). In decapod crustaceans, these cells can be

divided, according their morphology (presence of cytoplasmic granules) into three types: hyaline, semigranular and granular cells (Bauchau, 1981).

The hyaline cells play a major role in phagocytosis (Söderhäll *et al.*, 1986). The semigranular cells take part in encapsulation reactions and have a limited function in phagocytosis. Both granular cells and semigranular cells store the components of prophenoloxidase activating system and are capable of cytotoxic reaction (Smith and Söderhäll, 1983). The semigranular cells are the most sensitive and they are the first to respond to the lipopolysaccharides and β-1,3-glucans by degranulation and then, the components of the proPO system are released (Johansson and Söderhäll, 1985).

2.2.5.1.2 – The prophenoloxidase activating system (proPO)

The primary mediator of the cellular response to injury and disease in invertebrates is the pro-enzyme prophenoloxidase (proPO) activating system (Söderhäll *et al.*, 1994). This system consists of several proteins involved in the immune defence in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Sritunyalucksana, and Söderhäll, 2000), where proPO is released from haemocytes by an active degranulation process that can be stimulated by inflammatory agents such as lipopolysaccharide (LPS) or peptidoglycan (molecules of bacterial cell walls) and ß-1,3-glucan (molecules of fungal and yeast cell walls). Once released, ProPO is proteolytically converted, through cleavage of the enzyme at a specific site, to its active form PO, which is the central component of an enzyme cascade that has been identified in crustaceans (Cardenas *et al.*, 2000). The active form of the enzyme then functions to produce antimicrobial effects, wound repair, encapsulation, and phagocytosis.

2.2.5.1.3 – The coagulation system

One of the principal differences between vertebrates and arthropods is the fact that the body fluids in vertebrates are mostly confined to blood and lymphatic vessels, while arthropods have an open circulatory system. Therefore, after wounding, arthropods must produce a matrix that quickly stops the loss of haemolymph, but also aids in trapping

foreign organisms to prevent spreading throughout the haemocoel. Haemolymph clotting is thus an important part of innate immunity and is regulated in many cases by microbial elicitors (Jiravanichpaisal, 2005).

In crustaceans, the coagulation system involves plasma clotting protein and a haemocyte-derived transglutaminase. This clotting protein is synthesised in the hepatopancreas and released to the haemolymph. The transglutaminase is synthesised and stored in the haemocytes, and released to the plasma upon the activation of haemocytes. This enzyme covalently crosslinks the clotting protein molecules in the presence of calcium ions to form a soft gel at the wound sites (Bangyeekhun, 2002).

2.2.5.1.4 – Antimicrobial peptides

Antimicrobial peptides are a major component of the innate immune defense system in marine invertebrates. They are defined as molecules less than 10 kDa in mass which show antimicrobial properties (Boman, 1995) and provide an immediate and rapid response to invading microorganisms (Bartlett, 2002). The major classes of antimicrobial peptides include (i) α-helices, (ii) β-sheet and small proteins, (iii) peptides with thio-ether rings, (iv) peptides with an overrepresentation of one or two amino acids, (v) lipopeptides, and (vi) macrocyclic cystine knot peptides (Epand and Vogel, 1999).

There is evidence that antimicrobial peptides are widespread in invertebrates (15), especially in tissues such as the gut and respiratory organs in marine invertebrates, where exposure to pathogenic microorganisms is likely (Chisholm and Smith, 1992). These peptides generally act by forming pores in microbial membranes or otherwise disrupting membrane integrity (Tam *et al*., 2000). The value of antimicrobial peptides in innate immunity lies in their ability to function without either high specificity or memory, and their small size makes them easy to synthesize (Relf *et al*., 1999). In addition, many antibacterial peptides show remarkable specificity for prokaryotes with low toxicity for eukaryotic cells (Zasloff, 1992).

Prominent among crustacean antimicrobial peptides are the penaeidins, which display antifungal and antibacterial properties and were isolated from the haemolymph of the shrimp *Litopenaeus vannamei* (Destoumieux el al. 1997).

2.2.5.1.5 – Non-self recognition system

The innate immune system is based on recognition of molecules named pattern recognition receptors that are present on pathogenic microbes. These molecules are structural molecules of pathogens, but not of the host, which are shared by a large group of microbes and are essential for their survival (Medzhitov and Janeway, 2000; Janeway 2001). Example of such molecules are ß-1,3-glucans from fungi, lipopolysaccharide, peptidoglycan and lipoteichoic acid from bacteria, and double-stranded RNA from virus. Therefore, presence of microbial molecules is an indication of an infection, which allows the host to choose a sufficient mechanism to fight against a certain class of pathogens (Medzhitov and Janeway, 2000). The biological function of recognition molecules in innate immune reactions are (i) triggering of proteinase cascades and/or signalling pathways of the defence mechanisms, and (ii) clearance of microbial invaders from the blood system (Bangyeekhun, 2002).

2.2.5.1.6 – Proteinase inhibitors

Proteinase cascades, such as clotting cascades and the proPO system, need to be carefully regulated to prevent excessive activation of endogenous cascades and damage to host tissue (Jiravanichpaisal, 2005). Proteinase inhibitors are present in multiple forms in animals, plants and microorganisms. A number of proteinase inhibitors have been reported from invertebrates. Most of them have a common structural feature as one of well characterised families, such as Kazal, Kunitz, α-macroglobulin, serpin, metalloproteinase inhibitor and cysteine proteinase inhibitor (Bangyeekhun, 2002).

In the mechanism of inhibition, that is common among most proteinase inhibitors, the inhibitor molecule combines with the proteinase at the reactive site to block proteolytic activity. Invertebrate proteinase inhibitors can be found in plasma, haemocytes or cuticle. The gross biological function of proteinase inhibitor is to prevent unwanted proteolysis. Two central roles of proteinase inhibitors in invertebrate immunity are defence against microbial proteinases and regulation of endogenous proteinases (Kanost, 1999).

These substances play a key role to control and regulate the proPO system, avoiding the deleterious effects of its active components, particularly PO, which can produce highly toxic intermediates (Jiravanichpaisal, 2005).

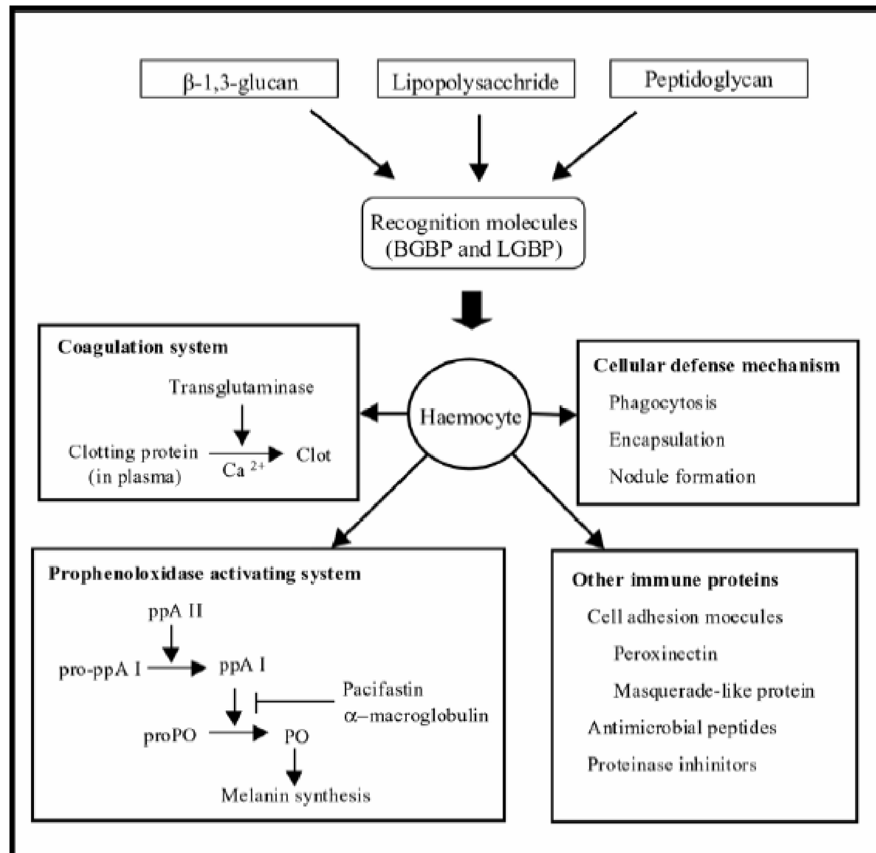

Fig. 10 – Schematic overview of crayfish defence reactions. In the presence of microbial organisms, the recognition molecules in the plasma participate in binding to the microbial cell wall components. Then the complexes bind to membrane receptors of the haemocytes and consequently activate the defence mechanisms. Haemocytes directly play a role in cellular defence mechanism or release humoral defence molecules, which lead to activation of the prophenoloxidase activating system and coagulation system (Bangyeekhun, 2002).

2.3 – Shrimp Farming

Shrimp farming started more than a century ago in Southeast Asia where farmers raised incidentally wild shrimp crops in tidal fish ponds (Rosenberry, 2004). Modern shrimp farming started in the early 1970s, and today, over fifty countries have shrimp farms. In the Eastern Hemisphere, Thailand, Vietnam, Indonesia, India and China are the leaders, but also Malaysia, Taiwan, Bangladesh, Sri Lanka, The Philippines, Australia

and Myanmar (Burma) have large industries. In the Western Hemisphere, Mexico, Belize, Ecuador and Brazil are the leading producers, and there are shrimp farms in Honduras, Panama, Colombia, Guatemala, Venezuela, Nicaragua and Peru. The shrimp importing nations (United States, Western Europe and Japan) specialize in high-tech "intensive" shrimp farming, but, thus far, their production has been insignificant. In the Middle East, Saudi Arabia and Iran produce the most farmed shrimp (Shrimp News International, 2004).

The shrimp farming process can be divided into three main phases, the hatchery, nursery and growout phase.

2.3.1 – Hatchery

The production cycle begins at the hatchery, where the shrimp seed is obtained from the broodstock. In many hatcheries, females with ripe, egg-laden ovaries (gravid females) are brought from the sea for spawning in captivity. As alternative, and due to reasons of price and availability, techniques were developed for inducing maturation of females in captivity. This procedure also allowed the establishment of breeding programs for fast growing, specific pathogen-free and/or resistant stocks.

Whether gravid shrimp are captured in the wild or matured in the hatchery, they invariably spawn at night, but with photoperiod manipulation, they can be induced to spawn at any time. Depending on a number of variables (temperature, species, size, wild/captive and number of times previously spawned), they produce between 50,000 and 1,000,000 eggs. After one day, the eggs hatch into nauplii, the first larval stage. Nauplii feed on their egg-yolk reserves for a couple of days. Next they pass through the next two main larval stages, the zoeae (which feed on microalgae and a variety of formulated feeds for three to five days) and mysis (feed on algae, formulated feeds and zooplankton for three or four days). Next they metamorphose into postlarvae. Postlarvae look like adult shrimp and feed on zooplankton, detritus and commercial feeds. Farmers refer to postlarvae as “PLs”, and as each day passes, the stages are numbered PL-1, PL-2, and so on. When their gills become branched (PL-13 to PL-17), they can be moved to the nursery or growout farm. From hatching, it takes about 25 days to produce a PL-15.

2.3.2 – Nursery

In modern semi-intensive farms, a nursery phase (between the hatchery and growout phases) is usually incorporated in the farm design. Nursery ponds may represent between 6 to 15% of all culture area. They are usually made with earthen embankments, and have at sizes that range from 0.04-1 ha. Stocking densities are typically 100-200 juveniles m^{-2}. In South-east Asia, nursery facilities also take the form of concrete tanks, concrete walled ponds with sand bottoms, staked net pens and floating cages (Wickins and Lee, 2002). The PLs are fed with a crumbled diet several times a day, where the protein levels range from 30 to 45%. The nursery phase should not exceed 25 days (Shrimp News International, 2004).

2.3.3 – Growout

The growout operation is stocked with postlarval shrimp and it takes from three to six months to produce a crop of market-sized shrimp. Northern China, the United States and Northern Mexico produce one crop per year, semi-tropical countries produce two crops per year, while farms closer to the equator have produced three crops a year, but rarely (Shrimp News International, 2004).

The two most practised production strategies are the extensive and intensive culture, however there are a numerous transitions between them. In extensive shrimp culture, shrimp are stocked at low densities (<25 PLs·m^{-2}) in large ponds or tidal enclosures in which little or no management is exercised or possible. Farmers depend almost entirely on natural conditions in extensive culture. Intensive shrimp culture is carried out in high densities (sometimes >200 PLs·m^{-2}) in intensively managed pens, ponds, tanks and raceways where a high level of investment is required (Rosenberry, 2001). Semi-intensive culture falls between these two extremes.

2.4 – Penaeid shrimp common diseases in farming conditions

The diseases of cultured penaeid shrimp include syndromes with infectious and non-infectious etiologies. Included among the infectious diseases of economic importance to cultured shrimp are those with viral, rickettsial, bacterial, fungal, protistan and metazoan etiologies. A number of non-infectious diseases are also of importance to the industry, and included among these are diseases due to environmental extremes, nutritional imbalances, toxicants, and genetic factors (Lightner and Redman, 1998).

2.4.1 – Viral diseases

Viruses are a group of organisms which must enter a host cell to replicate, since they lack the necessary biochemical machinery to manufacture proteins and metabolize sugars. Some virus also lack the enzymes required for nucleic acid replication, and are dependent on the host cell for these functions (Jiravanichpaisal, 2005).

Viral diseases are probably still underestimated in crustaceans, but nevertheless, they emerge as being responsible for serious enzootics or massive pandemics, on a regional scale in shrimp-farming countries. In 1989, 6 viruses were known to affect penaeid shrimp, but by 1997 more than 20 viruses were identified as having affected wild stocks and commercial production. The Office International des Epizooties (OIE) now lists seven viral diseases of shrimp which are considered to be transmissible and of significant socio-economic and/or public health importance (FAO, 2004). These viral diseases are:

- White Spot Disease (WSSV),
- Yellow Head disease (YHV),
- Taura Syndrome Virus (TSV),
- Spawnerisolated Mortality Virus Disease (SMV),
- Tetrahedral Baculovirosis (Baculovirus penaei - BP),
- Spherical Baculovirosis (*Penaeus monodon*-type baculovirus)
- Infectious Hypodermal and Haematopoietic Necrosis (IHHNV)

2.4.2 – Bacterial and fungal diseases

Bacteria, both gram-positive and gram-negative, are also etiological agents responsible for severe diseases in crustaceans (Söderhäll *et al*., 1993). These microorganisms act frequently as opportunistic follow-on to viral infection or environmental stress. Similar to bacteria, fungi often infect aquatic organisms as opportunistic invaders, but once established, they are also often fatal and difficult to treat. The most important bacterial and fungal diseases that have socio-economic impact in shrimp farming are:

- Vibriosis – *Vibrio* spp.;
- Necrotizing hepatopancreatitis (NHP) - Alfa proteobacteria (new genus);
- Rickettsial infection - Rickettsia or rickettsia-like microorganisms;
- Mycobacteriosis – *Mycobacterium marinum*, *Mycobacterium fortuitum, Mycobacterium* spp.;
- Larval mycosis – *Lagenidium* spp., *Sirolpidium* spp.;
- Fusarium disease - *Fusarium solani*, *F. moniliforme*;
- Crayfish plague - *Aphanomyces astaci*.

2.4.3 – Protozoan diseases

Among organisms causing diseases to shrimp, parasites, especially protozoan parasites form an important group. Although several diseases caused by parasites have been noticed in shrimp, often, chronic conditions caused by protozoan play a crucial role in shrimp production. The protozoa affecting shrimp can be grouped as parasites and commensals (Jithendran and Vijayan, 2001). Following are listed the major disease problems caused by the protozoa:

- Protozoan fouling - *Peritrichous* ciliates such *as Zoothamnium, Epistylis, Vorticella* and *Acinata*;
- Cotton shrimp disease - *Microsporeans* such as *Agmasoma*, *Ameson* and *Pleistophora*;

- Enterozoic cephaline gregarine infection - *Cephaline* gregarines such as *Nematopsis* and *Cephalolobus*;
- Invasive protozoan infection - Ciliate *protozoa*, *Paranophrys* and Paraoronema, *leptomonad*-like organisms.

2.4.4 – Non-infectious and toxic diseases

Non-infectious diseases are common in growout farms, as influences of nutritional factors, environmental factors such as temperature extremes and oxygen depletion, toxicity from biotic and abiotic origins, become critical during the lengthy culture period (Jithendran and Vijayan, 2001). The most common non-infectious pathologies are:

- Gas Bubble disease - caused by supersaturation of atmospheric gases, usually nitrogen, but occasionally oxygen;
- Haemocytic enteritis (HE) – caused by toxins released by certain blues-green algae blooms;
- Black gill disease – is associated to the presence of excessive levels of toxic substances such as nitrite, ammonia, heavy metals, crude oils in the culture water;
- Soft shell syndrome - caused by sudden fluctuation in water salinity, high soil pH, highly reducing conditions in soil, low organic matter in soil, low phosphate content and pesticide pollution in water, nutritional deficiency and insufficient water exchange;
- Muscle necrosis - is associated with poor environmental conditions such as low oxygen levels, and salinity or temperature shock.

2.5 - White Spot Syndrome Virus

White Spot Disease (WSD) is a pandemic disease of shrimp caused by a virus commonly known as White Spot Syndrome Virus (WSSV). First recorded in Taiwan in

1992 (Chou *et al*., 1995), the first major outbreaks were first detected in *Marsupenaeus japonicus* and *Fenneropenaeus chinensis* in Japan and China in 1993 (Nakano *et al.*, 1994; Zhan *et al*., 1998) and in the following 18 months the outbreak spread to the majority of the shrimp farming countries in Asia.

WSSV has a wide host range among decapod crustaceans (Lo *et al*., 1996), and is potentially lethal to most of the commercially cultivated penaeid shrimp species (OIE, 2003). Of all shrimp virus, WSSV has the largest impact on shrimp culture and remains a major problem up to the present day (Rosenberry, 2004).

2.5.1 – Taxonomy

Based on extensive phylogenetic analyses, and also on primary genomic structure and composition as well as the distinct morphology of the virion, the International Committee on Taxonomy for Virus (ICTV) approved a proposal in 2002 to accommodate WSSV in a new virus family called Nimaviridae, referring to the thread-like polar extension on the virus particle (Nima: Latin for thread). This virus family consists of a single genus (Whispovirus) and contains White spot syndrome virus I as its sole species so far (Mayo, 2002). Probably all WSSV isolates identified thus far are variants of the same species.

2.5.2 – Morphology

Electron microscopical studies on thin sections and viral suspensions obtained from infected shrimp revealed that the virion of WSSV is a large, ovoidal particle of about 275 nm in length and 120 nm in width, with a tail-like appendage at one end (Duran *et al*., 1997). It is formed by a road-shaped nucleocapsid with a tight-fitting capsid layer, surrounded by a loose-fitting trilaminar envelop, which consists mainly of the WSSV encoded proteins VP28 and VP19 (van Hulten *et al.*, 2000). VP28 is most likely located on the surface of the virus particle and plays a key role in the virus infection (van Hulten *et al.*, 2001b). Isolated nucleocapsids have a cross-hatched appearance and size of about 300 x 70 nm. The nucleocapsid is formed by stacks of rings (about 14 in total), which

are in turn formed by regular spaced globular subunits of about 8 nm in diameter, arranged in two parallel rows (Durand *et al.*, 1997).

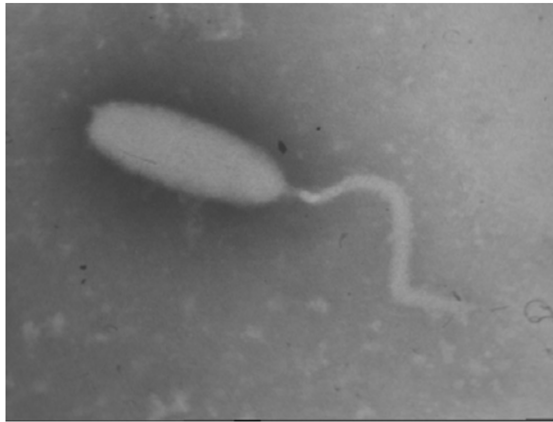

Fig. 11 – Image of a WSSV virion obtained by electron microscopy (source: Wageningen University, Laboratory of Virology)

2.5.3 – Genome

The virions of WSSV contain a circular, supercoiled, double-stranded DNA genome, originally estimated to be 300 kilobase pairs (kb) (Wang *et al.*, 2000). The genome contains 292,967 nucleotides encompassing 184 major open reading frames (ORFs). Of these, only 6% of the ORFs have putative homologues in databases, mainly representing genes encoding enzymes for nucleotide metabolism, DNA replication, and protein modification. The remaining ORFs are mostly unassigned, except for five, which encode structural virion proteins. Unique features of WSSV are the presence of a very long ORF of 18,234 nucleotides, with unknown function, a collagen-like ORF, and nine regions, dispersed along the genome, each containing a variable number of 250-bp tandem repeats (van Hulten, *et al.*, 2001a).

Between different geographic isolates of WSSV, a few restriction fragment length polymorphisms (RFLPs) were reported, indicating the presence of some genomic variation (Wang *et al.*, 2000).

2.5.4 – Epidemiology

This virus can be transmitted to benthic crustaceans and other fauna through different feeding pathways such as filter feeding, detritus feeding, and predation (Mortensen, 1993; Vijayan, *et al*., 2005). The transmission can occur horizontally either *per os* by predation on diseased individuals, but also by virus particles present in the water. Infection by the latter is thought to occur primarily through the gills, but may occur via other body surfaces as well (Chou *et al.,* 1998). No penaeid shrimp species are known to be resistant to WSSV infection (Lightner, 1996).

When viruses pass into the digestive tracts of other invertebrates (bivalves, polychaete worms), they can persist in the alimentary canal, potentially making the animal a passive carrier or vector of the virus. When these passive carriers are consumed by the shrimp, they can potentially infect the shrimp with WSSV (Vijayan *et al*., 2005). Also, a large number of other wild animals have been reported to be potential carrier of WSSV. These were not only shrimp, prawn and crab species but also planktonic organisms and insect larvae (Flegel and Alday-Sanz 1998).

Hence, the passage of the viral pathogen to shrimp broodstock in the hatchery through feeding of infected prey items is a realistic possibility (Vijayan *et al*., 2005). Once the broodstock is infected, the virus may also be transmitted from mother to offspring, although it is not a clear whether the WSSV virions are present inside the shrimp eggs (Peng *et al.,* 2001).

Frequent disease outbreaks in the shrimp farms of India and Asia lead to the offloading of dead and decayed shrimps carrying a heavy load of this virus into the coastal ecosystem. Horizontal transmission of WSSV from the affected shrimp farms to the neighbouring ecosystem has created a realistic scenario in which the receiving ecosystem carries the WSSV load in the form of live or dead tissues, dead and decomposed tissues and free virions (Mortensen, 1993). In addition, contrary to the common belief that free virus cannot survive in natural waters more than 24 hours, WSSV virions can remain infective in decaying tissues or in detritus for up to 4 days, (Bondad-Reantaso *et al*., 2001).

2.5.5 – Cytopathology and histopathology

Histopathology of the WSSV infection is characterized by the presence of cells with hypertrophied nuclei showing eosinophilic intranuclear inclusions and marginated chromatin, as WSSV DNA replication and virion morphogenesis take place (Durand *et al*., 1997; Wang *et al*., 2000). Inclusion bodies inside the nuclei are markedly distinct and bigger than the cowdry A-type inclusions characteristic of IHHNV (Wongteerasupaya *et al*., 1995). Nuclei of infected cells progressively become basophilic and hypertrophied because of the accumulation of intranuclear virions (Chang *et al*., 1996; Lo *et al*., 1996; Durand *et al*., 1996; 1997; Wang *et al*., 1998; Otta *et al*., 1999; Takahashi *et al*., 2000). In the late stage of infection, cells become degenerated, displaying cariorhexis and cellular disintegration which lead to the formation of necrotic areas characterized by vacuolization (Karunasagar *et al*., 1997; Kasornchandra *et al*., 1998).

WSSV targets tissues of ectodermal and mesodermal origin, such as epithelial and connective tissues of epidermis, stomach, gills, antennal gland, lymphoid organ and haemocytes, muscle, haematopoietic and nervous tissue, eye-stalk, heart, gonads, etc. (Chang *et al*., 1996; Durand *et al*., 1996; 1997; Mohan *et al*., 1998; Rajendran *et al*., 1999). No haemocytic infiltration can be seen in areas of necrotic tissues (Park *et al*., 1998; Flegel, 2001).

2.5.6 – Replication cycle

Although during the last decade, intensive efforts were undertaken for detection and characterization of in vivo WSSV infection in shrimp (Maeda, 2004) little is known about the molecular mechanisms underlying the WSSV life cycle and mode of infection.

Based on data from Ecobedo-Bonilla *et al.,* (2005), when *Litopenaeus vannamei* were infected with WSSV, the first infected positive cells were found at 12 hours post inoculation. This data indicates that the virus replication time may not be longer than 12 hours.

2.5.7 – General clinical signs

Infected animals show lethargic behaviour, such as lack of appetite and slow movement, and reddish to pink body discoloration. Characteristics for the WSSV infected shrimp are white spots on the exoskeleton. These spots are the result of calcified deposits that range in size from a few mm to 1 cm or more in diameter (Chou *et al*., 1995). However, in case of acute (experimental) infections the only signs of WSSV infection observed are lethargy and lack of appetite. White spots are also not evident in species like *L. vannamei*, even in normal farming conditions.

2.5.8 –Diagnostic methods

The earliest diagnostic methods developed for virus included the traditional methods of morphological pathology (direct light microscopy, histopathology, and electron microscopy), as well as enhancement and bioassay methods. While tissue culture is considered to be a standard tool in medical and veterinary diagnostic labs, it has never been developed as a useable, routine diagnostic tool for shrimp pathogens. As well, there are few antibody-based diagnostic tests available for the penaeid viral diseases (Lightner and Redman, 1998). PCR or RT-PCR methods are available for several of these viruses and some are in routine use by certain sectors of the industry. For others, specific DNA probes tagged with non-radioactive labels provide highly specific detection methods for application in dot blot formats with haemolymph or tissue extracts, and with routine histological sections using in situ hybridization (Lightner, 1996; Lightner and Redman, 1998).

2.5.8.1 – PCR (Polymerase Chain Reaction)

The polymerase chain reaction (PCR) is a method for amplification of a specific DNA sequence of interest. PCR will allow a short stretch of DNA (usually fewer than 3000 bp) to be amplified to about a million fold. The particular stretch of DNA to be amplified, called the target sequence, is identified by a specific pair of DNA primers, oligonucleotides usually about 20 nucleotides in length. The PCR product is amplified from the DNA template using a heat-stable DNA polymerase and using an automated thermal cycler. This device promotes the reaction through 30 or more cycles of denaturing, annealing of primers, and polymerization. After amplification by PCR, the products are separated by polyacrylamide gel electrophoresis and are directly visualized after staining with ethidium bromide.

In recent years, PCR has been used to detect WSSV in a very specific and sensitive manner (Lo *et al*., 1996; Nunan and Lightner, 1997; Kim *et al*., 1998). Nested or two-step PCR has the advantage of increasing the level of sensitivity over singlestep PCR. Nested PCR consists in the reamplification of the PCR product obtained in a single-step PCR reaction by using an aliquot of this first reaction as a template in a second round of amplification (Peinado-Guevara and Lopéz-Meyer, 2005). Frequently, when shrimps show clinical signs of the disease such as lethargy, reduction of food consumption, reddish coloration and white spots on the exoskeleton, WSSV is easily detected by single-step PCR (Lo *et al*., 1998). However, at low viral loads, WSSV is latent without causing disease symptoms in the shrimps, and can only be detected by nested PCR (Lo *et al*., 1996, 1998; Kim *et al*., 1998).

2.5.8.2 – IIF (Indirect immunofluorescence)

A number of antibody-based diagnostic methods have been developed and are described in the literature (Loh *et al*., 1998; Poulos *et al*., 2001; Shih *et al*., 2001) as a confirmation tool for virus infection in shrimp species.

One of the antibody-based assays that is being used for detecting WSSV in shrimp is indirect immunofluorescence (Poulos *et al*., 2001; Wang *et al*., 2002; Ecobedo-Bonilla *et al*., 2005; Rahman *et al*., 2005). This technique detects specific antigens in tissues. The

specific secondary antibodies are labelled with a compound (fluorescein isothiocyanate) that makes them glow an apple-green colour when observed microscopically under ultraviolet or blue light.

In the specific case of the detection of WSSV in *Litopenaeus vannamei* tissues, tissues from the pereon are embedded in methylcellulose and frozen at –20°C. Cryosections (5 to 6 µm) are made and fixed in absolute methanol at –20°C, washed with white phosphate buffered solution at 1% (WPBS), incubated for 1 h at 37°C with 2 mg ml^{-1} of the monoclonal antibody 8B7 specific for VP28 (Poulos *et al*., 2001), washed and incubated for 1 h at 37°C with 0.02 mg ml–1 of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (F-2761, Molecular Probes) in PBS, washed with PBS, rinsed in deionised water, dried and mounted. Slides are analyzed by fluorescence microscopy (Escobedo-Bonilla *et al*., 2005)

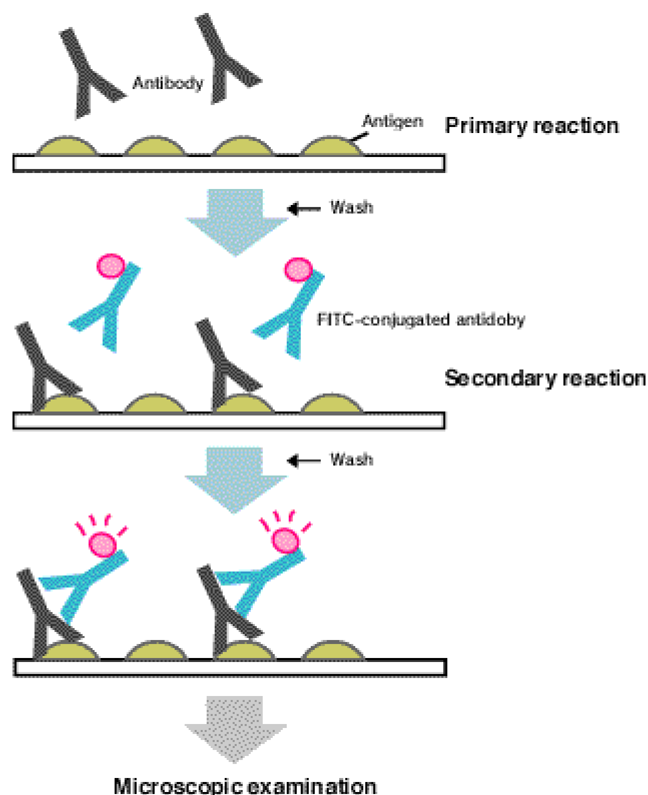

Fig. 12 – General principle of Indirect Immunofluorescence technique. Samples are added on a substrate slide for primary reaction of antibodies (primary antibodies) and antigens. After washing, conjugated antibodies (secondary antibodies) are added to make complexes of antigens - antibodies - conjugated antibodies. After washing, the fluorescence from FITC is observed by fluorescent microscope (MBL, 2005).

2.5.8.3 – Other methods

Several other diagnostic methods have been described for WSSV detection:

- Light and electron microscopy (Chou *et al*., 1995; Wongteerasupaya *et al*., 1995; Lightner, 1996; Durand *et al*., 1997)
- In-situ DNA hybridisation ((Chang *et al*., 1996; Durand *et al*., 1996)
- Dark-Field Microscopic Observation (Momoyama *et al*., 1995)
- Miniarray (Quere *et al*., 2002),
- Observation of tissues subjected to fixation or negative staining (Inouye *et al*.,1994),
- Reverse passive latex agglutination (Okumura *et al*., 2005)
- Bioassay (Nunan *et al*., 1998)
- Rapid staining Hematoxylin and Phloxine/Eosin (H&E) (Sheehan and Hrapchak, 1980).

2.5.9 – Tested strategies for WSSV control

Different approaches knew already some success to control WSSV, including (i) higher or lower than normal water temperatures (Vidal *et al*., 2001; Guan *et al*., 2003; Jiravanichpaisal *et al*., 2004), (ii) treatment with the immunostimulants peptidoglycan, lipopolysaccharide and β-1,3 glucan (Itami *et al*., 1998; Takahashi *et al*., 2000; Chang *et al*., 2003), (iii) vaccination with formalin inactivated bacteria over expressing WSSV proteins, siRNA and WSSV envelope proteins VP19 and VP28 (Namikoshi *et al*., 2004; Witteveldt *et al*., 2004; Musthaq *et al*., 2005; Westenberg *et al*., 2005) (iv) treatment with egg yolk antibodies (IgY) against WSSV (Kim *et al*., 2004) (v) feeding antiviral fucoidan, a sulfated polysaccharide extracted from Sargassum polycystum supplemented diet (Chotigeat *et al*., 2004) and (vi) treatment with cidofovir (antiviral) and a diet supplemented with *Spirulina platensis* (Rahman *et al*., 2005).

2.5.9.1 – WSSV control with temperature treatment

Few reports are available about the influence of temperature on viral diseases in aquatic animals (Amend, 1970; Dorson and Torchy, 1981; Castric and Kinkelin, 1984; Oseko *et al*., 1988; Sano *et al*., 1993; Kobayashi *et al*., 1999) and so far, only three reports on the WSSV infectivity in crustaceans are available. Vidal *et al*. (2001) have reported that hyperthermia was able to protect *Litopenaeus vannamei* from WSSV disease after challenge with WSSV. In *Marsupenaeus japonicus* that protection also occurs at low temperature (Guan *et al*., 2003). Jiravanichpaisal *et al*. (2004) reported that protection of WSSV infected freshwater crayfish occur at low temperatures.

In the study of Vidal *et al*. (2005) juveniles of the pacific white shrimp were infected with WSSV by oral and intramuscular route. Both the shrimp inoculated by oral and intramuscular route were divided in two groups, one maintained at ambient temperature (25.8ºC) and the other kept at higher temperature (32.3ºC). The results demonstrated a high degree of protection of the groups kept at high water temperature, as survival was always above 80% against 100% mortality obtained in those maintained at lower temperature.

Guan *et al*. (2003), tested the influence of four temperature levels (15, 23, 28 and 33ºC) on survival of WSSV infected *Marsupenaeus japonicus.* After virus injection, the four shrimp groups were maintained for 19 days at those temperature levels. The results demonstrate that WSSV infection can by controlled either by low and high temperature levels.

Jiravanichpaisal *et al*. (2004), used two species of freshwater crayfish, *Pacifastacus leniusculus* and *Astacus astacus,* for testing the effect of low water temperature on the development of WSSV infection. Crayfish were exposed to different temperatures (4, 12, 22ºC) after WSSV injection or oral exposure and the mortalities were recorded during 45 days. The results showed that the infection could be blocked at lower temperatures (4 and 12ºC), while at high temperature 100% mortality was reached. It was also observed that mortality could be delayed transferring moribund individuals to lower temperature.

2.5.10 – Socio-economic impact of WSSV

WSSV disease is responsible for direct losses of billions US$ per year in Asia and Latin America. For example, in Ecuador US$ 280 millions were lost in the first six months of its first appearance in 1999. In the export sector, shrimp exports fell from 115 000 metric tonnes (mt) in 1998 to 38 000 mt in 2000, and have only recovered slightly to 47 000 mt in 2002. This equates to a total direct loss of some 267 000 metric tonnes of shrimp worth nearly US$ 1.8 thousand million between 1999 and mid-2003. Although similar problems have occurred throughout Central and South America, Brazil and Venezuela remained several years free of WSSV due to a rapid and effective closure of their borders to all crustacean imports in 1999. However, recently, on 20 January 2005 the first occurrence of WSSV in Brazil was reported in *Litopenaeus vannamei*. After initial loses, United States also managed to eradicate WSSV from its shrimp culture industry in 1997 through the implementation of biosecurity measures, including the use of all SPF broodstock, although there are reports of its recent re-emergence in Hawaii in 2004 (Briggs *et al.*, 2004). Estimates for Asia include losses of over US$ 250 million for 1993 (continuing every year) in Mainland China, loosing 120 000 metric tonnes of production of *F. chinensis*, *M. japonicus* and *P. monodon* to WSSV (Jiang, 2000).

In addition to direct effects on production, the impacts of diseases are particularly felt by small-scale farmers who, especially in Asia, represent the backbone of many coastal communities. Their very livelihoods are threatened through reduced food availability, loss of income and employment, social disturbance and increased vulnerability. Crop losses to disease for this sector of society may determine whether or not those families are below the UN poverty threshold (Fegan *et al.*, 2001). In Mainland China, for example, the WSSV epidemic in 1993 affected the lives of 1 million people, and has continued to have effects to this day (Jiang, 2000). Similar effects have been noted from Latin American countries. In Ecuador for example, within the first year of the WSSV epidemic in 1999, the disease also lead to the loss of 26 000 jobs (13 percent of the labour force), the closure of 74 %t of the hatcheries, a 68 percent reduction in sales and production for feed mills and packing plants, 64 percent layoffs at feed mills and a total of 150 000 jobs lost in the shrimp farming industry (Alday de Graindorge and Griffith, 2000).

It is, however, difficult to accurately quantify the economic and social effect of this disease. WSD and the response of those depending on shrimp farming for their incomes are in a constant state of co-evolution. In such a relationship it is difficult to separate the effect of the disease from people's response to the disease. It also has been demonstrated that loss of a crop through WSD can result in extremely vulnerable farmers dropping to the lowest poverty levels, where their families are no longer food secure (Morgan, 2001).

2.6 – State of the art and future trends in WSSV research

Since the first record in Taiwan in 1992 (Chou *et al.* 1995), the White Spot Syndrome Virus (WSSV) epizootic quickly spread through many producing countries, costing billions of dollars in regional export earnings of which most of the economies have yet to recover. However, in all countries, university scientists, farm owners and technicians, NGOs and international development agencies are working on solutions to survive with WSSV (McClennen, 2004).

With the objective of finding solutions for this shrimp aquaculture threat, the reaction of the scientific community involved many different research areas.

Firstly, the development of specific techniques for WSSV detection, not only allowed the diagnosis of this pathological agent, but also provides powerful tools for other research areas. Amongst other less frequently used techniques, PCR (Nunan and Lightner, 1997; Kasornchandra *et al.*, 1998; Lo *et al.*, 2001), antibody-based assays (Poulos *et al.*, 2001; Shih *et al.*, 2001) and in-situ DNA hybridisation (Duran *et al.*, 1996; Numan *et al.*, 1997), are now used in routine.

Fields like molecular biology and molecular epidemiology have made some advances to understand the route and mechanisms of this viral infection, but nevertheless a lot of work still remains to be done. In contrast, extensive research on WSSV genetics and morphology has been done (Marks *et al.*, 2004; van Hulten, 2001; Wang *et al.*, 2000).

With respect to the development of control measures, several studies recently attempted "vaccination" or immunization of shrimp against WSSV infection. Although the results show some potential, they are still not always conclusive (Kim *et al.*, 2004;

Namikoshi *et al*., 2004; Witteveldt *et al*., 2004; Musthaq *et al*., 2005; Westenberg *et al*., 2005). On the other hand, some of the most interesting results were obtained by using both high (33ºC) and low (12ºC) temperature to control WSSV infection. Several studies clearly demonstrated that high temperature can "block" infection, or in other words, prevent mortality in infected animals (Vidal *et al*., 2001; Guan *et al*., 2003; Jiravanichpaisal *et al*., 2004). It however remains unclear how exactly temperature interacts with WSSV infection and also application of these findings in the field are limited. Therefore, further documentation of the use of environmental parameters (temperature, salinity, etc.) to control WSSV and its application in the field is considered timely.

In the future, it will also be necessary to direct research towards disease prevention and to propose new methodologies and concepts (standard operation protocols), in order to guarantee a better and responsible management by the farmers, and in this way minimise the impact of WSSV. As part of these strategies it is necessary to develop new "pond-side" detection techniques, to identify in an early stage this etiological agent and in this way avoid further spread of the disease. Also breeding efforts to generate resistant stocks are necessary, but there is a lack of basic information on challenge test strategies focused on genetic selection.

CHAPTER 3 - MATERIAL AND METHODS

The experiments were conducted at the facilities of the Laboratory of Aquaculture and Artemia Reference Center (ARC) and the Laboratory of Virology of the Ghent University.

Three different experiments were done:

Experiment 1

"Effect of high water temperature (33ºC) before and after inoculation, on White Spot Syndrome Virus (WSSV) infection in pacific with shrimp (*Litopenaeus vannamei*)."

This experiment aimed to determine the degree of protection that can be obtained, when WSSV infected SPF shrimps are submitted to high water temperature. It was also evaluated if the immune factor is enhanced by exposure at high water temperature before virus inoculation. Therefore the necessity of keeping the shrimp at high temperature before virus inoculation was also determined.

Experiment 2

"Effect of high water temperature (33ºC), on White Spot Syndrome Virus (WSSV) infection in pacific white shrimp (*Litopenaeus vannamei*), when applied at different times (12 and 24h) after virus inoculation."

In the first experiment the potential protection capacity of high water temperature on WSSV infected shrimps was evaluated. The temperature was however always done immediately after at the inoculation. In this second experiment the aim was to determine the effect of high water temperature when after inoculation, a certain period of viral replication (12 or 24 hours) was allowed.

Experiment 3

"Effect of cyclic variation of high water temperature (27ºC/33ºC), on White Spot Syndrome Virus (WSSV) infection in pacific white shrimp (*Litopenaeus vannamei*)."

In first 2 experiments once applied, high water temperature was continuously maintained until the end of the experiment.

This third experiment aimed to determine if the application of daily cyclic temperature regime with a limited number of hours at 33ºC is enough to prevent mortality in WSSV infected shrimps.

3.1 - Experimental animals

Specific pathogen-free (SPF) *Litopenaeus vannamei* Kona strain were used (mean body weight [MBW] = 20 g). These shrimps were imported as post-larvae (PL 8-12 with a mean body weight of 0.0013 g) from Molokai Seafarms in Hawaii. The Kona line originated from a small group of broodstock, which was originally derived from the West-coast of Mexico in 1989, cultured in the "Oceanic Institute" in Hawaii under the USDA Marine Shrimp Farming Programme. SPF shrimp are maintained under strict biosafety conditions to avoid possible contamination. As they originated from a small group of broodstock, the genetic variability among shrimps is limited and in that way the variation among individuals is reduced. The SPF status of the imported PL was checked before they were shipped from Hawaii on a sample of 10 individuals at the laboratory facilities of Moana Technologies (Hawaii). They were checked for almost all the main viruses such as WSSV, IHHNV, MBV, HPV, TSV, YHV and GAV and for some other pathogens (parasites and fungus) with PCR and with histopathology. All diagnostic techniques have confirmed that the PL's were not contaminated with known pathogenic organisms. After the arrival at ARC, a second sample of shrimp was send to confirm their health status (SPF status).

3.2 – Recirculation system to raise the shrimp

An isolated room at the ARC, equipped with a 3000-l raceway tank and 15 rectangular tanks of 250l, were used to raise the stock of SPF shrimps (Fig. 13). The tanks were filled with filtered natural seawater (Northsea water). Before use, high-pressure sand filter filtration was done to get rid of the suspended particles and coal filtration was used to avoid dissolved toxic materials. Finally the water was subjected to ultra violet filtration to eliminate microorganisms.

The shrimp were grown under standardised culture conditions. Constant temperature of 27 ± 1ºC was provided with heaters and a salinity of 35-37 gl^{-1} was maintained. Fluorescent light was used to illuminate the culture room and the bulbs were covered with brown paper to reduce the light intensity. The photoperiod was adjusted to have 12 hours of light.

The culture tanks were connected to a recirculation system consisting of a protein skimmer and a biological filter. The biological filter was inoculated with bacteria of the species *Nitrosomonas* and *Nitrobacter* that convert the excreted toxic nitric compounds that results from the shrimp metabolism. The levels of ammonium ($NH4^+$) and nitrite (NO_2) were checked daily using test kits. According to the daily water quality parameters measurement, a variable percentage of the water was exchanged. This way ammonium and nitrite levels were maintained below 0.5 mgl^{-1} and 0.05-0.15 mgl^{-1}, respectively. Compressed air was provided with air stones to maintain the water oxygen levels at optimum levels (7-10 mg/l). Biosecurity procedures (foot bath, daily disinfection of the area and material, limited number of people having access to the area and hand disinfection) were taken to guaranty the SPF status of the shrimp.

Fig. 13 – Recirculation system for raising shrimp at the the facilities of the Laboratory of Aquaculture and Artemia Reference Center. A – 250L tanks; B – Biological filter; C – 3000L raceway-tank

3.3 – Pre-challenge phase

As the actual WSSV challenge experiments were performed at a salinity of 15 gl^{-1}, before being transported to the facilities of the Laboratory of Virology in Merelbeke, the shrimp to be used in an experiment were first slowly acclimated to this lower salinity over a period of 5 days.

The required number of shrimp for an experiment were randomly selected and transferred into 250-l fibreglass tanks. Salinity was then lowered over a period of 4 days by daily replacing part of the water with water of a lower salinity. The acclimation tanks were equipped with small independent aquarium filters (Eheim filter with a capacity of 400 l/hour). Temperature in the tanks was maintained by aquaria heaters (300W capacity).

3.4 – Shrimp transport

The acclimatized shrimp were then transported by car from the ARC to the facilities of the Laboratory of Virology, where the challenge experiments were performed. For transportation, 20-l plastic buckets were used. Approximately 20 individuals were kept in one bucket. Buckets were filled with clean and well oxygenated 15 $mg.l^{-1}$ water at the required temperature.

3.5 – Challenge facilities

All challenge experiments were, as mentioned earlier, performed at the Laboratory of Virology of the Ghent University. A separated room, which is destined only to this type of experiment and this specific WSSV virus strain, was used.

3.6 – Virus stock preparation and titration

The same Thai WSSV strain was used in all experiments. This virus was obtained from Prof. Kenneth Söderhäll, from the Department of Comparative Physiology, Evolutionary Biology Centre, Uppsala University, Sweden.

ThisWSSV strain was isolated from naturally-infected *Penaeus monodon*. The virus isolate was passaged once in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal *et al.*, 2001). A gill suspension from crayfish (10^{-2} in L-15 medium) was kindly donated by P. Jiravanichpaisal and K. Söderhäll (Uppsala University, Sweden). It was diluted 10^{-1} in phosphate-buffered saline (PBS) pH 7.4, and 50 µl were injected intramuscularly into SPF *Litopenaeus vannamei* to amplify the virus. The inoculated shrimp were collected at 48 h post inoculation (hpi) and were frozen at –70°C. Tissues from these shrimps were analyzed by indirect immunofluorescence (IIF) to confirm WSSV infection. Thawed carcasses without hepatopancreas, gut and exoskeleton were minced. A 10^{-1} suspension was made in PBS and centrifuged (3000 × g at 4°C for 20 min). The supernatant was centrifuged (13 000 × g at 4°C for 20 min), filtered (0.45 µm) and aliquoted for storage at –70°C. The total volume was 250 ml. Samples from tissues used

to produce the viral stock were sent to Dr. James Brock (Moana Technologies LLC, Hawaii) for detection of the major viral pathogens of shrimp by polymerase chain reaction (PCR). PCR analysis confirmed the sole presence of WSSV DNA in the tissues (Escobedo-Bonilla *et al*., 2005).

The virus was titrated in vivo in SPF *Litopenaeus vannamei* by intramuscular and oral route. The median virus titer of infection by intramuscular route was $10^{6.0}$ shrimp infection doses 50% endpoint (SID_{50} ml^{-1}), according to the experimental protocol described by Escobedo-Bonilla *et al*. (2005), was calculated according to the Reed and Muench method (1938). From the titer, a dose of 30 SID_{50} in a volume of 50µl was determined to induce infection in all the inoculated shrimp in a reproducible manner and was therefore chosen as the standard dose for experimental WSSV challenge.

3.7 – Preparation of the virus inoculum

An aliquot of the virus stock was taken from the freezer and defrosted. It was then diluted to get 30 and/or 10000 SID_{50} per 50 µl of inoculum, according to the experiment in question. This was calculated considering the virus infectivity titer of $10^{6.0}$ SID_{50}.

The virus stock dilution was done with Phosphate Buffered Saline (PBS) (pH 7.4). Before preparing the inoculum, PBS was filtered through a membrane filter (0.45µm) to remove any possible particles in suspension and obtain a sterile medium for dilution.

Aliquots containing virus and aliquots containing PBS were placed in a container with ice cubes in order to keep the virus alive. This whole procedure was carried out under a laminar flow to avoid contamination.

3.8 – Shrimp inoculation

Twenty four hours after the shrimp were accommodated in the experimental challenge facilities, they were intramuscularly inoculated with a dose of 30 or 10000 SID_{50} in a volume of 50 µl, according to the experiment in question. For the inoculation, shrimp were caught with a smooth scoop net and placed on a sterile board with the left side of the body upwards. For inoculation a glass syringe with a 100µl capacity, with a minimum scale of 1µl and precision of 0.5 µl was used. The region of inoculation was

between the third and fourth abdominal segments and was always disinfected with cotton embedded in ethanol at 70% before injecting. The inoculation process was executed by two researchers. To avoid contamination between replications, treatments or controls, each person only took care of one specific shrimp treatment group. Immediately afterwards, the shrimp were placed into the respective treatment unit. All the manipulation procedures were realized with extreme caution in order to minimize handling stress to the animals. After the inoculation, the shrimp were fed to prevent cannibalism.

3.9 – Daily procedures during the challenge experiments

During the challenge experiments, the animals were fed twice a day with a commercial shrimp diet. The minimum amount of food (two pellets per shrimp) was given to maintain the water quality at optimum levels. The daily monitoring included the scoring of parameters such as temperature and ammonia, shrimp feeding, response of shrimp to mechanical stimuli, moulted shrimp. This scoring also was done two times per day. Based on the readings, water was partially exchanged. Dead individuals were collected twice a day for further analysis.

3.10 – Sample collection

During the experiment all the dead shrimp were collected for further confirmation of WSSV infection. At the end of the experiment, the survivors were euthanized and sampled for evaluation of WSSV infection status. An incision was made with a sterile scalpel blade in the medial line of the ventral region, from the posterior end to the anterior end. Then half of the cephalothorax but without the hard part (rostrum and legs), was separated and embedded in methylcellulose to cryopreserve. The embedding was made inside plastic laboratory flasks. To quickly freeze the samples, the flasks were partially introduced in a solution of ethanol and dry-ice. The rest of the body parts were packed in polythene bags for further use as required. Samples were stored in the freezer (-20ºC) till preparation of cryosections.

3.11 – Cryosections preparation

Cryosections were made from methylcellulose embedded tissues using the cryotome with a thickness of 5 μm and including most of the head tissues. After preparing the cryosections, they were fixated in absolute methanol at – 20ºC for twenty minutes. After that, sections were stored at -20ºC.

3.12 – WSSV infection detection

The presence of WSSV was detected by using the Indirect Immunofluorescence (IIF) technique. After sampling and processing, tissues from the pereon are embedded in methylcellulose and frozen at –20°C. Cryosections (5 to 6 μm) are made and fixed in absolute methanol at –20°C, washed with white phosphate buffered solution at 1% (WPBS), incubated for 1 h at 37°C with 2 mg ml^{-1} of the monoclonal antibody 8B7 specific for VP28 (Poulos *et al*., 2001), washed and incubated for 1 h at 37°C with 0.02 mg ml–1 of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (F-2761, Molecular Probes) in PBS, washed with PBS, rinsed in deionised water, dried and mounted. Slides are analyzed by fluorescence microscopy (Escobedo-Bonilla *et al*., 2005)

3.13 – Statistical analysis

The cumulative mortality and standard deviation of the 3 experiments were calculated for each treatment. The mean cumulative mortality was analysed by probit, which is a generalized linear model with a probit link function (Agresti 1996). After checking that no significant interactions existed between dose and time, the probit model had the form:

$$\text{Probit}(x) = \alpha + \beta\,(\text{time}) + \gamma\,(\text{treatment}) \qquad (1)$$

where α is the intercept, β is the rate of probability change of time (for a constant treatment), and γ is the rate of probability difference for each treatment (for a constant time).

The statistical software S-PLUS (S-PLUS v. 7.0) was used to calculate the parameters of the regression and to determine the median lethal time (LT_{50}) or the time at which 50% of the tested organisms died (Yi *et al*., 2003) for each treatment. Differences in the LT_{50} of treatments were evaluated by significance treatment in Equation 1 (significance level = 0.05) using the same statistical software.

3.14 – Experiment 1

"Effect of high water temperature (33ºC) before and after inoculation, on White Spot Syndrome Virus (WSSV) infection in pacific with shrimp (*Litopenaeus vannamei*)".

3.14.1 – Experimental design

Three temperature treatments and one control group were setup in this experiment based on water temperature before and after inoculation with WSSV: Water temperature of 33ºC before and after inoculation (Group A); 33ºC before inoculation and 27ºC after inoculation (Group B); 27ºC before inoculation and 33ºC after inoculation (Group C) 27ºC before and after inoculation (Control) (see also Fig. 14). For each temperature regime, there were two groups of shrimp. One group was injected with a low viral dose (30 SID_{50}) and the other with a high viral dose (10000 SID_{50}).

Two more groups were maintained at 27ºC before and after inoculation (infection controls). Of these, one was injected with the low dose and the other one with the high dose. These individuals were euthanized at two different timepoints: at 12 hours post inoculation (five shrimp from each group) and at 24 hours post inoculation (the remaining five shrimp from each group). These shrimp were analysed to prove they were indeed infected with WSSV.

For this experiment in total ten aquaria were used. Each experimental unit (aquarium) was stocked with ten shrimp, giving a total of 100 shrimp. This experiment was repeated three times in time. (table 1).

Clinical signs of disease and mortality were recorded twice a day for 144 hours after virus inoculation. Clinical signs recorded included lethargy, reduction on the food consumption and the absence of response when shrimp were mechanically stimulated.

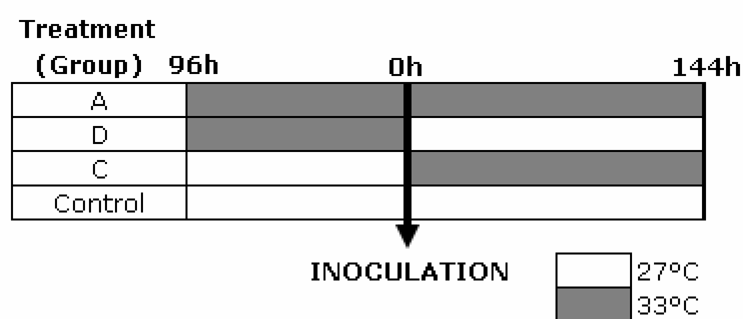

Fig. 14 – Schematic presentation of the different temperature treatments used in experiment 1

Table 1- Resume of the experimental design of experiment 1

Treatment (Group)	Number of shrimp		Temperature (ºC)	
	Low dose inoculation (30 SID50)	High dose inoculation (10000 SID50)	Before inoculation	After inoculation
A	10	10	33	33
B	10	10	33	27
C	10	10	27	33
Control	10	10	27	27
Infection control	10	10	27	27
Total	100			

3.14.2 – Experimental set-up and procedure

Before WSSV inoculation, at the ARC, a total of 110 shrimp were divided into two groups and acclimated to a salinity of 15 gl^{-1} over a period of four days following the procedure described above (section 3.3). During this 4 day acclimation period, one group was maintained at 27 °C, the other at 33 °C.

After acclimation, shrimp were transported to the Lab of Virology and divided over the experimental challenge units. Each experimental unit was composed of a 50-l glass aquarium, a mechanical filter, a heater, an aeration stone and a thermometer (Fig.15).

All the aquaria were filled with brakish water of 15 gl^{-1} salinity. This water was prepared using distilled water and artificial salt (Instant Ocean, Marine Systems).

All the materials used in this experiment were previously disinfected with a powerful disinfectant (AV5, Atlan'Tol Laboratory, Gent, Belgium) and dried during three days to ensure total virus elimination. Also strict biosecurity procedures were followed.

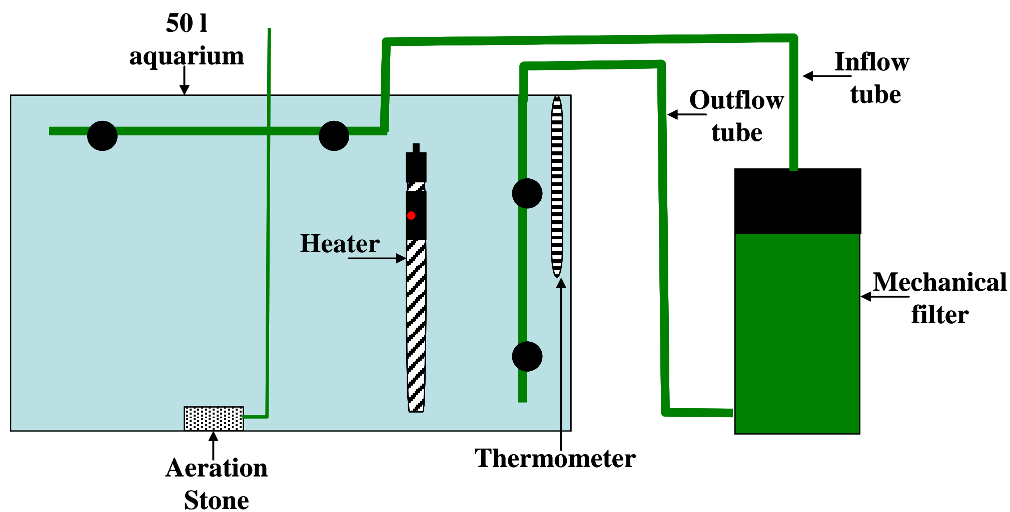

Fig. 15– Diagram of the experimental challenge units.

3.15 – Experiment 2

"Effect of high water temperature (33ºC), on White Spot Syndrome Virus (WSSV) infection in pacific white shrimp (*Litopenaeus vannamei*), when applied at different times (12 and 24h) after virus inoculation."

3.15.1 – Experimental design

Three temperature regimes were used in this experiment: in all regimes, temperature before WSSV inoculation was maintained at 27 °C, but then increased to 33ºC 0 hours after inoculation (Group A), 12 hours after inoculation (Group B) and 24 after inoculation (Group C) (see also Fig. 16) . For all temperature regimes, one group was injected with a low viral dose (30 SID_{50}) and the other with a high viral dose (10000 SID_{50}).

One control treatment was used, where the water temperature was maintained always at 27 °C (before and after inoculation) (Fig. 16).

Two more groups were maintained at 27ºC before and after inoculation (infection controls). Of these, one was injected with a low dose and the other one with a high dose. These individuals were euthanized at two different time points: at 12 hours post inoculation (five shrimp from each group) and at 24 hours post inoculation (the remaining five shrimp from each group). These shrimp were checked with IIF to prove the success of WSSV infection.

In this experiment in total eight aquaria were used. Each experimental unit (aquarium) was stocked with ten shrimp, making a total of 80 shrimp. This experiment was repeated three times.

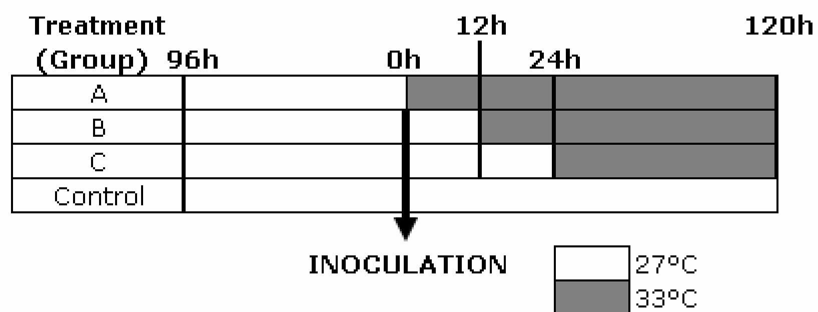

Fig. 16 – Schematic presentation of the temperature regime used in experiment 2

Table 2 – Resume of the experimental design of experiment 2

Treatment (Group)	**Number of shrimp**			
	Low dose inoculation (30 SID50)	**High dose inoculation (10000 SID50)**	**Temperatue before inoculation (ºC)**	**Switch to 33ºC (hours after inoculation)**
A	10	10	27	0
B	10	10	27	12
C	10	10	27	24
Control	10	10	27	–
Infection control	10	10	27	–
Total	100			

3.15.2 – Experimental procedure and set-up

The experimental setup was similar as described for experiment 1 in section 3.13.2.

3.16 – Experiment 3

“Effect of cyclic variation of high water temperature (33ºC), on White Spot Syndrome Virus (WSSV) infection in pacific white shrimp (*Litopenaeus vannamei*).”

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3.16.1- Experimental design

Three different cyclic temperature regimes were used in this experiment. In all treatments, temperature before inoculation and the first 12 hours after inoculation was kept at 27 °C. In the first regime (Treatment 1) , after these initial 12 hours, temperature was increased to 33 °C for 6 hours and again reduced to 27 °C for the following 18 hours; in the second regime (Treatment 2), after the initial 12 hours at 27 °C, first a 12 hour period at 33 °C and then 12 hours at 27 °C were applied; in the third regime (Treatment 3), shrimp were kept for 18 hours at 33 ºC and for only 6 hours at 27 °C. The objective of the initial 12-hour period at 27 °C was to allow for the virus to replicate before shifting to high temperature. Each temperature cycle was then repeated during five days (five cycles - 120 hours). The experiment total time was 132 hours (Fig. 17).

Five controls were used in this experiment. Three controls corresponding to each temperature regime (control 1, 2 and 3), one positive control (Control 4-water always at 27° C) and one negative control (Control 5-water always at 33° C) (see also Fig.17).

One more group was maintained at 27 ºC all the time. In this group, shrimp were euthanized at 12 hours after inoculation. The objective of this group was the same as above described for experiment 1, to confirm WSSV infection.

All the challenged shrimp were inoculated with a viral dose of 10000 SID_{50}, except for the temperature controls, which were inoculated with a saline solution (white PBS). One repetition was made for each of the three temperature regimes. The total number of experimental units was twelve. As each experimental unit consisted of ten shrimp, the total number of challenged individuals was 120 (Table 3).

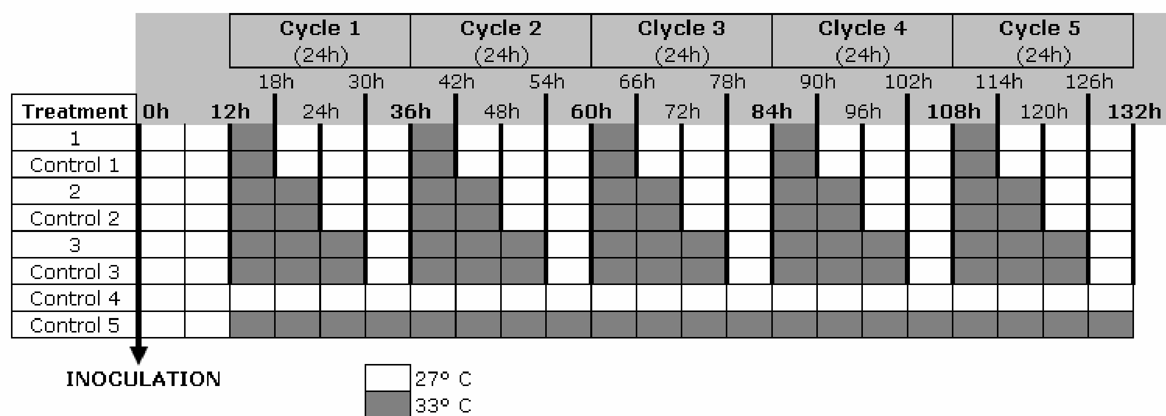

Fig. 17 – Schematic presentation of the temperature regimes used in experiment 3.

Table 3 – Resume of the experiment design of experiment 3

Treatment	**Number of shrimp**	**Exposure time per 24 hours (hours)**		**WSSV inculation at 10000 SID50**
		33ºC	**27°C**	
1	20 (2x10)	6	18	yes
2	20 (2x10)	12	12	yes
3	20 (2x10)	18	65	yes
Control 1	10	6	18	no
Control 2	10	12	12	no
Control 3	10	18	6	no
Control 4	10	0	24	yes
Control 5	10	24	0	yes
Infection control	10	0	24	yes
Total	120			

3.16.2 – Experimental procedure and setup

Before inoculation, a total of 120 shrimp were acclimatized at 27 °C to a salinity of 15 gl^{-1}over four days at the facilities of ARC, following the procedure described above (section 3.3).

Each experimental unit consisted of a 50l glass aquarium, a mechanical filter, two heaters, one aeration stone and one thermometer (Fig.18). After setup, all the aquaria were filled with brakish water of 15 gl^{-1} salinity, prepared as described above (section 3.13.2).

For performing the temperature cycles, both a water warming and a water cooling system was used.

The warming system consisted of two heaters, one calibrated previously at 33ºC, the other was regulated according to the temperature required in the cycle. For warming up the water to 33ºC, both heaters were plugged in, with the second one set for 33ºC. To average time needed to reach 33 °C this way was one hour. Afterwards, the temperature of 33ºC was maintained only with the previously calibrated heater, and the second heater was switched of.

The cooling system was composed of an aquarium pump (Eheim, Germany), a coil made from plastic aquarium tube and a beer cooling machine (Fig. 12). The pump pumped the warm aquarium water in the tube coil through the cooler and returned it to the aquarium. The beer cooling machine was set at 4ºC. For reducing the temperature the pump was turned on, and disconnected when the temperature reached 27ºC. On average, this process took 50 minutes. To maintain the water temperature at 27 °C, the variable temperature heater was then set at 27ºC and the 33ºC calibrated heater disconnected.

All the materials used in this experiment were previously disinfected with a powerful disinfectant (AV5, Atlan'Tol Laboratory, Gent, Belgium) and dried during three days to ensure total virus elimination. Strict biosecurity procedures were followed.

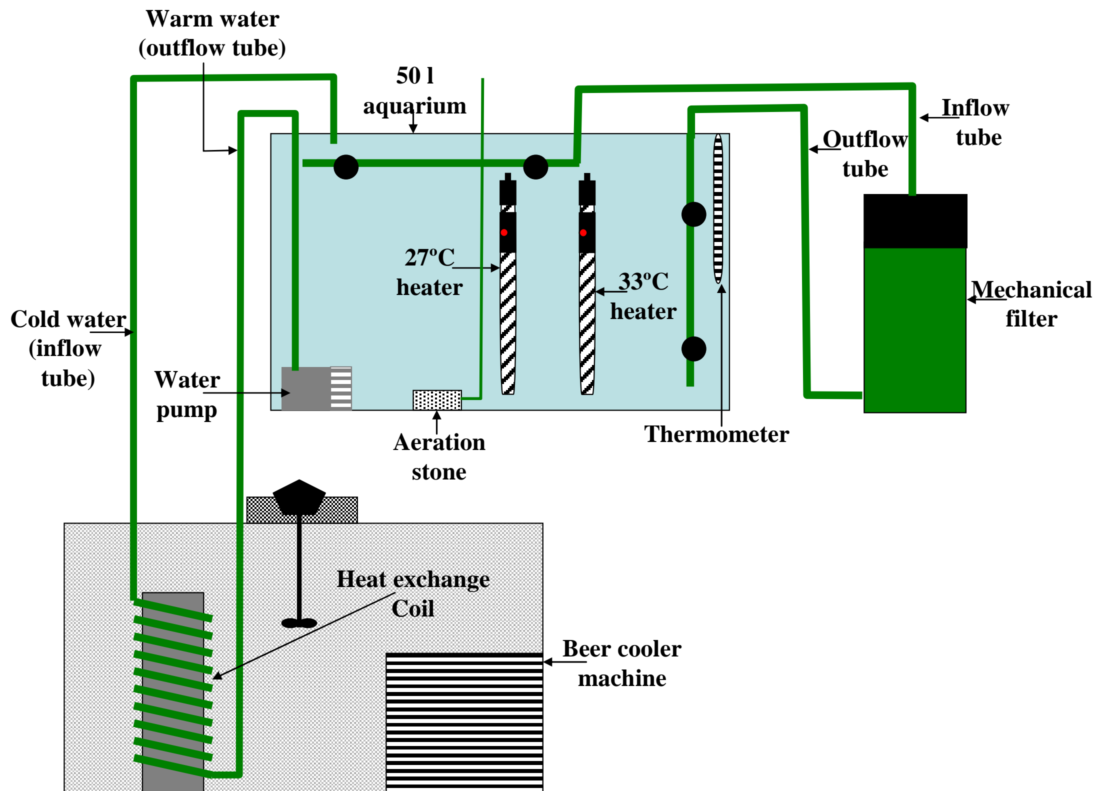

Fig. 18 – Schematic outline of the set-up used in experiment 3, with details of warming and cooling devices.

CHAPTER 4 – RESULTS

4.1 – Experiment 1

"Effect of high water temperature (33ºC), on White Spot Syndrome Virus (WSSV) infection in pacific with shrimp (*Litopenaeus vannamei*)."

4.1.1 - Clinical signs

Shrimp showed the first clinical signs of disease (lethargy and reduction of food consumption) in Group B (33/27ºC) and the Control (27/27ºC) at 24-36 hours after inoculation. Those injected with a high dose (10000 SID_{50}) showed in first place the clinical signs earlier (24h), and then those inject with low dose (30 SID_{50}) (36h). Lack of response to mechanical stimulus was usually the last clinical signs observed, and preceded death only 6 hours in general.

4.1.2 – Mortality

The first dead shrimp were found in Group B (33ºC/27ºC), Group C (27ºC/33ºC) and Control (27ºC/27ºC) injected with both low viral dose (30 SID_{50}) and high viral dose (10000 SID_{50}), at 36 hours post inoculation. In Group A (33ºC/33ºC), the first dead were only found at 60 hours when injected with a low dose, and at 80 hours when injected with a high dose (Fig. 19 and 20).

When injected with a low dose, Group B (33ºC/27ºC) and Control (27ºC/27ºC) reached 100% mortality at 96 and 144 hours post inoculation respectively. With the same dose, Group A (33ºC/33ºC) and C (27ºC/33ºC) reached a final cumulative mortality of 3% and 10% at 60 and 108 hours respectively (Fig. 19 and Table 4).

When injected with a high dose, Group B (33ºC/27ºC) and Control (27ºC/27ºC) both reached a final cumulative mortality of 100% at 60 hours post inoculation. With the same

dose, Group A (33ºC/33ºC) and C (27ºC/33ºC) reached the final mortality of 6% and 10% at 84 and 108 hours respectively (Fig. 20 and Table 4).

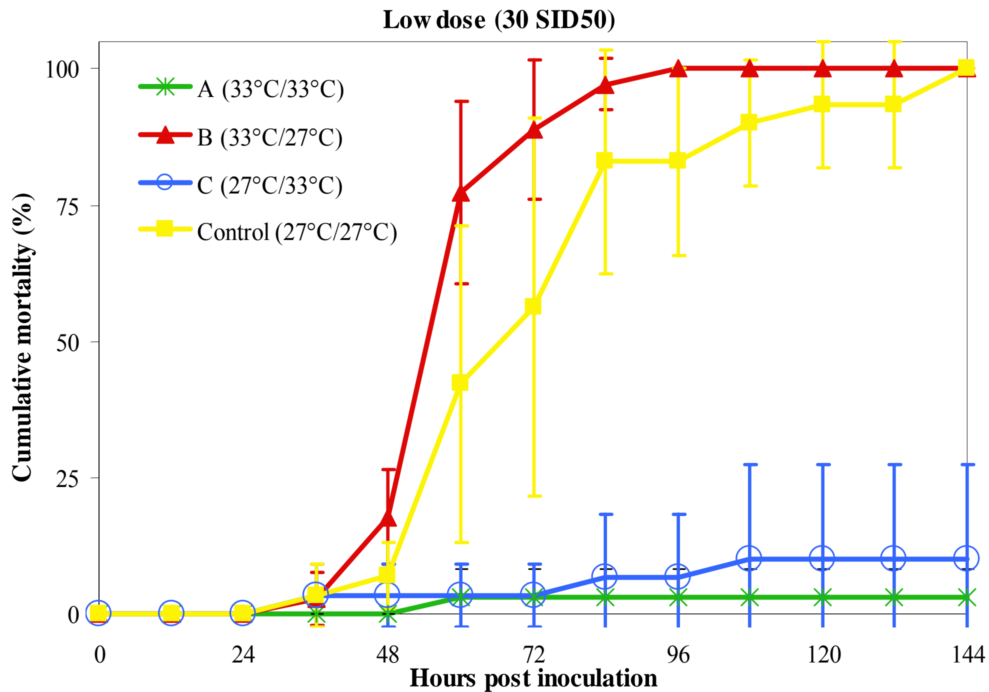

Fig. 19 – Cumulative mortality of shrimp challenged with a low dose (30 SID_{50}), subjected to different temperature regimes (Experiment 1)

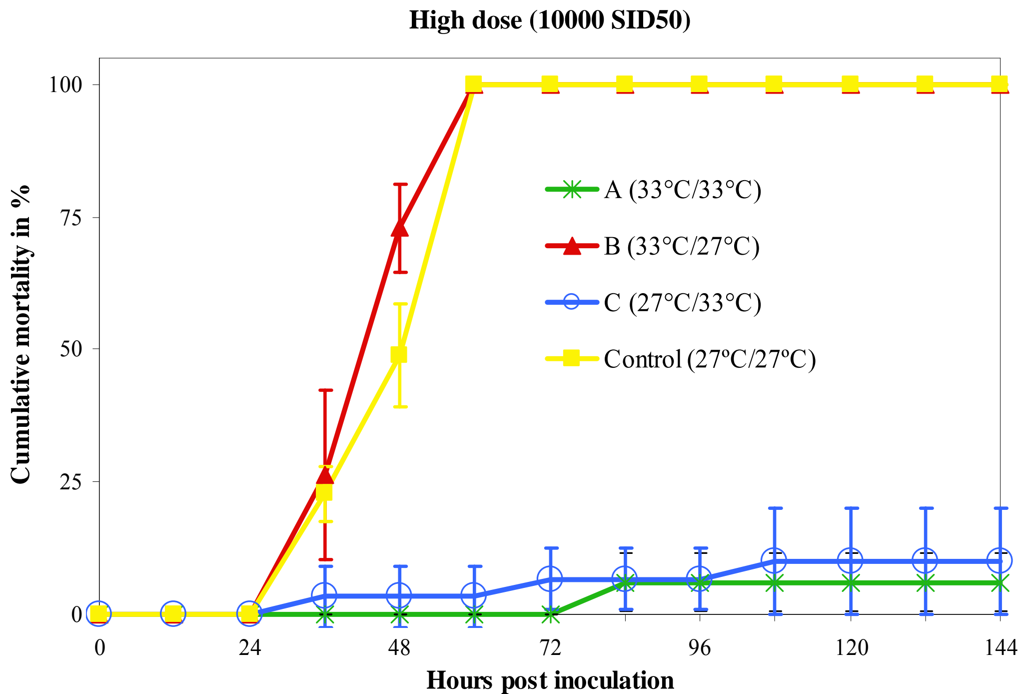

Fig. 20 - Cumulative mortality of shrimp challenged with a high dose (10000 SID_{50}), subjected to different temperature regimes (Experiment 1)

Table 4 – Cumulative mortality (%) found at hpi of shrimp inoculated with a low and high dose (30 and 10000 SID50), of WSSV subjected to different temperature regimes (experiment 1)

	Group	Cumulative mortality (%) (average from 3 repetitions)
Low dose (30 SID50)	A (33ºC/33ºC)	3
	B (33ºC/27ºC)	100
	C (27ºC/33ºC)	10
	Control (27ºC/27ºC)	100
High dose 10000 SID50	A (33ºC/33ºC)	6
	B (33ºC/27ºC)	100
	C (27ºC/33ºC)	10
	Control (27ºC/27ºC)	100

4.1.3 – WSSV detection by Indirect Immunofluorescence (IIF)

By detection with Indirect Immunofluorescence (Fig. 21), all dead shrimp found in the groups maintained at 27ºC after inoculation (Group B and Control) were WSSV positive, for both high and low WSSV dose. All survivors and dead shrimp found in groups maintained at 33ºC after inoculation (Group A and C) were WSSV negative, for both high and low dose. In all the infection controls euthanized at 12 and 24 hours, all the individuals were WSSV positive

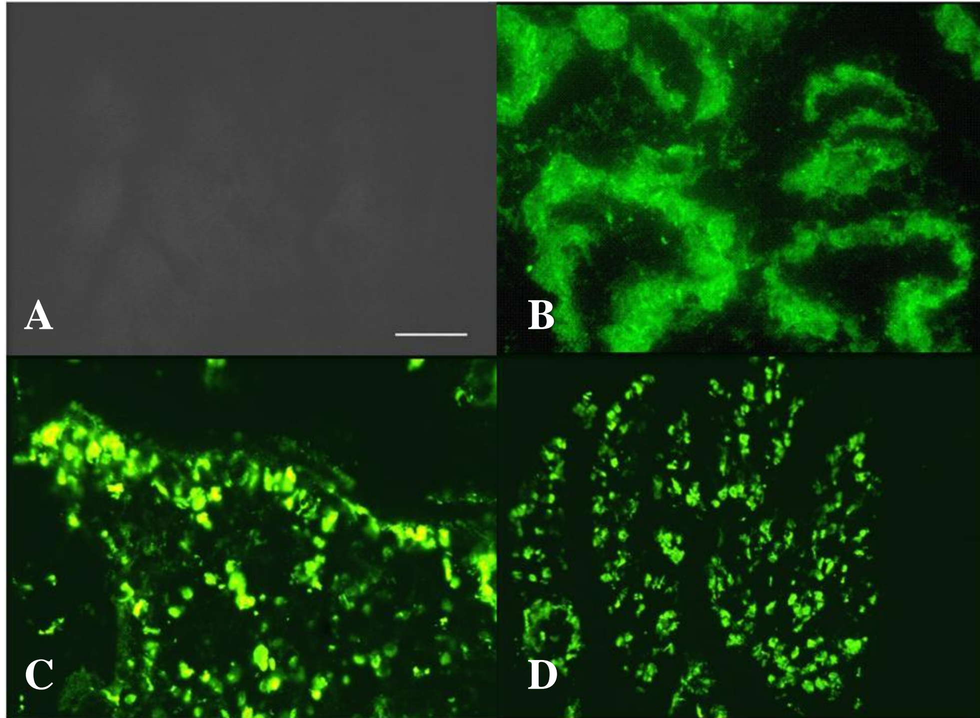

Fig. 21 – Indirect immunofluorescence microphotography showing infected and non-infected tissue from different body parts. A – uninfected tissue; B- Infected tissue form lymphoid organ; C - Infected tissue from stomach; D - Infected tissue from gill.

4.2 - Experiment 2

"Effect of high water temperature (33ºC) on White Spot Syndrome Virus (WSSV) infection in pacific white shrimp (*Litopenaeus vannamei*), when applied at different times (0, 12 and 24h) after virus inoculation."

4.2.1 - Clinical signs

Shrimp showed the first clinical signs of disease (lethargy and reduction of food consumption) in Group C (27ºC/33ºC-24hpi) and Control (27-27ºC) at 24-36 hours after inoculation. Those injected with high a dose (10000 SID_{50}) showed generally the clinical signs earlier (24h), than those injected with a low dose (30 SID_{50}) (36h). Lack of response to mechanical stimulus was shown only 6 hours before the recorded death.

4.2.2 – Mortality

The first dead shrimp were found at 36 hours in all the groups, both when injected with low (30 SID_{50}) and high dose (10000 SID_{50}).

In the groups injected with low dose, a final cumulative mortality of 10% was reached by the groups A (27ºC/33ºC-0hpi) and B (27ºC/33ºC-12hpi), at 108 hour post inoculation. Group C (27ºC/33ºC-24hpi) reached a final cumulative mortality of 24% at 84 hours post inoculation. At 144 hours post inoculation, the Control (27ºC/27ºC) reached 100% mortality (Fig. 22).

When injected with high dose, Group A reached a final cumulative mortality of 10% at 108 hours post inoculation, Group B reached 7% at 60 hours, Group C reached 90% at 84 hours and the Control reached 100% at 72 hours (Fig. 23).

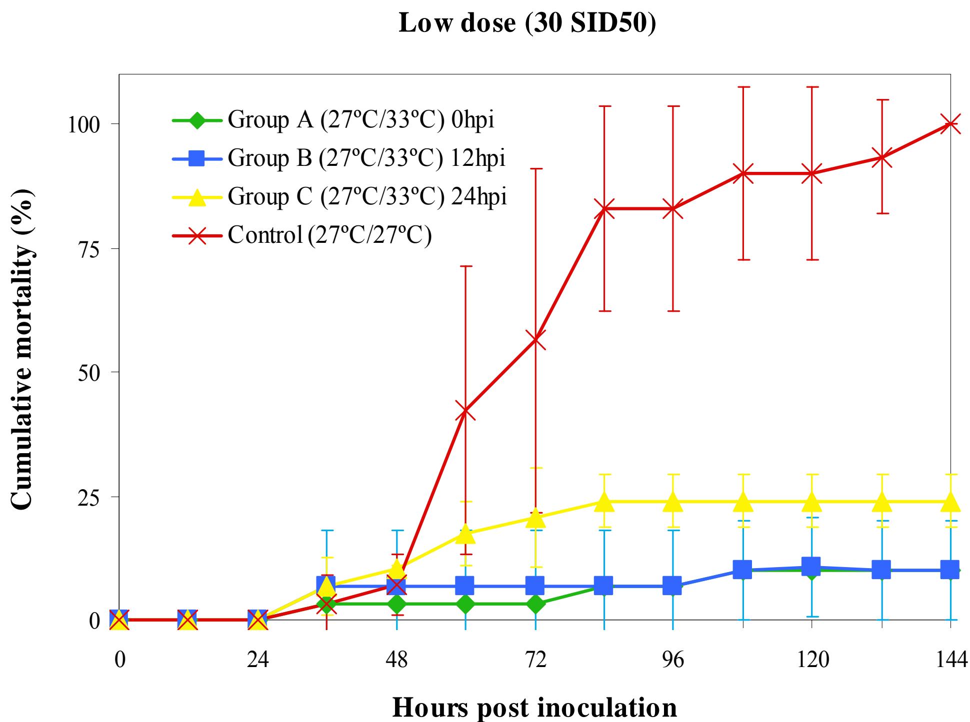

Fig. 22 – Cumulative mortality of shrimp challenged with a low dose (30 SID_{50}), subjected to different temperature regimes (Experiment 2)

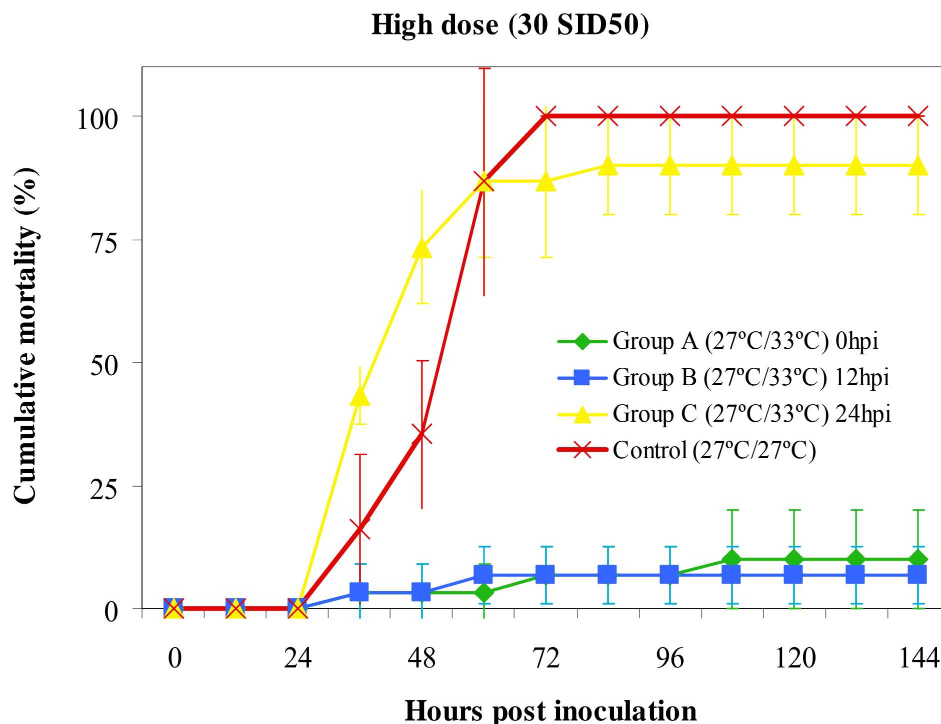

Fig. 23 – Cumulative mortality of shrimp challenged with a high dose (10000 SID_{50}), subjected to different temperature regimes (Experiment 2)

Table 4 – Percentage of cumulative mortality found for different temperature treatments in experiment 2

	Group	cumulative mortality (%) (average from 3 repetitions)
Low dose (30 SID50)	A (27ºC/33º-0hpi)	10
	B (27ºC/33ºC-12hpi)	10
	C (27ºC/33ºC-24hpi)	24
	Control (27ºC/27ºC)	100
High dose 10000 SID50	A (27ºC/33ºC-0hpi)	10
	B (27ºC/33ºC-12hpi)	7
	C (27ºC/33ºC-24hpi)	90
	Control (27ºC/27ºC)	100

4.2.3 – WSSV detection by Indirect Immunofluorescence (IIF)

By detection with Indirect Immunofluorescence, all analysed shrimp (dead and survivors) from the Group A (27ºC/33ºC- 0hpi) and Group B (27ºC/33ºC- 12hpi), either

injected with low and high dose, were WSSV negative. In the Group C (27ºC/33ºC- 24hpi) and Control (27ºC/27ºC) injected with low and high dose, all dead shrimp were WSSV positive and the survivors were WSSV negative. The analysed shrimps from the infection controls were WSSV positive.

4.3 - Experiment 3

“Effect of cyclic variation of water temperature (27ºC/33ºC), on White Spot Syndrome Virus (WSSV) infection in pacific white shrimp (*Litopenaeus vannamei*).“

4.3.1 - Clinical signs

Shrimp showed the first clinical signs of disease (lethargy and reduction of food consumption) at 24 h in Treatment 1 (6h at 33ºC) and Control 4 (24h at 27ºC), and at 36 hours in Treatment 2 (12h at 33ºC).

4.3.2 – Mortality

The first dead shrimp were found at 36 hours post inoculation in Treatment 1 (6h at 33ºC) and Control 4 (24h at 27ºC). In Treatment 2 (12h at 33ºC) the first dead shrimp were found at 48 hour post inoculation (Fig. 24).

Both Treatment 1 (6h at 33ºC) and Control 4 (24h at 33ºC) reached 100% of mortality at 60 and 96 hours post inoculation respectively. Treatment 2 (12h at 33ºC) attained 90% mortality at 96 hours. At 36 hours, dead shrimp was found in Control 3 (18h at 33ºC) (Table 5 and Fig. 24). In the rest of the groups no mortality was recorded.

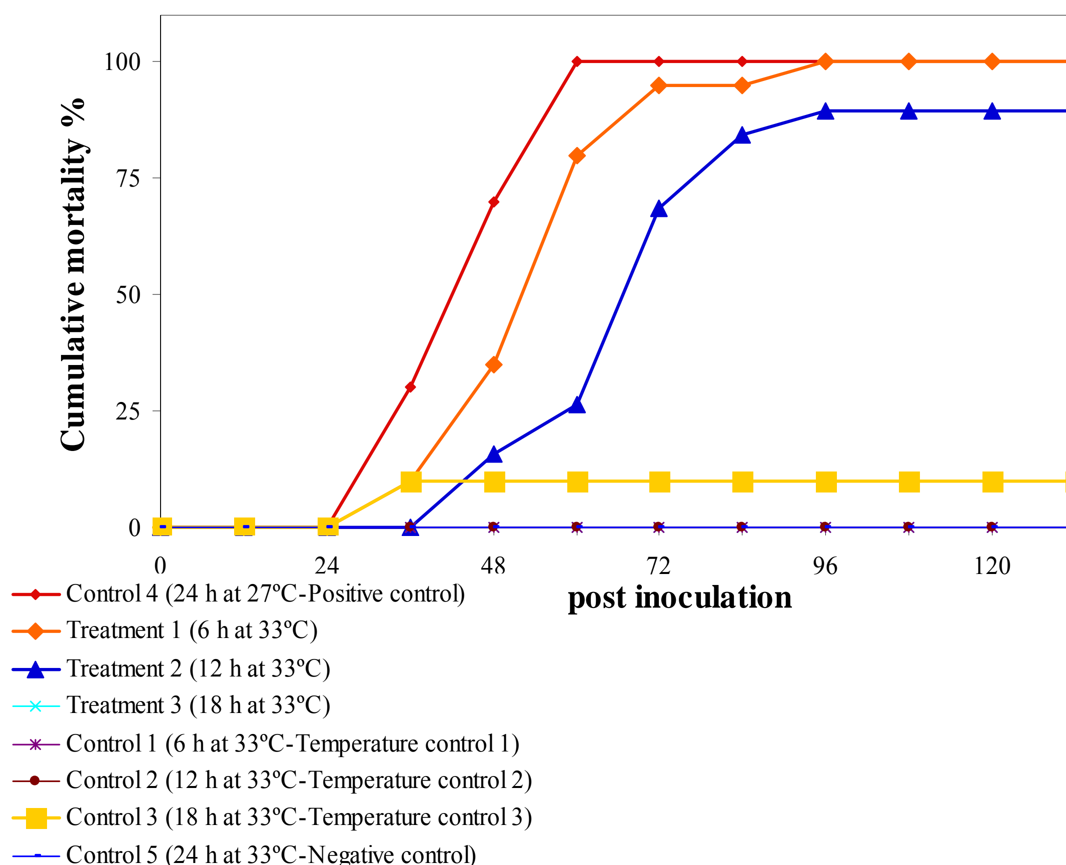

Fig. 24 – Cumulative mortality of shrimp challenged with to different cyclic temperature regimes (Experiment 3)

Table 5 - Percentage of cumulative mortality found for different temperature treatments cycles in experiment 3

Treatment	Time/temperature exposure	cumulative mortality (%)
1	6h at 33ºC	100
2	12h at 33ºC	90
3	18h at 33ºC	0
Control 1	6h at 33ºC-Temperature control 1	0
Control 2	12h at 33ºC- Temperature control 2	0
Control 3	18h at 33ºC - Temperature control 3	10
Control 4	24h at 27ºC-Positive control	100
Control 5	24h at 33ºC-Negative control	0

4.3.3 – WSSV detection by Indirect Immunofluorescence (IIF)

By immunofluorescence analysis all shrimp found in the Treatment 1 (6h at 33ºC) and Control 4 (24h at 27ºC) were WSSV positive. In Treatment 3 (18º at 33ºC) and temperature Control 1 (6h at 33ºC), 2 (12h at 33ºC), 3 (18h at 33ºC) and Control 5 (24h at 33ºC), all shrimp were WSSV negative, even the dead shrimp found in Control 3. In Treatment 2 (12h at 33ºC), the survivors were WSSV negative and the dead ones WSSV positive.

CHAPTER 5 – DISCUSSION

5.1 - Challenge model

Although the oral and waterborne route are considered the natural way of infection for WSSV in cultured shrimps (Chang et al, 1996), there still doesn't exist a well standardized method for using these virus inoculation methods accurately under experimental conditions. However, the method of intramuscular inoculation was described as being a good alternative (Vidal et al., 2001; Guan et al., 2003; Jiravanichpaisal et al., 2004; Escobedo-Bonilla et al., 2005). This method especially allows to obtain reproducible results between experiments.

Although the viral load in shrimp production conditions is still unknown, two extreme doses were used. A viral dose of 30 SID_{50} was estimated the minimum concentration needed to reach 100% mortality in the inoculated individuals (Escobedo-Bonilla et al., 2005). It was calculated that 10000 SID_{50} is the viral content of one gram of tissues from shrimp that died because of WSSV infection. So roughly, this dose might correspond to the viral dose taken by a shrimp when practising cannibalism in culture conditions.

A temperature of 27ºC was used as control, since this is the ideal temperature for culturing *Litopenaeus vannamei*. 33 ºC, although above the optimum temperature for *L. vannamei*, was determined by previous authors (Vidal et al., 2001; Guan et al., 2003) as being suitable to protect shrimp from WSSV infection.

5.2 – Experiment 1

In experiment 1, median lethal time (LT_{50} obtained by Probit analysis) in Group A (33ºC/33ºC), Group B (33ºC/27ºC) and Group C (27ºC/33ºC) injected with low dose, was significantly different ($P < 0.05$) from the Control (27ºC/27ºC). The LT_{50} in Treatment A and Treatment C were significantly higher than that of the Control, wheras in Group B it was significantly lower than in the Control. This means that, tanking into account both the percentage cumulative mortality and the mortality rate, Group A and C were able to significantly reduce the mortality progression when compared with the control, and oppositely in Group C mortality progression was increased.

When considereing the final cumulative mortality in Group B (33ºC/27ºC), it is obvious that previous (before inoculation) exposure to 33ºC, can not protect the shrimp from WSSV. It was even noted that shrimp in Group B had a higher mortality rate compared with the Control, which maybe can be attributed to additional susceptibility to WSSV due to temperature changing.

From a practical point of view, when considering the final cumulative mortalities, only the treatments A an C would be suitable for field application, or in other words, can effectively prevent mortality in WSSV infected shrimps. Group A and C were not significantly different from one another ($P > 0.05$), which again demonstrates that is not necessary to maintain high water temperature before inoculation, in order to obtain high survival.

Statistical analysis obtained from the groups injected with a high dose gave similar results to the low dose injected groups. Comparing the final cumulative mortality between all groups, it was noticed that the protection provided by high water temperature was not dependent on the viral dose. This assumption is valid at least for hyperthermia application immediately afterwards virus inoculation. Oppositely, a higher mortality rate was observed in Group B (33ºC/27ºC) and the Control (27ºC/27ºC) injected with a high dose compared with the same groups injected with a low dose. This fact suggests that mortality rate is dependent on the administrated viral dose.

By indirect immunofluorescence analysis, the capacity of hyperthermia to protect WSSV infected shrimp was confirmed, since both surviving and dead animals found in Treatment A (33ºC/33ºC) and C (27ºC/33ºC) were found to be WSSV negative. This means that the mortality obtained in these treatments originated from other reasons than WSSV infection. From this, it can also be concluded that high water temperature can totally block WSSV infection, when applied at least at the time of infection.

5.3 – Experiment 2

In Experiment 2, when challenged with a low viral dose, Group A (27ºC/33ºC-0hpi), Group B (27ºC/33ºC-12hpi) and Group C (27ºC/33ºC-24hpi), displayed a significantly different mortality pattern ($P < 0.05$) from the Control (27ºC/27ºC). No significant differences ($P > 0.05$) were found between Group A, B and C themselves. This means that

all treatments were equally able to significantly reduce mortality compared with the control. This also suggests that, when applying a low viral dose, even after allowing a period of viral replication of 12 or 24 hours, high water temperature can reduce mortality of previously WSSV infected shrimp.

The statistical analysis output obtained for the high dose injected groups was similar to those injected with a low dose in that all groups were significantly different ($P < 0.05$) from the Control. The mortality pattern in Treatment C was however also different from the one in Treatment A and B.

When comparing the final cumulative mortality obtained for Group A (27ºC/33ºC-0hpi) and B (27ºC/33ºC-12hpi), injected with high and low dose, it is clear that the protective affect of high temperature was not dose dependent in this case. Oppositely, mortality in Group C (27ºC/33ºC-24hpi) was clearly dose dependent. A practical interpretation of the previous results, suggests that hyperthermic treatment is not effective to prevent mortality after a longer period of viral replication (24 hours) when inoculated with a high viral dose (10000 SID_{50}). A higher mortality rate was also observed in the high dose Control (27ºC/27ºC), when compared with the low dose Control. From this, it can be concluded that the mortality outcome in WSSV infected shrimps under normal temperature conditions, is dependent on the inoculation dose.

The high mortality obtained in Group C (27ºC/33ºC-24hpi) when injected with a high dose, can maybe be explained from the cellular damage caused during this first 24 hour “incubation period”. When susceptible shrimp are submitted to a large period of viral replication (24 hours), even if afterwards hyperthermic treatment is applied, the cellular damage can already be too high for the shrimp to recover.

By indirect immunofluorescence analysis, the capacity of hyperthermia for protecting WSSV infected shrimp was confirmed as both in Group A (27ºC/33ºC-0hpi) and B (27ºC/33ºC-12hpi), both dead and surviving were WSSV negative. The results from the analysis of Group C (27ºC/33ºC-24hpi), demonstrate that the survivors were effectively protected from WSSV, as they were WSSV negative both for high and low dose. On the other hand all the dead shrimp in this treatment were WSSV positive, which confirms that under the conditions of this specific treatment, hyperthermia could not fully protect the animals.

5.4 – Experiment 3

For Experiment 3, no statistical analysis could be made, since it was only performed once, and consequently there are not enough data available for a correct statistical analysis.

Nevertheless, from the results it is clear there is a big difference between final cumulative mortality in Treatment 3 (18 hours at 33ºC), where no mortality was observed, and the Control 4 (27ºC/27ºC) were 100% mortality was reached. In Treatment 1 (6 hours at 33ºC) and Treatment 2 (12 hours at 33ºC) mortalities also amounted to 100% and 90% respectively, but mortality seemed to be delayed somewhat. These results seem to indicate that Treatment 3 is the only effective treatment for preventing mortality in WSSV infected shrimps, and thus a minimum of 18 hours per day at 33 °C is necessary. However, in the previous experiments, sometimes significant differences were found between the same temperature treatment (e.g. treatment 27ºC/33ºC 24hpi), when inoculated with a low or high viral dose. Because of this, and considering that only a high viral dose was used in experiment 3, it could be that Treatment 1 and/or Treatment 2 would be effective, if a low viral dose is used.

In this experiment, it was also proven the cyclic temperature variation and the inoculation procedure as such didn’t result in significant mortality, since in Control 1, 2 and 3 only one dead animal was found.

By indirect immunofluorescence analysis, it was confirmed that by maintaining the shrimp in daily cycles of 18 hours at 33ºC, WSSV infection can be prevented, as all the analysed shrimp were WSSV negative. In Treatment 2, all the dead shrimp were positive and oppositely, survivors were negative. This finding reconfirms the idea that the repetition of this temperature cycle with a low viral dose might result in higher survival rate, as even with a high dose this treatment proved capable to protect some infected shrimp. It was also confirmed that the dead individual found in Control 3 was not infected, which rejects the hypothesis of viral contamination of the control. Finally, all shrimp analysed in the infection control were WSSV positive, which proves the success of the inoculation procedure.

5.5 – General discussion

The logical sequence of experiments executed in the present study, clearly demonstrates the effectiveness of high water temperature (33ºC) to control WSSV infection in the pacific white shrimp (*Litopenaeus vannamei*), which confirms the results of previous studies on this subject (Vidal et al., 2001; Guan et al., 2003).

Data on the influence of temperature on WSSV infection are limited to only a few studies. To our knowledge, no information on the influence of factors such as virus dose, duration of virus replication before applying hyperthermia or the use of daily temperature variation existed. In the current study very conclusive results on these factors were obtained.

It could be proven that high water temperature can prevent mortality of WSSV infected shrimp, when the treatment is applied until 24 hours after virus inoculation, but only if the virus input is low (30 SID_{50}). With a higher virus input (10000 SID_{50}), temperature treatment was effective only when applied until 12 hours after inoculation. This could have been expected from the work of Escobedo-Bonilla (2005), which suggests the virus replication cycle takes approximately 12 hours. This also demonstrates that there is an influence of the viral dose on the progression of White Spot Disease. Also it has been clearly demonstrated that is not necessary to maintain high water temperature before virus inoculation. These facts reveal the potential of temperature manipulation, not only as a preventive measure, but to a certain extent, also as therapeutic measure.

Although the WSSV replication mechanism is still largely unknown, the influence of high temperature on viral replication has been reported (Yang, 1990). This can give some clues about how high water temperature affects WSSV infection. Most probably, high temperature (33ºC) inhibits virus replication by affecting some biological component (from the virus or from the shrimp) necessary in viral replication. The results of the present study, although not conclusive in this matter, suggests that the effect on mortality originates from inhibition of viral replication and not from a shrimp immunity response when induced to exposure to high water temperature. Present knowledge that the crustacean immune system, although very strong and efficient, does not possesses the capacity or specificity for responding to viral infections, supports this.

According to Vidal et al. (2001), when the virus inoculum itself was exposed to 33ºC during one hour, and then inoculated by intramuscular route in *Litopenaeus vannamei*,

100% mortality was obtained. The same author also reported that after keeping WSSV inoculated shrimp 40 days at high temperature (33ºC), when the temperature was reduced to 26ºC, 100% mortality was reached. This can to some extent explain the mortality obtained in Experiment 3, as similarly in our experiment shrimp were exposed to periods of high water temperature following by periods of low temperature. Where during high water temperature exposure, the virus is inhibited, but not inactivated (killed), when temperature is reduced to a lower level (27ºC) the virus becomes active again, being able to induce cellular damage.

Some studies however pointed out other ways in which high water temperature may influence the mortality of WSSV infected shrimp. It was reported that high temperature induces a higher rate of apoptosis in shrimp cells, which could be responsible for WSSV control in infected shrimp (Granja et al., 2003). Also it was suggested by Vidal et al. (2001), that several biologically active proteins, such as heat shock proteins, might modify the shrimp immune response to virus infection. Knowing that this kind of proteins have a considerable live time in the organism once produced, our study rather invalidates this hypothesis. After all, if heat sock proteins had some influence in WSSV infection control, then Group B (33ºC/27ºC) in experiment 1 probably should have had a better survival rate, as the shrimp were previously exposed to 33ºC water.

CHAPTER 6 – CONCLUSIONS

The main objective of this study was to further document the influence of high water temperature (33ºC) on survival of WSSV infected shrimp.

In Experiment 1, the effectiveness of high water temperature to control the mortality of shrimp infected with WSSV was clearly demonstrated. When temperature treatment is applied immediately after virus inoculation, the protection is effective even when submitting the shrimp to a very high viral dose.

In Experiment 2, it was shown that hyperthermic treatment has not only potential as a preventive intervention, but also to some extent as a therapeutic measurement. In this respect it was shown that hyperthermia, when applied 24 hours after inoculation with a low virus dose, was still effective. This experiment however also pointed out an important influence of the viral dose on outcome of the infection. For example, at a high virus dose, hyperthermia should be applied within the first 12 hours after inoculation.

Experiment 3, although results should be considered preliminary, suggest that high water temperature treatment could possibly be applied in a regime of daily cycles, and consequently opens new perspectives for further scientific work on this subject. When shrimp were subjected to a high virus dose, a minimum of 18 hours per day at 33 °C were necessary to offset mortality due to infection. Possible shorter periods at 33 °C would be required if shrimp were inoculated with a lower dose however.

The above clearly proves the effectiveness of high water temperature for preventing mortality in WSSV infected shrimp, and consequently the potential to apply management practices that increase water temperature in ponds to manage this devastating disease. However, under shrimp farming conditions, maintaining high water temperature for long periods of time, might not be economically feasible. Applying cyclic high temperature periods in the culture ponds, using solar energy during the daytime, may be a more realistic alternative. As in tropical or subtropical areas, water temperature can reach 33°C or higher for several hours during the day, but drops below 30°C during the night, this strategy might be feasible in the field, at a lower cost. In order to validate this assumption, the effect of cyclic temperature variations should be further documented (e.g. with different virus dose). Also climate conditions in shrimp producing countries should be studied. In order to obtain better conclusions about this matter, it would also be interesting to define the real load of virus that shrimp are exposed to in the field. These data would improve the design of

further challenge models, and in that way obtain more realistic results with better application in the field. Also it will be of major interest to direct research into the characterization of viral replication mechanisms, and more specifically on the influence of temperature on WSSV viral replication at the molecular level. This kind of knowledge can be decisive for the development of strategies for controlling WSSV outbreaks with temperature manipulation.

The technical aspects of temperature manipulation in growout ponds should also be looked at. As one of the major natural resource in shrimp producing countries is the solar power, the question will be how to apply it to increase the temperature in culture ponds. Naturally, this will be an engineering question.

REFERENCES

Agresti, A. (1996). An introduction to categorical data analysis. John Wiley & Sons, New York.

Alday de Graindorge, V. and Griffith, D. (2000). Ecuador. In: Thematic review on Management Strategies for Major Diseases in Shrimp Aquaculture. A component of the WB/NACA/WWF/FAO Program on Shrimp Farming and the Environment. Report of the workshop held in Cebu, Philippines from 28-30 November 1999. 17-19pp.

Amend, D.F. (1970). Control of infectious hematopoietic necrosis virus disease by elevating the water temperature. J Fish Res Board Canada, 27:265-70.

Bailey-Brock, J.H. and Moss, S. M. (1992). Penaeid taxonomy, biology and zoogeography. pp. 9-27. In Marine shrimp culture: principles and practices. Elsevier Science Publishers.

Bangyeekhun, E. (2002). Parasite on Crayfish, Characterisation of Their Pathogenesis, Host Interaction and Diversity. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 737. ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2002.

Bauchau, A. G. (1981). Crustaceans. In: Ratcliffe N. A. & Rowley, A. F. (editors). Invertebrate blood cells.Academic Press, London and New York, pp. 385-420.

Bartlett, T., Cuthbertson, B. J., Shepard, E., Chapman, R., Gross, P., Warr, G. (2002). Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, Litopenaeus vannamei and Litopenaeus setiferus. Mar. Biotechnol. 4:278–293.

Bell, T.A. and Lightner, D.V. (1988). A handbook of normal penaeid shrimp histology. The World Aquaculture Society, Baton Rouge, 114 pp.

Boman, H. (1995). Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. 13:61–92.

Bondad-Reantaso, M.G., McGladdery, S.E., East, I,, Subasinghe, R,P, (2001). Asia diagnostic guide to aquatic animal diseases. FAO Fish Tech Pap No 402, Suppl 2.

Briggs, M., Funge-Smith, S., Subasinghe, R., Phillips, M. (2004). Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. Food and Agriculture Organization of the United Nations Regional Office for Asia and the Pacific, Bangkok. RAP publication 2004/10.

Calderon, J., Bayot, P., Betancourt, I., Alday de Graindorge, V. (2000). Monitoring study of White Spot Syndrome Virus in Ecuador. AQUA 2000. Nice, France. European Aquaculture Society. Special Publications NO. 28: 106pp.

Cardenas, W. and Dankert, J.R. (2000). Cresolase, catecholase, and laccase activities in haemocytes of the red swamp crayfish: Fish and Shellfish Immunology, 10: 33-46.

Cardenas, W., Jenkins, J.A., Dankert, J.R. (2000). A flow cytometric approach to the study of crustacean cellular immunity. Journal of Invertebrate Pathology, 76 (2): 112-119.

Castric, J. and De Kinkelin, P. (1984). Experimental study of the susceptibility of two marine fish species, sea bass Dicentrarchus labrax and turbot Scophthalmus maximus, to viral hemorrhagic septicemia. Aquaculture, 41:203-12.

Cerenius, L. and Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. Immonol Rev, 198: 72-82.

Chang, C.F., Su, M.S., Chen, H.Y., Liao, I.C., (2003). Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish Shellfish Immunol., 15: 297–310.

Chang, P.S., Lo, C.F., Wang, Y.C., Kou, G.H., (1996). Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* in situ hybridization. Dis. Aquat. Org., 27: 131–139.

Chisholm, J. R. S. and Smith, V. J. (1992). Antibacterial activity in the hemocytes of the shore crab Carcinus maenas. J. Mar. Biol. Assoc., 72: 529–542.

Chou, H.Y., Huang, C.Y., Lo, C.F., Kou, G.H. (1998). Studies on transmission of White Spot Syndrome associated Baculovirus (WSBV) in *P. japonicus* via waterborne contact and oral ingestion. Aquaculture, 164: 263-276.

Chou, H.Y., Huang, C.Y., Wang, C.H., Chiang, H.C., Lo, C.F. (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Org., 23: 165–173.

Chotigeat, W., Tongsupa, S., Supamataya, K., Phongdara, A. (2004). Effect of fucoidan on disease resistance of black tiger shrimp. Aquaculture, 233: 23–30.

Dall, W. (1967). The functional anatomy of the digestive tract of a shrimp, Metapeneaus bennettae Racek and Dall (Crustacea: Decapoda: Penaedae). Aust J Zool, 15: 699-714.

Destoumieux, D., Bulet, P., Loew, D., VanDorsselaer, A., Rodriguez, J., Bachere, E. (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp Penaeus vannamei. J. Biol. Chem., 272: 28398–28406.

Dore, I. and Frimodt, C. (1987). An Illustrated Guide to Shrimp Of The World. Osprey Books, Huntington, NY, U.S.A., 229 pp.

Dorson, M. and Torchy, C. (1981). The influence of fish age and water temperature on mortalities of rainbow trout, Salmo gairdneri Richardson, caused by a European strain of infectious pancreatic necrosis virus. J Fish Dis, 4: 213-21.

Duran, S., Lightner, D.V., Redman, R.M., Bonami, J.R. (1997). Ultrastructure and morphogenesis of White Spot Syndrome Baculovirus (WSSV). Diseases of Aquatic Organisms, 29: 205-211.

Durand, S., Lightner, D.V., Nunan, L.M., Redman, R.M., Mari, J., Bonami J.R. (1996). Application of gene probes as a diagnostic tool for the white spot baculovirus (WSBV) of penaeid shrimp. Dis. Aquat. Org., 27: 59-66

Epand, R., and Vogel, H. (1999). Diversity of antimicrobial peptides and their mechanisms of action. Biochim. Biophys. Acta, 1462:11–28.

FAO (2002). The State Of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations, Rome, 2002.

FAO (2003). Health management and biosecurity maintenance in white shrimp (*Panaeus vannamei*) hatcheries in Latin America. FAO fisheries technical paper 450, Rome 2003.

FAO (2004). Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. RAP publication 2004/10.

FAO (2004). The state of world fisheries and aquaculture - SOFIA 2004. Food and Agricultural Organization of the United Nations, Rome, 2004.

Fegan, D., Arthur, J.R., Subasinghe, R.P., Reantaso, M.B., Alday de Graindorge, V., Phillips, M.J. (2001). Consultant report: A review of transboundary aquatic animal pathogen introductions and transfers. In: Report of the Puerto Vallata Expert Consultation. APEC/FAO/NACA/SEMERNAP, 2001. 132-175pp.

Flegel, T.W. (2001). The shrimp response to viral pathogens. in: C.L. Browdy and D.E. Jory (Eds.). The new wave, Proceedings of the special session on sustainable shrimp culture. Aquaculture 2001. The world aquaculture society. Baton Rouge, LA USA., 190-214pp.

Flegel, T.W. and Alday-Sanz, V. (1998). The crisis in Asian shrimp aquaculture: current status and future needs. Journal of Applied Ichthyology., 14: 269-273.

Fox, R. (2001). Invertebrate Zoology Laboratory Exercises, *Farfantepenaeus aztecus* (brown shrimp). In: Invertebrate Anatomy OnLine.
http://www.lander.edu/rsfox/310penaeusLab.html.

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapman, R.W., Warr, G.W. (2001). Immune Gene Discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, Litopenaeus vannamei, and the Atlantic White Shrimp, L. setiferus. Dev. Comp Immunol., 25(7):565-577.

Guan, Y., Yu, Z., Lia, C. (2003). The effects of temperature on white spot syndrome infections in *Marsupenaeus japonicus*. Journal of invertebrate pathology, 83: 257-260.

Hameed, A.S., Anilkumar, M., Stephen, R., Jayaraman, K., 1998. Studies on the pathogenicity of systemic ectodermal and mesodermal. Mesodermal baculovirus and its detection in shrimp by immunological methods. Aquaculture, 160: 31– 45.

Inouye, K., Miwa, S., Oseko, N., Nakano, H., Kimura, T., Momoyama, K., Hiraoka, M., (1994). Mass mortalities of cultured Kuruma shrimp *Penaeus japonicus* in Japan in 1993: electron microscopic evidence of the causative virus. Fish Pathol., 29: 149–158.

Itami, T., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., Takahashi, Y. (1998). Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture, 164: 277–288.

Janeway, C.A. (2001). How the immune system works to protect the host from infection: A personal view. Proc. Natl. Acad. Sci., 98: 7461-7468.

Jiang, Y. (2000). Mainland China. In: Thematic review on Management Strategies for Major Diseases in Shrimp Aquaculture. A component of the WB/NACA/WWF/FAO Program on Shrimp Farming and the Environment. Report of the workshop held in Cebu, Philippines from 28-30 November 1999.74-78pp.

Jiravanichpaisal, P. (2005). White Spot Syndrome Virus Interaction with a Freshwater Crayfish. Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 47. ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2005.

Jiravanichpaisal, P., Söderhäll, S., Söderhäll, I. (2004). Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. Fish & Shellfish Immunology, 17: 265-275.

Jiravanichpaisal, P., Bangyeekhun, E., Söderhäll, K., Söderhäll, I. (2001). Experimental infection of white spot syndrome virus (WSSV) in freshwter crayfish, Pacifastacus leniusculus. Diseases of Aquatic Organisms, 47(2): 151-157.

Johansson, M.W., Keyser, P., Sritunyalucksana, K., Söderhäll, K. (2000). Crustacean haemocytes and haematopoiesis. Aquaculture, 191: 2000 45–52.

Johansson, M.W. and Söderhäll, K. (1985). Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J. Comp. Physiol., 156B: 175-181.

Johnson, P. T. (1980). Histology of the blue crab, Callinectes sapidus. A model for the Decapoda. Praeger, New York, 440 pp.

Jithendran, K. P. and Vijayan, K. K. (2001). Parasitic and Non-Infectious Diseases. http://www.icar.org.in.

Kanost, M.R. (1999). Serine proteinase inhibitors in arthropod immunity. Dev. Comp. Immunol., 23: 291-301.

Karunasagar, I., Otta, S.K., Karunasagar, I. (1997). Histopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the west coast of India. Aquaculture. 153. 9-13.

Kasornchandra, J., Boonyaratpalin, S., Itami, T. (1998). Detection of white spot syndrome in cultured Penaeid shrimp in Asia: microscopic observation and polymerase chain reaction. Aquaculture, 164: 243–251.

Kautsky, N., Ronnback, P., Tedengren, M., Troell, M., (2000). Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture, 191: 145– 161.

Kim, C.K., Kim, P.K., Sohn, S.G., Sim, D.S., Park, M.A., Heo, M.S., Lee, T.H., Lee, J.D., Jun, H.K., Jang, K.L., (1998). Development of a polymerase chain reaction (PCR) procedure for the detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimp. J. Fish Dis., 21: 11– 17.

Kim, D.K., Jang, I.K, Seo, H.C., Shin, S.O., Yang, S.Y., Kim, J.W., (2004). Shrimp protected from WSSV disease by treatment with egg yolk antibodies (IgY) against a truncated fusion protein derived from WSSV. Aquaculture, 237: 21–30.

Kobayashi, T., Shiino, T., Miyazaki, T. (1999). The effect of water temperature on rhabdoviral dermatitis in the Japanese eel, Anguilla japonica Temminck and Schlegel. Aquaculture, 170:7-15.

Kurtz, J. (2005). Specific memory within innate immune systems. Trends Immunnol, 26: 186-192.

Lightner, D.V., Redman, R.M. (1998). Shrimp diseases and current diagnostic methods. Aquaculture 164 1998 201–220.

Lightner, D.V. (1996). A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA. 304 pp.

Lee, S.Y. and Söderhäll, K. (2001). Early events in crustacean innate immunity. Fish & Shellfish Immunology, 12: 421–437.

Lo, C.F., Peng, S.E., Kou, G.H. (2001). PCR screening for white spot syndrome virus (WSSV) in *Penaeus monodon* brooders: a general effort to combat shrimp WSS. In: Aquaculture and Fisheries Resources Management: Proceedings of the Joint Taiwan-Australia Aquaculture and Fisheries and Management Forum - TFRI Conference Proceedings 4, Liao I.C. & Baker J., eds. Taiwan Fisheries Research Institute, Keelung, Taipei China, 63-69pp.

Lo, C.F., Chang, Y.S., Chen, C.T., (1998). PCR monitoring of cultured shrimp for white spot syndrome virus (WSSV) infection in growth ponds. In: Flegel, T.W. (Ed.), Advances in Shrimp Biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok, 281– 286pp.

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H., (1996). White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp crabs and other arthropods. Dis. Aquat.Org., 27: 215–225.

Loh, P.C., César, E., Nadala, B.Jr., Tapay, L.M., Lu, Y. (1998). Recent developments in immunologically-based and cell culture protocols for the specific detection of shrimp viral pathogens. In: Advances in Shrimp Biotechnology, Flegel T.W., ed. National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand, 255-259pp.

Maedaa, M., Saitoh, H, Mizuki, E., Itami, T., Ohbad, M. (2004). Replication of white spot syndrome virus in ovarian primary cultures from the kuruma shrimp, *Marsupenaeus japonicus*. Journal of Virological Methods, 116: 89–94.

Marks, H., Goldbach, R.W., Vlak, J. M., van Hulten, M. C.W. (2004). Genetic variation among isolates of White spot syndrome vírus. Arch Virol, 149: 673–697.

Mayo, M.A. (2002). Virus taxonomy - Houston 2002. Archives of Virology, 147/5: 1071-1076.

McClennen, C. (2004). White Spot Syndrome Virus. The Economic, Environmental And Technical Implications On The Development Of Latin American Shrimp Farming. Master of Arts in Law and Diplomacy Thesis. The Fletcher School, Tufts University.

Medzhitov, R. and Janeway, C.A. (2000). Innate immune recognition: Mechanisms and pathways. Immunol. Rev., 173: 89-97.

Mohan, C.V., Shankar, K.M., Kulkarni, S., Sudha, P.M. (1998). Histopathology of cultured shrimp showing gross signs of yellow head syndrome and white spot syndrome during 1994 Indian epizootics. Diseases of aquatic organisms., 34: 9-12.

Momoyama, K., Hiraoka, M., Inouye, K., Kimura, T., Nakano, H., (1995). Diagnostic techniques of the rod-shaped nuclear virus infection in the Kuruma shrimp, *Penaeus japonicus*. Fish Pathol., 30: 263–269.

Morgan, K.L., Turnbull, J.F., Hao, N.V., Mohan, C.V., Corsin, F. (2001). An epidemiological approach to aquatic disease control: a study of the risk factors associated with outbreaks of White Spot Disease in artisanal systems in Vietnam and India. FINAL TECHNICAL REPORT Submitted to DFID.

Morales, J.C., (1991). Acuicultura Marina Animal. Madrid, España, Ediciones Mundi-Prensa, 150-159pp.

Mortensen, S.H. (1993). Passage of infectious pancreatic necrosis virus (IPNV) through invertebrates in an aquatic food chain. Dis Aquat Org, 16: 41 – 45.

Musthaq, S.S., Yoganandhan, K., Sudhakaran, R., Kumar, S.R., Hameed, A.S.S. (2005). Neutralization of white spot syndrome virus of shrimp by antiserum raised against recombinant VP28. Aquaculture*, In press.*

Nakano, H., Koube, H., Umezawa, S., Momoyama, K., Hiraoka, M., Inouye, K., Oseko, N. (1994). Mass mortalities of cultured kuruma shrimp, *Penaeus japonicu*s, in Japan in 1993 – epizootiological survey and infection trials. Fish Pathol, 29: 135-139.

Namikoshi, A., Wu, J.L., Yamashita, T., Nishizawa, T., Nishioka, T., Arimoto, M., Muroga, K. (2004). Vaccination trials with Penaeus japonicus to induce resistance to white spot syndrome virus. Aquaculture, 229: 25–35.

Nunan, L.M., Poulos, B.T., Lightner, D.V. (1998). The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. Aquaculture, 160: 19-30

Nunan, L.N., Lightner, D.V. (1997). Development of a nonradioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). J. Virol. Meth., 63: 193–201.

OIE (2003). Diagnostic Manual for Aquatic Animal Diseases, 4th Edition. Office International des Epizooties (OIE). Paris, France. 358 pp.

Okumura, T., Nagai, F., Yamamoto, S., Oomura, H., Inouye, K., Ito, M., Sawada, H. (2005). Detection of white spot syndrome virus (WSSV) from hemolymph of Penaeid shrimps *Penaeus japonicus* by reverse passive latex agglutination assay using high-density latex particles. Journal of Virological Methods, 124: 143–148.

Oseko, N., Yoshimizu, M., Kimura, T. (1988). Effect of water temperature on artificial infection of *Rhabdovirus olivaceus* (hirame rhabdovirus: HRV) to hirame (Japanese flounder, *Paralichtys olivaceus*). Fish Pathol, 23:125-32.

Otta, S.K., Shubha, G., Joseph, B., Chakraborty, A., Karunasagar, I., Karunasagar, I. (1999). Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. Dis Aquat Org, 38: 67–70.

Park, J.H., Lee, Y.S., Lee, S., Lee, Y. (1998). An infectious viral disease of penaied shrimp newly found in Korea. Diseases of aquatic organisms., 34: 71-75.

Peinado-Guevara, L.I. and López-Meyer, M., (2005). Detailed monitoring of white spot syndrome virus (WSSV) in shrimp commercial ponds in Sinaloa, Mexico by nested PCR. Aquaculture, 2005, *In press*.

Peng, S.E., Lo C.F., Lin, S.C., Chen, L.L., Chang, Y.S., Liu, K.F., Su, M.S., Kou1, G.H. (2001).Performance of WSSV-infected and WSSV-negative Penaeus monodon postlarvae in culture ponds. Dis Aquat Org, 46: 165–172.

Perez-Farfante, I. and Kensley, B. (1997). Penaeoid and Sergestoid Shrimps and Prawns of the World: Keys and Diagnoses for the Families and Genera. Memoires du Museum National d'Histoire Naturelle, volume 175, 233 pp.

Poulos, B.T., Pantoja, C.R., Bradley-Dunlop, D., Aguilar, J., Lightner, D.V. (2001). Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis Aquat Org, 47: 13–23.

Quere, R., Commes, T., Marti, J., Bonami, J.R., Piquemal, D., (2002). White spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus simultaneous diagnosis by miniarray system with colormetry detection. J. Virol. Meth., 05: 189–196.

Rahman, M.M., Escobedo-Bonilla, C.M, Wille, M., Alday Sanz, V., Audoorn, L., Neyts, J., Pensaert, M.B., Sorgeloos, P., Nauwynck, H.J. (2005). Clinical effect of cidofovir and a diet supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles. Aquaculture, *In press*.

Rajendran, K.V., Vijayan, K.K., Santiago, T.C., Krol, R.M. (1999). Experimental host range and histopathology of white spot syndrome virus (WSSV) infection in shrimp, prawns, crayfish and lobsters from India. Journal of fish diseases., 22: 183-191.

Relf, J., Chisholm, J., Kemp, G., Smith, V. (1999). Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, Carcinus maenas. Eur. J. Biochem., 264: 350–357.

Rosenberry, B. (2004). World shrimp farming 2004. In *Shrimp News Iinternational*. San Diego, California, USA.

Rosenberry, B. (2001). World Shrimp Farming 2000. Shrimp News International, San Diego, 324 pp.

Sano, N., Moriwake, M., Sano, T. (1993). Herpesvirus cyprini: thermal effect on pathogenicity and oncogenicity. Fish Pathol, 28:171-5.

Sheehan, D.C., and Hrapchak, B.B. (1980). Theory and Practice of Histotechnology. 2nd Edition. C.V. Mosby Co., St. Louis, MO.

Shih, H.H., Wang, C.S., Tan, L.F., Chen, S.N. (2001). Characterization and application of monoclonal antibodies against white spot syndrome virus. J. Fis. Dis., 24: 143-150.

Shrimp News International (2004). About shrimp farming. http://www.shrimpnews.com

Smith, V.J., and Söderhäll, K. (1983). Induction of degranulation and lysis of haemocytes in the freshwater crayfish, *Astacus astacus*, by components of the prophenoloxidase activating system in vitro. Cell Tissue Res., 233: 295-303.

Söderhäll, K., Smith, V.J., Johansson, M.W. (1986) Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: Evidence for cellular co-operation in the defence reactions of arthopods. Cell Tissue Res. 245: 43-49.

Söderhäll, K., Cerenius, L., Johansson, M.W. (1994). The Prophenoloxidase Activating System and Its Role in Invertebrate Defence. Primordial Immunity: Foundations For The Vertebrate Immune System Annals Of The New York Academy Of Sciences, 712: 155-161.

Söderhäll, K., Rantamaki, J., Constantinescu, O., (1993). Isolation of *Trichosporon beigelii* from the freshwater crayfish, *Astacus astacus*. Aquaculture, 116: 25–31.

Solis, N. B. (1988). Biology and Ecology. Chapter One., 3-36pp. In: Biology And Culture Of *Penaeus Monodon*. Brackishwater Aquaculture Information System, Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo, Philippines.

Song, Y. L. and Hsieh, Y. T. (1994). Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Developmental and Comparative Immunology, 18: 201-209.

Sritunyalucksana, K. and Söderhäll, K. (2000). The proPO and clotting system in crustaceans. Aquaculture, 191: 53–69.

Takahashi, Y., Kondo, M., Itami, T., Honda, T., Inagawa, H., Nishizawa, T., Soma, G.I., Yokomizo, Y. (2000). Enhancement of disease resistance against penaeid acute viraemia and induction of virus-inactivating activity in haemolymph of kuruma shrimp, *Penaeus japonicus*, by oral administration of Pantoea agglomerans lipopolysaccharide (LPS). Fish & Shellfish Immunology., 10: 555–558.

Tam, J., Lu, Y., Yang, J. (2000). Marked increase in membranolytic selectivity of novel cyclic tachyplesins constrained with an antiparallel two-B strand cysteine knot framework. Biochem. Biophys. Res. Commun., 267: 783–790.

Treece, D.G., and Yates, M.E. (1998). Laboratory manual for the culture of penaeid shrimp larvae. Marine Advisory Service, Sea Grant College Program, Texas, USA, 95pp.

Tsai, M.F., Lo, C.F., van Hulten, M.C.W., Tzeng, H.F., Chou, C.M., Huang, C.J., Wang, C.H., Lin, J.Y., Vlak, J.M., Kou, G.H. (2000). Transcriptional Analysis of the Ribonucleotide Reductase Genes of Shrimp White Spot Syndrome Virus. Virology, 277: 92-99.

van Hulten, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K., Vlak, J.M. (2001a). The White Spot Syndrome Virus DNA genome sequence. Virology 286: 7-22.

van Hulten, M.C.W., Vlak, J.M., (2001b). Identification and phylogeny of a protein kinase gene of white spot syndrome virus. Virus Genes, 22: 201– 207.

van Hulten, M. C. W., Westenberg, M., Goodall, S. D., and Vlak, J. M. (2000). Identification of two major virion protein genes of White Spot Syndrome virus of shrimp. Virology, 266: 227-236.

Vidal, O.M., Granja, C.B., Aranguren, L.F. (2001). A profound effect of hyperthermia on survival of Litopenaeus vannamei juveniles infected with white spot syndrome virus. Journal of the world aquaculture society, 32: 364-372

Vijayan, K.K., Raj, V.S., Balasubramanian, C.P., Alavandi, S.V., Sekhar, V.T., Santiago, T.C. (2005). Polychaete worms - a vector for white spot syndrome virus (WSSV). Diseases of Aquatic Organisms, 63: 107–111.

Wagner, E.K. and Hewlett, M.P. (1999). Basic virology. Blackwell science,inc., USA. 465pp.

Wang, C.H., Lo, C.F., Leu, J.H., Chou, C.M., Yeh, P.Y., Chou, H.Y., Tung, M.C., Chang, C.F., Su, M.S., Kou, G.H. (1995). Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis. Aquat. Org. 23, 239–242.

Wang, C.S., Tsai, Y.J., Chen, S.N., (1998). Detection of white spot disease virus (WSDV) infection in shrimp using in situ hybridization. J. Invertebr. Pathol., 72: 170–173.

Wang, Q., Nunan, L.M., Lightner, D.V., (2000). Identification of genomic variations among geographic isolates of White Spot Syndrome Virus using restriction analysis and southern blot hybridization. Dis Aquat Org, 43: 175-181.

Wang, Y.T., Liu, W., Seah1, J.N., Lam, C.S., Xiang, J.H., Korzh, V., Kwang, J. (2002). White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp Penaeus merguiensis. Dis Aquat Org, 52: 249–259.

Wickins, J.F. and Lee, D.O'C. (2002). Crustacean Farming Ranching and Culture. Second Edition. Blackwell Science, 9-29pp.

Witteveldt, J., Vlak, J.M., van Hulten, M.C.W. (2004). Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. Fish & Shellfish Immunology, 16: 571-579.

Westenberg, M., Heinhuis, B., Zuidema, D., Vlak. J.M., (2005). siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. Virus Research 114: 133–139.

Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Diseases of aquatic organisms, 21: 69-77.

Yang, F.H. (Ed.), 1990. Virology. Hunan science and technology press, Changsha, 173–247pp.

Yi, G., Qian, J., Wang, Z., Qi, Y., (2003). A phage-displayed peptide can inhibit infection by white spot syndrome virus of shrimp. J Gen Virol, 84: 2545-2553.

Zasloff, M. (1992). Antibiotic peptides as mediators of innate immunity. Curr. Opin. Immunol, 4: 3–7.

Zhan, W.B., Wang, Y.H., Fryer, J.L., Yu, K.K., Fukuda, H., Meng, Q.X. (1998). White spot syndrome virus infection of cultured shrimp in China. J Aquat Anim Health, 10: 405-410.

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