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Interaction between crayfish and some microorganisms; Effect of temperature

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Abstract

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Innate immunity, which constitutes the first line of defense in vertebrates, is the only immune system that invertebrates rely on to protect themselves from pathogens. The invertebrate immune system is composed of cellular and humoral components. Cellular immunity is phagocytosis, opsonization and encapsulation. The humoral part is mainly composed of the events taking place upon secretion of granules and the enzymes within that lead to the lysis of the pathogen by antimicrobial peptides (AMPs) and the melanization cascade. The Prophenoloxidase (proPO) activating system is an important pathway that is stored in the granules of semigranular and granular hemocytes (blood cells). These cells will degranulate and release the proPO system when activated upon pathogen recognition. This cascade results in the melanization reaction and to trap and eliminate pathogens.

White spot syndrome virus (WSSV) is a deadly pathogen mainly targeting crustaceans and causing huge economic losses since its first emergence in 1992 in Taiwan. It is known that WSSV disables the immune system of the host by interfering with the proPO cascade. Temperature is a restricting factor for the WSSV infections however it is not known if its affects are on host immunity or on the virus itself.

With the aim of elucidating WSSV infection, we studied the virus entry mechanisms. By crosslinking WSSV with the hemocytes we showed that a new clip-domain serine protease (*Plc*SP) plays an important role during the WSSV infection in crayfish by means of interacting with WSSV envelope protein VP28. Moreover, we have shown that the viral entry is inhibited at cold temperatures due to temperature's inhibitory effect on *Plc*SP expression. We also showed that by slowing down of the host's metabolism hence proliferation in host tissue either by low temperature or cell cycle inhibitors, we could inhibit WSSV replication once it has entered the host cell. We tested if the temperature effects host or pathogen, or both, we investigated the mortalities, phagocytosis, bacterial clearance, total hemocyte counts, degranulation and melanization rate of crayfish under a cold and warm temperature by using two strains of gram-negative bacteria and LPS. It is apparent that the cellular immunity is more effective at low temperature while the humoral immunity can become overactivated and toxic for the host at higher temperature. Furthermore, we aimed to study the cleavage specificity for *Plc*SP since it is predicted to be secreted from hemocytes and takes part in the serine protease cascade during melanization reaction.

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To my mom…

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Guo E, Korkut GG, Jaree P, Söderhäll I, and Söderhäll K. (2017). A Pacifastacus leniusculus serine protease interacts with WSSV. *Fish Shellfish Immunol. 68*, 211–219.

II Korkut GG, Söderhäll I, Söderhäll K, Noonin C. (2018). The effect of temperature on bacteria-host interactions in *Pacifastacus leniusculus*. (in press in *J. Invertebrate Pathol*)

III Korkut GG, Noonin C, Söderhäll K. (2018). The effect of temperature on WSD progression. (in press in *Dev. Comp. Immunol*.)

IV Korkut GG, Sirikharin R, Noonin C, Söderhäll K, Söderhäll I. (2018). Expression of an active and inactive clip-domain serine protease isolated from signal crayfish in an insect cell line. (manuscript)

Additional publication

Yang H, Kronhamn J, Ekström JO, Korkut, GG, and Hultmark D. (2015). JAK/STAT signalling in Drosophila muscles controls the cellular immune response against parasitoid infection. EMBO Rep. *16*, 1664–1672.

Contents

Abbreviations

ALF	Anti-lipopolysaccharide factor
AMP	Antimicrobial peptide
CBP	Chitin-binding protein
CHF	Crustacean hematopoietic factor
Clip-SP	CLIP-domain serine protease
c-Mas	Crayfish masquerade-like protein
CME	Clathrin-mediated endocytosis
CTL	C-type lectin
DOPA	Dihydroxyphenylalenine
dsRNA	Double stranded RNA
eSOD	Extracellular superoxide dismutase
Fc	*Fenneropenaeus chinensis*
FKBP	FK506-binding protein
GABARAP	Gamma-aminobutyric acid receptor associated protein
GC	Granular cell
HC	Hyaline cell
HEK	Human embryonic kidney
HPT	Hematopoietic tissue
HSC	Hematopoietic stem cell
ie1	Early viral gene 1
KPI	Kazal proteinase inhibitor
LGBP	Lipopolysaccharide binding protein
Lec	Lectin
LPS	Lipopolysaccharide
Lv	*Litopenaeus vannamei*
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight
MBL	Mannose binding lectin
MIP	Melanization inhibition protein
Mj	*Marsupenaeus japonicus*
MOI	Multiplicity of infection
MS	Mass spectrophotometry
Ni-NTA	Nickel nitrilotriacetic acid
PAMP	Pathogen associated molecular pattern
PCNA	Proliferating cell nuclear antigen
PGBP	Peptidoglycan binding protein

Pj	*Penaeus japonicus*
PKC	Protein kinase C
Pl	*Pacifastacus leniusculus*
*Plc*SP	*P. leniusculus* clip domain serine protease
Pm	*Peneus monodon*
PO	Phenoloxidase
proPPA/PPAE	Pro-prophenoloxidase activating enzyme
proPO	Prophenoloxidase
PRP	Pattern-recognition protein
ROS/RNS	Reactive oxygen species/reactive nitrogen species
RUNX	Runt related transcription factor
SEGS	Sulfo ethylene glycol bis (succinimidyl succinate)
Serpin	Serine protease inhibitor
SGC	Semigranular cell
SP	Serine protease
SPH	Serine protease homologue
STAT	Signal transducer and activator of transcription proteins
SOD	Superoxide dismutase
Trx	Thioredoxin
TSV	Taura syndrome virus
VP	Viral protein
WBP	WSSV binding protein
WSD	White spot disease
WSSV	White spot syndrome virus
YHV	Yellow head virus
2D gel	2-dimensional gel
βGBP	β-1,3-glucan binding protein

Introduction

All living organisms have developed immune systems to protect themselves against foreign microorganisms e.g. bacteria, fungi and viruses that they are constantly exposed to. Vertebrates, being evolutionarily more complex, have two different types of immunity: innate and adaptive. Innate immunity mounts a rapid and unspecific first line response, whereas adaptive immunity is more advanced in the sense that it can specifically recognize the pathogens and has a memory for faster responses at the second exposure by means of producing antibodies against the antigens (Janeway and Medzhitov, 2002)

Invertebrate immunity is composed only of innate immune system which is comprised of humoral and cellular systems. The cellular immunity works via phagocytosis of small pathogens and encapsulation of larger pathogens by the circulating hemocytes whereas humoral immune response corresponds to the other components of the hemolymph including clotting cascade, antimicrobial peptides, reactive oxygen intermediates and the proteins associated with the prophenoloxidase system which are found in the granules in hemocytes, mainly granular cells (Johansson and Söderhäll, 1985). These two systems work hand-in-hand to eventually destroy the invading pathogen. As the hemocytes sacrifice themselves to fight the intruding pathogens, the number of circulating hemocytes in the body cavity (hemocoel) will drop in number. This loss of hemocytes is then compensated by the activation of the HPT which will produce new hemocytes and release them into the circulation until homeostasis is reached (Janeway and Medzhitov, 2002; Söderhäll, 2016).

Temperature and its relationship with immunity have been investigated by many researchers (Hernroth and Baden, 2018; Marcogliese, 2008; Wojda, 2017). Crayfish adjust their body temperature according to their surroundings. It is especially important to understand the relationship of immunity to temperature as the global climate change is expected to increase the atmospheric temperature by up to 4°C in the next 100 years which will in turn increase water temperatures (IPCC, 2014).This change will not only inflict a temperature stress on aquatic organisms but also the increased CO_2 levels due to greenhouse effect will induce absorption of CO_2 by oceans will lead to ocean acidification (Caldeira and Wickett, 2003) and to an increase in available manganese in the waters (Post, 1999) and thus will increase hypoxia as the dissolved O_2 in the waters decrease (Diaz, 2001). Altogether, temperature change will have a negative impact on aquatic life, increasing the chance of transmission of pathogens to consumers via seafood.

By understanding crayfish immunity, we will be able to improve crustacean aquaculture which is an important source of income for shrimp and crayfish farmers, but more importantly we will be able to apply our knowledge to immune systems of other invertebrate animals.

An overview of innate immunity

A preliminary form of adaptive immunity emerges from lamprey onwards, who has lymphocyte (white blood cell) like cells (LLC) (Guo et al., 2009) to the jawed vertebrates with the emergence of V-D-J recombination for making antibodies by lymphocytes. Invertebrates have neither true antibodies nor lymphocytes to protect themselves from the pathogens or to remember a previously exposed pathogen to induce a more specific immune response. However, invertebrates have been around for millions of years because although essentially unspecific, they have a very rapid and effective innate immune system to fight foreign intruders. The innate immunity is composed of two main components which are cellular immunity which is generated by hemocytes to fight invaders, and humoral immunity which is generated by the synthesis and secretion of immune components of the blood cells, other tissues such as hepatopancreas and perhaps HPT. These two pathways work hand-in-hand.

These systems are evolutionarily conserved, hence the knowledge we gain from crayfish can be valuable for understanding immunity in other organisms. Lots of progress have been made in the last years understanding crayfish immunity (Cerenius and Söderhäll, 2018) since the first identification of products from fungi, beta-1,3-glucans that could activate prophenoloxidase and subsequent melanization (Unestam and Söderhäll,1977) to the generation of several crayfish transcriptomes in our lab and intense studies on how blood cells are produced (Söderhäll, 2016).

There are several pathogen associated molecular patterns (PAMPs) such as β-1,3 glucan from fungus, peptidoglycan by both gram-positive and gram-negative bacteria and LPS from gram-negative bacteria which are recognized by pattern-recognition proteins (PRPs) such as beta glucan binding protein (βGBP), peptidoglycan binding protein (PGBP), lipopolysaccharide and glucan binding protein (LGBP), thioester-containing proteins (TEP), fibrinogen-related proteins (FREP), masquerade-like proteins/serine protease homologues (SPH) and C-type lectins (Cerenius et al., 2010; Cerenius and Söderhäll,2018; Hillyer, 2010).

Upon recognition of PAMPs by PRPs on host cell, the immune system is activated. Phagocytosis, nodule formation, coagulation and encapsulation of the pathogens are the responsibilities of the cellular immunity. Production of antimicrobial peptides and production of toxic phenol intermediates or reactive oxygen species (ROS) and melanin pigments are associated with humoral immunity as schematized in Fig 1.

Several antimicrobial peptides have been identified in *P. leniusculus*. Astacidine 1 which is cleaved from hemocyanin (Lee et al., 2004), Astacidine 2 (Jiravanichpaisal et al., 2007), *Pl*-crustin 1, 2 and 3 (Donpudsa et al., 2010; Jiravanichpaisal et al., 2007; Sricharoen et al., 2005), proPO-PPA which is an N-terminal fragment of proPO after being cleaved by PPA (Jearaphunt et al., 2014) and hemagglutinin (Wang et al., 2001).

Phagocytosis is a highly conserved mechanism enabling rapid engulfment of pathogens by specialized cells, mainly semigranular cells and hyaline cells in *P. leniusculus* (Thörnqvist et al., 1994) through rearrangement of actin cytoskeleton and the membrane. The endosome engulfing the pathogen will then undergo maturation by decreasing its pH to be an early endosome which is to be fused with lysosome to generate a mature phagolysosome in which the invader will be killed by a highly acidic environment. The receptors for direct binding required have not been extensively studied but opsonins deposited on the pathogen surface help the phagocytosis.

Lectins are small sugar-binding multi-domain e.g. carbohydrate-recognition domain (CRD) containing proteins which can agglutinate cells. Lectins can be extracellular or intracellular. Depending on the function eight families have been classified. C-type lectins are the most diverse group of them belonging to extracellular group (Zelensky and Gready, 2005). They are Ca^{2+} dependent and involved in immunity in invertebrates (Weis et al., 1998). It has been reported that in addition to insects (Xia et al., 2018), crustaceans also encode C-type lectins (Liu et al., 2007b).

Invertebrate lectins have diverse functions like being involved in phagocytosis (Ling and Yu, 2006a; Luo et al., 2006), cell adhesion and nodule formation, encapsulation (Ling and Yu, 2006b), antibacterial activity (Schröder et al., 2003), activation of proPO system (Yu and Kanost, 2000) and during viral infections (Zhao et al., 2009). In *P. leniusculus*, mannose-binding lectin is a pattern recognition protein that is secreted from hemocytes upon pathogen encounter and is associated with regulation of the proPO-system (Sricharoen et al., 2005; Wu et al., 2013).

If the pathogen is too big to be phagocytosed it will be encapsulated by the hemocytes. Opsonization and encapsulation of the pathogen is required for this process so that the proPO system can kill the trapped pathogen. In *P. leniusculus*, masquerade-like protein I (Huang et al., 2000, Lee and Söderhäll,2001) and peroxinectin (Johansson et al.,1988, 1995) have been identified as the two cell adhesion molecules. Also, a PRP, LGBP has been reported to have opsonic and adhesion activity (Wang et.al JBC; Sricharoen et al., 2005c).

Although invertebrates lack adaptive immunity, some recent articles claim that immunological memory might exist but so far only one paper shows that memory against a Drosophila virus might exist (Tassetto et al., 2017). High specificity against virus was explained by RNA interference in *D. melanogaster* (Cherry and Silverman, 2006).

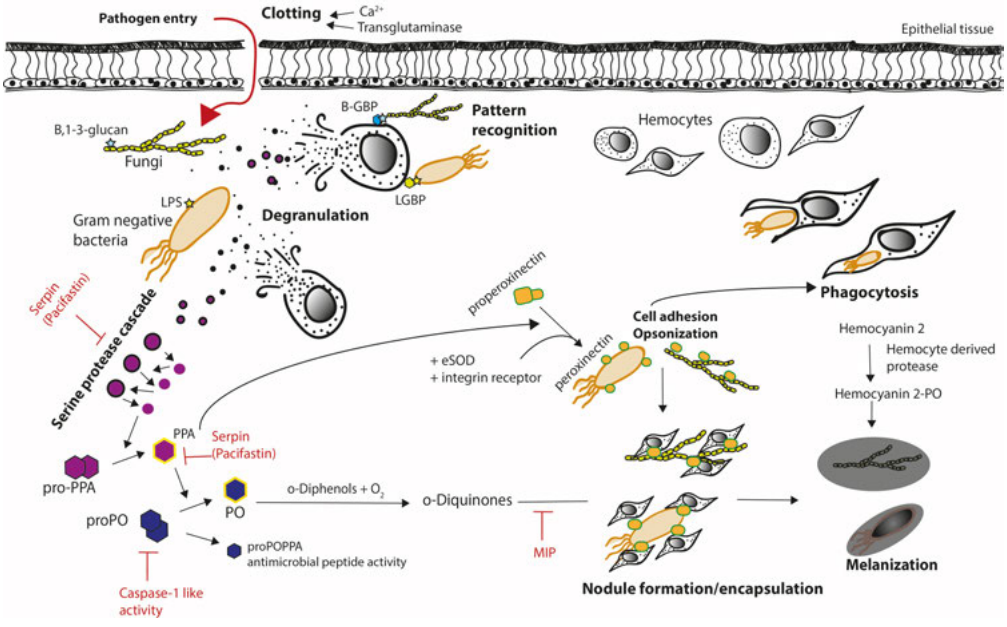

Figure 1: Schematic diagram for invertebrate immunity including cellular and humoral responses in crustacean.

Hemocytes and Hematopoietic Tissue

Innate immunity is sustained by circulating blood cells in crayfish as well as in other invertebrates. These blood cells are called hemocytes and perform phagocytosis, encapsulation and lysis of invasive organisms (Söderhäll and Cerenius, 1998). Hemocytes are produced throughout life time of the organism by a tightly regulated process called hematopoiesis, from self-renewing hematopoietic stem cells (HSCs) by a sheet-like actively proliferating tissue called hematopoietic tissue (HPT). There is around 1% of cells in circulation that can divide indicating that no or few hemocytes can proliferate after leaving HPT (Sequeira et al., 1996). These dividing cells are most likely a result of that morphotypes 1-2 can be released from HPT during stress or needle injections (Söderhäll, 2016). HPT is located on the dorsal side of the stomach of the crayfish (Chaga et al., 1995). It was shown by the ß-1,3-glucan injection that hemocytes are synthesized and partly differentiated in HPT (Söderhäll et al., 2003). However, the final differentiation is not completed until they are released into the hemolymph (Söderhäll et al., 2003). This system is strictly regulated and has been well studied yet the mechanisms are still not fully clear.

The two invertebrates in which this process has been well studied are fruit fly *Drosophila melanogaster* and the freshwater crayfish *Pacifastacus leniusculus*. Crayfish HPT is made up of small lobules containing HPT cells at different maturation stages, and all these are connected via a collagenous

connective tissue. In contrast to crayfish HPT, the hematopoiesis takes place in the lymph glands of *Drosophila*. Lymph glands are divided into zones based on their functions and differentiated stage of the cells: Medullary zone (MZ) is where the progenitor cells are situated; cortical zone (CZ) is where the differentiated cells are located. The posterior signaling center (PSC) is where the hematopoiesis in lymph glands are controlled (Williams, 2007). The hematopoietic tissue in mammals is the bone marrow where the blood cells differentiate moving against the oxygen gradient before they move into the blood vessels (Parmar et al., 2007).

Hematopoiesis continues throughout the lifetime of crayfish but the numbers of hemocytes can vary significantly as there is a simultaneous synthesis, maturation and apoptosis going on in HPT. For example, in the event of an infection the total hemocyte count (THC) will drop as the mature hemocytes in the circulation will sacrifice themselves to attack the pathogen (Persson et al., 1987). To compensate for the loss of those hemocytes, HPT will be stimulated to produce and release new hemocytes into the hemolymph. The proliferation/differentiation of the HPT cells as stem cells and stimulation of their secretion into the hemolymph by inhibiting transglutaminase activity (TGA) has been attributed to a protein called Astakine (Ast) which is homologous to vertebrate prokineticins (Söderhäll et al., 2005). It also controls the apoptotic rate by means of regulating CHF (Lin et al., 2011).

Based on the morphology, there are five types of cells residing in the HPT. Type 1 being the least differentiated precursor cell that gives rise to Type 2, 3 and 4 which are considered to be granular cell (GC) precursors due to presence of granules and Type 5 is thought to be the precursor for semigranular cells (SGCs) (Chaga et.al 1995). Based on granule density, nucleus size and staining properties, there are three types of circulating hemocytes present in crayfish that are analogous to the vertebrate leukocytes because of their role in non-self-recognition and elimination: Hyaline cells (HC), SGCs and GCs (Söderhäll and Smith, 1983). HC are small and do not have many granules. Their function is to be a player in phagocytosis. Furthermore, whether they are immature prohemocytes of SGC and GC is still debated (van de Braak et al., 2002; Lin et al., 2011). SGC are the most abundant cells with small granules. They are involved in the early recognition and encapsulation of the pathogen, as well as coagulation for example at the site of injury. GC are the ones with large granules containing antimicrobial peptides and the components of the proPO system. There are very few studies to understand hematopoiesis and more studies are required to understand the process of hemocyte maturation under normal and infection conditions, and this would be done by using the specific cell markers for different cell types discovered in our laboratory. We know the markers for HPT cells are PCNA and CHF; proPO, RUNX, CHF, peroxinectin and KPI for SGCs, and proPO, RUNX, peroxinectin, SOD and *Pl*-MBL for GCs (Lin et al., 2011; Sricharoen et al., 2005; Wu et al., 2008). However, the markers for the precursor cells are yet to be identified. (Söderhäll, 2016)

According to the proteomic analysis done on the vesicles of granular cells in *P. leniusculus*, the abundant proteins that are released by exocytosis of GCs include crayfish masquerade-like protein I (c-Mas I), pro-phenoloxidase, prophenoloxidase (proPO and peroxinectin) and antimicrobial peptide (AMP) which were identified by MALDI-TOF, crayfish masquerade-like protein II (c-Mas II), mannose receptor protein (MRP), vitelline membrane outer layer protein I (VMO-I) and antimicrobial peptide (AMP-16) which were identified by cloning (Sricharoen et al., 2005).

Our lab has developed the *in vitro* cell culture for HPT cells from crayfish, which has been a powerful tool to study hematopoiesis and gene functions by RNAi, or host-virus interactions with WSSV. (Jiravanichpaisal et al., 2006a; Söderhäll, 2013; Söderhäll et al., 2003)

Prophenoloxidase Activation Cascade

The proPO system in crustaceans and insects is function-wise similar to the complement system in vertebrates but it differs in that it is compartmentalized in hemocytes (Söderhäll,1982). In crustaceans, the proPO system is located in SGC and GC vesicles (Johansson and Söderhäll, 1985). Even small amount of a pathogen recognition will lead to hemocyte degranulation (exocytosis) of hemocytes (Sricharoen et al., 2005), that subsequently activate the serine protease cascade (Cerenius et al., 2008). This activation leads to conversion of proPPA into ppA, which in turn cleaves zymogen prophenoloxisase (proPO) into active phenoloxidase (PO) enzyme (Aspan et.al. 1995). ProPO is present in the hemocytes inactively until it is released into the hemocoel where it is activated in the presence of Ca^{2+} (Adachi et al., 2003; Ashida and Söderhäll, 1984). PO is an enzyme with tyrosinase activity that catalyses the conversion of monophenols to diphenols, then oxidize o-diphenols and tyrosine (Tyr) into DOPA, and DOPA into DOPA-quinones, the precursor of melanin (Aspan et al., 1995). During this activation pathway, other factors from humoral immunity are released to enhance the activity of the system such as helping with the degranulation. For example, an adhesion factor, peroxinectin, is released to induce the adhesion of pathogens to surfaces, which also stimulates the encapsulation reaction (Johansson et al., 1995). That is the aggregation of the SGCs on invasive particles that are bigger than 10 μm in diameter, to be trapped in a capsule made up of 5 to 30 layers of hemocytes without any intercellular spaces (Kobayashi et al., 1990). Melanization is the blackening reaction around the pathogen and melanizing and other intermediates in the DOPA-melanin pathway are toxic (Cerenius et al., 2010; Söderhäll and Ajaxon, 1982). Together with the production and release of reactive oxygen and nitrogen species (ROS/RNS) as byproducts (Cerenius et al., 2008; Söderhäll and Cerenius, 1998) released into the capsule, the pathogen trapped in this nodule will eventually be killed (Nappi and Vass, 1993; Nappi et al., 2009) (Fig 1).

Active ppA can also activate properoxinectin into active peroxinectin, an opsonic peroxidase with cell adhesion activity (Fig1) (Lin et al., 2007), that acts by binding to an extracellular superoxide dismutase (eSOD) and integrin on cell surface and stimulates the binding of the hemocytes to microbe (Johansson et al., 1995, Johansson et al., 1999). This complex formation on pathogen helps the invading microbes to be phagocytosed if they are small enough to be eaten by hemocytes (Cerenius et al., 2008, Cerenius and Söderhäll, 2004).

This system must be tightly regulated to prevent harming the host itself due to excessive production of toxic compounds. The negative regulation takes place at multiple steps first checkpoint being the serpins which block the serine protease cascade (Cerenius et al., 2010). Secondly, a protein with a protease inhibitor light chain and transferrin heavy chain called pacifastin inhibits the active ppA activity (Liang et al., 1997). Caspase1-like activity was found to reduce the activation of proPO into PO (Jearaphunt et al, 2014). Lastly, the formation of melanin from quinone compounds is controlled by the melanization inhibition proteins (MIPs) (Angthong et al., 2010; Söderhäll et al., 2009; Zhao et al., 2005). Pacifastin is the only serpin that has been identified in crustaceans and that is from crayfish *P. leniusculus* that inhibits *Pl*PPA1 (Liang et al., 1997) and forms a new family of proteinase inhibitors (Liang et al., 1997).

First invertebrate proPO was cloned from freshwater crayfish *P. leniusculus* in 1995 (Aspan et al., 1995). By *in vivo* proPO gene silencing, it has been proven that the phenoloxidase is an important player in the crayfish immunity (Liu et al., 2007a). Downregulating proPO gene via dsRNA in *M. japonicus* lead to an increase in the bacterial load and a reduced hemocyte number (Fagutao et al., 2009). Furthermore, the WSSV infection downregulated the proPO transcripts in several organisms as also when injected with bacteria (Jiravanichpaisal et al., 2006b; Sutthangkul et al., 2017).

Hemocyanin, the main oxygen carrier in arthropods, is also a precursor for antibacterial and antifungal peptides (Coates and Decker, 2017; Destoumieux-Garzón et al., 2001; Lee et al., 2003; Petit et al., 2016; Qin et al., 2018). In *P. leniusculus*, it exhibited antibacterial activity against both gram-positive and gram-negative bacteria, and antifungal activity against four fungal species (Choi and Lee, 2014; Lee et al., 2003). Furthermore, it has phenol oxidase activity after trypsin treatment (Lee et al., 2004). Also, hemocyanin possess antiviral activity against WSSV by interacting with WSSV (Lei et al., 2008; Zhang et al., 2004b)

Hemoglobin, the functional oxygen carrier equivalent of hemocyanin in vertebrates also has immune effector abilities. For example, in alligator *Alligator mississippiensis,* human, horse *Equus ferus caballus* and in cow *Bos taurus* the hemoglobulin tetramers show antimicrobial and antifungal activity. In catfish *Ictalurus punctatus*, hemoglobulin has an antiparasitic and antibacterial activity (Coates and Decker, 2017).

Serine Proteases

Extracellular proteases have a wide array of functions from food digestion, blood coagulation, digestion, fibrinolysis to immunity e.g. complement activation. They can form cascades by the activation of one protease activating the next zymogen in the next protease chain, and thereby amplifying the signals. They are one of the best studied family of enzymes in mammals, yet less is known about invertebrate SPs. Serine proteases are usually synthesized as zymogens which are then activated by proteolytic cleavage. They have a catalytic C terminus and a noncatalytic N terminus which is important for the activation of protein and regulation of enzymatic activity. Recognition of an PRP leads to the activation of extracellular SPs that initiates a chain reaction of SPs. Downstream of this cascade a pathogen is killed.

A large group of serine proteases are CLIP-domain serine protease (clip-SP) which also are involved in immunity and development. First clip-SP described when identifying a horseshoe crab *Tachypleus tridentatus* clotting enzyme (Muta et al., 1990). Clip-SPs have only been identified in arthropods. It has a characteristic paper-clip like configuration found at the N-terminal of SP domain or SPH, due to the six strictly conserved cysteine residues that form three internal disulphide bonds as well as a cationic glycine rich domain and a cationic proline rich domain (Jiang and Kanost, 2000). The C-terminal half is composed of typical serine protease domain and within this domain in crayfish SP the clip domain can be cleaved off and becomes antibacterial (Wang et al., 2001). The SP like domain contains an active site with His, Asp and Ser amino acid residues forming the catalytic triad. This site participates in the formation of transient acyl-enzyme intermediate between the substrate and the proteinase. Depending on the proteolytic activity of the SP-like domain, clip-SPs can be catalytic or non-catalytic like SPHs. SPHs lack the proteolytic activity due to mutations at their one or more catalytic triad residues and therefore function as cofactors for proPO activation, or they can negatively regulate the melanization response (Yu et al., 2003; Zhang et al., 2004a).

SPs are implicated to be involved in control of dorso-ventral patterning in embryonic development (Moussian and Roth, 2005), activation of Toll mediated response to microbial infections in *D. melanogaster*, and the melanization cascade in *P. leniusculus* (Aspan et.al. 1995) and *P. monodon* (Sutthangkul et al., 2015). In mosquito, *A. gambiae* it has been reported that two clip-domain serine proteases are secreted from the hemocytes and shown to be involved in the melanization cascade by dsRNA knockdown procedure (Volz et al., 2005).

Table 1: Serine proteases in crustaceans.

Serine Protease	Mechanism	Reference
*Pm*PPAE2	Regulation of melanization during WSSV infection first demonstrated. Binds WSSV453.	(Sutthangkul et al., 2015)
	Melanization is inhibited by this interaction.	(Sutthangkul et al., 2017)
*Pl*PPAF	Masquerade-like protein that mediates cell adhesion	(Huang et al., 2000)
*Pm*PPAE1	Immune responsive and involved in proPO activation	(Charoensapsri et al., 2009)
*Lv*PPA1	Immune responsive and involved in proPO activatio	(Jang et al., 2011)
*Pm*ClipSP1	Antibacterial, not involved in proPO	(Amparyup et al., 2010)
*Pm*ClipSP2	Pattern recognition, involved in proPO cascade	(Amparyup et al., 2013; Khorattanakulchai et al., 2017)
*Pm*Snake	Activates proPO, involved in serine protease cascade	(Monwan et al., 2017)
*Pl*PPA	Catalyze proPO to active PO. Antibacterial	(Wang et al., 2001)
*Pl*MasI	PRP in hemocytes. Binds ß-1,3-glucan, LPS and Gram-negative bacteria Opsonization, cell adhesion	(Lee and Söderhäll, 2001)
*Pl*SPHI	Released by exocytosis of granular cells	(Sricharoen et al., 2005)
*Pl*SPH2a/2b	Involved in proPO activation triggered by PGN without a PGNR	(Liu et al., 2011)

Serine protease homologues (SPHs) are serine proteases that show homology to serine proteases and have one or more CLIP domains which takes part in protein-protein interactions, antimicrobial activity and protease activity. However, the CLIP domains in SPH have a non-catalytic domain with a substation of one or more of the residues in the catalytic H-D-S (His57-Asp103-Ser195) triad therefore are inactive (Ross et al., 2003). These have been identified both in vertebrates and invertebrates but have different functions than serine proteases and they are involved in pattern recognition, opsonization and cell adhesion (Huang et al., 2000; Lee and Söderhäll, 2001), antimicrobial activity (Kawabata et al., 1996), regulation of proPO activity in different organisms. A SPH called masquerade (Mas) first identified in *D. melanogaster* is important for cell attachment in embryo (Murugasu-Oei et al., 1995). A SPH in crayfish is attached to bacteria and then cleaved into 4 fragments one being a cell adhesion molecule and one an AMP (Lee and Söderhäll, 2001; Wang et al., 2001). They are also crucial for the activation and regulation of the proPO cascade in insects (Cerenius et al., 2008)

White Spot Syndrome Virus

Viruses are the most common pathogens that the crustaceans are exposed to because they are present up to ten billion per liter of sea water (Fuhrman, 1999). Among these viruses, white spot syndrome virus (WSSV), yellow head virus (YHV) and Taura syndrome virus (TSV) are regarded as the most pathogenic for cultured penaeid shrimps causing high mortality rates. WSSV has a cumulative mortality of 100% in 3 -10 days after the onset of first visible gross signs. It has been causing serious economic losses by wiping out the shrimp farms all over the world since its first outbreak in Taiwan in 1992 (Chou et al., 1995). It has been shown that this virus has a broad host range from shrimp, crustaceans such as crayfish, crab and spiny lobster, to insects which makes more than 93 species of arthropods. Not necessarily all serve as hosts but also as natural carriers (Sánchez-Paz, 2010).

The main sign of infection is the presence of white spots on the shrimp cuticle in varying sizes from invisible to 3 mm in diameter, hence the name. These spots happen due to deterioration of transport canals; therefore calcium carbonate, proteins and chitin priming substances are deposited on the cuticle-cell interface (Wang et al., 1999). Other symptoms of the WSD include cuticle being easy to separate from epidermis, hepatopancreas is yellowish-white, hemolymph fails to coagulate, reduced food intake and locomotion, reddish body color, reduced food consumption and lethargy in moribund shrimp which are followed by 100% mortality in a week (Lightner, 1996).

The histopathological studies have shown that WSSV targets several tissues in *P. leniusculus* all of which are of ectodermal and mesodermal origin including gill lamellae, adipose connective tissue, cuticular epithelium under the shell and around stomach, hematopoietic tissue and also hemocytes – with a higher preference to SGCs than GCs (Jiravanichpaisal et al., 2001). When infected, the nuclei become hypertrophied with a marginalized chromatin and inclusion bodies appear which strongly stains eosinophilic during early stages of infection and basophilic at the later advanced stages of infection (Lightner, 1996).

WSSV is transmitted through ingestion of an infected animal since cannibalism is very common in crayfish, and through ingestion of WSSV particles in infected waters. Vertical transmission (from parent to child) is also possible since the virus particles were detected in oocytes. However no mature eggs show virions suggesting that these oocytes cannot make it into mature eggs (Pradeep et al., 2012).

WSSV Structure

It is an enveloped, rod-shaped, circular dsDNA virus with a 300kbp genome encoding 531 putative open reading frames (Escobedo-Bonilla et al., 2008). It belongs to a new family *Nimaviridae*, genus *Whispovirus*. It has a nucleocapsid, a tegument and an outer envelope with over 30 envelope proteins that aid

virus entry as well as other infection processes as shown in Figure 2 (Chou et al., 1995; Escobedo-Bonilla et al., 2008). It also has a tail-like appendage but the function of this is not known yet. Of all 40 characterized WSSV structural proteins, VP26 and VP28 constitute 60% of the virion's envelope (Tsai et al., 2006) and unsurprisingly, VP28 has been reported to be involved in cell attachment during virus infection (van Hulten et al., 2001; Yi et al., 2004).

The stability of WSSV was shown to be very low against heat, but it can be maintained in a frozen tissue for a very long time and still be active. In a dark sterile sea water, it can remain viable for up to 30 days but in ponds it can be inactivated in about 3 days due to UV radiation and heat (Edgerton, 2002).

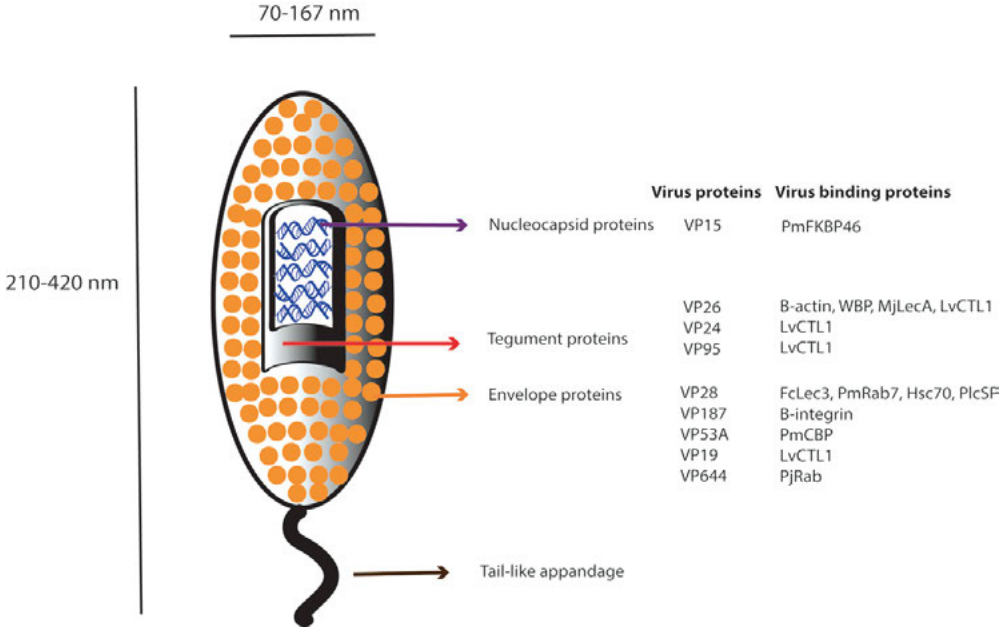

Figure 2: A schematic illustration of white spot syndrome virion and the virus-host protein interactions. Modified from Sritunyalucksana and colleagues. (Sritunyalucksana et al., 2012)

Entry pathways

Viruses use different strategies to enter host cells and transporting their DNA into host cytoplasm or nucleus. This makes them an attractive tool in biomedicine and biotechnology owing to their use in delivering drugs, vaccines, DNA and peptides. Liposomes loaded with components of virus to be delivered are called virosomes and they resemble the original viruses. The first step of viral entry is the binding of virus to receptor on target cell followed by fusion with cell membrane for enveloped viruses, and penetration of cell membrane by

non-enveloped viruses. The entry mechanism is dependent on the structure of the virus.

After crossing the basal membrane of the digestive tract, WSSV virions enters the hemolymph through which it can travel throughout the animal. WSSV then binds and enters to the target cell using its envelope and tegument proteins. In the past years, increasing number of virus binding proteins have been described (Ma et al., 2016; Verma et al., 2017).

Multiple protein interactions are required for the binding of integrin to viral protein for efficient binding and penetration of WSSV through membrane. Integrin on host cell surface acts as a receptor due to its recognition of Arg-Gly-Asp (RGD) motif on WSSV proteins. Not only RGD but KGD and SGD are also responsible for a firm interaction between integrin and VP187. An RGD, YGL and LDV motifs are required to bind VP26, VP31, VP37, VP90 and VP136 to ß-integrin in *L. vannamei* (Zhang et al., 2014).

The uptake of WSSV virions were observed both in HPT cells and hemocytes showing higher tropism to SGCs than to GCs (Jiravanichpaisal et al., 2001; Wang et al., 2002). However, it was shown that the virus progeny cannot be produced inside hemocytes but in HPT (Jiravanichpaisal et al., 2001; Wu et al., 2015), which has shown to be a very nice tool to study WSSV replication.

Protein kinase C (PKC) pathway is activated by the binding of peroxinectin, that is an opsonic and cell adhesive peroxidase, moreover this molecule is involved in cell spreading and degranulation (Johansson et al., 1995). The percentage of cell spreading and degranulation is lower in WSSV infected hemocytes (Jiravanichpaisal et al., 2006b). Therefore, WSSV infection might have an effect on PKC pathway.

Multiple endocytic routes have been described for WSSV. So far three different entry pathways have been studied: Clathrin mediated endocytosis (CME) (Huang et al., 2015) mediated by *Cq*-GABARAP was studied in crayfish *Cherax quadricarinatus* (Chen et al., 2016), cavaole-mediated endocytosis (Sahtout et al., 2001) and micropinocytosis.

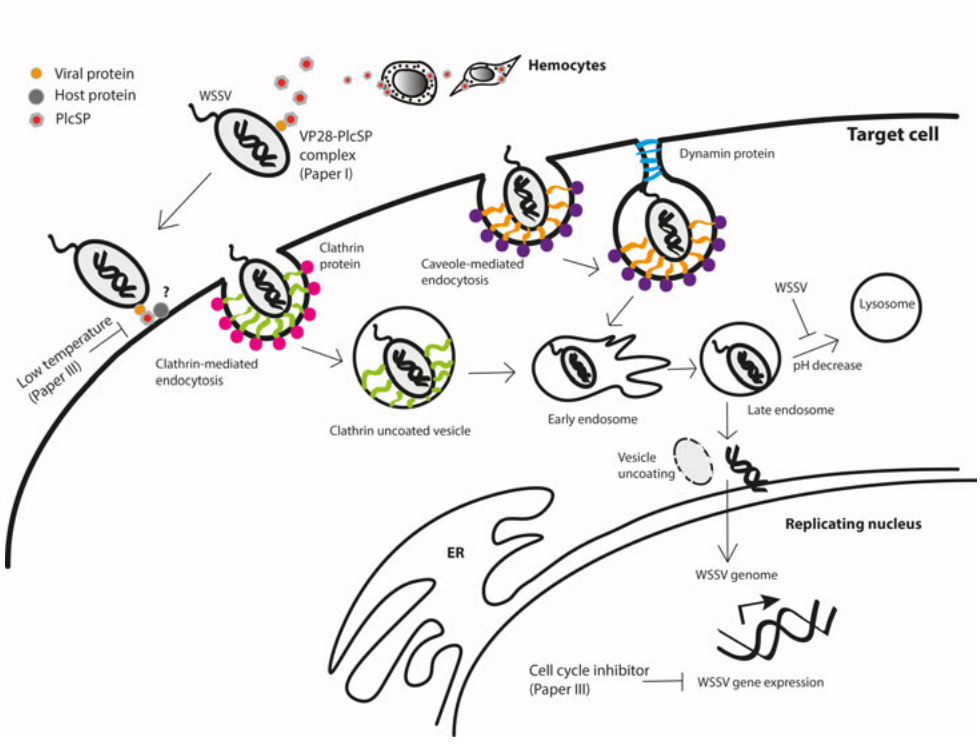

Figure 3: Schematic diagram depicting different entry routes for WSSV and the fate of virus inside the target cell (modified from Verma et al. 2017).

Upon uptake of WSSV into an early endosome by clathrin-mediated endocytosis, acidity with the endosome gradually increases as the endosome matures. Early endosomes are distinguished by the presence of Rab5 on their membrane which will be exchanged to Rab7 in mature endosomes by Rab-GTPases. *Pm*Rab7 of *P. monodon* has shown to be involved in cavaole-mediated endocytosis through its interaction with VP28 (Sritunyalucksana et al., 2006). The change in acidity in late endosomes will induce changes on WSSV such as uncoating of the virus envelope and escape of the viral DNA into cytosol. The viral genome will then direct itself towards the nucleus so that it can replicate its genome as depicted in Figure 3.

When WSSV is in cytoplasm, STAT will be activated, forms a dimer to go in the nucleus to activate the early viral gene WSSV ie1 promoter to start the viral replication (Li et al., 2009) this can be confirmed by the strong up-regulation of this pathway upon WSSV infection in *P. clarkii* (Du et al., 2016).

Host response

Several genes/proteins have been found to be involved in anti-WSSV responses in crustaceans. Anti-lipopolysaccharide factor (ALF) from crayfish *P.*

leniusculus was shown to have antiviral activity against WSSV (Liu et al., 2006; Tharntada et al., 2009) as well an ALF isolated from horseshoe crab *Limilus polyphemus* (de la Vega et al., 2008). An antiviral gene in *P. monodon* designated *Pm*AV was upregulated in *P. monodon* (Luo et al., 2003). A C-type lectin is upregulated by WSSV challenge, shows strong affinity to WSSV and interacts with envelope proteins (Li et al., 2014). Beta integrin interacts with WSSV envelope protein VP187. Silencing inhibits WSSV infection (Li et al., 2014). Hemocyanin found in blood, has antiviral properties (Adachi et al., 2003). Moreover, chitin binding protein (Huang et al., 2014), actin microfilaments (Xu et al., 2017; Ye et al., 2012), calreticulin (Chen et al., 2016; Wang et al., 2014), caspase-3 like gene in *L. vannamei* (Rijiravanich et al., 2008) and fortilin (Nupan et al., 2011; Tonganunt et al., 2008) are some of the other proteins that are involved in WSSV infection. Accordingly, it has been shown that in *L. vannamei*, the nucleoside diphosphate kinase activity has increased after WSSV infection which is involved in DNA replication by maintenance of deoxynucleotide triphosphate (dNTPs) (Liu et al., 2017)

As in response to other stressful conditions or infections, the number of circulating hemocytes in the hemolymph decreases rapidly during WSSV infection. For this reason, the production of new hemocytes is necessary to replace the lost hemocytes during an infection with a pathogen.

Peroxinectin activates the protein kinase C (PKC) pathway and results in the tyrosine phosphorylation of a ~80kDa protein thereby regulates cell attachment and spreading (Johansson et al., 1995). When peroxinectin treated, GCs and SGCs from WSSV infected crayfish lead to three types of cell reactions: spreading, no spreading and degranulation. WSSV was found to be more abundant in the non-spread cell groups. Therefore, PKC pathway could possibly be affected by WSSV (Jiravanichpaisal et al., 2006a).

Prevention of apoptosis and melanization

It has been shown that WSSV suppresses the immune system by inhibiting melanization reaction in shrimp (Sutthangkul et al., 2015), for its own benefit of exploiting the host for proliferating and spreading purposes. This was also the case with freshwater crayfish that the WSSV infection blocks the melanization reaction according to Jiravanichpaisal et al., 2006b. A reason for why the melanization reaction is inhibited by the WSSV could be the chelation of *Plc*SP by WSSV in the hemolymph as we have demonstrated in Paper I. Moreover, there are reports pointing to the involvement of serine proteases in viral infections such as YHV (Sriphaijit et al., 2007), dengue virus (Conway et al., 2014) and human metapneuma virus (Shirogane et al., 2008). A new study in *P. monodon* suggests that viral protein WSSV164 binds to *Pm*proPO1 and *Pm*proPO2 and thereby interferes with the melanization reaction (Sangsuriya et al., 2018).

According to (Shi et al., 2005), who showed that the virus replication does not take place in hemocytes or in the lymphoid organ (except for the connective tissue that surrounds the lymphoid organ tubules), they used DIG labelling and by in situ hybridization, they showed that the WSSV particles reside in the hemocytes of the WSSV infected animals. The particles do not go into the nucleus, nor are they destroyed by the immune system of the cell even when the cells were finally autolyzed. They note that the cells who contained these virus particles could be hyaline cells given that they have no or few granules in the cytoplasm also according to Johansson and colleagues (2000), these cell types are capable of phagocytosis. Zhu et al (2013) showed that WSSV inhibits phagosome maturation and digestion pathway.

Induction of apoptosis is regarded as one of the antiviral responses by the host. Upon recognition of the virus, the host will start the expression of pre-apoptotic proteins and change the mitochondrial membrane permeability and increase oxidative stress. Thereby the apoptotic program of the cell will be initiated.

Caspases are important regulators during apoptosis and hence, viruses target them. Initiator caspases are autocatalytic and effector caspases which are present as zymogens are activated by the active initiator caspases (Shi, 2002). WSSV can block the apoptosis of the infected cell by expressing two anti-apoptotic proteins in *M. japonicus*: anti apoptosis protein 1 (AAP-1) or WSSV449 – a caspase inhibitor and WSV222 – a E3 ubiquitin ligase by blocking an apoptotic inducer protein of shrimp TSL protein. A caspase was identified in *M. japonicus*, *Pj*Caspase, which was upregulated in survivors of WSD. When silenced, the viral copy number increased by inhibition of apoptosis, proving that induction of apoptosis is a good antiviral mechanism (Wang et al., 2008). Two viral proteins VP38 and VP41B are shown to bind *Pj*Caspase promoter as a repressor and activator, respectively. These opposite action of two viral proteins indicate that the virus facilitates its exit from the cell by inducing apoptosis at a later stage of infection (Jie et al., 2008; Xie et al., 2006).

Another antiapoptotic protein in *P. monodon* is *Pm*-fortilin which acts on mitochondria induced apoptosis (Tonganunt et al., 2008). In order to produce enough virions in the infected cell, WSSV will continue to prevent apoptosis from happening. The pro and anti-apoptotic proteins will compete during this process to determine the cell's fate. When the host is close to death, virus will exit the infected cell and spread its virions which will go in the hemolymph and continue to infect new cells. In order to do that, WSSV will then induce apoptosis as shown by the increased number of apoptotic cells with fragmented DNA (Sahtout et al., 2001) in the moribund *P. vannamei* and *P. monodon* (Sahtout et al., 2001) .

Treatment strategies

In contrast to the fact that invertebrates do not have an adaptive immunity, exposure to WSSV envelope proteins, for example, VP28, VP68, VP281 and VP466 antibodies when injected or orally administered, reduce or delay death in shrimp. (Witteveldt et al., 2004; Xu et al., 2006). Moreover, administration of inactivated WSSV has shown to be protective against WSSV when these animals were injected with active WSSV particles later on (Balasubramanian et al., 2006; Bright-Singh et al., 2005). Furthermore, it was shown that rabbit sera with anti-VP28 and anti-VP19 antibodies neutralized WSSV (Ha et al., 2008; Robalino et al., 2006). Preincubation of virus with an antimicrobial peptide, mytilin reduced shrimp mortality (Dupuy et al., 2004).

In addition, mi-RNA was shown to be an efficient treatment for WSSV. RNAi only interferes with the target viral gene in a sequence specific manner eliminating off-target effects, therefore siRNA and mRNA injections may be good and efficient therapeutics. Not only to WSSV but to YHV and TSV which are positive strand RNA viruses (Verbruggen et al., 2016). dsRNA exposure reduced the mortality in shrimp and crayfish that are later exposed to WSSV (Nilsen et al., 2017; Sarathi et al., 2010; Westenberg et al., 2005)

Herbal treatments from extracts from plants are also shown to be effective against WSSV such as main green tea catechin EGCG (Wang et al., 2017) and extract from seaweed (Balasubramanian et al., 2006). Notably all studies on treatment strategies have only been made by one group and not confirmed by other except for VP28 and some other proteins which will enhance resistance to WSSV and thus one needs to be careful in interpreting these suggested treatments.

Temperature and immunity

Freshwater crayfish is an ectotherm which means that its body temperature can change according to its surrounding temperatures and it can survive a temperature range between 4-30°C. The metabolism of crayfish is faster in warm temperatures meaning more energy consumption, while the crayfish in colder temperatures will devote its sources for growth and eat less, therefore the warmer temperatures were less likely to be preferred by crayfish. An experiment was performed about social hierarchies since crayfish are dominant hierarchical animals. The more dominant ones were reported to took the possession at the more comfortable temperature chamber over the other subordinate crayfish (Tattersall et al., 2012).

According to a recent study, the thermal tolerance of crayfish could be estimated from its electron transport system activity. Food consumption, respiration rates, difference between energy consumption and respiration costs were determined at a temperature range between 5°C and 30°C and ~20°C was shown to be the highest temperature for *P. leniusculus* for all these

parameters, all of which go down below and higher than that temperature (Simčič et al., 2014).

A previous transcriptome analysis of the initial stage of acute WSSV infection caused by temperature change shows that the genes involved in host cell cycle and positive regulation of cell death were downregulated and genes related to energy production were upregulated at the initial stage of infection (Sun et al., 2014).

Our knowledge on temperature's effect on pathogen infectivity is limited however most of the research is based on the growth rates of the pathogen, most of which show that the higher temperatures are better for the development of the pathogen for example in the case of West Nile virus, the incubation time decreases as the temperature warms from 10°C to 30°C (Reisen et al., 2006).

Aim of the present study

The purpose of this thesis is to understand the molecular mechanisms underlying WSSV entry and replication in the freshwater crayfish, *Pacifastacus leniusculus.* With the aim of revealing WSSV entry into the host cell, we looked at the proteins involved in virus attachment and a new clip-domain serine protease was identified (Paper I). This serine protease, which is possibly also involved in the melanization reaction, is further subjected to investigation for its role in serine protease cascade by studying its cleavage specificity (Paper IV).

As the increasing temperature around the globe is posing a threat to marine and aquatic life, the effect of temperature on WSSV infectivity was investigated (Paper II). In addition to the inhibition of WSSV infection at lower temperatures, we observed an inhibition of our previously discovered clip-domain serine protease, *Plc*SP. In order to understand the reason for high WSSV resistance at low temperatures the temperatures direct effect on the host immunity was studied. We also studied *P. leniusculus* immune responses using two gram-negative bacteria strains and lipopolysaccharide to elucidate the importance of temperature on host immunity regardless of its effect on pathogens (Paper III).

Results and discussion

A *Pacifastacus leniusculus* serine protease interacts with VP28 (Paper I)

With the aim of finding protein interactions between WSSV and host cell, we cross-linked WSSV with crayfish hemocytes using SEGS. Afterwards, we ran hemocyte lysates on SDS-PAGE and performed western blotting using anti-VP28 antibody to find the proteins which virus binds to. Infected granular (GCs) and semigranular cells (SGCs) both showed the same bands of sizes 40 kDa and 50 kDa when infected with WSSV. These bands were not visible in non-infected hemocytes. We performed a 2D gel analysis on the same samples only to find out that this spot was detectable with anti-VP28 only in the WSSV infected hemocytes.

We did a mass spectrum (MS) analysis to identify this protein. It turned out to be a new clip domain serine protease (*Plc*SP) in *P. leniusculus* hemocytes that can interact with WSSV envelope protein VP28. MS results point out to a six strictly conserved Cys residues, and the conserved His–Asp–Ser (H-D-S) motif in the C terminal catalytic domain suggesting that this is a new clip-domain serine protease. It has a 16-residue secretion signal peptide and therefore this should be a cytoplasmic protein which probably translocates onto the cell membrane to interact with VP28.

Nine different tissues were examined for their *Plc*SP expression. Relative tissue distribution results showed that *Plc*SP mRNA expression was almost 10 times higher in the *P. leniusculus* hemocytes compared to hepatopancreas, stomach, brain, hematopoietic tissue, intestine, heart, muscle and gill. Moreover, time course analysis was performed for the *Plc*SP expression. Crayfish were injected with WSSV and hemocytes were collected at 6, 12, 24-hour post-infection and qPCR analysis using VP28 primers show that the expression had increased in hemocytes upon WSSV challenge and that it peaks at 6 hpi.

Since *Plc*SP binds to VP28 of the WSSV, this interaction seems to be necessary for WSSV entry. Therefore, we used RNAi assay to knockdown this SP and we determined the WSSV replication by quantifying the VP28 expression in HPT *in vitro*. MOI measurements showed that the WSSV replication in HPT cell culture was 10 times lower in the ds*Plc*SP-RNAi cells compared to dsGFP-RNAi control group.

In addition to this, we showed that the spot detected in the 2D gel with the anti-VP28 antibody had shown a decrease in the intensity in the ds*Plc*SP-RNAi group compared to dsGFP-RNAi control group.

These finding prove our hypothesis that this new clip domain SP is an important player for WSSV infection and that it has an effect on the overall melanization cascade. Basically, our study confirms similar findings in a shrimp that WSSV interacts with a shrimp proteinase (Amparyup et al., 2010).

The effect of temperature on bacteria-host interactions in crayfish (Paper II)

We studied the effect of temperature on the defense system of the freshwater crayfish *Pacifastacus leniusculus*. Animals were challenged with two pathogenic Gram-negative bacteria *Aeromonas hydrophila* B1 and *Pseudomonas gessardii*, as well as the bacterial cell wall component lipopolysaccharide (LPS) at the same temperatures as in Paper II (6°C and 22°). We analyzed the growth curves for *A. hydrophila* and *P. gessardii* at cold and room temperature to find out that *P. gessardii* has a slightly slower growth in cold than at room temperature in contrast to *A. hydroph*ila which grows much slower in cold than room temperature. The immune responses were compared by means of differences in mortality, phagocytosis, bacterial clearance, and the melanization reaction of the hemolymph at these two temperatures. We observed that crayfish survived infections better at cold temperatures. The mortality rate was zero at 6°C following *A. hydrophila* and LPS injections. Whereas we observed delayed mortality for *P. gessardii*. Furthermore, *A. hydrophila* were completely cleared from crayfish after they had been kept at this low temperature for more than 9 days. This was shown by using a bacterial clearance assay and could be explained by a high phagocytic rate at 6°C compared to 22°C. The bacterial clearance rate was the same for both temperatures for *P. gessardii*. In order to find the reason for high mortality despite the equal bacterial clearance of *P. gessardii*, we checked the melanization rate of hemolymph from same animals but incubated at different temperatures. We observed a strong melanization reaction of hemolymph at 22°C when stimulated with LPS, *P. gessardii or A. hydrophila* in contrast to the same hemolymph at 6°C, which may explain the higher mortality rates at high temperature. Taken together, our results suggest that the cellular immunity is more effective at low temperature in this animal and pathogens are efficiently removed from the body by means of phagocytosis. We also showed that the humoral immunity is overactive at high temperature, which can lead to overproduction of toxic products for the host and probably contributes to the high mortality rates.

The effect of temperature on the WSD progression (Paper III)

In a previous study, the temperature dependence of WSD was demonstrated (Jiravanichpaisal et al., 2004). In order to understand why a low temperature is not permissive for WSD progression we studied the susceptibility of crayfish to WSSV infection at two different temperatures: 6°C and 22°C. We measured the mortality rates of WSSV injected animals in cold and room temperature and found that the WSSV injected crayfish could survive without any disease symptoms as long as they were maintained at cold temperature, while their room temperature counterparts developed the WSD and died in 4 days. Our hypothesis was that since WSSV needs proliferating cells for its own replication, decreasing the temperature would inhibit WSSV replication by means of decreasing proliferation rate of host HPT. In order to prove our hypothesis, we injected the crayfish with a mitotic inhibitor substance called demecolcine. Demecolcine injections postponed the deaths for 2 days compared to the control group who were injected with crayfish saline (CFS).

To further confirm that these differences of mortalities were due to virus replication, we checked the expression of VP28 gene in hemocytes and HPT of crayfish who were injected with WSSV but kept at different temperatures. The ones kept in cold had almost no VP28 expressed compared to moribund crayfish who were kept at room temperature. This result was demonstrated by qRT-PCR.

Furthermore, the VP28 expression was found to be lower in these animals receiving both injections with WSSV and demecolcine since cell proliferation was inhibited by demecolcine. We quantified WSSV copy numbers and found that virus entry was blocked at 6°C, but not in demecolcine treatments. We supported this result by quantifying the expression of a clip domain serine protease (*Plc*SP) which plays an important role for WSSV binding and it was inhibited at 6°C. Therefore, our hypothesis is that the WSSV needs proliferating cells to replicate, and an optimum temperature to successfully enter the host hematopoietic stem cells.

According to our results from Paper II, at cold temperature the virus is in the hemolymph not entering neither HPT nor the hemocytes-not even through phagocytosis. However according to Paper III, the phagocytosis is much better in the crayfish that are maintained at 6°C. This can be explained by different routes or mechanisms for the phagocytosis for bacteria and virus, since they are different in size just like our results for LPS in Paper III. The *Plc*SP or another protein which can act as an opsonizing agent and is downregulated in cold could be preventing the phagocytosis of the virus. Furthermore, the decrease of melanization reaction rate in cold temperature could as well be explained by the downregulation of *Plc*SP gene which is possibly involved in the serine protease cascade during melanization reaction.

Expression of an active and inactive clip-domain serine protease isolated from signal crayfish in an insect cell line (Paper IV)

In order to further understand the intrinsic role of *Plc*SP we started an experiment to find a putative endogenous substrate and cleavage specificity for this protease. The method that we intend to use is substrate phage display.

Substrate phage display method is a method to identify the substrate of a protein of interest (Matthews and Wells, 1993). It uses a phage library of 5 x 10^7 random peptide sequences followed by a His_6 -tag which are displayed on the surface of a phagemid particle, on C-terminal of capsid 10 protein as illustrated in Fig 4A. The peptide sequences are of 9 aa length, allowing us to characterize not only the cleavage site but the 4-5 amino acids both upstream and downstream of P1 and P1', which gives us the extended cleavage specificity of our serine protease. Each of these phages is allowed to bind to Ni-NTA agarose beads via their His_6 -tags, and unbound phages are removed. By adding the protease, we cleave the bond between the phage and the beads. When we centrifuge this solution the heavy Ni-NTA beads with bound phages that could resist the proteolysis of our enzyme will be precipitated at the bottom of the tube while the cleaved phages will be in the supernatant. So, we can collect the phages, which contain potential substrate sequences for our enzyme. These phages are propagated to *E. coli* so that we can expand and visualize them. The entire procedure is repeated 6 more times by using the expanded phages from each round. The phages collected from the last round of selection are plated on an LA-Amp plate for plaque assay. By isolating and sequencing individual plaques, we can obtain the 9 random amino acids that are cleaved by our protease.

To validate the peptide sequence we get from phage-display, another method called two-thioredoxin (trx) system is used (Ahooghalandari et al., 2013; Andersson et al., 2010; Gallwitz et al., 2012; Thorpe et al. 2012). The peptide sequence is synthesized as oligonucleotides and inserted between BamHI and SalI restriction sites between two trx proteins in a pET21 vector as shown in Fig 4b. The second trx has a His_6–tag, which makes purification easier. This construct is expressed and grown in *E. coli* Rosetta gami. The protein was purified by addition of Ni-NTA beads after the cells are sonicated to collect the intracellular protein so that the proteins could be eluted. By adding our serine protease to these eluted fractions and analyzing the proteins by SDS-PAGE gel under denaturing conditions, we can visualize with which of the eluted proteins were susceptible to cleavage by our protease by Coomassie blue staining of the gel.

With the aim of applying these techniques to our *Plc*SP, we constructed an active and an inactive form of *Plc*SP. These oligonucleotides were inserted in pCEP-Pu2 vector, which contains a powerful promoter, a start codon, a N-terminal His_6 -tag followed by an enterokinase cleavage site (DDDDK). The isolated plasmids were then transfected into competent *E. coli* DK1 cells to amplify the plasmid. The plasmids were isolated, washed and transfected to

HEK293 cell line by lipofectamine. Puromycin was added for selection of plasmid-positive cells. A confluent culture should have been obtained after 1 or 2 weeks. However, it was very hard to get the cells to survive after transfection of this serine protease. After months of trying to grow the transfected HEK293 cells, very few colonies could be grown.

The vector contains a secretion signal; therefore, our protein should be secreted into the conditioned culture medium, from which we can purify our protein by Ni-NTA bead purification. The purity and the concentration of the extracted protein were determined by SDS-PAGE gel and the activation of the recombinant protease is checked by chromogenic substrate assay. We observed a minute amount of protein on SDS-PAGE and the only activity we observed using chromogenic substrate assay was for enterokinase, the cleavage site that was already present in the plasmid.

The transfection experiments were repeated three times with different DNA preparations. All attempts to grow the active and inactive *Plc*SP failed in HEK293 cells, indicating that it could be toxic for mammalian cells. This could be due to differences in folding of the crayfish protein in the mammalian endoplasmic reticulum (ER), resulting in a failure to transport the proteins out of ER and eventually clogging the cell with misfolded protein aggregates and leading to death of the cell.

Knowing that recombinant *Plc*SP was not possible to express in mammalian cells, we have instead initiated attempts to express the protease in Sf9 insect cell line from the ovarian tissue of the Fall armyworm, *Spodoptera frugiperda,* using a baculovirus expression system with pFastBac1 vector and we have confirmed the expression of the recombinant *Plc*SP in this cell line.

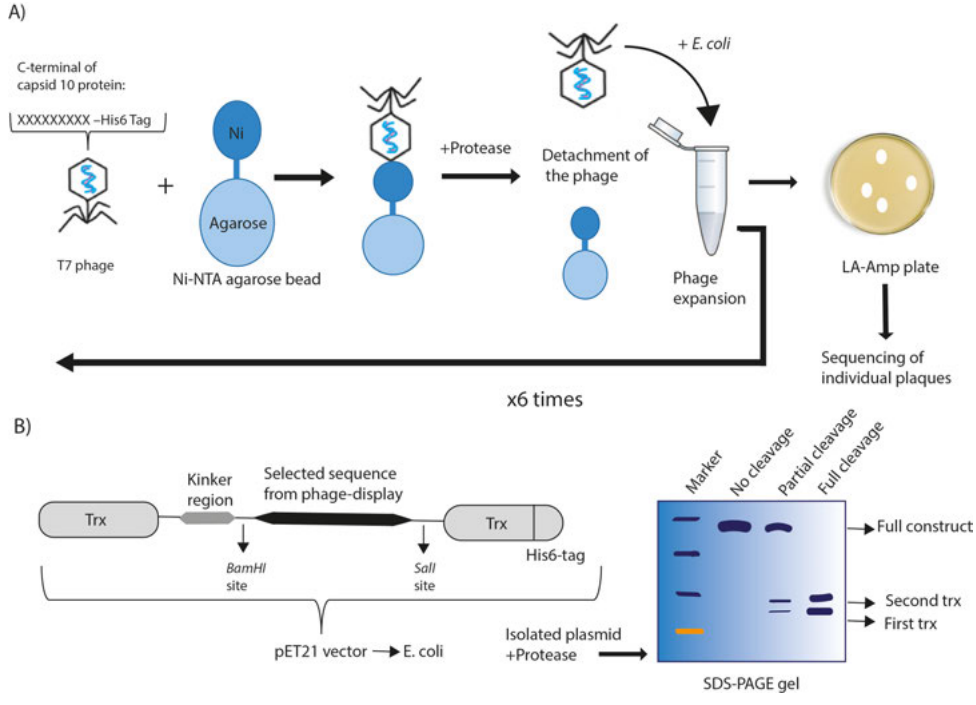

Figure 4: A schematic representation of the two methods, which are aimed to be used. A) Phage-display method to identify the cleavage specificity of an enzyme. B) Two thioredoxin (trx) approach to verify the peptide sequence obtained from phage-display method (Modified from Thorpe et al., 2016).

Concluding Remarks

My studies focused on understanding crayfish immunity with respect to temperature and WSSV infection in *P. leniusculus*. In the first study, we have elucidated a new WSSV interacting protein in the hemocytes and HPT. The new clip *Plc*SP participates in WSSV replication by providing the virus with an attachment site on the host cell membrane. This protein has a signal peptide and therefore we assume it to be secreted by the hemocytes upon infection with WSSV. By knocking down this gene, we could prevent WSSV infection. Therefore, this protein seems to be essential for a successful WSSV infection.

Moreover, we investigated the cleavage site for *Plc*SP which is probably involved in the serine protease cascade during prophenoloxidase activation cascade. By expressing an active and inactive form of this protein in insect cell line (Sf9 cells from fall armyworm) and by using phage-substrate display technique we will be able to identify what this protease cleaves in the cascade.

Secondly, we answered the question: Does WSSV needs host cells to be proliferating in order to replicate itself? In a quiescent state of host HPT, the virus will remain silent until the conditions are optimal i.e. the host metabolism is active. We demonstrated this by using a low and room temperature to check WSSV infection. In contrast to room temperature, at the lower temperatures in which the cell metabolism is quiescent, the infection was inhibited which we demonstrated by mortality and quantification of WSSV virions. Moreover, the virus was not even able to enter in neither HPT nor hemocytes. The expression of *Plc*SP which we have shown to be essential for viral entry is also inhibited at low temperatures which might explain our results. However, by inhibiting the cell cycle at M-phase using an inhibitor, we could not prevent viral entry but we could inhibit the viral replication in HPT cells. Hemocytes showed no viral particles. This might be due to the inhibition of HPT cells release from HPT and confirming that the HPT is the main target for the virus due to its highly proliferative abilities.

In order to understand if the temperature's direct effect is on the host immunity or the pathogen itself, we repeated the same experiment with two gram-negative bacteria and LPS, whereby we could demonstrate that the cellular immunity is enhanced at low temperatures while the melanization is much higher in room temperatures which lead to higher mortalities due to production of excessive toxic compounds which harms the host.

Svensk sammanfattning

Ryggradslösa djur använder sig enbart av sitt medfödda immunförsvar för att skydda sig mot patogener. Deras immunförsvar består av både cellulära och humorala komponenter. Det cellulära försvaret består huvudsakligen av fagocytos, opsonisering och inkapsling av patogener, medan det humorala försvaret främst består av produkter som t.ex. antimikrobiella peptider (AMP) och melaniseringsreaktionen som leder till att patogenen dör. Melanisering orsakas av det så kallade profenoloxidas (proPO)-aktiverande systemet och som är en viktig försvarsreaktion vars komponenter lagras i vesikler i semigranulära och granulära hemocyter (blodkroppar). Dessa celler degranulerar och frigör proPO-systemet när de aktiveras av en patogen, och en kaskad av proteaser aktiverar enzymet fenoloxidas och patogenen kan elimineras.

Vitfläcks-virus (WSSV) är en dödlig patogen som främst angriper kräftdjur och sjukdomen har orsakar enorma ekonomiska förluster inom vattenbruk sedan den först upptäcktes år 1992 i Taiwan. Det är känt att WSSV avaktiverar värdens immunsystem genom att störa proPO-kaskaden, men mekanismen för hur viruset tar sig in i cellerna och dödar dessa är fortfarande till stora delar okända.

Ökande vattentemperaturer som följer av globala klimatförändringar utgör ett hot mot vattenlevande organismer, däribland även sötvattenlevande djur. Förändringar i temperaturen kan vara en stressande faktor, som i sin tur påverkar djurens mottaglighet för sjukdomar. Kalla temperaturer är en begränsande faktor för utveckling av en WSSV-infektion men det är inte känt om detta beror på påverkan är på värdens immunitet eller på själva virusets förmåga att föröka sig.

Mina studier i denna avhandling fokuserar på att förstå hur temperaturen påverkar signalkräftans *Pacifastacus leniusculus* immunförsvar mot WSSV och bakterier.

I den första studien har vi funnit ett nytt WSSV-interagerande protein i kräftans blodkroppar, s.k. hemocyter och även i den blodkroppsbildande vävnaden (kallad HPT). Detta protein är ett proteolytiskt enzym som vi har kallat *Plc*SP och detta enzym hjälper viruset att komma in i cellen genom att binda det till cellmembranet. Detta protein har en s.k. signalpeptid och därför antar vi att den utsöndras av hemocyterna under en infektion med WSSV. Genom att slå ut genen som kodar för *Plc*SP kunde vi hindra WSSV att föröka sig i

kräftans blodkroppar och dess stamceller. Därför verkar detta protein vara nödvändigt för att en WSSV-infektion ska kunna etablera sig.

Proteolytiska enzymer klyver ofta andra proteaser (såkallade zymogener) så att dessa kommer i sin aktiva form, och våra försök visar att det är troligt att *Plc*SP är inblandat i den proPO-aktiverande serinproteaskaskaden. Därför har vi nu också påbörjat en undersökning av vilket endogent naturligt substrat som *Plc*SP klyver i kräftan. Det gör vi genom att tillverka ett aktivt *Plc*SP i ett insektcell-system (Sf9-celler) och sedan använda en speciell teknik kallad ”Phage-display” för att hitta det proteinsubstrat som binder starkt till och klyvs av *Plc*SP och därmed är ett troligt verkligt substrat.

Det är välkänt att temperaturen påverkar cellernas metabolism och att låg temperatur kan påverka cellernas delningshastighet. Därför undersökte vi om WSSV behöver värdceller som delar sig för att själv föröka sig. Vi kunde visa att WSSV-viruset kan ligga inaktivt i kräftan vid låg temperatur för att sedan bli aktivt när förhållandena och temperaturen är optimala för tillväxt. Genom att mäta mängden WSSV-partiklar och kräftornas dödlighet kunde vi visa att virusinfektionen hämmades vid låg temperatur när cellmetabolism och delningshastighet är låga. Dessutom kunde viruset inte ens komma in i varken HPT-celler eller i hemocyter i kyla. En förklaring till detta kan vara att proteaset *Plc*SP inte heller bildas i någon högre grad vid låga temperaturer. Genom att hämma celldelning kunde vi dock inte hindra att virus kom in i cellerna, men vi kunde förhindra deras förökning.

För att förstå om den låga temperaturen främst påverkar värdens immunförsvar eller patogenens förmåga att infektera värden, utformade vi liknande experiment med två gramnegativa bakterier och LPS, varigenom vi kunde visa att den cellulära immuniteten var bättre vid låga temperaturer med högre fagocytos medan melaniseringen är mycket högre i höga temperaturer och detta leder då till högre dödlighet på grund av produktion av alltför höga toxiska föreningar som skadar värden.

Sammanfattningsvis visar resultaten i denna avhandling att en allt högre vattentemperatur allvarligt kan försvåra för vattenlevande kräftdjur att överleva infektioner.

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References

Adachi, K., Hirata, T., Nishioka, T., and Sakaguchi, M. (2003). Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *134*, 135–141.

Ahooghalandari, P., Hanke, N., Thorpe, M., Witte, A., Messinger, J., and Hellman, L. (2013). Mutations in Arg143 and Lys192 of the Human Mast Cell Chymase Markedly Affect the Activity of Five Potent Human Chymase Inhibitors. PloS One *8*, e65988.

Amparyup, P., Promrungreang, K., Charoensapsri, W., Sutthangkul, J., and Tassanakajon, A. (2013). A serine proteinase *Pm*ClipSP2 contributes to prophenoloxidase system and plays a protective role in shrimp defense by scavenging lipopolysaccharide. Dev. Comp. Immunol. *41*, 597–607.

Amparyup, P., Wiriyaukaradecha, K., Charoensapsri, W., and Tassanakajon, A. (2010). A clip domain serine proteinase plays a role in antibacterial defense but is not required for prophenoloxidase activation in shrimp. Dev. Comp. Immunol. *34*, 168–176.

Andersson, M.K., Thorpe, M., and Hellman, L. (2010). Arg143 and Lys192 of the human mast cell chymase mediate the preference for acidic amino acids in position P2' of substrates. FEBS J. *277*, 2255–2267.

Angthong, P., Watthanasurorot, A., Klinbunga, S., Ruangdej, U., Söderhäll, I., and Jiravanichpaisal, P. (2010). Cloning and characterization of a melanization inhibition protein (*Pm*MIP) of the black tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol. *29*, 464–468.

Ashida, M., and Söderhäll, K. (1984). The prophenoloxidase activating system in crayfish. Comp. Biochem. Physiol. Part B Comp. Biochem. *77*, 21–26.

Aspan, A., Huang, T. S., Cerenius, L., and Söderhäll, K. (1995). cDNA Cloning of Prophenoloxidase from the Freshwater Crayfish Pacifastacus leniusculus and Its Activation. Proc. Natl. Acad. Sci. U. S. A. *92*, 939–943.

Balasubramanian, G., Sudhakaran, R., Syed Musthaq, S., Sarathi, M., and Sahul Hameed, A.S. (2006). Studies on the inactivation of white spot syndrome virus of shrimp by physical and chemical treatments, and seaweed extracts tested in marine and freshwater animal models. J. Fish Dis. *29*, 569–572.

van de Braak, C.B.T., Botterblom, M.H.A., Liu, W., Taverne, N., van der Knaap, W.P.W., and Rombout, J.H.W.M. (2002). The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*). Fish Shellfish Immunol. *12*, 253–272.

Bright-Singh, I.S., Manjusha, M., Pai, S.S., and Philip, R. (2005). Fenneropenaeus indicus is protected from white spot disease by oral administration of inactivated white spot syndrome virus. Dis. Aquat. Organ. *66*, 265–270.

Caldeira, K., and Wickett, M.E. (2003). Oceanography: Anthropogenic carbon and ocean pH. Nature *425*, 365.

Cerenius, L., Lee, B.L., and Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. *29*, 263–271.

Cerenius, L., and Söderhäll, K. (2018). Crayfish immunity - Recent findings. Dev. Comp. Immunol. *80*, 94–98.
Cerenius, L., Kawabata, S., Lee, B.L., Nonaka, M., and Söderhäll, K. (2010). Proteolytic cascades and their involvement in invertebrate immunity. Trends Biochem. Sci. *35*, 575–583.
Chaga, O., Lignell, M., and Söderhäll, K. (1995). The haemopoietic cells of the freshwater crayfish Pacifastacus leniusculus. Anim. Biol. *4*, 59–70.
Charoensapsri, W., Amparyup, P., Hirono, I., Aoki, T., and Tassanakajon, A. (2009). Gene silencing of a prophenoloxidase activating enzyme in the shrimp, Penaeus monodon, increases susceptibility to *Vibrio harveyi* infection. Dev. Comp. Immunol. *33*, 811–820.
Chen, R.Y., Shen, K.L., Chen, Z., Fan, W.W., Xie, X.L., Meng, C., Chang, X.J., Zheng, L.B., Jeswin, J., Li, C.H., et al. (2016). White spot syndrome virus entry is dependent on multiple endocytic routes and strongly facilitated by *Cq*-GABA-RAP in a CME-dependent manner. Sci. Rep. *6*, 28694.
Cherry, S., and Silverman, N. (2006). Host-pathogen interactions in *Drosophila*: new tricks from an old friend. Nat. Immunol. *7*, 911–917.
Choi, H., and Lee, D.G. (2014). Antifungal activity and pore-forming mechanism of astacidin 1 against *Candida albicans*. Biochimie *105*, 58–63.
Chou, H.Y., Wang, C.H., Chiang, H.C., and Lo, C.F. (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Organ. *23*, 165–173.
Coates, C.J., and Decker, H. (2017). Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin. Cell. Mol. Life Sci. *74*, 293–317.
Conway, M.J., Watson, A.M., Colpitts, T.M., Dragovic, S.M., Li, Z., Wang, P., Feitosa, F., Shepherd, D.T., Ryman, K.D., Klimstra, W.B., et al. (2014). Mosquito saliva serine protease enhances dissemination of dengue virus into the mammalian host. J. Virol. *88*, 164–175.
Destoumieux-Garzón, D., Saulnier, D., Garnier, J., Jouffrey, C., Bulet, P., and Bachère, E. (2001). Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. J. Biol. Chem. *276*, 47070–47077.
Diaz, R.J. (2001). Overview of Hypoxia around the World. J. Environ. Qual. *30*, 275–281.
Donpudsa, S., Rimphanitchayakit, V., Tassanakajon, A., Söderhäll, I., and Söderhäll, K. (2010). Characterization of two crustin antimicrobial peptides from the freshwater crayfish *Pacifastacus leniusculus*. J. Invertebr. Pathol. *104*, 234–238.
Du, Z., Jin, Y., and Ren, D. (2016). In-depth comparative transcriptome analysis of intestines of red swamp crayfish, *Procambarus clarkii*, infected with WSSV. Sci. Rep. *6*, 26780.
Dupuy, J.W., Bonami, J.R., and Roch, P. (2004). A synthetic antibacterial peptide from *Mytilus galloprovincialis* reduces mortality due to white spot syndrome virus in palaemonid shrimp. J. Fish Dis. *27*, 57–64.
Edgerton, B.F. (2002). Hazard analysis of exotic pathogens of potential threat to European freshwater crayfish. Bull. Fr. Pêche Piscic. 813–820.
Escobedo-Bonilla, C.M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M.B., and Nauwynck, H.J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J. Fish Dis. *31*, 1–18.

Fagutao, F.F., Koyama, T., Kaizu, A., Saito-Taki, T., Kondo, H., Aoki, T., and Hirono, I. (2009). Increased bacterial load in shrimp hemolymph in the absence of prophenoloxidase. FEBS J. *276*, 5298–5306.

Fuhrman, J.A. (1999). Marine viruses and their biogeochemical and ecological effects. Nature *399*, 541–548.

Gallwitz, M., Enoksson, M., Thorpe, M., and Hellman, L. (2012). The extended cleavage specificity of human thrombin. PloS One *7*, e31756.

Guo, P., Hirano, M., Herrin, B.R., Li, J., Yu, C., Sadlonova, A., and Cooper, M.D. (2009). Dual nature of the adaptive immune system in lampreys. Nature *459*, 796–801.

Ha, Y.M., Kim, Y.I., Kim, K.H., and Kim, S.K. (2008). Neutralization of white spot syndrome virus (WSSV) for *Penaeus chinensis* by antiserum raised against recombinant VP19. J. Environ. Biol. *29*, 513–517.

Unestam, T., Söderhäll, K. (1977). Soluble fragments from fungal cell walls elicit defence reactions in crayfish. Nature. *267*, 1476-4687 .

Hernroth, B.E., and Baden, S.P. (2018). Alteration of host-pathogen interactions in the wake of climate change – Increasing risk for shellfish associated infections? Environ. Res. *161*, 425–438.

Hillyer, J.F. (2010). Mosquito immunity. Adv. Exp. Med. Biol. *708*, 218–238.

Huang, J., Li, F., Wu, J., and Yang, F. (2015). White spot syndrome virus enters crayfish hematopoietic tissue cells via clathrin-mediated endocytosis. Virology *486*, 35–43.

Huang, P.Y., Leu, J.H., and Chen, L.L. (2014). A newly identified protein complex that mediates white spot syndrome virus infection via chitin-binding protein. J. Gen. Virol. *95*, 1799–1808.

Huang, T.S., Wang, H., Lee, S.Y., Johansson, M.W., Söderhäll, K., and Cerenius, L. (2000). A cell adhesion protein from the crayfish Pacifastacus leniusculus, a serine proteinase homologue similar to Drosophila masquerade. J. Biol. Chem. *275*, 9996–10001.

van Hulten, M.C., Witteveldt, J., Snippe, M., and Vlak, J.M. (2001). White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology *285*, 228–233.

IPCC (2014). Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Janeway, C.A., and Medzhitov, R. (2002). Innate immune recognition. Annu. Rev. Immunol. *20*, 197–216.

Jang, I.-K., Pang, Z., Yu, J., Kim, S.-K., Seo, H.-C., and Cho, Y.-R. (2011). Selectively enhanced expression of prophenoloxidase activating enzyme 1 (PPAE1) at a bacteria clearance site in the white shrimp, *Litopenaeus vannamei*. BMC Immunol. *12*, 70.

Jearaphunt, M., Noonin, C., Jiravanichpaisal, P., Nakamura, S., Tassanakajon, A., Söderhäll, I., and Söderhäll, K. (2014). Caspase-1-Like Regulation of the proPO-System and Role of ppA and Caspase-1-Like Cleaved Peptides from proPO in Innate Immunity. PLoS Pathog *10*.

Jiang, H., and Kanost, M.R. (2000). The clip-domain family of serine proteinases in arthropods. Insect Biochem. Mol. Biol. *30*, 95–105.

Jie, Z., Xu, L., and Yang, F. (2008). The C-terminal region of envelope protein VP38 from white spot syndrome virus is indispensable for interaction with VP24. Arch. Virol. *153*, 2103–2106.

Jiravanichpaisal, P., Bangyeekhun, E., Söderhall, K., and Söderhall, I. (2001). Experimental infection of white spot syndrome virus in freshwater crayfish *Pacifastacus leniusculus*. Dis. Aquat. Organ. *47*, 151–157.

Jiravanichpaisal, P., Lee, B.L., and Söderhäll, K. (2006a). Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology *211*, 213–236.

Jiravanichpaisal, P., Lee, S.Y., Kim, Y.-A., Andrén, T., and Söderhäll, I. (2007). Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. Dev. Comp. Immunol. *31*, 441–455.

Jiravanichpaisal, P., Sricharoen, S., Söderhäll, I., and Söderhäll, K. (2006b). White spot syndrome virus (WSSV) interaction with crayfish haemocytes. Fish Shellfish Immunol. *20*, 718–727.

Jiravanichpaisal, P., Söderhäll , K., and 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 Immunol. *17*, 265–275.

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

Johansson, M.W., Lind, M.I., Holmblad, T., Thörnqvist, P.O., and Söderhäll, K. (1995). Peroxinectin, a novel cell adhesion protein from crayfish blood. Biochem. Biophys. Res. Commun. *216*, 1079–1087.

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

Johansson, M.W., and Söderhäll, K. (1988). Isolation and purification of a cell adhesion factor from crayfish blood cells. J. Cell Biol. *106*, 1795–1803.

Kawabata, S., Tokunaga, F., Kugi, Y., Motoyama, S., Miura, Y., Hirata, M., and Iwanaga, S. (1996). Limulus factor D, a 43-kDa protein isolated from horseshoe crab hemocytes, is a serine protease homologue with antimicrobial activity. FEBS Lett. *398*, 146–150.

Khorattanakulchai, N., Amparyup, P., and Tassanakajon, A. (2017). Binding of *Pm*ClipSP2 to microbial cell wall components and activation of the proPO-activating system in the black tiger shrimp *Penaeus monodon*. Dev. Comp. Immunol. *77*, 38–45.

Kobayashi, M., Johansson, M.W., and Söderhäll, K. (1990). The 76 kD cell-adhesion factor from crayfish haemocytes promotes encapsulation in vitro. Cell Tissue Res. *260*, 13–18.

Lee, S.Y., and Söderhäll, K. (2001). Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. J. Immunol. Baltim. Md 1950 *166*, 7319–7326.

Lee, S.Y., Lee, B.L., and Söderhäll, K. (2003). Processing of an Antibacterial Peptide from Hemocyanin of the Freshwater Crayfish *Pacifastacus leniusculus*. J. Biol. Chem. *278*, 7927–7933.

Lee, S.Y., Lee, B.L., and Söderhäll, K. (2004). Processing of crayfish hemocyanin subunits into phenoloxidase. Biochem. Biophys. Res. Commun. *322*, 490–496.

Lei, K., Li, F., Zhang, M., Yang, H., Luo, T., and Xu, X. (2008). Difference between hemocyanin subunits from shrimp *Penaeus japonicus* in anti-WSSV defense. Dev. Comp. Immunol. *32*, 808–813.

Li, F., Li, M., Ke, W., Ji, Y., Bian, X., and Yan, X. (2009). Identification of the immediate-early genes of white spot syndrome virus. Virology *385*, 267–274.

Li, M., Li, C., Ma, C., Li, H., Zuo, H., Weng, S., Chen, X., Zeng, D., He, J., and Xu, X. (2014). Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp *Litopenaeus vannamei*. Dev. Comp. Immunol. *46*, 231–240.

Liang, Z., Sottrup-Jensen, L., Aspán, A., Hall, M., and Söderhäll, K. (1997). Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. Proc. Natl. Acad. Sci. U. S. A. *94*, 6682–6687.

Lightner, D.V. (1996). A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society.

Lin, X., Cerenius, L., Lee, B.L., and Söderhäll, K. (2007). Purification of properoxinectin, a myeloperoxidase homologue and its activation to a cell adhesion molecule. Biochim. Biophys. Acta *1770*, 87–93.

Lin, X., Söderhäll, K., and Söderhäll, I. (2011). Invertebrate hematopoiesis: an astakine-dependent novel hematopoietic factor. J. Immunol. Baltim. Md 1950 *186*, 2073–2079.

Ling, E., and Yu, X.Q. (2006a). Hemocytes from the tobacco hornworm *Manduca sexta* have distinct functions in phagocytosis of foreign particles and self-dead cells. Dev. Comp. Immunol. *30*, 301–309.

Ling, E., and Yu, X.Q. (2006b). Cellular encapsulation and melanization are enhanced by immulectins, pattern recognition receptors from the tobacco hornworm *Manduca sexta*. Dev. Comp. Immunol. *30*, 289–299.

Liu, H., Jiravanichpaisal, P., Cerenius, L., Lee, B.L., Söderhäll, I., and Söderhäll, K. (2007a). Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* Infection in a crustacean, *Pacifastacus leniusculus*. J. Biol. Chem. *282*, 33593–33598.

Liu, H., Jiravanichpaisal, P., Söderhäll, I., Cerenius, L., and Söderhäll, K. (2006). Antilipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish Pacifastacus leniusculus. J. Virol. *80*, 10365–10371.

Liu, H., Wu, C., Matsuda, Y., Kawabata, S.-I., Lee, B.L., Söderhäll, K., and Söderhäll, I. (2011). Peptidoglycan activation of the proPO-system without a peptidoglycan receptor protein (PGRP)? Dev. Comp. Immunol. *35*, 51–61.

Liu, P.-F., Liu, Q.-H., Wu, Y., and Huang, J. (2017). Increased nucleoside diphosphate kinase activity induces white spot syndrome virus infection in *Litopenaeus vannamei*. PloS One *12*, e0175741.

Liu, Y.-C., Li, F.-H., Dong, B., Wang, B., Luan, W., Zhang, X.-J., Zhang, L.-S., and Xiang, J.-H. (2007b). Molecular cloning, characterization and expression analysis of a putative C-type lectin (*Fc*lectin) gene in Chinese shrimp *Fenneropenaeus chinensis*. Mol. Immunol. *44*, 598–607.

Luo, T., Yang, H., Li, F., Zhang, X., and Xu, X. (2006). Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. Dev. Comp. Immunol. *30*, 607–617.

Luo, T., Zhang, X., Shao, Z., and Xu, X. (2003). *Pm*AV, a novel gene involved in virus resistance of shrimp *Penaeus monodon*. FEBS Lett. *551*, 53–57.

Ma, K., Guo, L., Xu, A., Cui, S., and Wang, J.-H. (2016). Molecular Mechanism for Stress-Induced Depression Assessed by Sequencing miRNA and mRNA in Medial Prefrontal Cortex. PloS One *11*, e0159093.

Marcogliese, D.J. (2008). The impact of climate change on the parasites and infectious diseases of aquatic animals. Rev. Sci. Tech. Int. Off. Epizoot. *27*, 467–484.

Matthews, D.J., and Wells, J.A. (1993). Substrate phage: selection of protease substrates by monovalent phage display. Science *260*, 1113–1117.

Monwan, W., Amparyup, P., and Tassanakajon, A. (2017). A snake-like serine proteinase (*Pm*Snake) activates prophenoloxidase-activating system in black tiger shrimp *Penaeus monodon*. Dev. Comp. Immunol. *67*, 229–238.

Moussian, B., and Roth, S. (2005). Dorsoventral axis formation in the Drosophila embryo--shaping and transducing a morphogen gradient. Curr. Biol. CB *15*, R887-899.

Murugasu-Oei, B., Rodrigues, V., Yang, X., and Chia, W. (1995). Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the Drosophila embryo. Genes Dev. *9*, 139–154.

Muta, T., Hashimoto, R., Miyata, T., Nishimura, H., Toh, Y., and Iwanaga, S. (1990). Proclotting enzyme from horseshoe crab hemocytes. cDNA cloning, disulfide locations, and subcellular localization. J. Biol. Chem. *265*, 22426–22433.

Nappi, A.J., and Vass, E. (1993). Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. Pigment Cell Res. *6*, 117–126.

Nappi, A., Poirié, M., and Carton, Y. (2009). The role of melanization and cytotoxic by-products in the cellular immune responses of Drosophila against parasitic wasps. Adv. Parasitol. *70*, 99–121.

Nilsen, P., Karlsen, M., Sritunyalucksana, K., and Thitamadee, S. (2017). White spot syndrome virus VP28 specific double-stranded RNA provides protection through a highly focused siRNA population. Sci. Rep. *7*, 1028.

Nupan, B., Phongdara, A., Saengsakda, M., Leu, J.H., and Lo, C.F. (2011). Shrimp *Pm*-fortilin inhibits the expression of early and late genes of white spot syndrome virus (WSSV) in an insect cell model. Dev. Comp. Immunol. *35*, 469–475.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc. Natl. Acad. Sci. *104*, 5431–5436.

Persson, M., Cerenius, L., and Söderhäll, K. (1987). The influence of haemocyte number on the resistance of the freshwater crayfish, *Pacifastacus leniusculus* Dana, to the parasitic fungus *Aphanomyces astaci*. J. Fish Dis. *10*, 471–477.

Petit, V.W., Rolland, J.-L., Blond, A., Cazevieille, C., Djediat, C., Peduzzi, J., Goulard, C., Bachère, E., Dupont, J., Destoumieux-Garzón, D., et al. (2016). A hemocyanin-derived antimicrobial peptide from the penaeid shrimp adopts an alpha-helical structure that specifically permeabilizes fungal membranes. Biochim. Biophys. Acta *1860*, 557–568.

Post, J.E. (1999). Manganese oxide minerals: Crystal structures and economic and environmental significance. Proc. Natl. Acad. Sci. *96*, 3447–3454.

Pradeep, B., Rai, P., Mohan, S.A., Shekhar, M.S., and Karunasagar, I. (2012). Biology, Host Range, Pathogenesis and Diagnosis of White spot syndrome virus. Indian J. Virol. *23*, 161–174.

Qin, Z., Babu, V.S., Wan, Q., Muhammad, A., Li, J., Lan, J., and Lin, L. (2018). Antibacterial activity of hemocyanin from red swamp crayfish (Procambarus clarkii). Fish Shellfish Immunol. *75*, 391–399.

Reisen, W.K., Fang, Y., and Martinez, V.M. (2006). Effects of temperature on the transmission of west nile virus by *Culex tarsalis* (Diptera: Culicidae). J. Med. Entomol. *43*, 309–317.

Rijiravanich, A., Browdy, C.L., and Withyachumnarnkul, B. (2008). Knocking down caspase-3 by RNAi reduces mortality in Pacific white shrimp *Penaeus (Litopenaeus) vannamei* challenged with a low dose of white-spot syndrome virus. Fish Shellfish Immunol. *24*, 308–313.

Robalino, J., Payne, C., Parnell, P., Shepard, E., Grimes, A.C., Metz, A., Prior, S., Witteveldt, J., Vlak, J.M., Gross, P.S., et al. (2006). Inactivation of White Spot Syndrome Virus (WSSV) by normal rabbit serum: implications for the role of the envelope protein VP28 in WSSV infection of shrimp. Virus Res. *118*, 55–61.

Ross, J., Jiang, H., Kanost, M.R., and Wang, Y. (2003). Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships. Gene *304*, 117–131.

Sahtout, A.H., Hassan, M.D., and Shariff, M. (2001). DNA fragmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). Dis. Aquat. Organ. *44*, 155–159.

Sangsuriya, P., Charoensapsri, W., Sutthangkul, J., Senapin, S., Hirono, I., Tassanakajon, A., and Amparyup, P. (2018). A novel white spot syndrome virus protein WSSV164 controls prophenoloxidases, *Pm*proPOs in shrimp melanization cascade. Dev. Comp. Immunol. *86*, 109–117.

Sarathi, M., Simon, M.C., Venkatesan, C., Thomas, J., Ravi, M., Madan, N., Thiyagarajan, S., and Sahul Hameed, A.S. (2010). Efficacy of bacterially expressed dsRNA specific to different structural genes of white spot syndrome virus (WSSV) in protection of shrimp from WSSV infection. J. Fish Dis. *33*, 603–607.

Sánchez-Paz, A. (2010). White spot syndrome virus: an overview on an emergent concern. Vet. Res. *41*, 43.

Schröder, H.C., Ushijima, H., Krasko, A., Gamulin, V., Thakur, N.L., Diehl-Seifert, B., Müller, I.M., and Müller, W.E.G. (2003). Emergence and disappearance of an immune molecule, an antimicrobial lectin, in basal metazoa. A tachylectin-related protein in the sponge Suberites domuncula. J. Biol. Chem. *278*, 32810–32817.

Sequeira, T., Tavares, D., and Arala-Chaves, M. (1996). Evidence for circulating hemocyte proliferation in the shrimp *Penaeus japonicus*. Dev. Comp. Immunol. *20*, 97–104.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell *9*, 459–470.

Shi, Z., Wang, H., Zhang, J., Xie, Y., Li, L., Chen, X., Edgerton, B.-F., and Bonami, J.-R. (2005). Response of crayfish, *Procambarus clarkii*, haemocytes infected by white spot syndrome virus. J. Fish Dis. *28*, 151–156.

Shirogane, Y., Takeda, M., Iwasaki, M., Ishiguro, N., Takeuchi, H., Nakatsu, Y., Tahara, M., Kikuta, H., and Yanagi, Y. (2008). Efficient multiplication of human metapneumovirus in Vero cells expressing the transmembrane serine protease TMPRSS2. J. Virol. *82*, 8942–8946.

Simčič, T., Pajk, F., Jaklič, M., Brancelj, A., and Vrezec, A. (2014). The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. J. Therm. Biol. *41*, 21–30.

Söderhäll, I. (2013). Recent advances in crayfish hematopoietic stem cell culture: a model for studies of hemocyte differentiation and immunity. Cytotechnology *65*, 691–695.

Söderhäll, I. (2016). Crustacean hematopoiesis. Dev. Comp. Immunol. *58*, 129–141.

Söderhäll, I., Bangyeekhun, E., Mayo, S., and Söderhäll, K. (2003). Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of *Pacifastacus leniusculus*. Dev. Comp. Immunol. *27*, 661–672.

Söderhäll, I., Kim, Y.-A., Jiravanichpaisal, P., Lee, S.-Y., and Söderhäll, K. (2005). An ancient role for a prokineticin domain in invertebrate hematopoiesis. J. Immunol. Baltim. Md 1950 *174*, 6153–6160.

Söderhäll, I., Wu, C., Novotny, M., Lee, B.L., and Söderhäll, K. (2009). A novel protein acts as a negative regulator of prophenoloxidase activation and melanization in the freshwater crayfish *Pacifastacus leniusculus*. J. Biol. Chem. *284*, 6301–6310.

Söderhäll, K., and Ajaxon, R. (1982). Effect of quinones and melanin on mycelial growth of *Aphanomyces spp.* and extracellular protease of *Aphanomyces astaci*, a parasite on crayfish. J. Invertebr. Pathol. *39*, 105–109.

Söderhäll, K., and Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. *10*, 23–28.

Söderhäll, K., and Smith, V.J. (1983). Separation of the haemocyte populations of Carcinus maenas and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol. *7*, 229–239.

Sricharoen, S., Kim, J.J., Tunkijjanukij, S., and Söderhäll, I. (2005). Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish. Dev. Comp. Immunol. *29*, 1017–1031.

Sriphaijit, T., Flegel, T.W., and Senapin, S. (2007). Characterization of a shrimp serine protease homolog, a binding protein of yellow head virus. Dev. Comp. Immunol. *31*, 1145–1158.

Sritunyalucksana, K., Wannapapho, W., Lo, C.F., and Flegel, T.W. (2006). PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. J. Virol. *80*, 10734–10742.

Sritunyalucksana, K., Utairungsee, T., Sirikharin, R., and Srisala, J. (2012). Virus-binding proteins and their roles in shrimp innate immunity. Fish Shellfish Immunol. *33*, 1269–1275.

Sun, Y., Li, F., Sun, Z., Zhang, X., Li, S., Zhang, C., and Xiang, J. (2014). Transcriptome analysis of the initial stage of acute WSSV infection caused by temperature change. PloS One *9*, e90732.

Sutthangkul, J., Amparyup, P., Charoensapsri, W., Senapin, S., Phiwsaiya, K., and Tassanakajon, A. (2015). Suppression of shrimp melanization during white spot syndrome virus infection. J. Biol. Chem. *290*, 6470–6481.

Sutthangkul, J., Amparyup, P., Eum, J.H., Strand, M.R., and Tassanakajon, A. (2017). Anti-melanization mechanism of the white spot syndrome viral protein, WSSV453, via interaction with shrimp proPO-activating enzyme, PmproPPAE2. J. Gen. Virol.

Tassetto, M., Kunitomi, M., and Andino, R. (2017). Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell *169*, 314-325.e13.

Tattersall, G.J., Luebbert, J.P., LePine, O.K., Ormerod, K.G., and Mercier, A.J. (2012). Thermal games in crayfish depend on establishment of social hierarchies. J. Exp. Biol. *215*, 1892–1904.

Tharntada, S., Ponprateep, S., Somboonwiwat, K., Liu, H., Söderhäll, I., Söderhäll, K., and Tassanakajon, A. (2009). Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from white spot syndrome virus infection. J. Gen. Virol. *90*, 1491–1498.

Thorpe, M., Akula, S., and Hellman, L. (2016). Channel catfish granzyme-like I is a highly specific serine protease with metase activity that is expressed by fish NK-like cells. Dev. Comp. Immunol. *63*, 84–95.

Thorpe, M., Yu, J., Boinapally, V., Ahooghalandari, P., Kervinen, J., Garavilla, L. de, and Hellman, L. (2012). Extended cleavage specificity of the mast cell chymase from the crab-eating macaque (Macaca fascicularis): an interesting animal model for the analysis of the function of the human mast cell chymase. Int. Immunol. *24*, 771–782.

Thörnqvist, P.O., Johansson, M.W., and Söderhäll, K. (1994). Opsonic activity of cell adhesion proteins and beta-1,3-glucan binding proteins from two crustaceans. Dev. Comp. Immunol. *18*, 3–12.

Tonganunt, M., Nupan, B., Saengsakda, M., Suklour, S., Wanna, W., Senapin, S., Chotigeat, W., and Phongdara, A. (2008). The role of Pm-fortilin in protecting shrimp from white spot syndrome virus (WSSV) infection. Fish Shellfish Immunol. *25*, 633–637.

Tsai, J.-M., Wang, H.-C., Leu, J.-H., Wang, A.H.-J., Zhuang, Y., Walker, P.J., Kou, G.-H., and Lo, C.-F. (2006). Identification of the Nucleocapsid, Tegument, and Envelope Proteins of the Shrimp White Spot Syndrome Virus Virion. J. Virol. *80*, 3021–3029.

de la Vega, E., O'Leary, N.A., Shockey, J.E., Robalino, J., Payne, C., Browdy, C.L., Warr, G.W., and Gross, P.S. (2008). Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (*Lv*ALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Mol. Immunol. *45*, 1916–1925.

Verbruggen, B., Bickley, L.K., van Aerle, R., Bateman, K.S., Stentiford, G.D., Santos, E.M., and Tyler, C.R. (2016). Molecular Mechanisms of White Spot Syndrome Virus Infection and Perspectives on Treatments. Viruses *8*.

Verma, A.K., Gupta, S., Singh, S.P., and Nagpure, N.S. (2017). An update on mechanism of entry of white spot syndrome virus into shrimps. Fish Shellfish Immunol. *67*, 141–146.

Volz, J., Osta, M.A., Kafatos, F.C., and Müller, H.-M. (2005). The Roles of Two Clip Domain Serine Proteases in Innate Immune Responses of the Malaria Vector *Anopheles gambiae*. J. Biol. Chem. *280*, 40161–40168.

Wang, R., Lee, S.Y., Cerenius, L., and Söderhäll, K. (2001). Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. Eur. J. Biochem. *268*, 895–902.

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

Wang, L., Zhi, B., Wu, W., and Zhang, X. (2008). Requirement for shrimp caspase in apoptosis against virus infection. Dev. Comp. Immunol. *32*, 706–715.

Wang, X.W., Xu, Y.H., Xu, J.D., Zhao, X.F., and Wang, J.X. (2014). Collaboration between a soluble C-type lectin and calreticulin facilitates white spot syndrome virus infection in shrimp. J. Immunol. Baltim. Md 1950 *193*, 2106–2117.

Wang, Y.G., Hassan, M.D., Shariff, M., Zamri, S.M., and Chen, X. (1999). Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. Dis. Aquat. Organ. *39*, 1–11.

Wang, Z., Sun, B., and Zhu, F. (2017). Epigallocatechin-3-gallate inhibit replication of white spot syndrome virus in *Scylla paramamosain.* Fish Shellfish Immunol. *67*, 612–619.

Weis, W.I., Taylor, M.E., and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. Immunol. Rev. *163*, 19–34.

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

Williams, M.J. (2007). Drosophila Hemopoiesis and Cellular Immunity. J. Immunol. *178*, 4711–4716.

Witteveldt, J., Cifuentes, C.C., Vlak, J.M., and van Hulten, M.C.W. (2004). Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. J. Virol. *78*, 2057–2061.

Wojda, I. (2017). Temperature stress and insect immunity. J. Therm. Biol. *68*, 96–103.

Wu, C., Söderhäll, I., Kim, Y.-A., Liu, H., and Söderhäll, K. (2008). Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, Pacifastacus leniusculus. Proteomics *8*, 4226–4235.

Wu, C., Charoensapsri, W., Nakamura, S., Tassanakajon, A., Söderhäll, I., and Söderhäll, K. (2013). An MBL-like protein may interfere with the activation of the proPO-system, an important innate immune reaction in invertebrates. Immunobiology *218*, 159–168.

Wu, J., Li, F., Huang, J., Xu, L., and Yang, F. (2015). Crayfish hematopoietic tissue cells but not hemocytes are permissive for white spot syndrome virus replication. Fish Shellfish Immunol. *43*, 67–74.

Xia, X., You, M., Rao, X.-J., and Yu, X.-Q. (2018). Insect C-type lectins in innate immunity. Dev. Comp. Immunol. *83*, 70–79.

Xie, X., Xu, L., and Yang, F. (2006). Proteomic analysis of the major envelope and nucleocapsid proteins of white spot syndrome virus. J. Virol. *80*, 10615–10623.

Xu, J.D., Jiang, H.S., Wei, T.D., Zhang, K.Y., Wang, X.W., Zhao, X.-F., and Wang, J.X. (2017). Interaction of the Small GTPase Cdc42 with Arginine Kinase Restricts White Spot Syndrome Virus in Shrimp. J. Virol. *91*.

Xu, Z., Du, H., Xu, Y., Sun, J., and Shen, J. (2006). Crayfish *Procambarus clarkii* protected against white spot syndrome virus by oral administration of viral proteins expressed in silkworms. Aquaculture *253*, 179–183.

Ye, T., Tang, W., and Zhang, X. (2012). Involvement of Rab6 in the regulation of phagocytosis against virus infection in invertebrates. J. Proteome Res. *11*, 4834–4846.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J., and Hu, L. (2004). Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. J. Biochem. Mol. Biol. *37*, 726–734.

Yu, X.Q., and Kanost, M.R. (2000). Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria. J. Biol. Chem. *275*, 37373–37381.

Yu, X.-Q., Jiang, H., Wang, Y., and Kanost, M.R. (2003). Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. *33*, 197–208.

Zelensky, A.N., and Gready, J.E. (2005). The C-type lectin-like domain superfamily. FEBS J. *272*, 6179–6217.

Zhang, G., Lu, Z.-Q., Jiang, H., and Asgari, S. (2004a). Negative regulation of prophenoloxidase (proPO) activation by a clip-domain serine proteinase homolog (SPH) from endoparasitoid venom. Insect Biochem. Mol. Biol. *34*, 477–483.

Zhang, J.Y., Liu, Q.H., and Huang, J. (2014). Multiple proteins of White spot syndrome virus involved in recognition of beta-integrin. J. Biosci. *39*, 381–388.

Zhang, X., Huang, C., and Qin, Q. (2004b). Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*. Antiviral Res. *61*, 93–99.

Zhao, M., Söderhäll, I., Park, J.W., Ma, Y.G., Osaki, T., Ha, N.-C., Wu, C.F., Söderhäll, K., and Lee, B.L. (2005). A novel 43-kDa protein as a negative regulatory component of phenoloxidase-induced melanin synthesis. J. Biol. Chem. *280*, 24744–24751.

Zhao, Z.Y., Yin, Z.X., Xu, X.-P., Weng, S.P., Rao, X.Y., Dai, Z.X., Luo, Y.-W., Yang, G., Li, Z.-S., Guan, H.-J., et al. (2009). A novel C-type lectin from the shrimp Litopenaeus vannamei possesses anti-white spot syndrome virus activity. J. Virol. *83*, 347–356.

Zhu, F., and Zhang, X. (2013). The Wnt signaling pathway is involved in the regulation of phagocytosis of virus in Drosophila. Sci Rep *3*, 2069.

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