

Chapter 1: Introduction

1.1 Problem statement

Ticks are obligate blood-feeding ecto-parasites that transmit pathogenic organisms such as viruses, bacteria and protozoa. They parasitize mammals, birds, amphibians and occasionally reptiles (Sonenshine, 1991). Tick-borne diseases greatly impact both human and animal health (Estrada-Peña & Jongejan 1999; Parola & Raoult, 2001). There are three distinct tick families, namely, the soft ticks (*Argasidae*), hard ticks (*Ixodidae*) and the *Nuttalliellidae*, containing only one species (Hill & Wikel, 2005; Sonenshine, 1991).

Much research has focused on control methods that include the use of acaricides and the development of anti-tick vaccines (de Castro & Newson, 1993; Sauer *et al.*, 1995; Vargas *et al.*, 2010; Willadsen, 2001). Both strategies are costly and suffer either from the emergence of acaricide resistance or ineffective cross-protection between different tick strains and species. Therefore, alternative methods to control ticks are needed (Sugumar *et al.*, 2010).

Recently, more investigations into tick immunity have been conducted and the regulation thereof could provide new insights into tick control that could be used for both medical and agricultural purposes (Nijhof *et al.*, 2010; Prudencio *et al.*, 2010; Sugumar *et al.*, 2010). These studies may also lead to the discovery of novel antibiotics (Steen *et al.*, 2006). Currently the widespread use of antibiotics has promoted the emergence of resistant bacteria. In the case of agricultural use this may lead to the spread of resistant bacterial strains to human populations (WHO 2002; Goossens *et al.*, 2005; Mathew *et al.*, 2007). The resistance problem demands that a renewed effort be made to seek antibacterial agents that are effective against resistant pathogenic bacteria. In this regard, ticks may provide a valuable source of antimicrobial agents that could be further developed for therapy. Furthermore, the relationship between tick immune responses and micro-organisms can be explored to limit disease transmission by ticks (Johns *et*

al., 2000, 2001a; Nakajima *et al.*, 2001, 2002, 2003a, b, c; Sonenshine & Hynes, 2008).

1.2 *Ornithodoros savignyi* as a model organism for this study

Ornithodoros savignyi is a soft tick that occurs throughout the North-western regions of Southern Africa. This tick is also known as “the eyed sand tampan”. During feeding the uniform integument which folds in on itself allows the tick to obtain a large quantity of blood in a short period of time (Mans *et al.*, 2002). When these ticks feed they secrete toxic substances which can be lethal to young animals (Neitz *et al.*, 1969; Howell *et al.*, 1975; Mans *et al.*, 2001, 2002).

These ticks exhibit a very selective and low incidence of pathogen transmission. They have however, been proven to be responsible for the transmission of pathogens such as Alkhurma hemorrhagic fever virus and *Borrelia* (Shanbaky & Helmy, 2000; Charrel *et al.*, 2007). *Ornithodoros moubata*, a close relative to *O. savignyi*, is able to transmit *B. duttoni* which cause relapsing fever.

Little is known about the innate immune defense mechanisms of *O. savignyi*. In a previous study, Olivier (2002) purified a Gram-positive antimicrobial peptide (AMP) from the hemolymph of *O. savignyi*, and the N-terminal sequence was obtained. The project was continued by an Honours student who identified 2 defensin isoforms using degenerate primers based on the findings of Olivier (2002).

Recently, Stopforth *et al.* (2010) used a proteomics approach to identify proteins secreted into the hemolymph of these ticks 2 hours after immune-challenge with the yeast, *Candida albicans*. Profiling of the proteins present in hemolymph of unchallenged versus challenged ticks using heat-killed yeast revealed five differentially expressed proteins. The modulated protein spots were subjected to tandem mass spectrometry analysis (MS/MS), but could not be positively

identified. These proteins could be involved in the tick immune response as they were not induced following aseptic injury.

1.3 Invertebrate innate immunity

To accomplish the requirements necessary for survival in nature, arthropods must be aware of their surroundings and respond accordingly. Since they do not have an adaptive immune system like mammals, they utilize defense mechanisms similar to the innate immune system of vertebrates to protect themselves from pathogens (Iwanaga & Lee, 2005; Jiggins & Kim, 2005). The key questions of innate immunity are firstly how can pathogenic organisms be detected by arthropods and secondly what types of defense strategies against different types of infection are involved. Much work has been done to identify the sensing molecules and downstream components involved in the immunity of the fruit fly, *Drosophila melanogaster* (Royet, 2004; Kim & Kim, 2005).

Two general responses against the invasion of micro-organisms exist within arthropods. These include cellular and humoral responses. Cellular responses are generally mounted in the arthropod hemolymph by hemocytes. Recognition of micro-organisms by hemocytes generally occurs via specific arthropod-derived proteins that bind to the microbial surface (Lee *et al.*, 1996; Kim *et al.*, 2000; Janeway & Medzhitov, 2002). Humoral responses are generally mediated by peptides such as defensins and lysozyme.

In *Drosophila*, pathogens sensed by specific receptors initiate signals via the Toll and/or IMD (immune deficiency) signaling pathways. These events often lead to transcription of genes involved in the production of AMPs as well as trigger responses such as the production of reactive oxygen species (ROS), melanization, phagocytosis, nodulation, encapsulation and iron sequestration (Fig 1.1).

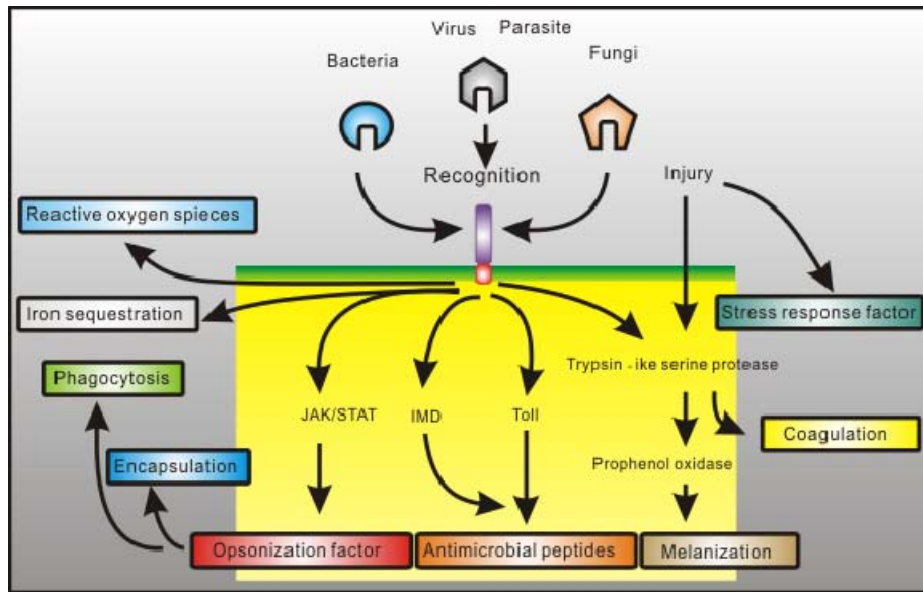


Figure 1.1 The intracellular and extracellular events taking place in arthropods after injury or pathogen recognition by cells.

1.3.1 Discrimination between self and non-self

The innate immune system in both vertebrates and non-vertebrates is able to identify micro-organisms as foreign by pattern recognition receptors (PRRs) of the host (Royet, 2004). The non-self molecules recognized by PRRs are generally present on the surface of microbes and are known as pathogen associated molecular patterns (PAMPs). Their recognition by PRRs activates the Toll-like and/or IMD pathways of host cells, especially in phagocytic hemocytes and the digestive cells lining the lumen of the midgut (Brennan & Anderson, 2004).

In arthropods such as *Drosophila*, PRRs which have been implicated in pathogen recognition include the peptidoglycan binding protein (PGBP) for Gram-positive bacteria (Kang *et al.*, 1998) and the Gram negative-binding protein (GNBP), which recognizes lipopolysaccharide (LPS) and β -1,3-glucan (Lee *et al.*, 1996; Kim *et al.*, 2000). PRRs differ in specificity due to the fact that different pathogenic organisms exhibit different PAMPs on their surface. In addition, a LPS binding protein (LBP) has been isolated from hemolymph of the American

cockroach *Periplaneta americana* (Jomori *et al.*, 1990). Lectins in turn recognize pathogen-specific carbohydrate moieties on the pathogen surface, thereby facilitating opsonization, phagocytosis and cytolysis (Janeway & Medzhitov, 2002).

The first peptidoglycan receptor protein (PGRP) was discovered in the silkworm, *Bombyx mori* (Yoshida *et al.*, 1996). Infection-induced proteins that bind to peptidoglycan (PGN), trigger the proteolytic melanization cascade. In *Drosophila* approximately 13 PGRPs have been identified which share a 160 amino acid PGN recognition domain. They occur as secreted (S) and transmembrane (L) forms (Kang *et al.*, 1998; Michel *et al.*, 2001; Werner *et al.*, 2000, 2003). PGRP-L consists of transmembrane and cytoplasmic domains and has different extracellular PGN recognition domains which trigger the IMD pathway (Choe *et al.*, 2002; Werner *et al.*, 2003). PGRP-L also plays an essential role in the recognition and distinguishing between Gram-negative and Gram-positive PGN (Werner *et al.*, 2003). PGRP-S consists of a single recognition domain and binds Gram-positive PGN with high affinity (Werner *et al.*, 2000; Michel *et al.*, 2001).

Lee *et al.* (1996) discovered a GGBP in the silkworm, *B. mori*. It is a 50 kDa hemolymph protein which has a strong affinity for the cell walls of Gram-negative bacteria, and is up-regulated during bacterial infection. The GGBP sequence contains a region displaying significant homology to the putative catalytic region of a group of bacterial β -1,3 and β -1,4 glucanases. Silkworm GGBP was also shown to have an amino acid sequence similar to the vertebrate LPS receptor CD14.

A cDNA sequence coding for GGBP has been characterized and identified as DGGBP-1 in *Drosophila*. It is either soluble or glycosylphosphatidylinositol-anchored and is expressed on *Drosophila* immunocompetent cells. It has a high affinity for LPS and β -1,3-glucan, but has no binding affinity for PGN, β -1,4-glucan or chitin (Kim *et al.*, 2000).

The LBP in the hemolymph of *P. americana* has a molecular mass of 450 kDa and is made up of 28 kDa subunits. This protein is highly specific for *Escherichia coli* K12 (Gram-negative bacteria), suggesting that this protein recognizes and binds to a specific carbohydrate structure of *E. coli* LPS (Jomori *et al.*, 1990).

Lectins are glycoproteins that play an important role in recognizing and binding to specific carbohydrate moieties commonly found on the cell walls of pathogens. They are also known as hemagglutinating agents. Lectins activate the complement pathway in both vertebrate and invertebrate animals, and also bind to microbes which enable hemocytes to recognize and engulf them in the opsonization process (Grubhoffer *et al.*, 2004). In addition, lectins are able to bind to one another. This was reported in the horseshoe crab, *Tachypleus tridentatus*, in which tachypleus lectin-1 (TPL-1) and tachypleus lectin-2 (TPL-2) form clusters with one another and bind to pathogens (Chen *et al.*, 2001). This clustering effect that immobilizes invading pathogens has been seen in insects and ticks (Gillespie *et al.*, 1997; Natori 2001; Rego *et al.*, 2005; 2006).

Tick lectins recognize a wide range of Gram-negative bacteria due to their specificity towards cell wall carbohydrate moieties such as LPS, sialic acid and N-acetyl-D-glucosamine (Grubhoffer *et al.*, 2004; Huang *et al.*, 2007). Several researchers have suggested their involvement in controlling pathogens and pathogen transmission. These molecules and other humoral immune factors in the hemolymph are produced and secreted into the hemolymph mainly by hemocytes and fat body cells (Grubhoffer *et al.*, 2004).

In hard ticks, *Ixodes ricinus* and *Rhipicephalus appendiculatus*, hemagglutination activity is found in the hemolymph, midguts and salivary glands (Kamwendo *et al.*, 1993, 1995; Kuhn *et al.*, 1996; Uhlír *et al.*, 1996). Lectins found in *I. ricinus* were later identified as Ixoderin A and Ixoderin B (Rego *et al.*, 2005).

In soft ticks, hemagglutination activity is found in the hemolymph of *O. tartakovskyi*, *O. tholozani (papillipes)*, *O. moubata* and *Argas polonicus* (Grubhoffer *et al.*, 1991; Grubhoffer & Dusbábek, 1991; Kovář *et al.*, 2000). In *O. moubata*, Dorin M is expressed in hemocytes, salivary glands and malphigian tubules, but not in the midgut or ovary (Rego *et al.*, 2006). Within the same species, two other lectins OMFREP and OmGalec, were also discovered with similar tissue distribution to Dorin M (Huang *et al.*, 2007; Rego *et al.*, 2005).

1.3.2 Cellular immune responses

The exact mechanism of response and signaling in ticks is not known, but it is clear that hemocytes are recruited and specific responses are mounted upon infection or induction with an immunogen (Johns *et al.*, 1998; Matsuo *et al.*, 2003). The hemocytes have been shown to participate in a variety of responses, including opsonization by PAMPs and lectins, nodulation, encapsulation and phagocytosis (Fig 1.2).

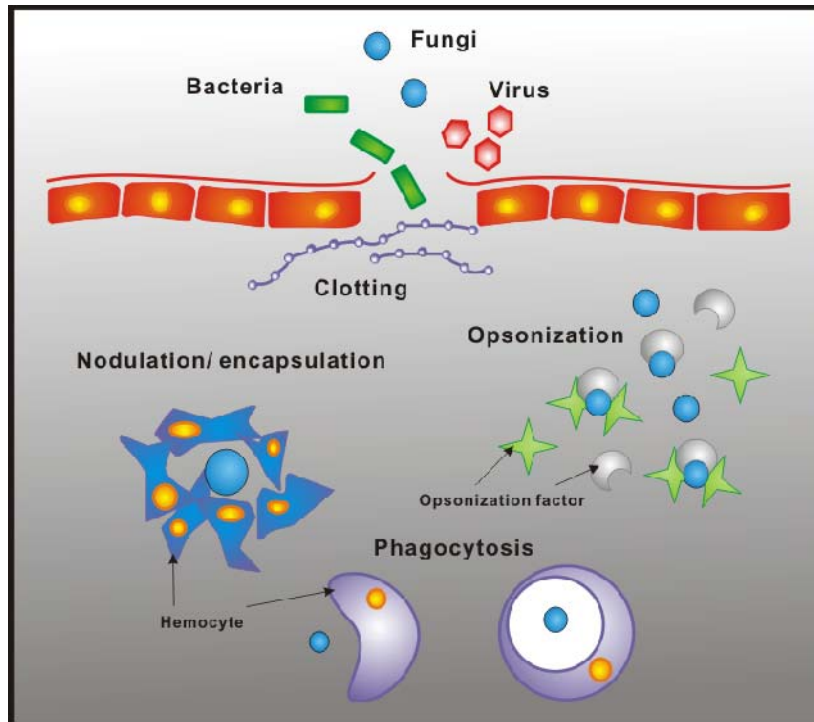


Figure 1.2: A schematic representation of cellular immune responses in arthropods. Once pathogens gain entry into the hemocoel, they activate a complex system of innate defense responses. Opsonization factor: PAMPs or lectins.

It has been reported that tick hemocytes function similarly to white blood cells. Four major cell types exist in tick hemolymph. These are the prohemocytes, non-granular plasmatocytes, granulocytes and spherulocytes (Kuhn 1996; Kuhn *et al.*, 1996; Borovicková & Hypsa, 2005). Hemocyte populations in the hard tick, *Dermacentor variabilis*, have been shown to increase 6.4 fold within 48 hours when challenged by injection with Gram-positive bacteria (*B. subtilis*) (Johns *et al.*, 1998). A similar finding was also observed in *D. variabilis* challenged with the spirochete, *Borrelia burgdorferi* (Johns *et al.*, 2000).

1.3.2.1 Phagocytosis

Phagocytosis is the cellular process of engulfing foreign molecules, which is similar to receptor-mediated endocytosis (Fig 1.2). The pathogens or foreign molecules are recognized and bind to the plasmatocyte or granulocyte cell surface receptors. This is followed by extrusion of pseudopods from the cells that

surround and engulf bound foreign molecules or pathogens (Lamprou *et al.*, 2007). The trapped molecules are internalized by endocytosis into a vesicle that fuses with lysosomes to form a phagolysosome. The foreign body is then destroyed via digestion with enzymes such as acid phosphatase and lysozyme. In ticks, both plasmatocytes and granulocytes are involved in phagocytosis of microbes (Johns *et al.*, 2001a). Three morphologically different cell types, granulocytes, plasmatocytes, and prohemocytes, were detected in hemolymph of *O. moubata*, and granulocytes and plasmatocytes showed phagocytic activity (Inoue *et al.*, 2001). In a study by Pereira *et al.* (2001) phagosomes from hemocytes of *Rhipicephalus (Boophilus) microplus* were able to use reactive oxygen species to eliminate the bacteria. More recently, Buresova *et al.* (2009), demonstrated that IrAM, an α 2-macroglobulin (α 2M) protease inhibitor, is involved in phagocytosis of *Chryseobacterium indologenes* by hemocytes in the hard tick *I. ricinus*.

1.3.2.2 Nodulation and encapsulation

In general nodulation is an event involving the clustering of hemocytes that attach to aggregated bacteria or microbes (Fig 1.2). Nodulation in insects is mediated by eicosanoids, prostaglandins and lipoxygenase-derived products (Miller *et al.*, 1994). Encapsulation occurs when the arthropod host is exposed to pathogens such as parasitoids and nematodes which are too large to be eliminated by phagocytosis or nodulation. During this process granulocytes degranulate and deposit a matrix-like material around the foreign molecules. Subsequently, plasmatocytes attach to the matrix, undergo apoptosis, become highly flattened and form a thick layer encapsulating the particle (Ceraul *et al.*, 2002). In insects, encapsulation is also associated with melanization.

Encapsulation in ticks has been demonstrated by Eggenberger *et al.* (1990). Plastic particles were injected into the hemocoel of *D. variabilis* and this resulted in a sequence of encapsulation events as seen previously in insects, but without melanization.

1.3.2.3 Melanization

Melanization is an encapsulation reaction mediated by phenol oxidase (PO) and plays a major role in the defense reactions of insects during wound healing and against pathogen transmission (Lackie, 1988; Sugumaran, 2002). After the recognition of PAMPs, a serine protease cascade activates a prophenoloxidase (proPO)-activating enzyme. This enzyme activates PO in a similar fashion causing the production of quinones in the presence of oxygen. The production of quinones leads to the formation of melanin (Fig 1.3). PO activity was found to be present in hemolymph plasma during ecdysis in 4th instar nymphs of *O. moubata* (Kadota *et al.*, 2002). The enzyme activity was not detected in the hemolymph or tissues of three ixodid ticks (Zhioua *et al.*, 1997).

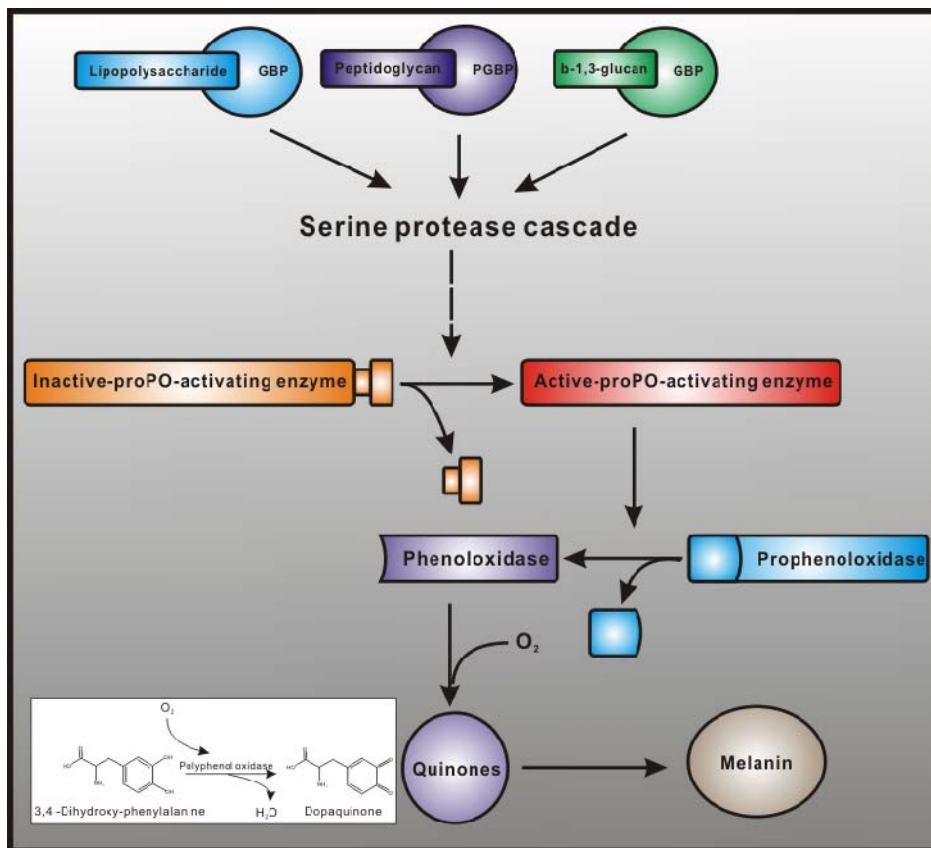


Figure 1.3 The prophenoloxidase activating system in arthropods (Adapted from Cerenius & Söderhäll 2004).

1.3.3 Humoral immune responses

Aside from the cellular responses of phagocytosis, encapsulation, nodulation, and melanization, the innate immune system of arthropods also involves humoral factors that not only identify foreign micro-organisms, but also play a role in the response to the micro-organism. These molecules can act directly on the microbe, or act as an adaptor to allow other molecules to act. They may also serve as opsonins, facilitating the effect of hemocytes on the microbe. Several classes of these molecules have been defined, including AMPs, serine proteases, protease inhibitors, lysozymes, lectins and iron sequestration molecules (Johns *et al.*, 2001a; Nappi & Christensen, 2005).

Peptide-mediated responses in *Drosophila* depend on the production of AMPs expressed in the fat body (the equivalent of the liver in mammals) and stored in the hemocytes (Kim & Kim, 2005; Lemaitre & Hoffmann, 2007). It remains unclear as to whether regulation of AMP levels is due to transcriptional up-regulation by the IMD or a similar pathway, or whether it is based on the release of AMPs from storage vessels in the hemocytes (Kim & Kim, 2005; Lemaitre & Hoffmann, 2007). In ticks, AMPs have been identified and characterized in hemolymph and midguts, but recently they have also been discovered in salivary glands (Sonenshine *et al.*, 2005; Fogaça *et al.*, 2006; Yu *et al.*, 2006).

1.3.3.1 AMPs

These have been found in various tissues and cell types in all species of life (Ganz, 1999; Hancock & Diamond, 2000; Lehrer & Ganz 2002; Zaiou & Gallo, 2002; Bulet *et al.*, 2004). AMPs are conserved components of the innate immune response (Brogden, 2005; Zasloff, 2002) and have been isolated from both hard and soft ticks (This aspect of the tick immune response will be extensively discussed in section 4.1).

AMPs are divided into subgroups on the basis of their amino acid composition and structure. They are generally small peptides consisting of 12 to 50 amino acids (Yeaman & Yount, 2003). These peptides include two or more positively charged residues provided by arginine, lysine and histidine in acidic environments, as well as containing a large proportion of hydrophobic residues (Sitaram & Nagaraj, 2002; Papagianni, 2003; Dürr *et al.*, 2006). Very often AMPs are unstructured in free solution and fold into their final conformation upon partitioning into biological membranes (Dhople *et al.*, 2006).

Bacterial cell membranes are rich in acidic phospholipid head groups (Chou *et al.*, 2008; Matsuzaki, 2008). Therefore, the outermost leaflets of the bacterial membrane bilayer are more prone to attack by the positively charged AMPs. For helical peptides, hydrophilic amino acid residues of AMPs are aligned along one side while the hydrophobic amino acid residues are aligned along the opposite side (Yeaman & Yount, 2003). This amphipathicity of the AMPs allows partitioning into the membrane lipid bilayer. The ability to associate with membranes is a definitive feature of AMPs although membrane permeabilization is not necessary. These peptides have a variety of antimicrobial activities ranging from membrane permeabilization to reacting with a range of cytoplasmic targets (Hancock & Rozek, 2002; Brogden, 2005).

The mechanism of action of AMPs is dependent on its amino acid composition (amphipathicity and cationic charge) and size (Oren & Shai, 1998; Huang *et al.*, 2004; Brogden, 2005). Most pore forming peptides cause depolarization of the normally energized bacterial membrane (Westerhoff *et al.*, 1989). Another proposed mechanism is the creation of physical holes that cause leakage of cellular contents (Shai, 1999). Other mechanisms include the activation of deadly processes such as induction of hydrolases that degrade the cell wall (Bierbaum & Sahl, 1985, 1987) and scrambling of the usual distribution of lipids between the leaflets of the bilayer, resulting in disturbance of membrane functions

(Matsuzaki, 1999). Three models have been proposed to explain the interaction of AMPs with biological membranes, namely the “barrel-stave”, “carpet” and “toroidal-pore” models (Fig 1.4).

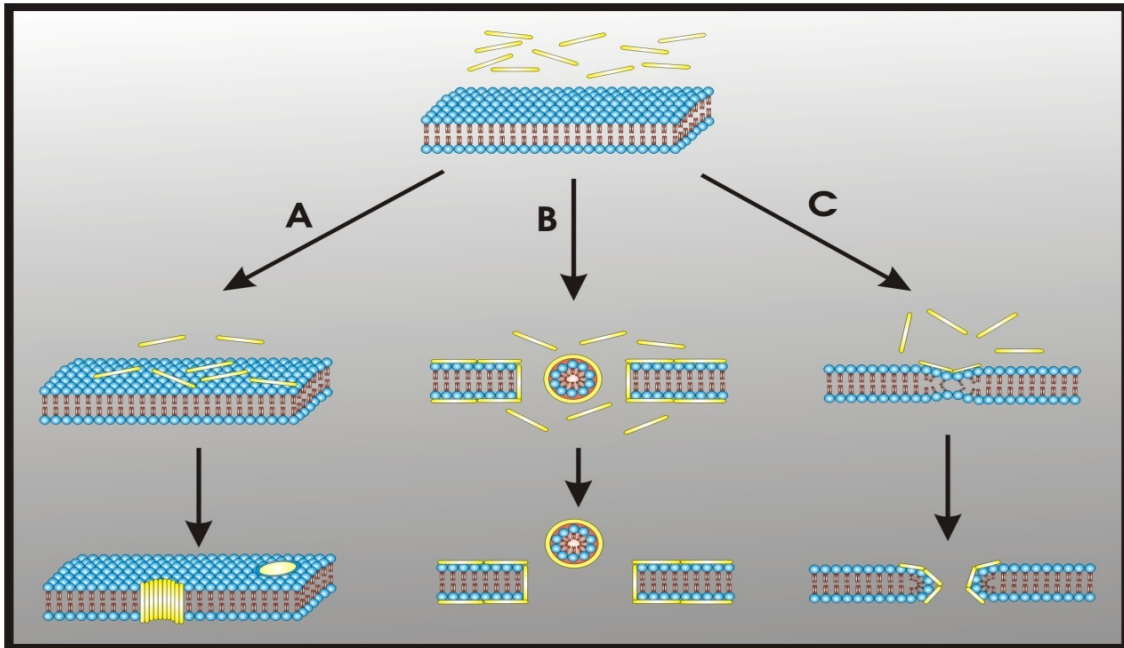


Figure 1.4: Extracellular mechanism of action of AMPs. A: barrel stave model. B: Carpet model. C: Toroidal-pore model (Adapted from Yang *et al.*, 2001; Brogden, 2005; Oren & Shai 1998; Shai, 1999).

In the barrel-stave model, peptide helices form a bundle in the membrane with a central lumen (Fig 1.4A). The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore (Yang *et al.*, 2001).

The carpet model suggests that peptides are electrostatically attracted to the anionic phospholipid heads at numerous sites covering the surface of the membrane in a carpet-like manner (Fig 1.4B). At high peptide concentrations, the bilayer is disrupted in a detergent-like manner, eventually leading to the formation of micelles (Shai, 1999; Ladokhin & White, 2001).

A toroidal-pore is formed when AMP helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water pore is lined by both the inserted peptides and the lipid head groups as shown in Fig 1.4C (Matsuzaki *et al.*, 1996, Yamaguchi *et al.*, 2002).

There have been increasing reports from studies on various organisms illustrating that AMPs also have intracellular targets (Fig 1.5). Intracellular targets include the activation of autolysins, inhibition of cell wall synthesis, alteration of the cytoplasm membrane polarity, inhibition of DNA, RNA and protein synthesis and inhibition of enzymatic activity (Brogden, 2005).

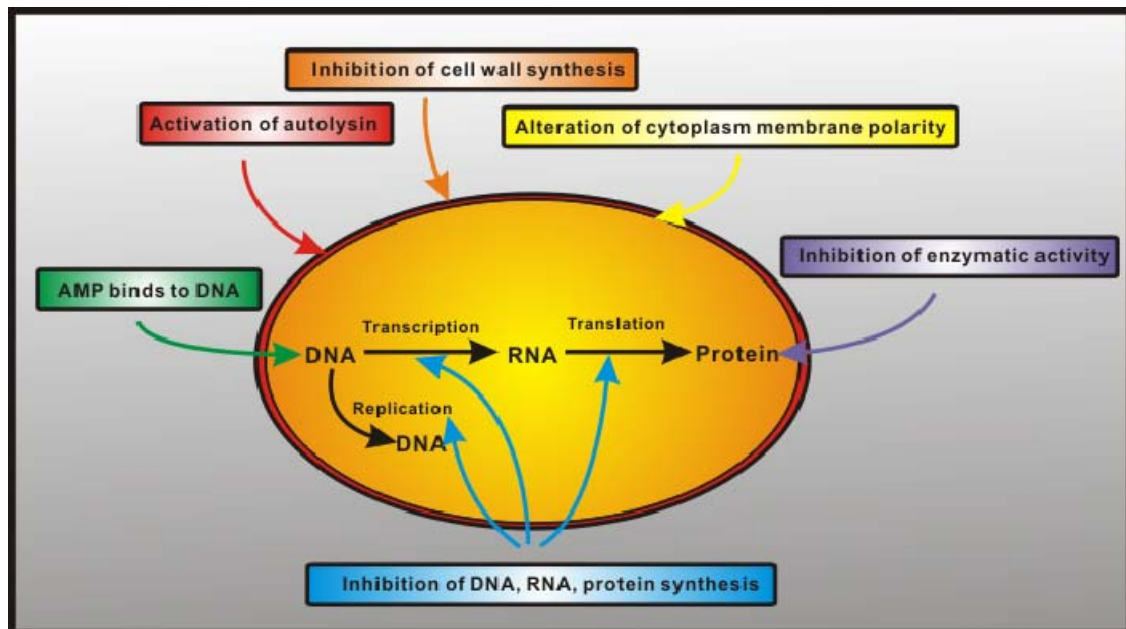


Figure 1.5 Intracellular mode of action of antimicrobial peptides. The intracellular targets and their effects are indicated in boxes (adapted from Brogden, 2005).

The defensins are a major family of AMPs. They are usually small in size and range from 67 – 92 amino acids (3 - 6 kDa) and include a region (37- 61 amino acids in length) which is cleaved to release the mature peptide. The arginine-rich and cationic peptides usually contain 6 – 8 cysteine residues which form the disulphide bridges. These bridges play a vital role in stabilization and

maintenance of the tertiary structure known as the “defensin fold” (Ganz, 2003). Ticks defensins will be discussed in section 4.1.

1.3.3.2 Lysozymes

These are small proteins with a molecular mass of ~ 14kDa, that catalyze the hydrolysis of β -1,4- glycosidic linkages between N-acetylmuramic acid (NAM) and N-acetyl glucosamine (NAG) in PGN, resulting in the disruption of the integrity of the cell wall of Gram-positive bacteria. Gram-negative bacteria are protected from lysozyme attack because of the presence of the outer cytoplasmic membrane (Salton & Kim, 1996).

Lysozymes are generally characterized as chicken (c), goose (g), or invertebrate (i) based on their possession of type specific amino acid sequence features and their species of origin (Nilsen & Myrnes, 2001). In arthropods, lysozyme is mostly found in hemocytes, midguts and salivary glands. While lysozymes function mainly as antimicrobials, other functions such as digestion, development, communication and reproduction have been described. In some insects such as *Drosophila* and house flies (*Musca domestica*), lysozymes are acidic proteins, which do not respond to bacterial infection (Kylsten *et al.*, 1992; Daffre *et al.*, 1994; Ito *et al.*, 1995).

In tick hemolymph, lysozyme may act synergistically with defensin to destroy Gram-negative bacteria, thereby broadening the range of bacteria inhibited by the activity of AMPs (Matsuzaki *et al.*, 1998; Johns *et al.*, 2001b). It is proposed that defensin affects the integrity of the outer membrane which allows lysozyme to reach and hydrolyze the inner PGN cell wall (Fig 1.6).

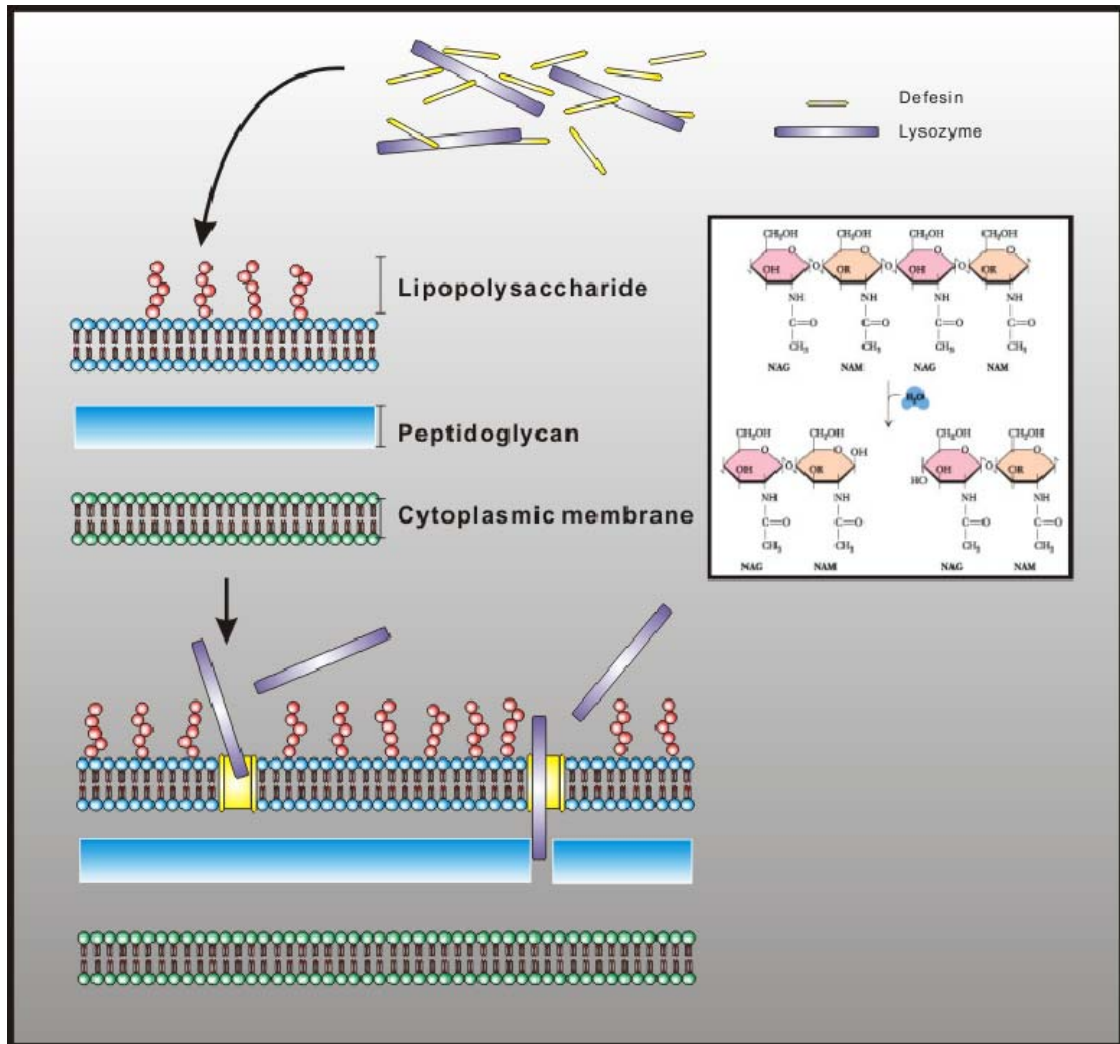


Figure 1.6 A schematic representation of the synergistic action of defensin and lysozyme against Gram-negative bacteria. Defensins permit lysozyme to infiltrate the outer LPS layer of Gram-negative bacteria and thus cleave the β -1,4-glycosidic bond between NAM and NAG in PGN.

In most ticks, lysozyme of the c-type is involved either in humoral innate immunity (induced in response to bacterial challenge) or has been evolutionary adapted to serve as digestive enzymes within the alimentary system (Table 1.1).

The mature lysozyme from digestive tissues in *O. moubata* contains 124 amino acids and belongs to the c-type. Transcript levels were elevated in response to feeding (Kopáček *et al.*, 1999; Grunclová *et al.*, 2003). This finding suggested

that c-type lysozyme possesses a combination of immune and digestive functions. However, its regulation in response to bacterial challenge has not been assessed.

Mattila *et al.* (2007) reported that lysozyme expression was not up-regulated in tick cell lines (IDE12 and DAE15) when challenged with *Rickettsia peacockii*. Expression was however up-regulated after stimulation of the cells with *E. coli* and *Micrococcus luteus*.

The mature c-type lysozyme of *D. variabilis* is 121 amino acids long. Transcript levels in hemolymph were increased 17 fold within 72 hours post-injection with *E. coli* (Simser *et al.*, 2004a). Lysozyme expression profiles showed levels of expression to be most abundant in hemolymph but present in very low levels in the midgut or other organs. Feeding of *D. variabilis* did not result in increased lysozyme expression (Simser *et al.*, 2004a; Sonenshine *et al.*, 2005). Lysozyme antimicrobial activity has been reported in the hemocytes of *I. ricinus*, *I. persulcatus* and cell lines derived from both *I. scapularis* and *D. andersoni* (Kuhn & Haug, 1994; Mattila *et al.*, 2007).

Table 1.1: Tick lysozyme

Species	Found	Antimicrobial	Gene bank accession code	Reference
<i>D. andersoni</i>	DAE 100 cell line, DAE 15 cell line	+	28883557	Simser <i>et al.</i> ,2004a; Mattila, <i>et al.</i> , 2007
<i>D. variabilis</i>	Hemocyte	+	27819105	Simser <i>et al.</i> ,2004b
<i>I. persulcatus</i>	Homogenate of tick	+		Podboronov, 1990; 1991
<i>I. ricinus</i>	Hemocyte	+		Kuhn & Haug, 1994
<i>I. scapularis</i>	IDE 12 cell line	+		Mattila <i>et al.</i> , 2007
<i>O. moubata</i>	Midgut	-	16326735	Kopáček <i>et al.</i> ,1999; Grunclová <i>et al.</i> ,2003

-: not determined

+ indicates antimicrobial activity

1.3.3.3 Proteases and Protease inhibitors

Serine proteases play an important role in the innate immune responses of hemolymph coagulation, melanization and AMP synthesis. In the mosquito, *Anopheles dirus*, serine proteases were up-regulated in response to invasion of the hemolymph by *Plasmodium yoelii* (Xu *et al.*, 2006). Similar findings were found in tick hemolymph when an immune-responsive factor D-like serine protease was expressed in hemocytes in response to *E. coli* challenge (Simser *et al.*, 2004b). The serine proteases are comprised of a clip domain, a feature that is highly conserved in *D. andersoni* and *D. variabilis* (Simser *et al.*, 2004a, b).

The arthropod host produces protease inhibitors that selectively target pathogen proteases in various ways (Armstrong, 2001). Some inhibitors that have been identified in ticks include both the serine proteinase inhibitors (serpins) and α 2M-type protease inhibitors. The α 2M functions in both entrapment and inactivation of the proteases secreted from the pathogen (Armstrong & Quigley, 1999). The α 2M identified from hemocytes of *O. moubata* is known as tick α 2M (TAM: Kopáček *et al.*, 2000). TAM inhibits trypsin and thermolysin cleavage of the high-molecular-weight substrate azocoll in a manner similar to that of bovine α 2M (Kopáček *et al.*, 2000). The mature protein is cleaved to form two subunits similar to the C3 and C4 complement components. Phylogeny analysis revealed that TAM is closely related to *Limulus* α 2M and displays the highest similarity to the partial sequence from *I. scapularis* (Valenzuela *et al.*, 2002; Saravanan *et al.*, 2003).

1.3.4 Coagulation

Coagulation is the first response to injury. In the horseshoe crab, *Tachypleus tridentatus*, transglutaminase and proxin are expressed in hemocytes during injury. Proxin is cross linked with the plasma protein coagulin to seal wounds. Stabilin, another coagulation factor derived from hemocytes interacts with proxin forming a fibril complex. In addition, stabilin can bind to both Gram-positive and Gram-negative bacteria to immobilize them within the clots (Matsuda *et al.*, 2007). To date, no coagulation studies in ticks have been performed and the molecular mechanism of coagulation remains unknown.

1.3.5 Iron sequestration

Iron plays an indispensable role in various physiological processes such as gene regulation and DNA synthesis. Since iron plays an instrumental role in organisms, including the host and invading pathogens, it is vital to understand how host proteins regulate both intracellular and intercellular iron levels (Andrews, 2000).

During an infection, both host and the invasive pathogen compete for iron. Pathogens usurp host iron by secreting siderophores that have a high affinity for iron. They are then transported into the bacteria through specific receptors (Andrews, 2000; Ratledge & Dover, 2000). Transferrin has been shown to inhibit the growth of bacteria by depleting iron which may be used for bacterial metabolism (Yoshiga *et al.*, 1997; Caccavo *et al.*, 2002; Paily *et al.*, 2007).

To date, invertebrate transferrin has been isolated from the cockroach (*Blaberus discoidalis*), mosquito (*Aedes aegypti*), silkworm (*B. mori*), fruit fly (*D. melanogaster*) and tobacco hornworm (*Manduca sexta*) (Jamroz *et al.*, 1993, Huebers *et al.*, 1988; Yoshiga *et al.*, 1997, 1999; Yun *et al.*, 1999, 2009). Transferrin synthesis and secretion was shown to be increased upon exposure to microbes and to elicit an immune response in *D. melanogaster*, *A. aegypti* and the honey bee, *Apis mellifera* (Yoshiga *et al.*, 1997, 1999; Kucharski & Maleszka,

2003). A recent study has shown that the promoter region of transferrin found in *A. aegypti* contains a putative NF- κ B-binding site, which is consistent with the postulated role of transferrin in insect innate immunity (Harizanova *et al.*, 2005). In ticks, *R. (B.) microplus*, levels of transferrin decreased significantly after feeding (Carvalho *et al.*, 2008).

1.3.6 Oxidative stress

In *D. variabilis*, the enzymes DvGST₁ and DvGST₂ which belong to the Glutathione S-transferase (GST) family, play a central role in removal of ROS and other harmful radicals. (Dreher-Lesnicks *et al.*, 2006). Both isoforms were identified in tick midguts and were up-regulated during blood feeding. DvGST₂ was also found in the ovaries (Dreher-Lesnicks *et al.*, 2006). A similar finding was also observed in *I. ricinus* when infected with *B. burgdorferi* (Rudenko *et al.*, 2005).

1.4 Aims of this thesis

The overall aim of this study is the biochemical and molecular characterization of putative immunoprotective molecules of the tick, *O. savignyi*. The investigations in Chapter 2 were aimed at identifying hemolymph plasma proteins with a putative Gram-negative recognition role. In Chapter 3 molecular characterization, bioinformatic analysis and expression profiling of a lipocalin-like hemolymph protein was undertaken. In Chapter 4 the biochemical isolation and characterization of a salivary gland Gram-positive antibacterial activity was attempted.

Part of this study was published in:

Paul H. Cheng, Ben J. Mans, Albert W. H. Neitz, Anabella R. M. Gaspar. *Experimental and Applied Acarology* (2010) 52:313-326. Savicalin, a lipocalin from hemocytes of the soft tick, *Ornithodoros savignyi*. DOI 10.1007/s10493-010-9368-6.

Various aspects were presented at scientific meetings:

Cheng P, Saunders M, Exley A, Neitz A & Gaspar A (2005). Proteomic and peptidomic analyses of the innate immune response of the soft tick, *Ornithodoros savignyi*. 5th International Conference on Ticks and Tick-borne Pathogens, Neuchatel, Switzerland. 29 Aug 2 September 2005 (P66).

CHENG PH, STOPFORTH E, NEITZ AWH & GASPAR ARM (2006). Antimicrobial activity in the soft tick, *Ornithodoros savignyi*. 20th SASBMB Conference, Pietermaritzburg, 2-5 July 2006 (P71).

SAUNDERS MG, CHENG PH, NEITZ AWH & GASPAR ARM (2006). Investigations into mechanisms in the hemolymph of the soft tick *Ornithodoros savignyi* to combat infection by micro-organisms. 20th SASBMB Conference, Pietermaritzburg, 2-5 July 2006 (P70).