# Chapter 5: Concluding discussion

Over the past few years, investigations in the field of tick innate immunity have increased considerably, but there still remains much to be done in order to understand the mechanisms and molecules involved. Knowledge obtained by these studies will lead to better control strategies against ticks as well as diseases they transmit. The current ideas regarding tick innate immunity were outlined in the introduction to this thesis. Survival of the tick in nature is dependent on its awareness and response to pathogens. Ticks utilize a defense mechanism similar to the innate immune system of vertebrates to protect themselves from pathogens (Johns *et al.,* 2000; 2001a, b; Nakajima *et al.,* 2001; 2002; 2003a; Sonenshine & Hynes, 2008). Information obtained from structure-function investigations of the molecules involved in tick innate immunity, may also be useful for the development of novel antimicrobial drugs or anti-tick vaccines.

A number of AMPs have been described from ticks including defensin from the hemolymph of *O. savignyi* (Olivier, 2002). No work has however, been done on the PRRs molecules from this tick species. The aim of the current study was therefore to investigate potential immunoprotective molecules that recognize and bind to Gram-negative bacterial surfaces. Chapter 2 describes the attempts to identify such molecules, while Chapter 3 describes the molecular characterization of a potential binding protein identified in Chapter 2. Due to numerous reports on the presence of potential AMPs secreted during feeding, the antimicrobial activity from the salivary glands of *O. savignyi* was investigated in Chapter 4.

Two high molecular mass HMM PRRs (Mr > 250 kDa) were identified from hemolymph, based on the ability to selectively elute tick-derived proteins from *E. coli* affinity beads using high ionic strength conditions (0.5 M NaCl). Previously, chaotropic and extreme pH conditions were used to elute surface binding

proteins from Gram- positive bacteria (Zhu *et al.,* 2005). These conditions were not suitable in the present study as it caused disruption of the Gram-negative bacteria. The high ionic strength method developed in the present study proved to be more effective and could in future be used to identify PRRs that recognize Gram-negative bacteria in other tick species. However, this method has some disadvantages, in that low molecular mass proteins from bacterial origin were also extracted. This could mask tick-derived proteins with similar molecular masses. Future studies may improve the current methods by using LPS rather than whole bacteria as affinity beads. This will eliminate bacterial background and allow bound proteins to be characterized without bacterial protein interferences (Mitsuzawa *et al.,* 2006). This strategy is based on biotinylated-LPS and the affinity of biotin which associates with streptavidin-resin and forms a hemolymph protein-LPS-biotin-streptavidin-resin complex (Fig 5.1).

The HMM proteins identified in this study are noteworthy. It is not known whether they are polymers composed of monomers or synthesized as one large protein. These monomers could be stored in the hemocytes and covalently linked in plasma following a challenge. Such a large protein was identified in the horseshoe crab and was shown to be composed of two monomers, coagulin and proxin. These molecules make up a complex coagulation network of non-covalent head-to-tail homopolymers (x-coagulin-coagulin-x). These polymers act in synergy with hemolymph plasma to trap, bind and immobilize microbes (Isakova & Armstrong, 2003). Alternatively, coagulin binds to proxin on the hemocytes by means of the action of a transglutaminase (TGase) (Osaki & Kawabata, 2004). It has been suggested that these polymers play a crucial role in the defense mechanism of the horseshoe crab (Armstrong & Armstrong, 2003; Matsuda *et al.,* 2007).

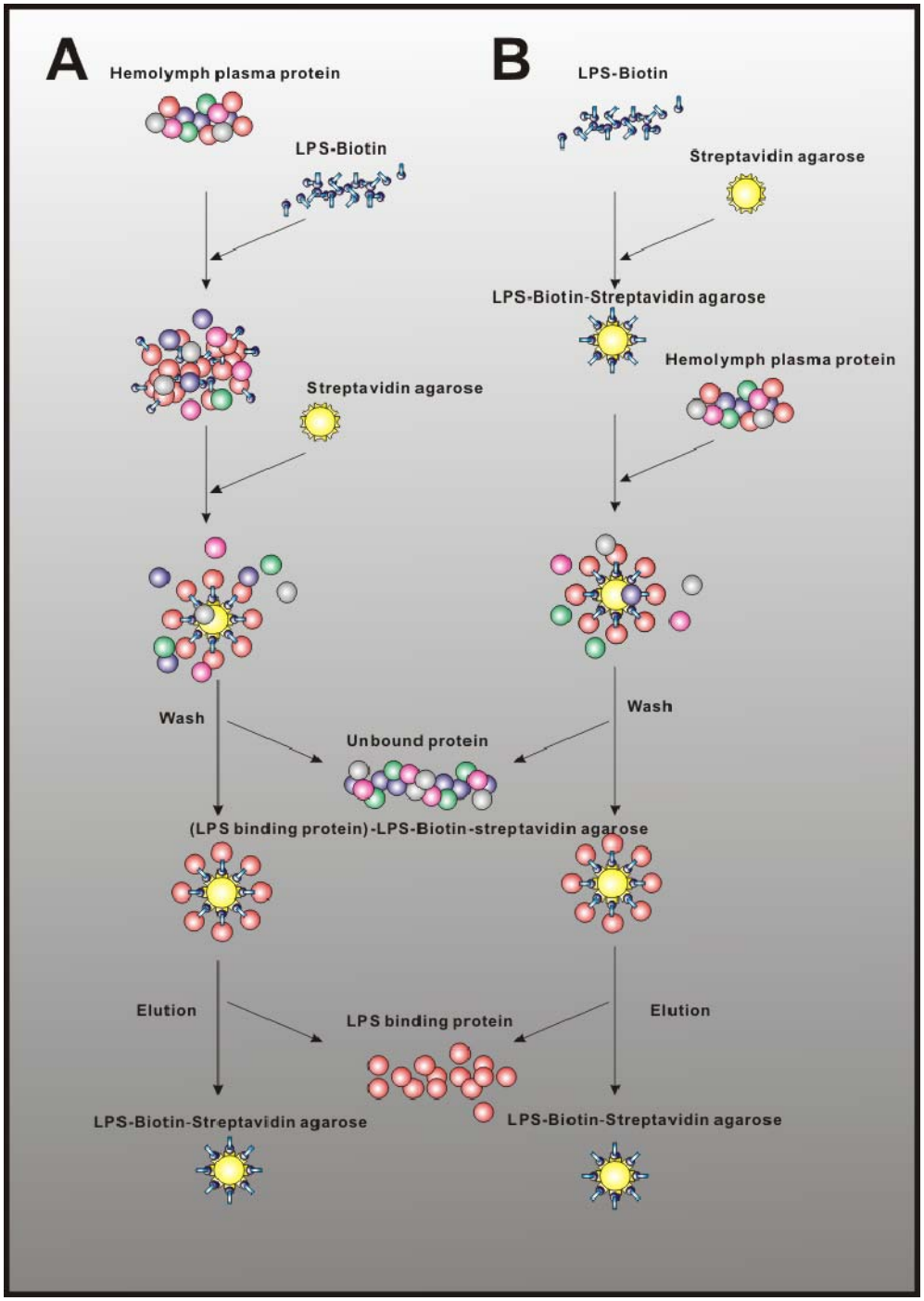


**Figure 5.1 Schematic representation of the LPS binding strategy.** A. Hemolymph plasma binds to LPS-biotin followed by subsequent binding to streptavidin B. Preparation of LPS-biotin-streptavidin beads prior to binding.

This might explain the absence of the HMM proteins when hemocyte extracts were analyzed. Future experiments could consider ways to activate hemocyte contents before analysis, thereby inducing the formation of HMM complexes. Alternatively, the HMM protein could be expressed in fat body tissue and secreted into the hemolymph. Future studies that use antibodies generated against the HMM proteins could resolve this.

The HMM proteins were further characterized using tryptic digestion followed by tandem MS/MS and *de novo* sequencing. While this yielded high quality ion spectra data, no homologous proteins were identified from the sequence database. This is most probably due to the current paucity in sequence data for tick hemolymph proteins. It is a well recognized current problem that the limited number of tick protein sequences available in databases is a drawback to the identification of tick proteins using a proteomics approach (Madden *et al.,* 2002; Oleaga *et al.,* 2007). The number of tick sequences in Genebank is however rapidly growing and MS/MS ion spectra data and *de novo* sequences may be archived and used to search databases in the future (Blackburn & Goshe, 2009; Shevchenko *et al.,* 2009). However until then, *de novo* sequences may be employed for the design of degenerate primers for cloning of the genes of the corresponding proteins by a PCR-based approach (Lingner *et al.,* 1997; Shevchenko *et al.,* 2001).

In the case of Protein X, a number of hits showed a similar peptide (WTDYYDRM) which had a low degeneracy. Due to its recurrence, we considered the possibility that this peptide would be a true sequence of Protein X and the degenerate primer designed from this sequence was used in an attempt to identify this molecule using cDNA derived from hemocytes. This strategy did not yield the expected sequence for Protein X, but rather a novel lipocalin-like sequence designated as savicalin which is described in Chapter 3.

The discovery of savicalin in hemocytes seems to be serendipitous because its expression profiles seem to correlate with responses towards bacterial challenge, even though it seems to be completely unrelated to the HMM proteins. However, it might not be surprising that savicalin could be involved in antimicrobial responses, because the primary function of hemocytes is related to the innate immune system of arthropods (Schlenke *et al.,* 2007). It can as such be expected that most hemocyte-derived proteins that are not involved in house-keeping functions will have some function related to the arthropod immune system.

Perhaps more surprising is the fact that savicalin is the first non-salivary gland lipocalin described in ticks. Lipocalins are the most abundant proteins found in tick salivary glands, in terms of transcript numbers, protein expression levels and numbers of family members (Mans *et al.,* 2008a, b). To date more than 300 lipocalin sequences (Table 3.1; 3.2 and Appendix I; II) have been deposited into the sequence databases and most have been indicated to have been derived from salivary glands (Mans *et al.*, 2008 a, b). This could suggest, perhaps erroneously, that tick lipocalins are limited to salivary glands and tick feeding.

However, a number of lipocalins have been identified in whole-body derived EST libraries from *R.* (*Boophilus*) *microplus* and *O. porcinus*, and have the potential to function in other facets of tick biology (Mans *et al.,* 2008a). In addition, salivary gland-derived lipocalin transcripts were also detected in midguts from the hard tick *I. ricinus* (Beaufays *et al.,* 2008a, b). However, the study described in this thesis is the first to assign a lipocalin-like molecule, savicalin, uniquely to non-salivary gland derived tissues and this includes hemocytes, midgut and ovaries of the soft tick *O. savignyi*. This provides evidence that tick lipocalins can also function in a non-feeding capacity in ticks.

Ticks are obligate blood-feeding parasites that interact with their hosts mainly at the feeding site. Soft ticks such as *O. savignyi* feed multiple times imbibing

relatively small amounts of blood that is utilized for laying small batches of eggs as well as for development and molting (Sonenshine, 1991; Mans & Neitz, 2004a). During feeding a salivary gland derived cocktail of bio-active proteins is secreted into the feeding site and this suppresses the host's immune and hemostatic defenses. Soft ticks may secrete up to 200 different proteins during the feeding process, all of which belong to various protein families (Mans *et al.,* 2008a; Francischetti *et al.,* 2008a, b). Characterization of the protein families found in salivary glands by proteomic methods have become a standard means to identify proteins secreted during feeding that are involved in the regulation of the host's defense mechanisms (Ribeiro & Francischetti, 2003; Francischetti *et al.,* 2009).

The presence of a signal peptide is indicative of the secretory nature of a salivary gland protein and implicates it as being functional at the feeding site in the host (Valenzuela *et al.,* 2002; Ribeiro *et al.,* 2006; Mans *et al.,* 2008a). Signal peptides also target secretory proteins to the secretory granules where proteins are stored as aggregated masses until needed (Mans *et al.,* 2001). These proteins do not have a house-keeping function (i.e: functions different to the tick-host interaction). A basic assumption is thus that salivary gland derived proteins are limited to the salivary gland and will not be present in other tissues. The absence of proteins in tissues other than the salivary gland is thus also used as evidence for potential roles in feeding (Stutzer *et al.,* 2008). It was, however, indicated that most protein families present in salivary glands are related to much larger families generally found in arthropods and that salivary gland proteins probably derived from these families with house-keeping function during the evolution of hematophagy in ticks (Mans & Neitz, 2004a; Mans *et al.,* 2008a). As such, protein family members should also exist in non- salivary gland tissues. Exon - intron gene structure similarities suggested that tick salivary gland-derived lipocalins are evolutionary related to lazarillo sequences from insects and it was therefore suggested that

such non-salivary gland derived lipocalins should also exist in ticks (Mans & Neitz, 2004a, b).

Homology modeling supports the inclusion of savicalin into the lipocalin family. The model and multiple alignments indicate the presence of five disulphide bonds. Two conserved disulphide bonds are found in hard and soft tick lipocalins. A third disulphide bond is shared with the TSGP4-clade of the leukotriene C4 binding soft tick lipocalins, while a fourth is shared with a lipocalin from the hard tick *I. scapularis*. The fifth disulphide bond is unique and links strands D-E in the beta-barrel lipocalin fold. Phylogenetic analysis showed that savicalin is a distant relative of salivary gland-derived lipocalins, but groups within a clade that is possibly non-salivary gland derived. It lacks the biogenic amine-binding motif associated with tick histamine and serotonin binding proteins. Expression profiles indicated that savicalin is found in hemocytes, midguts and ovaries, but not in the salivary glands. Up-regulation occurs in hemocytes after bacterial challenge and in midguts and ovaries after feeding. Given its tissue distribution and up-regulation of expression, it is possible that this lipocalin functions either in tick development following feeding or in an antimicrobial capacity.

There are similarities between hemocytes, midgut and ovaries that might explain the distribution of savicalin in these tissues. Note that antibodies against lipocalin-like proteins may cross-react with hemocytes, midgut and ovaries (Magnarelli *et al.,* 1991; Cristofoletti *et al.,* 2005; Wang *et al.,* 2007). This implies that hemocytes, midgut and ovaries share common antigenic determinants or that the same or similar proteins are expressed in these organs. It has also been shown that the number of hemocytes (nongranular cells and granule-scant basophilic granular cells) increases during ecdysis of fed nymphs and that basophilic granular cells increase in fed adult ticks and nymphs (Kadota *et al.,* 2003). Up- and down-regulation of savicalin could thus be closely related to

hemocyte numbers during ecdysis. Molecules known to influence tick development and ecdysis, include ecdysone and juvenile hormone.

Savicalin could also act as an antimicrobial by scavenging siderophores, as found for the mammalian lipocalin NGAL (Alpízar-Alpízar *et al.,* 2009). Given its up-regulation in hemocytes upon bacterial challenge, savicalin might also play a critical role in the defence against harmful pathogens. In this regard, it should be noted that ecdysone and juvenile hormone influence the innate immune system of insects and could be potential ligands for savicalin (Meister & Richards, 1996; Figueiredo *et al.,* 2006; Flatt *et al.,* 2008).

The innate immune responses associated with hemocytes and hemolymph function primarily to protect the tick against foreign invasions that gain access to the tick via breaches in the integument, genital pore or from the gut by crossing the midgut barrier. A significant amount of fluid in the form of blood is ingested during the feeding process, which is eventually digested in the midgut. Argasid ticks digest blood over a prolonged period (Sonenshine, 1991), which makes the midgut a potential ground for bacterial growth and invasion. To counteract this, ticks can secrete AMPs into the lumen of the gut (Nakajima e*t al.,* 2005). It is, however, known that salivary gland contents can be ingested with the blood-meal and has been suggested as a possible mechanism to maintain the fluidity in the gut after feeding (Bowman & Sauer, 2004). Secretion of AMPs in the saliva, could therefore serve to “sterilize” the feeding site and the ingested blood-meal from any microbial contamination. Ingestion of such AMPs with the blood-meal could also help to maintain the sterility after feeding has been completed.

This rationale prompted us to look into the potential for AMPs in the salivary glands (Chapter 4). Gram-positive antimicrobial activity was detected and purified using reversed-phase chromatography. Three potential proteins were visualized

using tricine SDS-PAGE of which one was savignin, the anti-thrombin inhibitor previously characterized by Nienaber *et al.* (1999).

Savignin was ruled out as an antimicrobial by introducing 10 kDa cut-off spin columns as an additional purification step. This yielded an antimicrobial fraction with molecular masses ranging from 0.99 ~ 7.182 kDa as determined by MALDI-MS. MS-MS analysis of the active fraction revealed the presence of actin-derived peptide fragments. In arthropods the up-regulation of full-length actin following challenge has been reported (Vierstraete *et al.,* 2004a, b; Paskewitz & Shi, 2005). Actin-derived peptide fragments have been observed in the hemolymph of septically challenged *Drosophila* (Verleyen *et al.,* 2006) as well as in a celomocyte extract of the starfish, *A. rubens* (Maltseva *et al.,* 2007; Li *et al.,* 2010).

The initial focal point of this study was to investigate proteinaceous antimicrobials. Some evidence has shown that insects produce antimicrobial metabolites (Leem *et al.,* 1996, 1999; Chiou *et al.,* 1998). Leem *et al.* (1996) first reported that the fleshfly, *Sarcophaga peregrine,* produces an inducible compound known as N-β-alanyl-5-S-glutathionyl-3,4-dihydroxy-phenylalanine. From larvae of the saw fly, *Acabtholyda parki*, *p*-hydroxycinnamaldehyde, an antimicrobial metabolite was identified (Leem *et al.,* 1999). Another two metabolites β-alanyl-tyrosine and 3-hydroxykynurenine were identified in the hemolymph of grey fleshy fly larvae, *Neobelliera bullata* (Chiou *et al.,* 1998). As such, other compounds or metabolites playing a role in anti-Gram positive activity may have been overlooked. In some cases, these compounds and metabolites could not be detected with a HPLC detector. Thus, more sensitive techniques such as LC-MS may be required for further studies.

This study utilized novel approaches that yielded some interesting results even if they may be deemed to be unconventional. The presence of heretofore unknown

HMM proteins in hemolymph that can bind to microbial surfaces has been detected using a bacterial bead and elution approach. The serendipitous discovery of a non-salivary gland lipocalin implicated in tick innate immunity has suggested novel functions for a very well characterized tick salivary gland family. At this stage one of the actin derived fragments could not be linked to the observed antimicrobial activity. It cannot be excluded that the other fragments are not involved. Their biological functions need to be investigated.