Chapter 1: Evolution of hematophagy in ticks

1.1.1 Ticks as a relevant problem

Man as manager of a variety of livestock has a moral responsibility to protect the animals under his care against damage caused by ectoparasites. Ticks are the most important ectoparasites in medical, veterinary and economical terms. Ticks transmit the highest number of pathogenic organisms that can kill and harm the host. These include anaplasmosis (*Boophilus* spp), babesiosis (*Boophilus* spp), cowdriosis (*Amblyomma* spp) and theileriosis (*Hyalomma truncatum*) (de Castro and Newson, 1993; Munderloh and Kurtti, 1995). Tick paralysis (Gothe and Neitz, 1991) and toxicoses (Gothe, 1999) also have significant impacts on lifestock mortality.

Although being obligate parasites, ticks are non-permanent feeders that can survive without their host for extended periods of time. Like predators, ticks are only in contact with their host for the short time during which they feed. For fast feeding ticks (Argasidae or soft ticks) this could be less than an hour and for slow feeders (Ixodidae or hard ticks) up to nine or ten days (Sauer *et al.* 1995). In many argasid and some ixodid species, new stages of development take place away from the host and after each molting stage a new host is found (Balashov, 1972). This relieves much of the burden other permanent parasites face, which have to depend on the host's fitness and viability for their own survival. This adaptation of ticks to survive away from their host also led to ticks being more aggressive than ectoparasites living permanently on their host (Lehmann, 1993). Due to this lack of dependence of ticks on host fitness, the tick burden itself can severely affect the host.

Natural infestations may involve thousands of ticks per animal. Anemia that follows from infestation can be severe. Salivary gland toxins secreted can cause loss of appetite which can aggrevate the anemia. This leads to fatigue and a weakened immune system. Even small numbers of ticks can have an impact on host fitness, especially in young animals (de Castro and Newson, 1993). It has been reported that calves infested with an average of 40 ticks gained 37 kg less in body mass than control calves (Kaufman, 1989). Wounds inflicted by ticks as well as skin damage due to scratching can lead to

secondary infections through miasis-producing flies. Other consequences include hair and weight loss, that leads to a decrease in the quality of hides and wool and reduced milk and meat production, respectively (de Castro and Newson, 1993).

Combined, these factors have a great economic impact on agriculture. In 1984, the United Nations Food and Agriculture Organization estimated an annual global loss of seven billion US dollars due to tick infestation and tick-borne diseases. This amount has increased even more in recent years (Sauer *et al.* 1995).

1.1.2 Tick control

Conventional methods of tick control make use of chemical acaricides, normally applied on the host through dipping or spraying practices. Although intensive use can be beneficial over a short period of time, it soon leads to the loss of protective immunity against ticks, due to acaricide resistance. Interruption in tick control programs also leaves livestock vulnerable to major outbreaks of tick-borne diseases or the build up of tick populations (de Castro and Newson, 1993). The increasing cost of development of new chemicals and residual contamination of meat and milk are other major drawbacks to this approach (de Castro and Newson, 1993). Pathogens are also becoming resistant to drugs directed against them and there is even emergence of new pathogenic strains (Wikel, 1996).

Anti-tick vaccines represent one of the most promising alternatives to chemical control (Willadsen, 2001). They have the advantage of target specificity, environmental safety, lack of human health risk and ease of administration and cost (Wikel, 1996). Cross reactivity between antigens of different species is an important consideration in the development of vaccines (Crause *et al.* 1994). This could be exploited as cross protection has been observed between different Ixodidae species (Opdebeeck, 1994; de Vos *et al.* 2001). One of the problems inherent in the strategy of vaccination against ticks is the inefficient or even non-existent anti-tick immunity in natural hosts (de Castro and Newson, 1993). This is attributed to either evasion of the host's immune system or its direct suppression (Sauer *et al.* 1995).

Two main strategies for vaccination development have been focused on, those based on the modulation of the host's immunocompetence and those that make use of novel antigens (Opdebeeck, 1994). The focus in the former is on the salivary secretions of the tick during feeding, as the saliva contains biologically active compounds necessary for suppression of the host's immune system (Sauer *et al.* 1995). The latter focus on those antigens that do not normally come into contact with the host's immune system and are appropriately called concealed antigens (Alger and Cabrera, 1972).

1.1.3 Concealed antigens as vaccine targets

The advantage of concealed antigens is that it bypasses the immune evasion response (Kaufman, 1989). It would be unlikely that the host have developed immune tolerance with respect to these antigens as opposed to those from the salivary glands. Concealed antigens include those from the midgut, which are involved in blood digestion, hemolymph, reproductive organs and synganglion. The different internal organs are validated as targets by the fact that ticks have an open circulatory system and that the host's immunoglobulins can pass through the gut barrier and retain their specificity (Opdebeeck, 1994; Munderloh and Kurtti, 1995). The efficacy of concealed antigens as vaccine targets was indicated by a reduced number of feeding ticks, reduced engorged body weights, increased feeding times, diminished production of ova and a decreased tick fecundity (Sauer *et al.* 1995). It was found that damage to the tick's midgut leads to leakage of host blood into the hemocoel. This is due to anti-midgut antibodies as well as the presence of host basophils, eosinophils and macrophages in the digestive tract of the tick (Opdebeeck *et al.* 1988). A recombinant midgut membrane protein (Bm86 from *Boophilus microplus*) has recently been expressed in a baculovirus system. Vaccination with this protein gave effective protection for cattle in the field as well as in pens with reduced tick reproductive capacity of 77% (Opdebeeck, 1994). This was also the first commercial available tick vaccine and it was recently shown that cross species protection could be attained using this vaccine (Willadsen *et al.* 1995; de Vos *et al.* 2001; Willadsen, 2001).

1.1.4 Exposed antigens as vaccine targets

Tick antigens that normally do invoke an immune response in the host enter through salivary secretions (Kaufman, 1989). The first anti-tick vaccine strategy was based on salivary gland extracts (Trager, 1939). This vaccine worked well in the laboratory setting, but poorly in the field. Some authors have argued that on principle a salivary gland derived antigen would be of limited value (Kaufman, 1989) and that ectoparasites and their hosts adapt in a complementary manner to reduce provocation of antagonistic reactions such as immune and pathogenic responses (Opdebeeck, 1994). Salivary gland secretions do however, perform a main function in the disarmament of the anti-hemostatic and inflammatory reactions of the host. Saliva is also the main vehicle for transmission of tick-borne pathogens, although pathogens may also be transmitted from the gut, as ticks do regurgitate bloodmeals (Munderloh and Kurtti, 1995). It was shown that immunosuppression of the host by tick saliva promotes both blood meal aquisition and the effective transmission of pathogens. Immunity to tick salivary gland extracts could have a protective effect against disease transmission and as such is worth investigating (Sauer *et al.* 1995).

Elevated immune responses against exposed antigens may circumvent immunosuppression (Opdebeeck, 1994). Artificial induced immunity with sufficient magnitude against the components which play a role in immunosuppression, could break immune system subversion. This could contribute significantly to the success of a multivalent vaccine. Advantages of using salivary gland extracts is that exposure to natural infestation has the capacity to boost vaccine immunity (Janse van Vuuren *et al.* 1992). It could also stimulate naturally acquired resistance mediated through reponses to antigens found in tick saliva. On the other hand, immediate or delayed hypersensitivity could occur on repeated exposure to salivary gland antigens. These should be screened for allergens and this necessitates the requirement for purified components (Opdebeeck, 1994). During feeding there is also the expression of numerous other proteins in the salivary glands of ixodids which have important functions in the feeding process and thus provide a new array of possible immunogens (Wikel, 1996). Despite arguments against the use of exposed antigens, a number of successes have been attained. These include vaccination stategies against *Ripicephalus appendiculatus* (Shapiro, Bascher

and Dobbelaere, 1987) and *Hyalomma anatolicum anatolicum* (Banerjee, Momin and Samantaray, 1990). In a recent study the vaccinal value of novel and salivary gland extracts were investigated for the argasid ticks *Ornithodoros moubata* and *Ornithodoros erraticus*. It was found that the immune response against salivary gland extract was much higher than the response toward novel antigens from the gut, ovaries or synganglion. Vaccination with salivary gland extracts also provided protection in swine against tick infestation (Astigarraga *et al.* 1996).

A combined approach of novel as well as exposed antigen vaccines may be the best strategy to follow. Molecular engineered vaccines containing immunogenic parts of both exposed and concealed antigens may be an exciting alternative to conventional approaches (Opdebeeck, 1994). For rational vaccine design a thorough knowledge of the properties of potential vaccine targets is needed. This necessitates a deeper understanding of tick-host interactions, which is at the heart a question of how ticks adapted to their blood-feeding environment.

1.2.1 Evolution of hematophagy

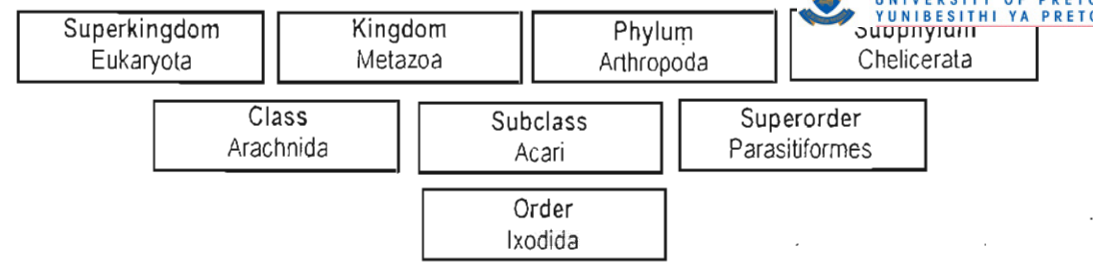
Hemostasis is an efficient defense mechanism that prevents blood-loss through an open wound. The vertebrate hemostatic system originated approximately 400 million years ago (MYA) (Doolittle and Feng, 1987). In contrast, hematophagy (blood-feeding behaviour) evolved independently at least six times in the approximately 15000 species and 400 genera of the hematophagous arthropods (Table 1.1) (Ribeiro, 1995). Many arthropods evolved blood-feeding capabilities during the Cretaceous and Jurassic Era's, (145-65 MYA) (Balashov, 1984). Adaptation of hematophagous arthropods to a blood-feeding environment thus entailed specific adaptation to an efficient existing hemostatic system. Elucidation of the evolutionary mechanisms of blood-feeding arthropods could further our understanding of the evolution of complex systems as is exhibited at the blood-feeding interface, as well as allow the development of new control strategies based on novel as well as shared mechanisms. The subject investigated in this thesis is the adaptation of ticks to a blood-feeding environment, with specific reference to salivary gland proteins.

Table 1.1: Arthropods that evolved hematophagy independently. Orders or genera suggested to have evolved blood-feeding behaviour independently are shown in bold. Adapted from Ribeiro (1995).

Insect or arachnid order	Family (common name)	Genera	Species
Diptera	Sandflies	6	70
	Blackflies	24	1571
	Horseflies	5	4400
	Tsetse flies	1	23
	Stable flies	3	50
	Mosquitoes	36	3450
Hemiptera	Bedbugs	23	91
	Bat bugs	5	32
	Kissing bugs	14	118
Anoplura	Lice	42	490
Siphonaptera	Fleas	239	2500
Acari	Soft ticks	3	178
	Hard ticks	12	657
Total number		417	14595

1.2.2 The Ixodida or ticks

Ticks are obligate hematophagous organisms and the most important ectoparasites of domestic animals. The Ixodida comprises three families (Fig. 1.1), the **Ixodidae** or hard ticks (Scutum present, short in female, long in male, capitulum at anterior of body in all stages), **Argasidae** or soft ticks (Scutum absent, capitulum on underside of body in nymphs and adults, anterior in larvae) and **Nuttalliellidae** that is monotypic (Hoogstraal, 1956). The Ixodida are part of the Phylum Arthropoda and group with the spiders, scorpions, whip-scorpions, sun spiders, harvestmen, hooded tick spiders and false scorpions in the Class Arachnida. Ticks are a small group of a much larger group of mites that group in the subclass Acari, more specifically in the superorder Parasitiformes. Parasitiformes are characterized by the presence of free coxae, a ventral anal opening covered by plates, a hypostome and a sclerotized ring surrounding the capitulum. Actinochitin is absent on the setae, distinguishing them from the other superorder Acariformes.



Family			
Ixodidae		Argasidae	Nuttalliellidae
Amblyomma (102)	*Anocentor* (1)	*Antricola* (14)	*Nuttalliella namaqua*
Aponomma (24)	*Anomalohimalaya* (1)	*Argas* (61)	
Boophilus (5)	*Cosmiomma* (1)	*Nothoaspis* (1)	
Dermacentor (32)	*Haemaphysalis* (150)	*Ornithodoros* (103)	
Hyalomma (25)	*Ixodes* (234)	*Otobius* (2)	
Margaropus (3)	*Nosomma* (1)		
Rhipicentor (2)	*Rhipicephalus* (76)		

Fig. 1.1: Hierarchy of the Ixodida. Approximate number of species is indicated in brackets next to the genus name (Personal communication, Dr. Lance Durden, 50th Annual Acarology Summer Program, Ohio State University, 2000).

1.2.3 The origin of ticks

Ticks were considered to be of the earlier lineages of terrestrial arachnids, with proposed origins in the Devonian (350-400 MYA) (Oliver, 1989), late Silurian (400 MYA) (Lindquist, 1984), late Permian (Hoogstraal and Aeschlimann, 1982) and late Paleozoic or early Mesozoic (225 MYA) (Hoogstraal, 1985; Balashov, 1989, 1994). These estimations were based on the assumptions that the most primitive group of ticks are associated with the most primitive group of hosts, and that ticks must have arisen approximately at the same time as their particular host group. It has been proposed that ticks originated more recently in the early Cretaceous (Fillipova, 1977) or late Cretaceous (120 MYA) (Klompen *et al.* 1996). The latter is based on comparison of the distribution of ixodids worldwide, where some of the presumably basal lineages are exclusively Australian and suggests a major role of Australia in the evolution of ixodids. The late Cretaceous was the last time that Australia was part of Gondwanaland and indicates that this period played an important role in the origin of the Australian lineages, and by extension the entire tick family (Klompen *et al.* 2000). This period also

saw the emergence of mammals and was closely followed by dinosaur extinction (65 MYA). This provokes tantalizing suggestions that ticks evolved at the dawn of the emergence of mammals.

1.2.4 Tick fossil records

Ixodid fossils were found in Baltic amber (*Ixodes succineus*, 30-40 MYA), Oligocene deposits (*Ixodes tertiarius*, 30 MYA), Dominican amber (*Amblyomma testudinis*, *Ornithodoros antiquus*, 30-40 MYA) and in the ear of a woolly rhinoceros (*Dermacentor reticulates*, 1-3 MYA (Scudder, 1885; Weidner, 1964; Lane and Poinar, 1986; Poinar, 1995). The oldest fossil to date is an argasid (*Carios jerseyi*) found in New Jersey amber, 90-94 MYA(Klompen and Grimaldi, 2001). If ticks originated 120 MYA, they already speciated by ~92 MYA into the main tick families, as well as argasid genus level.

1.2.5 Speciation of ticks

Phylogenetic analysis using 16S RNA placed the Argasinae basal to the Ixodidae, suggesting that ixodids were derived from an Argas-like ancestor (Black and Piesman, 1994). Phylogenetic analysis also suggested that Argasidae were paraphyletic (Crampton, McKay and Barker, 1996). However, re-analysis of ixodid and argasid relationships, using 18S RNA indicated strong support for the monophyly of Argasidae and Ixodidae (Black, Klompen and Keirans, 1997). It also placed Argasinae and Ornithodorinae as single monophyletic groups, respectively, inside the Argasidae, which is concurrent with original morphologically based phylogenies (Hoogstraal and Aeschlimann, 1982). The question raised here is whether ticks adapted to blood-feeding environment before or after speciation of the main tick families?

1.2.6 Ticks were free-living scavengers

Holothyrida forms with the Ixodida and Mesostigmata the Order Parasitiformes (Evans 1992). Phylogenetic analysis indicates that the Holothyrida rather than Mesostigmata are a sister-group to ticks (Dobson and Barker, 1999). It is of interest that the holothyrida are distributed only in areas that were part of Gondwanaland, which corresponds with the current ideas on tick origins (Klompen *et al.* 2000). Holothyrida is

a group of free-living mites, which mainly live on body fluids of dead organisms. It has been suggested that ticks shared this same trait before adaptation to a blood-feeding environment (Walter and Proctor, 1998).

1.2.7 Questions regarding tick evolution

The preceding sections lead to the following questions:

1) What was the nature of the ancestral tick before adaptation to a blood-feeding environment (i.e. which anti-hemostatic strategies were present or evolved *de novo*)?
2) Did ticks evolve anti-hemostatic strategies before or after divergence into the main tick families (i.e. did ticks evolve anti-hemostatic strategies more than once independently)?
3) How did ticks acquire novel anti-hemostatic strategies (i.e. what were the evolutionary mechanisms by which ticks adapted to an already present and complex hemostatic system)?
4) What was the driving force behind the evolution of hematophagy in ticks?

The answers to these questions can only be contemplated by a clear understanding of tick-host interactions: the interface where adaptation originated.

1.3.1 Tick-host interactions

To acquire a blood meal, ticks need to penetrate the host's skin and damage enough blood vessels for the release of blood. The depth of penetration and feeding time depends on the type of tick. Slow and fast feeding ticks have different mouthparts that cause different degrees of damage to the host (Binnington and Kemp, 1980). In ixodid ticks (slow feeders) the lesion develops gradually with the formation of a hematoma. It has been proposed that the main damage at the feeding lesion is caused by neutrophils attracted to the feeding site where they degranulate and cause inflammation (Ribeiro, 1987). Argasids feed rapidly with deep penetration of the host's skin and cause considerable damage. Bloodloss can still occur long after a tick has stopped feeding (Binnington and Kemp, 1980).

During probing for blood, capillary and small blood vessels are lacerated, host cells are ruptured and hemorrhage occurs. This increases the blood volume at the site of feeding and leads to activation of the host's defence mechanisms which include the hemostatic (blood coagulation and platelet aggregation) and the immune system (Ribeiro, 1987). Platelet aggregation represents the most immediate defence and is sufficient to arrest blood flow from small vessels (Law, Ribeiro and Wells, 1992). It is possible that platelet aggregation directed the evolution of the salivary gland proteins of all blood sucking arthropods to a greater extent than did blood coagulation (Ribeiro, 1987). Coagulation may thus be of lesser significance in the host's defence against blood sucking arthropods. It may play a more important role during the ingestion of the blood meal and it was suggested that anti-coagulant inhibitors from ticks have their main function in the gut where they serve to keep the blood in a fluid form (Bowman *et al.* 1997).

1.3.2 Platelet aggregation and its role in hemostasis

Platelet aggregation is essential for hemostasis (the cessation of bloodflow from a damaged vessel wall) by means of blood coagulation and formation of a fibrin clot that serves as a plug (Dam-Mieras and Muller, 1986). The whole blood coagulation cascade depends on a negatively charged membrane surface where the different serine proteases involved in the cascade can dock to be sequentially activated (Esmon, 1995). Normally the outer membranes of cells are depleted of negatively charged phospholipids. Platelets provide this membrane surface by the translocation of phosphatidyl serine from its inner to outer membrane during platelet activation and aggregation. Clotting proteins (fVII, fX and prothrombin) subsequently bind to the membrane through a Ca^{2+} bridge by means of the modified amino acid γ-carboxyglutamate (Gla) and are thus targeted and localized to the site of damage (Tans and Rosing, 1986).

1.3.3 Collagen and its role in hemostasis

Damage to the blood vessel wall exposes the collagen containing subendothelial layer. Collagen binds the plasma protein, von Willebrandt's factor (vWf), which changes conformation and activates platelets by binding to a specific vWf receptor (glycoprotein Ib) or to the main integrin involved in aggregation, $\alpha_{IIb}\beta_3$ (Hawiger, 1992). This restricts platelets to the site of injury and prevents thrombosis by localization of the clotting

cascade. Collagen also activates factor XII, the initiator of the intrinsic pathway of the blood-clotting cascade (Zwaal, Bevers and Comfrius, 1986).

1.3.4 Platelet activation

Unactivated platelets are discoid and smooth and upon activation change to a spherical shape with numerous pseudopods. Activated platelets secrete their granule contents and eventually aggregate to form a platelet plug. Platelets can be activated by a variety of compounds (ADP, collagen, thrombin, thromboxane A_2, epinephrine, platelet activating factor, thrombospondin) which binds to specific membrane receptors present on the platelet surface. Activation is mediated by signal transduction of the different receptors which activates either the cyclo-oxygenase or phospholipase C pathway, or inhibits adenylate cyclase (Fig. 1.2).

1.3.5 Inhibition of adenylate cyclase

Epinephrine, thrombin and ADP bind to Gi-coupled receptors, and prevent the synthesis of cAMP through inhibition of adenylate cyclase. Raised cAMP levels lower the intra-platelet free calcium levels necessary for shape change and granule secretion. Modulation of cAMP levels thus aid in platelet activation, although it does not cause platelet shape change.

1.3.6 The cyclo-oxygenase pathway

Collagen, epinephrine and thrombin receptors are all linked to G-proteins that activate phospholipase A_2, which cleaves phosphatidyl inositol and phosphatidyl choline to produce arachidonic acid. Cyclo-oxygenase then converts arachidonic acid to thromboxane A_2 (TXA_2) which is a platelet agonist as well as a vasoconstrictor. Overall, agonists exclusive to this pathway does not cause primary aggregation, but rather activation of platelets by TXA_2 and subsequent activation and aggregation via the PLC pathway.

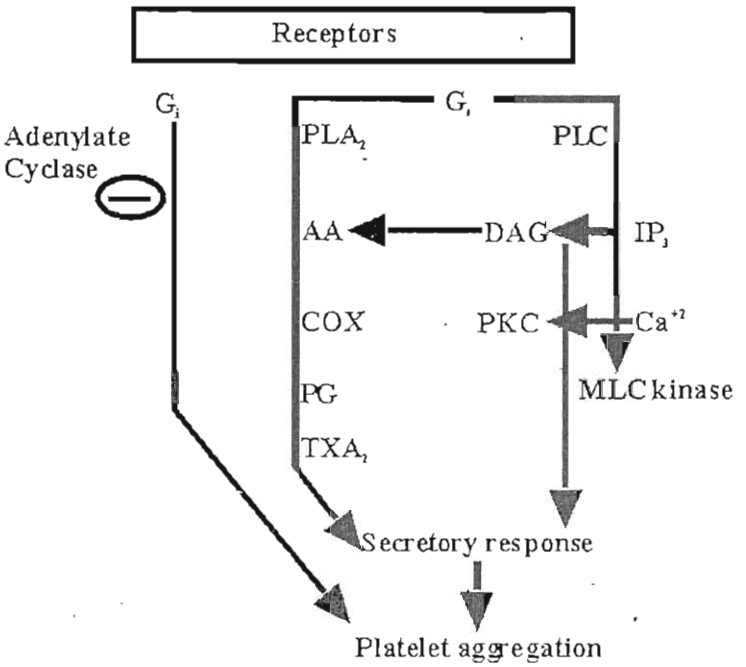


Fig. 1.2: Platelet activation mechanisms. Binding of different agonists to their specific receptors activates phospholipase A_2 (PLA_2) or phopholipase C (PLC) or inhibits adenylate cyclase. Arachidonic acid (AA) released by PLA_2 is converted to prostaglandins (PG) by cyclo-oxygenase (COX) with subequent conversion to thromboxane A_2 (TXA_2). PLC releases diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) from the platelet inner membrane. IP_3 induces release of Ca^{2+} from the dense tubular system and calcium activates myosin light chain kinase (MLC kinase), which phosphorylates myosin, leading to shape change. DAG in the presence of calcium activates protein kinase C (PKC), which phosphorylates a number of platelet proteins, leading to secretion of granules. Inhibition of adenylate cyclase leads to a relaxed control of intracellular calcium concentration, which promotes those processes activated by calcium (Hawiger, 1989).

1.3.7 The phospholipase C pathway (PLC)

Thrombin, ADP, PAF and TXA_2 receptors are linked to G-proteins that activate PLC leading to the formation of diacylglycerol (DAG) and inositol trisphosphate (IP_3) through hydrolysis of phosphatidyl inositol diphosphate. DAG in the presence of calcium activates phosphokinase C (PKC) causing degranulation of platelets. IP_3 releases Ca^{2+} from the dense tubular system and this activates myosin light chain kinase resulting in the phosphorylation of myosin (Hawiger, 1989). Activated myosin binds actin to form the actomyosin cytoskeleton which facilitates shape change and formation of focal adhesion points (FAP). These FAPs form on the ends of philopodia and consist of a multi-protein integrin $\alpha_{IIb}\beta_3$ complex (Jamaluddin, 1991). Due to multiple phosphorylation events in the

multi-protein complex, $\alpha_{IIb}\beta_3$ undergoes a conformational change, with exposure of a fibrinogen-binding site and subsequent platelet aggregation through linkage of platelets by fibrinogen (Clark *et al.* 1994). Fibrinogen binds to $\alpha_{IIb}\beta_3$ through two RGD integrin recognition motifs on its α-chain and one non-RGD containing sequence on its γ-chain (Hawiger and Timmons, 1992).

1.3.8 Secretion of granule components

Various biologically active compounds stored in α- and dense-granules are secreted during activation, which in turn, activate other platelets and induce inflammation (Table 1.2). Compounds of special interest for tick biology include ADP, which activates platelets and ATP, which causes mast cell degranulation and neutrophil aggregation. This leads to local inflammation and vasoconstriction (Ribeiro, 1989). Serotonin and thromboxane A_2 stimulate vasoconstriction, while serotonin also promotes vascular permeability for the infiltration of mast cells and macrophages (Bloom and Thomas, 1987).

Table 1.2: Biologically active compounds secreted by activated platelets. Adapted from Jamaluddin (1991).

Granule	Contents	Effect on hemostasis
Dense granules	ADP	Activates platelets
	ATP	Inflammation
	Ca^{2+}	Shape change of platelets
	Serotonin	Vasoconstriction and vascular permeability
α-granules	VWf	Binds platelets to collagen
	Fibrinogen	Platelet aggregation
	Fibronectin	Cell adhesion
	Plasminogen	Precursor of plasmin
	Thrombospondin	Activates coagulation
	Kininogen	Coagulation factor
	fV	Coagulation factor
	$\alpha_{IIb}\beta_3$	Platelet aggregation

1.3.9 Blood coagulation

The intrinsic pathway of blood coagulation starts with collagen-induced activation of fXII which activates fXI as well as kallikrein. Kallikrein cleaves a precursor to form bradikinin, a peptide causing inflammation, the sensation of pain and irritation (Ribeiro, 1989). The extrinsic pathway starts with the release of thromboplastin (tissue factor) from damaged endothelial cells, which activates factor VII (Fig. 1.3) (Bevers, Comfrius and Zwaal, 1993). Both pathways eventually coalesce in the formation of factor Xa which in turn

produces thrombin. Fibrinogen is then cleaved by thrombin to fibrin which forms a network, the main constituent of the blood clot together with platelets and erythrocytes (Jackson and Nemerson, 1980).

These events ultimately lead to edema, one of the signs many tick-resistant hosts display. Edema and associated irritation lead to host grooming, an important factor in the reduction of the tick burden (Wikel, 1996).

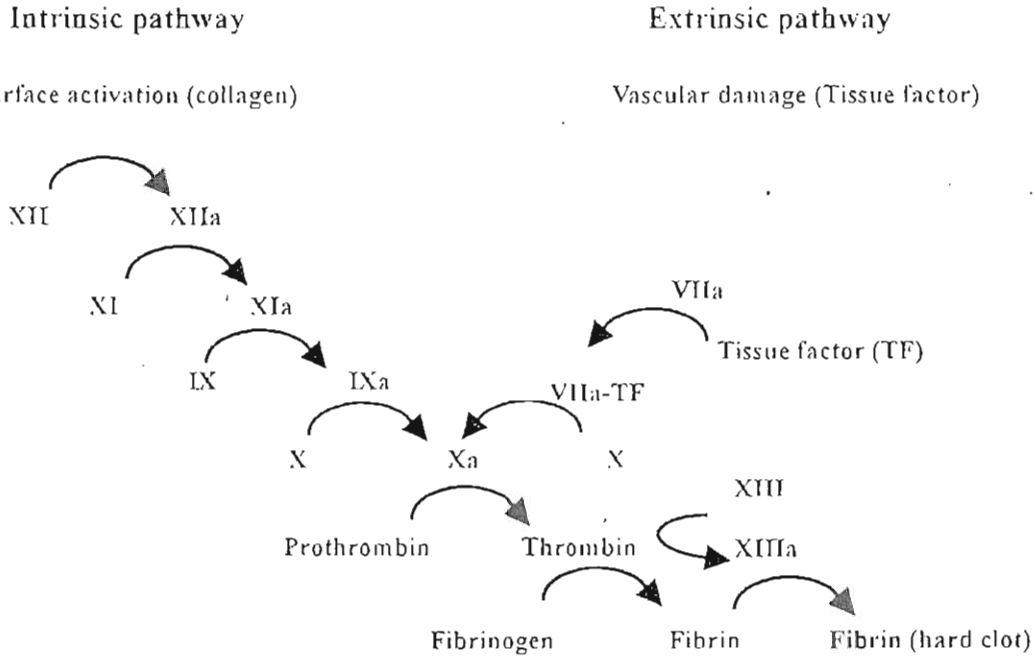


Fig. 1.3: The blood coagulation cascade. The intrinsic pathway is initiated by the binding of fXII to a negatively charged surface like collagen and is then activated by kallikrein. fXIIa then activates fXI as well as prekallikrein. This leads to eventual activation of fX. The extrinsic pathway is activated by trauma that releases tissue factor. This binds to fVIIa, that is activated by thrombin and together activates fX. Prothrombin is activated to thrombin by fXa which cleaves fibrinogen to fibrin that forms a network. Thrombin also activates fXIIIa which stabilizes the fibrin clot by crosslinkage. Adapted from Bloom and Thomas (1987).

1.4.1 Anti-hemostatic components produced by ticks

Ticks manage to down regulate the complex host immune and hemostatic mechanisms through secretion of bio-active components with a range of pharmacological properties which ease the burden of obtaining a bloodmeal.

1.4.2 Immunomodulatory components in tick saliva

Tick saliva contains prostaglandin E_2 (PGE_2) which promotes vasodilation, inhibits platelet aggregation, mast cell degranulation and T-lymphocyte activation. PGE_2 has been identified in the saliva of *Ixodes scapularis*, *Amblyomma americanum* and *B. microplus* (Dickinson *et al.* 1976; Ribeiro *et al.* 1985; Ribeiro, Makoul and Robinson, 1988; Ribeiro *et al.* 1992; Bowman *et al.* 1995). This alone counteracts a whole range of the host's defences. Anti-histamine is also present which prevents inflammation. Histamine-binding proteins that sequestrate histamine from the feeding-site have been identified in *R. appendiculatus* (Paesen *et al.* 1999; Paesen *et al.* 2000). The saliva of *I. scapularis* contain a carboxypeptidase activity which cleaves bradykinin and thus prevents irritation and pain (Ribeiro *et al.* 1985; Ribeiro and Mather, 1998). An anti-complement protein from the tick *I. scapularis* that inhibits erythrocyte lysis by human serum, has also been described (Valenzuela *et al.* 2000).

1.4.3 Blood clotting inhibitors from ticks

Numerous inhibitors for the different serine proteases involved in the clotting cascade have been described for hard and soft ticks (Table 1.2). Inhibitors of fV and fVII have been described for the hard tick *Dermacentor andersoni* (Gordon and Allen, 1991). Thrombin and fXa inhibitors have been described in a variety of hard ticks (Table 1.3). Tick anticoagulant peptide (TAP) and fXaI (factor Xa inhibitors) as well as ornithodorin and savignin (thrombin inhibitors) have been described for the soft ticks *O. moubata* and *Ornithodoros savignyi*, respectively (Waxman *et al.* 1990; Gaspar *et al.* 1996; Joubert *et al.* 1998; van de Locht *et al.* 1996; Nienaber, Gaspar and Neitz 1999; Mans, Louw and Neitz, 2002a).

Fibrinogen which serves as the connective link during platelet aggregation and which is the precursor for the fibrin network is also under attack during feeding. A fibrinogenolytic enzyme has been identified in the soft tick *O. savignyi* (Mahlaku, Gaspar and Neitz, 2000). Other enzymes present in the saliva of argasid ticks are the elastases and hyaluronidases (Neitz and Vermeulen, 1987). The function of these enzymes may be to assist in the enlargement of the feeding lesion through degradation of the extracellular-matrix, thus shortening the feeding time and promoting the formation of a hematoma (Kaufman, 1989).

Table 1.3: Anticoagulants from ticks. Adapted from Bowman *et al.* (1997).

Species	Source	Target	Mr (kDa)	Reference
R. appendiculatus	Salivary gland	fXa	65	Limo *et al.* 1991
H. truncatum	Salivary gland	fXa	17	Joubert *et al.* 1995
H. dromedarii	Nymphs	fXa	12, 16	Ibrahim *et al.* 2001
O. moubata	Whole tick	fXa	7	Waxman *et al.* 1990
O. moubata	Salivary gland	Thrombin	12	van de Locht *et al.* 1996
O. savignyi	Salivary gland	fXa	7	Gaspar *et al.* 1996; Joubert *et al.* 1998
O. savignyi	Salivary gland	Thrombin	12	Nienaber *et al.* 1999; Mans *et al.* 2002a
B. microplus	Salivary gland	fXa and thrombin	NA	Anastopoulos *et al.* 1991
B microplus	Whole larvae	Kallikrein	10, 18	Tanaka *et al.* 1999
B. microplus	Saliva	Thrombin	60	Horn *et al.* 2000
I. holocyclus	Salivary gland	fXa and thrombin	NA	Anastopoulos *et al.* 1991
H. longicornis	Salivary gland	fXa and thrombin	NA	Anastopoulos *et al.* 1991
A. americanum	Salivary gland	fXa and thrombin	NA	Zhu *et al.* 1997
I. ricinus	Whole tick	Thrombin	7	Hoffmann *et al.* 1991
D. andersoni	Salivary gland	fV and fVII	NA	Gordon and Allen, 1991

1.4.4 Platelet aggregation inhibitors from ticks

Moubatin, an inhibitor of collagen induced platelet aggregation has been identified in *O. moubata*, while tick adhesion inhibitor (TAI), inhibits the adhesion of platelets to matrix collagen (Waxman and Connolly, 1993; Karczewski *et al.* 1995). The thrombin inhibitors involved in the inhibition of the coagulation cascade also serve to inhibit thrombin-induced platelet aggregation (Nienaber, Gaspar and Neitz, 1999). Binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ are also inhibited by the RGD-containing inhibitors variabilin from *Dermacentor variabilis* and savignygrin from *O. savignyi*, as well as the RGD lacking inhibitor, disagregin from *O. moubata* (Karczewski, Endris and Connolly, 1994; Wang *et al.* 1996; Mans, Louw and Neitz, 2002b). Apyrase, an ATP-diphosphohydrolase enzyme inhibits platelet aggregation through hydrolysis of ADP (Law, Ribeiro and Wells, 1992). Apyrase activity has been found in the salivary gland extracts or saliva of *I. scapularis* and *O. moubata* and *O. savignyi* (Ribeiro *et al.* 1985; Ribeiro, Endris and Endris, 1991; Mans *et al.* 1998a; Mans *et al.* 1998b). It was also shown that apyrase could play a role in the disaggregation of aggregated platelets, which could serve an important function in dissolving platelet aggregates, during feeding (Mans *et al.* 2000). Apyrase activity appears

however, to be absent from the saliva of *A. americanum* (Bowman *et al.* 1997). PGI_2, PGD_2 and PGE_2 inhibit platelet aggregation by preventing ADP secretion during platelet activation (Ribeiro, Makoul and Robinson, 1988; Bowman *et al.* 1995, Aljamali *et al.* 2002). The various platelet aggregation inhibitors are summarized in Table 1.4.

Table 1.4: Platelet aggregation inhibitors from ticks. Adapted from Bowman *et al.* (1997).

Species	Compound	Target	Mr (kDa)	Reference
O. moubata	Apyrase	ADP	NA	Ribeiro *et al.* 1991
O. moubata	Moubatin	Collagen	17	Waxman and Connolly, 1993
O. moubata	TAI	Collagen	15	Karczewski *et al.* 1995
O. moubata	Disagregin	$\alpha_{IIb}\beta_3$	6	Karczewski *et al.* 1994
O. savignyi	Apyrase	ADP	67	Mans *et al.* 1998, 2000
O. savignyi	Savignygrin	$\alpha_{IIb}\beta_3$	7	Mans *et al.* 2002b
D. variabilis	Variabilin	$\alpha_{IIb}\beta_3$	5	Wang *et al.* 1996
I. dammini	PGI_2	PGI_2-receptor	NA	Ribeiro *et al.* 1988
A. americanum	PGD_2	PGI_2/D_2-receptor	NA	Bowman *et al.* 1995
A. americanum	PGE_2	PGE_2-receptor	NA	Aljamali *et al.* 2002

It is clear that there were a definite selective pressure on ticks to adapt to a blood-feeding environment and that an impressive array of anti-hemostatic components were evolved by ticks. The study of these salivary gland components could aid the understanding of the mechanisms that ticks evolved during their adaptation to a blood-feeding environment.

1.5.1 Argasidae as models of a primitive ancestor

The Argasidae are generally considered to be more primitive than the Ixodidae, by morphological, developmental and physiological criteria. The prototype ixodid ancestor is thus considered to be argasid-like (Oliver, 1989). Ixodids spend more time on the host, during which extensive changes take place in the salivary glands, while general argasid salivary gland structure is considered to be less complex than that of ixodids (Sauer *et al.* 1995). The study of argasid salivary gland biology is thus a good place to start when investigating tick adaptation to a blood feeding environment and the possible state of a primitive ancestor.

1.5.2 *Ornithodoros savignyi* as a model of argasid biology

Harry Hoogstraal penned the behaviour of the sand tampan in classic prose: "*at the Khartoum qaurantine one may see a long, seething line of thousands of hungry tampans helplessly confined to the shade of a row of acacia trees. A few yards away, separated only by the hot, nine o'clock sun, newly arrived cattle tied to a post fence tempt the tampans to cross the glaring strip. The next morning, in the coolness of seven o'clock, those tampans under the trees are all blood bloated and resting comfortably in the sand, others dragging back from their hosts across the now nonexistent barrier, and the legs of the cattle are beaded with yet other podshaped ticks taking their fill of blood in a regular line just above the hoof.*" (Hoogstraal, 1956). The ferociousness of these ticks can be attested by personal experience. Using the dry ice collection method to lure these tick from their hiding places, we were surrounded by ticks in a twenty meter radius around the dry ice container (Nevill, 1964). Ticks were running crazily this way and that way, seeking animals they could scent, in a futile attempt to be there first (personal observation, Upington, Western Cape Province, 1995). *O. savignyi* is of local economic importance in that it kills many domestic animals, especially young calves and lambs. Originally, the consensus was that these ticks kill their hosts through exsanguination, but it was later shown that death is caused by a toxin which may cause serious allergic reactions in humans (Howell, Neitz and Potgieter, 1975).

These ticks are relatively easily obtainable and they require little care and their salivary glands are of managable size. *O. savignyi* has a uniform integument which folds in on itself and allows the tick to obtain a large quantity of blood in a short feeding time (15-30 minutes). Males are smaller than females and lack the large genital opening that identify females (Fig. 1.4). *O. savignyi* is diploid (2n = 20), with the presence of sex-chromosomes, XY and XX for males and females, respectively (Howell, 1966a).

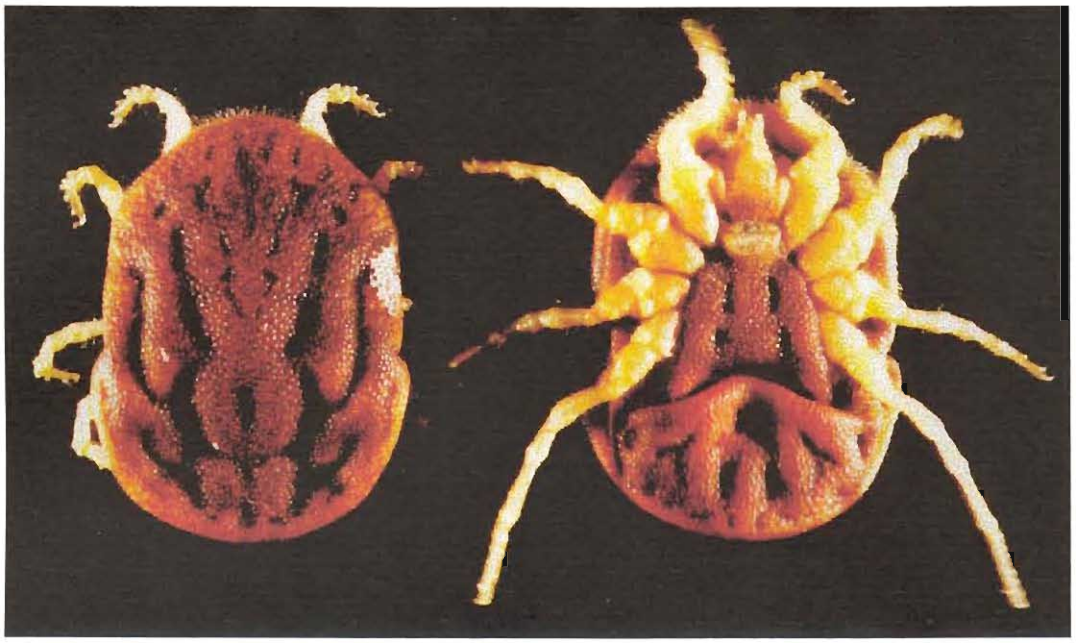


Fig. 1.4: External anatomy of a female *O. savignyi*. Note the folded integument, position of the capitulum and genital opening (Photograph:B.J Mans, 1996).

1.5.3 The relationship between *O. savignyi* and *O. moubata*

Extensive studies on anti-hemostatic components from the tick *O. moubata* have been conducted. A study of a closely related tick, like *O. savignyi*, could thus prove useful to gain comparative data, as there are few notable differences between these species. Morphologically they are similar, although *O. savignyi* has two pairs of eyes situated in its supracoxal folds, which is lacking in *O. moubata*. While *O. savignyi* burrows in sandy areas, *O. moubata* is endophilous (nest-dwelling). During its lifetime *O. moubata* is generally confined to a limited set of hosts that inhabit their burrow. *O. savignyi* is more predatory and will feed on any mammal in its proximity (Hoogstraal, 1956). *O. moubata* transmits *Borrelia duttoni*, which causes relapsing fever in humans and also transmits, african swine fever virus. In contrast, no pathogenic organisms have as yet been found associated with *O. savignyi*, although natural infection with Borrelia spirochetes have been reported (Helmy, 2000). *O. savignyi* do however, secrete toxic substances that can be lethal, especially in young animals. One adult tick or the salivary gland extract equivalent to half of a salivary gland can kill an adult mouse (20g) within 20 minutes (Mans *et al.* 2001, Mans *et al.* 2002). *O. moubata* however, does not secrete any toxins, exemplified by the feeding of 5 adult females, 10 males and 100 nymphs on an adult mouse to generate an immune response (Astigarraga *et al.* 1997).

1.5.4 Distribution of *O. savignyi*

O. savignyi is distributed through the North Western parts of South Africa and are also found in Egypt, Arabia, Ethiopia, Kenya and Zimbabwe (Paton and Evans, 1929). It lives in sandy regions where it resides below sand near trees, corrals and other places likely to attract animals. It has been reported that the incidence of these ticks has risen in regions where soil is more rocky as in kraals, which is probably due to attraction by domestic animals (Howell, Neitz and Potgieter, 1975).

1.5.5 Control of *O. savignyi*

The use of acaricides to control these ticks is limited due to the nature of their distribution over a large area and the fact that they do not reside on the host. Places where the soil has been treated with chemicals are avoided by these ticks. They can survive without feeding for up to five years, which makes starvation a null option. These ticks can have a lifespan of up to 15-20 years if they only feed occasionally (every 5-6 years) (Astigarraga *et al.* 1996). Long term biological control of these ticks would thus be a viable option.

1.5.6 Bio-active components from *O. savignyi*

O. savignyi is a rich source of bio-active components. An acidic toxin has been previously isolated and characterized in terms of its Mr (15400 Da), pI (5.01) (Neitz, Howell and Potgieter, 1969) and N-terminal amino acid sequence (Neitz *et al.* 1983). Re-purification confirmed the toxicity of this acidic toxin as well as a closely homologous non-toxin and the presence of yet another basic toxic activity (Mans *et al.* 2001). These proteins together with another highly abundant protein have been cloned and sequenced and shown to be part of the lipocalin family. It has also been suggested that these proteins could be involved in granule biogenesis (Mans *et al.* 2001). The toxins affect the cardio-vascular system rather than the nervous system, as is found in paralysis ticks (Mans *et al.* 2002). A factor Xa inhibitor has been identified, purified, characterized on kinetic level and has been cloned and expressed (Gaspar, Crause and Neitz, 1995; Gaspar *et al.* 1996; Joubert *et al.* 1998). Savignin, a thrombin inhibitor has been purified, kinetically characterized, cloned, sequenced and a molecular model has been proposed (Nienaber, Gaspar and Neitz, 1999; Mans, Louw and Neitz, 2002a). Apyrase, a ATP-diphosphohydrolase enzyme and platelet

aggregation inhibitor has been identified, purified, kinetically characterized and its disaggregation effect on aggregated platelets have been described (Mans *et al.* 1998a, Mans *et al.* 1998b, Mans *et al.* 2000). The savignygrins, a set of four platelet aggregation inhibitors proposed to target the platelet fibrinogen receptor, have been purified, characterized, cloned, sequenced, expressed and their relationship to the fXa and thrombin inhibitors elucidated (Mans, Louw and Neitz, 2002b; Mans, Louw and Neitz, 2002c).

1.6.1 Molecular evolution as mechanism of adaptation to a blood-feeding environment

Intrinsic to the word 'adaptation' is the notion of change, and applied to novel environments, the idea of acquisition of novel properties or functions. In biochemical terms, the study of the adaptation of organisms lies in the realm of molecular and protein evolution. As such adaptation entails the acquisition of novel protein functions. Several different modes for the acquisition of new protein function exist. An organism can utilize an existing function in a new way, for example, if apyrase had an intracellular function in the endoplasmic reticulum or Golgi-apparatus (Gao, Kaigorodov and Jigami, 1999; Zhong and Guidotti, 1999) and it acquires the signals for extracellular export, it might now also be involved in the regulation of platelet aggregation. Alternatively, a protein can through mutation acquire a novel function while still retaining its old function. This is referred to as gene sharing and such "moonlighting" proteins with multiple functions are discovered frequently (Jeffery, 1999). It has been estimated that there exists approximately a 1000-7000 different protein folds or topologies (Chothia, 1992; Orengo, Jones and Thornton, 1994). It has been predicted that there are at least 1709, 6241, 13601, 18424 and 40580 protein coding sequences in *Haemophilus*, yeast, fly, worm and human genomes (Rubin *et al.* 2000; Li *et al.* 2001). It should thus be clear that protein folds are utilized for more than one function. The problem that arises though, is how do new functions evolve from old folds? Protein function tends to be conserved, so that except for the case of gene sharing, proteins would not lose their original function to gain a new function. This phenomenon is referred to as purifying or negative selection in that any mutation that would be deleterious for protein function would be removed from the population. The answer to this lies in gene duplication, the single most important event in the generation of new protein functions (Ohno, 1970).

1.6.2 Gene duplication and the acquisition of novel protein function

Genes can be duplicated via unequal crossing-over during recombination, polyploidy (duplication of whole genomes), non-homologous chromosomal breakage and reunion or transposition (Maeda and Smithies, 1986). It has been estimated that gene duplication is a very common event during adaptive radiation (Ohno, 1970). In this regard, it has been estimated that there are at least 284, 1858, 8971, 5536 and 4519-15121 duplicated genes in the genomes of *Haemophilus*, yeast, fly, worm and humans, respectively (Rubin *et al.* 2000; Li *et al.* 2001). Gene duplication rates of 0.02-0.2 genes per million years have been estimated for different species, so that at least 50% of all genes in a genome are expected to duplicate and increase in frequency at least once every 35-350 million years. With a genome size of 15000 genes it can be expected that at least 60-600 duplicate genes arise in a pair of sister taxa per million years (Lynch and Conery, 2000). When genes duplicate and retain their original function, a whole set of the same genes form a multigene family that leads to expression of highly abundant proteins (such as histones) or high-level transcription (such as rRNAs). However, gene duplicates are normally functionally redundant at the time of origin and there are several possible outcomes for the evolution of such duplicate genes:

A) The vast majority of duplicated genes are silenced by deleterious mutations and the time it takes to silence a gene is considered to be relatively short (a few million years or less). B) Mutations in regulatory areas of the gene could lead to unique expression patterns in different tissues, where such a protein can acquire new functions due to a novel environment. C) During the acquisition of randomly sustained mutations, a duplicated redundant gene might acquire a novel, beneficial function that becomes fixed due to natural selection (Lynch and Conery, 2000).

The latter mode of functional acquisition has been described as the mutation during non-functionality model (MDN). It has been argued that this model is flawed and not supported by current evidence on gene duplication. It has been predicted that duplicated genes would be subjected to purifying selection, so that the "redundant" gene retains its function. In this case the rate of synonymous substitution always exceed that of non-synonymous substitution (Hughes, 1994). In order for new functions to evolve, positive selection would be needed so that the rate of non-synonymous to synonymous

substitution would be higher. It is expected that diversifying selection would operate shortly after gene duplication but would cease once a new function has evolved so that the genes would be subjected again to purifying selection. New models to account for the evolution of protein functions have been proposed:

A) New functions might first evolve (gene sharing) before the duplication event, as this would allow genes to each retain a function after duplication. A period of non-functionality as described for the MDN model is thus not required. Genes that do duplicate, which code for only one function, and are not subjected to purifying selection would thus be rapidly non-functionalized and become pseudogenes. B) A gene can also duplicate and insert into another gene sequence or be fused at the start or end of an existing gene. Hetero-multimeric proteins can form in this way, so that different functional domains are linked. Conversely, an internal-tandem duplication can lead to a homo-dimeric protein that contains two of the same structural domains. The duplicated domains can then either retain the same function or one domain can acquire a new one, leading to a multi-functional protein. Even here the protein that acquires the new function is postulated to go through a period of gene sharing. Gene duplication from individual domains can now take place to give single domain proteins with novel functions (Hughes, 1994). In these models positive Darwinian selection plays an important role in the evolution of novel protein functions. While this seems to contradict the now common accepted neutral theory of evolution, i.e. that random fixation of selectively neutral mutations is the predominant mode of evolution at molecular level (Kimura, 1983), there is really no contradiction, as the cases where duplicate genes assume separate functions will be in the minority of all gene duplications.

1.6.3 Orthologous genes

When a speciation event takes place, so that the same gene is now found in two distinct species, such "duplicated" genes are called orthologous genes. Orthologous genes represent the same phylogeny as is found for the organisms and can thus be used to construct species trees (Fitch, 2000). Proteins coded for by orthologous genes generally retain the same function and conserved structure. Due to functional constraints the ratio of nonsynonymous to synonymous substitution is usually a constant, low value. Identities in amino acid sequences of orthologous proteins are a good indication of

regions critical for structure or function. Functions for novel proteins that can be shown to be orthologs can thus be predicted with great confidence (Patthy, 1999).

1.6.4 Paralogous genes

Genes that duplicate and diverge in the same organism are called paralogous genes. Mixing of orthologous and paralogous genes can lead to a phylogenetic tree that has the correct sequence phylogeny, but does not indicate species phylogeny (Fitch, 2000). Generally paralogous genes change over time so that the encoded proteins have different functions although the same structural protein fold is retained. Residues that are essential for structure will be conserved, so that those regions that show marked differences are possible indications of new functional properties. Depending on the degree of divergence, paralogous proteins might have novel functions, while still retaining the original function. Predicting functions for novel paralogous proteins can be difficult and caution should be exercised when using homology to infer function. Because paralogous proteins generally diverge it can be difficult to detect distant homology with current homology-based methods and knowledge-based techniques might be needed to detect biological significant similarities (Patthy, 1999). Closely linked paralogous genes with significant similarity in sequence can undergo recombination, so that these genes evolve in a concerted fashion. This normally occurs close to the origin of duplication and ensures that divergence is slowed down, which reduces the chances for acquisition of new functions, but also protects redundant copies from rapid non-functionalization (Patthy, 1999). Paralogous genes in one species may thus share greater similarity than orthologous genes from different species, thereby complicating studies into the evolutionary relationships between proteins (Maeda and Smithies, 1986).

1.6.5 Alleles

Organisms that have more than one set of homologous chromosomes (diploid or polyploid) carry the same gene at the same chromosomal locus. If these genes differ due to mutation events, they are referred to as alleles. Due to sexual recombination, a population can consist of individuals that are either homozygous (same allele for a gene) or heterozygous (different alleles for a gene). The frequency of alleles in a

population is an indication of the polymorphism of such a population. When one allele is exclusively expressed in a heterozygous individual, such an allele is dominant, while the other non-expressed allele is recessive (Page and Holmes, 2000). If both alleles are expressed they are co-dominant. Depending on the frequency of different alleles in a population, protein isoforms that represent different alleles present in the population can be expected. Such isoforms can be encountered during a protein purification procedure so that care should be exercised when gene duplication events are considered.

1.6.6 Homology and analogy

Paralogous and orthologous genes are homologous. Homology indicates that two genes/proteins have descended from a common ancestor. In contrast, analogy indicates two genes that have descended convergently from unrelated ancestors. Two orthologous genes can independently attain a new function through divergence and as such be genetically homologous, but functionally analogous (Fitch, 2000). Homology is a term and the correct terminology to use when comparing protein sequences is to refer to percentage identity or similarity and not to percentage homology.

1.6.7 Detecting homology

Detecting homology is crucial when establishing the origins of novel protein function and when elucidating evolutionary adaptive mechanisms. Several criteria have been used to test whether two proteins are derived from a common ancestor: 1) similar DNA or protein sequences, 2) similar 3-D structures, 3) similar functions or protein-protein interactions. The criteria are in order of decreasing strength. If criterion 1 holds, the rest usually follows because similar sequences adopt similar structures and functions (Matthews *et al.* 1981). While this is the general case, care should be taken when inferring homology, as non-homologous proteins might either display similar functions or structures and cannot be considered to be strong evidence for homology on their own (Murzin, 1998). It should be clear that this problem is further compounded by the existence of paralogous proteins that have similar structures, but dissimilar functions.

1.6.8 Detecting homology by immunological means

The use of cross-reactivity between homologous proteins is an established method used to indicate sequence similarity and homology (Wilson, Carlson and White, 1977). It has been shown that the level of antigenic cross-reactivity correlate with the degree of similarity between two sequences (Van Regenmortel, Joisson and Wetter, 1993; Prager and Wilson, 1993). Immunological methods used to detect homology include microcomplement fixation, ELISA, immunoblotting and precipitin techniques. Microcomplement fixation can generally detect sequence identity above 70%, while ELISA and immunoblotting can detect sequence similarity between 30-40%, which is approximately the cut-off value for most orthologous proteins (Van Regenmortel *et al.* 1993). Care should however, be taken due to the fact that similar antigenic epitopes might occur in unrelated proteins. In this regard, immunoblotting after gel electrophoresis has the added advantage of identifying antigens also by their molecular masses and due to SDS and heat induced unfolding only sequence specific epitopes are detected and not conformational epitopes, that might be mimicked in various different sequences.

1.6.9 Detecting homology by database search

Pairwise alignments are normally used to determine the extent of identity or similarity between two proteins. To find similar proteins in the existing databases (SWISS-PROT or GenBank), a search tool such as BLAST (basic local alignment search tool) can be used, which employs a heuristic algorithm that can detect relationships between sequences which share only isolated regions of similarity (Altschul, 1990). Sequence databases are updated almost daily and share the sequence data submitted with other databases, so that they are in essence redundant and only differ in the way of annotation. Due to the size of sequence space, a sequence with significant similarity can be safely assumed to be a result of homology (Gogarten and Olendzenski, 1999). Problems arise in the "twilight zone" when sequence identity or similarity (20-35% sequence identity) is so low that alignment methods fail to indicate homology. Methods generally indicate homology above 30% sequence identity, while below 25% identity less than 10% are assigned homologous. However, the "more similar than identical" rule can be used to identify homologous proteins that fall into this area of false-positives and false-

negatives. Proteins that are homologous, normally have a higher similarity than identity, while the similarity and identity of non-homologous proteins does not significantly differ (Rost, 1999).

1.6.10 Detecting homology with multiple sequence alignment and phylogenetic analysis

Multiple alignments can be used to detect more distant relationships between proteins and possible motifs involved in structure or function. Used in conjunction with phylogenetic analysis this is a powerful method to investigate evolutionary relationships between homologous proteins. Closely linked to multiple alignment is the use of phylogenetic analysis to investigate possible evolutionary relationships between proteins. Thus, even if proteins are highly divergent, phylogenetic analysis can be used to infer homology.

1.6.11 Structure as means of detecting homology

Because structure evolves more slowly than sequence, protein structure is generally conserved even for highly divergent proteins. Assigning structures or folds to novel proteins by methods such as fold recognition by threading or molecular modeling can be valuable aids in detecting distant homology. The chances of finding a correct structure or fold is quite good, since the number of different protein-folds is limited and will improve as more protein structures are solved in the near future (Chothia and Lesk, 1986). However, the physico-chemical constraints of protein folding probably limited the number of protein folds in nature, so that different evolutionary families with unrelated functions can share a common structure. Consequently structural similarity do not necessarily imply homology (Todd, Orengo and Thornton, 1999). The same argument is valid for similar functions, but unrelated structure, so that structure as well as function should be considered when used to infer homology (Murzin, 1998). It has been shown that most similar structure pairs have less than 12% pairwise sequence identity, while the average sequence identity centered around 8%, which is comparable to sequence identities of unrelated proteins. In this "midnight zone", proteins could be similar due to convergent or divergent evolution. The levels of significant sequence identity and similarity that can be used to infer structural similarity depend on the alignment length of two sequences (Rost, 1997). Normally the longer the protein, the

lower the percentage identity that implies identical structure. The higher the percentage identity, the smaller would be the root mean square deviation (rmsd) of the coordinates of two structures. As a rule of thumb, RMSD values can be predicted for different levels of sequence identities: 80% (0.5-1.0Å), 40% (1.0-1.5 Å) and 20% (1.5-2.0 Å). Presently molecular modeling of novel sequences using known three-dimensional structures to obtain a structure model, can only be attained at higher than 25% sequence identity and structural models derived from lower identities should be treated with due caution if no other methods to detect homology are successful.

1.7 Aim of thesis

This thesis aims to look at adaptation of ticks to a blood feeding environment, in the light of gene duplication and gain/loss of protein function. Two protein families have been investigated. One concerns a family of platelet and blood coagulation inhibitors that possess the bovine pancreatic trypsin inhibitor fold (Part 1). The other family of proteins form part of the lipocalin family, which have diverse functions in the host, although evidence is presented of a universal function in tick salivary glands (Part 2).

PART 1

Evolution of soft tick anti-hemostatic factors of the BPTI-like superfamily

The subsequent three chapters discuss the characterization of the platelet aggregation inhibitor savignygrin and its relation to the BPTI-like anti-coagulants from the same tick species.

Chapter 2 focuses on the identification, cloning and sequence determination of the savignygrins, which exist as four conformational isoforms of which two are encoded by different genes. These proteins are shown to be homologous to disagregin, a platelet aggregation inhibitor previously described for *O. moubata*. The savignygrins possess a RGD motif that is absent in *O. moubata*.

Chapter 3 considers the cloning and molecular modeling of savignin, a thrombin inhibitor previously described for the tick *O. savignyi*. This inhibitor is shown to be homologous to ornithodorin, the thrombin inhibitor of *O. moubata*, for which a three-dimensional structure was described. Similar mechanisms of inhibition are predicted for these inhibitors. In addition, association between the separate domains of savignin is proposed, suggesting a dissociation step during binding to thrombin.

Chapter 4 investigates the evolutionary relationship between the platelet aggregation, thrombin and fXa inhibitors from *O. moubata* and *O. savignyi*. A model for the evolution of the respective inhibitor functions from a common ancestor is proposed and it is shown that savignygrin possesses a BPTI-like fold. The implications of this protein fold for the mechanism of action of savignygrin is considered and it is suggested that the RGD motif as well as downstream acidic residues are involved in integrin recognition.