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Faculty of Science


# Blood meal digestion in the hard tick *Ixodes ricinus*

Ph.D. Thesis

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## Annotation

Although several reports have described individual proteolytic enzymes from different tick species, the complex study focused on the digestive apparatus within one specific species at a defined feeding phase was still missing. Such a comprehensive approach is presented within this thesis. We have characterized the suite of digestive peptidases in the gut of semi-engorged *Ixodes ricinus* females and revealed their role in intracellular proteolysis. Moreover, we have mapped the morphological changes of female midgut during feeding and disclosed the specific roles of individual enzymes during this process.

**Declaration [in Czech]**

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Zdeněk Franta

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## List of papers and author's contribution

The thesis is based on seven papers concerning blood meal digestion in the hard tick *Ixodes ricinus*, which will be referred to in the text by their Roman numerals and four supplemental papers marked by capital letters:

**I.** Sojka D, Hajdusek O, Dvorak J, Sajid M, **Franta Z**, Schneider EL, Craik CS, Vancova M, Buresova V, Bogyo M, Sexton KB, McKerrow JH, Caffrey CR and Kopacek P, 2007. IrAE - An asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. Int J Parasitol 37, 713-724.
*Zdeněk Franta was responsible for designing the gene specific PCR primers, isolation of total RNA from tick tissues, synthesis of cDNA and performing the semi-quantitative RT PCR experiments.*

**II.** Sojka D, **Franta Z**, Horn M, Hajdusek O, Caffrey CR, Mares M, Kopacek P, 2008. Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases. Parasit Vectors 1, 7.
*Zdeněk Franta participated in sequencing of full clone genes of individual digestive enzymes, isolation of total RNA and cDNA synthesis, designing of gene specific primers, running semi-quantitative RT PCR experiments and revision of manuscript.*

**III.** Horn M, Nussbaumerova M, Sanda M, Kovarova Z, Srba J, **Franta Z**, Sojka D, Bogyo M, Caffrey CR, Kopacek P, Mares M, 2009. Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics. Chem Biol 16, 1053-1063.
*This work was mainly done in our collaborating laboratory at the Institute of Organic Chemistry and Biochemistry, Prague. Zdenek Franta participated in the study design, tick gut tissues preparation and manuscript revision.*

**IV.** **Franta Z**, Frantova H, Konvickova J, Horn M, Sojka D, Mares M, Kopacek P, 2010. Dynamics of digestive proteolytic system during blood feeding of the hard tick *Ixodes ricinus.* Parasit Vectors 3, 119.
*Zdeněk Franta participated in the designing of the study, expression, purification and refolding of recombinant IrCB and antibody preparation. ZF was responsible for performing and analyzes of mRNA expression profiles by qRT-PCR and manuscript preparation.*

**V.** **Franta Z,** Sojka D, Frantova H, Dvorak J, Horn M, Srba J, Talacko P, Mares M, Schneider E, Craik CS, McKerrow JH, Caffrey CR and Kopacek P, 2011. IrCL1 - the hemoglobinolytic cathepsin L of the hard tick, *Ixodes ricinus*. Int J Parasitol 41, 1253-1262
*Zdeněk Franta was responsible for management of experimental study, expression of recombinant IrCL1 in both expression systems, preparation of antibodies, biochemical characterization of active recombinant enzyme, qRT-PCR profiling, RNAi silencing and manuscript preparation.*

**VI.** Hajdusek O, Sojka D, Kopacek P, Buresova V, **Franta Z**, Sauman I, Winzerling J, Grubhoffer L, 2009. Knockdown of proteins involved in iron metabolism limits tick reproduction and development. Proc Natl Acad Sci U S A 106, 1033-1038.
*Zdeněk Franta was responsible for isolation of total RNA from tick tissues, synthesis of cDNA and performing the semi-quantitative RT PCR experiments.*

**VII.** Sojka D, **Franta Z**, Frantova H, Bartosova P, Horn M, Vachova J, O'Donoghue AJ, Eroy-Revelles AA, Craik CS, Knudsen G, Caffrey CR, McKerrow JH, Michael Mares M and Kopacek P. *Ir*CD1 – gut aspartic hemoglobinase of the tick *Ixodes ricinus* (in manuscript)

*Zdeněk Franta participated in the designing of the study, expression and refolding of recombinant enzymes and antibody preparation. ZF was responsible for performing and analyzes of mRNA expression profiles by qRT-PCR as well as semi quantitative RT PCR and manuscript preparation.*

**Supplemental papers:**

**A** Buresova V, **Franta Z,** Kopacek P, 2006. A comparison of *Chryseobacterium indologenes* pathogenicity to the soft tick *Ornithodoros moubata* and hard tick *Ixodes ricinus*. J Invertebr Pathol 93, 96-104.

*Zdeněk Franta was responsible for the maintaining of* Ornithodoros moubata *colony mortality studies, isolation of bacteria from tick tissues, antibiotic susceptibility tests, 16S rRNA sequencing and revision of manuscript.*

**B** Grunclova L, Horn M, Vancova M, Sojka D, **Franta Z**, Mares M and Kopacek P, 2006. Two secreted cystatins of the soft tick *Ornithodoros moubata*: Differential expression pattern and inhibitory specificity. Biol Chem 387, 1635-1644.

*Zdeněk Franta was responsible for designing the gene specific PCR primers, isolation of total RNA from tick tissues, synthesis of cDNA and performing the semi-quantitative RT PCR experiments*

**C** Buresova V, Hajdusek O, **Franta Z,** Sojka D and Kopacek P, 2009. IrAM-An alpha2-macroglobulin from the hard tick *Ixodes ricinus*: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. Dev Comp Immunol 33, 489-498.

*Zdeněk Franta was involved in full clone sequencing of IrAM molecule, expression of recombinant protein and antibody preparation, isolation of total RNA, cDNA synthesis and semi-quantitative RT PCR profiling.*

**D** Buresova V, Hajdusek O, **Franta Z**, Loosova G, Grunclova L, Levashina EA and Kopacek P, 2011. Functional genomics of tick thioester-containing proteins reveal ancient origin of the complement system. J Innate Immun 3, 623-630.

*Zdeněk Franta participated in isolation of total RNA from tick tissues, synthesis of cDNA, performing the semi-quantitative RT PCR and RNAi experiments*

## Contents

# INTRODUCTION

Acari are the largest and the most biologically diverse group of the arachnids. The subclass Acari is divided into two superorders, Acariformes and Parasitiformes, and includes both free living and parasitic forms. The members of Acari are distributed worldwide and inhabit aquatic as well as terrestrial niches.

Ticks, order Ixodida, superorder Parasitiformes, are obligatory hematophagous ectoparasites of terrestrial vertebrates. There are more than 896 identified tick species that are divided into three families. The family Ixodidae (hard ticks) consists of 702 species in 14 genera and the family Argasidae (soft ticks) comprises 193 species, but there is still disagreement regarding their genera classifications (Guglielmone et al., 2010). Both families differ significantly in many biological aspects as well as life strategies (**Tab 1**). The third and the smallest family of the order Ixodida is the family Nuttalliellidae, represented by a single species, *Nuttalliella namaqua*, which is most likely the closest living relative to the ancestral tick lineage and the evolutionary missing link between Argasidae and Ixodidae (Mans et al., 2011a).

The importance of ticks is not just due to their ability to negatively affect livestock productivity by imbibing large volumes of host blood, but they are also very important vectors of a wide variety of pathogens (comprising viruses, bacteria, fungi and protozoa) that are transmitted to human and/or domestic animals (de la Fuente et al., 2008).

## Argasidae (soft ticks)

Argasid ticks are large nidicolous ectoparasites inhabiting mostly arid and semi-arid areas of the world. The oval shaped body is characterized by a "leathery" cuticle without the sclerotized dorsal scutum. The mouth part (gnathosoma) of nymphs and adults is located in the ventral or subterminal part of the body (idiosoma) and is concealed from above by an idiosomas anterior region. The adult specimens show minimal sexual dimorphism and both sexes are capable of sucking blood. The life cycle can take several years, as ticks can survive long periods of starvation, and it is characterized by an inconsistent number of nymphal molts and several ovipositions by adult females. The number of nymphal stages, as well as ovipositions, depends on the amount of

blood meal taken up (Sonenshine, 1991). The maximum feeding period of all life stages takes about an hour and the adults are not dependent on mating, which can occur before as well as after the blood meal uptake.

**Table 1: Survey of major differences between Ixodidae and Argasidae.** Adapted according to Sonnenshine, 1991.

| | Ixodidae (Hard ticks) | Argasidae (Soft ticks) |
|---|---|---|
| Gnathosoma | Terminally located, visible from dorsal aspect | Subterminally located, not visible from dorsal aspect (except larvae) |
| Scutum | Present | Absent |
| Integument | Smooth or superficially folded, grows vastly during feeding | Leathery cuticle allows rapid but limited expansion |
| Life cycle | Three distinct life stages | Non-uniform number of nymphal stages |
| Feeding | One feeding per each life stage, secrete attachment cement, remove water from blood meal via the salivary glands | Multiple feedings during the nymphal and adult stages, no attachment cement, remove water from blood meal via coxal glands |
| Mating | Occurs mostly on the host, essential for feeding accomplishment | Occurs off the host, not important for feeding accomplishment |
| Oviposition | Lay 1 huge batch of eggs (up to 23 000) and then dies | Lay small batch of eggs (200-300) after each feeding |
| Host seeking | Ambush passing hosts. | Mostly nidicolous, attacks hosts in nest, caves, etc. |

## Ixodidae (hard ticks)

Ixodid ticks are found throughout the world and are obligatory blood sucking ectoparasites of all vertebrates. Their dorsoventrally flattened body is characterized by a terminally located gnathosoma and sclerotized dorsal

scutum. The ticks undergo a uniform life cycle consisting of eggs and 3 active stages: larvae, nymphs and adults. The blood meal is uptaken once per each life stage and is followed either by a molting step in larvae and nymphs or by oviposition and death in adult females. In contrast to soft ticks, hard ticks feed for several days and during this period they stay attached to the host and imbibe an enormous amount of host blood. Moreover, mating is a very important factor in the biology of hard ticks because only a fertilized female is able to finish feeding and lay a huge grist of eggs. With the exception of a few species, where the fed juveniles remain and develop on the host (1 host and 2 hosts ticks), most hard ticks follow the 3 host life cycle (each life stage feeds on a different host) and drop off of the host after each feeding.

***Ixodes ricinus***

The hard tick *Ixodes ricinus* belongs to the largest tick genus, *Ixodes*, which counts for 220 species that mainly feed on mammals and birds. *I. ricinus* is common across Europe and the surrounding areas of the Middle East and North Africa. It can easily be found from early spring until late fall in relatively humid areas of woodlands, forests or heaths. Its occurrence is bimodal, where the highest tick abundance can be monitored in the spring and again in the fall after a decline during the summer months.

The body of *I. ricinus* has a terminally located gnathosoma with a long cone shaped hypostom bearing multiple reversed hooks, a pair of chelicerae and a pair of palps. Sclerotized scutum is localized on the dorsal part of idiosoma and covers the whole body of males and about one third of the female body. The dorsal part of the female body that is posterior to the scutum is called the alloscutum and is characterized by innumerable fine striations, which represent superficial folds of the cuticular surface. The alloscutum can be enormously increased by the synthesis of a fresh cuticle during feeding (Sonenshine, 1991).

The life cycle of *I. ricinus* is completed in 2-6 years in nature and is a paradigmatic example of the 3 host cycle (**Fig 1**). Six legged larvae hatch from eggs, feed, drop off the host and molt into the eight legged nymphs. Nymphs seek for a new host, feed, drop off and turn into an adult male or female. Adult females seek for their final host on which to feed, while adult males do not need to feed, but instead search for a female for sexual reproduction. Mating is a very important factor of *I. ricinus* biology because only mated females can

accomplish feeding. Engorged fertilized female ticks fall off the host and lay down several thousands spherical eggs.

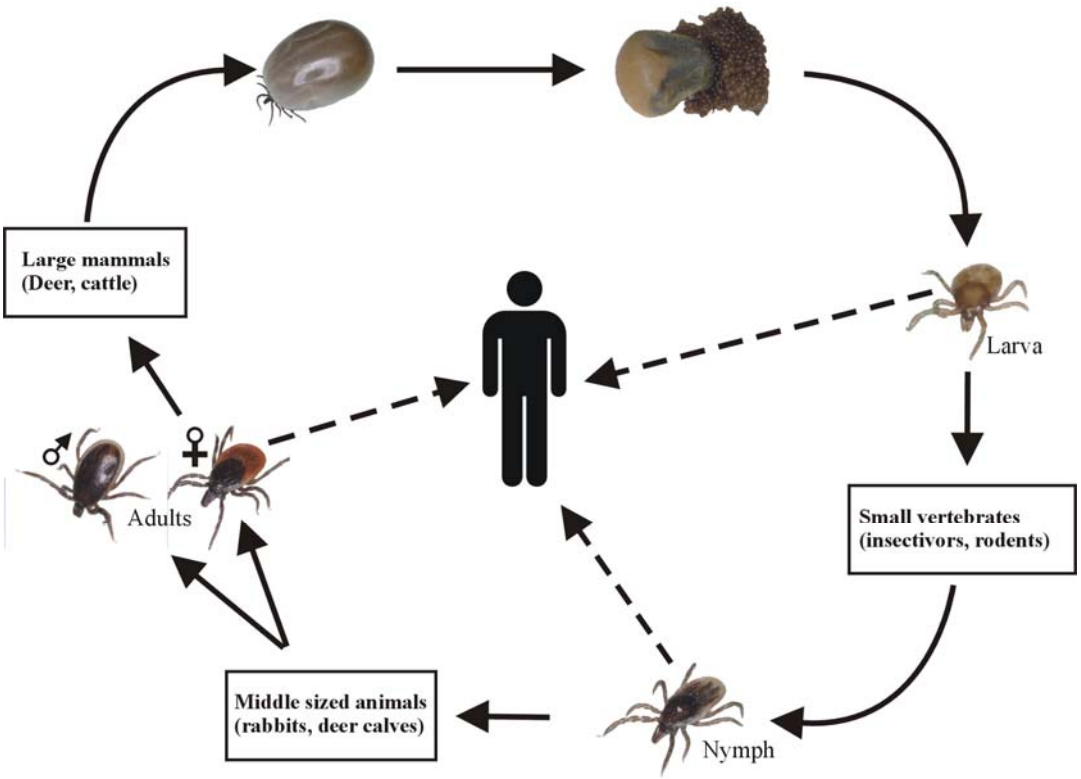



**Figure 1: Life cycle of *Ixodes ricinus*.** Typical hosts are listed in the boxes; humans are potential hosts for all developmental stages.

Host seeking, "questing", is a very important factor for life cycle completion. This behavior is typical of "hungry" ticks that have a high tendency to climb any favorable location in their environment where it may come into contact with the host. Once the tick reaches its spot, it stays in a typical position (**Fig 2**) and exposes its sensoric system – the Haller´s organ. It is located on each tarsus of the first pair of legs and it is highly sensitive to host odors, vibrations, temperature changes and other external cues (Sonenshine, 1991). Host preference varies among the particular *I. ricinus* life stages. Larvae prefer small vertebrates like insectivores or rodents. The nymphs feed mainly on middle sized animals, e.g. rabbits or fawns. Adult females seek for a final large mammalian host, typically deer or cattle.

A wide distribution area and different vertebrate hosts make *I. ricinus* the most important arthropod disease vector throughout Europe. It serves as a principal vector for the tick-borne encephalitis virus and the spirochetes of the *Borrelia burgdorferi sensu lato* complex, the causative agents of Lyme disease (Nuttall, 1999). Furthermore, *I. ricinus* transmits many tick-borne diseases such as babesiosis (caused by the protozoan *Babesia* spp.) or tularemia (caused by bacterium *Francisella tularensis*).

## BLOOD MEAL DIGESTION IN HARD TICKS

Hematophagy (blood-feeding habit) evolved independently more than 20 times within arthropods (Mans, 2011b). Furthermore, the processes of blood feeding and blood digestion evolved independently in both tick families (Mans and Neitz, 2004). Ticks differ greatly from hematophagous insects, where the blood meal digestion occurs rapidly in the gut lumen, predominantly by trypsin-like serine peptidases active within an alkaline pH range (Gooding, 1972). In ticks, the blood meal digestion is a slow process, taking place solely in the acidic endo/lysosomal compartments of midgut cells (Sonenshine, 1991). Tick proteolytic armament consists of cysteine and aspartic peptidases and resembles the digestive system of parasitic platyhelminthes or nematodes (**Paper II**). Blood meal digestion also results in the production of host hemoglobin fragments with antimicrobial activity (Fogaca et al., 1999; Sonenshine et al., 2005).

**Figure 2: Adult *I. ricinus* female searching for the host**

## Tick midgut

The digestive organ of hard ticks can be subdivided into 3 major compartments: the foregut, midgut and hindgut. These regions differ functionally as well as morphologically, with the midgut (**Fig 3**) acting as the executive organ region. The midgut consists of a short central part called the ventriculus (stomach) and several pairs of blind, tubular and highly branched diverticula (caeca) that penetrate into all body regions, creating the major organ of the tick female body. Although the stomach and caeca show different morphology, they are anatomically consistent and have an identical digestive function. The midgut wall consists of a single layer of epithelial cells that rest on the basal lamina, surrounding the inner space – the gut lumen. Outside the lamina, a network of muscles ensures the peristaltic movement of the ingested blood meal (Balashov et al., 1983). The inner epithelium is covered by a peritrophic matrix (Rudzinska et al., 1982; Zhu et al., 1991), creating a layer between the midgut contents and the epithelial wall. In contrast to blood feeding insects, the tick gut lumen is believed to be free of digestive enzymes and serves mainly as a storage organ (Coons et al., 1986).

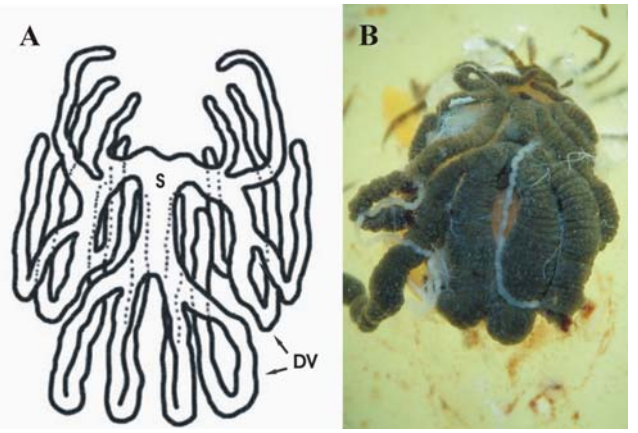



**Figure 3: Midgut of the hard tick *Ixodes ricinus*.**
**A** – Diagrammatic drawing of tick midgut; S – stomach; DV - diverticula.
**B** – An insight into the body cavity of semi-engorged *I. ricinus* female

## Hard ticks feeding strategy

*I. ricinus* is a typical long feeding ectoparasite, which stays attached to the host for several days and undergoes two different feeding periods (**Fig 4**). During the first hours after attachment (24-36 hours), ticks insert their mouthparts into the host skin and prepare for feeding, mainly by adjusting the feeding lesion and producing a "cement" to fix itself in the host skin (Sonenshine, 1991).

During the slow feeding period, ticks start to gradually uptake the host blood and the continuous intracellular digestion commences. Most of the energy utilized for the cuticle synthesis that is needed for the following rapid expansion (Sonenshine, 1991) is most likely gained from the host hemoglobin (Arthur, 1970).

The last phase of feeding is referred to as the rapid engorgement, since an adult fertilized female uptakes the major portion of the blood meal (about two thirds of the total blood volume) within 1-2 days and its overall size expands enormously (Sonenshine, 1991). Only previously mated females are capable of commencing this rapid engorgement. The fertilization apparently controls an unknown hormonal mechanism, which triggers the rapid blood sucking (Sonenshine, 1991). The completion of the rapid engorgement period is the usual signal for tick detachment.

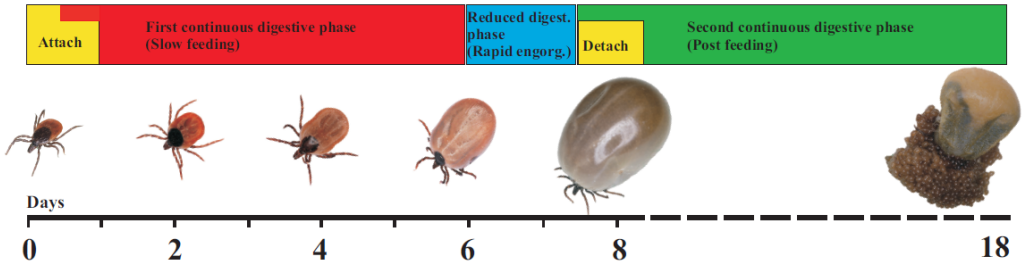



**Figure 4: The Overview of tick feeding periods.** An adult *Ixodes ricinus* female feeds for about 7 to 8 days. The slow feeding period (first continuous digestive phase) starts about one day post attachment and a female ingests about one third of the total blood meal. The major portion of the host blood (about two thirds) is ingested by the mated female during the rapid engorgement period correlating with a reduced digestive phase and taking place during the last 24-48 hours before the engorged tick drops off the host. Detachment triggers the post feeding period (second continuous phase of digestion), which last about 10 days and ends by oviposition and the female's death.

## Hemolysis

The first step of blood processing in ticks is the lysis of red blood cells (hemolysis), which occurs in the gut lumen. Since there are no remnants of the red blood cells, it seems that a complete digestion of the erythrocytes occurs rather than a simple lysis that leaves red cell ghosts (Hughes, 1954). The first described hemolytic activity occurred at an alkaline pH optimum and increased during blood feeding in the midgut homogenate of *Ixodes dammini* (Ribeiro,

1988). Later, Myoshi and his colleagues identified a cubilin-related serine peptidase (H1SP) as a specific hemolytic enzyme in *Haemaphysalis longicornis.* This enzyme was found in the gut lumen and its up-regulation after blood feeding was observed. Recombinant H1SP was shown to lyse host erythrocytes *in vitro* at pH 6. Moreover, the hemolytic role of endogenous H1SP was verified by gene specific RNA interference (Miyoshi et al., 2007).

Despite the generally accepted opinion that there are no extracellular protease activities in the tick gut, the above mentioned studies indicate that serine peptidases may play a specific role in the gut lumen. However, the molecular basis of hemolysis still remains unclear.

**The uptake of blood meal constituents**

The blood meal is taken up from the gut lumen by digestive cells either via fluid phase endocytosis (FPE) or by clathrin coated pits in a process termed receptor mediated endocytosis (RME). Intracellular digestion of the blood meal begins with the fusion of primary lysosomes and endosomes to form secondary lysosomes (digestive vesicles, vacuoles). This process of endocytosis and intracellular lysis is termed heterophagy (Sonenshine, 1991).

Lara et al. (2005) suggested that the different endocytic pathways (FPE and RME) in the digestive gut cells might be connected to the different intracellular handling of the two major proteins of the host blood - hemoglobin and serum albumin. Albumin seems to be taken up non-specifically by fluid phase endocytosis and is directed into small acidic vesicles. Hemoglobin, on the other hand, seems to be recognized by specific cell-surface receptors, which target it towards a population of large vesicles (Lara et al., 2005). Moreover, a long time before Lara's observation, Coons et al. (1986) had suggested that clathrin pits bind macromolecules, especially hemoglobin, to mediate endocytosis. The existence of a distinct apparatus for hemoglobin uptake and its processing has evolved in ticks as a detoxification mechanism that avoids the problem of a vast amount of potentially dangerous heme being released from the digested hemoglobin (Graca-Souza et al., 2006; Lara et al., 2005; Lara et al., 2003).

We propose that tick heterophagy is a rather complex process that possibly shares several important features with known mammalian endocytic mechanisms (for review, see Doherty and McMahon, 2009).

**Digestive phases**

Hard ticks undergo three digestive phases, which correlate with a feeding or post feeding period (**Fig 4**) and are initiated by specific events in the tick life cycle. Data presented in the following paragraphs are based on the observations from different hard tick species (reviewed by Coons and Alberti, 1999) and our results, which mapped the midgut morphology of *I. ricinus* females during the first continuous digestive phase and the reduced digestive phase (**Fig 5**), (**Paper IV**).

The midgut of an unfed female is very narrow and its epithelium consists mostly of undifferentiated reserve cells (RC) and a few remnant digestive cells from the nymphal stage. The reserve cells are also known as stem cells (Agbede and Kemp, 1985) or replacement cells (Tarnowski and Coons, 1989). Tick attachment triggers the first continuous digestive phase, lasting from about 24 hours after attachment until the onset of rapid engorgement (Tarnowski and Coons, 1989). The midgut epithelium responds to the blood uptake by undergoing morphological changes. The gut lumen slowly expands and the reserve cells start to grow and differentiate into the initial digestive cells (DCI), also known as prodigest cells (Agyei and Runham, 1995). DCI cells are characterized by a high concentration of rough endoplasmatic reticulum (RER) and numerous Golgi bodies, which are features typical of a cell either synthesizing proteins for secretion or active in endolysosomal digestion (Agyei and Runham, 1995). The fourth day post attachment, digestive cells (DC) grow in size and the first residual bodies filled with condensed waste heme products are formed in membrane delimited organelles called hemosomes (Lara et al., 2003). Then the first detached digestive cells (DDC) start to appear in the gut lumen. On the sixth day of post attachment, fully expanded digestive cells containing many mitochondria, endosomes and lipid vacuoles, as well as residual bodies, are present. More detached digestive cells are liberated into the gut lumen and driven by peristaltic waves into the rectal sac and defecated (Sonenshine, 1991). Exfoliated DDC cells are replaced by reserve cells, which differentiate into digestive cells and a new cycle of hemoglobin uptake and intracellular digestion begins (Coons et al., 1986).

The next digestive phase occurs only in mated females. It lasts for 12-48 hours and correlates with the rapid engorgement period. The tick midgut epithelium is overlaid with flattened digestive cells. Digestive cells do not pass

into the gut lumen and either reserve or initial digestive cells are no longer detected (Coons and Alberti, 1999). This phase is described in the literature as "the reduced digestive phase" (Coons and Alberti, 1999; Coons et al., 1986; Sonenshine, 1991), which is associated with a decrease in hemoglobinolysis inside the tick gut. Our data and a different methodical approach of their evaluation contradict this theory and instead showed that *I. ricinus* hemoglobinolysis is highly active towards the end of first continuous digestive phase and peaking in fully engorged ticks (**Paper IV**).

The final digestive phase is the second continuous phase. It is triggered by the detachment of the female tick from the host and concludes with oviposition and death. This phase is not as well described compared to the phases that occur during tick feeding and it is still not clear whether the digestion remains entirely intracellular or not. This final phase extends from the pre-oviposition through the oviposition period, during which the gained nutrients and energy are used for the production of vitellogenin and egg maturation (Coons et al., 1986).

**The knowledge on tick digestive enzymes by 2006 (prior to my PhD studies)**

For a relatively long time prior to my PhD studies, it had been known that the intracellular blood meal digestion in ticks occurs at an acidic pH and involves lysosomes in a process termed heterophagy. The first reports describing the tick digestive enzymes were published in the '70s and dealt with the *in vitro* activity of peptidases (Akov et al., 1976; Bogin and Hadani, 1973; Reich and Zorzopulos, 1978). Based on these works, Akov classified the blood digestive enzyme as cathepsin D (Akov, 1982). The first aspartic peptidases were then isolated and characterized from the gut of the partially engorged *Rhipicephalus appendiculatus* females. These enzymes, designated as 1 and 2, hydrolyzed hemoglobin at an acidic pH and were completely inhibited by pepstatin (Vundla et al., 1992). In the late '90s, the first assays of the tick midgut extracts with specific substrates and inhibitors indicated that aspartic (cathepsin D) and cysteine (cathepsin L) peptidases are the major hemoglobinolytic enzymes of *Rhipicephalus (Boophilus) microplus* females (Mendiola et al., 1996). In 1999, two cysteine peptidases identified from the midgut of *Haemaphysalis longicornis* by inhibition studies against the gelatin

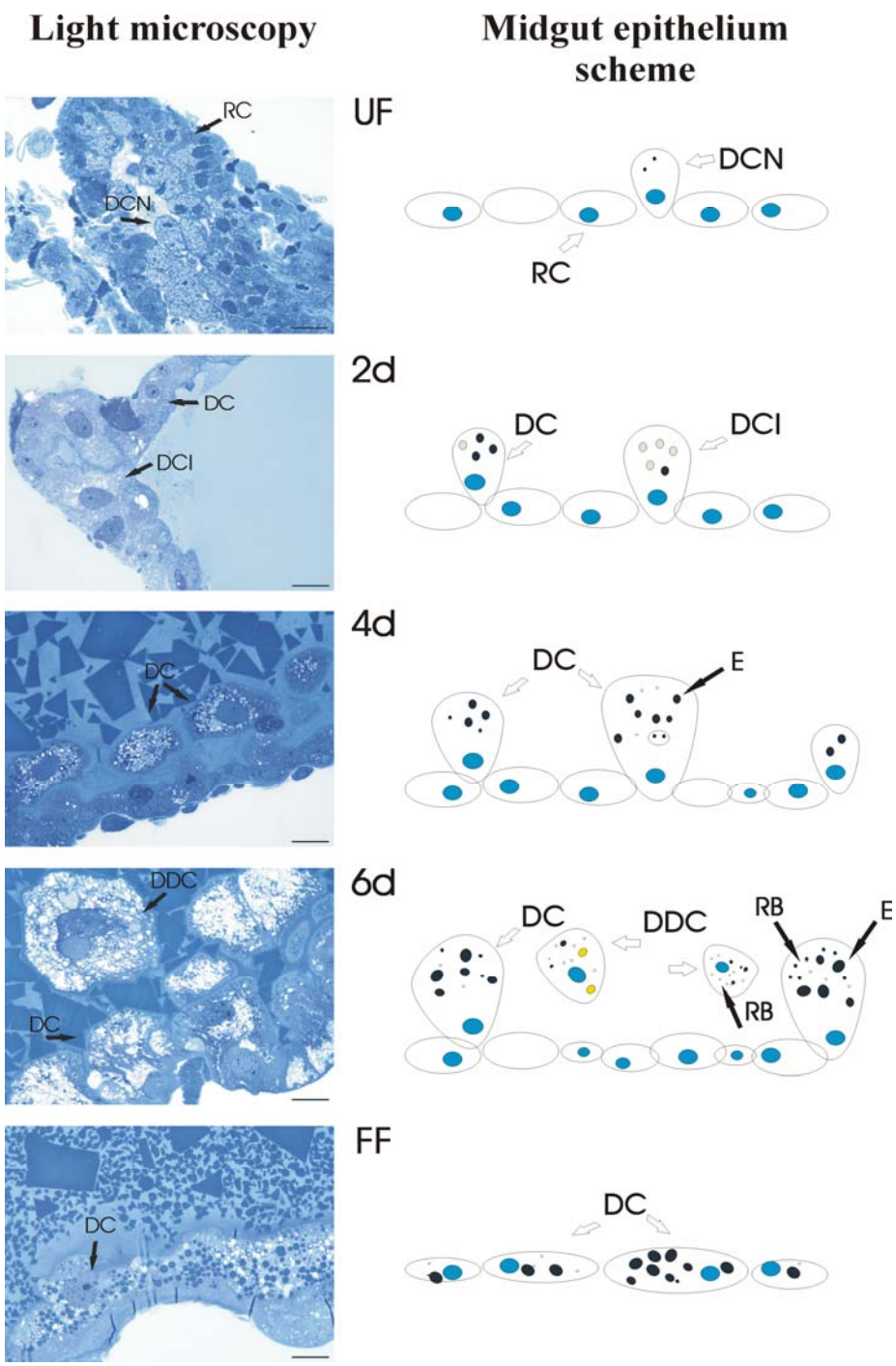



**Figure 5: Morphological changes of *I. ricinus* midgut tissue during the first continuous phase of digestion and the reduced phase of digestion as observed by light microscopy.**
Light microscope panel: The semi-thin sections were stained with toluidine blue; scale bar = 20µm. **UF** – unfed ticks; **2d**, **4d, 6d** – 2, 4, 6 days of feeding, respectively; **FF** – fully fed (engorged) ticks. Midgut epithelium scheme panel: **RC** – reserve cells (stem cells); **DCN** – digestive cells persisting from the nymphal stage; **DCI** – initial digestive cells (prodigest cells); **DC** – digestive cells; **DDC** - detached digestive cells; **RB** – residual bodies (hemosomes)

substrate (Mulenga et al., 1999b), were cloned and partially characterized (Mulenga et al., 1999a).

Another cysteine peptidase gene homologous to cathepsin L (BmCL1) was identified in *R. microplus* and expressed in an *Escherichia coli* system. The optimal enzymatic activity of BmCL1 against bovine hemoglobin has been recorded at an acidic pH (Renard et al., 2000). In 2002, RT PCR profiling, as well as an immunoblotting approach, confirmed the presence of BmCL1 in the gut of partially engorged *R. microplus* females (Renard et al., 2002). Later on, an aspartic peptidase termed "longepsin" was identified and partially characterized from *H. longicornis* (Boldbaatar et al., 2006). RT-PCR profiling and a western blot analysis confirmed the presence of both the mRNA and the protein in the tick midgut and salivary glands. Up regulation of longepsin during feeding was also observed. Furthermore, the recombinant enzyme was capable of digesting hemoglobin at acidic conditions (pH 3.5). Taken altogether, the role of longepsin in blood meal digestion was postulated (Boldbaatar et al., 2006).

The opinion that tick digestion is not executed by a single "hemoglobinase" enzyme but rather relies on a multi-enzyme network or cascade of aspartic and cysteine type peptidases was supported by a variety of digestive peptidase types reported from a number of model tick species **(Tab 2).** However, a comprehensive study demonstrating the existence of such a hemoglobinolytic armament in a single tick species was still missing.

In order to prove (or rule out) this hypothesis, we focused on describing of the entire proteolytic cascade/network in the tick gut at a defined feeding stage, namely the guts of semi-engorged females of our model tick *I. ricinus*. To accomplish this task, we have performed biochemical and genetic screens to identify tick digestive enzymes. Our results clearly demonstrate that hard ticks possess a hemoglobinolytic cascade, which consists of aspartic peptidase cathepsin D (clan AA), cysteine peptidases of papain type cathepsin B, L and C (clan CA) and an asparaginyl endopeptidase (AE, legumain; clan CD) (**Paper II**).

**Table 2: List of cysteine and aspartic peptidases identified from different tick species with a proposed role in hemoglobin digestion.**

| Tick species | Peptidase name | Specificity | Tissue | GenBank # | Reference |
|---|---|---|---|---|---|
| *Haemaphysalis longicornis* | Longepsin | Cathepsin D | Gut, salivary glands | AB218595 | (Boldbaatar et al., 2006) |
| *Rhipicephalus (Boophilus) microplus* | BmAP | Cathepsin D | Gut | FJ655904 | (Cruz et al., 2010) |
| *Ixodes ricinus* | IrCD | Cathepsin D | Gut | EF428204 | (Franta et al., 2010; Horn et al., 2009; Sojka et al., 2008) |
| *Ixodes ricinus* | IrCB | Cathepsin B | Gut | EF428206 | (Franta et al., 2010; Horn et al., 2009; Sojka et al., 2008) |
| *Haemaphysalis longicornis* | Longipain | Cathepsin B | Gut | AB255051 | (Tsuji et al., 2008) |
| *Ixodes ricinus* | IrCC | Cathepsin C | Gut, salivary glands, ovary, malph. tubules | EU128750 | (Franta et al., 2010; Horn et al., 2009; Sojka et al., 2008) |
| *Rhipicephalus (Boophilus) microplus* | BmCL1 | Cathepsin L | Gut | AF227957 | (Cruz et al., 2010; Renard et al., 2000; Renard et al., 2002) |
| *Haemaphysalis longicornis* | H1CPL-A | Cathepsin L | Gut | AB490783 | (Yamaji et al., 2009) |
| *Ixodes ricinus* | IrCL1 | Cathepsin L | Gut, salivary glands, ovary, malph. tubules | EF428205 | (Franta et al., 2010; Franta et al., 2011; Horn et al., 2009; Sojka et al., 2008) |
| *Ixodes ricinus* | IrAE | Legumain | Gut | AY584752 | (Franta et al., 2010; Horn et al., 2009; Sojka et al., 2007) |
| *Haemaphysalis longicornis* | H1Lgm | Legumain | Gut | AB279705 | (Abdul Alim et al., 2007; Alim et al., 2008a) |
| *Haemaphysalis longicornis* | H1Lgm2 | Legumain | Gut | AB353127 | (Alim et al., 2008a; Alim et al., 2008b) |

## The hemoglobinolytic cascade of hard ticks

Hemoglobin represents the major nutrient source for ticks. Its intracellular degradation is performed by an orchestrated cascade of peptidases that cleave the intact hemoglobin tetramers into smaller fragments that are used to fulfill tick's nutritional and energetic requirements. Using a partially fed *I. ricinus* female as a model organism, we deconvoluted the process of intracellular hemoglobinolysis and demonstrated that it is similar to organisms as phylogenetically distant as blood feeding nematodes, platyhelminthes or protists (**Fig 6**).

Host hemoglobin is primarily cleaved by endopeptidases of the aspartic and cysteine class (cathepsin D supported by cathepsin L and AE/legumain). In the next step, the hemoglobinolytic pathway is accomplished by the papain type cysteine peptidases, cathepsin B and cathepsin L, respectively. The resulting peptides are further processed into dipeptides by the exopeptidase activity of cathepsin B and cathepsin C (dipeptidyl-peptidase I). Finally, the terminal step culminates in the release of free amino acids by the specific activity of leucine amino-peptidase(s) and serine carboxy-peptidase(s) (**Paper III**).

## Aspartic peptidases

The aspartic peptidase activity of a Cathepsin D type (clan AA) peptidase is involved in the initial degradation of host hemoglobin in the tick gut cells (**Paper III**). Three *I. ricinus* cathepsin D encoding genes (IrCDs) that are orthologs to three cathepsin D isoforms identified in the *Ixodes scapularis* genome were cloned and sequenced (**Paper II, VII**). Phylogenetic analysis and *in-silico* structure modeling showed that the IrCD1 differs substantially from other tick cathepsin D peptidases. Contrary to IrCD2, which is expressed in several tick tissues, IrCD1 is expressed solely in the gut and its expression is clearly induced by feeding. Furthermore, IrCD3 is most likely not a hemoglobinolytic enzyme with specific roles in tick digestion due to its specific expression in ovaries **(Paper VII)**. The localization of IrCD1 to the digestive compartments of female gut cells was confirmed by indirect immunofluorescence microscopy using specific antibodies raised and then purified using the zymogene recombinantly expressed in *E. coli* (rIrCD1). We then determined that rIrCD1 is auto-activated and remains active and stable at acidic conditions. The screening of a novel synthetic dodecapeptidyl library

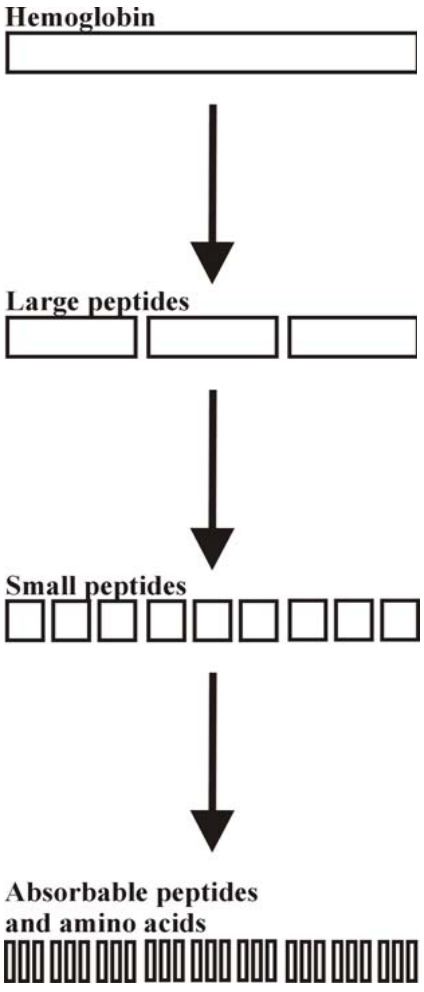
revealed that the substrate preferences of IrCD1 are hydrophobic residues at the P1 and P1′ position and confirmed that the enzyme acts as a strict endopeptidase. The hemoglobin cleavage pattern produced by the recombinant IrCD1 matches well with the cleavage map obtained with the cathepsin D activity from the gut extract. Moreover, the specific function of IrCD1 as the major tick aspartic hemoglobinase was confirmed by RNAi experiments (**Paper VII**).

Another aspartic peptidase was isolated from the gut of the tick *R. microplus* and designated as BmAP. The full-length sequence was obtained and characterized. The qRT PCR profiling revealed that BmAP is expressed solely in the gut tissue of fully fed ticks. The substrate specificity of native BmAP was mapped by PS-SCL (Positional scanning of synthetic combinatorial library (Choe et al., 2006)) and shown that the enzyme prefers amino acids with hydrophobic residues at the P1 subsite, which is in accordance with the specificity of most aspartic peptidases. Its role in the generation of hemocidins, the previously described antimicrobial fragments from hemoglobin alpha and beta chains, was also demonstrated (Cruz et al., 2010).

Phylogenetic analysis and amino acid sequence alignments (**Paper VII;** Fig 1, supplementary Fig 1) reveal that BmAP and longepsin are more closely related to the IrCD2 isoform, which is expressed in the guts and salivary glands of *I. ricinus* in fully engorged females. Based on these data and a different prediction of posttranslational changes, we propose that IrCD2 –like cathepsin D paralogues are putatively secreted/extracellular forms of cathepsin D. We suggest that in addition to a possible immunomodulative function in the saliva, IrCD2 also plays a digestive or immunoprotective role in the gut during the second continuous phase in the fully engorged females after the detachment from the host. Certainly, further studies need to be completed in order to confirm this hypothesis.

**Legumain-like cysteine peptidases**

Asparaginyl endopeptidase (legumain), termed IrAE, was the first member of the clan CD cysteine peptidases ever described in arthropods. The full mRNA/cDNA sequence was obtained from the *I. ricinus* gut-specific λ phage cDNA library by hybridization screening. Semi-quantitative RT PCR profiling showed that IrAE is expressed solely in the tick gut tissue. Specific




| | ***Ixodes ricinus***[1,2,] | ***Schistosoma mansoni***[5,6,12] | ***Necator americanus***[19,20,21] | ***Plasmodium falciparum***[36,37,38, 39] |
|---|---|---|---|---|
| Large peptides | Aspartic peptidase (IrCD1[a,2]) Cysteine peptidase of CA clan (IrCL1[3,2]) Cysteine peptidase of CD clan[b] (IrAE[4,2]) | Aspartic peptidase (SmCD[7]) Cysteine peptidase of CA clan (SmCB1[8,9], SmCL1[10]) Cysteine peptidase of CD clan[b] (SmAE1[11]) | Aspartic peptidase (NaAPR1[15], NaAPR2[16]) Cysteine peptidase of CA clan[c] (AcCP2[20]) | Aspartic peptidase (Plasmepsin1[22,25], 2[22,23], 4[22,24], HAP[22]) Cysteine peptidase of CA clan Falcipains[26] (2[27], 3[28], 2´[29]) Cysteine peptidase of CD clan[d] |
| Small peptides | Cysteine peptidase of CA clan (IrCB[2], IrCL1[3,2]) | Cysteine peptidase of CA clan (SmCB1[8,9], SmCL1[10]) | Cysteine peptidase of CA clan (NaCP3[18]) | Cysteine peptidase of CA clan (Falcipain2[27], 3[28],2´[29]) |
| Absorbable peptides and amino acids | (IrCB[2]) Dipeptidyl peptidase I (IrCC[2]) Metallopeptidase (LAP) Serine peptidase (SCP) | (SmCB1[8,9]) Dipeptidyl peptidase I (SmCC[13]) Metallopeptidase (SmLAP[14]) | Metallopeptidase (NaMEP1[19]) | Metallopeptidase (Falcilysin[30]) Dipeptidyl peptidase I (DPAP1[33,34]) Aminopeptidase (PfAPP[32], PfA-M1[32], PfM18AAP[35]) |

Continued on the next page

**Figure 6: The comparison of hemoglobinolytic cascades of the tick *Ixodes ricinus*, blood fluke *Schistosoma mansoni*, nematode *Necator americanus* and malaria parasite *Plasmodium falciparum*.**

A schematic diagram showing the hemoglobin digestion, which occurs in several steps and results in the production of dipeptides and amino acids. The primary cleavage of the hemoglobin molecule is generally executed by aspartic endopeptidases, as proposed by Horn for *I. ricinus*[1], Goldberg for *P.falciparum*[39], Williamson for *N. americanus*[21] and Brindley for *S. mansoni*[5], but cysteine peptidases of the CA clan can also participate in the initial step of cleavage as shown for *I. ricinus*, *P. falciparum* and *S. mansoni*. None of the *N. americanus* cysteine peptidases were shown to participate in the primary cleavage as it was shown for AcCP2, a cysteine peptidase from the nematode *Ancylostoma caninum*[20]. An Asparaginyl endopeptidase (a cysteine peptidase of the CD clan) was shown to trans-activate cathepsin B[9] and its putative role in the initial cleavage with other cysteine peptidases of the CA clan was proposed. The consequent cleavage of large peptides and the generation of small peptides in all parasitic species is maintained by cysteine endopeptidases of the CA clan. Exopeptidases act on the release of absorbable peptides and amino acids.

a – Sojka et al., *Ir*CD1 – gut aspartic hemoglobinase of the tick *Ixodes ricinus* (in manuscript)

b – SmAE was shown to trans-activate gut associated SmCB1 zymogen[9] to its active form and has been hypothesized to trans-activate other gut associated enzymes eg SmCL1[12]. Moreover IrAE from *I. ricinus* was capable to trans-activate the zymogen of *S. mansoni* cathepsin B1 (SmCB1)[4].

c – Although several cysteine proteases have been identified from *N. americanu,*[17] so far, none of them have been shown to digest native hemoglobin as was proved for the cysteine peptidase from *Ancylostoma caninum*[20].

d – The clan CD cysteine peptidases were predicted in *P. falciparum* genome sequence, but their role in hemoglobin degradation as well as biochemical characterization has not been proven yet[3]


References

1 - (Horn et al., 2009)
2 - (Franta et al., 2010)
3 - (Franta et al., 2011)
4 - (Sojka et al., 2007)
5 - (Brindley et al., 1997)
6 - (Delcroix et al., 2006)
7 - (Brindley et al., 2001)
8 - (Ghoneim and Klinkert, 1995)
9 - (Sajid et al., 2003)
10 - (Brady et al., 1999)
11 - (Caffrey et al., 2000)
12 - (Caffrey et al., 2004)
13 - (Butler et al., 1995)
14 - (McCarthy et al., 2004)
15 - (Williamson et al., 2002)
16 - (Williamson et al., 2003a)
17 - (Ranjit et al., 2006)
18 - (Ranjit et al., 2008)
19 - (Ranjit et al., 2009)
20 - (Williamson et al., 2004)
21 - (Williamson et al., 2003b)
22 - (Banerjee et al., 2002)
23 - (Goldberg et al., 1991)
24 - (Wyatt and Berry, 2002)
25 - (Gluzman et al., 1994)
26 – (Gamboa de Dominguez and Rosenthal, 1996)
27 - (Shenai et al., 2000)
28 - (Sijwali et al., 2001)
29 - (Singh et al., 2006)
30 - (Eggleson et al., 1999)
31 - (Rosenthal, 2004)
32 - (Dalal and Klemba, 2007)
33 - (Klemba et al., 2004)
34 - (Wang et al., 2011)
35 - (Teuscher et al., 2007)
36 - (Goldberg et al., 1990)
37 - (Rosenthal and Meshnick, 1996)
38 - (Francis et al., 1997)
39 - (Goldberg, 2005)

antibodies used to localize IrAE by indirect immunofluorescence and electron microscopy demonstrated the presence of the enzyme not only inside the tick digestive cells, but also on their surface and within the peritrophic matrix covering the gut epithelium. Recombinant IrAE expressed in the yeast *Pichia pastoris* was biochemically characterized and its *in vitro* hemoglobinolytic activity at an acidic pH was demonstrated (**Paper I**). Native enzyme was labeled in tick gut extracts by a specific and novel activity-based probe. Moreover, the AE activity was shown to be participating in the primary cleavage of the hemoglobin molecule (**Paper III**).

Two forms of asparaginyl endopeptidases were previously described from the tick *H. longicornis* and termed H1Lgm (Abdul Alim et al., 2007) and H1Lgm2 (Alim et al., 2008b). Both enzymes have been shown to have up-regulated expression in the gut after feeding (Alim et al., 2008a), thus their role in blood meal digestion was proposed. Both enzymes were recombinantly expressed and intriguingly were reported to digest bovine hemoglobin *in vitro* at an alkaline pH. The follow-up study demonstrated that RNA interference (RNAi) of both legumains resulted in the failure of tick repletion, a higher number of death ticks and the reduction of their weight. The damage to midgut tissue and the disruption of normal cellular remodeling were observed during feeding. Moreover, RNAi treated ticks were significantly delayed in oviposition with a reduced number of eggs that were structurally deformed and failed to hatch (Alim et al., 2009).

**Papain-type cysteine peptidases**

Specific fluorogenic substrates and selective inhibitors were utilized to reveal the major enzymatic activities inside the *I. ricinus* gut tissue. Three significant papain type cysteine peptidases of the clan CA, namely cathepsin L, cathepsin B and dipeptidyl-peptidase I cathepsin C, were identified and their role in intracellular hemoglobinolysis has been elucidated (**Paper III**).

A gene coding for a cathepsin L-like *I. ricinus* peptidase (later named as IrCL1) was first described in 2008 and shown to be expressed in the tick gut tissue (**Paper II**), where this cathepsin L activity present in tick gut extracts was found to be important in the initial phase of hemoglobin digestion (**Paper III**). Recently, we observed that the IrCL1 mRNA and protein levels are strongly up regulated after feeding (**Paper IV**) and then confirmed the

intracellular localization of the native enzyme within tick digestive cells by indirect immunofluorescence microscopy (**Paper V**). Recombinant IrCL1 expressed in the yeast *P. pastoris* was shown to be active at an acidic pH with an optimum of about pH 3.5, which corresponded to the optimum pH of cathepsin L in tick gut extracts. In addition, recombinant IrCL1 was able to digest bovine hemoglobin and albumin *in vitro*. Substrate specificity determined by PS-SCL and the inhibition profile revealed that IrCL1 has the same ligand binding characteristics as the cathepsin L subfamily of cysteine peptidases (**Paper V**).

The screening of EST databases (http://www.ncbi.nlm.nih.gov/) and a genome-wide dataset of the closely related species *I. scapularis* (http://iscapularis.vectorbase.org/) allowed us to identify two more cathepsin L like peptidase genes from *I. ricinus*, designated as IrCL2 and IrCL3, which were subsequently cloned and fully sequenced (unpublished data). Multiple amino acid alignments of all three isoforms clearly demonstrates that IrCL1 differs substantially from IrCL2 and IrCL3, as well as the previously described cathepsins L from other tick species in both their sequence and N-glycosylation pattern (**Paper V;** supplementary Fig S1). In order to demonstrate which of the IrCL forms is responsible for the gut-associated hemoglobinolytic cathepsin L activity, we performed gene specific RNAi silencing of IrCL1. It resulted in an almost complete depletion of cathepsin L activity in the guts of semi engorged *I. ricinus* females (**Paper V**). It remains to be verified whether IrCL2 or IrCL3 play any specific roles in blood digestion within other feeding periods/digestive phases of female ticks.

The previously identified cathepsin L from *R. microplus* (BmCL1) (Renard et al., 2000) was recently expressed in the yeast *P. pastoris* and its substrate preference was determined by the PS-SCL approach (Cruz et al., 2010). Moreover, its involvement in the specific endopeptidolytic generation of antimicrobial hemocidins (together with BmAP) has been demonstrated (Cruz et al., 2010). Another cathepsin L-like cysteine peptidase (H1CPL-A) up-regulated by blood feeding was identified from *H. longicornis* and functionally expressed. Recombinant H1CPL-A was capable of autocatalytic processing and hydrolyzed bovine hemoglobin as well as synthetic peptidyl substrates at an acidic pH. Its hydrolytic activity was inhibited by leupeptin, antipain and E-64, but remained unaffected by pepstatin (Yamaji et al., 2009).

A gene encoding for the cathepsin B-like cysteine peptidase was isolated from the gut-specific cDNA library of *I. ricinus* (**Paper II**). Native enzyme was detected in female gut tissue extracts by an activity-based probe labeling and its endopeptidase as well as exopeptidase activity during hemoglobinolysis was observed (**Paper III**). Active site titration with the CA-074 inhibitor (a specific inhibitor of cathepsin B) showed that IrCB is the most abundant enzyme during the whole feeding process. Thus, IrCB specific affinity purified rabbit polyclonal antibodies were used to demonstrate the intracellular localization of tick hemoglobinolysis during the whole feeding period. Moreover, strong up-regulation of both mRNA and protein levels upon feeding were monitored by qRT PCR and western blotting, respectively (**Paper IV**).

A cysteine peptidase classed as cathepsin B and termed "longipain" was identified and characterized from *H. longicornis*. The enzyme is solely expressed in the tick gut and up-regulated upon feeding. Recombinantly expressed longipain displayed an unusually broad pH range with two pH optima for ZFR-AMC and ZRR-AMC substrates, respectively. Longipain silencing by RNAi indicated that this peptidase is involved in blood meal digestion as well as in the transmission of *Babesia* spp. (Tsuji et al., 2008).

A gene coding for a dipeptidyl-peptidase I, cathepsin C, was isolated from *I. ricinus* (**Paper II**) gut-specific cDNA. Its role as an exopeptidase in the terminal stage of intracellular hemoglobinolysis (**Paper III**) and its up-regulation during blood feeding process was reported (**Paper IV**).

**Serine and leucine peptidases**

Although the hemoglobinolysis has been well described in many blood-sucking parasites (Delcroix et al., 2006; Williamson et al., 2003b), little is known about the terminal process of hemoglobin digestion and the liberation of free amino acids. Monopeptidases seem to play a role in this process within blood flukes (McCarthy et al., 2004) or *Plasmodium* parasites (Gavigan et al., 2001). Also, ticks possess several serine and leucine peptidases that have been identified so far.

Serine carboxypeptidase (H1SCP1), identified from *H. longicornis*, was shown to be strongly expressed in the gut tissue and up-regulated during feeding. Native H1SCP1 is supposed to be localized inside digestive cells and also on their surface as detected by immunoblotting. Recombinant enzyme was

shown to digest hemoglobin *in vitro* over a broad pH range and its role in blood meal digestion was suggested (Motobu et al., 2007).

Two serine peptidases, termed H1SP2 and H1SP3, from *H. longicornis* were identified by the same group and shown to be up-regulated upon blood feeding (Miyoshi et al., 2008). Independently, three serine peptidases were also identified from *Rhipicephalus appendiculatus*, but their biological function was not assessed (Mulenga et al., 2003).

H1LAP – a leucine amino peptidase from *H. longicornis* was localized in the cytosol of midgut cells and salivary glands by indirect immunofluorescence microscopy and was shown to be up-regulated during blood feeding. Recombinant H1LAP was active at an alkaline pH (optimum at pH 8) and its hydrolytic activity targeted the N-terminal amino acid (Hatta et al., 2006). Knockdown of H1LAP by RNAi lead to the reduction of its expression as well as enzymatic activity in the tick gut. Moreover, reduced oviposition and delayed egg laying upon the enzyme silencing was reported (Hatta et al., 2007). Later on, Hatta performed further studies of the H1LAP knockdown and hypothesized that H1LAP may play a role in the process of vitellogenesis by supplying free amino acids (Hatta et al., 2010).

We have also detected the activity of serine and leucine monopeptidases in the gut extracts of *I. ricinus* and thus postulated their role in the production of single amino acids from hemoglobin derived peptides. The pH optima measured for native enzymes in gut homogenates were much higher than those identified for all the key cysteine and aspartic peptidases of *I. ricinus* hemoglobinolysis, suggesting that the final peptidolysis is spatially separated from the above described lysosomal digestion (**Paper III**). No follow-up studies on these enzymes have been carried out to verify this hypothesis.

**Heme and iron detoxification**

The blood meal diet is a very complex and rich source of nutrients and energy, but it also constitutes an enormous amount of heme molecules - a potential source of free iron radicals that may cause severe damage to the proteins, lipids and DNA of the tick (Graca-Souza et al., 2006). The fate of heme (a prosthetic group containing iron) was well described in the cattle tick *Rhipicephalus microplus* (Lara et al., 2005; Lara et al., 2003). The ticks get rid of most of the heme in the form of aggregates by a specific detoxification

process, which occurs in specialized, membrane delimited, organelles called hemosomes (Lara et al., 2003). Since ticks apparently lack the heme synthetic pathway (Braz et al., 1999), the heme needed as a prosthetic group for tick enzymes is transported from the gut to various tissues with the aid of heme binding proteins present in the tick hemolymph, i.e. the heme-binding lipoprotein HeLp from *R. microplus* (Maya-Monteiro et al., 2000) or the hemelipoglyco-carrier protein CP described from *D. variabilis* (Gudderra et al., 2001). Moreover, vitellogenin, the precursor of the main yolk protein vitellin is also able to bind heme during vitellogenesis and serve as the major heme reservoir for the developing embryos (Logullo et al., 2002).

Based on the available data from the *I. scapularis* genome, as well as large data sets of transcriptomes from other tick species, it seems very likely that ticks also lost the capacity of heme degradation -for instance, ticks apparently do not possess the catabolic enzyme heme oxygenase. Therefore, ticks have to have a specific mechanism to acquire, transport and utilize the iron from non-heme origin.

In our laboratory, three primary proteins involved in the iron metabolism were described from the hard tick *Ixodes ricinus*. The intracellular ferritin (FER1) serves as the main iron storage protein inside tick cells and was shown to consist of 24 identical subunits, similar to the heavy chain of vertebrate ferritins (Kopacek et al., 2003). Further studies on tick iron metabolism led to the identification of the iron regulatory protein (IRP1) and a novel form of ferritin (FER2), which is expressed in the tick gut and secreted into the hemolymph (**Paper VI**). All proteins were recombinantly expressed and functionally characterized. The role of individual proteins in tick iron metabolism was demonstrated by specific RNAi experiments. The effect of FER1, IRP1 and FER2 silencing was evaluated and clearly demonstrated that all 3 genes have a high impact on *I. ricinus* feeding, survival, oviposition and larvae hatching. The most profound impact on tick feeding and further development was observed for the FER2 RNAi, as it presumably functions as a transporter of non-heme iron from the tick gut to the peripheral tissues. This makes this protein a very promising candidate for the development of an effective “anti-tick” vaccine (**Paper VI**), (Hajdusek et al., 2010).

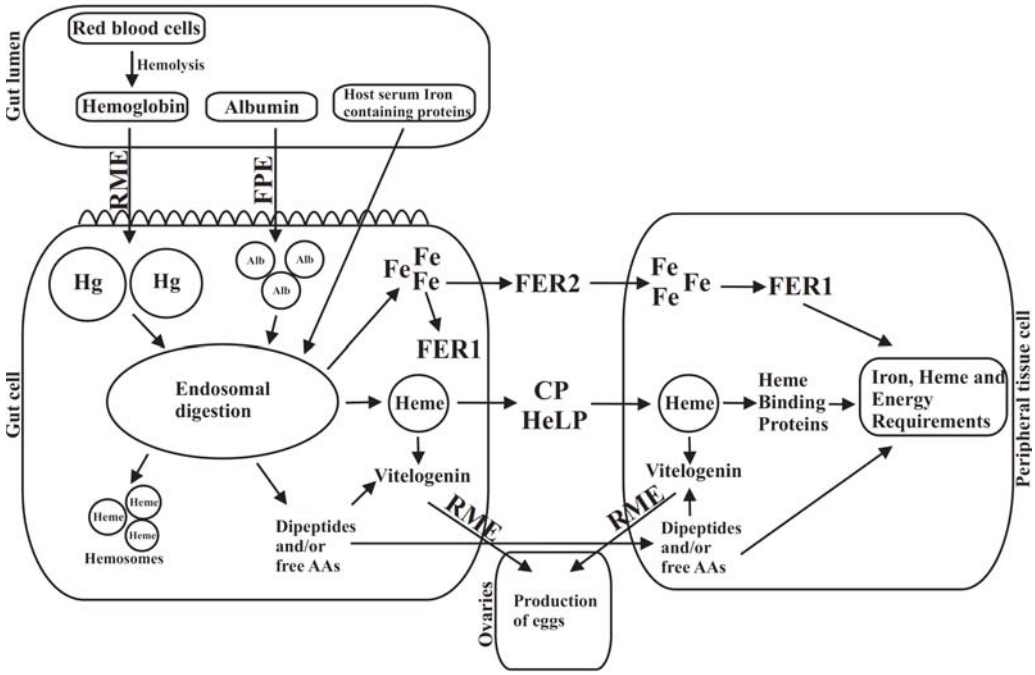



**Figure 7: Schematic diagram showing iron, heme and energy metabolism in ticks.** Two different endocytic pathways have been described for the two major protein constituents. Whereas albumin is uptaken by tick digestive cells via fluid phase endocytosis (FPE), hemoglobin, released from red blood cells by hemolysis, is uptaken via receptor mediated endocytosis (RME) (Lara et al., 2005). Intracellular digestion of host blood proteins lead to production of dipeptides and amino acids, which represent the major source of nutrients and energy for all metabolic processes inside the tick body. Most of the heme released by intracellular hemoglobinolysis is aggregated in hemosomes and removed with detached digestive cells by defecation. A portion of heme is bound to heme carrier proteins (CP, HeLP) and transported to various tick tissues to fulfill their cellular requirements. Another portion of heme is sequestered by vitellogenin, which is expressed in tick digestive cells or fat body. Vitellogenin -a yolk protein precursor, is consequently transported to the ovaries by RME. The non-heme iron released from host serum iron-containing proteins is bound by intracellular FER1 or loaded into FER2 and transported to other tick tissues.

## The "anti-tick" vaccination potential of recombinant hemoglobinases

After mosquitoes, ticks are the second most important vectors of human infectious diseases and they also represent important veterinary pests in the tropical and subtropical regions of the world. Acaricides were primarily used for tick control, but their use resulted in the selection of resistant ticks, as well as the environmental pollution and contamination of meat and milk products (George et al., 2004; Graf et al., 2004). The development of vaccines against tick proteins became an alternative strategy that represents a cheap non-contaminating possibility that is applicable to a wide variety of hosts

(Willadsen, 2004). Two types of antigens are used for vaccine development: (i) – exposed antigens that are usually expressed in tick salivary glands and secreted in the saliva during tick feeding; (ii) – concealed antigens that are hidden to the host immune system, but could be targeted by immunoglobulins ingested via a blood meal uptake (Nuttall et al., 2006). Interestingly, the only commercially available vaccine is of the concealed type, derived from the tick midgut protein Bm86 (Willadsen et al., 1995). The most limiting step in the development of "anti-tick" vaccines is the identification of new antigens, which usually requires a detailed knowledge of tick physiological processes.

Tick digestive peptidases play a crucial role in tick biology and as such seem to be promising candidates for the rational development of "anti-tick" vaccines. Although the vaccination potential of digestive peptidases from other blood feeding parasites has been extensively studied and shows promising results (Loukas et al., 2005; Loukas et al., 2004), it still remains rather unexplored in ticks. Therefore, a deeper understanding of the specific features in tick physiology like feeding, blood meal digestion and iron metabolism at a molecular level may result in the identification of new potential antigens.

# CONCLUDING REMARKS

Host blood processing is a crucial event in the tick physiology since host blood represents the ultimate source of nutrients and energy. Until recently, only a few digestive enzymes had been described in different tick species, but a complex study of the digestive apparatus within one particular species was missing. My Ph.D. thesis offers such a comprehensive analysis of the tick proteolytic machinery using the semi-engorged female of *Ixodes ricinus*, an important vector of Lyme disease and TBE virus in Europe, as a model organism.

In my thesis I show that the tick proteolytic armament consists mainly of aspartic and cysteine peptidases, which function in a semi-ordered pathway within a network that resembles the digestive system of parasitic protists, platyhelminthes and nematodes. Individual enzymes of the *I. ricinus* proteolytic machinery have been characterized and their intracellular hemoglobinolytic digestive roles were disclosed. We have also mapped the enzymatic and morphological changes that occur in the tick gut during feeding on the host and confirmed the intracellular localization of hemoglobinolysis.

Although the hemoglobinolytic cascade has been well defined, the fate of the second prominent blood protein, albumin, still remains unexplored. Lara et al., has shown that the uptake of the major blood proteins (hemoglobin and albumin) occurs via two different endocytic pathways. Host albumin is uptaken by fluid phase endocytosis and observed in small digestive vesicles. Whereas the uptake of host hemoglobin is executed via receptor mediated endocytosis and the protein is exclusively present inside the large vesicles (Lara et al., 2005). To this end, it is not known whether the tick hemoglobinolytic and albuminolytic armaments are consistent or if ticks possess parallel proteolytic pathways for hemoglobin and albumin degradation in the multi-enzyme digestive network as was shown for *Schistosoma mansoni* (Delcroix et al., 2006). Class specific substrates, inhibitors and active based probes were used to reveal the tick hemoglobinolytic cascade and the same procedures should be applied to deconvolute the *I. ricinus* albuminolysis.

The *in vitro* feeding of hard ticks developed by (Krober and Guerin, 2007) was also recently established in our laboratory. After optimalization, it should open new gates to study tick digestive enzymes, e.g. tracing the fate of

albumin and hemoglobin inside the tick gut by using their fluorescent derivatives or observing the role of different types of diet on different aspects of tick biology, such as feeding, oviposition or larvae hatching. It would be of great interest to see how the digestive enzymes or components of iron metabolism are expressed and regulated depending on the amount of uptaken hemoglobin (feeding with complete blood vs. feeding with blood plasma). Moreover, *in vitro* feeding should improve the efficiency of the currently used dose limited RNAi protocol. Nowadays, only about 0.5 µl of the 3µg/µl dsRNA per one female tick could be delivered to the coelome cavity using microinjection. *In vitro* feeding rapidly increases the total amount of dsRNA accessible to one tick, allowing us to perform simultaneously multiple RNAi knock-downs, which is currently not possible with our present methods. Thus, it should be possible to experimentally verify *in vivo* the well discussed redundancy among the individual digestive peptidases.

In our work, we have mapped the intracellular hemoglobinolytic cascade during feeding (the first continuous phase of digestion), but little is known about the digestive processes occurring during the second continuous phase of digestion. This phase starts after the detachment from the host, ends by female death and is characterized by massive oviposition (see above). In order to map the digestive enzymes during this period, we dissected the tick gut tissue 5 and 11 days post detachment from the host, separately collected the gut contents and gut tissues and used specific fluorogenic substrates to measure the enzymatic activities in these samples. Interestingly, our preliminary results indicate that blood digestion ceased to be strictly an intracellular event, as the enzymatic activities progressively moved into the gut lumen (**Fig 8**). The reason for this translocation remains unclear and requires further detailed studies. Although any impairment of the tick proteolytic cascade during the second continuous phase of digestion will have no effect on tick feeding success, it could greatly affect tick progeny and reduce the tick population, especially of one-host ticks.

We hope that the data presented within my PhD thesis offers better insight into the complicated story of tick digestion and will serve as the starting point in the development of new "anti-tick" vaccines targeting the tick digestive apparatus, which would ultimately help to control tick populations and the transmission of tick-borne pathogens.

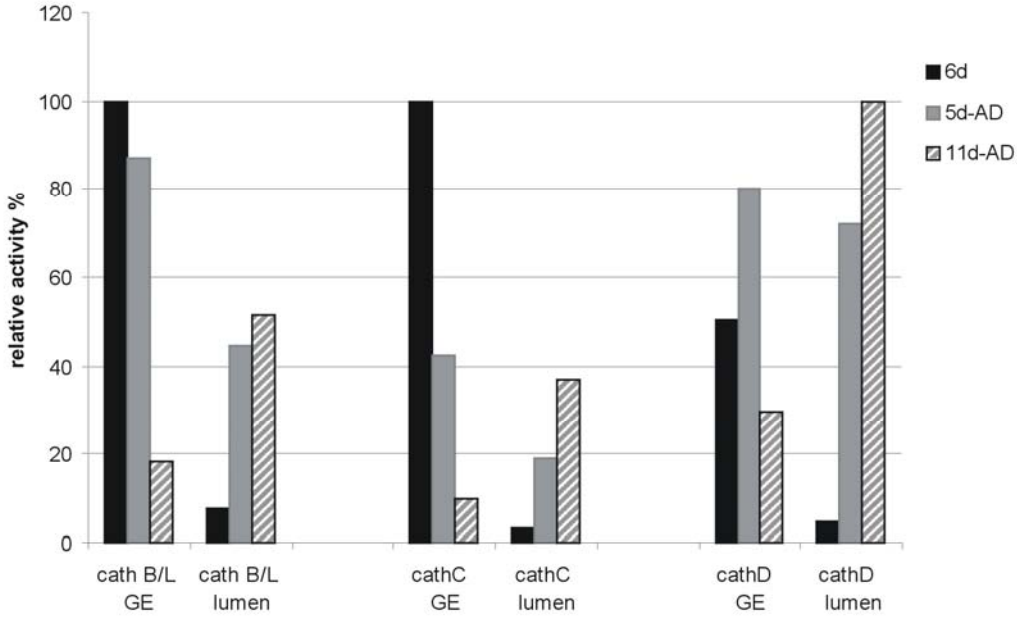



**Figure 8: The progressive change from the intracellular to the luminal digestion in fully engorged *Ixodes ricinus* females. .** Tick gut extracts (**GE**) or gut contents (**lumen**) were prepared from adult *I. ricinus* females at different time points. Six days post attachment (**6d**), five days after detachment from the host (**5d-AD**) or eleven days after detachment from the host (**11d-AD**). Proteolytic activities were measured using specific substrates, namely ZFR-AMC for cathepsin B and L (**cath B/L**), GR-AMC for cathepsin C (**cathC**) and Abz-KPAEFF*EL for cathepsin D (**cathD**).

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Sojka, D., Hajdusek, O., Dvorak, J., Sajid, M., Franta, Z., Schneider, E.L., Craik, C.S., Vancova, M., Buresova, V., Bogyo, M., Sexton, K.B., McKerrow, J.H., Caffrey, C.R., Kopacek, P., 2007. IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. Int J Parasitol 37, 713-724.

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Williamson, A.L., Brindley, P.J., Knox, D.P., Hotez, P.J., Loukas, A., 2003b. Digestive proteases of blood-feeding nematodes. Trends Parasitol 19, 417-423.

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Paper I

Sojka D, Hajdusek O, Dvorak J, Sajid M, **Franta Z**, Schneider EL, Craik CS, Vancova M, Buresova V, Bogyo M, Sexton KB, McKerrow JH, Caffrey CR and Kopacek P, 2007.
IrAE - An asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. Int J Parasitol 37, 713-724.

Abstract

Ticks are ectoparasitic blood-feeders and important vectors for pathogens including arboviruses, rickettsiae, spirochetes and protozoa. As obligate blood-feeders, one possible strategy to retard disease transmission is disruption of the parasite's ability to digest host proteins. However, the constituent peptidases in the parasite gut and their potential interplay in the digestion of the blood meal are poorly understood. We have characterized a novel asparaginyl endopeptidase (legumain) from the hard tick *Ixodes ricinus* (termed IrAE), which we believe is the first such characterization of a clan CD family C13 cysteine peptidase (protease) in arthropods. By RT-PCR of different tissues, IrAE mRNA was only expressed in the tick gut. Indirect immunofluorescence and EM localized IrAE in the digestive vesicles of gut cells and within the peritrophic matrix. IrAE was functionally expressed in *Pichia pastoris* and reacted with a specific peptidyl fluorogenic substrate, and acyloxymethyl ketone and aza-asparagine Michael acceptor inhibitors. IrAE activity was unstable at pH P 6.0 and was shown to have a strict specificity for asparagine at P1 using a positional scanning synthetic combinatorial library. The enzyme hydrolyzed protein substrates with a pH optimum of 4.5, consistent with the pH of gut cell digestive vesicles. Thus, IrAE cleaved the major protein of the blood meal, hemoglobin, to a predominant peptide of 4 kDa. Also, IrAE trans-processed and activated the zymogen form of *Schistosoma mansoni* cathepsin B1 – an enzyme contributing to hemoglobin digestion in the gut of that blood fluke. The possible functions of IrAE in the gut digestive processes of *I. ricinus* are compared with those suggested for other hematophagous parasites.

Paper II

Sojka D, **Franta Z**, Horn M, Hajdusek O, Caffrey CR, Mares M, Kopacek P, 2008.
Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases. Parasit Vectors 1, 7..

Abstract

BACKGROUND: Ticks are vectors for a variety of viral, bacterial and parasitic diseases in human and domestic animals. To survive and reproduce ticks feed on host blood, yet our understanding of the intestinal proteolytic machinery used to derive absorbable nutrients from the blood meal is poor. Intestinal digestive processes are limiting factors for pathogen transmission since the tick gut presents the primary site of infection. Moreover, digestive enzymes may find practical application as anti-tick vaccine targets.

RESULTS: Using the hard tick, *Ixodes ricinus*, we performed a functional activity scan of the peptidase complement in gut tissue extracts that demonstrated the presence of five types of peptidases of the cysteine and aspartic classes. We followed up with genetic screens of gut-derived cDNA to identify and clone genes encoding the cysteine peptidases cathepsins B, L and C, an asparaginyl endopeptidase (legumain), and the aspartic peptidase, cathepsin D. By RT-PCR, expression of asparaginyl endopeptidase and cathepsins B and D was restricted to gut tissue and to those developmental stages feeding on blood.

CONCLUSION: Overall, our results demonstrate the presence of a network of cysteine and aspartic peptidases that conceivably operates to digest host blood proteins in a concerted manner. Significantly, the peptidase components of this digestive network are orthologous to those described in other parasites, including nematodes and flatworms. Accordingly, the present data and those available for other tick species support the notion of an evolutionary conservation of a cysteine/aspartic peptidase system for digestion that includes ticks, but differs from that of insects relying on serine peptidases.

Paper III

Horn M, Nussbaumerova M, Sanda M, Kovarova Z, Srba J, **Franta Z**, Sojka D, Bogyo M, Caffrey CR, Kopacek P, Mares M, 2009.
Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics. Chem Biol 16, 1053-1063.

Abstract

Hemoglobin digestion is an essential process for blood-feeding parasites. Using chemical tools, we deconvoluted the intracellular hemoglobinolytic cascade in the tick Ixodes ricinus, a vector of Lyme disease and tick-borne encephalitis. In tick gut tissue, a network of peptidases was demonstrated through imaging with specific activity-based probes and activity profiling with peptidic substrates and inhibitors. This peptidase network is induced upon blood feeding and degrades hemoglobin at acidic pH. Selective inhibitors were applied to dissect the roles of the individual peptidases and to determine the peptidase-specific cleavage map of the hemoglobin molecule. The degradation pathway is initiated by endopeptidases of aspartic and cysteine class (cathepsin D supported by cathepsin L and legumain) and is continued by cysteine amino- and carboxy-dipeptidases (cathepsins C and B). The identified enzymes are potential targets to developing novel anti-tick vaccines.

Paper IV

**Franta Z**, Frantova H, Konvickova J, Horn M, Sojka D, Mares M, Kopacek P, 2010.
Dynamics of digestive proteolytic system during blood feeding of the hard tick *Ixodes ricinus.* Parasit Vectors 3, 119.

Abstract

BACKGROUND: Ticks are vectors of a wide variety of pathogens causing severe diseases in humans and domestic animals. Intestinal digestion of the host blood is an essential process of tick physiology and also a limiting factor for pathogen transmission since the tick gut represents the primary site for pathogen infection and proliferation. Using the model tick *Ixodes ricinus*, the European Lyme disease vector, we have previously demonstrated by genetic and biochemical analyses that host blood is degraded in the tick gut by a network of acidic peptidases of the aspartic and cysteine classes.

RESULTS: This study reveals the digestive machinery of the *I. ricinus* during the course of blood-feeding on the host. The dynamic profiling of concentrations, activities and mRNA expressions of the major digestive enzymes demonstrates that the de novo synthesis of peptidases triggers the dramatic increase of the hemoglobinolytic activity along the feeding period. Overall hemoglobinolysis, as well as the activity of digestive peptidases are negligible at the early stage of feeding, but increase dramatically towards the end of the slow feeding period, reaching maxima in fully fed ticks. This finding contradicts the established opinion that blood digestion is reduced at the end of engorgement. Furthermore, we show that the digestive proteolysis is localized intracellularly throughout the whole duration of feeding.

CONCLUSIONS: Results suggest that the egressing proteolytic system in the early stage of feeding and digestion is a potential target for efficient impairment, most likely by blocking its components via antibodies present in the host blood. Therefore, digestive enzymes are promising candidates for development of novel 'anti-tick' vaccines capable of tick control and even transmission of tick-borne pathogens.

Paper V

**Franta Z,** Sojka D, Frantova H, Dvorak J, Horn M, Srba J, Talacko P, Mares M, Schneider E, Craik CS, McKerrow JH, Caffrey CR and Kopacek P, 2011. IrCL1 - the hemoglobinolytic cathepsin L of the hard tick, *Ixodes ricinus*. Int J Parasitol 41, 1253-1262

Abstract

Intracellular proteolysis of ingested blood proteins is a crucial physiological process in ticks. In our model tick, *Ixodes ricinus*, cathepsin L (IrCL1) is part of a gut-associated multi-peptidase complex; its endopeptidase activity is important in the initial phase of hemoglobinolysis. We present the functional and biochemical characterization of this enzyme. We show, by RNA interference (RNAi), that cathepsin L-like activity that peaks during the slow feeding period of females is associated with IrCL1. Recombinant IrCL1 was expressed in bacteria and yeast. Activity profiling with both peptidyl and physiological protein substrates (hemoglobin and albumin) revealed that IrCL1 is an acidic peptidase with a very low optimum pH (3-4) being unstable above pH 5. This suggests an endo/lysosomal localization that was confirmed by indirect fluorescence microscopy that immunolocalised IrCL1 inside the vesicles of digestive gut cells. Cleavage specificity determined by a positional scanning synthetic combinatorial library and inhibition profile indicated that IrCL1 has the ligand-binding characteristics of the cathepsin L subfamily of cysteine peptidases. A non-redundant proteolytic function was demonstrated when IrCL1-silenced ticks had a decreased ability to feed compared with controls. The data suggest that IrCL1 may be a promising target against ticks and tick-borne pathogens.

Paper VI

Hajdusek O, Sojka D, Kopacek P, Buresova V, **Franta Z**, Sauman I, Winzerling J, Grubhoffer L, 2009.
Knockdown of proteins involved in iron metabolism limits tick reproduction and development. Proc Natl Acad Sci U S A 106, 1033-1038.

Abstract

Ticks are among the most important vectors of a wide range of human and animal diseases. During blood feeding, ticks are exposed to an enormous amount of free iron that must be appropriately used and detoxified. However, the mechanism of iron metabolism in ticks is poorly understood. Here, we show that ticks possess a complex system that efficiently utilizes stores and transports non-heme iron within the tick body. We have characterized a new secreted ferritin (FER2) and an iron regulatory protein (IRP1) from the sheep tick, *Ixodes ricinus*, and have demonstrated their relationship to a previously described tick intracellular ferritin (FER1). By using RNA interference-mediated gene silencing in the tick, we show that synthesis of FER1, but not of FER2, is subject to IRP1-mediated translational control. Further, we find that depletion of FER2 from the tick plasma leads to a loss of FER1 expression in the salivary glands and ovaries that normally follows blood ingestion. We therefore suggest that secreted FER2 functions as the primary transporter of non-heme iron between the tick gut and the peripheral tissues. Silencing of the fer1, fer2, and irp1 genes by RNAi has an adverse impact on hatching rate and decreases post bloodmeal weight in tick females. Importantly, knockdown of fer2 dramatically impairs the ability of ticks to feed, thus making FER2 a promising candidate for development of an efficient anti-tick vaccine.

Paper VII

Sojka D, **Franta Z**, Frantova H, Bartosova P, Horn M, Vachova J, O'Donoghue AJ, Eroy-Revelles AA, Craik CS, Knudsen G, Caffrey CR, McKerrow JH, Michael Mares M and Kopacek P.
*Ir*CD1 – gut aspartic hemoglobinase of the tick *Ixodes ricinus* (in manuscript)

Abstract

In the Lyme disease tick vector, *Ixodes ricinus*, gut associated Cathepsin D activity is part of the multienzyme digestive network degrading host hemoglobin. Three *I. ricinus* cathepsin D encoding genes (IrCDs) representing *Ixodes scapularis* genome paralogues were cloned. Phylogenetic analyses and *in-silico* structure models revealed that IrCD1 is distinct from other cathepsins D of tick origin. IrCD1 is only expressed in the gut of female parasites and is induced by feeding as measured by RT-PCR, qRT-PCR, immunoblotting and activity-based labeling. Using specific antibodies against the *E. coli*-expressed protein, IrCD1 was localized in the endosomes of gut cells. Consistent with the endo-lysosomal environment, mature rIrCD1 is auto-activated and remains active in acid conditions. Activity is inhibited by potato cathepsin D inhibitor and pepstatin. rIrCD1 hemoglobin cleavage pattern is identical to that produced by the native enzyme. Preference for hydrophobic residues at the P1 and P1′ position was confirmed by screening a synthetic dodecapeptidyl substrate library. Specific RNA interference of IrCD1in ticks decreased gut cathepsin D activity by >90% relative to ticks treated with non-tick-specific double-stranded RNA. IrCD1 is the major *I. ricinus* aspartic gut-associated hemoglobinase and a potential target for anti-tick interventions.

# FRONT PAGES OF SUPPLEMENTARY PAPERS

Paper A

Buresova V, **Franta Z**, Kopacek P, 2006.
A comparison of *Chryseobacterium indologenes* pathogenicity to the soft tick *Ornithodoros moubata* and hard tick Ixodes ricinus. J Invertebr Pathol 93, 96-104.

Abstract

A yellow-pigmented Gram-negative bacterium, *Chryseobacterium indologenes*, was found in the gut contents of about 65% of soft tick *Ornithodoros moubata* from a perishing laboratory colony. The isolated putative pathogen, *C. indologenes*, was susceptible to cotrimoxazol and addition of this antibiotic (Biseptol 480) to the blood meal significantly decreased the tick mortality rate. The artificial infection of healthy *O. moubata* by membrane feeding on blood contaminated with *C. indologenes* was lethal to all ticks at concentrations $>10^6$ bacteria/ml. On the contrary, a similar infection dose applied to the hard tick *Ixodes ricinus* by capillary feeding did not cause significant mortality. Examination of guts dissected from infected *O. moubata* and *I. ricinus* revealed that *C. indologenes* was exponentially multiplied in the soft tick but were completely cleared from the gut of the hard ticks within 1 day. In both tick species, *C. indologenes* were found to penetrate from the gut into the hemocoel. The phagocytic activity of hemocytes from both tick species was tested by intrahaemocoelic microinjection of *C. indologenes* and evaluated by indirect fluorescent microscopy using antibodies raised against whole bacteria. Hemocytes from both tick species displayed significant phagocytic activity against *C. indologenes*. All *O. moubata* injected with *C. indologenes* died within 3 days, whereas the increase of the mortality rate of *I. ricinus* was insignificant. Our results indicate that hard ticks possess much more efficient defense system against infection with *C. indologenes* than the soft ticks. Thus, *C. indologenes* infection has the potential to be a relevant comparative model for the study of tick immune reactions to transmitted pathogens.

Paper B

Grunclova L, Horn M, Vancova M, Sojka D, **Franta Z**, Mares M and Kopacek P, 2006.
Two secreted cystatins of the soft tick *Ornithodoros moubata*: Differential expression pattern and inhibitory specificity. Biol Chem 387, 1635-1644.

Abstract

Two genes coding for cysteine peptidase inhibitors of the cystatin family (Om-cystatin 1 and 2) were isolated from a gut-specific cDNA library of the soft tick *Ornithodoros moubata*. Both cystatins were clearly down-regulated after a blood meal. Om-cystatin 1 is mainly expressed in the tick gut, while Om-cystatin 2 mRNA was also found in other tick tissues. Authentic Om-cystatin 2 was significantly more abundant than Om-cystatin 1 in the gut contents of fasting ticks and was associated with hemosome- derived residual bodies accumulated in the gut lumen. Om-cystatin 2 was also expressed by type 2 secretory cells in the salivary glands of unfed ticks. The inhibitory specificity of recombinant Om-cystatins 1 and 2 was tested with mammalian cysteine peptidases, as well as endogenous cysteine peptidases present in the tick gut. Both cystatins efficiently inhibited papain-like peptidases, including cathepsin B and H, but differed significantly in their affinity towards cathepsin C and failed to block asparaginyl endopeptidase. Our results suggest that the secreted cystatin isoinhibitors are involved in the regulation of multiple proteolytic targets in the tick digestive system and tick-host interaction.

Paper C

Buresova V, Hajdusek O, **Franta Z,** Sojka D and Kopacek P, 2009.
IrAM-An alpha2-macroglobulin from the hard tick *Ixodes ricinus*: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. Dev Comp Immunol 33, 489-498

Abstract

The universal protease inhibitors of the $\alpha_2$-macroglobulin ($\alpha_2$M) family are evolutionarily conserved constituents of innate immunity, presumably because they guard organisms against undesired proteolytic attacks of a different origin. Here, we determined the primary structure of $\alpha_2$-macroglobulin from the hard tick *Ixodes ricinus* (IrAM) by sequencing of overlapping PCR products. Predicted bisulfide and glycosylation patterns, post-translational cleavage and alternative splicing within its 'bait region' demonstrate that IrAM is closely related to the $\alpha_2$-macroglobulin from the soft tick *Ornithodoros moubata*. The IrAM message is expressed in all tick developmental stages and tissues, except for the gut, and the protein was detected to be mainly present in the hemolymph. Silencing of IrAM by dsRNA interference markedly reduced the phagocytosis of a potential pathogen, *Chryseobacterium indologenes*, by tick hemocytes both in vitro and in vivo. In contrast, phagocytosis of the Lyme disease spirochete *Borrelia burgdorferi* or a commensal bacteria *Staphylococcus xylosus* was not affected by the IrAM knock-down. Similar results were obtained upon deactivation of all thioester proteins in tick hemolymph by methylamine. We have further demonstrated that phagocytosis of *C. indologenes* is dependent on an active metalloprotease secreted by the bacteria. These data indicate that interaction of tick $\alpha_2$- macroglobulin with a protease of an invading pathogen is linked with cellular immune response.

Paper D

Buresova V, Hajdusek O, **Franta Z**, Loosova G, Grunclova L, Levashina EA and Kopacek P, 2011.
Functional genomics of tick thioester-containing proteins reveal ancient origin of the complement system. J Innate Immun 3, 623-630.

Abstract

Ticks are important ectoparasites and vectors of multiple human and animal diseases. The obligatory hemophagy of ticks provides a formidable route for parasite transmission from one host to another. Parasite survival inside the tick relies on the ability of a pathogen to escape or inhibit tick immune defenses, but the molecular interactions between the tick and its pathogens remain poorly understood. Here we report that tick genomes are unique in that they contain all known classes of the $\alpha_2$-macroglobulin family ($\alpha_2$ M-F) proteins: $\alpha_2$-macroglobulin pan-protease inhibitors, C3 complement components, and insect thioester-containing and macroglobulin-related proteins. By using RNA interference mediated gene silencing in the hard tick *Ixodes ricinus* we demonstrated the central role of a C3-like molecule in the phagocytosis of bacteria and revealed nonredundant functions for $\alpha_2$ M-F proteins. Assessment of $\alpha_2$ M-F functions in a single organism should significantly contribute to the general knowledge on the evolution and function of the complement system. Importantly, understanding the tick immune mechanisms should provide new concepts for efficient transmission blocking of tick-borne diseases.

# CURRICULUM VITAE

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**Education**

1999-2003: Bachelor program, Faculty of Biological Science University of South Bohemia, České Budějovice, Czech republic
Bachelor thesis: Identification of bacteria in the gut contains of the soft tick *Ornithodoros moubata*
Supervisor: RNDr. Petr Kopáček CSc.

2003-2005: Master program: Parasitology, Faculty of Biological Science University of South Bohemia, Česke Budejovice, Czech Republic
Master thesis: "Mucin-like peritrophin" from the soft tick *Ornithodoros moubata*.
Supervisor: RNDr. Petr Kopáček CSc.

Since 2005: PhD program: Parasitology, Faculty of Sciences University of South Bohemia, Česke Budejovice, Czech Republic
PhD. Thesis: Blood-meal digestion in the hard tick *Ixodes ricinus*
Supervisor: RNDr. Petr Kopáček CSc.

2009: RNDr. Examination,
RNDr. Thesis: Mapping of hemoglobinolytic proteases in the gut of the hard tick *Ixodes ricinus*
Supervisor: RNDr. Petr Kopáček CSc

**Employment history**

Since 2005: Research assistant at Laboratory of vector immunology, Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic

**Teaching and mentoring experience**

2005: Teaching assistant, Faculty of Biological Science, University of South Bohemia, Czech Republic. *Biochemistry*, Fall 2005

2006 – 2009: Co-supervisor of Bachelor degree student Petr Franta (project: Exprese rekombinantního katepsinu C - trávicí exopeptidázy klíštěte *Ixodes ricinus*.), Faculty of Science, University of South Bohemia, Czech Republic.

2009 – 2012 Co-supervisor of Master degree student Petr Franta (project: Experimentální vakcinace králíků rekombinantními trávicími peptidázami klíštěte *Ixodes ricinus*), Faculty of Science, University of South Bohemia, Czech Republic.

**Other scientific training**

Summer school on molecular vector biology, České Budějovice, Czech Republic, 20.6.-24.6. 2005

Biology of parasitism – Modern approaches**,** summer course, Woods Hole, MA, USA 7.6. - 6.8. 2007

ICTTD Bioinformatics Workshop, České Budějovice, Czech Republic, 8.6.-15.6. 2008

**International research visits**

4.10.2008 - 9.1. 2009 The Laboratory of Dr. James McKerrow, The Sandler Center for Basic Research in Parasitic Diseases, UCSF, San Francisco, USA

18.8. - 2.10. 2009 The Laboratory of Dr. James McKerrow, The Sandler Center for Basic Research in Parasitic Diseases, UCSF, San Francisco, USA

**Publications**

2006

Buresova V, **Franta Z,** Kopacek P, 2006. A comparison of *Chryseobacterium indologenes* pathogenicity to the soft tick *Ornithodoros moubata* and hard tick *Ixodes ricinus*. J Invertebr Pathol 93, 96-104.

Grunclova L, Horn M, Vancova M, Sojka D, **Franta Z**, Mares M and Kopacek P, 2006. Two secreted cystatins of the soft tick *Ornithodoros moubata*: Differential expression pattern and inhibitory specificity. Biol Chem 387, 1635-1644.

2007

Sojka D, Hajdusek O, Dvorak J, Sajid M, **Franta Z**, Schneider EL, Craik CS, Vancova M, Buresova V, Bogyo M, Sexton KB, McKerrow JH, Caffrey CR and Kopacek P, 2007. IrAE - An asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. Int J Parasitol 37, 713-724.

2008

Sojka D, **Franta Z**, Horn M, Hajdusek O, Caffrey CR, Mares M, Kopacek P, 2008. Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases. Parasit Vectors 1, 7.

2009

Buresova V, Hajdusek O, **Franta Z,** Sojka D and Kopacek P, 2009. IrAM-An alpha2-macroglobulin from the hard tick *Ixodes ricinus*: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. Dev Comp Immunol 33, 489-498.

Hajdusek O, Sojka D, Kopacek P, Buresova V, **Franta Z**, Sauman I, Winzerling J, Grubhoffer L, 2009. Knockdown of proteins involved in iron metabolism limits tick reproduction and development. Proc Natl Acad Sci U S A 106, 1033-1038.

Horn M, Nussbaumerova M, Sanda M, Kovarova Z, Srba J, **Franta Z**, Sojka D, Bogyo M, Caffrey CR, Kopacek P, Mares M, 2009. Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by unctional proteomics. Chem Biol 16, 1053-1063.

2010

**Franta Z**, Frantova H, Konvickova J, Horn M, Sojka D, Mares M, Kopacek P, 2010. Dynamics of digestive proteolytic system during blood feeding of the hard tick *Ixodes ricinus.* Parasit Vectors 3, 119.

2011

Buresova V, Hajdusek O, **Franta Z**, Loosova G, Grunclova L, Levashina EA and Kopacek P, 2011. Functional genomics of tick thioester-containing proteins reveal ancient origin of the complement system. J Innate Immun 3, 623-630.

**Franta Z,** Sojka D, Frantova H, Dvorak J, Horn M, Srba J, Talacko P, Mares M, Schneider E, Craik CS, McKerrow JH, Caffrey CR and Kopacek P, 2011. IrCL1 - the hemoglobinolytic cathepsin L of the hard tick, *Ixodes ricinus*. Int J Parasitol 41, 1253-1262

**Manuscripts in late phase of preparation**

Sojka D, **Franta Z**, Frantova H, Bartosova P, Horn M, Vachova J, O'Donoghue AJ, Eroy-Revelles AA, Craik CS, Knudsen G, Caffrey CR, McKerrow JH, Michael Mares M and Kopacek P. *Ir*CD1 – gut aspartic hemoglobinase of the tick *Ixodes ricinus*

**Selected presentations**

2006 5th International Symposium on Molecular Insect Science, May 20-24, Tucson, Arizona, USA; Mapping of hemoglobin proteolysis in the hard tick *Ixodes ricinus* (poster).

2008 VIth International Conference on Ticks and Tick-borne Pathogenes, September 21-26, Buenos Aires, Argentina; Molecular and biochemical characterization of *Ixodes ricinus* cathepsin B and its role in hemoglobin degradation. (poster)

2009 34th FEBS Congress: Life´s Molecular Interactions, July 4 – 9, Prague, Czech Republic; Functional expression and characterization of cathepsin B and L from the gut of the tick *Ixodes ricinus*. (poster)

2010 The XIIth International Congress of Parasitology - ICOPA, August 15-20, Melbourne, Australia; Characterization and functional expression of *Ixodes ricinus* cathepsin L. (talk)

2011 VIIth International Conference on Ticks and Tick-borne Pathogenes, September 28.8.-2.9., Zaragoza, Spain; IrCL1 and IrCD1 – The acidic enzymes initiating the hemoglobin digestion in the tick gut. (talk)

## Ph.D. DEFENSE

**Date:** 14th of March 2012, České Budějovice, Czech Republic

**Reviewers:**

| | |
|---|---|
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