University of South Bohemia in České Budějovice

Faculty of Science

Factors regulating the expression and activity of digestive enzymes in the tick *Ixodes ricinus*

Master thesis

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Annotation:

The intracellular proteolysis of ingested meal plays an essential role in tick development. The thesis focuses on the factors influencing the expressions and activities of digestive enzymes in *Ixodes ricinus* females during the feeding and post-feeding period. We have revealed the effect of fertilization on blood feeding and digestion. The females cannot reach the rapid engorgement phase without being fertilized. The rate of mated females in the nature proved the presumption that mating can occur even off the host. Implementation of *in vitro* feeding technique further extended our current knowledge about tick digestive apparatus. Adult females were fed on hemoglobin-rich and hemoglobin-poor diet and the mRNA expression levels of digestive proteases were determined. In line with obtained data, we assumed that albuminolysis is conducted by the same or similar pathway as hemoglobinolysis. The gene silencing method and protein immuno-detection were used to unequivocally identify the isoforms of 'early expressed' *Ir*CL1 and 'late expressed' *Ir*CL3 isoform of cathepsin L.

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Abbreviations:

AMC – 7-amino-4-methylcoumarin

DC – digestive cells

DCM – dichloromethane

dsRNA – double stranded RNA

FF – fully fed females

FU – feeding units

G – gut

gfp – green fluorescent proten

***Ir*CB** – *Ixodes ricinus*, cathepsin B

***Ir*CL** – *Ixodes ricinus*, cathepsin L

***Ir*CC** – *Ixodes ricinus*, cathepsin C

***Ir*CD** – *Ixodes ricinus*, cathepsin D

***Ir*AE** – *Ixodes ricinus*, legumain (asparaginyl endopeptidase)

MT – Malpighian tubules

Ov – ovaries

qRT-PCR – quantitative real time PCR

PBS – phosphate saline buffer

RFU – relative fluorescent units

RNAi – RNA interference

RT – room temperature

SG – salivary glands

Tr-FB – trachea and fat body complex

UF – unfed females

WB – western blot

1. Introduction

Acari are the most diverse and abundant group of all arachnids and include terrestrial as well as aquatic species living virtually in every habitat. The group is divided into 2 superorders: Acariformes and Parasitiformes.

Ticks, order Ixodida, superorder Parasitiformes, are obligatory blood-sucking ectoparasites of terrestrial vertebrates. The order Ixodida contains 2 large families: Ixodidae family formed by 702 so far known species and Argasidae family comprising 193 species (Guglielmone et al., 2010). The family Nuttalliellidae represented by a single species, *Nuttalliella namaqua* has been considered as the evolutionary missing link between the other two families (Mans et al., 2011).

Beside the negative effect of imbining large volume of host blood, ticks are of the major concern given to their capability to transmit a wide variety of pathogens comprising bacteria, viruses and protozoa (Jongejan et al., 2004) causing serious diseases to human and domestic animals (de la Fuente et al., 2008). Ticks and tick-borne diseases affect animal and human health worldwide and are the cause of significant economic losses.

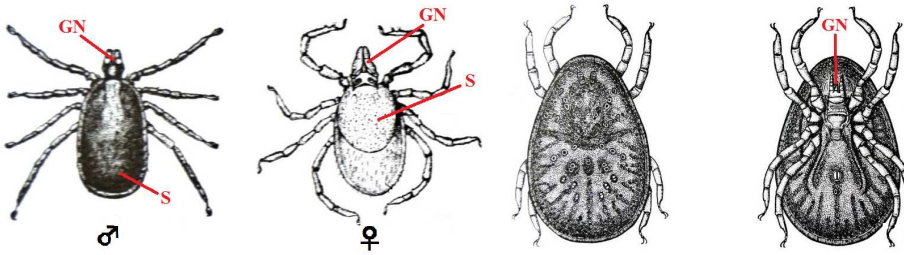
1.1. Argasidae (soft ticks)

Argasidae, also known as soft ticks, behave as nidicolous ectoparasitic species living in arid and semi-arid areas of the world. They have leathery cuticle without the presence of dorsal sclerotized scutum. Gnathosoma, the mouth part, is placed on ventral subterminal side of the body. Life cycle of soft ticks includes a blood-feeding larva, several nymphal stages (often 3 to 4) depending on the blood volume uptake during feeding. After molting from last nymphal stage to adult ticks, both sexes, with minimal sexual dimorphism, can suck blood. The feeding period usually takes about an hour. Blood uptake is not dependent on mating which occurs mostly off the host. Adult females feed repeatedly and lay a small batch of eggs after each blood-meal (Jongejan et al., 2004; Sonenshine et al., 2014).

1.2. Ixodidae (hard ticks)

Ixodidae, also known as hard ticks, are obligatory blood-feeding ectoparasites of all vertebrates. Their dorsoventrally flattened body has sclerotized scutum on dorsal side of the body. The scutum covers about 1/3 of nymphal and female body whereas the sclerotization of male body covers almost whole dorsal surface. All developmental stages (larvae, nymphs and adults) feed once and usually on different hosts. Males from the genus Ixodes do not suck blood; they only fertilize females. Ixodidae ticks are mainly three-host species with few exceptions of one- or two-host species. This characteristic depends on the number of host animals to which they attach within their life cycle (Jongejan et al., 2004). The feeding takes several days (6-8 days in case of females) when ticks can imbibe enormous blood volume facilitated by highly elastic cuticle. Larvae and nymphs molt to another instar after feeding. Females feed for consecutive production of huge batch of eggs and die (Schmidt et al., 1996; Sonenshine et al., 1991). Table 1 shows the major known differences between Argasidae and Ixodidae families.

Table 1. Major differences between Argasidae and Ixodidae family. Adapted according to Sonenshine et al., 1991; pictures adapted from Volf et al., 2007.

	Ixodidae	**Argasidae**
Body morphology		
Body shape	Dorsoventrally flattened	Oval shaped
Gnathosoma (GN)	Terminal	Ventral
Scutum (S)	Present (1/3 of female body, whole male body)	Absent
Life cycle	3 life stages (larvae, nymphs, adults)	More nymphal stages and ovipositions
Sex dimorphism	Males are markedly smaller than females and scutum covers their whole dorsal side	Almost none
Feeding strategy	Once per life stage, takes days	Multiple per life stage, takes about an hour
Oviposition	Female dies after 1 oviposition of huge batch of eggs, usually 1 000–10 000 eggs, (*Amblyomma* up to 23 000 eggs)	200-300 eggs after each feeding of females

1.3. *Ixodes ricinus*

Ixodes ricinus belongs to the largest tick genus, *Ixodes*, comprising more than 240 species (Jongejan et al., 2004). *I. ricinus* occurrence ranges across the Europe but it can also be found in the Middle East and North Africa. It prefers humid areas (mostly forests and grasslands) and mild climate; therefore the tick abundance increases mainly in the spring and subsequently in the fall. Another factor for tick occurrence is winter climate and it has been observed that mild winters resulted in a higher density of ticks during the year (Lindgren et al., 2000). *I. ricinus* has typical tree-host life cycle and every parasitic life-stage (except adult males) feeds on a different host. This complete cycle takes commonly 2-3 years. The six-legged larvae feed on small birds and rodents, drop off and molt into eight-legged nymphs which prefer bigger vertebrate hosts. After molting from nymphal stage, adult females usually feed on bigger wild or domestic animals. Humans can be host of every feeding instar (Volf et al., 2007).

I. ricinus capitulum has specific pentagonal basis with a long cone-shaped hypostome with concentric denticule rows (Mariana et al., 2008), a pair of palps and chelicerae. It lacks eyes but possess a sensory Haller's organ at the terminal segment of the 1st pair of legs. It serves as a detector of changes within the environment (such as temperature, carbon dioxide concentration, humidity and vibrations) while questing for the host. The process of attachment to the host has several important steps: i) the disruption of the host skin via hypostome and chelicerae and insertion to the host (Richter et al., 2013); ii) the attachment and fixation to the host skin by special cement-like protein produced by salivary glands (Goddard, 2008); iii) the salivary glands secretion of protein and lipid factors with anti-coagulative, anti-inflammatory and immunosuppressive effect to avoid the host immune response, host blood coagulation and vasoconstriction (Bowman et al., 2004; Sauer et al., 2000); iv) the osmoregulation and water return to the host assured by salivary glands and their aquaporine water channels (Bowman et al., 2004).

I. ricinus is a vector of several extremely dangerous diseases e.g. Lyme disease, caused by spirochetes of the *Borrelia burgdorferi sensu lato* complexand encephalitis, caused by tick-borne encephalitis flavivirus (Nuttall, 1999). Other rather neglected diseases transmitted by *I. ricinus* are babesiosis, caused by the malaria-like protozoan parasites of the genus *Babesia spp.* (Becker et al., 2009) and ehrlichiosis (also known as tick-borne fever) caused by the bacteria *Anaplasma phagocytophilum* (Stuen, 2007).

1.4. Reproduction

Soft ticks can mate, feed and oviposit small number of eggs several times during the adulthood. By contrast, hard ticks undergo this process only once in their lifespan laying a single massive batch of eggs. Ixodidae ticks from the genus *Dermacentor*, *Amblyomma*, and *Rhipicephalus* are sexually mature (capable to mate) immediately after molting from nymphs. In the genus *Ixodes*, gametogenesis is initiated during the molting period from nymphal stage to the adults and it is fully completed in males within this period. Females finish their gametogenesis after the feeding completion. Until then, the oocytes remain in a diapause, at an early previtellogenic developmental stage (Kiszewski et al., 2001). Fertilized females start to lay eggs several weeks after feeding completion. Each individual egg is coated with hydrophobic lipid-like secretion from Gene's organ, paired egg-waxing secretory organ, to protect eggs from dehydration (Booth, 1989; Sonenshine, 1991). Important regulating factors which play a crucial role in tick reproduction are pheromones, chemicals produced by one tick to influence the social and sexual behavior of others. Engorged and unengorged (questing) females emit volatile substance attracting males and inducing their pre-copulatory behavior e.g. localization of female and its gonopore, hypostom insertion into the female's gonopore (Bouman et al., 1999). Zeme et al. (2002) investigated the attractiveness of *I. ricinus* females in different feeding status (unfed, semi-engorged and engorged females). The study showed that males are attracted mainly to unfed and semi-engorged females. *Ixodes* female was also reported to be capable of repeated insemination, showing that multiple paternity in *I. ricinus* is quite likely to happen (Hasle et al., 2008).

1.5. Blood meal digestion

Hematophagy, the blood-feeding process, developed separately more than 20 times in arthropods such as ticks, mosquitoes, lice, moths and fleas (Mans, 2011). It plays an essential role in the development and reproduction of the blood-feeders and concomitantly, in the transmission of serious human and animal diseases. Blood-feeding habit of ticks serves as a source of nutrients and energy for basal metabolism and egg production (Sonenshine, 1991). Blood digestion in mosquitoes, sand flies, tse-tse flies and other blood-sucking insects, is a rapid process that takes place in the gut lumen and is mediated by trypsin-like serine proteases active in alkaline pH (Wu et al., 2009). By contrast in ticks, the blood digestion is a slower

process occurring intracellularly in the midgut cells (Sonenshine et al., 2014) and the digestion is accomplished by a network of acidic cysteine and aspartic proteases (Horn et al., 2009; Sojka et al., 2013).

1.5.1. Feeding periods of *I. ricinus* female

Blood feeding of *I. ricinus* female takes about 6-9 days. The feeding process may be divided into 3 consecutive stages: i) preparative attachment phase; ii) slow-feeding period; iii) the phase of rapid engorgement (Figure 1). During the preparative phase (first 24-36 hours) the female firmly attaches to the host by cement protein (Coons et al., 1986) and starts to feed. The attachment is followed by slow-feeding period when ticks consecutively uptake small amount of host blood. The blood digestion is initiated and slowly continues during this phase. Acquired energy from the host blood proteins (Arthur, 1970) is used on the cuticle synthesis as the female body largely expands during the last phase of feeding (Kaufman, 1989). The rapid engorgement phase, also named as 'big sip' (usually starts from the 5^{th} or 6^{th} day of feeding) refers to the time when females uptake of about two-thirds of the total blood meal and the rate of digestion increases rapidly. It is generally accepted presumption that rapid engorgement phase occurs only in previously fertilized females. After the completion of blood feeding, females detach from the host and digest the imbibed blood to have enough nutrient supply and energy for egg production and oviposition (Sonenshine, 1991).

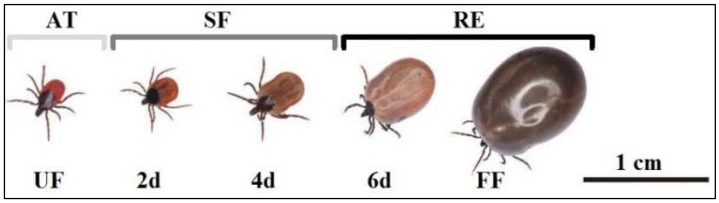


Figure 1. Feeding periods of adult *I. ricinus* female. Feeding process of adult female from the attachment to the detachment phase. **AT** – attachment preparatory phase, **SF** – slow-feeding period, **RE** – rapid engorgement phase, **UF** – unfed, **2d-6d** – days of feeding, **FF** – fully fed female.

1.5.2. Gut morphology and physiology

Tick gut is morphologically and functionally divided into 3 main sections: the foregut, midgut and hindgut. The intracellular digestion part principally occurs in the midgut (Figure 2), the major part of the gut which penetrates almost all body during the feeding. It comprises the central stomach (ventriculus) and several pairs of blind, cylindric furcate caeca (diventricula). The tick gut lumen is considered to be a storage place for nourishment uptake (Coons et al., 1999).

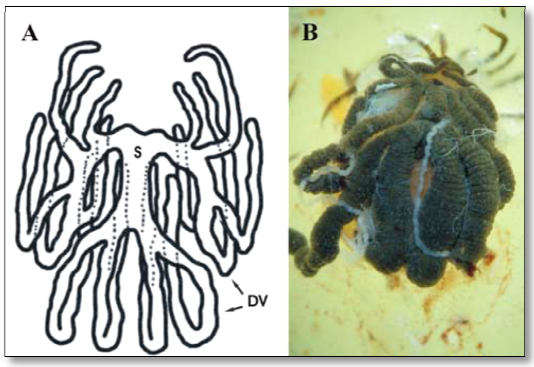


Figure 2. Midgut of *I. ricinus* (Franta, 2012). A – scheme of tick midgut, **S** – stomach, **DV** – diventriculae. **B** – dissected midgut from partially fed female (6 days of feeding). The white thin tubules around DV (in part B) are Malpighian tubules and trachea-fat body complex.

The gut epithelium consists of a single cell layer tacked to the basal lamina and inclosing the inner gut cavity – the gut lumen. Muscle fibers around the cell layer ensure the peristaltic movements of ingested alimentation (Balashov et al., 1983).

The whole midgut is covered by peritrophic matrix synthesized at the beginning of feeding. The matrix thickens with the growing amount of ingested blood (Zhu et al., 1991). Hegedus et al. (2009) showed that peritrophic membrane of arthropods serves as a barrier essential to prevent pathogens as well as indigenous bacteria from penetrating the gut epithelium. The protecting effect of peritrophic matrix was shown also in fruit fly *Drosophila melanogaster*, the common model of developmental biology (Kuraishi et al., 2013). Another recently published study (Narasimhan et al., 2014) presented peritrophic matrix in *Ixodes scapularis* as the shield of spirochetes protecting them against hostile environment of blood-filled gut lumen.

Adult hard ticks undergo 3 digestive phases corresponding to 2 feeding and 1 post-feeding period (slow-feeding, rapid engorgement and post-detachment period). Previously published papers focused on different species of hard ticks (e.g. *Dermacentor variabilis, Rhipicephalus*

appendiculatus) described many different morphological and functional features of digestive cells (Coons et al., 1999). Today's implicit theory is based on Coons et al. (1986) and assumes the existence of only one type of multifunctional digestive cells (DC) which undergo several differentiations during the digestive phases. The first continuous digestive phase evolves usually from the 2nd day of feeding towards the end of slow-feeding period. During this phase, the narrow gut lumen expands with the continuous diet uptake. Undifferentiated digestive cells (also called reserve cells; UDC) start to change into initial digestive cells where endosomes, lysosomes, hemosomes and lipid vacuoles are formed (Agyei et al., 1995; Lara et al., 2003). Endosomes, membrane-bounded compartments, transport diet components to the digestive cells. Lysosomes contain proteases that digest the ingested diet compartments. The excess of heme resulting from hemoglobin digestion forms non-crystaline aggregates stored in hemosomes. These intracellular organelles of digestive cells play a major role in heme detoxicifation (Lara et al., 2003). Four days after attachment, first residual bodies with condensed heme products in hemosomes start to appear together with detached digestive cells (DDC) (Lara et al., 2003). It was previously demonstrated that *R. microplus* lacks the heme synthesis pathway (Braz et al., 1999). In our laboratory we have recently approved, that heme biosynthesis is missing also in the *Ixodes* sp. ticks. Heme needed for tick metabolism and exogenous hemoproteins originates exclusively from the host hemoglobin and is consequently stored in eggs, bound to vitellins (Perner et al., manuscript in preparation). Second digestive phase starts 6 days post attachment and involves a lot of fully developed digestive cells (Figure 3). This phase of rapid digestion may occur likely only in fertilized females. More DDC are deplenished by peristaltic movements through the rectal sac (Sonenshine, 1991). The DDC are renewed by reserve digestive cells also prepared to differentiate. Last digestive phase is initiated right after detachment from the host. The acquired energy is used for vitellogenesis and oviposition (Coons et al., 1986).

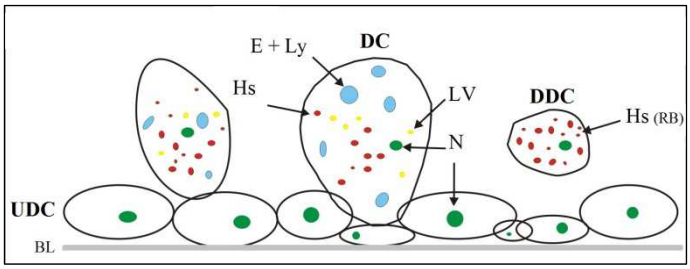


Figure 3. Midgut epithelium scheme of partially fed female. Midgut morphology of female in rapid-engorgement phase of feeding. **UDC** – undiferentiated digestive cells, **DC** – digestive cells, **DDC** – detached digestive cells, **BL** – basal lamina, **Hs** – hemosomes, **E + Ly** – endosomes and lysosomes, **LV** – lipid vacuoles, **N** – nucleus, **(RB)** – residual bodies (adapted from Franta et al., 2010).

1.5.3. Uptake of blood meal

Hemoglobin and albumin are the most abundant protein constituents of host blood and tick main source of nutrients and amino acids. Lara et al. (2005) reported 2 different types of endocytosis which serve to transport those macromolecules from the gut lumen to the digestive cells. Hemoglobin is transported via receptor-mediated endocytosis (including specific hemoglobin receptors), while albumin transport is assured by pinocytosis of small globules known as fluid phase endocytosis (Lara et al., 2005). The aggregations of transported vesicles fuse into endosomes, subsequently merge with primary lysosomes and create secondary lysosomes where the digestion is carried out in acidic pH (Lara et al., 2003; Sojka et al. 2013).

1.5.4. Tick digestion of host blood

In contrast to most hematophagous arthropods which digest the host blood extracellularly, tick digestion takes place inside the digestive cells of midgut epithelium. The only exception is the hemolysis which occurs in the midgut lumen (Sonenshine, 1991; Hovius et al., 2007). The hemolysis mechanism in ticks has not yet been completely understood, however, the presence of serine proteases (with optimum at pH 6.0) was shown in the midgut lumen during the hemolysis of host erythrocytes in *Haemaphysalis longicor*nis (Miyoshi et al., 2007) and *Dermacentor variabilis* (Tarnowski et al., 1989). Once the hemoglobin is released, it tends to form quite large crystals (Smit et al., 1977) with unclear function. The crystals may serve as a reserve stock of nutrients or a protection against a potential danger from hemoglobin excess (Sojka et al., 2013). Previous thought, that there exists only one digestive enzyme, referred to as hemoglobinase, was displaced by the evidence of a number of hemoglobinolytic proteases characterized in different tick species (Table 2). Intracellular blood digestion in ticks is processed by cysteine and aspartic proteases (Sojka et al., 2008; Horn et al., 2009). This digestive system more resembles nematodes and platyheminths (Williamson et al., 2003; Delcroix et al., 2006). Blood digestion in ticks is targeted to secondary lysosomes by heterophagy (i.e. fusion of endosomes with primary lysosomes) (Sonenshine, 1991).

Table 2. Cysteine and aspartic proteases from different hard tick species participating in blood digestion.

Species	Protease	Specificity	Tissue	Reference
Haemaphysalis longicornis	Longepsin	Cathepsin D	G, SG	Boldbaatar et al., 2006
	Longipain	Cathepsin B	G	Tsuji et al., 2008
	H1CPL-A	Cathepsin L	G	Yamaji et al., 2009
	H1Lgm	Legumain	G	Alim et al., 2008
Rhipicephalus microplus	BmAP	Cathepsin D	G	Cruz et al., 2010
	BmCL1	Cathepsin L	G	Cruz et al., 2010
Ixodes ricinus	IrCB	Cathepsin B	G	Franta et al., 2010
	IrCL	Cathepsin L	G, SG, Ov, MT	Franta et al., 2011
	IrCC	Cathepsin C	S, SG, OV, MT	Franta et al., 2010
	IrAE	Legumain	G	Sojka et al., 2007
	IrCD	Cathepsin D	G	Sojka et al., 2012

G – gut, **SG** – salivary glands, **Ov** – ovaries, **MT** – Malpighian tubules

In *I. ricinus*, the hemoglobinolysis occurs at the acidic range 3.5–4.5 pH of the digestive vesicles (Sojka et al., 2013). Hemoglobin digestion is a process of consequent cleavages (mediated by several digestive enzymes) into large protein fragments, small hemoglobin-derived peptides and dipeptides or single amino acids (Horn et al., 2009). Figure 4 presents the hemoglobinolytic pathway and shows formation of heme aggregates and antimicrobial fragments (hemocidins). Antimicrobial fragments may be acquired from different parts of hemoglobin protein molecule and have antimicrobial activity against Gram-positive bacteria and fungi (Fogaça et al., 1999; Cruz et al., 2010). The initial cleavage is assured by 3 endopeptidases: cathepsin D (*Ir*CD), cathepsin L (*Ir*CL) and legumain (*Ir*AE). Small hemoglobin fragments are then derived from large fragments by cathepsin B (*Ir*CB) and cathepsin L endopeptidase activity. The final cleavage into dipeptides is made by exopeptidase activity of cathepsin C (*Ir*CC) and cathepsin B (Sojka et al., 2013). The final cleavage into single amino acids is assured by monopeptidases, namely a serine carboxy-peptidase and a leucine metallo-aminopeptidase (Horn et al., 2009).

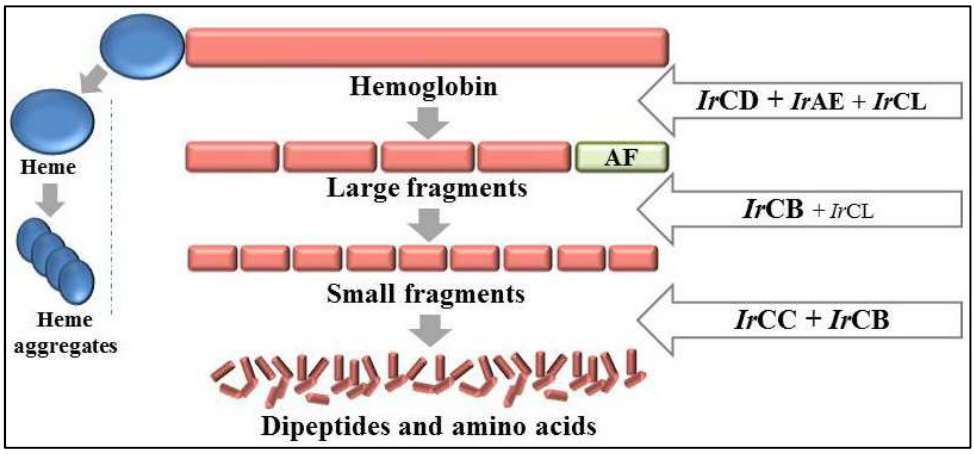


Figure 4. Pathway of *I. ricinus* hemoglobinolysis (adapted from Horn et al., 2009). A model for the proteolytic pathway of hemoglobin cleavage in differentiated digestive cells of midgut epithelium. Big blue oval represent the heme prosthetic group forming heme aggregates accumulated finally in hemosomes (Lara et al., 2003). ***Ir*CD** – cathepsin D (Sojka et al., 2012), ***Ir*AE** – legumain (Sojka et al., 2007), ***Ir*CL** – cathepsin L (Franta et al., 2011), ***Ir*CB** – cathepsin B and ***Ir*CC** – cathepsin C (Sojka et al., 2008; Franta et al., 2010), **AF** – antimicrobial fragments.

1.6. Digestive proteases

In our laboratory, we have focused on the research of 5 major types of digestive proteases in *I. ricinus* tick described in detail further in the text.

1.6.1. Aspartic proteases

Three paralogs of aspartic cathepsin D endopeptidases (clan AA, family A1[*]) were identified in the genome dataset of *Ixodes scapularis* : ISCW013185, ISCW003823, and ISCW023880 named as cathepsin D1, cathepsin D2 and cathepsin D3. Accordingly, orthologs of the cathepsins D isoforms were identified in *I. ricinus* tick and named cathepsin D1 (*Ir*CD1), cathepsin D2 (*Ir*CD2) and cathepsin D3 (*Ir*CD3) – for GenBank numbers see table 5. *Ir*CD1 is synthesized in digestive cells in the form of pre-proenzyme. This precursor of inactive proenzyme (~47 kDa) includes a signal sequence for the transport to the lysosomes where it is activated by autocatalytic reaction in acidic pH to form an active *Ir*CD (~40 kDa) (Sojka et al., 2012). *Ir*CD1 (along with cysteine proteases *Ir*AE and *Ir*CL) initiates the hemoglobin cleavage into the secondary large fragments – the polypeptides (Horn et al., 2009).

[*] - http://merops.sanger.ac.uk/cgi-bin/clansum?clan=AA

1.6.2. Legumain-like asparaginyl endopeptidases

Legumain-like asparaginyl endopeptidase (*Ir*AE) was identified in *I. ricinus* by Sojka et al. (2007). *Ir*AE belongs to the cysteine proteases from clan CD, family C13[*]. Its pro-enzyme has molecular weight of approximately 47 kDa and the molecular mass of the active *Ir*AE is ~38-40 kDa. *Ir*AE is active at acid pH, optimally at 4.0-4.5 pH (Sojka et al., 2007). The enzyme has strict cleavage specificity with the asparagine residue at the P1 position (Chen et al., 2000). IrAE participates in cleavage of hemoglobin into large fragments. In our laboratory, we currently distinguish 2 paralogs of legumain: *Ir*AE1 (GenBank accession No. AY584752) (Sojka et al., 2007) and *Ir*AE2 which has not yet been deposited in GenBank (*I. ricinus* sequence was determined based on the *I. scapularis* legumain with GenBank accession No. XM_002402043).

[*] - http://merops.sanger.ac.uk/cgi-bin/famsum?family=C13

1.6.3. Papain-like cysteine proteases

Papain-like cysteine proteases belong to the clan CA, family C1[*]. Sojka et al. (2008) and Franta et al. (2010) identified 3 major types of papain-like proteases in *I. ricinus*: cathepsin L (*Ir*CL), cathepsin B (*Ir*CB) and cathepsin C (*Ir*CC).

[*] - http://merops.sanger.ac.uk/cgi-bin/famsum?family=C1

The sequence, phylogenetic relationship and characterization of *Ir*CL was described by Sojka et al. (2008). The moleucular mass of the proenzyme is ~40 kDa and active form *Ir*CL is ~28 kDa. This endopeptidase is active in acidic pH (optimum 4.0). *Ir*CL cleaves the hemoglobin into large fragments and also participates in the cleavage into small peptides (Horn et al., 2009). The detailed biochemical and functional characterization of *Ir*CL1 was described by Franta et al. (2011). Later, two other isoforms of cathepsin L were identified in our laboratory. Further studies suggest that *Ir*CL3 likely plays a role in the later stage of blood digestion while the function of *Ir*CL2 still remains unclear.

*Ir*CB has a dual function in hemoglobinolysis. It acts as an endopeptidase, cleaving the large hemoglobin fragments into small fragments, and also exerts an exopeptidase (carboxy-dipeptidase) activity in the last part of hemoglobin cleavage. Regarding the amount of each protease, *Ir*CB is the most abundant enzyme in the hemoglobinolytic pathway

(Horn et al., 2009). *Ir*CB proenzyme has about 36 kDa and its active enzyme is 30-32 kDa (Sojka et al., 2008).

*Ir*CC cysteine protease is an exopeptidase that cleaves dipeptides from the substrate N-terminus, therefore it is also named as dipeptidyl peptidase I. *Ir*CC is active mainly during the cleavage of small hemoglobin peptides into dipeptides (Sojka et al., 2008; Horn et al., 2009).

1.7. Artificial (*in vitro*) feeding of ticks

The attempts to feed hard ticks *in vitro* on membranes date back to 1956, when the air cell membrane was made from an embryonated hen egg (Pierce and Pierce, 1956). First silicone membrane for tick feeding was made by Habedank and Hiepe in 1993 and *I. ricinus* tick was firstly successfully fed *in vitro* in 1996 (Kuhnert, 1996). The development of artificial feeding techniques is an extremely difficult process. The complexity of tick feeding behavior bears many variables (e.g. duration of feeding, temperature, humidity, olfactory attachment stimuli, blood supplements etc.) which must be optimized to achieve similar efficiency to compare *in vitro* feeding as the *in vivo* feeding. However, artificial feeding brings many advantages such as reduced number of laboratory animals, the ability to define the nutrient diet source and the conditions (Kröber and Guerin, 2007). Nowadays, the in vitro feeding techniques based on Kröber and Guerin (2007) offer promising alternatives to explore the tick-host-pathogen interactions. Finally, the automation and miniaturization of *in vitro* feeding in multi-well plates would help to establish high-throughput systems to test products against ticks and the pathogens they transmit (Kröber and Guerin, 2007).

2. Objectives

- To reveal the effect of fertilization of *Ixodes ricinus* females on blood feeding
- To measure the activity of digestive enzymes (namely cathepsin B, L, C and legumain) in virgin and mated females during blood feeding period
- To detect tissue specificity for individual isoforms of digestive enzymes (namely cathepsins B, L1, L3, C, D1, D2, D3 and legumains AE1, AE2) by quantitative real-time PCR method
- To disclose the dynamic expression profiles of these digestive enzymes depending on two diet types: hemoglobin-rich (whole blood) and hemoglobin-poor (serum), using *in vitro* membrane feeding technique
- To explore the effectiveness of RNA interference for further studies of genes expressed during on-host feeding and off-host digestion periods

3. Materials and methods

3.1. Collecting and rearing ticks

Ixodes ricinus virgin females used in this work were reared in the animal facility of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice. Ticks were kept in optimal conditions: 25 °C and 96% humidity. To prevent mating, adult *I. ricinus* females and males were separated immediately after molting from nymphal stage. Subsequently, both groups were let 5 weeks to mature before the following feeding/mating experiments.

For other experiments in the thesis, *I. ricinus* females were collected by flagging in the forests around České Budějovice, Czech Republic.

Females were naturally fed on the guinea pigs (25 females each) in the presence of the same amount of males. In case of virgin females, they were fed without the males to prevent from mating. Females were forcibly removed from the guinea pigs at specific time points during feeding (see results 4.1). Unfed females from every studied group were included for consequent methods and experiments.

3.2. Tick gut dissection and its homogenization for activity assays

Pooled gut tissues from each time point (from each group) were dissected under the stereo microscope with LED illumination (Stemi DV4, Zeiss) and were used for activity assays of digestive peptidases. Dissected gut tissues were washed in sterile PBS (Table 3) to remove excessive blood from gut lumen without the disruption of gut epithelial cells.

Dissected gut samples were distributed in 300 µl of ice-cold Na-acetate buffer (Table 3) in 1.5 ml Eppendorf tube and homogenized by 3 times repetition of freezing in liquid nitrogen and subsequent grinding with sterile plastic pestles. Next, the plastic pestles were washed with 200 µl Na-acetate buffer and the homogenates were supplemented with CHAPS (Table 3) to a final concentration of 1%. Finally, the samples were 30 minutes incubated in thermomixer (Eppendorf) (4 ºC, 1400 rpm) and cleared by centrifugation (16 000×g, 4 ºC, 10 min) in Multifuge 3 (Hereaus). The supernatant aliquots were stored in -80 ºC until being used for activity measurements.

Table 3. Solutions for tissue dissection and homogenization

Solution	Composition
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 in distilled H_2O
Na-acetate	0.1 M sodium acetate buffer, pH 5.0
CHAPS	10% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (SIGMA-ALDRICH) in distilled H_2O

3.3. Determination of individual peptidase activities

The level of peptidases during feeding was analyzed by activity profiling in gut extracts. Relative enzymatic activity of *Ir*CB, *Ir*CL, *Ir*CC and *Ir*AE was detected by specific fluorogenic substrates using the Infinite M 200 (Tecan) 96-well microplate fluorimeter. Table 4 summarizes the specific substrates, pH and inhibitors used for measuring the activity of individual peptidases in 360/465 nm excitation/emission. Gut extracts were appropriately diluted in the assay buffers to achieve approximately 1500 RFU/min. Enzyme activity was measured for at least 10 minutes and calculated from the linear portion of the kinetic curve. All measurements were performed in three technical replicates. The measured activities were mathematically normalized per one tick gut (RFU/min/gut).

Table 4. Assay conditions for the fluorimetric measurements of digestive peptidases activities

Enzyme	Assay buffer	pH	Substrate (final concentration)	Shielding inhibitor (final concentration)
***Ir*CB**	0.2 M NaH_2PO_4 2.5 mM DTT[a] 1 M EDTA	5.5	Z-Arg-Arg-AMC* (5 μM)	-
***Ir*CL**	0.05 M citric acid 0.1 M NaH_2PO_4 0.1 M NaCl 2.5 mM DTT[a]	4.0	Z-Phe-Arg-AMC* (5 μM)	CA-074 (2.5 μM) [b]
***Ir*AE**	0.05 M citric acid 0.1 M NaH_2PO_4 0.1 M NaCl 2.5 mM DTT[a]	4.0	Z-Ala-Ala-Asn-AMC* (10 μM)	CA-074 (2.5 μM) [b]
***Ir*CC**	0.1 M NaH_2PO_4 0.025 M NaCl 2.5 mM DTT[a]	5.5	Gly-Arg-AMC* (40 μM)	-

* – AMC fluorogenic substrates (Bachem), [a] - dithiothreitol (Thermo Scientific), [b] – specific inhibitor of cathepsin B (Sigma-Aldrich)

3.4. *In vitro* tick feeding

3.4.1. Diet preparation

Bovine blood was acquired from the slaughterhouse, České Budějovice. It was immediately, cooled on ice and defibrinated by stirring. Next, the remaining fibrinogen was disposed by filtration through the sterile strainer and the blood was supplemented with sterile-filtered glucose (final concentration 2 g/l) and stored at 4 °C (Kuhnert, 1996). Serum was prepared from filtered blood by centrifugation (2 500×g/10 min, slow deceleration), subsequent serum phase collection and additional centrifugation (10 000×g/10 min). Final serum phase was glucose-supplemented (final concentration 2 g/l) and stored at 4 °C.

3.4.2. Cow-hair extract preparation

The volume of 250 ml of dichloromethane (DCM) was added to 50 g of cow hair and left to shake for 20 min/RT. The half volume was collected; the second half was replenished with 100 ml of fresh DCM and shaken for additional 20 min. The process was repeated twice. All collected solutions were combined and solid parts were disposed by filtration. The concentration of cow-hair scent extract was assessed by weighing the dry matter from 1 ml of the extract.

3.4.3. Preparation of feeding units

Feeding unit (FU) preparation mostly followed the published *in vitro* feeding assay protocol (Kröber and Guerin, 2007); except the using of plastic FU (Miroslav Kubík – PLEXI, České Budějovice) instead glass FU. Preparation of 10 membranes included 15 g of the silicone (Wacker silicone E4), 5 g of the silicone oil (FLUKA DC 200 silicone oil), 0.15 g of the color paste (Wacker FL colour paste) and 2.9 g of n-hexane (VWR chemicals). The upper edges of lens cleaning papers (Tiffen) were attached to the smooth plastic board. The prepared silicone mixture was spread with silicone spatula over the lens papers and was let dry overnight. The membrane thickness was measured with micrometer on 4 different places within the paper range and only those with thickness of 60 – 100 µm were used for FU completion. Subsequently, thin silicone layer was applied onto the FU bottom rim; FU were stuck to the membranes and let harden. Membranes were cut to adjust the FU shape. For tick attachment support, the circular grids were stuck to the FU bottom and the plastic tile spacers were glued to the grid center for elasticity reduction (Figure 5). Before tick placing, each FU membrane

was perfumed with 640 ng of cow scent (appropriate amount of cow-hair extract on the membrane was let dry overnight).

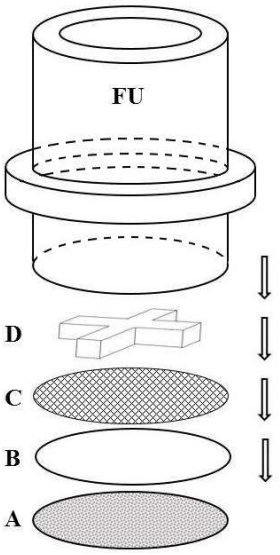


Figure 5. Feeding unit folding. All components used for FU preparation. Arrows show the folding concept of FU. **A** – lens cleaning paper, **B** – silicone membrane, **C** – plastic grid, **D** – plastic tile spacer, **FU** – feeding unit.

3.4.4. Tick emplacement into feeding units

Feeding units with the same parameters were prepared for each diet. Fifteen adult tick females were put into each FU and were let 24 hours to attach to the membrane. Afterwards, non-attached females were taken out. The equal number of adult tick males was put inside each FU to ensure full engorgement of the females. The females were fed, forcibly removed at specific time points or let finish the feeding.

3.4.5. Diet supply

Both diets (blood and serum – Figure 6) were changed every 8 hours. Each well was filled with 3.1 ml of diet and subsequently supplemented with ATP* (final concentration 1 mM) and gentamicin (Sigma) antibiotic (final concentration 5 µg/ml). Each FU was washed in sterile 0.9% NaCl before the transfer to the fresh diets. To assure natural-like conditions during feeding, plastic plates with diet and FUs were placed in water bath filled with distilled water (37 °C).

* - 10mM ATP (FLUKA), filter-steriled, (0.22 µm filter)

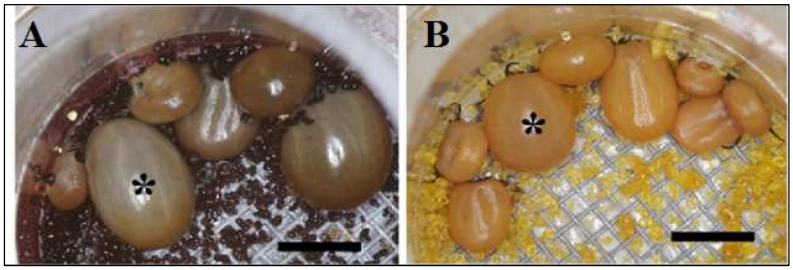


Figure 6. Females fed *in vitro* on blood and serum diet. A – Blood fed females **B** – Serum fed females. ***** - Fully engorged females. Scale bar – 0.5cm.

3.5. Tick dissection for RNA isolation and reverse transcription

Sterile PBS in DEPC H_2O* was used to dissect tissues (gut, salivary glands, ovaries, trachea-fat body complex, Malphigian tubes and rest of the bodies) from each time point (from each group) under the stereo microscope with LED illumination (Stemi DV4, Zeiss). To remove excessive blood from gut lumen or from blood-contaminated tissues, the tissues were washed in PBS (in DEPC H_2O) without the disruption of gut epithelial cells. Next, total RNA was isolated following the protocol (NucleoSpin® RNA, Macherey-Nagel) except elution volume which was 40 µl / sample. To verify the isolation, samples concentrations were measured by the spectrophotometer (NanoDrop™ 1000) and subsequently the quality of RNA was checked on the 1% agarose gel (see results, Figures 13, 14). Isolated RNA samples were used for cDNA synthesis following the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) protocol using the anchored-oligo $(dT)_{18}$ primer.

* - see PBS in table 3 + diethyl pyrocarbonate 2000x diluted in distilled H_2O, autoclaved, filtered (0.22 µm filter)

3.6. Quantitative real-time PCR

The cDNA tissue samples served as templates for the following expression analyses by quantitative real-time PCR (qRT-PCR) using the Roche LightCycler 480 instrument and SYBR green chemistry. For primers used for individual proteases and reference genes, see Table 5.

A universal scheme for 1 reaction master mix (total volume 25 µl):

12.5 µl Master mix (FastStart Universal Sybr Green Master, Rox)

0.25 µl Forward primer (100µM)

0.25 µl Reverse primer (100µM)

10 µl H_2O (nuclease free)

2 µl template

Reaction conditions followed protocol (95 °C/10s – denaturation, 60 °C/10s – annealing, 72 °C/10s – extension) in 50 cycles and included the melting curve analysis. The expression profiles from adult *I. ricinus* females tissues were normalized to actin (reference gene) and the gut tissue samples expression profiles were normalized to elongation factor 1α (Urbanova et al., 2014).

Table 5. List of qRT-PCR primers used for individual proteases and reference genes expression assay

Gene	GenBank accession No.	Primer names	Forward primer	Reverse primer	Amplicon length
IrCB	EF428206	B-RealTime-S B-RealTime-AS	tcaacaagatcaacacaacttgg	tcatggagatggatttgtcg	60 nt
***Ir*CL1**	EF428205	CatL1-qPCR-F CatL1-qPCR-R	agaaccagggacagtgtgga	ctcttgcggaagtgctgtc	76 nt
***Ir*CL3**	XM_002405329*	*Ir*CL3-RealTb-S *Ir*CL3-RealTb-AS	aatgcgtcttcgacaagtcc	gtctcgtcgcctgtctgc	73 nt
***Ir*AE1**	AY584752	*Ir*AE-RT-S *Ir*AE-RT-AS	cgaaaccgtgctttcctg	tcagtcttctcagcgtcacc	77 nt
***Ir*AE2**	XM_002402043*	*Ir*AE2-RT-S *Ir*AE2-RT-AS	cattcttgcaagcggtga	gctcggtttttcaacggtaa	72 nt
***Ir*CC**	EU128750	CathC_S-realT CathC_AS-realT	caccaagaacagggtgaagaa	ctcgcaaccctgagagtagg	76 nt
***Ir*CD1**	EF428204	CathD1-RT-S CathD1-RT-AS	gacagaaggcggacagtacc	cggaaattgtgaaggtgacat	74 nt
***Ir*CD2**	HQ615697	CatD2-qPCR-F CatD2-qPCR-R	agtgccctttcaccaacatt	ctcgacttgcggctgtagta	61 nt
***Ir*CD3**	HQ615698	*Ir*CD3-RT-S *Ir*CD3-RT-AS	ctcaagatcacgcagtttgg	aatgtccagccccacaaag	60 nt
EF1α	GU074769.1	EF F EF R	acgaggctctgacggaag	cacgacgcaactccttcac	80 nt
Actin	AY898751.1	Actin F Actin R	gatcatgttcgagaccttca	cgatacccgtggtacga	92 nt

EF1α – elongation factor 1α (reference gene), * - *Ixodes ricinus* sequences were determined based on the *I. scapularis* genome sequencing, not yet deposited in the GenBank.

3.7. SDS-polyacrylamide gel electrophoresis and Western blot

Solutions used for this method are described in the Table 6. Gut tissues dissected from 6 females per each time point were homogenized in Na-acetate buffer (without adding CHAPS – see chapter 3.2). Gut extracts were then appropriately diluted in sample reduction buffer, denatured (10 min/100 °C) and centrifuged (16 000×g/5 min). Sample volume was calculated to contain ~0.13 gut per lane. Protein marker was used as a standard. The SDS-polyacrylamide gel electrophoresis (SDS PAGE) was run in 5% - 17.5% polyacrylamide gradient gel in electrophoretic buffer (100 V/10 min and subsequently 200 V). Proteins were subsequently transferred from the gel to the polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) previously immersed in methanol. The western blot sandwich was built from filter papers, PVDF membrane and gel pre-soaked in blotting buffer. Protein transfer was run in constant electric current (150 mA/90 min). Protein marker transferred to the membrane was stained in staining solution and the background was washed-out in destaining solution. The rest of the membrane was incubated 1 hour/RT in the blocking buffer. Blocked membranes were incubated 2 hours/RT separately in primary *Ir*CL1 and *Ir*CL3 antibodies[*] diluted in blocking buffer (1:50). Before applying the secondary antibodies, the membranes were thoroughly washed 3 times for 10 min in PBS-Tween. Secondary anti-rabbit antibodies conjugated with peroxidase (Anti-Rabbit IgG (whole molecule)-Peroxidase, Sigma-Aldrich) were diluted in PBS-Tween (1:2000) and incubated 1 hour/RT. To remove excess unbound antibodies, the membranes were carefully washed 5 times for 10 min in PBS-Tween. Finally, the specific antigens were detected by the peroxidase reaction using DAB substrate buffer.

[*] - Primary antibodies were previously prepared in our laboratory according to the protocol described in Kopacek et al., 2003, Franta et al., 2011.

Table 6. Solutions for SDS PAGE and Western blot analysis

Solution	Composition
Sample reduction buffer	0.75 M Tris/HCl, pH 6.8, 5% SDS (w/v), 50% (v/v) glycerol, 32 mM DTT, 0.25% (w/v) bromophenol blue
Electrophoretic buffer	25 mM Tris, 192 mM glycine, 0.1% SDS (w/v)
Staining solution	0.05% Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid
Destaining solution	50% (v/v) methanol, 10% (v/v) acetic acid
Blotting buffer	25 mM Tris, 192 mM glycine, 20% methanol, 0.04% (w/v) SDS
Protein marker (LMW)	LMW Electrophoresis Calibration Kit (GE Healthcare)
PBS-Tween	1× PBS, 0.05% (v/v) Tween 20
Blocking buffer	5% (w/v) nonfat skim milk in PBS-Tween
DAB substrate buffer	100 mM Tris-HCl pH 7.5 with added H_2O_2 (100 µl/100 ml) and 3-3'diaminobenzidine (DAB) (50 µg/100 ml)

3.8. Silencing gene expression by RNA interference

RNA interference (RNAi) gene silencing was performed following the protocol routinely used in our lab (Buresova et al., 2009; Hajdusek et al., 2009). Briefly, 403 bp long *Ir*CL1 fragment was amplified from partially fed female gut tissue cDNA using specific forward 5'-ATGGGCCCCAGTGTGGATCCTGCTGG-3' and reverse 5'-ATTCTAGAGC TCGTCGTACACACCG-3' primers (previously designed Franta et al, 2011). Fragment (376 bp) used for *Ir*CL3 silencing was amplified using designed forward 5'-ATGGGCCCCAACAGCTTCAGAGGAGTCAG-3' and reverse 5'-ATTCTAGAGA AGACGCATTGACCATCCTC-3' primers. Both primer pairs included *Apa*I and *Xba*I restriction sites (underlined). The amplified *Apa*I/*Xba*I restricted PCR products were cloned into restricted linearized pll10 plasmids, subsequently transformed into One Shot® TOP10 Chemically Competent *E. coli* and grown overnight on LB plates in the presence of ampicillin (100µg/ml). White colonies were tested for positivity by PCR using M13 forward and reverse primers from TOPO cloning kit (Invitrogen). Following the protocol, pll10 plasmids were isolated by NucleoBond® Xtra Midi/Maxi kit (Macherey-Nagel). Purified linearized plasmids with the *Ir*CL1/*Ir*CL3 inserts served as templates for ssRNA synthesis by MEGA script T7 transcription kit (Ambion). Purified ssRNAs were equally hybridized overnight and the dsRNA quality was checked on 1% agarose gel. dsRNA (0.5 µl of 3 µg/µl) was injected into the hemocoel of unfed female ticks using a micromanipulator (Narishige) under the stereomicroscope. Control ticks were injected with the same volume of previously synthesized *gfp* dsRNA. Injected females

were allowed to rest for 3 days and then placed on guinea pigs to naturally feed. Gut tissues from injected females were dissected at specific feeding time points and were processed to RNA isolation and reverse transcription (as described above). Each time point sample from each group was made in biological triplicate. Prepared cDNAs were used for gene silencing verification by qRT-PCR using SYBR green dye and gene-specific primers (Table 5). The relative expression of *Ir*CL1, *Ir*CL3 and *gfp* genes was normalized to the elongation factor 1α (reference gene).

4. Results

4.1. The effect of fertilization of tick *Ixodes ricinus* females on blood feeding and digestion of host blood

This part of the work is focused on the fertilization influence of *I. ricinus* females and its impact on the dynamics of digestive enzymes activities during blood-feeding period on the host. To prevent mating, adult *I. ricinus* females were separated from males immediately after molting from nymphal stage. Virgin females were allowed to feed naturally on laboratory guinea pigs (25 females on each). First group of virgin females was fed without the presence of males, second group was fed together with males as the control (mated females).

4.1.1. Virgin and mated female weights comparison

Firstly, mated and virgin females were forcibly removed from the guinea pigs at specific time points of feeding (2, 4, 6 days post attachment). Secondly, the rest of females from both groups (virgin and mated females) were let to accomplish the feeding. Unfed, fed and fully fed females (8 females/group/time point) were weighed during the feeding (Figure 7). The whole experiment was repeated 3 times.

The weights of virgin and mated females showed no difference in unfed females and in females 2 days after attachment to the host. The first evident weight difference between virgin and mated females was observed after 4 days of feeding. The biggest weight differences of both groups were detected 6 and 8 days after attachment that corresponded to the rapid-engorgment phase (Franta et al., 2010). Mated females weighed almost 10 times more than virgin females at the same time points. All mated and virgin females detached from the guinea pigs 8 days post attachment. However, virgin females did not evidently reach the rapid engorgement phase and were not capable to fully engorge (Figure 7, 8).

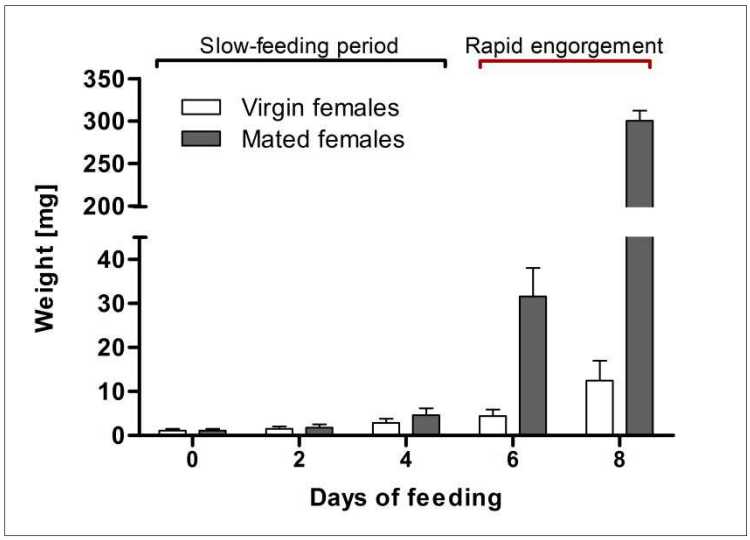


Figure 7. Virgin and mated females weights comparison. For each time point (**0** – unfed; **2, 4, 6, 8** days of feeding), 8 females from both groups (virgin/mated females) were weighed. The experiment was made in three biological replicates. Error bars represent the standard errors of three independent biological replicates. Mated females were capable to imbibe the largest portion of blood volume during rapid engorgement phase. Virgin females did not get into this stage.

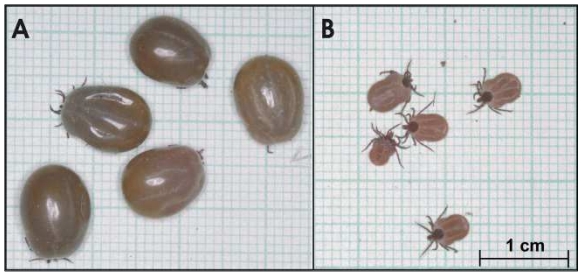


Figure 8. Size differences of virgin and mated *I. ricinus* females. Differences were assessed after 8 days of feeding. **A** - fully fed mated females, **B** - virgin females.

4.1.2. Activity profiles of digestive enzymes of virgin and mated *I. ricinus* females during blood-feeding on the host

Virgin and mated female gut extracts were used to measure the activity of 4 major digestive peptidases: cathepsin B (*Ir*CB), cathepsin L (*Ir*CL), cathepsin C (*Ir*CC) and legumain (*Ir*AE). Female gut tissue extracts were prepared from virgin and mated females at specific time points: unfed females; females 2, 4, 6, 7 days post attachment and fully fed females. The experiment was performed in 3 biological replicates: set A, B and C (set A includes females only from the 5^{th} day of feeding to fully fed females). Specific fluorogenic substrates (Table 4) were used to detect the activity of digestive peptidases in relative fluorescent units (RFU).

*Ir*CB activity was negligible in unfed females and 2 days post attachment to the host in all sets (Figure 9). The low level activity of *Ir*CB in virgin females did not remarkably change during the whole feeding period. It slightly increased the 6th day of feeding in virgin females set A and C, and in fully fed virgin females from set B. By contrast, the activity of *Ir*CB in mated females dramatically increased four days post attachment in all sets, reaching its maxima in semi-engorged females (6th day of feeding). Fully fed mated females had the relative activity of *Ir*CB at about 60 % of their measured maxima.

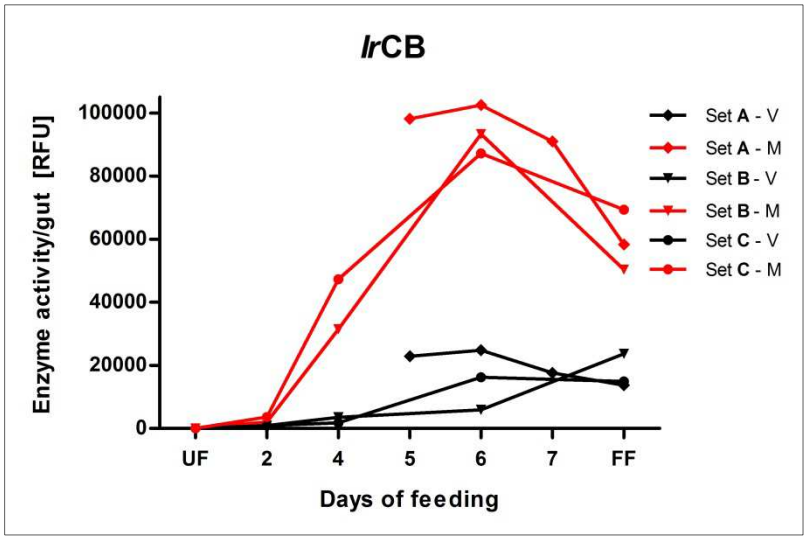


Figure 9. *Ir*CB dynamic profiles in gut extracts from virgin and mated females during feeding. Relative enzymatic activities measured in the gut homogenates were normalized per one gut tissue. Lines show 3 sets (**A, B, C**) of virgin and mated females *Ir*CB activities which correspond to three biological replicates. The points display average activities obtained from three technical replicates (standard deviations were ≤5% of the average values and are not shown). **V** - virgin females, **M** – mated females, **UF** – unfed, **FF** – fully fed.

*Ir*CL activity in virgin females was imperceptible in first two days of feeding, gently increased after 4 days of feeding, but its maxima (the 6th day of feeding) corresponded to approximately 20 % of the mated females maxima (Figure 10). The exponential increase of *Ir*CL proteolytic activity in mated females reached the maxima in semi-engorged females (the 6th day of feeding) and decreased in fully fed females from set B and C to 50 % of its maxima. *Ir*CL activity in set A of fully fed mated females decreased rapidly almost to zero values.

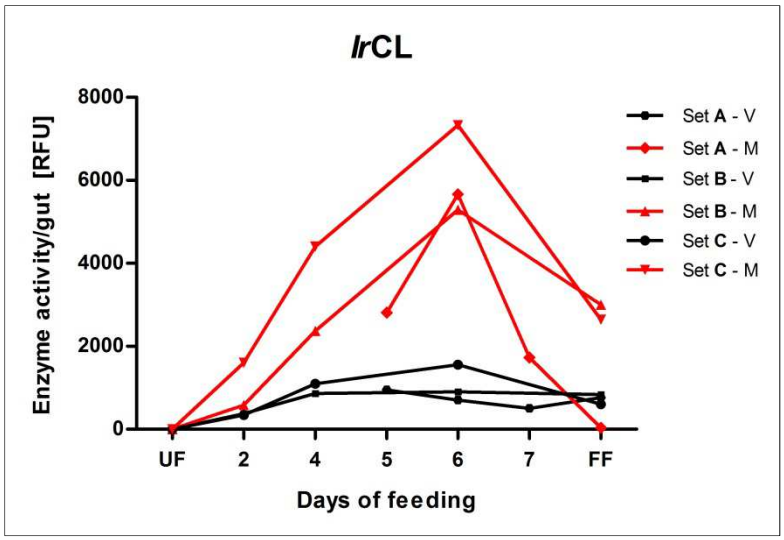


Figure 10. *Ir*CL dynamic profiles in gut extracts from virgin and mated females during feeding. Relative enzymatic activities measured in the gut homogenates were normalized per one gut tissue. Lines show 3 sets (**A, B, C**) of virgin and mated females *Ir*CL activities which correspond to three biological replicates The points display average activities obtained from three technical replicates (standard deviations were ≤5% of the average values and are not shown).**V** - virgin females, **M** – mated females, **UF** – unfed, **FF** – fully fed.

Virgin females had very low and constant level of *Ir*AE activity during the whole feeding on the host (Figure 11). By contrast, the activity of *Ir*AE in mated females was increasing from the 2nd day towards the 6th day of feeding. Afterwards, the activity in fully fed mated females steeply declined in all sets, mainly in set A.

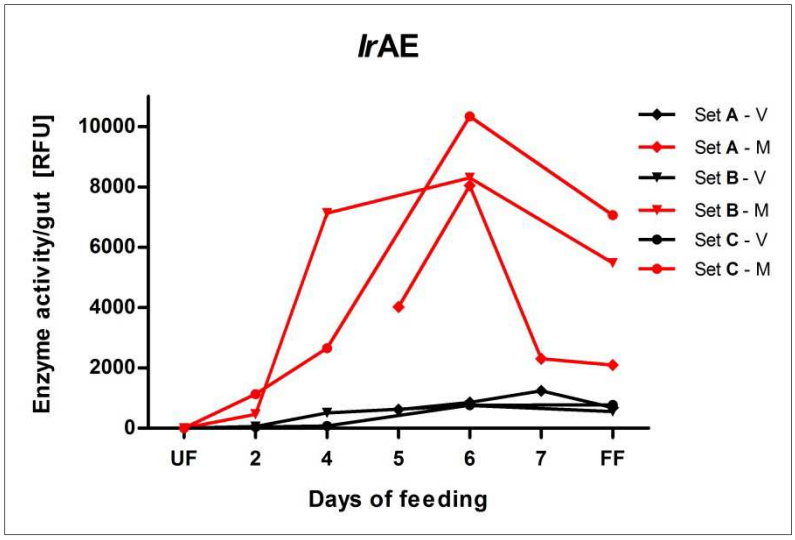


Figure 11. *Ir*AE dynamic profiles in gut extracts from virgin and mated females during feeding. Relative enzymatic activities measured in the gut homogenates were normalized per one gut tissue. Lines show 3 sets (**A, B, C**) of virgin and mated females *Ir*AE activities which correspond to three biological replicates. The points display average activities obtained from three technical replicates (standard deviations were ≤5% of the average values and are not shown).**V** - virgin females, **M** – mated females, **UF** – unfed, **FF** – fully fed.

*Ir*CC activity in virgin females was slowly increasing from unfed females towards the end of feeding (Figure 12). The activity of *Ir*CC in mated females started to rapidly increase from the 4th day of feeding; reaching its maxima 6 days after attachment to the host and it stayed at high level activity in fully fed females. The maximal activity level of *Ir*CC in virgin females was almost 4 times smaller than in mated females.

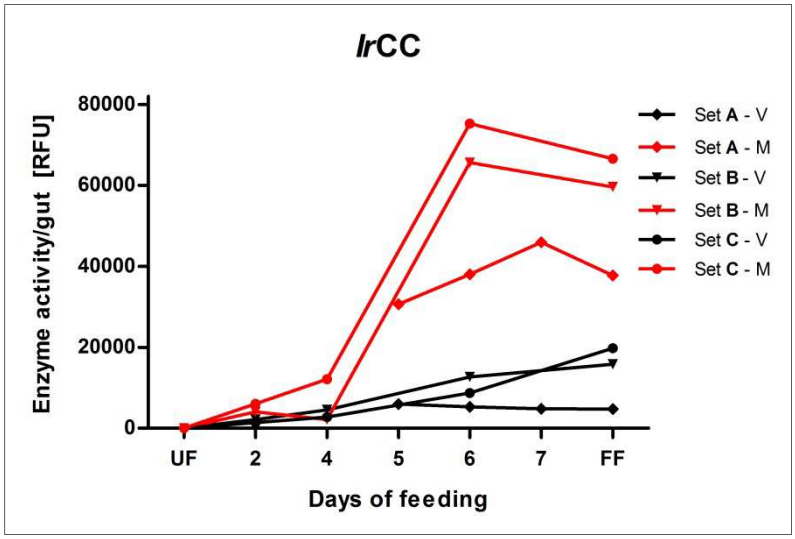


Figure 12. *Ir*CC dynamic profiles in gut extracts from virgin and mated females during feeding. Relative enzymatic activities measured in the gut homogenates were normalized per one gut tissue. Lines show 3 sets (**A, B, C**) of virgin and mated females *Ir*CC activities which correspond to three biological replicates. The points display average activities obtained from three technical replicates (standard deviations were ≤5% of the average values and are not shown). **V** - virgin females, **M** – mated females, **UF** – unfed, **FF** – fully fed.

4.1.3. The rate of fertilized females from the nature

Our results confirmed that entering the rapid engorgement stage of feeding is conditioned by the previous fertilization of female. The long-term practice, used in our or other laboratories, is based on the presumption that female mating occurs mainly during on-host feeding. To assess the rate of fertilized females from the nature, fifty *I. ricinus* females were collected by flagging in locations around České Budějovice, Czech Republic. Half of them were fed on guinea pig without males; second half was fed together with males as the control group. Subsequently, the numbers of females from both groups were compared. The experiment was repeated twice.

At least 92% of females collected from the nature fully engorged when fed without males (Table 7) which was almost identical with the number of females that could fully engorge

in the presence of males. These results clearly demonstrated that fertilization of females occurs in the nature almost exclusively prior to attachment to the host.

Table 7. Number of completely engorged females from the nature

Experiment	FE females fed without males	FE females fed with males (control)
1st	**23** of 25	**24** of 25
2nd	**22** of 25	**23** of 25

FE – fully engorged females (rest of females did not complete engorgement).

4.2. Tissue specificity of peptidases in *I. ricinus* female tick during blood feeding

Previously published tissue expression profiles of *I. ricinus* digestive peptidases were achieved mainly by reverse transcription PCR using a limited number of tissues (Sojka et al., 2008). This part of my work extends and precise our previous data on tissue expression profiles of digestive peptidase by exploiting quantitative real-time PCR (qRT-PCR). The main goal was to reveal tissue specificity of each known peptidase isoforms: cathepsin B (*Ir*CB), cathepsin L1 and L3 (*Ir*CL1, *Ir*CL3), legumain 1 and 2 (*Ir*AE1, *Ir*AE2), cathepsin C (*Ir*CC), and cathepsin D1, D2 and D3 (*Ir*CD1, *Ir*CD2 and *Ir*CD3). *I. ricinus* female ticks were fed on guinea pig in the presence of the same amount of males. Nine females were forcibly removed 4 days after attachment; other 9 females were allowed to accomplish feeding. Tissues (gut, salivary glands, ovaries, trachea-fat body complex (Tr-FB), Malpighian tubules and rest of the bodies) from these time points were dissected in 3 biological replicates (3 females each) and were processed for total RNA isolation. RNA quality was checked on 1% agarose gel giving a clear 18S ribosomal band, whereas the 28S ribosomal band is less visible as is the frequent case reported also for other arthropods (Winnebeck et al., 2010) (Figure 13, 14). After successful RNA isolation, single stranded cDNA was reversibly transcribed and expressions of peptidases were detected by qRT-PCR in biological and technical triplicates.

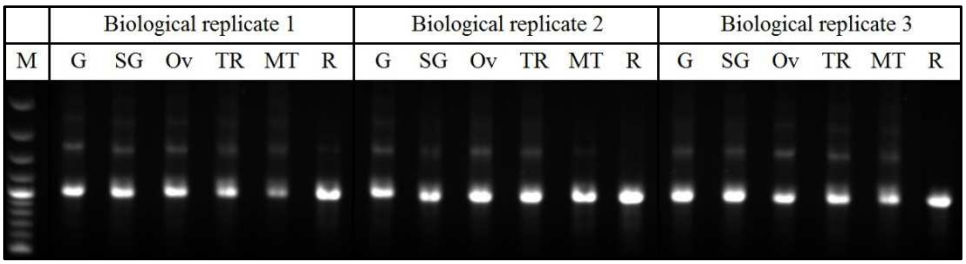


Figure 13. Quality check of total RNA isolated from tissues of 4 days fed females. Three biological tissue replicates (200 ng/lane) on 1% agarose gel. **M** – 100 bp marker (GeneRuler^{TM} 100 bp; MBI Fermentas), **G** – gut, **SG** – salivary glands, **Ov** – ovaries, **TR** – trachea-fat body complex, **MT** – Malpighian tubules, **R** – rest of the bodies including the cuticle.

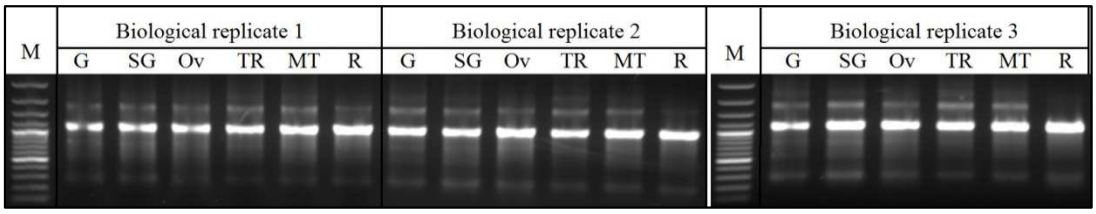


Figure 14. Quality check of total RNA isolated from tissues of fully fed females. Three biological tissue replicates (500 ng/lane) on 1% agarose gel. **M** – 100 bp marker (GeneRuler^{TM} 100 bp; MBI Fermentas), **G** – gut, **SG** – salivary glands, **Ov** – ovaries, **TR** – trachea-fat body complex, **MT** – Malpighian tubules, **R** – rest of the bodies including the cuticle.

*Ir*CB, *Ir*CL3, *Ir*AE1, *Ir*AE2 and *Ir*CD1 were expressed solely in gut tissues while *Ir*CL1, *Ir*CC and *Ir*CD2 were not strictly specific only to gut tissues; they were expressed also in salivary glands and Tr-FB (Figure 15). *Ir*CD3 was equally expressed in all examined tissues except for the rest of the body. The mRNA expressions were evidently up-regulated during the feeding period. All mRNA expressions were up-regulated in fully fed females except *Ir*CL1 gut tissues which were more up-regulated 4 days after attachment than in fully fed females.

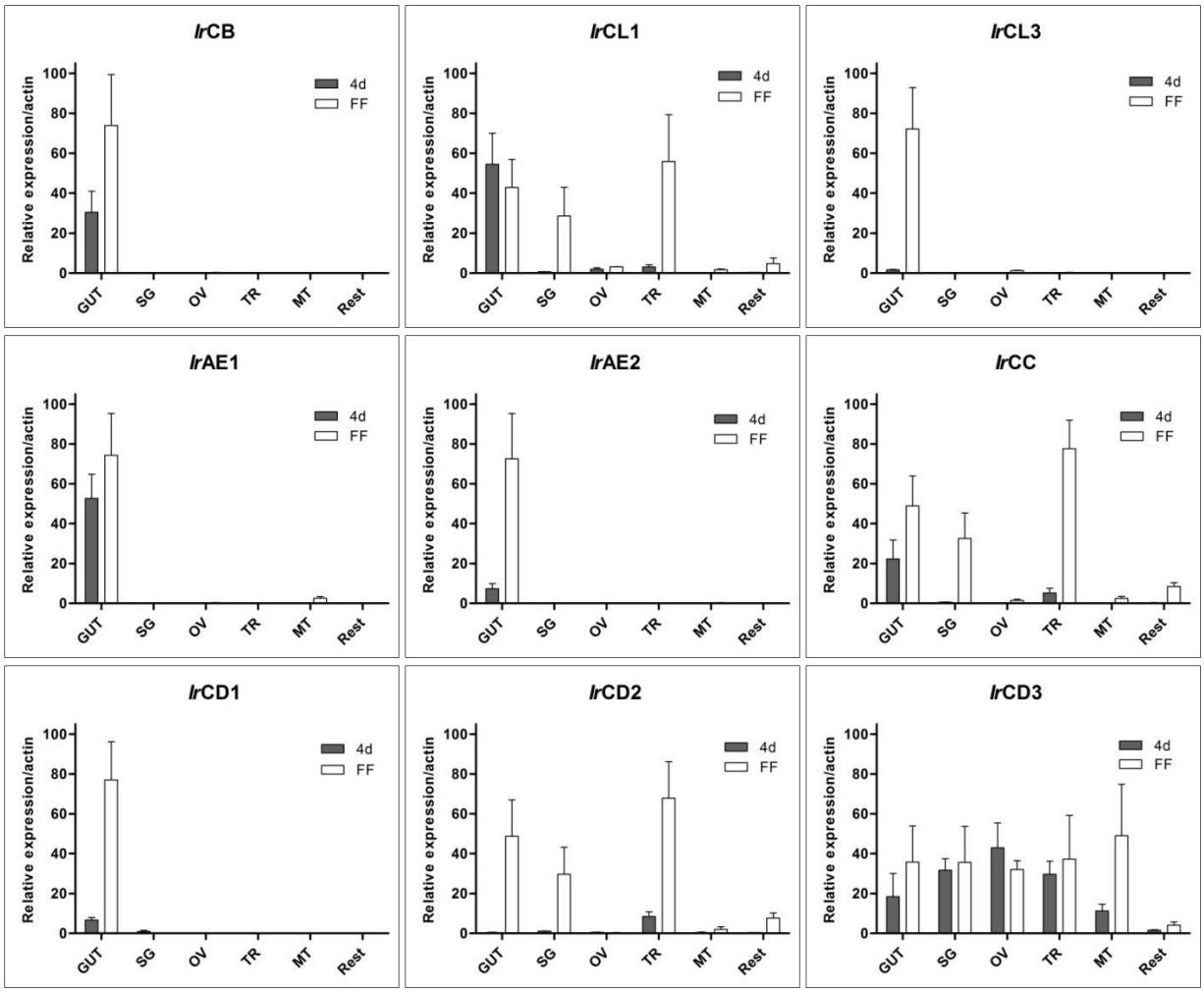


Figure 15. Peptidases mRNA expressions of 6 tissues detected four days after female attachment to the host and in fully fed females. Tissues were dissected and processed for total RNA isolation (3x 3 females – biological triplicate); cDNA was synthesized from RNA. Enzyme gene expressions were determined by qRT-PCR, using actin as a reference gene. Error bars represent the standard errors of three independent biological replicates. Expressions were related to the maximum measured level set as 100%. **4d** – 4 days of feeding, **FF** – fully fed females; **SG** – salivary glands, **OV** – ovaries, **TR** – trachea-fat body complex, **MT** – Malpighian tubules, **Rest** - rest of the body including the cuticle.

4.3. Pilot screening of digestive peptidases expression after differential *in vitro* feeding on blood and serum

To assess whether the expression of tick digestive peptidases depends on the presence of hemoglobin, an *in vitro* membrane feeding system was implemented allowing feeding of ticks on whole blood and serum (hemoglobin-rich and hemoglobin-poor diet, respectively). *In vitro* fed *I. ricinus* females were able to accomplish feeding on both diets until full engorgement (Figure 6). Pools of gut tissues were dissected (5 females in each pool) and processed for isolation of total RNA checked on agarose gel. The samples were obtained from unfed females, 3 time points of *in vitro* feeding period (3 and 5 days of feeding, fully fed females) and from 2 time points after the detachment from the membrane (2 and 6 days after detachment). Next, cDNA was reversibly transcribed. The mRNA expression of each peptidase (*Ir*CB, *Ir*CL1, *Ir*CL3, *Ir*AE1, *Ir*AE2, *Ir*CC, *Ir*CD1, *Ir*CD2 and *Ir*CD3) was determined by qRT-PCR

Unfed females had minimal mRNA expression level for all tested peptidases (Figure 16). The up-regulation of gene expressions towards the end of feeding period was noticed almost in every protease except the expression level of *Ir*CL3 and *Ir*CD2. Up-regulation of *Ir*CL3 and *Ir*CD2 gene expression was observed after the detachment from the membrane. The results proposed that *Ir*CC was more up-regulated in blood fed than in serum fed females. *Ir*CL3 expression was highly up-regulated in serum fed females post detachment. The differences in other peptidases between blood fed and serum fed females were not significant. These findings were used as a pilot survey and required verification by following *in vitro* feeding experiment performed in 3 independent biological replicates.

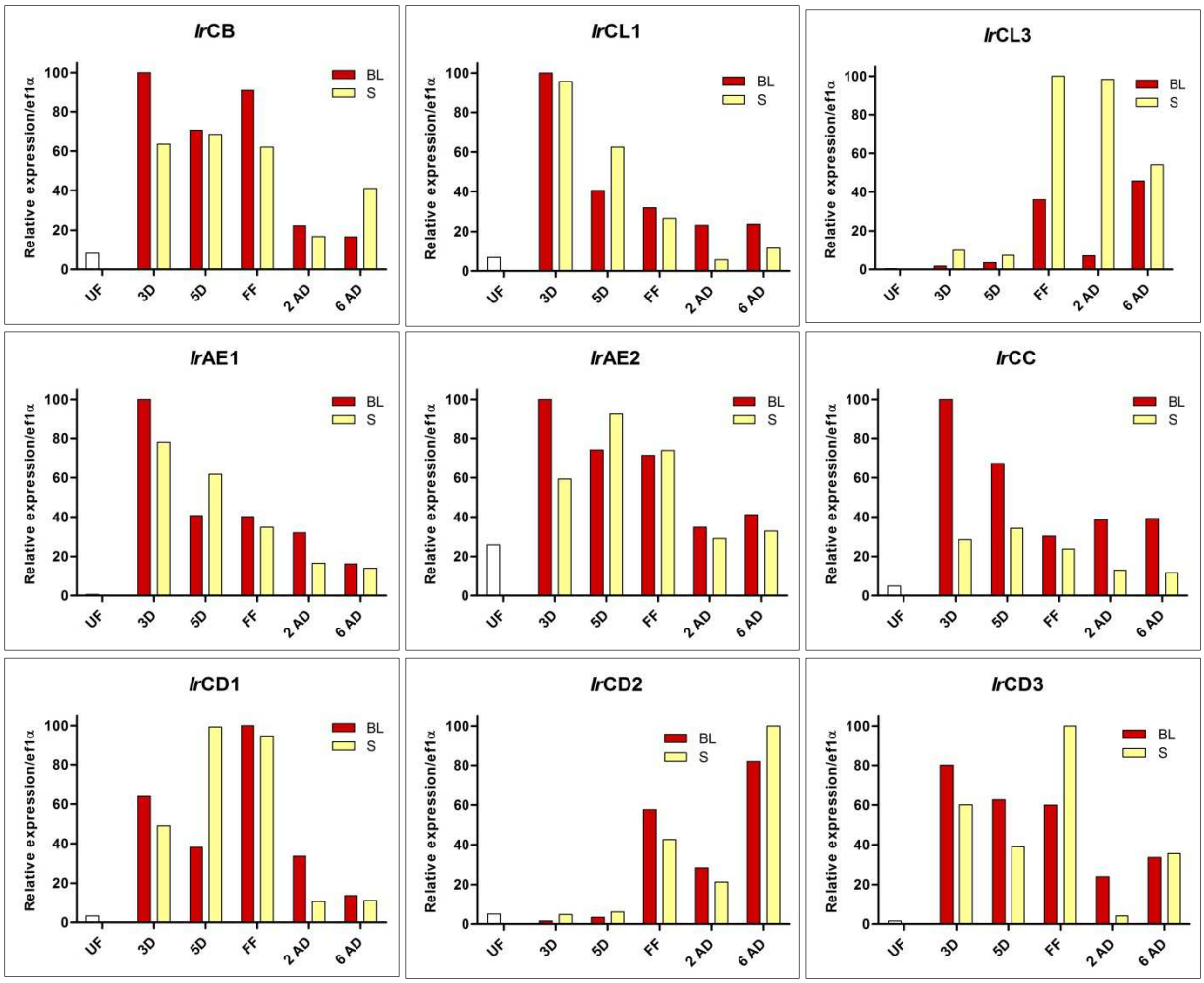


Figure 16. Expression of digestive peptidase mRNAs in *I. ricinus* females during *in vitro* feeding on the whole blood and hemoglobin-depleted serum: Analysis in pooled samples. Gut tissues were dissected and total RNA isolated from a pool of 5 females in each time point; cDNA was synthesized from RNA by reverse transcription. Gene expression of the enzymes was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Expressions were related to the maximum measured level set as 100 %. **BL** – blood fed females, **S** – serum fed females; **UF** – unfed female, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** - fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the host.

4.4. Expression of digestive peptidases in response to hemoglobin-rich and hemoglobin-poor diet

Previous results described mRNA expression levels of major digestive peptidases in pooled gut tissues from blood and serum *in vitro* fed females. Next, we decided to verify those results in three independent biological replicates. Total RNA from 9 female gut tissues from each time point and both diet types (blood and serum) were isolated (3 females for each replicate), checked on agarose gel and processed to reverse transcription (cDNA). Dynamic profiles acquired by qRT-PCR are further shown in the chapter separately for each peptidase.

*Ir*CB was slightly transcribed in unfed females (Figure 17). The mRNA level of *Ir*CB was markedly up-regulated in blood fed ticks, reached its maxima already five days post attachment and decreased in fully fed females post detachment. In contrast, mRNA level of serum fed ticks was significantly lower for all examined time intervals. The mRNA maximum level in blood fed females was ~4 times larger than the maximal level in serum fed females. This result implied that expression of *Ir*CB, the major peptidase in the *I. ricinus* digestive machinery, was substantially dependent on the presence of hemoglobin in the tick diet.

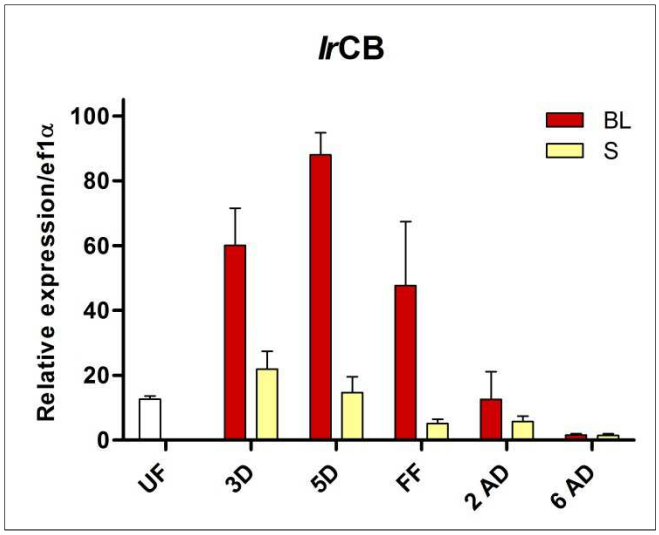


Figure 17. *I. ricinus* cathepsin B (*Ir*CB) mRNA expression dynamics in blood and serum *in vitro* fed females. *Ir*CB mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CB was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** - 2 and 6 days after detachment from the membrane.

*Ir*CL1 mRNA expression ratio in blood fed females did not show significant difference between blood and serum fed females during *in vitro* feeding and post-detachment period (Figure 18). *Ir*CL1 dynamic profile of mRNA expression displayed expression maxima the 5th day post attachment. The mRNA expression dropped dramatically in fully fed ticks and held at the same level post detachment from the membrane.

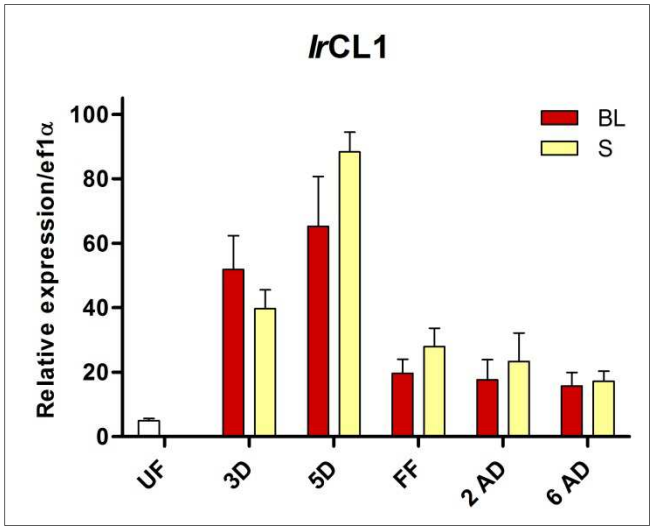


Figure 18. *I. ricinus* cathepsin L1 (*Ir*CL1) mRNA expression profile in blood and serum *in vitro* fed females. *Ir*CL1 mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CL1 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

*Ir*CL3 mRNA expression was marginally perceptible up to 5 days after attachment, raised to its maxima in fully engorged blood fed females and 2 days post detachment in serum fed females (Figure 19). This dynamic profile further supports our concept that *Ir*CL3 functions as a 'late' peptidase playing a role mainly during the off-host digestive phase. There was an evident up-regulation of *Ir*CL3 mRNA level in fully engorged females fed on blood compared to those fed on serum, suggesting its possible regulation by hemoglobin presence.

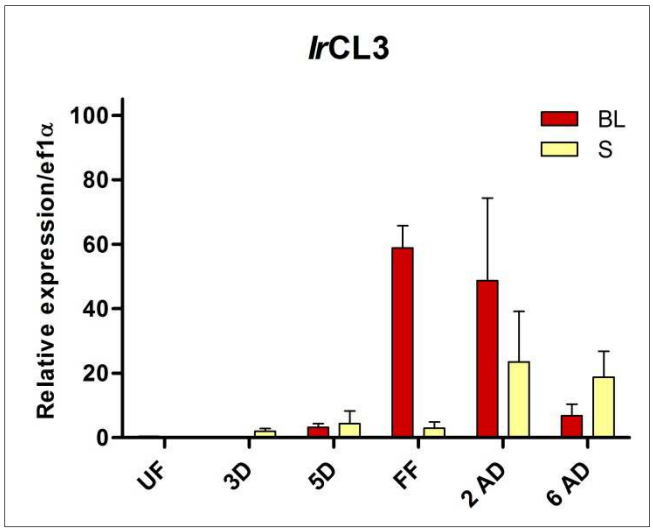


Figure 19. *I. ricinus* cathepsin L3 (*Ir*CL3) mRNA expression dynamics in blood and serum *in vitro* fed females. *Ir*CL3 mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CL3 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

*Ir*AE1 mRNA expression profile (Figure 20) is equal in both types of diet (blood and serum). The expression starts on zero values in unfed females then increases rapidly from the 3rd day post attachment to its maxima 5 days post attachment and subsequently declines in fully fed females and females 2 and 6 days post detachment to 30 % of its maxima.

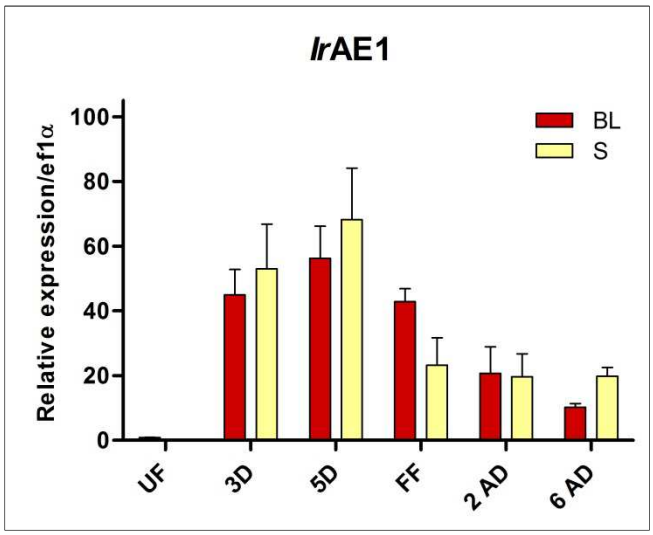


Figure 20. *I. ricinus* legumain 1 *(IrAE1)* mRNA expression dynamics in blood and serum *in vitro* fed females. *IrAE1* mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of

IrAE1 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %. **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D,5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

*Ir*AE2 was the only peptidase with relatively high expression in unfed females (Figure 21). Blood fed females had a rising tendency from 3 days post attachment to 2 days post detachment. *Ir*AE2 expression 6 days post detachment was almost equal to the maxima in 2 days post detachment. Serum fed females *Ir*AE2 expression slightly diminished from unfed to fully fed females and repeated this trend post detachment. The maxima reached in serum fed females corresponded to 50 % of maxima measured in blood fed females.

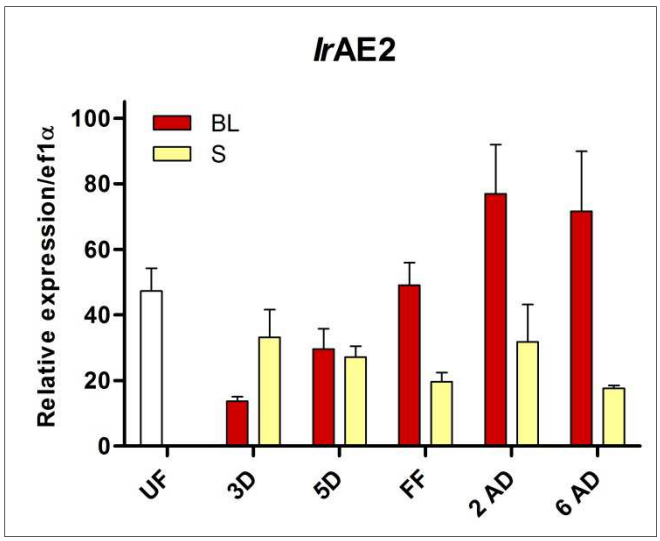


Figure 21. *I. ricinus* legumain 2 (*IrAE2)* mRNA expression dynamics in blood and serum *in vitro* fed females. *Ir*AE2 mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*AE2 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

*Ir*CC mRNA expression was evident from the 3rd day of feeding in both diet types of feeding (Figure 22). The expression stayed at similar level during feeding on blood and increased slowly in post-detachment period reaching its maxima 6 days after blood meal. The *Ir*CC expression in serum fed females had one peak the 5th day of feeding. After detachment, it

raised continually to the maximal expression (2 times higher than in blood fed ticks) reached 6 days after feeding completion.

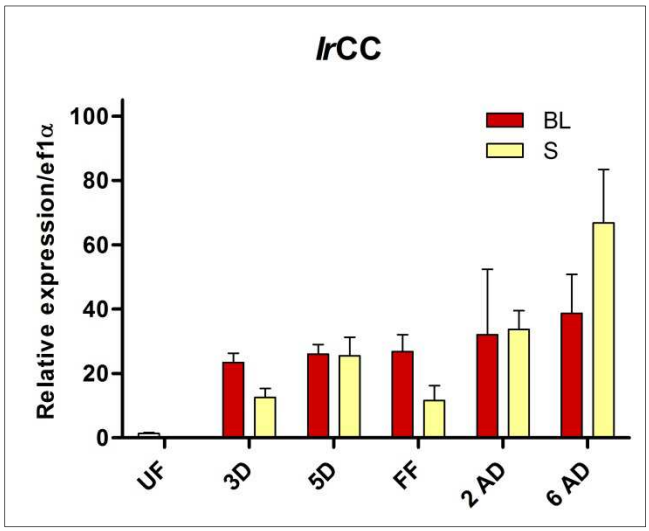


Figure 22. *I. ricinus* cathepsin C (*Ir*CC) mRNA expression dynamics in blood and serum *in vitro* fed females. *Ir*CC mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CC was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

*Ir*CD1 mRNA expression of blood fed females linearly grew throughout the whole feeding period, stayed at the maxima level 2 days post detachment and steeply declined 6 days post detachment (Figure 23). By contrast, *Ir*CD1 expression in serum fed females displays two maxima: first peak 5 days post attachment and second peak, 2 days after detachment. Steep decrease 6 days after detachment was evident in serum fed females as well.

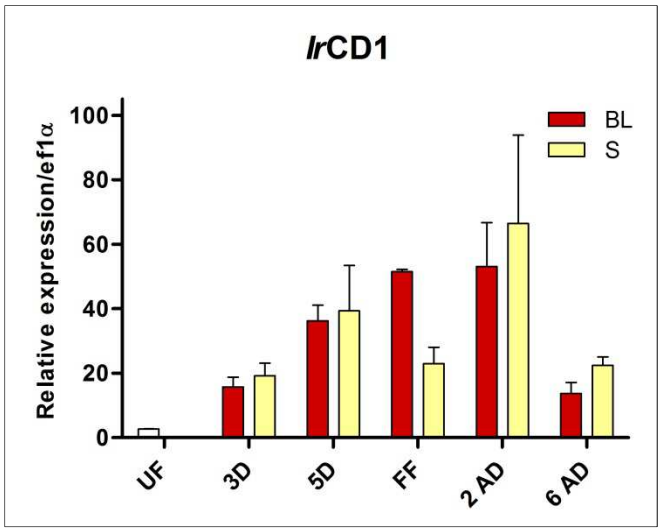


Figure 23. *I. ricinus* cathepsin D1 (*Ir*CD1) mRNA expression profile in blood and serum *in vitro* fed females. *Ir*CD1 mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CD1 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

*Ir*CD2 was second peptidase after *Ir*CL3 which was clearly up-regulated in post-detachment period, more markedly in blood-fed females (Figure 24). *Ir*CD2 expression level was negligible during the whole feeding period. It started to increase in fully fed females and steeply raised 2 days post detachment (staying at the same level in serum fed females 6 days post detachment). Following declined expression 6 days after detachment in blood fed females was comparable to expression level in fully engorged females fed on blood.

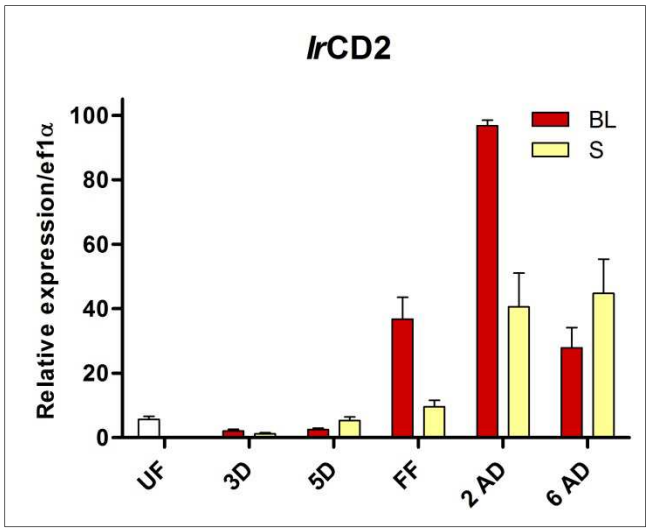


Figure 24. *I. ricinus* cathepsin D2 (*Ir*CD2) mRNA expression profile in blood and serum *in vitro* fed females. *Ir*CD2 mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CD2 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %. **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2AD, 6AD** – 2 and 6 days after detachment from the membrane.

*Ir*CD3 had very low expression level throughout the whole feeding on the membrane as well as 2 days post detachment in both diet types (Figure 25). The only time point with high level of expression was 6 days after detachment from the membrane in blood fed females.

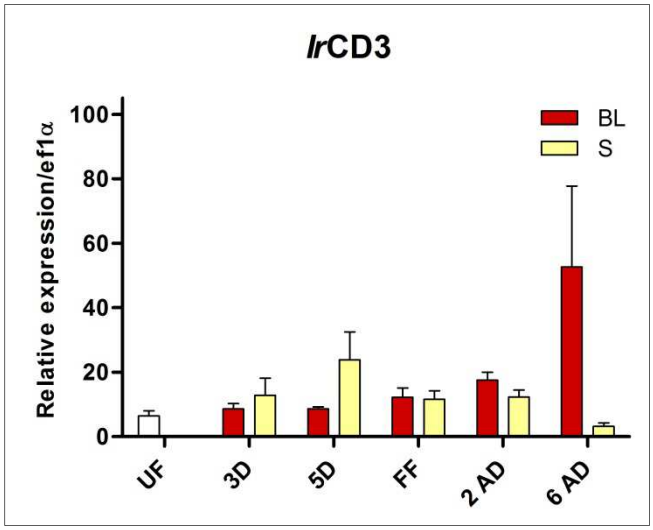


Figure 25. *I. ricinus* cathepsin D3 (*Ir*CD3) mRNA expression profile in blood and serum *in vitro* fed females. *Ir*CD3 mRNA expression was measured in three biological replicates of gut tissues (3 guts in each). Total RNA was isolated, checked on 1% agarose gel; cDNA was reversibly synthesized. Gene expression of *Ir*CD3 was determined by qRT-PCR (in technical triplicates), using elongation factor 1α as a reference gene.

Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **BL** – blood fed females, **S** – serum fed females; **UF** – unfed females, **3D, 5D** – 3 and 5 days of feeding on the membrane, **FF** – fully fed females, **2 AD, 6 AD** – 2 and 6 days after detachment from the membrane.

4.5. *Ir*CL1 and *Ir*CL3 immuno-detection (western blot analysis)

Based on previous results, *Ir*CL1 and *Ir*CL3 dynamic profiles of mRNA expression showed different up-regulation during feeding and post feeding period (Figure 18, 19). *Ir*CL1 gene expression was upregulated during feeding period, mainly from the 3rd to the 5th day of feeding, whereas the mRNA expression of *Ir*CL3 was upregulated in fully fed females and in post-detachment period. This experiment should confirm/confute this statement at the protein level by western blot (WB) immuno-detection. *I. ricinus* females fed on guinea pigs were forcibly removed and gut tissues were dissected 6 and 8 days post attachment (5 females each). Other females were allowed to finish feeding and 5 females were dissected in 4 time points: 5, 7, 10 and 15 days post detachment. Samples for WB analysis were electro-transferred onto a PVDF membrane (0.13 gut applied per lane) and immuno-detected by specific *Ir*CL1 and *Ir*CL3 antibodies.

*Ir*CL1 immuno-detection (Figure 26) distinguished 3 major bands. The size of the largest band (~40 kDa) corresponded to the theoretical molecular weight of *Ir*CL1 inactive pro-enzyme, whereas the middle-size band (~28 kDa) correlated with the predicted mass of the *Ir*CL1 activated enzyme. The smallest bands apparently represented the inactive cleaved form of *Ir*CL1. Double bands probably correspond to two slightly different cleavage products of *Ir*CL1.

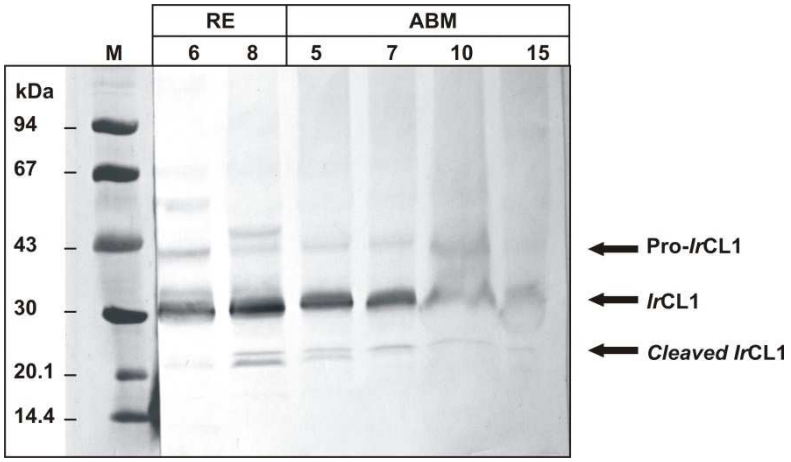


Figure 26. Immuno-detection of cathepsin L1 (*Ir*CL1) in the gut of *Ixodes ricinus* female during feeding and post-detachment period. Western blot analysis of *Ir*CL1 in gut homogenates (0.13 gut/lane) electro-transferred onto PVDF membrane. Primary rabbit antibody against recombinant *Ir*CL1 (dilution 1:50). Secondary antibody – swine anti-rabbit-peroxidase conjugate (dilution 1:2000). **M** – LMW protein markers, **RE** – rapid engorgement (**6, 8** - days of feeding), **ABM (5, 7, 10, 15)** – days after blood meal. **Pro-*Ir*CL1** – inactive pro-enzyme of *Ir*CL1, ***Ir*CL1** – active form of *Ir*CL1.

*Ir*CL3 immuno-detection (Figure 27) recognized 3 band sizes. The largest band (~40 kDa) correlated to the theoretical molecular weight of *Ir*CL3 inactive pro-enzyme, whereas the middle-size band (~32 kDa) corresponded to the *Ir*CL3 activated enzyme. The smallest bands visible 10 and 15 days post detachment apparently represented the inactive cleaved form of *Ir*CL3.

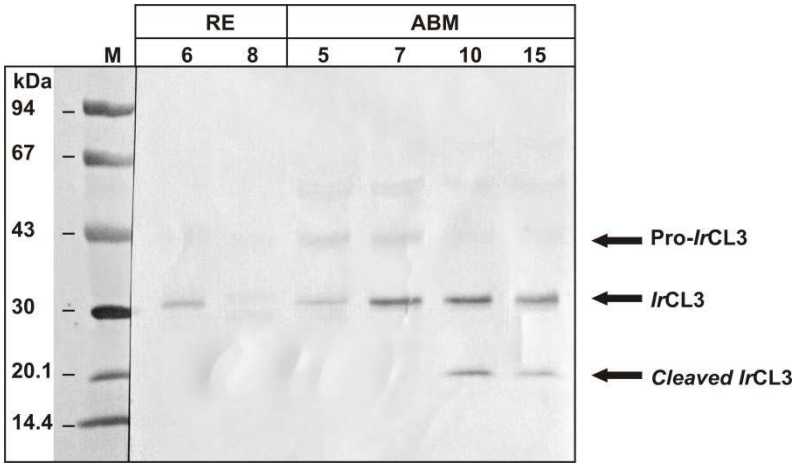


Figure 27. Immuno-detection of cathepsin L3 (*Ir*CL3) in the gut of *Ixodes ricinus* female during feeding and post-detachment period. Western blot analysis of *Ir*CL3 in gut homogenates (0.13 gut/lane) electro-transferred onto PVDF membrane. Primary rabbit antibody against recombinant *Ir*CL3 (dilution 1:50). Secondary antibody – swine anti-rabbit-peroxidase conjugate (dilution 1:2000). **M** – LMW protein markers, **RE** – rapid engorgement (**6, 8** - days of feeding), **ABM (5, 7, 10, 15)** – days after blood meal. **Pro-*Ir*CL3** – inactive pro-enzyme of *Ir*CL3, ***Ir*CL3** – active form of *Ir*CL3.

4.6. *Ir*CL1 and *Ir*CL3 RNA silencing

In order to unequivocally distinguish *Ir*CL1 and *Ir*CL3, RNA silencing method should be used. However, it was not clear, whether injection of dsRNA into unfed females will maintain the reduced mRNA levels of *Ir*CL1 and *Ir*CL3 during the entire period of on-host feeding and off-host digestion. Therefore, I first analyzed the effectiveness of *Ir*CL1 and *Ir*CL3 RNAi that would be used for further investigation of *Ir*CL1/*Ir*CL3 function in blood digestion. RNAi was accomplished by injecting of prepared *Ir*CL1 and *Ir*CL3 dsRNA (Figure 28) into 25 unfed female ticks per each gene. *Gfp* dsRNA, previously prepared in our lab, was injected into 25 unfed female ticks as a control group. Females were fed on 3 guinea pigs (1 guinea pig/gene) three days after the dsRNA injection. Total RNA was isolated from each dissected gut tissue in four time points (5 guts/time point): 6 and 8 days post attachment and 2 and 6 days post detachment in each group. The dynamic expression of peptidase knockdown (KD) was detected by qRT-PCR. *Ir*CL1 and *Ir*CL3 KD expressions were compared with the KD control group (*gfp*).

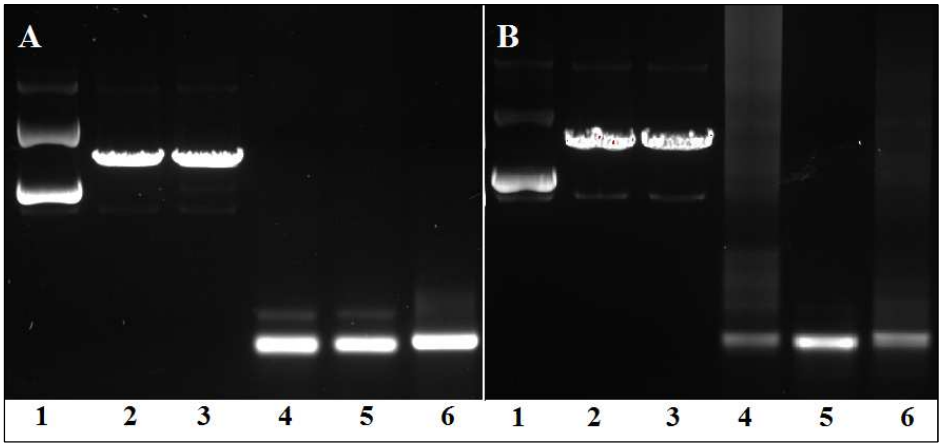


Figure 28. *Ir*CL1 and *Ir*CL3 dsRNA control. Check of the dsRNA quality on the 1% agarose gel. **A -** *Ir*CL1 dsRNA control, **B** – *Ir*CL3 dsRNA control, **1** - unlinearized Pll10 plasmid with cloned *Ir*CL1/*Ir*CL3 insert, **2** - *Apa*I linearized Pll10 plasmid, **3** - *Xba*I linearized plasmid, **4** - ssRNA cut with APA restrictase, **5** - ssRNA cut with XBA restrictase, **6** - dsRNA

The decrease in *Ir*CL1 transcript level (after *Ir*CL1 RNAi) was significant (60-70 % reduction) in 6 and 8 days of feeding (Figure 29). The *Ir*CL1 silencing could not be clearly demonstrated for later time intervals where expression of *Ir*CL1 in both *gfp* control and *Ir*CL1 KD was too low.

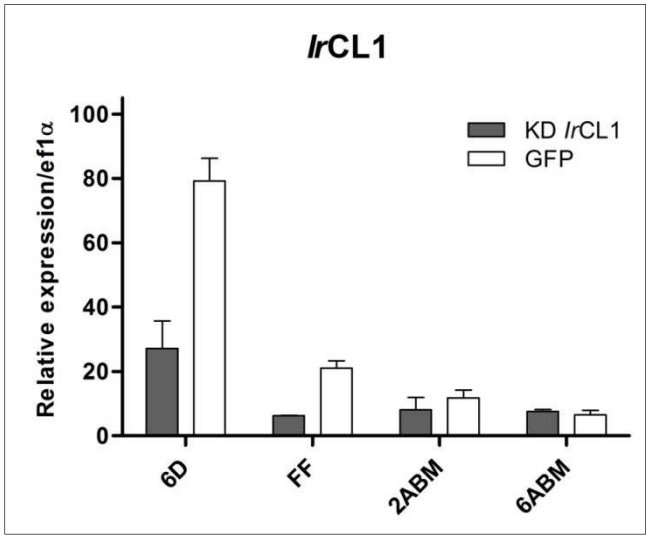


Figure 29. RNA interference silencing of *I. ricinus* cathepsin L1 (*Ir*CL1). Adult *I. ricinus* females (25 ticks per each group) were injected with *IrC*L1 ds RNA (dsRNA) or *gfp* dsRNA as a control group, and allowed to feed naturally on guinea pigs. Gene expression of *Ir*CL1 and *gfp* was determined by qRT-PCR (in five biological replicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **KD *Ir*CL1** – knockdown of cathepsin L1, ***GFP*** – control group, **6D** – 6 days of feeding, **FF** – fully fed females, **2ABM** – 2 days after blood meal, **6ABM** – 6 days after blood meal.

Expression level of *Ir*CL3 was very low during feeding period and the effect of KD was not apparent (Figure 30). *IrCL3* mRNA was mainly expressed after detachment from the host and its RNA silencing resulted in only about 1/3 and 2/3 reduction 2 days and 6 days after blood meal, respectively. This result indicated that for effective *Ir*CL3 RNA silencing, a re-injection of dsRNA into fully engorged females would be necessary.

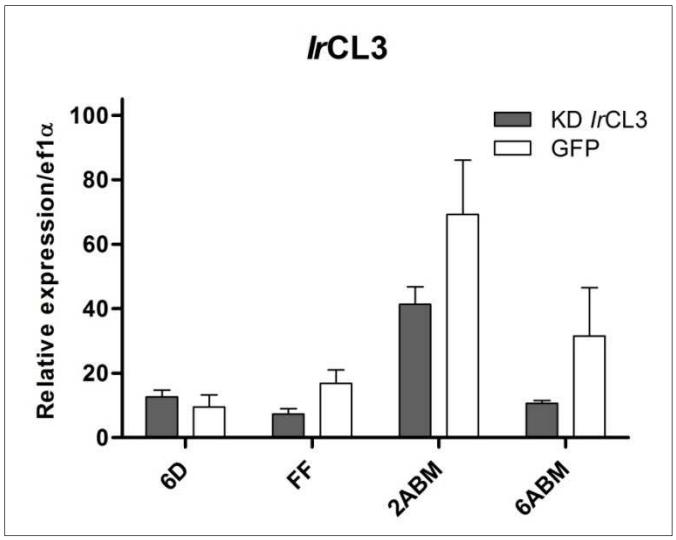


Figure 30. RNA interference silencing of *I. ricinus* cathepsin L3 (*Ir*CL3). Adult *I. ricinus* females (25 ticks per each group) were injected with *IrC*L1 ds RNA (dsRNA) or *gfp* dsRNA as a control group, and allowed to feed naturally on guinea pigs. Gene expression of *Ir*CL3 and *gfp* was determined by qRT-PCR (in five biological replicates), using elongation factor 1α as a reference gene. Error bars represent the standard errors of three independent biological replicates (expressions were related to the maximum measured level set as 100 %). **KD *IrC*L3** – knockdown of cathepsin L3, **GFP** – control group, **6D** – 6 days of feeding, **FF** – fully fed females, **2 ABM** – 2 days after blood meal, **6 ABM** – 6 days after blood meal.

5. Discussion

This work was focused on the factors influencing the expression and activity of digestive enzymes in *I. ricinus* females during the feeding and post-feeding period. Tick blood digestion is an intracellular process occurring in acidic vesicles of digestive cells (Sonenshine, 1991). Digestion of hemoglobin in *I. ricinus* has been studied in our lab for almost a decade and progressively revealed the involvement of a complex multi-enzyme network of cysteine and aspartic proteases (reviewed in Sojka et al., 2013). We have outlined the activity and gene transcription of five major digestive proteases (*Ir*CB, *Ir*CL, *Ir*CC, *Ir*AE and *Ir*CD) during blood feeding on the host (Franta et al., 2010; Konvickova, 2013). The aim of this work was to further extend our current knowledge about the proteolysis in the *I. ricinus* blood digestive system.

One of the main tasks of this work was to enlighten the effect of fertilization of *I. ricinus* females on blood feeding and digestion of host blood. Firstly, the groups of naturally fed virgin and mated females were compared in body weights. Virgin females weighed 10 times less than mated females and they did not evidently reach the rapid engorgement phase. This proved the generally accepted idea that fertilization is absolutely required for females to be able to reach the rapid engorgement phase of feeding (Sonenshine, 1991). Secondly, the dynamic profiling of digestive peptidases (*Ir*CL, *Ir*CB, *Ir*AE, *Ir*CC) further strengthened this opinion in both, virgin and mated females. Almost negligible protease activities were detected in virgin females, whereas the enzymatic activities of mated females (the control groups) showed the substantial activity increase typically from the day 4 of feeding towards the end of the feeding. In control groups, the measured values and dynamic profiles of digestive enzymes activities corresponded to our previous proteolytic data (Franta et al., 2010; Konvickova, 2013). Finally, the fertilization rate of females collected from the nature was observed. At least 92 % of females fed without the males were able to reach the rapid engorgement stage demonstrating that they were fertilized before the feeding. The similar ratio of successful feeding was obtained for the control group of females fed in the presence of males. Despite the majority of females were obviously fertilized from the nature, the females in the control groups were evidently mating again with the males on the host (guinea pig). This observation supported the data published by Hasle et al. (2008), who reported that multiple paternity in *I. ricinus* is likely to happen. It is also in line with the work by Kiszewski et al. (2001) who proposed that ticks from genus *Ixodes* may copulate in the absence of hosts as well as during female engorging. Altogether, these results corroborate

that hemoglobinolytic apparatus in adult ticks is hormonally regulated following the female fertilization. On the other hand, it is still unresolved issue, which mechanism triggers the up-regulation of hemoglobinolytic enzymes activity during the feeding and molting of the *I. ricinus* nymphs described earlier (Konvickova, 2013).

For deeper understanding of *I. ricinus* digestive enzymes and their function, the tissue specificity screening of all studied protease isoforms (*Ir*CB; *Ir*CL1, *Ir*CL3; *Ir*AE1, *Ir*AE2; *Ir*CC, *Ir*CD1, *Ir*CD2, *Ir*CD3) was performed. Each individual protease was detected in partially fed (4 days post attachment) and fully engorged females tissues by qRT-PCR. The mRNA expression levels of *Ir*CB, *Ir*CL3, *Ir*AE1, *Ir*AE2 and *Ir*CD1 were observed solely in the gut suggesting their exclusive role in blood-meal digestion. *Ir*CL3, *Ir*CC and *Ir*CD2 were detected not only in the gut tissues but also in salivary glands and trachea-fat body complex. These proteases probably undertake different functions in different tissues. The results presented here confirmed previously published data from partially fed (5 days post attachment) females (Sojka et al., 2007; Sojka et al., 2008; Franta et al., 2011; Sojka et al., 2012) and extended them with the proteases expression profiles in trachea-body complex and rest of the bodies. The only protease with mRNA expression detected in all tissues (except the rest of the body) was *Ir*CD3. This result revises the previous report that *Ir*CD3 isoform is expressed solely in the ovaries (Sojka et al., 2012).

Lara et al. (2005) suggested that hemoglobin and albumin proteolysis is spatially separated in different digestive vesicles of *R. microplus* digestive cells. The hemoglobin is taken by a receptor-mediated endocytosis, whereas the albumin is absorbed by a fluid-phase pinocytosis (Lara et al., 2005). In order to investigate the specific features and possible differences of hemoglobin and albumin digestion in *I. ricinus*, we exploited the recently implemented *in vitro* feeding technique developed for this species (Kröber and Guerin, 2007). Using *in vitro* feeding, we succeeded to feed *I. ricinus* females on the whole blood (hemoglobin-rich) and serum (hemoglobin-poor) diets and compared the expression differences of above mentioned digestive proteases during the feeding and post-feeding period.

At first, the pooled gut tissues from blood-fed and serum-fed females dissected at different time points were used for the preliminary analysis. Data obtained by qRT-PCR screening displayed only marginal differences in the relative expressions of most digestive proteases for both examined diet types. The dynamic expression profiles of digestive enzymes corresponded to those observed in naturally fed females (Sojka et al., 2013). The only

apparent difference was obtained in *Ir*CL3 and *Ir*CC expression levels. *Ir*CL3 results revealed up-regulation in serum-fed females, while *Ir*CC seemed to be up-regulated in blood-fed females. In order to verify this pilot screening, another experiment was performed in three independent biological replicates carried out in the same feeding conditions. In contrast to the previous findings, *Ir*CC expression did not show any clear up-regulation in blood-fed females. *Ir*CL3 expression levels contradicted the serum-fed up-regulation shown in the pooled gut tissues. On the contrary, this experiment from individual replicates showed the conclusive up-regulation in blood-fed females. Regardless the served diet, the dynamic profiles of *Ir*CL1 and *Ir*CL3 expression profiles confirmed again that *Ir*CL1 and *Ir*CL3 function as the 'early expressed' and 'late expressed' isoforms, respectively. These results are consistent with two peaks of *Ir*CL activities already measured in naturally fed females (Konvickova, 2013). First peak monitored in semi-engorged females probably reflects to the activity of *Ir*CL1 isoform while the second peak observed in off-host period in last few days right before oviposition follows the expression of 'late' *Ir*CL3 isoform. Regarding the *Ir*AE1 and *Ir*AE2 isoforms, they also seemed to work slightly in tandem as early and late proteases (*Ir*AE1 and *Ir*AE2, respectively). *Ir*AE activities measured in the previous work (Konvickova, 2013) depicted two individual peaks in the partially fed females and right at the end of feeding period. That could also signify the role of two different isoforms (*Ir*AE1 and *Ir*AE2, respectively).

Furthermore, *Ir*CB and *Ir*CD2 were expressed more in females fed on hemoglobin-rich diet. *Ir*CB expression increased rapidly in blood-fed females from the 3rd to the 5th day of feeding and linearly decreased from fully fed females to zero values 6 days post detachment. While *Ir*CB is the most abundant peptidase of the hemoglobinolytic pathway (Franta et al., 2010), the mRNA expression of *Ir*CB in serum-fed females persevered in relatively low level and reached its maxima in the 3rd day of feeding. The maxima corresponded to only about 20 % of maxima obtained in blood-fed females. *Ir*CD2 expression level increased in fully engorged females fed on hemoglobin-rich diet and reached its maxima 2 days post detachment. The enhancement of *Ir*CD2 mRNA expression at the end of feeding was also described by Sojka et al. (2012). Considerable *Ir*CD2 expression differences between blood-fed and serum-fed ticks were observed for fully engorged females and 2 days after detachment suggesting that the hemoglobin presence influences mainly the early stage of *Ir*CD2 transcription.

The results of biological triplicates of serum-fed and blood-fed females indicated that despite the differences in expression levels of all studied isoforms, none of the enzyme mRNAs is completely absent in the serum-fed females. Therefore we assume that the albuminolysis is conducted by the same or similar multi-enzyme network as hemoglobinolysis described earlier (Horn et al., 2009). However, this hypothesis has to be confirmed by the measurement of enzyme activities in gut homogenates from serum-fed ticks. Another step to progress our knowledge of hemoglobin and albumin proteolysis in *I. ricinus* is offered by transcriptomics. We have recently accomplished a next generation sequencing of guts from blood-fed and serum-fed *I. ricinus* females. The Hi-Seq and MiSeq data (Illumina platform) are currently being analyzed and put in correlation with here presented data acquired by qRT-PCR.

Last but not least, the conspicuous distribution of 'early expressed' *Ir*CL1 and 'late expressed' *Ir*CL3 seen in mentioned qRT-PCR profiles was further studied (Figure 18, 19). The protein immuno-detection supported that *Ir*CL1 is the isoform active during on-host feeding whereas *Ir*CL3 plays this role in fully fed females and during off-host digestive stage. The confirmation of *Ir*CL1/*Ir*CL3 up-regulation during/post feeding on the protein and mRNA levels led us to the requirement to unequivocally identify the *Ir*CL isoforms by RNAi. According to the qRT-PCR results, the *Ir*CL1 RNAi worked well in the comparison with *gfp* control and corresponded to the silencing efficiency demonstrated previously by Franta et al. (2011). Unfortunately, the *Ir*CL3 was not sufficiently silenced and as only a limited decrease in mRNA expression was detected (Figure 30). The gene silencing technique (Buresova et al., 2009; Hajdusek et al., 2009) brought back our long-lasting concern, that a single dsRNA injection into unfed females is not capable of maintaining the reduced mRNA levels during the whole process of off-host digestion. Therefore, re-injection of dsRNA after the completion of feeding should be the first and necessary step which might solve this problem for our future analysis. Another important issue to be studied is whether RNAi silencing of one peptidase mRNA would be compensated by up-regulation of another isoform or even different type of peptidase from the digestive apparatus.

In vitro feeding method, albeit quite demanding, would be very helpful technique for further investigations of all vital nutritional components needed for the tick development and reproduction. The ability to modify the served diet in a defined way (e.g. by adding specific peptidase inhibitors) opens up a new perspective for further investigation of tick digestive system. More generally, artificial feeding of ticks could be exploited for testing anti-tick, compounds, vaccines and importantly, minimize the amount of treated laboratory animals.

6. Conclusion

The main aim of this work was to reveal the effect of fertilization and diet types on the feeding and digestion of *I. ricinus* females.

We showed that fertilization is essential for females to be able to reach rapid engorgement phase and hereby proved the generally accepted presumption that only fertilized females can complete the feeding. The activities of digestive enzymes in virgin females were almost negligible compared to the mated females, suggesting that up-regulation of blood digestion in adult females is hormonally controlled.

Expression profiles of the main *I. ricinus* digestive proteases were detected in tissue samples from partially and fully fed females naturally fed on the guinea pigs. The majority of tested proteases were expressed solely in the gut and only few of them were expressed also in other tissues.

The dynamic expression profiles of all studied digestive proteases were observed in females fed *in vitro* on hemoglobin-rich and hemoglobin-poor diet. The results of artificial feeding disclosed several proteases (*Ir*CB, *Ir*CL3, *Ir*AE2, *IrCD2*) which were up-regulated in the presence of hemoglobin-rich nourishment. Regardless their mRNA levels, all proteases detected in blood-fed females were also present in serum-fed females suggesting that albuminolysis is conducted by the same or at least similar enzymatic network as hemoglobinolysis. Artificial feeding can bring new findings and knowledge about tick digestion and results from this work represent the first attempt towards the understanding of regulation of *Ixodes ricinus* digestive apparatus.

Gene silencing of *Ir*CL1 isoform of cathepsin L was successful, while the knockdown efficiency of later expressed *Ir*CL3 was much lower. This result underscores the necessity of dsRNA re-injection for silencing the genes important for off-host digestion and vitellogenesis.

7. References

Alim, M.A., Tsuji, N., Miyoshi, T., Islam, M.K., Hatta, T., Yamaji, K., Fujisaki, K. (2008). Developmental stage- and organ- specific expression profiles of asparaginyl endopeptidases/legumains in the ixodid tick *Haemaphysalis longicornis*. *J Vet Med Sci.* 70: 1363-1366.

Arthur, D.R. (1970). Tick feeding and its implications. *Adv Parasitol*, 8, 275-292.

Agyei, A.D., Runham, N.W. (1995). Studies on the morphological changes in the midguts of two ixodid tick species *Boophilus microplus* and *Rhipicephalus appediculatus* during digestion of the blood meal. *International Journal for Parasitology*, *25*(1), 55–62. http://doi.org/10.1016/0020-7519(94)00114-4

Balashov, I.U.S., Raikhel, A.S., Hoogstraal, H. (1983). An Atlas of ixodid tick ultrastructure, Special publication / Entomological Society of America, *College Park, Md.*, pp. ix, 289 p. 281 folded p. of plates.

Becker, C.A.M., Bouju-Albert, A., Jouglin, M., Chauvin, A., Malandrin, L. (2009). Natural transmission of zoonotic *Babesia spp.* By *Ixodes ricinus* ticks. *Emerging Infectious Diseases*, *15*(2), 320–322. http://doi.org/10.3201/eid1502.081247

Booth, T.F. (1989). Wax lipid secretion and ultrastructural development in the egg-waxing (Gene's) organ in ixodid ticks. *Tissue Cell*. ;21(1):113-22. http://www.ncbi.nlm.nih.gov/pubmed/2772908

Bouman, E.A.P., Zemek, R., Dusbabek, F., Socha, R. (1999). Sexual behaviour of the sheep tick, *Ixodes ricinus* (L.) (Acari, Ixodidae). *Proceedings of the 3rd Intrernational Conference on Urban Pests*, (March 1997), 255–260. http://www.icup.org.uk/reports%5CICUP425.pdf

Bowman, A.S., Sauer, J.R. (2004). Tick salivary glands: function, physiology and future. *Parasitology*, 129 Suppl, S67–S81. http://doi.org/10.1017/S0031182004006468

Braz, G.R.C., Coelho, H.S.L., Masuda, H., Oliveira, P.L. (1999). A missing metabolic pathway in the cattle tick *Boophilus microplus*. *Current Biology*, *9*(13), 703–706. http://doi.org/S0960-9822(99)80312-1 [pii]

Boldbaatar, D., Sikalizyo Sikasunge, C., Battsetseg, B., Xuan, X., Fujisaki, K. (2006). Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. *Insect Biochem Mol Biol*. 36: 25-36.

Buresova, V., Hajdusek, O., Franta, Z., Sojka, D., Kopacek, P. (2009). IrAM-An alpha2-macroglobulin from the hard tick *Ixodes ricinus*: Characterization and

function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. *Developmental and Comparative Immunology*, *33*(4), 489–498. http://doi.org/10.1016/j.dci.2008.09.011

Chen, J. M., Fortunato, M., Barrett, a J. (2000). Activation of human prolegumain by cleavage at a C-terminal asparagine residue. *The Biochemical Journal*, *352 Pt 2*, 327–334. http://doi.org/10.1042/0264-6021:3520327

Coons, L.B., Rosell-Davis, R., Tarnowski, B.I. (1986). Blood meal digestion in ticks. In Morphology, Physiology, and Behavioural Biology of Ticks. Edited by Sauer J.R., Hair J.A. New York: *Ellis Horwood Ltd., John Wiley & Sons*. 248-279.

Coons, L.B. and Alberti, G. (1999). The Acari-Ticks. In Microscopic Anatomy of Invertebrates, Vol. 8B, Chelicerata Arthropoda, F.W. Harrison and R. Foelix, eds. *New York: Wiley-Liss.* pp. 267–514.

Cruz, C.E., Fogaça, A.C., Nakayasu, E.S., Angeli, C.B., Belmonte, R., Almeida, I.C., Miranda, A., Miranda, M.T., Tanaka, A.S., Braz, G.R., Craik, C.S., Schneider, E., Caffrey, C.R., Daffre, S. (2010). Characterization of proteinases from the midgut of *Rhipicephalus (Boophilus)* microplus involved in the generation of antimicrobial peptides. *Parasit Vectors*. 3: 63.

de la Fuente, J., et al. (2008). Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci*. 13: 6938-6946.

Delcroix, M., Sajid, M., Caffrey, C.R., Lim, K.C., Dvorak, J., Hsieh, I., Bahgat, M., Dissous, C., McKerrow, J.H. (2006). A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. *Journal of Biological Chemistry*, 281(51), 39316–39329. http://doi.org/10.1074/jbc.M607128200

Fogaça, A.C., da Silva Jr., P.I., Miranda, M.T.M., Bianchi, A.G., Miranda, A., Ribolla, P.E.M., Daffre, S. (1999). Antimicrobial activity of a bovine hemoglobin fragment in the tick *Boophilus microplus*. *The Journal of Biological Chemistry*, 274, 25330-25334 doi: 10.1074/jbc.274.36.25330.

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*. *Parasites & Vectors*, *3*, 119. http://doi.org/10.1186/1756-3305-3-119

Franta, Z., Sojka, D., Frantova, H., Dvorak, J., Horn, M., Srba, J., Talacko, P., Mares, M., Schneider, E., Craik, Ch.S., McKerrow, J.H., Caffrey, C.R., Kopacek, P. (2011). IrCL1 - The haemoglobinolytic cathepsin L of the hard tick, *Ixodes*

ricinus. *International Journal for Parasitology*, *41*(12), 1253–1262. http://doi.org/10.1016/j.ijpara.2011.06.006

Franta, Z. (2012). Blood meal digestion in the hard tick *Ixodes ricinus*. PhD. thesis, Ceske Budejovice, Univerity of South Bohemia. 20.

Goddard, J. (2008). Infectious Diseases and Arthropods. 2. s.l. *Humana Press*. 81-82.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D. a., Petney, T.N., Estrada-Pena, A., Horak, I.G., Shao, R.F., Barker, S.C. (2010). The argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: A list of valid species names. *Zootaxa*, (C), 1–28. http://doi.org/10.1023/A:1025381712339

Habedank, B. and Hiepe, T. (1993) *In-vitro*-Futterung von Zecken, *Dermacentor nuttalli*, Olenev 1928 (Acari: Ixodidae) uber eine Silikonmembran. *Dermatol. Monatsschr*. 179, 292–295

Hajdusek, O., Sojka, D., Kopacek, P., Buresova, V., Franta, Z., Sauman, I., Winzeling, J., Grubhoffer, L. (2009). Knockdown of proteins involved in iron metabolism limits tick reproduction and development. Proceedings of the *National Academy of Sciences* of the United States of America, *106*(4), 1033–1038. http://doi.org/10.1073/pnas.0807961106

Hasle, G., Røed, K.H., Leinaas, H.P. (2008). Multiple paternity in *Ixodes ricinus* (Acari: Ixodidae), assessed by microsatellite markers. *The Journal of Parasitology*, *94*(2), 345–347. http://doi.org/10.1645/GE-1280.1

Hegedus, D., Erlandson, M., Gillott, C., Toprak, U. (2009). New insights into peritrophic matrix synthesis, architecture, and function. *Annu Rev Entomol*. 54:285–302.

Horn, M., Nussbaumerova, M., Sanda, M., Kovarova, Z., Srba, J., Franta, Z., Sojka, D., Bogyo, M., Caffrey, C.R., Kopacek, P., Mares, M. (2009). Hemoglobin Digestion in Blood-Feeding Ticks: Mapping a Multipeptidase Pathway by Functional Proteomics. *Chemistry and Biology*, *16*(10), 1053–1063. http://doi.org/10.1016/j.chembiol.2009.09.009

Hovius, J.W., van Dam, A.P., Fikrig, E. (2007). Tick-host-pathogen interactions in Lyme borreliosis. *Trends Parasitol*. 23: 434-438.

Jongejan, F., Uilenberg, G. (2004). The global importance of ticks. *Parasitology*, *129*, 3–14. http://doi.org/10.1017/S0031182004005967

Kaufman, W.R. (1989). Tick-host interaction: a synthesis of current concepts. *Parasitol. Today*. 5: 47–56.

Kiszewski, A. E., Matuschka, F., Spielman, A. (2001). Mating Strategies and Spermiogenesis in Ixodid Ticks. *Annual Review of Entomology*, *46*, 167–182.

Konvickova, J. (2013). Dynamika aktivit trávicích proteáz ve střevě klíšťat *Ixodes ricinus* během sání na hostiteli. Bachelor thesis, *Faculty of Science*, Univerity of South Bohemia in Ceske Budejovice. 14-35.

Kopacek, P., Zdychova, J., Yoshiga, T., Weise, C., Rudenko, N., Law, J.H. (2003). Molecular cloning, expression and isolation of ferritins from two tick species- *Ornithodoros moubata* and *Ixodes ricinus*. *Insect Biochemistry and Molecular Biology*, *33*(1), 103–113. http://doi.org/10.1016/S0965-1748(02)00181-9

Kröber, T., Guerin, P.M. (2007). *In vitro* feeding assays for hard ticks. *Trends in Parasitology*, *23*(9), 445–449. http://doi.org/10.1016/j.pt.2007.07.010

Kuhnert, F. (1996). Feeding of hard ticks *in vitro*: new perspectives for rearing and for the identification of systemic acaricides. *ALTEX*, 13, 76–87

Kuraishi, T., Hori, A., Kurata, S. (2013). Host-microbe interactions in the gut of *Drosophila melanogaster*. *Frontiers in Physiology*, *4 DEC*(December), 1–8. http://doi.org/10.3389/fphys.2013.00375

Lara, F.A., Lins, U., Paiva-Silva, G., Almeida, I.C., Braga, C.M., Miguens, F.C., Oliveira, P.L., Dansa-Petretski, M. (2003). A new intracellular pathway of haem detoxification in the midgut of the cattle tick *Boophilus microplus*: aggregation inside a specialized organelle, the hemosome. *J Exp Biol.* 206: 1707-1715.

Lara, F.A., Lins, U, Bechara, G.H., Oliveira, P.L. (2005). Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. *J Exp Biol*. 208: 3093-3101.

Lindgren, E., Tälleklint, L., Polfeldt, T. (2000). Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick *Ixodes ricinus*. *Environmental Health Perspectives*, *108*(2), 119–123. http://doi.org/10.1289/ehp.00108119

Mans, B.J. (2011). Evolution of vertebrate hemostatic and inflammatory control mechanisms in blood-feeding arthropods. *Journal of Innate Immunity*, *3*(1), 41–51. http://doi.org/10.1159/000321599

Mariana, I., Mitrea, I.L., Minculescu, F., Buzatu, M.C. (2008). Comparative morphological study on two species (*Ixodes ricinus* and *Dermacentor marginatus*) From Romania By Light and Scanning Electron Microscopy. *Veterinary Medicine 65*(2), 75–79.

Miyoshi, T., Tsuji, N., Islam, M.K., Huang, X., Motobu, M., Alim, M.A., Fujisaki, K. (2007). Molecular and reverse genetic characterization of serine proteinase-

induced hemolysis in the midgut of the ixodid tick *Haemaphysalis longicornis. J Insect Physiol.* 53(2):195-203. PMID: 17275020

Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-Epstein, R., DePonte, K., Fish, D., Fikrig, E. (2014). NIH Public Access. *Cell Host Microbe*, *15*(1), 58–71. http://doi.org/10.1016/j.chom.2013.12.001.Gut

Nuttall, P.A. (1999). Pathogen-tick-host interactions: *Borrelia burgdorferi* and TBE virus. *Zentralblatt Fur Bakteriologie : International Journal of Medical Microbiology*, 289 (5-7), 492–505. http://doi.org/10.1016/S0934-8840(99)80002-4

Pierce, A.E. and Pierce, M.H. (1956) A note on the cultivation of *Boophilus microplus* (Canestrini, 1887) (Ixodidae: Acarina) on the embryonated hen egg. *Aust. Vet. J.* 32, 144–146.

Richter, D., Matuschka, F., Spielman, A., Mahadevan, L. (2013). How ticks get under your skin : insertion mechanics of the feeding apparatus of *Ixodes ricinus* ticks. *Proc R Soc B* 280: 20131758. http://dx.doi.org/10.1098/rspb.2013.1758

Sauer, J.R., Essenberg, R.C., Bowman, A.S. (2000). Salivary glands in ixodid ticks: Control and mechanism of secretion. *Journal of Insect Physiology*, 46(7), 1069–1078. http://doi.org/10.1016/S0022-1910(99)00210-3

Schmidt, G.D., Roberts, L.S., Janovy, J., (1996). Gerald D. Schmidt and Larry S. Roberts' Foundations of parasitology. Wm. C. Brown, 5m Dubuque (USA), 603-611.

Smit, J.D. et al. (1977) Haemoglobin crystals in the midgut of the tick *Ornithodorus moubata* Murray. *Nature* 266, 536–538.

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.

Sojka, D., Franta, Z., Horn, M., Hajdusek, O., Caffrey, C.R., Mares, M., Kopácek, 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.

Sojka, D., Franta, Z., Frantova, H., Bartosova, P., Horn, M., Vachova, J., O'Donoghue, A.J., Eroy-Revele, A.A., Craik, Ch.S., Knudsen, G.M., Caffrey, C.R., McKerrow, J.H., Mares, M., Kopacek, P. (2012). Characterization of gut-associated cathepsin D hemoglobinase from tick *Ixodes ricinus* (CD1). *Journal of Biological Chemistry*, *287*(25), 21152–21163. http://doi.org/10.1074/jbc.M112.347922

Sojka, D., Franta, Z., Horn, M., Caffrey, C.R., Mares, M., Kopacek, P. (2013). New insights into the machinery of blood digestion by ticks. *Trends in Parasitology*, 29(6), 276–285. http://doi.org/10.1016/j.pt.2013.04.002

Sonenshine, D.E. (1991). Biology of ticks. *Oxford University Press*, New York. First ed., Vol. 1.

Sonenshine, D.E., Roe, R.M. (2014). Biology of ticks. *Oxford University Press*, New York. Second ed., Vol. 1.

Stuen, S. (2007). *Anaplasma Phagocytophilum* - The most widespread tick-borne infection in animals in Europe. *Veterinary Research Communications*, *31*(SUPPL. 1), 79–84. http://doi.org/10.1007/s11259-007-0071-y

Tarnowski, B.I., Coons, L.B. (1989). Ultrastructure of the midgut and blood meal digestion in the adult tick *Dermacentor variabilis. Exp Appl Acarol.* 6(4):263-89.

Tsuji, N., Miyoshi, T., Battsetseg, B., Matsuo, T., Xuan, X., Fujisaki, K. (2008). A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. *PLoS Pathog.* 4: e1000062.

Urbanova, V., Sima, R., Sauman, I., Kopacek, P. (2014). Thioester-containing proteins of the tick *Ixodes ricinus* : Gene expression , response to microbial challenge and their role in phagocytosis of the yeast *Candida albicans*. *Dev. Comp. Immunol.*, 48, 55–64. http://doi.org/10.1016/j.dci.2014.09.004

Volf, P., Horak, P., et al. (2007). Paraziti a jejich biologie. Triton, Praha/Kromeriz: 260-264.

Williamson, A.L., Brindley, P.J., Knox, D.P., Hotez, P.J., Loukas, A. (2003). Digestive proteases of blood-feeding nematodes. *Trends in Parasitology*, 19(9), 417–423. http://doi.org/10.1016/S1471-4922(03)00189-2

Winnebeck, E.C., Millar, C.D., Warman, G.R. (2010). Why does insect RNA look degraded? *Journal of Insect Science (Online)*, *10*(159), 159. http://doi.org/10.1673/031.010.14119

Wu, D.D., Wang, G.D., Irwin, D.M., Zhang, Y.P. (2009). A profound role for the expansion of trypsin-like serine protease family in the evolution of hematophagy in mosquito. *Molecular Biology and Evolution*, *26*(10), 2333–2341. http://doi.org/ 10.1093/molbev/msp139

Yamaji, K., Tsuji, N., Miyoshi, T., Islam, M.K., Hatta, T., Alim, M.A., Anisuzzaman, Takenaka, A., Fujisaki, K. (2009). Hemoglobinase activity of a cysteine protease from the ixodid tick *Haemaphysalis longicornis. Parasitol Int.* 58: 232-237.

Zeme, R., Bouman, E.A., Socha, R., Dusbabek, F. (2002).The effect of feeding status on sexual attractiveness of *Ixodes ricinus* (Acari: Ixodidae) females. *Exp Appl Acarol*. 27(1-2):137-49. PMID: 12593518

Zhu, Z., Gern, L., Aeschlimann, A.A. (1991). The peritrophic membrane of *Ixodes ricinus*. *Parasitology Research*. 77; 7, 635-641.