University of South Bohemia in České Budějovice
Faculty of Science

Johannes Kepler University Linz
Faculty of Engineering and Natural Sciences

Functional analysis of fibrinogen-related proteins (FREPs, Ixoderins) of the tick *Ixodes ricinus* and their function in pathogen transmission

Master thesis

Bc. Helena Mondeková, BSc

Cross-border joint Master's program Biological Chemistry

Supervisor: RNDr. Ondřej Hajdušek, Ph.D.
Supervisor specialist: RNDr. Radek Šíma, Ph.D.

České Budějovice

2015

Master thesis:

Mondeková H., 2015: Functional analysis of fibrinogen-related proteins (FREPs, Ixoderins) of the tick *Ixodes ricinus* and their function in pathogen transmission. MSc. thesis, in English. - 58 pp., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic and Faculty of Engineering and Natural Sciences, Johannes Kepler University, Linz, Austria

Annotation:

This study is focused on characterization of fibrinogen-related proteins (FREPs) from the tick *Ixodes ricinus* using molecular methods - PCR, cloning, qRT-PCR, RNA interference via dsRNA synthesis and injection, and also pathogen (*Borrelia* sp.) transmission on animal model.

Financial sources: GACR: 570/3181, 570/3179; SGA 2014

University of South Bohemia in České Budějovice, Declaration:

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my master thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 14. 12. 2015

….....

Helena Mondeková

Johannes Kepler University Linz, Sworn Declaration:

I hereby declare under oath that the submitted Master's degree thesis has been written solely by me without any third-party assistance, information other than provided sources or aids have not been used and those used have been fully documented. Sources for literal, paraphrased and cited quotes have been accurately credited.

The submitted document here present is identical to the electronically submitted text document.

České Budějovice, 14. 12. 2015

….....

Helena Mondeková

Acknowledgment:

Hereby, I would like to thank to my supervisors and colleagues from the Laboratory of Tick Transmitted Diseases. The biggest thanks go to Ondra Hajdušek for being good head of the Laboratory, always having overview of what is going on, keeping experiments and data in order, motivating others to do their best and always being personally interested and involved. Ondra deserves big "thank you" also for being good teacher and mentor, for passionately sharing his knowledge in the field as well as providing great tips and tricks for the lab work. Apart from being great scientist, Ondra is very good manager and I think he managed even my personality very well.

My great thanks go further to Radek Šíma; not only for being good mentor and teaching me a lot of things, but also for being always friendly guy with good mood and eternal smile. He taught me how to analyze data, how to interpret it and how not to get lost in all the data sheets. Most importantly, Radek managed to push me through the animal experiments with no harm done on my psychics or the animals' welfare, which is a great job.

I would like to thank to Verča Urbanová for introducing me to the world of microscopy, tricky staining and ticks dissections and injections. Verča has all my respect for being the best time manager I have met so far. She has showed me what does it mean to be super-effective in the lab.

My big thanks belong to Adla Harcubová. Adla was a person checking on me to avoid stupid mistakes, showing me the clean, tidy and organized laboratory work. She always answered all my questions about procedures, uncertainties and weird results. Adla also helped me a lot during my work with mice. Thank you, Adla.

Thanks to Gábi Loosová, who took me under her wings when I started to work in the lab and showed me all around; and for all the small talks, which make the working days easy. Thanks to Honza Erhart for maintaining and taking great care of tick breeding facility and for being ready to provide ticks in short time any time during the year. Thanks to Maruška Jalovecká, Zuzka Zemanová, Jirka Ťápal and Terka Pospíšilová for creating great atmosphere in the lab. Thank you, guys; it has been great pleasure to work with you.

Finally, I would like to thank the Institute of Parasitology for being my pleasant work-home for many years, for the technical and financial support and for making me feel very welcome there.

List of Abbreviations

aa	amino acids
act	gene coding for Actin
ef1	gene coding for Elongation factor 1
FReD	fibrinogen-related domain
FREP	fibrinogen-related protein
GFP, *gfp*	green fluorescent protein, gene coding for green fluorescent protein
GlcNAc	*N*-acetylglucosamine
GalNAc	*N*-acetylgalactosamine
G(-), G(+)	gram-negative, gram-positive
IMD pathway	immune deficiency pathway
ISAMP	*I. scapularis* antimicrobial peptide
IXO, *ixo*	Ixoderin protein, gene coding for Ixoderin
KD	gene knockdown, term is used synonymously to gene silencing via RNAi
MBL	mannose-binding lectins
MCR	macroglobulin complement-related proteins
PAMP	pathogen-associated molecular pattern
PAZ	Piwi/Argonaute/Zwille
PRM	pattern recognition molecule
PRR	pattern-recognition receptor
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	reactive oxygen species
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
TEPs	thioester-containing proteins
gfp KD = *gfp* RNAi	control knockdown of *gfp* gene, term is used synonymously to *gfp* silencing via RNAi
ixo KD = *ixo* RNAi	knockdown of *ixoderin* gene, term is used synonymously to *ixoderin* silencing via RNAi
triple KD = triple RNAi	triple knockdown of *ixoderins A*, *B* and *C* genes together, term is used synonymously to their silencing via RNAi
E, UL, FL, UN, FN, M, UF, HF-6D, FF	eggs, unfed larvae, fed larvae, unfed nymphs, fed nymphs, males, unfed females, half-fed females (6 days fed females), fully fed females
GUT, SG, OVA, HEM, TRA, MT, REST	gut, salivary glands, ovaria, hemolymph, trachea, Malpighian tubules, rest of the body

Table of Contents

1. Introduction and Objectives

Ticks are blood-feeding ectoparasites able to transmit various pathogens including viruses, bacteria, protozoa, fungi and nematodes (Jongejan and Uilenberg, 2004). In Europe, Lyme disease, caused mostly by spirochetes *Borrelia burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* (Radolf and Samuels, 2010), and tick-borne encephalitis (TBE), caused by tick-borne encephalitis virus (TBEV), belong to the most important and the most common vector borne diseases. While there is an effective vaccine against TBE, no safe and effective vaccine against Lyme disease exists so far.

The ability of ticks to transmit pathogens is closely related to their immune system. Tick immune system comprises of cellular and humoral part, which act in interplay (reviewed by Hajdusek et al., 2013). Lectins represent an important part of the humoral immune system of invertebrates; these carbohydrate-binding proteins possess the ability of specific saccharides recognition (Dong and Dimopoulos, 2009). The majority of lectins in ticks are fibrinogen-related proteins (FREPs) containing a fibrinogen-related domain (FReD) (Rego et al., 2006). In *Ixodes ricinus* (a commonly occurring tick throughout Europe), these FREPs are named Ixoderins and phylogenetically sorted into two groups: A and B (Rego et al., 2005). Kopacek et al. (2010) further report existence of third group C. Recent study of Kopacek and colleagues (2012) suggests that Ixoderins could play a role in the tick primitive complement activation or have other signaling function.

Fibrinogen-related proteins functional characterization in ticks is therefore crucial for understanding their role in pathogen transmission and vector-pathogen relationships. Also, these might be potential targets for vaccine development. The main objective of this thesis is to reveal the functions of Ixoderins in the tick *I. ricinus* in relation to various pathogens transmitted by this vector with main focus on *B. afzelii*. This was achieved by qRT-PCR analysis of expression pattern, RNA interference (RNAi) and subsequent experiments on *I. ricinus* abilities to eliminate or block transmission of pathogens.

2. Literature Review

Ticks can be found on every continent and can cause various diseases of different severity. The viral diseases vary throughout the world from relatively mild tick-borne encephalitis to very serious Crimean-Congo hemorrhagic fever. The bacterial diseases transmitted by ticks in Europe and North America include Lyme disease (caused by *B. burgdorferi* sensu lato), human granulocytic anaplasmosis or rickettsiosis, tularemia or tick-transmitted veterinary important diseases like anaplasmosis, African swine fever or protozoan babesiosis or theileriosis (Estrada-Pena and Jongejan, 1999; Jongejan and Uilenberg, 2004).

Furthermore, ticks are not only source of various pathogens; they do cause harm already directly by blood consumption. During massive tick infestation, simple blood loss causes significant annual damages on livestock in many countries worldwide. In humans, also uninfected ticks (especially members of family Argasidae) can cause irritation, rash or blisters in the bite spot, or other allergic reactions and in some cases paralysis or toxicosis (Estrada-Pena and Jongejan, 1999).

2.1 Tick *Ixodes ricinus*

I. ricinus belongs to phylum Arthropoda, class Arachnida, subclass Acari, order Parasitoformes and suborder Ixodida. Three families are distinguished within the suborder: Nuttalliellidae (single species), Argasidae (soft ticks, 193 species) and Ixodidae (hard ticks, 702 species) (Guglielmone et al., 2010). *I. ricinus* is the primary vector of TBE virus in Europe as well as of spirochetes of *B. burgdorferi* sensu lato (s. l.) complex causing Lyme disease. Furthermore, it may transmit *B. miyamotoi* causing relapsing fever (Platonov et al., 2011), *Anaplasma phagocytophilum* causing human granulocytic anaplasmosis (Stuen et al., 2013), *Rickettsia rickettsii* causing Rocky Mountain spotted fever (Parola et al., 2013) or *Babesia divergens and B. microti* causing babesiosis in Europe (Rizzoli et al., 2014).

This most abundant tick in Europe with distribution from Ireland and south of Scandinavia to Russia and Mediterranean (Estrada-Pena et al., 2006) undergoes a three-host life cycle as other species in the genus *Ixodes*. The developmental stages of larva and nymph feed on two different hosts for 2-4 and 3-5 days, respectively, and molt to the next stadium after dropping-off the host. The adult females feed for 8-10 days to engorgement. After copulation, they lay a batch of eggs. Adult males do not feed and only fertilize the female

before feeding or during their feeding on the host (Suss, 2003).

Various species of vertebrates serve as hosts for *I. ricinus*, including mammals, birds and reptiles. Host sizes range from small rodents, which are preferred by larvae, to animals as large as deer, which are preferably infested by adult females (Mejlon and Jaenson, 1997). The whole life cycle of *I. ricinus* can take from two up to six years (Suss, 2003), in laboratory conditions it is possible to proceed through the full developmental cycle in one year.

2.2 Innate immune system

The invertebrate immune system lacking the adaptive immunity may be considered less advanced, imperfect and evolutionary outdated. However, the innate immune system represents an ancient defense mechanism, which have undergone more than 1 billion years of evolution and which is conserved among various organisms from plants over fruit flies to mammals (Hoffmann et al., 1999).

The innate immune system contains central mechanism common to all multicellular organisms, which seems to be conserved (Hoffmann et al., 1999). Its crucial role lies in self/non-self recognition using pattern recognition molecules (PRM) or pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002) and induction of adequate response in the organism. Among invertebrates, arthropods are the best studied group and their innate immunity system comprises of mechanisms including antimicrobial peptides, hemolymph coagulation, cell agglutination, phagocytosis, phenol-oxidase-mediated melanization and others (Kawabata and Tsuda, 2002).

The innate immunity fights the first-line battle against pathogens not only in insects, but also in humans. In vertebrates, the response is mediated by signaling pathways starting from PAMP recognition on host´s germline-encoded pattern-recognition receptors (PRRs) and ending by release of cytokines, chemokines, antimicrobial molecules and type I interferons (reviewed by Kumar et al., 2011). The vertebrate innate immunity key role is therefore not only in recognition, but also in production of antimicrobial peptides and stimulation of an adaptive immunity via co-stimulatory molecules (Akira et al., 2006; Hoffmann et al., 1999).

2.3 Innate immune system of ticks

The general understanding of tick immune system is still mediocre when compared to studied arthropods like *Drosophila* or horseshoe crab. However studies conducted on other invertebrates and availability of *I. scapularis* genome and RNAi methods usable in ticks (De La Fuente et al., 2007) have boosted progress in tick immune system research.

The tick innate immune system comprises of cellular and humoral part and it seems that unlike other arthropods ticks do not possess the phenol-oxidase system. While the cellular responses are represented by phagocytosis, encapsulation and nodulation of foreign particles, the humoral immunity consists of antimicrobial peptides, hemolymph clotting, lysozymes, production of reactive oxygen species (ROS), lectins, complement-like proteins and PRRs (Kopacek et al., 2010). The cellular part of the immune system is active in hemolymph, where phagocytic cells and opsonins (lectins and complement-like proteins) can be found. The humoral part permeates the whole organism: the hemolymph-clotting mechanism is placed in the hemolymph, the reactive oxygen species are found in the tick gut and antimicrobial peptides can be isolated from various tissues.

So far, the signaling pathways known to be involved in regulation of the arthropod innate immunity are Toll pathway, immune deficiency (IMD) pathway and JAK-STAT pathway (Ferrandon et al., 2007). The putative complements of the Toll pathway have been found in *I. scapularis* genome: Toll-like receptor, nuclear factor κB, Cactus (nuclear factor-κB inhibitor). Furthermore, the JAK-STAT pathway is an important defense mechanism against *Anaplasma* sp. in *I. scapularis* (Liu et al., 2012). Putative Caspar and Relish related to *D. melanogaster* IMD pathway were discovered in ticks as well, although the Imd protein was not revealed.

2.3.1 Immunity in tick gut

The pathogen acquired from the host during feeding or from another tick during co-feeding has to pass through tick innate immune system in order to survive in the tick. The first site of interaction with the pathogen is the tick gut where the blood meal is accumulated. Unlike other blood feeding arthropods, ticks digest intracellularly in lysosome-like vesicles and hence the blood does not encounter digestive proteases in the gut lumen (Sojka et al., 2013; Sonenshine, 1991). Therefore, ticks have to possess a different mechanism to regulate diverse intestinal microflora. Some of the microbe-regulation factors are antimicrobial

peptides called defensins. These proteins, found in midgut of *I. ricinus*, might play a role in antimicrobial defense, but their activity is to be proven (Nakajima et al., 2003a; Rudenko et al., 2005). Another important antimicrobial activity in the tick gut is provided by hemocidins - large peptide fragments of hemoglobin found in both soft and hard ticks (Nakajima et al., 2003b; Sonenshine et al., 2005). These fragments show their activity against G(+) bacteria and some fungi. Hemoglobin digestion, its subsequent detoxification, reactive oxygen species (ROS) formation and elimination or utilization plays ultimate role in tick survival and the ROS can play possible role in microbial community maintenance or in pathogen elimination, which needs to be further explored.

2.3.2. Immunity in tick salivary glands

Tick saliva, containing hundreds of anti-hemostatic, anti-inflammatory and immunomodulatory molecules, plays an important role during tick feeding on the host. These molecules modulate the immune response and hemostasis of the host (Francischetti et al., 2009). Although various antimicrobial peptides were found to be expressed in salivary glands, clear evidence for their interaction with pathogens is still missing. On the other hand, several saliva proteins were found to act in favor of the pathogen – Salp15 from *I. scapularis* and its homologue from *I. ricinus* are protecting *B. burgdorferi* s. l. from antibody mediated killing in the host (Hovius et al., 2008).

The only protein isolated from tick saliva reported to act against G(-) and G(+) bacteria was a 5.3 kDa peptide ISAMP (*I. scapularis* Antimicrobial Peptide) (Pichu et al., 2009). ISAMP is however expressed not only in salivary glands, but also in hemocytes and fat body. It belongs to the group of 5.3 kDa large antimicrobial peptides in *I. scapularis*, which are controlled by JAK/STAT signaling pathway and at least some of them act against *A. phagocytophilum* (Liu et al., 2012).

2.3.3 Immunity in tick hemolymph

2.3.3.1 Phagocytosis by hemocytes

Several types of hemocytes in tick hemolymph present another immunity barrier. Some of these cells have the ability of phagocytosis, namely plasmatocytes and granulocytes I (Borovickova and Hypsa, 2005). Phagocytosis is important for cleaning out foreign material including pathogens, which managed to get from the gut to the hemolymph. The coiling

phagocytosis of *Borrelia* (Rittig et al., 1996) plays certain role in *B. burgdorferi* s. l. clearing from the hemolymph. The phagocytosis is far more promoted after borrelial infection in the vector *Dermacentor variabilis* (immunocompetent to *Borrelia* sp.) than in *I. scapularis* Nevertheless, phagocytosis itself is not sufficient for *Borrelia* sp. elimination. Other borreliacidal factors from tick plasma contributes to *D. variabilis* immunocompetence (Johns et al., 2001a). In case of the pathogen Anaplasma phagocytophilum, phagocytosis even contributes to spread of the pathogen (Liu et al., 2011). Therefore, although some of the microbes undergo engulfment, they may also escape and avoid disintegration by phagocytes, or they may use the phagocytosis as a way to infect the organism or spread within it.

2.3.3.2 Antimicrobial molecules

Multiple antimicrobial molecules can be found in hemolymph. Defensins and defensin-like peptides, which were reported in both soft and hard ticks (Gudderra et al., 2002), are cationic cysteine-rich peptides containing around 30-40 amino acids. These peptides destroy bacterial plasma membrane of G(+) bacteria via depolarization and channel formation (Cociancich et al., 1993; Nakajima et al., 2003a). Their role in tick is however very complex and not simply predictable. Defensin named varisin isolated from *D. variabilis* shows on one hand activity against *B. burgdorferi* s. l. (Johns et al., 2001b), However, on the other hand, its knockdown led to unexpected decrease in numbers of *Anaplasma marginale* during infection (Kocan et al., 2008). Other antimicrobial cysteine-rich peptides with different mechanisms of action were isolated from various tick species including *Amblyomma hebraeum* (Lai et al., 2004) and *R. microplus* (Fogaca et al., 2004, 2006).

2.3.3.3 Primitive complement system

The evolutionary ancient and conserved primitive complement system in ticks was recently described by Kopacek and colleagues (2010). The components of the complement system are represented by different groups of thioester-containing proteins (TEPs). These were found and described among diverse animal species including nematodes, insects, mollusks, fish, birds and mammals (Nonaka, 2000). The phylogenetic search among *Drosophila melanogaster* and *Anopheles gambiae* revealed that TEPs evolved independently, possessing however the same features and functions. In invertebrates, three distinguished groups of TEPs were described so far: (i) α2-macroglobulins (broadly specific proteinase inhibitors); (ii) C3-components of the complement system and (iii) insect TEPs

(Blandin and Levashina, 2004). Recently, a fourth related group (iv) macroglobulin complement-related proteins (MCR) was described (Kopacek et al., 2010). In the genome of *I. scapularis*, three proteins belonging to α2-macroglobulins were found. Three other proteins belong to C3-components, one protein to insect TEPs and another two form the branch of MCR proteins. Furthermore, C3 convertase related gene was found in the *I. scapularis* genome (Kopacek et al., 2012).

Several invertebrate molecules from the complement-like system were proved to be active against the invading microbes. For example *Candida albicans* is phagocytosed based on signaling by Macroglobulin complement related molecule (dTEP6) in *D. melanogaster*. dTEP2 and dTEP3 from *D. melanogaster* are mediators of phagocytosis of *Escherichia coli* and *Staphylococcus aureus*, respectively (Stroschein-Stevenson et al., 2005). IrAM from *I. ricinus* is a TEP α2-macroglobulin, which deficiency leads to failing phagocytosis of *Chryseobacterium indologenes* (Buresova et al., 2009). Its function in the tick is however very complex as the main function of α2-macroglobulins found in soft and hard ticks is a non-specific defense against all exogenous and endogenous proteases causing proteolysis in the tick. Therefore, not only protease inhibition fulfills the protective function, but also the foreign protease recognition by α2-macroglobulins leads to cellular immune response.

2.3.3.4 Microbe-recognition molecules

The specific defense against foreign microorganisms in the tick is based on recognition of PAMPs by tick lectins (Natori, 2001), which appear to be invertebrate analogues of vertebrate immunoglobulins. The PAMPs are found exposed on the surface of pathogens, being usually part of the cell walls, plasma membranes or other outer surfaces with clearly distinguishable structures like carbohydrates, lipopolysaccharides, peptidoglycans or lipoproteins. Tick lectins are commonly found in hemolymph or hemocytes, however, their presence in other cells and tissues in not an exception. A large portion of lectins is represented by FREPs, which can be seen as the humoral part of the innate immune system, are repeatedly reported to be implicated in PAMPs recognition (Adema et al., 1997; Dong and Dimopoulos, 2009; Gokudan et al., 1999; Hanington and Zhang, 2010; Kawabata and Tsuda, 2002; Zhang et al., 2008a). However, some carbohydrate-binding proteins like Hemelipoglycoprotein from *D. marginatus* (Sterba et al., 2011) or OmGalec from *Ornithodoros moubata* (Huang et al., 2007) do not contain fibrinogen-related domains. OmGalec contains tandem of GLECT domains (galectin/galactose-binding lectin), which

bind exclusively β-galactosides like glucose.

Lectins have sophisticated functions in various organisms and their importance for immune system is clearly demonstrated in the lectin complement pathway in humans. Moreover, it is known that this pathway can be activated not only by mannose-binding lectins (MBL), but also by ficolins, which structurally resemble the MBL and which possess the fibrinogen-related domain (Matsushita et al., 2001).

2.4 Fibrinogen-related proteins

2.4.1 Fibrinogen-related domain

Proteins with fibrinogen-related domain are called fibrinogen-related proteins. Their biological role varies as evolution has led to modifications of their functions among different organisms. FReD consists of 220-250 amino acids (aa) among which 40 residues are highly conserved including four conserved cysteines preserving its secondary structure (Holmskov et al., 2003). Furthermore, 24 invariant residues are present in FReD, which serve as FReD recognition pattern (Doolittle, 1992). The FReD corresponds to the globular C-terminal half of the β and γ chains of fibrinogen (Lu and Le, 1998; Yee et al., 1997), therefore it is called fibrinogen-related domain, previously referred to as fibrinogen-like domain (abbreviation FBG). It consists of three subdomains. Subdomain A is approximately 50 aa long, located at the N-terminal end of the FReD and contains two cysteine residues located 28±2 aa apart, which form disulfide bond with each other. The subdomains B and P are intimately linked together forming a ca. 150 aa long sequence. The P subdomain serves usually as a site of bindings and interactions and also undergoes slowest evolutionary change. It contains two cysteines 12 aa apart, which form another disulfide bond. Calcium-binding site is commonly located in the vicinity of this bond (Doolittle et al., 2012). The FReD has been so far always found on the C-terminus of the protein. All the known structures of FReD consist of central larger subdomain B, which is built up from twisted seven-stranded antiparallel β-sheets and two or three α-helices (Kairies et al., 2001). These are surrounded by globular units of P subdomain and A subdomain. Due to the evolution of FReD and its combination with other domains, the FREPs targets for binding changed from carbohydrates to peptides (as seen in vertebrate fibrinogen). It is likely that the change of target-type was caused by alterations in subdomain P of FReD. For example, human γ-fibrinogen C-terminal fragment possesses highly negative surface potential, while Tachylectin 5A (B) possesses hydrophobic groove in the same area (Wang et al., 2005). Recently, FReDs were classified into class I, which

harbors evolutionary older FReDs being present in both chordate and non-chordate animals, and class II, which consists of later evolved FReDs that are found in chordates only. The major difference can be found in the subdomain P: class II contains longer aa sequences between the two disulfide bonds (one in subdomain A and the other in subdomain P) compared to the class I members. Another aa insertion occurs at the C-terminus in class II FReDs (Doolittle et al., 2012). Class II representative is for example human fibrinogen, while human ficolins, as well as horseshoe crab Tachylectins, belong to class I.

The fibrinogen-domain structure of human fibrinogen, horseshoe crab Tachylectin 5A and predicted structure of mosquito FReDs were compared and found to be structurally related in both B and P subdomains. Although the FReDs sequence diversity is high, it remains incorporated in the shared architectural blueprint (Wang et al., 2005).

2.4.2 FREPs among organisms

FREPs among all organisms are predominantly extracellular and diffusible proteins (Doolittle et al., 2012), although Wang and colleagues (2005) reported, that only 14 out of 53 mosquitos FREPs contain signal peptides. FREPs are commonly described as lectins. It is even possible that fibrinogen itself originated from a mannose-binding lectin (Doolittle et al., 1997). This theory is supported by generally accepted hypothesis that the fibrinogen coagulation function was obtained phylogenetically recently, as the first evidence for such functions was found in deuterostomes. It is further hypothesized that new functions were obtained with increasing number of FReD sequences encoded in the genome of more complex organisms, which allowed diversification and gain of new functions via connection to other domains. FREPs of evolutionary older organisms retained their original non-self recognition function (reviewed by Doolittle et al., 2012; Hanington and Zhang, 2010). The FReD tandem duplication events in various organisms led in some cases to extreme sequence diversity among and within species. In Pacific oyster (*Crassostrea gigas*), about 190 FREPs were identified (Huang et al., 2015).

In vertebrates, the FREPs consist of such diverse proteins like ficolins (innate immunity), fibroleukins (inflammatory response), tenascins (cell-cell and cell-extracellular matrix interactions), angiopoietins (angiogenesis), angioarrestins (anti-angiogenesis), fibrinogens (fibrin clot formation), and others (reviewed by Doolittle et al., 2012).

In human ficolins as well as in horseshoe crab Tachylectins, the FReD is responsible for Ca^{2+}-dependent *N*-acetyl group binding (Lu and Le, 1998). More specifically they bind *N*-

acetylglucosamine (GlcNAc) and other carbohydrates as well as non-carbohydrates containing *N*-acetyl group (Endo et al., 2007; Kairies et al., 2001; Matsushita et al., 1996; Thiel, 2007). These proteins are therefore not typical lectins, as they bind not only carbohydrates. Human ficolins are multimeric proteins containing FReD on the C-terminus and collagen-like domain on the N-terminus. Ficolins have evolved to recognize surface carbohydrates and they resemble MBLs and are as well capable of complement activation via the lectin pathway. Furthermore, ficolins trigger primitive opsonophagocytosis (Endo et al., 2007). Ficolin FReDs form "bouquet arrangement" – clusters of three units of ficolins linked through their collagen domains (Lu et al., 2002).

FREPs in the horseshoe crab *Tachypleus tridentatus* are represented by Tachylectins 5A and 5B found in the plasma. Tachylectins specifically recognize *N*-acetyl-group of any molecule (both carbohydrates and non-carbohydrates) and agglutinate human erythrocytes. Tachylectin 5A is considered to be a principal innate immune protein. It binds to G(-) and G(+) bacteria and triggers release of large defensins (Gokudan et al., 1999). Tachylectins do not contain collagen-like domain like ficolins and they arrange to tetrameric structures (Kairies et al., 2001; Kawabata and Tsuda, 2002), However, human ficolins are structurally still more closely related to Tachylectins than to human fibrinogen γ chain (Kairies et al., 2001). *N*-acetyl-binding site of Tachylectin 5A is a hydrophobic funnel formed by Tyr_{210}, Tyr_{236}, Tyr_{248} and His_{220} and a methyl site of Ala_{237}. The Ca^{2+} ligand-binding site located nearby the *N*-acetyl-binding funnel is structurally shared with ficolins (Kairies et al., 2001). In fact, all the so far known FREPs structures contain Ca^{2+} ligand-binding site in the vicinity of the main binding funnel (Doolittle et al., 2012). The Ca^{2+}-dependent manner of carbohydrate binding in ficolins and Tachylectins resembles the C-type lectin-carbohydrate interactions.

In freshwater mollusk *Biomphalaria glabrata*, lectins with FReDs produced in hemocytes are induced by the presence of bacteria or trematode parasites, to surface of which some of these FREPs bind (Adema et al., 1997). In total, 13 FREP subfamilies were established in *B. glabrata* by Zhang and Locker (2004). Some were characterized more closely revealing an extraordinary diversity of the FREP families in this and related organisms together with high FReD conservation. Moreover, FREP subfamilies show different expression patterns. For example, FREP 2 and 4 proteins expressions (containing FReD on the C-terminus and one immunoglobulin superfamily on the N-terminus) were strongly up-regulated after snail exposure to *Echinostoma paraensei* and were found to be over-expressed in schistosome-resistant BS-90 snails (Hertel et al., 2005). Also, genetically

diversified FREP 3 subfamily plays a role in adult snails resistance against *E. paraensei* (Hanington et al., 2010). Several assays further demonstrated specificity of different FREPs in recognition of different groups of pathogens (G(+), G(-) bacteria and yeast). However, FREPs play a role in ontogenesis of *B. glabrata* as well (Zhang et al., 2008b). It was shown that FREPs can create large clusters of diverse fibrinogen-related domains in *B. glabrata* and *A. gambiae* (Christophides et al., 2002; Loker et al., 2004).

Mosquito FREPs are highly expressed by hemocytes following *Plasmodium* infection. Nearly sixty putative FREP genes were found in *A. gambiae.* Their products act complementary and synergistically. Considering the number of FREPs, these proteins are the largest pattern recognition receptor gene family in this organism (Dong and Dimopoulos, 2009; Wang et al., 2005). However, Doolittle and colleagues (2012) described that *A. gambiae* possesses only 29 FReDs of full length, therefore some of the suggested FREPs contain only portions of FReDs as previously reported by Wang and colleagues (2005). The same authors have also shown that some FREPs contain two or even three FReDs. They further conducted a search in *A. gambiae* expressed sequence tag database and found only 21 FREP genes to have transcripts. Nevertheless, most of these proteins undergo increased expression after being challenged with bacteria, fungi or *Plasmodium* parasites. Some of these are even responsible for immune homeostasis maintenance. RNAi-silencing has shown that FREP FBN8 acts in anti-plasmodium defense, FBN9 possesses a strong anti-plasmodium activity and interact with both G(+) and G(-) bacteria and FBN39 protects the mosquito against *Plasmodium falciparum* infection (Dong and Dimopoulos, 2009; Dong et al., 2006). Another mosquito, *Armigeres subalbatus*, produces FREP “aslectin” that binds GlcNAc and is probably involved in antimicrobial response (Wang et al., 2004).

Fourteen FReD containing genes were identified in *D. melanogaster* (Lu et al., 2002; Middha and Wang, 2008). Demonstrating the variability of FREPs functions among organisms, some of FREPs in *D. melanogaster* are involved in organ development like FREP "scabrous", which complex with Notch receptor influencing eye development (Lee et al., 1996; Powell et al., 2001).

Furthermore, recombinant FREP from *Branchiostoma belcheri* acts as a pattern recognition receptor and a bacteriolytic agent against G(+) and G(-) bacteria (Fan et al., 2008). In crayfish *Pacifastacus leniusculus,* FReD plays role in melanization inhibition via regulation of the prophenoloxidase activation cascade (Soderhall et al., 2009). FREP active against *E. coli* and *S. aureus* was identified in a sponge *Suberites domuncula*. Expression of this Tachylectin-related lectin rises upon challenge with lipopolysaccharides and activates

MAPK (mitogen-activated protein kinases) pathway, while GlcNAc treatment abolishes this signaling (Schroder et al., 2003). Surprisingly, no conserved FReDs were revealed in nematodes *Caenorhabditis elegans*, *C. briggsae* and *Brugia malayi* (Rego et al., 2005).

2.4.3 FREPs in ticks

Tick FREPs contain the same fibrinogen-related domain like vertebrate ficolins, however, similarly to Tachylectins, they miss the collagen domain on the N-terminus (Rego et al., 2006). Presence of FREPs proteins and/or FREPs gene sequences was confirmed in ticks *O. moubata* (Kovar et al., 2000), *I. ricinus* and *I. scapularis* (Rego et al., 2005), *D. marginatus*, *Rhipicephalus appendiculatus*, *R. pulchellus*, and *R. sanguineus* (Sterba et al., 2011). Further unidentified lectins (possible FREPs) were found in ticks *O. tartakovskyi*, *O. pappillipes*, and *Argas polonicus* (Grubhoffer et al., 1991).

So far, the best characterized tick FREP is a sialic acid-binding Dorin M from *O. moubata*, which was isolated and characterized by Kovar and colleagues (2000) and was found to be the predominant plasma lectin (Rego et al., 2006). It is a glycoprotein of 37 kDa, which forms aggregates of molecular weight up to 640 kDa. Nevertheless, the subunits are not linked covalently, as no free cysteine is present in the sequence, which could allow formation of disulfide bridge outside the secondary structure. In its deglycosylated form, Dorin M reaches 29.1 kDa and contains three predicted N-linked glycosylation sites (NHS/NGS sequence). While the C-terminus contains the conserved FReD sequence, the non-conserved N-terminus contains a substitution of N-terminal glutamine to pyroglutamate (Kovar et al., 2000; Rego et al., 2006).

Dorin M is expressed in hemocytes and salivary glands of *O. moubata*. However, using immunofluorescence and confocal microscopy, Dorin M was detected in cytoplasm of hemocytes implying that this protein is synthesized there (Kovar et al., 2000; Rego et al., 2006). Strong hemagglutination activity of Dorin M can be inhibited by GlcNAc and GalNAc (*N*-acetylgalactosamine) as it is for Tachylectins. Furthermore, the strongest inhibitor is N-acetyl-neuraminyl lactose. This implies that the N-acetyl residue is the key recognition pattern for Dorin M in *O. moubata* similarly to Tachylectins in *T. tridentatus* (Kawabata and Tsuda, 2002; Kovar et al., 2000). However, in contrary to Tachylectins, the hemagglutination activity is not dependent on the presence of Ca^{2+} ions, which corresponds to the documented change in this ligand-binding aa sequence (Rego et al., 2006). The predicted binding site for Dorin M based on an overall similarity to Tachylectin 5A is

formed by Tyr $_{199, 219, 235-237, 239}$ His$_{203}$ and Ala$_{220}$. Similar binding site was predicted for OMFREP: residues of Tyr $_{215, 257, 259, 261}$ and His$_{225}$.

OMFREP is another FREP from *O. moubata*, which is similar to Dorin M (sequence similarity 65%) including its expression pattern (Rego et al., 2006). Its molecular weight was determined to be 28.9 kDa, with one predicted cleavage site and two glycosylation sites. Sterba et al. (2011) were studying FREPs and hemagglutination activity in *Rhipicephalus* species and in *D. marginatus* and found that sialic acid, sialylated glycoproteins and *N*-acetylated saccharides were the best inhibitors of this activity. Furthermore, multiple FREP genes were further found in the genome of *I. scapularis* and *I. ricinus* and named Ixoderins (Rego et al., 2005). It is hypothesized, that at least some of the tick FREPs play role in the tick innate immunity (Kopacek et al., 2012).

2.4.3.1 Ixoderins

FREPs of *I. ricinus* and *I. scapularis* are named Ixoderins and sorted in three groups: A, B and C. It is known that in the tick *I. ricinus* more Ixoderins are present in group B, more Ixoderins are predicted in group A and Ixoderin C (IXOC) is the only one predicted in group C so far (Kopacek et al., 2010; Rego et al., 2005).

Ixoderin A (IXOA) and Ixoderin B (IXOB) were identified in the tick *I. ricinus* by Rego and colleagues (2005). *Ixoderin A* (*ixoA*) expression was found in hemocytes, salivary glands and midgut. IXOA molecular weight is 30.8 kDa and it possesses one predicted cleavage site (A18-N19) and two glycosylation sites (110-112; 150-152). *Ixoderin B* (*ixoB*) expression was found only in salivary glands. IXOB molecular weight is 32.6 kDa and possesses one predicted cleavage site (G13-D14) and two glycosylation sites (51-53; 132-134).

IXOA is the main lectin in *I. ricinus* hemolymph (Hajdusek, unpublished results). It shows close relation to OMFREP and Dorin M as well as to Tachylectins. Furthermore, the four cysteines within FReD serving for disulfide bridges are conserved among these proteins. This suggests similar structure of these molecules and therefore similar function. One of these conserved cysteines is replaced by proline in IXOB. Further substitutions were revealed in motif ADGIEW otherwise conserved among chelicerates. IXOB was found to be closely related to *I. pacificus* salivary gland protein (Rego et al., 2005). Several forms of Ixoderin B were predicted in the *I. scapularis* genome (Kopacek et al., 2010) and found and sequenced in *I. ricinus* genome (*ixo B, B3, B4, B5, B6*) by Rego et al. in 2006 (unpublished results, sequences deposited in NCBI database). Carbohydrate-binding site predicted for

OMFREP is very similar to the one predicted for IXOA ($Tyr_{214, 256, 258, 260}$ and His_{224}) (Rego et al., 2006).

Rego and colleagues (2005) suggested that the tick-derived FReDs sequences might have originated from an ancestor immune protein. They further assume that while Dorin M, OMFREP and IXOA belong to the conserved sequences, the cluster of proteins containing modified FReD and being expressed solely in salivary glands (Ixoderins B or *I. pacificus* salivary gland protein) might have evolved in different direction and serve for a different purpose.

2.5 *Borrelia burgdorferi* sensu lato spirochetes

The tick *I. ricinus* is a vector of spirochetes of the *B. burgdorferi* sensu lato (s. l.) complex, the causative agent of Lyme disease (borreliosis).

The genus *Borrelia* belongs to the phylum Spirochaetes, class Spirochaete, order Spirochaetales and family *Spirochaetaceae*. The species complex of *B. burgdorferi* s. l. consists of currently at least 20 defined genospecies (Casjens et al., 2011; Ivanova et al., 2014; Skuballa et al., 2012). Lyme disease is caused mainly by *B. burgdorferi* sensu stricto (s. s.) in USA and by *B. burgdorferi* s. s., *B. afzelii* and *B. garinii* in Europe (Radolf and Samuels, 2010), although other genospecies from this complex were also found to be pathogenic in human (Stanek and Reiter, 2011).

Borreliae are bacteria with mean length of 20 μm and diameter of 0.2 μm. Their screw-like shape and 7-14 periplasmic flagella allow these bacteria to move relatively quickly (Barbour and Hayes, 1986). Borreliae genome is stored in form of a chromosome of 911 kbp and multiple (minimum of 16) linear or circular plasmids of variable size. These plasmids combined contain additional genetic information of at least 533 kbp and undergo frequent horizontal gene transfer (Casjens et al., 2012; Fraser et al., 1997).

Although sometimes controversially placed among gram-negative (G(-)) bacteria, borreliae are very unique bacteria in lacking lipopolysaccharides in their cell walls (Takayama et al., 1987). Nevertheless, several glycolipids are surface-exposed parts of the borrelial membrane – namely BbGL-I and BbGL-II (6-*O*-acyl-β-D-galactopyranoside and 1,2-di-*O*-acyl-3-*O*-α-D-galactopyranosyl-*sn*-glycerol respectively) acting in a similar way as lipopolysaccharides and inducing immune reaction in infected vertebrates (Ben-Menachem et al., 2003).

Several outer surface proteins are commonly used for serotyping and are potentially usable for vaccine development - OspA, OspB and OspC are major outer surface proteins of *Borrelia* species (reviewed by Kenedy et al., 2012). While the sequentially similar lipoproteins OspA and OspB maintain the *Borrelia* species colonization in the tick vector, with tick receptor for OspA (TROSPA) mediating the borrelial attachment to the tick gut, OspC is employed in transmission to the vertebrate host (Pal et al., 2004). *ospA* and *ospB* are highly expressed in unfed ticks, but their expression is down regulated within 36-48 hours after tick attachment to the host. In parallel, *ospC* expression is nearly not detectable in unfed ticks, while its expression rises upon blood feeding. It has been shown that this switch in outer surface proteins production is partly based on temperature change – OspC is produced at 32-37°C and not at lower temperatures (Schwan et al., 1995). Furthermore, OspC was found to bind to tick salivary protein Salp15, which is produced in higher amounts in *B. burgdorferi* infected ticks and which protects the spirochetes from recognition by host immune system via protection from antibody-mediated killing (Ramamoorthi et al., 2005).

The different genospecies of *B. burgdorferi* s. l. prefer different vertebrate host species (concept of selective transmission), which is associated with host complement systems being able to clear out the specific genospecies not only from the infested host, but also from the feeding tick. While *B. afzelii* spirochetes are killed in the ticks feeding on birds, *B. garinii* and *B. burgdorferi* s. s. survive and infect the bird host (Kurtenbach et al., 2002). Incompetent hosts for *B. burgdorferi* s. l. are large ungulates, like deer (Jaenson and Talleklint, 1992).

While it is known, that ticks provide several advantages to borreliae starting from their transmission to the host and ending by their protection from the host immune system, it remains unknown, whether borreliae provide some advantages to the ticks in return. Certain evidence for this was reported by (Gassner and Hartemink, 2013; Herrmann and Gern, 2010, 2013; Herrmann et al., 2013) showing that *B. burgdorferi* s. l. infected *Ixodes* ticks survive longer in challenging environment (temperature and humidity), have higher energy reserves and are generally more active.

2.6 RNA interference

RNA interference (RNAi) is a widely used technique of reverse genetic allowing for simple determination of gene function in the organism. Its mechanism is based on an ancient system of RNAi machinery present in all eukaryotic organisms studied so far and serving as defense against endogenous and exogenous pathogenic nucleic acids as well as a regulator of gene expression (Hannon, 2002). RNAi has quickly become standard tool for functional genomics studies on ticks. The principle underlying this mechanism is conserved among eukaryotes, although the individual proteins functions may differ (Hoa et al., 2003). RNAi in ticks is fully self-sufficient, all the components (dicers, argonauts, dsRNA-binding proteins, and others) necessary for effective post-transcriptional silencing were found in the genome of *I. scapularis* (Kurscheid et al., 2009), it is expected that the tick RNAi machinery is similar to the one of *D. melanogaster* and *A. gambiae*. The exact mechanism of RNAi in ticks was proposed by De La Fuente and colleagues (2007) and reworked by Hajdusek (2009).

The RNAi can be triggered by the foreign dsRNA (viral or artificially prepared) entering the cell or by the nuclear DNA processed to miRNA or rasiRNA (Fig. 1). For the genetic knockdown, highlighted pathway in Fig. 1 is used. Exogenous dsRNA is cleaved by tick Dicer-2 protein into short interfering RNAs (siRNAs - 19-23 nucleotides long) after entering the cytosol. The Dicer-2 captures and guides the siRNA to formatted RNA-induced silencing complex (RISC), which incorporates given siRNA and its complementary mRNA is degraded by this complex (Hajdusek, 2009; De La Fuente et al., 2007).

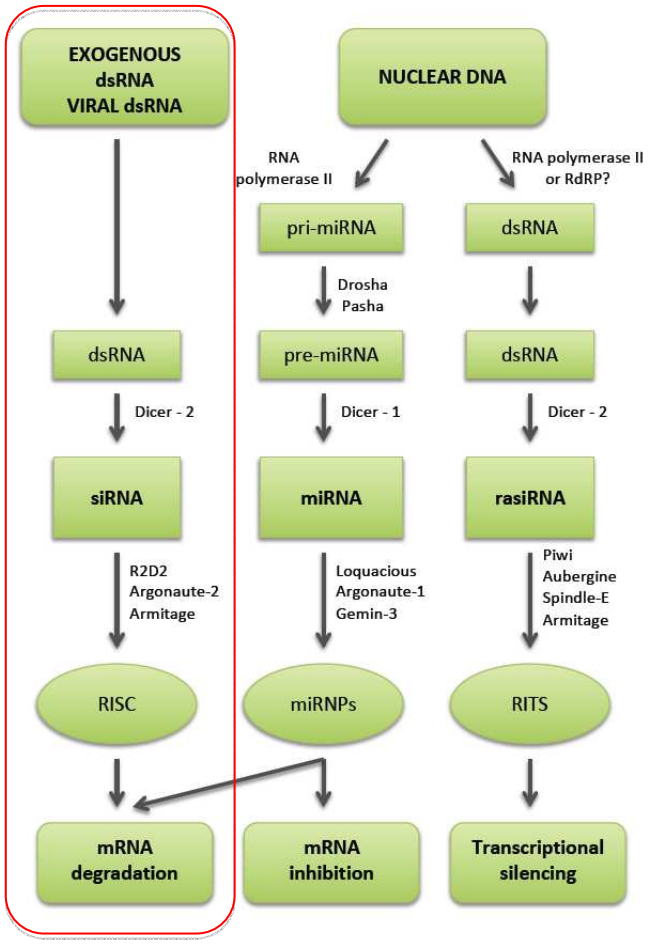


Figure 1: Postulated model of RNAi pathway triggered by dsRNA in ticks. The pathway used for genetic knockdown is highlighted in red. Modified with permission from (Hajdusek, 2009).

The gene silencing takes advantage of individual components being naturally present in the tick organism. The artificially prepared dsRNA homologous to gene of interest is processed as any other potentially dangerous foreign dsRNA in the tick. The injection of dsRNA therefore results also in degradation of naturally produced mRNA and subsequent underproduction or even total elimination of coded proteins.

Although there are several methods of dsRNA insertion into the tick, the injection into hemocoel was found to be most effective in adults and nymphs. This method also allows for full control over amount of dsRNA injected resulting into reliable and reproducible results (De La Fuente et al., 2006).

3. Materials and methods

3.1 Materials

3.1.1 Animals

Adult ticks of the species *I. ricinus* were collected in nature near České Budějovice, Czech Republic by flagging method. For gene expression level determination, phagocytosis experiment and knockdown verification, 25 female ticks were fed on laboratory guinea pigs in presence of the same number of males for 5-6 days (resulting in semi-engorged females).

Nymphal ticks as well as other developmental stages were obtained from the breeding facility of the Institute of Parasitology, Biology Center, CAS. Nymphs are bred in glass boxes with air humidity around 95% at constant temperature of 26°C and with photo-period light: dark – 12h: 12h. Nymphs were used for pathogen transmission experiment and for triple knockdown verification. Mice (CD1) were used for nymphal ticks feeding. Mice used for the pathogen transmission experiment were females of inbred C3H/N strain from Charles River Laboratories (VELAZ, Czech Republic). Experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 359/2012 Sb. and with the decree 419/2012 Sb. of Ministry of Agriculture on the protection of experimental animals (including relevant EU regulations).

3.1.2 Primers

Primers (Tab. 1) were designed accordingly to the *ixoA* (AY341424) sequence from *I. ricinus, ixoB* (AY643518) sequence from *I. ricinus* and *ixoC* (XM_002405041) analogue sequence from *I. scapularis*, all available in NCBI nucleotide database. Primers for qRT PCR were designed using online ROCHE system (http://lifescience.roche.com/shop/en/us/overviews/brand/universal-probe-library) or online software Primer3 (primer3.uu.ee). Primers for RNAi (dsRNA synthesis) were designed manually. These RNAi primers introduce ApaI and XbaI restriction sites (GGGCCC and TCTAGA, respectively) to the sequence for site-specific cloning into pll10 plasmid. The synthesized primers were supplied by GENERI BIOTECH (Hradec Králové, Czech Republic).

Table 1: List of primers. Light gray highlighted nucleotides are inserted overhangs for cloning via ApaI and XbaI restriction enzymes

Name	Sequence	Description
ixoderin A - I. ricinus (AY341424)		
IXOARNAI-F	ATGGGCCCATGCTCCTGGGCATCCTCAT	Primers for cloning and dsRNA synthesis (position 1-243 in AY341424)
IXOARNAI-R	ATTCTAGAATAGACCGATGTTCCCTTGG	
IR3	AGGGAACATCGGTCTATTGC	qRT primers for gene expression determination
IR4	TGGCCTCTGTTCTGAATAACG	
IR199	TGTGCTGCAACCTACAAAGG	qRT primers for gene expression determination; verification set for RNAi
IR200	CATTGAGGTTGCTGATGTGG	
ixoderin B - I. ricinus (AY643518)		
IXOBRNAI-F	ATGGGCCCATGTTCGTAGCATTCCTCTTC	Primers for cloning and dsRNA synthesis (position 1-249 in AY643518)
IXOBRNAI-R	ATTCTAGATGCTGCATCTACCGGAGTGC	
IR153	ATTCAACGTCGGACCGAAT	qRT primers for gene expression determination; verification set for RNAi; *ixoB* and *ixoB4* specific
IR154	CGCTCATAATCTCGTTCCTTTT	
IR308	CTGGTGGTTCAAGGAATGCA	qRT primers for gene expression determination; verification set for RNAi; *ixo*B specific
IR309	TGATGTTTTCAATTCCAAAGCGT	
ixoderin C - I. scapularis (ISCW009412)		
IXOCRNAI-F	ATGGGCCCCTCTCAGCTTCGAACACGA	Primers for cloning and dsRNA synthesis (position 1256-1536 in ISCW009412)
IXOCRNAI-R	ATTCTAGACTGTGTCTCCTCAGACTGT	
IR23	CACCCTGGAGACCTTCGAG	qRT primers for gene expression determination
IR24	TCTCAGAACCCACGACGAA	
IR201	AACACTCCATCGACCAAAGG	qRT primers - verification set for RNAi
IR202	CTTCATCTCCACCGCCTTC	

Name	Sequence	Description
qRT primers for gene expression quantification - *I. ricinus* housekeeping genes		
ACT F	CGACATCAAGGAGAAGCTCTG	*actin* (AJ889837)
ACT R	GTCGGGAAGCTCGTAGGAC	
EF1 F	ACGAGGCTCTGACGGAAG	*elongation factor 1* (GU074828)
EF1 R	CACGACGCAACTCCTTCAC	
qRT primers for quantification of *B. afzelii* and bacteria in ticks		
FlaF1A	AGCAAATTTAGGTGCTTTCCAA	*flagellin B. burgdorferi* sensu lato (conserved among *Borrelia* species) (Schwaiger et al., 2001)
FlaR1	GCAATCATTGCCATTGCAGA	
Fla Probe1	TGCTACAACCTCATCTGTCATTGTAGCA TCTTTTATTTG	
Mm actin F	AGAGGGAAATCGTGCGTGAC	*B-actin Mus musculus* (NM007393.3) (Dai et al., 2009)
Mm actin R	CAATAGTGATGACCTGGCCGT	
Mm actin P	CACTGCCGCATCCTCTTCCTCCC	
Q16S-F	TCCTACGGGAGGCAGCAGT	16S ribosomal subunit (conserved among bacteria) (Nadkarni et al., 2002)
Q16S-R	GGACTACCAGGGTATCTAATCCTGTT	
Q16S-P-FAM	CGTATTACCGCGGCTGCTGGCAC	

3.1.3 Plasmids

Plasmid pll10 was used, which is a modified pBluescript-based vector with two T7 promoters in reverse orientation (Levashina et al., 2001). Plasmids carrying parts of genes for *ixoA*, *ixoC* and *ixoB* were previously created in the laboratory, named plOH34, plOH35 and plOH36, respectively, and stored at -80°C and further used.

3.1.4 Bacteria and other microorganisms

E. coli (One Shot TOP10 Chemically Competent *E. coli* from Invitrogen – Life Technologies) were used for transformation. *B. afzelii* (strain CB43 isolated from *I. ricinus* from Novohradské hory, Czech Republic) was used in all experiments. Further *C. albicans*

(source: Institute of Parasitology, Biology Center CAS, České Budějovice) was used in in-vitro phagocytic assay.

3.1.5 Commercial sets and chemicals

Table 2: List of commercial sets.

Name	Producer
NucleoBond Xtra Midi	Macherey-Nagel
NucleoSpin Gel and PCR clean-up	Macherey-Nagel
MEGAscript T7 High Yield Transcription Kit	Ambion
NucleoSpin RNA	Macherey-Nagel
NucleoSpin Plasmid	Macherey-Nagel
NucleoSpin Tissue	Macherey-Nagel

Table 3: List of chemicals.

Experiment name	Chemicals used	Producer
HAEMOLYMPH COLLECTION FOR TOTAL RNA ISOLATION	TRIzol®	Life Technologies
HAEMOLYMPH COLLECTION FOR PHAGOCYTOSIS ASSAY	Medium L15 + 10% BOFES (pH6.8)	
CLONING AND SEQUENCING	LB medium with ampicillin (AMP)	Amresco
	Restriction enzymes: ApaI, XbaI + Buffer Tango	Thermo Scientific
	Agar plates with LB + AMP	Amresco
	Glycerol	
	SOC medium	Invitrogen
	T4 ligase + Buffer 2x	
dsRNA PREPARATION	Restriction enzyme: ApaI + Buffer Tango	Thermo Scientific
	Restriction enzyme: XbaI + Buffer Tango	Thermo Scientific
	Proteinase K	Sigma Aldrich

	10% SDS solution	
	Phenol-chloroform	Sigma Aldrich
	Chloroform	Lach:ner
	Isopropanol	Lach:ner
	Ethanol	Lach:ner
	RNA loading dye	Ambion
	GeneRuler 100bp Plus	Fermentas
PCR	FastStart MasterMix	Roche
ELECTROPHORESIS	Agarose	Sigma Aldrich
	Ethidium Bromide	
TOTAL RNA ISOLATION	TRI Reagent solution	Sigma Aldrich
	Chloroform	Lach:ner
	Isopropanol	Lach:ner
	Ethanol	Lach:ner
QRT-PCR	FastStart Universal SYBR Green Master	Roche
	FastStart Universal Probe Master (ROX)	Roche
PHAGOCYTOSIS	4% Formaldehyde	
	BSA (Bovine Serum Albumin)	Sigma-Aldrich
	PBS (Phosphate-buffered saline)	
	Primary antibody - polyclonal rabbit anti *B. burgdorferi*	Produced by Parasitology Institute, BC AS
	Secondary fluorescent antibody - ALEXA 594	Life technologies
	Secondary fluorescent antibody - ALEXA 488	Life technologies
	1% BSA in 1% Triton^{TM} X-100 in PBS	Sigma-Aldrich
	0.1% Triton^{TM} X-100 in PBS	Sigma-Aldrich
	DAPI (1,4-diamino-2-fenylindol)	Sigma-Aldrich
	DABCO (3,3-diaminobenzidin)	Sigma-Aldrich
METHYLAMINE TREATMENT	0.2 M methylamine	
	0.2 M glycine	

3.2 Methods

3.2.1 Cloning and sequencing

Small amounts of frozen bacterial stocks were scratched and transferred into 100 ml liquid LB medium with ampicillin. After overnight incubation at 37°C, plasmids were isolated from the bacteria using NucleoBond Xtra Midi kit (Macherey-Nagel). Presence of desired sequences of *ixoA* and *ixoC* in correct position in the plasmids was confirmed by sequencing.

Desired *ixoB* sequence was not confirmed in the previously cloned plasmid. Therefore, its nucleotide sequence (249 bp) suitable for RNA interference was amplified by standard PCR with 30 cycles and annealing temperature 55ºC. For this procedure, cDNA from salivary glands was used as a template and primers containing restriction sites for ApaI and XbaI enzymes (IXOBRNAI F and R) were used for amplification. The DNA band was cut of the agarose gel and purified using NucleoSpin - Gel and PCR clean-up kit (Macherey-Nagel). The PCR product (20 μl) and the plasmid pll10 (3 μl) were cleaved with ApaI and XbaI restriction enzymes (2 hours at 37°C) and purified using NucleoSpin - Gel and PCR clean-up kit (Macherey-Nagel). PCR product and plasmid were ligated for 1 hour at room temperature followed by overnight ligation at 4ºC. 50 μl of One Shot TOP10 Chemically Competent *E. coli* were transformed with the whole ligation mixture (10 μl). The transformed bacteria were plated on LB-agar plates with ampicillin and grown overnight. MidiPrep was prepared from selected colony and the plasmid was isolated using NucleoBond Xtra Midi kit (Macherey-Nagel). Presence and position of *ixoB* sequence was confirmed by sequencing. Sequencing was done by SEQme company (Czech Republic). Plasmid stocks of 100 μl were prepared for *ixoA* (plOH34 - 2793 μg/ml), *ixoB* (plOH36 - 2575 μg/ml) and *ixoC* (plOH35 - 2858 μg/ml).

3.2.2 RNAi silencing

3.2.2.1 dsRNA preparation

The three purified plasmids carrying individually *ixoA, ixoB* and *ixoC* desired sequences were cleaved once with ApaI and once with XbaI (separately). A total of 30 μg of each plasmid were used for each linearization. The linearized plasmids were purified as follows: plasmids were incubated with Proteinase K in presence of 0.5% SDS. Linearized plasmids were extracted by phenol-chloroform extraction, precipitated by isopropanol, washed with

ethanol and dissolved in DEPC water. ssRNA was synthesized overnight using a MEGAscript T7 Transcription kit (Ambion) according to manufacturer's instructions, purified linearized plasmids were used as templates. ssRNA was treated with DNAse and ammonium acetate and then extracted with phenol/chloroform extraction. Isopropanol was used to precipitate ssRNA, which was subsequently dissolved in DEPC (Diethylpyrocarbonate - treated) water. Hybridization of corresponding complementary ssRNAs (1:1 ratio, 3 μg/μl each) was carried out overnight in water bath with decreasing temperature (95 °C decreasing to 20 °C). The integrity and size of dsRNAs were checked on agarose gel, their concentration were measured and diluted to 3 μg/μl concentration and then used for RNA interference in female ticks. Control *gfp* dsRNA was prepared using the same method from linearized plasmid pll6 (Levashina et al., 2001) by Veronika Urbanova and stored at -80 ºC. dsRNA nucleotide sequence for *ixoB* was blasted against *I. ricinus* FREPs sequences and proved to be effective against all *ixoderins B* (*ixoB* and *ixoB2* - *ixoB6*).

3.2.2.2 Tick injection

The dsRNA for each *ixoderin* (300-500 nl; 3 μg/μl) was injected into the hemolymph (in coxa III) of unfed *I. ricinus* female using a micromanipulator (Narishige). Control ticks were injected with the same volume of *gfp* dsRNA. Injected ticks rested for 24 hours in humid chamber. Afterwards, ticks were fed on guinea pigs for 5-6 days to semi-engorgement. Ticks were further used for hemolymph collection and tissue dissection.

For triple RNAi (*ixoderins A, B* and *C*); together 66 nl – 22 nl of each dsRNA (mixed before) was injected into coxa III of unfed *I. ricinus* nymphs using the same instrumentation. Control nymphs were injected with 66 nl of *gfp* dsRNA. Nymphs were let to recover for 3 days in humid chamber and then were fed on CD1 mice for 3-4 days until repletion. Afterwards, nymphs were used for total RNA isolation.

3.2.3 Hemolymph collection and tissue dissection

Tick hemolymph was collected from a cut on a front leg. Each experimental group comprised of 25 semi-engorged tick females (*gfp* dsRNA injected, non-injected or *ixoderin A, B* or *C* dsRNA injected). For phagocytosis assay, hemolymph was collected into 1 ml of media L15 + 10% BOFES (pH6.8). For RNA isolation, hemolymph of 25 semi-engorged females was collected into TRIzol (250 μl).

Additionally, 10 semi-engorged female ticks for each experimental group were

dissected on Petri dish filled with wax under a binocular dissection microscope. Tissues were washed in PBS (DEPC water used for preparation) during and after dissection. Ovaria, salivary glands, Malpighian tubules, trachea, gut and rest of the body were separated for total RNA isolation.

The total RNA for different tick stages was isolated from pools of ticks in different developmental stadia: half of the batch of eggs, 20 unfed larvae, 20 fed larvae, 10 unfed nymphs, 10 fed nymphs, 10 males, 5 unfed females, 5 females fed for 6 days and pool of 5 fully fed females. The total RNA from *ixoderins A, B* and *C*-silenced nymphs (triple KD) and control nymphs (*gfp* KD) was isolated from pools of 5 whole fully fed nymphs.

3.2.4 Total RNA isolation and cDNA preparation

Total RNA from tissues and from the whole tick bodies was isolated using NucleoSpin RNA II kit (Macherey-Nagel) according to manufacturer's instructions. RNA concentration was measured on spectrometer. Total hemolymphal RNA was obtained by chloroform extraction from TRIzol. RNA was precipitated using isopropanol, washed with 80% ethanol and dissolved in water. Hemolymphal RNA concentration was measured using spectrometer BioPhotometer plus (Eppendorf). RNA was treated with DNAse in DNAse buffer, precipitated by isopropanol, washed by 80% ethanol and dissolved in DEPC water. RNA concentration was measured spectrometrically.

Isolated RNA was reverse-transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Anchored-oligo (dT)18 primers were used. The cDNAs were diluted 10x in the end and stored at -80°C.

3.2.5 qRT-PCR

First, gene specific primers were tested in conventional PCR (annealing temperature 60°C, 40 cycles) on *I. ricinus* control cDNA (*gfp* KD). Then, quantitative reverse transcription PCR (qRT-PCR) was used for determination of relative gene expression in different tissues of semi-engorged female ticks. Further, this method was used for determination of relative gene expression in different tick stages. In both, tissue and stages profile determination, FastStart Universal SYBR Green Master (ROCHE) and gene specific primers IR3+IR4, IR153+IR154, IR23+IR24 for *ixoA, ixoB* and *ixoC*, respectively, and gene specific primers ACT F+R and EF1 F+R for housekeeping genes *actin (act)* (AJ889837) and

elongation factor 1 (ef1) (GU074828) (Nijhof et al., 2009), respectively, were used in 0.4 μM concentrations in 25 μl reactions.

qRT-PCR pipetting was prepared using epMotion 5070 (Eppendorf). The runs were performed and analyzed using Light-cycler 480 (ROCHE). All samples were analyzed in triplicates. The threshold cycles were determined as the resulting arithmetic means of three measurements in triplicates. Melting curves for each sample was checked. The relative expression levels were calculated based on expressions of housekeeping genes.

qRT-PCR was further used for *ixoderins* silencing verification in tissues of semi-engorged tick females and for verification of triple RNAi of all three *ixoderins* in nymphs. For gene silencing verification, FastStart Universal SYBR Green Master (ROCHE) and gene specific primers IR199+IR200, IR153+IR154, IR201+IR202 for *ixoA*, *ixoB* and ixo*C* respectively and ACT F+R for *act* were used in 0.4 μM concentrations in 25 μl reactions. The relative expressions of *ixoderins* were calculated based on *act* expression. The gene-silencing efficacy was expressed in percentage of gene expression silencing against *gfp* RNAi control group.

3.2.6 Phagocytic assay with double staining of phagocytosed and free pathogens

Hemolymph was collected from 25 semi-engorged females of *ixoA*, *ixoB* and *ixoC* knockdown group as well as from *gfp* KD control group into 1 mL L15 medium with 10% BOFES (filtered over 22 μm filter, pH adjusted to 6.8 using 1 M HCl). Hemocytes concentration was determined by counting them in Bürker chamber. Determined volume of hemolymph was placed onto a round microscope cover slip in order to obtain 4 x10^4 hemocytes on each slip.

3.2.6.1 Phagocytosis of *Borrelia afzelii*

Borrelia afzelii CB 43 strain spirochetes (10^6 bacterial cells estimated by counting under microscope) were added to the hemolymph and mixed properly. The volume was filled up to 250 μl with L15 medium with 10% BOFES. The hemocytes were allowed to adhere to the cover slide and to phagocyte *B. afzelii* for 2 hours at 28°C in a humid dark chamber. The slides were quickly washed with PBS and then fixed with 4% formaldehyde for 30 minutes and washed again 3 x 10 minutes with PBS.

Phagocytosis of spirochetes was detected by indirect immunofluorescence (an example of phagocytosis as seen under the confocal microscope in Fig. 2). The cover slip was treated with primary antibody (polyclonal rabbit anti-*B. burgdorferi*, 1:200 in PBS) for 1 hour at room temperature. After another three washing steps with PBS, secondary fluorescence antibody ALEXA 594 (red dye, 1:500 in PBS) was added and incubated for 1 hour. Another washing steps with PBS were performed. The hemocytes were permeabilized overnight at 4°C with 1% BSA in 1% Triton X-100 in PBS. Primary antibody (polyclonal rabbit anti-*B. burgdorferi* 1:200 in 0.1% Triton X-100 in PBS) was added and the cover slip was incubated for 1 hour at room temperature. Three times ten minutes washing with 0.1% Triton X-100 in PBS was done. Then, the cover slip was incubated with secondary anti rabbit antibody ALEXA 488 (green dye, 1:500 in 0.1% Triton X-100 in PBS) for 1 hour at room temperature. After another washing steps, cell nuclei were stained with DAPI for 10 minutes. The preparations were then mounted with 2.5% DABCO on the microscope slide. Approximately six cover slides were prepared for each *ixoderin* RNAi-mediated knockdown. Each *ixoderin* knockdown was performed three times.

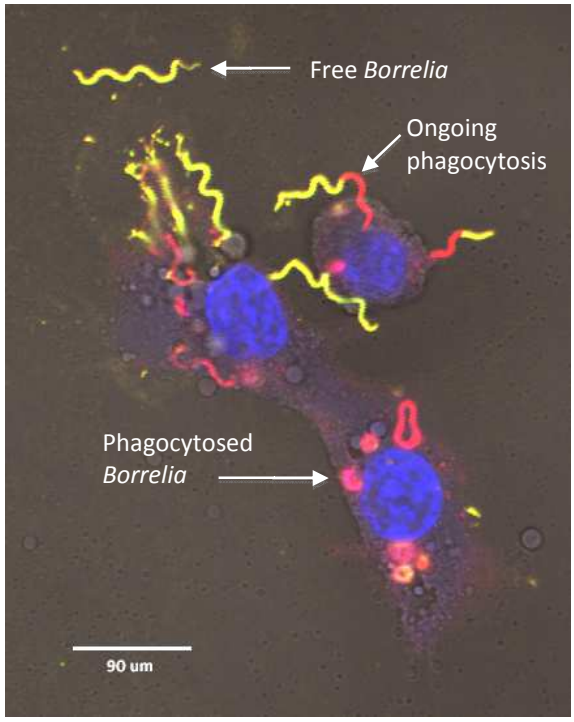


Figure 2: Example of *B. afzelii* phagocytosis by tick hemocytes under confocal microscope, merged picture. The red spirochetes (or their parts) are already phagocytosed, while the yellow spirochetes are not. The nuclei are stained in blue.

3.2.6.2 Phagocytosis of *Candida albicans*

The hemocytes on the cover slide were let to adhere for 15 minutes at 30°C. Paraformaldehyde-pretreated *Candida* cells were then added and incubated for 1 h 45 min at 30°C. The 30 minutes long 4% formaldehyde fixation step followed immediately. The cover slides were then 2x washed with PBS for 10 minutes, the cell nuclei were stained with DAPI (2.5 µg DAPI in 1 ml distilled water) for 10 minutes and the slides were then washed once again with PBS for 10 minutes. Finally, the cover slides were mounted with 2.5% DABCO on a microscope slide. Approximately six cover slides were prepared for each gene.

The preparations were examined with the fluorescence microscope Olympus BX 60 with dual filter (source of fluorescence: Olympus U-RFL-T). A total of 100 hemocytes were examined for each slide and number of hemocytes with engulfed or partially engulfed spirochetes was counted. The phagocytic index was then calculated as a percentage of phagocyting hemocytes. Resulting phagocytic indexes were then related to the *gfp* KD control indexes (*gfp* KD control taken as 100%) and the relative phagocytoses was calculated. Pictures were taken on the confocal microscope Olympus FW1000 with red, green and DAPI filter and bright field and assembled using FluoView FV10-ASW 4.0 (version for Windows, Olympus Corporation) and Photoshop 7 CE (version for Windows, Adobe Systems Inc.).

3.2.7 Transmission of *Borrelia afzelii*

A total of 100 *I. ricinus* nymphs infected with *B. afzelii* CB 43 were separated into test and control group (each of 50 nymphs). The control group was injected with 66 nl / nymph of dsRNA for *gfp* RNAi, the test group was injected with mix of dsRNA for *ixoderins A, B* and *C* (22 nl each). Both, test and control groups were allowed to feed on C3H/N mice (10 nymphs per one mouse). As reaching the fully fed stage, the nymphs were collected and weighed. DNA was isolated from individual nymphs using NucleoSpin Tissue kit (Macherey-Nagel). The DNA presence in every sample was verified by a standard PCR with primers for tick *actin*. Presence of spirochetes was detected by a standard PCR with primers FlaF1A + FlaR1 for *B. burgdorferi flagellin* detection. The positive samples were further investigated by qPCR to absolutely quantify *Borrelia* sp. genome copies. FastStart Universal Probe Master (ROX) (ROCHE), primers FlaF1A + FlaR1 and Fla probe1 were used. The total number of *Borrelia* sp. genome copies was determined per tick (per 60 μl of DNA isolate) against external *B. burgdorferi flagellin* standard curve.

Individual mice ear biopsies were collected once per a week for four weeks following the tick engorgement. After the final ear biopsy, the mice were sacrified and tissue samples from urinary bladder, joint and heart were dissected. DNA was isolated from all the tissues using NucleoSpin Tissue kit (Macherey-Nagel). Numbers of *B. afzelii* present in the tissue samples were quantified using qPCR, in which FastStart Universal Probe Master (ROX) (ROCHE), primers FlaF1(A) + FlaR1 and probe Fla probe1 for *B. burgdorferi flagellin* detection and primers Mm actin F + Mm actin R and probe Mm actin P for *Mus musculus actin* detection were used. The number of borreliae was determined per 10^5 *M. musculus*

actin copies. Murine *actin* was absolutely quantified against *M. musculus actin* standard curve.

Furthermore, general number of bacteria per nymph was absolutely quantified by qPCR using the same method with different primers: q16S-F, q16S-R and different probe q16S-P-FAM. These primers and probe were designed to detect the 16S ribosomal subunit of bacteria, which is highly conserved among the bacterial species. The absolute quantification was done against external 16S ribosomal subunit standard curve.

3.2.8 Database search and phylogenetic analysis

Protein sequence of IXOA from *I. ricinus* (AAQ93650) was used as a template for BLAST analysis against *I. scapularis* genome (vectorbase.org) in order to obtain all the protein and nucleotide sequences of fibrinogen related proteins from *I. scapularis*. Further, FREPs related sequences of other organisms were added to the list. These were Dorin M and OMFREP from *O. moubata*, Ixoderins A, B, B3, B4, B5 and B6 from *I. ricinus*, recently obtained sequence of Ixoderin C from *I. ricinus* (Kotsyfakis et al., 2015) (Transcriptome Shotgun Assembly), Tachylectin 5A and 5B from *T. tridentatus*, FBN8, FBN9 and FBN39 from *A. gambiae*, fibrinogen and ficolin from *Homo sapiens*, FREP 2, 3 and 4 from *B. glabrata*. The complete list can be found in SupTab. 1 in the supplement. For each protein sequence, signal sequence was analyzed using online programs PSORT (http://psort.hgc.jp/form.html) and SIGNAL P (http://www.cbs.dtu.dk/services/SignalP/). Conserved domain sequences were found out for each protein sequence using online BLAST "conserved domain sequence" (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

First, protein sequences were processed in BioEdit using ClustalW alignment. Some sequences were not of sufficient quality and therefore these were excluded from the alignment (ISCW000158, ISCW013797, ISCW012248). The sequence alignments were manually checked.

In order to determine the phylogenetic relationship among tick FREPs, only the common conserved FReD sequences were used; in most of the sequences the A subdomain was missing, thus this part was cut-off in the remaining sequences. On the other hand, in order to determine the relationships among FREPs from various organisms, the full FReD sequence as described by (Doolittle, 1992; Doolittle et al., 2012) was used for alignment, keeping the sequences with missing parts. The sequence ISCW022063 was omitted from full FReD sequence comparison as its A domain varies greatly from the others A domains while

its C-terminal B and P part remains common with other FREPs.

The files were further proceeded in MEGA 6 (Tamura et al., 2013) according to protocol from Hall (2013). The best models for maximum likelihood trees were estimated and the maximum likelihood trees were created with default set up with Bootstrap of 500 replicates. The picture was adjusted in Inkscape (version 2 for Windows, Free Software Foundation, Inc., Boston, MA, USA).

3.2.9 Data analysis

The obtained data were processed using GraphPad Prism 4 (version for Windows, GraphPad Software), where statistical analyses were run and graphs produced. p-values of <0.05 was considered statistically significant. One-way ANOVA (Tukey test) or unpaired t-test (two-tailed, F-test used to compare variances) was used for sample groups comparison. Data normalization was done via logarithm for numbers of bacteria and *Borrelia* spirochetes in nymphs due to high variances of the compared groups of samples. The error bars show standard errors of the means in the graphs as three independent biological replicates were used.

4. Results

4.1 Genome analysis

In order to determine evolutionary relationships among all FREPs from *I. scapularis* and several known FREPs from other tick species and in order to find a developmental context between FREPs from ticks and other organisms, maximum likelihood phylogenetic trees were created based on amino acids sequences alignments. The sequence alignment used for the phylogenetic tree construction can be seen in SupFig. 1 in the supplement. The resulting tree of selected tick FREPs (Fig. 3) demonstrates that these protein sequences are grouped into three phylogenetically distant groups: Ixoderins A, Ixoderins B and Ixoderins C.

Ixoderins A from *I. ricinus* and *I. scapularis* were found to be related to OMFREP and Dorin M from the soft tick *O. moubata*. The name Ixoderin A denotes two analogues from *I. ricinus* and *I. scapularis*. However, *I. scapularis* genome contains multiple (7) Ixoderins A. Out of these, the second closest was named Ixoderin A2. Its expression and expression of its *I. ricinus* analogue would be silenced by the same dsRNA used for *ixoA*. The group of Ixoderin B protein sequences consists of FREPs from *I. scapularis* (15) and *I. ricinus* (5). Two proteins from *I. scapularis* (ISCW003711 and ISCW008812) are rather distant from the rest of the group. Interestingly, these two proteins are the only Ixoderins B from *I. scapularis*, which do possess full fibrinogen-related domain, like all true Ixoderins B from *I. ricinus*. All other *I. scapularis* Ixoderins B lack the A subdomain of FReD. One Ixoderin C representative (analogue) was found in both studied *Ixodes* tick species and one additional Ixoderin C-related sequence was found in the *I. scapularis* genome.

In the context of other organisms (tree in Fig. 4, based on alignment showed in SupFig. 2 in the supplement), Ixoderin A group seems to be present in both hard and soft ticks and is more closely related to the FREPs from other organisms. Ixoderin B group creates a clearly separated group, so far containing exclusively representatives from *Ixodes* ticks. Ixoderin C group of protein sequences was separated from the other groups of Ixoderins. The phylogenetic position of Ixoderins C is uncertain due to low bootstrap value.

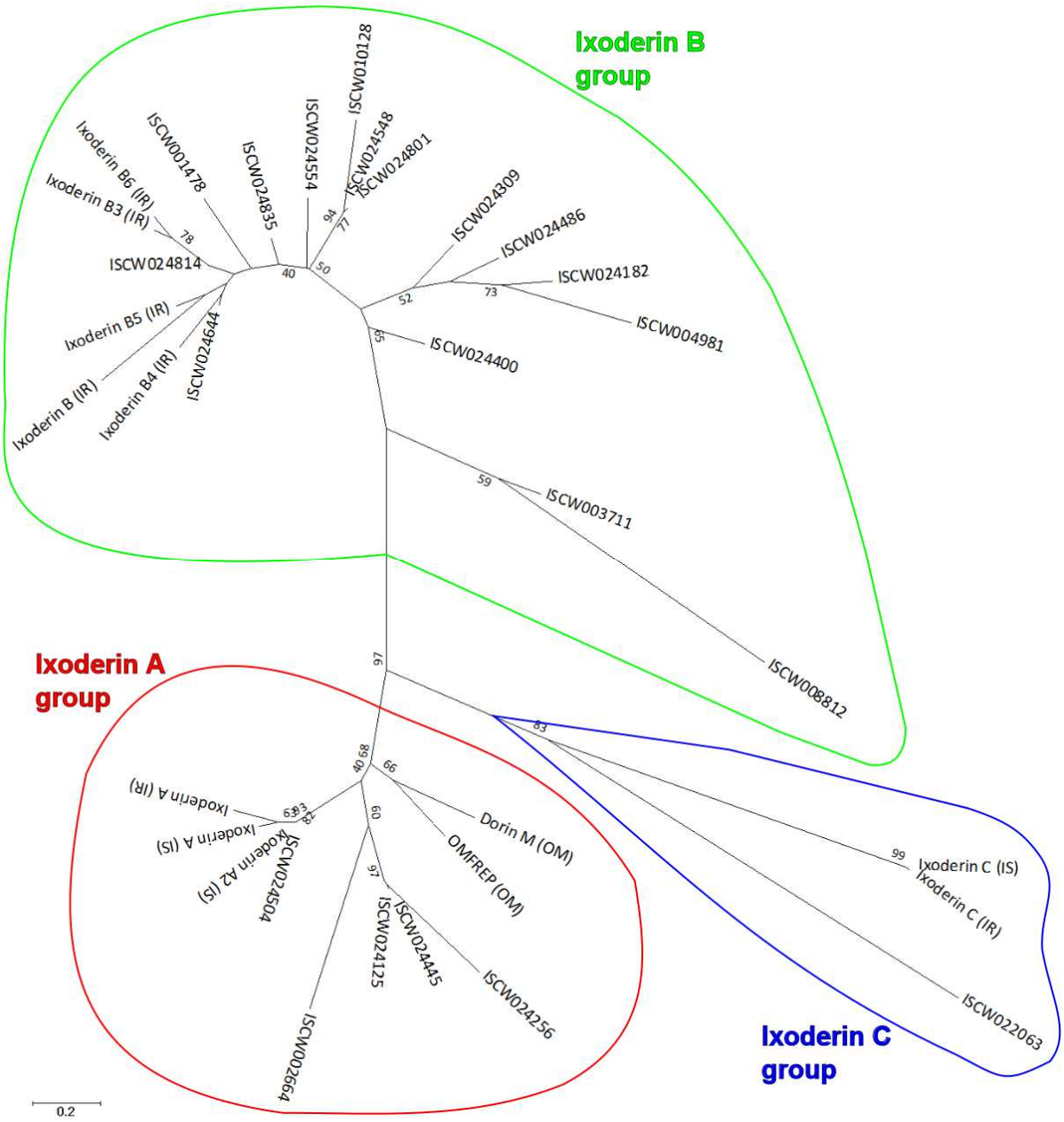


Figure 3: Phylogeny of tick FREPs. Unrooted maximum likelihood tree was constructed using common part of FReD of tick FREPs. Numbers at the branches denote Bootstrap values. The scale bar denotes the length of branch representing 0.2 substitutions per site. IS - *Ixodes scapularis*, OM - *Ornithodoros moubata*, IR - *Ixodes ricinus*, sequence names starting with ISCW belong also to *Ixodes scapularis* genome.

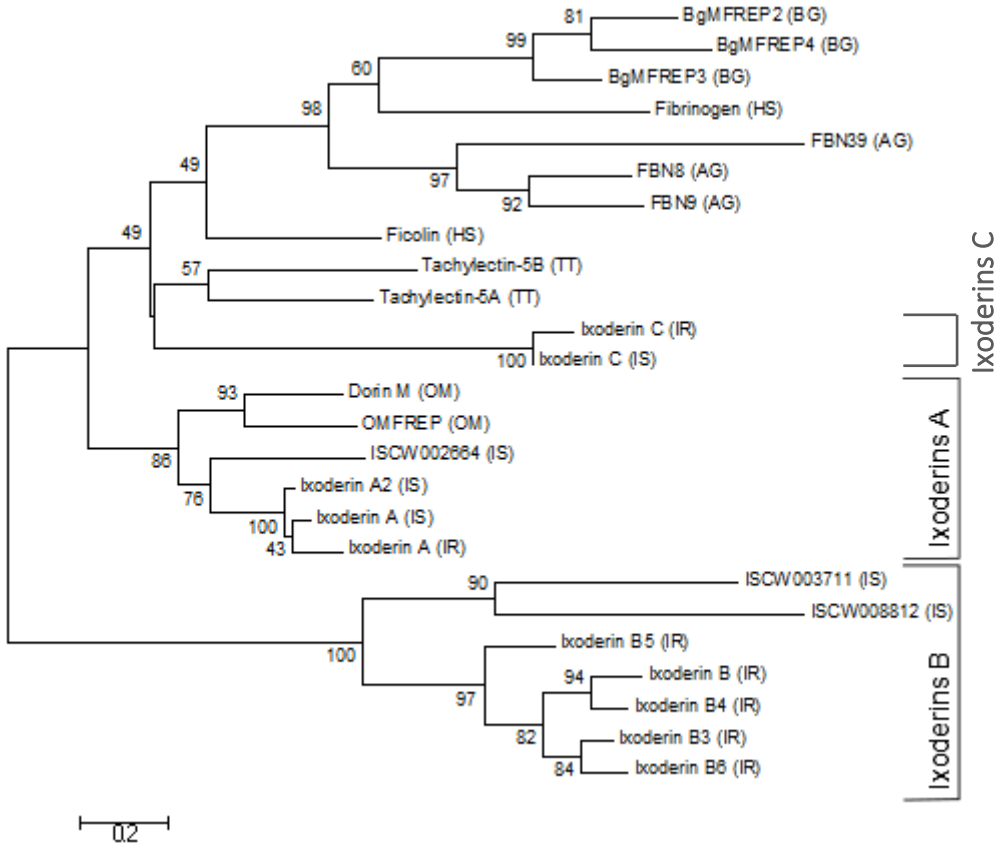


Figure 4: Phylogeny of tick FREPs put into context with selected organisms. Unrooted maximum likelihood tree was constructed using full-length fibrinogen-related domain sequences in FREPs of ticks and selected organisms. Numbers at the branches denote Bootstrap values. The scale bar denotes the length of branch representing 0.2 substitutions per site. IS - *Ixodes scapularis*, OM - *Ornithodoros moubata*, IR - *Ixodes ricinus*, TT - *Tachypleus tridentatus*, AG - *Anopheles gambiae*, HS - *Homo sapiens*, BG - *Biomphalaria glabrata*.

4.2 Comparison of *ixoderin* expression levels among different tissues of *I. ricinus*

qRT-PCR was used to determine relative gene expression in different tissues of semi-engorged female ticks based on comparison with housekeeping gene *actin* (Fig. 5).

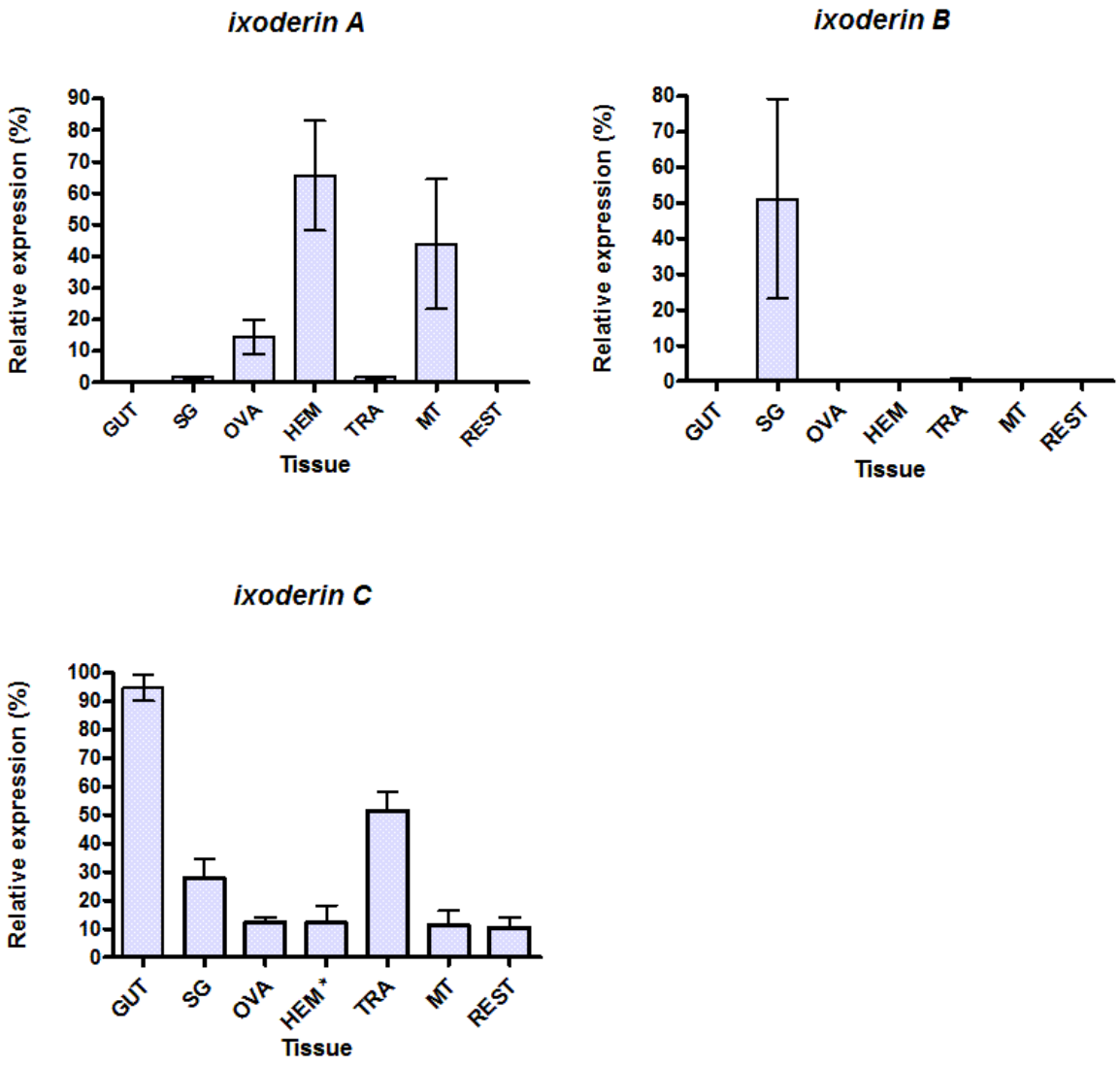


Figure 4: The relative expression of *ixoderins A, B* and *C* against housekeeping *actin* in tissues of half-fed tick females. The highest value is set to 100%. The error bar represents the standard error of mean from three independent biological replicates – pools of ticks. The asterisks labeled (*) tissues/cells represent the results from only two replicates. GUT – gut, SG – salivary glands, OVA – ovaria, HEM – hemolymph, TRA – trachea, MT – Malpighian tubules, REST – rest of the body.

Increased expression levels of *ixoA* were recorded in ovaria, hemolymph and Malpighian tubules. Elevated levels of *ixoB* were found solely in the salivary glands while increased expression levels of *ixoC* were detected in all the examined tissues. Considerable variability was found in the level of expression of the same *ixoderin* sequence among different tissues. The variability in expression among the three testing groups of ticks per tissue (each tissue was analyzed in three independent pools of 10 ticks) was higher in the case of *ixoA* and *ixoB* compared to *ixoC* as seen in Tab 4.

Table 4: The variability in *ixoderins* expressions among three independent biological samples – pools of ticks. The results are relative, based on housekeeping gene *actin* levels. GUT – gut, SG – salivary glands, OVA – ovaria, HEM – hemolymph, TRA – trachea, MT – Malpighian tubules, REST – rest of the body, " – " – inconsistent value

Tissues	*ixoderin A* expression (%)			*ixoderin B* expression (%)			*ixoderin C* expression (%)		
	Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3
GUT	0.28	0.18	0.05	0.30	0.00	0.00	85.66	98.40	100.00
SG	1.48	2.19	1.53	50.70	2.85	100.00	16.38	39.69	27.55
OVA	25.58	9.28	8.48	0.02	0.00	0.19	15.35	12.88	8.13
HEM	45.17	100.00	51.64	0.00	0.00	0.00	6.04	18.30	-
TRA	2.21	1.75	1.06	0.01	0.00	1.28	61.84	53.96	39.00
MT	45.80	78.64	7.47	0.00	0.00	0.00	21.32	8.88	3.18
REST	0.32	0.44	0.25	0.00	0.00	0.01	4.93	17.08	9.58

4.3 Comparison of *ixoderin* expression levels among different developmental stages of *I. ricinus*

Levels of *ixoderin* genes expression were compared among the developmental stages of *I. ricinus*. qPCR was used for determination of relative gene expression in different stages of the tick development compared to expression level of housekeeping gene *elongation factor 1*. The expression of *ixoA* increased after feeding more than ten times (Fig. 6) in all tested developmental stages. Also relatively high expression level was recorded in eggs. *Ixoderin B* expression in different tick stages exhibits relatively low values. An relative high expression level was observed in one pool of fed tick females, while in another pool, no expression of *ixoB* was observed (SupFig. 3B in the supplement). Hence the resulting mean has an extremely high standard error (SupFig. 3A in the supplement). In order to see the variability in expression among stages, the high expression value in the second pool of fed females was removed and new 100% level was adjusted (Fig. 6). In this graph (Fig. 6), the unfed larvae show about 42% higher gene expression than fed larvae. The unfed nymphs express *ixoB* on the level of 30% compared to the highest measured expression value. The fed nymphs do not show any *ixoB* expression. On the contrary, unfed females show generally lower expression rate than the 6-days fed females. The results of fully fed females are not conclusive as one pool did not show any *ixoB* expression and the second pool showed extremely high expression.

Ixoderin C mRNA was recorded in all the developmental stages tested, in unfed as well as in fed ticks. Although the expression levels of *ixoC* were considerably variable, no particular pattern in expression during tick development was observed (Fig. 6).

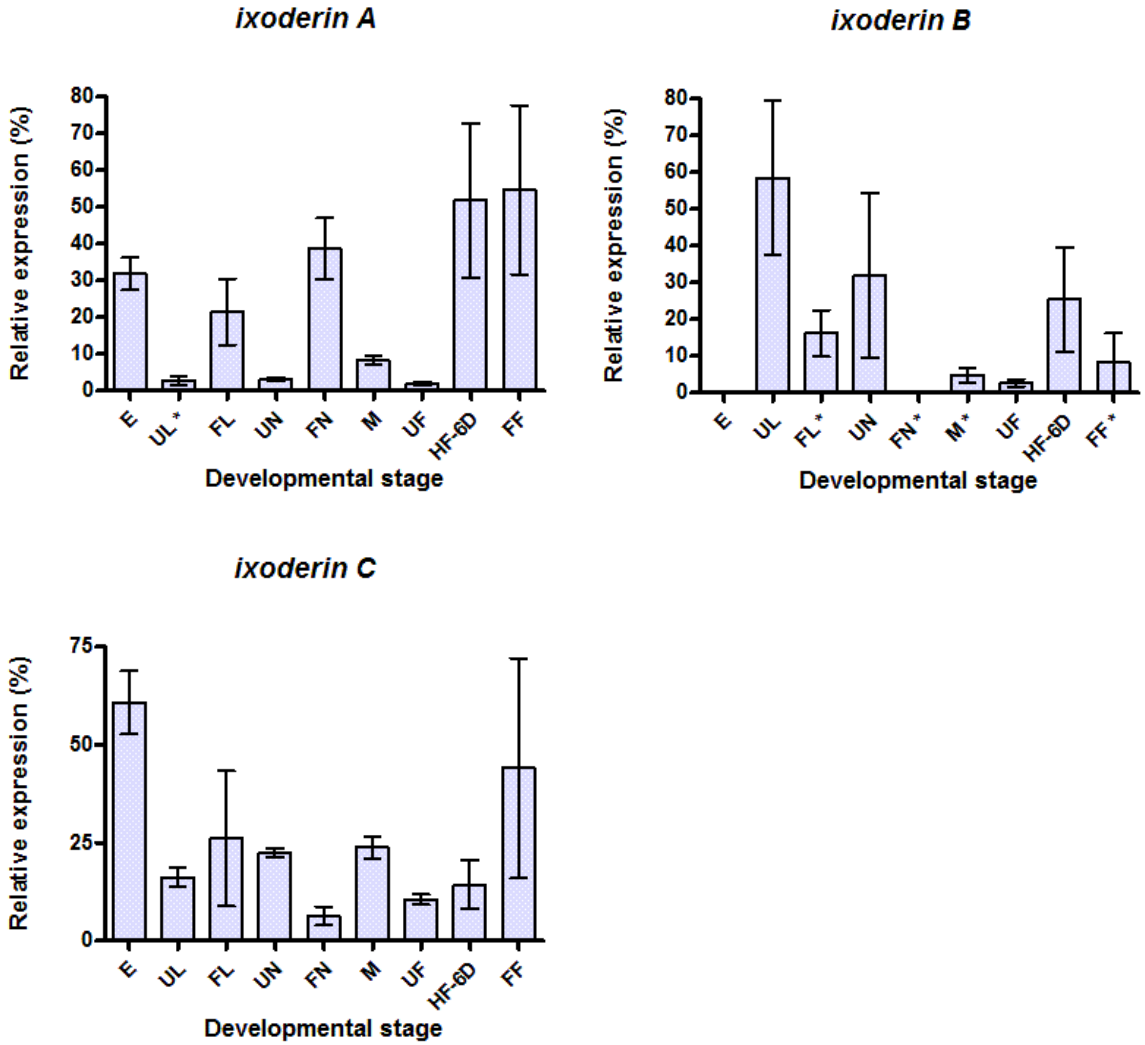


Figure 6: The relative expression of *ixoderins A*, *B* and *C* against *elongation factor 1* in different tick developmental stages. The highest value is set to 100%. The error bar represents the standard error of mean from three independent biological replicates – pools of ticks. The asterisks labeled (*) stages represent the results from only two replicates. Developmental stage: E - eggs, UL - unfed larvae, FL - fed larvae, UN - unfed nymphs, FN - fed nymphs, M - males, UF - unfed females, HF-6D - 6 days fed females, FF - fully fed females.

4.4 Knockdown verification

4.4.1 dsRNA synthesis

An injection of prepared dsRNA for RNAi of individual *ixoderins* into unfed tick females was used for knockdown of *ixoderin A*, all *ixoderins B* and *ixoderin C*. Triple-KD with mixture of all dsRNA for *ixoderins* was carried out in unfed nymphs. The overview of the dsRNA synthesis procedure products can be seen in Fig. 7. The efficiency of the knockdowns was verified by qRT-PCR (subchapters 4.4.2 and 4.4.3).

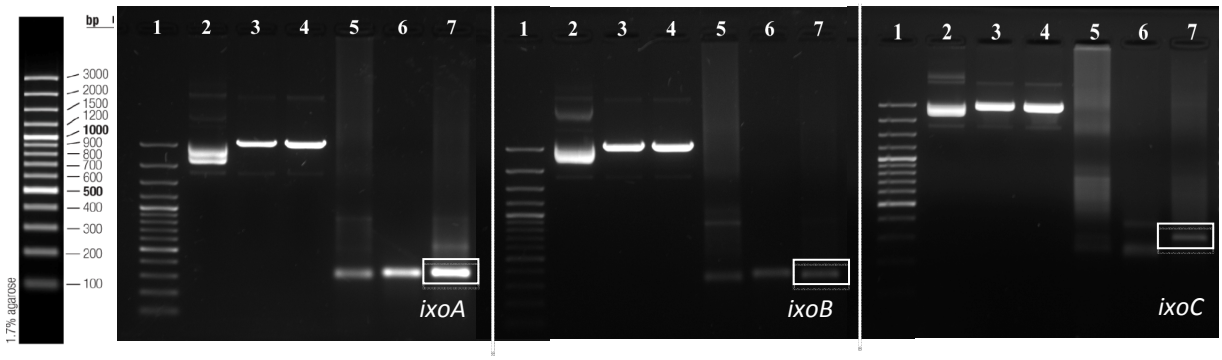


Figure 7: Preparation of dsRNA for knockdown of *ixoA*, *ixoB* and *ixoC*. GeneRuler 100bp Plus (Fermentas) is presented in the first line. The process of dsRNA synthesis is shown for each *ixoderin*: column 1 - marker, 2 - circular plasmid, 3 - ApaI cut plasmid, 4 - XbaI cut plasmid, 5 - ssRNA from ApaI cut plasmid, 6 - ssRNA from XbaI cut plasmid, 7 - dsRNA

4.4.2 Knockdown in tick females

In order to determine whether the KD was efficient, cDNA prepared from ticks after KD was used as a template for qRT-PCR to estimate the level of *ixoderins* expression. The estimated levels of *ixoderins* mRNA expression were compared to control groups of ticks injected with *gfp* dsRNA. Both *ixoderins* expression levels (after *ixoderin* KD and *gfp* control) are relative, related to the housekeeping gene *actin*. The changes in expression level in tissues after knockdown of individual *ixoderins* are summarized in Tab. 5.

The gene silencing of *ixoA* was generally very successful. The gene silencing ratio ranged in different tissues from 33.6x to 2.1x decrease against control samples. The effect of the knockdown was observed in all the tissues including tissues with high expression levels (OVA, HEM, MT). The relevant expression tissue for KD verification of *ixoB* is salivary glands, where the observed decrease is 96.0% (25.2x). The efficiency of *ixoC* silencing corresponds to approximately 3x decrease of the expression level in the tissues with high expression levels (GUT, SG, TRA).

Table 5: Summary of individual *ixoderins* expression level change after knockdown in tissues of half-fed tick females. The results are relative, based on housekeeping gene *actin* levels. The tissues where given *ixoderin* is mainly expressed are highlighted by green color. GUT – gut, SG – salivary glands, OVA – ovaria, HEM – hemolymph, TRA – trachea, MT – Malpighian tubules, REST – rest of the body

***ixoA*, *ixoB* and *ixoC* level in tissues after individual knockdowns (%)**			
Tissue	Expression change in *ixoderin A* after KD	Expression change in *ixoderin B* after KD	Expression change in *ixoderin C* after KD
GUT	**-2.3x**	+11.9x	**-2.9x**
SG	**-3.4x**	**-25.2x**	**-2.9x**
OVA	**-9.1x**	**-1.6x**	**-17.1x**
HEM	**-9.8x**	+4.2x	+1.2x
TRA	**-33.6x**	**-87.5x**	**-2.8x**
MT	**-21.8x**	**-5.2x**	**-3.2x**
REST	**-2.1x**	**-1.3x**	**-3.5x**

4.4.3. Triple knockdown in tick nymphs

In order to determine, whether the triple KD is able to efficiently silence expression of all three genes together, we injected dsRNA of all *ixoderin* genes into nymphs of *I. ricinus*. The whole bodies were used for RNA isolation and cDNA synthesis. The results of estimated expression levels are seen in Tab. 6.

ixoA expression was decreased by 97.1% (34.7x) after KD. Expression of *ixoB* decreased by 64.8% (2.8x) and expression of *ixoC* by 83.2% (5.9x). The triple-KD is therefore fully functional and multiple genes can be knocked-down simultaneously.

Table 6: Summary of individual *ixoderin* expression level change in whole nymphs after triple knockdown.

***Ixoderins* expression level in whole nymphs after triple knockdown (%)**			
Ixoderin	Control KD (*gfp*)	Triple KD (*ixo A+B+C*)	Expression change
A	100.00	2.88	**-34.7x**
B	100.00	35.15	**-2.8x**
C	100.00	16.84	**-5.9x**

4.5 In vitro phagocytosis of *Candida albicans*

To reveal the influence of *ixoderins* gene silencing on pathogen phagocytosis by tick hemocytes, we conducted in vitro phagocytic assays with different pathogens.

Concerning phagocytosis of *C. albicans*, in ticks with *ixoA* RNAi, phagocytosis of *C. albicans* was significantly ($p<0.01$) decreased and reached only 75.5% of the phagocytic index of the control group. RNAi of *ixoderin B* showed even higher phagocytosis suppression to 51.8% ($p<0.001$) (Fig. 8). RNAi of *ixoderin C* did not show any significant influence on the phagocytosis of *C. albicans*.

4.6 In vitro phagocytosis of *Borrelia afzelii*

RNAi of any *ixoderin* did not show any significant change in the efficiency of phagocytosis of *B. afzelii* (Fig. 8). RNAi of *ixoderin A* showed slight increase in phagocytosis to 104% against 100% of *gfp* RNAi control. RNAi of *ixoderin B* demonstrated slight increase in phagocytosis to 114.6% against control *gfp* silenced group (set to 100%) and *ixoC* RNAi demonstrated slightly decreased phagocytosis to 93%. None of the differences was found statistically significant.

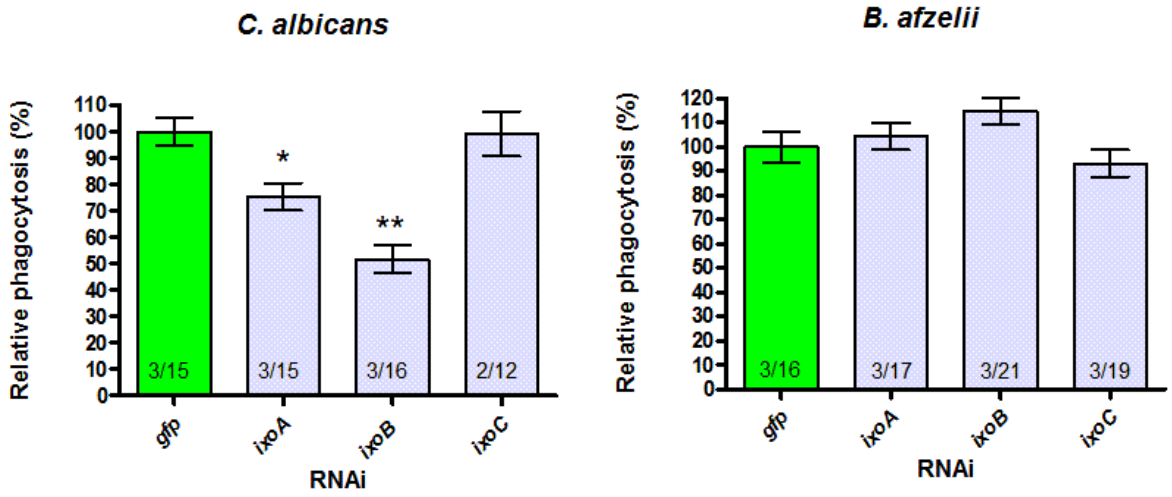


Figure 8: The influence of *ixoderins* silencing on in vitro phagocytosis of *Candida albicans* and *Borrelia afzelii* by tick hemocytes. The numbers on the column base indicate the number of independent experiments / number of hemocytes counts. The error bar represents the standard error of mean of hemocytes counts. The asterisks (*) and (**) mark statistically significant results at $p<0.01$ and $p<0.001$, respectively.

4.7 Effect of Ixoderins on *Borrelia afzelii* transmission and tick feeding

Although phagocytosis of *B. afzelii* was not influenced by KD of *ixoderins* in in vitro assay, Ixoderins might act as opsonins for direct pathogen lysis. Therefore, we have investigated the potential role of *ixoderins* in general survival and transmission of *B. burgdorferi*. In the transmission experiment, infected nymphs after KD of all three *ixoderins*, were fed on infection susceptible mice.

First, the influence of *ixoderins* gene expression silencing on blood feeding was inspected. The engorged nymphs after triple KD were weighed and their weight, which serves as an indicator of the nymph fitness and health, was compared to the control sample group. No significant difference was observed. The mean values reached 3.4 mg for the control (*gfp* KD) group and 3.3 mg for the test group (Fig. 9A). The weight distribution distinguished two groups – one with lower weight, from which adult males will be molted; one with higher weight, from which adult females will be molted (Dusbabek, 1996; Knight et al., 1978).

When the weights of fully fed female nymphs were compared between control and test group, no significant difference was found. Nevertheless, the difference in weights between male nymphs in control and test group was found significant ($p<0.05$) (Fig. 9B).

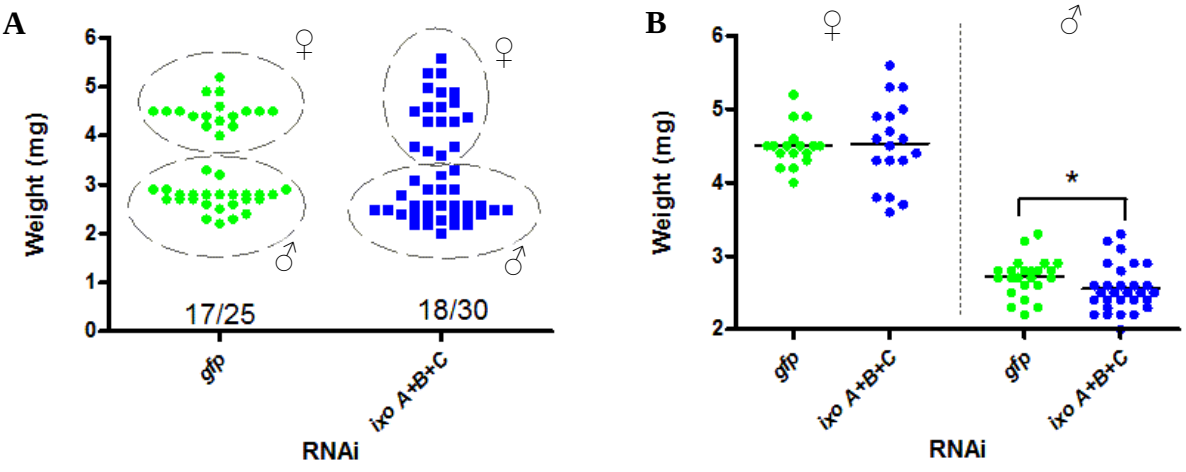


Figure 9: The influence of *ixoderins* triple silencing (*ixo A+B+C*) on the nymphal weight after feeding. *gfp* silenced nymphs serve as negative control. The asterisk (*) marks statistically significant result ($p<0.05$). **A)** All nymphs together. **B)** Female nymphs and male nymphs are separated.

After weighing, all the fully fed nymphs were tested by conventional PCR for presence of *Borrelia* sp. using *flagellin* gene. The prevalence in the control group reached 86% (within 42 nymphs collected), whereas it was 75% (48 nymphs collected) in the test group. There were at least four infected nymphs (with detectable borreliae) on each mouse.

The qPCR was run to absolutely quantify borreliae in each nymph using external *B. burgdorferi flagellin* standard. It showed no significant difference in the number of *Borrelia* spirochetes (genome copy number) in control and test group (Fig. 10).

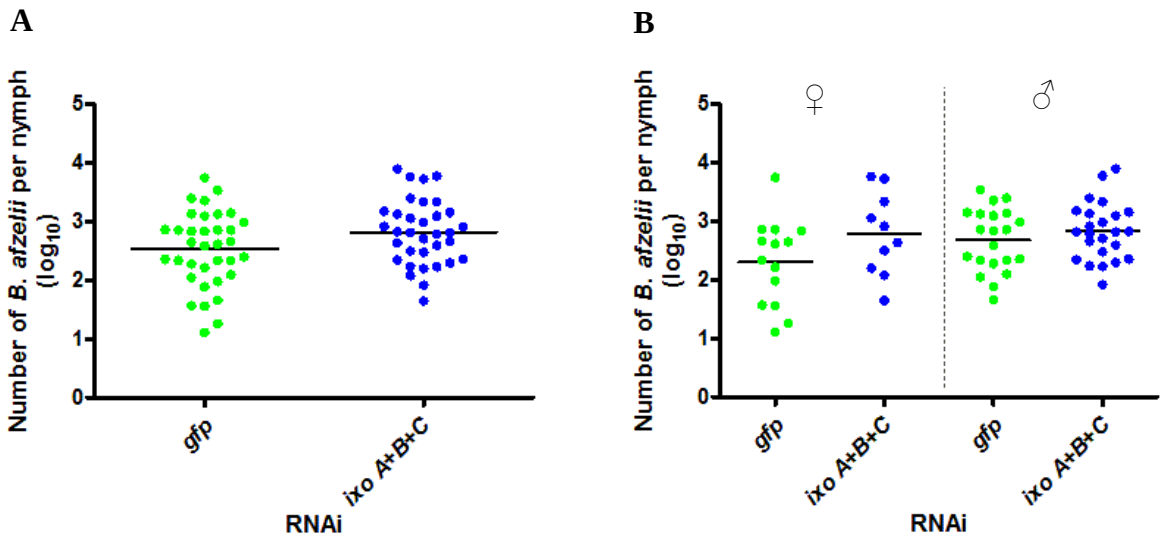


Figure 10: The influence of *ixoderins* triple silencing (*ixo A+B+C*) on number of *B. afzelii* genome copy number present in the nymphal body after feeding. The number of *Borrelia* is absolutely quantified using external *B. burgdorferi flagellin* standard. *gfp* silencing serves as negative control. **A)** All nymphs together. **B)** Female nymphs and male nymphs are separated.

Using primers for the highly conserved bacterial 16S ribosomal subunit, we further determined the total amount of bacteria present in the same nymphs. No significant influence of *ixoderins* gene expression silencing on the amount of bacteria was observed (Fig. 11). Interestingly, there is a significant difference in number of bacteria between female nymphs and male nymphs, regardless on *gfp* KD or triple KD of *ixoderins A, B* and *C* (Fig. 12).

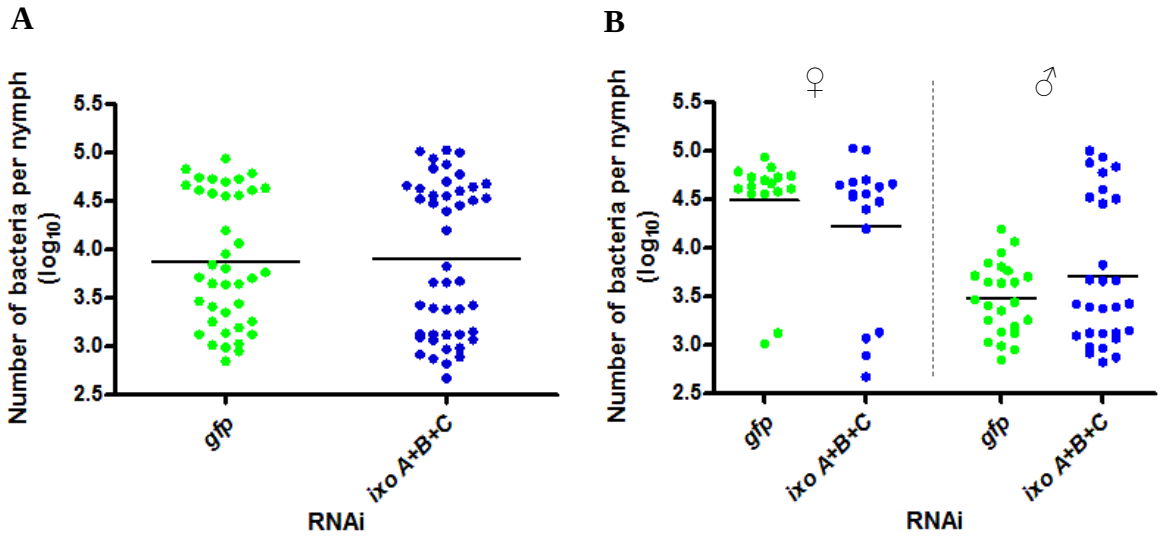


Figure 11: The influence of *ixoderins* triple silencing (*ixo A+B+C*) on number of bacteria genome copy number present in the nymph body after feeding. The number of bacteria is absolutely quantified using external 16S ribosomal subunit standard. *gfp* silencing serves as negative control. **A)** All nymphs together. **B)** Female nymphs and male nymphs are separated.

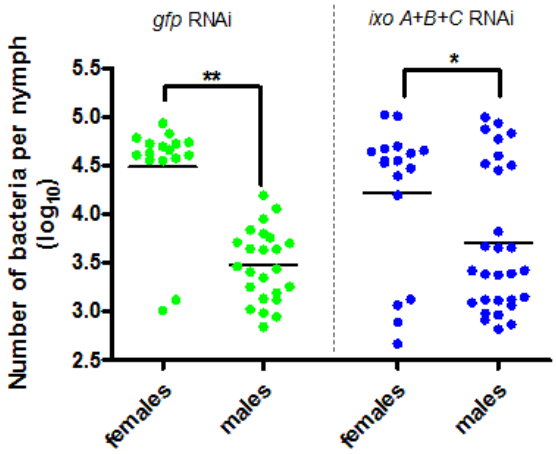


Figure 12: The difference in bacteria genome copy number between female and male nymphs. The number of bacteria is lower in males regardless the genetic knockdown. The asterisks (*) and (**) mark statistically significant results at $p<0.05$ and $p<0.0001$, respectively.

In order to find out, whether there is any influence of the knockdown on *B. afzelii* transmission, the mice that the ticks were fed on were examined for the presence of borrelial DNA. Ear biopsies were taken each week for four consecutive weeks. The *B. afzelii* proliferation in the mouse was very similar (not significantly different) in both test and control groups of mice. No evidence of borrelial DNA was observed in the ear biopsies after the first week, while already in the second week, high number of *Borrelia* spirochetes was detected (Fig. 13A). The number of spirochetes in the ears then decreased within the next two weeks. The infection amplitude was observed in the second week after the tick feeding. The final quantification of *B. afzelii* in the inner organs four weeks after feeding did not show significant differences between the control and test group (Fig. 13B).

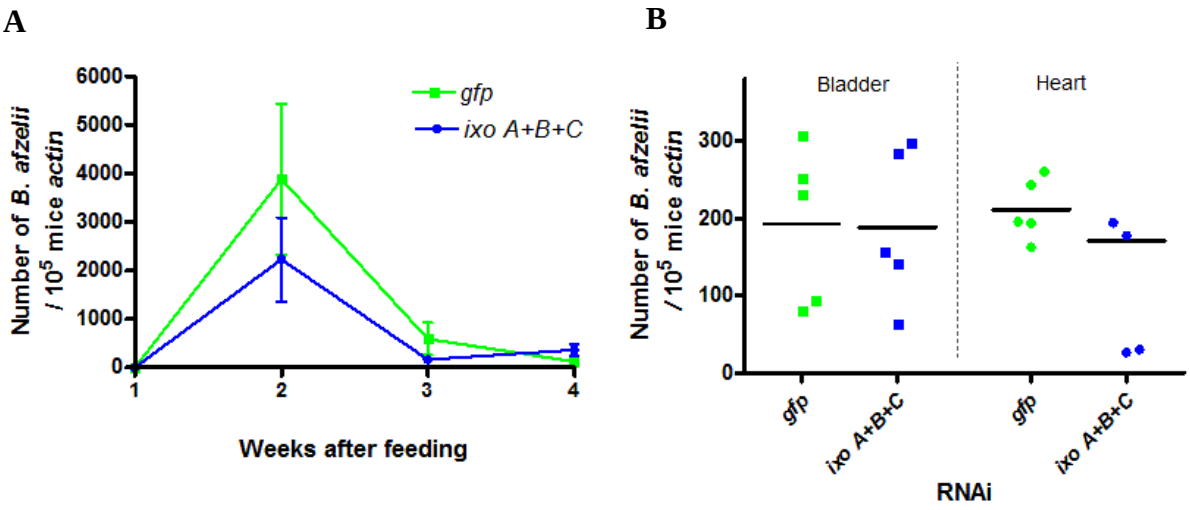


Figure 13: **A)** The course of infection in mice after feeding of *B. afzelii* infected nymphs. *gfp* (green line): average of samples from five mice, on which infected control *gfp* KD nymphs were fed. *ixo A+B+C* (blue line): average of samples from five mice, on which infected *Ixoderins* KD nymphs were fed. **B)** Quantification of *B. afzelii* genome copy number in urinary bladder and heart (from five mice each group) four weeks after feeding. *gfp*: control group of mice, on which infected control *gfp* KD nymphs were fed. *ixo A+B+*C: test group of mice, on which infected *Ixoderins* KD nymphs were fed.

5. Discussion

Lectins being able to recognize self and non-self carbohydrate motifs play important role in innate immunity of invertebrates (Natori, 2001). Substantial part of these lectins contains fibrinogen-related domain, which defines them as fibrinogen related proteins. Several FREPs among arthropods were proved to have major immune function in defense against bacteria and protozoa, which suggest their conserved role among invertebrates (Adema et al., 1997; Dong and Dimopoulos, 2009; Fan et al., 2008; Gokudan et al., 1999; Hanington et al., 2010; Hertel et al., 2005; Wang et al., 2004). There is little known about tick FREPs and about whether they play a role in the pathogen transmission between vectors and hosts (Kopacek et al., 2012). The *Ixodes* FREPs named Ixoderins are supposed to form three distinguished groups. The role of this thesis was to elucidate differences among them, to assess their probable roles in the tick organism and in the pathogen-vector-host interaction.

The phylogenetic analysis confirmed existence of three groups of Ixoderins denoted as A, B and C (Kopacek et al., 2012) and further revealed that Ixoderins B have clearly evolved into closed and separated group with high diversity. Unsurprisingly, all Ixoderins have their analogues in *I. ricinus* and *I. scapularis* species. Ixoderins A are diverse group closely related to FREPs from other tick species (Kovar et al., 2000; Rego et al., 2006), while low diverse Ixoderin C group may represent a link between tick FREPs and FREPs from other organisms. Surprisingly, Ixoderins B were found only in *Ixodes* species so far. Important portion of Ixoderins B lack the N-terminal A subdomain, which is likely to be caused by incomplete sequence entries as these do not contain methionine on the N-terminus. The missing subdomain however does not participate in carbohydrate binding and evolves faster than the binding site of FReD (Doolittle et al., 2012), which might lead to its elimination from some FREPs. Some of the Ixoderins B further contain mutations in one of the cysteines, which must alter the structure and hence function of these proteins.

Due to the knowledge of complete *I. scapularis* genome sequence, an exhaustive list of FREPs from *I. scapularis* was obtained and used in the analysis. However, transcriptome shotgun assembly database of *I. ricinus* (Kotsyfakis et al., 2015) was not completed at the time of our work, therefore only available *I. ricinus* FREP sequences from NCBI database were used. Ixoderin C from *I. ricinus* was added to the analysis as the only found representative of its group in *I. ricinus*, however, many Ixoderins A would be definitely found in *I. ricinus* transcriptome and probably even some more Ixoderins B.

The expression profiling of *ixoderins* representatives from each group confirms the phylogenetic diversification: the groups (A, B and C) vary also in amount of expression among tick tissues and developmental stages. *Ixoderin A* is expressed predominantly in hemolymph as previously reported (Rego et al., 2005), in Malpighian tubules and in ovaria. Its expression is clearly induced by feeding, which corresponds to the situation in some FREPs from *Anopheles* species (Wang et al., 2005) and to analogous protein from *Rhipicephalus appendiculatus* (Grubhoffer and Kovar, 1998). Furthermore, the finding that Ixoderin A is the main lectin in *I. ricinus* plasma (Hajdusek, unpublished results) demonstrates the high importance of FREPs in the tick and predicts its role in immune responses and in processes associated with blood uptake. Findings of Kuhn et al. (1996) about certain sialic acid-binding lectin in *I. ricinus* therefore correspond to Ixoderin A.

The phylogenetically distinct *Ixoderins B* are specifically expressed in salivary glands and due to their diversity we assume their primary role lies in non-self recognition of different carbohydrate structures (Ixoderins B might be shipped to hemolymph) or they may play a role at the tick-host interface, being produced in tick saliva. Rather unequal expression pattern among different ticks suggest that there are multiple genes of *Ixoderins B* expressed differently among tick populations. The *Ixoderin C* is expressed in all the tissues of adult tick and, interestingly, is highly expressed in eggs, which suggests its developmental role corresponding to e.g., FREPs from *Drosophila* (Powell et al., 2001).

RNAi-mediated knockdown of *ixoderins* led to reduced phagocytosis of some pathogens. Ixoderin A and more significantly Ixoderin B contributes to phagocytosis of *Candida albicans* - a representative of fungal infection causing agent. IXOA and IXOB act therefore similarly to Macroglobulin complement related molecule (dTEP6) in *D. melanogaster* (Blandin and Levashina, 2004). It was previously shown that knockdown of the *ixo A* and *ixo B* led to decrease in phagocytosis of G(-) bacteria *Chryseobacterium indologenes*, which means that these Ixoderins may act (in *I. ricinus*) synergistically with IrAM (TEP α2-macroglobulin), which deficiency leads to failing phagocytosis of *Chryseobacterium indologenes* (Buresova et al., 2009). Knockdown of *ixo A* further caused decreased phagocytosis of G(-) bacteria *Escherichia coli* (Urbanova, unpublished results). The expected immune functions of Ixoderins (tick FREPs) was therefore confirmed, with suggestion that Ixoderins act as opsonins similarly/in synchrony to thioester-containing proteins, although their role is not such extensive as in *Anopheles gambiae* (Dong and Dimopoulos, 2009). On the other hand, phagocytosis of *Borrelia afzelii* remained unchanged after *ixoderins* knockdown. The probable cause is missing recognizable pattern on the

surface of *Borrelia* spirochetes as these bacteria do lack lipopolysaccharides and present mainly lipoproteins (Radolf and Samuels, 2010), while FReD is in arthropods conservatively recognizing sugar residues. Borreliae are therefore phagocytosed by hemocytes, but other molecules mediate this engulfment. Plenty of other pathogens remain to be tested in our phagocytic assay to demonstrate, which pathogens are further recognized by Ixoderins.

Although phagocytosis of *B. afzelii* was not altered by *ixoderins* knockdown, it did not exclude their possible role in *Borrelia* spirochetes transmission (e.g. by direct killing of spirochetes after opsonization). The transmission experiment conducted in order to resolve this uncertainty showed that although *ixoderins* knockdown leads to decreased number of *B. afzelii* transmitted from the vector to the host, it does not protect the host from infection. Interestingly, the number of *Borrelia* spirochetes detected in nymphs after feeding on mice was similar in all the nymphs, although the nymph weights differ between males and females (Dusbabek, 1996; Knight et al., 1978), meaning that males harbor spirochetes (in the midgut) in higher concentration than females. However, the number of all bacteria detected in the same nymphs corresponds to the expected difference between male and female nymphs; the male nymphs contain fewer bacteria as they weight less.

Ixoderins from *I. ricinus* generally follow the arthropod FREPs properties, whose primary role is defense (Hanington and Zhang, 2010). Ixoderin A, the main lectin in plasma, functions as opsonin of some pathogens and is very likely involved in hemostasis maintenance similarly to *Anopheles* FREPs (Dong and Dimopoulos, 2009). Ixoderin B, the strict salivary glands protein also opsonizes some pathogens. Ixoderin C might play role in development similarly to FREPs from *Drosophila* (Lee et al., 1996). None of the Ixoderins recognizes *B. afzelii* nor affects *Borrelia* spirochetes transmission. Nevertheless, fibrinogen-related domain containing proteins in ticks might act in synchrony with thioester-containing proteins in opsonization of foreign organisms and particles. This work contributes to revealing the tick immune system, its capabilities and restrictions and function in transmission of pathogens. Our understanding of tick immunity is crucial for finding of possible targets for fight with ticks and tick-borne diseases.

6. References

Adema, C.M., Hertel, L. a, Miller, R.D., and Loker, E.S. (1997). A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. Proc. Natl. Acad. Sci. U. S. A. *94*, 8691–8696.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783–801.

Barbour, A.G., and Hayes, S.F. (1986). Biology of Borrelia species. Microbiol. Rev. *50*, 381–400.

Ben-Menachem, G., Kubler-Kielb, J., Coxon, B., Yergey, A., and Schneerson, R. (2003). A newly discovered cholesteryl galactoside from Borrelia burgdorferi. Proc. Natl. Acad. Sci. *100*, 7913–7918.

Blandin, S., and Levashina, E.A. (2004). Thioester-containing proteins and insect immunity. Mol. Immunol. *40*, 903–908.

Borovickova, B., and Hypsa, V. (2005). Ontogeny of tick hemocytes: a comparative analysis of Ixodes ricinus and Ornithodoros moubata. Exp. Appl. Acarol. *35*, 317–333.

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

Casjens, S.R., Fraser-Liggett, C.M., Mongodin, E.F., Qiu, W.-G., Dunn, J.J., Luft, B.J., and Schutzer, S.E. (2011). Whole Genome Sequence of an Unusual Borrelia burgdorferi Sensu Lato Isolate. J. Bacteriol. *193*, 1489–1490.

Casjens, S.R., Mongodin, E.F., Qiu, W.-G., Luft, B.J., Schutzer, S.E., Gilcrease, E.B., Huang, W.M., Vujadinovic, M., Aron, J.K., Vargas, L.C., et al. (2012). Genome stability of Lyme disease spirochetes: comparative genomics of Borrelia burgdorferi plasmids. PLoS One *7*, e33280.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G., et al. (2002). Immunity-Related Genes and Gene Families in Anopheles gambiae. Science (80-.). *298*, 159–165.

Cociancich, S., Ghazi, A., Hetru, C., Hoffmann, J.A., and Letellier, L. (1993). Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in Micrococcus luteus. J. Biol. Chem. *268*, 19239–19245.

Dai, J., Wang, P., Adusumilli, S., Booth, C.J., Narasimhan, S., Anguita, J., and Fikrig, E. (2009). Antibodies against a Tick Protein, Salp15, Protect Mice from the Lyme Disease Agent. Cell Host Microbe *6*, 482–492.

Dong, Y., and Dimopoulos, G. (2009). Anopheles Fibrinogen-related Proteins Provide Expanded Pattern Recognition Capacity against Bacteria and Malaria Parasites. J. Biol. Chem. *284*, 9835–9844.

Dong, Y., Aguilar, R., Xi, Z., Warr, E., Mongin, E., and Dimopoulos, G. (2006). Anopheles gambiae Immune Responses to Human and Rodent Plasmodium Parasite Species. PLoS Pathog *2*, e52.

Doolittle, R.F. (1992). A detailed consideration of a principal domain of vertebrate fibrinogen and its relatives. Protein Sci. A Publ. Protein Soc. *1*, 1563–1577.

Doolittle, R.F., Spraggon, G., and Everse, S.J. (1997). Evolution of vertebrate fibrin formation and the process of its dissolution. Ciba Foundation, ed. (Chichester: John Wiley & Sons),.

Doolittle, R.F., McNamara, K., and Lin, K. (2012). Correlating structure and function during the evolution of fibrinogen-related domains. Protein Sci. *21*, 1808–1823.

Dusbabek, F. (1996). Nymphal sexual dimorphism in the sheep tick Ixodes ricinus (Acari: Ixodidae). Folia Parasitol. (Praha). *43*, 75–79.

Endo, Y., Matsushita, M., and Fujita, T. (2007). Role of ficolin in innate immunity and its molecular basis. Immunobiology *212*, 371–379.

Estrada-Pena, A., and Jongejan, F. (1999). Ticks Feeding on Humans: A Review of Records on Human-Biting Ixodoidea with Special Reference to Pathogen Transmission. Exp. Appl. Acarol. *23*, 685–715.

Estrada-Pena, A., Venzal, J.M., and Sanchez Acedo, C. (2006). The tick Ixodes ricinus: distribution and climate preferences in the western Palaearctic. Med. Vet. Entomol. *20*, 189–197.

Fan, C., Zhang, S., Li, L., and Chao, Y. (2008). Fibrinogen-related protein from amphioxus Branchiostoma belcheri is a multivalent pattern recognition receptor with a bacteriolytic activity. Mol. Immunol. *45*, 3338–3346.

Ferrandon, D., Imler, J.-L., Hetru, C., and Hoffmann, J.A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat. Rev. Immunol. *7*, 862–874.

Fogaca, A.C., Lorenzini, D.M., Kaku, L.M., Esteves, E., Bulet, P., and Daffre, S. (2004). Cysteine-rich antimicrobial peptides of the cattle tick Boophilus microplus: isolation, structural characterization and tissue expression profile. Dev. Comp. Immunol. *28*, 191–200.

Fogaca, A.C., Almeida, I.C., Eberlin, M.N., Tanaka, A.S., Bulet, P., and Daffre, S. (2006). Ixodidin, a novel antimicrobial peptide from the hemocytes of the cattle tick Boophilus microplus with inhibitory activity against serine proteinases. Peptides *27*, 667–674.

Francischetti, I.M.B., Sa-Nunes, A., Mans, B.J., Santos, I.M., and Ribeiro, J.M.C. (2009). The role of saliva in tick feeding. Front. Biosci. (Landmark Ed. *14*, 2051–2088.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K., et al. (1997). Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature *390*, 580–586.

Gassner, F., and Hartemink, N. (2013). Tick - Borrelia interactions: burden or benefit? W. Takken, and C.J.M. Koenraadt, eds. (Springer), p. 259 pages.

Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoe, Y., Wai, S.N., Iwanaga, S., and Kawabata, S. (1999). Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. Proc. Natl. Acad. Sci. U. S. A. *96*, 10086–10091.

Grubhoffer, L., and Kovar, V. (1998). Arthropod lectins: affinity approaches in the analysis and preparation of carbohydrate binding proteins. Tech. Insect Immunol. FITC *5*, 47–57.

Grubhoffer, L., Veres, J., and Dusbabek, F. (1991). Lectins as the molecular factors of recognition and defence reactions in ticks. Mod. Acarol. *2*, 381–388.

Gudderra, N.P., Sonenshine, D.E., Apperson, C.S., and Roe, R.M. (2002). Hemolymph

proteins in ticks. J. Insect Physiol. *48*, 269–278.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Pena, A., Horak, I.G., Shao, R., and Barker, S.C. (2010). The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world : a list of valid species names.

Hajdusek, O. (2009). Functional analysis of metabolic and immune proteins in the tick Ixodes ricinus by RNA interference (Ph.D. thesis, University of South Bohemia, Faculty of Science, Ceske Budejovice, Czech republic).

Hajdusek, O., Sima, R., Ayllon, N., Jalovecka, M., Perner, J., de la Fuente, J., and Kopacek, P. (2013). Interaction of the tick immune system with transmitted pathogens. Front. Cell. Infect. Microbiol. *3*.

Hall, B.G. (2013). Building Phylogenetic Trees from Molecular Data with MEGA. Mol. Biol. Evol. *30*, 1229–1235.

Hanington, P.C., and Zhang, S.-M. (2010). The Primary Role of Fibrinogen-Related Proteins in Invertebrates Is Defense, Not Coagulation. J. Innate Immun. *3*, 17–27.

Hanington, P.C., Forys, M.A., Dragoo, J.W., Zhang, S.-M., Adema, C.M., and Loker, E.S. (2010). Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. Proc. Natl. Acad. Sci. *107*, 21087–21092.

Hannon, G.J. (2002). RNA interference. Nature *418*, 244–251.

Herrmann, C., and Gern, L. (2010). Survival of Ixodes ricinus (Acari: Ixodidae) Under Challenging Conditions of Temperature and Humidity Is Influenced by Borrelia burgdorferi sensu lato Infection. J. Med. Entomol. *47*, 1196–1204.

Herrmann, C., and Gern, L. (2013). Survival of Ixodes ricinus (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations. Ticks Tick. Borne. Dis. *4*, 445–451.

Herrmann, C., Voordouw, M.J., and Gern, L. (2013). Ixodes ricinus ticks infected with the causative agent of Lyme disease, Borrelia burgdorferi sensu lato, have higher energy reserves. Int. J. Parasitol. *43*, 477–483.

Hertel, L.A., Adema, C.M., and Loker, E.S. (2005). Differential expression of FREP genes in two strains of Biomphalaria glabrata following exposure to the digenetic trematodes Schistosoma mansoni and Echinostoma paraensei. Dev. Comp. Immunol. *29*, 295–303.

Hoa, N.T., Keene, K.M., Olson, K.E., and Zheng, L. (2003). Characterization of RNA interference in an Anopheles gambiae cell line. Insect Biochem. Mol. Biol. *33*, 949–957.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R. a. B. (1999). Phylogenetic Perspectives in Innate Immunity. Science (80-.). *284*, 1313–1318.

Holmskov, U., Thiel, S., and Jensenius, J.C. (2003). COLLECTINS AND FICOLINS: Humoral Lectins of the Innate Immune Defense. Annu. Rev. Immunol. *21*, 547.

Hovius, J.W., Schuijt, T.J., Groot, K.A. de, Roelofs, J.J.T.H., Oei, G.A., Marquart, J.A., Beer, R. de, Veer, C. van't, Poll, T. van der, Ramamoorthi, N., et al. (2008). Preferential Protection of Borrelia burgdorferi Sensu Stricto by a Salp 15 Homologue in Ixodes ricinus Saliva. J. Infect. Dis. *198*, 1189–1197.

Huang, B., Zhang, L., Li, L., Tang, X., and Zhang, G. (2015). Highly diverse fibrinogen-related proteins in the Pacific oyster Crassostrea gigas. Fish Shellfish Immunol. *43*, 485–490.

Huang, X., Tsuji, N., Miyoshi, T., Nakamura-Tsuruta, S., Hirabayashi, J., and Fujisaki, K. (2007). Molecular characterization and oligosaccharide-binding properties of a galectin from the argasid tick Ornithodoros moubata. Glycobiology *17*, 313–323.

Ivanova, L.B., Tomova, A., González-Acuña, D., Murúa, R., Moreno, C.X., Hernández, C., Cabello, J., Cabello, C., Daniels, T.J., Godfrey, H.P., et al. (2014). Borrelia chilensis, a new member of the Borrelia burgdorferi sensu lato complex that extends the range of this genospecies in the Southern Hemisphere. Environ. Microbiol. *16*, 1069–1080.

Jaenson, T.G.T., and Talleklint, L. (1992). Incompetence of Roe Deer as Reservoirs of the Lyme Borreliosis Spirochete. J. Med. Entomol. *29*, 813–817.

Janeway, C.A., and Medzhitov, R. (2002). Innate Immune Recognition. Annu. Rev. Immunol. *20*, 197–216.

Johns, R., Ohnishi, J., Broadwater, A., Sonenshine, D.E., Silva, A.M. De, and Hynes, W.L. (2001a). Contrasts in Tick Innate Immune Responses to Borrelia burgdorferi Challenge: Immunotolerance in Ixodes scapularis Versus Immunocompetence in Dermacentor variabilis (Acari: Ixodidae). J. Med. Entomol. *38*, 99–107.

Johns, R., Sonenshine, D.E., and Hynes, W.L. (2001b). Identification of a defensin from the hemolymph of the American dog tick, Dermacentor variabilis. Insect Biochem. Mol. Biol. *31*, 857–865.

Jongejan, F., and Uilenberg, G. (2004). The global importance of ticks. Parasitology *129 Suppl*, S3–S14.

Kairies, N., Beisel, H.-G., Fuentes-Prior, P., Tsuda, R., Muta, T., Iwanaga, S., Bode, W., Huber, R., and Kawabata, S. (2001). The 2.0-Å crystal structure of tachylectin 5A provides evidence for the common origin of the innate immunity and the blood coagulation systems. Proc. Natl. Acad. Sci. U. S. A. *98*, 13519–13524.

Kawabata, S., and Tsuda, R. (2002). Molecular basis of non-self recognition by the horseshoe crab tachylectins. Biochim. Biophys. Acta - Gen. Subj. *1572*, 414–421.

Kenedy, M.R., Lenhart, T.R., and Akins, D.R. (2012). The role of Borrelia burgdorferi outer surface proteins. FEMS Immunol. Med. Microbiol. *66*, 1–19.

Knight, M.M., Norval, R.A.I., and Rechav, Y. (1978). The Life Cycle of the Tick Hyalomma marginatum rufipes Koch (Acarina: Ixodidae) under Laboratory Conditions. J. Parasitol. *64*, 143–146.

Kocan, K.M., de la Fuente, J., Manzano-Roman, R., Naranjo, V., Hynes, W.L., and Sonenshine, D.E. (2008). Silencing expression of the defensin, varisin, in male Dermacentor variabilis by RNA interference results in reduced Anaplasma marginale infections. Exp. Appl. Acarol. *46*, 17–28.

Kopacek, P., Hajdusek, O., Buresova, V., and Daffre, S. (2010). Tick innate immunity. Adv. Exp. Med. Biol. *708*, 137–162.

Kopacek, P., Hajdusek, O., and Buresova, V. (2012). Tick as a model for the study of a primitive complement system. Adv. Exp. Med. Biol. *710*, 83–93.

Kotsyfakis, M., Schwarz, A., Erhart, J., and Ribeiro, J.M.C. (2015). Tissue- and time-dependent transcription in Ixodes ricinus salivary glands and midguts when blood feeding on the vertebrate host. Sci. Rep. *5*.

Kovar, V., Kopacek, P., and Grubhoffer, L. (2000). Isolation and characterization of Dorin M, a lectin from plasma of the soft tick Ornithodoros moubata. Insect Biochem. Mol. Biol.

30, 195–205.

Kuhn, K.H., Uhlir, J., and Grubhoffer, L. (1996). Ultrastructural localization of a sialic acid-specific hemolymph lectin in the hemocytes and other tissues of the hard tick Ixodes ricinus (Acari; Chelicerata). Parasitol. Res. *82*, 215–221.

Kumar, H., Kawai, T., and Akira, S. (2011). Pathogen Recognition by the Innate Immune System. Int. Rev. Immunol. *30*, 16–34.

Kurscheid, S., Lew-Tabor, A.E., Valle, M.R., Bruyeres, A.G., Doogan, V.J., Munderloh, U.G., Guerrero, F.D., Barrero, R.A., and Bellgard, M.I. (2009). Evidence of a tick RNAi pathway by comparative genomics and reverse genetics screen of targets with known loss-of-function phenotypes in Drosophila. BMC Mol. Biol. *10*, 26.

Kurtenbach, K., Schafer, S.M., Sewell, H.-S., Peacey, M., Hoodless, A., Nuttall, P.A., and Randolph, S.E. (2002). Differential Survival of Lyme Borreliosis Spirochetes in Ticks That Feed on Birds. Infect. Immun. *70*, 5893–5895.

De La Fuente, J., Almazan, C., Blas-Machado, U., Naranjo, V., Mangold, A.J., Blouin, E.F., Gortazar, C., and Kocan, K.M. (2006). The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood ingestion and reproduction. Vaccine *24*, 4082–4095.

De La Fuente, J., Kocan, K.M., Almazan, C., and Blouin, E.F. (2007). RNA interference for the study and genetic manipulation of ticks. Trends Parasitol. *23*, 427–433.

Lai, R., Takeuchi, H., Lomas, L.O., Jonczy, J., Rigden, D.J., Rees, H.H., and Turner, P.C. (2004). A new type of antimicrobial protein with multiple histidines from the hard tick, Amblyomma hebraeum. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *18*, 1447–1449.

Lee, E.C., Hu, X., Yu, S.Y., and Baker, N.E. (1996). The scabrous gene encodes a secreted glycoprotein dimer and regulates proneural development in Drosophila eyes. Mol. Cell. Biol. *16*, 1179–1188.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., and Kafatos, F.C. (2001). Conserved Role of a Complement-like Protein in Phagocytosis Revealed by dsRNA Knockout in Cultured Cells of the Mosquito, Anopheles gambiae. Cell *104*, 709–718.

Liu, L., Narasimhan, S., Dai, J., Zhang, L., Cheng, G., and Fikrig, E. (2011). Ixodes scapularis salivary gland protein P11 facilitates migration of Anaplasma phagocytophilum from the tick gut to salivary glands. EMBO Rep. *12*, 1196–1203.

Liu, L., Dai, J., Zhao, Y.O., Narasimhan, S., Yang, Y., Zhang, L., and Fikrig, E. (2012). Ixodes scapularis JAK-STAT Pathway Regulates Tick Antimicrobial Peptides, Thereby Controlling the Agent of Human Granulocytic Anaplasmosis. J. Infect. Dis. *206*, 1233–1241.

Loker, E.S., Adema, C.M., Zhang, S.-M., and Kepler, T.B. (2004). Invertebrate immune systems--not homogeneous, not simple, not well understood. Immunol. Rev. *198*, 10–24.

Lu, J., and Le, Y. (1998). Ficolins and the fibrinogen-like domain. Immunobiology *199*, 190–199.

Lu, J., Teh, C., Kishore, U., and Reid, K.B.M. (2002). Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. Biochim. Biophys. Acta - Gen. Subj. *1572*, 387–400.

Matsushita, M., Endo, Y., Taira, S., Sato, Y., Fujita, T., Ichikawa, N., Nakata, M., and Mizuochi, T. (1996). A Novel Human Serum Lectin with Collagen- and Fibrinogen-like Domains That Functions as an Opsonin. J. Biol. Chem. *271*, 2448–2454.

Matsushita, M., Endo, Y., Hamasaki, N., and Fujita, T. (2001). Activation of the lectin complement pathway by ficolins. Int. Immunopharmacol. *1*, 359–363.

Mejlon, H.A., and Jaenson, T.G.T. (1997). Questing behaviour of Ixodes ricinus ticks (Acari: Ixodidae). Exp. Appl. Acarol. *21*, 747–754.

Middha, S., and Wang, X. (2008). Evolution and potential function of fibrinogen-like domains across twelve Drosophila species. BMC Genomics *9*, 260.

Nadkarni, M. a., Martin, F.E., Jacques, N. a., and Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology *148*, 257–266.

Nakajima, Y., Ishibashi, J., Yukuhiro, F., Asaoka, A., Taylor, D., and Yamakawa, M. (2003a). Antibacterial activity and mechanism of action of tick defensin against Gram-positive bacteria. Biochim. Biophys. Acta - Gen. Subj. *1624*, 125–130.

Nakajima, Y., Ogihara, K., Taylor, D., and Yamakawa, M. (2003b). Antibacterial Hemoglobin Fragments from the Midgut of the Soft Tick, Ornithodoros moubata (Acari: Argasidae). J. Med. Entomol. *40*, 78–81.

Natori, S. (2001). Insect Lectins and Innate Immunity. G. Beck, M. Sugumaran, and E.L. Cooper, eds. (Springer US), pp. 223–228.

Nijhof, A.M., Balk, J.A., Postigo, M., and Jongejan, F. (2009). Selection of reference genes for quantitative RT-PCR studies in Rhipicephalus (Boophilus) microplus and Rhipicephalus appendiculatus ticks and determination of the expression profile of Bm86. BMC Mol. Biol. *10*, 112.

Nonaka, M. (2000). Origin and Evolution of the Complement System. P.D.L. Du Pasquier, and P.G.W.L. M.D, eds. (Springer Berlin Heidelberg), pp. 37–50.

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., deSilva, A.M., Bao, F., Yang, X., Pypaert, M., Pradhan, D., et al. (2004). TROSPA, an Ixodes scapularis Receptor for Borrelia burgdorferi. Cell *119*, 457–468.

Parola, P., Paddock, C.D., Socolovschi, C., Labruna, M.B., Mediannikov, O., Kernif, T., Abdad, M.Y., Stenos, J., Bitam, I., Fournier, P.-E., et al. (2013). Update on Tick-Borne Rickettsioses around the World: a Geographic Approach. Clin. Microbiol. Rev. *26*, 657–702.

Pichu, S., Ribeiro, J.M.C., and Mather, T.N. (2009). Purification and characterization of a novel salivary antimicrobial peptide from the tick, Ixodes scapularis. Biochem. Biophys. Res. Commun. *390*, 511–515.

Platonov, A.E., Karan, L.S., Kolyasnikova, N.M., Makhneva, N.A., Toporkova, M.G., Maleev, V. V., Fish, D., and Krause, P.J. (2011). Humans Infected with Relapsing Fever Spirochete Borrelia miyamotoi, Russia. Emerg. Infect. Dis. *17*, 1816–1823.

Powell, P.A., Wesley, C., Spencer, S., and Cagan, R.L. (2001). Scabrous complexes with Notch to mediate boundary formation. Nature *409*, 626–630.

Radolf, J.D., and Samuels, D.S. (2010). Borrelia: molecular biology, host interaction and pathogenesis (Caister Academic Press).

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X.F., Fish, D., Anguita, J., Norgard, M. V., Kantor, F.S., Anderson, J.F., et al. (2005). The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature *436*, 573–577.

Rego, R.O.M., Hajdusek, O., Kovar, V., Kopacek, P., Grubhoffer, L., and Hypsa, V. (2005).

Molecular cloning and comparative analysis of fibrinogen-related proteins from the soft tick Ornithodoros moubata and the hard tick Ixodes ricinus. Insect Biochem. Mol. Biol. *35*, 991–1004.

Rego, R.O.M., Kovar, V., Kopacek, P., Weise, C., Man, P., Sauman, I., and Grubhoffer, L. (2006). The tick plasma lectin, Dorin M, is a fibrinogen-related molecule. Insect Biochem. Mol. Biol. *36*, 291–299.

Rittig, M.G., Kuhn, K.-H., Dechant, C.A., Gauckler, A., Modolell, M., Ricciardi-Castagnoli, P., Krause, A., and Burmester, G.R. (1996). Phagocytes from both vertebrate and invertebrate species use “coiling” phagocytosis. Dev. Comp. Immunol. *20*, 393–406.

Rizzoli, A., Silaghi, C., Obiegala, A., Rudolf, I., Hubalek, Z., Foldvari, G., Plantard, O., Vayssier-Taussat, M., Bonnet, S., Spitalska, E., et al. (2014). Ixodes ricinus and Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New Hazards and Relevance for Public Health. Front. Public Heal. *2*.

Rudenko, N., Golovchenko, M., Edwards, M.J., and Grubhoffer, L. (2005). Differential Expression of Ixodes ricinus Tick Genes Induced by Blood Feeding or Borrelia burgdorferi Infection. J. Med. Entomol. *42*, 36–41.

Schroder, H.C., Ushijima, H., Krasko, A., Gamulin, V., Thakur, N.L., Diehl-Seifert, B., Muller, I.M., and Muller, W.E.G. (2003). Emergence and Disappearance of an Immune Molecule, an Antimicrobial Lectin, in Basal Metazoa A TACHYLECTIN-RELATED PROTEIN IN THE SPONGE SUBERITES DOMUNCULA. J. Biol. Chem. *278*, 32810–32817.

Schwaiger, M., Peter, O., and Cassinotti, P. (2001). Routine diagnosis of Borrelia burgdorferi (sensu lato) infections using a real-time PCR assay. Clin. Microbiol. Infect. *7*, 461–469.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. (1995). Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. Proc. Natl. Acad. Sci. U. S. A. *92*, 2909–2913.

Skuballa, J., Petney, T., Pfaffle, M., Oehme, R., Hartelt, K., Fingerle, V., Kimmig, P., and Taraschewski, H. (2012). Occurrence of different Borrelia burgdorferi sensu lato genospecies including B. afzelii, B. bavariensis, and B. spielmanii in hedgehogs (Erinaceus spp.) in Europe. Ticks Tick. Borne. Dis. *3*, 8–13.

Soderhall, I., Wu, C., Novotny, M., Lee, B.L., and Soderhall, K. (2009). A Novel Protein Acts as a Negative Regulator of Prophenoloxidase Activation and Melanization in the Freshwater Crayfish Pacifastacus leniusculus. J. Biol. Chem. *284*, 6301–6310.

Sojka, D., Franta, Z., Horn, M., Caffrey, C.R., Mares, M., and Kopacek, P. (2013). New insights into the machinery of blood digestion by ticks. Trends Parasitol. *29*, 276–285.

Sonenshine, D.E. (1991). Biology of ticks, vol. 1 and 2 (Oxford University Press, New York).

Sonenshine, D.E., Hynes, W.L., Ceraul, S.M., Mitchell, R., and Benzine, T. (2005). Host blood proteins and peptides in the midgut of the tick Dermacentor variabilis contribute to bacterial control. Exp. Appl. Acarol. *36*, 207–223.

Stanek, G., and Reiter, M. (2011). The expanding Lyme Borrelia complex--clinical significance of genomic species? Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. *17*, 487–493.

Sterba, J., Dupejova, J., Fiser, M., Vancova, M., and Grubhoffer, L. (2011). Fibrinogen-related proteins in ixodid ticks. Parasit. Vectors *4*, 127.

Stroschein-Stevenson, S.L., Foley, E., O'Farrell, P.H., and Johnson, A.D. (2005). Identification of Drosophila Gene Products Required for Phagocytosis of Candida albicans. PLoS Biol *4*, e4.

Stuen, S., Granquist, E.G., and Silaghi, C. (2013). Anaplasma phagocytophilum—a widespread multi-host pathogen with highly adaptive strategies. Front. Cell. Infect. Microbiol. *3*.

Suss, J. (2003). Epidemiology and ecology of TBE relevant to the production of effective vaccines. Vaccine *21 Suppl 1*, S19–S35.

Takayama, K., Rothenberg, R.J., and Barbour, A.G. (1987). Absence of lipopolysaccharide in the Lyme disease spirochete, Borrelia burgdorferi. Infect. Immun. *55*, 2311–2313.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. *30*, 2725–2729.

Thiel, S. (2007). Complement activating soluble pattern recognition molecules with collagen-like regions, mannan-binding lectin, ficolins and associated proteins. Mol. Immunol. *44*, 3875–3888.

Wang, X., Rocheleau, T.A., Fuchs, J.F., Hillyer, J.F., Chen, C.-C., and Christensen, B.M. (2004). A novel lectin with a fibrinogen-like domain and its potential involvement in the innate immune response of Armigeres subalbatus against bacteria. Insect Mol. Biol. *13*, 273–282.

Wang, X., Zhao, Q., and Christensen, B.M. (2005). Identification and characterization of the fibrinogen-like domain of fibrinogen-related proteins in the mosquito, Anopheles gambiae, and the fruitfly, Drosophila melanogaster, genomes. BMC Genomics *6*, 114.

Yee, V.C., Pratt, K.P., Cote, H.C., Trong, I.L., Chung, D.W., Davie, E.W., Stenkamp, R.E., and Teller, D.C. (1997). Crystal structure of a 30 kDa C-terminal fragment from the gamma chain of human fibrinogen. Struct. (London, Engl. 1993) *5*, 125–138.

Zhang, S.-M., and Loker, E.S. (2004). Representation of an immune responsive gene family encoding fibrinogen-related proteins in the freshwater mollusc Biomphalaria glabrata, an intermediate host for Schistosoma mansoni. Gene *341*, 255–266.

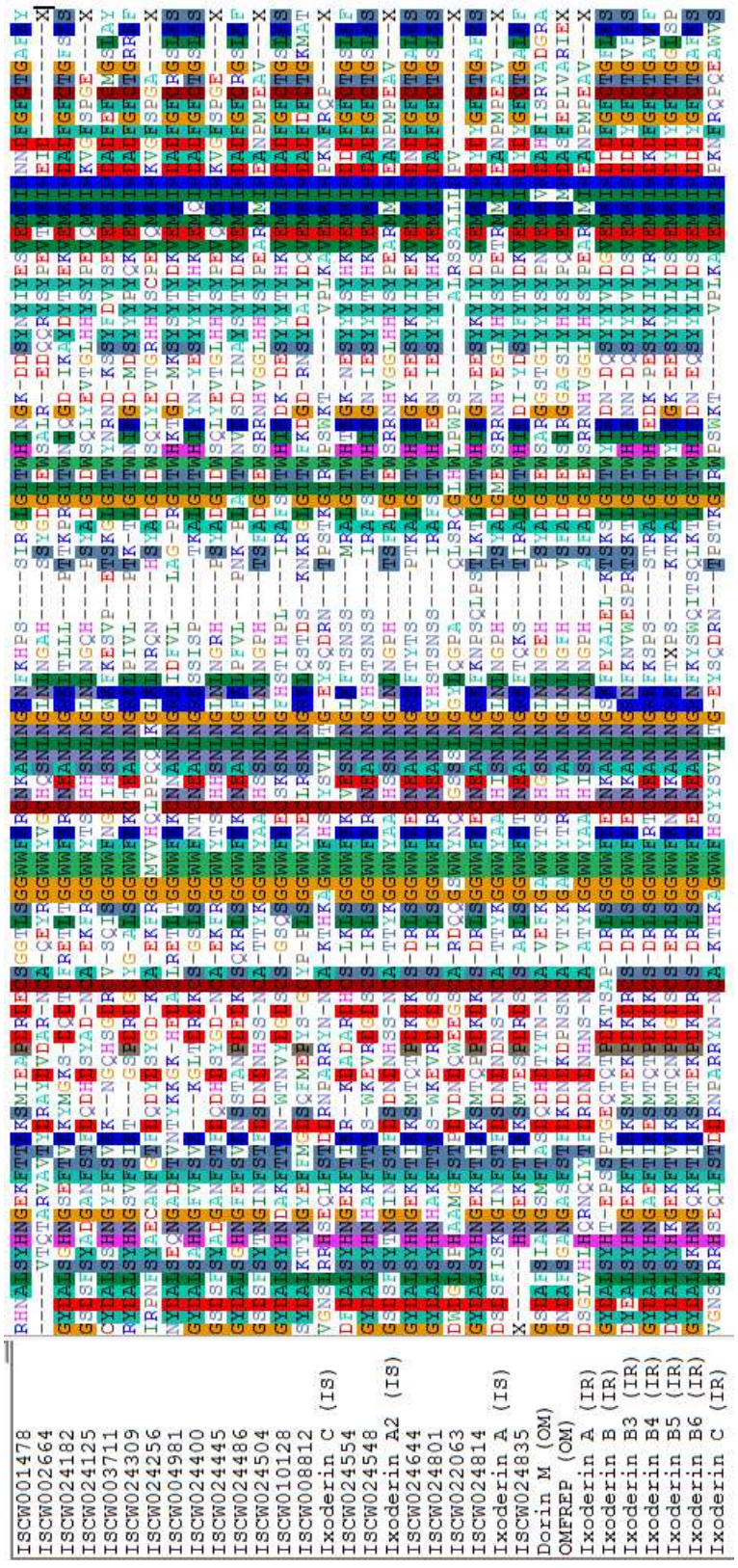
Zhang, S.-M., Zeng, Y., and Loker, E.S. (2008a). Expression profiling and binding properties of fibrinogen-related proteins (FREPs), plasma proteins from the schistosome snail host Biomphalaria glabrata. Innate Immun. *14*, 175–189.

Zhang, S.-M., Nian, H., Zeng, Y., and DeJong, R.J. (2008b). Fibrinogen-bearing protein genes in the snail Biomphalaria glabrata: Characterization of two novel genes and expression studies during ontogenesis and trematode infection. Dev. Comp. Immunol. *32*, 1119–1130.

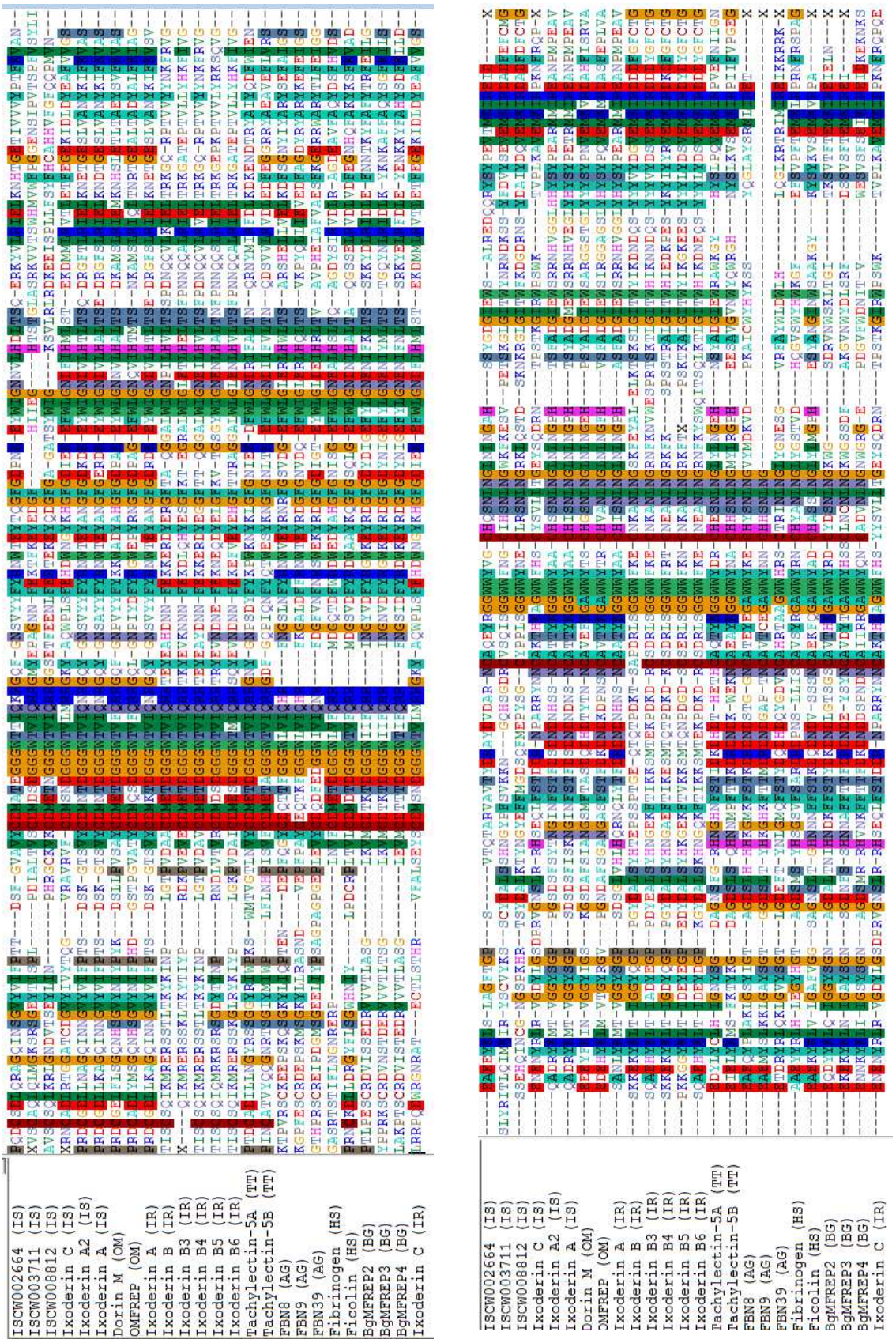
7. Supplement

Supplement Table 1: Complete list of FREPs used for phylogenetic analysis, with their access numbers for either www.vectorbase.org or http://www.ncbi.nlm.nih.gov/. SignalP – program for signal aa sequence detection, PSORT – program for protein localization in the cell based on signal aa sequence.

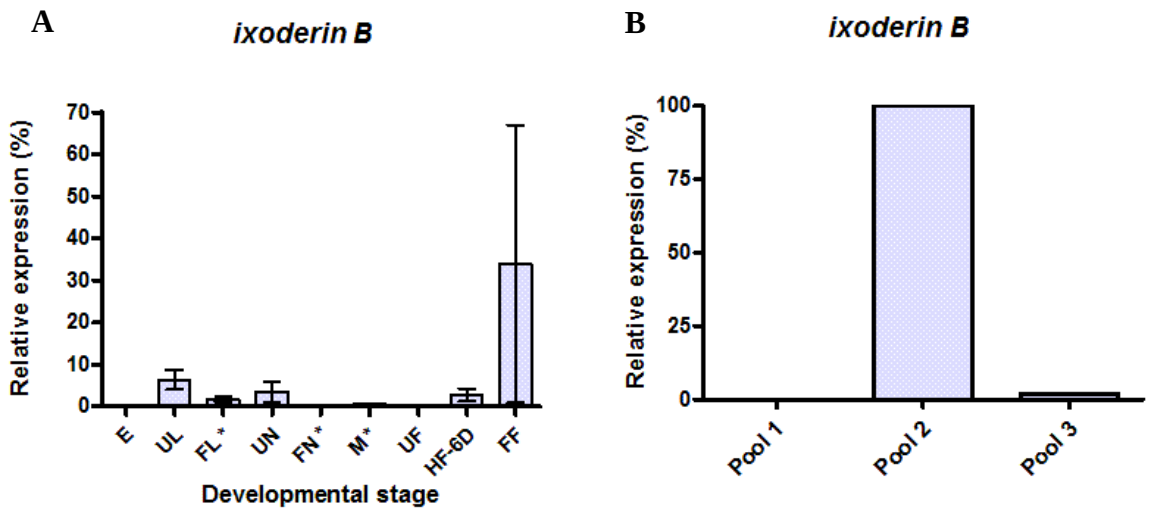
NAME	Gene ID	Protein ID	Signal peptide sequence	Organism
	ISCW000158	ISCW000158-PA	SignalP: no	*Ixodes scapularis*
	ISCW001478	ISCW001478-PA	SignalP: no; PSORT: Mitochondrial matrix	*Ixodes scapularis*
	ISCW002664	ISCW002664-PA	SignalP: no	*Ixodes scapularis*
	ISCW024182	ISCW024182-PA	SignalP: no	*Ixodes scapularis*
	ISCW024125	ISCW024125-PA	SignalP: no	*Ixodes scapularis*
	ISCW003711	ISCW003711-PA	SignalP: no	*Ixodes scapularis*
	ISCW024309	ISCW024309-PA	SignalP: no	*Ixodes scapularis*
	ISCW024256	ISCW024256-PA	SignalP: no	*Ixodes scapularis*
	ISCW004981	ISCW004981-PA	SignalP: no	*Ixodes scapularis*
	ISCW024400	ISCW024400-PA	SignalP: no	*Ixodes scapularis*
	ISCW024445	ISCW024445-PA	SignalP: no	*Ixodes scapularis*
	ISCW024486	ISCW024486-PA	SignalP: no	*Ixodes scapularis*
	ISCW024504	ISCW024504-PA	SignalP: no	*Ixodes scapularis*
	ISCW010128	ISCW010128-PA	SignalP: no	*Ixodes scapularis*
	ISCW008812	ISCW008812-PA	SignalP: no	*Ixodes scapularis*
Ixoderin C	ISCW009412	ISCW009412-PA	SignalP: yes; PSORT: Outside	*Ixodes scapularis*
	ISCW024554	ISCW024554-PA	SignalP: no	*Ixodes scapularis*
	ISCW024548	ISCW024548-PA	SignalP: no	*Ixodes scapularis*
Ixoderin A2	ISCW024686	ISCW024686-PA	SignalP: no	*Ixodes scapularis*
	ISCW024644	ISCW024644-PA	SignalP: no	*Ixodes scapularis*
	ISCW024801	ISCW024801-PA	SignalP: no	*Ixodes scapularis*
	ISCW022063	ISCW022063-PA	SignalP: no	*Ixodes scapularis*
	ISCW024814	ISCW024814-PA	SignalP: no	*Ixodes scapularis*
	ISCW012248	ISCW012248-PA	SignalP: yes; PSORT: Plasma membrane	*Ixodes scapularis*
	ISCW013797	ISCW013797-PA	SignalP: yes; PSORT: Outside	*Ixodes scapularis*
Ixoderin A	ISCW013746	ISCW013746-PA	SignalP: yes; PSORT: Outside	*Ixodes scapularis*
	ISCW024835	ISCW024835-PA	SignalP: no	*Ixodes scapularis*
DORIN M	AY333989.1	AAP93589.1	SignalP: yes; PSORT: Outside	*Ornithodoros moubata*
OMFREP	AF527411.1	AAM88421.1	SignalP: yes; PSORT: Outside	*Ornithodoros moubata*
Ixoderin A	AY341424.1	AAQ93650.1	SignalP: yes; PSORT: Outside	*Ixodes ricinus*
Ixoderin B	AY643518.3	AAV41827.2	SignalP: yes; PSORT: Outside	*Ixodes ricinus*
Ixoderin B3	EF063561.1	ABO09952.1	SignalP: no	*Ixodes ricinus*
Ixoderin B4	EF063562.1	ABO09953.1	SignalP: no	*Ixodes ricinus*
Ixoderin B5	EF063563.1	ABO09954.1	SignalP: no	*Ixodes ricinus*
Ixoderin B6	EF063564.1	ABO09955.1	SignalP: no	*Ixodes ricinus*
Tachylectin 5A	AB024737.1	BAA84188.1	SignalP: yes; PSORT: Endoplasmic reticulum	*Tachypleus tridentatus*
Tachylectin 5B	AB024738.1	BAA84189.1	SignalP: yes; PSORT: Outside	*Tachypleus tridentatus*
FBN8	AGAP011223	AGAP011223-PA	SignalP: no; PSORT: Microbody (peroxisome)	*Anopheles gambiae*
FBN9	AGAP011197	AGAP011197-PA	SignalP: no; PSORT: Microbody (peroxisome)	*Anopheles gambiae*
FBN39	AGAP000806	AGAP000806-PA	SignalP: yes; PSORT: Outside	*Anopheles gambiae*
Fibrinogen	X71937.1	CAA50740.1	SignalP: no; PSORT: Microbody (peroxisome)	*Homo sapiens*
Ficolin	S80990.1	AAB50706.1	SignalP: yes; PSORT: Outside	*Homo sapiens*
BgMFREP 2	AY012700.1	AAK13550.1	SignalP: yes; PSORT: Outside	*Biomphalaria glabrata*
BgMFREP 3	AY028461.1	AAK28656.1	SignalP: yes; PSORT: Outside	*Biomphalaria glabrata*
BgMFREP 4	AY012701.1	AAK13551.1	SignalP: yes; PSORT: Outside	*Biomphalaria glabrata*
Ixoderin C	GANP01004287.1	JAB80181.1	SignalP: yes; PSORT: Nucleus	*Ixodes ricinus*



Supplement Figure 1: The clustalW alignment (in BioEdit) of common part of fibrinogen-related domains in FREPs of ticks. Shaded threshold 35%. "X" is inserted manually at the beginning and the end of the sequences instead of a gap. IS: *Ixodes scapularis*; OM: *Ornithodoros moubata*; IR: *Ixodes ricinus*; sequence names starting with ISCW belong to *Ixodes scapularis* genome.



Supplement Figure 2: The clustalW alignment (in BioEdit) of full-lenght fibrinogen-related domains in FREPs of ticks and selected organisms. Shaded treshold 35%. "X" is inserted manually at the beginning and the end of the sequences instead of a gap. IS: *Ixodes scapularis*; OM: *Ornithodoros moubata*; IR: *Ixodes ricinus*; TT: *Tachypleus tridentatus*; AG: *Anopheles gambiae*; HS: *Homo sapiens*; BG: *Biomphalaria glabrata*



Supplement Figure 3: **A)** The relative expression of *ixoderin B* against *elongation factor 1* in different tick developmental stages. The highest value is set to 100%. The error bar represents the standard error of mean from three independent biological replicates – pools of ticks. The asterisks labeled (*) stages represent the results from only two replicates. Developmental stage: E - eggs, UL - unfed larvae, FL - fed larvae, UN - unfed nymphs, FN - fed nymphs, M - males, UF - unfed females, HF-6D - six days fed females, FF - fully fed females. **B)** The relative expression of *ixoderin B* against *elongation factor 1* in fully fed tick females demonstrating high variability of *ixo B* expression among ticks. Each pool corresponds to one pool of five ticks.