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Nutritional requirements of ticks

Biology of haem in the tick *Ixodes ricinus*

Ph.D. Thesis

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Annotation: Ticks acquire nutrients only by a parasitic nature of feeding on animals, including humans. During this process, a wide array of pathogens is transmitted. Ticks of the Ixodidae family receive exactly one blood meal in each active developmental. Knowing the trophic dependence of tick metabolism on the host blood meal components may enable discovering processes essential for the tick physiology and development. Exploiting a membrane system of tick feeding and whole blood fractionation, we have revealed that adult ticks need to acquire host haemoglobin-derived haem so that they can produce viable larvae, and reproduce. Haem is not further catabolised in ticks, and iron is thus acquired *via* independent route with the host serum transferrin as a source molecule. Using RNA-seq, we compared transcriptome compositions between guts of blood- and serum-fed ticks. We identified fifteen gut transcripts that change their levels with respect to the presence/absence of dietary red blood cells. Glutathione S-transferase, one of the identified encoded molecules, shows a clear haemin-responsive expression at both transcript and protein levels. Its apparent haem-binding properties suggest that this protein is directly involved in haem homeostasis maintenance within the tick gut. The ultimate goal of such research is to identify and verify targets that, when blocked, would render the acquisition and/or distribution system of haem in ticks non-functional. This would represent a novel way of anti-tick interventions in veterinary and human medicine.

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Declarations

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List of papers and author's contributions

The Ph. D. thesis is based on following papers:

Paper I

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Jan Perner participated in: conception and design, acquisition of data, analysis and interpretation of data, drafting or revising the article.

Paper II

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Jan Perner designed and conducted the experiments and wrote the manuscript.

Paper III

Kopáček P, **Perner J**, Sojka D, Šíma R, Hajdušek O (in press) Molecular targets to impair blood meal processing in ticks. Ectoparasites: Drug discovery against moving targets (Wiley-Blackwell).

Paper IV

Hajdušek O, Šíma R, **Perner J**, Loosová G, Harcubová A, Kopáček P (2016) Tick iron and heme metabolism - New target for an anti-tick intervention; Ticks and Tick Borne Diseases 7, 565-572 (IF = 2.69).

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Paper V

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Jan Perner participated in: partial data acquisition and commenting on the manuscript draft.

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Jan Perner participated in: co-writing the dispatch paper.

Foreword

Parasitism is, to a large extent, governed by a nutritional dependence of the parasite on its host. Many metazoan species turned to blood-feeding habit during evolution, at least for some stages of their life cycle. The family of hard ticks (Ixodidae) receive exactly one blood meal in each active developmental stage as their only source of nutrients. Haemoglobin thus represents a staple diet component that ticks co-evolved with and gained distinct adaptations for its disposal and acquisition. In this thesis, the trophic relationship between ticks and haem as an essential metallonutrient[1] will be addressed.

[1] The term „metallonutrient“ or „metallolipid“ was coined to emphasise the requirments of haem active mobilisation instead of an image of inert cofactor burried in haemoprotein pockets (Reddi and Hamza, 2016).

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Introduction

Haem across Tree of Life

It is now widely acknowledged that biochemistry coevolved with geochemistry, and it was therefore speculated that the formation of the metallic cofactors coincided with the availability of the corresponding metals in the primitive sea (Ilbert and Bonnefoy, 2013). The presence of iron atoms in primordial chemistry indicates the participation of iron in the transition from geochemistry to biochemistry. Later in evolution, iron was incorporated into a tetrapyrrole ring to form a molecule of haem. The appearance of haem and other modified tetrapyrroles such as chlorophyll, sirohaem, vitamin B_{12}, coenzyme F_{430}, and haem d_1 in all domains of life underpin a wide range of their essential biological functions (Bali et al., 2011). Most prokaryotes, and all but one eukaryotic organism (Kořený et al., 2012), require haem for living. Most organisms obtain haem from endogenous enzymatic biosynthetic pathway. Haem biosynthesis has been elucidated in eukaryota and most bacteria, where the classic pathway for its biosynthesis is advanced via an ordered and sequential side chains modifications of a tetrapyrrole precursor, followed by oxidation and ferrochelation (Layer et al., 2010). In contrast, archea haem biosynthesis proceeds *via* an alternative route that arose independently of the classic pathway (Storbeck et al., 2010).

Prokaryota - haem biosynthesis and degradation

Haemin[1] structure was solved by Prof. Hans Fischer in 1929 (Figure 1). For this remarkable achievement at that time, and also the notion that structures of both haemin and chlorophyll share structural similarity of a tetrapyrrole macrocycle, he was awarded the Nobel Prize in 1930.

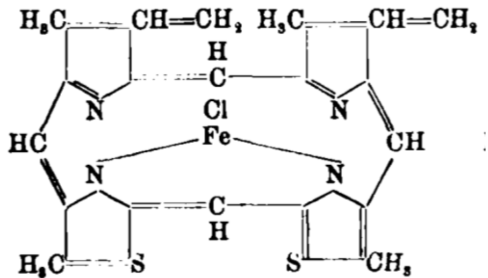

Figure 1. "Häminformel". First published haemin molecular formulation (Fischer and Zeile, 1929).

[1] Protoporphyrin IX containing a ferric iron ion with chloride ligand.

In living systems, haem is used as a prosthetic group of cytochromes, which are required for electron transfer and the final reduction of the terminal electron acceptor (O'Brian and Thöny-Meyer, 2002). Haem proteins can also serve as sensors for diatomic gases such as O_2, CO, or NO in signal transduction pathways (Rodgers, 1999). Exogenous haem serves as a nutritional iron source to some pathogenic bacteria that invade and colonise eukaryotic hosts (Barber and Elde, 2015).

Most bacteria, with a few phylogenetically unrelated exceptions like *Streptococcus* spp., *Lactobacillus* spp., *Borrelia burgdorferi*, or *Treponema palidum* (Gruss et al., 2012), synthesise tetrapyrrole backbone *de novo* via subsequent enzymatic steps commencing by formation of δ-aminolevulinic acid (ALA) either from carboxylic and amino acids (C4 pathway) or from nucleic and amino acids (C5 pathway). Among prokaryotes, ALA synthase (ALA-forming enzyme of the C4 pathway) is restricted only to the α-proteobacteria, while the most prokaryotes synthesise ALA by the C5 pathway. As mitochondria of eukaryotic cells have descended from α-proteobacteria, the eukaryotic ALA synthase has clear origin in that group, while algae and plants retained C5 pathway which has clear origins in photosynthetic bacteria (O'Brian and Thöny-Meyer, 2002). Deficiency of haem biosynthesis in bacterial mutants results in poorly grown colonies devoid of catalase and cytochromes (Kohler et al., 2003; Sasarman et al., 1968). ALA, the sole source of all carbon and nitrogen atoms required for a tetrapyrrolic macrocycle formation, is further metabolised to form uroporphyrinogen III, the final precursor to all tetrapyrroles, *via* three enzymatic steps. Uroporphyrinogen III is then either methylated to form precorrin 2, a precursor for vitamin B_{12}, sirohaem, haem d_1, and coenzyme F_{430}, or is metabolised *via* further three enzymatic steps to form protoporphyrin, the last common precursor for (bacterio)chlorophyll and haem *b*, which then chelates magnesium or iron by magnesium- or ferro-chelatase, respectively. Haem *b* can be incorporated directly into apo-proteins or it can serve as a precursor for the formation of different cytochrome haems *c*, *d*, *e*, and *a*.

Haem can be degraded by haem oxygenase (HO) which, in prokaryotes, is a soluble enzyme of ~25 kDa (Frankenberg-Dinkel, 2004). One major role of prokaryotic HOs has been attributed to synthesis of phycobilin chromopohores of light-harvesting phycobilinoproteins of cyanobacteria

(Frankenberg-Dinkel, 2004). Second big group of HOs with well described biological function is their participation of HOs in iron acquisition for pathogenic bacteria (Frankenberg-Dinkel, 2004). Such HOs were suggested to be suitable drug targets against the particular bacterial pathogens.

Eukaryota - haem biosynthesis and degradation

Most eukaryotes obtain haem from endogenous enzymatic biosynthetic pathway. It is firmly established that mitochondria are derived from endosymbiotic α-proteobacteria (Yang et al., 1985). Haem biosynthesis in heterotrophic eukaryotes is, therefore, mediated by the homologous enzymatic set of α-proteobacteria, the C4 pathway[2] (Figure 2), a part of which is carried out by cytosol-localised enzymes, while the rest of the biosynthetic apparatus still reside in the mitochondria.

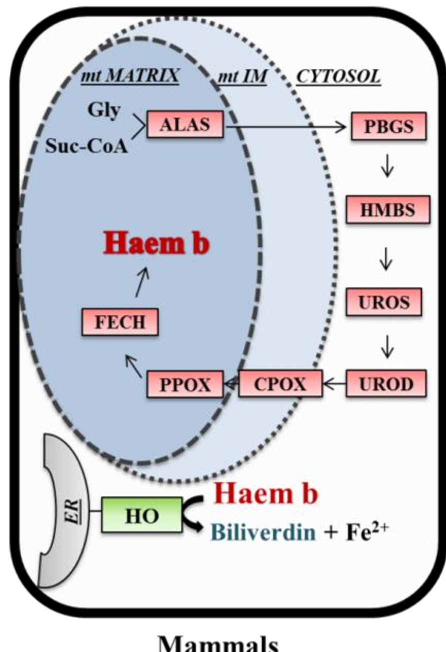

Figure 2. Depiction of the C4 pathway (Shemin pathway) of haem biosynthesis. Mammals harbor α-proteobacterial apparatus for haem biosynthesis which comprises 8 enzymatic steps localised into mitochondria and cytosol. mt - mitochondria, IM - intermembrane space, ER – endoplasmic reticulum; CO - carbon monoxide, Gly - glycine, Suc-CoA - succinyl coenzyme A, ALAS - 5-aminolevulinate synthase, PBGS - porphobilinogen synthase, HMBS - hydroxymethylbilane synthase, UROS - uroporphyrinogen synthase, UROD - uroporphyrinogen decarboxylase, CPOX - coproporphyrinogen oxidase, PPOX - protoporphyrinogen oxidase, FECH - ferrochelatase; HO - haem oxygenase.

[2] C4 pathway is also referred to as Shemin route. It is named after David Shemin who noted that glycine is the starting molecule for haem biosynthesis. In 1945, he swallowed 66g of ^{15}N-labelled glycine over a three-day period. He took regular blood samples from himself and discovered that ^{15}N atoms ended up incorporated in porphyrin ring.

All enzymes in the haem biosynthetic pathway have been crystallised and the crystal structures have permitted detailed analyses of enzyme mechanisms (Ajioka et al., 2006). A short description of individual enzymes involved in haem biosynthesis that follows is based on two excellent reviews by Severance & Hamza and from Martina & Dieter Jahn´s group (Heinemann et al., 2008; Severance and Hamza, 2009). *(i)* ALA synthase (ALAS) is a pyridoxal-5´-phosphate-dependent enzyme, i.e. utilizing an active form of vitamin B_6 as a cofactor. In most vertebrates, ALAS is coded by two gene isoforms *alas1* (ALAS-N) and *alas2* (ALAS-E), where the former is expressed ubiquitously, while the latter is expressed only in erythroid cells *via* an iron-responsive-element-regulated fashion (Cox et al., 1991). While *alas1* transcription (Srivastava et al., 1988; Yamamoto et al., 1988), translation (Yamamoto et al., 1983), mRNA (Hamilton et al., 1991), and protein (Kubota et al., 2016) stability are compromised by haem as a negative feedback regulation, such mechanisms are not desirable in erythroid cells where *alas2* expression is not affected by haem. *(ii)* Porphobilinogen synthase catalyses the asymmetric condensation of two ALA molecules to yield first pyrrole derivative, porphobilinogen. *(iii)* Porphobilinogen deaminase catalyses the polymerisation of four porphobilinogen molecules to form a linear tetrapyrrole, hydroxymethylbilane. *(iv)* Uroporphyrinogen III synthase catalyses the cyclisation of hydroxymethylbilane to form the first cyclic intermediate of the pathway, uroporphyrinogen III. *(v)* Uroporphyrininogen III decarboxylase catalyses the sequential decarboxylation of the four acetate residues producing corresponding methyl groups to form the product coproporphyrinogen III. (vi, vii, viii) Coproporphyrinogen III oxidase, Protoporphyrinogen IX oxidase, and Ferrochelatase are mitochondria-localised enzymes and were proposed to assemble a complex allowing a substrate channelling (Medlock et al., 2015). Two different types of coproporphyrinogen III oxidase are found in nature; one is oxygen-dependent (coded by a *hemF gene*) and the other is oxygen-independent (coded by a *hemN gene*). The two enzyme forms show no obvious amino acid sequence identity, indicating an independent evolution. Protoporphyrinogen IX oxidase catalyses the aromatisation of protoporphyrinogen IX to protoporphyrin IX. Ferrochelatase is responsible for the final insertion of ferrous iron into porphyrin macrocycle to generate protohaem (haem *b*).

Haem oxygenase, the haem-deconstructing enzyme (Beale and Yeh, 1999), is a slightly larger enzyme in eukaryotes of ~36 kDa with a C-terminal membrane anchor (Frankenberg-Dinkel, 2004). Humans and mice possess two well-characterised HO enzymes: HO-1, which is called “inducible”, and HO-2, which is constitutively expressed in most tissues (Balla et al., 2007). In mammals, haem homeostasis is based primarily on haem biosynthesis *de novo* and HO-mediated haem degradation. Dysfunction of the haem recycling system in haem oxygenase 1-deficient mice had profound effects on macrophage viability and tissue iron distribution (Kovtunovych et al., 2010). The majority of body iron and biliverdin/bilirubin (85–95%) is derived from recycled senescent red blood cells. Senescent red blood cells are phagocytosed by spleen and bone marrow macrophages. Between 10 and 20 percent of senescent red blood cells rupture in circulation, the released haemoglobin is complexed with serum haptoglobin, and the complex is scavenged by macrophages *via* a scavenging haemoglobin-haptoglobin receptor (Kristiansen et al., 2001). Haemoglobin is then hydrolysed in macrophages phago-lysosomes, and haem is subsequently exported to a cytoplasm for a HO-mediated degradation (White et al., 2013).

Eukaryota - haem acquisition and distribution in parasitic protists

Some eukaryotic organisms have lost the capacity to form haem molecules *de novo* and are, therefore, reliant on acquisition of haem from the environment. One big group that has lost genes encoding haem biosynthesis is the Kinetoplastida class, which includes human and animal parasites of the genera *Trypanosoma* and *Leishmania* (Kořený et al., 2013). It was expected that the parasites would meet their intracellular haem demand by uptake of dietary haemoglobin, despite the fact that these parasites do not live within red blood cells. The presence of a parasite glycoprotein receptor, which binds the haemoglobin-haptoglobin complex present in host plasma, was first identified in *Trypanosoma brucei* (Vanhollebeke et al., 2008). The cattle parasite *Trypanosoma congolense*, however, expresses a homologous receptor only in the insect (*Glossina pallidipes*) stage and displays high affinity to unbound haemoglobin rather than haemoglobin-haptoglobin complex (Lane-Serff et al., 2016). The authors suggest that, during the evolution, the protein changed from an insect-stage expressed haemoglobin receptor into a haemoglobin-haptoglobin receptor, as haemoglobin is always complexed with haptoglobin in a

mammalian bloodstream (Lane-Serff et al., 2016). *Leishmania donovani*, a protozoan parasite causing human visceral leishmaniasis, was reported to acquire haem by a different, sequentially unrelated protein (Krishnamurthy et al., 2004). Both parasites of the genera *Trypanosoma* and *Leishmania* were recently shown to transport haem to cytosol, upon being released in the acidic digestive vacuole, by *Tb*HRG and *Lm*HRG (Cabello-Donayre et al., 2016), respectively. These proteins are orthologous to haem responsive gene 1, *hrg-1*, first identified in *Caenorhabditis elegans* (Rajagopal et al., 2008). Another bloodstream parasite, belonging to the Apicomplexa phylum, *Plasmodium falciparum*, the causative agent of malaria in humans, codes for haem biosynthesis but does not require its activity during the blood stages (Ke et al., 2014; Nagaraj et al., 2013; Sigala et al., 2015). Recent results suggest that the parasite can scavenge host haem to satisfy metabolic requirements during blood-stage infection (Sigala et al., 2015). The tetrapyrrole uptake mechanism of the parasite can be also harnessed for a photodynamic therapy against the disease (Sigala et al., 2015). The copious amounts of potentially cytotoxic haem, which are released within the food vacuole upon haemoglobin hydrolysis, form haemozoin biominerals in *Plasmodium* parasites (Goldie et al., 1990; Sigala and Goldberg, 2014; van Dooren et al., 2012). Haem metabolism of *Plasmodium,* mainly interference with haem biomineralisation process, is a major antimalarial target (Padmanaban and Rangarajan, 2000).

Haem biology in metazoan parasites

Metazoan parasites are cosmopolitan agents or vectors of "anthroponoses" or "zoonoses", targeting primarily human species as exclusive hosts or non-human species as natural reservoirs, respectively. Metazoan parasites encompass predominantly the phylogenetic groups of Arthropods, Nematodes, and Platyhelminthes (Figure 3). Platyhelminthes can cause severe pathologies and diseases in both human and livestock. Schistosomiasis, for example, is a disease with around 200 million people in Africa being infected. Many of these cases are associated with severe morbidity. Long-term organ damage is often irreversible and chronic (WHO, June 2016). The parasitic nematodes (filaria) cause many pathologies in humans and wildlife such as lymphatic filariasis, ancylostomiasis, or trichinosis. The WHO neglected tropical diseases roadmap set the target date for achieving elimination of lymphatic filariasis by 2020 (WHO, October 2016). Arthropods are the most notorious vectors of diseases. According to World Health Organisation (WHO), vector-borne diseases account for more than 17% of all infectious diseases, causing more than one million deaths annually (WHO, February 2016).

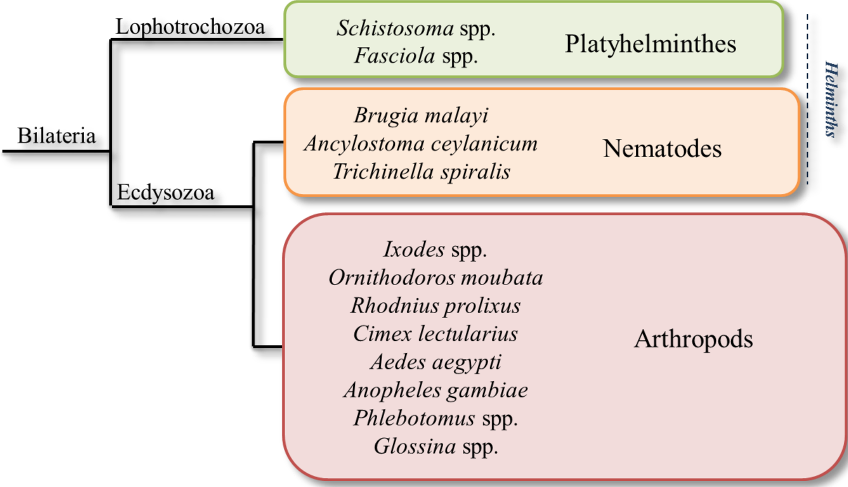

Figure 3. A simplified phylogenetic tree of Metazoan parasites. Metazoan parasites represent both vectors of diseases and direct disease causing agents. Vectors belong mainly to Arthropod species, while infectious agents rather belong to Nematodes and Platyhelminthes (collectively known as Helminths).

Parasitism is a heterotrophic mode of nutrient acquisition from a so-called host. As the relationship between the parasite and its host is largely a nutritional one (Dalton et al., 2004),

they have evolved a number of specific modifications that secure optimal nutrient acquisition for survival and reproduction. Blood meal ensures its consistent composition (both molecular and cellular) and provides a comprehensive source of nutrients (Dalton et al., 2004). Red blood cells comprise nearly half of the volume of whole blood. Red blood cells are rich source of haemoglobin exceeding about twice a protein content of serum[3]. This generous source of protein is utilised by all parasites as a source of amino acids. After its hydrolysis vast amounts of haem are released. Most parasites evolved mechanisms to efficiently dispose of excess haem, however some parasites became dependent on host haemoglobin-derived haem supply. These parasites thus need to operate very delicate system whereby they acquire finely defined quantity of haem and efficiently dispose of the rest, as it can cause cell damage and tissue injury through reactive oxygen species formation, ultimately resulting in oxidative stress (Chiabrando et al., 2014).

The research of molecular network participating in haem acquisition, transportation, or detoxification represents an under-investigated area in metazoan parasites. A greater understanding of the mechanisms that the parasites exploit to secure the haem-driven processes in the organism and, at the same time, circumvent deleterious effects of elevated intracellular haem levels, represent a promising direction of further research.

With the advent of genome databases, complete sets of genes, that code for proteins likely involved in haem biology, can be listed. Using a comparative genomics, a clear illustration of partial or complete loss of genetic make-up for haem biosynthesis in unrelated metazoan parasites can be drawn (Figure 4). Given that haem cleavage represents the main mechanism for iron ions liberation that constitutes a bioavailable iron pool in insects and mammals (Soares and Hamza, 2016; Zhou et al., 2007), it is unlikely that Myriapoda, Chelicerata, and Nematoda will depend on haem-iron source due to the absence of haem oxygenase coding gene (Figure 4). Collectively, haem intracellular homeostasis in selected groups of parasites will certainly depart from a mammalian system, where haem homeostasis is maintained by regulated haem biosynthesis and haem degradation. In contrast, some Metazoan parasites will exploit independent haem and iron acquisition routes with their subsequent systemic distributions.

[3] Haemoglobin is present in blood at roughly 150g/l, while all serum proteins account for 70g/l.

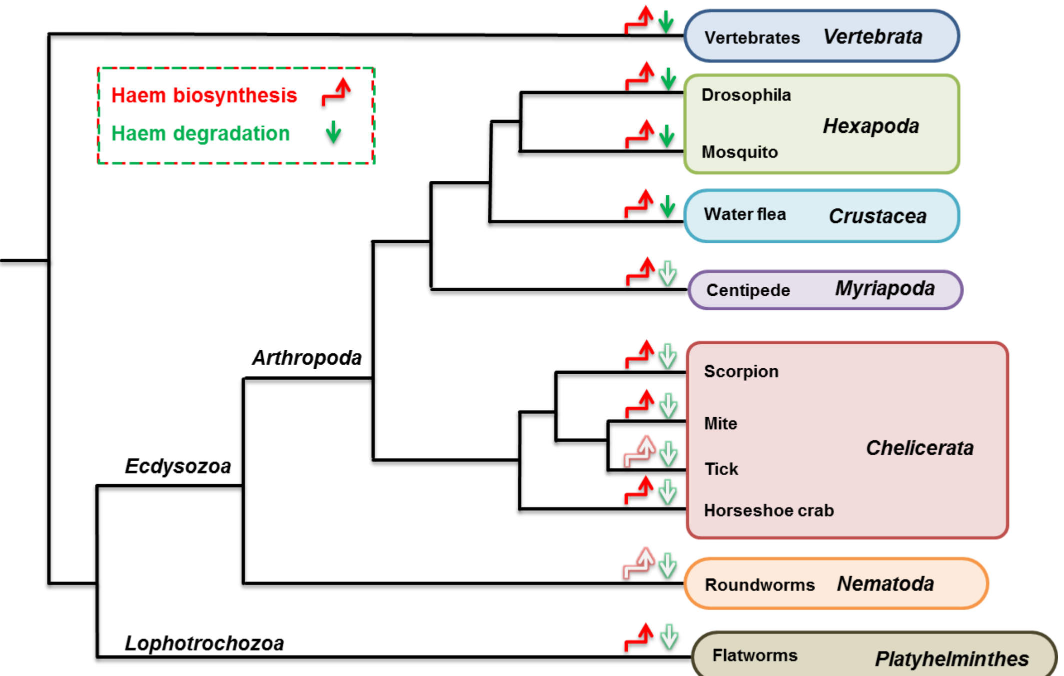

Figure 4. A simplified phylogenetic tree of protostomes bilateria with indicated haem biosynthesis and haem oxygenase-mediated degradation of haem. Genome-wide screening revealed that haem biosynthesis (red arrows), as well as haem-oxygenase mediated haem degradation (green arrows), are lost in contemporary species of selected groups; the tree visualisation adopted from (Qu et al., 2015).

Haem biology in Platyhelminthes

Approximately three quarters of known Platyhelminthes are parasitic. Schistosomes are formidable multicellular blood parasites. In 1852, Theodor Bilharz discovered *Distomum haematobium* (now *Schistosoma haematobium*), the first *Schistosome* identified as a cause of human schistosomiasis (Sandbach, 1976). There are three major species human-infective species; *Schistosoma mansoni and S. haematobium* are endemic to Africa, the Middle East and/or South America, whereas *Schistosoma japonicum* is confined to Asia. These parasites go through a complex life cycle necessitating a freshwater snail as an intermediate host. Their definite host cycle starts by penetrating the human skin as cercariae in freshwater, transforming into skin schistosomules, and then migrating in the vasculature where they develop further into male and female immature worms. Then, dimorphic males and females[4] mate and migrate together to mesenteric venules, where they intensely reproduce (Ressurreição et al., 2016). The

[4] A female can produce hundreds of eggs each day, many of which are released in the faeces or urine enabling parasite transmission.

paired adult parasites can remain for decades in the blood stream, showing their highly adapted interaction with the vertebrate host (Cantacessi et al., 2016). Schistosome females can take up blood meal nutrients by absorption through a tegument layer, or are digested in the gut lumen depending on the nutrient solubility (Chitnis et al., 2014). Schistosomes are dependent on both serum and cellular nutrients. Immature schistosomes, for example, are dependent on host transferrin iron for early development within the mammalian host (Clemens and Basch, 1989). Sequestred red blood cells release haemoglobin that is digested in the gut lumen by a series of proteases (Brindley et al., 1997; Gobert et al., 2015). Most of the liberated haem crystalise in a haemozoin-like structure in the parasite gut lumen (Oliveira et al., 2005). Formation of this haemozoin-like strucure, resembling haemozoin from *Plasmodium* parasites, could be prevented by chloroquine treatment, but with no further impact on parasite fecundity (Oliveira et al., 2004). A fraction of released haem was proposed to be taken up by adult schistosomes and transported into ovaries as an essential nutrient for embryogenesis (Toh et al., 2015). Given that the genes encoding proteins participating in haem biosynthesis are conserved in all the available schistosome genomes (Berriman et al., 2009; Young et al., 2012; Zhou et al., 2009) indicates that the parasites are dependent, at least in one life stage, on haem *de novo* biosynthesis (Figure 5). It is still not clear whether the haem uptake and haem *de novo* biosynthesis represent redundant mechanisms in adult schistosomes.

Both free-living and parasitic flatworms do not code for haem-degrading enzyme haem oxygenase (Figure 5). The annelid *Helobdella robusta*, however, does code for haem oxygense (Simakov et al., 2012), confirming the presence of this gene in the Lopohotrochozoa group and suggesting its loss in the Platyhelminthes lineage.

The liver flukes *Fasciola hepatica* and *Fasciola gigantica* infect livestock and humans worldwide. Two genome assemblies of *F. hepatica* are available; one from the University of Liverpool (BioProject PRJEB6687, Cwiklinski et al., 2015), and the other was performed at Washington University (BioProject PRJNA179522). *Fasciola* parasites undergo similarly complex life cycle as *Schistosoma* parasites. In contrast to *Schistosomes*, the adult liver flukes reside in the bile ducts of infected mammals, which pass immature *Fasciola* eggs in their faeces. The rest of their life cycle takes place in aquatic environment. The diet of the adult fluke essentially comprises blood.

The host blood is digested in the gut lumen of the fluke. Residual waste, including haematin, appears in the gut lumen rather than in the gut cells, so its elimination is simply a matter of regurgitation (Halton, 1997). Whether a fraction of haem is also imported from gut lumen is not clear. It was reported, however, that *F. hepatica* codes for a unique haem-binding protein MF6p/*Fh*HDM-1 that may participate in haem trafficking across the parasite tissues (Martinez-Sernandez et al., 2013).

Schematic representation of the genetic make-up for haem biosynthesis and degradation in selected Platyhelminthes species is shown in Figure 5.

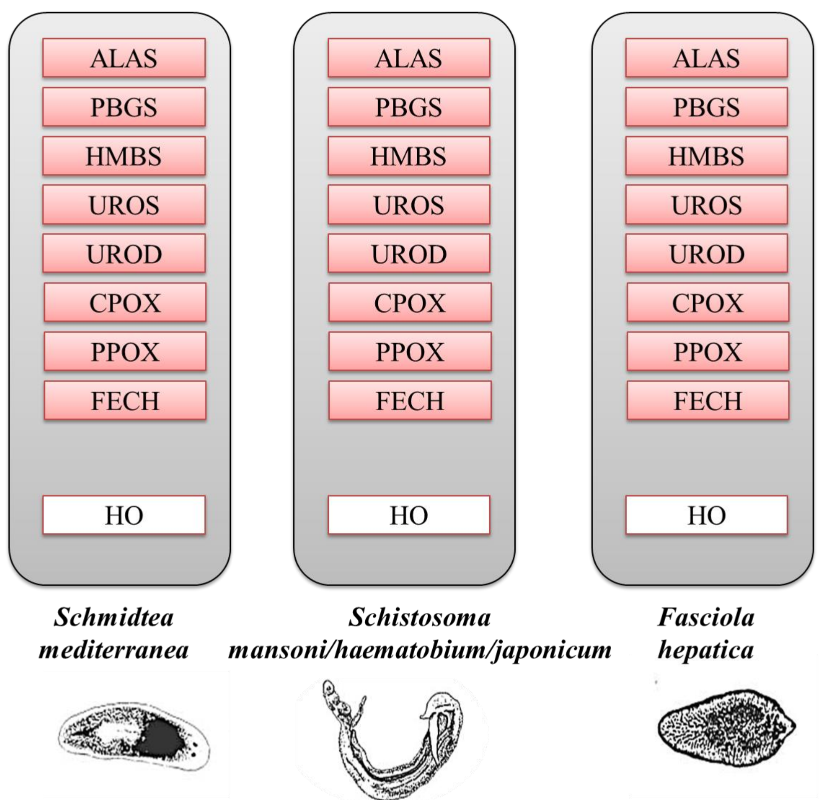

Figure 5. **Genome prediction of genes encoding proteins of haem biosynthesis and degradation in platyhelminthes.** Haem biosynthesis and degradation is conducted *via* sequential enzymatic steps and single-enzyme reaction, respectively. Genes were mined in the genome of free living flatworm *Schmidtea mediterranea* (Robb et al., 2007): ALAS: SMU15001551; PBGS: SMU15014408; HMBS: SMU15030126, SMU15000117, SMU15000570, SMU15025315; UROS: SMU15028091, SMU15014268; UROD: SMU15012633; CPOX: SMU15037876; PPOX: SMU15001566; FECH: SMU15001135. The mined genes of *Schistosoma* species were retrieved from the genomes of *S. mansoni*, *S. haemotobium*, and *S. japonicum*, respectively. ALAS: Smp_045260, MS3_00729, Sjp_0050580; PBGS: Smp_045810, MS3_05595, Sjp_0075570; HMBS: Smp_095130, MS3_04981, Sjp_0002380; UROS: Smp_079840, MS3_08353, Sjp_0011310; UROD: Smp_143740, MS3_03715, Sjp_0084260; CPOX: Smp_162280, MS3_01956, Sjp_0044310; PPOX: Smp_068250, MS3_02152, Sjp_0048650; FECH: Smp_140790, MS3_07149, Sjp_0114760. The mined genes of *Fasciola hepatica* were retrieved from the available transcriptome BioProjectID: PRJNA330752. Drawings of flatworms were taken from (Young et al., 2012).

Haem biology in nematodes

Parasitic nematodes are roundworms causing infections in humans and cattle. Some roundworms require an insect vector to complete their cycle (mosquitoes or black flies). Lymphatic filariasis is a disease caused by *Wuchereria bancrofti* or *Brugia malayi.* Adult *Brugia* parasites reside in the lymphatic system. The adults produce microfilariae which migrate from lymph into the blood stream. Microfilariae can be then transmitted by mosquitoes (*Mansonia* and *Aedes* sp.) where they develop into infective larvae. The parasitic, as well as free-living, nematodes do not code for haem biosynthetic enzymes (Figure 6). A nutritional dependence on exogenous haem supply is therefore expected. Given that the adult stages reside off the bloodstream, it reasonable to assume that the parasites cannot acquire large quantities of dietary haem. Based on the *B. malayi* genome, the parasite also lack genes encoding proteins participating in haem biosynthesis (Ghedin et al., 2007). The only encoded protein of haem biosynthesis pathway is ferrochelatase, which is clearly of bacterial origin (Wu et al., 2013). Yet, the activity of the gene product was shown to be essential for parasite activity and survival (Wu et al., 2013). Last year, it was reported that the parasite *B. malayi* acquire exogenous dietary haem (Luck et al., 2016). However, the study presumes the haem content in the lymph to be a third of the haem content. Such presumption needs to be re-examined. An alternative theory as to where adult filariae might source haem from suggests an endosymbiotic supply. Supporting evidence that *B. malayi* might actually acquire haem from *Wolbachia* endosymbiont came from assessment of haem biosynthetic pathway inhibitors in viability assays. Using succinyl acetone and N-methyl-mesoporphyrin treatment, both male and female adults reduced their motility over the course of ten days (Wu et al., 2009). It is not shown, however, whether the milimolar and submilimolar concentrations of the inhibitors specifically inhibited only enzymes of haem biosynthesis. In another roundworm species, *Dirofilaria immitis*, it was reported that, in microfilaria stage, transcripts encoding haem biosynthesis and haem export are highly abundant in its *Wolbachia* endosymbiont (Luck et al., 2014), which might suggest that these microfilariae might source haem from internal dwelling bacteria. Given that the microfilariae are the only stages that inhabit the host blood stream, the whole picture of haem acquisition in filariae remains counter-intuitive, bizzare, and far from being clear.

The two hookworm species causing the most human infections are *Necator americanus* and *Ancylostoma duodenale*. These parasites are responsible for anaemia-causing hookworm disease. Unfortunately, neither of these can be cultured *in vitro*, however the ability to culture *Ancylostoma ceylanicum* in golden hamster allows it to be used as a model system for the human-specific hookworms (Schwarz et al., 2015). The parasite lives hooked to a host intestine for blood acquisition. The parasite also lacks genes encoding proteins participating in haem biosynthesis except for the gene encoding uroporphyrinogen III decarboxylase (Schwarz et al., 2015) (Figure 6). Consequently, it is presumed that *A. ceylanicum* is dependent on host haem acquisition; a presumption supported by a typical representation of intestinal transcripts coding for haemoglobin proteases and haem-binding glutathione S-transferases (Wei et al., 2016).

Trichinella spiralis is a common wild life parasite of intestinal tract and muscles. Encyst-released larvae invade small intestine mucosa where they develop into adult worms. Females then produce larvae that migrate to striated muscles where they encyst. From the available genome (Mitreva et al., 2011), it is obvious that even this nematode species does not code for enzymes participating in haem biosynthesis (Figure 6). A host supply of haem is therefore expected.

Irrespective of the parasite niche, it is apparent that all nematode species, parasitic or not, lack the genetic coding for a complete haem biosynthetic and degradative apparatus. Given that the pathway is conserved in Hexapodes, within an Ecdysozoan branch, it is likely that the common ancestor of these species has the genetic make-up that was lost only after nematode speciation. What was the selection pressure for this occurance is not clear. A schematic representation of the genetic make-up for haem biosynthesis and degradation in nematodes is shown in Figure 6.

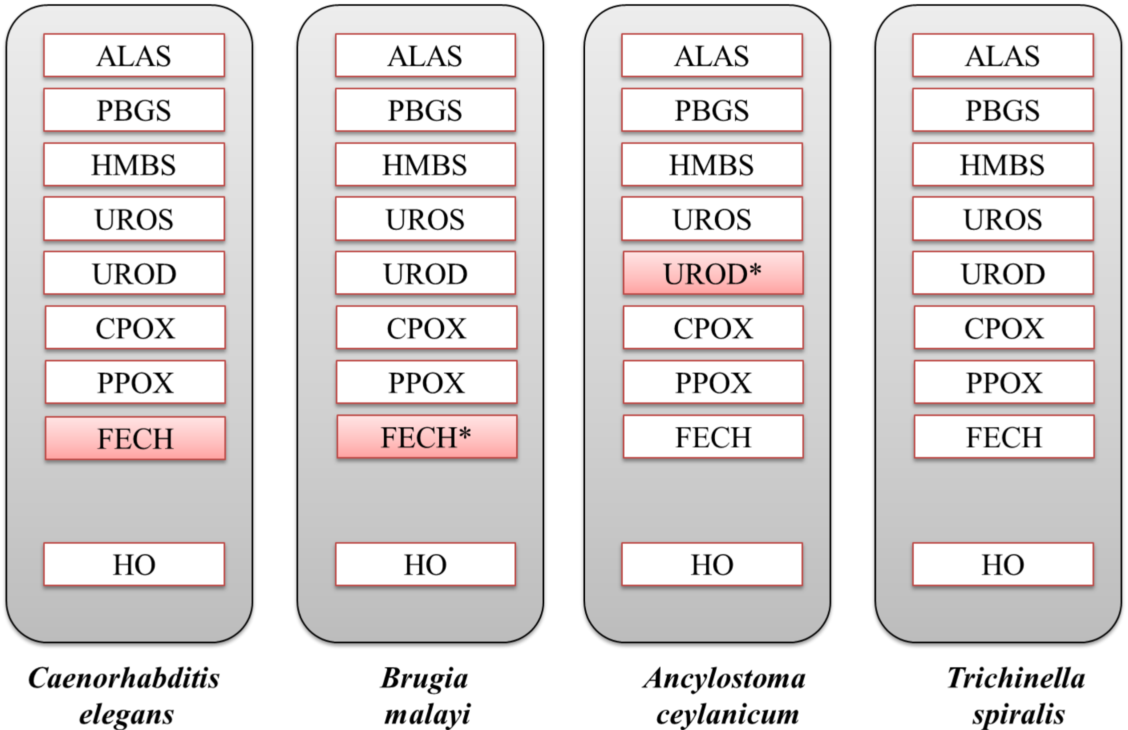

Figure 6. Genome prediction of genes encoding proteins of haem biosynthesis and degradation in nematodes. Haem biosynthesis and degradation is conducted *via* sequential enzymatic steps and single-enzyme reaction, respectively. Genes were mined in the genomes of *Caenorhabditis elegans* (a free-living nematode), *Bugia malayi*, *Ancylostoma ceylanicum*, and *Trichinella spiralis*. UROD*: EPB65962; FECH*: ADI33748; FECH: NM_059622.4. * denotes bacterial origin. For full names of individual abbreviations, please see Figure 2.

Haem biology in arthropods

The habit of blood feeding evolved independently several times among the > 14000 species across 400 genera of haematophagous arthropods (Ribeiro, 1995). Insect vectors transmit diseases such as malaria, dengue, schistosomiasis, human African trypanosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis and onchocerciasis, with reported more than 1 billion cases globally every year (WHO, February 2016), while ticks transmit borreliosis, tick-borne encephalitis, or babesiosis.

Blood-feeding mosquitoes spend most of their lives in immature non-blood-feeding stages. Mostly only adult females of some species feed host blood. Adult mosquito females digest haemoglobin extracellularly in intestine of neutral pH by trypsin-like peptidases (Brackney et al., 2010). Large quantities of haem are released upon digestion of red blood cell haemoglobin in the midgut of mosquitoes. It was suggested that the peritrophic matrix, which develops along

the mosquito intestinal lining, binds the released haem molecules and that haem accumulates in electron-dense aggregates at the luminal face of the peritrophic matrix in both *Aedes* and *Anopheles* mosquitoes (Okuda et al., 2005; Pascoa et al., 2002). Specifically, it was shown that *Aedes aegypti* intestinal mucin 1 (*Ae*IMUC1) could be the main haem-binding protein in the peritrophic matrix (Devenport et al., 2006). Given that haem does not seem to cross the peritrophic matrix of mosquitoes, it may not be expected that the maternal haem would impact embryogenesis of mosquitoes, as observed in hemipterans and ticks (see below). Sand flies of the genus *Phlebotomus* and *Lutzomiya* are small blood-feeding dipteran insects that transmit leishmaniasis. Similarly to mosquitoes, it appears that haemoglobin digestion occurs only extracellularly and excessive haem is stored in gut lumen (Guzman et al., 1994). Analogously, peritrophin of sand flies was suggested to be a haem-binding protein (Pimenta et al., 2013). The tsetse fly of the genus *Glossina* is the vector of sleeping sickness; both males and females take blood-meal as the only nutrient source and transmit the parasites (Bates et al., 2012). The flies apparently digest host blood meal extracellularly and excrete haematin with other waste products (Baylis and Nambiro, 1993).

Haematophagous Hemiptera, such as *Rhodnius* or *Cimex*, feed host blood in all their developmental stages. *R. prolixus* digest host haemoglobin extracellularly using lysosomal type peptidases of mainly Cathepsins B and L classes (Ribeiro et al., 2014). Doing so, digested haemoglobin releases the prosthetic group of haem that accumulates in a haemozoin-like crystal formations (Oliveira et al., 2005). The identification of haemoxisome inside the gut of *R. prolixus*, however, suggests that at least part of the host haemoglobin is digested intracellularly (Roberto Silva et al., 2006). Even though these parasite code for a complete haem biosynthetic apparatus, and also inhibition of the haem biosynthesis at the porphobilinogen synthase step decrease fecundity of the females (Braz et al., 2001), silencing of circulatory haem-binding protein (RBHP) resulted in laying non-colored infertile eggs (Walter-Nuno et al., 2013). These seemingly contradictory results rather substantiate the redundancy of the haem acquisition system in ovaries of blood-feeding hemipterans. Nevertheless, the fact that the *de novo* biosynthesis in ovaries could not replace the portion of delivered dietary haem indicates that oocyte maturation is rather dependent on maternal haem delivery. It needs to be noted that no

biliverdin-like traces could be identified in developing embryos, showing that delivered haem is strictly needed as a cofactor, and not a source of bioavailable iron (Walter-Nuno et al., 2013). Bed bug (*Cimex lectularius*) is a parasitic insect that feeds exclusively on host blood (mostly human). From their midgut infrastructure, it is apparent that, upon blood-feeding, they store excess haem in their gut in haemoxisome-like structure (similarly to the one of *R. prolixus*), suggesting an intracellular haemoglobin digestion (Azevedo et al., 2009). Despite the fact that the Cimicidae and the Triatominae do not share a common hematophagous ancestor, this research revealed structural similarities in their midguts, and shared many adaptations for blood feeding (Azevedo et al., 2009). The convergent evolution is also apparent at the molecular level as they both code for nitrophorins, salivary gland haemoproteins that release NO stores into feeding site as a main vasodilator (Ribeiro and Walker, 1994; Valenzuela and Ribeiro, 1998).

Ticks are blood-feeding arthropods that belong to the Chelicerate lineage. Ticks can be divided into hard ticks (Ixodidae), soft ticks (Argasidae), and Nuttalliellidae (Mans et al., 2016). Ticks feed blood in each active developmental stage: larva, nymph, and adult. With that, ticks transmit a broad array of pathogens ranging from viruses and bacteria to single-cell protists. Genome of the deer tick *I. scapularis* was sequenced and recently published (Gulia-Nuss et al., 2016). The genome assembly and gene annotations brought valuable data that enabled much deeper insight into tick biology. To address specific tissue expressions, many transcriptomes (mainly assembled *de novo*) became available for many tissues of several tick species (de Castro et al., 2016; Kotsyfakis et al., 2015a; Kotsyfakis et al., 2015b; Schwarz et al., 2014; Xu et al., 2016; Zhu et al., 2016). Genomes available for non-blood feeding mites allow comparisons aiming to reveal blood-feeding adaptations of ticks (Grbić et al., 2011; Hoy et al., 2016). Before the *I. scapularis* genome data were available, a lack of haem biosynthetic activity was reported in ovaries of the *Rhipicephalus microplus* ticks (Braz et al., 1999). It was suggested, at that time, that ticks might obtain haem from host haemoglobin. It was later discovered that digest cells of *R. microplus* are able to internalise and hydrolyse exogenous haemoglobin, and detoxify liberated haem by a unique mechanism of haemosome formations (Lara et al., 2003; Lara et al., 2005). The conclusive proof came with the availability of *I. scapularis* genomic data when it became evident that most of the genes encoding haem biosynthesis proteins were missing

(Figure 7). The non-blood-feeding relative of ticks, herbivorous *Tetranychus urticae*, codes for the whole haem biosynthetic machinery (Figure 7) suggesting their common ancestor did operate this pathway, and only tick speciation led to a gradual loss of the pathway. Collectively, the data indicate that ticks, during their blood-feeding evolution, lost genes encoding haem biosynthetic apparatus and, instead, became dependent on a haem acquisition system in the midgut with subsequent systemic distribution. Several haem-binding proteins have been described in ticks to date (Donohue et al., 2009; Gudderra et al., 2001; Logullo et al., 2002; Maya-Monteiro et al., 2000), yet the haem-binding capacity is lacking in the orthologous proteins of tick non-blood feeding relatives *Varroa destructor* (Cabrera et al., 2013) and *T. urticae* (Cabrera et al., 2009). Most of these proteins are large lipoproteins (e.g. carrier protein, vitellogenin) belonging to the large lipid transfer protein superfamily (Donohue et al., 2009). These proteins participate in haem storage, enabling ticks to survive long periods without feeding, and/or haem transport, compensating for the loss of haem *de novo* biosynthesis. The molecular route of haem, from digestive vesicle into haemolymph and ovaries, was only recently revealed. An ATP-binding cassette transporter (ABCB10) was characterised in *R. microplus* ticks, and suggested to participate in translocation of haem from digestive vesicles to cytosol of tick digest cells (Lara et al., 2015).

Schematic representation of the genetic make-up for haem biosynthesis and degradation in blood-feeding arthropods is shown in Figure 7.

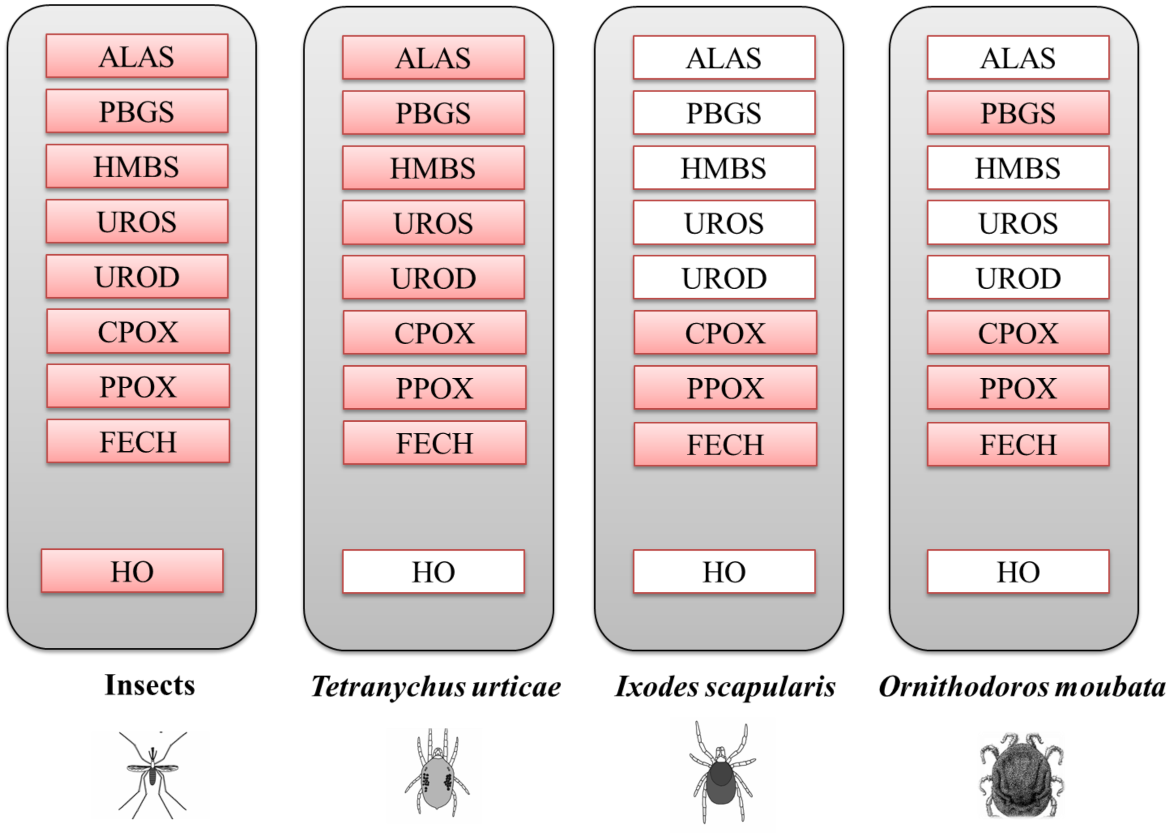

Figure 7. **Genome prediction of genes encoding proteins of haem biosynthesis and degradation in arthropods.** Haem biosynthesis and degradation is conducted *via* sequential enzymatic steps and single-enzyme reaction, respectively. Genes were mined in the genomes of *Anopheles gambiae* (as an insect representative), *Tetranychus urticae*, *Ixodes scapularis*, and in the in-house Argasidae database (Mans et al., 2016) for *Ornithodoros moubata*. The genomic accession numbers for individual genes: ALAS: AGAP003184, tetur32g00320; PBGS: AGAP010935, tetur32g00590; HMBS: AGAP011080, tetur24g02360; UROS: AGAP008064, tetur04g03030; UROD: AGAP011895, tetur19g03090; CPOX: AGAP004749, tetur04g09527, ISCW010977; PPOX: AGAP003704, tetur10g04900, ISCW023396; FECH: AGAP003719, tetur04g02210, ISCW016187; HO: AGAP003975. For full names of individual abbreviations, please see Figure 2. Drawings of selected blood-feeding arthropods were taken from (Perner et al., 2016b) and Institute of Tropical Medicine in Antwerp (permission granted).

Thesis objectives

Thesis objectives

Parasitism is conditioned by host nutrients acquisition, which determines the dependence of the parasite on its host. Haem, as an inevitable by-product of host haemoglobin hydrolysis, constitutes an essential molecule for some unicellular, as well as for multicellular parasites. Most of the acquired haem, however, is disposed of in the parasites, using distinct adaptive mechanisms. In the Introduction, a summary of a current knowledge of haem detoxification and acquisition by metazoan parasites is described. For a summarising table, please see Figure 10.

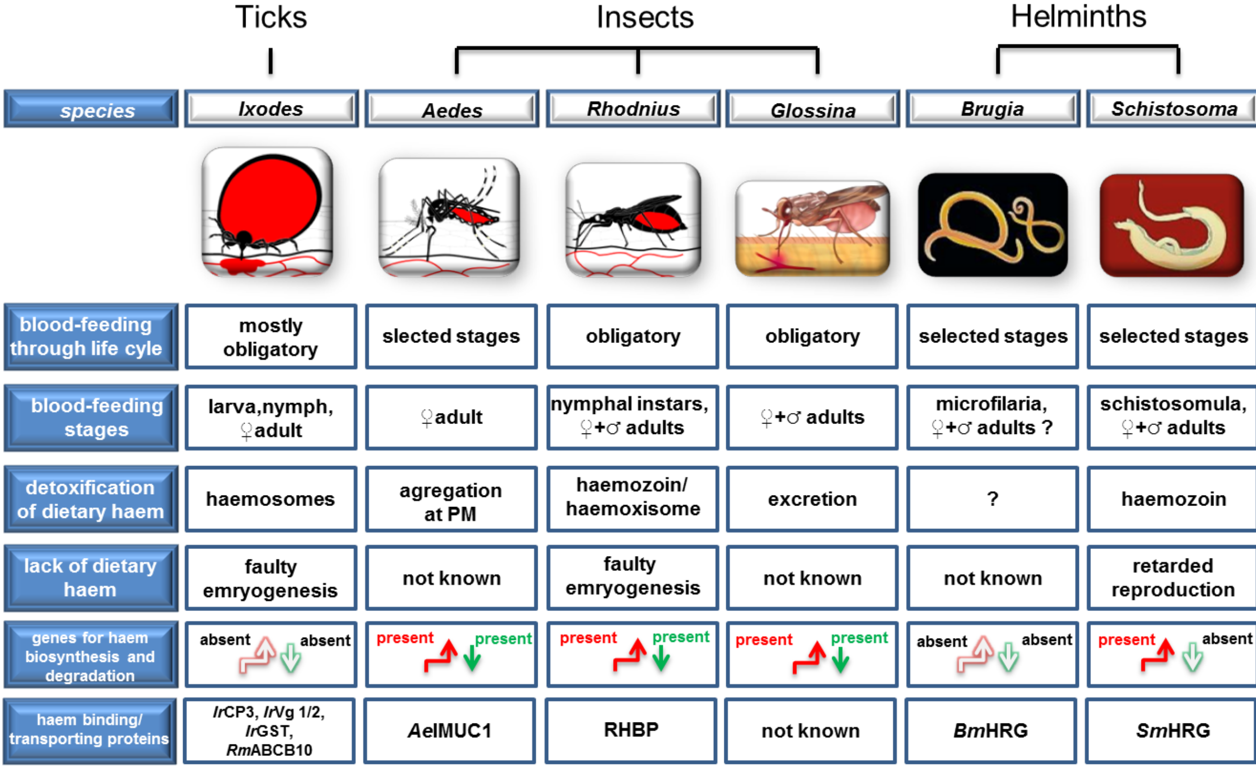

Figure 10. An overview of haem biology among metazoan parasites. Not all life stages of the blood-feeding parasites actually receive nutrients from a host blood. Therefore, only blood-feeding stages of the parasites are highlighted and adaptations for haem biology are stated. *Ir*CP3 and *Ir*VG1/2 are *Ixodes ricinus* haem-binding lipoproteins (Perner et al., 2016b). *Ir*GST is *I. ricinus* glutathione S-transferase (Part II). *Rm*ABCB10 is a *Rhipicephalus microplus* ABC transporter of haem (Lara et al., 2015). *Ae*IMUC1 is *Aedes aegypti* intestinal mucin 1 (Devenport et al., 2006). RHBP is *Rhodnius prolixus* haem-binding protein (Oliveira et al., 1995). *Bm*HRG is *Brugia malayi* haem-responsive gene 1 (Luck et al., 2016). *Sm*HRG is *Schistosoma mansoni* haem-responsive gene 1 (Toh et al., 2015). Parasite drawings were taken from (Kopáček and Perner, 2016; Raper et al., 2013; Sojka et al., 2013).

What makes the tick, in nutritional sense, a parasite is a basic question that the thesis attempts to address. So far, very little is known as to what single blood constituents are necessary for tick feeding physiology, inter-stadial development, and/or reproduction. Using blood fractionation and artificial feeding system of nymphal and adult *I. ricinus* ticks, we have been able to start answering these questions.

In this thesis, I have summarised our work, both published and unpublished, that helped to understand biological and molecular aspects of haem biology in ticks. Initially, our first objective was to set a biological concept. We decided to try exploiting the membrane feeding system and carrying out comparative analyses between blood- and serum-fed ticks. First, we had to optimise a method to receive virtually haemoglobin-free serum. Once the protocol was optimised, our initial expectation was that the absence of red blood cells, a predominant source of amino acids and haem, would have large impact on tick feeding physiology and that ticks would not be able to finish feeding and lay eggs. That was not confirmed, however. Serum-fed ticks were nearly identical in achieved weights and size of egg clutches as the blood-fed ticks (Appendix I). The impact of haem on tick biology became evident when no larvae hatched out of clutches of serum-fed ticks. Failure of adult serum-fed ticks to reproduce served as a proof of metabolic auxotrophy of *I. ricinus* ticks for dietary haem. To provide a direct link between host haemoglobin and haem necessary for tick embryogenesis, a rescue experiment was conducted, where haemoglobin supplementation in bovine serum rescued the lethal phenotype of “serum” eggs, unambiguously proving that host haem is essential for tick embryogenesis. Making use of human dietary nomenclature, we can call haem an essential vitamin for ticks.

Why ticks and not other mites lost haem biosynthesis, whereas both free-living as well as parasitic nematodes lost it, is not completely clear. It can be argued that strict blood-feeding habit of ticks might have favoured such loss of genes. Ticks, with a guaranteed supply of host haemoglobin, could have perhaps allowed collecting mutations in genes encoding haem biosynthesis proteins, as they could replace their endogenous *de novo* haem biosynthesis by acquisition of dietary haem. When challenged by an unnatural situation of serum feeding, these ticks could not respond to that and we could observe the lethality of haem-deprived embryos. Even though the partial genetic loss is evident from *I. ricinus* genomic and transcriptomic data

(Cramaro et al., 2015; Schwarz et al., 2014), why the genes encoding last three enzymes of the pathway were retained in the genome and are expressed in the transcriptomes is not clear. It is reasonable to conclude that the same will hold true even for other tick species, and that the phenomenon would be "pan-tick" (Braz et al., 1999; Mans et al., 2016).

The next objective was an attempt to deconvolute the array of midgut processes involved during blood-feeding of ticks. Capturing the repertoire of midgut mRNA molecules and comparing them between blood- and serum-fed ticks, by RNA-seq analyses, we could identify transcripts that change their levels in a red blood cells responsive manner. The identification of transcripts with elevated or lowered levels in midguts of blood-fed ticks, in comparison to those of serum-fed ticks, was an indicative proof of involvement of the encoded proteins in haem biology. The indicative suspicion had to be further validated using methods of biochemistry and biophysics. Glutathione S-transferase (GST) is an enzyme that is present in tick midguts of blood-fed ticks at substantially increased levels, contrary to low levels in midguts of serum-fed ticks. Previous reports showed that GSTs of other organisms displayed high affinity binding with haem (Al-Qattan et al., 2016; Hiller et al., 2006; van Rossum et al., 2004; Zhan et al., 2005). Therefore, we sought to characterise the relationship between tick GST and haem. Exploiting the characteristic absorbance properties of haem in the visible spectrum of light, we could demonstrate that tick GST interacts with haem *in vitro*, as evidenced by apparent absorbance maximum shifts. Whether the GST actually binds haem *in vivo*, within the tick digest cells, and acts as a *bona fide* haem scavenger has not been conclusively shown. It is also not clear if the enzyme catalyses a transfer of glutathione on haem, or whether it is a terminal binding.

In the third quarter of this thesis, current proteinaceous targets in ticks are described. Candidate molecules, assumed to participate in iron or haem homeostasis in ticks, were selected and tested for essentiality by RNA interference. The transcript expression profiles were also described. As serum feeding was shown to be sufficient to provide ticks with amino acids for vitellogenesis, it raised a question as to how the serum proteins are hydrolysed. It was shown, however, that the digesting apparatus of serum albumin does not differ from that of host haemoglobin. Also, the transcripts and enzymes are not responsive to dietary red blood cells at transcript levels as well as in enzymatic activities, respectively.

In the final part of this thesis, our current activities are outlined and our objectives for further research utilising a membrane feeding as a research platform are set. Fractionation of serum and observation of nutritional dependence of tick on individual components, tick salivary adaptive measures, or pathogen transmission kinetics could be assessed by this approach.

Part I

Nutritional dependence of ticks on host haemoglobin

Paper I

Perner J, Sobotka R, Šíma R, Konvičková J, Sojka D, Oliveira PL, Hajdušek O, Kopáček P (2016)
Acquisition of exogenous haem is essential for tick reproduction.
eLife 2016;5:e12318.

Unpublished data to Paper I

Distribution and storage of maternal haem in ticks

Manuscript in preparation I

Perner J, Hatalová T, Kučera M, Kopáček P.
Impact of serum-feeding on development of *Ixodes ricinus* nymphs.

Preface

It was reported that the cattle tick *R. microplus* had a dysfunctional haem biosynthesis in its ovaries, and the authors suggested a that dietary uptake of haem and its inter-tissue trafficking, a mechanism very uncommon at that time, might be responsible the high haem content in tick eggs (Braz et al., 1999). To support their suggestion, we wanted to experimentally show that dietary haemoglobin is the source of abundant haem deposits in eggs. Nearly a decade ago, a team of Dr. Kröber and prof. Guerin from the University of Neuchâtel advanced membrane feeding of hard ticks, including a system suitable for *I. ricinus* ticks (Kröber and Guerin, 2007). The main obstacles that had to be overcome were appropriate attractant formulation, perfecting mechanical properties of a membrane, and sustainable sterility over an 8-day period of blood-feeding. We have managed to learn and adopt this technique to test our hypotheses regarding tick-host nutritional dependence. To inspect the nutritional demand of ticks towards host haemoglobin-haem, we have exploited an artificial membrane feeding system for adult ticks and made a comparative study between blood- and serum-fed ticks (Paper I). We have revealed a strict reliance of ticks on host haemoglobin for haem acquisition and transportation into ovaries to facilitate tick embryogenesis. By transcriptomic data (Perner et al., 2016a; Schwarz et al., 2014), we confirmed presence of only three transcripts from the standard eukaryotic haem biosynthetic pathway. This is in line with a genetic loss evidenced in the genome of *I. scapularis*. From transcriptomic, genomic and biochemical data, we also revealed the absence of haem oxygenase-mediated haem degradation indicating that ticks need to acquire iron from a non-haem source. By supplementing diet with excess amounts of iron-saturated holo-transferrin, we showed that host transferrin is the source of bioavailable iron for ticks. We thus managed to deliver experimental proof to previously described presumption of haem and iron independent acquisition and transportation routes in ticks (Hajdušek et al., 2009). While the iron inter-tissue trafficking was described (Hajdušek et al., 2009), haem transportation was not elucidated in *I. ricinus* ticks. Using a combination of VIS-absorbance, selectively stained native-PAGEs and RNAi, we managed to identify predominant haem-binding proteins in haemolymph and ovaries. While carrier protein 3 is a predominant haemoprotein in haemolymph of ticks fed for six days, a vitellogenin (*Ir*Vg) complex is a predominant

haemoprotein in ovaries dissected six days after ticks drop off. As only gene silencing of *ir-vg1*/*vg2* and not of *ir-cp3* led to decreased haem deposits in ovaries, we speculate that haem is being delivered from midgut into ovaries in a vitellogenin complex of their holo- state. This is in contrast with previous observation, when the tick *R. microplus* was injected HeLp, an *Ir*-CP3 homologue, with iron labelled haem that was later detected in ovaries homogenates (Maya-Monteiro et al., 2000). It was suggested that HeLp would deliver haem into ovaries and only then would be haem scavenged by vitellins, a proteolytically cleaved vitellogenins with potentially exposed haem-binding sites. The transporting role of is thus not fully resolved. Whether vitellogenins form holo-proteins in the gut, haemolymph, or ovaries is not known. Whether haem-binding vitellogenin complex is formed as a heterodimer (*Ir*Vg1-*Ir*Vg2 complex) or two homodimers (*Ir*Vg1-*Ir*Vg1 and *Ir*Vg2-*Ir*Vg2 complex) needs to be also examined. A few pilot unpublished experiments regarding that issue are shown in a short section following Paper I. Reviewers comments and authors´ responses to Paper I first submission, as well as its resubmission, including additional experiments and data are shown in Appendix I.

Unlike adult *I. ricinus* females, the *I. ricinus* nymphs, when serum-fed, do not display any obvious deviation in their development. The serum fed nymphs can successfully molt into adults, and when blood-fed in adult stage, they are able to mate, fully engorge, and lay viable eggs. This indicates that dietary haem does not play a role in trans-stadial development, does not affect spermatogenesis in males, and does not affect reproductive capacity of females. Such an observation suggests that the main role of haem in tick biology is its indispensable participation during embryogenesis. Therefore, haem acquisition and inter-tissue trafficking in adult ticks might represent a valuable target due to its biological essentiality, utilising mechanisms being unique and not present in tick hosts.

Paper I

Perner J, Sobotka R, Šíma R, Konvičková J, Sojka D, Oliveira PL, Hajdušek O, Kopáček P (2016)
Acquisition of exogenous haem is essential for tick reproduction.
eLife 2016;5:e12318.

RESEARCH ARTICLE

Acquisition of exogenous haem is essential for tick reproduction

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Abstract Haem and iron homeostasis in most eukaryotic cells is based on a balanced flux between haem biosynthesis and haem oxygenase-mediated degradation. Unlike most eukaryotes, ticks possess an incomplete haem biosynthetic pathway and, together with other (non-haematophagous) mites, lack a gene encoding haem oxygenase. We demonstrated, by membrane feeding, that ticks do not acquire bioavailable iron from haemoglobin-derived haem. However, ticks require dietary haemoglobin as an exogenous source of haem since, feeding with haemoglobin-depleted serum led to aborted embryogenesis. Supplementation of serum with haemoglobin fully restored egg fertility. Surprisingly, haemoglobin could be completely substituted by serum proteins for the provision of amino-acids in vitellogenesis. Acquired haem is distributed by haemolymph carrier protein(s) and sequestered by vitellins in the developing oocytes. This work extends, substantially, current knowledge of haem auxotrophy in ticks and underscores the importance of haem and iron metabolism as rational targets for anti-tick interventions.
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Introduction

Haem, the heterocyclic tetrapyrrole that conjugates divalent iron, is an essential molecule for most aerobic organisms, as a prosthetic group of numerous enzymes involved in a variety of biological processes such as cellular respiration, detoxification of xenobiotics or redox homeostasis (***Furuyama et al., 2007***; ***Kořený et al., 2013***). Most organisms synthesise their own haem by an evolutionarily conserved multi-enzymatic pathway occurring in the mitochondria and cytosol. Only a few haem auxotrophs lacking functional haem biosynthesis have been described to date. Among these rare organisms that are reliant on the acquisition of exogenous haem are, for instance, a protozoan parasitic apicomplexan *Babesia bovis* (***Brayton et al., 2007***), and kinetoplastid flagellates of the genus *Trypanosoma* and *Leishmania* (***Kořený et al., 2010***). Some haem auxotrophs, such as the filarial nematode parasite *Brugia malayi* (***Ghedin et al., 2007***; ***Wu et al., 2009***), acquire haem from their endosymbionts, while others, such as the free-living nematode *Caenorhabditis elegans* (***Rao et al., 2005***) obtain haem from ingested bacteria. The inability to synthesise haem *de novo* was also biochemically demonstrated for the cattle tick *Rhipicephalus (Boophilus) microplus* (***Braz et al., 1999***).

In contrast to its benefits, haem is also cytotoxic, where free haem catalyses the generation of reactive oxygen species (ROS), causing cellular damage, mainly through lipid peroxidation (***Jeney et al., 2002***; ***Klouche et al., 2004***; ***Graca-Souza et al., 2006***). Therefore, in all living organisms, free intracellular haem has to be maintained at a low level via strictly regulated homeostasis

eLife digest Ticks are small blood-feeding parasites that transmit a range of diseases through their bites, including Lyme disease and encephalitis in humans. Like other blood-feeders, ticks acquire essential nutrients from their host in order to develop and reproduce.

Iron and haem (the iron-containing part of haemoglobin) are essential for the metabolism of every breathing animal on Earth. Most organisms obtain iron by degrading haem and, reciprocally, most of the iron in cells is used to make haem. However, an initial search of existing genome databases revealed that ticks lack the genes required to make the proteins that make and degrade haem.

Perner et al. wanted to find out if ticks can steal haem from the host and use it for their own development. To achieve this, Perner et al. exploited a method of tick membrane feeding that simulates natural feeding on a host by using a silicone imitation of a skin and cow smell extracts ("l´ odeur de vache"). Ticks were fed either a haemoglobin-rich (whole blood) or a haemoglobin-poor (serum) diet. This experiment revealed that ticks can develop normally without haemoglobin, but female ticks fed a haemoglobin-poor diet lay sterile eggs out of which no offspring can hatch.

Further investigation showed that haemoglobin is vitally important as a source of haem but not as a source of the amino acids needed to produce the vitellin proteins that nourish embryos. As ticks are not armed with the ability to degrade haem, they do not acquire iron from the host haem but rather from a serum transferrin, a major iron transporter protein found in mammalian blood. Further experiments revealed that ticks have evolved proteins that can transport and store haem and so make the obtained haem available across the whole tick body.

Overall, Perner et al.'s findings suggest that targeting the mechanisms by which ticks metabolise haem and iron could lead to the design of new "anti-tick" strategies.

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(**Ryter and Tyrrell, 2000**; **Khan and Quigley, 2011**). This task is a critical challenge for haematophagous parasites, such as the malarial *Plasmodium*, blood flukes or triatominae insects that acquire large amounts of haem from digested haemoglobin (**Oliveira et al., 2000**; **Pagola et al., 2000**; **Paiva-Silva et al., 2006**; **Toh et al., 2010**). Maintenance of haem balance is even more demanding for ticks, as their blood meal exceeds their own weight more than one hundred times (**Sonenshine and Roe, 2014**). Despite its importance, the knowledge of haem acquisition, inter-tissue transport and further utilisation in ticks is fairly limited. Haemoglobin, the abundant source of haem for these animals, is processed intracellularly in tick gut digest cells by a network of cysteine and aspartic peptidases (**Sojka et al., 2013**). Excessive haem is detoxified by aggregation in specialised organelles termed haemosomes (**Lara et al., 2003**; **2005**) and its movement from digestive vesicles is mediated by a recently described ATP-binding cassette transporter (**Lara et al., 2015**). Only a small proportion of acquired haem is destined for systemic distribution to meet the metabolic demands of tick tissues (**Maya-Monteiro et al., 2000**).

In the present work, we have screened available tick and mite genomic databases and found that ticks have lost most genes encoding the haem biosynthetic pathway. All mites also commonly lack genes coding for haem oxygenase (HO) that catalyzes haem catabolism, raising the question of iron source for these organisms. Using in vitro membrane feeding of the hard tick *Ixodes ricinus* (**Kröber and Guerin, 2007**), the European vector of Lyme disease and tick-borne encephalitis, we performed differential feeding of females on haemoglobin-rich and haemoglobin-depleted diets. These experiments conclusively proved that ticks completely rely on the supply of exogenous haem to accomplish successful reproduction and that iron required for metabolic processes in tick tissues does not originate from haem. We propose that the unique maintenance of systemic and intracellular haem homeostasis in ticks represents a specific adaptation to their parasitic life style, and as such offers promising targets for anti-tick intervention.

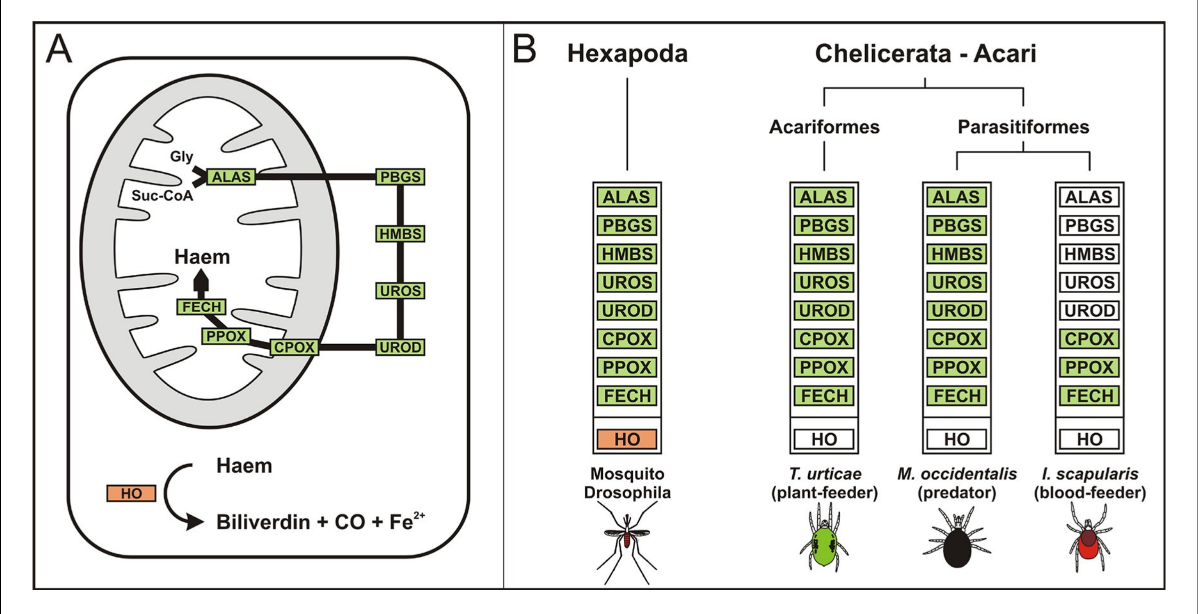

Figure 1. Evolution of haem biosynthetic and degradative pathways. (**A**) General scheme of haem biosynthetic and degradative pathways in the eukaryotic cell. Haem biosynthesis (upper) is a series of eight reactions beginning in the mitochondria by condensation of succinyl coenzyme A with glycine, continuing in the cell cytoplasm, and finishing in the mitochondria with the final synthesis of the haem molecule. Haem degradation (lower) is mediated by haem oxygenase in the cell cytoplasm, releasing a ferrous iron, biliverdin, and carbon monoxide. (**B**) Evolution of haem biosynthetic and degradative pathways in arthropods, according to the available genomic projects. Similarly to vertebrates, hexapods (insects) including blood feeding mosquitoes (red-coloured body), possess all enzymes for haem biosynthesis and degradation. Chelicerates lack haem oxygenase, indicating iron acquisition from sources other than haem. Plant-feeding mites (green-coloured body) of the superorder Acariformes, as well as mite-predating mites (black-coloured body) of the superorder Parasitiformes, possess a complete set of genes for haem biosynthesis. Ticks, which feed solely on blood (red-coloured body) retained only the last three enzymes (mitochondrial) of the pathway. CO - *carbon monoxide*, Fe^{2+} - *ferrous iron*, Gly - *glycine*, Suc-CoA - *succinyl coenzyme A*, ALAS - *5-aminolevulinate synthase*, PBGS - *porphobilinogen synthase*, HMBS - *hydroxymethylbilane synthase*, UROS - *uroporphyrinogen synthase*, UROD - *uroporphyrinogen decarboxylase*, CPOX - *coproporphyrinogen oxidase*, PPOX - *protoporphyrinogen oxidase*, FECH - *ferrochelatase*; HO - *haem oxygenase*. Enzyme nomenclature and abbreviations according to (**Hamza and Dailey, 2012**)

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The following figure supplements are available for figure 1:

Figure supplement 1. Phylogenetic tree of selected coproporphyrinogen-III oxidases.

DOI: 10.7554/eLife.12318.004

Figure supplement 2. Phylogenetic tree of selected protoporphyrinogen oxidases.

DOI: 10.7554/eLife.12318.005

Figure supplement 3. Phylogenetic tree of selected ferrochelatases.

DOI: 10.7554/eLife.12318.006

Figure supplement 4. Phylogenetic tree of selected 5-aminolevulinate synthases.

DOI: 10.7554/eLife.12318.007

Figure supplement 5. Phylogenetic tree of selected uroporphyrinogen decarboxylases.

DOI: 10.7554/eLife.12318.008

Results

Ticks have an incomplete pathway for haem biosynthesis

The availability of the genome-wide database for the deer tick *Ixodes scapularis* (**Gulia-Nuss et al., 2016**) made it possible to analyse the overall genetic make-up for enzymes possibly participating in haem biosynthesis and compare this data with other mites and insects (Hexapoda). Complete haem biosynthetic and degradative pathways are present in insects, represented by the genomes of the fruit fly *Drosophila melanogaster* (**Adams et al., 2000**) and the blood-feeding malaria mosquito, *Anopheles gambiae* (**Holt et al., 2002**) (**Figure 1A,B**). The canonical haem biosynthetic pathway is also fully conserved in the genomes of the herbivorous mite *Tetranychus urticae*, and the predatory

Figure 2. Impact of dietary haemoglobin on tick feeding, oviposition, embryogenesis, and larval hatching. (**Membrane feeding**) - membrane feeding in vitro of *Ixodes ricinus* females on whole blood (Blood-fed), serum (Serum-fed) and on serum supplemented with 10% bovine haemoglobin (Serum + 10% Hb). For dietary composition, see ***Figure 2—figure supplement 1***. (**Oviposition**) – representative females laying eggs. (**Embryogenesis**) – microscopic examinations of embryonal development in eggs laid by differentially fed females; 1w, 2w – 1 week, 2 weeks after oviposition, respectively. Note, no embryos developed in eggs from serum-fed ticks, while embryogenesis was rescued in serum + 10% Hb-fed ticks. (**Larval hatching**) – Laid eggs were incubated to allow larval hatching. Note, no larvae hatched out of eggs laid by serum-fed females and the hatching was fully rescued in serum + 10% Hb-fed ticks. Similar rescue effects were also observed for ticks fed on serum supplemented with 1% and 0.1% Hb (see ***Figure 2—figure supplement 2***)

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The following figure supplements are available for figure 2:

Figure supplement 1. Diets used for tick membrane feeding and faecal examination.

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Figure supplement 2. Rescue experiments with sub-physiological levels of haemoglobin.

DOI: 10.7554/eLife.12318.011

mite *Metaseiulus occidentalis*, but is substantially reduced in the genome of the obligatory blood-feeding tick, *I. scapularis* (**Figure 1B**). The tick genome contains only genes encoding the last three mitochondrial enzymes of haem biosynthesis, namely, coproporphyrinogen-III oxidase (CPOX, [Vectorbase: ISCW010977], **Figure 1—figure supplement 1**), protoporphyrinogen oxidase (PPOX, [Vectorbase: ISCW023396, **Figure 1—figure supplement 2**), and ferrochelatase (FECH, [Vectorbase: ISCW016187], **Figure 1—figure supplement 3**). Corresponding orthologues could be also found in the *I. ricinus* transcriptome (**Kotsyfakis et al., 2015**) (GenBank Ac. Nos JAB79008, JAB84046 and JAB74800, respectively). Phylogenetic analyses confirmed that these genes cluster together with other Acari homologues (**Figure 1—figure supplements 1–3**, respectively). Another two gene sequences related to 5-aminolevulinate synthase (ALAS, Vectorbase: ISCW020754) and uroporphyrinogen decarboxylase (UROD, Vectorbase: ISCW020804) are clearly bacterial and most likely originate from bacterial contamination of the genomic DNA (**Figure 1—figure supplement 4** and **Figure 1—figure supplement 5**, respectively). This conclusion was further corroborated by the fact that these genes do not contain introns and are flanked by other bacterial genes in the corresponding genomic regions.

Despite an incomplete haem biosynthetic pathway, the *I. scapularis* genome contains at least 225 genes encoding a variety of enzymes utilizing haem as a cofactor, such as respiratory chain cytochromes, catalase, and a large family of cytochrome P450 genes (**Supplementary file 1**). Hence, ticks must possess efficient mechanisms for the acquisition of exogenous haem, together with its intra- and extra-cellular transport to produce endogenous haemoproteins.

Host blood haemoglobin is expendable for tick feeding and oviposition but essential for embryonic development

In order to determine the origin of haem required for tick basal metabolism and development, we exploited an in vitro membrane feeding system developed by Kröber and Guerin (**Kröber and Guerin, 2007**). We fed *I. ricinus* females with whole blood (BF ticks), and, in parallel, with haemoglobin-free serum (SF ticks) (**Figure 2** and **Figure 2—figure supplement 1**). Serum-fed ticks were capable of fully engorging and laying eggs similar to BF ticks (**Figure 2**). However, striking differences were observed in embryonic development and larval hatching. Embryos in eggs laid by BF females developed normally as described for naturally-fed ticks (**Santos et al., 2013**) and gave rise to living larvae (**Figure 2**). In contrast, no embryonic development was observed in colourless eggs laid by SF ticks, and accordingly, no larvae hatched from these eggs (**Figure 2**). To prove that haemoglobin alone, and no other component of red blood cells, is required for successful tick development, a rescue experiment was performed. From the fifth day of membrane feeding (prior to the females commencing the rapid engorgement phase), the serum diet was supplemented with 10%, 1%, or 0.1% pure bovine haemoglobin and ticks were allowed to complete feeding (S+Hb-F ticks). The presence of haemoglobin in the diet rescued the competence of embryos to develop normally and the number of larvae hatching from eggs laid by S+Hb-F ticks was comparable with BF ticks (**Figure 2**, bottom panels). The same rescue effect was observed for ticks fed on 1% and 0.1% haemoglobin (**Figure 2—figure supplement 2**) demonstrating that as little as one hundredth of the physiological concentration of haemoglobin in the diet is sufficient to maintain tick reproduction.

Haemoglobin is an indispensable source of haem, a replaceable source of amino acids, but not a source of iron for ticks

After a blood meal, the physiology of an adult female tick is dominated by its reproductive effort as up to half of the weight of a fully engorged female is used in the production of thousands of eggs (**Sonenshine and Roe, 2014**). To disclose the importance of haemoglobin in tick reproduction, we first determined haem levels in eggs obtained from both BF and SF ticks. The concentration of haem *b* (the form of haem present in haemoglobin) was determined by reverse-phase HPLC (**Figure 3A** and **Figure 3—figure supplement 1**). Eggs laid by BF ticks contained 669 ± 45 pmol haem *b*/mg eggs, whereas eggs laid by SF ticks contained virtually no haem (only 3 ± 1.6 pmol haem *b*/mg eggs). Eggs from the rescue experiment (S+Hb-F ticks) contained only slightly decreased haem levels (508 ± 79 pmol haem *b*/mg eggs) compared to BF ticks. Eggs from ticks fed with sub-physiological levels (1% and 0.1%) of haemoglobin contained gradually decreasing haem levels (471 ± 17 and

Figure 3. Determination of haemoglobin-derived nutrients in ticks (haem, amino acids, iron). (**A**) Levels of haem *b* were determined by HPLC in egg homogenates from ticks fed on whole blood (BF) serum (SF), and serum supplemented with 10%, 1% or 0.1% bovine haemoglobin (S+10%Hb, S+1%Hb and S+0.1% Hb, respectively; rescue experiments). Data (mean values ± SEM) were acquired from homogenates of three independent clutches of eggs. Representative chromatograms detecting haem *b* in egg homogenates are shown for BF ticks, SF ticks, and S+10% Hb - fed ticks, see ***Figure 3—figure supplement 1***. (**B**) Quantitative Western blot analyses detecting levels of vitellin 1 and vitellin 2 in egg homogenates using antibodies raised against vitellin precursors - vitellogenins (*Ir*Vg1, *Ir*Vg2). Bar charts depict the mean levels ± SEM of the particular vitellin in the egg homogenates from three different clutches of BF ticks or SF ticks (see ***Figure 3—figure supplement 2***). Representative Western blot detection is shown below the bar chart. (**C**) Quantitative Western blot analyses detecting ferritin1 (*Ir*Fer1) in the gut, ovary, and salivary gland homogenates from BF and SF ticks. Bar charts depict the mean ± SEM levels of *Ir*Fer1 in the tissue homogenates prepared from three independent tissue pools (see also ***Figure 3—figure supplement 2***). Representative Western blot detections for guts, ovaries and salivary glands are shown below the bar charts. (**D**) GF-AAS elemental analysis of iron in ovaries and salivary glands pools. Each data point represents a pool of five tissues dissected from BF and SF partially engorged ticks (fed for 6 days). Iron content is expressed in ppm (ng Fe per mg of dry tissue). Main and error bars indicate group means and SEM, respectively.

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The following figure supplements are available for figure 3:

Figure supplement 1. HPLC analysis of haem b in tick egg homogenates.

DOI: 10.7554/eLife.12318.013

Figure supplement 2. Full appearance of SDS-PAGE and Western blot analyses shown in the ***Figure 3***.

DOI: 10.7554/eLife.12318.014

Figure supplement 3. Detection of biliverdin IX derivatives in *Ixodes ricinus* and *Aedes aegypti*.

DOI: 10.7554/eLife.12318.015

229 ± 97 pmol haem *b* /mg eggs, respectively) (***Figure 3A***), but were still capable completing development and producing viable larvae (***Figure 2—figure supplement 2***).

Vitellins, the major tick egg yolk proteins, account for more than 90% of the protein content of a mature egg (***James and Oliver, 1997***; ***Logullo et al., 2002***). In contrast to haem concentrations, no apparent differences were observed in vitellin levels in eggs from BF and SF ticks, as determined by quantitative Western blot analysis (***Figure 3B***) with specific antibodies raised against recombinant vitellin precursors, *I. ricinus* vitellogenin 1 (*Ir*Vg1) and vitellogenin 2 (*Ir*Vg2) (***Supplementary file 2***; ***Figure 3—figure supplement 2***). This result implies that haemoglobin is replaceable by serum proteins as a nutritional source of amino acids needed for vitellogenesis.

Genome-wide analyses of *I. scapularis* and other mites revealed a common unique feature; the gene encoding haem oxygenase (HO) is missing, pointing to a lack of enzymatic degradation of haem in these Acari representatives (***Figure 1B***). HO-mediated haem degradation results in the equimolar release of iron and the linear tetrapyrrole product, biliverdin (***Khan and Quigley, 2011***). Gut homogenates from fully engorged *I. ricinus* females were analysed by HPLC for the presence of biliverdin IX (***Figure 3—figure supplement 3***). With the detection limit as low as 5 pmol, no trace of biliverdin IX or modified biliverdin showing a bilin-like light absorbance near 660nm was detected in *I. ricinus* gut homogenates. In contrast to ticks, the presence of biglutamyl biliverdin IX in whole body extracts of the blood-fed mosquito, *Aedes aegypti* (***Pereira et al., 2007***), was confirmed by our method exploiting diode-array detection (***Figure 3—figure supplement 3***). The lack of HO thus poses a question of the iron source for ticks. Iron availability in tick tissues was examined using two independent methods: (i) The presence of iron was indirectly tested by monitoring the levels of intracellular Ferritin 1 (*Ir*Fer1). Under iron deficiency, the translation of *ir-fer1*mRNA is suppressed by binding of the iron regulatory protein (IRP1) to its 5'-located iron-responsive element, whereas at high iron levels, the proteosynthesis of *Ir*Fer1 is up-regulated (***Kopáček et al., 2003***; ***Hajdusek et al., 2009***). Homogenates of guts, ovaries, and salivary glands were analysed by quantitative Western blotting using *Ir*Fer1-specific antibody (***Figure 3C*** and ***Figure 3—figure supplement 2***). *Ir*Fer1 levels were lower in guts and about equal in ovaries and salivary glands of BF compared to SF ticks (***Figure 3C*** and ***Figure 3—figure supplement 2***); (ii) The elemental iron concentration in tick tissues was determined directly by graphite furnace atomic absorption spectrometry (GF-AAS). As this method is not able to distinguish between iron of haem and non-haem origins, only salivary glands and ovaries dissected from partially engorged BF and SF ticks were used for the analysis to avoid distortions caused by the presence of haemoglobin in the samples. Despite large variations within individual biological replicates, the average iron concentration in either tissue was independent of haemoglobin in the tick diet (***Figure 3D***). These results conclusively proved that the bioavailable iron in tick tissues originates from host serum components rather than from haemoglobin-derived haem.

Haemoglobin-derived haem is transported from the gut to the ovaries

Guts dissected from partially-engorged *I. ricinus* females, and ovaries dissected 6 days after detachment (AD) from both BF and SF ticks displayed similar overall morphologies, except for colour (***Figure 4***). Accordingly, haem-containing haemosomes were not observed in the digest cells from SF ticks (***Figure 4***). Haemolymph collected from BF ticks displayed a typical haem light absorbance maximum (Soret peak) around 400 nm, which is not present in haemolymph from SF ticks (***Figure 5A***). This observation demonstrates that haem present in the haemolymph of fully engorged females originated only from the blood meal of adults, and not from previous feeding at the nymphal stage. We estimate that out of approximately 10 µmol of total haem acquired from a tick blood meal, only about 100 nmol (~1%) needs to be transported to the ovaries within a period of several days.

*Ir*CP3 is the major haem-binding protein in *I. ricinus* haemolymph

Haem inter-tissue distribution and storage is facilitated by haem-binding protein(s). In the cattle tick *R. microplus*, the most abundant haemolymph protein, named HeLp, was reported to bind haem in the haemocoel (***Maya-Monteiro et al., 2000***). The genome of *I. scapularis* contains at least five genes related to HeLp, annotated as carrier proteins (*cp1–5*). In *I. ricinus,* we identified and sequenced the *cp3* orthologue, further referred to as *ir-cp3* (GenBank KP663716). Expression

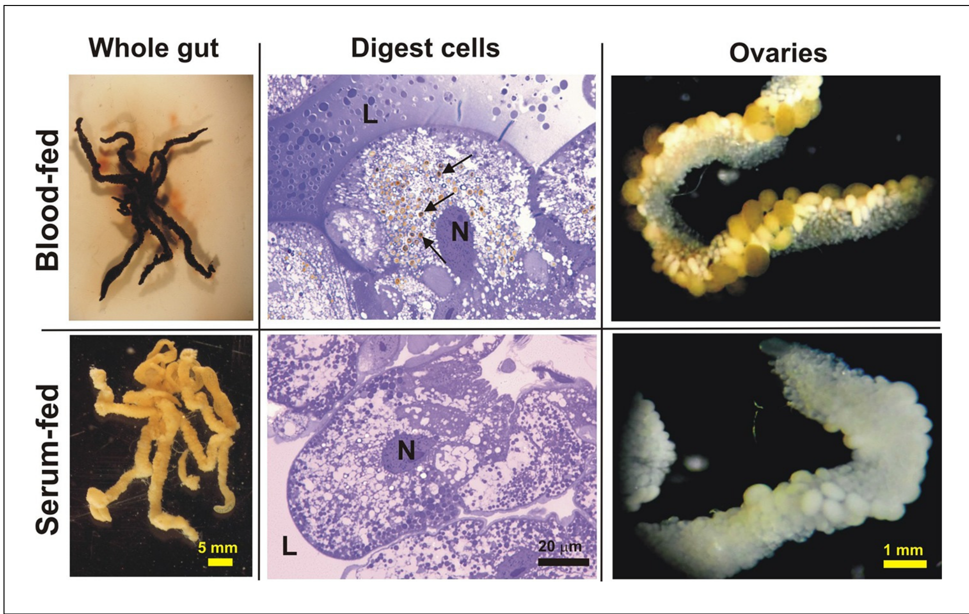

Figure 4. Appearance of the tick gut, digest cells, and ovaries from blood- and serum-fed ticks. Whole guts from blood-fed (BF) and serum-fed (SF) partially engorged females (fed for 6 days) were dissected and semi-thin sections of digest cells were prepared and stained with toluidine blue. L - lumen; N - nucleus; arrows point to developing haemosomes that were present only in digest cells of BF ticks. Ovaries were dissected from BF and SF fully engorged females 6 days after detachment from the membrane.
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profiling over *I. ricinus* developmental stages and tissues revealed that *ir-cp3* mRNA was consistently up-regulated by blood-feeding and was predominantly expressed in the trachea-fat body complex and, to a lesser extent, in salivary glands and ovaries of adult females (***Figure 5—figure supplement 1***). SDS PAGE and Western blot analysis revealed that *Ir*CP3 was most abundant in tick haemolymph (***Figure 5—figure supplement 1***), where its levels were not affected by the presence or absence of haemoglobin in the tick diet (***Figure 5B,C***). Native pore-limit PAGE, followed by detection of haem via its peroxidase activity with 3,3′-diminobenzidine (DAB), showed that haem was associated with the ~ 300 kDa band of *Ir*CP3 only in the haemolymph from BF ticks (***Figure 5C***, DAB panel). RNAi-mediated silencing of *ir-cp3* in *I. ricinus* females (*ir-cp3* KD) resulted in the disappearance of the haem Soret peak (***Figure 5D***), a substantial (~80%) reduction in *Ir*CP3 levels on SDS PAGE (***Figure 5E***), and the absence of *Ir*CP3-associated DAB stained haem on the native gel (***Figure 5F***). These results collectively demonstrate that *Ir*CP3 is the major haem-binding protein in *I. ricinus* haemolymph.

Vitellins are the major haem-binding protein in *I. ricinus* ovaries

Extracts from *I. ricinus* ovaries were colourless until the 3rd day after detachment (AD) from the host, and then the Soret peak absorbance gradually increased, indicating an increase in haem concentration up to 8 days AD (***Figure 6A***). SDS PAGE and Western blot analysis of ovary homogenates revealed that levels of *Ir*Vg1- and *Ir*Vg2-derived proteolytic products gradually increased after tick detachment whereas *Ir*CP3 remained constant (***Figure 6—figure supplement 1***). Native pore-limit PAGE followed by DAB-based haem co-detection and Western blot analyses confirmed that the appearance of haem in tick ovaries was coincident with the occurrence of vitellins (***Figure 6B***). *I. ricinus* vitellogenin genes (*ir-vg1* and *ir-vg2*) are exclusively expressed in fully engorged females, predominantly in the gut, salivary glands and trachea-fat body complex, but not in the ovaries (***Figure 6—figure supplement 2***). As vitellins are predominantly found in ovaries, their precursors (vitellogenins) must be transported from their site of synthesis to the ovaries.

RNAi-mediated silencing of *ir-vg1* and *ir-vg2* resulted in a substantial decrease in mRNA levels of both vitellogenin genes in gut tissues, and the same dual silencing effect was also observed at the protein level for *Ir*Vg1 and *Ir*Vg2 in tick ovary homogenates (***Figure 6—figure supplement 3***). This

Figure 5. *Ir*CP3 is the major haem-binding protein in *I. ricinus* haemolymph. (**A-C**) *Ir*-CP3 and haem levels in haemolymph collected from blood-fed (BF) and serum-fed (SF) partially engorged females. (**A**) Absorbance spectra of haemolymph samples from BF and SF females. (**B**) SDS-PAGE of haemolymph samples from BF and SF ticks. Protein profiles were visualized using the TGX Stain-Free technology (TGX). (**C**) Native pore-limit PAGE of heamolymph proteins stained with Coomassie (CBB) and specific co-detection of haem using peroxidase reaction with 3,3′-diaminobenzidine (DAB). (**D-F**) Effect of RNAi-mediated silencing of *ir-cp3* on the *Ir*-CP3 and haem levels in tick haemolymph. Unfed *I. ricinus* females were injected with *gfp* dsRNA (gfp, control group) or with *ir-cp3* dsRNA (*ir-cp3* KD group) and ticks were allowed to feed naturally on guinea pigs until partial engorgement (fed for 6 days). (**D**) Absorbance spectra of haemolymph samples from from g*fp* control and *ir-cp3* KD silenced ticks. (**E**) SDS-PAGE of haemolymph proteins (10 µl, 1:20 dilution) collected from g*fp* control and *ir-cp3* KD ticks. Protein profiles were stained with Coomassie (CBB). (**F**) Native pore-limit PAGE of heamolymph proteins from *gfp* control and *ir-cp3* KD ticks. Protein profiles were stained with Coomassie (CBB) and haem was co-detected using DAB.
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The following figure supplement is available for figure 5:

Figure supplement 1. Stage and tissue expression of *I. ricinus* haemolymph carrier protein (IrCP3).
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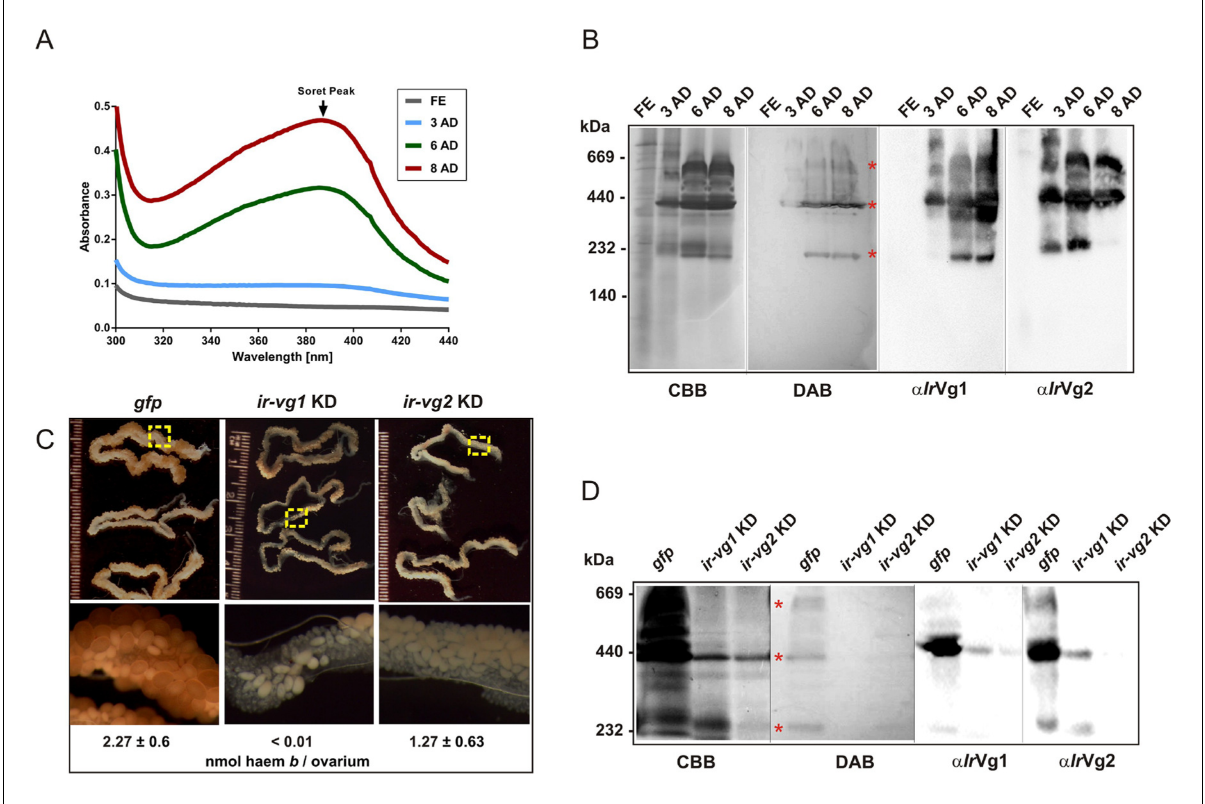

Figure 6. Vitellins are the major haem-binding proteins in tick ovaries. (**A-B**) Haem accumulation in tick ovaries occur concurrently with the appearance of vitellins. Ovaries were dissected from *I. ricinus* females at subsequent time-points after detachment (AD) from the host: FE - fully-engorged; 3 AD, 6 AD, 8 AD - 3, 6, and 8 days AD, respectively. (**A**) Absorbance spectra of ovaries homogenates show gradually increasing Soret peak following the 3rd day AD. (**B**) Native pore-limit PAGE of ovaries homogenates stained with Coomassie (CBB), co-detection of haem-associated peroxidase activity with 3,3′-diaminobenzidine (DAB), and Western blot analyses of vitellogenin 1- and vitellogenin 2- cleavage products (α*Ir*Vg1 and α*Ir*Vg2, respectively). Note that the native *Ir*Vg1- and *Ir*Vg2-specific bands correspond to the positions of the major haemoproteins in tick ovaries (red asterisks). (**C-D**) RNAi-mediated silencing of *I. ricinus* vitellogenin 1 and 2. Unfed *I. ricinus* females were pre-injected with *gfp* dsRNA (control, *gfp*), *ir-vg1* dsRNA (*ir-vg1* KD), and *ir-vg2* dsRNA (*ir-vg2* KD), allowed to feed naturally on guinea pigs and then re-injected after detachment from the host with the same amount of dsRNA. (**C**) Effect of *I. ricinus* vitellogenin 1 and 2 RNAi-mediated silencing on ovaries appearance and haem levels. Bottom panels show the detailed view of ovary parts depicted by the yellow dashed squares above. Levels of haem *b* were determined by HPLC in three independent homogenates of ovaries dissected from each tick group 6 days after detachment. (**D**) Native pore-limit PAGE of ovaries homogenates (10 μg protein per lane) dissected 6 days AD from control (*gfp*), *ir-vg1* KD and *ir-vg2* KD ticks. Gels were stained with Coomassie (CBB) for proteins, 3,3′-diaminobenzidine for peroxidase activity of haem (DAB, red asterisks), and Western blot analyses were performed with antibodies against vitellogenin 1 (α*Ir*Vg1) and vitellogenin 2 (α*Ir*Vg2).

DOI: 10.7554/eLife.12318.019

The following figure supplements are available for figure 6:

Figure supplement 1. SDS-PAGE and Western blot analyses of ovary homogenates from I. ricinus.

DOI: 10.7554/eLife.12318.020

Figure supplement 2. Stage and tissue expression of *I. ricinus* vitellogenin 1 (*Ir*Vg1) and vitellogenin 2 (*Ir*Vg2).

DOI: 10.7554/eLife.12318.021

Figure supplement 3. RNAi-mediated silencing of *I. ricinus* vitellogenin 1 and 2.

DOI: 10.7554/eLife.12318.022

result points to a mutual regulation of both genes by an as yet unknown mechanism. More importantly, silencing of both *ir-vg1* and *ir-vg2* led to impaired development of tick ovaries and concomitant reduction in haem content in this tissue (***Figure 6C***). Additionally, native pore-limit PAGE followed by DAB-staining and Western blotting (***Figure 6D***) showed that decreased levels of both *Ir*Vg1 and *Ir*Vg2 in ovary homogenates from *ir-vg1* and *ir-vg2* KD ticks were associated with the disappearance of DAB-stained haem. These results collectively show that vitellins are the major haem-

binding proteins in *I. ricinus* ovaries and imply that the majority of haem is transported, along with vitellogenins, to the developing oocytes after tick detachment from the host.

Discussion

The previous report on non-functional haem biosynthesis in the cattle tick *R. microplus* (**Braz et al., 1999**) prompted us to screen available mite genomes (namely: *I. scapularis, T. urticae*, and *M. occidentalis*) and reconstitute their gene repertoires for enzymes of the haem biosynthetic and degradative pathways. We found that 5-aminolevulinate synthase, together with the whole cytoplasmic segment of the haem biosynthetic pathway, is completely missing in hard ticks, but is present in other mites. Therefore we hypothesise that during evolution, ticks have lost most of the genes encoding haem biosynthesis as a consequence of their strict haematophagy.

Only three genes encoding the vestigial mitochondrial enzymes of the haem biosynthetic pathway, namely PPOX, CPOX and FECH, have been retained in the *I. scapularis* genome (**Gulia-Nuss et al., 2016**) (**Figure 1B**) and their orthologues were also identified in midgut and salivary gland transcriptomes of *I. ricinus* (**Kotsyfakis et al., 2015**) (**Figure 1—figure supplements 1–3**). PPOX transcripts were also found in salivary gland transcriptomes from various species of the genus *Amblyomma* (**Garcia et al., 2014**; **Karim and Ribeiro, 2015**). The same partial reduction in genomic coding for haem biosynthesis has been reported for a unicellular parasite, *Leishmania major*, in which the intracellular amastigote form expresses an active PPOX that likely sequesters the haem precursor coproporphyrinogen III from the macrophage cytosol to complete synthesis of its endogenous haem (**Zwerschke et al., 2014**). Another haem auxotroph, the nematode *Brugia malayi*, was suggested to bypass its incomplete haem biosynthetic pathway using tetrapyrrole intermediates from endosymbionts (**Wu et al., 2009**). Two lines of evidence suggest that PPOX, CPOX and FECH are not involved in haem biosynthesis in adult ticks: (i) Earlier, it was reported for *R. microplus* that no radioactively labelled δ-aminolevulinic acid was incorporated into haem present in haemolymph and ovaries (**Braz et al., 1999**); (ii) Recently, we have shown that RNAi-mediated silencing of the terminal FECH did not exert any effect on tick engorgement, oviposition, and larval hatching (**Hajdusek et al., 2016**). This data suggests that these remnants of the haem biosynthetic pathway in *I. ricinus* do not contribute to the tissue haem pool that sustains successful reproduction. Therefore the reason for retaining genes encoding the last three enzymes of the haem biosynthetic pathway in ticks remains obscure and should undergo further investigation.

The differential in vitro membrane feeding of *I. ricinus* females on whole blood (BF) or haemoglobin-free serum (SF) allowed us to investigate the importance of haemoglobin acquisition and inter-tissue transport of dietary haem in the hard tick *I. ricinus,* in an as yet unexplored way. These experiments surprisingly revealed that haemoglobin, which makes up about 70% of total blood proteins, is not a necessary source of amino acids for vitellogenesis (**Figure 2** and **Figure 3**). Moreover, we have unambiguously demonstrated that haem in tick eggs originates entirely from host haemoglobin acquired during female feeding on hosts. Serum-fed *I. ricinus* were capable of full engorgement and oviposition, however embryonal development and larval hatching was aborted (**Figure 2**). The capability of tick embryos to develop viable progeny could be fully rescued by addition as little as about 1% of the physiological concentration of haemoglobin (0.1% in serum) (**Figure 2—figure supplement 2**). In contrast to ticks, serum-fed triatomine *Rhodnius prolixus* were capable of laying eggs and giving rise to viable larvae (**Machado et al., 1998**). As *Triatominae* insects possess a complete haem biosynthetic pathway (**Kanehisa and Goto, 2000**), they can apparently reproduce even in the absence of dietary haem.

In the majority of animals studied so far (including insect blood-feeders), haem degradation represents the main source of iron, and conversely, iron is mainly utilised for *de novo* haem biosynthesis (**Zhou et al., 2007**; **Gozzelino and Soares, 2014**). Although it has been reported that, under certain conditions, haem can be degraded non-enzymatically (**Atamna and Ginsburg, 1995**), haem degradation-based on haem oxygenase (HO) is the most physiologically relevant (**Khan and Quigley, 2011**). We found that the HO gene was missing in the tick genome and correspondingly, the haem degradation product, biliverdin IX, could not be found in *I. ricinus* gut homogenates (**Figure 3—figure supplement 3**). We further noted that the absence of the HO gene is a common feature in other mite genomes (**Figure 1B**) and respective HO orthologues could not been found even in non-Acari genomes: the chelicerate genome of *Stegodyphus mimosarum* (**Sanggaard et al., 2014**) and the

myriapode genome of *Strigamia maritima* (**Chipman et al., 2014**). The apparent absence of HO transcripts in two color-polymorphic spiders of the genus *Theridion* is in agreement with the notion that these animals do not produce bilin pigments as haem degradation products (**Croucher et al., 2013**). As HO gene is present in the genomes of Hexapoda (**Adams et al., 2000**; **Holt et al., 2002**) and Crustacea (**Colbourne et al., 2011**), we hypothesise that the loss of HO is an old ancestral trait of Chelicerata and Myriapoda that are phylogenetically supported as sister groups (**Dunn et al., 2008**). Such a finding raises the question of dietary iron source for these animals, since iron is an essential electron donor/acceptor involved in vitally important physiological processes such as energy metabolism, DNA replication, and oxygen transport (**Hentze et al., 2004**; **Dunn et al., 2007**).

Earlier, we and others reported that successful tick development and reproduction is strictly dependent on the availability of iron and maintenance of its systemic homeostasis (**Hajdusek et al., 2009**; **Galay et al., 2013**). Here, we demonstrate that levels of intracellular ferritin, as an indicator of bioavailable iron, as well as the concentration of elemental iron, do not significantly differ in tick tissues dissected from BF and SF females (**Figure 3C,D**). These results further support the conclusion that bioavailable iron does not originate from haemoglobin-derived haem, but rather from serum iron-containing proteins, most likely host transferrin (**Hajdusek et al., 2009**; **Galay et al., 2014**; **Mori et al., 2014**). However, an unequivocal identification of the source(s) of bioavailable iron for tick metabolic demands has to await the implementation of a chemically defined artificial tick diet, as recently reported for the mosquito *Aedes aegypti* (**Talyuli et al., 2015**).

The entire dependence of ticks on haem derived from host haemoglobin underscores the importance of a deeper understanding of haem inter-tissue transport from the site of haemoglobin digestion in the gut to ovaries and other peripheral tissues. In the triatomine bug, *R. prolixus*, a 15-kDa haemolymphatic haem-binding protein (RHBP) was reported to transport haem to pericardial cells for detoxification and to growing oocytes for yolk granules as a source of haem for embryo development (**Walter-Nuno et al., 2013**). The haem transport and/or binding in ticks is mediated by HeLp/CPs and vitellins (**Maya-Monteiro et al., 2000**; **Logullo et al., 2002**; **Boldbaatar et al., 2010**; **Smith and Kaufman, 2014**), that belong to the family of large lipid transfer proteins (LLTP) known to facilitate distribution of hydrophobic molecules across circulatory systems of vertebrates, as well as invertebrates (**Smolenaars et al., 2007**). Vitellogenins are reported to be expressed only in fertilised fully-fed females, whereas HeLp/CPs are expressed ubiquitously in various stages, including adult males, and tissues (**Donohue et al., 2008**; **Donohue et al., 2009**; **Khalil et al., 2011**; **Smith and Kaufman, 2014**). Based on these criteria, we clearly distinguished the *I. ricinus* carrier protein *Ir*CP3 from two vitellogenins, *Ir*Vg1 and *Ir*Vg2 (**Figure 5—figure supplement 1**; **Figure 6—figure supplement 2**) and demonstrated that during tick feeding, most haem in haemolymph is bound to *Ir*CP3. The haem is mainly transported to the developing ovaries during the off-host digestive phase, however the proportion of haem transported by *Ir*CP3 or vitellogenins remains to be investigated. In ovaries, haem is sequestered by vitellins serving as haem-storage proteins for embryonal development. Further studies of the native arrangement and haem-binding capabilities of tick vitellins are needed to determine whether one or both vitellin apoproteins are involved in haem binding.

Collectively, our results demonstrate that ticks lack functional haem biosynthesis, recycle dietary haem originating from digested haemoglobin, and the acquired haem does not contribute to the cellular iron pool. Therefore, haem and iron metabolism in ticks constitute a major departure from its canonical functioning described for other eukaryotic cells, where haem and iron homeostasis is based on balancing the flux between the opposing haem biosynthetic pathway and the HO-based degradative pathway. Further investigations of the exact molecular mechanisms involved in haem inter-tissue transport, intracellular trafficking, and compartmentation within the tick digest cells, promise to identify vulnerable targets in tick haem auxotrophy. This may lead to novel strategies for controlling ticks and the diseases that they transmit.

Materials and methods

Tick maintenance and natural feeding

A pathogen-free colony of *Ixodes ricinus* was kept at 24°C and 95% humidity under a15:9-hr day/night regime. Twenty five females and males were placed into a rubber ring glued on the shaven

back of guinea pigs and ticks were allowed to feed naturally for a specified time or until full engorgement (7–9 days). Partially or fully engorged ticks were then either dissected or kept separately in glass vials until oviposition and larval hatching. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 095/2012.

Tick membrane feeding in vitro

Membrane feeding of ticks in vitro was performed in feeding units manufactured according to the procedure developed by Kröber and Guerin (**Kröber and Guerin, 2007**). Whole bovine blood was collected in a local slaughter house, manually defibrinated and supplemented immediately with sterile glucose (0.2% w/vol). To obtain serum, whole blood samples were centrifuged at 2 500 × g, for 10 min at 4˚C and the resulting supernatant was collected and centrifuged again at 10 000 × g, for 10 min at 4˚C.

Diets were then supplemented with 1 mM adenosine triphosphate (ATP) and gentamicin (5 µg/ml), pipetted into the feeding units and regularly exchanged at intervals of 12 hr. For feeding, fifteen females were placed in the feeding unit lined with a thin (80–120 µm) silicone membrane, previously pre-treated with a bovine hair extract in dichloromethane (0.5 mg of low volatile lipids) as described (**Kröber and Guerin, 2007**). After 24 hr, unattached or dead females were removed and an equal number of males were added to the remaining attached females. For rescue experiments, pure bovine haemoglobin (Sigma, St. Louis, MO, H2500) was added to the serum diet since the 5th day of feeding at a concentration of 10%, 1%, or 0.1% (w/vol) and then feeding was resumed until tick full engorgement.

Tissue dissection, haemolymph collection, and extraction of total RNA

Naturally or in vitro fed *I. ricinus* females were forcibly removed from the guinea pig or membrane at a specified time of feeding, or collected at a specified time after detachment. Haemolymph was collected into a glass capillary from the cut front leg, pooled, and used for subsequent experiments. Other tissues, namely ovaries, salivary glands, gut, tracheae with adjacent fat body cells, Malpighian tubules, and the remaining tissues tagged as 'rest' were dissected on a paraplast-filled Petri dish under a drop of DEPC-treated PBS. Total RNA was isolated from dissected tissues using a NucleoSpinRNA II kit (Macherey-Nagel, Germany) and stored at –80˚C prior to cDNA synthesis. Total RNA from haemolymph was isolated using TRI reagent (Sigma). Single-stranded cDNA was reverse-transcribed from 0.5 µg of total RNA using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany). For subsequent applications, cDNA was diluted 20 times in nuclease-free water.

Genome and transcriptome data mining

The search for tick genes encoding enzymes possibly involved in the haem biosynthetic and haem degradative pathways, a BLAST search using mosquito (*Anopheles gambiae*) genes was performed in the genome-wide database of *Ixodes scapularis* (https://www.vectorbase.org/organisms/ixodes-scapularis). Genes encoding canonical haemoproteins were identified based on their genomic annotation. Other mite genomes, namely *T. urticae* (**Grbić et al., 2011**) and *M. occidentalis*, were mined in available databases http://metazoa.ensembl.org/Tetranychus_urticae/Info/Index/ and http://www.ncbi.nlm.nih.gov/bioproject/62309, respectively. Additionally, transcriptomes available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) were screened using the BLAST® program. Metabolic pathways were reconstituted according to the Kyoto Encyclopedia of Genes and Genomes (**Kanehisa and Goto, 2000**).

Expression and purification of recombinant proteins and production of antibodies

Gene products of 1806 bp, 2070 bp, 2151 bp, and 519 bp encoding fragments of *I. ricinus* carrier protein CP3 (*ir-cp3), I. ricinus* vitellogenin 1 (*ir-vg1*), vitellogenin 2 (*ir-vg1*), and complete ferritin 1 (*ir-fer1*), respectively, were amplified from a whole body cDNA library using primers designed according to corresponding *I. scapularis* orthologues or the *ir-fer1* sequence (for primer sequences, see **Supplementary file 3**). Resulting amplicons were purified using the Gel and PCR Clean-up kit

(Macherey-Nagel), cloned into the pET100/D-TOPO vector of Champion pET directional TOPO expression kit (Invitrogen, Carlsbad, CA), and expressed using *E. coli* BL 21 Star (DE3) chemically competent cells. Expressed fusion proteins were purified from isolated inclusion bodies in the presence of 8M urea using a 5 ml HiTrap IMAC FF (GE Healthcare Bio-Sciences AB, Sweden) metal-chelating column charged with Co^{2+} - ions and eluted with an imidazole gradient. The recombinant proteins (for sequences, see ***Supplementary file 2***) were refolded by gradually decreasing the concentration of urea, finally dialyzed against 150 mM Tris/HCl, 150 mM NaCl, pH = 9.0, and used to immunize rabbits as described previously (***Grunclová et al., 2006***). The immune sera against *Ir*CP3, *Ir*Vg1, *Ir*Vg2 and *Ir*Fer1, tagged as α*Ir*CP3, α*Ir*Vg1, α*Ir*Vg2 and α*Ir*Fer1, were collected, aliquoted, and stored at –20˚C until use.

Tissue and developmental stage expression profiling by quantitative real-time PCR

cDNA preparations from developmental stages and tissues were made in independent triplicates and served as templates for the following quantitative expression analyses by quantitative real-time PCR (qPCR). Samples were analysed using a LightCycler 480 (Roche) and Fast Start Universal SYBR Green Master Kit (Roche). Each primer pair (for the list of qPCR primers, see ***Supplementary file 3***) was inspected for its specificity using melting curve analysis. Relative expressions of *ir-cp3*, *ir-vg1* and *ir-vg2* were calculated using the ΔΔCt method (***Pfaffl, 2001***). The expression profiles from adult *I. ricinus* female tick tissues were normalized to *actin* and the developmental stage expression profiles were normalized to *elongation factor 1 (ef1)* (***Nijhof et al., 2009***; ***Urbanová et al., 2014***).

RNAi

A 521-bp fragment of *ir-cp3* (corresponding to positions 2688–3208 bp, GenBank KP663716), a 301-bp fragment of *ir-vg1* (corresponding to positions 2277–2577 bp of *I. scapularis* orthologue ISCW013727), a 303-bp fragment of *ir-vg2* (corresponding to positions 801–1103 bp of *I. scapularis* orthologue ISCW021228) were amplified from tick gut cDNA and cloned into the pll10 vector with two T7 promoters in reverse orientations (***Levashina et al., 2001***), using primer pairs CP3-F_RNAi, CP3-R_RNAi (***Supplementary file 3***) containing the additional restriction sites ApaI and XbaI. dsRNA of *ir-fer1* and *ir-irp* were synthesized as described (***Hajdusek et al., 2009***). Purified linear plasmids served as templates for RNA synthesis using the MEGAscript T7 transcription kit (Ambion, Lithuania). dsRNA (~1 μg in 350 nl) was injected into the haemocoel of unfed female ticks using Nanoinject II (Drummond Scientific Company, Broomall, PA). Control ticks were injected with the same volume of *gfp* dsRNA synthesized under the same conditions from linearized plasmid pll6 (***Levashina et al., 2001***). After 24 hr of rest in a humid chamber at room temperature, ticks were allowed to feed naturally on guinea pigs. The gene silencing was verified by qPCR and/or Western blot analyses.

Reducing SDS-PAGE and Western blot

Tissue homogenates were prepared in 1% Triton X-100 in PBS supplemented with a Complete™ cocktail of protease inhibitors (Roche) using a 29G syringe, and subsequently subjected to three freeze/thaw cycles using liquid nitrogen. Proteins were then extracted for 1 hr at 4˚C and 1 200 rpm using a Thermomixer comfort (Eppendorf, Germany). Samples were then centrifuged 15 000 × g, for 10 min at 4˚C. Protein concentrations were determined using the Bradford assay (***Bradford, 1976***). Electrophoretic samples for SDS-PAGE were prepared in reducing Laemmli buffer supplemented with β-mercaptoethanol. Ten micrograms of protein were applied per lane unless otherwise specified. Proteins were separated on gradient (4–15%) Criterion TGX Stain-Free Precast gels (BioRad, Hercules, CA) in Tris-Glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/vol) SDS, pH 8.3) and visualized using TGX stain-free chemistry (BioRad). Proteins were transferred onto nitrocellulose using a Trans-Blot Turbo system (BioRad). For Western blot analyses, membranes were blocked in 3% (w/vol) non-fat skimmed milk in PBS with 0.05% Tween 20 (PBS-T), incubated in immune serum diluted in PBS-T (α*Ir*Fer1-1:50, α*Ir*Vg1-1:1 000, α*Ir*Vg2-1:1 000, α*Ir*CP3-1:1 000), and then in the goat anti-rabbit IgG-peroxidase antibody (Sigma) diluted in PBS-T (1:50 000). Signals were detected using ClarityWestern ECL substrate, visualized using a ChemiDoc MP imager, and analysed using Image Lab Software (BioRad).

Normalisation of Western blot analyses of gut homogenates were conducted using antibodies against *Ir*CP3, and homogenates of ovaries and eggs were normalised against the whole lane protein profile. Membrane stripping was carried out in a solution of 2% (w/vol) SDS and 0.5% (vol/vol) β-mercaptoethanol, and membranes were incubated for 1 hr at room temperature.

Pore-limit native PAGE and detection of haem via peroxidase activity

Tissue homogenates were prepared as described above in Tris-Borate-EDTA (TBE) buffer (0.09M Tris, 0.08M boric acid, 2mM EDTA) supplemented with a Complete^{TM} protease inhibitor cocktail (Roche). Electrophoretic samples for pore-limit native PAGE were supplemented with 10% (vol/vol) glycerol and 0.001% (w/vol) bromophenol blue. Samples were run in 4–16% Bis-Tris gel (Invitrogen) at 150 V in a cold room for 12 hr. Proteins were stained with Coomassie Brilliant Blue R-250 (CBB). For visualisation of haem-associated peroxidase activity, the gel was rinsed in water and then incubated in 100 mM sodium acetate pH 5.0 with 0.2% (w/vol) 3,3′-diaminobenzidine (DAB) and 0.05% (vol/vol) hydrogen peroxide (***McDonnel and Staehelin, 1981***). Alternatively, proteins were transferred onto nitrocellulose using a Trans-Blot Turbo system (BioRad) and used for Western blot analyses as described above.

Light absorbance

Homogenates of five ovaries were prepared as described above in 400 μl TBE buffer and briefly spun down. Haemolymph samples were diluted 1:4 in TBE. Collected faeces (10 mg) were homogenised in 100 μl of TBE buffer and briefly spun down. Supernatants from all samples were applied in a 2 μl-drop on a NanoQuant Plate (Tecan, Austria) and absorbance over the UV-VIS spectrum was scanned using the model Infinity 200 M Pro microplate reader (Tecan).

Haem *b* quantification

One dissected ovary, or 10 mg of eggs, was manually homogenised in methanol / 0.2% NH_4OH (vol/vol) and centrifuged (15 000 × g, 10 min). The supernatant was discarded and haem was extracted from the pellet in 80% acetone / 2% HCl (vol/vol). The extract was immediately separated by HPLC on a Nova-Pak C18 column (4-μm particle size, 3.9 × 75 mm; Waters, Milford, MA) using a linear gradient of 25–100% (vol/vol) acetonitrile/0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min at 40˚C. Haem *b* was detected by a diode array detector (Agilent 1200; Agilent Technologies, Santa Clara, CA) and quantified using an authentic haemin standard (Sigma, H9039).

Detection of biliverdin IX

Tick guts (wet weight ~20 mg) were dissected from naturally fed ticks 5 days after detachment from the guinea pig and homogenized individually in 100 μl of sterile PBS. For a positive control, 13 *Aedes aegypti* females were allowed to feed on mice and homogenized the 3rd day after feeding in 200 μl of sterile PBS. The samples were centrifuged (15, 000 × g, 10 min), supernatants were extracted in 80% acetone / 2% HCl (vol/vol) and separated by HPLC on a Zorbax Eclipse plus C18 column (3.5 μm particle size 4.6 x 100 mm, Agilent). A linear gradient (0–100%, 20 min) of solvent A (methanol: acetonitrile: 0.01 M sodium acetate pH 3.65; 1:1:2) and solvent B (acetonitrile / 0.1% TFA) at a flow rate of 0.6 ml/min at 40˚C was used. Biliverdin IX and haem *b* were detected simultaneously using an Agilent 1200 diode array detector at wavelengths of 660 nm and 375 nm, respectively.

Analysis of elemental iron

I. ricinus females were membrane fed on a blood or serum diet for 7 days until partial engorgement. Ovaries and salivary glands were dissected, taking special care to avoid contamination with gut contents, and washed in ultrapure 150 mM NaCl (TraceSELECT, Fluka, Switzerland). Pools of tissues, collected from 5 females, were spun down briefly to remove excess saline, and freeze-dried. The dry tissue samples were weighed on microbalances (with microgram precision) and submitted for elemental analysis using graphite furnace atomic absorption spectroscopy, kindly performed by Prof. Hendrik Küpper, Institute of Plant Molecular Biology, BC CAS, České Budějovice. The iron concentrations obtained were expressed in parts per million (ppm) related to the dry weight of tissues.

Statistics

Data were analysed by GraphPad Prism 6 for Windows, version 6.04 and an unpaired Student's t-test was used for evaluation of statistical significance.

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Author contributions

JP, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; RSo, RSi, JK, Participated in the experimental design, Approved the manuscript, Acquisition of data, Analysis and interpretation of data; DS, Participated in the experimental design, performed experiments, Approved the manuscript, Conception and design, Analysis and interpretation of data; PLdO, Analysis and interpretation of data, Drafting or revising the article; OH, Approved the manuscript, Conception and design, Acquisition of data, Analysis and interpretation of data; PK, Conception and design, Analysis and interpretation of data, Drafting or revising the article

Ethics

Animal experimentation: All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No.095/2012.

Additional files

Supplementary files

• Supplementary file 1. Prediction of genes coding for haemoproteins in the genome of *I. scapularis* and their putative function in tick metabolism.

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- Supplementary file 2. Design, sequences, and sequence similarities of recombinant Vitellogenin_N domains of IrCP3, IrVg1, and IrVg2 used for raising specific antibodies.

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- Supplementary file 3. Oligonucleotides used in this work.

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Figure supplements

Acquisition of exogenous haem is essential for tick reproduction

Jan Perner *et al*

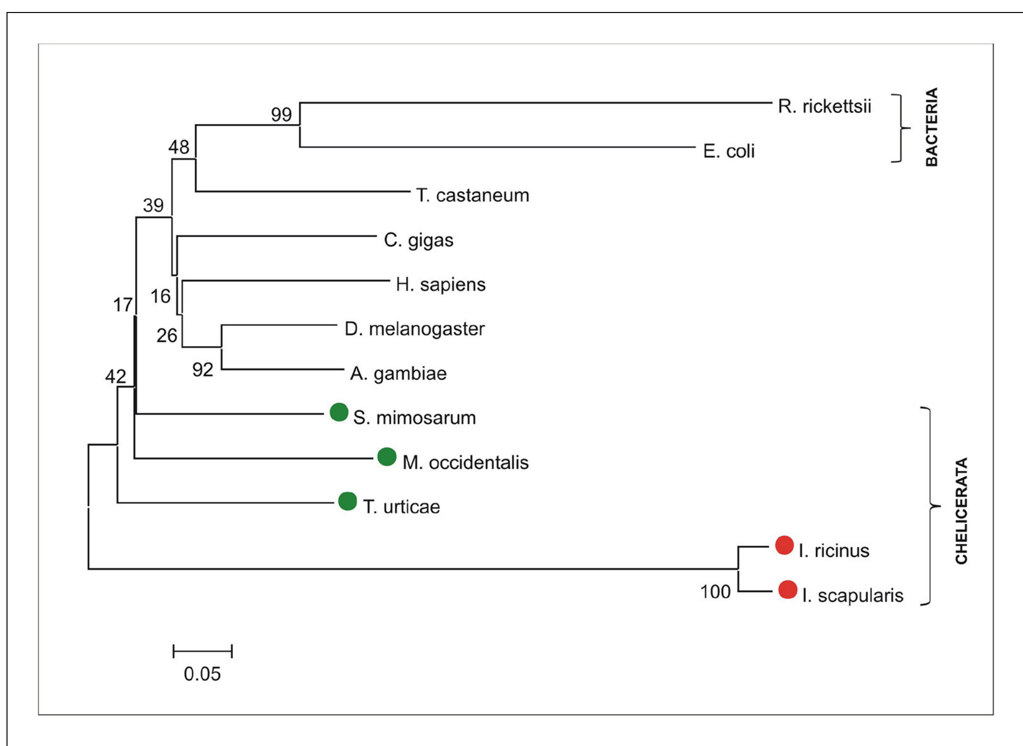

Figure 1—figure supplement 1. Phylogenetic tree of selected coproporphyrinogen-III oxidases. Unrooted tree of coproporphyrinogen-III oxidase (CPOX) amino acid sequences reconstructed using the Neighbor Joining method (NJ) based on alignment using ClustalX. The *Ixodes scapularis* and *Ixodes ricinus* CPOXs are distant from bacterial, but also from vertebrate, and invertebrate homologues, whose phylogenies cannot be clearly resolved (low bootstrap). Red dots indicate CPOX of ticks and green dots indicate CPOX of other chelicerates. Numbers at branches represent bootstrap supports using NJ criteria with 1000 replicates. The horizontal bar represents a distance of 0.05 substitutions per site. R. ricketsii (*Rickettsia rickettsii*, bacteria, WP_012151472), E. coli (*Escherichia coli*, bacteria, WP_001625620), T. castaneum (*Tribolium castaneum*, red flour beetle, XP_008201513), C. gigas (*Crassostrea gigas*, pacific oyster, EKC32626), H. sapiens (*Homo sapiens*, ENSG00000080819), D. melanogaster (D*rosophila melanogaster*, fruitfly, FBgn0021944), A. gambiae (*Anopheles gambiae*, malaria mosquito, AGAP004749), S. mimosarum (*Stegodyphus mimosarum*, social spider, KFM71890), M. occidentalis (*Metaseiulus occidentalis*, western predatory mite, XP_003744828), T. urticae (*Tetranychus urticae*, two-spotted spider mite, tetur04g09527), I. ricinus (*Ixodes ricinus*, castor been tick, JAB79008), I. scapularis (*Ixodes scapularis*, deer tick, ISCW010977).

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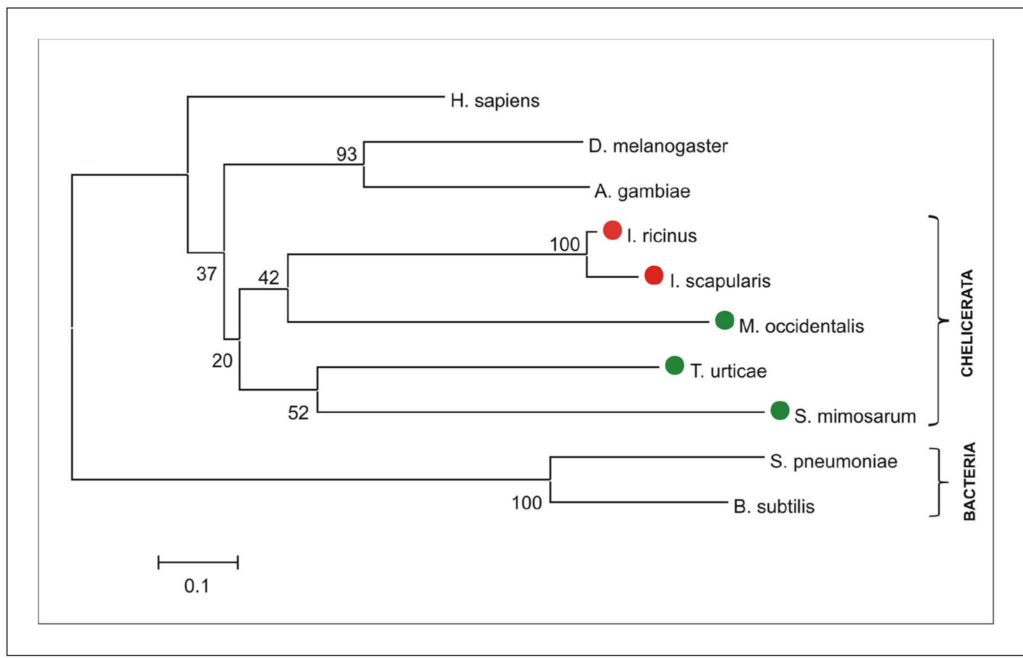

Figure 1—figure supplement 2. Phylogenetic tree of selected protoporphyrinogen oxidases. Unrooted tree of protoporphyrinogen oxidase (PPOX) amino acid sequences reconstructed using the Neighbor Joining method (NJ) based on alignment using ClustalX. The *Ixodes scapularis, Ixodes ricinus*, vertebrate, and invertebrate PPOXs, whose phylogenies cannot be clearly resolved (low bootstraps) are distant from bacterial homologues. Red dots indicate CPOX of ticks and green dots indicate CPOX of other chelicerates. Numbers at branches represent bootstrap supports using NJ criteria with 1000 replicates. The horizontal bar represents a distance of 0.1 substitutions per site. H. sapiens (*Homo sapiens*, ENSG00000143224), D. melanogaster (*Drosophila melanogaseter*, fruitfly, FBgn0020018), A. gambiae (*Anopheles gambiae*, malaria mosquito, AGAP003704), I. ricinus (*Ixodes ricinus*, castor been tick, JAB84046), I. scapularis (*Ixodes scapularis*, deer tick, ISCW023396), M. occidentalis (*Metaseiulus occidentalis*, western predatory mite, XP_003740594), T. urticae (*Tetranychus urticae*, two-spotted spider mite, tetur10g04900), S. mimosarum (*Stegodyphus mimosarum,* social spider, KFM82234), S. pneumoniae (*Streptococcus pneumoniae*, bacteria, CGG00621), B. subtilis (*Bacillus subtilis*, bacteria, WP_032725328).
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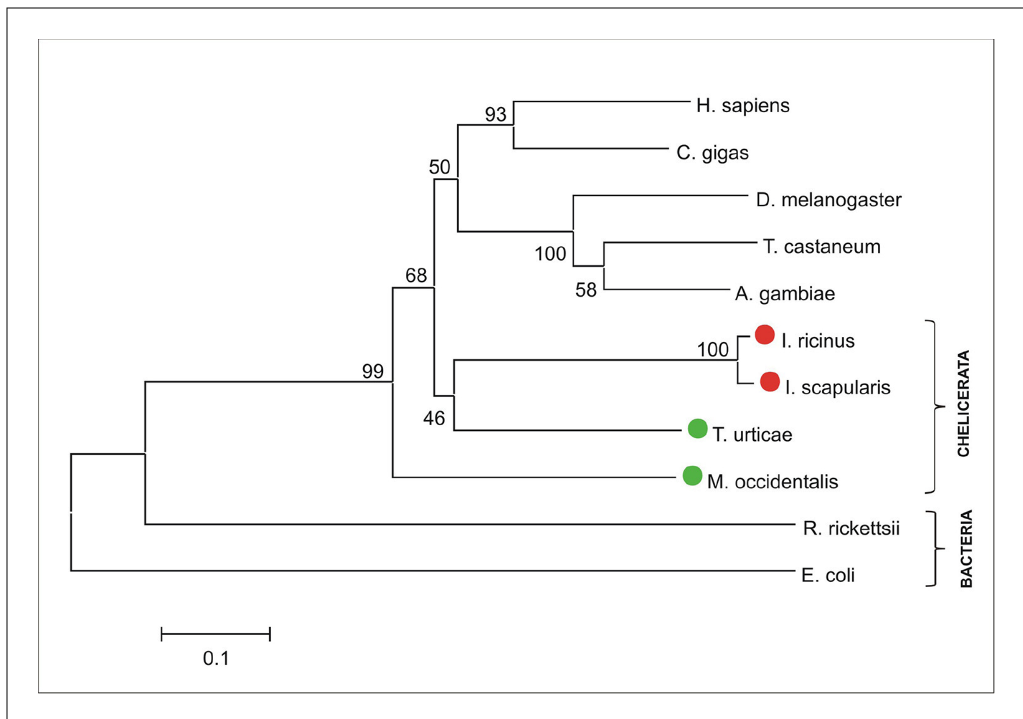

Figure 1—figure supplement 3. Phylogenetic tree of selected ferrochelatases. Unrooted tree of ferrochelatase (FECH) amino acid sequences reconstructed using the Neighbor Joining method (NJ) based on alignment using ClustalX. The *Ixodes scapularis* and *Ixodes ricinus* FECHs clusters together with other chelicerate homologues. Red dots indicate CPOX of ticks and green dots indicate CPOX of other chelicerates. Numbers at branches represent bootstrap supports using NJ criteria with 1000 replicates. The horizontal bar represents a distance of 0.1 substitutions per site. H. sapiens (*Homo sapiens*, ENSG00000066926), C. gigas (*Crassostrea gigas*, pacific oyster, EKC30122), D. melanogaster (*Drosophila melanogaster*, fruitfly, FBgn0266268), T. castaneum (*Tribolium castaneum*, red flour beetle, XP_008193416), A. gambiae (*Anopheles gambiae*, malaria mosquito, AGAP003719), I. ricinus (*Ixodes ricinus*, castor been tick, JAB74800), I. scapularis (*Ixodes scapularis*, deer tick, ISCW016187), T. urticae (*Tetranychus urticae*, two-spotted spider mite, tetur04g02210), M. occidentalis (*Metaseiulus occidentalis*, western predatory mite, XP_003748486), R. rickettsii (*Rickettsia rickettsii*, bacteria, WP_012262655), E. coli (*Escherichia coli*, bacteria, ACI87485).

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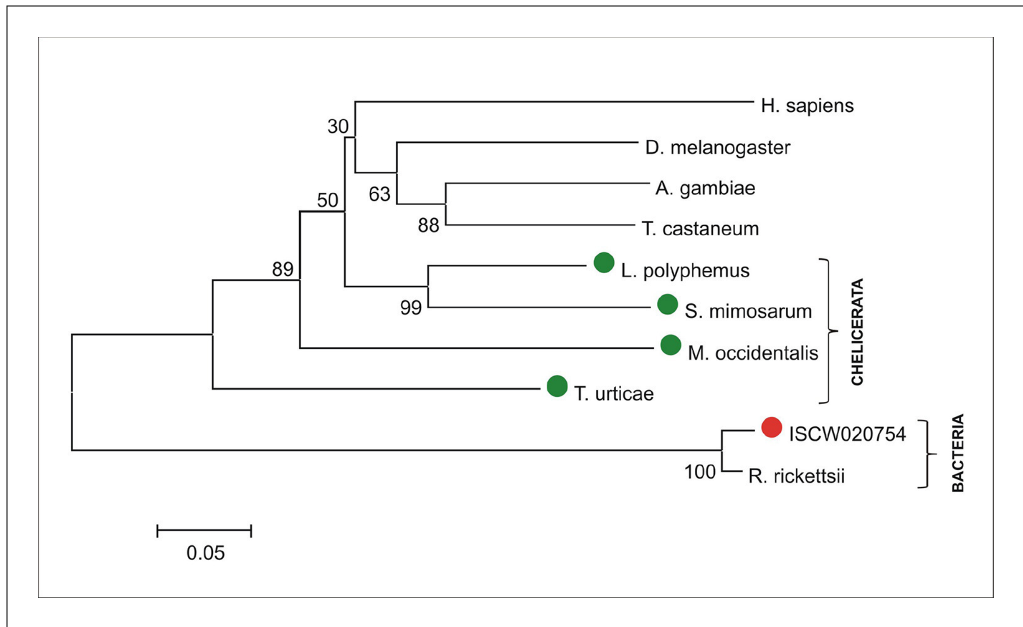

Figure 1—figure supplement 4. Phylogenetic tree of selected 5-aminolevulinate synthases. Unrooted tree of 5-aminolevulinate synthase (ALAS) amino acid sequences reconstructed using the Neighbor Joining method (NJ) based on alignment using ClustalX. The ISCW020754 annotated in the *Ixodes scapularis* genome as a putative serine palmitoyltransferase is clearly a bacterial gene, homologous to Rickettsia, symbionts of ticks. Red dot indicates ISCW020754 sequence from the tick genome, green dots indicate chelicerate ALASs. Numbers at branches represent bootstrap supports using NJ criteria with 1000 replicates. The horizontal bar represents a distance of 0.05 substitutions per site. H. sapiens (*Homo sapiens*, CAA42916), D. melanogaster (*Drosophila melanogaster*, fruitfly, CAA74915), A. gambiae (*Anopheles gambiae*, malaria mosquito, AGAP003184), T. castaneum (*Tribolium castaneum*, red flour beetle, TC013340), L. polyphemus (*Limulus polyphemus*, atlantic horseshoe crab, AAD20805), S. mimosarum (*Stegodyphus mimosarum*, social spider, KFM81891), M. occidentalis (*Metaseiulus occidentalis*, western predatory mite, XP_003744200), T. urticae (*Tetranychus urticae*, two-spotted spider mite, tetur32g00320), ISCW020754 (annotated *Ixodes scapularis* gene, deer tick, ISCW020754), R. ricketsii (*Rickettsia rickettsii*, bacteria, WP_014363330).

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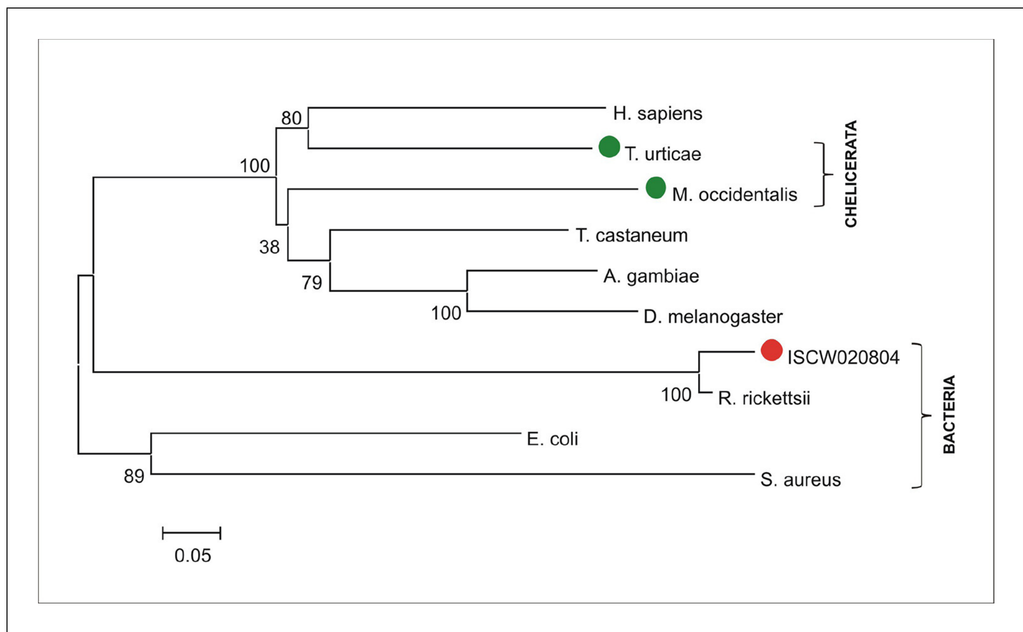

Figure 1—figure supplement 5. Phylogenetic tree of selected uroporphyrinogen decarboxylases. Unrooted tree of uroporphyrinogen decarboxylase (UROD) amino acid sequences reconstructed using the Neighbor Joining method (NJ) based on alignment using ClustalX. The ISCW020804 annotated in the *Ixodes scapularis* genome is clearly a bacterial gene homologous to Rickettsia, symbionts of ticks. Red dot indicates ISCW020804 sequence from the tick genome, green dots indicate chelicerate URODs. Numbers at branches represent bootstrap supports using NJ criteria with 1000 replicates. The horizontal bar represents a distance of 0.05 substitutions per site. H. sapiens (*Homo sapiens*, NP_000365), T. urticae (*Tetranychus urticae*, two-spotted spider mite, tetur19g03090), M. occidentalis (*Metaseiulus occidentalis*, western predatory mite, XP_003740745), T. castaneum (*Tribolium castaneum*, red flour beetle, XP_972457), A. gambiae (*Anopheles gambiae*, malaria mosquito, XP_320631), D. melanogaster (*Drosophila melanogaster*, fruitfly, ACH92415), I. scapularis (*Ixodes scapularis*, deer tick, ISCW020804), R. ricketsii (*Rickettsia rickettsii*, bacteria, WP_014362650), E. coli (*Escherichia coli*, bacteria, WP_000137647), S. aureus (*Staphylococcus aureus,* bacteria, KLN00580).
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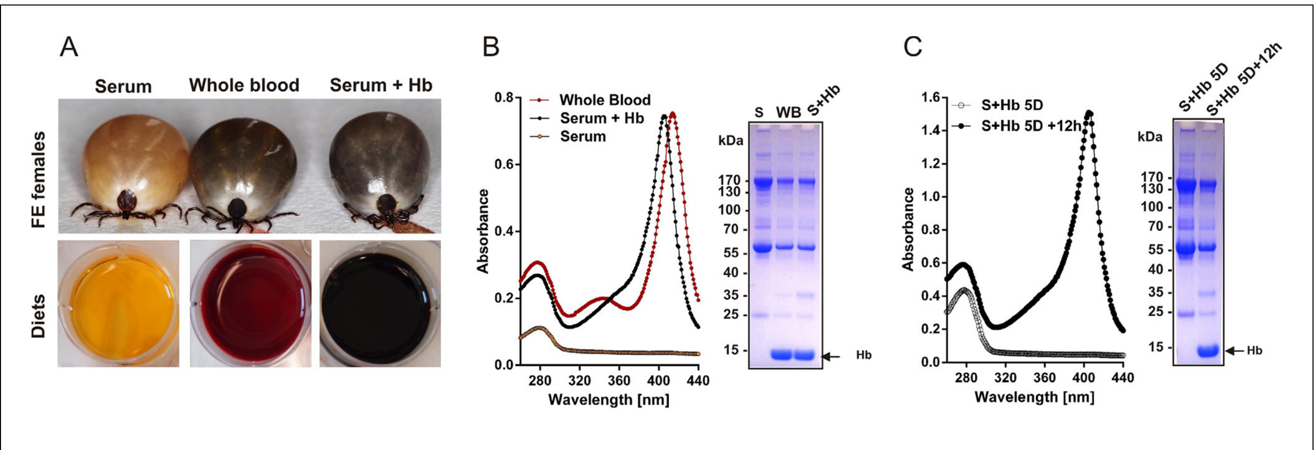

Figure 2—figure supplement 1. Diets used for tick membrane feeding and faecal examination. (**A**) Females of *I. ricinus* were membrane fed until full engorgement (FE) using whole blood, serum, and serum supplemented with 10% (physiological concentration) of pure bovine haemoglobin (Serum +Hb). (**B**) Composition of diets. Equal levels of haemoglobin in whole blood and reconstituted Serum+Hb were verified by spectrophotometry (absorbance at ~ 400 nm - Soret peak) and by SDS-PAGE of diets (arrow points to haemoglobin band). (**C**) Faecal examination. To ensure complete passage of Hb through the digestive tract, faeces were inspected 12 hr after serum supplementation with Hb. Examination of faecal extracts by spectrophotometry (absorbance at ~ 400 nm - Soret peak) and by SDS PAGE confirmed the availability of supplemented Hb before ticks commence a rapid engorgement phase ('big sip'). Note that the protein profile of faeces was almost identical to that of the applied meal.
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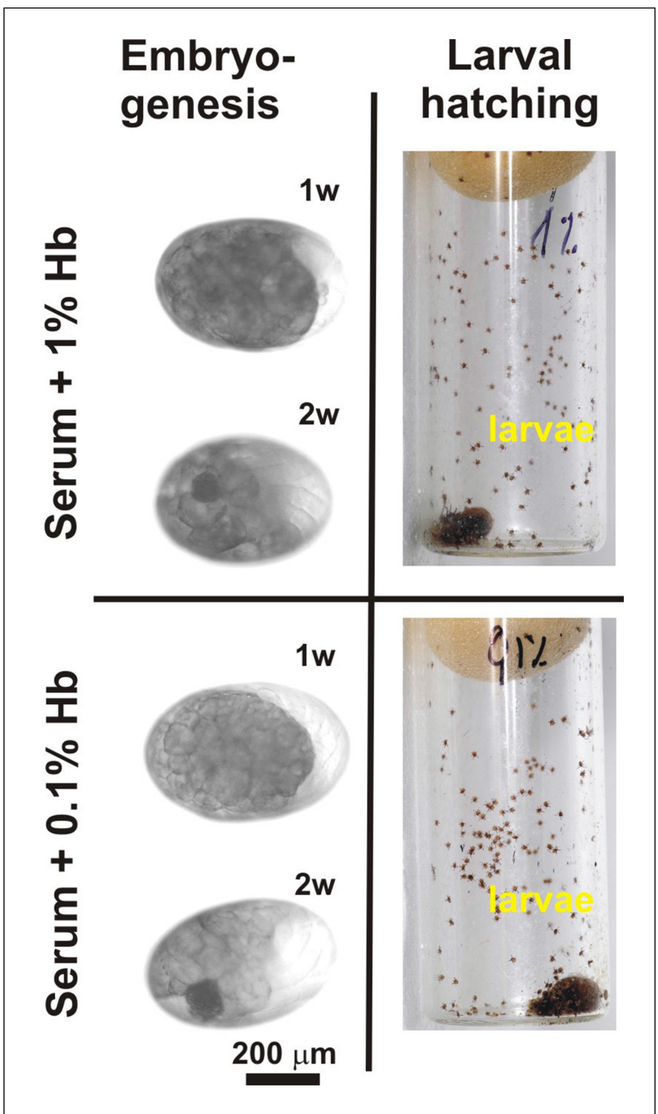

Figure 2—figure supplement 2. Rescue experiments with sub-physiological levels of haemoglobin. Embryonal development and larval hatching was fully rescued in *I. ricinus* females fed on serum supplemented with 1% or 0.1% bovine haemoglobin. (**Embryogenesis**) – microscopic examination of embryonal development in eggs laid by differentially fed females; 1w, 2w – 1 week, 2 weeks after oviposition, respectively. (**Larval hatching**) – Laid eggs were incubated to allow larval hatching. Note that tick reproduction was not affected if only one hundredth of the natural haemoglobin concentration was present in the tick diet.

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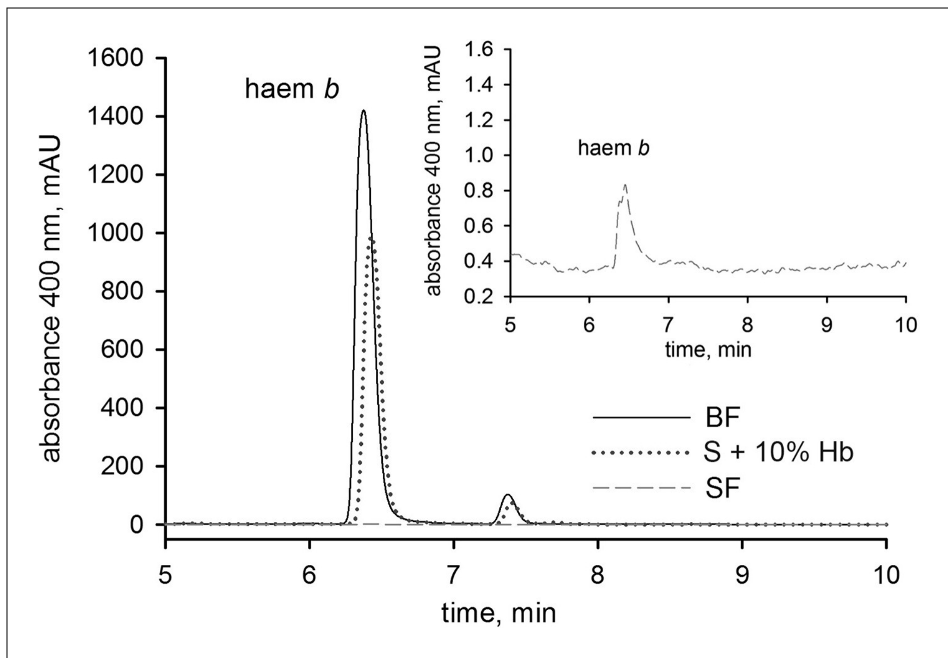

Figure 3—figure supplement 1. HPLC analysis of haem b in tick egg homogenates. Homogenates prepared from 10 mg of eggs were collected from three independent egg clutches laid by *I. ricinus* females fed on different diets. Representative chromatograms are shown detecting haem *b* in egg homogenates of ticks fed on the whole blood (BF) and haemoglobin-free serum (SF) and serum supplemented with 10% of haemoglobin (S+10% Hb). The inset shows the zoom-in of haem *b* detection in SF ticks; note the different y-axis scales. For details, see Material and methods.

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Figure 3—figure supplement 2. Full appearance of SDS-PAGE and Western blot analyses shown in the ***Figure 3***. SDS-PAGE analyses were carried out on homogenates prepared from three independent tissue preparations from ticks fed on whole blood (BF) or serum (SF). Protein profiles were visualized using the TGX Stain-Free technology (TGX) and the BioRad ChemiDoc MP imager. (**A**) SDS-PAGE of homogenates of freshly laid eggs, and Western blot analyses detecting levels of vitellin 1 (Vn1) and vitellin 2 (Vn2) using specific antibodies against vitellogenin 1 (α*Ir*Vg1) and vitellogenin 2 (α*Ir*Vg2). (**B**) Control SDS-PAGE (upper panel) and corresponding Western blot (lower panel) for identification of ferritin 1 (Fer1) by RNAi. Western blot analysis of Fer1 levels was performed using specific antibodies against recombinant *I. ricinus* ferritin1 (α*Ir*Fer1). No cross-reacting band was present in the

Figure 3—figure supplement 2 continued on next page

Figure 3—figure supplement 2 continued

whole blood or serum diet. The Fer1-specific band was clearly present in gut homogenates from naturally fed ticks, pre-injected with *gfp* dsRNA (*gfp*) but completely absent in ticks pre-injected with *ferritin 1* dsRNA (*fer1*-KD). RNAi-mediated silencing of iron-regulatory protein (*irp*-KD) caused a marked Fer1 up-regulation. (**C–E**) SDS-PAGE (upper panels) and corresponding Western blots (lower panels) used for quantification of Fer1 levels in tissue homogenates dissected from partially engorged BF and SF ticks (fed for 6 days).

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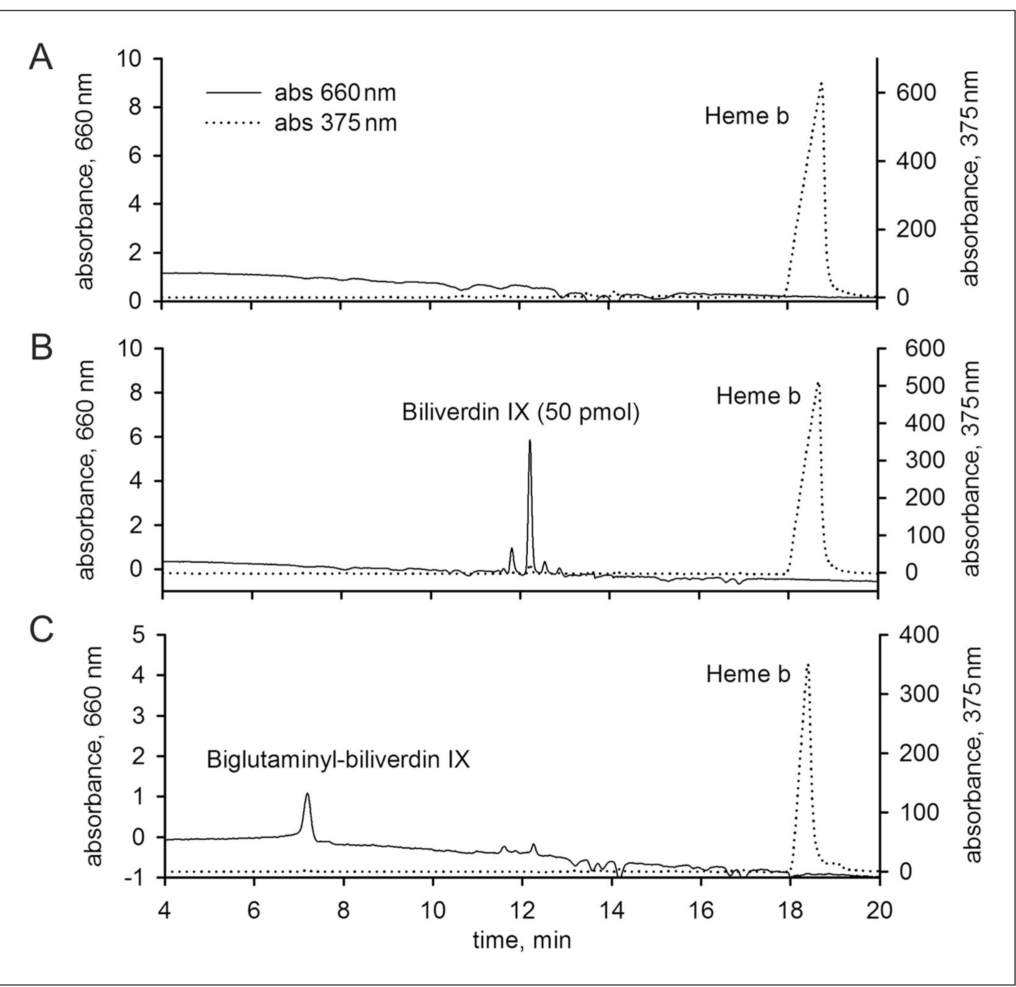

Figure 3—figure supplement 3. Detection of biliverdin IX derivatives in *Ixodes ricinus* and *Aedes aegypti*. The HPLC using a diode array detector was set to enable a simultaneous determination of haem *b* and biliverdin IX compounds at wavelengths of 375 nm and 660 nm, respectively. For details, see Materials and methods. (**A**) *I. ricinus* gut extracts from fully engorged females 5 days after detachment from the host. (**B**) *I. ricinus* gut extracts from fully engorged females 5 days after detachment from the host and spiked with 50 pmol of biliverdin IX standard. (**C**) Whole body extracts from naturally fed *Aedes aegypti* mosquitoes allowed to digest blood for three days were used as a positive control. The presence of biglutaminyl-biliverdin IX as a haem *b* degradation product (***Pereira et al., 2007***) was detected.

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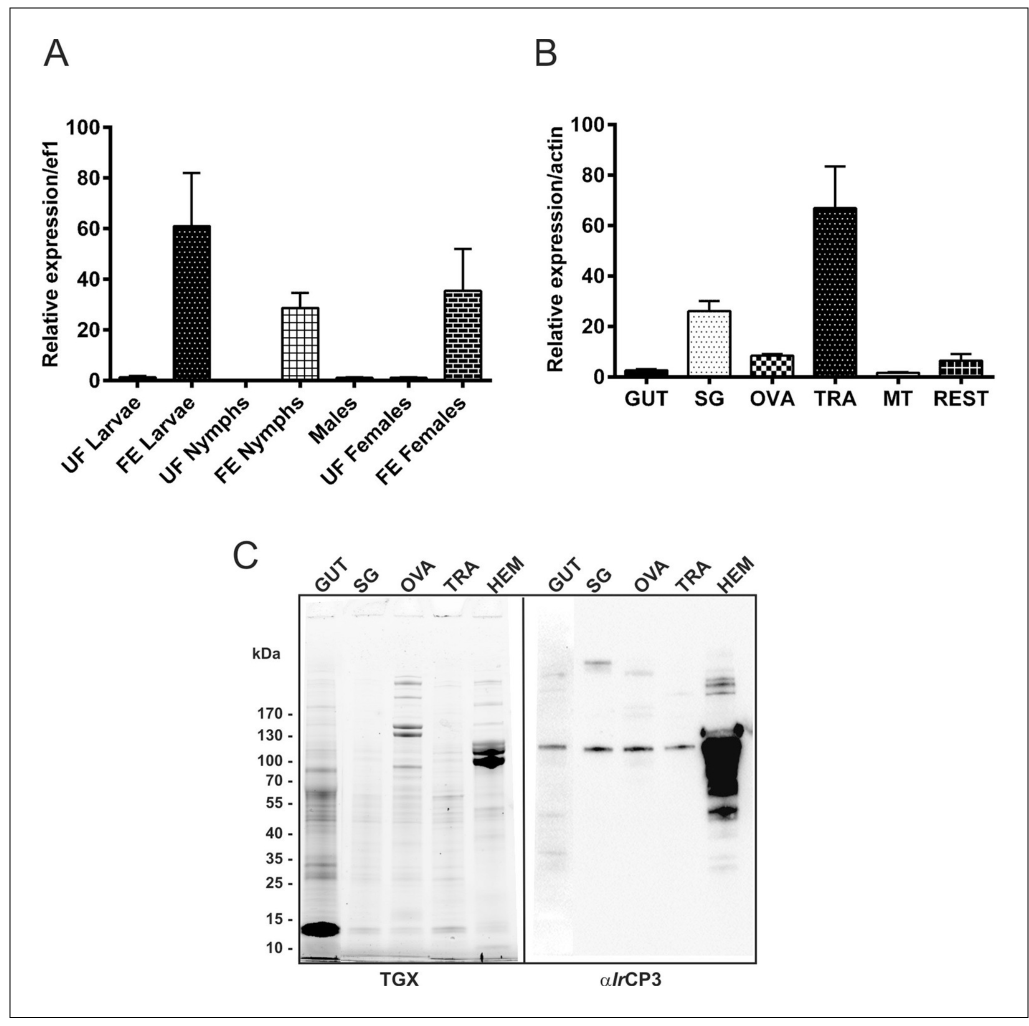

Figure 5—figure supplement 1. Stage and tissue expression of *I. ricinus* haemolymph carrier protein (IrCP3). (**A**) qPCR analyses of *ir-cp3* expression in developmental stages of *I. ricinus*. (**B**) qPCR analyses of *ir-cp3* expression in tissues dissected from fully engorged females. Data were obtained from three independent cDNA sets, and normalised to *elongation factor 1 (ef1*) or *actin*. UF - unfed; FE - fully engorged; SG - salivary glands, OVA - ovaries; TRA - trachea-fat body complex; MT - Malpighian tubules; REST - remaining tissues. (**C**) SDS-PAGE separation of tissues dissected from *I. ricinus* females 6 days after detachment, visualized using the TGX Stain-Free technology (TGX), and corresponding Western blot analyses of *Ir*CP3 detected with specific antibodies (α*Ir*CP3). SG - salivary glands, OVA - ovaries; TRA - trachea-fat body complex; HEM - haemolymph. Gut homogenate (50 µg of protein) or other tissue homogenates (10 µg of protein) were loaded per lane.
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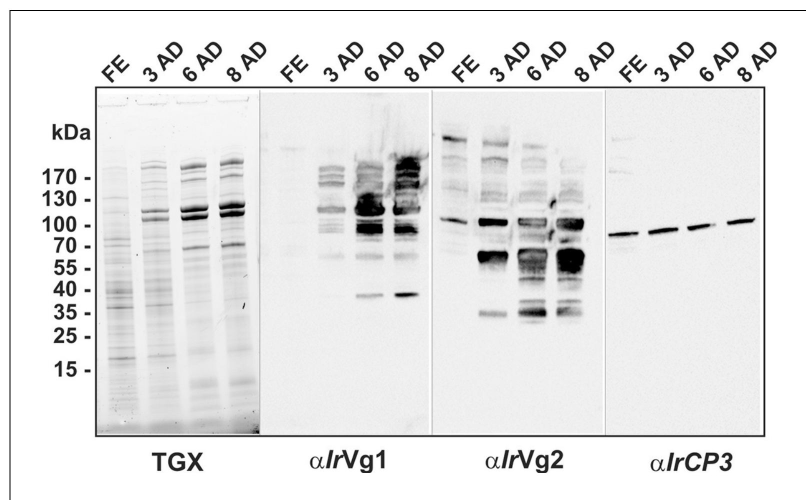

Figure 6—figure supplement 1. SDS-PAGE and Western blot analyses of ovary homogenates from I. ricinus. Ovaries were dissected from *I. ricinus* females at subsequent time-points after detachment (AD) from the host: FE - fully-engorged; 3 AD, 6 AD, 8 AD - 3, 6, and 8 days AD, respectively. Protein profiles of ovaries homogenates were visualised using TGX Stain-Free technology (TGX) and corresponding Western blots of vitellogenin 1-, vitellogenin 2-derived cleavage products and *Ir*CP3 were detected using α*Ir*Vg1, α*Ir*Vg2, and α*Ir*CP3 specific antibodies, respectively. Note the gradual increase in both vitellins but a constant level of *Ir*-CP3.
DOI: 10.7554/eLife.12318.020

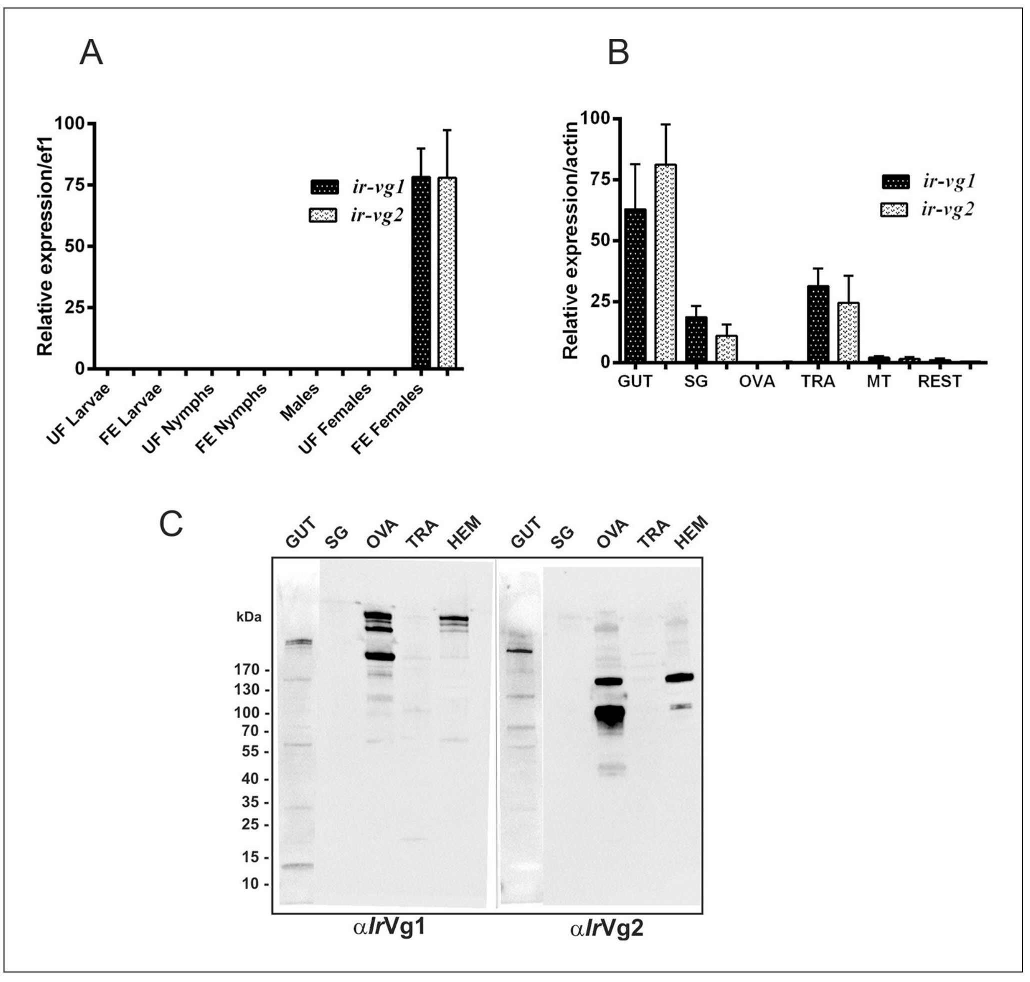

Figure 6—figure supplement 2. Stage and tissue expression of *I. ricinus* vitellogenin 1 (*Ir*Vg1) and vitellogenin 2 (*Ir*Vg2). Stage and tissue expression of *I. ricinus* vitellogenin 1 (*Ir*Vg1) and vitellogenin 2 (*Ir*Vg2). (**A**) qPCR analyses of *ir-vg1* and *ir-vg2* expression in developmental stages of *I. ricinus*. (**B**) qPCR analyses of *ir-vg1* and *ir-vg2* expression in tissues dissected from ticks 4 days after detachment. Data were obtained from three independent cDNA sets, and normalized to *elongation factor 1 (ef1)* or *actin*. UF - unfed; FE - fully engorged; SG - salivary glands, OVA - ovaries; TRA - trachea-fat body complex; MT - Malpighian tubules; REST - remaining tissues. (**C**) Western blot analyses of *Ir*Vg1, and *Ir*Vg2 detected with specific antibodies (α*Ir*Vg1), and (α*Ir*Vg2), respectively. SG - salivary glands, OVA - ovaries; TRA - trachea-fat body complex; HEM - haemolymph. Gut homogenate (50 µg of protein) or other tissue homogenates (10 µg of protein) were loaded per lane.

DOI: 10.7554/eLife.12318.021

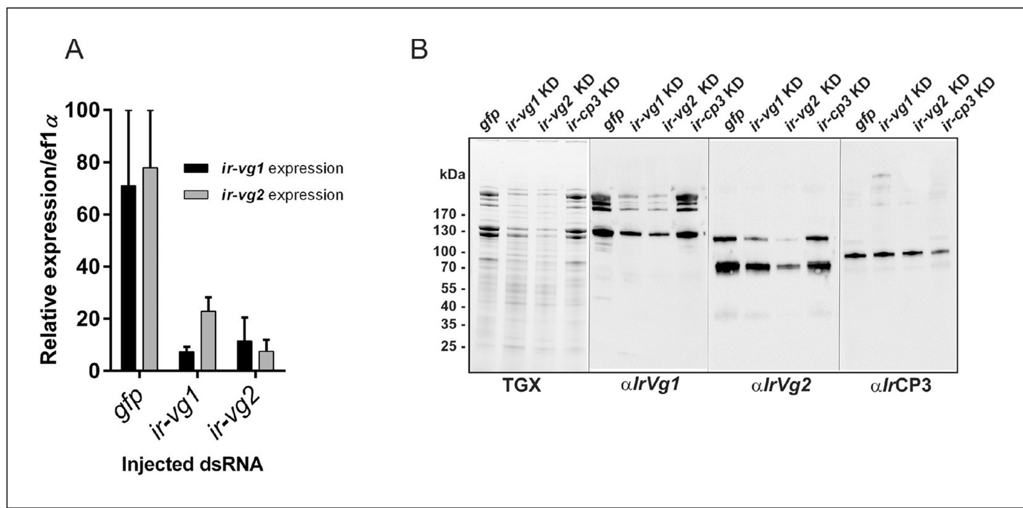

Figure 6—figure supplement 3. RNAi-mediated silencing of *I. ricinus* vitellogenin 1 and 2. Unfed *I. ricinus* females were pre-injected with *gfp* dsRNA (*gfp*), *ir-vg1* dsRNA (*ir-vg1* KD), *ir-vg2* dsRNA (*ir-vg2* KD), and *ir-cp3* dsRNA (*ir-cp3* KD), allowed to feed naturally on guinea pigs and re-injected immediately after detachment (AD) with the same amount of dsRNA. Tissues were dissected 6 days AD. (**A**) qPCR analysis of *ir-vg1* and *ir-vg2* gene expression in the tick gut upon RNAi-mediated silencing. Note the mutual co-silencing of both genes. (**B**) SDS-PAGE protein profiles and corresponding Western blot analyses of ovary homogenates dissected from *gfp, ir-vg1* KD, *ir-vg2* KD, and *ir-cp3* KD ticks. Proteins were visualized using the TGX Stain-Free technology (TGX), and Western blots of *Ir*Vg1, *Ir*Vg2, and *Ir*CP3 were detected with specific antibodies (α*Ir*Vg1), (α*Ir*Vg2), and (α*Ir*CP3), respectively. Note the mutual co-silencing of *Ir*Vg1 and *Ir*Vg2 at the protein level.
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Unpublished data to Paper I

Distribution and storage of maternal haem in ticks

Unpublished data to Perner et al., 2016, eLife 5:e12318

Distribution and storage of maternal haem in ticks

Haem levels in tick haemolymph throughout and after feeding

We have shown that Soret peak of haem absorbance increases in ovaries homogenates only after tick drop off. Whether this is caused by selective import or simply by availability of haem transporter in the haemolymph was examined. Using VIS-absorbance, we detected increased Soret abosrbence in haemolymph of the dropped-off ticks, indicating that haem dedicated for transportation into ovaries is available in the haemolymph only after tick drop-off, indirectly suggesting that haem is imported with its carries once it is available in the haemolymph (Fig. 1).

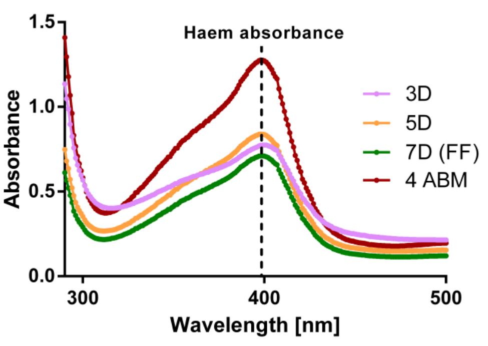

Figure 1. VIS-absorbance of *I. ricinus* haemolymph of indicated days (D) of feeding or after blood meal (ABM). Soret peak of haem absorbance indicates an increase in haem levels in tick haemolymph after tick drop-off.

Presence of vitellins in tick haemolymph throughout and after feeding

To test whether vitellogenins appear in the haemolymph also only after drop off, we have subjected tick haemolymph, taken from different stages of feeding and after drop-off, to reducing SDS-PAGE and Western blot analyses. We have confirmed that both vitellogenins (*Ir*Vg1 and *Ir*Vg2) appear in the haemolymph only after tick drop off, not exluding vitellogenins as potential haem transporters from midgut into ovaries (Fig. 2).

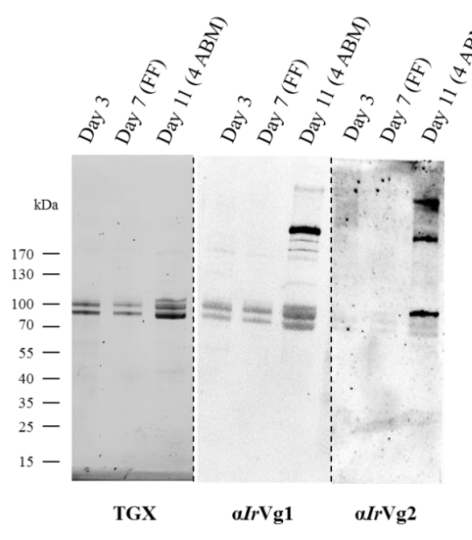

Figure 2. SDS-PAGE and Western blot of *I. ricinus* haemolymph of indicated days of feeding or after blood meal (ABM). Protein profiles were visualised using TGX Stain-Free technology (TGX) and corresponding Western blots were detected using αIrVg1, αIrVg2 antibodies.

Composition of haemoprotein vitellin complex

To further evidence, how gene silencing efects the haemoprotein complexes in egg homogenates, we subjected the egg homogenates, of given RNAi-silenced ticks, to selectively-stained native-PAGEs. These electrophoretic separations were then blotted and subjected to Western blot analyses. To see what individual proteins participate in complex formation, we have sliced out individual bands and ran them on reducing SDS-PAGE with subsequent Western blot analyses. We have revealed that the predominant haemoprotein complex of about 400 kDa is formed of *Ir*Vg1 and *Ir*Vg2. While *Ir*Vg2 seems to be required for complex dimer formation, *Ir*Vg1 monomer is suficient for haem-binding (Fig. 3, band 6), while *Ir*Vg2 monomer is not (Fig. 3, band 5). It indicates that the vitellogenin haemprotein complex is a heterodimer with only *Ir*Vg1 having a haem-binding capacity.

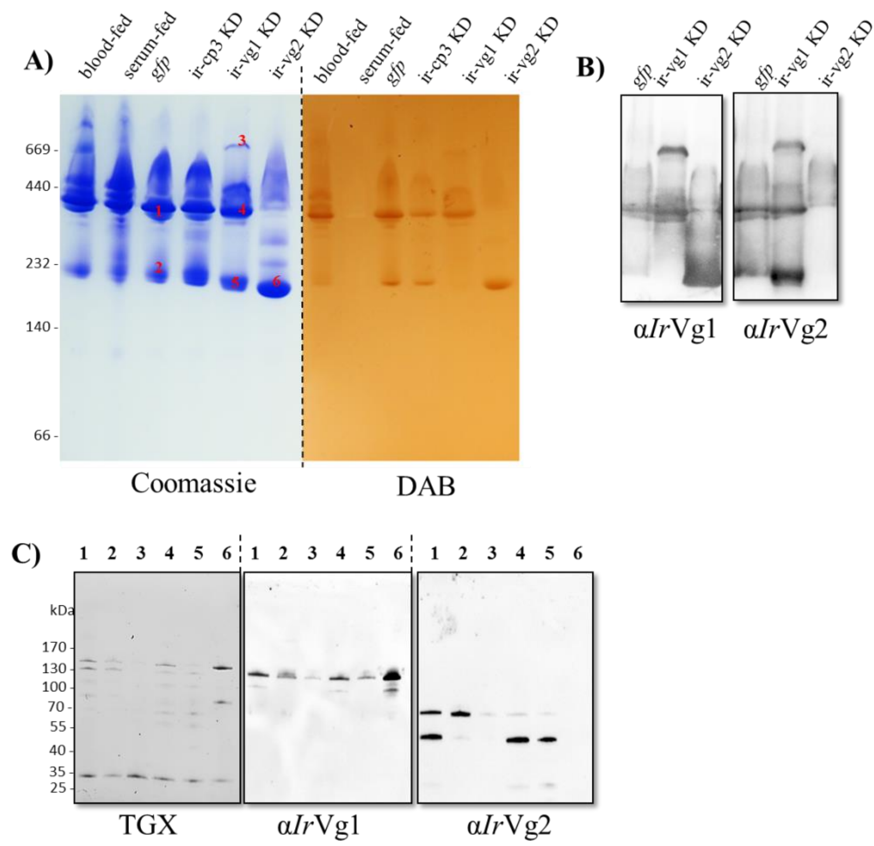

Figure 3. Native PAGE and Western blot of egg homogenates of artificially-fed or RNAi-silenced ticks. A) Native pore-limit PAGE of egg homogenates stained with Coomassie, co-detection of haem-associated peroxidase activity with 3,3′-diaminobenzidine (DAB). **B)** Western blot analyses of tick vitellins, vitellogenin 1- and vitellogenin 2- cleavage products (α*Ir*Vg1 and α*Ir*Vg2, respectively). **C)** Reducing SDS-PAGE and Western blot analyses of excised bands. Protein profiles were visualised using TGX Stain-Free technology (TGX) and corresponding Western blots were detected using αIrVg1, αIrVg2 antibodies. KD – knocked-down ticks by RNAi interence of genes: carrier protein 3 (*cp3*), vitellogenin 1 (*vg1*), and vitellogenin 2 (*vg2*).

Manuscript in preparation I

Perner J, Hatalová T, Kučera M, Kopáček P.

Impact of serum-feeding on development of *Ixodes ricinus* nymphs.

Impact of serum-feeding on development of *Ixodes ricinus* nymphs

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Abstract

Ticks have lost the endogenous haem biosynthetic pathway during evolution. Instead of biosynthesis, ticks acquire haem from host haemoglobin and recycle it as a prosthetic group needed for their endogenous haemoproteins. Reduced levels of dietary haemoglobin led to aborted larvae hatching suggesting a critical involvement of host haem in the embryogenesis of ticks. In contrast, host haemoglobin could be fully replaced by plasma proteins in the provision of amino acids. The role of host haemoglobin has been studied in the adult stages of the European Lyme disease vector *Ixodes ricinus* as of yet. We have developed a system suitable for artificial feeding of *I. ricinus* nymphs that allows us studying nutritional dependence of *I. ricinus* nymphal stages. Nymphs are capable feeding on bovine serum and molt into adult stages. These adults can fully engorge and lay viable eggs when blood-fed. RT-qPCR analyses of midgut transcripts implicated in adaptations to blood-feeding of adult stages display haemoglobin-responsive expression also in tick nymphal stages.

Introduction

Ixodes ricinus ticks are vectors of borreliosis in Europe. Adult *I. ricinus* ticks were shown to be directly dependent on acquisition of host haemoglobin-derived haem (Perner et al., 2016b). Failure of inter-tissue distribution of haem after tick-drop off aborts embryogenesis and leads to oviposition of infertile eggs. Interference of such haem transportation mechanism hinders tick reproduction and may be utilised to reduce tick populations in restricted areas.

Nymphal stages of *I. ricinus* ticks are notorious for pathogen transmission in humans. However, very little is known as to what molecules they need to acquire from the host so they can successfully feed, transmit pathogens, or molt into adult stage. In the present study, we elucidated the impact of dietary haem on nymphal feeding capabilities as well as of the molted adults. We noted that, unlike adult females, tick nymphs do not require haem in order to feed or develop into adults. Interstadial haem(oglobin) deposits are also not required for further adult feeding.

A comparative RNAseq study quantifying transcripts in the midgut of blood- and serum-fed *I. ricinus* adult females identified several transcripts responsive to presence of dietary red blood cells. We have tested, by RT-qPCR, the expression change of the adult differentially expressed transcripts on RNA isolates from blood- and serum-fed nymphs. We revealed that, also in nymphs, transcripts encoding glutathione S-transferase, sulfotransferase, and peritrophic matrix chitin-binding protein were up-regulated in blood-fed nymphs, while transcripts encoding cytochrome P450 and phospholipid hydroperoxide glutathione peroxidase were up-regulated in serum-fed nymphs.

Results and Discussion

Membrane blood- and serum-feeding of *I. ricinus* nymphs

Artificial feeding system of *Ixodes ricinus* adult stages has been recently exploited to study tick nutritional requirements(Perner et al., 2016b). However, it was not possible to feed nymphs using this system (Kröber and Guerin, 2007). Therefore we had to optimise this system so that nymphs are attracted to attach to the membrane, pierce it, and finish the feeding on artificial diet. Even though we managed to achieve a feeding success for *I. ricinus* adults to be 70 % and 62 % for blood-feeding and serum-feeding, respectively (Perner et al., 2016b), the feeding success of nymphal stages was lower, namely 25 % and 45% for blood and serum, respectively (Fig. 1). Even so, the high number of nymphs fitting the feeding unit enables acquisition of more data in shorter period of time despite the smaller feeding success rate. While the adult *I. ricinus* ticks take on average about 10 days to finish the feeding, more than 50% of *I. ricinus* nymphs finish feeding up to day 6 (Fig. 1).

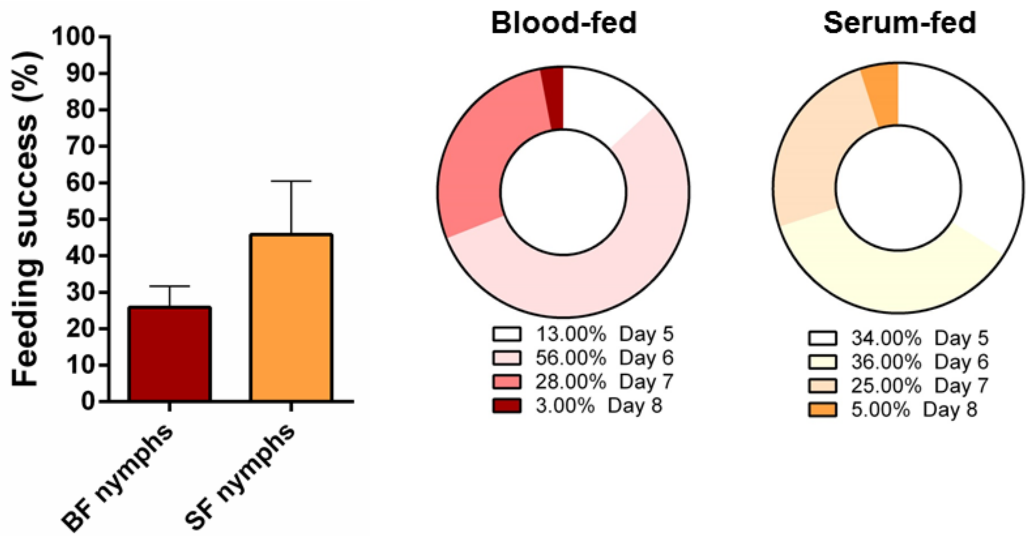

Figure 1. Feeding success of *Ixodes ricinus* nymphs. The bar graph represents feeding success of all *I. ricinus* nymphs. Mean and SEM are shown. Pie charts depict the percentage representation of a given day for *I. ricinus* nymphs detachment after full engorgement.

Molting of blood- and serum-feeding of *I. ricinus* nymphs

Unlike adult *I. ricinus* ticks where only females imbibe host blood, both *I. ricinus* nymphal males and females feed naturally host blood. The achieved weights display sex-specific pattern with female nymphs feeding into around 4 mg and male nymphs feeding into nearly half of female size (Fig. S1). Both female and male nymphs managed to finish blood- and serum-feeding (Fig. 2). Fed nymphs achieved comparable weights irrespective of served diets (Fig. S1). Blood-fed nymphs (*n*Blood-fed) and serum-fed nymphs (*n*Serum-fed) were then allowed to molt into adults. Both *n*Blood-fed and *n*Serum-fed nymphs managed to molt into adult females and males without any apparent developmental aberration. However, in both adult males and females midgut tissues lack typical coloration caused by haem(oglobin) deposits (Fig. 2). This fact enabled us to further study the role of host haem, deposited from previous stage feeding, on the physiology of adult *I. ricinus* ticks.

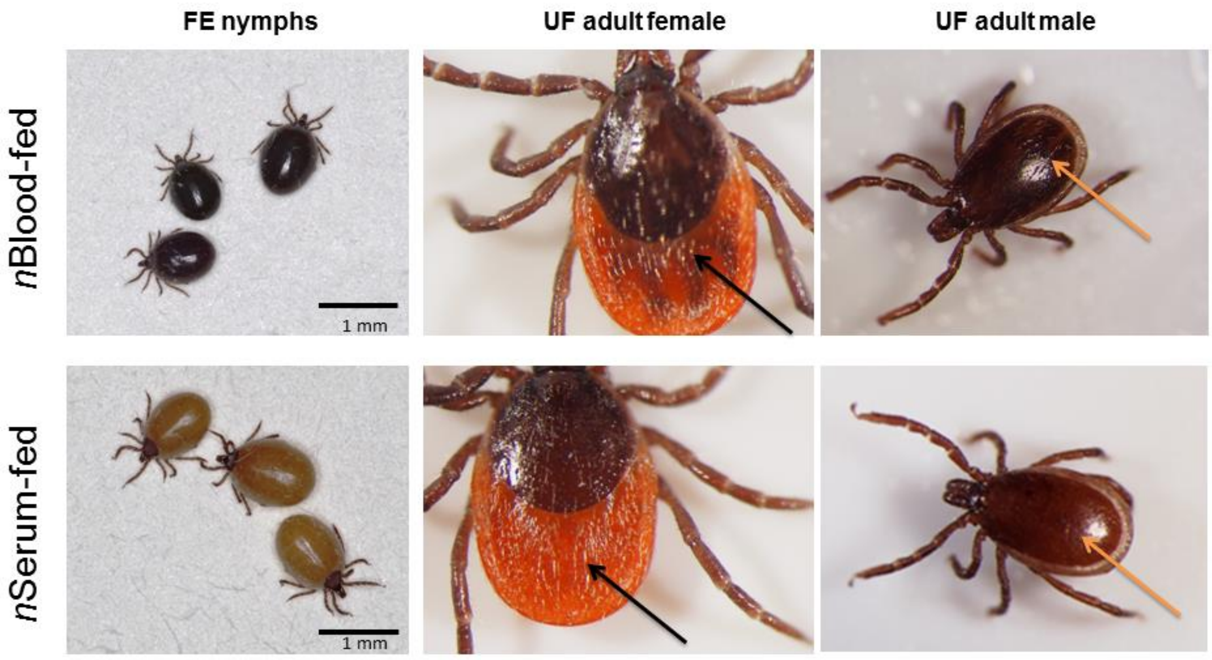

Figure 2. Molting of *I. ricinus* nymphs. Fully engorged blood-fed nymphs (*n*Blood-fed) and serum-fed nymphs (*n*Serum-fed) developed into adult females and males (middle and right panels, respectively). Arrows point to haem(oglobin) rich or poor midgut tissue.

Haem midgut deposits are not required for following natural feeding of adult *I. ricinus* ticks

To test whether absence of host haemoglobin in nymphal stage feeding interferes with the feeding and reproducing capabilities of adult ticks, we have fed *n*Blood-fed and *n*Serum-fed ticks naturally on guinea pigs. We have noted that *n*Serum-fed ticks are able to attach to a host and progress in feeding similarly as *n*Blood-fed ticks (Fig. 3A). Even though unfed adult males do not differ in weight irrespective of the way of nymphal feeding (artificially blood-fed, artificially serum-fed, wild collected), females coming from artificial feeding of nymphs weighted less that those collected in the wild (Fig. S2). However, unfed adults of *n*Blood-fed and *n*Serum-fed ticks did not differ (Fig. S3). Upon engorgement, however, *n*Blood-fed ticks weighted more than *n*Serum-fed ticks (Fig. 3B), indicating a possible role of haem in inter-stage development of ticks.

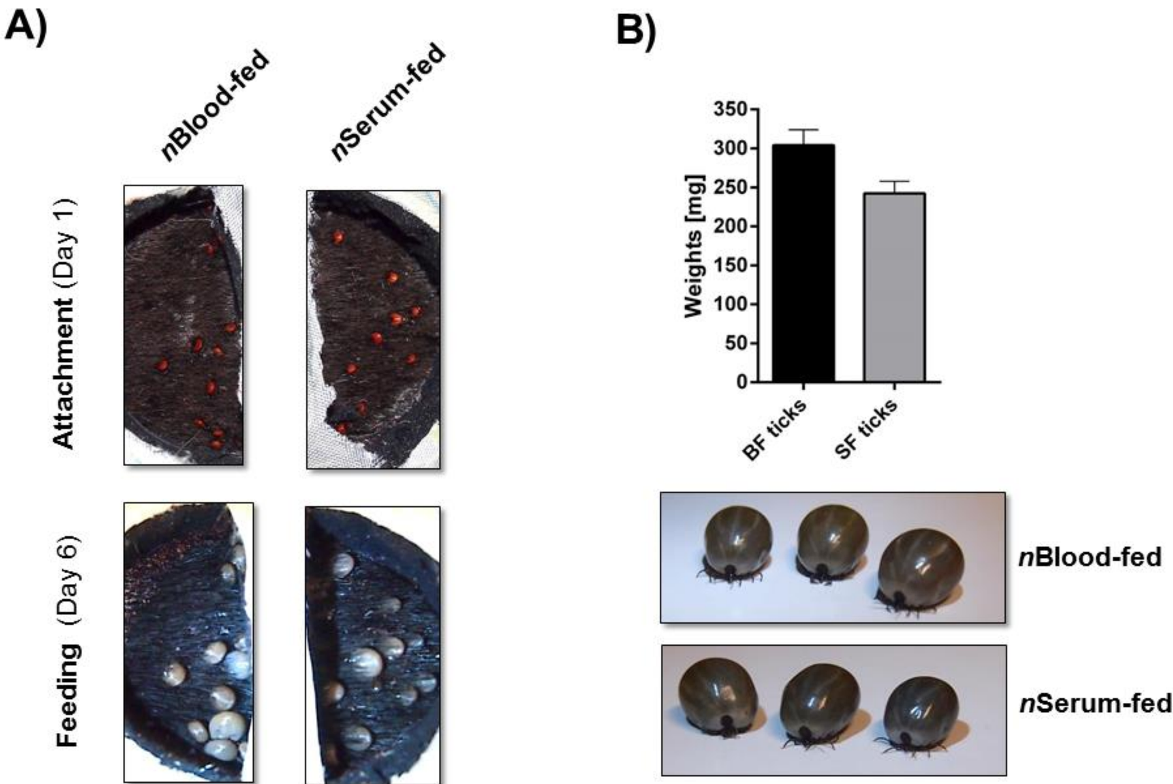

Figure 3. Natural feeding of adult originating from blood- and serum-fed nymphs. Blood-fed nymphs (*n*Blood-fed) and serum-fed nymphs (*n*Serum-fed) developed into adult females. Resulting unfed ticks were allowed to feed naturally on guinea pigs. **A)** Ticks were tested for capability of feeding initiation (top panel) and feeding progression (bottom panel). **B)** The bar graph represents the weights of fully engorged adults originating from *n*Blood-fed and *n*Serum-fed ticks. Mean and SEM are shown. Three representative fully engorged adult females are shown.

Reproduction of adult ticks originating from blood- and serum-fed *I. ricinus* nymphs

Adult ticks acquire haem upon host haemoglobin hydrolysis in tick midgut and transport it into ovaries to enable tick embryogenesis and larvae hatching (Perner et al., 2016b). To test whether this system is developed irrespective of absence of dietary haemoglobin during nymphal stage, we have let the *n*Blood-fed and *n*Serum-fed naturally fed ticks to lay eggs (Fig. 4). Both *n*Blood-fed and *n*Serum-fed ticks managed to lay eggs with *n*Blood-fed tick egg clutches being slightly bigger than those of *n*Serum-fed ticks (Fig. S3). We have reported that serum feeding of adult *I. ricinus* stages led to aborted embryogenesis caused by the lack of haem molecules deposited in laid eggs (Perner et al., 2016b). Using UV-Vis absorbance, we have quantified haem in egg homogenates of the *n*Blood-fed and *n*Serum-fed naturally fed ticks. Irrespective of nymphal fed diet, both *n*Blood-fed and *n*Serum-fed ticks managed to deposit nearly nmol of haem in mg of eggs (Fig. S4). Using light microscopy, eggs were inspected one and two weeks after being laid and typical embryonic formation was observed (Fig. 4). Accordingly, viable larvae were hatched out of these clutches (Fig. 4).

Figure 4. Oviposition, embryogenesis, and larvae hatching in adults originating from blood- and serum-fed nymphs. Blood-fed nymphs (*n*Blood-fed) and serum-fed nymphs (*n*Serum-fed) developed into adult females. Resulting unfed ticks were allowed to engorge and lay eggs (left panel). Using light microscopy, eggs were inspected for embryogenesis (middle panels). Fraction of hatched viable larvae is shown in left panels.

Transcript levels in response to dietary haemoglobin in midgut of blood- and serum-fed *I. ricinus* nymphs

Using RNA-seq, we have recently identified contigs differentially expressed in blood- and serum-fed adult *I. ricinus* ticks (Perner et al., 2016a). To test whether these genes are also differentially expressed in the midgut of fully engorged blood- and serum-fed nymphs, we have analysed the levels of the respective transcripts by RT-qPCR. We have revealed that the five selected genes are upregulated in similar fashion as in the midgut of adult blood- and serum-fed ticks with cytochrome p450 and phospholipid hydroperoxide glutathione peroxidase being up-regulated by serum-feeding, while glutathione S-transferase, sulfotransferase, and peritrophic matrix chitin-binding protein being up-regulated by blood-feeding (Fig. 5A). These data correlate well together (Fig. 5B) and indicate the encoded proteins of these genes participate in the processes evolved to enable the blood-feeding habit of ticks.

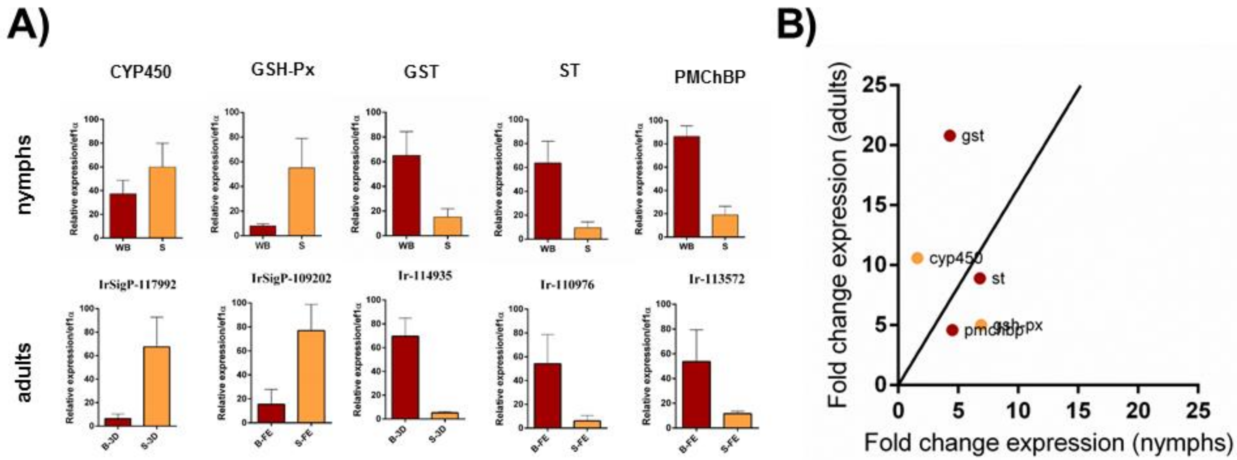

Figure 5. RT-qPCR analyses of haemoglobin responsive genes from the gut of fully engorged nymphs. A) The bar graph represents relative expression values of selected genes in fully engorged *I. ricinus* nymphs (top panel). Mean and SEM are shown, n = 3 (biological replicates). Relative expression values for *I. ricinus* adults are extracted from (Perner et al., 2016a). **B)** Correlation plot of individual relative expression means for selected genes and contigs. CYP450 - cytochrome p450, GSH-Px - phospholipid hydroperoxide glutathione peroxidase, GST - glutathione S-transferase, ST - sulfotransferase, PMChBP - peritrophic matrix chitin-binding protein.

Material and Methods

Tick maintenance and natural feeding

These ticks were kept at 24 °C and 95% humidity under a 15:9-hour day/night regime. Adult *I. ricinus* were also collected in the wild near Ceske Budejovice. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 357 095/2012. The study was approved by the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (CAS) and Central Committee for Animal Welfare, Czech Republic (protocol no. 1/2015).

Adult tick membrane feeding

Membrane feeding of ticks *in vitro* was performed in feeding units manufactured according to the procedure developed by Kröber and Guerin (Kröber and Guerin, 2007). Whole bovine blood was collected in a local slaughter house, manually defibrinated and supplemented immediately with sterile glucose (0.2% w/vol). To obtain serum, whole blood samples were centrifuged at 2 500 × g, for 10 min at 4°C and the resulting supernatant was collected and centrifuged again at 10 000 × g, for 10 min at 4°C. Diets were then supplemented with 1 mM adenosine triphosphate (ATP) and gentamicin (5 μg/ml), pipetted into the feeding units and regularly exchanged at intervals of 12 hr. For feeding, fifteen females were placed in the feeding unit lined with a thin (80–120 μm) silicone membrane, previously pre-treated with a bovine hair extract in dichloromethane (0.5 mg of low volatile lipids) as described (Kröber and Guerin, 2007). After 24 hr, unattached or dead females were removed and an equal number of males were added to the remaining attached females.

Nymphal tick membrane feeding

Silicone membrane is spread on a specific mash that enables even and thin membrane formation. Feeding unit is pre-initiated by non-defined adult females attractants. Diets were then supplemented with 1 mM adenosine triphosphate (ATP) and gentamicin (5 μg/ml), pipetted into the feeding units and regularly exchanged at intervals of 12 hr. For feeding, thirty nymphs were placed in the feeding unit lined with a thin (< 80 μm) silicone membrane, previously pre-treated with a bovine hair extract in dichloromethane (0.5 mg of low volatile lipids) as described (Kröber and Guerin, 2007).

Tissue dissection, extraction of total RNA, and cDNA synthesis

In vitro fed *I. ricinus* nymphs were collected from feeding units upon completion their feeding. Nymphal midguts were dissected on a paraplast-filled Petri dish under a drop of DEPC-treated PBS. Total RNA was isolated from dissected tissues using a NucleoSpinRNA II kit (Macherey-Nagel, Germany) and stored at –80°C prior to cDNA synthesis. Single-stranded cDNA was reverse-transcribed from 0.5 μg of total RNA

using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany). For subsequent applications, cDNA was diluted 20 times in nuclease-free water.

Reverse Transcription - quantitative PCR (RT-qPCR)

cDNA preparations from midguts were made in independent triplicates and served as templates for subsequent quantitative expression analyses by RT-qPCR. Samples were analysed using a LightCycler 480 (Roche) and Fast Start Universal SYBR Green Master Kit (Roche). Each primer pair was inspected for its specificity using melting curve analysis. Relative expressions were calculated using the ΔΔCt method. The expression profiles from adult *I. ricinus* nymphal midguts were normalised to elongation factor (*ef-1α*).

Haem quantification

Collected tick eggs (10 mg) were placed in 1.5 ml plastic Eppendorf tube and homogenised in 50 μl distilled H_2O using a plastic pestle. Ice cold basic methanol (950 μl) was added into the homogenate and centrifuged at 10 000 × g for 2 minutes at 4 °C. Supernatant (basic methanol) was carefully discarded without disturbing the pellet. One millilitre of acid acetone was added into plastic tube to extract haem by shaking at room temperature for 10 minutes. After extraction, the plastic tube was centrifuged at 16 000 × g for 2 minutes at 4 °C. Haem was detected at 385 nm in quartz 1 ml cell using the UV-Vis spectrophotometer (Shimadzu) and quantified using an authentic haemin standard (Sigma, H9039).

Supplementary data

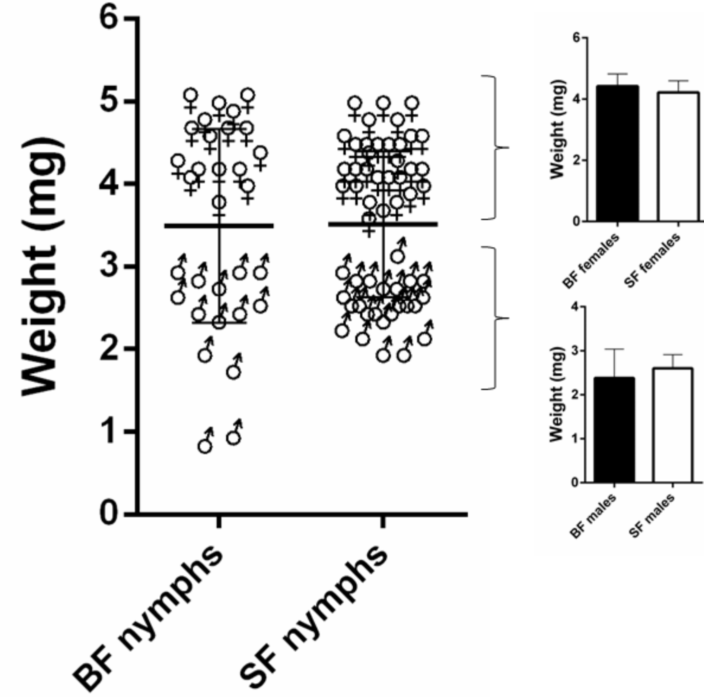

Figure S1. Weights of fully engorged *Ixodes ricinus* nymphs. The point graph represents weights of all *I. ricinus* fully engorged nymphs. Symbols represent expected sex of each nymph. Bar represents a mean of the dataset. Bar graphs depict comparison of fully engorged nymphs of a given sex. Mean and SEM are shown.

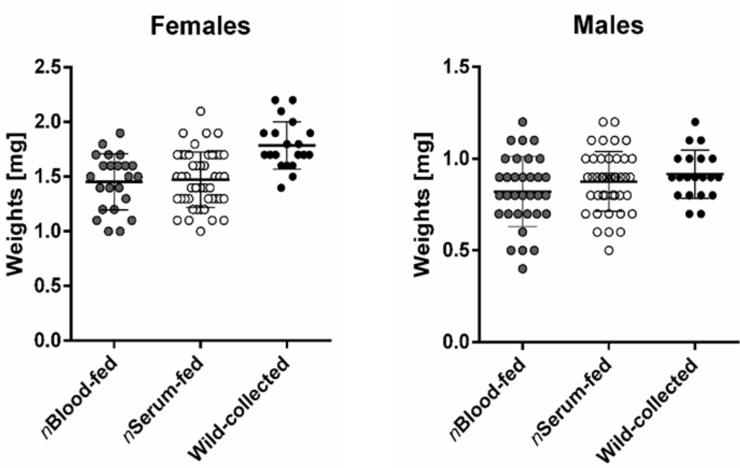

Figure S2. Weights of unfed *Ixodes ricinus* adults molted from artificially and naturally fed nymphs. The point graphs represent weights of individual unfed *I. ricinus* adults molted from nymphs fed artificially either bovine blood (*n*Blood-fed) or serum (*n*Serum-fed), or from nymphs collected in the wild and fed naturally on guinea pigs. Mean bars and SEM are shown.

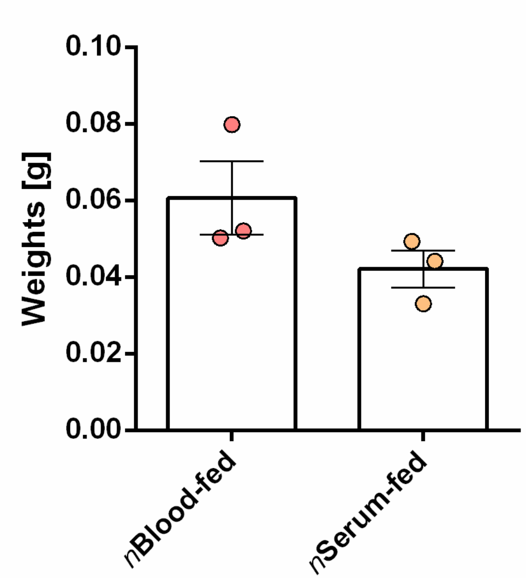

Figure S3. Weights of egg clutches from naturally fed *Ixodes ricinus* adults molted from artificially fed nymphs. The point graphs represent weights of individual egg clutches of *I. ricinus* adults molted from nymphs fed artificially either bovine blood (*n*Blood-fed) or serum (*n*Serum-fed). Adults were fed naturally on guinea pigs. Mean and SEM are shown.

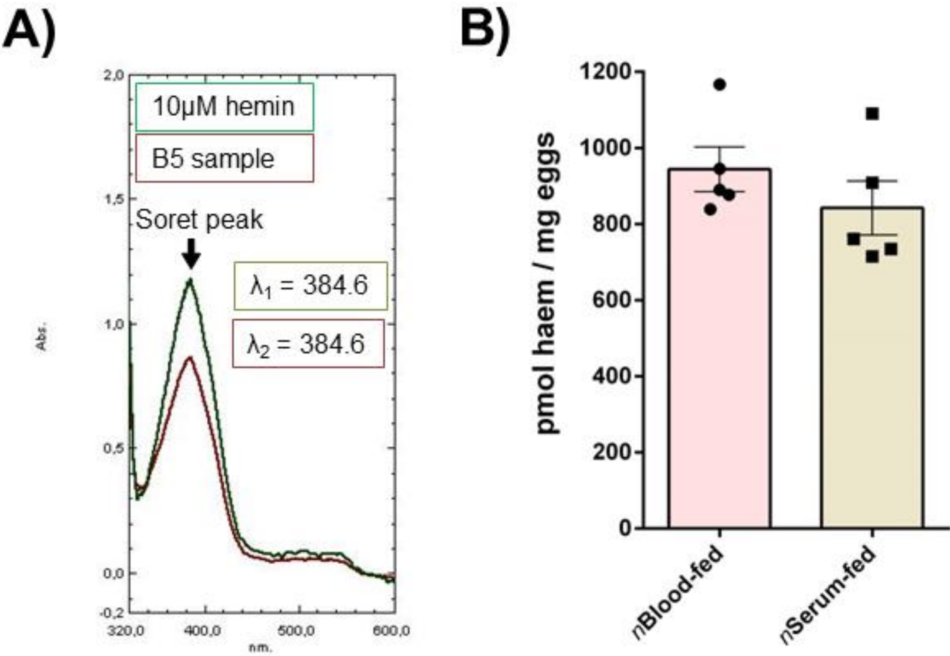

Figure S4. Quantification of haem in tick egg homogenates. A) UV-Vis absorbance spectrum of commercial haemin standard and a representative extract of egg homogenate. **B)** Normalisation of acquired haem amounts per mg of tick eggs, n = 5 (independent tick egg clutches).

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Acknowledgements

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Part II

Molecular adaptations to haemoglobin digestion in ticks

Paper II

Perner J, Provazník J, Schrenková J, Urbanová V, Ribeiro JM, Kopáček P (2016)
RNA-seq analyses of the midgut from blood- and serum-fed *Ixodes ricinus* ticks.
Sci. Rep. 6, 36695.

Manuscript in preparation II

Perner J, Hatalová T, Brophy P, Kopáček P.
GST as a putative endogenous haem-binder in tick gut.

Preface

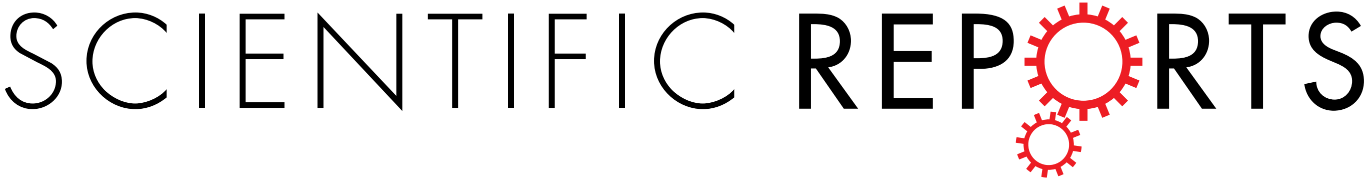
Single tick RNA-seq analyses were conducted to explore blood-feeding adaptations of ticks. This trasncriptomic project should have provided an insight into what protein-encoding transcripts are expressed, in red blood cells-responsive manner, that might facilitate haem acquisition, detoxification, and further dispatch from the midgut. We compared midgut transcriptomes of blood- and serum-fed adult *I. ricinus* females dissected either 3 days or 8 days (full engorgement) after feeding initiation (Perner et al., 2016a) - Paper II. A specific cohort of one egg clutch progenies was used for this project to prevent numerous nucleotide polymorphisms that would complicate transcriptome *de novo* assembly. Using MiSeq reads, prof. José Ribeiro managed to carry out a neat re-assembly with already existing *I. ricinus* transcriptomes (Kotsyfakis et al., 2015a; Schwarz et al., 2014). On that, our HiSeq reads were mapped and quantified. Identified differentially expressed genes were verified by RT-qPCR on identical RNA isolates (technical validation) and also on RNA isolates from an independent blood-/serum-feeding experiment of tick collected in the wild (biological validation). From the Gene Ontology analysis, it is apparent that the predominant functions of midgut of ticks fed for 3 days are cysteine-type endopeptidase, glutathione transferase, and glutathione peroxidase activities, while the predominant functions of midgut of ticks fed for 8 days are glutathione peroxidase, flavine adenine dinucleotide binding, and serine-type endopeptidase inhibitor activities (Paper II). Despite identifying a large numbers of transcripts that were present only at a certain time point of feeding (Day 3 vs Day 8) in both blood- and serum-fed ticks, a mere fifteen transcripts were identified to be differentially expressed in the gut of either blood-fed ticks or serum-fed ticks (Paper II).

One of the transcripts that is markedly up-regulated in the presence of haemoglobin in the tick diet encodes glutathione S-transferase. The level of this transcript was elevated in the gut of blood-fed ticks not only at days three and eight, but throughout the entire blood-feeding. To see, if this protein participates in haem-homeostasis maintenance in the tick midgut, a recombinant version of this protein was expressed in *E. coli*. This protein displayed high affinity towards haem-related compounds in *in vitro* activity assays, VIS-absorbance, and affinity

chromatography. Whether the haem-binding occurs in the *in vivo* environment remains to be investigated. RNAi analysis effectively reduced expression of the protein product, but did not result in any impact on weights of engorged ticks, declining the ambitions of this gene product as an anti-tick target.

Paper II

Perner J, Provazník J, Schrenková J, Urbanová V, Ribeiro JM, Kopáček P (2016) RNA-seq analyses of the midgut from blood- and serum-fed *Ixodes ricinus* ticks. Sci. Rep. 6, 36695.



OPEN

RNA-seq analyses of the midgut from blood- and serum-fed *Ixodes ricinus* ticks

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Adult females of the genus *Ixodes* imbibe blood meals exceeding about 100 times their own weight within 7–9 days. During this period, ticks internalise components of host blood by endocytic digest cells that line the tick midgut epithelium. Using RNA-seq, we aimed to characterise the midgut transcriptome composition in adult *Ixodes ricinus* females during early and late phase of engorgement. To address specific adaptations to the haemoglobin-rich diet, we compared the midgut transcriptomes of genetically homogenous female siblings fed either bovine blood or haemoglobin-depleted serum. We noted that tick gut transcriptomes are subject to substantial temporal-dependent expression changes between day 3 and day 8 of feeding. In contrast, the number of transcripts significantly affected by the presence or absence of host red blood cells was low. Transcripts relevant to the processes associated with blood-meal digestion were analysed and involvement of selected encoded proteins in the tick midgut physiology discussed. A total of 7215 novel sequences from *I. ricinus* were deposited in public databases as an additional outcome of this study. Our results broaden the current knowledge of tick digestive system and may lead to the discovery of potential molecular targets for efficient tick control.

Ticks acquired the habit of blood feeding more than 100 million years ago and are the main vectors for pathogens of humans and livestock globally[1,2]. Unlike blood-feeding mosquitoes, all tick life stages feed exclusively on host blood; adult *Ixodes* spp. females feed on their hosts for 7–9 days. As tick feeding progresses, tick digest cells develop along the tick gut epithelium[3], where nutrient endocytosis and lysosome maturation facilitate intracellular digestion[4]. Extensive characterisations of tick midguts have been conducted in various tick species, at both transcript[5–9] and protein[6,9] levels, using massive parallel sequencing and mass spectrometry, respectively. All these studies have been carried out using pooled samples of midgut preparations dissected from a number of ticks fed naturally on laboratory animals. This approach, however, does not reveal expression of novel transcripts induced by blood meal components.

Using an artificial feeding system implemented for the European Lyme disease vector *Ixodes ricinus*[10], we have recently demonstrated that ticks fed on red blood cell (RBC)-depleted serum can successfully engorge and lay eggs. These eggs, however, were sterile and unable to give rise to viable progeny, indicating the ultimate dependence of ticks on haem acquisition from host haemoglobin to sustain embryonic viability[11]. We hypothesised that blood meal depletion of RBCs (serum feeding) might reveal adaptive traits that have enabled ticks to digest haemoglobin intracellularly, with concomitant haem acquisition and transport to the haemocoel, supplying haem metabolic demands in peripheral tissues or detoxification of haem excesses via transport to haemosomes[12]. To reveal if adult female ticks express specific transcripts associated with blood meal processing in response to dietary haemoglobin we have subjected gut RNA extracts from individual blood- and serum-fed ticks to Illumina RNA sequencing (RNA-seq) and have compared their assembled transcriptomes.

To characterise the two important phases of blood-meal uptake and digestion[3], namely the slow phase (feeding initiation) and rapid phase (preceding tick detachment from the host) of engorgement, we have isolated RNA from the midgut of ticks fed for 3 days and 8 days. The midgut transcriptomes from single *I. ricinus* females

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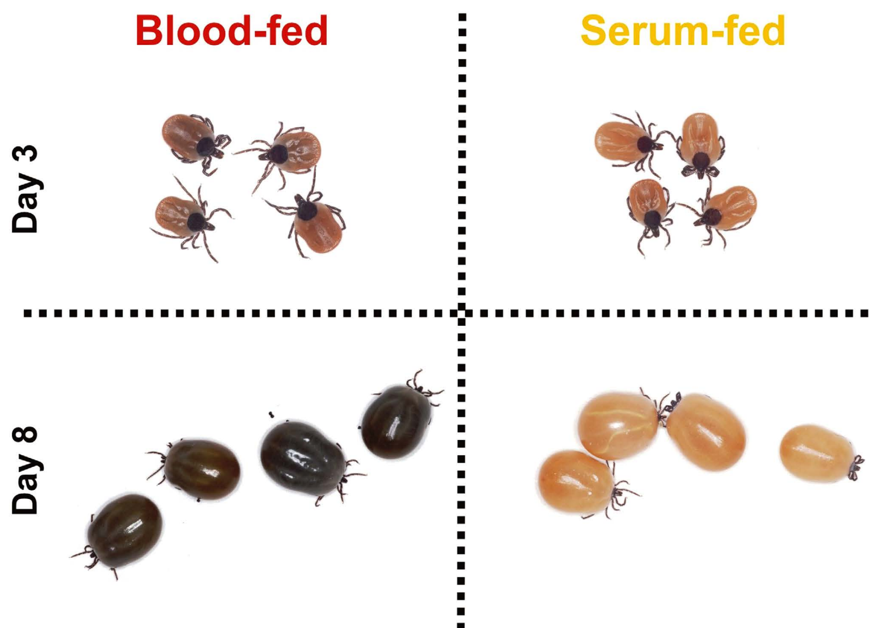


Figure 1. Blood- and serum-fed adult *Ixodes ricinus* females used in this study. First-generation siblings *I. ricinus* females were membrane-fed for 3 days (partial engorgement) or 8 days (full engorgement) with either reconstituted bovine blood or bovine serum. At particular time points, ticks were dissected and individual midgut caeca were used for RNA extractions. Resulting RNA extracts from individual ticks were used for RNA-seq analyses.

revealed substantial temporal differences in gene expression between these two phases. However, the number of genes whose expression was affected by the presence/absence of haemoglobin in the diet was surprisingly low. These findings may help to better understand the physiological processes that are indeed crucial for tick feeding and reproduction.

Results and Discussion

Sample preparation and RNA-seq design. We have recently demonstrated, using artificial membrane feeding[10], that ticks require dietary haemoglobin as their ultimate source of haem since they are not capable of haem biosynthesis[11]. Apart from the fact that feeding ticks on haemoglobin-depleted serum led to aborted embryogenesis, no other obvious physiological effect was observed during the process of tick feeding and oviposition. Using RNA-seq analysis, we have examined transcriptomic changes in the adult tick gut in response to blood-feeding (BF) and serum-feeding (SF) in a temporal-dependent manner. In order to increase the consistency and integrity of RNA-seq data and minimise individual-specific deviations in expression among tick females, we have raised, under laboratory conditions, a cohort of genetically related adult *I. ricinus* siblings (first generation sisters). Ticks were dissected at two time points: day 3 of feeding (3D), which corresponds to the to the slow-feeding phase and day 8, representing fully engorged females (FE)[3,13]. Four females were dissected per time point and per diet (Fig. 1) with each female being represented by a single cDNA library (in total, 16 libraries were prepared). For library preparation, only females with similar weights were selected (Supplementary Figure S1). A catalogue of individual females selected for library preparations was prepared and library names were allocated (Supplementary Figure S1). RNA extractions were performed from single midgut caeca comprising developed digest cells containing both small and large digestive vesicles[14] from both BF and SF ticks (Fig. 2).

Tick gut transcriptome re-assembly and mapping of reads. *De novo* assembly of the *I. ricinus* midgut transcriptome was recently performed for the early stage of adult female feeding (up to 36 hours after attachment)[7]. Our libraries were sequenced using a MiSeq protocol yielding 300 nt transcripts that aided re-assembly of longer transcripts[7,15]. From MiSeq sequencing, nearly 3 million reads per library, averaging 280 bp in length, were obtained. HiSeq sequencing yielded an average of 13 million single-end reads per library, averaging 120 bp in length. A summary of the reads, after removal of Illumina primers and trimming low quality base (smaller than 20) values, is provided in the Supplementary Information (Supplementary Tables S1 and S2) for MiSeq and HiSeq protocols, respectively. HiSeq reads were then mapped onto our midgut transcriptome re-assembly. The coding sequences were deposited in DDBJ/ENA/GenBank under accession number GEFM00000000 as a Transcriptome Shotgun Assembly project; BioProject: PRJNA311553. The generated contigs, their respective expression values, and putative protein characteristics are presented in a hyperlinked Excel spreadsheet (further referred to as Source data 1) so that comparisons of contig levels can be made between individual ticks. Sequences were deposited on the NIH exon server and are available at: http://exon.niaid.nih.gov/transcriptome/Ixric-MG/Ir-web.xlsx.

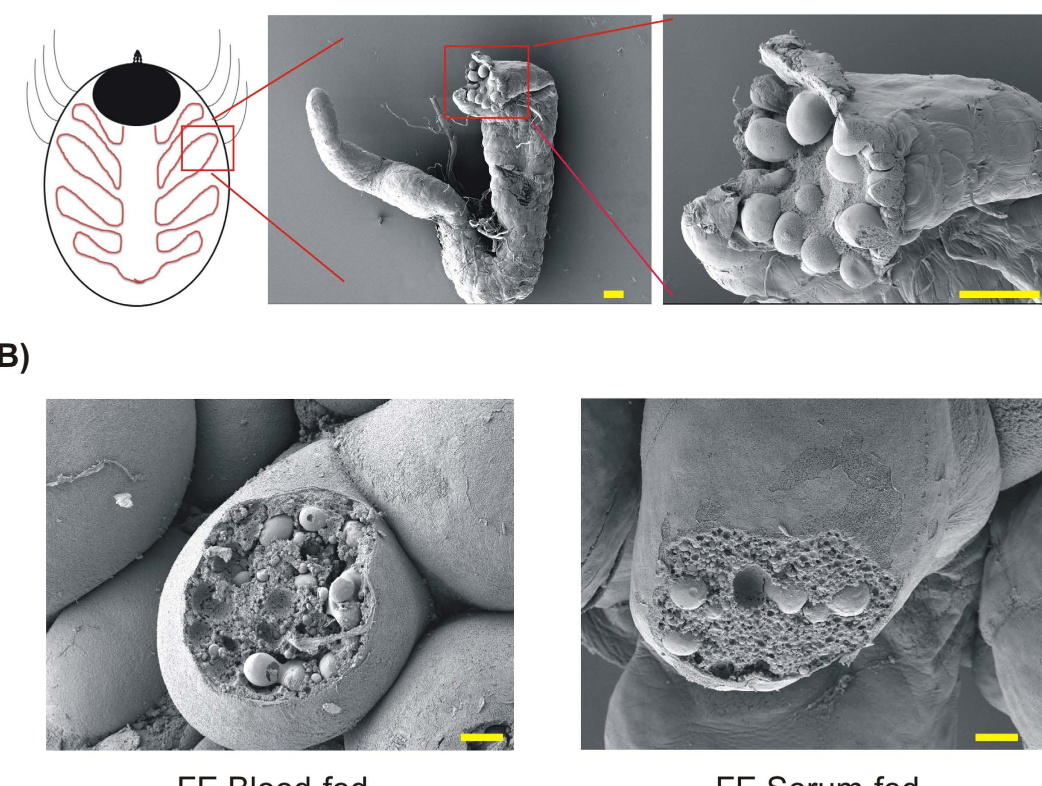


Figure 2. Scanning electron microscopy of tick gut caecum and digest cells. (A) Illustration of tick gut caecum dissected from a partially-fed adult *I. ricinus* female. Such caeca were used for RNA-seq analyses. Scale bars indicate 100 µm. **(B)** Manually disrupted digest cells maturing along tick midgut epithelium from blood- (left) and serum-fed (right) fully engorged adult *I. ricinus* females. Note that digest cells from either tick contain both small and large digestive vesicles. Scale bars indicate 10 µm.

Tick gut transcriptome composition in response to time or diet. To visualise similarities between individual libraries, a multidimensional scaling (MDS) plot was generated. Visualisation indicates that tick gut transcriptomes are subject to distinct temporal-dependent changes (Fig. 3A). The generated heat map shows that differentially expressed gene transcripts derived from the gut of Day 3 ticks cluster well together, while individual transcripts derived from the gut of FE ticks cluster amongst themselves (Fig. 3B). EdgeR analysis, using a set of 13,437 transcripts (RPKM $\geq$ 1 in at least one library), identified 2,676 transcripts that were differentially expressed when the time variable was considered (using an FDR of 0.05 or smaller) (Fig. 3C), while only 15 transcripts were found to be differentially expressed (using an FDR of 0.05 or smaller) (Fig. 3D) when the variable diet was considered. We have listed the most up-regulated contigs (RPKM $\geq$ 3) in the midgut at day 3 or day 8 of feeding in Tables 1 and 2, respectively. Some of the "early gut transcripts" encode enzymes generally associated with the physiology of tick salivary glands, such as phospholipase A2 or glycine-rich secreted cement protein. To confirm our observation, we have generated tissue-specific cDNA sets from both Day 3 and Day 8 ticks to test tissue-specific expression. We have confirmed that most of the listed up-regulated transcripts displayed a gut-specific expression pattern. Only contigs IrSigP-110295 and IrSigP-109251 were shown to be predominantly expressed in the fat body-trachea-associated complex of Day 3 ticks (Supplementary Figure S2). To further confirm that expression of these genes is linked to the slow feeding phase of engorgement, we have run an RT-qPCR analysis of cDNA samples prepared from the tick midgut during and after feeding. We confirmed that all genes were expressed only during the slow feeding phase (Supplementary Figure S2). Most of the contigs that were up-regulated in Day 8 ticks were gut-specific, except for contigs Ir-121498, Ir-113115, Ir-97790 and Ir-113452 (Supplementary Figure S3). The tissue profile of contig IrSigP-108669, encoding vitellogenin 2, displays gut and trachea-fat body complex-specific expression[11]. To further confirm that expression of these genes was linked to the rapid phase of engorgement, we performed an RT-qPCR analysis of cDNA samples prepared from the tick midgut during and after feeding. We confirmed that all of these genes were expressed only during the rapid engorgement phase and after tick drop-off (Supplementary Figure S3). We have also identified the most abundant contigs overall that are listed in the Source data 1 (columns GA–GP).

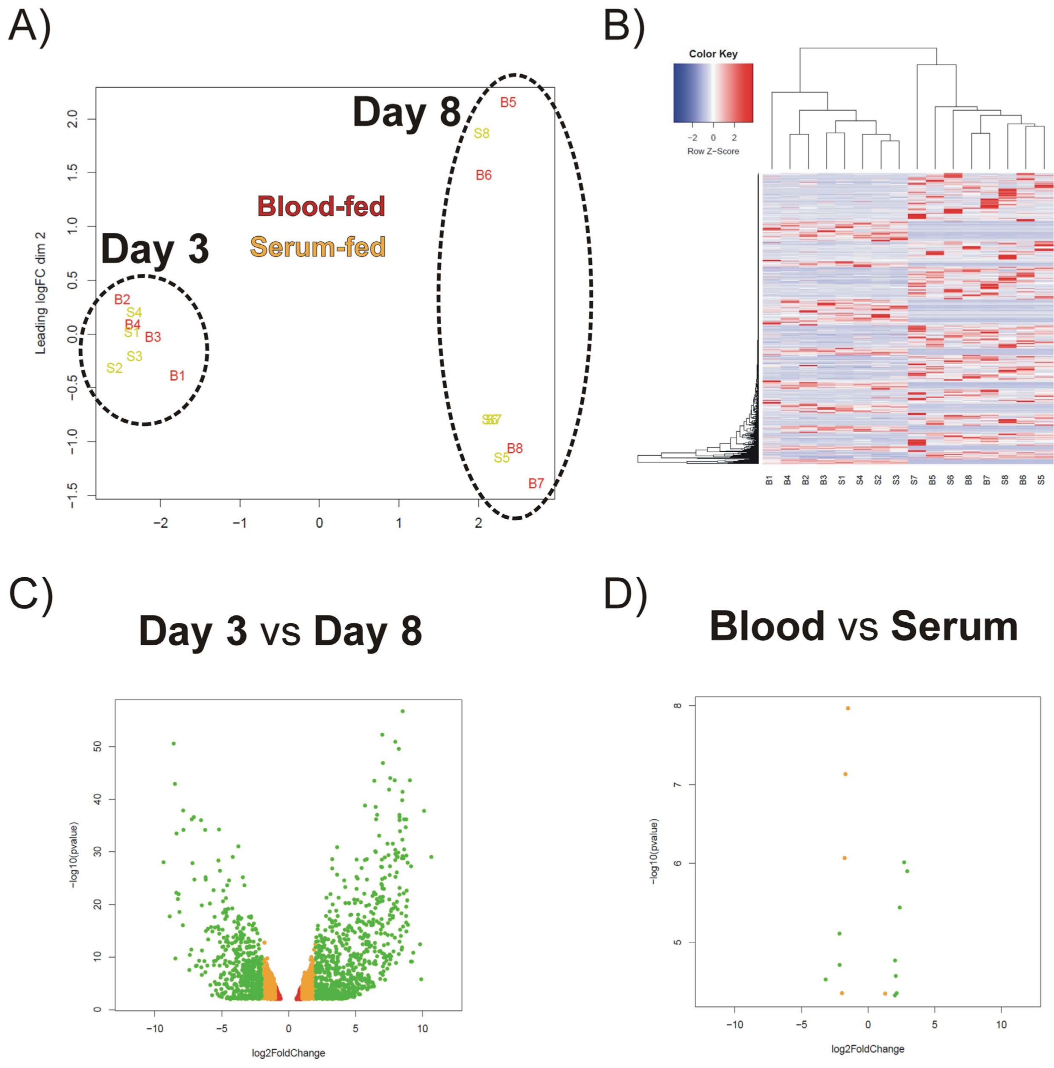


Figure 3. Transcriptome analysis. The transcriptomes from individual blood-fed (B1–B8) and serum-fed (S1–S8) ticks were characterised using an EdgeR package. **(A)** MDS plot of 16 libraries indicating the related nature of transcriptomes from a given time-point of feeding. **(B)** Heat map of differentially expressed intestinal contigs in individual libraries. The gene expression (RPKM) values for each gene were normalised to the standard normal distribution in order to generate Z-scores. **(C)** Volcano plot visualising the number of differentially expressed genes (2676) with respect to tick feeding stage. **(D)** Volcano plot visualising the number of differentially expressed genes (15) with respect to tick RBCs (+/−) diet composition.

Contig Name	Encoded protein	Fold Change	E value	Coverage (%)	Protein Database
Ir-111829	Phospholipase A2	2131	0	109.8	NR-LIGHT
Ir-103540	Sphingomyelin phosphodiesterase	669	0	44.6	NR-LIGHT
Ir-110985	Acid sphingomyelinase	614	0	66	KOG
Ir-108861	Lipase	492	0	98	NR-LIGHT
IrSigP-110295	Phosphoenolpyruvate synthase	451	1e-179	81	NR-LIGHT
IrSigP-109251	Glycine-rich secreted cement protein	221	0	102	NR-LIGHT
Ir-109833	Glycine-rich secreted cement protein	119	0	80.1	SWISSP
Ir-114115	Sulfotransferase	94	0	63.6	ACARI
IrSigP-114509	Phosphatidylinositol- phospholipase c domain	81	0	100	ACARI
Ir-98671	Arginine kinase	68	1e-173	99	SWISSP
Ir-100072	Estradiol 17-beta-dehydrogenase 8	50	5e-046	98	SWISSP

Table 1. Overview of contigs[1] over-represented in libraries B1–B4 and S1–S4 (partially-fed stage) over libraries B5–B8 and S5–S8 (fully-engorged stage). [1]RPKM ≥3, E value ≤1e-15, and coverage ≥50%.

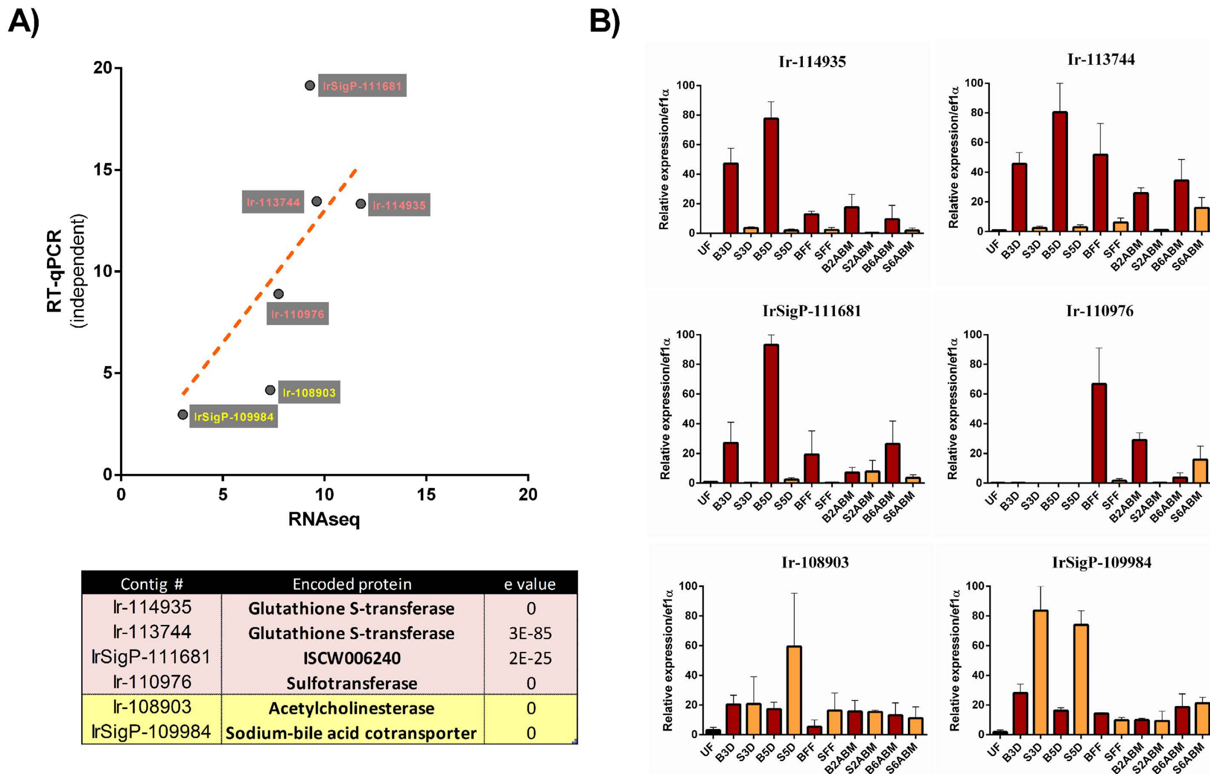
Contig Name	Encoded protein	Fold Change	E value	Coverage (%)	Protein Database
IrSigP-107534	Vesicular amine transporter	634	0	87	NR-LIGHT
Ir-121498	Acyl-CoA synthetase	520	0	99	NR-LIGHT
Ir-113115	JH acid methyltransferase	441	7e-96	60	ACARI
Ir-97790	GSH peroxidase	339	9e-62	97	ACARI
Ir-113156	Kunitz 80	325	2e-40	80	ACARI
IrSigP-108669	Vitellogenin 2	211	0	102	NR-LIGHT
Ir-109200	Organic anion transporter	109	0	87	KOG
Ir-118795	Estradiol 17-beta-dehydrogenase 8	99	2e-49	97	SWISSP
Ir-113345	15-OH prostaglandin dehydrogenase	91	7e-44	90	KOG
Ir-113452	SEC14	85	3e-35	93	KOG
IrSigP-113423	Acetylcholinesterase	43	0	91	KOG
Ir-108832	Carrier protein 6	26	0	90	NR-LIGHT

Table 2. Overview of contigs[1] over-represented in libraries B5–B8 and S5–S8 (fully-engorged stage) over libraries B1–B4 and S1–S4 (partially-fed stage). [1]RPKM ≥3, E value ≤1e-15, and coverage ≥50%.

Encoded protein[1]	Contig name[2]	B vs S logFC[3]	Blood 3D	Blood FE	Serum 3D	Serum FE
Sulfotransferase	Ir-110976	2,94	0,24	32,24	0,04	4,16
Secreted protein	IrSigP-111681	2,7	2,58	12,13	0,71	1,31
GST	Ir-114935	2,38	1898,43	438,95	161,11	205,02
	Ir-113744	2,15	670,28	168,16	69,73	90,69
TNF receptor-associated factor	Ir-113246	2,08	6,71	2,96	1,33	0,87
Alpha-macroglobulin	IrSigP-1636	2,02	0,65	0,89	0,19	0,28
Signal transduction protein	Ir-5162	2,01	1,42	0,63	0,33	0,17
DRAM	IrSigP-114688	1,28	42,59	48,93	21,35	20,05
Sodium-dependent glucose transporter	Ir-115811	-1,51	1,93	1,12	4,68	4,46
Sodium-bile acid cotransporter	IrSigP-109984	-1,69	10,27	3,38	31,79	13,57
Hypothetical secreted protein precursor	IrSigP-10741	-1,76	7,80	1,98	21,95	10,00
PHGPx	IrSigP-109202	-1,96	60,05	536,62	235,94	2300,35
Secreted protein precursor	IrSigP-114886	-2,14	27,80	0,94	100,23	8,73
Acetylcholinesterase	Ir-108903	-2,14	0,71	0,55	5,17	1,95
3-hydroxysteroid dehydrogenase	Ir-113126	-3,18	0,47	1,78	9,09	7,64

Figure 4. Overview of average RKPM values for contigs differentially expressed between serum- and blood-fed ticks. Encoded proteins and their respective average RPKM values in particular libraries are listed. [1]Abbreviations: GST-glutathione S-transferase; TNF-tumor necrosis factor; DRAM-damage-regulated autophagy modulator; PHGPx-Phospholipid hydroperoxide glutathione peroxidase; [2]For contig names, sequences and other and RKPM data, see the Source data 1 at http://exon.niaid.nih.gov/transcriptome/Ixric-MG/Ir-web.xlsx; [3]BvsS logFC represents the base 2 logarithm of the fold change in transcript expression when the Blood treatment was compared with the Serum treatment. 3D, FE-indicate time periods of feeding 3 days and 8 days (fully engorged), respectively. Background color coding: No colour-low expression; Grey colour-insignificant differences; Red vs. blue-significant differences.

Transcriptomic changes in response to blood meal. Although the gut transcriptomes showed substantial feeding phase dependent changes, only few transcripts displayed differential expression in response to dietary RBCs (+/−). The fifteen transcripts with statistically significantly different expressions between BF and SF ticks are shown in Fig. 4. Four of the differentially expressed genes encode enzymes participating in antioxidant or detoxification networks, namely two paralogues of glutathione S-transferase (GST), one homologue of sulfotransferase (SULT), and one homologue of phospholipid hydroperoxide glutathione peroxidase. Three of the differentially expressed genes encode membrane proteins: a sodium-bile acid cotransporter, a sodium-dependent glucose transporter, and DNA damage-regulated autophagy modulator protein (DRAM). The remaining differentially expressed genes encode acetylcholinesterase, alpha-macroglobulin, 3-hydroxysteroid dehydrogenase, and unknown proteins IrSigP-111681, IrSigP-10741, and IrSigP-114886. To validate our RNA-seq data, a



Contig #	Encoded protein	e value
Ir-114935	Glutathione S-transferase	0
Ir-113744	Glutathione S-transferase	3E-85
IrSigP-111681	ISCW006240	2E-25
Ir-110976	Sulfotransferase	0
Ir-108903	Acetylcholinesterase	0
IrSigP-109984	Sodium-bile acid cotransporter	0

Figure 5. Biological (independent) RT-qPCR validation of RNA-seq data. (A) Correlation between fold change (up-regulation) of RT-qPCR (independent cDNA sets from unrelated ticks collected in the wild) and RNA-seq data on selected transcripts. The table of selected differentially expressed genes for blood-fed ticks and serum-fed ticks is shown below the graph. **(B)** RT-qPCR analyses of differentially expressed genes in the tick midgut dissected from *I. ricinus* females during and after feeding. Data were obtained from three independent cDNA sets, and normalised to *elongation factor 1* (*ef1α*). UF-unfed; B-blood-fed; S-serum-fed; 3D, 5D, FF-indicate time periods of feeding 3 days, 5 days, 8 days (fully fed), respectively; 2 ABM and 6 ABM indicate time periods after blood meal, 2 days and 6 days, respectively. Mean and SEM are shown, n = 3 (biological replicates).

direct RT-qPCR analysis was carried out using RNA samples used for library construction and this confirmed the differential expression of selected contigs, although the RT-qPCR analysis tended to slightly overestimate differences in transcript levels (Supplementary Figure S4). To verify the biological validity of the differentially expressed genes, we have performed RT-qPCR of selected contigs on cDNA originating from unrelated ticks collected in the wild and fed with different batch of reconstituted bovine blood or serum, using the membrane feeding system. We have plotted their mean up-regulation values obtained from RT-qPCR against those obtained from RNA-seq (Fig. 5A). To extend the time-points, we inspected their expression profiles throughout the entire blood- or serum-feeding period and up to six days after detachment (Fig. 5B). We determined that both GST encoding contigs (Ir-114935 and Ir-113744) and contig IrSigP-111681 were consistently up-regulated throughout blood-feeding. A SULT-encoding contig (Ir-110976) was up-regulated by blood-feeding at FE and 2 days after blood meal (2 ABM). Acetylcholinesterase-encoding contig (Ir-108903) did not show any significant up-regulation during serum-feeding, while the sodium-bile acid co-transporter-encoding contig (IrSigP-109984) was up-regulated at days 3 and 5 of serum feeding (Fig. 5B). To reveal whether diet composition induced expression changes in gut-specific or more systemically expressed genes, we carried out an RT-qPCR analysis of selected differentially expressed genes in various tick tissues. While GSTs and SULT displayed gut-specific expression, contigs encoding an unknown protein, the sodium-bile acid cotransporter, and acetylcholinesterase displayed more systemic expression (Supplementary Figure S5). Below, we briefly discuss the known homologues of those differentially expressed genes affected by a blood or serum diet.

Glutathione S-transferase (GSTs) was shown to be up-regulated in blood-fed ticks. GSTs, formerly called ligandins, are notorious for showing affinities to a broad array of ligands. Several classes of GSTs were reported to have high affinities to haem-like compounds[16]. Interestingly, levels of GST contigs were shown to be up-regulated in a comparative analysis of *C. elegans* fed on medium containing differing levels of supplemented haemin[17]. Here, we have shown that two contigs (Ir-114935 and Ir-113744) were up-regulated upon blood-feeding. BLAST analysis of these sequences against the available *I. scapularis* genome[18] revealed that Ir-113744 is most likely an intron-containing version of Ir-114935, which might have originated from pre-mRNA co-isolation.

Sulfotransferase (Ir-110976) was shown to be up-regulated by blood-feeding in Day 8 ticks. These enzymes catalyse the transfer of sulfonic groups to a broad range of substrates. The resulting molecules become more soluble in the cellular environment and are easier to metabolise/detoxify[19]. It is tempting to speculate that the

encoded sulfotransferase may have an affinity towards haem in the tick digest cells and may facilitate its dispatch/detoxification. Further work is needed to explore the affinity of sulfotransferase towards an array of substrates in order to obtain a better insight into its biological function.

DNA damage-regulated autophagy modulator protein (IrSigP-114688; DRAM) was up-regulated in blood-fed ticks at both time points, Day 3 and Day 8. The encoded protein has an estimated molecular weight of 27 kDa and 6 predicted membrane helices (Source data 1, Column N and S, respectively). A related molecule was reported to display stress-responsive transcriptional suppression in human cells[20]. This result indicates that the serum-fed ticks may be subject to enhanced oxidative stress in their intestinal digest cells. A similar hypothesis proposed that sugar-fed mosquitoes, in comparison to blood-fed mosquitoes, experience enhanced oxidative challenge in their midgut[21].

Acetylcholinesterase (Ir-108903), in contrast, was up-regulated in serum-fed ticks. The encoded enzyme suggests a possible involvement in neurological perception of received diets. Detailed screening of acetylcholinesterase inhibitors may be a fruitful strategy towards formulation and exploitation of anti-tick intervention initiatives. Such screening was recently performed against acetylcholinesterase from the herbivorous mite *Tetranychus cinnabarinus*[22].

Sodium-dependent glucose transporter (Ir-115811) was up-regulated in Day 3 and Day 8 serum-fed females. The encoded protein, with an estimated molecular weight of 50 kDa and 12 predicted membrane helices (Source data 1, Column N and S, respectively), belongs to families of major facilitators. Members of these families in herbivorous mites were reported to show significant changes in their transcriptional responses to xenobiotics[23,24].

Phospholipid hydroperoxide glutathione peroxidase (IrSigP-109202) was shown to be up-regulated in serum-fed Day 8 ticks. These enzymes are engaged in decomposing lipid hydroperoxides. It was reported that protein levels and enzymatic activity of phospholipid hydroperoxide glutathione peroxidase from *Schistosoma japonicum* can be stimulated by incubation of adult worms with millimolar amounts of oxidative molecules, such as paraquat or hydrogen peroxide[25].

Sodium-bile acid cotransporter (IrSigP-109984) was shown to be up-regulated in Day 8 serum-fed ticks. The encoded ~50 kDa protein contains 9 predicted membrane helices (Source data 1, Column N and S, respectively). The sodium-bile acid cotransporter is a member of the solute carrier protein family 10 (SLC 10) that comprises influx transporters of bile acids, steroidal hormones, various drugs, and several other substrates[26].

Gene ontology analysis. Gene ontology (GO) analysis examines a representative set of contigs in particular metabolic networks. We examined GO across Day 3 and Day 8 libraries to obtain a general overview of physiological processes predominating at each time point. The GO classification considers 3 categories: molecular function, component (cellular localization), and biological process. To assess the enrichment of molecular function and biological process categories between Day 3 and Day 8 ticks, we examined GO term annotations associated with the encoded contigs. The most substantial enrichments ($p < 0.001$, $n = \geq 5$, enrichment ≥ 1.5) in function and process are shown in Fig. 6. These include enrichments in the molecular functions of chitin binding, cysteine-type endopeptidase, NADH dehydrogenase (ubiquinone), low-density lipoprotein receptor, and lipase activities in the tick gut at day 3 (Fig. 6A), while serine-type endopeptidase inhibitor, methyltransferase, acetylcholinesterase, glutathione transferase, iron binding, monooxygenase, and receptor activities were found to be enriched in the tick gut at day 8 (Fig. 6E). Several enrichments were detected in biological processes, out of which iron transport and catabolic processes of carbohydrates, lipids, and proteins stand out in the tick gut at day 3 (Fig. 6B), while negative regulation of peptidase activity, response to oxidative stress, and xenobiotic metabolism were enriched in the tick gut at day 8 (Fig. 6F). When absolute representation in molecular functions was considered, cysteine-type endopeptidase, glutathione transferase, and glutathione peroxidase activities predominated in libraries of Day 3 ticks (B1–B4 and S1–S4), while glutathione peroxidase activity, flavin adenine dinucleotide binding, and serine-type endopeptidase inhibitor activity prevailed in libraries of Day 8 ticks (B5–B8 and S5–S8; Fig. 6C,G, respectively). When absolute representation in biological processes was considered, DNA-templated regulation of transcription, protein catabolic processes, and glutathione metabolic processes were over-represented in libraries of Day 3 ticks (B1–B4 and S1–S4), while DNA-templated regulation of transcription, negative regulation of peptidase activity, and neuron projection morphogenesis predominated in libraries of Day 8 ticks (B5–B8 and S5–S8; Fig. 6D,F, respectively). Although the supplementation of tick diets with gentamicin, necessary to prevent their bacterial decay, may affect expression of some genes responsive to the presence of microbial population in the tick gut, the presence of the antibiotics throughout the feeding in both blood and serum should not substantially misrepresent the obtained data for such comparisons.

RNA-seq analyses have recently enabled exhaustive descriptions of steady-state transcriptome compositions with a remarkable dynamic range between the most abundant and lowly expressed genes, ranging over more than five orders of magnitude. The RNA transcript level of a gene is determined by its regulation at multiple levels, including transcriptional initiation, elongation, splicing, export and degradation[27]. An early belief that there is little correlation between mRNA and protein abundance was recently challenged and, depending on the biological system, mRNA levels were shown to explain up to 90% of changes in protein levels[28]. It can, therefore, be assumed that the transcriptome is a strong predictor of the proteome[29]. Nevertheless, the hierarchical regulation that affects metabolic fluxes in the central metabolism of eukaryotes was reported to be predominantly influenced by post-transcriptional regulation[30]. Therefore, the data collected by GO analysis should serve as a guide that needs to be complemented with protein/metabolite level studies to fully infer physiological processes in the tick gut. Below, we briefly discuss selected categories to gain a better insight into individual functions and processes in tick physiology.

Chitin metabolism. Blood-feeding arthropods synthesise a physical barrier along the intestinal lining, called a peritrophic matrix[31,32], which protects intestinal epithelial cells from a blood meal and tick-borne pathogens.

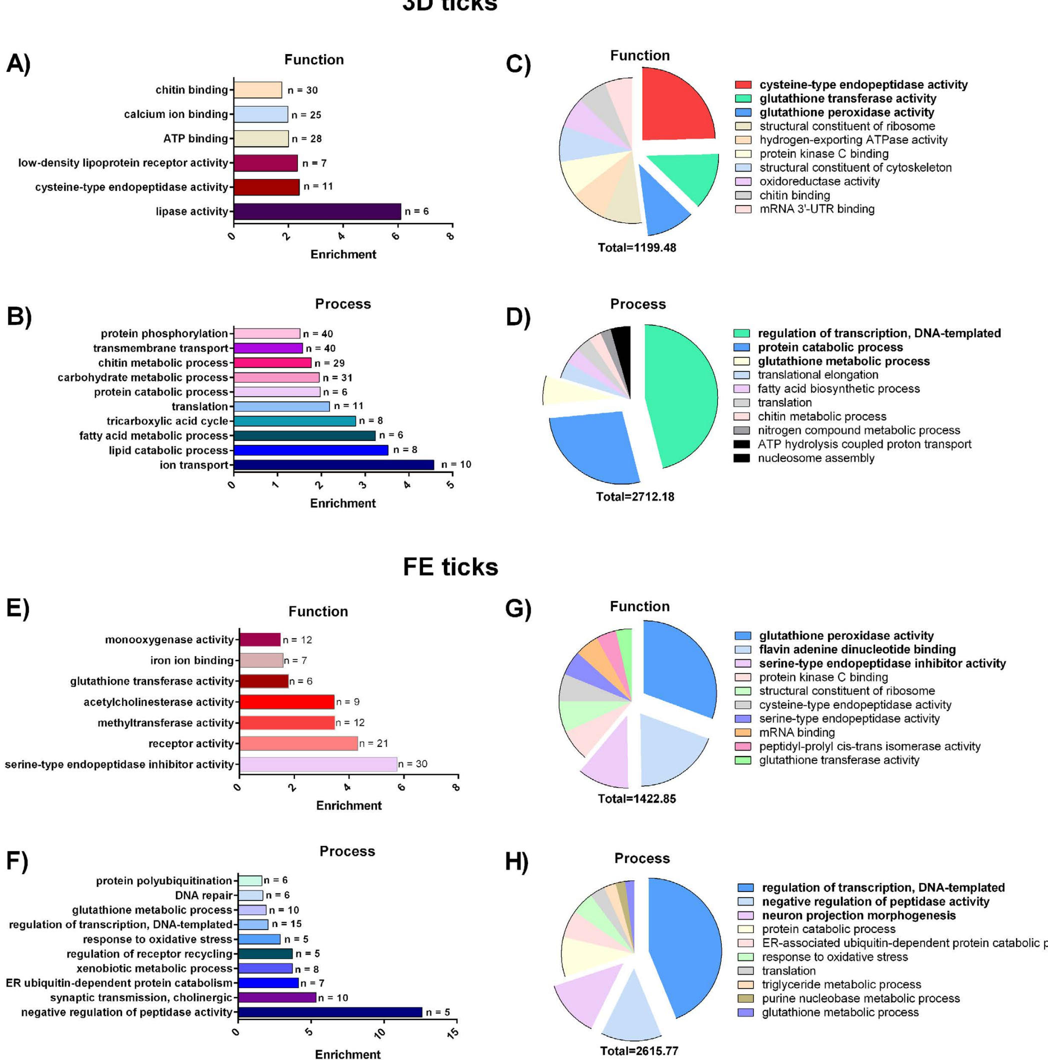


Figure 6. Gene ontology analysis. Bar graphs show the enriched GO terms of molecular function **(A,E)** and biological process **(B,F)** for 3D ticks **(A,B)** and FE ticks **(E,F)**; n indicates the number of contigs concerned. Results are shown only for E value match of $<$1e-15 and n $\geq$5. Pie charts show absolute representation of GO terms of molecular function **(C,G)** and biological process **(D,H)** for 3D ticks **(C,D)** and FE ticks **(G,H)**; sum of RPKM values are shown below the charts. The three most represented categories are highlighted.

Chitin, a linear homopolymer of N-acetyl-β-D-glucosamine, is an important component of such a matrix. We presume that chitin is synthesised endogenously in the tick midgut through a pathway that is conserved throughout lower eukaryotes such as fungi, arthropods, nematodes, or protists[33]. Mapping the chitin biosynthetic pathway in the tick midgut (RPKM $\geq$3, E value $\leq$1e-15, and coverage $\geq$50%; criteria used all the way through the manuscript for mapping and identification of transcripts), we found a single orthologue of glucosamine-phosphate N-acetyltransferase (Ir-114042), UDP-N-acetylglucosamine pyrophosphorylase (Ir-117513), and chitin synthase (Ir-108417). As chitin biosynthesis is absent in vertebrates, chitin biosynthesis and maintenance of chitin integrity is an attractive target not only against fungi[34], but also against blood-feeding arthropods in order to interfere with their feeding and/or compromise their vectorial capacity[32].

Lipid metabolism. Blood meal plasma contains an abundant lipid fraction of heterogeneous structures including sterols, glycerolphospholipids, glycerolipids, or sphingolipids[35]. Some lipidic compounds are found in higher concentrations in a blood meal cellular fraction, as is the case of sphyngomyosins in erythrocyte membranes[36]. We have noted that lipid catabolism is enriched on day 3 of feeding. There are several copies of phospholipase A2 in the genome of *I. scapularis*[18]. Here, we identified a secreted form of phospholipase A2 (Ir-111829) that is strictly regulated in a temporal-dependent and tissue-specific manner with the Day 3 gut being its prime site of expression. Phospholipase A2 activity was already detected in the secreted saliva of Ixodid ticks[37]. The authors also showed that phospholipase A2 caused haemolysis of sheep red blood cells in an *in vitro* assay and speculated that the enzyme could participate in tick digestive processes *in vivo*[38].

The low density lipoprotein (LDL) receptor binds LDL, a rich source of host cholesterol, and transports it into cells by endocytosis. Unlike vertebrates, arthropods are incapable of cholesterol biosynthesis *de novo* and must, therefore, acquire it from their diet. In arthropods, exogenous cholesterol is required for endogenous ecdysteroid biosynthesis[5]. Ecdysteroids play a fundamental role in both post-engorgement salivary gland degeneration[39] and vitellogenesis[40]; for a review, please see[41]. We have found two contigs coding for putative LDL receptor-related proteins: IrSigP-98036 and Ir-112187 that most likely belong to one gene transcript that is orthologous to the *I. scapularis* gene ISCW021767 [VectorBase]. Upon digestion of host lipoproteins, cholesterol is likely bound to tick endogenous lipoproteins and dispatched into the haemolymph for delivery to peripheral tissues. Tick carrier protein, named HeLp, was shown to bind both cholesterol and cholesterol esters in the haemolymph of the tick *Rhipicephalus microplus*[42]. We have identified its putative orthologue (Ir-108832) that is enriched in the gut transcriptomes of Day 8 ticks. Also, cholesterol transport protein and mitochondrial cholesterol transporter were found (IrSigP-116437 and IrSigP-86065, respectively). These proteins likely participate in cholesterol transportation and maintenance of cholesterol homeostasis. The levels of two contigs coding for a putative triglyceride lipase/cholesterol esterase (Ir-107663 and IrSigP-103478) were found to be increased on day 3 of feeding. Interestingly, we have also identified a contig encoding 7-dehydrocholesterol reductase (Ir-120421), the enzyme catalysing the final step of cholesterol biosynthesis.

Protein hydrolysis. Ticks and other blood-feeding organisms have evolved an array of lysosomal cysteine proteases (reviewed in ref. 13) that intracellularly digest the protein-rich blood diet within their endo-lysosomal system. Here, we have found contigs coding for members of a papain-type cysteine-protease and asparaginyl-endopeptidase (legumain) families to be among the most abundantly expressed contigs (Source data 1, Column GA–GP). This finding underscores a critical involvement of protein digestion in tick physiology. One of the principal questions we asked was whether the presence/absence of haemoglobin in the tick diet would affect the levels of transcripts encoding digestive enzymes in the tick gut since serum proteins represent roughly only a third of the total blood protein content. The intracellular digestive apparatus of ticks is localised within the digest cell of the midgut epithelium (Fig. 2) and consists of a network of acidic aspartic peptidases of cathepsin D-type, cysteine exo- and endo-peptidases of cathepsin B, L and C-types (papains), and asparaginyl endopeptidases (AE) of legumain-type[13,43,44]. The contigs assigned to the *I. ricinus* digestive enzyme isoforms were identified and selected from the Source data 1 and displayed in Fig. 7. RPKM values suggest that there is no apparent difference in levels of digestive peptidase-encoding transcripts between BF and SF ticks, thereby suggesting that their expression is apparently independent of haemoglobin. These results confirm our previous data obtained by qRT-PCR dynamic expression profiling of *I. ricinus* females differentially fed on reconstituted blood or serum[45]. The only exception was reported for *Ir*CB1 that was expressed significantly more in BF ticks on the 5th day of feeding[45]; this could not be verified in the present study, which compared only midgut transcriptomes from *I. ricinus* females fed for 3 and 8 days (fully engorged). Figure 7 clearly indicates that apart from the digestive peptidases up-regulated during the early stages of feeding, there exists another set of "late" peptidase isoforms (*Ir*CD2 and *Ir*CL3) that most likely play a role in blood digestion upon the tick's drop-off from the host[46,47]. Expression of some peptidase isoforms such as *Ir*CD3[46] or putative longipain[48] in the *I. ricinus* midgut was negligible, suggesting that these enzymes are not involved in blood-meal digestion. Additionally, we have identified a contig of an as yet undescribed tick cathepsin F (Ir-119743). Whether or not this enzyme is involved in blood meal processing remains to be examined.

Besides the most abundant cysteine and aspartic proteases, contigs encoding two types of serine proteases involved in the tick digestive system[13,43] were also identified: (i) Serine carboxypeptidases were reported to function in the tick as C-terminal monopeptidases, liberating amino-acids from haemoglobin-derived fragments[43]. The serine carboxypeptidase *Hl*SCP1 from *Haemaphysalis longicornis* was characterized and reported to be up-regulated during the course of blood feeding[13,49]. The *I. ricinus* orthologue encoded by contig Ir-111222 was also up-regulated in FE ticks while the other isoenzyme encoded by contig Ir-115255 seems to be expressed in the early feeding stage. (ii) Cubilin-related serine peptidase, composed of an N-terminal CUB domain followed by a low-density receptor domain and C-terminal catalytic active serine protease domains were described in *H. longicornis*[50]. This protein is secreted into the gut lumen where it reportedly functions in haemolysis of RBCs in the initial stage of blood-meal processing[13,51]. In line with its proposed function, two *I. ricinus* sequences related to cubilin-related serine peptidases were up-regulated during the early feeding stage (Fig. 7).

The *I. ricinus* midgut transcriptomes contain a number of other genes encoding serine proteases with conserved catalytical triad H/D/S. Most of the genes encoding serine proteases were markedly up-regulated in the late stage of feeding, whereas some displayed stable expression or slightly increased expression during the early feeding stage (Fig. 7). Two genes encoding trypsin-like serine proteases, tagged as *Hl*SP2 and *Hl*SP3, with a proposed function in blood digestion, were described in *H. longicornis*. Expression of these genes increased during feeding and the recombinant enzymes had a mildly acidic pH optimum, ~5.0, corresponding to their presumed function in the digest cells and/or midgut lumen[48]. By contrast, our previous results based on selective peptidase inhibitors suggest that serine proteases do not substantially contribute to haemoglobinolytic activity in midgut homogenates from semi-engorged *I. ricinus* females[43]. Therefore, it is possible that the serine proteases expressed towards the end of feeding are active during the off-host stage of blood digestion or have some other, as yet unknown function. Other contigs (Ir-108132 and Ir-114599) that are markedly expressed in *I. ricinus* midgut and up-regulated during feeding, code for non-active chymotrypsin-C homologs having the catalytic triad H/D/S replaced by the residues K/D/L. Corresponding orthologues are present in the *I. scapularis* genome (genes ISCW004734 and ISCW015350, respectively). Earlier, we cloned a related gene from the soft tick *Ornithodoros moubata* (GenBank AAQ82934). An interesting feature of these proteins is the presence of a histidine/aspartic acid-rich motif in the central region of the molecule. A similar motif has been found in hebraein, an 11 kDa

Encoded protein[1]	Contig name[2]	Blood 3D	Blood FE	Serum 3D	Serum FE
*Ir*CD1	IrSigP-107463	123,02	169,39	131,76	143,08
*Ir*CD2	IrSigP-117733	1,95	47,049	4,14	49,36
*Ir*CD3	IrSigP-108122	0,68	1,00	0,91	1,33
*Ir*AE1	IrSigP-107919	484,15	88,13	760,96	270,86
	Ir-112007	512,06	92,40	789,15	264,69
*Ir*AE2	IrSigP-112176	15,35	18,42	16,19	23,55
*Ir*AE3	IrSigP-92701	6,60	1,80	9,64	10,95*
*Ir*CB1	Ir-109850	517,24	118,91	609,36	238,43
	IrSigP-116754	486,46	84,68	560,70	149,04
*Ir*CB2	Ir-114164	76,69	23,06	184,89	64,69
Ir-putative longipain	IrSigP-56451	0,70	0,73	0,29	0,33
*Ir*CL1	Ir-114583	1622,13	495,76	2634,35	1059,36
	Ir-116896	1645,55	493,19	2475,38	967,17
	Ir-116895	1113,543	342,25	1752,34	665,91
	Ir-112868	1559,09	461,16	2315,82	870,70
*Ir*CL3	IrSigP-112645	0,67	38,07	13,68	85,82
*Ir*CC	IrSigP-115186	250,26	204,55	154,99	197,2
Ir-serine carboxypeptidase	Ir-111222	27,43	108,38	31,46	91,21*
	Ir-115255	24,40	5,54	53,25	9,72
Cubilin-related serine proteases	IrSigP-111833	102,11	19,82	162,60	19,90
	IrSigP-108777	310,92	28,35	789,69	37,72
Serine proteases (catalytic triad H/D/S conserved)	Ir-107856	0,47	154,36	0,32	258,92
	Ir-107858	0,80	53,51	1,44	223,37
	IrSigP-98327	0,27	42,67	0,28	32,95
	IrSigP-112743	0,85	66,77	1,61	47,90
	Ir-113501	277,04	281,83	182,45	169,31
	Ir-120940	25,05*	6,45	13,46	14,76
	IrSigP-116770	41,70	62,59*	64,93*	38,94*
	IrSigP-107768	75,24	18,75	140,43	11,96
	IrSigP-108104	42,29	4,71	43,14	2,7
	IrSigP-121503	115,31*	4,91	26,11	1,47
Chymotrypsin-like homologs (HD repeats)	Ir-108132	221,88	2297,76	480,98	2586,31
	Ir-114599	58,35	821,93	111,78	1029,02

Figure 7. Overview of average RKPM of putative digestive peptidases. Encoded proteins and their respective average RPKM values in particular libraries are listed. [1]Abbreviations and GenBank Accession Nos: *Ir*CD–*I. ricinus* cathepsin D, *Ir*CD1 (EF428204); *Ir*CD2 (HQ615697), *Ir*CD3 (HQ615698); *Ir*AE–*I. ricinus* asparaginyl endopeptidase (legumain), *Ir*AE1 (AY584752), *Ir*AE2 (ortholog *of I. scapularis* XM_002402043); *Ir*AE3 (unpublished); *Ir*CB–*I. ricinus* cathepsin B, *Ir*CB1 (EF428206); *Ir*CB2 (unpublished); *Ir*-putative longipain (orthologue of *I. scapularis* XM_002433755); *Ir*CL–*I. ricinus* catheppsin L, *Ir*CL1 (EF428205); *Ir*CL3 (ortholog of *I. scapularis* XM_002405329); *Ir*CC–*I. ricinus* cathepsin C (EU128750); [2]For contig names, sequences and other and RKPM data, see the Source data 1 at http://exon.niaid.nih.gov/transcriptome/Ixric-MG/Ir-web.xlsx. Background color coding: No colour-low expression; Grey colour-insignificant differences; Red vs. blue-substantial differences. *-non homogenous expression over four biological replicates.

antimicrobial protein from the hard tick, *Amblyomma hebraeum* (Lai *et al.*, 2004). Whether or not the non-active chymotrypsin homologs play a role in antimicrobial defense within the tick awaits further investigation.

Even though protein hydrolysis yields amino acids essential for vitellogenesis, it also serves as a carbon source for other pathways[52]. During the latter processes, amino acids are deaminated and excessive amino acid-derived nitrogen is disposed of. Most terrestrial invertebrates excrete uric acid or other purines as waste products of nitrogen digestion (uricotelic or purinotelic organisms). These organisms, including ticks, have utilised a pre-existing pathway of purine biosynthesis for disposal of ammonia ions[53]. In purinotelic spiders, mites, and ticks, guanine is the main product of nitrogen digestion[54,55]. Here, we have found contigs encoding a full pathway of purine biosynthesis (Ir-110951, Ir-109322, Ir-111239, Ir-108035, Ir-116366, Ir-110916, Ir-107659, Ir-112139, Ir-108403, Ir-109488 and IrSigP-108704). Even though synthesised purines play important roles as putative excessive nitrogen disposal molecules, synthesised and/or salvaged purines also participate in ribonucleotide synthesis for transcription within digest cells, tetrahydrofolate biosynthesis, or as a pheromone signal upon excretion[55].

Negative regulation of proteolytic activity. Contig levels encoding secreted protease inhibitors were substantially enriched in Day 8 ticks. It was recently reported that the midgut transcriptome of *I. ricinus* contains a number of highly expressed protease inhibitors of different classes, including cystatins, serpins, or peptides containing trypsin inhibitor-like (TIL) or Kunitz domains[7]. As the previous study was focused on transcriptomes from early stage feeding (up to 36 hours in adult ticks), roughly corresponding to our Day 3 libraries, our data has allowed disclosure of a further remarkable increase in expression of the majority of putative protease inhibitors, as most of them were up-regulated at full engorgement (Fig. 8).

Encoded protein[1]	Contig name[2]	Blood 3D	Blood FE	Serum 3D	Serum FE
Cystatin-type1	Ir-112024	164,06	109,03	211,25	469,22*
	Ir-116079	129,36	83,88	160,26	335,49*
Cystatin-type2	Ir-119134	23,07	113,38*	16,51	137,45*
	Ir-119135	39,07	172,17*	24,27	209,49*
Serpin	Ir-111795	3,64	800,37	3,78	1205,22
	IrSigP-108039	5,67	1571,75	6,18	1421,99
	Ir-111998	5,45	1196,34	4,83	1367,55
BPTI/Kunitz (boophilin-related)	Ir-119569	102,24	95,67	111,23	74,88
	Ir-110707	52,54	32,69	78,95	64,76
TIL-domain peptides	Ir-108216	24,61	4609,81	24,71	1912,78
	Ir-108275	37,89	6175,27	34,63	3097,01
	Ir-117752	19,21	3025,73	21,65	1769,63
	Ir-116209	3,55	1119,44	4,56	816,83
	Ir-117015	11,44	1504,32	9,56	627,50

Figure 8. Overview of average RKPM of selected protease inhibitors. Encoded proteins and their respective average RPKM values in particular libraries are listed. [1]Abbreviations: BPTI-bovine pancreatic trypsin inhibitor, TIL-trypsin inhibitor-like domain (cysteine rich); [2]contig names, sequences and other and RKPM data, see the Source data 1 at http://exon.niaid.nih.gov/transcriptome/Ixric-MG/Ir-web.xlsx. Background color coding: Grey colour-insignificant differences; Red vs. blue-substantial differences. *-non homogenous expression over four biological replicates.

The role of midgut cystatins is likely associated with the regulation of digestive cysteine peptidases and is presumed to prevent their undesired proteolytic activity within the mature digest cells of the midgut epithelium[3,13,56]. We noted a marked up-regulation of serpins and putative TIL-domain protease inhibitors in fully engorged BF and SF ticks. We and others have also noted a number of midgut-specific contigs annotated as 'Kunitz-type' peptides identified in the *I. ricinus* midgut transcriptome[7]. However, based on a "conserved domain database" search, their sequences do not unequivocally encode an unambiguous Kunitz domain. The genes represented by contigs Ir-119569 and Ir-110707 each contain two BPTI/Kunitz domains and are related to *boophilin*, a Kunitz-type inhibitor from the midgut of the cattle tick *R. microplus*[57]. It was recently suggested that boophilin inhibits proteolytic enzymes of host origin and thereby controls undesired blood coagulation or complement activation within the tick gut lumen[58]. We assume that the tick midgut serine protease inhibitors that are strikingly up-regulated in fully engorged females may function against exogenous serine proteases, either to control haemostasis or inhibit premature digestion of blood-meal stored in the midgut lumen.

Response to oxidative stress (redox homeostasis, antioxidant defence, and detoxification). In addition to the maintenance of endogenous redox homeostasis, ticks must cope with the oxidative challenges caused by the host immune system and digestion of a pro-oxidative haemoglobin-containing diet. We have summarised contigs encoding proteins likely participating in redox homeostasis, antioxidant defence, and detoxification in Fig. 9. The glutathione and thioredoxin pools are responsible for the activity of antioxidant enzymes in respective cellular compartments. Glutathione is synthesised in two enzymatic steps, catalysed by dimeric glutamate-cysteine ligase (regulatory and catalytic subunit) and glutathione synthetase. In our midgut libraries of adult *I. ricinus* females, we could find contigs encoding glutathione synthetase (Ir-109549) and a regulatory subunit of glutamate-cysteine ligase (Ir-109549) but we failed to identify the catalytic subunit of this enzyme that was, in contrast, present in the salivary gland transcriptome (BioProject:PRJNA 177622). Unlike glutathione, thioredoxin is a thiol-containing peptide encoded by a single gene. Here, we have identified contigs encoding both cytosolic and mitochondrial putative thioredoxins (Ir-100771 and Ir-121036, respectively), both of which seem to be evenly expressed in all libraries. Both thiols (glutathione and thioredoxin) are recycled by respective reductases at the expense of NADPH. Yeast cells code for two sets of reductases, glutathione reductase and thioredoxin reductase, with affinities to distinct thiols in order to restore their reduced state[59]. Platyhelminth parasites, however, were reported to code for an enzyme glutathione-thioredoxin reductase, displaying affinities towards both oxidised thiols in order to restore their reduced forms[60]. Here, we have found a contig (Ir-111289) encoding a putative glutathione-thioredoxin reductase. NADPH is synthesised predominantly in the pentose phosphate pathway by its two enzymes, glucose-6-dehydrogenase and 6-phosphogluconate dehydrogenase. Contigs encoding homologous proteins were identified as Ir-114475 and Ir-116172, respectively (Fig. 9).

Aerobic organisms have evolved an efficient set of enzymes that can deal with the reactive metabolites of oxygen respiration. Catalase, the first-line antioxidant enzyme that ensures the removal of hydrogen peroxide molecules, is encoded by a single gene in the *I. scapularis* genome [VectorBase; ISCW017124]. Therefore two

	Encoded protein[1]	Contig name[2]	Blood 3D	Blood FE	Serum 3D	Serum FE
REDOX	Glu-6-P DH	Ir-114475	8,84	4,70	15,51	9,04
	6-PG DH	Ir-116172	12,24	3,73	14,25	5,53
	Glu-Cys ligase (reg.)	Ir-113189	10,60	11,96	8,44	13,43
	Glutathione synthetase	Ir-109549	14,46	6,60	12,79	8,38
	Thioredoxin	Ir-100771	13,20	7,30	12,72	9,92
	Thioredoxin (mt)	Ir-121036	9,00	4,22	9,35	5,81
	Trx-GSH Reductase	Ir-111289	19,89	13,73	16,00	15,33
ANTIOXIDANT	Catalase	Ir-107724	47,09	161,76	37,63	190,19
		Ir-107725	47,50	158,89	37,39	185,98
	PHGPx	IrSigP-109202	60,05	536,62	235,94	2300,35
		Ir-109203	76,67	840,26	298,22	2529,45
		Ir-113312	33,83	648,47	53,89	973,72
		IrSigP-115731	27,72	490,24	49,65	883,98
	Peroxiredoxin (1-Cys)	Ir-114582	466,52	152,35	438,47	128,59
	Peroxiredoxin (2-Cys)	Ir-101807	15,00	1,74	8,68	3,94
	Cu-Zn SOD	IrSigP-107870	29,00	99,93	36,13	211,80
	Cu-Zn SOD	Ir-109675	138,20	89,16	157,03	118,09
	Mn SOD	Ir-108054	22,59	12,84	23,99	17,93
DETOXIFICATION	CYP450	IrSigP-117992	6,86	0,86	41,70	2,67
	CYP450	Ir-108673	2,83	45,25	3,87	98,54
	CYP450	Ir-113896	46,86	23,11	66,61	21,79
	CYP450	Ir-110111	209,76	22,14	183,71	48,21
	Flavin-containing monooxygenase	IrSigP-112323	1,56	618,27	1,09	419,06
		IrSigP-111018	0,91	295,68	0,67	221,42
		IrSigP-109207	1,51	433,00	1,10	313,03
	δ/ε GST	Ir-114935	1898,43	438,95	161,11	205,02
		Ir-113744	670,28	168,16	69,73	90,69
	μ GST	Ir-111209	60,56	27,62	70,77	28,78
	μ GST	Ir-119644	220,39	97,98	211,63	140,44
	ξ GST	Ir-99420	6,04	13,28	4,13	23,02
	ξ GST	Ir-112382	25,47	68,67	17,27	64,49
	microsomal GST	Ir-108334	125,70	69,64	75,09	139,77
	Sulfotransferase	Ir-110976	0,24	32,24	0,04	4,16
	Sulfotransferase	IrSigP-108278	13,28	256,76	7,39	358,19
	Sulfotransferase	IrSigP-109501	2,01	9,24	1,68	24,84

Figure 9. Overview of average RKPM values for contigs encoding redox, antioxidant, and detoxification proteins. Encoded proteins and their respective average RPKM values in particular libraries are listed. [1]Abbreviations: Glu-6-P DH-Glucose-6-phosphate dehydrogenase; 6-PG DH-6-phosphogluconate dehydrogenase; Glu-Cys ligase-Glutamate-cysteine ligase; Trx-GSH Reductase-Thioredoxin Glutathione Reductase; PHGPx-Phospholipid hydroperoxide glutathione peroxidase; SOD-Superoxide dismutase; CYP450-Cytochrome P450; GST-Glutathione S-transferase; [2]For contig names, sequences and other and RKPM data, see the Source data 1 at http://exon.niaid.nih.gov/transcriptome/Ixric-MG/Ir-web.xlsx. Background colour coding: Grey colour-insignificant differences; Red vs. blue-substantial differences.

identically expressed contigs identified in our libraries (Ir-107724 and Ir-107725), likely encode one protein product. Despite the fact that catalase is a haem-containing enzyme, we did not observe any expressional difference of the contigs in relation to dietary RBCs (+/) (Fig. 9). The second group of hydrogen peroxide-removing enzymes is represented by non-haem peroxidases. They are divided into two major subfamilies: (i) glutathione peroxidases (GPx) and (ii) thioredoxin peroxidases (peroxiredoxins). Our data show that contigs (IrSigP-109202, Ir-109203, Ir-113312 and IrSigP-115731) encoding putative *I. ricinus* phospholipid-hydroperoxide glutathione peroxidases (PHGPx), together with catalase, were significantly up-regulated in Day 8 ticks (Fig. 9). The PHGPx class enzymes specifically protect the cell by reducing hydroperoxides of phospholipids, thereby preventing membrane lipoperoxidation. They were also suggested to be involved in resistance to acaricides in the tick *R. microplus*[61]. Our results suggest that the reduction of hydrogen peroxide and phospholipid peroxides is more important during the rapid engorgement and/or off-host digestive phases. Peroxiredoxins contain one or two reactive cysteines (1-Cys or 2-Cys) in their active sites. Here, we have identified one contig (Ir-101807) encoding 2-Cys peroxiredoxin and one highly expressed contig (Ir-114582) encoding a protein with homology to 1-Cys peroxiredoxin. The latter is an orthologue of *I. scapularis* immuno-dominant salivary gland antigen, tagged as Salp25D[62]. This protein, which exerts glutathione peroxidase activity, has been reported to facilitate the acquisition of *Borrelia burgdorferi* spirochetes by the tick[62,63]. Superoxide dismutase (SOD) participates in cellular antioxidant defence as it converts highly reactive superoxide radicals to peroxides[64]. SODs are metallo-enzymes that contain Cu, Zn, or Mn in their cores. The *I. ricinus* midgut transcriptomes analysed here contain two contigs encoding Cu-Zn SOD (IrSigP-107870 and Ir-109675) and one contig encoding a putative mitochondrial Mn SOD (Ir-108054) out of which only the former seems to be more highly expressed in Day 8 ticks.

The detoxification of xenobiotics, takes place in two phases: (phase I)-biotransformation of xenobiotics via insertion or uncovering of reactive hydrophilic moieties in their structures via oxidation, reduction, or hydrolysis;

(phase II)-xenobiotics or their metabolites undergo conjugation reactions with endogenous compounds, e.g. glutathione or saccharides that enhance their excretion[65]. Cytochromes P450 (CYPs), member of the phase I detoxification system form a large family of membrane proteins. Genes encoding CYPs have apparently undergone several multiplications resulting in a several hundreds of *cyp* genes being present in the genome of *I. scapularis*[18]. Here, we have found four moderately expressed contigs (IrSigP-117992, Ir-108673, Ir-113896 and Ir-110111) encoding CYPs. Although CYPs represent another group of proteins that needs haem as a prosthetic group, no apparent relationship between their expression and dietary RBCs (+/−) was noted. Another phase I detoxification enzyme is flavin-containing monooxygenase (FMO), here encoded as one protein product by contigs IrSigP-112323, IrSigP-111018 and IrSigP-109207 (Fig. 9). In contrast to CYPs, FMO utilizes flavin adenine dinucleotide as a prosthetic group and is markedly up-regulated in fully engorged ticks whereas its expression in the early stage of feeding is marginal.

A family of glutathione S-transferases (GSTs) comprise several classes of enzymes, some of which participate in the phase II detoxification system. GSTs catalyse conjugation of reduced glutathione to an array of xenobiotic substrates. Two GST contigs (Ir-114935 and Ir-113744, likely encoding one protein product), share homology with the δ and ε class of GSTs. As discussed above, this particular GST showed quite rare but obvious up-regulation in the presence of dietary haemoglobin (see also Fig. 4). By contrast, no apparent diet-dependent expression was noted for two other contigs (Ir-111209 and Ir-119644) encoding μ class GSTs, two contigs (Ir-99420 and Ir-112382) encoding ξ class GSTs, and one contig (Ir-108334) encoding microsomal GST (belonging to a MAPEG family of proteins). Sulfotransferases (SULT) catalyse the conjugation of sulfuryl groups to a broad array of substrates, often small insoluble molecules, in order to increase their water solubility and decrease their biological activity. Three contigs (Ir-110976, IrSigP-108278 and IrSigP-109501) encode three distinct SULTs out of which the former transcript seemed to be up-regulated in the presence of haemoglobin (see above and the Fig. 4).

Iron binding and haem homeostasis. We recently demonstrated that ticks do not acquire bioavailable iron from haemoglobin, but most likely from host transferrin (Tf)[11]. It is therefore, not surprising that transcripts encoding iron metabolism proteins are not transcriptionally regulated by the presence of dietary haemoglobin (Fig. 10). Mammals internalise Tf through a well described Tf receptor-mediated pathway. Similarly with predictions in insects[66], we could not find any sequence related to the transferrin receptor in the *I. scapularis* genome[18] nor in our transcriptomic database (Source data 1). It is only speculative that ticks acquire host transferrin through non-specific endocytosis, similarly to that suggested for host serum albumin[14]. Upon Tf degradation in lysosomes, iron may be transferred to the cytosol through a divalent metal transporter DMT, also termed Malvolio. A contig (Ir-109409) encodes an nramp domain-containing protein that is homologous to insect Malvolio and mammalian DMT-1 protein, both of which have been implicated in intestinal iron absorption and systemic trafficking[67,68]. The *I. ricinus* homologue was recently shown to be expressed throughout tick developmental stages and tissues[69]. This work also revealed that RNA-mediated silencing of tick *dmt-1* had no impact on adult *I. ricinus* engorgement or reproductive success[69]. The tick midgut transcriptome also confirmed our previous results[70] that transcription of the intracellular "storage" ferritin *Ir*-Fer1 (Ir-108507) and the secreted "transporting" ferritin *Ir*Fer2 (Ir-108441) were independent of the course of feeding and further corroborates our data that tick ferritin expression does not respond to dietary RBCs (+/−). The protein levels of *Ir*Fer1 are regulated at the translational level via binding of the iron-responsive protein (IRP) to the iron-responsive element at the 5′ end of *irfer1* mRNA[71]. IRP is an inactive form of the cytosolic aconitase (Ir-109153), which is produced under conditions of iron deficiency. Expression of cytosolic aconitase seems to be slightly up-regulated in fully engorged ticks, independent of haemoglobin. Conversely, mitochondrial aconitase (Ir-116255) was evenly expressed in all transcriptomes. Mitoferrin, a transporter protein facilitating the import of ferrous iron into the mitochondrial matrix, is encoded by the contig Ir-92695 (Fig. 10). We have also noted low levels of contigs encoding transferrin 2, a homologue of insect transferrin 2 (also tagged melanotransferrin)[72,73]. Recent work, however, did not confirm the participation of this protein in iron homeostasis or immune defence in the *I. ricinus* tick[69].

Several types of haem transporter proteins have been, thus far, described in eukaryotic cells: (i) haem carrier protein 1, (ii) haem responsive genes, (iii) feline leukemia virus subgroup C receptor and (iv) ABC transporters. For a more comprehensive review, please see refs 74 and 75. Haem carrier protein (HCP-1) was first described in mouse intestine as a homologue of bacterial metal tetracycline transporters[76]. Using BLAST analysis of the bacterial metal tetracycline transporter (AAG43220), we have identified three contigs (Ir-109069, Ir-112177 and Ir-116070) encoding homologous transmembrane proteins. Two further contigs were detected as homologous to putative HCP-1 (Ir-111111 and IrSigP-5644). In studies exploiting microarray assays in *C. elegans* worms cultured under elevated/reduced levels of haemin, haem-responsive genes (hrg´s) were discovered[77,78]. Both HRG-1 and HRG-4 were shown to be essential for haem homeostasis in *C. elegans* and both endogenous proteins were shown to have affinities towards haemin. While HRG-1 was shown to localize in endosomal-lysosomal organelles, HRG-4 was found to localize to the plasma membrane[78]. Recently, the possible *hrg-1* homologue was detected in *I. ricinus* and its levels were reported to be constantly increasing with tick feeding during each developmental stage, with the ovaries of partially and fully engorged females being the prime site of expression[69]. However, in a present study, *irhrg*-1(Ir-4974) showed stable expression in the tick midgut regardless of the feeding time course or diet (Fig. 10). Even though the putative four transmembrane domains were conserved in *Ir*HRG-1, amino acid side chains histidine-90 or the FARKY sequence at the C-terminal, proposed to interact with haem, were not conserved[78]. Therefore, involvement of *Ir*HRG-1 in haem homeostasis in tick tissues remains speculative. Feline leukemia virus subgroup C receptor 1(FLVCR1) was identified in mammalian cells as a member of a major facilitator family of transmembrane transporters and was shown to function as a haem exporter[79]. We have found a contig (Ir-115770) encoding a putative FLVCR that shares 50% and 48% amino acid identities with human FLVCR1 (AAH48312) and FLVCR2 (AAH19087), respectively. A multidrug resistance protein (MRP-5), which belongs to the MRP/ABCC family, was described in *C. elegans* and was implicated in haem export from the worm

Encoded protein[1]	Contig name[2]	Blood 3D	Blood FE	Serum 3D	Serum FE
*Ir*Fer1	Ir-108507	391,56	267,80	369,82	479,07
*Ir*Fer2	Ir-108441	113,27	79,97	100,62	102,35
c-Aconitase (*Ir*IRP)	Ir-109153	9,51	20,59	10,45	25,39
Malvolio (DMT-1)	Ir-109409	4,93	9,44	3,14	11,87
Zinc/iron transporter	IrSigP-113475	5,80	96,69	10,86	69,20
	Ir-116008	5,48	2,37	3,11	11,13
Zinc/iron transporter	IrSigP-117442	4,42	1,77	3,43	2,78
	IrSigP-117453	2,07	0,92	1,88	1,71
Mitoferrin	Ir-92695	3,73	1,62	3,01	2,16
m-Aconitase	Ir-116255	14,08	17,72	13,20	27,13
Tf2 (melano-Tf)	Ir-105628	0,54	0,42	0,31	0,55
HCP-1	Ir-109069	5,40	4,02	3,73	4,96
HCP-1	Ir-112177	1,68	1,64	1,54	2,10
HCP-1	Ir-116070	4,83	5,56	3,93	4,74
PCFT/HCP-1	Ir-111111	2,36	3,70	2,12	5,21
	IrSigP-5644	1,81	3,06	1,80	3,02
MRP-5/ABCC5	Ir-4633	0,48	0,29	0,42	0,52
ABC B10	Ir-109605	4,77	3,88	5,46	5,27
Hrg-1	Ir-4974	16,86	9,26	13,49	9,29
FLVCR	Ir-115770	5,61	2,02	4,43	2,14
*Ir*CPOX	IrSigP-4249	0,95	0,54	0,58	0,49
*Ir*PPOX	Ir-101433	2,75	0,85	2,23	1,51
Haem *o* synthase	Ir-114670	2,69	1,79	2,48	2,09
	IrSigP-115465	4,06	2,46	3,48	2,38

Figure 10. Overview of average RKPM values for contigs encoding proteins involved in iron and haem meatbolism. Encoded proteins and their respective average RPKM values in particular libraries are listed. [1]Abbreviations and GenBank Accession Nos: *Ir*Fer1-*I. ricinus* ferritin 1 (AF068224); *Ir*Fer2-*I. ricinus* ferritin 2 (EU885951), *Ir*IRP-*I. ricinus* iron regulatory protein/cytoplasmic aconitase (EU885952); DMT-divalent metal transporter; m-Aconitase-mitochondrial aconitase; Tf-transferrin; HCP-haem carrier protein; PCFT-proton-coupled folate transporter; MRP-multidrug resistance protein; Hrg-haem responsive gene; FLVCR-group C feline leukemia virus receptor; *Ir*CPOX-*I. ricinus* coproporphyrinogen oxidase; *Ir*PPOX-*I. ricinus* protoporphyrinogen oxidase; [2]For contig names, sequences and other and RKPM data, see the Source data 1 at http://exon.niaid.nih.gov/transcriptome/Ixric-MG/Ir-web.xlsx. Background color coding: No colour-low expression; Grey colour-insignificant differences; Red vs. blue-substantial differences.

intestine[80]. A contig (Ir-4633) encoding a tick MRP-5 homolog has been identified. In addition, we found that contig Ir-109605 encoded a protein homologous to the recently reported ABC transporter (ABC B10) that participates in haem transport and detoxification in digest cells of the tick *R. microplus*[81]. Despite the proposed roles of these molecules in maintenance of haem homeostasis, expression of neither MRP-5 -nor ABC B10-related genes seem to be affected by the presence/absence of haemoglobin in the diet. Recently, we have shown that ticks *I. scapularis* and *I. ricinus* do not code for a complete protein set participating in haem biosynthesis[11]. Here, we have found only contigs encoding two out of three vestigial mitochondrial enzymes of the haem biosynthetic pathway, namely coproporphyrinogen oxidase (CPOX) and protoporphyrinogen oxidase (PPOX), whereas a transcript encoding terminal ferrochelatase was absent from our midgut libraries (Fig. 10). Contigs encoding haem *o* synthase were present in our transcriptome (Fig. 10), indicating that acquired host haem is further endogenously metabolised. However, none of these transcripts encoding proteins involved in haem metabolism seemed to be significantly affected by the course of feeding or RBCs (+/−) diet.

Conclusion

This work is, to our knowledge, the first report describing the use of artificial tick feeding linked with high-throughput transcriptomic analysis aimed at addressing biologically relevant questions such as the tick response to RBC +/− in the diet. In addition, this study has allowed us to perform an as yet unexplored comparison of gene expression in the midgut of individual adult *I. ricinus* females during their early and late stages of feeding. Although we have found a surprisingly low number of transcripts responsive to the presence/absence of haemoglobin, we have identified a great number of contigs associated with temporal-dependent expression in the tick midgut. We believe that our work may pave the way towards experimental manipulation of tick diets in order to enhance our current knowledge of the tick digestive system and tick metabolic demands, with the potential to discover novel target molecules for efficient anti-tick interventions.

Methods

Tick maintenance and natural feeding. Larvae from one egg clutch were fed on guinea pigs, under laboratory conditions, to obtain nymphs. These nymphs were fed identically to obtain a genetically-related cohort of adult *Ixodes ricinus* females. These ticks were kept at 24 °C and 95% humidity under a 15:9-hour day/night regime. Adult *I. ricinus* were also collected in the wild near Ceske Budejovice. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 357 095/2012. The study was approved by the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (CAS) and Central Committee for Animal Welfare, Czech Republic (protocol no. 1/2015).

Tick artificial membrane feeding. Artificial membrane feeding was performed in feeding units manufactured according to the protocol developed by Kröber and Guerin[10] and adjusted for serum feeding as previously reported[11]. Briefly, serum was obtained from fresh bovine blood samples by centrifugation at 2 500 × g, for 10 min at 4 °C and then the collected serum was spun again at 10 000 × g, for 10 min at 4 °C. The serum was sterile filtered using a 0.22 μm membrane filter (Merck Millipore). To reconstitute the blood without host white cells (reconstituted blood), the serum was supplemented with red blood cells previously washed three times in sterile PBS (haematocrit 45%). Diets were supplemented with ATP (1 mM) and gentamicin (5 μg/ml) and regularly exchanged at 12 h intervals. Females were allowed to attach in 12 h and all unattached ticks were then removed. Males were added at the beginning of the day 3 to complete mating.

Scanning electron microscopy. Gut caeca were dissected from BF ticks at day 3 of feeding. The tissue was immediately fixed in 2.5% glutaraldehyde in phosphate-glucose buffer (PGB, 0.1M sodium-phosphate, pH 7.2, 4% w/v glucose) for 4 h at room temperature. Tissues were then rinsed with PGB (3 × 15 min). Post-fixation treatment was performed using 2% OsO_4 for 2 h at room temperature. After washing in PGB, specimens were dehydrated with a graded series of acetone (30–100%) for 15 min at each step, critical point dried (CPD2 Pelco TM) and gold coated using a Baltec SCD 050 sputter coater. The samples were examined in a JSM 7401- F FE-SEM (JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 3 kV using GB-low mode.

Tick gut preparation and total RNA extraction. Tick females were forcibly removed from the membrane at specified time-points and individual females were dissected under ice-cold DEPC-treated PBS so that clear gut preparations were obtained for RNA extraction. The purity of dissected midguts was examined and all contaminating tissues were removed. Gut preparations were homogenized in RA1 buffer (Machery Nagel) supplemented with β-mercaptoethanol using a 29G syringe. Total RNA was then extracted using a Nucleospin RNA kit (Machery Nagel).

Illumina Sequencing. Total RNA samples were submitted to the North Carolina State Genomic Sciences Laboratory (Raleigh, NC, USA) for Illumina RNA library construction and sequencing. The amplified library fragments were purified and checked for quality and final concentration by using an Agilent 2100 Bioanalyser with a High Sensitivity DNA chip (Agilent Technologies, USA). The final quantified libraries were pooled in equimolar amounts for sequencing on one lane, according to the MiSeq protocol, and single libraries on four lanes of an Illumina HiSeq 2500 DNA sequencer, utilising a 125 bp single end sequencing flow cell with a HiSeq Reagent Kit v4 (Illumina, USA). Flow cell cluster generation for the HiSeq2500 was performed using an automated cBot system (Illumina, USA). The Real Time Analysis (RTA), version 1.18.64 software package was used to generate raw bcl, or base call files, which were then de-multiplexed by sample into fastq files for data submission using bcl2fastq2 software version v2.16.0. The raw fastq files were deposited in the Sequence Read Archives (SRA) of the National Center for Biotechnology Information (NCBI) under accession number SRP070144 of Bioproject PRJNA311553 and Biosample SAMN04485566.

Bioinformatics Analyses. Assembly of all reads was carried out as described previously using the Abyss and Soapdenovo-Trans assemblers with every o kmer ending in 1 and 5 (-k program switch) from 21 to 95[15]. Resulting contigs were re-assembled by a pipeline of blastn and a cap3 assembler. Coding sequences were extracted based on blastx results deriving from several database matches, including a subset of the non-redundant protein database of the NCBI containing tick and other invertebrate sequences, as well as the Swissprot and Gene Ontology (GO) databases. Open reading frames larger than 150 nt were also extracted if they had a signal peptide indicative of secretion, as evaluated by version 3.0 of the SignalP program. Reads from each library were mapped back to the CDS by blastn with a word size of 25 and allowing one gap. Reads were mapped up to a maximum of five different CDS if the blast scores were the same for all matches. The edgeR program was used in ancova mode to detect statistically significant differentially expressed genes according to time or diet variables[82]. EdgeR inputted the read matrix for genes having at least one library expressing an FPKM (fragments per thousand nucleotides per million reads) equal or larger than 10. For heat map display of the CDS temporal expression, Z scores of the FPKM values were used. Heatmaps were produced using gplots and heatmap.2 programs with R[82]. All deduced coding sequences and their reads are available for browsing in hyperlinks to several databases (Source data 1).

Gene ontology analysis. To identify Gene ontology (GO) terms that are differentially expressed in the set of differentially expressed genes, we normalized the RPKM reads for each transcript by dividing them by the average RPKM of the 16 values, and then submitted the pooled values for each of the 4 experimental groups for Kruskal-Wallis analysis (using the R package). The resulting p values were corrected for multiple testing using the Bonferroni implementation in R. Of the 2,685 differentially expressed genes, 1,506 had a match to a GO functional annotation (E-value ≤1e-15) leading to the characterisation of 1,047 transcripts associated with 312 GO functions that were significantly different among the 4 treatments. A total of 1,521 differentially expressed genes matched GO sequences having a component annotation (E-value ≤1e-15). Of these, 1,266 sequences were

significantly associated with 108 GO component terms. Finally, 1,530 transcripts matched GO sequences (E-value ≤1e-15) having a process annotation. Of these, 926 had statistical significance, grouping into 328 processes.

cDNA synthesis, and reverse transcription-quantitative PCR (RT-qPCR). cDNA preparations from tissues were made in independent triplicates and served as templates for subsequent quantitative expression analyses by RT-qPCR. Samples were analysed using a LightCycler 480 (Roche) and Fast Start Universal SYBR Green Master Kit (Roche). Each primer pair (for the list of qPCR primers, see Supplementary Table S3) was inspected for its specificity using melting curve analysis. Relative expressions were calculated using the ΔΔCt method. The expression profiles from adult *I. ricinus* female tick tissues were normalised to *actin* or *ef-1α*.

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Author Contributions

J.Pe. designed and conducted the experiments and wrote the manuscript, J.Pr. assisted in bioinformatics analyses, J.S. conducted the scanning electron microscopy, V.U. assisted in sample preparation, J.M.R. performed the bioinformatics analyses and co-wrote the manuscript, P.K. conceived the study and co-wrote the manuscript. All authors read and reviewed the manuscript.

Additional Information

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Manuscript in preparation II

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GST as a putative endogenous haem-binder in tick gut.

Glutathione S-transferase as an endogenous haem-binder in the tick gut

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Abstract

The tick *Ixodes ricinus* was shown to lack a functional haem biosynthetic pathway. To go around that, ticks acquire haem that is released upon intracellular haemoglobin hydrolysis in tick digest cells. Even though a fraction of the acquired haem is further trafficked across the tick body, the vast majority of intracellular haem is detoxified in the tick midgut. Using comparative RNAseq analyses of the tick midguts between blood- and serum-fed ticks, several transcripts were shown to be elevated in response to the presence/absence of dietary red blood cells. Glutathione S-transferase (GST) transcript was shown to have substantially enriched in the gut of blood-fed ticks, while transcript levels were marginal in the gut of serum-fed ticks. To gain an insight into the biological role of this protein, a recombinant version was characterised *in vitro*. The recombinant GST is expressed into a soluble fraction of *E. coli* and is active with model substrate for glutathione conjugation. The recombinant protein showed haem-binding capacity *in vitro* using VIS-absorbance, enzymatic activity assays, and displayed high affinity binding to haemin agarose. RNAi analysis, substantially decreased GST expression in tick midgut but showed no clear impact on tick feeding physiology.

Introduction

Ticks are blood-feeding arthropods that infest on cattle and men globally. They undergo a life cycle of larvae, nymphs, and adults each of which requires one blood meal as the only source of nutrients. Adult females, especially, imbibe large quantities of host blood exceeding up to hundred times their unfed weight. The host serum and red blood cells proteins are internalised by tick midgut (Lara, 2005) and hydrolysed in endo-lysosomal system (Sojka et al., 2013). Haemoglobin degradation thus inevitably leads to release of vast amounts of haem within tick digest cells. In the tick *Rhipicephalus microplus*, it was suggested that liberated haem is transported from digestive vesicle to cytosol of digest cell by an ATP-binding cassette (ABCB10) (Lara et al., 2015). The next fate of cytosolic haem has not yet been described.

The tick *Ixodes ricinus* has been shown to have lost genes encoding both haem biosynthetic apparatus and haem degradation enzyme, haem oxygenase (Perner et al., 2016b). Ticks thus need to acquire haem only from host haemoglobin (Perner et al., 2016b). Intracellular haem, therefore, cannot be further catabolised and need to complex with binding partners in order to aggregate as a means of detoxification or further dispatched for systemic inter-tissue distribution. To test, which transcripts change their levels in response to haemoglobin in form of dietary red blood cells, midguts of blood- and serum-fed ticks were subjected to RNAseq analyses. Transcript encoding a delta glutathione S-transferase (GST) was found to be substantially enriched in midguts of blood-fed ticks (Perner et al., 2016a).

Delta GST appears to be one of the major detoxification enzymes in the tick midgut (Perner et al., 2016a). GSTs from other organisms were shown to interact with lipid peroxidation products, haem-related molecules, as well as chemotherapeutic agents (Brophy et al., 1990; van Rossum et al., 2004; Zhan et al., 2005). In the present study, kinetic parameters of recombinant tick GST and haem binding capacity was investigated *in vitro*. Whether this enzyme is essential for tick survival by binding excess haem in tick digest cell cytosol was assessed by RNA interference.

Results and Discussion

Preparation of rGST and production of antibodies

To elucidate a function of the protein, we have expressed a His-tagged recombinant version in *E. coli* expression system (rGST). The overexpression was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Even though some leaky expression was noticed without IPTG induction, a clear signal around a predicted size 29.6 kDa was detected in induced cells. An electrophoretic separation *E. coli* soluble extract showed an apparent band in induced culture as evidenced by Coomassie-stained SDS-PAGE as well as by His-probing of blotted samples (Fig. 1A). The protein was purified by Ni^{2+}-IMAC chromatography under native conditions (Fig. 1A). The purified protein was active with a model 1-chloro-2,4-dinitrobenzene substrate (CDNB). A substantial enrichment in specific CDNB-activity was observed in eluted fraction indicating a yield of pure active protein. Even though the protein failed to purify exploiting endogenous affinity towards glutathione affinity columns, glutathione or S-hexyl glutathione sepharose (Fig. S1), the His-tag exploiting purification resulted in a clear and straightforward purification procedure yielding high amounts of active protein. The pure protein was then used for recombinant protein characterisation and polyclonal antibody production in rabbit.

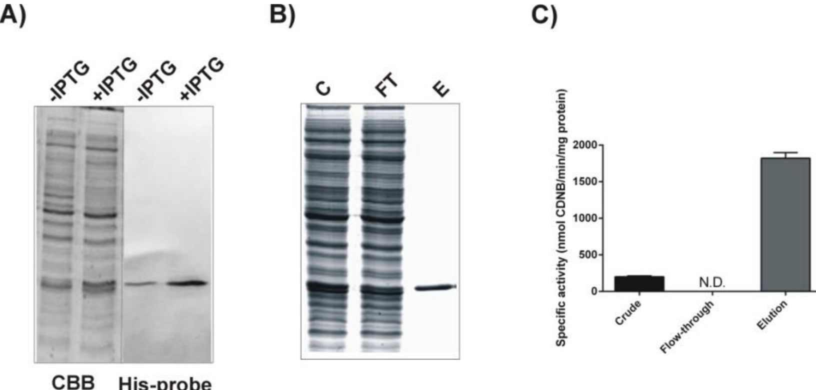

Figure 1. Over-expression induction, Ni^{2+}-IMAC purification, and activity measurement. *E. coli* BL21 cells were induced by 0.1 mM IPTG. **(A)** Crude homogenates were separated by reducing SDS-PAGE and were probed for His-tag. **(B)** The induced cell homogenate was then applied to native Ni^{2+}-IMAC affinity chromatography and the outcome evidenced by SDS-PAGE. **(C)** Fractions were then subject to activity assay utilising a 1-chloro-2,4-dinitrobenzene (CDNB) as a model substrate. Mean and SEM are shown, n = 3. CBB - Coomassie Brilliant Blue, C - crude extract, FT - flow through, E - elution.

Haemin-induced expression in the tick gut

We have recently identified a blood induced expression of a transcript encoding Glutathione S-transferase (*Ir*GST) in the midgut of the tick *Ixodes ricinus*. To confirm that haem moiety, and no other component of red blood cell, induces the *Ir*GST transcription, we have conducted an artificial membrane feeding with serum supplemented with haemoglobin or haemin itself. We have revealed, by RT-qPCR, that *ir-gst* is up-regulated by dietary haemoglobin and haemin (Fig. 2A). Also, its protein product, *Ir*GST, as evidenced by Western blotting (Fig. 2B, C), is upregulated by dietary haemin. A clear correlation between *Ir*GST expression and haemin dietary levels was observed (Fig. 2C).

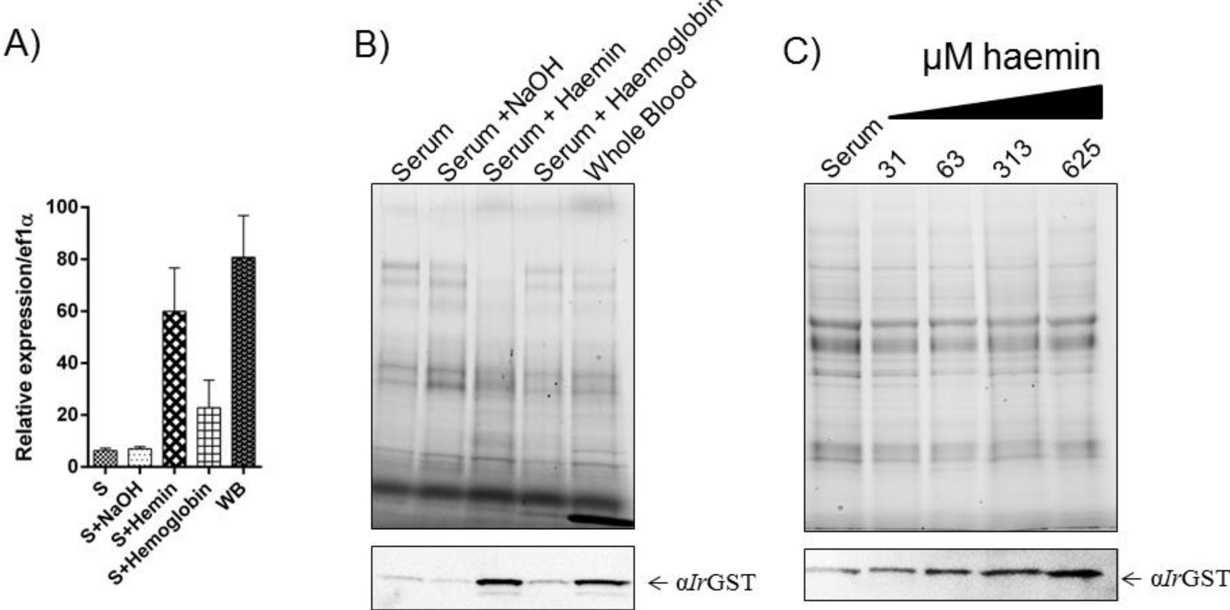

Figure 2. Analysis of *Ir*GST expression by RT-qPCR and Western blotting. To test if haemin specifically induces the expression of *Ir*GST, we have analysed its expression in the tick midgut. **A)** RT-qPCR analysis of tick midguts fed for 5 days on serum and serum supplemented with appropriately diluted NaOH (haemin solvent), 37.5 μM haemin, 1% haemoglobin (equimolar haem concentration), or whole blood. Expression data are normalised against elongation factor 1 alpha. Shown data represents three biological replicates. Mean and SEM is shown. **B)** Midgut homogenates of ticks fed for 5 days on serum and serum supplemented with NaOH (haemin solvent), 37.5 μM haemin, 1% haemoglobin (equimolar haem concentration), or whole blood were separated on reducing SDS-PAGE and Western Blot analysis was performed using specific rabbit anti-serum (1:5000). **C)** Midgut homogenates of ticks fed for 5 days on serum and serum supplemented an increasing amounts of dietary haemin supplementations were separated on reducing SDS-PAGE and Western Blot analysis was performed using specific rabbit anti-serum (1:5000).

Recombinant protein expression, purification, and substrate profiling

To reveal the kinetic parameters of the recombinant *Ir*GST, assays with typical GST substrates were carried out and compared to a recombinant GST of a fluke *Fasciola gigantica*. GSTs display a wide range of enzymatic activities, each of which is represented by a model substrate enabling to get insight into a potential enzymatic activity with natural substrates *in vivo* (Brophy et al., 1990). It was shown that recombinant *Ir*GST display GSH-conjugating activity using CDNB as a model substrate (Fig. 3A) as well as peroxidase activity with the model lipid hydroperoxide substrate cumene hydroperoxide (Fig. 3C), while no activity towards reactive carbonyles was detected using trans-2-nonenal as a model substrate (Fig. 3B). We have noted, however, high inhibitory properties of haemin towards GSH-conjugating activity of *Ir*GST (Fig. 3D).

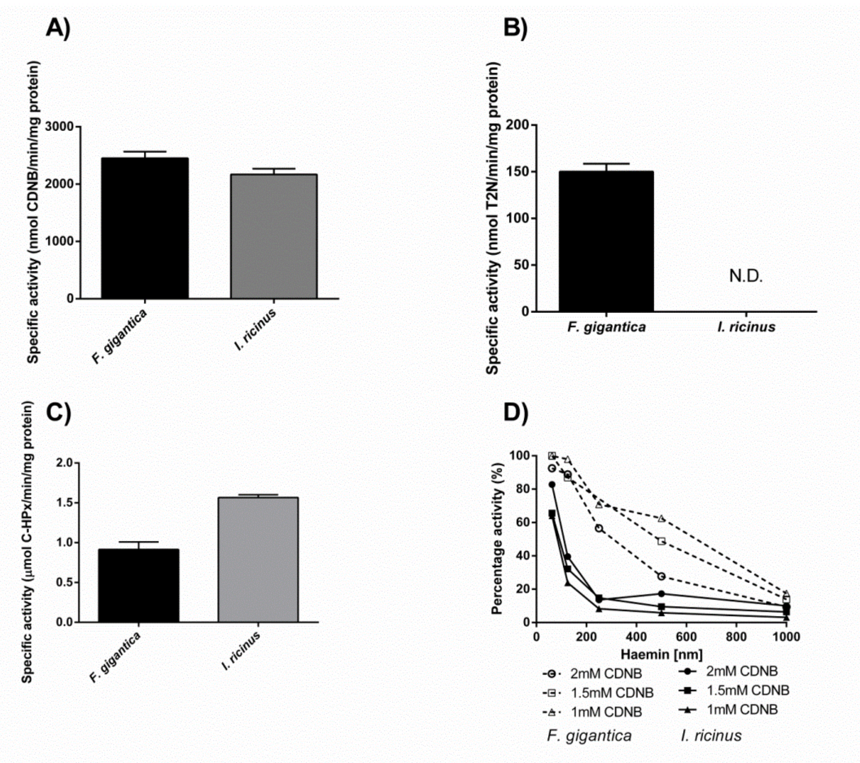

Figure 3. Comparative substrate profiling. Recombinant GST proteins of *Fasciola gigantica* and *Ixodes ricinus* were tested for specific activities in spectrophotometric assays using model GST substrates. **A)** 1-chloro-2,4-dinitrobenzene (CDNB) was used to test GSH-conjugation activity. **B)** Trans 2-nonenal (T2N) was used to test GST-mediated reactions with reactive carbonyls. **C)** Cumene Hydroperoxide (C-Hpx) was used to test peroxidase activity. **D)** Inhibition assays of haemin on CDNB activity of *F. gigantica* and *I. scapularis* GST.

Haem binding assays

To determine the haemin-binding properties of the recombinant *Ir*GST, VIS-spectrophotometry was used. While haemin alone had an absorption maximum around 385 nm, equimolar mixture of haemin and recombinant *Ir*GST exhibited a red-shifted absorption maximu to λ = 422 nm (Fig. 4A). To examine the binding capacity of recombinant *Ir*GST to haemin, different molar ratios were prepared to identify the point of saturation of around λ = 421 nm. It was shown that recombinant *Ir*GST display 1:1 binding with haemin (Fig. 4B), indicating that one GST molecule may bind only one haem also *in vivo*. To determine if haem-related compounds have any effect on CDNB activity, different tetrapyrroles in a concetration range were used in a CDNB activity assay. It was shown that free haemin-chloride and haematin-hydroxide inhibited the GST activity with apparent IC_{50} around 200µM haemin or haematin (Fig. 4C). Iron-free Protoporphyrin IX (PPIX) did not induce inhibition suggesting that the iron ion participates in the inhibition (Fig. 4C). Also, myoglobin-bound haem did not induce any inhibiton suggesting the requirment of free haem availibility for the GSH-haem binding to happen (Fig. 4C). To gain a more accurate inhibition constant of haemin (Ki) a series of different substrate (CDNB) concentrations was used a resulting inhibited activities were plotted using a Dixon plot (Fig. 4D). Ki of around 42 nm haemin indicates a strong binding affinity of *Ir*GST.

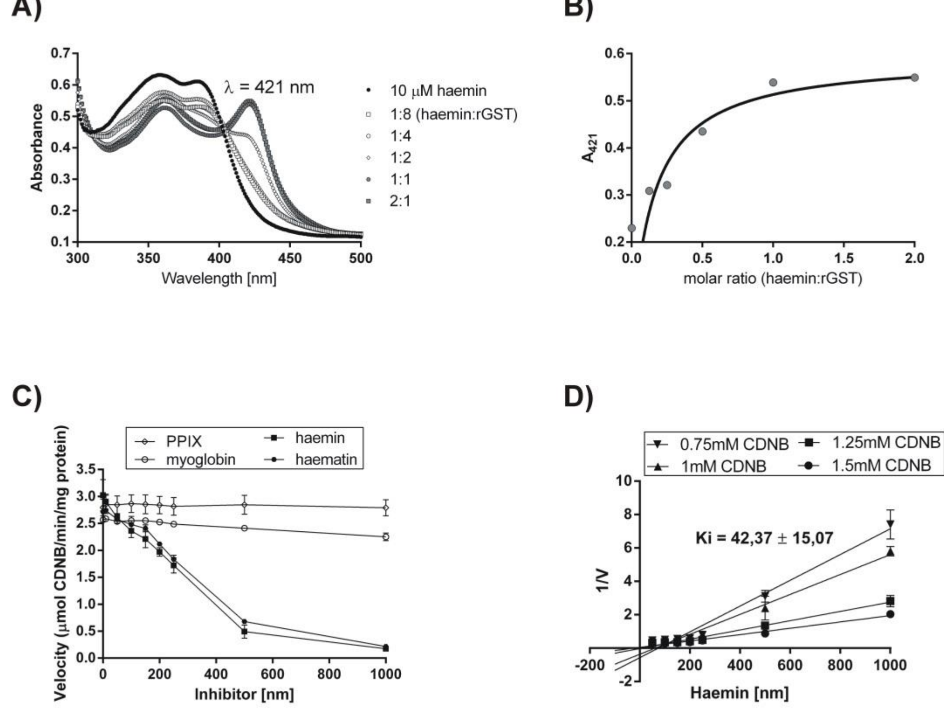

Figure 4. Assessment of haemin binding by VIS-spectrophotometry. To characterise the haem binding properties of recombinant GST (rGST), the characteristic absorbance properties of haemin were exploited. **A)** Titration of *Ir*GST was carried out in a range of molar ratios with haemin. Haemin was kept constant at 3 µM. **B)** A plot of absorbance values in relation to molar ratios of haemin and rGST. **C)** CDNB assays testing inhibitory properties of equimolar concentrations of haemin, haematin, protoporphyrin IX (PPIX), and myoglobin. **D)** Dixon plot of inhibitory properties on GST activity under different substrate (CDNB) concentrations. Calculated inhibitory constant is indicated (nM).

Haemin-agarose pull-down

To further support evidence of haem binding of the *Ir*GST, *E. coli* crude extract was incubated with haemin-agarose and bound proteins were eluted by series of a urea wash. Haemin agarose depletion of the recombinant protein was evidenced by Bradford assay (Fig. 5A), CDNB-specific activity (Fig. 5B), SDS-PAGE (Fig. 5C) and mass spectrometry (Fig. 5D). The fact that a tick haem-binding protein can be enriched from a bacterial crude preparation supports the prospect of purifying other tick haem-binding proteins from cellular fractions of tick tissues.

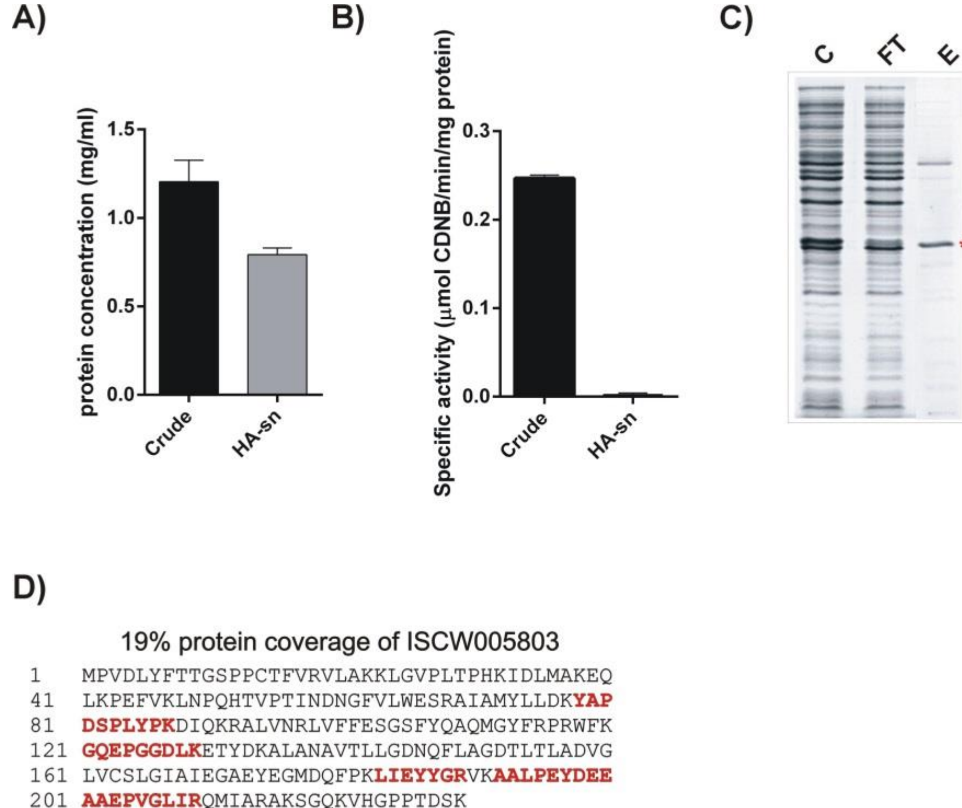

Figure 5. Haemin-agarose affinity chromatography. Induced *E. coli* homogenate was subjected to affinity pull-down by incubating the homogenate with haemin-agarose (HA) beads. **A)** Bradford assay indicating a substantial loss of a protein in a supernatant (sn) after the incubation. **B)** CDNB activity of the supernatant after the incubation. **C)** SDS-PAGE of the crude *E. coli* homogenate, the flow through, and elution (1M urea). An asterisk indicates a band excised for mass-spectrometry finger-printing. **D)** GST protein sequence with highlighted sequences (in red) received after tryptic digests and mass-spectrometry. CBB - Coomassie Brilliant Blue, C - crude extract, FT - flow through, E - elution.

RNAi analysis

To study a physiological role of *Ir*GST *in vivo*, a knock-down of this transcript was obtained using RNAi. Using a specific anti-serum against recombinant *Ir*GST, we proved a clear downregulation of *Ir*GST in tick midgut throughout the feeding was shown by Western Blotting (Fig. 6A). Weights of adult *I. ricinus* ticks did not deviate in received weights throughout the feeding compared to control (Fig. 6B).

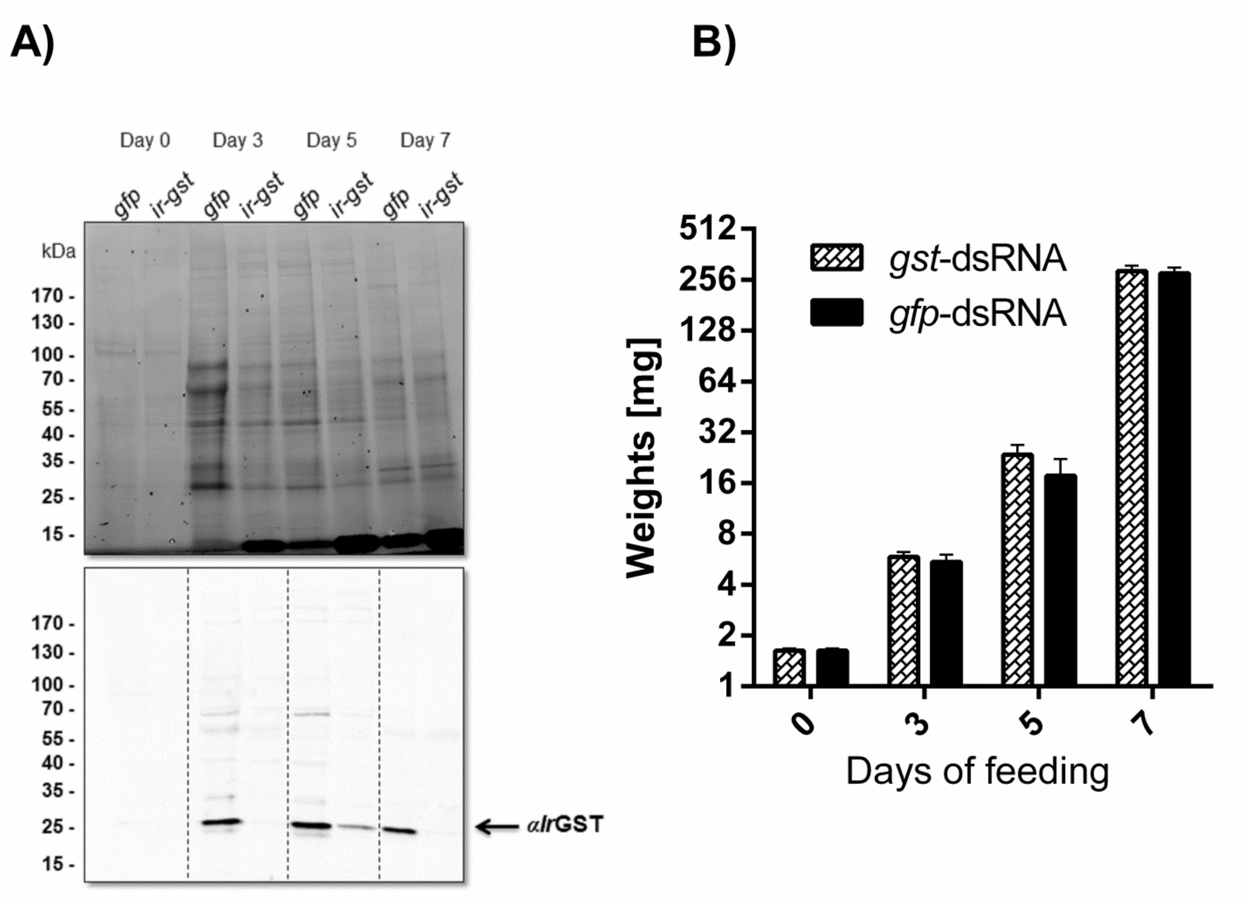

Figure 6. RNAi analysis. Adult *I. ricinus* ticks were injected *gst* or *gfp* (control) dsRNA and rested for 24 hours (Day 0). Ticks (n = 25) were then placed on a rabbit and allowed to fully engorge. Under indicated time-points (Day 3, Day 5, and Day 7) ticks were forcibly removed, weighed out, and their midguts dissected ($n \geq 3$). **A)** Tick midgut homogenates were separated by reducing SDS-PAGE and GST expression was analysed by Western blotting using specific rabbit anti-serum (1:5000). **B)** Column graph of tick weights before feeding and from individual time-points of feeding. Mean and SEM is shown.

Material and Methods

Heterologous expression in E. coli and purification

Tick GST was amplified from Day 3 gut specific cDNA using Ir-114935 specific primers (Perner et al., 2016a). PCR reaction was separated on 1% agarose gel and a corresponding band of ~580bp was excised. DNA was extracted from a gel using Gel extraction kit (Roche) and the insert was cloned into a pet100 vector (Invitrogen) following manufacturers' protocol. Plasmid was transformed into TOP10 *E. coli* cells and positive clones were selected on ampicillin LB plates. Colony PCR was used to verify positive clones. Two positive clones were cultured in 400 ml LB^{amp} medium to yield high plasmid yield. Plasmids were isolated using midi-prep (Roche) and sequences verified. A positive clone was transformed into *E. coli* BL21 Star (DE3) chemically competent cells (Invitrogen) and transformation efficiency was verified. Cells were grown in LB^{amp} medium at 37°C and when reached OD = 1.6, the culture was supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (final concentration). The cells were cultured overnight and the harvested cells were suspended in PBS and homogenised using sonication cycles. Using a 2-ml Ni^{2+}-IMAC bed, 25 ml of cellular homogenate was applied on the matrix. The resin was washed with 20 bed volumes of 20 mM phosphate buffer pH = 6.0, 500 mM NaCl, 20 mM imidazol, 10% glycerol and 0.5% Triton X-100 (v/v) to remove unspecifically bound proteins. The recombinant was then liberated from the resin by 100 mM imidazol.

Tick maintenance and natural feeding

Ticks were kept at 24 °C and 95% humidity under a 15:9-hour day/night regime. Adult *I. ricinus* were also collected in the wild near Ceske Budejovice. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 357 095/2012. The study was approved by the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (CAS) and Central Committee for Animal Welfare, Czech Republic (protocol no. 1/2015).

Tick membrane feeding in vitro

Membrane feeding of ticks in vitro was performed in feeding units manufactured according to the procedure developed by Kröber and Guerin (Kröber and Guerin, 2007). Whole bovine blood was collected in a local slaughter house, manually defibrinated and supplemented immediately with sterile glucose (0.2% w/vol). To obtain serum, whole blood samples were centrifuged at 2 500 × g, for 10 min at 4°C and the resulting supernatant was collected and centrifuged again at 10 000 × g, for 10 min at 4°C. Diets were then supplemented with 1 mM adenosine triphosphate (ATP) and gentamicin (5 µg/ml), pipetted into the feeding units and regularly exchanged at intervals of 12 hr. For feeding, fifteen females were placed in the feeding unit lined with a thin (80–120 µm) silicone membrane, previously pre-treated with a bovine hair extract in dichloromethane (0.5 mg of low volatile lipids) as described (Kröber and Guerin, 2007). After 24 hr, unattached or dead females were removed and an equal number of males were added to the remaining attached females. For diet supplementation, pure bovine haemoglobin (Sigma) and haemin (Sigma) was used. Haemin was dissolved in 100 mM NaOH and 62.5 mM haemin stock was prepared. Haemin was diluted down 37.5 µM equalling a haem equimolarity with 1% haemoglobin (w/v).

Tissue dissection and extraction of total RNA

In vitro fed *I. ricinus* females were forcibly removed from the membrane on day 6 of feeding. Tick midgut was dissected on a paraplast-filled Petri dish under a drop of DEPC-treated PBS. Total RNA was isolated from dissected tissues using a NucleoSpinRNA II kit (Macherey-Nagel, Germany) and stored at –80°C

prior to cDNA synthesis. Single-stranded cDNA was reverse-transcribed from 0.5 μg of total RNA using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany). For subsequent applications, cDNA was diluted 20 times in nuclease-free water.

Reducing SDS-PAGE, Western blot and His-probing

Tick midgut homogenates were prepared in 1% Triton X-100 in PBS supplemented with a CompleteTM cocktail of protease inhibitors (Roche) using a 29G syringe, and subsequently subjected to three freeze/thaw cycles using liquid nitrogen. Proteins were then extracted for 1 hr at 4°C and 1 200 rpm using a Thermomixer comfort (Eppendorf, Germany). Samples were then centrifuged 15 000 × g, for 10 min at 4°C. Protein concentrations were determined using the Bradford assay (Bradford, 1976). Electrophoretic samples for SDS-PAGE were prepared in reducing Laemmli buffer supplemented with β-mercaptoethanol. Ten micrograms of protein were applied per lane unless otherwise specified. Proteins were separated on 12.5 % polyacrylamide gels in Tris-Glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/vol) SDS, pH 8.3) and visualized using TGX stain-free chemistry (BioRad) or Coomassie staining. Proteins were transferred onto nitrocellulose using a Trans-Blot Turbo system (BioRad). For Western blot analyses, membranes were blocked in 3% (w/vol) non-fat skimmed milk in PBS with 0.05% Tween 20 (PBS-T), incubated in immune serum diluted in PBS-T (α*Ir*GST) and then in the goat anti-rabbit IgG-peroxidase antibody (Sigma) diluted in PBS-T (1:50 000). Signals were detected using ClarityWestern ECL substrate, visualized using a ChemiDoc MP imager, and analysed using Image Lab Software (BioRad). To detect His-tags, a membrane was incubated with HisProbe™-HRP conjugate (ThermoFisher Scientific) 1:5000 for one hour. Signal was visualised using SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific).

Substrate specificities in activity assays

The substrate specificities of the tick GST was tested with natural and model substrates. All enzyme assays were carried out in a UV/VIS Gilford Response spectrophotometer and were measured over 3 min at 25°C. GST enzymatic activity towards the model substrate, CDNB, was assayed in 100 mM potassium phosphate buffer pH 6.5 containing 1mM GSH and 1mM CDNB at 340 nm (Ɛ = 9.6 x 106 cm^{-2} mol^{-1}) according to the method as described in (Habig et al., 1974). GST was also tested for GSH-dependent peroxidase activity according to the method of Jaffe and Lambert, 1986. The assay was carried out in 50mM phosphate buffer pH 7.0 containing 1mM GSH, 0.2mM NADPH, 0.5 U glutathione reductase, and 1.2 mM cumene hydroperoxide. The reaction was measured at 340 nm (Ɛ = 6.22 x 106 cm^{-2} mol^{-1}) over 3 minutes. GST activity towards trans-2-nonenal was determined as described in Brophy et al., 1989. The reaction composed of 100 mM potassium phosphate buffer, pH 6.5, 1 mM GSH, and 0.23 mM trans-2-nonenal. The reaction was measured at 225 nm (Ɛ = -19.2 x 106 cm^{-2} mol^{-1}) over 3 min.

Determination of kinetic parameters and inhibition studies

The apparent Km and apparent Vmax determinations with CDNB as a substrate for GST assays were performed in a triplicate with varying concentrations of CDNB and constant GSH (1 mM), or constant CDNB (1 mM) and varying concentrations of GSH. Kinetic constants were calculated by non-linear regression analysis of the experimentally measured activities. Data were fitted to the Michaelis-Menten equation using GraphPad Prism 6.0 software. The interaction of the recombinant GST protein towards haem-related compounds was investigated by inhibition of CDNB activity assay. Haemin and haematin were dissolved in 100 mM NaOH, PPIX was dissolved in DMSO (10 mg/ml) and further diluted in 100 mM NaOH, and haemoglobin was dissolved in dH_2O.

VIS spectrophotometry

The absorption spectrum properties of hemin were determined by recording the absorption spectra of hemin in the absence or presence of the recombinant in 20 mM sodium phosphate buffer (pH 8.0) with 50 mM NaCl. The molar ratio of hemin to proteins was 1:1. Hemin was incubated for 15 min with the recombinant protein. The spectra were recorded between 300 and 450 nm on a UV-1800 spectrophotometer (Shimadzu).

Haemin affinity chromatography

E. coli soluble homogenate (50 mM Tris, pH = 8.0, 0.5 M NaCl; 1.5 ml) was incubated with 50 µl of commercial haemin-agarose (Sigma) for one hour with slow rotation. Agarose beads were then allowed to settle and supernatant removed. The agarose was then transferred to an empty column (BioRad) and substantially washed. Specifically bound proteins were then eluted with 2 M urea.

Preparation of samples for mass spectrometry

Protein band was excised from Coomassie stained gel using a 1ml-tip. The gel was incubated with 50 mM ammonium bicarboante and 100% acetonitrile (1:1) solution for 15 minutes at 37°C to destain the gel. The gel was dehydrated in 100% acetonitrile for 30 minutes at 37°C. The gel was rehydrated in trypsin solution (100 ng/µl) in ammonium bicarbonate and left for 45 minutes at 8°C and further incubated at 37°C over-night. Supernatant was removed and gel was rinsed several time alternately with acetonitrile and 50 mM ammonium bicarbonate. Samples were then vacuum-dried and resuspended 1% formic acid (w/w).

RNA interference

dsRNA of *ir-gst* and *gfp* were synthesised as described (Hajdušek et al., 2009). Purified linear plasmids served as templates for RNA synthesis using the MEGAscript T7 transcription kit (Ambion, Lithuania). dsRNA (~1 µg in 350 nl) was injected into the haemocoel of unfed female ticks using Nanoinject II (Drummond Scientific Company, Broomall, PA). Control ticks were injected with the same volume of *gfp* dsRNA. After 24 hr of rest in a humid chamber at room temperature, ticks were allowed to feed naturally on guinea pigs. The gene silencing was verified by Western blot analysis.

Supplementary data

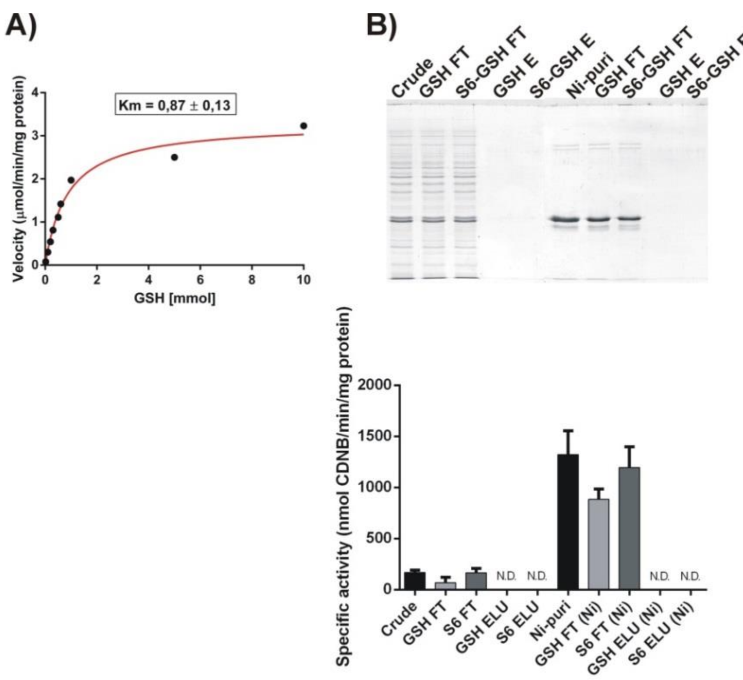

Figure S1. Binding of *Ir*GST to glutathione or S-hexyl glutathione. (A) Activity measurements with titrated glutathione (GSH) concentrations to calculate Michaelis-Menten constant. **(B)** Induced *E. coli* fraction as well as Ni^{2+}-IMAC purified *Ir*GST were subject to glutathione or S-hexyl glutathione (S6) agarose. Individual fractions were examined by SDS-PAGE and activity measurements. Mean and SEM are shown, n =3. FT - flow-through, E - elution, N.D. – not determined with a given detection limit.

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Part III

Molecular targets affecting ticks and transmission of tick-borne pathogens (co-authored articles)

Paper III

Kopáček P, Perner J, Sojka D, Šíma R, Hajdušek O (in press)
Molecular targets to impair blood meal processing in ticks.
Ectoparasites: Drug discovery against moving targets (Wiley-Blackwell).

Paper IV

Hajdušek, O., Šíma, R., Perner, J., Loosová, G., Harcubová, A., Kopáček, P., 2016.
Tick iron and heme metabolism - New target for an anti-tick intervention.
Ticks and Tick-borne Diseases 7, 565-572.

Paper V

Sojka, D., Pytelková, J., Perner, J., Horn, M., Konvičková, J., Schrenková, J., Mareš, M., Kopáček, P., 2016.
Multienzyme degradation of host serum albumin in ticks.
Ticks and Tick-borne Diseases 7, 604-613.

Paper VI

Kopáček, P., Perner, J., 2016.
Vector Biology:
Tyrosine Degradation Protects Blood Feeders from Death via *La Grande Bouffe*.
Current Biology 26, R763-R765.

Preface

Ticks can harm their host directly by infestation (causing anaemias, secondary infections) or by concomitant direct pathogen transmission. Both aspects are sought to be prevented by anti-tick intervention. Two predominant modes of intervention are represented by a) small compound (drug) interaction with tick protein(s) leading to interruption of tick feeding and/or pathogen transmission, and b) by immunological response to a tick antigen, whereby the antibody-antigen complex prevents the antigen exerting its biological activity and ultimately leading to interruption of tick feeding and/or pathogen transmission. To reveal novel proteinaceous targets, RNA interference has a good potential for validation of their indispensability in tick physiology and/or pathogen transmission.

To consolidate the current knowledge of tested or new promising proteinaceous targets in ticks, we have written a book chapter titled Molecular targets to impair blood meal processing in ticks (Kopáček et al., in press) - Paper III in a book called Ectoparasites: Drug discovery against moving targets (Wiley-Blackwell). Not only modes of actions are discussed, but also the potential of tick tissues at the feeding interface (saliva or midgut) for target/antigen “fishing” is considered.

In the study by (Hajdušek et al., 2016) - Paper IV, selected proteins in the haem/iron network were validated using both RNAi experiments and vaccination trials. None of the selected targets reached desired phenotypes or protectivity. In a study by (Sojka et al., 2016) - Paper V, digestive apparatus of host serum albumin in tick midgut was described (Sojka et al., 2013). The rationale of the study stemmed from the fact that, in the *R. microplus* ticks, it was shown that the acquisition route of albumin is different to acquisition route of haemoglobin (Lara et al., 2005). Differences at molecular levels in protein hydrolysis were thus expected. It was shown, however, that serum albumin is digested under acidic conditions by identical papain-type peptidases as host haemoglobin. The absence of dietary red blood cells did not substantially change the transcript levels of selected proteases or the activities of the respective enzymes.

It has been recently reported that blood-feeding arthropods display specific sensitivity to tyrosine accumulation contrary to their non-haematophagous counterparts (Sterkel et al., 2016). We have provided a short dispatch paper (Kopáček and Perner, 2016) - Paper VI to the

original work focused on *Rhodnius prolixus*, *Aedes aegypti*, and *R. microplus*. Verification of essentiality, using RNAi, of endogenous tyrosine degradation pathway in *I. ricinus* is currently ongoing.

Paper III

Kopáček P, Perner J, Sojka D, Šíma R, Hajdušek O (in press)
Molecular targets to impair blood meal processing in ticks.
Ectoparasites: Drug discovery against moving targets (Wiley-Blackwell).

Molecular targets to impair blood meal processing in ticks

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Abstract

Feeding and digestion of host blood are key physiological processes providing essential nutrients for the development and fecundity of ticks. Ingested host blood, which exceeds the weight of unfed females by more than one hundred times, is concentrated and stored in the tick gut lumen, gradually being taken up by digestive cells, and intracellularly digested by a multi-enzyme network of acidic aspartic and cysteine endo- and exo-peptidases. Digestion of hemoglobin, the major protein component of blood, results in the release of a vast excess of potentially toxic heme. In most eukaryotic cells, heme and iron homeostasis is based on a balanced flux between heme biosynthesis and heme degradation, mediated by heme oxygenase. In contrast, ticks are not capable of synthesizing or degrading heme. Therefore, ticks have evolved specific molecular mechanisms of heme and iron acquisition, detoxification, intracellular trafficking, and inter-tissue transport. This chapter reviews current knowledge on the molecular mechanisms of these processes and discusses their potential as targets for anti-tick interventions.

Introduction

Ticks (Acari, Ixodida) are ectoparasites that have adapted to an obligate blood-feeding lifestyle (hematophagy) during their evolution from ancestral mites. About nine hundred species have been described and these are divided into two major families – the hard ticks (Ixodidae, ~ 700 species) and soft ticks (Argasidae ~200 species) [1, 2]. Of special interest is a monotypic family, *Nuttalidae*, represented by a single species *Nuttaliella namaqua* that is hypothesized to represent an evolutionary link between these two families [3, 4]. Ticks of both families are dangerous vectors of a wide variety of pathogens, causing severe infectious diseases of humans as well as wild and domestic animals [5]. Although hard and soft ticks may have followed independent paths towards hematophagy [6] and differ in many physiological and developmental aspects [7], they still share several common traits that offer targets for rational and specific tick control. Major physiological differences in feeding strategies of soft and hard ticks can be summarized as follows: In soft ticks, the larvae, several nymphal stages and adults of both sexes take their blood meal rapidly by feeding on the host for up to one hour. In adult females, feeding and oviposition are cyclic processes, and feeding performance is not necessarily related to mating status. In contrast, hard ticks feed only once per life stage (larva, one nymphal stage, and adult) for long periods of several days. An adult, mated female can engorge a huge amount of host blood that exceeds more than one hundred times the weight of the unfed tick. The substantial amount of imbibed blood is then digested and converted into a large number (several thousands) of laid eggs, following which the hard tick female dies.

What all ticks noticeably have in common is the process of blood meal digestion that clearly distinguishes ticks from their vertebrate hosts as well as from blood-feeding insects that have digestive apparatus based on neutral or alkaline serine proteases. In contrast, ticks digest blood intracellularly in the acidic lysosome-like vesicles of digestive cells lining the midgut epithelium [7]. The multi-enzymatic machinery of acidic aspartic and cysteine peptidases involved in blood meal processing in ticks resembles the digestive system in other, evolutionary distant blood-feeding parasites, such as the malaria-causing *Plasmodium*, flatworms or nematodes [8].

The blood meal, the ultimate source of nutrients for all tick developmental stages, is a rather unhealthy diet that required ticks to evolve specific adaptations to cope with excessive amounts of blood. Hemoglobin, the major protein constituent (accounting for about 60% of total blood proteins), has been assumed to be the main source of amino-acids for tick proteosynthesis and vitellogenesis. Digestion of hemoglobin brings about the release of its prosthetic group – heme, which, when unbound, is toxic through its involvement in the formation of free oxygen radicals via the Fenton reaction [9]. One adult hard tick female, such as *Ixodes ricinus*, can imbibe and concentrate up to 1 ml of host blood containing ~150 mg of hemoglobin, out of which heme comprises 6 mg. In the case of complete degradation of acquired heme, about 500 μg of ferrous iron would be released. These extremely large amounts of pro-oxidative molecules would clearly represent a lethal burden for a ~2 mg organism, as is the case of the unfed female tick. This simple consideration indicates how

important in tick physiology are efficient “waste management” strategies for superfluous blood-meal components.

In the majority of eukaryotic organisms, heme and iron homeostasis is based on balancing the flux between heme biosynthesis and its degradation, mediated by heme oxygenase. An earlier biochemical study on the cattle tick *Rhipicephalus (Boophilus) microplus* [10] and a recent genome-wide analysis of *Ixodes scapularis* genome [11] revealed that at least hard ticks lack a functional pathway for biosynthesis of endogenous heme [12]. Ticks have retained only the last three mitochondrial enzymes (coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase) out of seven enzymes that make up the canonical heme-biosynthetic pathway that was present in tick mite ancestors [12]. The most likely explanation for the loss of heme biosynthesis during tick evolution is their exposure to a vast surplus of heme originating from digested host hemoglobin. On the other hand, the inability to synthesize endogenous heme makes ticks completely dependent on the acquisition of host-derived heme for use as a prosthetic group for proteosynthesis of their own hemoproteins. Besides lacking heme biosynthesis, ticks are also unable to degrade heme as the gene coding for the key enzyme – heme oxygenase (HO), is apparently not present in their genome [11]. This trait, however, does not seem to have evolved as an adaptation to hematophagy, since HO is also absent from the genomes of other non-hematophagous mites and possibly also chelicerates [12].

Taken together, the physiological processes of ticks associated with digestion of huge amounts of blood meal, elimination of waste products and excessive water, lack of heme biosynthesis and catabolism, and unique mechanisms for acquisition and inter-tissue distribution of heme or iron, all constitute a major departure from their vertebrate hosts (Figure 1). There are basically two ways to effectively target tick Achilles’ heels: ‘anti-tick’ vaccines and acaricides. The development of effective ‘anti-tick’ vaccines or highly selective and environmentally friendly acaricides depends on the discovery of suitable protective antigens or distinctive traits in tick physiology. This effort comprises a variety of strategies including the rational approach (the primary focus of this chapter) as well as high-throughput approaches such as functional genomics, vaccinomics and tick-host-pathogen interactomics [13-15]; anti-tick vaccines are discussed further in Chapter xx of this volume.

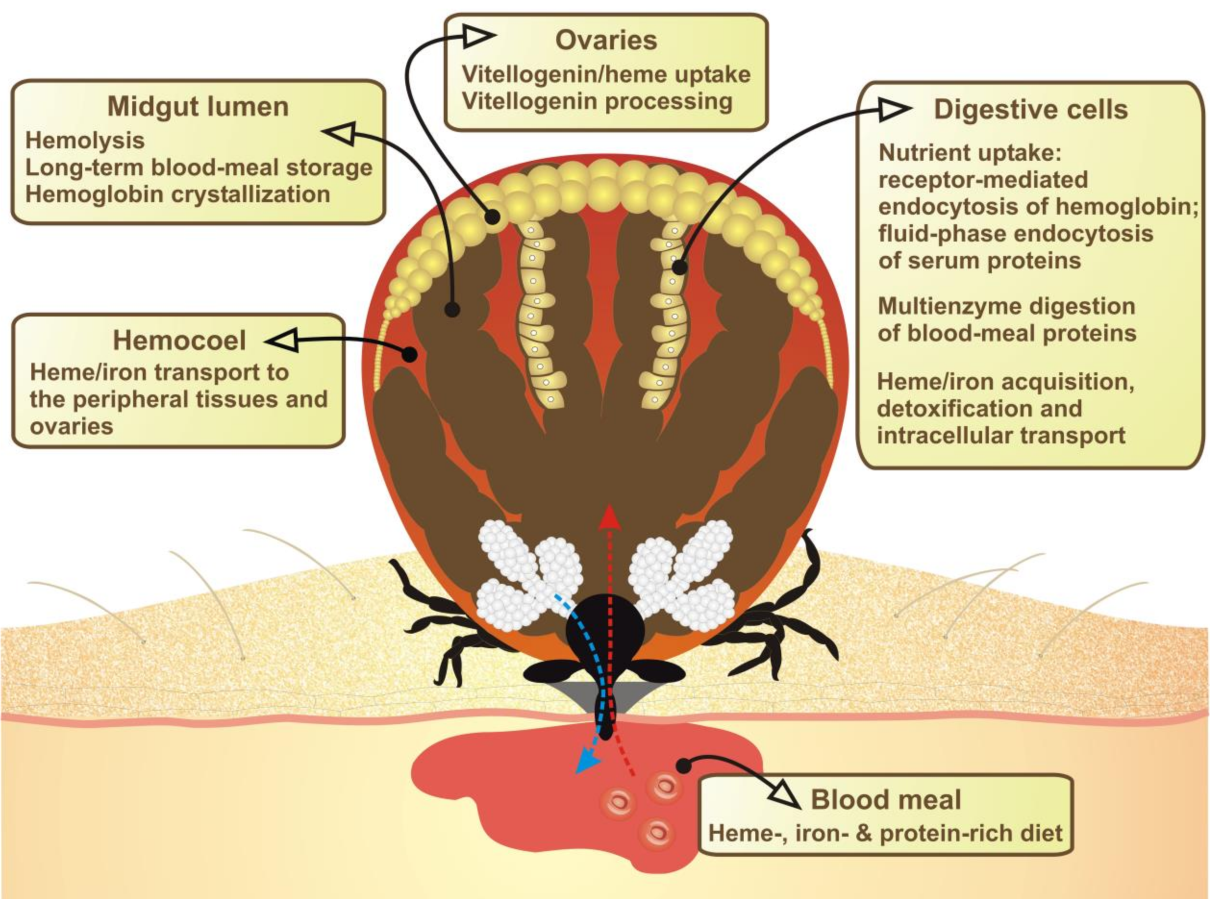

Figure 1 Physiological processes associated with blood meal processing in ticks. A schematic overview of tick tissues and related processes of blood meal uptake, digestion, heme and iron metabolism, detoxification and inter-tissue transport that may serve as rational targets for 'anti-tick' intervention. The red and blue arrows indicate blood meal uptake and reverse water secretion, respectively.

Processes associated with blood feeding and digestion

Attachment and on-host feeding

Blocking of tick attachment to the host and impairing their long undisturbed feeding is intuitively the most straightforward 'anti-tick' strategy. Therefore, a focus on the tick-host interface and the role of tick saliva components in modulation of host hemostasis, inflammation and innate (complement-based) or acquired (antibody-based) immunity represents the largest and most explored field of tick research. However, it is out of the scope and aim of this chapter to cover the complexity of bioactive factors present in tick saliva and we can only refer to several recent comprehensive reviews that deal with this extensive area of research [14, 16-18].

Nevertheless, identification of suitable targets in the rich cocktail of compounds secreted by the tick salivary glands is quite problematic given a high redundancy of multi-genic protein families [14]. The molecules present in tick saliva come into contact with the host immune system (exposed antigens) and should be capable of eliciting an antibody response. Yet the counter-action of tick immune modulators usually suppresses the host's ability to prevent or reduce tick attachment and feeding during repeated exposure to tick infestation [19]. By contrast, blood meal digestion and associated processes located inside the tick are mediated by molecules that are not exposed to the host immune system (concealed antigens). Therefore, tick infestation on a host vaccinated with a recombinant concealed antigen might have a protective effect if the specific antibodies present in ingested blood meal block a physiologically important mechanism. The feasibility of targeting concealed antigens has been successfully tested by the use of the only existing 'anti-tick' commercial vaccine against the cattle tick *R. microplus,* based on the midgut membrane protein Bm-86 [20, 21].

Blood meal uptake

Processes of blood meal uptake and digestion differ significantly between the two evolutionary distinct Argasidae and Ixodidae as well as between the individual developmental stages of hard ticks [7]. However, morphological, tissue, cellular and molecular backgrounds of these processes are best explored for female hard ticks, which are thus mainly discussed in this chapter.

Following attachment to the host, blood meal uptake occurs in two phases: (i) a **slow feeding period** (taking about 6 days post-attachment in the case of *Ixodes* sp. ticks), during which time about one-third of total blood meal is ingested and the tick body size continuously grows; (ii) **rapid engorgement,** occurring 12-24 hours prior to detachment, in which the main portion (about 2/3) of the host blood is imbibed [8, 22]. Rapid engorgement is conditioned by female fertilization that might occur during feeding in the presence of the male on the host. After the fully engorged female drops off the host, the ingested blood meal is gradually digested and the pool of free amino acids is mainly utilized for synthesis of yolk proteins, – vitellogenins (Vg), that takes place mainly in the fat body and midgut. Following transport to ovaries, vitellogenins are proteolytically processed to vitellins (Vn), the major storage proteins for embryonic development and larval stages [7, 12].

Mating factors

Rapid engorgement of females, conditioned by mating, is triggered by the factor **voraxin,** which is passed from males to females during copulation [23]. *Amblyomma hebraeum* voraxin is composed of two independent peptides, AHEFα and AHEFβ, of MW 16.1 kDa and 11.6 kDa, respectively. Interestingly, rabbits vaccinated with a mixture of recombinant AHEFα and AHEFβ were highly protected against infestation with mated *A. hebraeum* females as only 25% were capable of full engorgement [23]. The vaccination potential of voraxin was also successfully tested against *Rhipicephalus appendiculatus* infested on rabbits immunized with a recombinant homolog of voraxinα from this tick species. Fully engorged *R. appendiculatus* females were reduced in weight by ~40% and also the efficacies of subsequent oviposition and larval hatching were markedly lowered [24].

Secretion of blood meal water

The imbibed blood meal is concentrated and stored in the gut lumen and excessive water is excreted via mechanisms that substantially differ between *N. namaqua*, soft ticks and hard ticks, providing another piece of evidence in support of independent evolution of hematophagy among these tick families [3, 4]. The ancestral mode of secreting excessive water of blood meal origin via the Malpighian tubules was described in the "living fossil" tick *N. namaqua* [4]. In soft ticks, about 40% of blood meal water is secreted via the coxal glands that are, by contrast, absent in hard ticks [7]. Hard ticks are capable of removing 60%-70% of blood meal water by salivation during the rapid engorgement phase. The water is transported from the gut contents via the hemolymph to the salivary glands and its secretion to the host is dependent on the presence of prostaglandin E_2, synthesis of which is specific for the salivary glands of hard ticks [25, 26]. The water channels that allow this massive reverse water flow through the hydrophobic lipidic membranes are named **aquaporins** [27]**.** The potential of aquaporins as anti-tick molecular targets was recently demonstrated for several tick species [28-31]. A vaccine based on the recombinant aquaporin 1 from the cattle tick *R. microplus* (RmAQP1) exerted high protection against this species as only about one third of ticks infested on vaccinated cattle completed feeding [30].

Digestion of blood meal in the tick gut

The entire process of blood digestion takes place in the tick gut, the largest organ (>80%) of the tick body (Figure 1). Blood digestion occurs in the central part of the gut (midgut) that is branched into individual protuberances (caeca). In contrast to blood-feeding insects, the tick gut lumen serves mainly as a storage organ and the host blood is degraded intracellularly in the digestive cells of the midgut epithelium [7, 32]. Blood digestion in ticks is a slow and gradual process that allows for the survival of immature as well as adult ticks for long periods of time (months to years) of starvation. The blood-filled midgut lumen is surrounded by a thin layer of histologically distinguishable epithelial cells and a thin outer layer of muscle fibers. The inner epithelial surface is covered by a peritrophic

matrix (PM) composed of mucopolysaccharides underpinned by a chitin network. PM has been described in several hard and soft tick species and is repeatedly formed in each instar during blood meal ingestion [7]. Therefore, the endogenous tick **chitinase** likely plays an important role in inter-stage molting and turnover of PM. Chitinase cloned and characterized from the hard tick *Haemaphysalis longicornis* is a protein of MW 116 kDa that contains one chitin binding peritrophin A domain and two glycosyl hydrolase family 18 chitin binding domains, further corroborating the role of the enzyme as a chitin hydrolase in the tick life cycle [33]. The potential of chitinase as a bioacaricide component was tested using a recombinant baculovirus expressing *H. longicornis* chitinase, giving promising results in increased tick mortality, especially in combination with the pyrethroid flumethrin [34].

The existing nomenclature of tick gut cells is very inconsistent (gastrointestinal, basal, secretory and other cell types described differently in various tick species), when in reality this may only reflect one type of cell that undergoes asynchronous differentiation during blood meal uptake and digestion. The original basal cells of the intestinal epithelium change to digestive cells that first multiply their proteosynthetic apparatus, then secrete components of the PM on the surface and begin to digest hemoglobin [35]. During preparation for rapid engorgement (see below), the cell filled with condensed heme leaves the epithelial layer and migrates towards the inner intestinal lumen. This phenomenon is not apparent in fully-engorged females where digestion occurs in all intestinal cells [22].

The imbibed host blood is stored in the gut lumen and the major protein component – hemoglobin, is gradually released from lysed red blood cells (RBC). Hemolysis is not merely an osmotic or mechanical destruction of erythrocytes, but more likely a complex biochemical process involving extracellular hemolysins. Hemolytic activity of unspecified origin was described in the midgut of *Ixodes dammini* (nowadays *I. scapularis*) [36]. Later, a multi-domain cubulin-like serine protease from *H. longicornis,* tagged as **HlSP,** was reported to exert hemolytic activity in both *in vivo* and *in vitro* experiments, suggesting its role in initiation of the whole hemoglobinolytic process [37, 38]. Hemoglobin released from RBC of some vertebrates tends to form relatively large protein crystals in the tick gut lumen, most likely as a result of a high protein concentration that follows blood meal water secretion [39]. A physiological rationale for this fascinating phenomenon remains unclear, but could possibly be long-term preservation of hemoglobin as a nutrient source and/or physiological protection against potential risks from excess hemoglobin and released free heme.

The process of uptake and digestion of blood-meal proteins, termed **heterophagy,** in ticks consists of at least two parallel endocytic mechanisms (Figure 2), as elegantly demonstrated by Lara et al. who monitored the intracellular fate of fluorescently labeled hemoglobin and albumin in a primary culture of intestinal cells from *R. microplus* [40]. Serum albumin (and possibly other serum proteins) is non-specifically transported into the population of small acidic vesicles within tick digestive cells by fluid phase endocytosis (FPE), whereas hemoglobin seems to be recognized specifically by as yet unidentified cell surface receptor(s) and transported via clathrin-coated pits to large endosomal

vesicles (receptor-mediated endocytosis, RME) [40, 41]. Intracellular transport and degradation of these two major host blood proteins thus occurs separately (Figure 2). The need for specific recognition and endocytosis of hemoglobin evolved most likely as a response to the toxicity of free heme released upon the enzymatic cleavage of hemoglobin. Ticks digestive cells adopted a mechanism of heme detoxification by heme accumulation and aggregation within specialized membrane organelles – hemosomes [42] (Figure 2). Although hemoglobin seems to be a replaceable source of amino-acids, its absence in the tick diet leads to defects in tick embryogenesis [12]. Hence, molecular identification and targeting of the gut surface localized hemoglobin receptor by a recombinant vaccine has great potential to control tick reproduction.

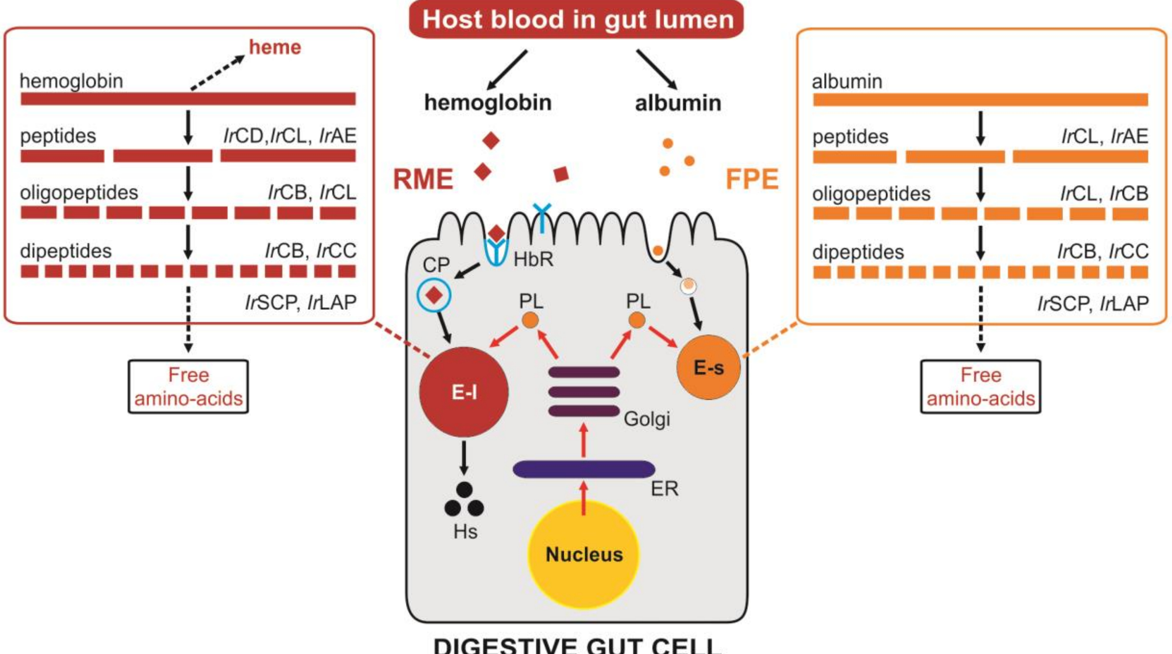

Figure 2 The current model of uptake and digestion of major host blood proteins by *Ixodes ricinus* digestive cells. Red arrows – expressional and secretory pathway of hydrolases involved in protein digestion. Black arrows – endocytic pathways of hemoglobin and serum albumin. **RME** – receptor mediated endocytosis of hemoglobin; **FPE** – fluid phase endocytosis of albumin (and other serum proteins). **HbR** – hemoglobin receptor (putative, yet not identified); **CP** – coated pits, **E-l** – large endosomal vesicles containing hemoglobin; **E-s** – small endosomal vesicles containing dissolved serum albumin [40]; **Hs** – hemosomes containing condensed heme [42]; **Golgi** – Golgi apparatus; **ER** – endoplasmic reticulum; **PL** – primary lysosomes. *Ir*AE – *I. ricinus* legumain/AE; *Ir*CD – *I. ricinus* cathepsin D; *Ir*CB – *I. ricinus* cathepsin B; *Ir*CC – *I. ricinus* cathepsin C; *Ir*SCP – *I. ricinus* serine carboxypeptidase; *Ir*LAP – *I. ricinus* leucine aminopeptidase. For details, see the text.

The previous concept of blood digestion in tick digestive cells by a single "hemoglobinase" was replaced by the current model of multi-enzyme system that comprises a complex of acidic cysteine and aspartic peptidases involved in processing of host hemoglobin [8, 43, 44] and albumin [41]. Complexes comprising clan CA (cathepsin B, L and C), clan CD (asparaginyl endopeptidase - legumain) and clan AA (cathepsin D) peptidases are also present in the unicellular malarial agent, *Plasmodium* sp. as well as in the intestines of parasitic platyhelminthes and nematodes (for review see [8]). Blood digestion based on acidic lysosomal proteases apparently predates the evolution of the pancreas and serine-based extracellular digestion used in most blood-feeding insects such as mosquitoes, fleas, lice, sandflies or tse-tse flies.

Despite spatial separation, digestion of hemoglobin and albumin seems to be performed by the same cadre of peptidases [8, 41, 43]. The initial cleavage of hemoglobin is performed by three endopeptidases, namely cathepsin D (CatD) and cathepsin L (CatL) with a supportive (possibly activating) role of asparaginyl endopeptidase (AE) [43]. By contrast, CatD does not seem to be necessary for the initial cleavage of albumin [41]. The large protein fragments are further cleaved by the endo-peptidolytic activity of cathepsin B (CatB), the most abundant peptidase in the system. The dipeptides are cleaved from the small fragments by the exo-peptidolytic activity of CatB and Cathepsin C (CatC). The free amino acid residues are liberated by monopeptidases, comprising a class of serine carboxypeptidases (SCP) and leucine aminopeptidases (LAP) [8, 43] (Figure2).

Individual enzymes involved in blood digestion were characterized in several tick species. The hard tick *I. ricinus* possesses three **cathepsin D** isoenzymes tagged as *Ir*CD1, 2 and 3, out of which only *Ir*CD1 is solely expressed in the gut tissue of partially engorged females [45]. *Ir*CD1 is auto-catalytically activated from its zymogen upon cleavage of its N-terminal pro-part. The activated enzyme preferentially cleaves hemoglobin as well as synthetic substrates between large hydrophobic amino-acids in the P1 and P1' position [45]. RNAi silencing of *ircd1* gene expression resulted in a substantial reduction of CatD activity in the midgut of semi-engorged *I. ricinus* females. The *Ir*CD2 isoform that is expressed in the gut of fully engorged females (post rapid engorgement) is phylogenetically more related to tick aspartic peptidases *Bm*AP from *R. microplus* [46] and longepsin from *H. longicornis* [47]. *Bm*AP was found to be responsible for generation of heme-derived antimicrobial fragments (hemocidins) that were isolated from the gut of fully engorged *R. microplus* females [46, 48].

Asparaginyl endopeptidases (legumains, AEs) were shown to have a supportive role in the initial phases of hemoglobinolysis and albuminolysis [41, 43]. The *I. scapularis* genome contains nine genes encoding AEs, out of which four seem to be exclusively expressed in the midgut (Hartmann et al., unpublished data). *Ir*AE1from *I. ricinus*, the first ever identified and characterized invertebrate AE, is localized intracellularly in digestive cells as well as extracellularly within the peritrophic matrix covering the midgut epithelium of semi-engorged females [49]. *Ir*AE1 has a strict cleavage specificity

for asparagine at the P1 position and is irreversibly inactivated at pH > 6.0. Functional characterization of two gut-associated legumains, *Hl*Lgm and *Hl*Lgm2 from *H. longicornis* by RNAi displayed a phenotype in gut cell remodeling and reduced tick post-engorgement weight, oviposition and hatching rate [50-52].

A cysteine endopeptidase of **cathepsin L**-type is involved in the initial cleavage of blood meal proteins and is capable of substituting for the activity of *Ir*CD1 in hemoglobin digestion after its specific inhibition [43]. At least three isoenzymes of CatL could be identified in the *I. scapularis* genome [11]. The enzyme responsible for CatL activity in the midgut of *I. ricinus* (*Ir*CL1) is markedly up-regulated during the slow feeding phase, has a very low pH optimum (pH 3-4), and undergoes autocatalytic activation [53], features described also for *Hl*CPL-A, the CatL from the *H. longicornis* midgut [54]. Expression and activity of *Ir*CL1 is markedly reduced in fully engorged females [22, 53] and its role is most likely substituted by expression of the isoform *Ir*CL3 during the off-host digestive phase [55]. The *Ir*CL1 ortholog in *R. microplus* (*Bm*CL1), together with *Bm*AP, was reported to be involved in production of hemoglobin-derived hemocidins [46].

Cathepsin B of the papain family of cysteine peptidases is capable of both endo- and exopeptidase activity. *Ir*CB1 is the most abundant component of the *I. ricinus* digestive apparatus [22, 43, 44]. Western blotting analysis of midgut homogenates using *Ir*CB1-specific antibodies detected the zymogen, prevailing intermediates and active enzyme of ~38 kDa, ~33kDa, and ~31 kDa, respectively [22]. The enzyme activity of the native CatB in the *I. ricinus* midgut extracts has a pH optimum at 5.5-6.0, significantly higher than the pH optima of the initial peptidases *Ir*CD1, *Ir*CL1 and *Ir*AE1. A search of the *I. scapularis* genome, as well as in the rich transcriptomic data from *I. ricinus,* revealed the existence of another two isoforms of CatB, tagged as *Ir*CB2 and *Ir*CB3. Transcripts encoding *Ir*CB1and *Ir*CB2 are expressed more highly in the course of feeding than in the fully engorged females [56]. *Ir*CB3 is orthologous to the *H. longicornis* CatB, termed longipain, which was reported to be involved in blood processing and was shown to exert a babesiacidal effect by killing the midgut stage of *Babesia* parasites in *H. longicornis* [57]. By contrast, expression of *ircb3* mRNA in the *I. ricinus* midgut is marginal and, therefore, the role of this isoenzyme in *I. ricinus* remains unclear [56].

A papain family cysteine peptidase, **Cathepsin C** (aka dipeptidyl-peptidase I), is involved in the terminal phase of blood protein digestion by cleavage of dipeptides from fragments produced by the upstream endopeptidases. Only a single gene encoding CatC is present in the *I. scapularis* genome, and accordingly only one type of transcript could be found in *I. ricinus* midgut transcriptomes [56]. The gene encoding *I. ricinus* CatC (*Ir*CC) zymogen (~50 kDa) is mainly expressed in the tick gut but transcripts are also present in other tissues [44]. The pH optimum of native CatC activity in *I. ricinus* gut extracts is similar to that of CatB, pointing to a pH shift towards neutral values along with the blood meal processing [43].

Monopeptidase activities of **serine carboxypeptidase** and **leucine aminopeptidase** types have been detected in *I. ricinus* midgut homogenates [43]. The serine carboxypeptidase *Hl*SCP1 was shown to liberate free amino acids from the blood meal-derived peptides in the digestive vesicles of *H. longicornis.* The enzyme is induced by blood meal and is active over a broad range of acidic and neutral pHs [58]. The leucine aminopeptidase termed *Hl*LAP from the same tick species, a member of the M17 family of cytosolic aminopeptidases, was found to be mainly expressed in the cytosol of midgut epithelial cells [59]. The transcription of its encoding gene peaks during the post-feeding period [60]. A follow-up study revealed that *Hl*LAP is also localized to the ovarian cells, indicating its role in the supply of free amino acids for the developing oocytes [61]. The pH optimum and cytosolic localization of *Hl*LAP thus support the concept that at least part of the blood meal processing (cleavage of dipeptides to free amino-acids) takes place in the cytosol of digestive gut cells.

Except for the above mentioned RNAi silencing of *hllgm*, *hllgm2* and *longepsin* in *H. longicornis* [51, 57], other attempts to silence genes encoding individual components of tick digestive machinery by RNAi usually did not substantially affect tick fitness and fecundity, despite a clear reduction in transcription, protein content and activity of the targeted enzymes in gut tissue extracts from partially engorged females [45, 53]. Vaccination of laboratory animals with individual recombinant digestive enzymes, as well as with a mixed cocktail of recombinant antigens, did not exert any significant protective effect against *I. ricinus* infestation [13]. The limited potential of targeting the tick digestive apparatus may be explained by redundancies in the system, which was also demonstrated *in vitro* by the specific inhibition of individual enzymes that did not prevent completion of hemoglobinolysis or albuminolysis [41, 43].

Heme detoxification and intracellular transport

Ticks detoxify the majority of heme liberated from digested hemoglobin in the digestive vesicles via its accumulation in specialized, membrane-delimited organelles called hemosomes that were first described from the gut cells of the cattle tick *R. microplus* [40, 42]. Heme detoxification via formation of hemosomes (also tagged as residual bodies in soft ticks [7]) is a process functionally analogous to hemozoin formation in other hematophagous parasites such as the malarial *Plasmodium*, triatominae bug *Rhodnius prolixus* or the flatworm *Schistosoma mansoni* [42]. In contrast to hemozoin, which consists of a crystalline form of heme, the tick hemosomes contain non-crystalline heme aggregates [42].

The mechanism of intracellular heme trafficking within the tick digestive cell is unknown and its depiction in Figure 3 is only putative. In the model heme auxotrophic nematode *Caenorhabditis elegans*, heme acquisition was shown to be mediated by the heme responsive gene (***hrg-1***) [62]. Proteins homologous to HRG-1 were also described in the unicellular, heme auxotrophic parasites of the genus *Trypanosoma* and *Leishmania* [63, 64] which, similarly to ticks, exploit a mechanism of acquisition of exogenous heme via receptor-mediated endocytosis and lysosomal digestion of

hemoglobin. These protozoan parasites transport heme required for synthesis of their endogenous hemoproteins from the endolysosomes to the cytosol via HRG transporters; these present very promising targets for rational development of anti-parasitic drugs [65]. One gene related to *hrg-1* (ISCW001847) was identified in the *I. scapularis* genome [11] and its corresponding ortholog in *I. ricinus* (Gen Bank GEFM01005533) was found to be expressed in ovaries and the midgut of adult females, in all developmental stages [66]. The gene encodes a protein of 204 amino acids, with no signal sequence and one HRG-superfamily domain. RNAi-mediated silencing of putative tick *hrg-1* had no marked effect on the number and weight of females that completed feeding. However, the number of females capable of laying eggs was reduced by 30%, suggesting that functional HRG-1 is involved in tick reproduction. As the conservation of HRG-1 molecules among metazoans is rather low, more detailed characterization and functional studies of putative tick HRG-1 is needed to conclude that this molecule is indeed the transporter of heme from digestive vesicles (endolysosomes) to the cytoplasm of tick digestive cells (Figure 3). Heme detoxification via its transport from the digestive vesicles to the hemosomes has been recently reported to be mediated by the **ATP Binding Cassette** (**ABC**) transporter (Figure 3) that is also involved in detoxification of amitraz, the acaricide used for the control of the cattle tick population [67]. It remains to be examined whether the tick ABC transporter(s) might also be involved in extracellular heme export, as was recently demonstrated in *C. elegans* for MRP-5, a multi-drug resistance protein belonging to the ABC transporter family [68].

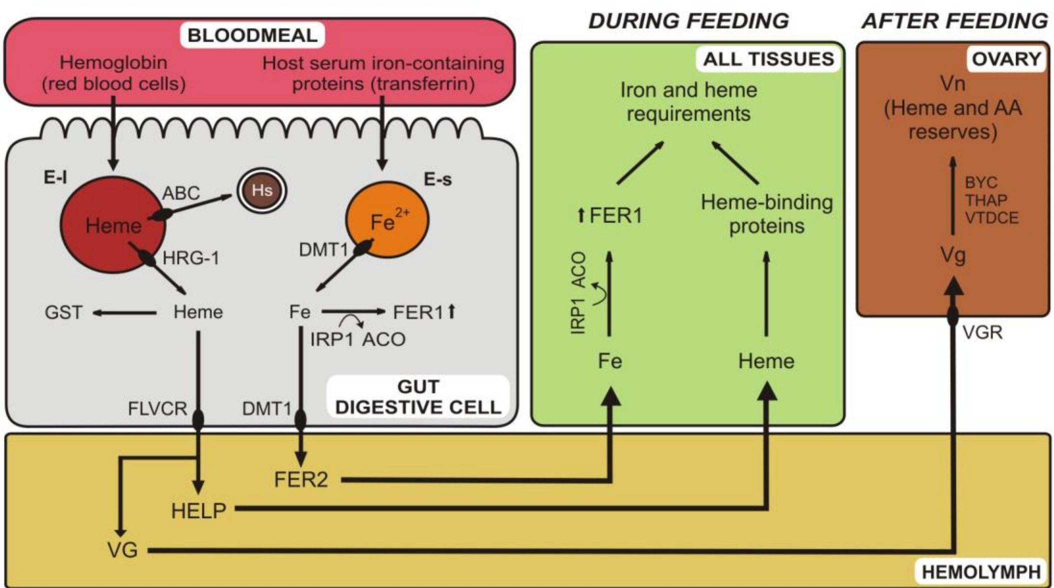

Figure 3 A model of putative tick iron and heme metabolic pathways. Iron and heme pathways in ticks are independent as ticks are not capable of heme degradation given the absence of heme oxygenase. Iron is most likely acquired from the host serum transferrin digested in the acidic environment of small endosomal vesicles (**E-s**) of midgut digestive cells. Upon reduction, ferrous iron is transported from the lysosome by the divalent metal transporter 1 (**DMT1**). Once in the cytoplasm,

Fe^{2+} ions are scavenged by intracellular ferritin 1 (**FER1)**, whose translation is strictly regulated by the cytoplasmic Aconitase/Iron responsive protein1 (**ACO/IRP1)**, which senses the iron cellular level. Iron destined to be delivered to the peripheral tissues is transported from digestive cells via DMT1 to the hemolymph and bound to the iron secreted ferritin 2 (**FER2**) that functions as an iron transporter. The excessive iron in peripheral tissues is scavenged and stored in Fer1. **Heme** released from the digested host hemoglobin in the large endosomal vesicles (**E-l**) is transferred to the cytoplasm via the heme responsive gene 1 transporter (**HRG-1)**. Ticks detoxify the majority of acquired heme by an ABC transporter (**ABC**)-mediated transport to hemosomes. Glutathione-S-transferase(s) (**GST**) serves as an intracellular scavenger of free heme. A small portion of acquired heme required for proteosynthesis of endogenous hemoproteins is exported from the digestive cells to the hemocoel by **FLVCR** transporter. In hemolymph, heme is bound by the abundant carrier protein(s), heme-lipo-glycoprotein (**HELP**), which serves in all developmental stages both as a scavenger of excessive heme and transporter into peripheral tissues. In the post-repletion period of fully engorged females, most of the heme is bound to vitellogenins (**Vg)** and transported to the ovaries to supply heme metabolic demands of developing embryos and larvae. After entry into the developing oocytes via vitellogenin receptor (**VGR**), Vg is proteolytically processed to vitellins (**Vn**) by aspartic proteases **BYC** and **THAP**, and cathepsin L-like activity of **VTDCE**. For details, see the text.

In mammalian macrophages, heme originating from degradation of red blood cells is exported via **FLVCR** (the cell surface receptor for feline leukemia virus, subgroup C) [69, 70]. The gene (ISCW022805) coding for a protein related to FLVCR was also identified in the genome of *I. scapularis*. The putative tick FLVCR is a protein of 413 amino acids that clearly belongs to the large and diverse group of secondary transporters of the major facilitator superfamily (MSF), and contains a signal sequence peptide, ten trans-membrane motifs, and predicted target sequences for localization to the cellular plasma membrane or Golgi apparatus vesicles [66]. The BLAST search displayed up to 50% sequence identity to FLVCR-related proteins of other organisms such as the horseshoe crab or *Drosophila* sp. Expression of a corresponding *flvcr* ortholog in *I. ricinus* developmental stages or tissues of adult female was undetectable by qRT-PCR analysis, and RNAi-mediated silencing of *flvcr* in this species revealed no phenotype . However, it is possible that the RNAi experiment did not hit the right target as transcriptomes from *I. ricinus* midgut or *I. scapularis* synganglion contain transcripts coding for different FLVCR-related proteins of the MSF family (Gen Bank GEFM01002811, GANP01002798 and GBBN01005999, respectively).

Glutathione S-transferases (GSTs) form a family of enzymes that catalyze the reaction of xenobiotics as well as endogenous molecules with reduced glutathione and thus facilitate their solubility and biological detoxification [66, 71]. GSTs were proposed to bind heme in several blood-feeding parasites, the nematodes *Haemonchus contortus* [72] and *Ancylostoma caninum* [73], or the malarial parasite *P. falciparum* [74]. As such, GSTs have long been in focus as promising anti-

parasitic targets [73, 75] and GST, together with the digestive CatD-like peptidase Na-ASP1, eventually became a leading vaccine candidate in vaccine development against the human hookworm *Necator americanus* [76]. In ticks, the GSTs were characterized in *H. longicornis* and *R. appendiculatus* [77]. Vaccination of cattle with recombinant GST from *H. longicornis* exerted a high (about 50%) protection against infestation with *R. microplus,* given the cross-reactivity of anti GST-antibodies in both species [78]. On the other hand, identification of the best 'anti-tick' target among the tick GST family might be a quite demanding task, as the *I. scapularis* genome, for example, contains more than 40 genes annotated as putative *gst* [11]. It remains to be investigated whether any tick GSTs participate in heme binding and/or metabolism in the tick gut.

Inter-tissue transport of heme

Despite heme auxotrophy of ticks, more than 200 genes encoding enzymes that utilize heme as a cofactor were identified in the genome of *I. scapularis* [12]. Among the hemoproteins that are vitally important for basic metabolism of ticks are: respiratory chain cytochromes, catalase, and a large number of genes encoding members of the cytochrome P450 family. Certainly, to fulfill the heme demands for synthesis of endogenous hemoproteins in all tick developmental stages, effective transport and distribution of heme from the site of hemoglobin digestion to the peripheral tissues has to be secured throughout the tick life cycle. Moreover, it was recently demonstrated by artificial membrane feeding of ticks on hemoglobin-depleted serum that the presence of heme in developing ovaries is absolutely necessary for successful embryogenesis [12]. Therefore, efficiently targeting heme transport to the ovaries of mated, fully engorged females can substantially reduce tick populations, a goal of particular importance for one host-tick species, the cattle tick *R. microplus*. The inter-tissue transport or scavenging of heme in all tick stages is facilitated by the abundant **hemolymph carrier proteins** (CPs) also termed HeLp (for hemo-lipo-glycoprotein) [79], whereas heme transport to the ovaries of fully engorged females seems instead to be mediated by **heme-binding vitellogenins** (Vgs) [12, 80-82] (Figure 3). Tick CPs and vitellogenins belong to the same family of large lipid transfer proteins known to facilitate the circulation of hydrophobic molecules across bodies of vertebrate as well as invertebrate animals [83]. To clearly distinguish between genes encoding tick CPs from those encoding Vgs, their expression profile has to be known. Whereas HeLp/CPs are expressed ubiquitously in all tick stages including males, Vgs are expressed only in fertilized, fully engorged females [12]. The genome of *I. scapularis* contains at least five carrier proteins (*cp1-5*) [11]. In *I. ricinus*, the *cp3* ortholog, tagged as *ircp3,* was identified and sequenced (GenBank KP663716). It encodes a protein of 1537 amino-acid residues including the signal peptide with a theoretical MW of about 175 kDa. In accord with aforementioned criteria, the *ircp3* is expressed in all developmental stages and is up-regulated by blood feeding. In adult females, *ircp3* is mainly expressed by the fat body associated with trachea, and to a lesser extent also in salivary glands and ovaries. Silencing of *ircp3* by RNAi reduced the amount of the protein in hemolymph by about

80% and correspondingly lowered the concentration of associated heme [12]. On the other hand, silencing of *ircp3* did not markedly reduce the amount of heme present in tick ovaries (Perner, unpublished data). The *I. scapularis* genome encodes two vitellogenin molecules *Is*Vg1(ISCW013727) and *Is*Vg2 (ISCW021228) that differ by the absence of the DUF1943 domain in the latter. The corresponding *I. ricinus* orthologs are preferentially synthesized in the tick gut and fat body. Their silencing by RNAi revealed that heme transport to developing ovaries occurs mainly during the off-host digestive phase and is dependent on vitellogenins [12]. Preliminary results further suggest that *Ir*Vg1 has a higher affinity for heme binding than *Ir*Vg2 (Perner, unpublished data). Whether the DUF1943 domain that is also present in *Ir*CP3 is responsible for heme-binding capacity remains to be examined. It is also possible that in the native state, *Ir*Vg1 and *Ir*Vg2 form a functional heterodimer capable of heme binding and transport.

The crucial function in vitellogenin and most likely also vitellogenin-bound heme uptake to tick ovaries is mediated by the **vitellogenin receptor (VgR),** which was characterized in three hard-tick species, the American dog tick *Dermacentor variabilis* [84], the Asian hard tick *H. longicornis* [85], and in the African bont tick *A. hebraeum* [86]. Tick VgRs are large membrane proteins, of about MW 200 kDa, sharing the common multi-domain architecture comprising two ligand binding sites, two EGF-precursor domains, an O-sugar binding domain, a transmembrane domain and a cytoplasmic C-terminal tail [84-86]. In all these studies, the function of VgR was examined by RNA interference, clearly demonstrating that VgR knockdown led to impaired development of heme-depleted oocytes, finally resulting in limited egg production. These findings present tick VgRs as a good target capable of reducing tick reproduction. However, the key to the rational control of vitellogenesis and vitellogenin/heme uptake by tick oocytes seems to be rather in targeting hormonal regulation of Vg and VgR expression, which is still inadequately understood [87, 88].

Embryogenesis and fertility

Rational targeting of oogenesis and embryogenesis would be specifically important for the control of the cattle tick *R. microplus* population. To date, three enzymes processing vitellogenins in *R. microplus* eggs have been characterized: (i) An aspartic peptidase, **BYC** (Boophilus yolk pro-cathepsin D) was isolated from tick eggs [89] and its molecular cloning revealed that BYC lacks the highly conserved second catalytic Asp residue that is essential for CatD-type endoproteolytic activity [90]. Despite this, the recombinant as well as isolated native BYC exerted limited proteolytic activity, which is actually a desired feature for the slow degradation of vitellin in the course of embryonal development [90]. (ii) **THAP**, a tick-heme binding aspartic peptidase isolated and cloned from *R. microplus* eggs has conserved both catalytic Asp residues and is specifically active against hemoproteins [91]. The authors conclude that THAP uses heme bound to vitellins as a docking site to increase the specificity of degradation of its physiological substrate – vitellin and regulation of gradual heme supply for the developing embryos [91]. (iii) Another enzyme proposed to be involved in vitellin

processing in *R. microplus* eggs has been described as CatL-like vitellogenin degrading cysteine endopeptidase (**VTDCE**) [92]. This enzyme was purified from tick eggs and characterized as CatL based on its substrate/inhibitor specificity. However, the molecular mass of the purified protein did not match the size of CatL-type peptidases and recent molecular cloning revealed that VTDCE is more related to tick antimicrobial peptides of microplusin and/or hebraein types [93]. Vaccination of cattle with native or recombinant BYC and isolated VTDCE conferred only limited protection (some 25%) in overall efficacy against *R. microplus* ticks [94-96].

Iron acquisition and metabolism

Iron is an essential element that acts as an electron donor/acceptor involved in vitally important physiological processes across the whole animal kingdom. Iron or iron-sulfur (Fe-S) clusters are core components of many enzymes functioning for instance in the respiratory chain of mitochondria, DNA biosynthesis and energy metabolism [97]. The major source of iron for most known animals, including hematophagous insects, originates from heme degradation that is catalyzed by HO [98]. As mentioned above, this enzyme is, however, absent from the tick genome [11]. Artificial membrane feeding *I. ricinus* females on hemoglobin-depleted serum revealed that the amount of iron in tick tissues does not depend on hemoglobin in the diet, experimentally proving that hemoglobin-derived heme is not a source of iron for ticks [12]. The lack of heme degradation thus raises the question of the dietary source of iron for ticks, which is most likely explained by acquisition of a sufficient amount of non-heme iron from the host serum transferrin [99-101] (Figure 3). Mammalian cells uptake iron from circulating transferrin via receptor-mediated endocytosis of the transferrin/transferrin receptor complex, followed by iron release in the mildly acidic environment of endosomes [102, 103]. A similar mechanism of iron acquisition from host transferrin was also described for the blood-stream form of the sleeping sickness agent, *Trypanosoma brucei.* However, the transferrin receptor of this protozoan parasite is structurally completely different from its mammalian counterpart [104, 105]. No protein related either to mammalian or trypanosomal transferrin receptor could be found by BLAST searches of the available tick genome and/or transcriptome databases. It is possible that the release of iron from the host transferrin occurs in the acidic environment of digestive vesicles along with digestion of other serum proteins. Released iron must be first reduced to Fe^{2+} before its transport from the lysosome to the cytoplasm, mediated in other organisms by the **divalent metal-transporter (Dmt1),** also tagged as malvolio in *Drosophila* [106]. A gene encoding a putative *dmt1*/*malvolio* homologue was identified in the *I. ricinus* midgut transcriptomes (GenBank GANP01004329 or GEFM01002799). The tick *dmt1* gene was shown to be expressed in all developmental stages and tissues. However, its silencing by RNAi did not result in any obvious phenotype [66, 107].

Free iron is potentially toxic for all living cells as it participates in the formation of free oxygen radicals and its intracellular levels must therefore be strictly maintained at low levels [108]. This function is carried out by the intracellular iron storage protein ferritin, referred to here as **ferritin1**

(Fer1) [Figure 3]. Fer1, first characterized in the hard tick *I. ricinus* and the soft tick *Ornithodoros moubata,* shared high sequence similarities and were closely related to the mammalian heavy-chain ferritins, including the typically conserved motifs for ferroxidase center [109]. Tick Fer1 are proteins of MW about 20 kDa, which, in the native state, form homopolymers of MW about 500 kDa, most likely composed of 24 subunits as in vertebrates. The 5'-untranslated regions of tick *fer1* mRNA contains a stem-loop structure of an iron responsive element (IRE) (the only IRE found in the tick genome), which points to the regulation of Fer1 at the translational level by an **iron responsive protein** (IRP1) [Figure 3]. Increasing levels of intracellular iron allow insertion of newly synthesized Fe-S clusters into IRP, which then becomes an active cytoplasmic aconitase that subsequently detaches from *fer1* mRNA IRE thereby allowing its translation. This was experimentally proved by RNAi silencing of the *I. ricinus irp1* gene, which resulted in a marked increase in Fer1 protein levels in tick tissues [12, 100]. The *irp1* KD did not affect tick feeding but exerted a clear impact on tick reproduction as larval hatching from laid eggs was significantly reduced [100]. Unlike vertebrates, ticks possess another form of heavy chain-type ferritin, called **ferritin2 (Fer2)** that is synthesized mainly in the tick gut and secreted into the hemolymph [100] [Figure 3]. RNAi KD of *fer2* resulted in a substantial decrease in Fer1 levels in tick peripheral tissues, suggesting that Fer2 plays a role in iron inter-tissue transport [100]. It remains an unresolved issue whether iron is loaded into Fer2 inside the midgut cells or if iron is first secreted to the hemolymph and subsequently scavenged by Fer2. Impairment of iron storage and transport by RNAi KD of tick ferritins 1 and 2 severely affects tick development and reproduction as first demonstrated in *I. ricinus* [100] and later in *H. longicornis* [110, 111]. Fer2 possesses all the important attributes of a suitable concealed antigen as it has no counterpart in mammals, is encoded by a single gene and is mainly expressed in the tick gut where it comes into direct contact with ingested host blood. This all makes Fer2 a promising candidate for development of an 'anti-tick' vaccine. The concept of using recombinant tick Fer2 for vaccination of animals against tick infestation was successfully demonstrated on laboratory rabbits against *I. ricinus* [112] and *H. longicornis* [113]. More importantly, vaccination of cattle with recombinant Fer2 from *R. microplus* exerted a protective effect against this one-host tick species that was comparable with the commercial vaccine based on Bm-86 [112].

The *I. scapularis* genome contains a gene encoding a putative **transferrin** (GenBank XM_002400404), but a phylogenetic analysis of this gene revealed that it is most closely related to the insect type 2 transferrins (Tf2) (also termed melanotransferrins) [66, 107]. The function of Tf2 remained obscure until a study showed that *Drosophila* Tf2, capable of binding iron, is a component of epithelial septate junctions and apparently does not play a role in inter-tissue iron transport [114]. In line with this finding, RNAi KD of the *tf2* ortholog in *I. ricinus* did not affect iron supply into tick tissues, as monitored by the levels of Fer1 [66, 107].

Conclusions

Although our understanding of blood meal digestion and associated physiological processes in the tick gut has progressed remarkably during the past decade, it nevertheless remains limited mainly to adult hard tick females during their on-host feeding phase. In contrast, we know almost nothing about blood digestion in fully engorged females following their detachment from the host. An in-depth knowledge of molecules and processes capable of transforming the huge amount of ingested blood into the imposing egg mass laid by the females would be especially useful for the control of tick reproduction and for reducing their population in the field. In order to protect hosts from tick infestation and transmission of tick-borne diseases, it would, however, be particularly important to effectively impair blood uptake and digestion during the early stages of feeding of both adult and immature ticks. Preliminary results suggest that the activities of digestive enzymes stay low in virgin females and are only up-regulated in fertilized females [55]. Therefore, male factors such as voraxins [23], most likely linked with hormonal control, trigger the exponential up-regulation of digestive enzymes during the slow-feeding period in fertilized females [22]. But a similar up-regulation of the digestive apparatus, which is obviously independent of mating status, also occurs in the nymphal stage (Konvičková, unpublished results). What is then the triggering stimulus? Assuming that nutrient sensing is responsible, what blood meal component is important for that? These questions might be experimentally addressed using *in vitro* membrane feeding techniques (see Chapter xx by Tyson and Nijhof). The necessary prerequisite for such experiments is developing a chemically defined diet, similar to that recently implemented for the mosquito *Aedes aegypti* [115]. The other possibility of sensing incoming blood could be via neuropeptidergic signaling, facilitated by the complex system of neurons present in the tick hindgut [116].

Blood-feeding arthropods are challenged not only by a surplus of heme and iron but also by a huge amount of amino acids originating from their protein-rich diet. Hence, functional catabolism of amino acids is also essential for their survival, as recently demonstrated for the triatominae bug *R. prolixus* by RNAi silencing and/or inhibition of the tyrosine degradative pathway in this insect [117]. Disabling tyrosine detoxification by specific inhibitors also caused premature death of *A. aegypti* and of the cattle tick *R. microplus,* suggesting the potential of using these compounds to selectively target arthropod blood feeders [117].

In addition to the molecules and processes described in this chapter, there will definitely appear in the future many other targets that may eventually turn out to be the right keys to reach the ultimate goal – discovery of effective anti-tick vaccines and/or selective acaricides protecting us and our household animals against ticks and infectious diseases that they transmit.

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Paper IV

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Tick iron and heme metabolism – New target for an anti-tick intervention

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ABSTRACT

Ticks are blood-feeding parasites and vectors of serious human and animal diseases. *Ixodes ricinus* is a common tick in Europe, transmitting tick-borne encephalitis, Lyme borreliosis, anaplasmosis, or babesiosis. Immunization of hosts with recombinant tick proteins has, in theory, the potential to interfere with tick feeding and block transmission of pathogens from the tick to the host. However, the efficacy of tick antigens has, to date, not been fully sufficient to achieve this. We have focused on 11 *in silico* identified genes encoding proteins potentially involved in tick iron and heme metabolism. Quantitative real-time PCR (qRT-PCR) expression profiling was carried out to preferentially target proteins that are up-regulated during the blood meal. RNA interference (RNAi) was then used to score the relative importance of these genes in tick physiology. Finally, we performed vaccination screens to test the suitability of these proteins as vaccine candidates. These newly identified tick antigens have the potential to improve the available anti-tick vaccines.

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1. Introduction

Ticks are blood-feeding ectoparasites transmitting viral, bacterial, and protozoal diseases to humans and animals (Hajdusek et al., 2013; Jongejan and Uilenberg, 2004). In Europe, Lyme borreliosis (LB) and tick-borne encephalitis are the most frequent human diseases transmitted by ticks (Mansfield et al., 2009; Rizzoli et al., 2011). Ticks and tick-borne diseases also greatly affect livestock, limiting production in many areas around the world. Protection against ticks and economic losses on animals are estimated to amount to billions of US dollars every year (Sonenshine and Roe, 2014). The castor bean tick (*Ixodes ricinus*) is commonly found in Europe and is closely related to the blacklegged tick *I. scapularis*, a vector of LB in the USA, and whose genomic sequence has been recently released (Lawson et al., 2009). Because of a high gene similarity (more than 95% of the nucleotide sequence), genomic information on this species can be directly applied to *I. ricinus* research.

Immunization with tick proteins to protect hosts against tick feeding or transmission of pathogens is a challenge not only for livestock production, but is also important for human health (Merino et al., 2013; Moyer, 2015). Because vaccine production is not as difficult or expensive as production of acaricides (Bowman and Nuttall, 2008), scientists are encouraged to find suitable tick antigens that could be used for vaccine development. The first commercialized vaccine (TickGUARD, Gavac), which protected cattle from *Rhipicephalus microplus*, was based on the tick midgut protein BM86 (Willadsen et al., 1995). This vaccine also interfered with transmission of babesiosis (de la Fuente et al., 2007). However, the BM86 vaccine is specific against cattle tick (*Rhipicephalus* spp.) infestations only with limited efficacy against other tick species (de la Fuente and Kocan, 2003). Protection based solely on vaccination therefore requires the identification of new, more efficient antigens.

RNA interference (RNAi) technologies have raised new options for screening tick genes as new vaccine candidates. Using RNAi we have previously described the basic pathway of iron metabolism in *I. ricinus* and identified a crucial protein, ferritin2 (FER2), with a novel function (Hajdusek et al., 2009). Immunization of cattle or rabbits with recombinant FER2 dramatically reduced tick feeding, tick weight after feeding, and the fertility of various tick species. The protective efficacies were similar to those obtained with a commercial vaccine based on BM86 (Hajdusek et al., 2010). Recently, vaccinations with recombinant FER2 and ferritin1 (FER1) were shown to reduce tick feeding and oviposition, and hatching, respectively, in the hard tick *Haemaphysalis longicornis* (Galay et al., 2014), reinforcing the potential of tick ferritins as universal vaccination antigens applicable against multiple tick species.

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Iron is an indispensable inorganic element in most organisms. Because of its redox properties, iron serves as an electron donor and acceptor in various metabolic processes. Iron or iron-sulfur clusters are core components of many enzymes involved e.g., in the respiratory chain of mitochondria or DNA biosynthesis. Heme, a prosthetic group that holds an iron atom at the center of a porphyrin ring, is a key component of hemoproteins e.g., for transport of oxygen, electron transfer in mitochondria, or defense mechanisms against oxidative stress. Importantly, iron participates in the formation of toxic radicals that cause substantial damage to proteins, lipids, and DNA. For this reason, iron and heme homeostasis is maintained in every organism by an orchestrated set of sophisticated proteins handling their uptake, utilization, transport, and storage (Hamza and Dailey, 2012; Hentze et al., 2004).

Here, we screened for new vaccine candidates in the tick iron and heme metabolic pathways. The genes identified in the available tick sequence databases were initially molecularly characterized in silico and their expression profiles were determined in different tick stages and tissues by relative quantitative real-time PCR (qRT-PCR). RNAi was then used to score their importance during and after tick feeding. Rabbits were vaccinated with recombinant proteins and potential anti-tick antigens were evaluated. From this novel investigation of iron and heme metabolism in ticks we identified several genes that effected tick feeding, oviposition, and hatching. Production of new vaccine antigens that interfere with tick iron and heme metabolism could help in strategies to fight ticks and tick-transmitted diseases.

2. Materials and methods

2.1. Ticks

Adult females and males of *I. ricinus* were collected by flagging around Ceske Budejovice, Czech Republic and were used for RNAi (guinea pigs) and vaccination (rabbits) experiments. Ticks were maintained in wet chambers with a humidity of about 95%, temperature 24 °C, and day/night period set to 15/9 h. To obtain tick developmental stages for qRT-PCR experiments, females were fed on laboratory guinea pigs in the presence of males. Larvae were fed on guinea pigs, nymphs were fed on guinea pigs or rabbits. Molted adult females (pathogen free) were used for pathogen injection/feeding experiments (Urbanova et al., 2015). All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 021/2012.

2.2. Identification and characterization of genes

To identify tick genes possibly involved in iron and heme metabolism, we performed a BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) using homologous arthropod gene sequences as baits (Supplementary Table 1). Full sequences of the genes (from *I. scapularis* or *I. ricinus*) were screened in silico for putative signal sequences (SignalP; www.cbs.dtu.dk/services/SignalP/), cellular localizations (PSORT; http://psort.hgc.jp/form.html), the presence of transmembrane motifs (TMHMM; http://www.cbs.dtu.dk/services/TMHMM/), and glycophosphatidylinositol anchors (big-PI; http://mendel.imp.ac.at/sat/gpi/gpi_server.html). The phylogenetic analysis in Fig. 4A was carried out as described previously (Sojka et al., 2007).

2.3. Relative expression profiling by quantitative real-time PCR (qRT-PCR)

Total RNA, isolated from *I. ricinus* developmental stages, tissues, and unfed adults injected or fed with different pathogens, was prepared as described previously (Urbanova et al., 2014, 2015). All RNA samples were prepared in biological triplicates, transcribed into cDNA (Roche), and analyzed by quantitative real-time PCR using a LightCycler 480 (Roche) and SYBR green chemistry as described previously (Urbanova et al., 2015). For the primers used in qRT-PCR, see Supplementary Table 2. Relative expression was calculated using the mathematical model of Pfaffl (Pfaffl, 2001) and normalized to *elongation factor1* (Nijhof et al., 2009). RT-PCR profiling shown in Fig. 2B was performed as described previously (Hajdusek et al., 2009).

2.4. Impact of gene silencing on tick feeding and development

234–521 bp (for details see Supplemental Table 2) gene-specific double-stranded RNAs (dsRNA) were synthesized and injected into adult females of *I. ricinus* as described previously (Hajdusek et al., 2009). Injected ticks (25 per group) were mixed with an equal number of males, placed in cylinders on the backs of guinea pigs and allowed to feed naturally until repletion. After feeding, ticks were visually checked, weighed, and put into separate vials to evaluate oviposition and hatching. Ticks injected with dsRNA against green-fluorescence protein (GFP) served as a control in all experiments. Gene silencing was checked by qRT-PCR in midgut, salivary glands, and ovaries of half-fed ticks (pool of tissues dissected from five females). In Fig. 4B, homogenates of RNAi-silenced tick tissues were assayed by Western Blot analysis using anti-FER1 antibody (Kopacek et al., 2003) to evaluate involvement of *transferrin2* knock-down (KD) in the transport of iron from the tick gut to peripheral tissues, as described previously (Hajdusek et al., 2009).

2.5. Effect of vaccination on tick feeding and development

Full-length (whole coding sequence without signal peptides) or partial gene sequences were amplified from *I. ricinus* cDNA (for details see Supplemental Table 2), cloned into the expression vector pET100 (Invitrogen, containing N-terminal His-tag), expressed in *E. coli* BL21 (Invitrogen), purified, and refolded (Hajdusek et al., 2010). To immunize rabbits, 1 ml of the recombinant protein (100 μg/ml) was mixed 1:1 with Freund's adjuvant and injected subcutaneously in three doses (weeks 1, 3, and 6) as described previously (Hajdusek et al., 2010). Negative controls were injected with adjuvant/saline (50 mM Tris pH 9, 150 mM NaCl). Two weeks after the last immunization, a sample of rabbit blood was taken from the ear to check development of specific antibodies before and after vaccination by Western Blot analysis. Rabbits were then infested with 25 *I. ricinus* pairs per animal, in glued cylinders. Engorged ticks were visually checked, weighed, and put into separate vials to evaluate ensuing oviposition and hatching.

3. Results

3.1. Identification and molecular characterization of proteins possibly involved in iron and heme metabolism

We have screened available *I. ricinus* and *I. scapularis* sequences accessible in GenBank to identify tick proteins that are likely to participate in the maintenance of iron and heme homeostasis. In addition to three previously identified iron metabolism genes, *ferrtin1*, *ferritin2*, and *iron-regulatory protein1* (*irp1*) (Hajdusek et al., 2009), we identified two new genes of iron metabolism and six genes of heme metabolism with clear sequence homologies to other invertebrates (Table 1). Our in silico analysis revealed that divalent metal transporter1 (DMT1) was a 563 amino acids (aa) protein containing 10 transmembrane motifs (TMM) with predicted localization to peroxisomes or the plasma membrane. Transferrin2 (TF2) was a 793 aa protein containing a predicted signal peptide,

Table 1
Tick iron and heme metabolism genes screened from the available *I. scapularis* and *I. ricinus* genomic and EST projects assigned to GenBank (NCBI).

Gene name	Abbreviation	Reference sequence	Length (aa)	Signal sequence	Localization	TMM/GPI
*ferritin1**	*fer1*	AF068224	172	No	CYT	0
*ferritin2**	*fer2*	ACJ70653	196	Yes	OUT	0
*iron regulatory protein1**	*irp1*	ACJ70654	890	No	CYT	0
divalent metal transporter1	*dmt1*	GANP01004329	563	No	PER/PM	10
transferrin2	*tf2*	GBBN01012231	793	Yes	OUT	0/1
ferrochelatase	*fech*	ISCW016187	384	No**	MIT	0
feline leukemia virus subgroup C receptor-related protein	*flvcr*	ISCW022805	413	Yes	PM/GA	10
heme-binding lipoprotein	*help*	GANP01014013	1537	Yes	PM/OUT	0
heme-responsive gene1	*hrg1*	ISCW001847	204	No	PM/GA	4
vitellogenin1	*vg1*	ISCW013727	1936	Yes	PM	1/1
vitellogenin2	*vg2*	ISCW021228	1644	Yes	OUT	0

Abbreviations: *, previously analyzed by (Hajdusek et al., 2009); **, mitochondrial targeting sequence; CYT, cytoplasm; OUT, outside; PER, peroxisome; PM, plasma membrane; MIT, mitochondria; GA, Golgi apparatus; aa, amino acids; TMM, transmembrane motif; GPI, glycophosphatidylinositol anchor.

one glycophosphatidylinositol (GPI) membrane anchor, with predicted extracellular localization (secretion). We failed to find any homolog of a gene encoding the transferrin receptor (TFR). Ferrochelatase (FECH), an enzyme that in other organisms takes part in the final step of heme biosynthesis, was a 384 aa protein containing a predicted mitochondrial targeting sequence. In agreement with previous reports, a homolog of heme oxygenase (HO) was not found in the available tick transcriptomes (Hajdusek et al., 2009). Feline leukemia virus subgroup C receptor-related (FLVCR) protein contained 413 aa, a signal sequence peptide, 10 TMMs, and was targeted to the cellular plasma membrane or vesicles of the Golgi apparatus. A gene encoding a protein homologous to heme-binding lipoprotein (HELP) (Maya-Monteiro et al., 2000), previously called hemelipoglyco-carrier protein (CP) (Donohue et al., 2009) was identified. This was a 1537 aa protein containing a signal peptide and was predicted for insertion in the plasma membrane or secretion from the cell. Several similar homologs of this protein (e.g., ISCW021709, ISCW014675, ISCW012424, and ISCW02170) exist in the genome database of *I. scapularis*. Heme-responsive gene1 (HRG1) was a putative heme membrane transporter. It was 204 aa long, comprised four TMMs, and was predicted to be localized in the plasma membrane or Golgi apparatus. Vitellogenin1 (VG1) and vitellogenin2 (VG2) were proteins homologous to the invertebrate vitellogenins; *vg1* encoded a 1936 aa protein with a putative signal peptide sequence, one TMM, and a GPI anchor localized at the C-terminus of the protein. VG1 was predicted to have a plasma membrane localization. The second vitellogenin, VG2, comprising 1644 aa, also possessed a signal sequence peptide, but lacked TMMs or GPI anchors, and was predicted to be a secreted protein.

3.2. Tissue and developmental stage profiling by relative qRT-PCR

To ensure that the previously selected genes were expressed in feeding ticks so that host antibodies could target the tick antigens, we performed relative qRT-PCR profiling of different developmental tick stages and adult female tissues during and after feeding. We found that all genes were up-regulated by blood feeding in adult females and many of them were also up-regulated in feeding larvae and nymphs (Fig. 1). Expression of *vg1* and *vg2* was detected mainly in the fed females, pointing to their importance as suppliers of nutrition for the developing ovaries. Except for *fer1*, expression of all genes was low in *I. ricinus* males (not feeding) compared to other stages. Several genes were notably highly transcribed in developing eggs.

Gene expression in the tissues of feeding and engorged tick females was measured. Presumably, iron and heme metabolism genes should be mainly expressed in the midgut, which deals with excess iron and heme during blood digestion. In agreement with our previous observations (Hajdusek et al., 2009) we found that *fer2* was solely expressed in midgut tissue. Although *fer1*, *iron regulatory protein1* (*irp1*), *dmt1*, and *fech*, were also expressed in the midgut tissue, their mRNAs were ubiquitously presented in other tissues indicating their general role in tick physiology. Notably, *tf2* and *hrg1* were expressed mainly in ovaries and *help* in the fat body and salivary glands. In the feeding ticks we observed that *vg1* was expressed mainly in the midgut and ovaries, while *vg2* showed expression only in the midgut. However, expression of vitellogenins in feeding ticks was marginal compared to their expression four days after repletion, where both genes were similarly and massively (up to 362 thousand times) up-regulated (Table 2) in most tissues except the ovary. Remarkably, *dmt1*, *fech*, and *hrg1* were up-regulated at least five times in certain tissues. In general, we found that most of the identified genes were expressed in feeding ticks, could play a role in iron or heme metabolism, and are possible candidates for RNAi and vaccination trials.

3.3. Assessment of TF2 biological function

To consider the role of the newly identified *transferrin* homolog in the tick genome we performed a series of experiments. Firstly, we observed that phylogenetically, the tick transferrin clustered with insect type 2 transferrins (Fig. 2A). In a subsequent experiment (Fig. 2B) *tf2* was silenced by RNAi and the increase in FER1 protein was measured in tick tissues where it functions as an iron supply sensor (Hajdusek et al., 2009). However, the level of FER1 in peripheral tissues, as well as the level of FER2 in the tick plasma, remained unchanged after KD, demonstrating that TF2 was not involved in iron absorption, transport, or distribution between tissues. Moreover, we did not detect any effect of *tf2* silencing on tick feeding or fecundity (Table 3). To assess possible roles of TF2 in tick immunity, adult unfed ticks were injected or capillary-fed with different pathogens (Urbanova et al., 2015) and the levels of *tf2* up-regulation were checked 12 h after treatment (Fig. 2C and D). However, *tf2* expression remained unchanged after immune challenge suggesting that an immune role of TF2 was not likely. In summary, we could not confirm a role of TF2 in iron distribution or immune defense and its function in ticks remains unknown.

3.4. Silencing of genes by RNA interference and their effect on tick feeding and development

As a first step in the scoring of new anti-tick vaccine candidates (de la Fuente et al., 2005) all identified iron and heme metabolism genes were silenced by RNAi. The dsRNA-injected ticks were rested for one day and then fed on guinea pigs until repletion. The levels of gene silencing were checked by qRT-PCR in midgut, salivary glands, and ovaries of half-fed females and compared to the GFP dsRNA controls. Except for *flvcr*, which could not be analyzed because of

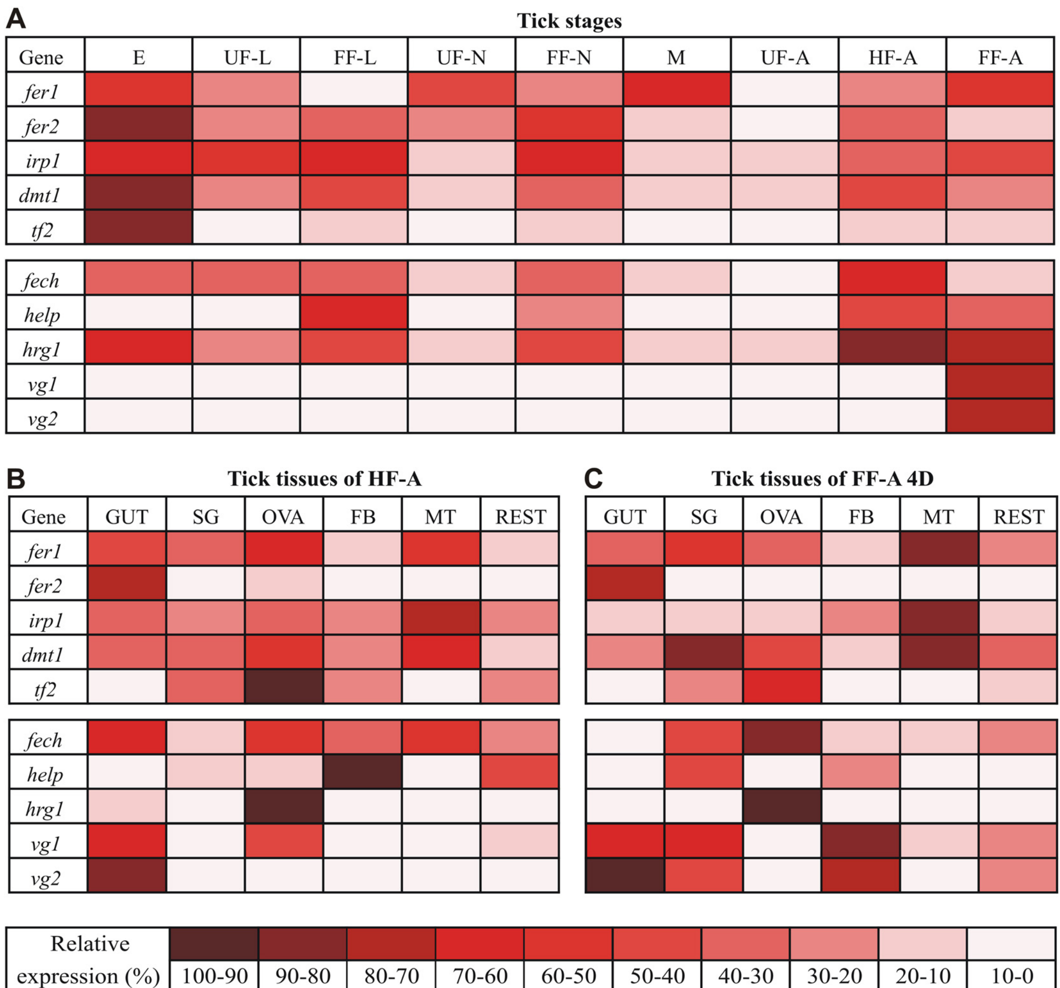

Fig. 1. Relative expression of iron and heme metabolism genes in *I. ricinus*. Quantitative real-time PCR (qRT-PCR) profiling of iron (upper rows) and heme (lower rows) metabolism genes in (A) developmental stages, (B) tissues of adult half-fed (day 5) ticks, and (C) tissues of adult ticks 4 days after repletion. The expression was normalized to the elongation factor1 (*ef1*) housekeeping gene. The results represent the mean of three independent biological replicates (five females per replicate), where the highest individual reading for a particular gene across a panel (of all analyzed cDNAs, including the biological triplicates) was set at 100% and all other values were expressed relative to this. Levels of gene expressions are not comparable between individual panels (for comparison see Table 2). Results of *flvcr* expression are not shown because of inconsistency of obtained results. E, eggs; UF-L, unfed larvae; FF-L, fully-fed larvae; UF-N, unfed nymphs, FF-N, fully-fed nymphs; M, males; UF-A, unfed adults; HF-A, half-fed adults; FF-A, fully-fed adults; FF-A 4D, fully-fed adults four days after repletion; GUT, midgut; SG, salivary glands; OVA, ovary; FB, fat body (from around the main tracheal trunks); MT, Malpighian tubules; REST, rest of the body.

previously observed inconsistencies in qRT-PCR expression results, KDs of other genes caused more than 50% transcript reductions (data not shown). The effects of gene silencing on tick feeding and fecundity are summarized in Table 3. From the newly identified genes, the number of fully engorged fed ticks was reduced only in *help* KD compared to the GFP dsRNA control. The weights of fed ticks were not reduced below control levels, but KDs of *help* and *hrg1* decreased the percentage of females capable of laying eggs. In conclusion, RNAi silencing of newly identified iron and heme metabolism genes did not result in a phenotype comparable to

Table 2
Up-regulation (times-up-regulated) of iron and heme metabolism gene expression calculated from the tissues of half-fed adult ticks and tissues of adult ticks four days after repletion.

Gene	GUT	SG	OVA	FB	MT	REST
fer1	1.22	2.58	1.01	1.80	2.65	3.50
fer2	0.42	0.18	0.31	0.10	0.25	0.30
irp1	0.77	1.54	0.71	3.04	3.20	1.87
dmt1	2.35	**8.79**	2.92	1.53	4.83	**8.83**
tf2	0.25	2.78	2.79	0.59	0.69	1.72
fech	1.75	**64.35**	**28.20**	**8.58**	3.37	**25.34**
help	0.05	2.31	0.02	0.28	0.88	0.06
hrg1	0.50	**5.38**	3.10	2.55	1.24	**6.40**
vg1	**16,450.77**	**362,197.67**	**1496.27**	**223,117.92**	**39,630.38**	**44,858.25**
vg2	**155.53**	**34,229.64**	**3733.91**	**23,996.93**	**13,023.06**	**9578.50**

Abbreviations: in bold, at least five-times up-regulated genes; GUT, midgut; SG, salivary glands; OVA, ovary; FB, fat body (from around the main tracheal trunks); MT, Malpighian tubules; REST, rest of the body.

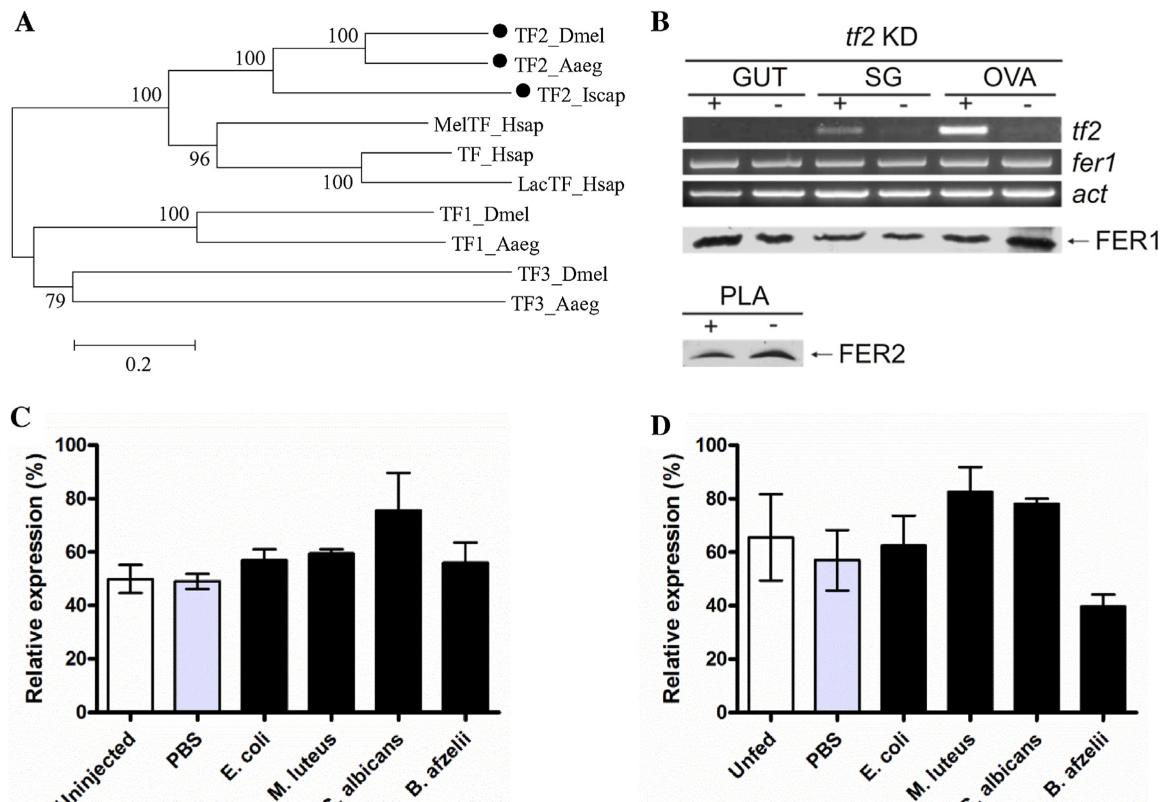

Fig. 2. Role of transferrin2 in tick physiology. (A) Phylogenetic tree of tick, human, and insect transferrins. Unrooted tree of transferrin (TF) amino acid sequences reconstructed using the Neighbor Joining method (NJ) based on alignment using ClustalX. Tick TF2 belongs to insect transferrins2 (black dots). Numbers at branches represent bootstrap support using NJ evolution criterion with 1000 replicates each. The horizontal bar represents a distance of 0.2 substitutions per site. TF2_Iscap (*Ixodes scapularis* GBBN01012231), TF_Hsap (*Homo sapiens* NP_001054), LacTF_Hsap (*Homo sapiens* NP_002334), MelTF_Hsap (*Homo sapiens* NP_005920), TF1_Aaeg (*Aedes aegypti* XP_001647719), TF2_Aaeg (*Aedes aegypti* XP_001662114), TF3_Aaeg (*Aedes aegypti* XP_001661801), TF1_Dmel (*Drosophila melanogaster* AAF48831), TF2_Dmel (*Drosophila melanogaster* AAF49900), TF3_Dmel (*Drosophila melanogaster* AAF58039). (B) RNAi silencing of *tf2* in partially engorged (fed for 5 days) ticks. KD of *tf2* reduced *tf2* mRNA levels while expression of *fer1* and *actin* (loading control) was unchanged (rows *tf2*, *fer1*, and *act*, respectively). When *tf2* was silenced, protein levels of FER1 in various tissues did not change (Western blot in the row FER1) indicating that TF2 is not involved in iron transport. Silencing of *tf2* does not change the level of FER2 (iron transporter) in the tick plasma (hemolymph). + *gfp* dsRNA control; − *tf2* silencing; GUT, gut tissue; SG, salivary glands; OVA, ovaries; PLA, plasma. (C and D) Expression level of *tf2* in adult unfed ticks 12 h after (C) injection or (D) feeding of Gram-negative bacteria (*Escherichia coli*), Gram-positive bacteria (*Micrococcus luteus*), yeast (*Candida albicans*), and Borrelia spirochetes (*Borrelia afzelii*). The expressions were compared to the PBS-injected and uninjected control ticks and normalized by using the *ef1* house-keeping gene. Five *I. ricinus* females were used in three independent biological replicates. The highest individual reading for a particular gene across a panel (of all analyzed cDNAs, including the biological triplicates) was set at 100% and all other values were expressed relative to this.

previously published results on *fer1*, *fer2*, or *irp1* (Hajdusek et al., 2009), however we found that silencing of *help* and *hrg*1 notably affected tick feeding and development.

3.5. Vaccination of rabbits and its effect on tick feeding and development

Recombinant proteins were prepared and immunization experiments were carried out with rabbits to determine vaccination efficacies of the new antigens. Full length (without signal sequence) or parts of genes (Supplemental Fig. 2) were cloned into expression plasmids and expressed as recombinant proteins in *E. coli*. The proteins were column-purified, refolded, their identities checked by protein mass spectrometry, and used for rabbit immunizations. Unfortunately, proteins containing TMM (DMT1, FLVCR, and HRG1) could not be expressed and purified in this expression system under our conditions, probably because of their affinity for bacterial membranes (Daley et al., 2005). Other proteins were expressed

Table 3
Effect of iron and heme metabolism gene silencing on tick feeding (guinea pigs), oviposition, and larval hatching.

Type of injected dsRNA	*N*	Number of engorged ticks	Average tick weight (g; S.D.)	Oviposition	Hatching
gfp (all experiments)	13	228 of 325 (70%)	0.302 (0.060)	190 of 228 (83%)	155 of 190 (82%)
dmt1	2	39 of 50 (78%)	0.288 (0.069)	33 of 39 (85%)	28 of 33 (85%)
tf2	2	41 of 50 (82%)	0.279 (0.075)	38 of 41 (93%)	37 of 38 (97%)
fech	1	22 of 25 (88%)	0.323 (0.065)	19 of 22 (86%)	17 of 19 (90%)
flvcr	2	35 of 50 (70%)	0.314 (0.051)	32 of 35 (91%)	28 of 32 (88%)
help	2	**33 of 50 (66%)**	0.293 (0.067)	**24 of 33 (73%)**	23 of 24 (96%)
hrg1	1	21 of 25 (84%)	0.300 (0.051)	**15 of 21 (71%)**	n.d.
vg1	1	20 of 25 (80%)	0.292 (0.053)	19 of 20 (95%)	19 of 19 (100%)
vg2	1	25 of 25 (100%)	0.251 (0.070)	24 of 25 (96%)	23 of 24 (96%)

Abbreviations: *N*, number of biological replicates; in bold, values >10% lower than in the control dsGFP group; n.d., not determined.

Table 4
Effect of vaccination with iron and heme metabolism recombinant proteins on tick feeding (rabbits), oviposition, and larval hatching.

Vaccination	*N*	Number of engorged ticks	Average tick weight (g; S.D.)	Oviposition	Hatching
CTRL (saline; all experiments)	9	200 of 225 (90%)	0.268 (0.070)	154 of 200 (77%)	133 of 154 (86%)
FER1	2	47 of 50 (94%)	0.308 (0.084)	43 of 47 (92%)	**30 of 43 (70%)**
IRP1	2	49 of 50 (98%)	0.327 (0.063)	45 of 49 (92%)	41 of 45 (91%)
TF2	2	40 of 50 (80%)	0.316 (0.082)	31 of 40 (76%)	26 of 31 (84%)
FECH	1	21 of 25 (84%)	0.305 (0.059)	**13 of 21 (62%)**	11 of 13 (85%)
HELP	2	41 of 50 (82%)	0.294 (0.091)	34 of 41 (83%)	32 of 34 (94%)

Abbreviations: N, number of biological replicates; in bold, values >10% lower than in control CTRL (saline) group.

and purified without difficulty, in quantities suitable for vaccination (milligrams). The recombinant proteins (except for IRP1) were readily refolded and remained soluble after freezing. Vaccination of rabbits was successful and sera from all animals showed strongly positive reactions against the recombinant proteins (data not shown). After tick infestation we observed that immunization with FECH markedly decreased (reduction >10% below the control level) oviposition, while vaccination with FER1 affected larval hatching (Table 4). In summary, two new antigens (FECH and FER1) showed an apparent effect on tick fecundity, however they did not reach efficacies previously observed after vaccination with FER2 (Hajdusek et al., 2010).

4. Discussion

Ticks do not consume any other food except host blood. During tick feeding, blood becomes concentrated in the midgut and ticks could ingest up to several hundred times their unfed weight during a single blood meal (Sonenshine and Roe, 2014). For that reason, ticks must have evolved strategies to manage excess iron and heme released after the host blood is digested. On the one hand, iron and heme must be kept and used by the tick for its own purposes, but on the other hand, excess needs to be safely detoxified and excreted. Here, we summarize recent knowledge about iron and heme metabolism in ticks and supplement this with new data obtained in this work (Fig. 3). We suggest that acquisition of iron from the host blood is limited to several iron-binding proteins in the host plasma e.g., host transferrin or ferritin. Iron can be released from these proteins in lysosomes by degradation or by low pH. Free iron must first be reduced to Fe^{2+} by an as yet unknown tick ferrireductase. Iron can then be transported from the lysosome by DMT1. Tick DMT1 is homologous to the insect iron, manganese, and copper cellular importer, DMT1 (Bettedi et al., 2011). Vertebrate DMT1, also known as natural resistance-associated macrophage protein2 (NRAMP2), is responsible for iron uptake by enterocytes and cellular iron acquisition during the transferrin receptor cycle (West and Oates, 2008). We found that in ticks, this gene is expressed in all tissues (Fig. 1) and is up-regulated during and after feeding, especially in salivary glands and ovaries (Table 2).

Intracellular iron probably does not accumulate in the cell cytoplasm but, rather, is detoxified by the intracellular FER1 and stored. Cytoplasmic iron could also be loaded into the heavy chain-type FER2 and transported via hemolymph into peripheral tissues to fulfill iron requirements. It is currently unknown whether iron is loaded into FER2 inside the midgut cells or if the iron is secreted to the hemolymph (by DMT1) and subsequently scavenged by FER2. We also do not know how iron is delivered from FER2 to the peripheral tissues. FER2 is probably absorbed by the target cell into lysosomes, where iron is released after FER2 degradation, reduced by ferrireductase, and transported to the cytoplasm by DMT1. Once inside the cytoplasm, iron is sensed by IRP1 and detoxified and stored by FER1 for further use.

In vertebrates, distribution of non-heme iron is accomplished by serum transferrin (Dunn et al., 2007; Hentze et al., 2004). Functions of insect transferrins are uncertain and some authors also

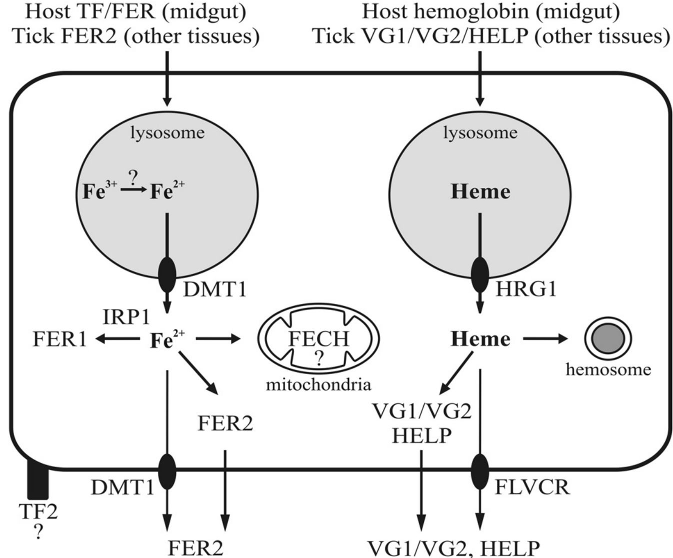

Fig. 3. A model integrating the studied proteins into tick iron and heme metabolism. Tick tissues acquire iron from the host TF/FER (in the midgut) or from tick FER2 (in other tissues). Heme can be obtained from the host hemoglobin (in the midgut) or from tick HELP/VG1/VG2 (in other tissues). The proteins degraded in lysosomes release iron or heme, which is transported into the cytoplasm by DMT1 or HRG1, respectively. Ferrireductase, reducing Fe^{3+} to Fe^{2+} before transport, is currently unknown. Cytoplasmic iron can be stored by FER1 (IRP1 serves as an iron sensor and regulates *fer1* expression), exported into mitochondria, or accumulated into FER2 and secreted from the cell into the tick plasma. Alternatively, iron can be exported through the membrane by DMT1 and directly bound to FER2 in the plasma. Involvement of TF2 in iron metabolism is not probable and its real function remains unknown. In the heme pathway, most heme is detoxified by aggregation in hemosomes. A small portion of the host heme is used by the tick to supplement its heme requirements. It can be bound to VG1/VG2 or HELP and secreted into the plasma or directly transported from the cytoplasm out of the cell, bound to VG/VG2 or HELP, and distributed in the tick body.

suggest their role in immunity (Ong et al., 2006; Zhou et al., 2009). Although the identified tick TF2 was a strong candidate for another iron transporter in ticks, its role in iron metabolism was disproved by RNAi (Fig. 2B). Moreover, expression of *tf2* was not stimulated after immune challenge (Fig. 2C and D), probably excluding its immune function. Tick TF2 contains a putative signal and GPI anchor sequence (Table 1) and is thus predicted to be anchored on the surface of cell, probably of the developing egg (Fig. 1). Therefore, the function of TF2 remains largely unknown and further experiments are needed to clarify its role in tick physiology.

Proteins involved in heme export after host hemoglobin breakdown in the lysosomes are currently unknown. *hrg1* is a membrane transporter, in humans shown to be essential for heme transport from the phagolysosome of macrophages during erythrophagocytosis (White et al., 2013). We found that tick *hrg1* was mainly expressed in ovaries (Fig. 1) and could be important for acquisition of heme imported into the eggs, as KD of *hrg1* by RNAi reduced tick oviposition (Table 3). Some portion of heme is not aggregated in hemosomes, but the tick uses it for its own purposes. It is excreted

from the midgut cell into the hemolymph by an as yet unknown transporter. Tick FLVCR is a homolog of the vertebrate membrane transporter of heme, where it actively exports heme from the cell and acts as an cellular overflow valve reducing excess manufactured heme (West and Oates, 2008). However, we observed that expression of tick FLVCR was below measurable levels and KD of this gene had no effect on ticks, eliminating FLVCR from this function in ticks. As a final step, heme is transported by HELP and VGs into peripheral tissues and ovaries, where it fulfills heme requirements or is incorporated into maturing eggs, respectively (Gudderra et al., 2002).

Heme synthesis is an ancient pathway found in the vast majority of organisms on the planet (Koreny et al., 2013). In the genome of *I. scapularis* we found only the last three genes of the heme biosynthetic pathway (coproporphyrinogen III oxidase, protoporphyrinogen oxidase, and ferrochelatase (FECH)). *fech* is up-regulated by feeding and expressed mostly in ovaries and salivary glands of replete females (Fig. 1). Although silencing by RNAi did not show any effect on tick feeding or further development, vaccination with recombinant FECH decreased oviposition of fed females. The function of this gene is currently unknown, but clearly invites further attention, not only from a physiological aspect, but also from an evolutionary standpoint.

We and others have previously shown by RNAi that KD of tick iron metabolism genes (*fer1*, *fer2*, and *irp1*) had an adverse effect on tick feeding, decreased post-bloodmeal weights, reduced the number of eggs, and influenced hatching (Galay et al., 2013; Hajdusek et al., 2009). It was thus foreseeable that KD of other genes involved in iron metabolism might have similar or even more dramatic effect on ticks. We also expected significant effects after KDs of genes involved in the heme acquisition pathway. Surprisingly, although gene expressions were considerably reduced by the KDs, we found that only KDs of *help* and *hrg1* had some effect on ticks, although not as dramatic as the effects seen with other previously studied genes. An explanation for the weak phenotypes could be slow turnover of some proteins, especially of the membrane receptors, or some leftover mRNA in the cell after KD that could maintain a basic level of the proteins in ticks. Previously, vaccination of rabbits or cows with recombinant FER2 greatly reduced survival and fecundity of ticks fed on these animals (Hajdusek et al., 2010). Out of the proteins studied in this work, vaccination with recombinant FECH and FER1 seemed to have an impact on tick fecundity and development. It remains to be investigated whether a combination of these proteins with FER2 or other available antigens could improve the overall efficacy of anti-tick and anti-transmission vaccines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2016.01.006.

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Paper V

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Multienzyme degradation of host serum albumin in ticks

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ABSTRACT

Host blood proteins, represented mainly by hemoglobin and serum albumin, serve as the ultimate source of amino acids needed for *de novo* protein synthesis during tick development and reproduction. While uptake and processing of hemoglobin by tick gut cells have been studied in detail, molecular mechanisms of host serum albumin degradation remain unknown. In this work, we have used artificial membrane feeding of *Ixodes ricinus* females on a hemoglobin-free diet in order to characterize the proteolytic machinery involved in albuminolysis. Morphological comparisons of ticks fed on whole blood (BF) and serum (SF) at microscopic and ultrastructural levels showed that albumin and hemoglobin have different trafficking routes in tick gut cells. Analysis *in vitro* with selective inhibitors demonstrated that albumin is degraded at an acidic pH by a network of cysteine and aspartic peptidases with predominant involvement of cysteine cathepsins having endo- and exopeptidase activities. The cleavage map of albumin and the roles of individual peptidases in albumin degradation were determined. These results indicate that the albuminolytic pathway is controlled by the same proteolytic system that is responsible for hemoglobinolysis. This was further supported by the overall similarity of gut peptidase profiles in SF and BF ticks at the transcriptional and enzymatic activity levels. In conclusion, our work provides evidence that although hemoglobin and albumin are transported differentially during heterophagy they are digested by a common multienzyme proteolytic network. This central digestive system, critical for successful blood feeding in tick females, thus represents a valuable target for novel anti-tick interventions.

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Introduction

Blood feeding (hematophagy) is an essential physiological process for ticks since host blood serves as their ultimate source of energy and nutrients utilized in their development and reproduction. Processing of blood in ticks differs greatly from that in other hemathophagous arthropods. In blood-sucking insects, blood protein digestion proceeds rapidly in the gut lumen and is carried out mainly by alkaline serine proteases (Briegel and Lea, 1975). In contrast, blood digestion in ticks is a much slower process that occurs inside the acidic vesicles of tick gut cells (Grandjean and Aeschlimann, 1973). Notably, the virtual absence of proteolytic enzymes in the gut contents creates a highly favorable environment for the survival of ingested microorganisms (Sonenshine and Roe, 2014) and represents one of the key factors making ticks potent vectors of an enormous number of human and domestic animal diseases.

Hard ticks (Ixodidae) feed only once per life-stage (larvae, nymph, adult female or male) and females die several days after laying down a large clutch of eggs (Coons and Alberti, 1999). Feeding of females lasts for several days and consists of a slow feeding period, taking approximately 6–9 days, followed by a rapid engorgement occurring 12–24 h prior to detachment from hosts and accounting for about two-thirds of the total blood volume ingested during the slow feeding period. Only mated females can proceed to rapid engorgement indicating a yet uncharacterized physiological control mechanism.

Albumin and hemoglobin serve as the main sources of amino acids for blood-feeding organisms since these proteins account for more than 80% of the total protein content of vertebrate blood. Digestive gut cells take up blood proteins by heterophagy (Sonenshine and Roe, 2014), which in ticks is comprised of at least two distinct endocytic mechanisms. As demonstrated by the tracking of fluorescent hemoglobin and albumin in a primary gut cell line from *Rhipicephalus (Boophilus) microplus* (Lara et al., 2005), the two

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major blood proteins, albumin and hemoglobin, are not handled the same way by the tick gut digestive cells. While albumin is taken up by fluid-phase endocytosis and is directed into a population of small acidic vesicles, hemoglobin is recognized by specific cell-surface receptor(s), which target it to a population of large endosomal vesicles (Lara et al., 2005). This is supported by early ultrastructural studies on tick gut epithelial cells that describe coated pits in the midgut epithelium (Coons et al., 1986). Receptor-mediated endocytosis and a separate processing pathway for hemoglobin most likely evolved as a detoxification mechanism against the potentially dangerous heme that is released upon digestion of hemoglobin (Graca-Souza et al., 2006; Lara et al., 2003).

Existing knowledge on tick digestive proteolytic enzymes began to be assembled from the 1980s to 1990s. First reports included individually characterized 'hemoglobinases' of various classes from different tick species, providing a rather fragmented picture of the tick hemoglobinolytic system (reviewed in (Sojka et al., 2013)). Focusing on one tick species, the major European Lyme disease vector, *Ixodes ricinus*, at a single well-defined life stage (partially-engorged female), we have previously employed a number of reverse genetic and proteomic approaches to globally profile the hemoglobinolytic machinery in the gut (Horn et al., 2009; Sojka et al., 2008, 2013). A mechanistic model for the uptake and proteolytic degradation of hemoglobin degradation in the digestive cells of *I. ricinus* was presented recently (Sojka et al., 2013). Briefly, endopeptidases, aspartic cathepsin D, supported by cysteine cathepsin L and legumain, are responsible for the primary hemoglobin cleavage events. Endopeptidolytic activity of cathepsin B participates in the production of smaller secondary fragments. The pool of peptide fragments released by these endopeptidases is degraded by the action of exopeptidases through the dipeptidase activities of cathepsins B and C (Horn et al., 2009. The unbound heme moiety forms aggregates that accumulate in the hemosomes (Lara et al., 2003).

In the present study we fill some of the gaps in knowledge on the two separate trafficking pathways of hemoglobin and serum albumin by mapping tick gut cell peptidases responsible for the digestion of serum albumin. Our primary task was to determine whether the component peptidases of the multienzyme hemoglobinolytic network were also involved in the degradation of host albumin. To address these fundamental questions we exploited our recently developed protocol for tick artificial membrane feeding on whole blood and hemoglobin-free serum (Perner et al, submitted manuscript). This powerful tool, combined with molecular approaches previously employed in the mapping of digestive hemoglobinolysis in *I. ricinus*, enabled a comprehensive analysis of albumin degradation in the same species and a comparison of both proteolytic pathways.

Materials and methods

Tick collection, maintenance and feeding

Adult *I. ricinus* females and males originating from the pathogen-free colony of Institute of Parasitology, BC CAS were used throughout the study. Ticks were maintained in glass vials at 24 °C, 95% humidity, and 15:9-h day/night regime. For natural feeding, 25 females were allowed to feed on the backs of guinea pigs, in the presence of 25 males, until full engorgement, or were forcibly removed from the host at the specified feeding phase. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 095/2012. For artificial membrane feeding, the procedure developed by Krober and Guerin (2007) was adapted as described elsewhere (Perner et al., submitted manuscript). Briefly, bovine blood, acquired from the local slaughterhouse, was manually defibrinated by stirring and the remaining clot was removed using a sterile strainer. The hemoglobin-free serum was prepared by centrifugation at 2500 × *g* for 10 min at 4 °C and the supernatant was centrifuged again at 10,000 × *g* for 10 min at 4 °C. Both the whole blood and serum were supplemented with filter-sterilized glucose to a final concentration of 2 g/l. Immediately prior to feeding, both blood and serum meals were supplemented with adenosine triphosphate (1 mM) and gentamicin (5 μg/ml), 3.1 ml of the meal was pipetted into each feeding unit adapted for a 6-well cultivation plate and regularly exchanged at intervals of 12 h. Fifteen *I. ricinus* females were placed into one feeding unit lined with a thin (80–120 μm) silicone membrane, previously pre-treated with a bovine hair extract (Krober and Guerin, 2007). An equal number of males were added to the feeding units 24 h post-attachment of females to the membrane. Females were allowed to feed until full engorgement or removed from the membrane at the specified time of feeding.

Preparation of tick gut homogenates for biochemical and proteomic analyses

Twelve feeding units were prepared for two groups of ninety *I. ricinus* females, either fed on whole blood (blood-fed, BF) or serum (serum-fed, SF). In both groups, about 50% of the females attached and successfully fed on the applied diet. Partially-engorged females were removed from the membrane, individually weighed and gut tissues (from each group) were dissected under a stereo microscope with LED illumination (Stemi DV4, Zeiss). Dissected gut tissues were washed in sterile PBS to remove excess blood from the gut lumen without disruption of gut epithelial cells. Pools of gut tissues were prepared from each BF and SF group and stored at −80 °C until homogenization. Gut tissue extracts (400 μg of protein/ml) were prepared by homogenization of the pooled gut tissue in 50 mM Na-acetate, pH 4.5 and 1% CHAPS on ice. The extract was cleared by centrifugation (16,000 × *g*, 10 min, 4 °C), filtered through an Ultrafree-MC 0.22 μm filter (Millipore) and stored at −80 °C.

Tick gut dissection for RNA isolation and reverse transcription

For each specified time point, guts from nine BF/SF *I. ricinus* females were dissected and divided into three independent biological replicates (3 guts per sample). Sterile diethyl pyrocarbonate (DEPC)-treated PBS was used for dissection and washing of gut tissues. Total RNA was isolated from dissected guts using a NucleoSpin® RNA II kit (Macherey-Nagel). The quantity and quality of isolated total RNA was verified using a NanoDropTM 1000 spectrophotometer and 1% agarose gel electrophoresis, respectively. Isolated RNA samples were stored at −80 °C prior to cDNA synthesis. Single-stranded cDNA was reverse-transcribed from 0.5 μg of total RNA using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche). For subsequent applications, cDNA was diluted 20 times in nuclease-free water.

Microscopy techniques

Partially-engorged *I. ricinus* females were dissected in 10 mM Hepes buffer, pH 7.4. Tick gut tissue was transferred to a freshly prepared fixative (4% formaldehyde, 0.1% glutaraldehyde in 10 mM Hepes buffer, pH 7.4) and incubated at room temperature for 1 h. Samples were further washed with 0.1 M Na-phosphate buffer (PB), pH 7.4, containing 4% glucose. Tissues were transferred to pre-cooled cryogenic vials and dehydrated in acetone at −10 °C (30%, 50%, 70%, 80%, 90% and 95% for 30 min at each step). The samples were rinsed three times in anhydrous acetone at room

temperature and infiltrated stepwise in acetone mixed with Embed 812 epon resin (EMS) (acetone:epon ratios of 2:1, 1:1, 1:2, at −10 °C for 1 h at each step followed by 100% SPI-epon resin overnight). Embedded samples were thermo-polymerized at 62 °C for 48 h. Semi-thin sections (400 nm) were stained with toluidine blue for 1 min at 40 °C for light microscopy observations. Ultrathin sections (90 nm) were picked up on copper grids, contrasted in ethanolic uranyl acetate and in lead citrate for 30 and 20 min, respectively, and observed in a JEOL 1010 transmission electron microscope. LR white resin tick gut preparations were prepared as described previously (Franta et al., 2010) and used for TEM immunostaining of serum albumin and hemoglobin. Sections were blocked in 3% BSA, 1×PBS, 0,05% Tween 20 (10 mM Na-phosphate, 0.5 M NaCl, pH 7.4, 0.05% Tween-20) for 1 h and subsequently exposed to primary antibodies (anti-hemoglobin AJ1339a, 1:50 in 1×PBS, AbGent) for 3 h. Grids were washed in 1×PBS and incubated with secondary antibody solution containing 10 mM PBS and 6 nm colloidal gold coupled to protein A (1:40 in 1×PBS, Aurion) and incubated at RT for 1 h. For double staining of hemoglobin and serum albumin sections were washed (3 × 3 min) with 1×PBS, 0,05% Tween 20, 1×PBS, and distilled H_2O. Grids were flipped over and incubated again in blocking solution (0.02 M glycine in 1×PBS, 0.05% Tween 20) for 1 h and in the anti-bovine serum albumin primary antibody (B7276, 1:30 in 1×PBS, Sigma-Aldrich) for 3 h. After several washing steps with 1xPBS, 15 nm colloidal gold (Goat anti Rabbit, 1:40 in 1×PBS, Aurion) was added and incubated at room temperature for 1 h and followed with additional washes with 1xPBS. After immunolabeling, sections were contrasted in ethanolic uranyl acetate for 5 min, lead citrate for 3 min and observed in a JEOL 1010 transmission electron microscope.

Profiling of tick peptidases with substrates and inhibitors

Proteolytic activities were identified and characterized by hydrolysis of the following substrates: 50 mM Z-Arg-Arg-AMC for cathepsin B (Barrett and Kirschke, 1981), 50 mM Z-Phe-Arg-AMC for cathepsins L and B (Barrett and Kirschke, 1981), 50 mM Gly-Arg-AMC for cathepsin C (McGuire et al., 1997), 40 mM Abz-Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu for cathepsin D (Masa et al., 2006), 30 mM Z-Ala-Ala-Asn-AMC for legumain (Kembhavi et al., 1993). Activity measurements were performed at 35 °C using an aliquot of the gut tissue extract (100 to 2000-fold diluted stock solution) in 0.1 M Na-acetate pH 4.0 (for cathepsin D), pH 5.0 (for legumain), pH 5.5 (for cathepsins B, C and L) including 2.5 mM DTT (for cysteine peptidases) and 25 mM NaCl (for cathepsin C). For activity assays in the presence of peptidase inhibitors, an aliquot of the extract was pre-incubated (15 min at 35 °C) in the assay buffer with the inhibitor: 10 mM E-64 for papain-type cysteine peptidases (Barrett et al., 1982), 10 mM CA-074 for cathepsin B (Murata et al., 1991), 10 mM Ala-Hph-VS-Ph for cathepsin C (Kam et al., 2004), 1 mM Aza-N-5s for legumain (Gotz et al., 2008), and 10 mM pepstatin for cathepsin D (Knight and Barrett, 1976). Proteolytic activity was continuously measured after addition of substrate in a Tecan Infinity M1000 fluorescence reader at 320 nm excitation and 420 nm emission wavelengths (for Abz-containing substrate) or at 360 nm excitation and 465 nm emission wavelengths (for AMC-containing substrates). Legumain was measured in the presence of 10 mM E-64 to prevent confounding proteolysis by papain-type cysteine peptidases. All measurements were performed in technical triplicate.

Quantification of serum albumin degradation

Digestion of human serum albumin (3–5 μg) was performed with BF tick gut tissue extracts (0.3–2.0 μg of protein) in 50 mM Na-acetate pH 3.0–5.0, 50 mM MES pH 6.0 or 50 mM Tris–HCl pH 7.0–8.0 including 2.5 mM DTT, in a total volume of 15 μl for 15 min to 6 h at 35 °C. For serum albumin degradation in the presence of peptidase inhibitors, an aliquot of the extract was pre-incubated (15 min at 35 °C) in buffer at pH 4.0 with the inhibitor: 10 μM E-64, 10 μM pepstatin, 10 μM CA-074, 1 μM Aza-N-5s. For quantification, aliquots of the digest were subjected to derivatization with fluorescamine to quantify the newly formed amino-terminal ends; the rate of degradation was determined using aliquots withdrawn at different time points (up to 4 h) (Horn et al., 2009). The fluorescence signal was measured using a Tecan Infinity M1000 fluorescence reader at 370 nm excitation and 485 nm emission wavelengths. All measurements were performed in triplicate. For SDS–PAGE visualization of hydrolysis, serum albumin digests were resolved by Laemmli SDS–PAGE 15% polyacrylamide gel electrophoresis under reducing conditions and protein was stained with Coomassie Blue G250. Selected serum albumin fragments were analyzed by N-terminal protein sequencing (after electroblotting of SDS–PAGE gels) using a Procise 494 cLC protein sequencer (Applied Biosystems).

Identification of serum albumin fragments

Human serum albumin (15 μg) was incubated with BF tick gut tissue extracts (0.3 μg of protein) in 50 mM Na-acetate, pH 4.0 including 2.5 mM DTT, in a total volume of 90 μl for 30 min at 35 °C. The extract was pre-incubated with different combinations of inhibitors (10 μM pepstatin, 50 μM E-64, 1 μM Aza-N-5s) for 15 min at 35 °C in the same buffer. Finally, the reaction mixture was treated with 10 μl of 50% formic acid. Detergents in reaction mixtures were removed using HiPPR Detergent removal spin columns (Thermo Scientific). Reaction mixtures were analyzed by LC–MS/MS analysis performed on an UltiMate 3000 RSLCnano system (Dionex) coupled to a TripleTOF 5600 mass spectrometer with a NanoSpray III source (AB Sciex). The peptides were separated on an Acclaim PepMap100 analytical column (3 μm, 25 cm × 75 μm ID, Thermo Scientific) using a formic acid/acetonitrile system. Full MS scans were recorded from 350 to 1250 *m/z*, with up to 15 candidate ions per cycle being subjected to fragmentation. In MS/MS mode the fragmentation spectra were acquired within the mass range of 100–1600 *m/z*. Protein Pilot 4.5 software (AB Sciex) was used for peptide identification.

Analysis of peptidase gene expression by quantitative real-time PCR (qPCR)

Tick gut cDNA preparations from independent triplicates for each time-point served as templates for expression analyses by quantitative real-time PCR (qPCR). Samples were analysed using a LightCycler 480 (Roche) and Fast Start Universal SYBR Green Master Kit (Roche). Information on qPCR primers is shown in Table 1. Each primer pair was inspected for its specificity using melting curve analyses. Reaction conditions in 50 cycles were the following: denaturation, 95 °C/10s; annealing, 60 °C/10s; extension, 72 °C/10s. Relative expression of peptidase genes was calculated using the ΔΔCt method (Pfaffl, 2001) and normalized to *elongation factor 1* (*ef1*) (Nijhof et al., 2009; Urbanova et al., 2014).

Statistics

Enzymatic activity and expression data were analyzed using an unpaired Student's *t*-test with Welsh's correction using the significance value set generally as $P < 0.05$ if not indicated differentally. For bar graphs, means ± SEM are used. All statistics were performed using GraphPad Prism 6 for Windows, version 6.04.

Table 1
Oligonucleotides used for qRT-PCR expression analyses.

Gene	GenBank Acc. no.	Forward primer 5′–3′	Reverse primer 5′–3′	Amplicon length (nt)
*Ir*CB	EF428206	tcaacaagatcaacacaacttgg	tcatggagatggatttgtcg	60
*Ir*CL	EF428205	agaaccagggacagtgtgga	ctcttgcggaagtgctgtc	76
*Ir*CC	EU128750	caccaagaacagggtgaagaa	ctcgcaaccctgagagtagg	76
*Ir*CD	EF428204	gacagaaggcggacagtacc	cggaaattgtgaaggtgacat	74
*Ir*AE	AY584752	cgaaaccgtgctttcctg	tcagtcttctcagcgtcacc	77
EF1α[a]	GU074769.1	acgaggctctgacggaag	cacgacgcaactccttcac	80

[a] EF1α—elongation factor 1α (reference gene).

Results

SF and BF ticks—Differences in morphology and intracellular trafficking of blood proteins

Adult *I. ricinus* females were artificially fed in a membrane feeding unit containing whole blood (BF ticks) or blood serum only (SF ticks). Both groups were morphologically identical except for the body color when BF ticks were brown-red due to the presence of hemoglobin in the diet, while SF ticks were a light yellow color (Fig. 1A and B). To be consistent with the hemoglobinolytic model established for partially-engorged females, most of the experiments in this work were performed with partially-engorged females fed for 5 days (Sojka et al., 2008). The only observed difference was the slightly slower feeding process for SF females, which displayed a significantly lower tick body weight when collected at the 5th day of feeding (Fig. 1C).

Gut cells showed overall identical morphologies by the light microscopy of toluidine blue stained semi-thin gut sections from BF and SF tick groups (Fig. 2A and B). Of significant difference was the presence of membrane-delimited organelles with condensed heme in the center in gut cells of BF ticks, which we propose to be the newly forming hemosomes (Lara et al., 2003), indicating ongoing digestion of hemoglobin. The ultrastructural TEM images of BF tick gut cells (Fig. 2C) displayed a massive uptake of hemoglobin through coated pit-endocytosis and hemoglobin storage, and possibly processing in large electron-dense endosomes. This process was clearly absent in SF tick gut cell (Fig. 2D). Double immunolabeling with anti-hemoglobin antibodies (6 nm gold particles) and serum albumin (15 nm gold particles) (Fig. 2E) confirmed the specific presence of hemoglobin in the electron-dense intracellular vesicles that are obviously absent in SF tick gut cells (Fig. 2D). Double immunolabeling of albumin and hemoglobin also confirmed that *I. ricinus* gut digestive cells transport the two major blood protein components to different endocytic vesicles.

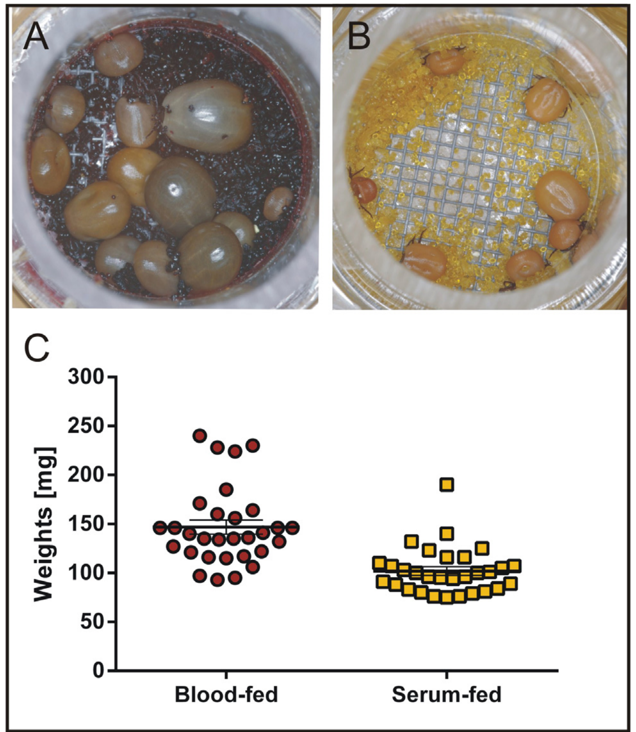


Fig. 1. *Differential in vitro membrane feeding of I. ricinus females.* Panel *A*—Feeding unit with *I. ricinus* ticks fed on whole bovine blood (BF). Panel *B*—Feeding unit with ticks fed on bovine serum (SF). Panel *C*—body weights of *in-vitro* blood-fed (BF) and serum-fed (SF) partially- engorged *I. ricinus* females used for determination of enzymatic activities in tick gut extracts. The mean weights and SEM of BF and SF ticks were 146.8 ± 7.3 mg and 101.9 ± 4.4 mg, respectively. The difference was statistically significant (Student's *t*-test) with *P* value <0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Serum albumin degradation in the tick gut occurs at an acidic pH

Proteolytic degradation of serum albumin in gut tissue extracts was investigated *in vitro* using SDS–PAGE analysis of serum albumin fragments. Degradation was most efficient at an acidic pH of 3.0 and 4.0, and was negligible at pH 5.0 and higher pH values (Fig. 3A). The time course of serum albumin degradation at acidic pH demonstrates a gradual hydrolysis of serum albumin (67 kDa) into initial fragments of about 50 kDa that are gradually processed by limited proteolysis (to ~45 kDa species) and then efficiently removed and converted into small fragments (below 15 kDa) (Fig. 3B). A comparison of albuminolytic activities in BF and SF tick gut extracts showed no significant differences in activity profiles and protein degradation patterns.

Serum albumin is degraded by multiple peptidases

The contribution of individual peptidase activities to serum albumin degradation was evaluated *in vitro* by analyzing the impact of selective inhibitors of major peptidases previously identified in the gut tissue of partially-engorged *I. ricinus* females (Horn et al., 2009) (Fig. 4A and B). Two different assays were used: (i) quantification of serum albumin fragments by fluorescamine derivatization to determine degradation rate and (ii) SDS–PAGE visualization of the fragmentation pattern of serum albumin. The fluorescamine derivatization assay showed that the application of E-64 (targeting papain-type cysteine peptidases) dramatically reduced the rate of serum albumin digestion to about 15% (Fig. 4A). Selective inhibition of papain-type peptidases cathepsins B and C, performing exopeptidase activities, with CA-074 and Ala-Hph-VS-Ph resulted in about a 45% and 40% decrease in the rate of albuminolysis, respectively. Inhibition of cathepsin D-type aspartic peptidases by pepstatin resulted in about a 10% reduction in albuminolysis and was additive when pepstatin was combined with E-64. Legumain inhibition by Aza-N-5s had an insignificant effect on the rate of albuminolysis.

SDS–PAGE visualization of the prolonged digest further demonstrated that papain-type peptidases play a major role in serum albumin degradation (Fig. 4B, lines 2 and 3). However, complete

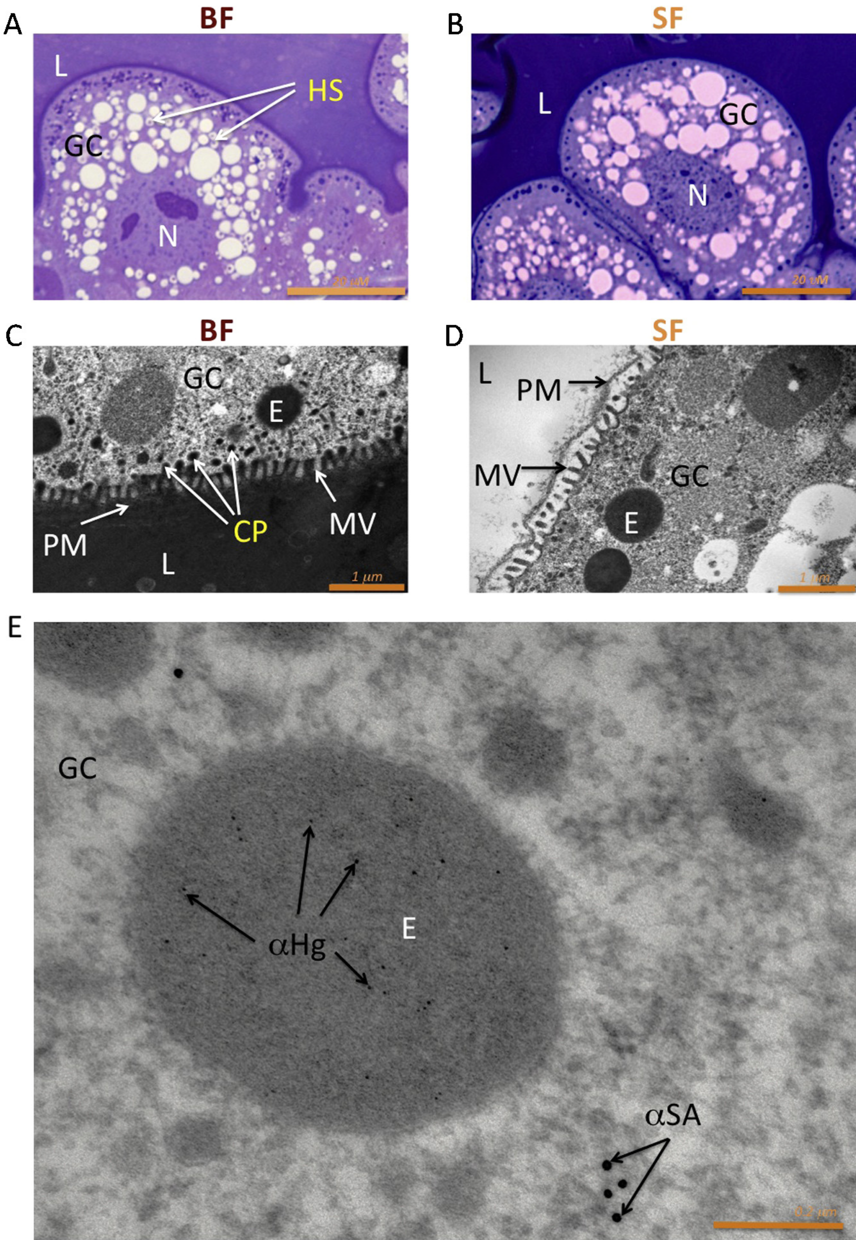
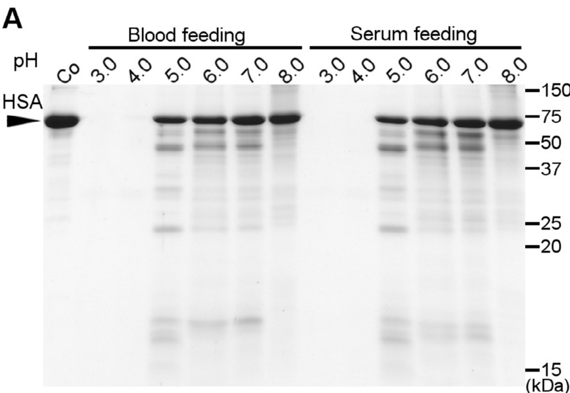


Fig. 2. *Microscopic tracking of hemoglobin and albumin in digestive gut cells of BF and SF tick females.* Panel *A*, *B*—toluidine blue stained sections of tick gut epithelium from BF (*A*) and SF (*B*) partially-engorged ticks under a light microscope. Cells are not different except for the clear presence of emerging hemosomes in the BF gut preparations, indicating ongoing hemoglobin digestion. Panels *C, D*—transmission electron microscopy (TEM) of ultrathin sections of gut cells from partially-engorged BF (C) and SF (D) *I. ricinus* females. Note that coated pits, indicating enormous endocytosis of hemoglobin through specific recognition by an uncharacterized clathrin-pathway associated receptor in the BF gut cells (C), are clearly missing in gut cells of ticks fed a hemoglobin-free (serum only) diet (D) although some dense large endosomes persist and could contain residual hemoglobin stored from nymphal feeding. Panel *E*—double immunostaining of ultrathin TEM preparations from blood-fed ticks. Note that albumin and hemoglobin are transported through different tick gut intracellular pathways and are stored and possibly processed in distinct types of endosomes. The staining also indicates that the coated pits highlighted in panel C specifically carry hemoglobin. Abbreviations: *BF*—whole-blood fed ticks, *SF*—serum only fed ticks, *L*—gut lumen, *GC*—gut cell, *N*—nucleus, *PM*—perithrophic matrix, *MV*—microvilli, *E*—hemoglobin containing endosomes, *αHg*—immunostaining of hemoglobin (6 nm diameter beads), *αSA*—immunostaining of serum albumin (15 nm diameter beads). Highlighted in yellow: *HS*—emerging hemosomes with condensed heme in the center and *CP*—coated pits—receptor mediated endocytosis of hemoglobin.

inhibition of albuminolysis was observed only when E-64 was combined with pepstatin and Aza-N-5s, thus targeting all three peptidase groups (Fig. 4B, line 7). Cathepsin D alone (Fig. 4B, line 8) and legumain alone (Fig. 4B, line 6) could also degrade serum albumin; however, their proteolytic contribution was much less than that of the papain-type peptidases (Fig. 4B, line 9). Fig. 4B (line 9) shows that papain-type peptidases were able to cleave serum albumin to produce large fragments (45–50 kDa) and were also responsible for their efficient removal and the generation of smaller peptides. Individual or combined actions of cathepsin D and legumain provided only limited proteolysis of serum albumin associated with the accumulation of the large fragments of about 45–50 kDa (and their complementary fragments in the range of 20–25 kDa) (Fig. 4B, lines 3, 6 and 8).

Proteolytic cleavage map of serum albumin

The tick gut tissue extract was treated with combinations of three selective peptidase inhibitors (E-64, pepstatin, and Aza-N-5s) to obtain peptidase-specific digests of serum albumin, driven by



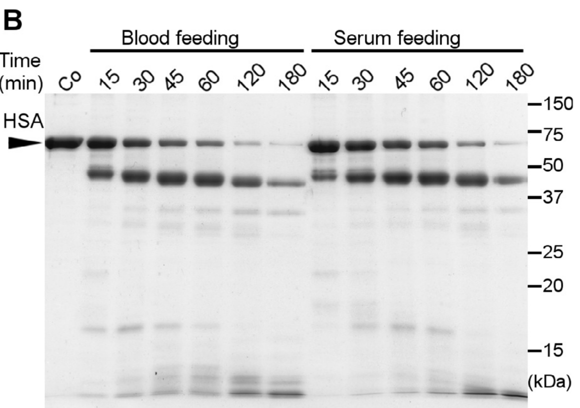


Fig. 3. *Human serum albumin degradation assays.* Panel *A*—Serum albumin was digested (6 h) *in vitro* with the gut tissue extract (1.2 μg of protein) from BF and SF *I. ricinus* partially-engorged females at various pH values. Panel *B*—The time course of human serum albumin degradation performed at pH 4.0 with gut extracts (0.3 μg protein) of BF and SF *I. ricinus* partially-engorged females. The digests were subjected to SDS–PAGE; the human serum albumin substrate (HSA mark) and its degradation products are visualized by protein staining. The non-digested control (Co) is indicated.

papain-type cysteine peptidases, cathepsin D-type aspartic peptidases, and legumains. The resulting fragments were characterized by mass spectrometry, and the cleavage sites in the serum albumin sequence are identified in Supplemental Fig. S. The data were used for analysis of cleavage site specificity of participating peptidases (Fig. 5A). A dense series of cleavage sites (∼75% of cuts) was found for papain-type peptidases, which exhibited less well-defined specificities. The cleavage sites of cathepsin D (∼16% of cuts) contained hydrophobic residues in the P1 and P1′ positions with a preference for Leu in P1. Legumain cleavage (∼9% of cuts) targeted predominantly Asn and Asp residues in the P1 position.

Furthermore, the proteolytic cleavage map of serum albumin (Supplemental Fig. S) derived from a short-term digest, was used for analysis of initial fragmentation events in the molecule. Fig. 5B shows the general distribution of cleavage sites in the 3D model of serum albumin and identifies regions susceptible or resistant to proteolysis. The highest frequency of cleavage events was found between residues 375 and 488 in the C-terminal half of the molecule.

Absence of hemoglobin in the tick diet has only limited influence on intestinal peptidase activities and their mRNA expression

The distribution of peptidases was analyzed by activity profiling in extracts from gut tissue of partially-engorged *I. ricinus* females. Five major digestive peptidases present in this tissue (Horn et al., 2009) were monitored using a set of specific peptidyl substrates (Fig. 6) and their identity was further confirmed by sensitivity to selective peptidase inhibitors listed in Table 2. Cathepsin D activity, measured with the substrate Abz-Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu, was strongly inhibited (more than 80%) by the class-selective inhibitor pepstatin. Cleavage of the substrate Z-Phe-Arg-AMC by cathepsins L and B was highly sensitive to the class-selective inhibitor E-64, and cathepsin B activity analyzed with the substrate Z-Arg-Arg-AMC was efficiently blocked by the inhibitor CA-074. Cathepsin C activity was assayed with the substrate Gly-Arg-AMC

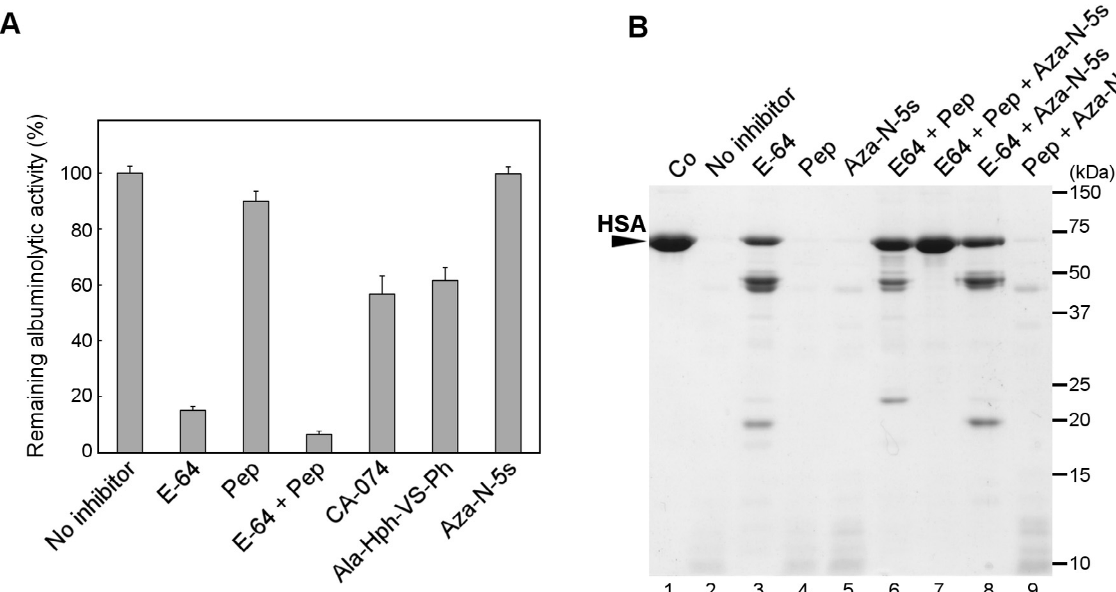


Fig. 4. *Inhibition of HSA degradation by selective peptidase inhibitors.* Human serum albumin was digested *in vitro* with the gut tissue extract from *I. ricinus* partially-engorged (naturally) females at pH 4.0. The extract was pre-incubated with the selective peptidase inhibitors (or their mixtures) prior to initiation of digestion. A glossary of inhibitors (and target enzymes): pepstatin (Pep) (aspartic peptidases), E-64 (papain-type cysteine peptidases), CA-074 (cathepsin B), Ala-Hph-VS-Ph (cathepsin C), Aza-N-5s (legumain). Panel *A*—the degradation rate of serum albumin was determined using the fluorescamine derivatization assay. Inhibition of digestion is expressed as remaining degradative activity relative to the uninhibited control (100%). The mean values ± SE are given. Panel *B*—the serum albumin digest (treated with 1.2 μg of protein extract for 2.5 h) was subjected to SDS–PAGE; the serum albumin substrate (HSA mark) and its degradation products are visualized by protein staining. The non-digested control (Co) is indicated.

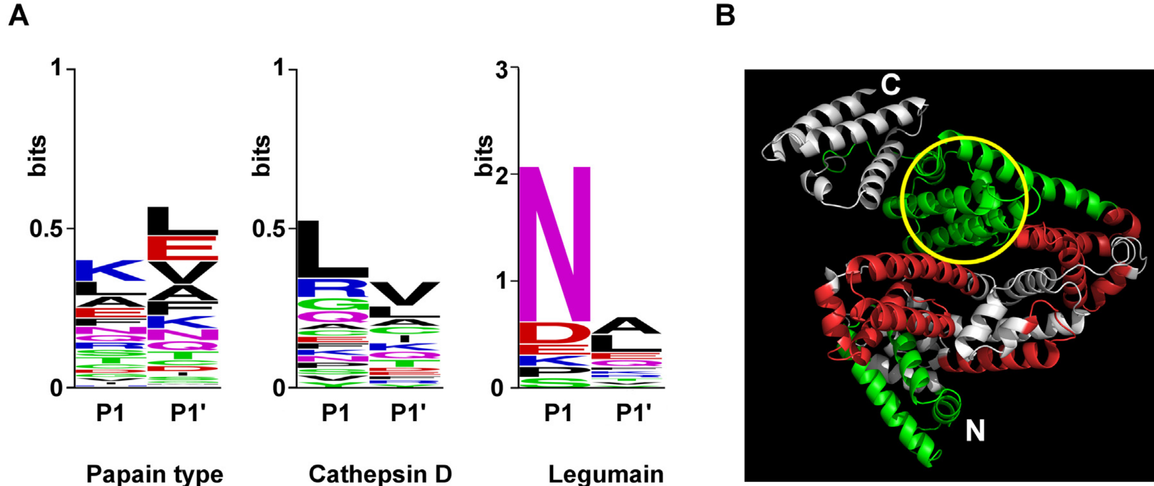


Fig. 5. *Proteolytic cleavage map of the human serum albumin molecule.* Panel *A*—Serum albumin was digested *in vitro* with the gut tissue extract from BF *I. ricinus* partially-engorged females at pH 4.0 for 30 min. The extract was pre-incubated with selective inhibitors of key albuminolytic peptidases to obtain peptidase-specific digests. These digests were driven by papain-type peptidases (pepstatin and Aza-N-5s treated extract), legumain (pepstatin and E-64 treated extract), and cathepsin D (E-64 and Aza-N-5s treated extract). The cleavage sites were identified by mass spectrometry (Supplementary Fig. S2), and the P1–P1′ subsite specificities of individual peptidases were inferred. The plots generated by WebLogo (Crooks et al., 2004) depict relative entropies between the observed and background distributions of amino acids at each subsite. The overall height of each letter stack indicates the sequence conservation at that position, whereas the height of amino acid symbols within the stack reflects the relative frequency of the corresponding residue at that position. Amino acid color coding: acidic (red), basic (blue), neutral (purple), polar (green) and hydrophobic (black). Panel *B*—Localization of cleavage sites (from panel *A*) is presented in the 3D ribbon model of human serum albumin (PDB code 1BM0). The molecule is colored according to relative frequency of the cleavage events: region susceptible and resistance to proteolysis are in green and red, respectively; partially susceptible regions are in white. The region of initial proteolytic attack is marked by a yellow circle. The amino and carboxyl termini of the molecule are indicated by N and C, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

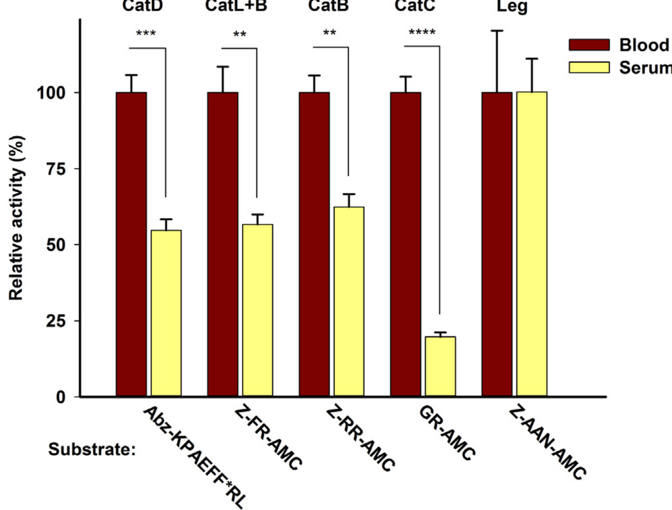


Fig. 6. *Substrate profiling of tick gut peptidases.* The major activities attributed to cathepsins (Cat) D, B, L and C, and legumain (Leg) were monitored. Proteolytic activities in the three extracts of five guts dissected from BF or SF *I. ricinus* females were measured using a continuous fluorometric assay with specific peptidase substrates (as indicated). Data are normalized to one tick, and BF values are set to 100%. The mean values of three biological replicates ± SEM are given. **, *** and **** show the statistically significant differences between BF and SF ticks with *P* values <0.003, <0.001 and <0.0001, respectively.

Table 2
Inhibitor profiling of BF and SF gut peptidases.

Target protease	Inhibitor[a]	Inhibition (%)[b]	
		Blood feeding	Serum feeding
Cathepsin D	Pepstatin (10 μM)	83.5 ± 5.0	91.7 ± 6.3
Cathepsins L + B	E-64 (10 μM)	100 ± 0.2	100 ± 0.7
Cathepsin B	CA-074 (10 μM)	97.7 ± 0.3	98.0 ± 0.6
Cathepsin C	Ala-Hph-VS-Ph (10 μM)	99.6 ± 0.3	100 ± 0.4
Legumain	Aza-N-5s (1 μM)	99.4 ± 1.0	99.5 ± 1.2

[a] Proteolytic activities in the gut tissue extracts from BF and SF *I. ricinus* were measured using continuous fluorimetric assays with specific peptidase substrates (see Fig. 4) in the presence of selective peptidase inhibitors (at indicated concentration) relevant to the target enzyme.
[b] The values are expressed as percentage of inhibition of the uninhibited activity (N.I.) set as 100%. The mean values ± and standard deviations from technical triplicates are given.

and was inhibited by Ala-Hph-VS-Ph. Legumain activity, measured with Z-Ala-Ala-Asn-AMC, was sensitive to the specific inhibitor Aza-N-5s. Using these assays, we compared individual peptidase activities in gut extracts from BF and SF *I. ricinus*. Fig. 6 shows that both extracts contained these activities, however there were differences in their levels. Activities in SF ticks were reduced by about half for cathepsin D, ∼38% for cathepsin L and B, ∼45% for cathepsin B, and by ∼80% for cathepsin C; no significant difference in activity was observed for legumain. The data were normalized per tick gut but consistent results were achieved using normalization per protein concentration in the extracts (data not shown). In summary, all five major endo- and exopeptidases in *I. ricinus* gut tissue were demonstrated in BF and SF ticks, including (i) aspartic peptidase class, cathepsin D of the AA clan, and (ii) cysteine peptidase class, cathepsins B, L and cathepsin C (dipeptidyl peptidase I) of the CA clan (papain-type), and asparaginyl endopeptidase (legumain) of the CD clan.

Based on the results of biochemical peptidase profiling, genes encoding individual peptidases playing roles in hemoglobin degradation in partially engorged females, namely IrCD, IrCL, IrAE, IrCB, IrCC (reviewed in (Sojka et al., 2013)), were analyzed for their expressional response to the presence/absence of hemoglobin in the diet (Fig. 7). The dynamic profiling of expression in the course of feeding and after detachment from the membrane show that all peptidases were upregulated by feeding at the same time in both groups of BF and SF ticks. The only statistically significant difference was observed for IrCB mRNA levels in partially engorged females (the day 5 of feeding), which were about 4-fold higher in BF ticks compared to SF ticks.

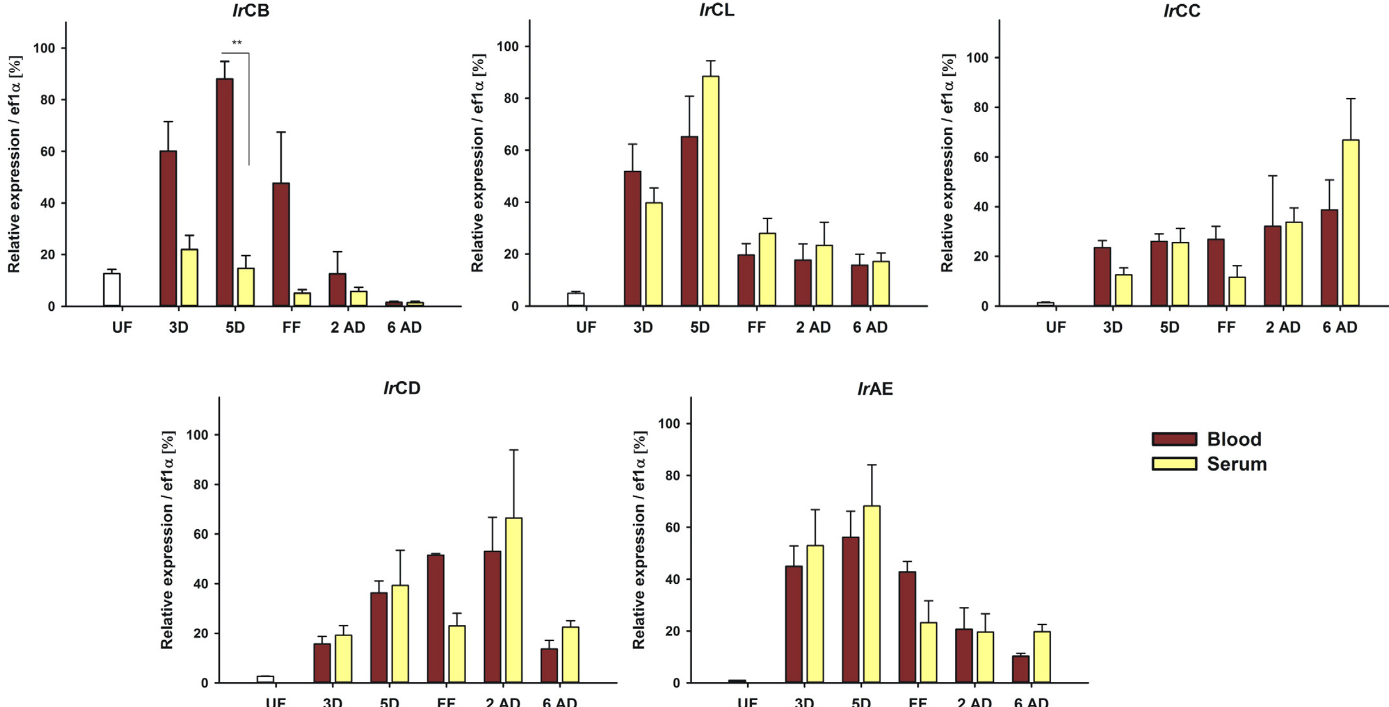


Fig. 7. *Dynamics of I. ricinus digestive peptidase gene expression in response to whole-blood or a serum-only diet.* Guts were dissected from SF and BF *I. ricinus* females at the specified time of feeding and after detachment from the membrane. Digestive peptidase mRNA levels were analyzed *via* qPCR using *I. ricinus* elongation factor 1α as a reference gene. Gene abbreviations and GenBank accession numbers are listed in Table 1. Expression levels are related to the maximal measured values (100%). The bars are means of three independent biological replicates (each containing gut cDNA from three females) and the error bars represent the calculated SEM. ** indicates the statistically significant difference with a P value = 0.0015. UF—unfed females; 3D, 5D—3 and 5 days feeding on the membrane; FF—fully-fed females; 2 AD, 6 AD—2 and 6 days after detachment from the membrane.

Discussion

The overall goal of this work was to compare the proteolytic degradation of serum albumin and hemoglobin inside tick gut cells in order to determine similarities or differences between the processing pathways of the two major host blood proteins. Our results with BF and SF ticks clearly demonstrate that digestive gut cells from BF ticks transport hemoglobin through small electron-dense compartments (coated pits) towards a population of large endosomes (Fig. 2C). Coated pits are clearly missing from the ultrastructural images of gut cells from SF ticks, confirming that hemoglobin is not trafficked from the gut lumen to their gut epithelium (Fig. 2D). Some large electron-dense endosomes did persist in SF tick gut cells and could perhaps contain undigested hemoglobin stored from nymph-stage feeding. Our results are consistent with the ultrastructural description of clathrin-coated pits and the transport of hemoglobin to a population of large gut cell endosomes, previously reported in other tick species (Coons et al., 1986; Lara et al., 2005). Heme condensation and the creation of hemosomes (Lara et al., 2003) in BF tick gut cells (Fig. 2A) indicate an ongoing degradation of hemoglobin in BF ticks only, while SF ticks uptake and degrade albumin and other serum proteins. This validates the results of comparative studies using BF and SF tick gut tissue extracts discussed below (Figs. 4, 6 and 7).

TEM immunostaining of hemoglobin and serum albumin confirm the exclusive presence of hemoglobin in the electron-dense coated pits and large endosomes of BF tick gut cells (Fig. 2E). Furthermore, simultaneous TEM localization to separate endosomes demonstrates different endocytic pathways for hemoglobin and albumin inside the *I. ricinus* gut cells in accordance with previous experimental findings from *B. (R.) microplus* (Lara et al., 2005). This result further underscores the need to compare proteolytic mechanisms that might differ between these two endocytic pathways.

Results of *in vitro* degradation of human serum albumin by gut tissue extracts from BF and SF ticks clearly demonstrate that albuminolysis in tick gut cells occurs under acidic conditions consistent with the acidic intracellular compartmental environment. Furthermore, no substantial differences were observed when comparing albumin fragmentation patterns in SF and BF tick gut extracts. This indicates that tick intestinal proteolysis works in the same manner regardless of the presence or absence of hemoglobin in the tick meal (Fig. 3A). Total albuminolytic rate measured by quantification of serum albumin fragments in SF ticks represents only about 73% (±8) % of the activity in BF ticks (see Section 2). The observed differences between BF and SF ticks may be due to differences in average tick weight on the collection day (Fig. 1C) since activity of all tested peptidases increased exponentially towards the end of the slow feeding period (Franta et al., 2010; Sojka et al., 2013).

As demonstrated by the *in vitro* effect of selective inhibitors on the rate of degradation and fragmentation pattern of serum albumin (Fig. 4), the general features of albumin proteolysis by tick gut proteases were similar to those observed in the proteolytic processing of hemoglobin (Horn et al., 2009). Albumin degradation was predominantly carried out by papain-like cysteine peptidases (cathepsins B, C and L) but a complete inhibition of the degradative process could only be achieved when the papain-family specific inhibitor E64 was combined with cathepsin D and legumain inhibitors. A detailed proteomic study was aimed at elucidating cleavage sites in serum albumin when attacked by the key albuminolytic peptidases (Fig. 5, Supplemental Fig. S). A dense series of cleavage sites of papain-type peptidases with less defined specificity requirements reflects the fact that several peptidases participated in this digestion. Predominant cleavage of papain-type tick albuminases after a Lys residue is in agreement with the general preference of these enzymes for basic residues at P1 (Franta et al., 2011; Choe et al., 2006; McGuire et al., 1997). The preference

of gut aspartic peptidases of the cathepsin D-type for hydrophobic residues at P1 and P1′ positions, with a preference for Leu in P1, correspond to the cleavage pattern determined for *I. ricinus* cathepsin D1 (Sojka et al., 2012). Asn and Asp residues in the P1 position of tick gut legumain is in agreement with specificity of tick gut legumain IrAE for hydrolysis of asparaginyl bonds and also aspartyl bonds at a lower pH (Rotari et al., 2001; Sojka et al., 2007). Importantly, the cleavage patterns for the three albuminolytic activities were analogous to those identified by the same method for hemoglobinolysis in *I. ricinus* gut tissue (Horn et al., 2009).

General differences between the processing of albumin and hemoglobin by tick gut cells were determined by the cleavage maps of hemoglobin (Horn et al., 2009) and serum albumin molecules (Supplemental Fig. S) exposed to inhibitor-delimited peptidase class activities of tick gut tissue extracts. While the primary "attack" on hemoglobin was carried out by cathepsin D, serum albumin was initially cleaved mainly by papain-type endopeptidases. We propose that the region between residues 375 and 488 in the C-terminal half of the HSA molecule is the initial site of proteolytic attack, leading to the formation of large fragments of about 45–50 kDa (and complementary fragments in the range of 20–25 kDa). This is in agreement with the fragmentation pattern observed in SDS–PAGE experiments (Figs. 3B and 4B) and also with N-terminal sequencing of the large fragments, which revealed that they have an intact N-terminus of the serum albumin molecule. The proteolysis-susceptible region, composed of six α-helices, was attacked by all three groups of albuminolytic peptidases (Fig. 5 B and Supplemental Fig. S), and its cleavage might start the gradual unfolding of the serum albumin substrate, making it prone to subsequent deep degradation by papain-type peptidases. Cysteine and aspartic protease cooperate in the two parallel degradative proteolytic pathways for host hemoglobin and serum albumin. This was previously studied in *Schistosoma mansoni* (Delcroix et al., 2006), where the primary cleavage of hemoglobin was also facilitated by cathepsin D as in hookworms (Williamson et al., 2003) and malaria causing *Plasmodia* (Goldberg, 2005), while serum albumin was primarily attacked by papain-type cathepsin B and L. We propose that differences in the initial processing of hemoglobin and serum albumin, in both ticks and blood flukes, are rather substrate-specific than being caused by a different composition of multi-peptidase digestive matrixes at the site(s) of albumin and hemoglobin degradation.

We analyzed changes in mRNA expression and enzyme activity of individual tick gut peptidases in response to the presence/absence of hemoglobin in the tick meal. Interestingly, as seen from the qRT-PCR comparison (Fig. 7), while the patterns of individual cysteine and aspartic peptidase mRNA levels were almost identical over the several-days of SF and BF female feeding, *I. ricinus* cathepsin B (IrCB) was the only significant peptidase gene/mRNA that was upregulated by hemoglobin in the tick diet and thus might have a more important role in hemoglobinolysis than in the degradation of serum albumin. Comparison of BF and SF peptidase activities (Fig. 6) showed that, except for legumain, all other peptidases were generally more active when hemoglobin was present in the tick meal. Substantial differences were found for cathepsins D and C (about 40–50% and more than 75% reduced activity in SF ticks, respectively). These differences may reflect specific requirements for albumin proteolysis or may be related to the previously discussed delay in feeding of SF ticks compared to BF females (Fig. 1C).

In summary, this work demonstrates that although hemoglobin and serum albumin follow different intracellular endocytic routes in tick gut cells, they are digested by an identical proteolytic enzyme network comprising peptidases of cysteine and aspartic classes. We assume that the mechanism of endocytosis and processing of the major blood protein components is evolutionary conserved among different tick species and that toxicity of large amounts of heme released during processing of hemoglobin inside the tick gut cells is the main reason for the evolution of a specific endocytic pathway for hemoglobin. In our future research we would like to determine individual roles of all isoenzymes of the tick gut digestive peptidase complex and to further explore the anti-tick application potential of specific peptidase inhibitors and recombinant protein based vaccines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.12.014.

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Paper VI

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Vector Biology:
Tyrosine Degradation Protects Blood Feeders from Death via *La Grande Bouffe*.
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Vector Biology: Tyrosine Degradation Protects Blood Feeders from Death via *La Grande Bouffe*

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Blood-feeding arthropods digest vast amounts of host-blood nutrients. A new study suggests that tyrosine degradation is essential for the survival of blood-fed kissing bugs, mosquitoes, and ticks. This finding presents a promising target for the control of these disease vectors.

Blood-feeding arthropods, such as mosquitoes, tse-tse flies, sand flies, kissing bugs, and ticks, transmit a broad variety of infectious diseases, which have a severe impact on human health and the agricultural economy worldwide [1]. According to the World Health Organization [2], "vector-borne diseases account for more than 17% of all infectious diseases, causing more than one million deaths annually." Diseases such as malaria, Chagas disease, leishmaniasis, Dengue fever, Lyme disease, schistosomiasis, sleeping sickness and, most recently, infections caused by the Zika virus affect hundreds of millions of people around the globe. Therefore, scientists from various research fields join forces in a common effort to find vulnerable targets to combat blood-feeding arthropods and the infections they transmit. Even though parasitic arthropods differ in many biological aspects, reflecting their independent evolutionary routes towards hematophagy (blood-feeding lifestyle), they all share one common trait — they all eat a lot! Can you imagine gorging yourself on food 100 times your own weight? Such gluttony would certainly kill the gourmands, as artistically portrayed in Marco Ferreri's classic film *La Grande Bouffe*. The reason why blood feeders eat as much as they do in a single blood meal is that blood feeding on the host is a risky maneuver, and a second opportunity to feed may not arise. Is there a common toolkit necessary for the successful digestion of such a large, nutrient-full meal? In this issue of *Current Biology*, Sterkel *et al.* [3] report on the discovery of the vital importance of functional tyrosine detoxification for blood-feeding arthropods [3].

Early studies by the research group of Pedro Oliveira significantly contributed to our current knowledge of how hematophagous arthropods have adapted to their bloody diet, mainly focusing on heme-detoxification mechanisms [4,5]. Heme, an iron-containing pro-oxidative molecule, is a component of host hemoglobin, the major protein constituent of blood that enables oxygen transport in the body. Vast amounts of heme from digested hemoglobin present a serious challenge for blood-feeding arthropods. Kissing bugs of the genus *Rhodnius* were shown to make crystals of excessive heme in a hemozoin-like structure [6]. Mosquitoes break heme down into a distinct degradation product [7]. Ticks store excessive heme in an organelle-like compartment called the hemosome [8,9]. As ticks lack heme biosynthesis [10], a small portion of acquired heme is used for the assembly of their endogenous hemoproteins. Failure of heme acquisition and inter-tissue trafficking leads to aborted embryogenesis in ticks [11]. In addition to heme and iron metabolism, the tyrosine-degradation pathway now described by Sterkel *et al.* [3] reveals a novel Achilles' heel in the physiology of blood-feeding arthropods.

Tyrosine is a semi-essential amino acid that can be either synthesized from phenylalanine or directly acquired from the diet. Eukaryotic proteins, including blood proteins, are not particularly rich in tyrosine (about 3% of total amino acids). Yet previous tissue-specific transcriptomic analyses in *Rhodnius prolixus* revealed an over-representation of transcripts encoding enzymes involved in tyrosine degradation in the digestive tract [12]. In order to elucidate the physiological relevance of this finding, Sterkel *et al.* [3] performed a series of functional studies focused on enzymes participating in tyrosine metabolism. The tyrosine-degradation pathway consists of five enzymatic steps, each of which is catalyzed by a discrete protein encoded by a single gene. The authors performed RNAi-mediated silencing of all five genes, yet only the knockdown of those genes encoding tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate dioxygenase (HPPD), the first two enzymes in the tyrosine-catabolism pathway, resulted in a marked reduction of survival of *R. prolixus* females following a blood meal (Figure 1). The authors reasoned that the observed lethal phenotype was most likely caused by the formation of crystals resulting from the accumulation of tyrosine upon digestion of vast amounts of host blood. Such a mechanism may remotely resemble gout, the human disorder caused by excessive meat consumption that is manifested by formation of uric-acid crystal deposits in the joints.

The authors also examined the functions of other genes involved in alternative pathways of tyrosine metabolism, for example, prophenoloxidases or tyrosine hydroxylase. However, RNAi-mediated knockdown of these genes did not exert a similar killing effect as that observed for knockdown of TAT and HPPD [3]. This observation further corroborates the hypothesis that the accumulation of tyrosine, rather than the absence of a tyrosine-derived molecule, mediates the lethality associated with inhibition of the tyrosine-degradation pathway.

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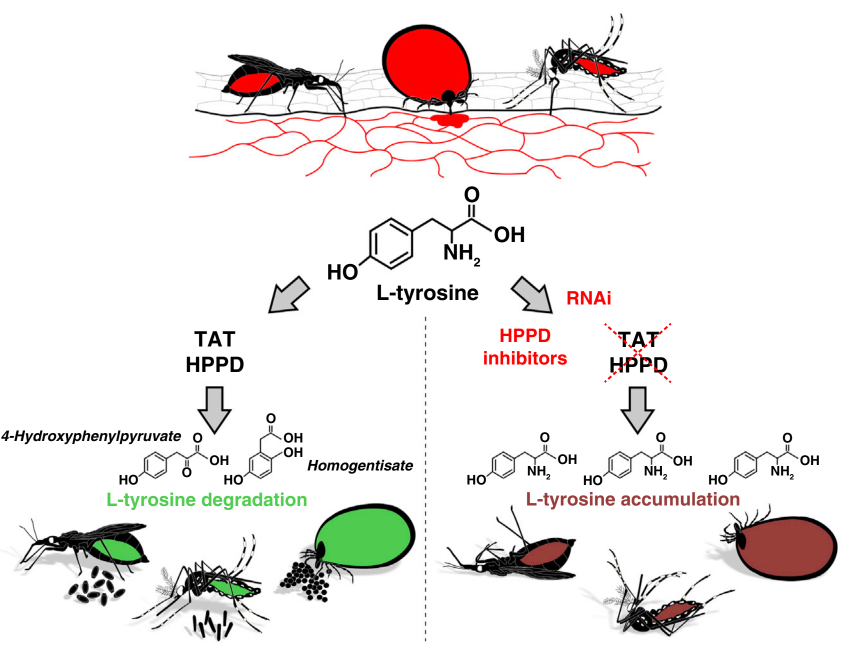

Figure 1. The essential nature of tyrosine detoxification in blood-feeding arthropods.
Blood-feeding arthropods such as (left to right) the kissing bug *R. prolixus* (Chagas-disease vector), the mosquito *Ae. aegypti* (vector of yellow fever and other infectious agents), and the cattle tick *R. microplus*, have to cope with vast amounts of nutrients acquired from a protein-rich blood meal. L-tyrosine, a semi-essential amino acid liberated upon digestion of host blood proteins, is further catabolized via a conserved pathway of five enzymatic steps. The first two enzymes of the tyrosine-catabolism pathway are tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate dioxygenase (HPPD). RNAi-mediated silencing of the gene encoding HHPD, or treatment with an HHPD inhibitor, is lethal for blood feeders due to the accumulation of crystals formed from an excess of non-degraded tyrosine. Image courtesy of Martina Hajduskova (www.biographix.cz).

In addition to using RNAi, the authors took the advantage of the availability of specific HPPD inhibitors [13,14] that are ordinarily used as a herbicide (mesotrione) or as a drug (nitisinone/ Orfadin®) taken by patients with hereditary tyrosinemia type I (a genetic defect in the tyrosine-degradation pathway). Mesotrione administered to *R. prolixus* either by injection or via artificial membrane feeding caused the premature death of blood-fed adult and nymphal stages of kissing bugs, similar to that observed following RNAi-mediated knockdown of the genes encoding TAT or HPPD. Moreover, further experiments demonstrated that inhibition of tyrosine degradation with mesotrione kills other blood-feeding disease vectors including *Aedes aegypti* or the cattle tick *Rhipicephalus microplus* (Figure 1). Most importantly, HPPD inhibition had no impact on two non-hematophagous insects studied, the milkweed bug (*Oncopeltus fasciatus*) and the mealworm beetle (*Tenebrio molitor*). These results substantiate the pivotal point of the study: impairing tyrosine degradation selectively kills blood-feeding arthropods.

Additional experiments by Sterkel *et al.* [3], oriented towards practical applications of their discovery, revealed that a mesotrione dose lethal for mature or immature kissing-bug stages could be absorbed through their cuticle via topical application, and that blood feeding on mice that had been administered nitisinone orally was also lethal. This signifies a fundamental prerequisite towards the development of novel insecticides based on HPPD inhibition. At present, over 6,000 HPPD inhibitors, with different chemical properties, have been developed in the search for a potent and environmentally friendly herbicide [13]. A systematic screening of appropriate candidates will be a useful starting point towards bringing the discovery of Sterkel *et al.* [3] to fruition in the development of a selective insecticide that specifically kills blood-feeding arthropods. Given that the phenotype is brought about only after a blood meal (Figure 1), such an insecticide or acaricide might be exploited mainly to reduce the population of disease vectors in the most invaded areas.

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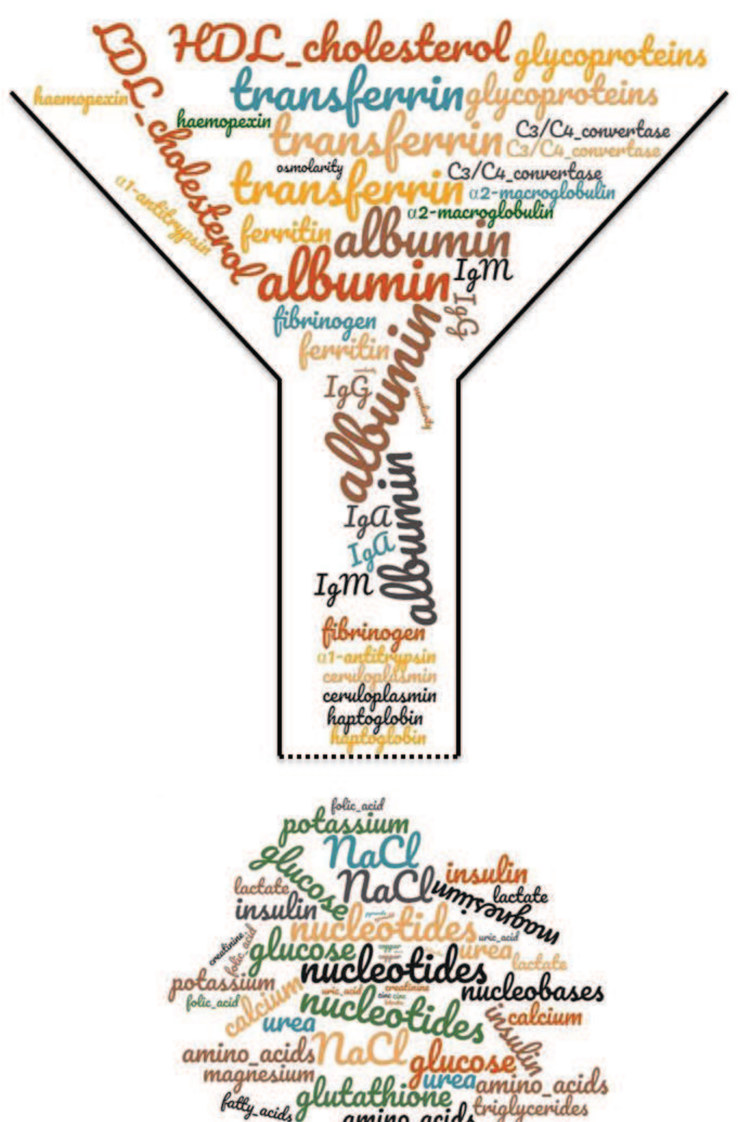
Part IV

Ongoing studies utilising a tick membrane feeding (unpublished data)



Interference with iron acquisition impairs tick feeding

We have experimentally demonstrated in Paper I (Appendix I) that host transferrin contributes to the bioavailable iron pool in the tick midgut. Feeding ticks on serum supplemented with 3 mg/ml of commercial bovine holo-transferrin increased IrFer1 expression in tick midgut during tick feeding and did not have any adverse effect on tick feeding physiology. Analogous supplementation with bovine apo-transferrin led to decreased IrFer1 expression in the tick midgut and, mainly, to impaired feeding progression of ticks. However, we failed to confirm such observation with dialysed (MWCO 10 kDa, PBS) apo-transferrin. Even though both suppliers, Sigma and Rocky mountain biochemicals, claim to extensively dialyse before lyophilisation and shipment, clearly an unknown variable affects the results. Either there are still traces left in the protein preparation of ethylenediaminetetraacetic acid used for stripping transferrin off the iron, or the protein supplementation in liquid form behaves differently than direct dissolving.

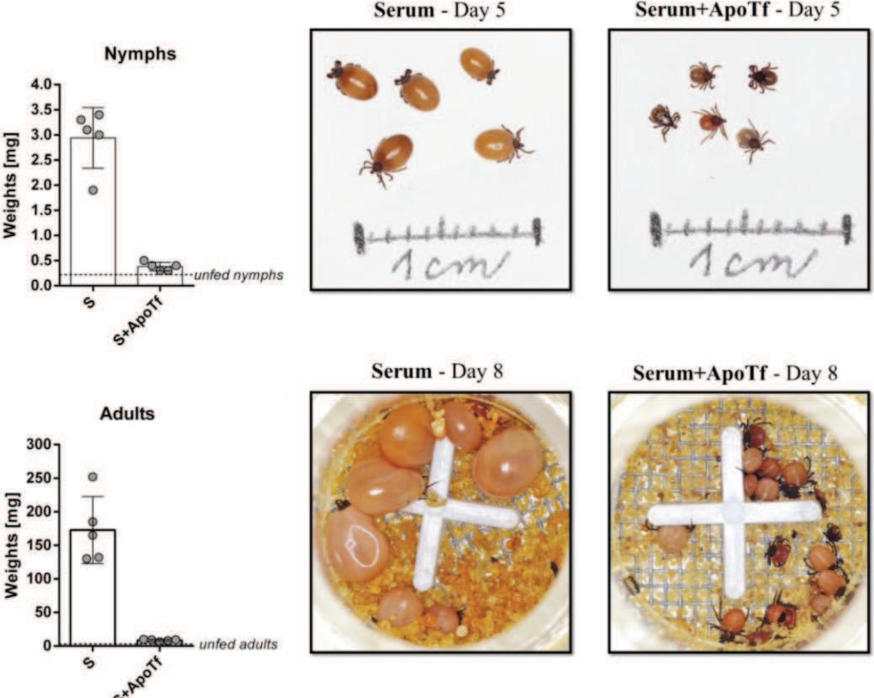


Figure 8. Membrane feeding of *Ixodes ricinus* ticks Apo-transferrin supplemented serum. Nymphs (top panel) and adult (bottom panel) *Ixodes ricinus* ticks were fed on serum supplemented with 3 mg/ml commercial bovine Apo-transferrin. Both tick stages fail to feed on the supplemented diet. Achieved fully engorged weights are shown in bar graphs (left panels) and pictures of feeding units with fully engorged ticks fed serum (middle pannels) or serum supplemented with Apo-transferrin (right panels) are shown.

Serum fractionation to assess nutritional requirements of the *I. ricinus* ticks

We have managed, so far, to get an insight into a tick dependency on dietary haem and what tick molecules interact with haem. Even though we showed that serum-feeding prevents tick reproduction, due to ovarian haem deficiency, the serum-feeding had no impact on feeding progression itself. It implies that essential nutrients required for tick feeding are serum components. To identify essential feeding molecule(s), we proceeded with serum fractionation. Size separation was one of the fractionation steps we utilised. Using a 10 kDa cut off, *I. ricinus* adults were fed low molecular fraction of serum (≤10 kDa), supplemented with a physiological haematocrit of red blood cells (RBCs). These ticks manage to initiate the feeding, but fail to progress in feeding stages. When the low molecular diet was switched for whole blood, ticks resumed feeding and progressed to full engorgement, unlike those that remained on low molecular diet (Figure 9). It indicates that a low molecular serum fraction (≤10 kDa) is essential for tick feeding initiation, but high molecular serum fraction (≥10 kDa) is essential for tick feeding progression. Further research into what components of low and high molecular serum fractions propel tick feeding initiation and progression, respectively, is currently ongoing.

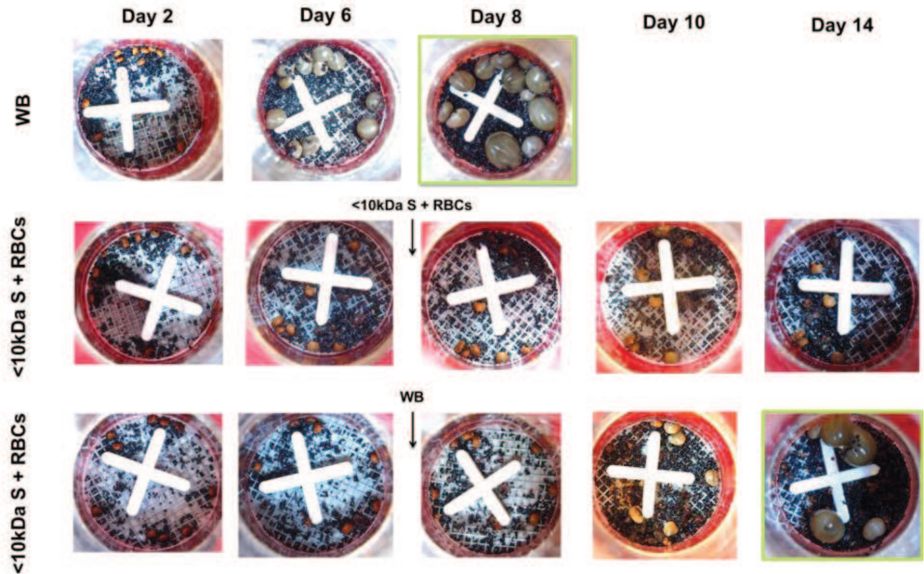


Figure 9. Membrane feeding of *Ixodes ricinus* adults fractionated diet. Adult *Ixodes ricinus* females were fed whole blood (WB, top panel) or low molecular weight serum fraction of bovine serum (<10kDa S) supplemented with red blood cells (RBCs, middle and bottom panel). One group resumed feeding the "<10kDa S" diet (middle panel), while the other switched for whole blood after Day 6 (bottom panel). Pictures of feeding units with ticks that managed to fully engorge are highlighted in green.

Tick membrane feeding to investigate salivary secretion

Tick membrane feeding system as described in (Kröber and Guerin, 2007) allows not only modulation of diets for ticks, but also allows a collection of tick secretion material or pathogen detection. Tick saliva contains a complex potion of pharmacologically active non-peptidic as well as peptidic components (Francischetti et al., 2009). As only protein-rich diets are currently used for tick feeding, proteomic analysis of early secretory material would seem non-feasible. Protein-free diet that would enable collection and identification of tick peptidic secretory material needs to be formulated. It was also discovered that many small RNAs have been found in externally secreted fluids across taxa (LeBoeuf et al., 2016). Tick feeding well, again, might be utilised for collection of tick-specific miRNA. It needs to be noted that role of these extracellular miRNAs remains uncertain (Turchinovich et al., 2012).

In collaboration with prof. José Ribeiro, we conducted a transcriptomic study exploring expression of salivary gland transcripts in response to active host immunity (BioProject PRJNA 312361). Artificially-fed ticks (on reconstituted rabbit blood with its heat inactivated serum) and naturally-fed ticks (on laboratory rabbit) were dissected at 24 h, 48 h, and 72 h after feeding initiation. Salivary glands preparations were subjected to RNA-seq analyses and transcriptome compositions were compared. We have revealed that, for instance, several transcripts encoding metalloproteases or 18.3 kDa Basic tail proteins were substantially up-regulated in ticks fed on a rabbit for 24 hours (Table 1). The encoded proteins may be essential for tick attachment and feeding initiation on host and, in that respect, may represent suitable anti-tick targets.

Table 1. Overview of RKPM values for contigs differentially expressed between membrane- (M) and rabbit-fed (R) ticks. Ticks were fed for 24 hours. Three libraries (1–3) were used for each mode of feeding. Each library is derived from a single tick pair of salivary glands. $p < 0.05$, fold change (FC) ≥ 5, coverage ≥ 50, mean RPKM in R ≥ 10.

Link to Pep	Comments	Evalue	% Coverage	M24_1 RPKM	M24_2 RPKM	M24_3 RPKM	R24_1 RPKM	R24_2 RPKM	R24_3 RPKM	T test R/M 24	FC R/M 24
Ir-249265	Secreted metalloprotease	0	100	31,2	13,4	8,6	175,6	125,4	87,7	0,013	7
Ir-SigP-242556	18.3 kda subfamily of the Basic tail superfamily	2,00E-59	100	3,2	2,4	2,1	17011,5	6302,5	15365,9	0,018	4956
Ir-249264	Secreted metalloprotease	0	76,5	7,7	20,5	1,9	47,5	36,3	66,8	0,019	5
Ir-SigP-241765	antigen 5 protein - signalP detected	2,00E-90	86	2,4	6,4	0,2	77,2	202,6	141,6	0,019	47
Ir-SigP-219629	18.3 kda subfamily of the Basic tail superfamily	3,00E-55	99,3	0,4	0,2	0,2	2695,6	987,3	2594,7	0,019	7525
Ir-261824	anticomplement protein 2	9,00E-92	91,5	0,0	0,1	0,1	91,5	32,2	91,2	0,022	1171
Ir-226907	Secreted metalloprotease	0	85,2	4,9	0,1	5,7	20,1	15,9	33,0	0,023	6
Ir-SigP-258570	18.3 kda subfamily of the Basic tail superfamily	1,00E-48	100	3,6	3,2	2,9	430,8	166,1	510,8	0,025	114
Ir-SigP-369	Secreted metalloprotease	0	100	48,8	0,1	7,2	172,3	145,1	319,6	0,026	11
Ir-237695	Secreted metalloprotease	0	89,8	9,7	0,1	12,3	34,2	29,0	57,8	0,026	5
Ir-369	Secreted metalloprotease	0	95,7	44,9	0,0	5,2	156,2	132,5	296,7	0,028	12
Ir-1315	secreted protein precursor	0	85,6	2,2	6,0	0,1	98,1	60,6	34,9	0,028	23
Ir-241765	antigen 5 protein - signalP detected	0	100	1,7	5,2	0,1	42,8	159,7	111,9	0,039	45
Ir-SigP-229700	Ticsk ixostatin	3,00E-17	103,1	0,0	0,0	0,1	32,3	17,6	8,6	0,048	720
Ir-SigP-239926	anticomplement protein IxAC-B5 precursor	8,00E-67	70,3	337,0	87,9	109,7	1444,0	1074,8	479,2	0,048	6

Tick membrane feeding to test a character of host anti-tick response

We and others (Schorderet and Brossard, 1993) showed that repeated natural feeding of adult *I. ricinus* ticks on rabbits gradually leads to a reduction of engorged tick weights (Figure 10 A). To disclose if the effect is brought about by a complex immune response including participation of cellular fractions or whether the phenotype is caused by a shear antibody-antigen complex formation (i.e. the repeated feeding of ticks turn the host blood immunologically toxic), we collected the blood from the repeatedly infested rabbits and fed the ticks these blood meals on the artificial feeding system. In this experiment, blood is manually defibrinated, where most of white cells are removed. The experimental blood from immunised rabbits was served to adult *I. ricinus* ticks and blood from naïve rabbits was used as a control. Using the artificial feeding system, no clear differences were observed in feeding progression and achieved weights of fully engorged ticks in this comparison to control (Figure 10 B,C). It is suggestive that a more complex immune response stands behind the reducing effect on tick engorgement. Further tests for the most prominent antigens identifications are ongoing.

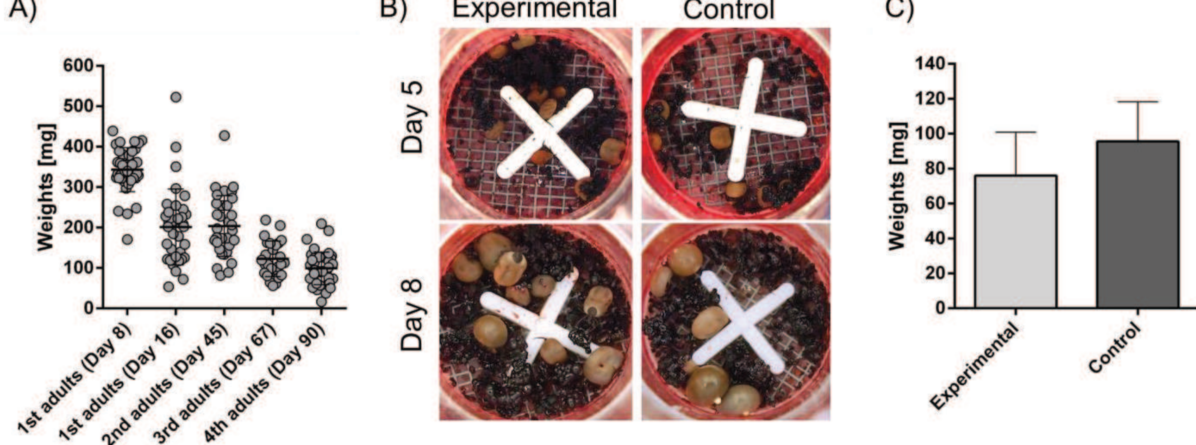


Figure 10. Membrane feeding of *Ixodes ricinus* adults on the blood from repeatedly-infested rabbit. (A) *I. ricinus* adult females were fed on a laboratory rabbit in subsequent feedings with 2–3 week gaps among the feedings. Adult females were placed in two chambers on rabbit sides (25 ticks into each chamber). When allowed, two immediately subsequent feedings were performed without a time gap. n ≥ 30. **(B, C)** *I. ricinus* adults were fed on a reconstituted rabbit blood (haematocrit 60%) either from the previously infested rabbit, repeated adult *I. ricinus* feeding (experimental), or from a naïve rabbit (control). **B)** Pictures of feeding units during the course of artificial feeding. **C)** Weights of dropped-off females and females removed on Day 10, n ≥ 6.

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Thesis references

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Appendix I

Authors response to Paper I decision letter

Acquisition of exogenous haem is essential for tick reproduction

Author response

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[Editors' note: the author responses to the first round of peer review follow.]

While one cannot fail to recognize the large amount of time and effort that went into the work and reviewers appreciate attention to an important topic, a number of substantial concerns were raised. […] Specific comments from the reviewers are appended below.

Summary of the major changes, new data, and additional experiments carried out to improve the merit of our work presented in the new manuscript.

1) Data mining

In addition to the tick *Ixodes scapularis* genome, the genome-wide search has been performed in available genomes of other related mites namely the herbivorous mite *Tetranychus urticae* and the predatory mite *Metaseiulus occidentalis*. This mining, completed by a detailed phylogenetic analysis, confirmed that while non-hematophagous mites contain all genes coding for complete haem biosynthesis, the *I. scapularis* genome has retained only three genes encoding vestigial mitochondrial enzymes of haem biosynthesis, namely coproporphyrinogen-III oxidase (CPOX), protoporphyrinogen oxidase (PPOX) and ferrochelatase (FECH). Two other genes present in *I. scapularis* genome: δ- aminolevulinic acid synthase (ALAS) and uroporphyrinogen decarboxylase (UROD) are clearly of bacterial origin.

Another new discovery made by the genome wide searches was that gene coding for haem oxygenase is commonly missing also in other mites and possibly in chelicerates and myriapodes in general.

Therefore, we conclude that lack of haem oxygenase in ticks is not likely an evolutionary adaptation to the blood-feeding but rather an old ancestral trait of the last common ancestor of Chelicerata and Myriapoda.

2) Membrane feeding and rescue experiments

In-vitro membrane feeding of *I. ricinus* females was complemented by a rescue experiment performed by addition of different concentration of commercial bovine haemoglobin to the serum diet.

This conclusively confirmed that dietary haemoglobin is essential as a source of haem for embryonic development and tick reproduction. Full rescue of fertility is obtained with less than 1% of the physiological concentration of hemoglobin in blood.Under this condition, where haemoglobin contribution as an amino acid source is negligible, a significant amount of haem is still transferred to the eggs allowing normal embryo development. The embryonic development in tick eggs was examined by microscopy and the results are included in relevant figures.

3) The iron-level analysis in tick tissues

The analysis of elemental iron in tick peripheral tisssues was performed by a highly sensitive method of graphite-furnace atomic absorption spectroscopy (GF AAS) that allows determination of elemental iron concentration in a sub-milligram range of starting biological material (as is the case of pooled and dried tick tissues). Since GF AAS cannot distinguish between haem and non-haem iron, only ovaries and salivary glands dissected from partially engorged BF or SF ticks were analysed. The obtained results confirmed the previous data based on quantitative monitoring of ferritin 1 levels in tick tissues proving that haem is not a source of bioavailable iron for ticks.

4) Lack of haem oxygenase was confirmed by HPLC analysis of heam-derived pigments

In order to provide a stronger evidence that haem is not catabolised in ticks, we have adapted an HPLC method for detection of biliverdin IX in the tick gut homogenates. This analysis confirmed that no haem degradation product was present in the guts of fully engorged ticks, while we could detect biglutaminyl-biliverdin IX metabolite in homogenates from blood-fed mosquitoes, used as a positive control.

5) Removal of ferrochelatase functional assessment by RNAi

Data showing that RNAi-silencing of ferrochelatase had no effect on tick feeding success, oviposition, and reproduction, raised another questions about its physiological role, that we were not able to answer. As we felt that these data were contributing to a loss of focus on the main conclusions of this work, we used them in another manuscript with a distinct focus(Hajdusek et

al. “Tick iron and heme metabolism -- new target for an anti-tick intervention”, currently under revision). We refer to these results in the Discussion with a tentative reference as (Hajdusek et al., submitted manuscript).

6) The manuscript was largely re-written to improve the overall readability, figures re-arranged to include new data, and the obtained results accordingly re-interpreted.

Reviewer #1:

Major concerns:

1) Paragraph one, subheading “Host blood haemoglobin is expendable for tick feeding and oviposition but essential for reproduction”, Figure 2: What is truly interesting about this work is that haem seems to play a critical role in tick reproductive physiology. This speculation is derived from the key finding that unlike with blood, ticks fed on serum are unable to give rise to larvae. However, serum-fed ticks are not only deprived of hemoglobin from RBCs but also lack many other cellular/molecular components present in the blood. If haem is indeed the blood factor (absent in serum-fed ticks) that resulted in reproduction defects, then the authors could restore these defects with biochemical complementation by adding physiological concentrations of haem (or hemoglobin) to the serum used for tick feeding. This is a critical experiment that needs to be performed to support the authors' major conclusion.

We agree with the reviewer that the lack of experiment with addition of haem or haemoglobin into the serum diet to rescue tick reproduction was a critical drawback. We performed this experiment with addition of physiological concentration (10%) of pure bovine haemoglobin and demonstrated that the embryonic development and larvae hatching was fully restored (see bottom panels of Figure 2). In addition, we performed this experiment with sub-physiological concentrations of haemoglobin (1% and 0.1%) and could demonstrate that as little as about one hundredth of haemoglobin levels in tick diet is sufficient to sustain their reproduction. We agree with the reviewer that supplementation the serum with haem (haemin) would be interesting. However, such an experiment requires a demanding optimization because of haem binding capacity of serum proteins (e.g. haemopexin, albumin).

Therefore simple addition of haemin into serum may lead to artefactual results which would be difficult to interpret.

2) Paragraph one, subheading “Haemoglobin is an indispensable source of haem, replaceable source of amino acids, and not a source of iron for ticks”, Figure 3: The proposition that haemoglobin is not a source of iron for ticks is another novel aspect of the study. However, the conclusion is based on indirect evidence that needs to be further substantiated. Iron concentration could be measured in tick tissues. The authors need to show that the amount of

non-haem (serum) iron in blood-fed ticks is comparable to that of the total iron in serum-fed ticks.

We agree with the reviewer that the proof of bio-available iron content based on monitoring intracellular ferritin (Fer1) in tissues (such as used in our previous work, Hajdusek et al., PNAS, 2009) is indirect and requires substantiation. Therefore, we performed a determination of total elemental iron in tick tissues by GF AAS (see above, paragraph 3). This analysis confirmed that, apart from bioavailable iron, there is also no significant difference in levels of elemental iron in tissues from BF and SF ticks (Figure 3D).

3) The Method/Results section relevant to the in vitro membrane feeding system is severely deficient in details that would permit other labs to reproduce the work. The authors should mention the differences (in any) in feeding time, engorgement rates, percentage of egg-laying females in comparing blood with serum feeding. This would suggest how serum feeding impacts tick engorgement, growth, and embryogenesis.

There were no obvious differences in the time-course of membrane feeding, feeding success, weights of engorged females and egg clutches sizes between BF and SF ticks. These experiments were repeated many times with reproducible results (see Author response image 1 below).

	Feeding duration (no. of days, mean ± SEM, n = 60)	Attachment rate (%, mean)	Engorgement success (%, mean)	Egg laying females (%, mean)	Clutch weight (mg, mean ± SEM, n = 26)
BF ticks	9.65 ± 0.077	76.7	91.3	85.4	107 ± 17
SF ticks	9.56 ± 0.090	68.3	90.8	72.6	88 ± 5

Author response image 1.

Table 1: Overview of the feeding and egg laying parameters of membrane-fed *I. ricinus* females.

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4) The defect in larval hatching for serum-fed ticks is an interesting finding that also lack details and should be elaborated further. No evidence is provided other than an undefined image (Figure 2, bottom right panel) and a statement "[…]whereas no larvae hatched out of SF ticks' eggs". If these represent immature eggs, at least a thorough histological analysis would be useful.

We agree that our previous statement that "no larvae hatched out of eggs laid by SF ticks" was insufficient as a proof of impaired tick reproduction. Therefore, we performed a light microscopic examination of developing embryos in eggs laid by BF- and SF- ticks, as well as in the rescue experiment with different amount of haemoglobin in the serum diet (S+Hb ticks). We noted that embryonic development was blocked only in eggs from SF ticks (see Figure 2 and Figure 2—figure supplement 2).

Reviewer #2:

Major comments

1) While the authors show that blood cells are required for larval viability, it is not conclusively shown that this is heme-dependent. Exogenous heme can easily be added back into serum to establish whether hatching of progeny can be rescued, showing this phenotype is specifically due to a lack of heme and not due to another components of the hematocrit. Additionally, the efficiency of ferrochelatase knockdown is not shown. Protoporphyrin IX and iron can be added back to the serum to analyze whether the ferrochelatase is truly functional and lysates can be directly analyzed for FECH activity.

We appreciate the ideas of the reviewer how to proof the lack of haem biosynthesis in ticks. However, addition of just soluble haemin or haem precursor into the diet is a kind of oversimplification that does not take in account the complexity of tick digestive system. Haem is released from digested haemoglobin inside the digestive vesicles of the tick gut digest cells after its receptor-mediated endocytosis involving clathrin-coated pits. Therefore we used for the rescue experiment the pure commercial haemoglobin to demonstrate that haemoglobin alone (together with other serum components) and no other component of the red blood cells is required for successful tick reproduction. The consequence 'no haemoglobin in the diet – no haem in eggs – no embryonic development' is to our opinion a strong evidence that haem needed for tick reproduction originates exclusively from the host haemoglobin. Certainly, we cannot completely rule out that haem might be provided from other resources during the whole developmental cycle of the hard ticks, especially during the long inter-stage periods of starvation.

In the previous manuscript, the efficiency of ferrochelatase knockdown by RNAi was shown in the Figure 1—figure supplement 1, panel D. The information that ferrochelatase RNAi had no obvious effect on tick fecundity was included among screening of other genes/proteins possibly playing a role in tick haem and iron metabolism and was recently submitted to another journal. As mentioned above, this part was completely removed from the current version of the manuscript and we only refer to this result in the Discussion to support the conclusion that the retained haem biosynthetic genes do not play a role in the synthesis of endogenous haem in adult ticks.

2) Genomic analyses and measuring ferritin levels is not sufficient to say the tick has no mechanism for removing iron from heme. There are examples of non-canonical heme oxygenases, for example in bacteria. To more definitively show that heme can utilized as an iron source, iron and heme-free serum should be supplemented with iron, heme, or both and then measurements of non-heme vs heme iron be performed in various tissues. Since HPLC data are already shown for heme levels, a direct measurement of heme degradation products / biliverdin can be made by HPLC. Changes in Ferritin levels are not a great indicator of iron levels, as mammalian Ftns are also acute phase proteins. Is Fer1/2 regulated by stress as by heme starvation?

We agree with the reviewer that determination of bio-available iron by monitoring levels of intracellular ferritin 1 is only indirect proof of the lack of haem catabolism in ticks. Therefore, we performed additional experiments based on determination of elemental iron in tick tissues by GF-AAS (see above the paragraph 3 and response to the Reviewer #1, Major concern 2). Obtained results confirmed that iron levels in tick tissues are independent on the haemoglobin presence in tick diet.

Additional HPLC analysis of tick gut extract (see the paragraph 4 above) confirmed the absence haem- derived biliverdin in line with our previous hypothesis that lack of haem oxygenase in ticks is associated with the need to obtain iron from serum components and not from haem.

3) The functional relationship between the homologs CP3, Vg1 and Vg2 is unclear. It is postulated that CP3 is the main heme carrier protein in the hemolymph but the reduction in heme content in eggs is minimal when CP3 is knocked-down, and it is not shown whether viable larvae arise from these eggs. If developing embryos are completely reliant on maternally-derived heme, there must be another mechanism for maternal heme delivery to ovaries. Does the ricinus genome code for additional CPs like scapulus? Additionally, if Vg1 and Vg2 bind heme after heme deposition in the ovaries, why would knockdown result in reduced heme in this tissue? What happens to intestinal heme content when CP3, Vg1 and Vg2 are knocked down? One

might expect heme accumulation in the intestine or hemolymph if a heme carrier is depleted, depending on which tissue the carrier protein acquires heme.

We agree with the reviewer that the relation between the haem-binding lipoproteins might seem unclear. In order to distinguish these two groups of lipoproteins, the knowledge of their stage and tissue expression profiles is needed. RNAi-mediated silencing of IrCP3 led to about 80% reduction of IrCP3 in tick haemolymph and only to about 50% reduction of haem in laid eggs. This can be explained either by the incomplete depletion of IrCP3 from tick haemolymph in KD ticks or by contribution of other CPs homologues possibly present in *I. ricinus* haemolymph. Alternatively, the haem transporting role is taken over by vitellogenins which presence in ovaries coincides with appearance of haem. Since we are aware about the uncertainty behind the haem inter-tissue transport in ticks, we interpret our obtained results in a more careful way and leave the ultimate answers to our future research. Here, we present only the unambiguous results: IrCP3 is the main haem-binding protein in *I. ricinus* haemolymph while vitellins are the main haem-binding protein in the ovaries.

Reviewer #3:

Although manipulative experiments are elaborate and describe the transport of heme to the eggs, evolutionary scenario suffers from incompleteness.

First, it is known that the genome of Ixoides scapularis is not completely finished and is not annotated at the level representing the standard in the field.listed genes were incompletely predicted and supported by few RNAseq (please compare T. urticae homologs) it cannot be excluded that the remaining 3 genes are present but in the portion of I. scapularis genome that has not been completed or not well annotated.

This should provide just a guidance and authors should confirm all these cases by proper alignment etc.

[...] Thus, an additional analysis and re-interpretation of data is necessary to support major conclusions of this paper.

We are grateful to the reviewer for his/her guide and advice regarding the mapping the haem biosynthetic pathway in *I. scapularis* using the orthologues from another mite, namely *T. urticae*. We agree that *I. scapularis* genome is still not perfectly annotated and contains a lot of gaps. In the previous manuscript, we only referred to the genes of haem biosynthetic pathway present in the KEGG database. Based on the reviewer's suggestion, we performed a much more detailed data mining and BLAST analyses in available mite genomes as described in the paragraph 1 of this appendix.

Moreover, we confirmed transcripts of identified genes sequences by finding the corresponding orthologues in *I. ricinus* tissue transcriptomes. This led to additional identification of PPOX as a third gene of the tick haem biosynthetic pathway that was not revealed by KEGG. The reviewer found also other two genes related to *T. urticae* ALAS and UROD. Our phylogenetic analysis unambiguously revealed that these two genes are of bacterial origin.

A great hint from the reviewer #3 was the note about the lack of haem oxygenase (HO) in *T. urticae*. We further confirmed that absence of HO is a common feature among other Acari. This certainly makes indefensible our previous hypothesis that lack of HO presents another feature of tick adaptation to the haem-rich diet. In the light of a general absence of HO in Acari genomes, we may rather speculate that the inability to acquire iron from haem pushed the tick ancestor into haematophagy, and allowed the loss of haem synthesis as HO-mediated haem degradation is the major source of iron in most organisms studied up to now.

[Editors' note: the author responses to the re-review follow.]

1) Data presented in Figure 2 suggest critical involvement of hemoglobin in tick development. As the manuscript discuss heme auxotrophy, please clarify why reconstitution experiments involved supplementation of hemoglobin, rather than heme, to the SF media. Also clarify whether there is a correlation between numbers of live/hatched progeny with heme concentration. This is important because low heme may support hatching of larvae in the initial stages but may not be able to keep up as embryos utilize the limited supply of heme, as demonstrated by a previous article by Walter-Nuno et al., JBC 2013.

Supplementation of serum with haemin (soluble haem chloride) instead of haemoglobin (Hb) would be a more straightforward rescue experiment. However, addition of haemin into the diet is a non-physiological over-simplification that does not take into account the complexity of the tick digestive system. Unlike haematophagous insects, ticks digest blood meal intracellularly and haem is released from digested haemoglobin inside the digestive (lysosome-like) vesicles of the tick gut digest cells. This was the main reason why we performed the rescue experiment using pure commercial haemoglobin. Consequently, 'no haemoglobin in the diet – no haem in the eggs – no embryonic development' is, in our opinion, strong enough evidence that haem needed for tick reproduction originates exclusively from host haemoglobin and no other component of the host blood.

Irrespective of these considerations, we tried to verify what would happen if ticks were fed on serum supplemented with haemin, as suggested by the reviewer. Therefore, we performed the experiment as suggested by the reviewer. We prepared diets with 625µM (HH – high haemin) and 62.5µM (LH – low haemin) haemin in the serum, corresponding to a concentration of 1% and 0.1% w/v haemoglobin, respectively. Ticks were fed for the first five days on pure serum

and from day 6, they received haemin-supplemented sera. Ticks were able to fully engorge and lay eggs under both conditions. Laid eggs were used for determination of haem concentrations by HPLC (Author response image 2). Interestingly, haem concentrations were about the same in eggs from HH- and LH-fed females. We have no clear interpretation of this result. We assume that haemin uptake might be facilitated through haem-binding serum proteins (e.g. albumin, haemopexin). Similar haem concentrations in egg clutches of HH and LH ticks may be attributed to limited haem-binding capacity of serum proteins.

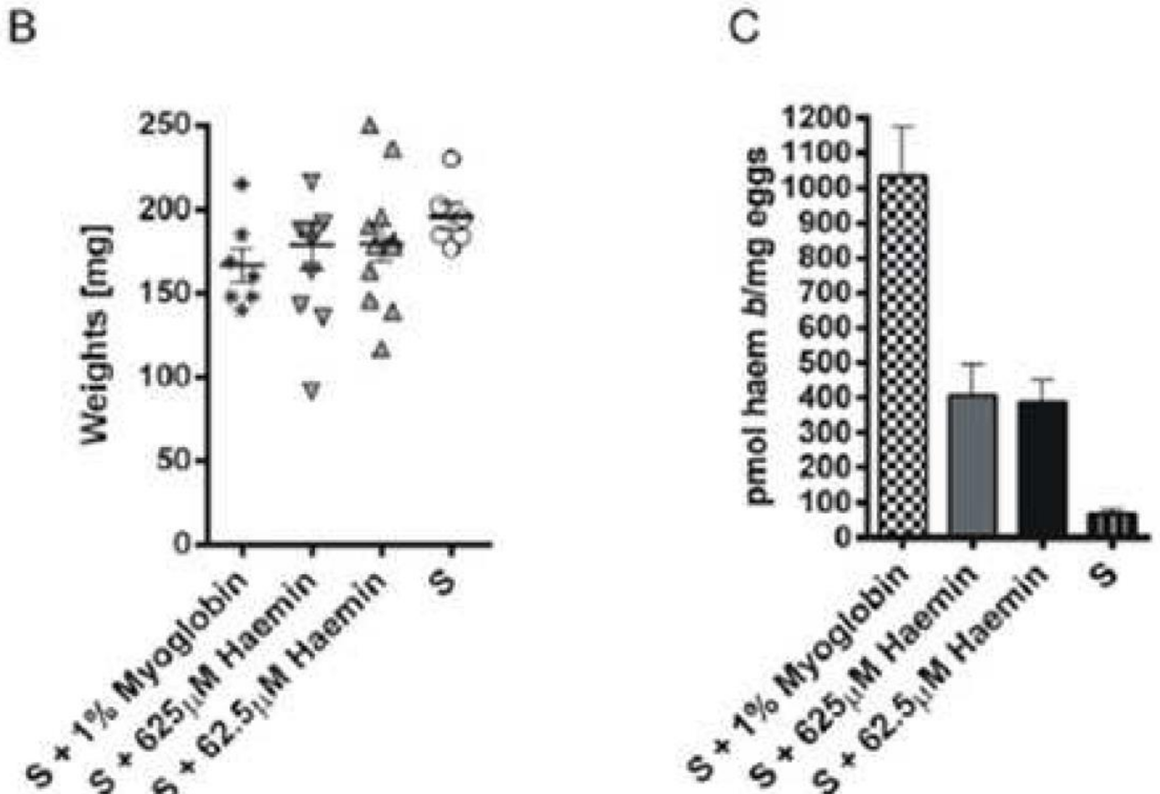
A quantitative correlation between haemoglobin (haem) concentration in the diet and the number of hatched living larvae is quite difficult to determine. We routinely estimate the larval hatching rate by crosses +, ++, and +++ for low, medium, and high numbers, respectively. There is great variability in larval numbers, even for naturally fed ticks, and we further noted that there are also seasonal variations in clutch sizes and the time needed for larval hatching. Therefore, it is not feasible to reliably evaluate differences in hatching from in vitro fed females in relation to haemoglobin concentrations over a reasonable time frame.

In the case of the triatominae bug *Rhodnius prolixus* mentioned by the reviewer, RNAi-mediated silencing of haemolymphatic haem-binding protein (RHBP) resulted in the laying of viable red eggs and non-viable white eggs in earlier and later stages of oviposition, respectively (Walter-Nuno et al., 2013). In contrast, ticks fed on different haemoglobin/haemin concentrations laid fairly homogenously colored clutches of eggs (Figure 2, Figure 2—figure supplement 2 and Author response image 2). To this end, we did not observe any obvious difference in larval hatching from individual parts of any egg clutch.

2) Perhaps the most interesting discovery here is that hemoglobin is required for embryo viability without serving as an iron or as an amino acid source. Then why is Hb required? Some additional experimentation could be conducted with their in vitro membrane feeding system, for example, adding other globular proteins or myoglobin to determine more specifically why Hb is required.

We believe that we have sufficiently demonstrated in our manuscript that haemoglobin is strictly required only as a source of haem needed as the prosthetic group for endogenous haemoproteins (see Supplementary file 1). Nevertheless, we thank the reviewer for his/her proposal to perform an additional experiment to replace haemoglobin with myoglobin in the diet. Despite this non-physiological situation, it appears that ticks, indeed, are capable of acquiring haem from myoglobin and can transport it to the developing oocytes (Author response image 2). As in the case of haemin, it is not clear whether the uptake of myoglobin by tick digestive cells follows the proposed haemoglobin pathway via the putative specific receptor-mediated

endocytosis or is absorbed non-specifically by fluid-phase pinocytosis proposed for serum proteins. Further studies are planned to answer this question.



Author response image 2.

Experimental feeding in vitro of *I.* ricinus females on serum supplemented with myoglobin or haemin.

Ticks were membrane fed in vitro on bovine serum (S). Pure equine myoglobin (Sigma, M0630) or haemin (Sigma, H9039) of specified concentrations were added to the serum diet from the 6th day of feeding and feeding was then resumed until tick full engorgement. The fully engorged females were weighed, allowed to lay eggs, and haem concentrations in eggs were determined by HPLC. (**A**) – representative females fed on respective diets laying eggs; note the different female coloration due to distinct amounts of haem in the diet, yet egg clutches are similarly coloured. (**B**) Weights of fully engorged females fed on respective diets; each symbol presents the weight of one fully engorged female; bar charts depict the mean ± SEM. (**C**) Levels of haem b were determined by HPLC in egg homogenates from ticks fed on sera supplemented with 1% w/v myoglobin, 625 μM haemin, 62.5 μM haemin, or pure serum. Data (mean values ± SEM) were acquired from homogenates of three independent clutches of eggs. For further details see the Material and Method section.

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3) The proper way to determine whether a heme degradation system exists is to first deplete all sources of inorganic iron followed by supplementation of varying concentrations of heme as the sole iron source (Figure 3). Under these conditions, it is possible that ticks may be able to degrade heme to acquire iron. This result will indicate that heme degradation is conditional and induced when iron is limiting and that a non-canonical enzyme might be performing this function (as is found in several bacteria). Please clarify and discuss these possibilities.

This is definitely a correct suggestion, but unfortunately unfeasible under the current state-of-the-art of tick artificial feeding. Our efforts to manipulate the serum diet usually resulted in a failure of tick feeding. For instance, ticks do not feed on serum that has been dialyzed (removal of low molecular weight components). On the other hand, ticks can be partially fed on a serum ultrafiltrate (< 3 kDa), however they do not commence the rapid engorgement ('big sip') phase in the absence of serum proteins. Hence, we have so far failed to establish conditions of limited iron supply in the tick diet. Currently, implementation of a defined artificial tick diet, similar to that found for *Aedes aegypti* (Talyuli et al., 2015), seems to be the only way to experimentally approach the question of the source of iron and other essential nutritional components.

4) The AAS result does not distinguish whether the iron was derived from Hb or other serum components (such as Tf). The result in Figure 3D is just suggestive, because SF conditions have the same iron and BF. Again, the only way to demonstrate this is to remove all sources of heme and inorganic iron followed by titrating heme and/or iron back.

We believe that this comment probably resulted from our unclear explanation of the experiment shown in the Figure 3D. Exactly because AAS is not able to distinguish between haem and non-haem iron, we chose to measure total iron in peripheral tissues (ovaries and salivary glands) dissected from semi-engorged females where still no haem is transferred to ovaries. If iron transported to these peripheral tissues by secreted ferritin 2 (Hajdusek et al., 2009) originated from haem degradation in guts, one should expect that the amount of iron in tissues from BF ticks would be much higher than found in SF ticks. Our AAS analysis therefore, excluded this hypothesis, providing additional evidence that haem is not a source of bioavailable iron in ticks. We have modified the corresponding sentence in Results to better clarify the rationale of this experiment:

"As this method is not able to distinguish between iron of haem and non-haem origin, only salivary glands and ovaries dissected from partially engorged BF and SF ticks were used for the analysis to avoid distortions caused by the presence of haemoglobin in the samples".

5) Please clarify this statement "an efficient inter-tissue heme distribution system", when only 100 nmol of heme is being utilized from a blood meal to be transported to the ovaries. What was the method used to measure this?

We thank the reviewer for pointing out the obvious contradiction in this sentence. We have re-phrased it accordingly:

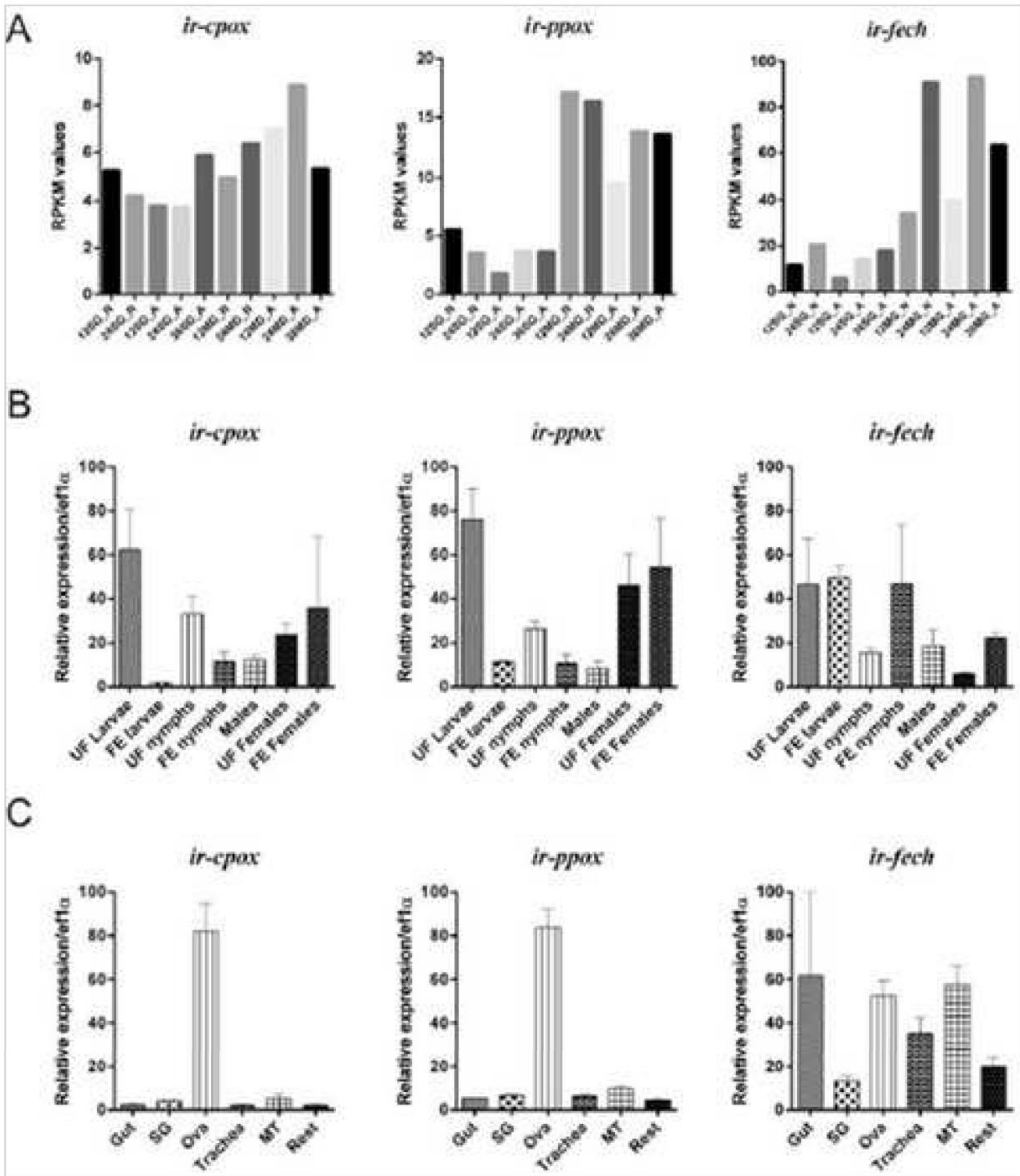
"We estimate that out of approximately 10 μmol of total haem acquired from a tick blood meal, only about 100 nmol (~1%) needs to be transported to the ovaries within a period of several days[…]"

Our estimation of the quantity of total haem acquired from a blood meal (~10 μmol) was based on the following calculation: A fully engorged female imbibes approximately 1 ml of blood meal containing ~150 mg haemoglobin (2.32 μmol) and each haemoglobin molecule contains four haem rings (~10 μmol). The average haem content in eggs from ticks fed on whole blood was approximately 600 pmol/mg, which, when multiplied by the weight of a typical egg clutch (100 –150 mg) yields 100 nmol of haem.

6) The authors should discuss why the ticks have retained the last three enzymes in heme biosynthesis – could these enzymes serve another function? Could heme precursors from the host enter into this partial pathway? What is their expression over the course of tick feeding and development? Please refer to whether any of these genes (transcripts) were identified in published studies (especially recent RNA-Seq studies) involving Ixodes ticks.

We have examined the available RNA-Seq data from *I. ricinus* salivary glands and midgut transcriptomes (Kotsyfakis et al., 2015) and found that *ir-cpox, ir-ppox* and *ir-fech* are indeed

expressed in this species (Author response image 3A). To obtain a deeper insight into putative functions of encoded proteins, we have carried out expression profiling of *ir-cpox, ir-ppox*, and *ir-fech* over tick active developmental stages (larvae, nymphs, adult males and females) (Author response image 3B) and tissues dissected from partially-engorged females (Author response image 3C). Genes *ir-cpox* and *ir-ppox* showed the highest expression in unfed larvae and in ovaries from partially-engorged females, while the *ir-fech* transcript was detected in all cDNA sets examined. As all three genes have retained mitochondrial target sequences (based on TargetP prediction), we speculate that the encoded proteins might have adopted certain functions in mitochondrial biology, possibly distinct from haem biosynthesis. Similar qPCR expression profiles of *ir-cpox* and *ir-ppox* suggest that at least two encoded proteins (CPOX and PPOX) may be part of the complex metabolon (Medlock et al., PLoS One, 2015).


A
ir-cpox
ir-ppox
ir-fech
RPKM values
B
ir-cpox
ir-ppox
ir-fech
Relative expression/ef1α
UF Larvae
FE larvae
UF nymphs
FE nymphs
Males
UF Females
FE Females
C
ir-cpox
ir-ppox
ir-fech
Relative expression/ef1α
Gut
SG
Ova
Trachea
MT
Rest

Author response image 3.

Expression of coproporphyrinogen-III oxidase (ir-cpox), protoporphyrinogen oxidase (ir-ppox), and ferrochelatase (ir-fech) in the tick *Ixodes ricinus*.

(**A**) Expression profiles of nymphal and female adult *I. ricinus* stages over initial phases of feeding from available RNAseq data (Kotsyfakis et al., 2015). SG – salivary glands; MG – gut; N – nymph; A – adult; 12, 24, 36 – 12, 24, 36 hours of feeding. (**B**) qPCR analyses of *ir-cpox, ir-ppox*, and *ir-fech* expression profiles over active developmental stages of *I. ricinus* and (**C**) over tissues dissected from partially-engorged females fed for 6 days (**C**). Data were obtained from three independent cDNA sets, and normalized to elongation factor 1α (*ef1α*). UF – unfed; FE – fully engorged; SG – salivary glands, OVA – ovaries; Trachea – trachea-fat body complex; MT – Malpighian tubules; Rest – remaining tissues. Mean values +/- SEM are shown.

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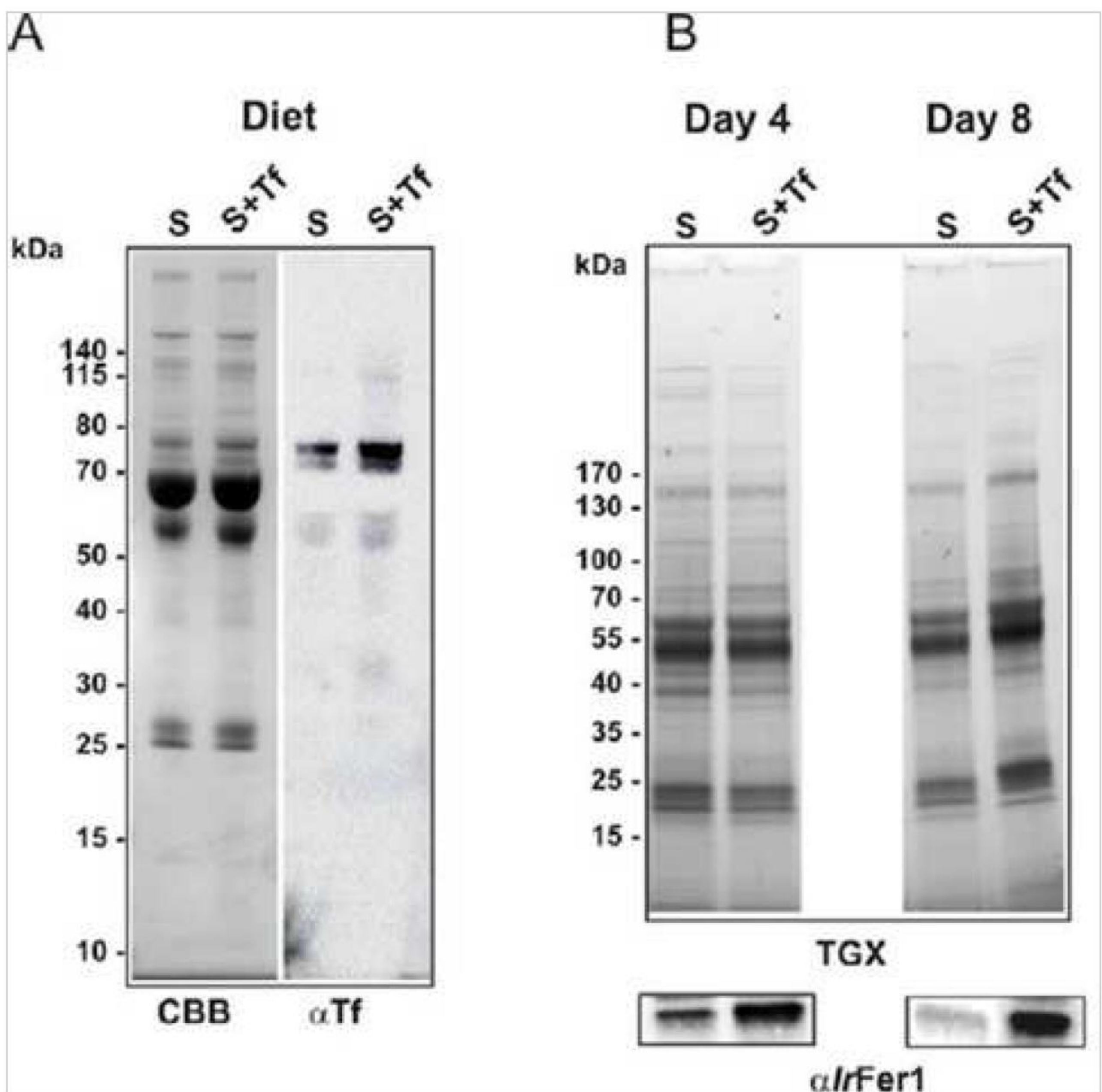
7) The authors should provide some possibilities for where egg Fe is coming from if not Hb. They suggest other serum proteins such as transferrin. Could they test this by manipulating transferrin content in their in vitro assay?

As already mentioned in our response to point 2, we could not find any way to deplete transferrin from bovine serum without the manipulation leading to a failure of tick feeding. Instead, we increased the amount of transferrin in bovine serum by addition of 3 mg/ml of commercially available holo-transferrin. Addition of this iron-saturated transferrin to the serum diet led to a corresponding increase in intestinal ferritin 1 used for monitoring the levels of bioavailable iron, as described earlier (Hajdusek et al., 2009) (Author response image 4). This experiment indicates that ticks are indeed capable of acquiring iron from host transferrin. However, it is still not clear whether the host transferrin is the exclusive source of bioavailable iron for ticks. Answering this question on the source of iron for ticks lacking the haem oxygenase

gene is a challenging task for our future research, possibly based on using defined artificial diets as discussed in our response to point 3.



Author response image 4.

Increased transferrin levels in serum led to higher levels of ferritin 1 in tick gut.

Ticks were fed on serum or on serum supplemented with 3 mg/ml of bovine holo-Transferrin (Sigma, T1283). This addition increased the concentration of transferrin in serum approximately 2-fold, whereas the amount of transferrin iron was increased 3–4 fold (iron saturation of natural transferrin in serum is usually about 30%). (**A**) SDS PAGE of diets (10 µg per lane) used for the experiment: S – serum; S+Tf – serum with added 3 mg/ml of iron-saturated transferrin, stained with Coomassie blue (CBB) and Western blot with anti-transferrin specific antibodies (αTf). (**B**) SDS PAGE of gut homogenates dissected on the 4th day of feeding (Day 4) and from fully engorged females (Day 8) visualized using the TGX Stain-Free technology (TGX) and Western blot detection of tick ferritin 1 levels using specific antibodies against recombinant *I. ricinus* ferritin1 (αIrFer1).

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8) The hypothesis in L72-274 stating that hemoglobin and serum proteins are endocytosed within gut cells via distinct mechanisms is not supported by solid experimental data, so please modify the statement.

The reviewer is correct in saying that the concept of separate haemoglobin uptake via receptor-mediated endocytosis involving clathrin-coated pits and fluid-phase endocytosis for serum proteins (e.g. albumin) has not yet been unambiguously proved. Also the haemoglobin receptor expected to be present on tick digest cells has not yet been identified. Due to the uncertainty in this matter, the corresponding part of the text was removed from the Discussion.

9) Please clarify the statement "These results collectively show that vitellins are the major haemoproteins". Does vitellin actually function with heme bound, or is it a storage molecule for heme?

We agree with the reviewer that vitellins are not haemoproteins in the strict sense as they apparently do not need haem as a prosthetic group for their function. The same is true for HeLp, the haemo-lipoprotein in tick hemolymph *Ir*CP3. Given their lipophilic character, these low-density lipoproteins function as haem scavengers, and most-likely as transporters and storage proteins for insoluble haem. Based on this, we replaced the term 'haemoprotein' by the term 'haem-binding proteins' where appropriate.

10) The authors say "these experiments revealed that haemoglobin was, surprisingly, not strictly required as a source of amino acids for vitellogenesis (Figure 2 and Figure 3)." Please clarify why this is surprising.

Based on the fact that haemoglobin makes up about 70% of total blood proteins, it has long been assumed that haemoglobin was an indispensable source of amino acids for the production of yolk proteins. For this reason, our research over the past decade was focused mainly on haemoglobin digestion as a potential target for anti-tick intervention. However, the results

presented in this work show that ticks can produce an equal amount of vitellogenins and eggs even in the absence of haemoglobin. In order to make this surprising result more obvious we changed the corresponding sentence in the following way (see lines 264–266) 'These experiments surprisingly revealed that haemoglobin, which makes up about 70% of total blood proteins, is not a necessary source of amino acids for vitellogenesis (Figure 2 and Figure 3)".

Finally, the discussion needs to be more cohesive and better link various results on tick metabolism and development into one complete story.

We thank the reviewers for this helpful suggestion. We have removed some parts that were not tightly associated with our results and that possibly would be more appropriate to be discussed in a review article concerning other haem auxotrophic organisms. We believe that the discussion is now more focused on blood-feeding arthropods without losing general interest for other biologists.

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Appendix II

Curriculum Vitae

Curriculum Vitae

Name:	Mgr. **Jan Perner**
Date of Birth:	20. 8. 1986
Address:	Verneřice 200, Hrob 417 04
Telephone Number:	+420 606 629 139
E-mail:	perner@paru.cas.cz

Education:

2011 – now **PhD studies in Molecular Biology and Biochemistry**
(Faculty of Science, University of South Bohemia, České Budějovice, CZ)

2008 – 2011 **Master's degree in Experimental Biology**
(Faculty of Science, University of South Bohemia, České Budějovice, CZ)

- Erasmus programme (University of Glasgow, Scotland, GB):
  Biochemistry and Molecular Biology of Parasites (Prof. Müller)
  Chemotherapy, Resistance, and Parasite Control (Prof. Barrett)
- Defended Master's Thesis:
  „Phenotyping of a glutamate dehydrogenase *a* null mutant of *Plasmodium falciparum*"

2005 – 2008 **Bachelor's degree in Biology**
(Faculty of Science, University of South Bohemia, České Budějovice, CZ)

- Defended Bachelor's Thesis:
  „Characterisation of cystatins from the tick *Ixodes ricinus*"

Work Experience:

2011 – now **Based in the laboratory of Dr. Kopáček**
(Institute of Parasitology, Biology Centre ASCR, České Budějovice, CZ)

2015 **Placement in the laboratory of prof. Peter Brophy**
(Aberystwyth University, Wales, GB; 3 months)
- Project: „Haem-binding properties of a tick glutathione S-transferase"

2013 **Placement in the laboratory of prof. Pedro L. Oliveira**
(UFRJ, Rio de Janeiro, BR; 1 month)
- Project: „Training for metabolite detections in the *Aedes aegypti* and *Rhodnius prolixus*"

2009 – 2010 **Placement in the laboratory of prof. Sylke Müller**
(University of Glasgow, Scotland, GB; 10 months)
- Project:„Phenotyping of a glutamate dehydrogenase *a* null mutant of *Plasmodium falciparum*"

Received Grants:

2016 – 2018 **TACR** (Technology Agency of the Czech Republic)

- Project: „*In vitro* feeding system for validating anti-tick chemicals and vaccines" (Investigator: Petr Kopáček, co-investigator: **Jan Perner**)

2014 **GAJU** (Grant Agency of University of South Bohemia)

- Project: „Differential gut transcriptomics of blood- and serum-fed sheep ticks" (Investigator: **Jan Perner**)

Selected meeting talks:

2016
- Tetrapyrroles, Chemistry & Biology of Gordon Research Conference (Newport, RI, USA)
- Biochemický sjezd (Praha, CZ)

2014
- International Conference of Parasitology (Mexico City, MEX)

Publications:

Perner J, Sobotka R, Šíma R, Konvičková J, Sojka D, Oliveira PL, Hajdušek O, Kopáček P (2016) Acquisition of exogenous haem is essential for tick reproduction; eLife 5:e12318.

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