University of South Bohemia in České Budějovice
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The role of dietary haemoglobin in physiology and development of *Ixodes ricinus* nymphal stage

Bachelor thesis

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Annotation

Obligatory blood feeding ensures consistent composition (both molecular and cellular) and provides a comprehensive source of nutrients. Ticks imbibe host blood meal in their all active stages. For that reason, they were hypothesised to loose some biosynthetic pathways at the expense of developing acquisition and transporting networks. Availability of genomic and transcriptomic data of *Ixodes* spp. ticks revealed that they have lost genes encoding enzymes, participant in haem biosynthesis. Using an artificial membrane feeding system of hard ticks, it was demonstrated that adult *I. ricinus* females acquire haem from a host haemoglobin and transport extracted haem into ovaries. Ovaries devoid of host haem deposits failed to undergo embryogenesis and halted tick reproduction. Questions how juvenile stages of ticks require haem and for what purposes is a focus of this bachelor thesis.

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Contents

1. Introduction

1.1.   Ticks

Ticks are obligatory blood-sucking ectoparasites belonging to the subclass Acari.   All developmental stages are dependent on sucking blood from their hosts, ranging from wild animals, companion animals to humans. A broad host range specificity stands behind the fact that ticks are one of the largest group, which transmits disease-causing pathogens. Ticks, order Ixodida, superorder Parasitiformers, contains two large families - Ixodidae and Argasidae.

Argasidae, also known as soft tick, live in animal burrows, caves, and dens. They are living in a hot and dry area around Globe. Soft ticks have wrinkled body without sclerotised scutum. Their mouth part, gnathosoma, is placed on the underside of the body. Their life-cycle includes larva, 3 or 4 nymphal instar stages, and adult ticks. All stages are addicted to sucking blood. The feeding periods takes from 30 minutes to 2 hours. Adult females feed repeatedly and lay eggs after each blood-feeding (Sonenshine and Roe, 2014).

Ixodidae, also known as hard ticks, consist of 14 genera. They have sclerotised dorsal scutal plate and dorsoventral flattened body. Genus *Ixodes* have sexual dimorfism. The scutum covers just about 1/3 female body. The rest of body creates elastic cuticle so they can imbibe enormous blood volume. Males are markedly smaller and scutum covers their whole body. Adult *Ixodes* sp. males do not suck blood, they only fertilise females. Ixodidae life cycle includes 3 developmental stages - larvae, nymphs, and adults. All life stages feed once and usually on different hosts. Females feed for consecutive 6–8 days. After this period, they lay eggs and die (Schmidt et al., 1996; Sonenshine et al., 1991).

1.2.   *Ixodes ricinus*

I. ricinus belongs to the largest tick group of Ixodidae, genus *Ixodes.* More than 240 species belong in this genus (Jongejan et al., 2004). *I. ricinus* inhabits most of Europe and it can be found in the Middle East and North Africa. It prefers mild climate, mostly grasslands and forest. The ticks are the most active in spring, furthermore in autumn. Another factor for tick

abundance is winter climate. It has been observed that after mild winter there is a large amount of tick during the year (Lindgren et al., 2000).

I. ricinus lacks eyes but they are reliant on the sensory Haller's organ at the terminal segment on the first pair of legs. It serves as a detector of environment changes, like humidity, temperature, CO_2 concentration, and vibration. Ticks also have specific morphological adaptations such as the hypostome. With chelicerates and a pair of palps, hypostome is one of the mouth part components. Hypostome is a long cone-shaped tube with backwards-turned teeth like a harpoon. It helps to attach to the host, disrupts host skin, and facilitates host blood uptake.

I. ricinus is a typical three-host parasite. Every life-stage feeds on different host. Larvae feed on small birds and rodents. After feeding, they molt into nymphs. Nymhal stages prefer rodents and bigger vertebrates. After molting from nymphal stage, adult females feed on big wild or domestic animals (Volf et al., 2007).

Although adult males do not engorge blood meal, we can find them on the host too. They look out for females. Only after fertilisation, females can fully engorge. This process is called the rapid engorgement. After rapid engorgement, females fall away from the host, digest the imbibed blood, process it to eggs, which are layed after about two to three weeks, and then the females die.

I. ricinus is a vector of several pathogens causing dangerous diseases. For example, Lyme disease is caused by the bacterial spirochetes of the *Borrelia burgdorferi sensu lato* complex and Meningoencephalitis, caused by the tick-borne encephalitis flavivirus (Nuttall, 1999). *I. ricinus* is a vector of rare diseases too. One of these is ehrlichiosis, also known as tick-borne fever. It is caused by the bacteria *Anaplasma phagocytophilum* (Stuen, 2007). Other rare disease transmitted by *I. ricinus* is babesiosis. This malaria-like illness is caused by parasitic protozoa of the genus *Babesia* spp. (Becker et al., 2009).

1.3. Components of vertebrate blood

Blood is a complex nutrient-rich mixture, consistent in composition, and continuously renewable for the life of the host (Toh et al., 2010). Blood represents an important part of inner environment of organism. It is a suspension of blood elements - erythrocytes, leukocytes, and thrombocytes suspended in blood plasma that is a yellow viscous fluid.

Plasma consists of 90% water, the rest is composed by proteins, inorganic salts, and trace amounts of other compounds. The term serum is used for blood plasma from which the clotting proteins have been removed. The predominant part of blood is constituted by erythrocytes (also known as red blood cells). Erythrocytes contain blood haemoglobin. Haemoglobin is a haem-containing protein, which transports oxygen in the body. As the main component of host blood meal, it serves as a rich source of amino acids and haem for all haematophagous organisms. Most of the haematophagous organisms turned to haematophagy (blood-feeding habit) independently (Mans, 2011) and, therefore, they deal with nutrient acquisition by distinct mechanisms.

1.4. Blood digestion

1.4.1 Blood-feeding mosquitoes

Mosquitoes constitute the family Culicidae, which is distributed globally. The most abundant genera are *Aedes* and *Culex*. Mosquitoes are vectors of many harmful diseases such as malaria, yellow fever, West Nile virus, dengue fever, filariasis, Zika virus, and others. For this reason, they are regarded as the deadliest animal family in the world. Mosquitoes spend most of their life like immature larvae in water. Mosquitoes are typical solenophagous organisms (feeding directly from blood vessels). They penetrate host skin, find vein, and take up blood meal straight from the vein (Volf et al., 2007). Received blood meal is subsequently digested extracellularly in the gut lumen. Mosquito sense components of blood-meal and use a so called "early trypsin" as a signal transducer for expression of blood digesting "late trypsin" (Barillasmury et al., 1995). They used trypsin-like serine proteases active in alkaline pH (Wu et al., 2009).

1.4.2 Tsetse flies

Tsetse flies belong to the order Diptera, family Glossinidae and genus *Glossina*. The Tsetse fly is an obligatory parasite and both males and females feed only blood from vertebrate animals. Tsetse flies are vectors of *Trypanosoma* spp. causing sleeping sickness in humans and Nagana in wild animals as well as in economically important livestock (Volf et al., 2007). Tsetse flies digest blood extracellularly and they excrete excessive haemin with faeces (Baylis and Nambiro, 1993).

1.4.3. "Kissing bugs"

"Kissing bugs" are true bugs from order Hemiptera, suborder Heteroptera, family Reduviidae, and subfamilies Triatominae. The genera of *Triatoma* and *Rhodnius* are most significant as vector of diseases. They live in Latin America and they are vectors of Chagas disease. All stages of these bugs feed on hosts. They attack hosts during their sleep and excrete the rest of the previous diet together with metacyclic stages of *Trypanosoma* in the course of feeding (Volf et al., 2007). True bugs digest blood extracellularly using lysosomal peptidase, primarily Cathepsins B and L (Ribeiro et al., 2014).

1.4.4. Ticks

Hard tick, unlike haematophagous insects, feed on host for a long-time period (typically 6 – 9 days). Blood digestion is a slower process, and ticks digest blood intracellularly in the midgut cells (Sonenshine and Roe, 2014). The digestion is completed by a network of acidic cysteine and aspartic proteases (Sojka et al., 2013). After the feeding, females detach from the host and digest the imbibed blood, which serves as a nutrient and energy supply for eggs production and oviposition (Sonenshine et al., 1991). Acquired haem, from digested haemoglobin, is essential for embryonic development in ovaries (Perner et al, 2016a).

1.5. Feeding periods of *I. ricinus*

In contrast to mosquitos or tsetse flies, which suck blood meal just for a few seconds to couple of minutes, ticks feed on host blood several days. Blood feeding of *I. ricinus* nymphs takes approximately 3 – 4 days. Adult females feed on their host twice as long, approximately 6 – 9 days. We can separate the feeding process into 3 sequential phases: First is preparative attachment phase. Ticks are seeking the right place with thin skin and then they firmly attach to the host. This phase takes 24 – 36 hours (Coons et al., 1986). Second phase is a slow-feeding period. In this phase, ticks suck a small volume of host blood. Blood digestion begins immediately after sucking and slowly continues during this phase. Final stage is a phase of rapid engorgement. This phase starts from the 5th or 6th day of feeding. Females´ body expands largely during this phase because female sucks in about two-thirds of the total blood meal. Phase of rapid engorgement starts only if the female has been fertilised previously. After the blood feeding is finished, the fully engorged females detach from the host and commence the phase of off-host blood digestion leading to the egg production (Sonenshine, 1991).

1.6. *In vitro* (artificial) feeding

Nowadays, there are many laboratories around the world which study dangerous diseases transmitting vectors. Scientists used many laboratory animals in trials. To control the studies, these animals must be kept in small climatic boxes, where stable environment is maintained. The costs of setting conditions are very high. The ethical aspect of using experimental animals cannot be easily justified. An *in vitro* feeding platform is an alternative to *in vivo* trials. Another advantage an *in vitro* feeding is the control over dietary nutrient, volume of intake blood, and other aspects of feeding (Kröber and Guerin, 2007).

Every haematophagous insect has its own strategy of sucking blood. Mosquitoes suck blood just a few second. Therefore mosquitoes can feed on drop of blood or with glass feeder funnel (Gunathilaka et al., 2017). *Glossina* feed repetitively and after that it is capable of lay eggs. Artificial feeding is done with shaved skins of the rabbit. In the beginning of artificial feeding era, scientists used a shaved skin of the animals. Blood in blood reservoir is put into water bath, which keeps the temperature constant (Moloo, 1971). Due to contamination issues from biological skins, most of mosquito and tse-tse flies feeding systems are based on synthetic membranes, like parafilm or silicone membranes.

1.6.1 *In vitro* feeding of hard ticks

First *in vitro* feeding attempts of ticks on membrane are documented in 1956 (Pierce and Pierce, 1956). First silicone membrane was made by Habedank and Hiepe in 1993. *I. ricinus* was firstly successfully fed *in vitro* (Kuhnert, 1996). The artificial feeding of ticks is very complicated especially because of the long time-period needed to complete the full engorgement. It must be well optimised in order to allow comparison of *in vitro* and *in vivo* feeding data. It must be ensured many variables like temperature, humidity, olfactory attachment stimuli, and all blood supplements. Of a special concern is maintenance of the blood meal sterility in the course of feeding. The biggest improvement in membrane of hard ticks was described a decade ago by Kröber and Guerin (2007).

1.7. Nutritional requirements studied by *in vitro* feeding system

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 the total protein content in serum. This most abundant protein is utilised by all parasites as a source of amino acids. After haemoglobin hydrolysis vast amounts of haem are released. Most parasites evolved mechanisms to efficiently dispose of excessive haem, however some parasites became dependent on host haemoglobin-derived haem supply.

In the post-genomic era, it became apparent that ticks have reduced some biosynthetic pathways at the expense of building acquisition and transporting networks. By artificial feeding, it was demonstrated that adult ticks need to acquire host haemoglobin in order to sustain their cellular demand for haem as a cofactor. Deprivation their meal of red blood cells caused failure of embryogenesis in ticks (Perner et al., 2016a).

In vitro membrane feeding was long unavailable for *I. ricinus* ticks, with first attempts resulting in low attachment and feeding success rates. Recent optimisations allowed investigating the dietary dependence of *I. ricinus* nymphal stages on host haemoglobin. First experiments trying to address this issue are the basis of this bachelor thesis.

2. Thesis objectives

- optimise a membrane feeding platform for nymphal stages of *I. ricinus* ticks
- perform a comparative study for serum and blood fed nymphs
- analyse expression of selected genes, known to be differentially expressed in gut of adult blood-fed ticks vs serum-fed ticks

3. Material and methods

Table 1. Material used in this work

Artificial feeding	
Silicone	silicone E4 (Wacker)
Silicone oil	DC 200 silicone oil (FLUKA)
Colour paste	FL colour paste (Wacker)
Hexane	95 % n-Hexane (Lach-Ner)
cow scent	Dichlormethane for analysis (MERCK) + cut animal (cow) hair (Kröber and Guerin, 2007)
Diet supplements	
Gentamicin	0.5 % gentamicin (Sigma)
Glucose	0.2 % glucose (Sigma)
ATP	10mM ATP (FLUKA), sterile, filtred (0.22 μm filter)
NaCl	0.9 % NaCl
Dissection for RNA isolation	
DEPC-treated PBS	8 % NaCl, 0.2% KCl, 1.8 % Na_2HPO_4, 0.14 % KH_2PO_4 in 1000 ml distilled H_2O + 0.1% diethyl pyrocarbonate in destilled H_2O, pH 7, autoclaved
RNA isolation, cDNA synthesis	
RNA isolation kit	NucleoSpin® RNA (MACHEREY-NAGEL)
cDNA synthesis kit	Transcriptor High Fidelity cDNA Synthesis kit
DNA electrophoresis, PCR	
1x TAE buffer	40 mM Tris-acetate, 1 mM EDTA, pH 8.0
DEPC H_2O	0.1% diethylpyrocarbonate diluted in distilled water
EtBr	Ethidium bromide 0.5 μl/ml
6x Loading Dye (Fermentas)	10 mM Tris/HCl (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylencyanol, 60 % glycerol, 60 mM EDTA
DNA Ladder	GeneRuler^{TM} 100 bp DNA Ladder (MBI Fermentas)
PCR Master Mix	FastStart PCR Master (Roche)
qPCR, chemistry	
RT-qPCR Master Mix	FastStart SYBR Green Master (Roche)
pure H_2O	PCR Ultra H_2O (TOP-BIO)
Haem extraction	
Haemin	Haemin from bovine, ≥ 90 % (SIGMA)
basic MeOH	Methanol + 0.2 % NH_4
acidic Acetone	80 % Acetone + 2 % HCl

Table 2. Primers used in experiments

Used primers	
elongation factor (EF)	F: ACGAGGCTCTGACGGAAG
	R: CACGACGCAACTCCTTCAC
actin	F: CGACATCAAGGAGAAGCTCTG
	R: GTCGGGAAGCTCGTAGGAC
glutathione S-transferase (GST)	F: GGTTCGTCCTGTGGGAAAG
	R: ACTGTCCGGGGCGTACTT
sultotransferase (ST)	F: GGTCGACAGAGTTCCCTACG
	R: TCCCACGGGTTACGATAGAC
cytochrome P450 (CYP450)	F: GCGAAGGAGCAGGTTCTCT
	R: AGATTTGGTTCGGGTCCAG
phospholipid-hydroperoxide glutathione peroxidase (PHGP)	F: CTGGAAGTACCTCAAGGAAAGG
	R: CTGTCCATTCTTGTCCACCA
peritrophic membrane chitin binding protein (PMChBP)	F: CCCGATGAACGTGCTCTT
	R: AGCGTCGAACAAGGGAACT

3.1. Ticks

Ticks, used for all experiments, were from tick colonies of the Institute of Parasitology or collected by flagging in forest around České Budějovice. Tick colonies were maintained at 24 °C and 95 % humidity.

3.2. *In vitro* feeding

3.2.1. Feeding unit preparation

Feeding units (FU) were prepared according to published *in vitro* feeding assay protocol (Kröber and Guerin, 2007), but the glass FU was exchanged for plastic FU. To prepare
8 − 10 membranes was used 15 g of the silicone (Elastisol E4, Waker), 0.15 g of the colour paste, 5 g of the silicon oil, and 2.9 g of the hexane was used. All of this was mixed in a plastic bowl to create a homogenous mixture. This mixture was spread over the rectangle mash and then let dry overnight. The thickness of the membrane was measured with a micrometre on 3 places and only those about 130 µm and less were used for FU completion. Every bottom rid of FU was cleaned and thin layer of silicon was applied onto them. FU was glued to the membrane and let harden for few hours or overnight. Membranes were trimmed according to shape of the FU and in the middle of the FU was glued a plastic cross (Fig. 1).

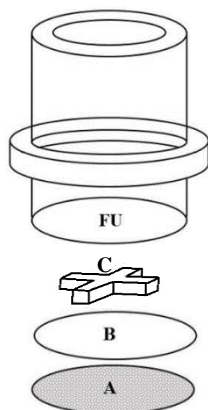The FU membranes were perfumed with 640 ng of cow scent per FU at least 3 hours before application of tick.

Figure 1. Feeding unit composition. Schematic depiction of feeding unit comonents. FU - Feeding unit, A - fine mash circle, B - silicon membrane, C - plastic cross

3.2.2. Diet preparation

The bovine blood was collected in the local slaughterhouse (Jihočeská masna s.r.o.) and it was manually defibrinated. Sterile-filtered glucose (final concentration 0.2 %) and gentamicin (final concentration 5 µg/ml) was added in laboratory. Serum was prepared from the bovine blood. Blood was poured into 50 ml tubes and centrifuged at 2 500 × g for 10 minutes in 4 °C with slow deceleration. Serum phase was collected and transferred into new tubes. It was then centrifuged at 10 000 × g for 10 minutes in 4°C and the supernatant was transferred into new tubes. Sterile-filtered glucose was added into serum to final concentration 0.2 %.

Both types of diets (blood and serum) were stored at 4 °C. Diets were served in six-well feeding plates. Before the FU submerge, they were added 31 µl of ATP (final concentration 1mM) and 3.1 µl of gentamicin (final concentration 5 µg/ml). The diet (WB, S) volume of 3.1 ml was pipetted into each well and the whole plate was gently shaked the whole plate to mix diet with ATP and gentamicin. Both types of diets were changed every 12 hours.

3.2.3. Nymphs application

Feeding units with similar parameters were prepared for each diet. Five adult females were added into each FU, diets were applied, and after 2 – 3 days in water bath with 37 °C, 30 nymphs were added into each FU. The nymphs were then allowed to finish the feeding.

3.3. Total RNA isolation and complementary DNA (cDNA) synthesis

Whole nymphal gut was dissected using binocular microscope (Stemi DV4, Zeiss) under DEPC-treated PBS. Rinsed gut from three nymphs were pooled and homogenized in RA1 buffer (RNA isolation kit) using an insulin syringe (29G). Total RNA was extracted by RNA isolation kit according to manufacturer´s protocol and its concentration was measured by photometer (NanoDrop^{TM} 1000). The integrity purity of RNA was checked out on 1 % agarose gel, where 100 ng of each RNA sample was loaded into the well. 100 ng of prepared total RNA was used for synthesis cDNA with cDNA synthesis kit (Table 1). cDNA samples were used as templates for RT-qPCR analyses.

3.4. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Gene expressions were quantified from 20 × diluted cDNA templates in a volume of 2 µl per 25 µl PCR run set-up mix using LightCycler® 480 SYBR Green Master in LightCycler® 480 System qPCR machine (Roche). Each sample was analysed in technical triplicate. Primers used for gene amplification are listed in Table 2. Relative values were normalised according to amplification of the cDNA for *Ir elongation factor* and *Ir actin*. Data were expressed as relative expression to the samples with highest expression levels - 100%.

3.5.   Haem quantification

In 1.5 ml plastic eppendorf tube, 10 mg of eggs were homogenised in 50 µl H_2O with plastic pestle. 950 µl of ice cold basic methanol was added into the homogenate and it was centrifuged at 10 000 × g for 2 minutes at 4 °C. Supernatant (basic methanol) was taken away very carefully, not to disturb the pellet. One millilitre of acid acetone was added into plastic tube to extract haem. After extraction, the plastic tube was centrifuged at 16 000 × g for 2 minutes at 4 °C. The supernatant (acid acetone) was transferred into new plastic tube and the absorbance was read at 385 nm in quartz cuvette by the UV-Vis spectrophotometr (Shimadzu). Acid acetone was used as a blanc.

4. Results

4.1. Artificial feeding of *Ixodes ricinus* nymphs

Artificial feeding system allows a controlled platform of tick feeding in laboratory condition. This system was tested on adult stage of the *I. ricinus* tick according to Kröber and Guerin assay (Kröber and Guerin, 2007). We have adjusted this system so that we are able to feed *I. ricinus* nymphs. Artificial feeding system of *I. ricinus* nymphs is a little different to feeding of adults. First, the design of membranes is different, in such a way that the membranes are thinner and more flexible (Kucera M, 2015). Further difference can be found in feeding success and length of feeding. Feeding success of *I. ricinus* adults is in the range of about 70 % for blood-fed (BF) and 62% for serum-fed (SF) (Perner at al., 2016a). Feeding success of nymphs is still substantially smaller: 26 % for BF and 46 % for SF -ticks (Fig. 2A). To reverse this downside, we can compensate the loss by putting more nymphs on the membrane. While the feeding of *I. ricinus* females takes on about 10 days, more than a half of *I. ricinus* nymphs finish feeding until day 6 (Fig. 2B).
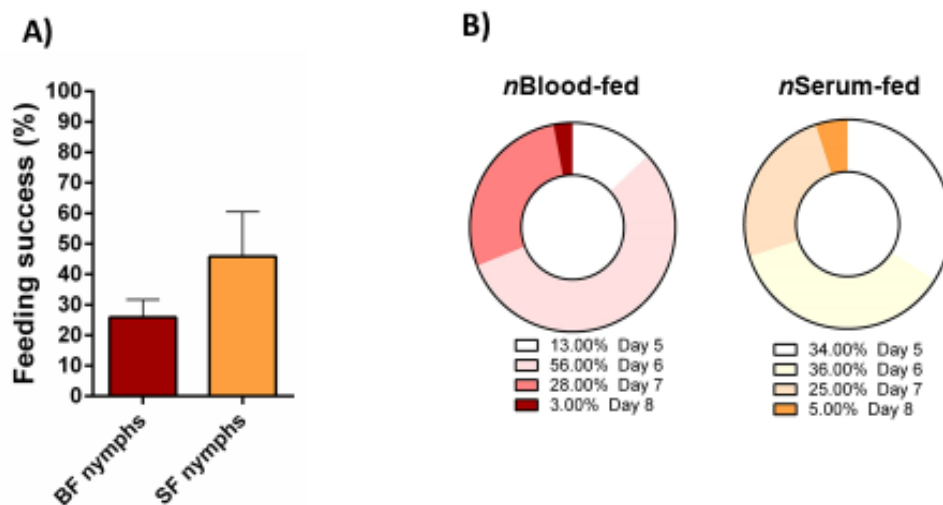

Figure 2. Feeding success of *Ixodes ricinus* nymphs. Nymphs were fed on blood (BF) and serum (SF) diets by artificial feeding system. **A)** The bar graph represents success of feeding of *I. ricinus* nymphs. Mean and SEM are show, n = 100 (number of observed ticks). **B)** Pie graphs denote the percentage ratio of a given days needed for *I. ricinus* nymphs after full engorgement.

4.2. Differences between sex after feeding on blood and serum diets

In adult stage, only tick females can feed a host blood, but both *I. ricinus* nymphal males and females feed. We put nymphs on the membranes and let them feed on the blood or serum diets. Both female and male nymphs finished feeding without complications. After feeding we weighted out all nymphs and we compared the weights between blood- and serum-fed nymphs (Fig. 3A). The weight of fully engorged nymphs displays sexual dimorphism (Dusbabek, 1996). Weight of the nymphs female reached around 4 mg, but males nymphs were nearly half of female size (Fig. 3B). Both blood- and serum-fed nymphs could molt into adults without any obvious developmental aberration, with the only difference being the disappearance of visible haem(oglobin) deposits highlighting the contours tick midgut in serum-fed ticks (Fig. 4). We did not observe any weight difference between unfed adults coming from blood-fed (*n*Blood-fed) and serum-fed (*n*Serum-fed) nymphs, but we could see that wild collected female ticks were heavier than those coming from artificially fed nymphs (Fig. 5A). The difference was not that pronounced in males (Fig. 5B).
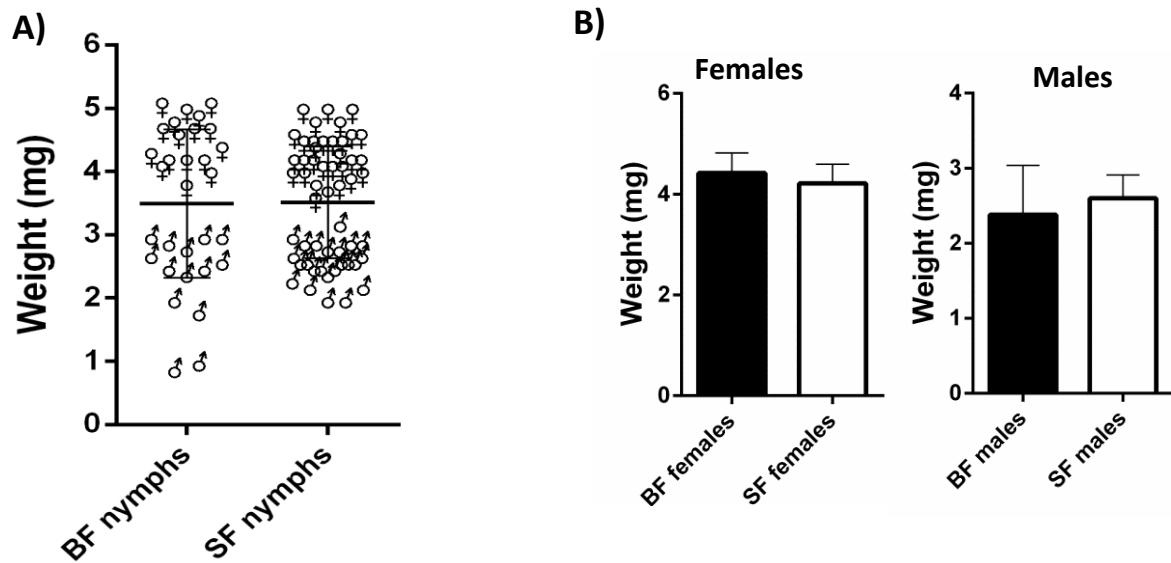

Figure 3. Weight of fully engorged *I. ricinus* nymphs. Nymphs were fed on blood (BF) and serum (SF). They were weighted up immediately after feeding and the weights were recorded. **A)** The point graph shows the weight of all fully engorged nymphs. Symbols represent sex specificity in the weight of fully engorged nymphs. **B)** Bar graphs compared females and male nymphs in depending on the diets. SEM and mean are show.
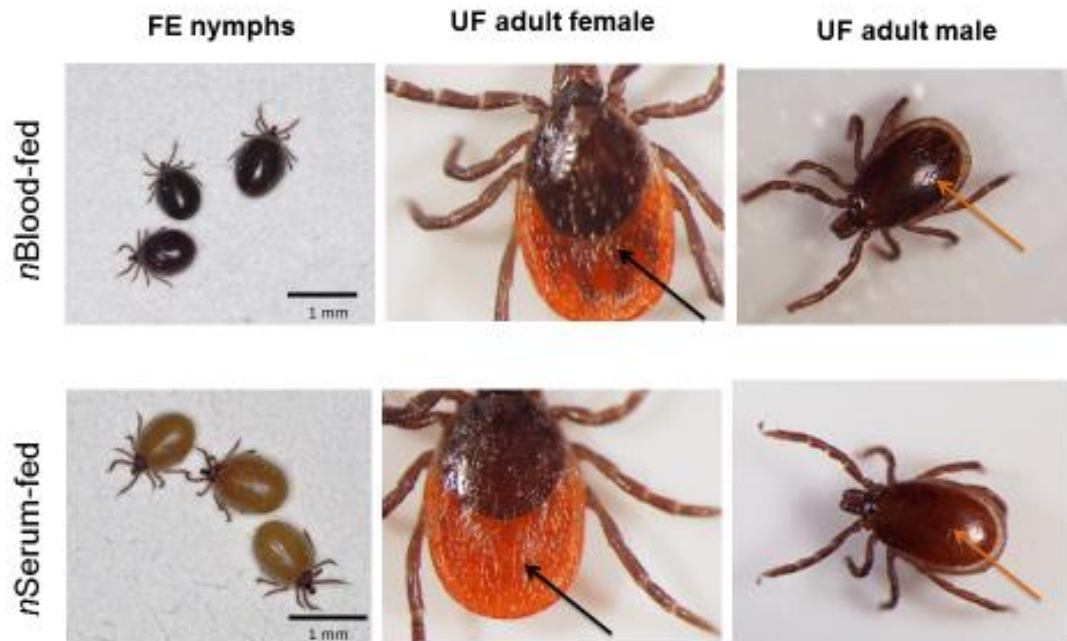
Figure 4. Molting of *I. ricinus* nymphs in dependence on diets. Nymphs were fed on blood or serum diets. Fully engorged blood-fed (*n*Blood-fed) and serum-fed (*n*Serum-fed) nymphs (left panels) were let molt into adults (middle and right panels). We observed if this ticks have some evident developmental aberration. Arrows indicate the haem(oglobin) rich or poor gut.
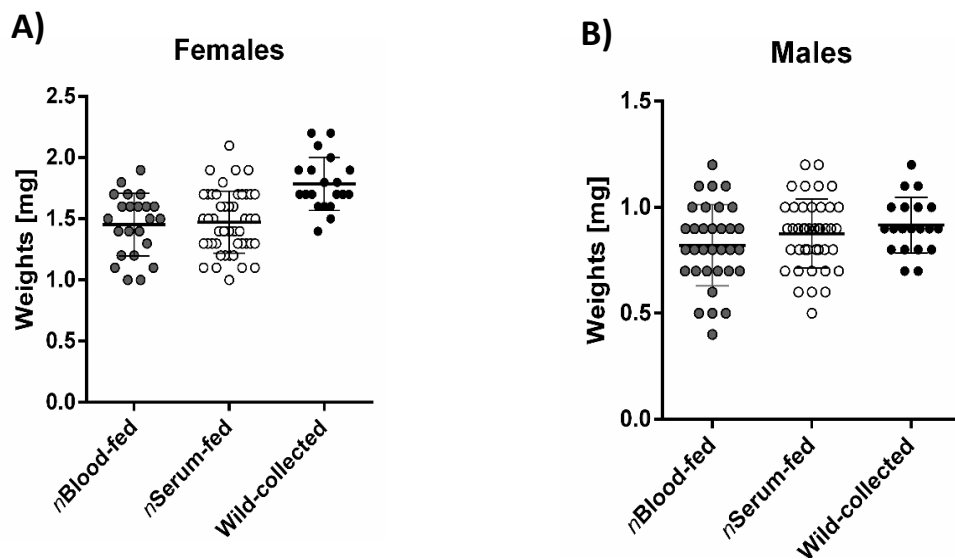

Figure 5. Weights of unfed *I. ricinus* adults after molting. Graphs show weights of the unfed female and male adults after molting. Weights are compared to the weights of molted wild collected nymphs from wild and fed on the guinea pig. **A)** The point graph depicts weights of molted female ticks. **B)** The point graph depicts weights of molted male ticks. Mean and SEM are shown.

4.3. Absence of haem in the nymphal stage does not affect natural blood-feeding of adult *I. ricinus* tick

To test if absence of host haemoglobin in nymphal stage interferes with the feeding and reproducing of adult tick, we have fed *n*Blood-fed and *n*Serum-fed ticks naturally on guinea pigs. We have observed that *n*Serum-fed ticks are able to attach to a host and finish the feeding similarly to *n*Blood-fed ticks (Fig. 6A). Upon full engorgement, the weight of adult *n*Blood-fed ticks was slightly higher than *n*Serum-fed ticks (Fig. 6B).
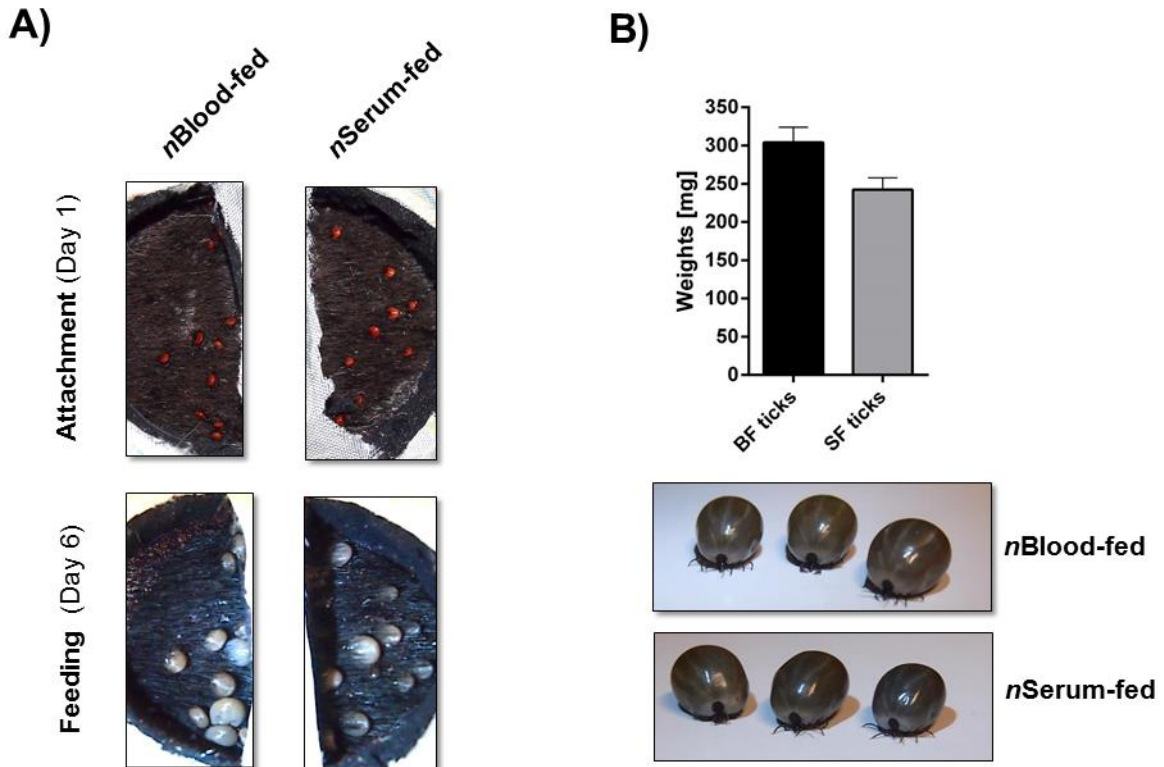

Figure 6. Natural feeding of adult stages of the nymphs feeding on blood (*n*Blood-fed), and on serum (*n*Serum-fed). Blood-fed (*n*Blood-fed) and serum-fed (*n*Serum-fed) nymphs were allowed to molt into adults. We examined if the *n*Blood-fed and *n*Serum-fed ticks are able to feed naturally on guinea pigs. **A)** Ticks were tested for ability to start feeding (top panel) and successive progress in feeding (bottom panel). **B)** The bar graph shows the weight of fully engorged adult *n*Blood-fed and *n*Serum-fed females. SEM and mean are shown. Three representatives from both groups are shown.

4.4. Reproduction of *n*Blood-fed and *n*Serum-fed tick

Adult form of *I. ricinus* ticks need to acquire haem from host haemoglobin for correct embryogenesis and larval hatching (Perner, 2016a). We tested if absence of haemoglobin in the nymphal stage interferes with consecutive reproduction. We have let the adult females,

originating from nymphs fed on blood (*n*Blood-fed) or serum (*n*Serum-fed), to feed naturally and lay eggs (Fig. 7). Both *n*Blood-fed and *n*Serum-fed ticks were able to lay eggs. The *n*Blood-fed tick egg clutches were slightly bigger than the egg clutches of *n*Serum-fed tick (Fig. 8). One and two weeks after eggs were laid, we checked eggs with light microscopy and observed a typical embryonic formation (Fig. 7). Subsequently larvae were able to hatch out of these clutches (Fig.7).
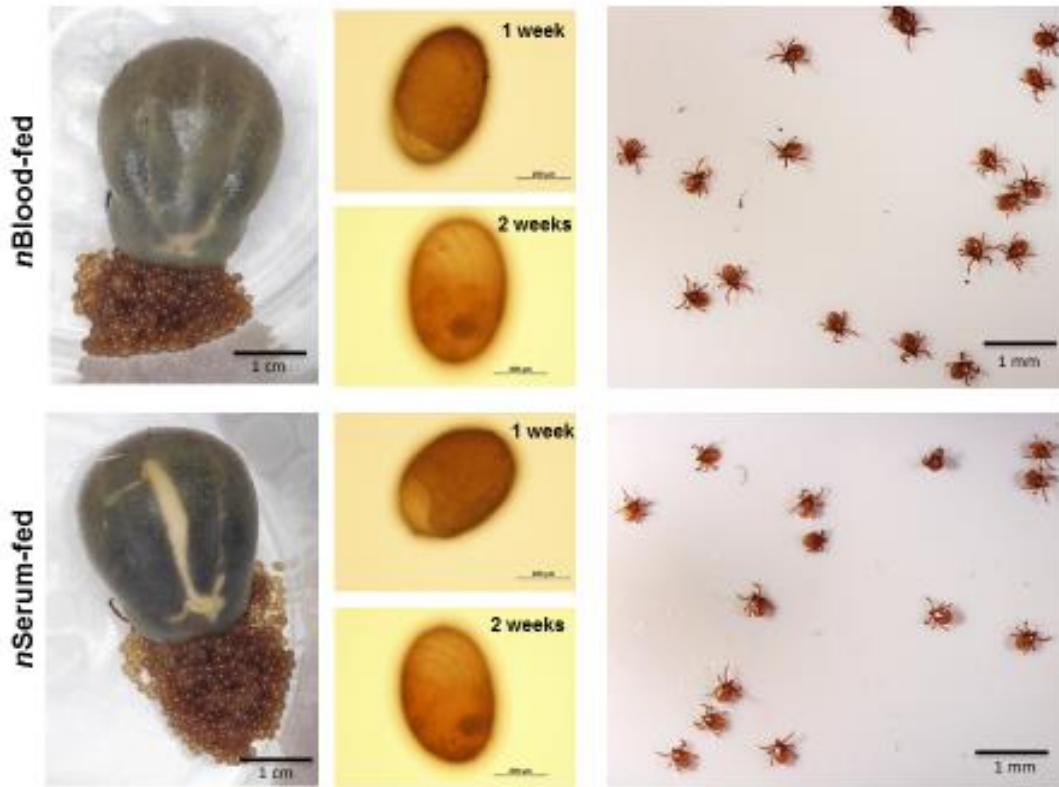

Figure 7. Laying eggs, embryogenesis, and larvae hatching in adult females molted from blood- and serum-fed nymphs. Blood-fed (*n*Blood-fed) and serum-fed (*n*Serum-fed) nymphs develop into adult females. After naturally feeding the *n*Blood-fed and the *n*Serum-fed tick were able to lay eggs (left panel). Eggs were checked for embryogenesis (middle panel). Larvae were able to hatch out from both, *n*Blood-fed and *n*Serum-fed, tick clutches (right panel).
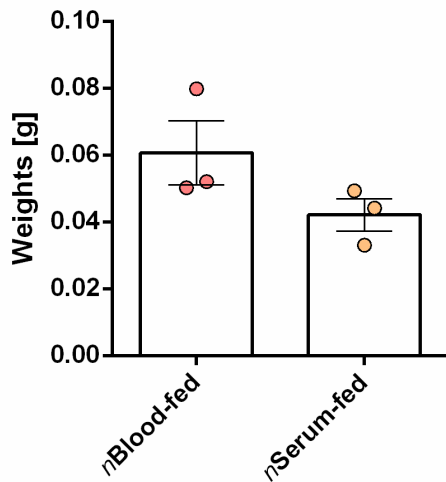
Figure 8. Weights of egg clutches from naturally fed adult _I. ricinus_ females molted from blood- (_n_Blood-fed) and serum-fed (_n_Serum-fed) nymphs. The point graph show weights of individual egg clutches of _I. ricinus_ adult from _n_Blood-fed and _n_Serum-fed nymphs. Adults were naturally fed on guinea pigs. SEM and mean are shown.

4.5. Extraction and quantification of haem in tick eggs

It was previously determined that most of the ~400 nm Vis absorbance (Soret peak) of tick egg extract is due to absorption spectra of haem deposits. Therefore, we can easily quantify haem by Vis absorbance from shear acetone extracts from crude egg homogenates. We naturally fed females molted from _n_Blood-fed and _n_Serum-fed nymphs on guinea pigs and we let them lay eggs. We homogenised the eggs from _n_Blood-fed and _n_Serum-fed clutches and measured the extract absorbance of each extract. Subsequently, we compared the absorbance values of extracts to haemin standard curve to quantify haem content per mg of egg clutch. We observed that the haemin and the extract from egg homogenate had Soret peak at the same wavelength 384.6 nm (Fig. 9A), confirming the haem identity in the extracts. Concentration of haem in the _n_Blood-fed tick eggs was slightly higher than in _n_Serum-fed tick eggs (Fig. 9B). However, this difference did not affect the further development of _I. ricinus_ ticks.
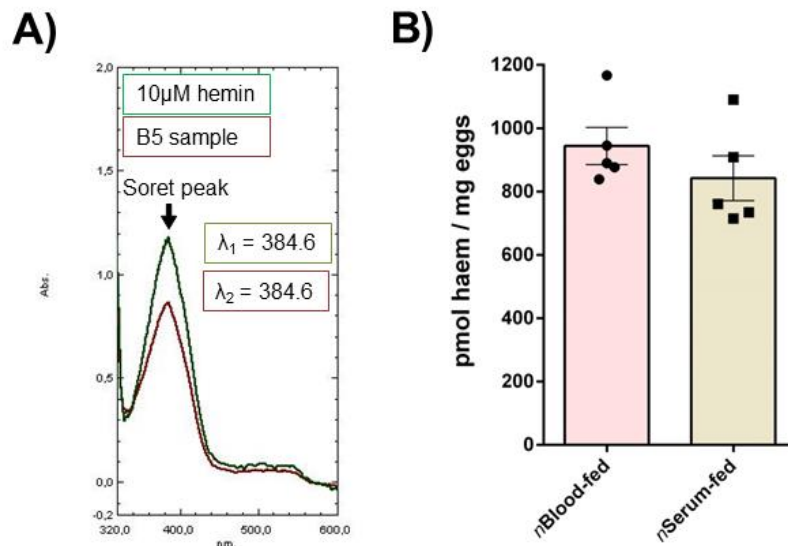
Figure 89. Quantification of haem in tick eggs. A) UV-Vis absorbance spectrum of hemin standard and a one extract of egg homogenate. **B)** Normalisation concentration of haem per mg of tick eggs, n = 5 (separate egg clutches). SEM and mean are show.

4.6. Expression of midgut genes in response to the presence haemoglobin in diet

By using RNA-seq, there were previously identified contigs, which have different expression in midgut of the blood- and serum-fed adult *I. ricinus* ticks (Perner at al., 2016b). Subsequently, we tested if these genes display also different expression in the midgut of fully engorged blood- and serum- fed nymphs. We picked out five genes from the transcriptome of the midgut from blood- and serum-fed adults *I. ricinus* tick (Perner at al., 2016b) and analysed the level of the transcripts in nymphs by RT-qPCR. We found out that these nymphal genes are up-regulated in analogous way as they do in the midgut of blood- and serum-fed adult ticks. Cytochrome P450 (CYP450) and phospholipid hydroperoxide glutathione peroxidase (GSH-Px) were upregulated by serum-feeding, while glutathione S-transferase (GST), sulfotransferase, (ST) and peritrophic matrix chitin-binding protein (PMChBP) were upregulated by blood-feeding. (Fig. 10A) These data, obtained from nymphs, correlate well with the data obtained from the adults (Fig. 10B).

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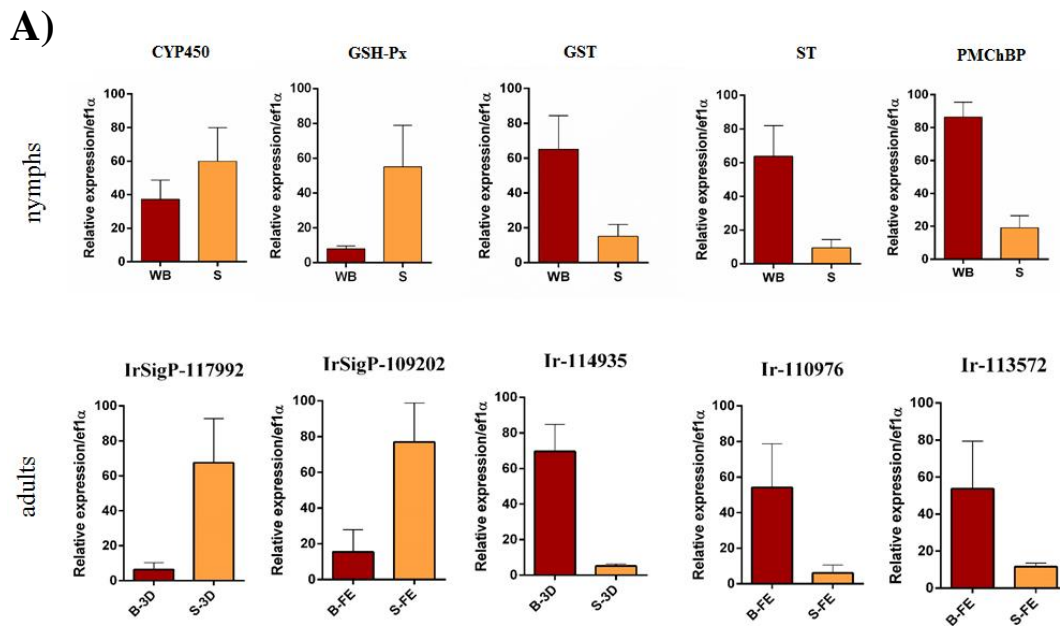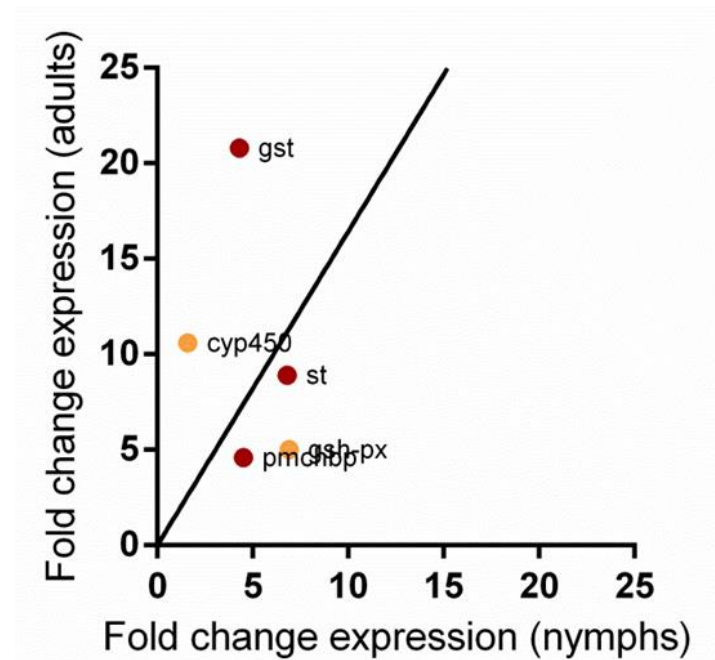
Figure 10. RT-qPCR analyses of haemoglobin responsive genes from the midgut of fully engorged nymphs. A) The bar graph shows relative expression of selected genes in fully engorged *Ixodes ricinus* nymphs (top panel). SEM and mean are show, n = 3 (biological replicates). Bottom panel shows relative expression of the same genes in adult of *I. ricinus* females. Relative expression for *I. ricinus* adults are overtaken from Perner at al. 2016b. **B)** Correlation plot of particular expression for selected nymphal genes and contigs.

5. Discussion

This work was focused on the impact of host haemoglobin at *I. ricinus* nymphal stages. We looked at obvious changes in morphology, physiology, and development upon nymphal serum feeding in comparison to blood-fed nymphs. Digestion of haemoglobin has been studied in our lab for many years, but vast majority of our results has been gained only from adults. In our previously published studies, we contributed to the knowledge of haem and iron metabolism in *I. ricinus* females (Perner et al., 2016a; Hajdusek et al., 2009). I followed up this study in an attempt to extend the knowledge of haem biology in tick nymphal stages.

At first, we had to adapt silicon membrane to match the nymphs. Nymphal hypostome is shorter than that of adult females, therefore the silicone membrane must be thinner. In spite of the effort, the feeding success still ranges around 40 % which is still comparable or better than the feeding success achieved in other laboratories (Dr. Ard Nijhoff, personal communication). Several parameters remain to be tested in order to achieve similar success rate for nymphs comparable to adults. For instance, does the perfume composition and concentration need to be further optimised? Another reason why we have so different feeding success between nymphs and adults is humidity. At the moment, it is difficult to maintain the constant level of humidity in our *in vitro* condition. Nymphs are very prone to change humidity and most of them die before feeding.

We showed that nymphs can feed *in vitro*. Their feeding period and their weight are comparable with data from nymphs fed naturally on animals. We can not see any significant difference between nymphs fed on the whole blood and nymphs fed on serum. Both type of nymphs, blood-fed and serum-fed, molt into adults without any obvious developmental aberrations.

Expression of midgut genes shows that nymphal genes are up-regulated in analogous way as in the midgut of blood- and serum-fed adult ticks. Sulfotransferase (ST), glutathione S-transferase (GST), and peritrophic matrix chitin-binding protein (PMChBP) were up-regulated by blood-feeding. Cytochrome P450 (CYP450) and phospholipid hydroperoxide glutathione peroxidase (GSH-Px) were upregulated by serum-feeding. These data from nymphs correlate well with the data obtained from the adults (Perner et al., 2016b). Pilot RNAi analyses of these five genes did not show any phenotype on nymphal feeding and molting into adults (data not shown).

We know why adult females need haem. When adult females feed on diet without haemoglobin, they can finish the feeding and lay eggs, but the embryos are not able to develop within the eggs. It means that adult females need haem for embryogenesis (Perner et al., 2016a). What impact does haem have on the nymphs that do not reproduce? According to the results presented in my thesis, it looks as if nymphs did not need high amounts of haem for their feeding-linked processes and further development. But is it really that simple? Adult females require very low levels of haemoglobin to secure embryogenesis above 0.1 % haem supplement in serum (Perner et al., 2016a). We are not able to get rid of all haem from blood just by centrifugation. So it is possible that the residual amount of haem is still sufficient for nymphs. Further experiments, with rigorously stripped serum of haemoglobin or with artificial feeding medium free of haemoglobin are necessary to learn more.

Another explanation might be that nymphs have enough haem from the previous blood meal in larval stage. Ticks have specialised sac in gut. This sac is called endosome. We can find this sac in tick gut which fed on serum too (Sojka et al., 2015). They store the haemoglobin in endosome and during nymphal feeding, they digest these reserves to release haem as a demanded cofactor for cellular processes. We can infer that, thanks the endosome, ticks are able to starve for so long and, maybe in connection with residual haemoglobin, it created sufficient amount to appropriate development of the nymphs. Having said that, unfed adult female gut homogenate originating from serum- and blood-fed nymphs, contained comparable amounts of apparent Hb band, which was evidenced by reducing SDS-PAGE (data not shown), which would indicate that haem is not stored in a bound form of haemoglobin complex.

Mitochondria are another option for haem storage. The hypothesis would have it that, after larval feeding, mitochondria will load their haemoproteins, which will be only recycled during nymphal existence, and no further exogenous haem influx, or very little of it, will be required. Adults must feed blood and refill the endogenous haem pool to form new organism. Such demand cannot be covered from endogenous source and, therefore, we could observe phenotype of embryonic lethality upon serum feeding.

6. Conclusion

The main purpose of this work was to reveal the impact of haemoglobin on development and physiology *I. ricinus* nymphal stages. We can conclude that nymphal *I. ricinus* ticks do not require high amounts of haem (derived from physiological levels of host haemoglobin) to sustain correct morphology, propel relevant physiological and developmental processes. However, the nymphal digestive tract is not inert to dietary haem sensing as they express the same battery of differentially expressed genes as adult stages. We, therefore, speculate that nymphs operate at very low exogenous requirements and may go about temporary exogenous haem unavailability (serum feeding) by utilisation of their endogenous haem stores.

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