University of South Bohemia in České Budějovice

Faculty of Science

The importance of the insulin receptor signaling pathway in physiology of *Ixodes ricinus* ticks

Master thesis

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České Budějovice 2019

Master thesis:

Kozelková T., (2019). The importance of the insulin receptor signaling pathway in physiology of *Ixodes ricinus* ticks. Ms. Thesis, in English - 74 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

In this thesis, the function of the insulin receptor signaling pathway (ISP) in hard ticks *Ixodes ricinus* was analyzed. Ticks are obligatory blood-feeding (hematophagous) ectoparasites, capable of transmitting a wide variety of pathogens comprising bacteria, viruses, and protozoa that affect animals and humans. The parasite is strictly bonded with its host through a unidirectional transfer of nutrition for its survival, development, and reproduction. The ISP is a highly conserved system, which regulates a variety of physiological and anabolic processes in response to the available nutrition. The aim of the thesis was to examine the function of several key components of this pathway, which had been identified in the midgut transcriptome, namely insulin receptor (*Ir*InR), protein kinase B called AKT (*Ir*AKT), and the target of rapamycin (*Ir*TOR). The subsequent objective was to assess the expression profiles in tick tissues of these components, during tick feeding and after detachment using qRT-PCR. Furthermore, the phenotype using RNAi knockdown, injection with insulin receptor antagonist (IRA), and the artificial feeding with the AKT and TOR inhibitors were verified. Finally, the immunization of rabbits with *Ir*InR recombinant protein and the tick infestation were carried out.

Financial support

This work was funded by the Grant Agency of the Czech Republic, Project No. 18-01832S to Petr Kopáček.

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Tereza Kozelková

Acknowledgements

First, I would like to express my deep gratitude to Dr. Petr Kopáček for the opportunity to become a member of his team, for his scientific guidance, useful comments and suggestions, patience and his valuable time. I would also like to thank my supervisor Dr. Lenka Grunclová for her assistance, her help in the stage of designing most of my experiments and for her support in the area of methodical issues. Big thanks belong to all members of Dr. Kopáček's team for their assistance during the experiments, useful advice and for making a friendly atmosphere. Special thanks go to Matěj and Terka for their help with the *in vitro* feeding experiments.

Special acknowledgements (in Czech):

Tímto bych ráda poděkovala svým rodičům za jejich láskyplnou a bezmeznou podporu, trpělivost a důvěru ve mě vloženou. Dále bych chtěla poděkovat svému dědovi za jeho životní nadhled, zájem o moje studium a optimismus. Neposlední dík patří všem mým přátelům, spolužákům a kolegům, se kterými jsme společně prožívali celé studium.

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1. Introduction

1.1. Ticks

Ticks are obligatory blood-feeding (hematophagous) ectoparasites capable of transmitting a wide variety of pathogens comprising bacteria, viruses, and protozoa that affect animals and humans (de la Fuente et al., 2008). They are the second most dangerous vectors of arthropod-borne pathogens after mosquitoes (Sonenshine, 1991).

Ticks belong to the class Arachnida, subclass Acari, order Ixodida. The order Ixodida consists of two main large families Argasidae (soft ticks) and Ixodidae (hard ticks) (Mans, 2011). An evolutionary missing link between these families is the family Nuttalliellidae represented by the monospecific genus *Nuttalliella* (Mans, 2011; Horak et al., 2002).

1.1.1. Argasidae

Argasidae are present mainly in hot and dry areas around the Globe (Sonenshine, 1991). The world's fauna of the Argasidae consists of four generas, namely *Argas*, *Carios*, *Ornithodoros* and *Otobius* (Jongejan and Uilenberg, 2004).

They are referred to as the soft ticks because their cuticle lacks the dorsal sclerotized scutum (Fig. 1). The gnathosoma (mouth part) is placed on the ventral side of the body and it is difficult to see it from a dorsal view (Fig. 1)

Immature developmental stages of soft ticks include larvae that hatched from laid eggs and three or four nymphal stages (depending on the volume of blood uptake during blood-feeding). Subsequently, the last nymphal stage molts to the adult males and females which could be hardly distinguished just only by the appearance of the genital pore. Both sexes ingest blood. Females are able to feed repeatedly on the host and lay a small amount of the eggs after each blood meal. In contrast to hard ticks, soft ticks feed for relatively short time on their host (up to one hour) (Sonenshine, 1991).

1.1.2. Ixodidae

Ixodidae, is the largest and economically the most important family with 13 genera and approximately 650 species (Nava et al., 2009). Almost 80 % of the World's ticks fauna belongs to the Ixodidae (Jongejan and Uilenberg, 2004). The most important genera are *Amblyomma*, *Dermacentor*, *Haemaphysallis*, *Hyalomma*, *Rhipicephalus* and *Ixodida* (Horak et al., 2002).

The sclerotized scutum covers about one third of nymphal or adult female body and whole body of the males. Thus, the Ixodidae are referred to as the hard ticks. There are obvious sexes dimorphisms in the presence of the scutum furthermore, adult males are evidently smaller than females (Sonenshine, 1991).

Their life cycle includes three life stages (larvae, nymphs, and adults). All life stages, except for adult males from the genus *Ixodes*, suck host blood. Males just fertilize females to ensure their full repletion. In addition, Ixodidade are divided into 3-host, 2-host and single host parasites, depending on the number of host animals they attach to during their life cycle. The complete life cycle usually takes 2-3 years (Sonenshine, 1991). Six-legged larvae hatched from the eggs feed on small vertebrates, molt to the eight-legged nymphs that prefer feeding on a bigger vertebrate host. After dropping off the host, the nymphs molt to the adult stage. The cycle is closing when a male fertilizes an adult female that drops off the host, lays a batch of thousands of eggs and subsequently dies (Sonenshine, 1991).

Ixodidae have at the terminal segment of the first pairs of legs Haller's organ. It facilitates the ticks seeking the host because of the detection of scents, humidity, temperature and CO_2 (Sonenshine, 1991). The main part of the mouth component (gnathosoma) is called the hypostome consisting of chelicerates (first pair of the mouth limbs) and a pair of palps (second pair of the mouth limbs). Hypostome has a harpoon-like structure and mediates the attachment of the ticks to their hosts, disrupts the host skin and helps ingest host blood (Sonenshine, 1993).

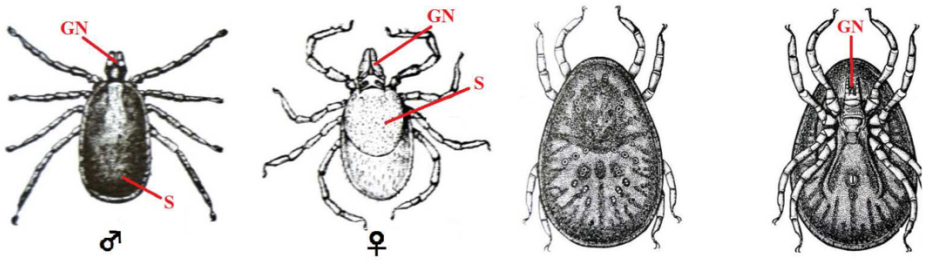

Figure 1: Body morphology of Argasidae and Ixodidae (adapted from Volf and Horak., 2007).
Argasidae (soft ticks) cuticle lacks sclerotized scutum, gnathosoma is placed on the ventral side of the body. The sclerotized scutum covers about one third of female body and whole male body, thus are Ixodidae referred as the hard ticks (Sonenshine, 1991).
From the left: male of the Ixodidae, female of the Ixodidae, dorsal view of the Argasidae and ventral view of the Argadidae. GN: gnathosoma, S: scutum.

1.2. *Ixodes ricinus*

Ixodes ricinus belongs to the largest genera *Ixodida*, which served as a model organism for this master thesis. This tick species occurs in areas across Europe but also the Middle East and North Africa with the preference of humid areas (mostly forests and grasslands) and mild climate. Tick activity increases mainly in the spring compared to the fall (Sonenshine, 1993). *Ixodes ricinus* is a typical representative of the 3-host tick (Fig. 2). Each of the parasitic stages, except for adult males, feeds on a host. *I. ricinus* is a vector of numerous extremely dangerous diseases including Lyme disease caused by the spirochetes of the genus *Borrelia* sp., tick borne encephalitis transmitting by flavivirus, Babesiosis as well as Ehrlichiosis (Sonenshine and Roe, 2014; Sonenshine, 1991).

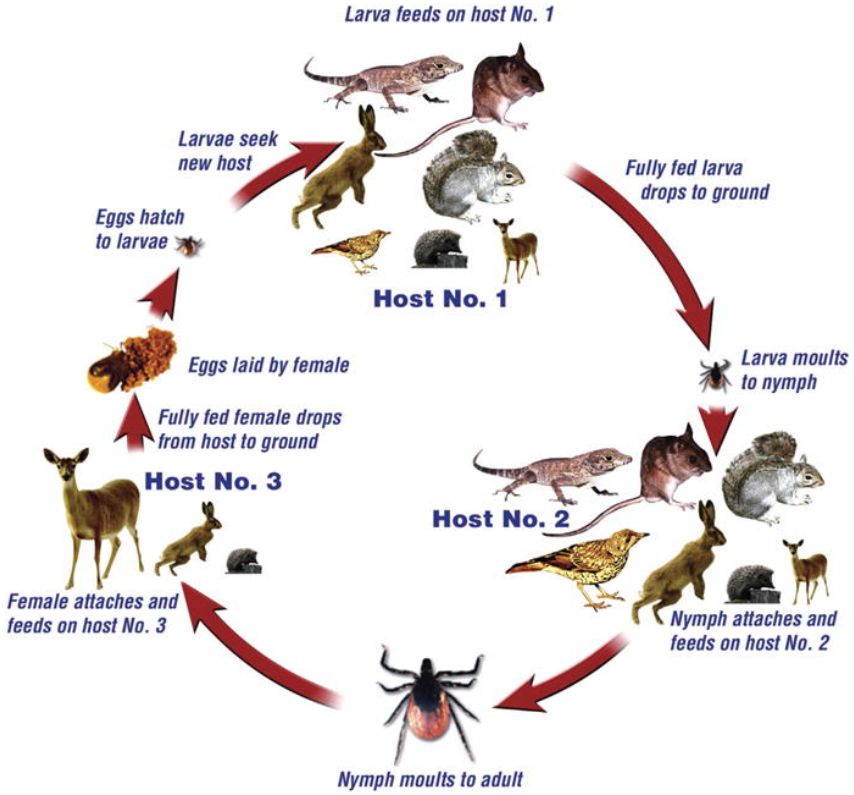

Figure 2: The life cycle of hard tick *Ixodes ricinus* (taken from Gray et al., 2016).
Ixodes ricinus is the 3-host life tick and its life cycle including larvae, nymphs and adults. Larva, hatched from the laid egg, prefers to feed on small vertebrates, molt to the nymph which feeds on a small or bigger vertebrate host. Adult female prefers big vertebrate host. The life cycle is closing when a male fertilizes an adult female that after engorgement drops off the host, lays a batch of thousands of eggs and subsequently dies (Sonenshine, 1991).

1.2.1. Feeding periods of *Ixodes ricinus*

In contrast to the soft ticks, hard ticks feed much longer. Nymphal feeding takes typically from 3 to 4 days, adults females feed twice longer approximately 6 – 9 days (Sonenshine, 1991). The feeding process might be divided into three phases (Fig. 3). First is the preparative **attachment phase** during which ticks find an appropriate place on the host where the skin is thin, and firmly attach the host by cement protein and start to feed (Coons et al., 1986). This is followed by the **slow-feeding phase.** Females suck a small volume of host blood, digestion of which is initiated and continues slowly. The final stage is the period of **rapid engorgement**. Females body size and weight expand noticeably during this phase that usually starts on the 5th or 6th day of feeding. Within about next 24 hours, females uptake about two thirds more of the host blood (called as "big-sip") than in the previous phases, and the digestion increases rapidly. The third rapid engorgement phase occurs exclusively after the females have been fertilized either before attachment or during blood feeding on the host. Fully fed females drop off their hosts, digest the ingested blood and prepare for the oviposition and die after laying eggs (Sonenshine, 1991).

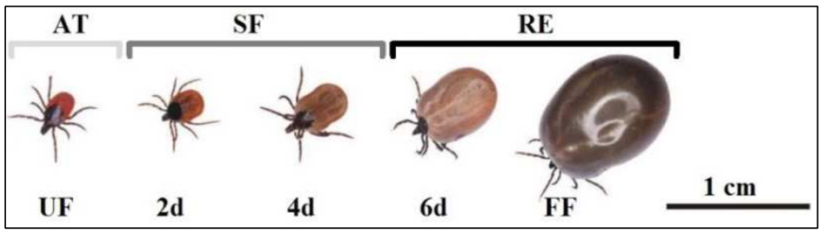

Figure 3: Feeding periods of *Ixodes ricinus* (adapted from Sojka et al., 2013).
AT: attachment phase, SF: slow-feeding phase, RE: rapid engorgement, UF: unfed, 2d: two days of feeding, 4d: four days of feeding, 6d: six days of feeding, FF: fully fed female.

1.3. Digestion of the imbibed blood

It is assumed that the tick gut lumen is the main store organ of the imbibed blood (Coons et al., 1986). Most of the hematophagous arthropods (such as insect blood-feeders) digest host blood extracellularly in the gut lumen (Okuda et al., 2005). By contrast, digestion in ticks is a slow process occurring intracellularly in the gut epithelium cells (Sonenshine, 1991). The hemoglobin digestion in ticks is an acidic process that occurs at pH of 3.5 – 4.5. The gradual hemoglobin degradation is mediated by a network of endo- and exo- peptidases, mainly of cysteine classes (cathepsin B, L, C and legumain) and aspartic peptidase (cathepsin D) (Sojka et al., 2013; Horn et al., 2009). Hemoglobin digestion is initiated by cathepsin D that results

in release of high amount of heme and formation of large hemoglobin fragments that exerts antibacterial activity (Fogaça et al., 1999). The major portion of potentially toxic heme is detoxified as an aggregate forming in the specialized organelles named hemosomes (Lara et al., 2003). The large hemoglobin fragments are further cleaved by cathepsin B and L and finally to the dipeptides and amino acids (Horn et al., 2009).

An enzymatic machinery involved in the hemoglobinolysis is well described. However, nothing is known about how the digestive system in ticks is set off and regulated. It is hypothesized, that the nutrient sensing and uptake may play an essential role of the digestion processes (Sojka et al., 2013).

1.4. The impaction of nutrients for the reproduction and development

Uptake of essential nutrients was mainly studied in model non-parasitic organisms. The effect of nutrition on egg development is obvious (Mirth et al., 2019). A decrease of laid eggs was observed in starving fruit flies (*Drosophila melanogaster*) females. Besides amino acids, also carbohydrates and lipids were shown to be essential for egg development (Mirth et al., 2019). Post and Tatar (2016) demonstrated that the metabolism and longevity of the fruit fly depend on a diet with a different ratio of carbohydrates and proteins. These types of diets widely involved the production of insulin-like peptides leading to the activation of the insulin receptor signaling pathway.

The parasite is strictly bonded with its host through unidirectional transfer of nutrition for its survival, development, and reproduction (Halton, 1997). Nutrients, obtained from blood-meal diet, play an essential role in the function of the hematophagous parasites. The host-parasite nutritional relationships evolved during the evolution of parasitic lifestyle (Dalton et al., 2004). For reproduction and egg development, mainly amino acids and lipids are needed (Hansen et al., 2014; Dalton et al., 2004). The major source of the amino acids for the hematophagous parasites such as platyhelminths comes from red blood cells after the hemoglobinolysis, and from soluble serum proteins that are present also in the host blood (Dalton et al., 2004).

Nutrition sensing acts through signaling pathways, controlling the cells homeostasis in the invertebrate and vertebrate organism. Nutrition initiates an activation of the insulin receptor signaling pathway via the expression of the insulin-like peptides (ILP). Amino acids act through the TOR (target of rapamycin) pathway to influence the oviposition (Mirth et al., 2019; Koyama and Mirth, 2016; Hansen et al., 2014; Badisco et al., 2013).

1.5. Insulin-like peptides

The nutritional status of the organism is responsible for the levels of insulin-like peptides (ILPs), that are synthesized in neurosecretory cells in brain of many insects (Koyama and Mirth, 2016; Vafopolou, 2014; Badisco et al., 2013). Insulin was believed to be a strictly typical vertebrate hormone however, ILPs also appear to be native to protostomian invertebrates, such as mollusks, nematodes and insects (Claeys et al., 2002). Insulin is one of the most studied hormone and was discovered in 1921 by the Frederick Grant Banting and Charles Best (Banting et al., 1922). It is a pancreatic hormone produced by the Pancreatic islets and disorders in its production leads to the serious illness *Diabetes melitus* (Claeys et al., 2002).

Invertebrates ILPs, which act as the agonists of the insulin receptor signaling pathway, were for the first time isolated in 1984 from silkworm *Bombyx mori* (Nagasawa et al., 1984), and are characterized by a specific disulfide bond arrangement (Hansen et al., 2014). ILPs play a crucial role in physiological processes (reproduction, lifespan, metabolism etc.) (Hernández-Sánchez et al., 2008; Nagata et al., 2008; Wu and Brown, 2006).

In many studies, invertebrates ILPs were widely discussed. Eight ILPs were identified in *D. melanogaster* (Badisco et al., 2013), *B. mori* has an ILP called bombyxin (Nagata et al., 2008). In parasitic invertebrates, four ILPs of mosquito *Aedes aegypti* were described (Hansen et al., 2014). Recently, four genes encoding ILPs have been identified and characterized from genome in the hard tick *Ixodes scapularis* (Sharma et al., 2019). It was demonstrated similarities in insects ILPs and vertebrates insulin-like growth factors (Vafopolou, 2014). Some of the ILPs of mosquito *A. aegypti* seemed to be similar to human insulin (Hansen et al., 2014).

Insect ILPs are synthesized by the brain neuroendocrine cells (Vafopolou, 2014; Badisco et al., 2013), however some of them appeared to be produced by fat body or other tissues (Hansen et al., 2014; Badisco et al., 2013; Koyama et al., 2013). Similarly, described ILPs of *I. scapularis* are mainly transcribed in synganglion (brain) but in salivary glands as well (Sharma et al., 2019).

1.6. Insulin receptor signaling pathway

The uptake of the vertebrates insulin and invertebrate ILPs are mediated by cells via the insulin receptor (InR) (Badisco et al., 2013; Hernández-Sánchez et al., 2008). Vertebrates possess three different InRs that could bind the insulin or insulin-like growth factors (Hernández-Sánchez et al., 2008). It has been believed, that invertebrates InR encoding one gene only (Hernández-Sánchez et al., 2008). More recently, duplication of InR gene has been reported in some early insects and cockroaches (Kremer et al., 2018).

Insulin receptor signaling pathway (ISP) regulates a variety of physiological and anabolic processes in response to nutrition; and is evolutionarily strongly conserved among the metazoan organisms (Badisco et al., 2013). The ISP interacts with many other signaling pathways, for example with target of rapamycin (TOR) pathway or Forkhead box-related transcription factors (FOXO). TOR signaling pathway acts as the major sensor of nutrition. FOXO is an important regulator of stress tolerance, longevity and growth (Vafopolou, 2014).

1.6.1. The cascade of the insulin receptor signaling pathway

Insulin receptor (**InR**) is a transmembrane component (Fig. 4), sometimes referred to as tyrosine kinase, and it encodes two subunits, α and β (Badisco et al., 2013). The α subunit and a part of the β subunit are extracellular (Defferrari et al., 2018). The α subunit ensures the peptides-binding specificity. The β subunit mediates the signal downstream to the other components of the ISP (Badisco et al., 2013). These two subunits are bound via disulfide bridges (Wu and Brown, 2006). Fruit fly InR and human InR are highly similar to each other, and furthermore, it was demonstrated that fruit fly InR was capable of binding human insulin (Wu and Brown, 2006; Claeys et al., 2002). This just confirms the high evolutionarily conservation of the ISP.

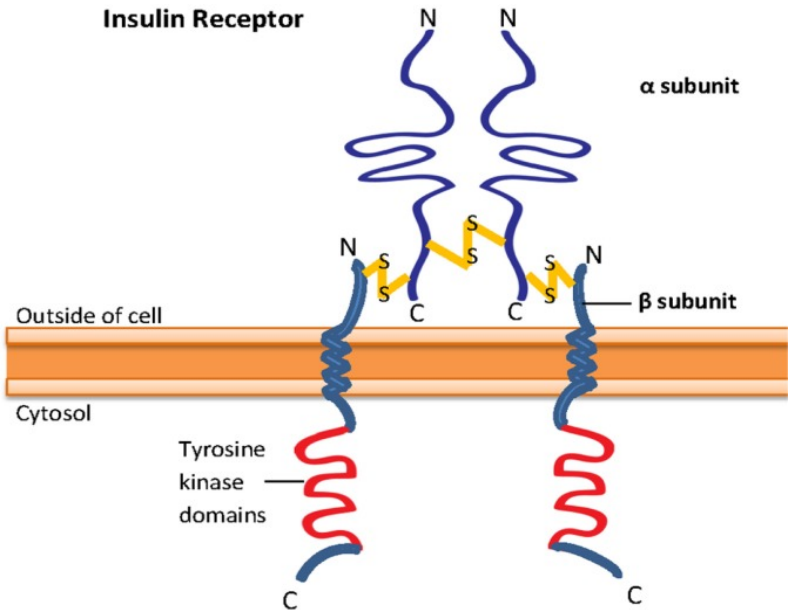

Figure 4: The schematic presentation of the insulin receptor (InR) (taken from Mangmool et al., 2017). Insulin receptor is a transmembrane component consists of extracellular α subunit and β subunit which presences in extracellular and in intracellular part as well (Badisco et al., 2013). These two subunits are bound via disulfide bridges (Wu and Brown, 2006).

Upon binding of ILPs to α subunit, the β subunit undergoes the autophosphorylation of specific tyrosine residues. The activated InR consequently triggers the insulin receptor substrate (IRS) (Badisco et al., 2013). In mammals two pathways could be potentially initiated. The activated IRS could either bind the growth factor receptor-bound protein (GRB2) or the phosphatidylinositol-3-kinase (PI3K) (Laplante and Sabatiny, 2009; Wu and Brown, 2006). In *D. melanogaster* two signaling pathways could be activated as well. The Ras-MAPK and the PI3K/PKB, respectively (Badisco et al., 2013). For the graphic presentation of the ISP see Figure 5. In this thesis, I have focused on the PI3K/PKB part of the pathway.

The activated PI3K acts as a catalysator of the consequent reaction. The phosphatidylinositol-4,5-diphosphate (PIP2) phosphorylates to a phosphatidylinositol-3,4,5-triphosphate (PIP3). Phosphatase and tensin homologue (PTEN) could inhibit such phosphorylation as well as decrease the concentration of PI3K in the cell. The higher concentration of the PIP3 in the cell activates the phosphoinositide-dependent protein kinase (PDK) which consequently sets off the protein kinase B (PKB) that is also called **AKT** (Badisco et al., 2013). Such activated proteins subsequently, influence other proteins related to glucose uptake, lipid synthesis or gene expression. These actions are generally associated with insulin influence (Wu and Brown, 2006).

AKT impacts the phosphorylation of the Tuberous sclerosis 1,2 (TSC1,2) complex, therefore this complex is inactivated. That leads to the indirect activation of the Target

of Rapamycin (**TOR**) (Badisco et al., 2013). Consequently, S6 kinase (S6K) is stimulated by activated TOR (Wu and Brown, 2006). In mammals, the TOR is activated through the complex Rheb. It seems that in mammalian ISP some sort of negative feedback exists. The activated S6K could inhibit the IRS by its phosphorylation thus decrease its stability (Laplante and Sabatini, 2009).

Additionally, mammals have at least two TOR complexes. The mTORC1 seems to have similar functions as the invertebrate TOR. The functions of the second complex, the mTORC2, are known very little (Laplante and Sabatini, 2009). Two TOR genes were identified in silkworm *B. mori* as well (Zhou et al., 2010).

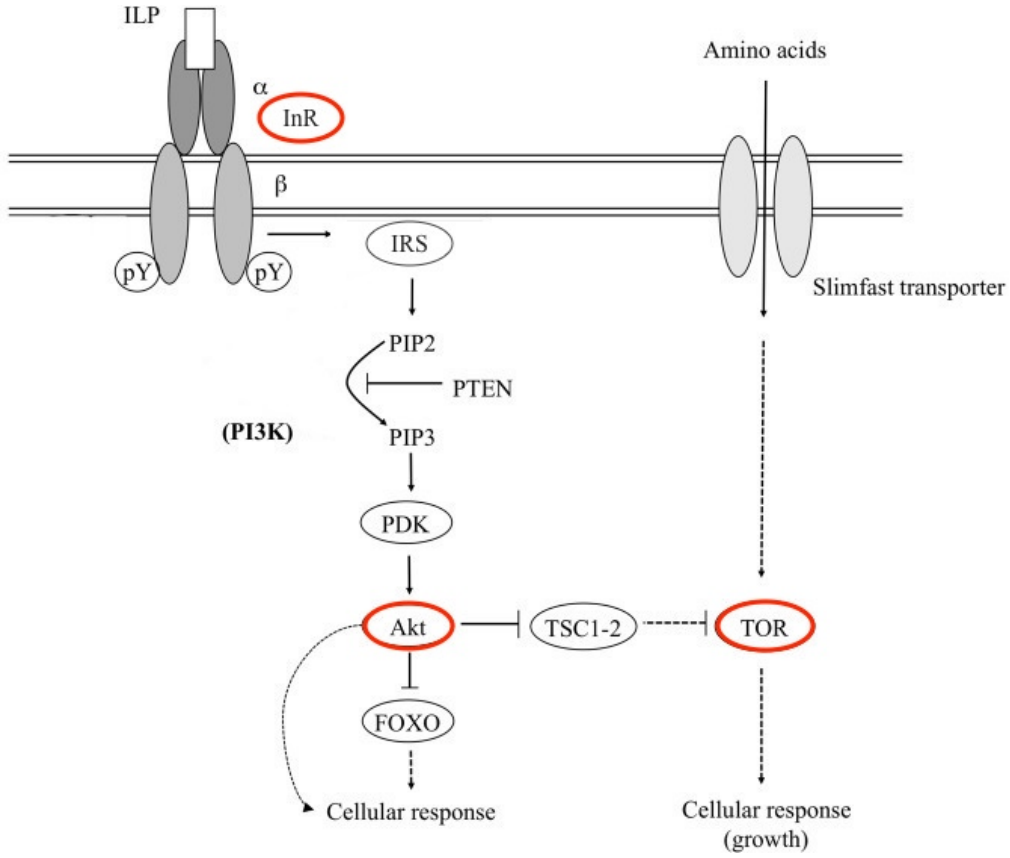

Figure 5: The schematic presentation of the insulin receptor signaling pathway (adapted from Badisco et al., 2013).
The insulin signaling pathway has been described in mammals with orthologous components for *D. melanogaster* and other insect. For more descriptions see text above. Dashes lines indicate the indirect interactions. ILP: insulin-like protein, InR: insulin receptor, IRS: insulin receptor substrate, PIP2: phosphatidylinositol-4,5-bisphosphate, PIP3: phosphatidylinositol-3,4,5-triphosphate, PTEN: phosphatase and tensin homologue, PI3K: phosphatidylinositol-3-kinase, PDK: phosphoinositide-dependent protein kinase, AKT: protein kinase B, FOXO: forkhead box-related transcription factors, TSC1-2: tuberous sclerosis 1,2 complex, TOR: target of rapamycin.

1.6.2. Insulin receptor signaling pathway in ticks

Only few components of the ISP have been described and functionally characterized in ticks. Umemiya-Shirafuji et al. (2012a, b) described homologues of AKT (*HlAKT*) and TOR (*HlTOR*) from the hard tick *Haemaphysalis longicornis*. Physiological functions of these homologues were assessed using RNAi silencing. The RNAi knockdown of the *HlAKT* showed the importance of this gene in completion of the blood-feeding, development and reproduction of the females of ticks (Umemiya-Shirafuji et al., 2012a). The RNAi silencing of the *HlTOR* resulted in the impaired oviposition of the female tick. In addition, these authors demonstrated that the treatment of *H. longicornis* with rapamycin, which acts as the TOR inhibitor (Ballou and Lin, 2008), decreased the expression of the genes encoding the vitellogenesis (Umemiya-Shirafuji et al., 2012b).

Another recent study has reported the role of three components of the ISP and TOR signaling pathways (AKT, TOR and glycogen synthase kinase - GSK3) in embryogenesis of the cattle tick *Rhipicephalus microplus* (Waltero et al., 2019). RNAi silencing of TOR in living females significantly impaired hatching of living larvae from the laid eggs. Furthermore, *in vitro* experiments showed, that TOR inhibition by rapamycin significantly affected viability of tick embryonic cell line BME26 (Waltero et al., 2019).

In our laboratory, several components of the ISP of *Ixodes ricinus* had been identified in the midgut transcriptome, including full sequences of the InR (*Ir*InR), the AKT (*Ir*AKT) and the TOR (*Ir*TOR) (Perner et al., 2016a). In this thesis, my aim was to verify the physiological function of *Ir*TOR and *Ir*AKT and, more importantly, to provide molecular and functional characterization of *Ir*InR that had never been described before in any tick species.

2. Aims of work

1) To identify homologues of important genes of the insulin receptor signaling pathway in available transcriptomes of *Ixodes ricinus*. Namely: insulin receptor (InR), protein kinase B called AKT and target of rapamycin (TOR).
2) To determine the expression profiles of tissue and feeding stages of these molecules using qRT-PCR.
3) To find and verify the phenotypes in the physiology of ticks of *Ir*InR, *Ir*AKT, and *Ir*TOR using RNAi silencing.
4) To prepare *Ir*InR recombinant protein and test its vaccination potential to protect the host against the ticks.
5) To identify an authentic *Ir*InR in tick tissues using SDS-PAGE and Western Blot analysis.
6) To alternatively assess the function of the *Ir*InR, *Ir*AKT, and *Ir*TOR, by injection with insulin receptor antagonist (IRA) and *in vitro* feeding with AKT and TOR inhibitors.

3. Materials and methods

3.1. Ticks and animals

Adult females and males of *Ixodes ricinus* were collected by flagging around České Budějovice, the Czech Republic. Thereafter, ticks were used for RNAi and infestation experiments. Ticks were maintained in separate vials with a humidity of about 95 %, temperature 24 °C and day/night period set to 15/9 h. To obtain ticks tissues for all experiments, females were fed naturally on laboratory guinea pigs or rabbits for a particular period of time. For the RNAi experiments guinea pigs were used. Rabbits were used for the vaccination experiments. All laboratory animals were treated in accordance with the Animal Protection Law of Czech Republic No. 246/1992 Sb., ethics approval No. 25/2018.

3.2. Identification and characterization of genes

The transcripts annotated as InR, AKT, and TOR were identified in the midgut transcriptomes from partially or fully fed *I. ricinus* females (Perner et al., 2016a) and their homology to the corresponding genes from other organisms were confirmed by BLAST analyses (National Center for Biotechnology Information (NCBI), National Institute of Health; https://blast.ncbi.nlm.nih.gov/Blast.cgi). For the alignment of the sequences, the ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/) using ClustalW method was used and presented using the BoxShade (https://embnet.vital-it.ch/software/BOX_form.html) software. Signal peptide was predicted using SinalP server (http://www.cbs.dtu.dk/services/SignalP/).

3.3. Tissues dissection, isolation of RNA and complementary cDNA transcription

Tissues (ovaries, salivary glands, tracheae with fat body, midgut, Malpighian tubes and the rest of the body) were dissected from *I. ricinus* females at specified time points under a drop of DEPC-treated PBS (8 % NaCl, 0.2 % KCl, 1.8 % Na_2HPO_4, 0.14 % KH_2PO_4 in 1 000 ml of 0.1 % diethylpyrocarbonate treated distilled H_2O, pH = 7, autoclaved). The following feeding intervals of ticks were examined: unfed, fed for 1, 3, 5 days, and fully fed, and ticks 3, 6 and 12 days after detachment. Total RNA was extracted from different tissues or feeding intervals of *I. ricinus* using Nucleo-SpinRNA II Kit (Macherey-Nagel, Germany) and its concentration was determined using Nanodrop (Thermo Fisher Scientific). RNA adjusted to the concentration of 200 ng/µl were transcribed into cDNA using Transcriptor High-Fidelity cDNA

Synthesis Kit (Roche Diagnostics, Germany) using oligo-dT primers according to the manufacturer's manual.

3.4. Relative expression profiling by quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA samples, prepared from *I. ricinus* tissues as described above, were analyzed in independent technical and biological triplicates by qRT-PCR using LigtCycler 480 (Roche) and Fast Star Universal SYBR Green Master Mix (Roche). For the reaction set-up and amplification program see Tables 1 and 2. Primers *InR F*, *InR R*, *AKT F*, *AKT R*, *TOR F*, and *TOR R* were designed using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer 3 Input (http://primer3.ut.ee/). The primers are listed in Table 13. The relative expression was calculated using the mathematical model of ΔΔCt method (Pfaffl, 2001) and normalized to elongation factor α1 (*ef*α1) (Nijhof et al., 2009) using *EF F* and *EF R* primers, which are listed in Table 13.

Table 1: RT-qPCR reaction set-up

Reagent	Volume
Master Mix	12,50 µl
Primers (100 µM)	0,25 µl + 0,25 µl
Template	5 µl
MiliQ PCR H_2O	7 µl

Table 2: RT-qPCR amplification program

		Temperature	Time	Number of cycles
Hold stage	Initial denaturation	95 °C	5 min	1
PCR stage	Denaturation	95 °C	20 s	50
	Annealing	60 °C	30 s	
	Elongation	72 °C	30 s	
Melting curve stage		95 °C	15 s	1
		60 °C	1 min	
		95 °C	15 s	

3.5. Standard polymerase chain reaction (PCR) and electrophoresis

Total RNA, isolated from *I. ricinus* tissues, was transcribed into cDNA as described above and used as a template for PCR reactions. Taq-Man Purple Polymerase (Top-Bio) was used in a master mix. Details of the reaction set-up and amplification program are listed in Tables 3 and 4. For the primers see Table 13. Subsequently, the PCR products were subjected to agarose gel (1 % agarose in TAE buffer with EtBr) under constant voltage of 100 V. For more details see Table 5. PCR products were visualized using the UV transilluminator.

Table 3: Standard PCR reaction set-up

Reagent	Volume
Polymerase (Top-Bio)	4 µl
Buffer (Top-Bio)	5 µl
dNTP's (Top-Bio)	1 µl
Primers (100 µM)	0,5 + 0,5 µl
Template	10 µl
PCR H_2O	29 µl

Table 4: Standard PCR amplification program

	Temperature	Time	Number of cycles
Initial denaturation	94 °C	10 min	1
Denaturation	94 °C	45 s	25-30
Annealing	55 °C	45 s	
Elongation	72 °C	1 min	
Final elongation	72 °C	10 min	1

Table 5: Chemicals used for electrophoresis

Chemicals	Compound
1× TAE buffer	40 mM Tris-acetate, 1 mM EDTA, pH 8.0
EtBr	Ethidium bromide 0.5 µg/ml
6× Loading Dye (Invitrogen)	10 mM Tris/HCl (pH 7.6), 0.03 % (w/v) bromophenol blue, 0.03 % (w/v) xylencyanol, 60 % (v/v) glycerol, 60 mM EDTA
DNA Ladder (Thermo Fisher Scientific)	GeneRulerTM 100 bp DNA Ladder Plus
RNA Ladder (Thermo Fisher Scientific)	High Range RNA Ladder

3.6. dsRNA synthesis

Total RNA was isolated from the ovaries, dissected from semi-engorged females, transcribed into cDNA and used as a template for PCR reaction (Tab. 3, 4). Primers were designed based on respective sequences using Primer Blast, Primer 3 Input, and Restriction Mapper (http://www.restrictionmapper.org/).

The dsRNAs were prepared as described in Hajdušek et al. (2009). The PCR products that were amplified using *InRapa*, *InRxba, AKTapa, AKTxba, TORapa*, and *TORxba* primers (Tab. 13) were subjected to 2 % agarose gel (Tab. 5), purified using the Gel and PCR Clean-up Kit (Macherey-Nagel) and restricted using ApaI and XbaI restriction enzymes (Thermo Fisher Scientific) (Levashina et al., 2001). Simultaneously, plasmid PlL10 was restricted with the same restriction enzymes (Tab. 6). The reactions were incubated at 37 °C for 2 hours. Consequently, the restricted PCR products and plasmid were purified as described above.

Table 6: Restriction reaction set-up

	PCR product	Plasmid PlL10
Buffer TANGO 10x (Thermo Fisher Scientific)	3 µl	3 µl
Enzymes (ApaI, XbaI) (Thermo Fisher Scientific)	1 + 1 µl	1 + 1 µl
Template	20 µl	3 µl
H_2O	5 µl	22 µl

The PCR products were ligated into restricted plasmid PlL10. For the details see Table 7. Ligation reaction was incubated at room temperature for 1 hour and at 16 °C overnight.

Table 7: Ligation reaction set-up

Buffer TANGO 2x (Thermo Fisher Scientific)	5 µl
Restricted plasmid PlL10	2 µl
Restricted PCR product	2 µl
T4 ligase (Thermo Fisher Scientific)	1 µl

The plasmid construct was transformed to the competent TOP10 cells (Invitrogen). Cells were cultivated on LB plates. Grown colonies were verified using standard PCR (Tab. 3, 4) to examine the construct. The colonies containing vector were incubated in 4 ml of LB with 4 µl of ampicillin (50 mg/ml) overnight at 37 °C (200 rpm). The plasmid DNA was isolated using High Pure Plasmid Isolation kit (Roche) and submitted for DNA sequencing to SeqMe (https://www.seqme.eu/cs/). Positive clones, verified by sequencing, were incubated in 100 ml of LB with 100 µl of ampicillin (50 mg/ml) overnight at 37 °C (200 rpm). The plasmid was isolated using Nucleo Bond Xtra Midi kit (Macherey-Nagel).

The plasmid, containing the *Ir*InR, *Ir*AKT or *Ir*TOR DNA sequences, was restricted with restriction enzymes ApaI and XbaI (Thermo Fisher Scientific) separately (Tab. 8), incubated at 37 °C for 2 hours and purified by classical phenol-chloroform method. Twenty-five µl of proteinase K (20 mg/ml proteinase K in 150 µl 10 mM Tris/HCl, pH 8.0 and 2 mM $CaCl_2$) and 3.75 µl of 10 % SDS (sodium dodecyl sulfate) were added to the restricted plasmid and incubated at 50 °C for 30 minutes. Consequently, 80 µl of phenol-chloroform (Sigma-Aldrich) was added, vortexed and spun at 13,300 x g for 5 minutes. Then, 80 µl of chloroform (Lach-Ner) was added to the aqueous phase, vortexed and spun. Finally, 80 µl of isopropanol (Lach-Ner) was added to the aqueous phase, gently mixed and incubated at -20 °C for 30 minutes. Subsequently, the samples were spun at 13,300 x g for 30 minutes at 4 °C. Pellet was washed in 80 % cooled ethanol and spun at 13,300 x g for 8 minutes at 4 °C, dried and resuspended in 20 µl of RNAse free water – DEPC water (0.1 % diethylpyrocarbonate diluted in distilled water).

Table 8: Restriction reactions set-up

Buffer TANGO 10x (Thermo Fisher Scientific)	5 µl
Enzymes (ApaI or XbaI) (Thermo Fisher Scientific)	6 µl
Plasmid	10 ng
H_2O	up to 50 µl

After the purification, the plasmid was used for the synthesis of the ssRNA fragments using MEGAscript™ T7 kit (Invitrogen) (Tab. 9) and incubated at 37 °C overnight. Two µl of the DNAse was added to the ssRNA and incubated at 37 °C for 15 minutes. Then, 230 µl of DEPC H_2O, 30 µl of ammonium acetate and 300 µl of phenol-chloroform (Sigma-Aldrich) were added to the samples, vortexed and spun at max rpm for 5 minutes. Three hundred µl of chloroform (Lach-Ner) was added to the aqueous phase, vortex and spun at 13,300 x g for 5 minutes. Subsequently, 220 µl of isopropanol (Lach-Ner) was added to the aqueous phase, gently mixed and incubated at -20 °C for 30 minutes. Then, the samples were spun at 13,300 x g for 30 minutes at 4 °C. The pelet was resuspended in 20 µl of DEPC H_2O. The concentrations of the ssRNAs were checked using Nonodrop (Thermo Fisher Scientific).

Table 9: ssRNA synthesis set-up

dNTPs	16 µl (4 µl of each)
Buffer	4 µl
Linearized plasmid	16 µl
Enzyme mix	4 µl
DEPC H_2O	up to 40 µl

The ssRNA ApaI and ssRNA XbaI of *Ir*InR, *Ir*AKT or *Ir*TOR were hybridized in ratio 1:1 (ApaI:XbaI) overnight in boiled water. The final concentration of each dsRNA was adjusted to 3 µg/µl. Each dsRNA was checked on agarose gel (Tab. 5). For control experiments, the green fluorescent protein (GFP) dsRNA prepared as described in Hajdušek et al. (2009) was taken from the stocks present in our laboratory.

3.7. RNA interference (RNAi)

Unfed *I. ricinus* females (25 per a group) were injected with 0.5 µl of *Ir*InR dsRNA, *Ir*AKT dsRNA, *Ir*TOR dsRNA or GFP dsRNA for control. The injected ticks (25 per a group) were kept at 24 °C to rest for one day and then, placed in glued cylinders on shaven backs of guinea pigs with an equal number of males to fed naturally. Three ticks from each group were forcibly removed after three days of blood feeding to examine efficiency of the genes knockdowns. Gene silencing was checked in ovaries by qRT-PCR. The engorged ticks were visually checked, weighed and maintained in separate vials as described above to assess the oviposition and hatching. The laid eggs were weighed as well. All results were related to the GFP-control ticks. The RNAi experiment was repeated three times to obtain three independent biological replications.

3.8. Injection with insulin receptor antagonist

The insulin receptor antagonist (IRA) (S961; Schäffer et al., 2008) (Phoenix Europe GmbH) was diluted in 1 x TBS (0.05 M TrisBase, 0.15 M NaCl, pH = 7.65) to the final concentration 100 nM. Consequently, ticks were injected with 0.4 µl (200 ng) with IRA. As a control, ticks injected with 1x TBS were used. The injected ticks (15 per a group) were kept at 24 °C to rest for one day and then, placed in halved glued cylinder on shaven back of guinea pig with an equal number of males (15 per a group) and allowed to feed spontaneously. The engorged ticks, that naturally dropped off their host, were visually checked, weighed and maintained in separate vials as described above for oviposition and hatching. The laid eggs were weighed as well.

3.9. Expression and purification of recombinant protein

Gene product of 906 bp encoding the 33 kDa fragment of the *Ir*InR N-terminal extracellular portion was amplified using *InR_pET100S2* and *InR_pET100AS2* primers (Tab. 13).

The ovarian cDNA from 5 days-fed females was used as a template. Resulting amplicons were purified using Gel and PCR clean-up kit (Macherey-Nagel). For the expression of *Ir*InR recombinant protein, an *E. coli* bacterial expression system (Champion^{TM} pET directional expression kit, Invitrogen) was used. N-Terminal 6X His-tagged fusion protein was prepared using a pET100/D-TOPO expression vector. The resulting expression constructs were transformed into TOP10 cells (Invitrogen) and submitted for sequencing to SeqMe using T7 forward and T7 reverse sequencing primers. The correct constructs, verified by sequencing, were transformed into BL21 Star^{TM} (DE3) *E. coli*. The sequences of the resulting N-terminal tagged fusion *Ir*InR products were MRGSH HHHHH GMASM TGGQQ MGRDL YDDDD KDHPF (6X His Tag) followed by the *Ir*InR specific amino acid sequence. The expression of recombinant protein was carried out in 10 ml of LB medium containing 10 µl of ampicillin (50 mg/ml) and 200 µl 1M glucose incubated at 37 °C (200 rpm) overnight. Subsequently, the culture was transformed to the 200 ml of LB medium with 200 µl of ampicillin (50 mg/ml) and 4 ml 1 M glucose and incubated at 37 °C (200 rpm) overnight. Glucose was used to increase the cells proliferation. Bacterial culture was centrifuged at 4, 000 x g for 10 minutes, washed and put into fresh LB medium with 1mM IPTG (Isopropyl β-d-1-thiogalactopyranoside) overnight to increase the expression. The cells were centrifuged at 4, 000 x g for 10 minutes. The pellet was resuspended in three different isolation buffers (Tab. 10) and each solution was sonicated. The cells lysate was divided on the individual fractions (cytoplasmic fraction, membrane fraction and inclusion bodies). Fractions were examined using SDS-PAGE electrophoresis and Western Blot analysis using Anti-His-Tag antibodies (see below).

The $(His)_6$-tagged fusion protein was purified from isolated inclusion bodies using Co^{2+} chelation chromatography in the presence of 8M urea using AKTA FPLC liquid chromatograph (GE Healthcare). The recombinant protein was eluted with linear gradient of imidazole. Fractions, containing the recombinant protein, were checked by SDS-PAGE electrophoresis and by Western Blot analysis, pooled and dialyzed against dialysis buffers (Tab. 11). During the refolding, the concentration of urea was decreased gradually from 8 M urea to 0 M urea. The buffers were regularly changed every 12 hours.

Table 10: Isolation buffers

Resuspention buffer	20 mM Tris-Cl, pH 8
Isolation buffer	20 mM Tris, 2 M urea, 0.5 M NaCl, 10 mM imidazole, 2 % triton, pH8
Solubilization buffer	20 mM Tris, 6 M quanidin hydrochlorid, 10 mM imidazole, 1 mM mercaptoethanol

Table 11: Refolding buffers

Buffer A	150 mM NaCl, 50 mM Tris-HCl, 8 M urea, 0,2 mM mercaptoethanol, pH 9
Buffer B	20 % glycerol, 150 mM NaCl, 50 mM Tris-HCl, 0,2 mM mercaptoethanol, pH 9
Buffer C	150 mM Tris-HCl, 150 mM NaCl, pH 9

3.10. SDS PAGE and Western Blot analysis

Recombinant protein was analyzed by SDS-PAGE using NuPAGE 4 – 12 % Bis-Tris Protein Gel (Thermo Fisher Scientific) or Criterion™ TGX Stain-Free™ Precast Gel (Bio-Rad). The gels were stained using Coomassie Brilliant Blue R-250 or visualized using the TGX Stain-Free™ technology. For Western blotting, gels were blotted on a PVDF (polyvinylidene difluoride) membrane (Immobilon Milipore), using the Trans-Blot Turbo system (Bio-Rad). The membrane was blocked in 3 % solution of non-fat milk in PBS-T (1x PBS with 0.05 % Tween 20 (Sigma-Aldrich)) for one hour at 4 °C, washed in PBS-T three times for 10 minutes and incubated in the primary antibody overnight at 4 °C. The primary antibody was either Anti-His-Tag (Sigma-Aldrich), Anti-*Ir*InR serum from immunized rabbit or the Ig fraction isolated from immune serum in 1 % non-fat milk in PBS-T at the dilution specified below. Then, the membrane was washed in PBS-T twice for 10 minutes and consequently, incubated in secondary antibody (Anti-Rabbit IgG-peroxidase, Sigma-Aldrich) in 1 % non-fat milk in PBS-T for 45 minutes. In the case of Anti-His-Tag (Sigma-Aldrich), the secondary antibody was Anti-Mouse IgG-peroxidase (Sigma-Aldrich). The membrane was washed in PBS-T for 30 and 10 minutes. After the final wash, the membrane was visualized by ChemiDoc™ Imaging System (Bio-Rad) using the Immobilon® Classico Western HRP Substrate (Milipore).

3.11. Production of antibodies

The recombinant protein (100 μg/ml) was mixed with incomplete Freud´s adjuvant (Sigma-Aldrich) (1:1) and used to immunize rabbit in four doses (weeks 1, 3, 5 and 7). Blood was collected from the immunized rabbit 2 weeks after the last immunization and kept in room temperature for one hour and then, at 4 °C overnight to fully clot. Serum was obtained by centrifugation at 5,000 x g for 15 minutes at 4 °C and used as a primary antibody during the Western Blot analysis or further processed for the isolation of the Ig fraction.

3.12. Preparation of immunoglobulin (Ig) fraction

Ig fractions were prepared as described in Pěničková (2009). Briefly, one volume of immune serum was mixed with two volumes of Na-acetate buffer (50 mM, pH 4) and the majority of the serum proteins was precipitated with caprylic acid. Small aliquots of caprylic acid were gradually added into the stirred serum-buffer mixture until the final concentration 25 μl per ml was reached. The precipitation further continued for 1 hour under constant stirring at room temperature, the precipitate was removed by centrifugation at 5,000 x g, the supernatant containing the Ig fraction was filtrated through the filter paper and dialyzed against 5 mM Na_2HPO_4 overnight at 4 °C. The prepared Ig fractions were used as a primary antibody for the Western Blot analysis.

3.13. Detection of authentic *Ir*InR in tick tissues

Unfed uninjected females or *Ir*InR dsRNA or GFP dsRNA injected females of *I. ricinus* were removed from the guinea pig after 7 days of feeding. Dissected tissues (ovaries, salivary glands, tracheae with fat body, midgut and Malpighian tubes) from three females were homogenized in 1x PBS, 0.25 M DTT and 1 x NuPage sample buffer (Invitrogen) and boiled at 100 °C for 5 minutes. Detection of *Ir*InR was performed by Western Blot analysis using the Ig fraction as a primary antibody, at the dilution specified below.

3.14. Effect of vaccination on ticks infestation

Three rabbits were immunized as described above. One negative control rabbit was injected with incomplete Freud´s adjuvant (Sigma-Aldrich) only. After the last immunization, blood sample was collected from the rabbits ears to examine specific antibodies by Western Blot analysis.

Unfed *I. ricinus* females were placed in two cylinders on shaven backs of immunized rabbits with an equal number of males (50 pairs of *I. ricinus* per rabbit) and allowed to feed naturally till repletion. The engorged ticks were visually checked, weighed and maintained in separate vials as described above for oviposition, weighing egg clutches and larval hatching scoring.

3.15. Tick membrane feeding

Feeding units (FU) were prepared as described by Kröber and Guerin (2007) with some minor modification according to Kučera (2015). Briefly, silicone paste consisting of silicone, silicone oil and hexane was spread over a rectangular mesh matrix and let to dry overnight. Only membranes not exceeding a thickness of 130 μm were used for completing FUs by sticking them on the bottom part of the plastic FUs. *In vitro* feeding experiments were performed according to the procedure as described Perner et. al (2016b).

Bovine blood was collected in a local slaughterhouse (Jihočeská masna s.r.o.), manually stirred in order to completely defibrinate it and supplemented gentamicine (5 μg/ml) to prevent bacterial growth. Diets consisting of 3.1 ml of blood, 1 mM ATP, gentamicine (5 μg/ml) and tested concentrations of rapamycin (Vézina et al., 1975) (Sigma-Aldrich) or AKT inhibitor (A-443654, Han et al., 2007) (APExBio) (Tab. 12) was applied into the wells of the 6-well cell culture plate (Corning® Costar®). An equivalent volume of solvents DMSO (dimethyl sulfoxide) and 100% ethanol was added into the blood as sham controls for rapamycin and AKT inhibitor respectively. Freshly prepared blood diets were exchanged regularly every 12 hours and the feeding units and tick-mouthparts were washed with 0.9 % NaCl. The membrane feeding was performed at 37 °C using a water bath thermostat and continued until full repletion.

Fifteen adult females were placed per FU. Two days after their attachment, the equivalent number of males was added to ensure full repletion of females. The fully-engorged ticks, that spontaneously dropped off the membrane, were visually checked, weighed and maintained in separate vials to assess the oviposition.

Table 12: Final concentrations of the dietary components in FU

Treatments	Final concentrations
Rapamycin	100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.1 μM, 10 nM
AKT inhibitor	100 μM, 10 μM, 1 μM, 0.1 μM, 10 nM

3.16. Statistics

Data were analyzed by GraphPad Prism 6 for Windows, version 6.04. For error bar graphs and means ± SD were used.

Table13: Used primers

	dsRNA primers	qRT-PCR primers	Primers used for the expression of recombinant protein
InR	InRapa attctagaCATACGTCTCCTTGGCAAGC InRxba atgggcccACAAGATTGCGGTGGTCAA	InR F TCAAGTATGTCATCAGCGGC InR R GAGGTACGAGGTGTGGTAGA	InR_pET100S2 caccCCCAACCTGTGCCACGCTG InR_pET100AS2 tcaCTTGGGGTTGATGTGGAAG
AKT	AKTapa atgggcccCATGTTCAGCGTAGAGTCTG AKTxba attctagaTGACCACTTTCTTCTTGAGG	AKT F GACTTTGGGCTCTGTAAGG AKT R CCGCACATCATCTCATACAT	X
TOR	TORapa atgggcccAGGTGCTTGGAGAATGGGAA TORxba attctagaTACTCCTCCATCGTCTCCCA	TOR F ACTACACCAGATCCCTCGCT TOR R CCATGGCGTTGATGAGCATG	X
EF	X	EF F ACGAGGCTCTGACGGAAG EF R CACGACGCAACTCCTTCAC	X

4. Results and discussion

4.1. Analysis of the sequences and alignments

In the midgut transcriptome of *I. ricinus*, several components of the ISP had been identified (*Ir*InR, *Ir*AKT, *Ir*TOR) (Perner et al., 2016a). The complete coding sequences for *I. ricinus* InR, AKT, and TOR were deposited in the GenBank under the accession numbers MN207065, MN207064, and MN207063, respectively (Supplement 1).

4.1.1. *Ir*InR

The midgut transcript encoding *Ir*InR (Ir-120837) contained the full coding sequence without the predicted signal peptide by SignalP server (http://www.cbs.dtu.dk/services/SignalP/) (Perner et al., 2016a). The identical gene, that contained the signal sequence (according to the SignalP), had been identified in the transcriptome from salivary glands (transcript Ir-SigP-26449_FR1_99-1649) annotated as insulin-like growth factor receptor (InGFR) (Perner et al., 2018). The presence of the insulin-like peptides (ILPs), which bind to the InR, in hard tick *I. scapularis* has been described (Sharma et al., 2019). Additionally, Mulenga and Khumthong (2010) identified the insulin-like growth factor binding proteins in tick *Amblyomma americanum*.

At the moment, we are not able to distinguish InR and InGFR, furthermore, it seems that these two receptors have the same structure and it is probably not possible to classify them based on their sequences. According to the BLAST and SignalP, InR of the *I. scapularis* (accession number: XP_029828634.1) lacks the signal peptide while InGFR (accession number: XP_002416224.1) contains the signal peptide.

We assumed that these two receptors may be differentiated by the signal peptide. Thus, we deduced that midgut transcript (Ir-120837) (Perner et al., 2016a), which lacks signal peptide, encoding the *Ir*InR while salivary gland transcript (Ir-SigP-26449_FR1_99-1649) (Perner et al., 2018) is encoding InGFR of *I. ricinus* as it was annotated.

The full-length cDNA of *Ir*InR (identified in the transcriptome from the midgut) encodes precursor containing both α and β subunit. The length of the *Ir*InR cDNA sequence is 4 491 bp encoding 1 496 amino-acid residues with a theoretical mass of 165 kDa. Based on the amino-acid sequence alignment, the *Ir*InR subunits are cleaved after the arginine-rich motive and the theoretical masses of the α and β subunits are 80.6 kDa and 65 kDa, respectively. The amino acid sequence is highly conserved, which was shown by the alignment with other

organisms (Supplement 2.1). According to the BLAST, the *Ir*InR gene contains many conserved domains (Fig. 6), for example, Furin-like cysteine-rich region, Protein tyrosine kinase or Catalytic domain of Insulin Receptor-like Protein Tyrosine Kinase (BLAST).
The presence of the domains proves that the ISP is a highly conserved system.

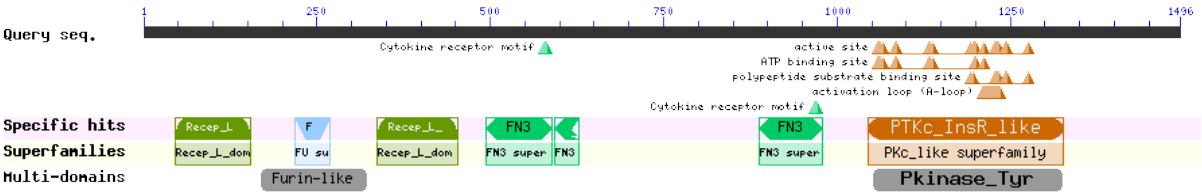

Figure 6: Domain structure of the *Ir*InR (taken from BLAST).

4.1.2. *Ir*AKT

The midgut transcript encoding *Ir*AKT (Ir-100439), contained the full coding sequence (Perner et al., 2016a). The gene *Ir*AKT is encoded by 1 593 bp long nucleotide sequence.
The sequence encoding 531 amino-acid residues of about the predicted mass 60 kDa.
The encoded protein lacks the signal peptide and consists of highly conserved regions as PH (Pleckstrin homology) domain, which belongs to the PH-like superfamily. Furthermore, Protein kinase superfamily, which is predominantly composed of the catalytic serine/threonine kinase domain (Supplement 2.2). The sequence contains one hydrophobic motif (Fig. 7). The deductive amino acid sequence alignment of *Ir*AKT showed high homology with other organisms (Supplement 2.2).

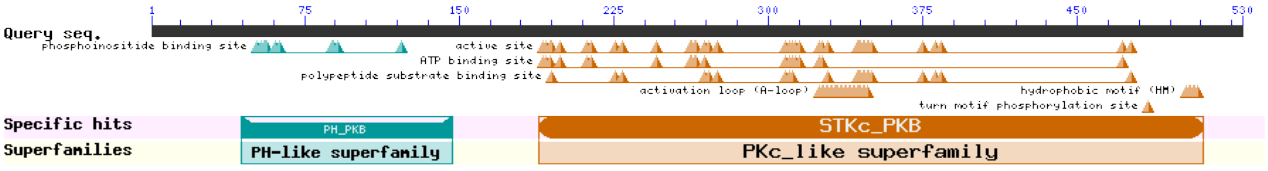

Figure 7: Domain structure of the *Ir*AKT (taken from BLAST).

4.1.3. *Ir*TOR

The transcript encoding *Ir*TOR (IrSigP-108190) was only partial sequence spanning the central region of the protein. The full sequence of *Ir*TOR was completed using overlapping transcripts IrHemSgMg-240809 from *I. ricinus* hemocytes (Kotsyfakis et al., 2015) and Ir-238238 from salivary glands (Perner et al., 2018) that encode the N-terminal and the C-terminal parts of the TOR protein, respectively.

The length of the *Ir*TOR nucleotide sequence is 7 518 bp encoding 2 506 amino-acid residues. The theoretical protein mass predicted from amino acid sequence is 284 kDa and lacks signal peptide. The alignment of the *Ir*TOR with other species resulted in high homology (Supplement 2.3). *Ir*TOR contains only one large domain, namely Phosphatidylinositol kinase domain, which belongs to the TEL1 superfamily (Fig. 8) (BLAST). Furthermore, according to the BLAST, *Ir*TOR consists of the FAT superfamily and Protein kinase superfamily (BLAST).

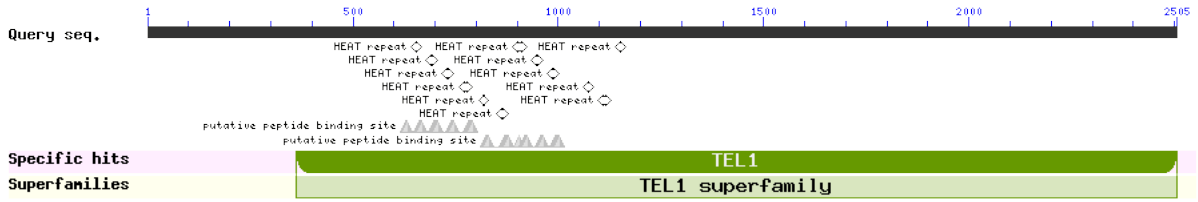

Figure 8: Domain structure of the *Ir*TOR (taken from BLAST).

4.2. Tissue relative expression profiling by qRT-PCR

Total RNA, isolated from tissues dissected from semi-engorged *I. ricinus* females, was transcribed into cDNA as described above and used as a template for the qRT-PCR reactions using specific gene primers *InR F, InR R, AKT F, AKT R, TOR F* and *TOR R*. It was found that mRNA level of all investigated genes was the highest in the ovaries of semi-engorged females (Fig. 9). Additionally, *Ir*InR was expressed in the midgut and in the rest of the body as well. The higher *Ir*AKT mRNA level was also detected in the midgut. Besides ovaries, *Ir*TOR was to lower extend equally expressed in all examined tissues (Fig 9).

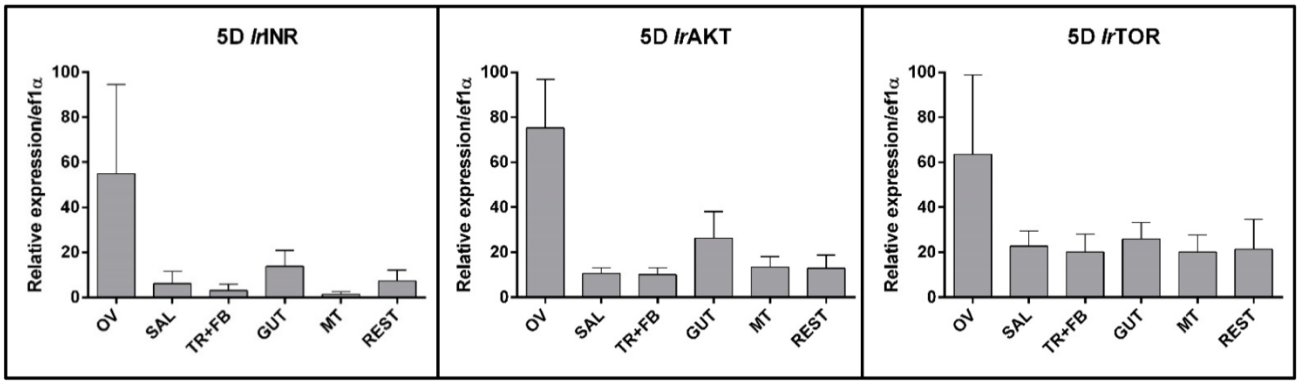

Figure 9: Tissues relative expression profiling by qRT-PCR.
Quantitative real-time PCR (qRT-PCR) profiling of *Ir*InR, *Ir*AKT and *Ir*TOR in tissues. Each data point represents the mean of biological and technical independent triplicates, bars indicate the standard deviation; OV: ovaries, SAL: salivary glands, TR+FB: trachea + fat body, GUT: the midgut, MT: Malpighian tubules, REST: the rest of body.

Based on our knowledge, expression of the InR in another tick species have never been described before. In a model organism *Drosophila melanogaster*, InR was transcribed in nurse cells of ovaries but also in the nervous system of adult females (Wu and Brown, 2006). InR of hematophagous kissing bug *Rhodnius prolixus* was expressed in all investigated tissues, but mainly in the central nervous system (Defferrari et al., 2018).

AKT of the hard tick *Haemaphysalis longicornis* was expressed in all examined tissues including ovaries, while the expression of AKT in the mosquito *Aedes aegypti* was demonstrated in ovaries of non-oogenic females only (Riechle and Brown, 2003). By contrast, in larvae of silkworm *Bombyx mori* the slightly lower expression of the AKT was examined in ovaries and testis compared to another tissues (Nagata et al., 2008).

In hard tick *H. longicornis,* the highest expression of TOR was observed in ovaries (Umemiya et al. 2012b) which is in agreement with our results. In silkworm *B. mori*, TOR was mostly expressed in nervous system (Zhou et al., 2010).

Among the mentioned organisms the components are presented mainly in the nervous system but in other tissues as well. In addition, insulin-like peptides (ILPs) are synthesized in neurosecretory cells in brain of many insects (Koyama and Mirth, 2016; Vafopolou, 2014; Badisco et al., 2013). It is in line with results recently published for the tick *Ixodes scapularis,* where four ILPs were reportedly expressed mainly in synganglion (brain) and salivary glands of the unfed females (Sharma et al., 2019). Badisco et al. (2013) claimed that accepted nutrients are stored into various organs. It may prove the complexity of the action place of the ISP among the organisms.

Our obtained data, however, showed the highest expression of the *Ir*InR, *Ir*AKT, and *Ir*TOR in ovaries. It seems that the ISP plays an essential role in ticks reproduction. Furthermore, each gene was also expressed in the midgut which is in line with the original identification of these genes in the midgut transcriptome (Perner et al., 2016a). Unlike the above discussed organisms, our data could not show the expression of the ISP in the tick nervous system as we did not assess their expression in ticks synganglion (brain), which is very difficult to dissect reliably. However, the rest of the body we examined, that contained synganglion, did not indicate the high levels of respective mRNAs.

4.3. Relative expression dynamics in ovaries by qRT-PCR during feeding and after detachment

Based on the tissue profiling results, the ovarian cDNA was chosen to investigate the expression dynamics of ISP components in the course of tick feeding and after detachment off the host. cDNA templates were prepared as described above from tick ovaries, dissected in the following stages: unfed ticks, ticks fed for 1, 3, 5 days, and fully fed ticks, and ticks 3, 6 and 12 days after detachment. Relative expressions of the *Ir*InR, *Ir*AKT, and *Ir*TOR were gradually increasing during the feeding course and reached their maxima at the fully fed stage. After detachment, the expression of *Ir*InR remained more than less stable, mRNA levels of *Ir*AKT were fluctuating, and the expression of *Ir*TOR seemed to be decreasing towards the 12 days after detachment (Fig. 10).

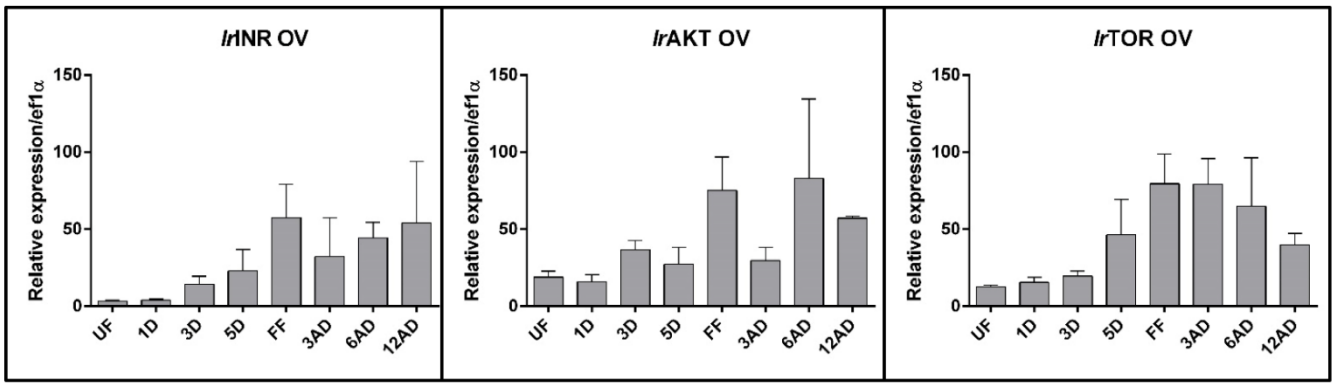

Figure 10: Relative expression profiling by qRT-PCR during feeding and after detachment.
Quantitative real-time PCR (qRT-PCR) profiling of *Ir*InR, *Ir*AKT and *Ir*TOR in ovaries. Each data point represents the mean of biological and technical independent triplicates, bars indicate the standard deviation; UF: unfed ticks, 1D: one day of feeding, 3D: three days of feeding, 5D: five days of feeding, FF: fully fed females, 3AD: three days after detachment, 6AD: six days after detachment, 12AD: twelve days after detachment.

We presumed the increasing expression of ISP components during the feeding according to Umemiya et al., (2012a). These authors demonstrated that the expression of AKT in hard tick *H. longicornis* was higher in fully fed stage compared to the unfed stage (Umemiya et al. 2012a). After detachment, the expression of *Ir*TOR decreased towards the 12 days after detachment. However, expressions of *Ir*InR and *Ir*AKT after detachment did not prove this decreasing trend (Fig. 10).

The amount of ILPs in *I. scapularis* in response to the feeding is still not clear (Sharma et al., 2019). The nutritional status of the organism is responsible for the levels of ILPs which act as agonists of the ISP (Koyama and Mirth, 2016; Vafopolou, 2014; Badisco et al., 2013).
It is still an unresolved question whether the increasing expression of ISP components during feeding is caused by initial digestion of the uptaken nutrients, by the opposite, whether the increased expression of the ISP up-regulates the digestive system of ticks (Sojka et al., 2013).

4.4. Silencing of genes by RNAi

To assess the function of the ISP, we carried out RNAi silencing of three key genes in this pathway. The dsRNAs were synthesized as described by Hajdušek et al. (2009). Amplified PCR products of *Ir*InR, *Ir*AKT and *Ir*TOR, using *InRapa*, *InRxba, AKTapa, AKTxba, TORapa,* and *TORxba* primers, were restricted using Apal and Xbal restriction enzymes, purified and ligated into restricted PlL10 plasmid. The plasmid constructs were transformed to the competent TOP10 cells and then, positive clones, verified by sequencing, were purified. Consequently, the plasmids, containing the *Ir*InR, *Ir*AKT or *Ir*TOR DNA sequences, were restricted with

restriction enzymes ApaI and XbaI separately, purified by the classical phenol-chloroform method and used for the synthesis of ssRNA fragments. Purified ssRNA fragments were hybridized. The final concentration of each dsRNA was adjusted to 3 µg/µl. The quality of each step of dsRNA synthesis was checked on the agarose gel and shown in Fig 11.

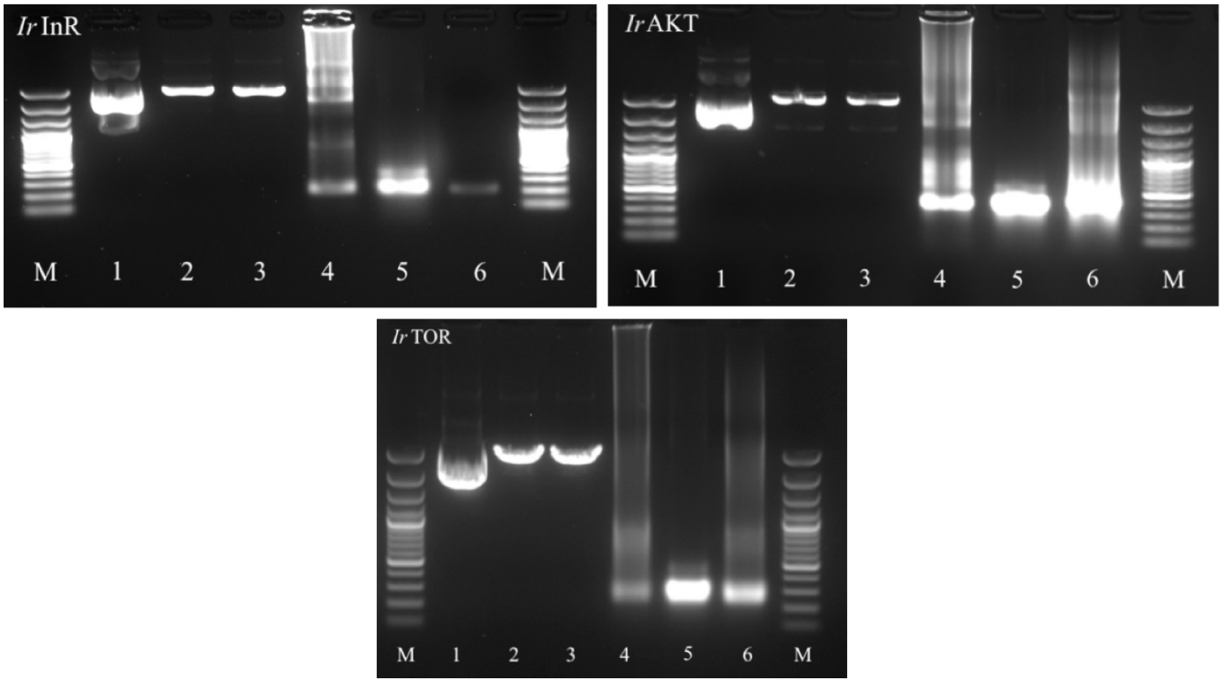

Figure 11: Quality control of dsRNAs preparations.
For the control of dsRNA quality, electrophoresis analysis was used. The final concentration of each dsRNA was 3 µg/µl; 1: nonlinearizied PlL10 plasmid with *Ir*Inr, *Ir*AKT or *Ir*TOR insert, 2: ApaI linearizied PlL10 plasmid, 3: XbaI linearizied PlL10 plasmid, 4: ssRNA ApaI, 5: ssRNA XbaI, 6: dsRNA, M: marker (100 bp RNA Ladder).

Unfed females were injected with 0.5 µl of *Ir*InR, *Ir*AKT or *Ir*TOR dsRNA, kept to the rest for one day and put on the shaven backs of guinea pigs with the equal number of males to naturally feed. The control group of ticks was injected with GFP dsRNA. The RNAi experiment was repeated three times to obtain three independent biological replications.

4.4.1. Efficiency of RNAi knockdowns

After three days of feeding, three females were removed from the guinea pigs, their ovaries were dissected as described above and used for the verification of the RNAi silencing.
The reduction of expression of all genes was confirmed by qRT-PCR using *InR F, InR R, AKT F, AKT R, TOR F,* and *TOR R* primers and compared to the control GFP group. The best RNAi silencing was achieved in the second RNAi experiment, in which the transcription of *Ir*InR, *Ir*AKT, and *Ir*TOR was reduced to 14 %, 2 %, and 14 %, respectively (Fig. 12). Silencing

in the first and the third experiments were not as effective as during the second one.
In the first experiment, the expression of *Ir*InR, *Ir*AKT, and *Ir*TOR was reduced to 28 %, 1 %, and 3 % respectively, and in the third experiment reduced to 87 %, 2 %, and 32 %, respectively. Apparently, *Ir*InR was not successfully silenced in the third RNAi experiment. However, *Ir*AKT was silenced with high efficiency in all three RNAi experiments. It seems, that the RNAi works individually for each gene. Joga et al. (2016) claimed that exist some barriers in the efficiency of the RNAi in insects.

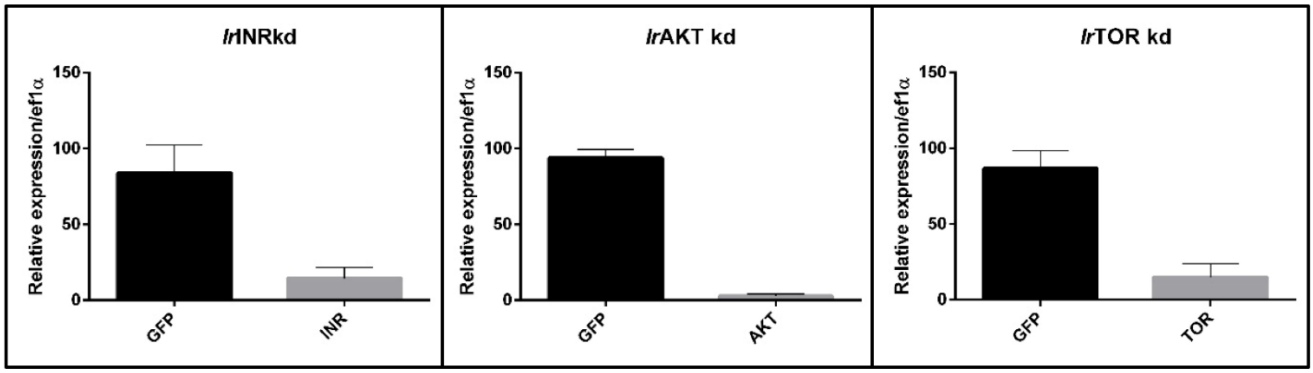

Figure 12: Efficiency of RNAi knockdowns.
The silencing of the genes *Ir*InR, *Ir*AKT, and *Ir*TOR was verified by qRT-PCR after three days of feeding in ovaries from three randomly selected females. These graphs show the knockdowns of the second replication of the RNAi experiment. Bars indicate the standard deviation.

4.4.2. RNAi and its effect on tick phenotype

The fully fed ticks were visually checked, weighed and maintained in separate vials for oviposition and hatching as described above. Considerable differences were found in the body weights of each dsRNA injected engorged females group in comparison with the control (GFP) dsRNA injected females group. The most obvious differences on tick phenotype were found in the second RNAi experiment (Supplement 3).

The average weight of the fully fed control ticks reached around 300 mg, whereas weights of *Ir*InR and *Ir*TOR dsRNA injected groups were slightly lower (Fig. 13). To our knowledge, RNAi knockdown of the InR had not been assessed in any tick species before. In the kissing bug *R. prolixus* the InR RNAi injected group weighed slightly lower than the control group in agreement to our results. In addition, it was demonstrated that RNAi knockdown of the InR resulted in lower phosphorylation of the AKT gene (Defferrari et al., 2018) which we did not assess. Additionally, it was demonstrated that TOR RNAi engorged females of hard tick *H. longicornis* had lower body weight than the control ticks (Umemiya et al., 2012b), in accordance with our experiment.

The most apparent differences were observed upon *Ir*AKT dsRNA injection. The body size and weights of *Ir*AKT dsRNA injected ticks were significantly lower. The average females weight reached only about 50 mg (Fig. 13). Besides the effect on ticks weights, *Ir*AKT RNAi silencing ticks also considerably prolonged the feeding duration from ten to twelve days in comparison with GFP control ticks and also ticks, injected with *Ir*InR or *Ir*TOR dsRNAs (Fig. 13). *Ir*AKT dsRNA group of ticks was not able to complete their blood-feeding. After dropping out from the host, the ticks had such a similar size as during the slow-feeding phase. *Ir*AKT seems to be essential for ticks to reach the rapid blood-feeding phase. Our explanation confirms the experiment performed by Umemiya et al. (2012a). Knockdown of the AKT in hard tick *H. longicornis* demonstrated the important role in blood feeding as well. Ticks did not finish their feeding successfully, therefore they were unable to continue in reproduction (Umemiya et al., 2012a) which is in agreement with our results. Additionally, *Ir*AKT injected group did not succeed in oviposition.

Most of the *Ir*InR and *Ir*TOR dsRNA injected females succeeded in oviposition. The laid eggs had slightly lower weights (not statistically significant) than the control group (Fig. 14). Hatching of *Ir*InR and *Ir*TOR dsRNA injected females were successful and did not show any differences in comparison to the control group of ticks. The RNAi knockdown of InR in mosquito *A. aegypti* demonstrated the reduced transcription of the vitellogenin genes (Roy et al., 2007). Umemiya et al. (2012b) demonstrated that TOR RNAi *H. longicornis* ticks did not lay eggs. We had not confirmed this result. A recent study reported that TOR RNAi silencing in the hard tick *Rhipicephalus microplus* did not show any differences in laying eggs in accordance with our results. Despite the weights of the laid eggs were similar to the control group, it was demonstrated significantly impaired hatching of living larvae from the laid eggs (Waltero et al., 2019). The knockdown of TOR in another hematophagous species, mosquito *A. aegypti*, showed the inhibition of vitellogenin genes, lower amount of eggs and inhibition of eggs development thus the end of the reproduction cycle (Hansen et al., 2004; Roy et al. 2007) in agreement to Umemiya et al. (2012b) results.

It seemed that the impact of the TOR to oviposition is variable among ticks species. Surprisingly, a recent study demonstrated that hard ticks *H. longicornis* seem to be able to complete their oogenesis without previous mating (Mihara et al., 2018; Kiszewski et al., 2001) which is in striking contrast with the results observed in our laboratory (unpublished). Any parthenogenesis was not observed both in *I. ricinus* and in *R. microplus* (Kiszewski et al., 2001). Our hypothesis is that TOR could have different impaction to ticks reproduction.

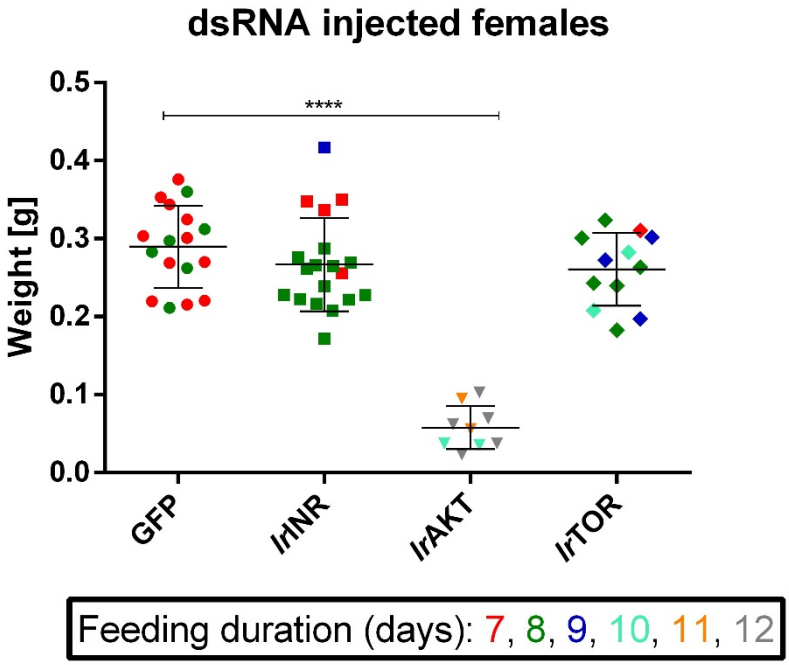

Figure 13: The weights of the RNAi fully fed females.
Graph shows weights of *Ir*InR, *Ir*AKT, and *Ir*TOR fully fed females from the second replication of the RNAi experiment. GFP dsRNA injected ticks were used as a control. Feeding duration is visualized with different colour of each data point. Data was analyzed using ANOVA test with P value <0.0001, followed by Sidak´s multiple comparisons (*Ir*InR and *Ir*TOR weights did not show any significant differences).

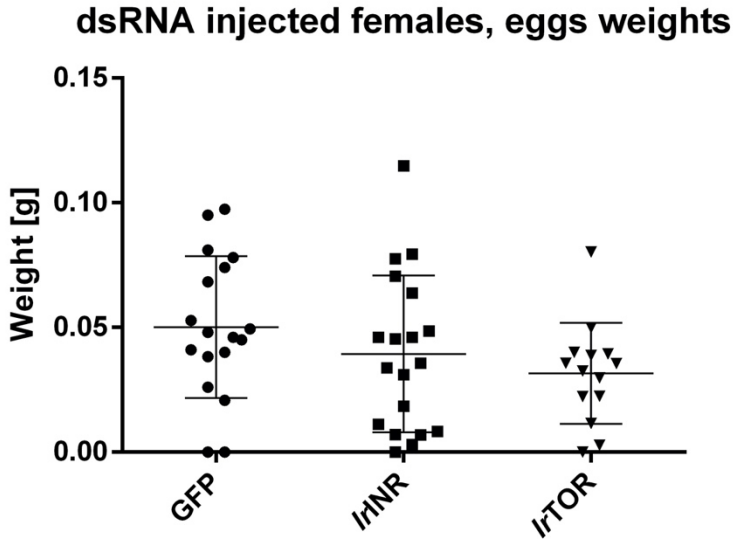

Figure14: The weights of the eggs of the dsRNA injected females.
Graph shows weights of the eggs of the dsRNA injected females from the second RNAi experiment. Data does not show any significant differences.

The RNAi experiment were carried out three times to obtain three independent biological replications. However, as mentioned above, the RNAi silencing in the experiments one and three was not as successful as in the second replication. The summary of weights and feeding time from all three biological replications is shown in the Figure 15. In the third RNAi experiment *Ir*InR dsRNA injected ticks were not evaluated for weighing, oviposition, weighing egg clutches and larval hatching scoring because *Ir*InR in the third RNAi experiment was not silenced. The *Ir*InR and *Ir*TOR dsRNA injected females weights were slightly lower than the control group. The average weight of the *Ir*AKT dsRNA injected females was significantly lower and was only about 150 mg compared to the weight of the GFP control group (~300 mg). However, some of the *Ir*AKT dsRNA injected ticks reached the same weight as the control ticks suggesting, that RNAi was not efficient at all in these ticks (Fig. 15).

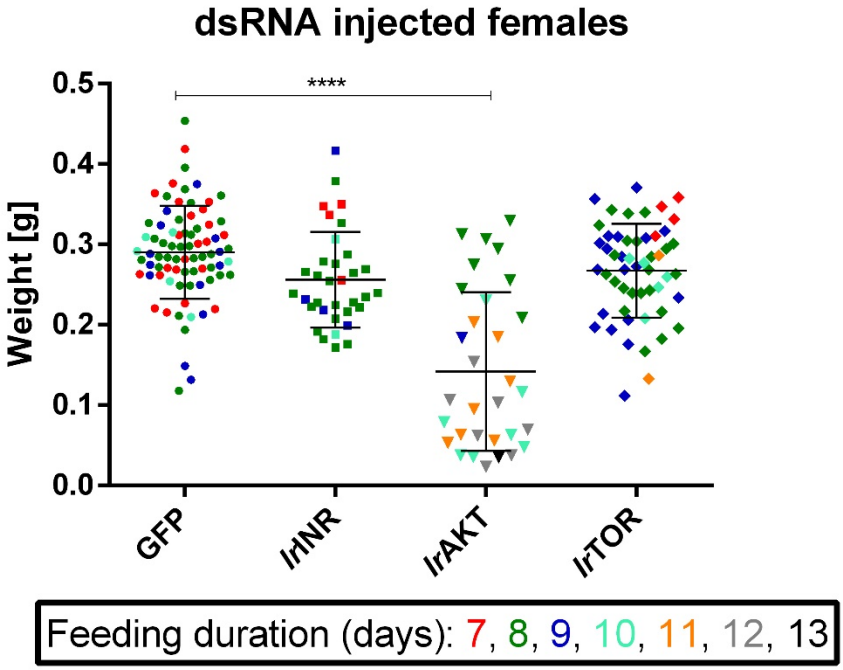

Figure 15: The weights of the RNAi fully fed females.
Graph shows weights of *Ir*InR, *Ir*AKT, and *Ir*TOR fully fed females from all three biological replications of RNAi experiments. GFP dsRNA injected ticks were used as a control. Feeding duration is visualized in the graph with different colours of each data point. Data was analyzed using Kruskal-Wallis test with P value <0.0001, followed by Dunn´s multiple comparisons (*Ir*InR and *Ir*TOR weights did not show any significant differences).

4.4.3. Relation between the observed phenotypes and the level of the RNAi knockdown

In order to demonstrate the connection between the observed phenotype and the level of RNAi silencing, the females injected with *Ir*InR dsRNA with apparently different body size were examined individually. Ticks were visually divided into large and small *Ir*InR groups. The knockdowns were verified in ovaries of each *Ir*InR group by qRT-PCR, using *InR F* and *InR R* primers, after seven days of feeding. The different levels of silencing of each group are shown in Figure 16.

As expected, our results showed the close connection between the level of knockdown and the body size. This may be the proof of the different efficiency of the RNAi in each organism as declared Joga et al. (2016) in insect. On the other hand, the impact of the ISP as a regulator of cell growth shows the mutations in the ISP in *D. melanogaster*. The changes in the ISP resulted in its smaller but evolved body. On the other hand, the overexpression of insulin-like peptides (ILPs) stimulates the growth of its body and increases cell number in the organism (Nijhout, 2003). The ISP regulates many physiological and anabolic processes in response to nutrition, which activates the pathway via the expression of the ILPs (Badisco et al., 2013). Upon the InR RNAi, the ISP may not be triggered thus the processes in the organisms may be disturbed and consequently, the body stops growing.

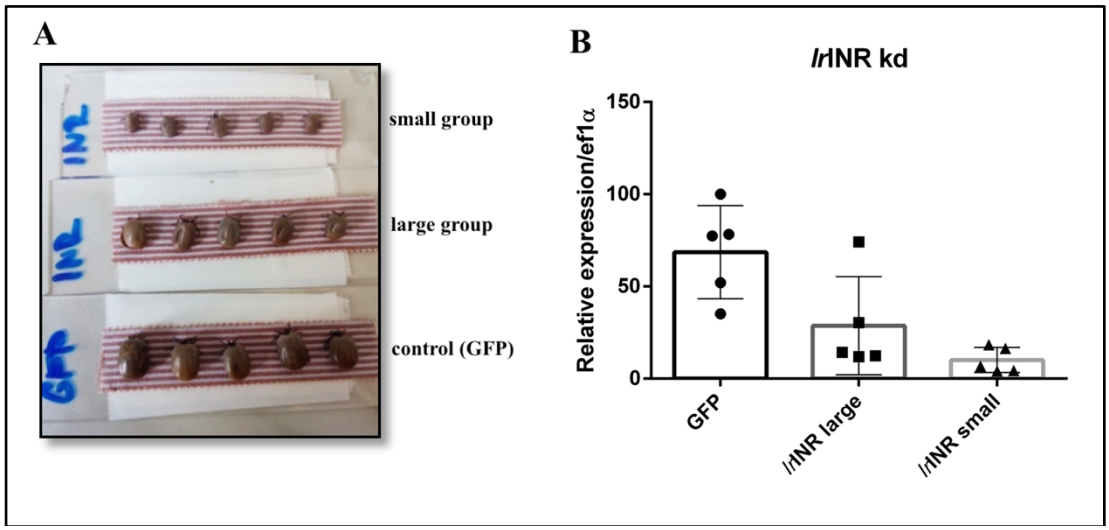

Figure 16: Relation between body size and the level of RNAi knockdown of the *Ir*InR.
The silencing of the *Ir*InR was verified after seven days of feeding in ovaries by qRT-PCR. A: Photo of individual control (GFP dsRNA) females and two groups (large and small) of *Ir*InR dsRNA injected females used for the analysis. B: Graph of the relative mRNA expressions of each group of individual ticks.

4.5. Expression and purification of recombinant *Ir*InR fragment

We designed a recombinant protein covering of about 33 kDa of the N-terminal extracellular portion of *Ir*INR for raising specific antibodies and experimental vaccination of rabbits (Supplement 2.1). Gene sequence of *Ir*InR was amplified using *InR_pET100S2* and *InR_pET100AS2* primers and purified. N-Terminal 6X His-tagged fusion protein was prepared using a pET100/D-TOPO expression vector. The correct construct, verified by sequencing, was transformed into *E. coli* cells BL21. The expression of recombinant protein was carried out in LB medium containing ampicillin (50 mg/ml) with glucose, to increase cell proliferation, or with IPTG, to increase the expression. Then, the recombinant protein was purified, from isolated inclusion bodies, using Co^{2+} chelation chromatography in the presence of 8M urea and eluted with a linear gradient of imidazole (Fig. 17). Fractions containing the recombinant protein were checked by SDS-PAGE electrophoresis and by Western Blot analysis using mice Anti-His tag antibody and refolded by dialysis against gradually decreasing the concentration of urea.

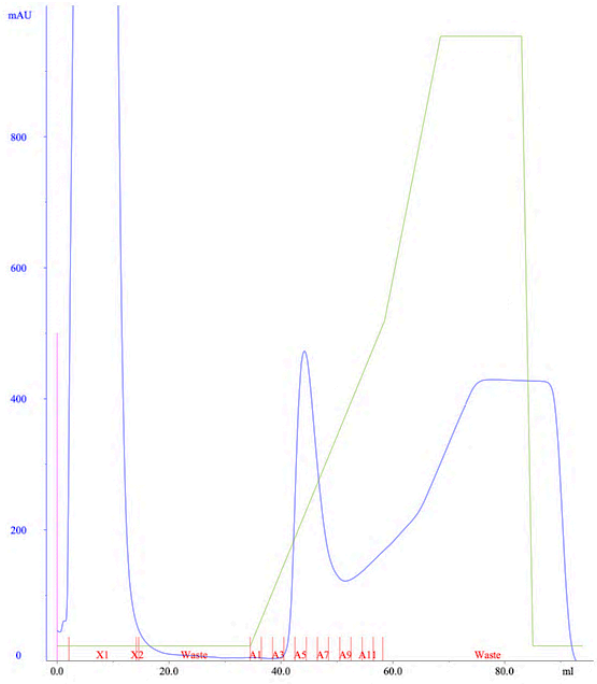

Figure 17: Chromatogram from the purification of the *Ir*InR recombinant protein using FPLC liquid chromatograph.

Blue line: UV
Green line: concentration of imidazole
Red legend: number of fractions

The *Ir*InR recombinant protein was prepared for the immunization of the rabbit. Two weeks after the last immunization, blood was collected from the rabbit to obtain the Anti-*Ir*InR serum. The quality of the immune serum was analyzed using SDS-PAGE electrophoresis

and Western Blot analysis with different concentration of the recombinant protein. Anti-*Ir*InR serum was used (1:5 000) as a primary antibody (Fig. 18).

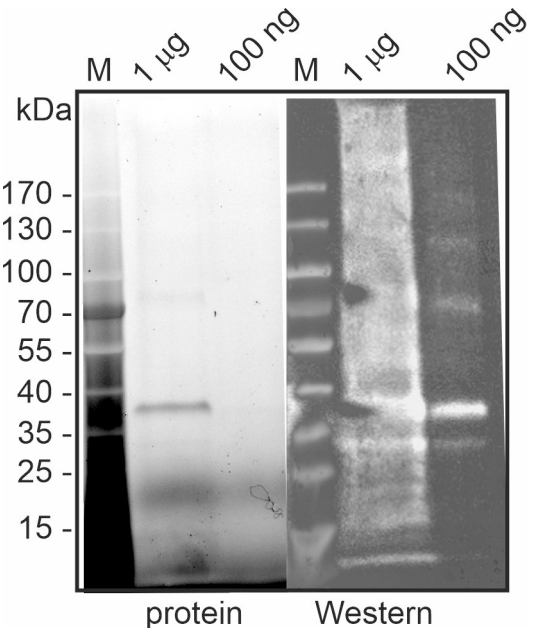

Figure18: Prepared *Ir*InR recombinant protein.
SDS-PAGE electrophoresis and Western Blot analysis of different concentrations of the *Ir*InR recombinant protein. Protein load was visualized using the TGX Stain-Free™ gel. Primary antibody: Anti-*Ir*InR serum 1:5 000, secondary antibody: Anti-Rabbit/peroxidase conjugate 1:5 000.

4.6. Detection of authentic *Ir*InR in tick tissues

Examined tissues were dissected from *I. ricinus* females fed for 7 days on guinea pigs and analyzed using SDS-PAGE and Western Blot analysis. The immune antiserum recognized several bands in tick tissues, out of which the protein of about 80 kDa was detected mainly in salivary glands, Malpighian tubules, ovaries, and to lesser extent also in the midgut. These bands seemed to have mass corresponding to the expected mass of *Ir*InR α-subunit (80 kDa) (Fig. 19).

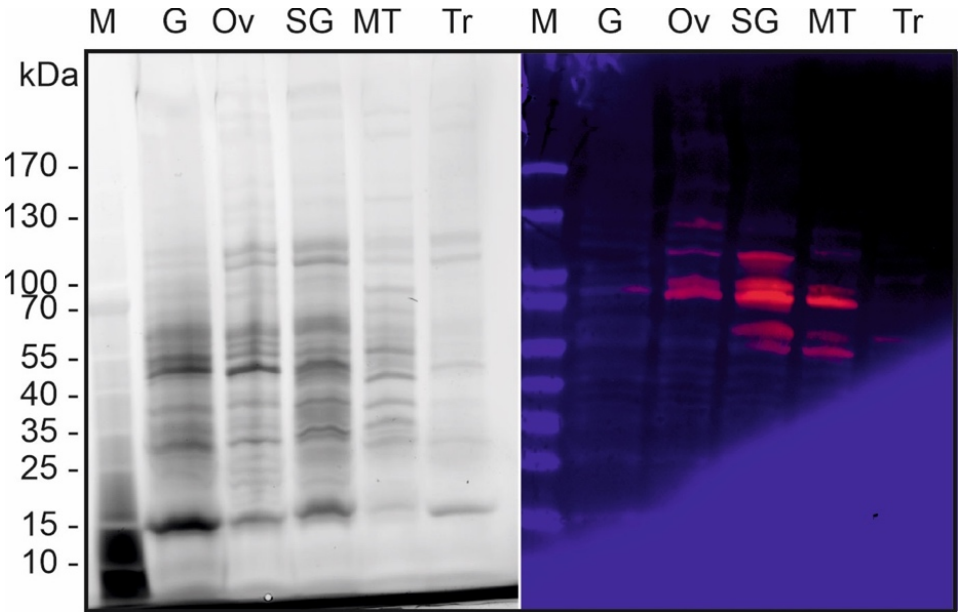

Figure 19: Detection of the *Ir*InR in tissues.
Protein load, visualized using the TGX Stain-Free™ technology, and Western Blot of the tissues protein localization. Primary antibody: *Ir*InR Ig fraction 1:10, secondary antibody: Anti-Rabbit/peroxidase conjugate 1:5 000. M: marker; G: midgut; Ov: ovaries; SG: salivary glands; MT: Malpighian tubules; TR: trachea and fat body.

In order to obtain more reliable identification of an authentic *Ir*InR in tick tissues, ovaries and salivary glands from ticks injected with *Ir*InR dsRNA or GFP dsRNA were compared (Fig 20). Ovaries were used because of the highest relative expression of the *Ir*InR mRNA, and the salivary glands due to the most intense protein band presumably identified as *Ir*INR α subunit (Fig. 19).

This 80 kDa protein and band of about 55 kDa were slightly attenuated in ovaries of *Ir*InR silenced ticks, and in addition, bands of about 35 and 170 kDa disappeared upon RNAi silencing.

Any apparent differences in protein intensity was not observed in salivary glands of the GFP control and *Ir*InR silenced ticks. In contrast to the previous Western Blot shown in Fig. 19, the band of about 80 kDa was much less intense and instead, a band of about 55 kDa was mainly recognized by the anti-*Ir*InR antibodies. Obtained data surprisingly showed that the major signal in salivary glands was detected at different mass (~55 kDa) than in the ovaries (~80 kDa). At the moment, we have no trustworthy explanation for this apparent discrepancy. We may speculate about some proteolytic cleavage in the tissue samples after dissection or just admit, that our antibodies are not specific enough and recognize artifacts given the high sensitivity of the fluorescent Western blot imaging. In order to resolve this issue, Western Blot analysis (with *Ir*InR and GFP injected ticks) was repeated using the affinity purified Ig fractions, on the sepharose column with bound recombinant *Ir*InR protein,

as described by Pěničková (2009). The purified Ig fractions were consequently used as a primary antibody in ratio 1:10. Unfortunately this attempt failed, as any marked differences were not observed on Western blot profiles from *Ir*InR silenced and control tick ovaries (data not shown).

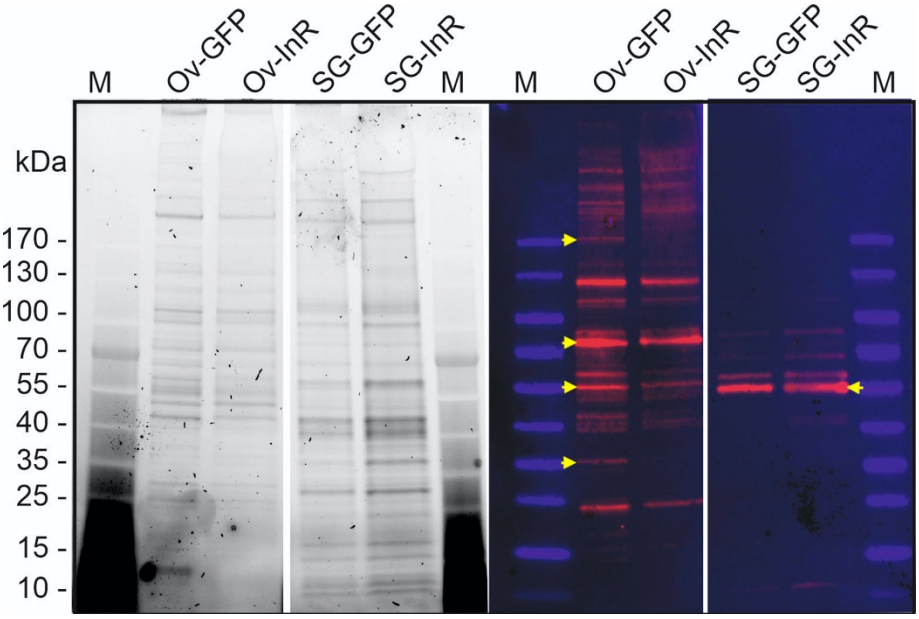

Figure 20: Detection of the *Ir*InR in tissues from RNAi silenced ticks and control ticks.
Protein load, visualized using the TGX Stain-Free™ technology, and Western Blot of the ticks injected with InR or GFP dsRNA. Primary antibody: *Ir*InR Ig fraction 1:50, secondary antibody: Anti-Rabbit/peroxidase conjugate 1:10 000. M: marker; Ov-GFP: ovaries from ticks injected with GFP dsRNA; Ov-InR: ovaries from ticks injected with InR dsRNA; SG-GFP: salivary glands from ticks injected with GFP dsRNA; SG-InR: salivary glands from ticks injected with InR dsRNA.

4.7. Experimental vaccination of rabbits with recombinant *Ir*InR

Even though, that RNAi silencing of *Ir*InR did not result in a striking phenotype, we could not in advance rule out the possibility, that vaccination with recombinant *Ir*InR protein will not affect tick feeding or oviposition. Therefore, we performed the experimental vaccination of rabbits with following ticks infestation.

Rabbits were immunized by *Ir*InR recombinant protein in four doses. For ticks feeding, three immunized rabbits and a one control rabbit, which was immunized with incomplete Freud´s adjuvant only, were used.

After the last immunization, the blood was taken from rabbits ears to examine the production of the antibodies using SDS-PAGE electrophoresis and Western Blot analysis.

It was verified that the rabbits produced antibodies against *Ir*InR recombinant protein (Fig. 21). Two weeks after the last immunization, ticks were placed on the shaven backs of rabbits and allowed to feed naturally.

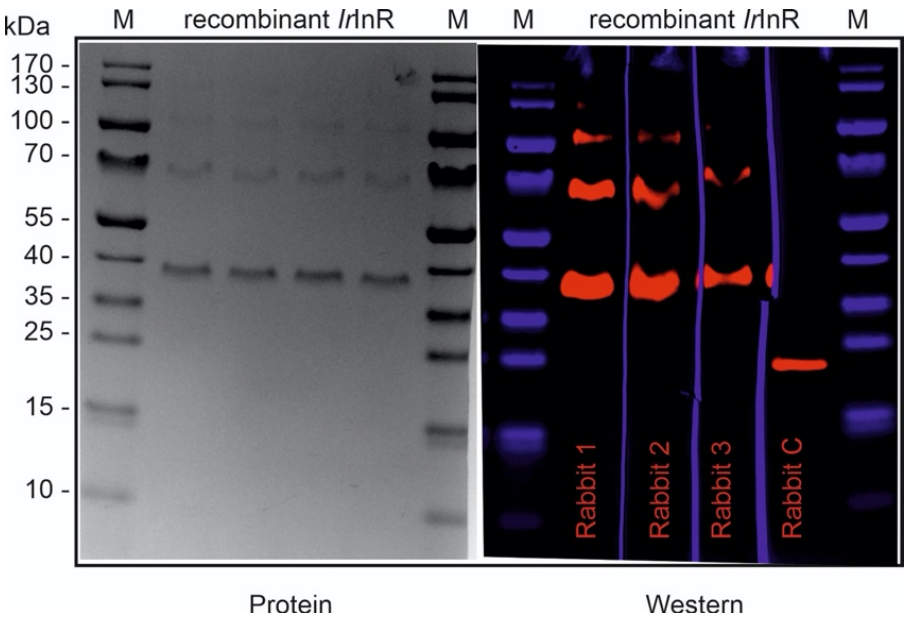

Figure 21: Verification of the antibodies of the immunized rabbits.
Protein load, stained using Coomassie Brilliant Blue, of the *Ir*InR recombinant protein (200 ng of protein per lane) and Western Blot analysis with the sera of the immunized rabbits. Primary antibody: Anti-*Ir*InR sera 1:5 000, secondary antibody: Anti-Rabbit/peroxidase conjugate 1:5 000. M: marker.

Ticks infesting on immunized rabbits did not show any weight differences compared to the ticks, that were feeding on the control rabbit. The weights of fully fed females were similar to the control group (Fig. 22). Curiously, ticks fed on the immunized rabbit No. 3, weighed even more, than the control group. In line with these results, ticks from all experimental groups did not exert any impairment in oviposition (Fig. 23) nor in the hatching success from the laid eggs.

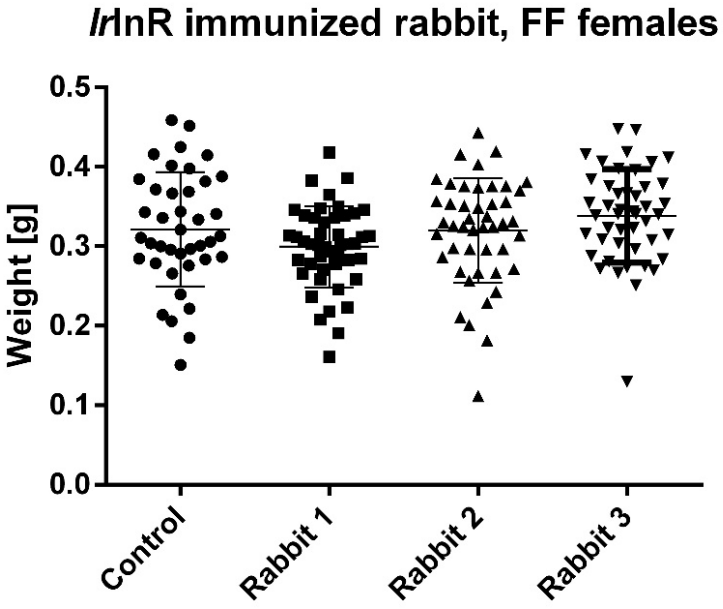

Figure 22: Weights of fully fed females infested on immunized rabbits.
Graph shows the weights of the fully fed females that were feed on the immunized rabbits. Data did not show any significant differences.

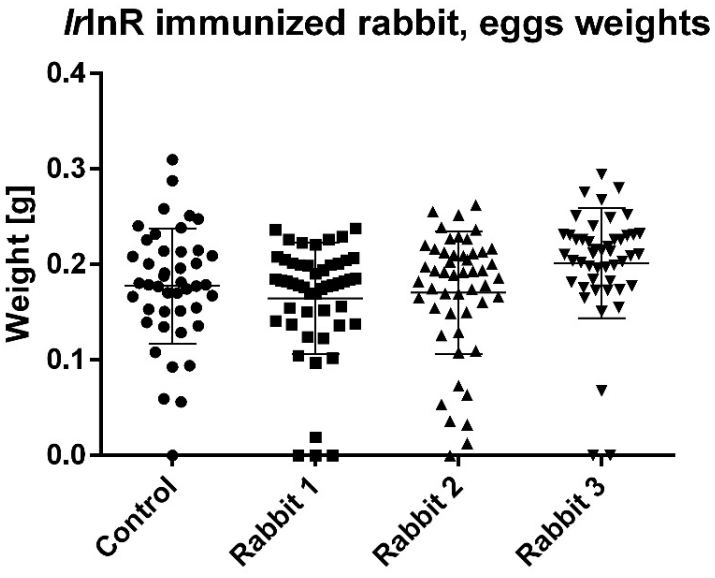

Figure 23: Weights of eggs laid by the ticks infested on immunized rabbits.
Graph shows weights of the eggs of females that were feed on the rabbits. Data did not show any significant differences.

4.8. Injection with insulin receptor antagonist

The commercially available insulin receptor antagonist (IRA) (S961; Schäffer et al., 2008) is a peptide expressed in *E. coli* bacterial expression system as a fusion protein. The affinity of the IRA to the human, rat and pig InR was reported to be slightly higher than of the insulin (Schäffer et al., 2008).

Injection of the IRA was tested as an alternative experimental approach how to eliminate the function of the *Ir*InR. The IRA was diluted in 1xTBS to the final concentration 100 nM. Consequently, 15 unfed females were injected with 0.4 µl (200 ng) of IRA or 1xTBS, which was used as a control, allowed to rest for one day and put on the shaven back of guinea pig in the halved cylinder with the same number of males to naturally fed till repletion. The fully fed females were weighed and maintained as described above.

The weights of IRA injected ticks seemed to be slightly lower than the TBS control group (Fig. 24 A), however the number of females that accomplished feeding did not allow to perform the evaluation of statistical significance. Besides one IRA injected female, all ticks of each group laid eggs (Fig. 24 B) and hatched successfully. It seemed that IRA was able to partly inhibit the function of the *Ir*InR. Unfortunately, because of the IRA prize, we could not effort to test higher concentrations of injected IRA and increase the number of ticks. Therefore, this pilot experiment does not allow to make any convincing conclusions.

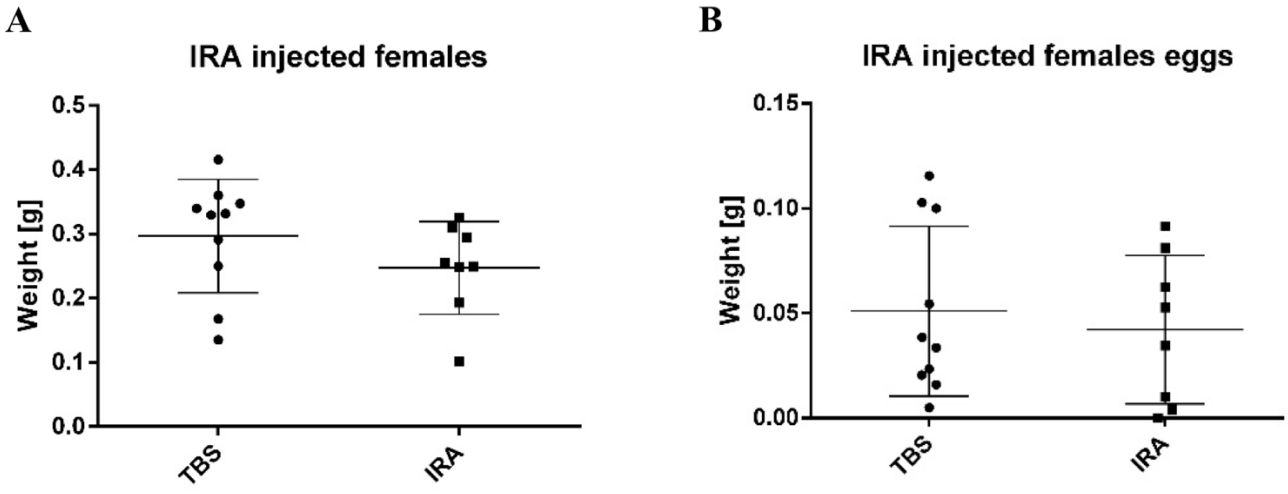

Figure 24: Injection with IRA.
A: The weights of the fully fed females injected with 1x TBS or IRA (S961) (200 ng). Data did not show any significant differences. B: The weights of eggs laid by females injected with 1xTBS or IRA. Data did not show any significant differences.

4.9. Membrane tick feeding

In order to alternatively assess the function of the *Ir*AKT and *Ir*TOR, ticks were artificially fed with different concentrations of specific inhibitors of AKT (A-443654; Han et al., 2007) and TOR (rapamycin; Vézina et al., 1975) added to the blood diet. As solvent controls ethanol and DMSO were used. Ticks were fed in feeding units (FU) and the blood diet, supplemented with tested inhibitors, was regularly changed every 12 hours. The engorged ticks were weighed and maintained as described above. Two independent experiments with the rapamycin were carried out. In the case of the AKT inhibitor, only one experiment was performed given the high cost of this compound.

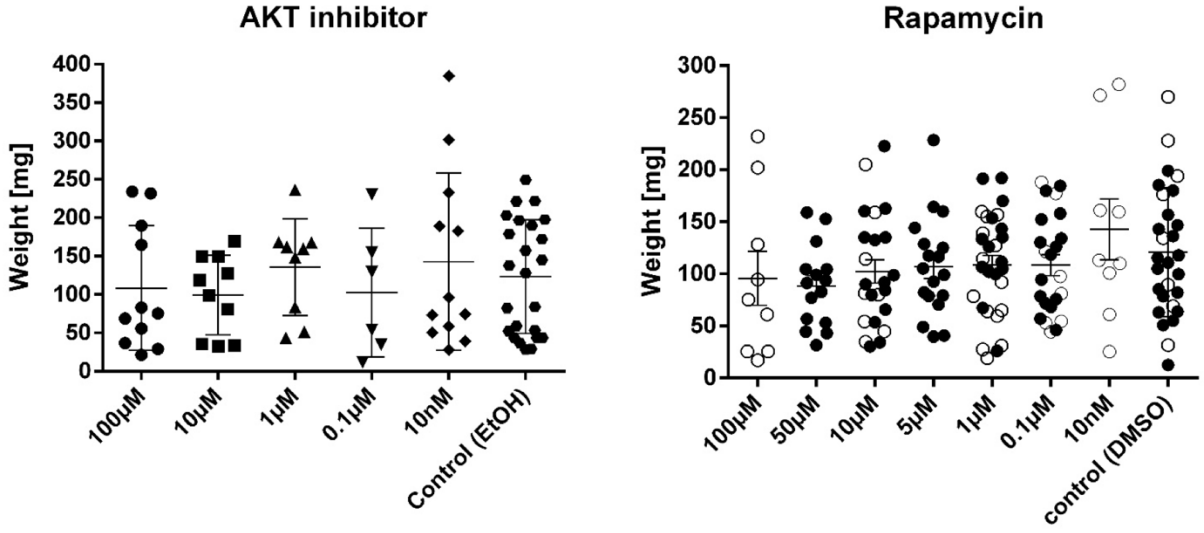

Figure 25: The weights of the fully fed females artificially fed on the diet with AKT and TOR inhibitors. Graphs show weights of fully fed females of different groups of concentrations of the AKT inhibitor (A-443654) and rapamycin. Data did not show any significant differences. In the case of rapamycin, two independent experiments are differentiated using empty (first experiment) and full (second experiment) data points.

Feeding with AKT inhibitor did not show any striking differences in engorged ticks weights compared to the control group (ethanol). In the case of 100 μM, 10 μM, and 0.1 μM concentrations, the weights were slightly lower compared to the control group, while ticks fed with 1 μM and 10 nM concentrations of the AKT inhibitor reached higher weights than the control group. (Fig. 25).

In the experiment with rapamycin, the fully fed control group of ticks (DMSO), which spontaneously dropped of the membrane, reached the average weight of about 125 mg. Ticks fed with different concentrations of rapamycin had slightly lower weights, while ticks fed with

10 nM concentration of rapamycin reached higher weights than the control group. Feeding ticks on different rapamycin concentrations did not yield any consistent results pointing to the rapamycin toxicity to ticks. (Fig. 25).

We have further evaluated the oviposition success of females fed with AKT inhibitor or rapamycin (Tab. 14).

In the case of AKT inhibitor, the results are obscured by a relatively low oviposition success in the control (ethanol) treated ticks, suggesting that this solvent might not be appropriate for membrane feeding experiments. Han et al. (2007) studied the mechanism of AKT inhibitor (A-443654) in human cancer cell lines. These authors demonstrated that AKT inhibitor was able to inhibit the phosphorylation of the downstream components in the ISP. No effect of this compound on the tick feeding and oviposition could be explained by low concentration and/or low stability of this inhibitor in the blood-meal diet.

In the case of rapamycin feeding, the first experiment indicated that oviposition was impaired in all tested concentration compared to the control. Unfortunately, the differences were not that apparent in the second experiment (Tab. 14). Rapamycin is immunosuppressive component produced by *Streptomyces hygroscopicus*, acts as the main inhibitor of the TOR activity (Ballou and Lin, 2008; Vézina et al., 1975). In the mosquito *A. aegypti* the treatment of fat bodies with rapamycin (150 nM) was assessed. It was demonstrated the inhibition of synthesis of vitellogenin and S6K kinase, which is another downstream component of TOR pathway (Roy et al., 2007; Hansen et al., 2004). In the tick *H. longicornis*, the treatment with rapamycin confirmed the reduction of the vitellogenin and S6K kinase as well (Umemiya et al., 2012b). Rapamycin treatment of tick embryonic cells BME-26 derived from *R. microplus* showed the reduction of the cells viability (Waltero et al., 2019). In our membrane feeding experiment with *I. ricinus*, we did not observe any significant differences in weights of engorged ticks in comparison to the control group.

Tab 14: The success in oviposition of artificially fed ticks

Treatments	100µM	50µM	10µM	5µM	1µM	0.1µM	10nM	Control
AKT inhibitor	6/11	X	3/10	X	7/9	4/4	5/12	11/24
Rapamycin (1)	4/7	X	2/7	X	4/13	4/8	4/7	7/8
Rapamycin (2)	X	8/14	6/15	13/18	7/15	3/14	X	8/12

5. Conclusion

The aim of this thesis was to verify the physiological function of *Ir*TOR and *Ir*AKT. More importantly, focused on examination of the molecular and functional characterization of the *Ir*InR which had never been described before in any type of tick species. The sequences were analyzed and subsequently conserved domains as well as superfamilies were described. Alignments of each gene showed a high homology with other species.

The tissues relative expression, examined by qRT-PCR, showed the highest level of mRNA in ovaries of semi-engorged ticks. The ovarian mRNA of each gene was examined during tick feeding and after its detachment. Increasing expression trend from unfed stage to full fed stage of each gene was observed. After the ticks detachment, the mRNA levels of *Ir*InR and *Ir*AKT were fluctuating, while *Ir*TOR mRNA level was decreasing towards 12 days after detachment. RNAi silencing was performed in unfed adult females. The weights of *Ir*InR, *Ir*AKT, and *Ir*TOR fully fed females were lower compared to the control (GFP) group. Most significant differences were performed in *Ir*AKT dsRNA group. The body size and weights of *Ir*AKT dsRNA injected ticks were significantly lower. Furthermore, these injected ticks had the longest feeding duration, but they were not able to complete their blood-feeding. Additionally, *Ir*AKT injected group did not succeed in oviposition. In addition, connection between the body size and level of knockdown was confirmed upon injection with *Ir*InR dsRNA.

Recombinant protein of *Ir*INR fragment was expressed, purified, refolded and used for the immunization of the rabbits. Ticks fed on the immunized rabbits did not show any differences in comparison with tick fed on a control rabbit.

Detection of the authentic *Ir*InR protein in tick tissues was assessed. *Ir*InR protein was recognized in salivary glands, ovaries and Malphigian tubules of around 80 kDa. Ovaries and salivary glands were examined using SDS-PAGE and Western Blot analysis upon injection with *Ir*InR dsRNA or GFP dsRNA. Some differences in protein intensity were observed in ovaries upon RNAi, while no differences in salivary glands were detected.

As another option how to alternatively assess the function of the *Ir*InR, *Ir*AKT, and *Ir*TOR, an injection with insulin receptor antagonist (IRA) and *in vitro* feeding with AKT and TOR inhibitors were carried out. No influence of these compounds on ticks phenotype was observed.

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7. Supplement

7.1. The deposited complete coding sequences for *Ir*InR, *Ir*AKT, and *Ir*TOR in the GenBank

GenBank flat file:

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2019
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REFERENCE   1  (bases 1 to 7518)
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Kopacek,P.
  TITLE     Functional mapping of the insulin signaling pathway components
in
            the hard tick Ixodes ricinus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 7518)
  AUTHORS   Kozelkova,T., Perner,J., Grunclova,L., Ribeiro,J.M. and
Kopacek,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-JUL-2019) Institute of Parasitology, Biology
Centre
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2019
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REFERENCE   1  (bases 1 to 1593)
  AUTHORS   Kozelkova,T., Perner,J., Grunclova,L., Ribeiro,J.M. and
Kopacek,P.
  TITLE     Functional mapping of the insulin signaling pathway components
in
            the hard tick Ixodes ricinus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1593)
  AUTHORS   Kozelkova,T., Perner,J., Grunclova,L., Ribeiro,J.M. and
Kopacek,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-JUL-2019) Institute of Parasitology, Biology
Centre
            CAS, Branisovska 31, Ceske Budejovice 370 05, Czech Republic
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2019
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cds.
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VERSION     MN207065
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Arachnida;
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REFERENCE   1  (bases 1 to 4491)
  AUTHORS   Kozelkova,T., Perner,J., Grunclova,L., Ribeiro,J.M. and
Kopacek,P.
  TITLE     Functional mapping of the insulin signaling pathway components
in
            the hard tick Ixodes ricinus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 4491)
  AUTHORS   Kozelkova,T., Perner,J., Grunclova,L., Ribeiro,J.M. and
Kopacek,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-JUL-2019) Institute of Parasitology, Biology
Centre
            CAS, Branisovska 31, Ceske Budejovice 370 05, Czech Republic
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2461 gagtactcca tacgggtgct ggcctgtcac cagaagctca agagcaagta ctacaggggg
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2761 aagatggtct cccaggagaa gttcaagccg ttccagtttt gcgtgtcgca ccacgagttc
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4441 aacggacgca tgtgcttctc gcagcagggc agtcggacta ccgcttgcta g
//
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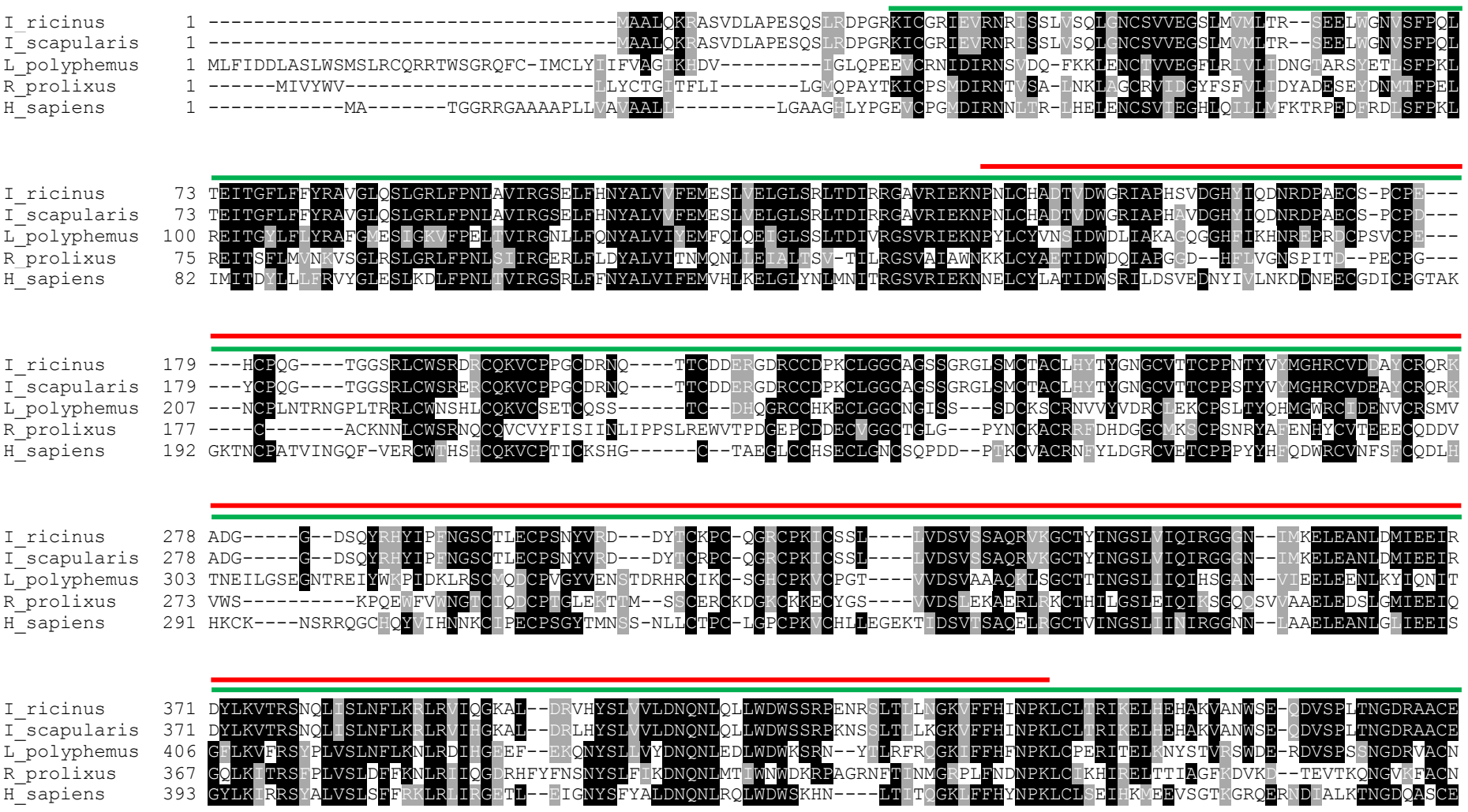
7.2. Alignments

7.2.1. *Ir*InR

The alignment of the deduced amino acid sequence of *Ir*InR with InR of black-legged tick *Ixodes scapularis* (accession number: XP_002416224.1), InR of horseshoe crab *Limulus polyphemus* (accession number: XP_022253681.1), InR of kissing bug *Rhodnius prolixus* (avaible on www.uniprot.org under the classification T1HQC7) and InR of *Homo sapiens* (accession number: P06213.4). For the alignment of the sequences, the ClustalOmega using ClustalW method was used and presented using the BoxShade software.

Recombinant protein is indicated with a red line (position 141-442). Green line indicates the α subunit (position 25-745), blue line indicates the β subunit (position 748-1338). Orange line represents transmembrane region (position 986-1013). Black star indicates arginine-rich cleavage.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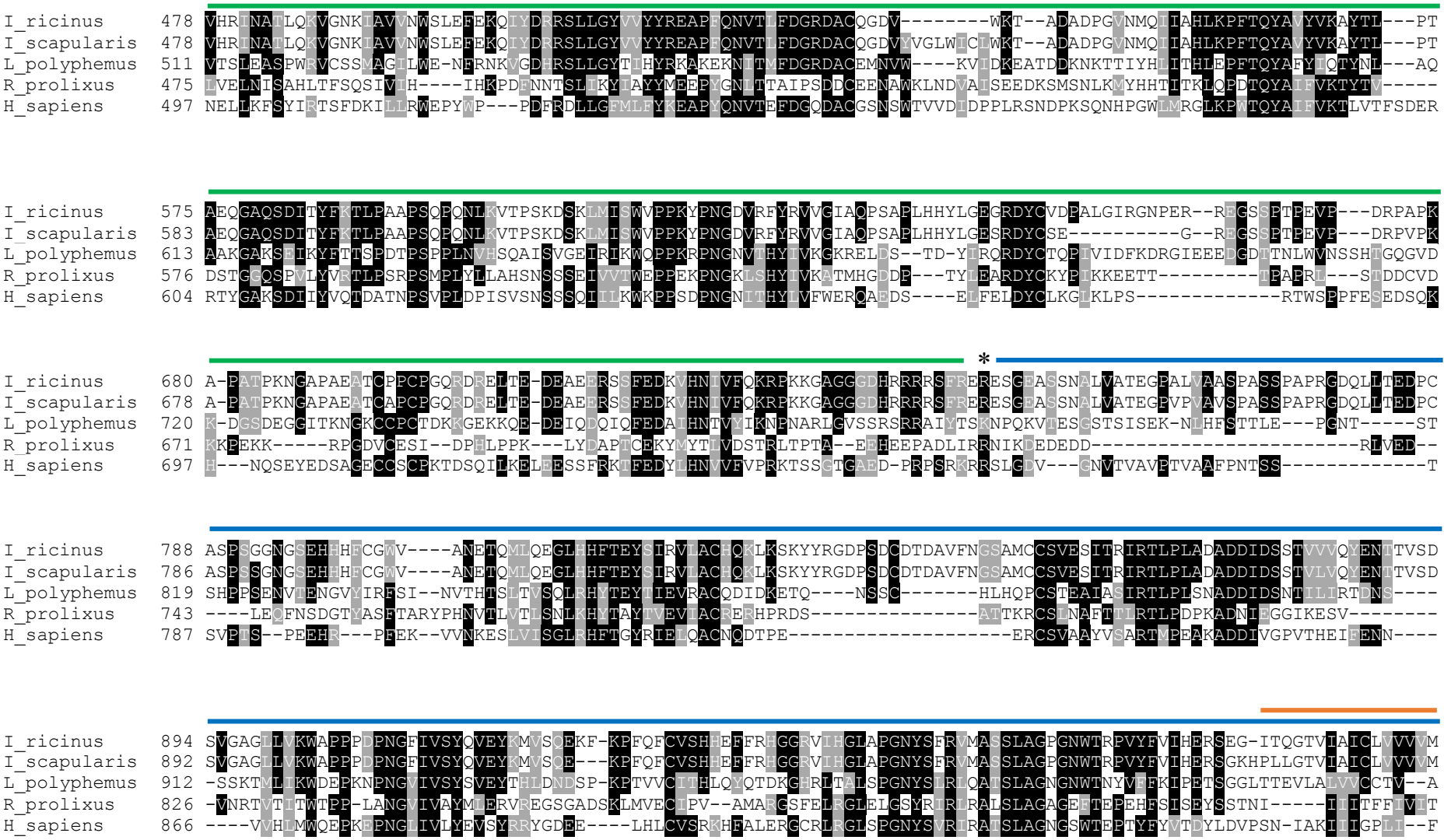
I_scapularis
L_polyphemus
R_prolixus
H_sapiens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I_scapularis 478 VHRINAILQRVGNKLAVVNWSLEFEKQIYDRRSLLGYVVYYREAPFQNVTLFDGRDACQGDVYVGLWICLWKT--ADADPGVNMQIIAHLKPFTQYAVYVKAYTL---PT
L_polyphemus 511 VTSLEASPWFVCSSMAGILWE-NFRNKVGDHRSLLGYTIHYRKAKEKNITMFDGRDACEMNVW----KVIDKEATDDKNKTTIYHLITHLEPFTQYAFYIQTYNL---AQ
R_prolixus 475 LVELNISAHLTFSQSIVIH----IHKPDFNNTSLIKYIAYYMEPYGNITTAIPSDDCEENAWKLNDVAISEEDKSMSNLKMYHHTITKLQPDTQYAIFVKTYTV-----
H_sapiens 497 NELLKFSYIRTSFDKILLRWEPYWP---PDFRDLLGFMLFYKEAPYQNVTEFDGQDACGSNSWTVVDIDPPLRSNDPKSQNHPGWLMRGLKPWTQYAIFVKTLVTFSDER
I_ricinus 575 AEQGAQSDITYFYTLPAAPSQPQNLKVTPSKDSKLMISWVPPKYPNGDVRFYRVVGIAQPSAPLHHYLGEEGRDYCVDPALGIRGNPER--REGSSPTPEVP---DRPAPK
I_scapularis 583 AEQGAQSDITYFYTLPAAPSQPQNLKVTPSKDSKLMISWVPPKYPNGDVRFYRVVGIAQPSAPLHHYLGEESRDYCSE----------G--REGSSPTPEVP---DRPVPK
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R_prolixus 576 DSTGGQSPVLYVFTLPSRPSMPLYLLAHSNSSSEIVVTWEPPEKPNGKLSHYTVKATMHGDDP---TYLEARDYCKYPIKKEETT---------TPAPRL---STDDCVD
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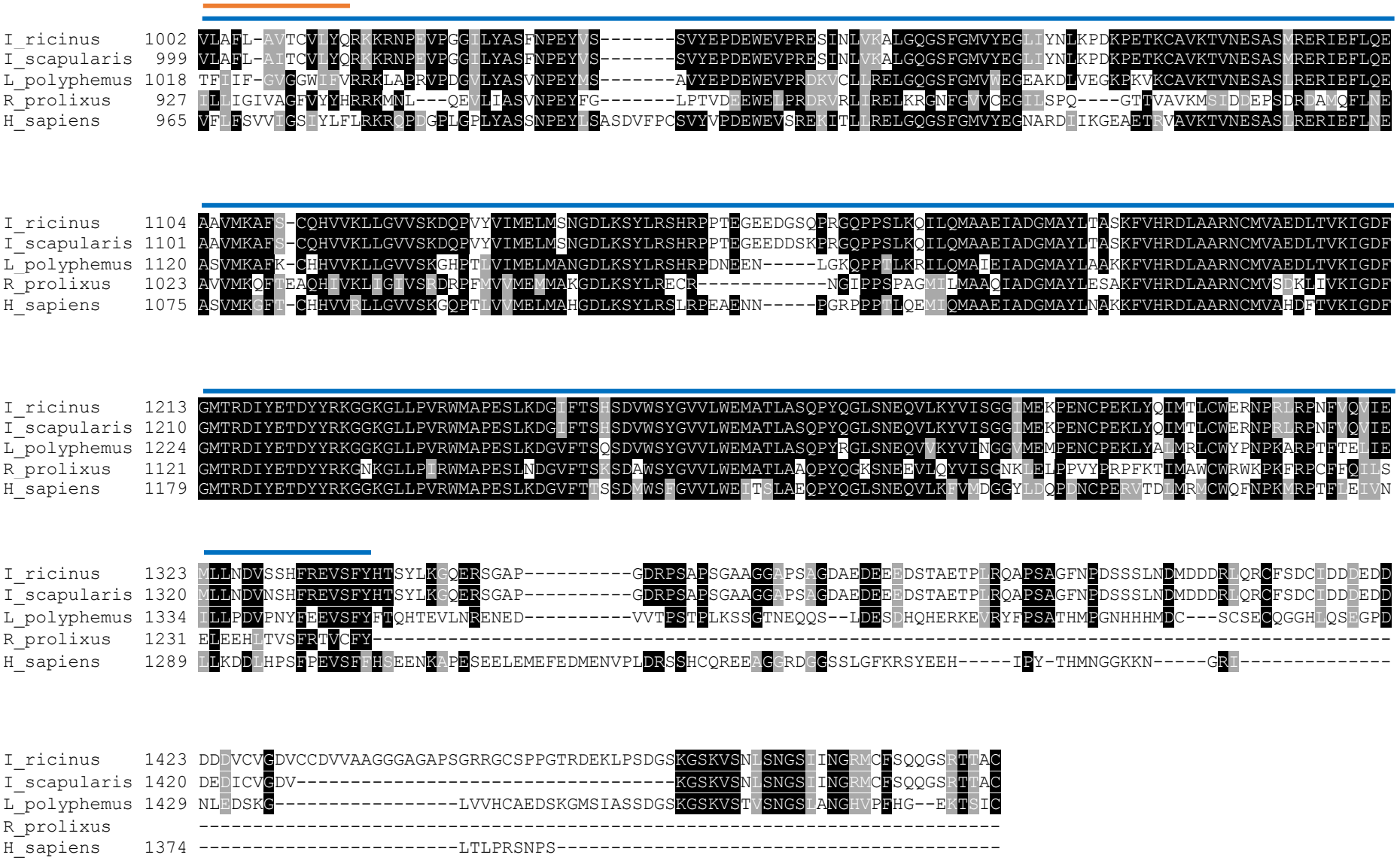
R_prolixus 826 -VNRTVTITWTPP-LANGVIVAYMLERVREGSGADSKLMVECIPV--AMAKGSFELRGLELGSYRIRLRALSLAGAGEFTEPEHFSISEYSSTNI-----IIITFFIVIT
H_sapiens 866 ----VVHLMWQEPKEPNGLIVLYEVSYRRYGDEE----LHLCVSRKHFALERGCRLRGLSPGNYSVRIRAISLAGNGSWTEPTYFYVTDYLDVPSN-IAKIIIGPLI--F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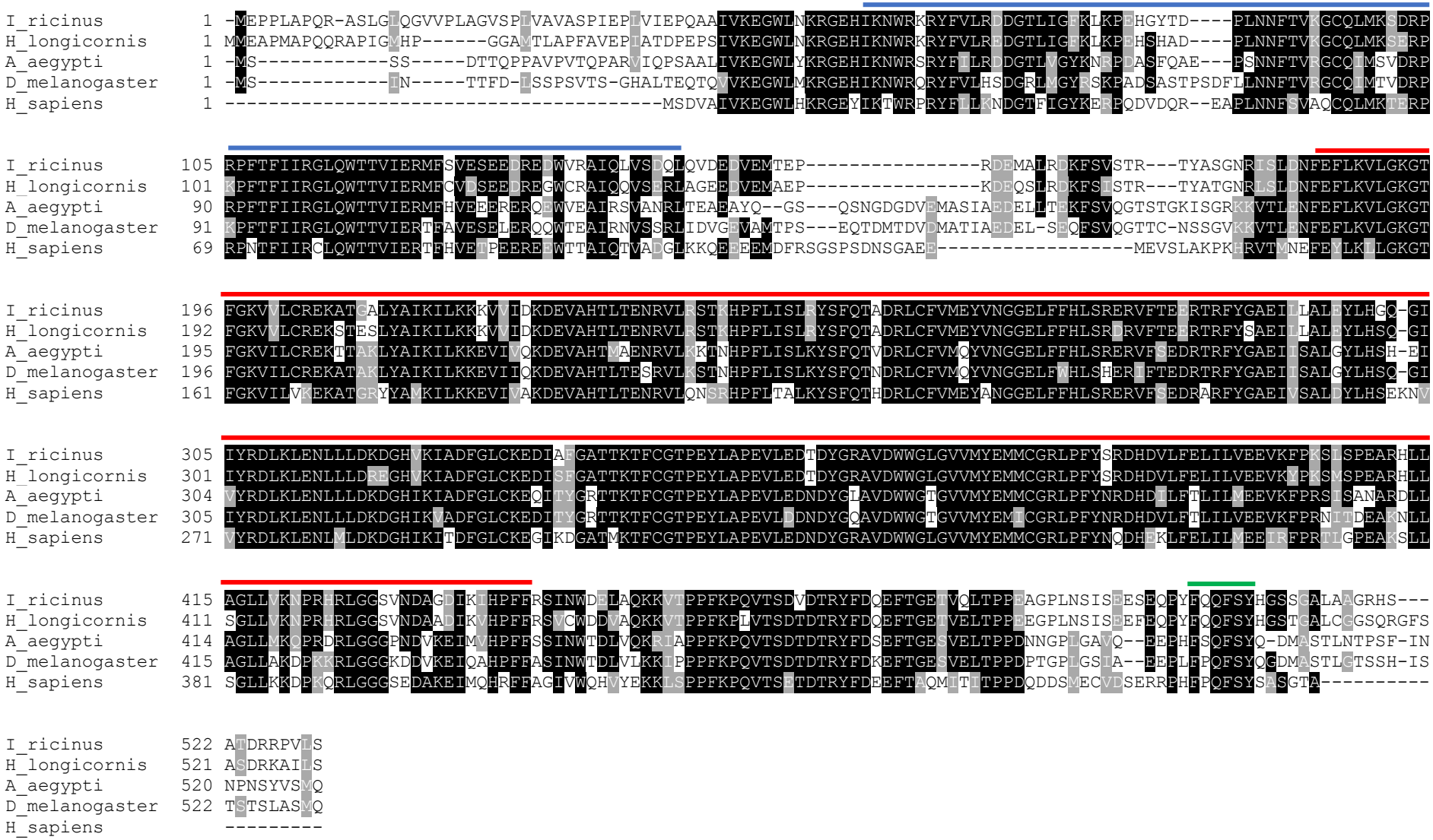
R_prolixus 1231
H_sapiens 1289
I_ricinus 1423
I_scapularis 1420
L_polyphemus 1429
R_prolixus
H_sapiens 1374

7.2.2. *Ir*AKT

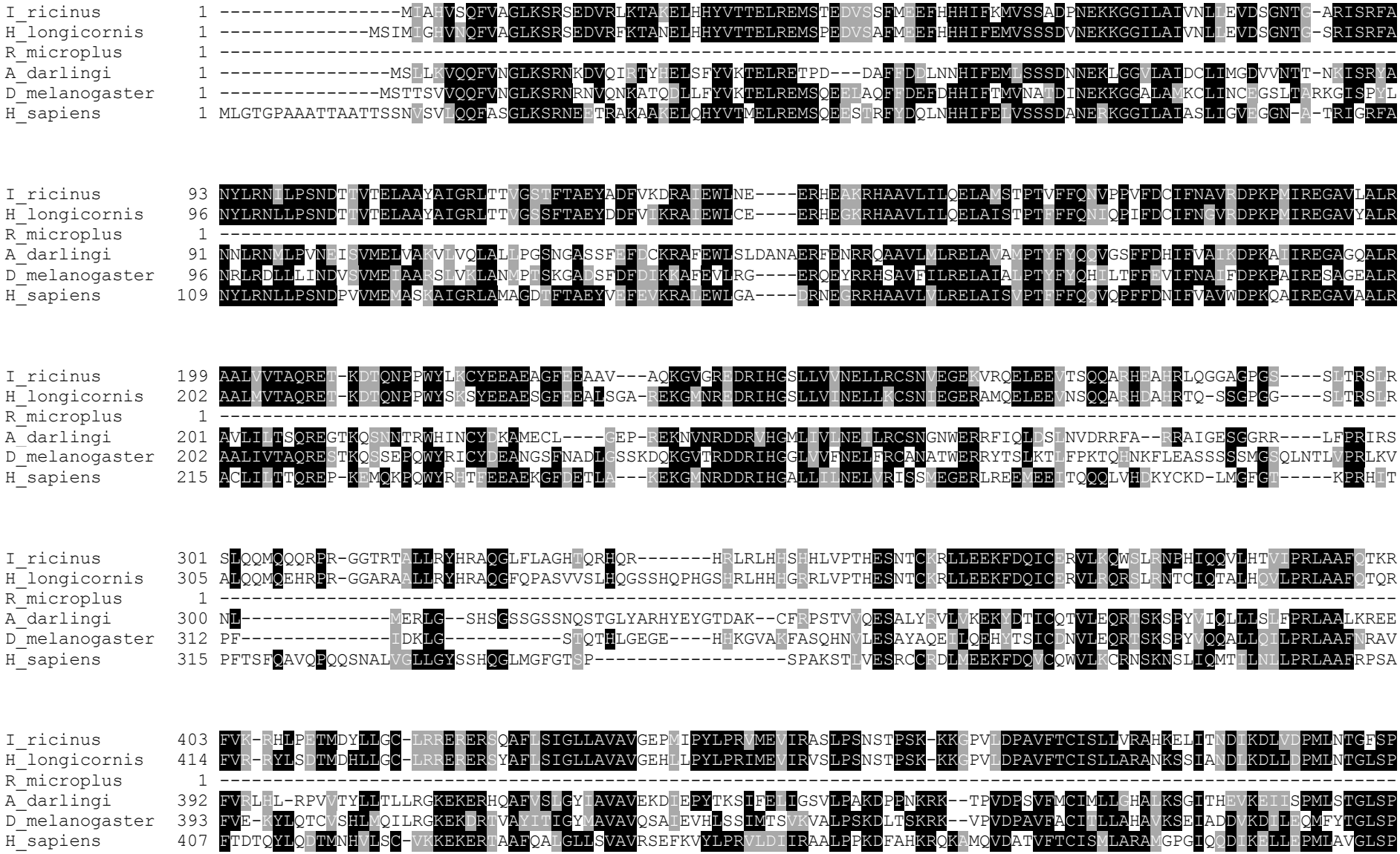
The alignment of the deduced amino acid sequence of *Ir*AKT with *HIAKT* of cattle tick *Haemaphysalis longicornis* (accession number: AB601888.1), AKT of mosquito *Aedes aegypti* (accession number: AAP3765), AKT1 isoform A of fruit fly *Drosophila melanogaster* (accession number: NP_732114.1) and AKT1 of *Homo sapiens* (accession number: NP_001014431.1). For the alignment of the sequences, the ClustalOmega using ClustalW method was used and presented using the BoxShade software.

Blue line indicates the PH domain (position 57-146). Red line indicates the serine/threonine kinase domain (position 185-442). The hydrophobic motif is indicated with green line (FQQFSY, position 503-508).


I_ricinus
H_longicornis
A_aegypti
D_melanogaster
H_sapiens

7.2.3. *Ir*TOR

The alignment of the deduced amino acid sequence of *Ir*TOR with TOR of cattle tick *Haemaphysalis longicornis* (accession number: AB716688.1), TOR of mosquito *Anopheles darlingi* (accession number: ETN59302.1), partial sequence of TOR of kissing bug (accession number: MK598842), TOR of fruit fly *Drosophila melanogaster* (accession number: NP_001260427.1) and TOR of *Homo sapiens* (accession number: NP_004949.1). For the alignment of the sequences, the ClustalOmega using ClustalW method was used and presented using the BoxShade software.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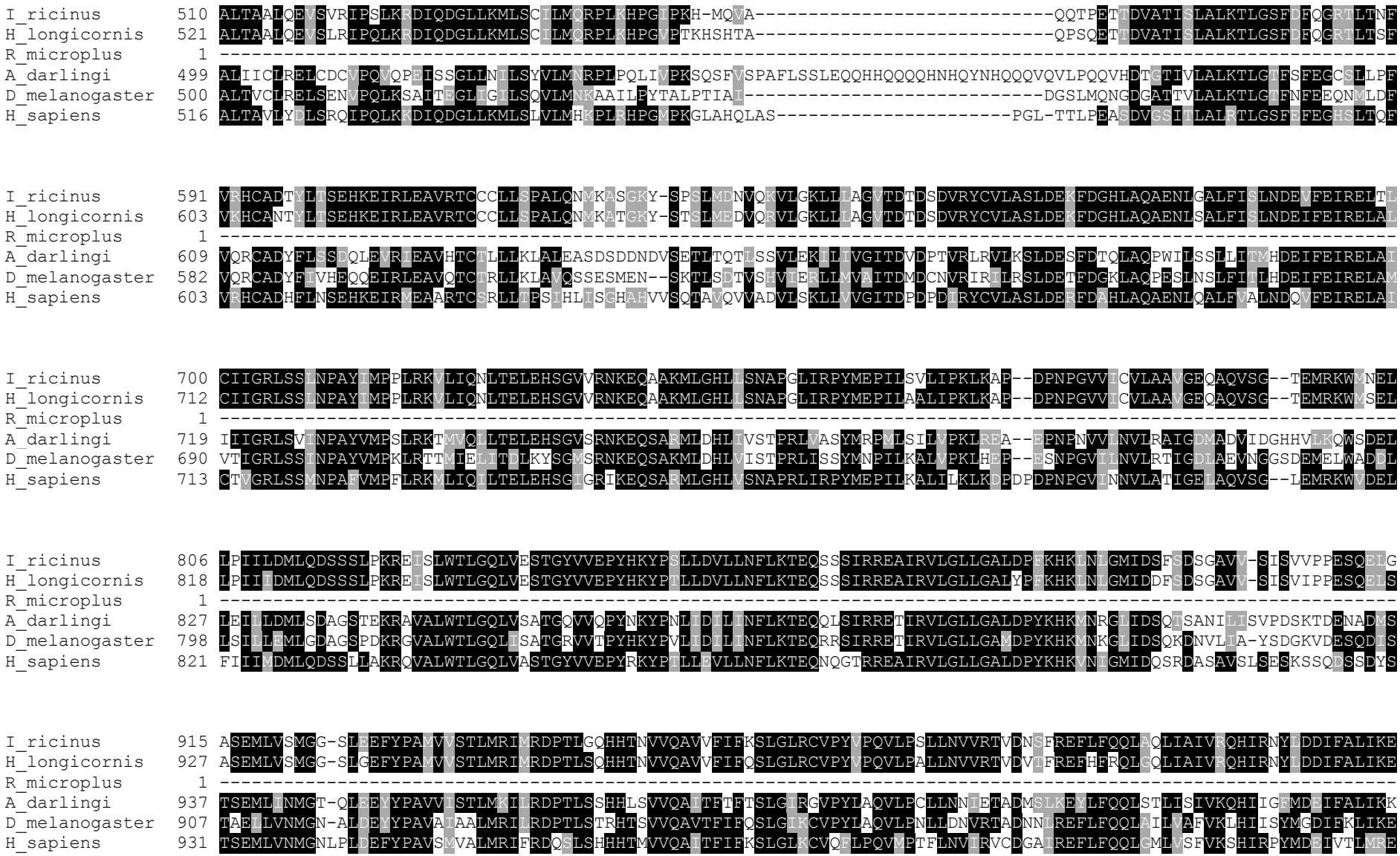
H_longicornis
R_microplus
A_darlingi
D_melanogaster
H_sapiens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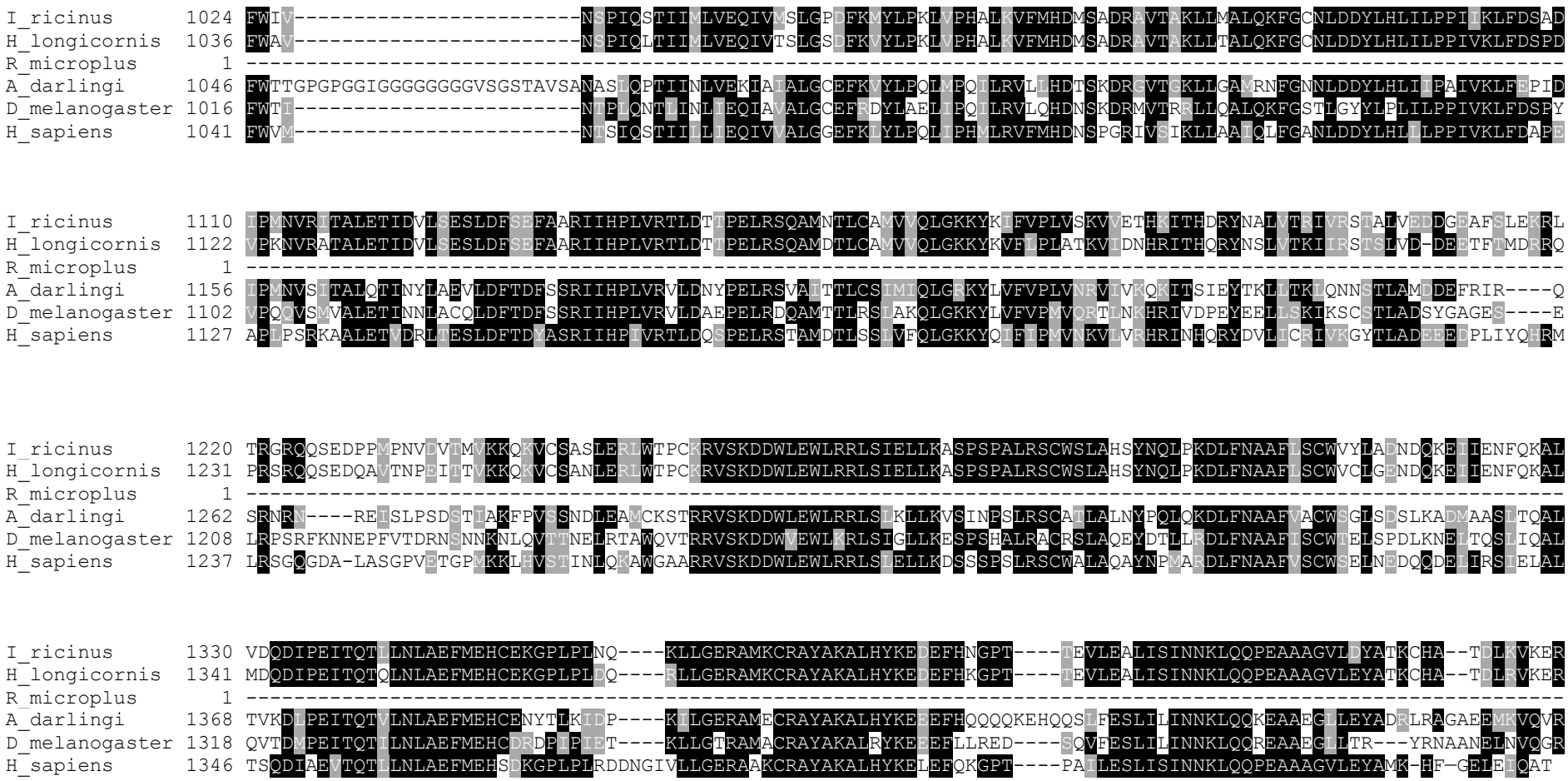
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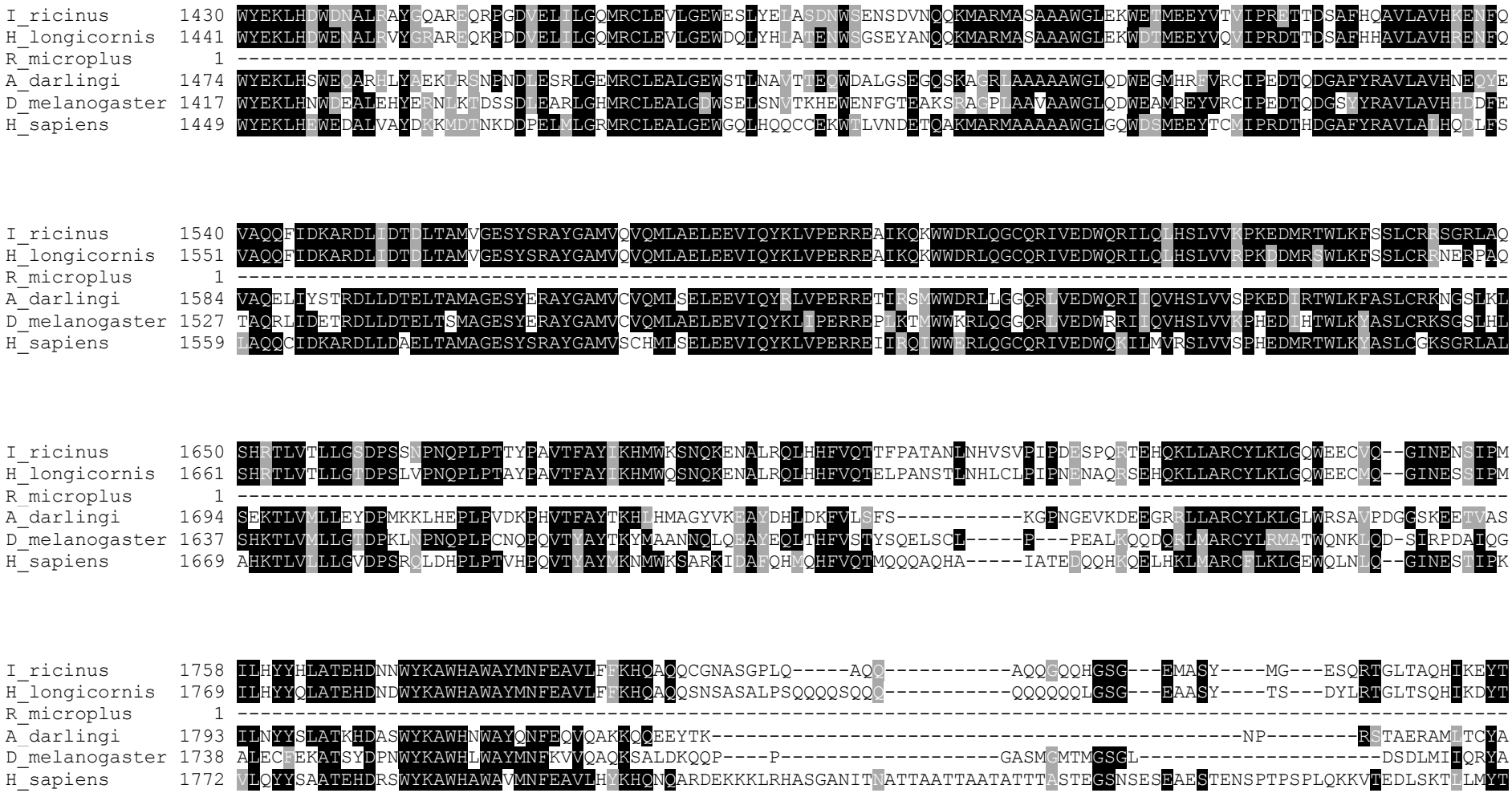
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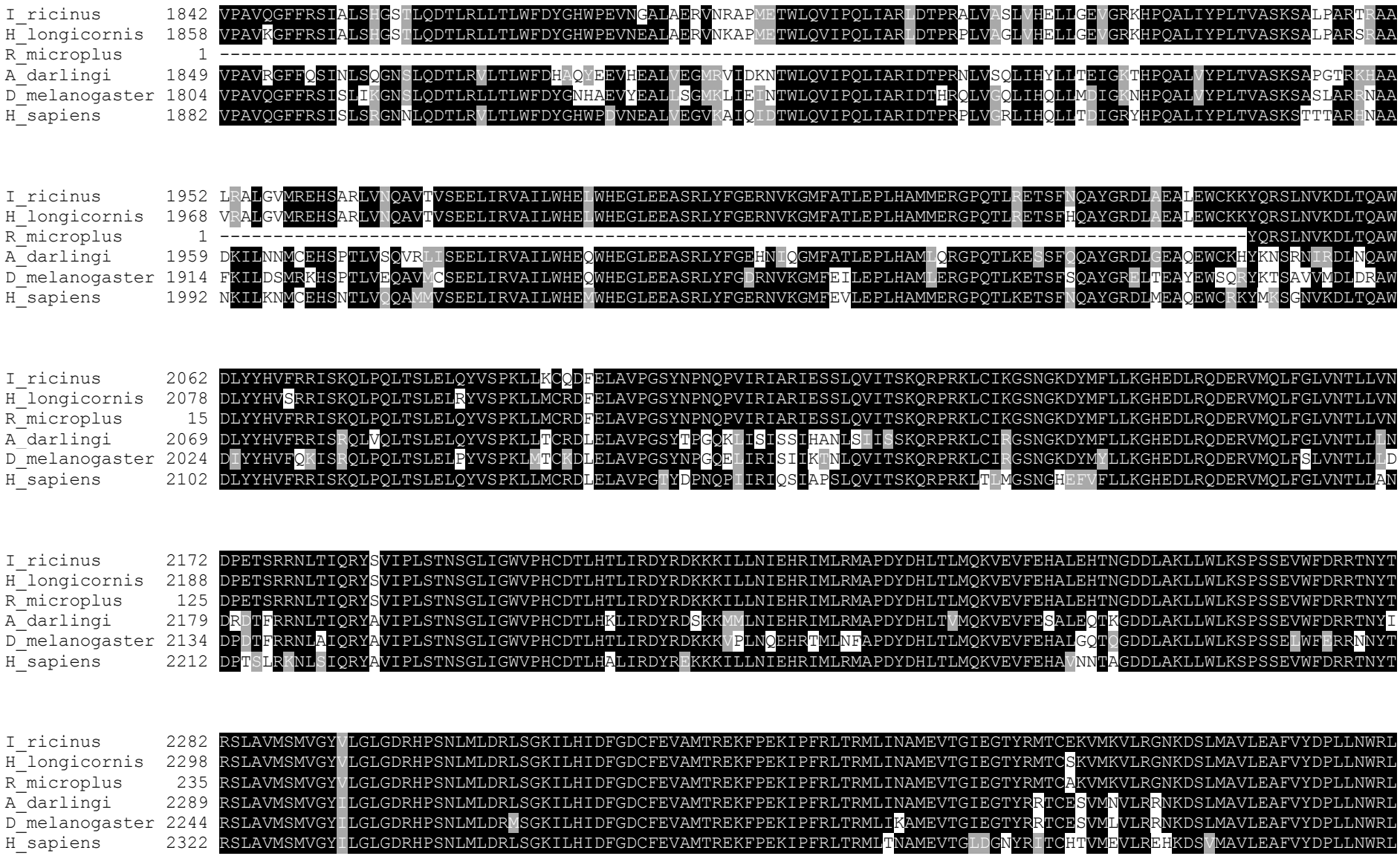
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R_microplus 1 ----------
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R_microplus 345 MDAQPKVKHSK-TRGGSAPCSHDQGDIL----------EAVDIGSQPTA-----KKAV---DAV----------SGIG-----DSE-------G
A_darlingi 2399 LEADRLRFSKNAGDMEGASGSMDDDTMLSYNARRDARMNELNAGLIARAPPGSNAAAGNAALNAMVTIPNGKTGANGAAGATAATAAALAAGTGAGDVPDGAAFAPPATN
D_melanogaster 2354 LDVDKKGNDAVAGA--GAPGGRGGSGMQD----------SLS----NSVED-SLPMA--------KSKPYD----------PTLQ-----QG-----GLHN
H_sapiens 2432 MDTNTKGNKRSRTRTDSYSAG-QSVEIL----------DGVELGEPAHK-----KTGTIVPESI----------HSFI-----GDG-------L
I_ricinus 2444 AQPEALNKKALAIINRVRDKLTGRDFAPDETLDVPEQVELLIKQATSHENLCQCYIGWCPFW
H_longicornis 2462 AQPEALNKKALAIINRVRDKLTGRDFAPDETLDVPEQVELLIKQATSHENLCQCYIGWCPFW
R_microplus 398 GQPEALNKKALAIINRVRDKLTGRDFAPDETLDVPEQVELLIKQATSHENLCQCYIGWCPFW
A_darlingi 2509 NPADVTNKKARAIVDRVKDKLTGKDFGKPEPVAVNRQIDLLIQQATNNENLCQCYIGWCPFW
D_melanogaster 2410 NVADETNSKASQVIKRVKCKLTGTDFQTEKSVNEQSQVELLIQQATNNENLCQCYIGWCPFW
H_sapiens 2488 VKPEALNKKALQIINRVRDKLTGRDFSHDDTLDVPTQVELLIKQATSHENLCQCYIGWCPFW

7.3. Photos of the dsRNA injected groups of ticks

7.3.1. GFP dsRNA injected (control) group of ticks

after 3 days of feeding

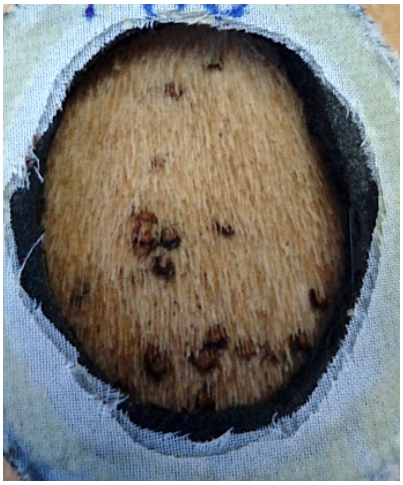
after 7 days of feeding

7.3.2. *Ir*InR dsRNA injected group of ticks

after 3 days of feeding

after 7 days of feeding

7.3.3. *Ir*AKT dsRNA injected group of ticks



after 3 days of feeding

after 7 days of feeding

7.3.4. *Ir*TOR dsRNA injected group of ticks

after 3 days of feeding

after 7 days of feeding