University of South Bohemia in České Budějovice
Faculty of Science

Identification of the tick NF-κB immune pathway read-out genes in *Ixodes ricinus*

Bachelor thesis

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Annotation

Bachelor thesis demonstrates identification of the tick immune read-out genes in the tick *Ixodes ricinus* up-regulated after stimulating of the NF-κB pathway by using molecular methods- (PCR, qRT-PCR and RNA interference via dsRNA injection).

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Miriama Pekľanská

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1. Introduction

1.1. Ticks

Ticks are a group of blood feeding (haematophagous) ectoparasites (Chelicerata, Arachnida) transmitting a number of different types of pathogens comprising viruses, bacteria, fungi and protozoa to their vertebrates hosts. They are vectors of human and domestic and wild animal diseases (Fuente, 2008). Different life cycle strategies and numerous morphological and physiological features clearly divide ticks into two main families, Argasidae and Ixodidae, known also as soft and hard ticks, respectively. The third family, Nuttalliellidae, including a single tick *Nuttalliella namaqua*, sharing the same features related to both hard and soft ticks (Estrada-Pena, 2015).

1.1.2. Soft ticks (Argasidae)

Argasidae ticks comprise 193 species and include genera *Antricola, Argas, Nothoaspis, Ornithodoros,* and *Otobius*, which are extremely different from one to another (Guglielmone, 2010). Soft ticks do not possess a scutum, a hard plate on their back. Their nymphs and adults are morphologically very similar. The soft tick surface has leathery covering that can rapidly expand. They enlarge up to ten times their body mass within a few hours, sometimes within minutes during feeding (Estrada-Pena, 2015). Their body is often wrinkled. To acquire a blood meal, ticks insert their highly specialized mouthparts (located anterioventrally) through the host skin and, depending on the species, anchor them in the skin by attachment cement (Sonenshine, 1991). The life cycle of Argasids involves three developmental stages: one larval stage, many nymphal stages and an adult stage (male and female). The female and male are mating off the host, female then lays eggs (200-300 eggs during her live) (Sonenshine & Roe, 2014b). The length of feeding is short, nymphs and adults of both sexes take their blood within a few minutes (Binnington & Kemp, 1980). This clearly determines course of the pathogen transmission. The soft ticks transmit pathogens immediately after the feeding starts (Hajdušek et al., 2013; Sojka et al., 2013).

1.1.3. Hard ticks (Ixodidae)

Ixodidae ticks consist of 702 species in 14 genera: *Amblyomma*, *Anomalohimalaya*, *Bothriocroton*, *Cosmiomma*, *Cornupalpatum*, *Compluriscutula*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Margaropus*, *Nosomma*, *Rhipicentor*, and *Rhipicephalus* (Guglielmone, 2010). Hard ticks possess hard dorsal shield called a scutum. The scutum covers entire dorsal surface of the adult males. In larvae, nymphs and adult females is the scutum substantially reduced, so their sexual dimorphism is apparent (Sonenshine & Roe, 2014a). They feed only once per a life stage, females feed for a longer period (several days to weeks) and enlarge up to 100 times their unfed body weight because of consuming large amount of blood (Sonenshine & Roe, 2014b). The length of feeding determines the pathogen transmission which usually happens several days after attachment (Hajdušek et al., 2013).

1.2. Life cycle of Ixodidae

The Ixodidae species have four developmental stages. The life cycle encounters the egg stage and three active stages- larva, nymph, and adult (males and females). The life cycle may take several months or years and depends on several circumstances like climatic conditions or accessibility of the hosts (Bowman & Nuttall, 2008).

The ticks require a blood meal from the vertebrate host during each stage. It is necessary for the larvae and nymph to molt to the next life stage and for the adult female to lay eggs (Hofmeester et al., 2016). Their period of feeding is relatively long. The females remain attached to the host for 5-12 days (unless they do not mate, in which case they may remain attached for several weeks). The male of Ixodid ticks feed for 3-5 days. He does not require too much blood for survive. His main role is to fertilize the feeding female (Mehlhorn, 2001; Anderson & Magnarelli, 2008). One of the significant females features is mating. After mating and the last dropping off their host, fully-fed females lay their eggs in grass or on leaf liver and die (Balashov, 1972).

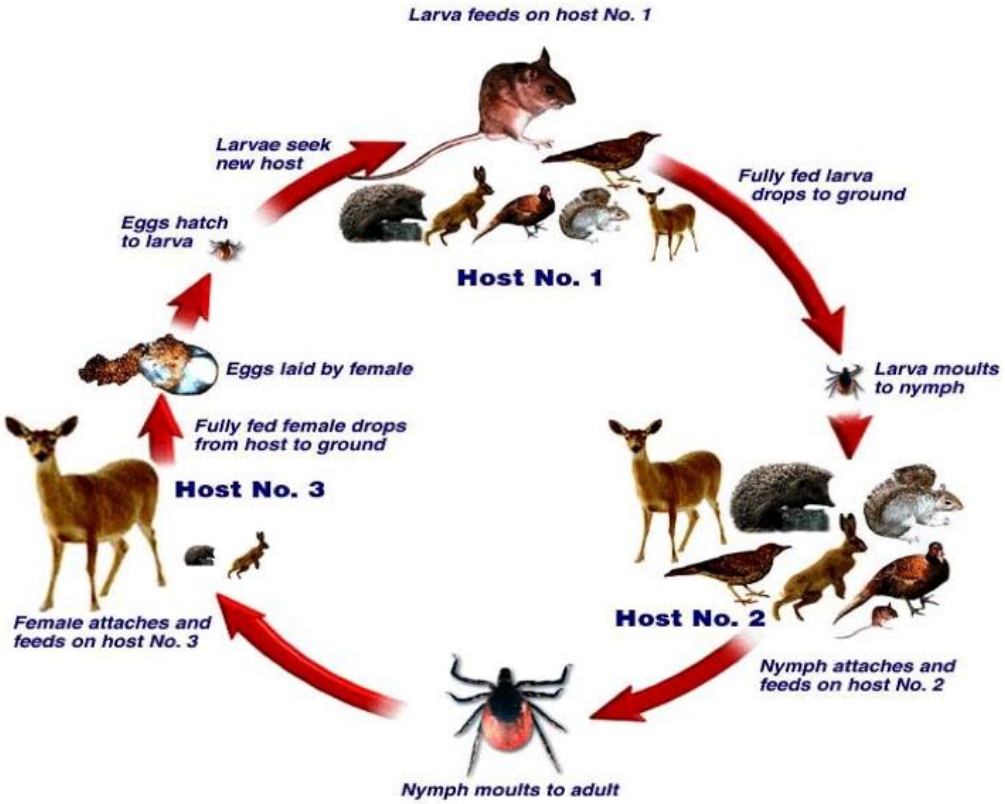


Figure 1: The scheme of life cycle of Ixodidae ticks with relative sizes of animals approximates their significance as hosts for the different tick life cycle stages. *Ixodes ricinus* has to undergo three-host stages during his lifecycle. Larval ticks drop off the host after 3-5 days of feeding on usually a small mammal or bird and molt into nymphs (Nuss et al., 2017). Subsequently, the unfed nymphs feed on small mammals such as rodents to achieve the fully-fed stage. After feeding, nymphs drop off the host and molt to the adult stage. The adults find another host, usually a big mammal. For the reproduction, adult females require a blood meal, so they mate and feed, drop off the host with laying thousands of eggs and die (Sonenshine & Roe, 2014a). The scheme was designed by Dr. Jeremy Gray and Bernard Kaye.

1.3. *Ixodes ricinus* as a vector

Ticks transmit the largest number of pathogens among all arthropod disease vectors and are second only to mosquitoes in their significance to human health. Pathogens causing tick-borne diseases might be imported by the migration of animals such as birds. This demonstrates the ability of ticks to spread over long distances together with a variety of tick-borne diseases (Kuo et al., 2017). The tick *I. ricinus* is very widespread and abundant ixodid tick in Europe. It is a vector of emerging zoonotic pathogens including spirochetes *Borrelia burgdorferi* sensu lato, the agents of Lyme borreliosis (Gern & Rais, 1996). Other pathogens transmitted by *I. ricinus* are *Anaplasma phagocytophilum* causing human granulotic anaplasmosis (HGA) and tick-borne fever (TBF) and *Babesia* spp (Cotté et al., 2008). Intraerythrocytic piroplasms of the genus *Babesia* cause a zoonosis called Babesiosis. Over 30 human cases of Babesiosis have been reported over the past 50 years in Europe (Bonnet et al., 2007).

Moreover, *I. ricinus* tick is the major vector of TBE (tick-borne encephalitis) virus causing neurological disease in humans or louping ill virus (LIV) causing neurological disease in sheep (de la Fuente et al., 2017). The incidence of TBE has significantly increased in the Czech Republic over the last decade (Daniel et al., 2003).

1.4. Immune system of ticks

1.4.1. Innate immune system

The immune response plays the most important role for effective survival of all living organisms. Invertebrates lack true adaptive immunity and they entirely depend on the primitive immunity called innate immunity. There are various immune mechanisms exerted by invertebrates that act potentially against various bacterial, fungal and viral pathogens (Hoffmann & Reichhart, 2002). The knowledge on the tick innate immunity, the tick immune pathway, is not fully explained, but we are capable to compare the tick innate immunity with other invertebrates (Syed Musthaq & Kwang, 2015). Good research conditions to obtain new knowledge about the invertebrate immunity and immune mechanisms were provided by model organism, such as fruit fly *Drosophila melanogaster* (Ferrandon et al., 2007) and other arthropods, such as the horseshoe crab and freshwater crayfish (Kopacek et al., 2012).

The arthropod innate immunity in reaction to the pathogen attack can be categorized as cellular or humoral. The humoral immunity employs the innate cascades, such as Toll and immune deficiency (IMD) pathways. These two are the best studied immune signalling pathways in the arthropod immunity. The third immune signalling pathway called JAK-STAT pathway plays an important role in immunity, cell division, cell death and tumour formation (Ferrandon et al., 2007; Chávez et al., 2017). The Toll and IMD pathways in invertebrates are initiated by the pathogen-associated molecular patterns (PAMPs), which are sensed by the pattern recognition receptors (PRRs) (Chávez et al., 2017). This recognition step activates various defence mechanisms in a complicated manner to eliminate the pathogen. Humoral immune responses are initiated by PAMPs and orchestrate production of a wide spectrum of microbicidal AMPs (antimicrobial peptides), pattern-recognition proteins and effector molecules such as lectins and complement-related proteins (Hajdušek et al., 2013; Syed Musthaq & Kwang, 2015).

The research on tick innate immunity has rapidly expanded during the last decade. It is proved, that ticks produce antimicrobial peptides (AMPs) e.g., *defensins* after activation of the immune system by Gram-positive bacteria and fungi. (Sonenshine et al., 2002). In the genome of *Ixodes scapularis* we can recognize two distinct multigene families of defensin-like peptides (DLPs): a) scapularisins, which share big sequence similarity to antibacterial ancient invertebrate-type defensins (AITDs) and were studied in this work and b) scansins, which are distantly related to AITDs (Wang and Zhu, 2011). Other AMPs produced in common with other arthropods are lysozymes or tick-specific molecules (e.g. microplusins) that are secreted to the gut lumen, hemolymph or saliva and they directly kill, entrap or inhibit the pathogens (Urbanová et al., 2015).

Ticks possess the evolutionarily oldest components of invertebrate immunity- a complement system, where belong thioester-containing proteins (TEPs) (Kopacek et al., 2012). The evidence of primordial complement dates back more than 1 billion years (Buresova et al., 2011). They are grouped into four major classes known from vertebrates and arthropods*:* (i) α_2M (macroglobulins), (ii) C3/C4/C5 complement, (iii) TEP (thioester-containing protein) and (iv) MCR (macroglobulin-complement-related) groups (Buresova et al., 2011). The tick genome encodes nine members representing all major groups of α_2M-F: (i) three different α2-macroglobulins (ii) three proteins related to C3-complement

component; (iii) one insect-type TEP (thioester-containing protein) and (iv) two macroglobulin-complement-related (MCR) molecules (Urbanová et al., 2017).

In ticks have been described other molecules related to the components of vertebrate or invertebrate complement systems, such as Factor D, homologs of Limulus Factor C and Factor C2/Bf (Factor B). These molecules are related to the central complement of mammalian component C3, C2/Factor B (IrC2/Bf) convertases, or ficolin-like lectins (Honig Mondekova et al., 2017; Urbanová et al., 2018).

1.4.2. Signalling pathways

The major signalling pathways involved in the innate immunity of arthropods (Toll and IMD pathways) have been studied in the fruit fly, *D. melanogaster*, the best-studied insect model organism. The bacterial or fungal infection triggers activation of the intracellular signalling pathways (Frolet et al., 2006). In 2011, Jules A. Hoffman was awarded by the Nobel Prize for discoveries concerning the activation of innate immunity, activation of the Toll pathway in microbial infection. He infected fruit flies with bacteria or fungi and discovered, that Toll mutants died because they could not mount an effective defence.

The Toll pathway was discovered and its function was described in *D. melanogaster* (Hoffmann, 2003) and also in the mosquito *Anophles gambiae*, the major vector for the protozoan malaria parasite *Plasmodium* (Frolet et al., 2006). In ticks, the Toll pathway and its function is not fully described. For this reason, I will describe current understanding of Toll pathway described in *D. melanogaster*.

The activation of Toll pathway occurs when Spätzle protein is cleaved by the indirect action of pathogens, when PGRPs (peptidoglycan recognition proteins) recognize bacterial peptidoglycan and activate proteases that cleave Spätzle protein (Dziarski & Gupta, 2006). After this cleavage, the cleaved Spätzle protein binds to Toll receptors and actives the NF-κB transcription factors. In the adult fruit flies, the transcription factor is called Dif (Dorsal-related immunity factor)/Dorsal. Dorsal is homologous to transcription factor Relish 1 (Rel1) in ticks. Dif and Dorsal regulate expression of *defensins* and other AMPs. The activation of Dorsal requires phosphorylation and degradation of its inhibitor Cactus. In ticks, we have detected over 20 genes coding transcription factor Cactus. Cactus causes translocation of Dif/Dorsal to the NF-kB binding sites in the nuclear DNA and up-regulation of AMPs expression (Hoffmann, 2003).

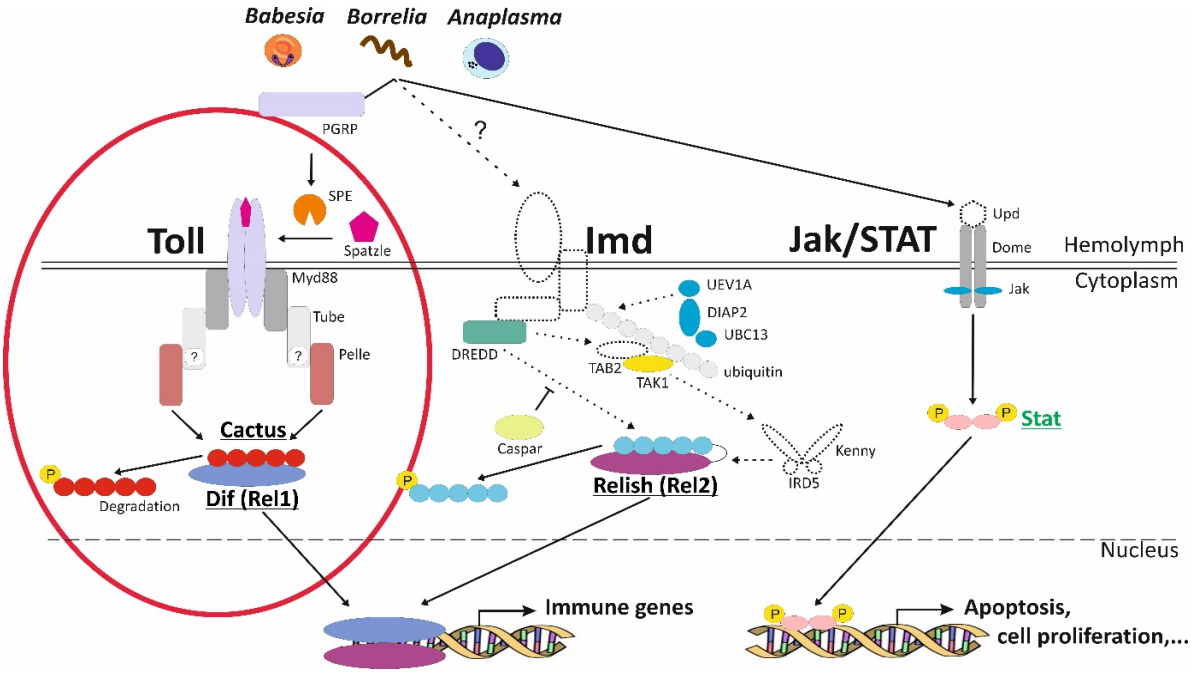


Figure 2: A scheme of the putative tick pathways (Toll, Imd and Jak/Stat pathways) based on the *Ixodes scapularis* genome data search. Adapted from Hajdušek, unpublished data.

1.5. RNA interference

During the last few years, new molecular tools such as the RNA interference (RNAi) were discovered. This discovery brought benefits for the study of genes functions, which represents a valuable alternative for analysis of the gene functions in eukaryotes and also became an important scientific tool in the cell cultures and living organism (Chavez-Pena & Kamen, 2018). RNAi is a post-transcriptional process activated by double-stranded RNA (dsRNA), which leads to gene silencing in a sequence-specific manner. RNAi was solved and used on the nematode model organism *Caenorhabditis elegans* to manipulate gene expression. The injection of exogenous double-stranded RNA (dsRNA) into the nematode lead to the silencing of the endogenous homolog mRNA (Fire et al., 1998). Further analysis was performed in the fruit fly *D. melanogaster*, which contributed greatly toward understanding RNAi pathway (Elbashir et al., 2001).

RNAi became an important standard tool for functional genomics researches in ticks, other methods of genetic manipulations are rather limited. Using this tool enables us to study

functions of the tick genes and understand immune pathway on the tick-pathogen interface (Hajdušek, 2009).

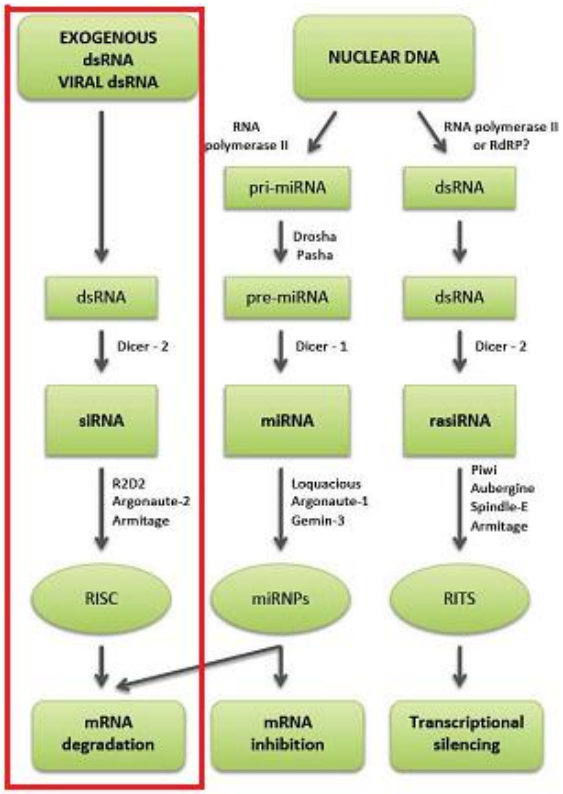


Figure 3: The scheme of RNAi pathways in ticks. The picture adapted from (Hajdušek, 2009).

Figure 3 displays the basic ways of the RNAi pathway. The exogenous dsRNAs are cleaved by Dicer 2 (member of RNase III family) into small dsRNAs fragments called small interfering RNAs (siRNAs) (Fuchs et al., 2004, Hajdušek, 2009). Then, siRNAs are loaded into the RNA-mediated silencing complex (RISC, a macromolecular complex possessing endonuclease activity) and there, they become active. RISC binds the protein called Argonaute-2 (endonuclease) and the guide strand of siRNAs. After RISC targeting mRNA with its complementary sequence, the target mRNA is cleaved and degraded (Hajdušek, 2009, Chavez-Pena & Kamen, 2018).

2. Objectives

- Identification and description of defensin genes in the genome of *Ixodes scapularis*.

- Design of gene-specific defensin primers for qRT-PCR.

- Quantification of *defensins* and other immune genes (*c3-1*, *c3-2*, *c3-3* and *factor b*) expressions by using cDNA prepared from the fully-fed male nymphs with silenced Toll pathway components by RNAi (identification of the tick immune pathways read-out genes).

3. Materials and methods

3.1. Biological material

Adult females and males of *Ixodes ricinus* were collected by flagging method in nature around Ceske Budejovice, Czech Republic. Nymphal ticks of *I. ricinus* obtained from the breeding facility of the Institute of Parasitology, Biology Centre, CAS, were used for the experiment. The nymphs were maintained at 95% relative humidity within glass vials in wet chambers containing 3% KCl solution. The temperature of the breeding facility was 24°C with photoperiod cycle 15h/9h (light/dark). Four groups of nymphs were injected with dsRNA (double-stranded RNA) to knockdown (KD) the corresponding genes. After 3-days rest, the nymphs were allowed to feed on the non-infected BABL/c mice.

The engorged nymphs were divided into two groups (females and males). The fully-fed males of *I. ricinus* were used to make cDNAs in order to verify knockdown efficacy followed by determination of the target gene expression levels.

3.2. Tick injection

The dsRNAs were prepared previously in the laboratory and were injected into nymph hemocoel using Narishige microinjector. The dsRNAs groups encountered: GFP- (green fluorescence protein) for control, Relish 1 (Rel1), Cactus 1-9 (Cac1-9; a mixture of 9 previously selected Cactus genes with a similar domain structure), Rel1 + Cac1-9. 32nl of the dsRNA was injected for the single KD, 64nl for the double KD. The injection was performed by Dr. Ondřej Hajdušek. After the injection, 20 viable ticks were taken for the further experiment.

3.3. Tick sorting

After feeding on mice, 80 engorged nymphs were divided into females and males according to their weights (Dusbábek, 1996). The nymphs with the weight $\leq$ 4 mg were considered as males. Five engorged males from each of the dsRNA groups with the highest weights were used for RNA extractions.

3.4. RNA isolation

The total RNA was extracted from the whole body of each chosen male tick using a NucleoSpin RNA (Macherey Nagel) according to the manufacturer protocol. In the last step, the RNA was eluted twice with the same volume of RNase-free H_2O (40µl) to obtain higher

yield. The concentration of RNA was measured on spectrometer NanoDrop^{TM} 1000 and integrity was verified on the 1% agarose gel. The obtained RNAs were labelled and stored at -80°C.

3.5. cDNA preparation

The cDNA was synthesized by reverse-transcription from the isolated RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) according to the manufacturer protocol (20µl reactions, random hexamers). 0.5 µg of the isolated RNA was used for each reaction. The cDNAs were diluted 10x, verified by PCR and stored at -80°C.

3.6. *In silico* work

3.6.1. Genome analysis

The genome of *Ixodes scapularis* (VectorBase, https://www.vectorbase.org/) was searched for defensin-like genes (containing defensin domain). For each homolog, NF-κB binding sites were screened 3000 bp (if possible) upstream the start codon using the Consite program (http://consite.genereg.net). The sequences for *D. melanogaster* transcription factors Dorsal 1 and Dorsal 2 (tick homologues of Rel1) were indicators for the tick NF-κB binding sites. The search was performed for antisense (-) and for sense (+) strands.

3.6.2. Phylogenetic tree

The construction of the phylogenetic tree was based on the primary amino acid sequences of defensin genes. The sequences were aligned and manually checked using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic tree was created using the Neighbor Joining method in the program Mega 4 (http://www.megasoftware.net/). The sequences used for the phylogenetic tree construction are shown in Table IV of the Supplement.

3.6.3. Design of primers

The specific primers were designed for three selected defensin genes accordingly to the general rules for primers design. The forward primers were designed by Primer 3 (http://primer3.ut.ee/), the gene-specific reverse primers were designed manually and checked in the same program. The primers were tested by PCR and used for qRT-PCR. The other primers used for qRT-PCR and RNAi were designed in advance in the laboratory by

Dr. Ondřej Hajdušek. Sequences of primers with their product sizes are shown in Table III of the Supplement.

3.7. Standard PCR

The standard PCR was performed to check quality of the newly transcribed cDNAs. The volume reaction was 10µl and contained 2µl of cDNA, 0.5µl of each gene specific primers (*elongation factor*- IR525+IR526), 5µl of FastStart PCR MasterMix (Roche) and 2µl PCR water. Sequences of primers are shown in Table III of the Supplement. The programme for PCR was performed with following conditions: initialisation at 96°C for 10 minutes, 40 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, elongation at 72°C for 1 minute and the final extension at 72°C for 10 minutes.

The designed primers were verified by standard PCR. The volume reaction was 10µl and contained 2µl of cDNA (fully-fed non-infected nymphs). Furthermore, the reaction included 0.5µl of each specific primers, 5µl of FastStart PCR MasterMix (Roche) and 2µl of PCR water. The programme for PCR reaction was used with the same conditions as described above. The primers were also used to check presence of the corresponding genes in the tick genomic DNA. The reaction proceeded with the same conditions as the previous PCR. 10µl reaction contained 2µl of gDNA. The programme for PCR was used with the same conditions as for the previous PCR reactions. The sequences of designed primers are shown in Table III of the Supplement.

3.8. Gel electrophoresis

The results of PCRs were visualized by the 1% agarose ethidium bromide-stained gel electrophoresis in TAE buffer. 10µl of the PCR reaction was mixed with the DNA-loading dye (Top-Bio) and subsequently loaded on the gel. The PCR product sizes were cross-checked according to the 100 bp DNA ladder (Thermo Scientific).

3.9. Quantitative Real-time PCR

3.9.1. Determination of the target gene expression levels

The reaction volume for Quantitative Real-time PCR (qRT-PCR) was 25µl and contained 12.5µl of FastStart Universal SYBR green Master; Rox (Roche); 0.5µl of forward and 0.5µl of reverse primer, 5µl of cDNA template prepared from the male tick. The remaining volume was adjusted with sterile water. All samples were analysed in triplicates using LightCycler 480 (Roche).The melting curves were checked for every sample. The relative expression profiles were normalized to the expression of housekeeping gene (*elongation factor* IR525+IR526). Amplification program for the qRT-PCR was performed with following conditions: initialisation at 95°C for 10 minutes, 55 cycles of denaturation at 95°C for 15s, and the final step with annealing and elongation at 60°C for 1 minute. The sequences of primers are shown in Table III of the Supplement.

3.9.2. Verification of the gene knockdowns after RNAi silencing

The efficiency of RNA interference (RNAi) was checked by qRT-PCR in individual male ticks. The knockdown validation was performed for *rel1* (IR13+IR14) and *cac2* (IR35+IR36) on GFP, Rel1, Cac1-9 and Cac1-9 + Rel1 cDNA groups. The reaction volume was 25µl and contained 12.5µl of FastStart Universal SYBR green Master; Rox (Roche), 1µl of forward and 1µl of reverse primer, 5µl of cDNA template prepared from the male tick and 5.5µl of sterile water. All samples were analysed in triplicates using LightCycler 480 (Roche). The melting curves were checked for every sample. The relative expression profiles were calculated based on housekeeping gene which was *elongation factor* (IR525+IR526) and the gene silencing efficiency was calculated against GFP. Amplification program for qRT-PCR was performed with following conditions: initialisation at 95°C for 10 minutes, 55 cycles of denaturation at 95°C for 15s, and the final step with annealing and elongation at 60°C for 1 minute. The sequences of primers are shown in Table III of the Supplement.

3.10. Statistical analysis

The statistics and graphs were performed using GraphPad Prism 6 (version 6.01 for Windows, GraphPad Software, USA). The obtained data were analyzed by One-way ANOVA using nonparametric test (Kruskal-Wallis test). $P<0.05$ was considered to be statistically significant. The error bars represent standard errors from five independent biological replicates.

4. Results

4.1. Gene selection

Tick immune genes *defensins* (*def*) were chosen to determine levels of the relative gene expression after stimulation of NF-κB pathway, They were found in the *In silico* genomic screens. Besides the defensin genes, the gene expression was measured for C3 protein family (C3-1, C3-2, C3-3) and Factor B, a component of the alternative pathway of complement activation. The biological functions of these immune molecules have been published and they are mentioned in the chapter 1.5.1. Innate immune system in the Introduction.

4.2. Genome analysis

The genome of *I. scapularis* was analyzed to identify tick *defensins*. The *In silico* analysis performed using the *I. scapularis* genome database (https://www.vectorbase.org/) showed, that the genome contains 9 defensin genes encoding for proteins containing single defensin-related domain. The search for the NF-κB binding sites (Dorsal 1, Dorsal 2) 3000 bp upstream the start codon detected different number of NF-κB binding sites for each defensin gene. The identified genes, their lengths of upstream sequence, and number of NF-κB binding sites for each defensin gene are shown in Table I.

Table I: The number of predicted transcription factor (*D. melanogaster* Dorsal 1, Dorsal 2) binding sites (BS) in the upstream sequence of identified defensin genes. (+) sense strand, (-) antisense strand, (bp) base pair, *available length in the genomic database.

Gene ID	Upstream length (bp)	Dorsal 1 (+)	Dorsal 1 (-)	Dorsal 2 (+)	Dorsal 2 (-)	BS
defensin 1 ISCW024015	479*	8	1	0	1	**2**
defensin 2 ISCW016747	3000	3	1	2	1	**6**
defensin 3 ISCW024381	1000*	0	0	0	0	**0**
defensin 4 ISCW005926	3000	3	3	4	2	**8**
defensin 5 ISCW005927	3000	2	5	2	4	**10**
defensin 6 ISCW005928	3000	2	1	3	3	**4**
defensin 7 ISCW011162	3000	1	2	1	3	**5**
defensin 8 ISCW022594	3000	2	0	0	0	**2**
defensin 9 ISCW022102	3000	2	1	1	3	**5**

The sequence of *defensin 1* and *defensin 3* upstream the start codon (used for the NF-κB binding sites prediction) was not 3000 bp long as for the other genes. The length of the sequence was 479 bp and 1000 bp, respectively. The number of BS for each defensin gene varies with the total number of transcription factors. In some cases, 1 BS has specific sequence for more than 1 transcription factor.

The table I demonstrates that each defensin gene with the same length of analyzed sequence has different number of NF-κB biding sites. *Defensin 3* does not contain any NF-κB biding sites, a gene with the highest number of NF-κB biding sites is *defensin 5*.

The phylogenetic tree was created to display the evolutional relationship amongst identified defensin genes. The phylogenetic tree was created for 9 defensin genes on the basis of their amino acid sequences. It clearly clustered the defensin genes into three groups (Group A, Group B, Group C) as shown in Figure 4. The Group A contained four defensin genes: *def 1*, *def 3*, *def 2*, *def 8*. The Group B contained also four defensin genes: *def 4*, *def 6*, *def 5*, *def 7*. The last group, Group C, contained just one defensin gene, *def 9*. (*def*) defensin gene.

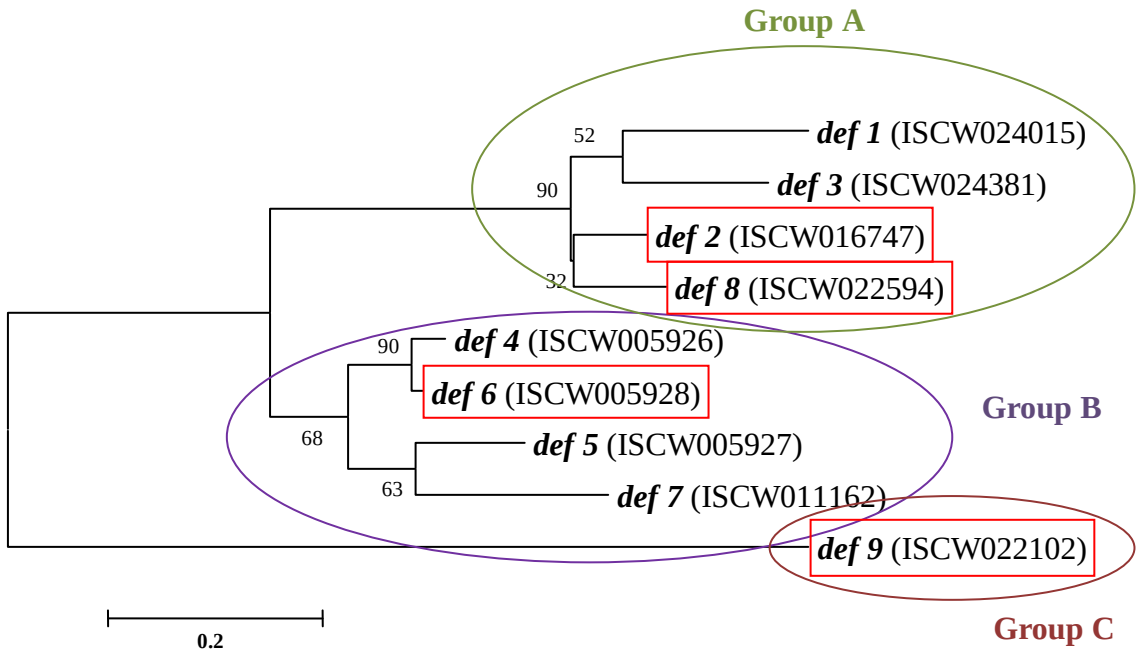


Figure 4: The phylogenetic tree of tick defensin genes. The identified 9 defensin genes were clustered into three groups named as Group A, Group B, and Group C. The primary amino acid sequence alignments used for the phylogenetic analysis contained only the defensin-related domain. The phylogenetic tree was constructed using the program Mega 4. Flattened circles indicate genomic tick sequences of *I. scapularis*. Numbers at the branches denote bootstrap values calculated from 1000 repetitions. The gene names enclosed by the red rectangles were chosen for the further analysis by qRT-PCR. Bar: 0.2 substitutions per site.

For the futher qRT-PCR analysis, one defensin gene was chosen from each group. From the Group A were chosen *defensin 2* (6 BS) with the average number of NF-κB biding sites and *defensin 8* (2 BS) with low number of NF-κB biding sites. From the Group B we selected *defensin 6* (4 BS) and from the Group C we took the only available defensin gene, *defensin 9* (5 BS). For these defensin genes (*defensin 2*, *defensin 8*, *defensin 9*) were designed specific primers. The specific primers for *defensin 6* have been previously designed and provided by Dr. Ondřej Hajdušek.

4.3. Verification of primers

Initially, the designed primers were verified by PCR using cDNA prepared from the fully-fed non-infected nymphs. The expected amplicon sizes were 101 bp, 114 bp, and 103 bp, respectively. The primer sequences with the corresponding product sizes are shown in Table III of the Supplement. The resulted PCR products of verification primers are shown in Figure 5.

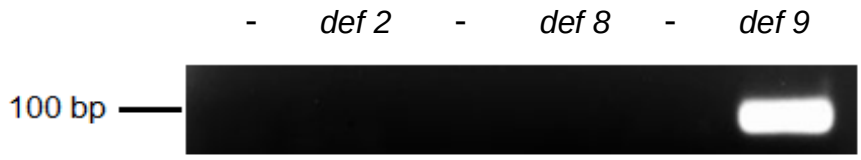


Figure 5: Verification of defensin gene (*def 2, def 8, def 9*) primers using cDNA as a template. The gel electrophoresis of PCR reaction showed, that only specific primers for *defensin 9* worked as expected. The *defensin 2* and *defensin 8* expression was too low for the analysis or designed primers were not specific. (*def*) defensin gene, (-) negative control, (bp) base pair.

To avoid the expression problem, an additional PCR reaction was performed to check specificity of the designed primers using genomic DNA as a template. Each of three defensin genes was tested on tick gDNA. The expected amplicon size for *defensin 2* was 685 bp, for *defensin 8* was 1096 bp, and for *defensin 9* was 2766 bp. The expected product sizes are shown in Table III of the Supplement. The PCR results are shown in Figure 6.

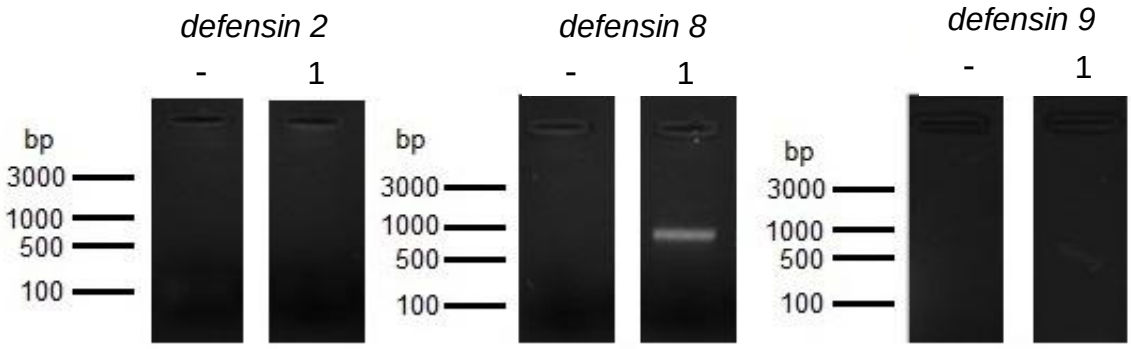


Figure 6: Verification of defensin gene (*def 2, def 8, def 9*) primers using gDNA as a template. The gel picture demonstrates that verification with the gDNA was successful only for *defensin 8* with product size of 1096 bp. (1) gDNA, (-) negative control, bp (base pair).

The verification of primers for *defensin 2* on cDNA and gDNA was unsuccessful. It means, that the primers were probably not well designed (did not work). The results for *defensin 8* showed that these primers worked, but this *defensin* was not expressed. The primers for *defensin 9* worked only on cDNA. It means, that the primers for *defensin 9* were well designed, but the PCR was not able to amplify the sequence from gDNA containing an intron (the product was probably bigger than predicted 1096 bp). In conclusion, for the further measurement of relative expression we chose the newly designed primers for *defensin 9* (IR696+IR697) along with the primers for *defensin 6* (IR21+IR22) predesigned by Dr. Ondřej Hajdušek.

4.4. Knockdown (KD) of genes by RNAi silencing

The experimental nymphs were divided into four groups GFP, Rel1, Cac1-9 and Cac1-9 + Rel1. For each group, we injected a specific dsRNA into the nymphal hemocoel of 20 nymphs per group. After the inoculation and feeding on mice, fully-fed nymphal ticks dropped off the host were divided into females and males according to their weights. The accurate number of engorged nymphs duration of the feeding and their weights after feeding are shown in Tables V, VI, VII, VIII of the Supplement. The overall success of feeding and a border between the weight of females and males is shown in Figure 7.

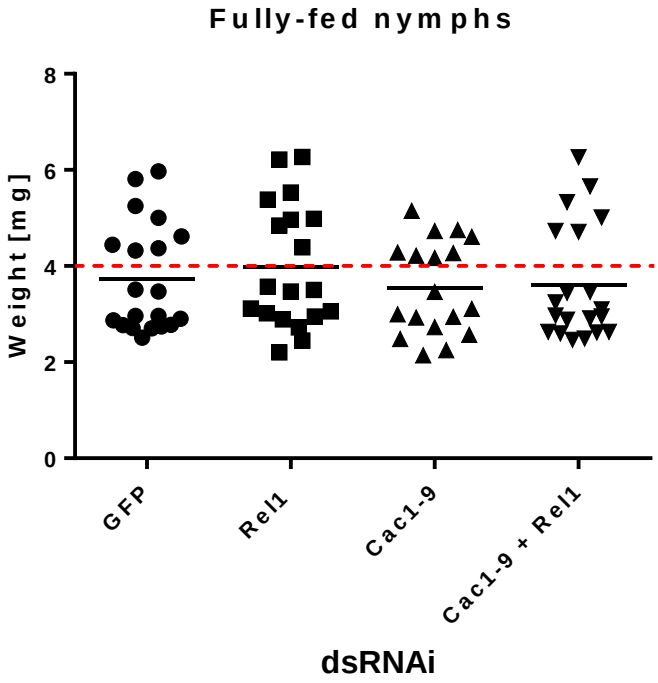


Figure 7: Sorting of the fully-fed gene-silenced nymphs according to their weights. Dashed line clustered the ticks into two groups- females and males. Ticks under the red line were taken as males. Black lines determine average weights of the fully-fed nymphs.

From each group were chosen 5 male ticks with the highest weights. The total RNAs were extracted from individual whole male tick bodies. Subsequently, the RNA was transcribed into cDNA. Table II shows, which males were selected per each dsRNAi group and the corresponding RNA concentrations. Figure 8 shows integrity of the isolated RNAs and associated PCR controls of the transcribed cDNA. All RNAs and cDNAs were found to be prepared in good quality.

Table II: The selected male nymphal ticks from each dsRNAi group with their RNA and cDNA numbers, their weights and RNA concentrations. (mg) milligrams, (ng/µl) nanogram/microliter.

Nymph ID	RNA/cDNA number	Weight (mg)	Concentration (ng/µl)
GFP group			
1.20	RNA 874/cDNA 915	3.51	110.5
1.13	RNA 875/cDNA 916	3.47	82.5
1.15	RNA 876/cDNA 917	2.96	77.8
1.9	RNA 877/cDNA 918	2.96	85.5
1.12	RNA 879/cDNA 919	2.90	97.5
Rel1 group			
2.11	RNA 881/cDNA 920	3.57	91.6
2.14	RNA 882/cDNA 921	3.50	136.5
2.9	RNA 883/cDNA 922	3.47	72.9
2.7	RNA 884/cDNA 923	3.12	97
2.10	RNA 885/cDNA 924	3.06	111.6
Cac1-9 group			
3.18	RNA 886/cDNA 925	3.46	101.4
3.7	RNA 887/cDNA 926	3.11	103
3.10	RNA 889/cDNA 927	3.00	106.9
3.17	RNA 890/cDNA 928	2.94	99
3.6	RNA 891/cDNA 929	2.93	76.3
Cac1-9 + Rel1 group			
4.17	RNA 892/cDNA 930	3.46	62.6
4.18	RNA 894/cDNA 931	3.44	50.8
4.13	RNA 895/cDNA 932	3.24	95
4.8	RNA 896/cDNA 933	3.10	67.5
4.19	RNA 897/cDNA 934	2.97	102.6

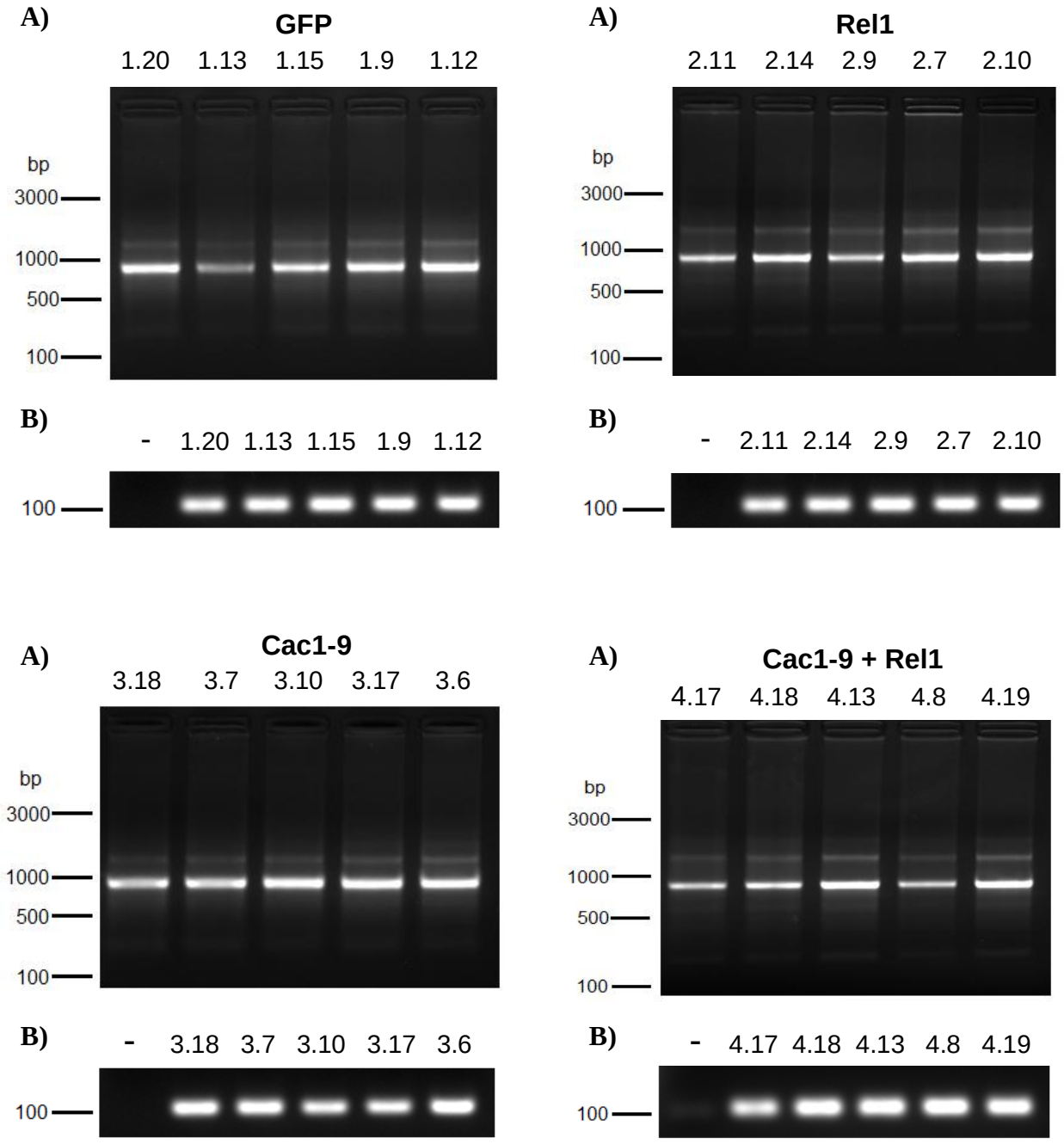


Figure 8: Verification of the RNA integrity and cDNA quality for each of the knockdown group (GFP, Rel1, Cac1-9, Cac1-9 + Rel1). A) Gel electrophoresis profiles of the extracted RNAs. **B)** Control PCR performed on the transcribed cDNA. For the verification of cDNAs were used primers (IR524+IR525) against the tick *elongation factor* (Table III of the Supplement). For both verifications were used 5 samples of male ticks with the highest weight. (-) negative control, (bp) base pair.

4.5. Knockdown validation in male ticks

The KD efficacy was verified by the relative qRT-PCR for *rel1* (IR13+IR14) and for *cac2* (IR35+IR36). As a template for the reaction we used cDNAs prepared from male ticks. The silencing level in each group was compared to the GFP RNAi control group. The expression levels were related to the tick *elongation factor* (housekeeping gene). The successful percentage reductions of gene transcripts are shown in Figure 9.

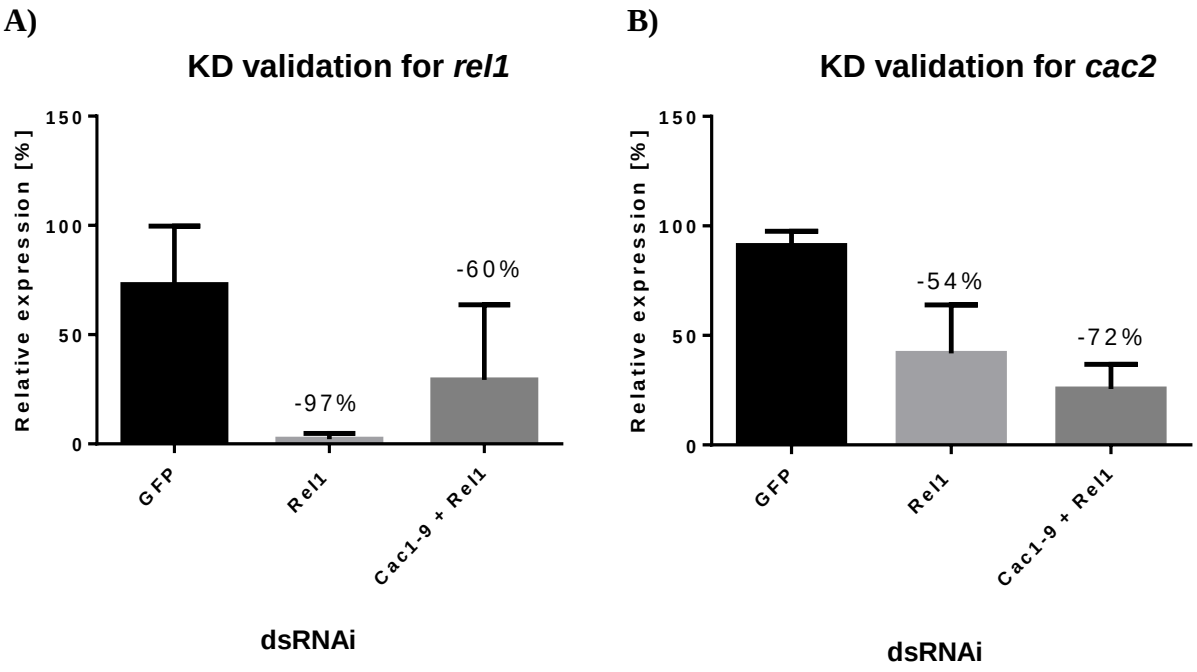
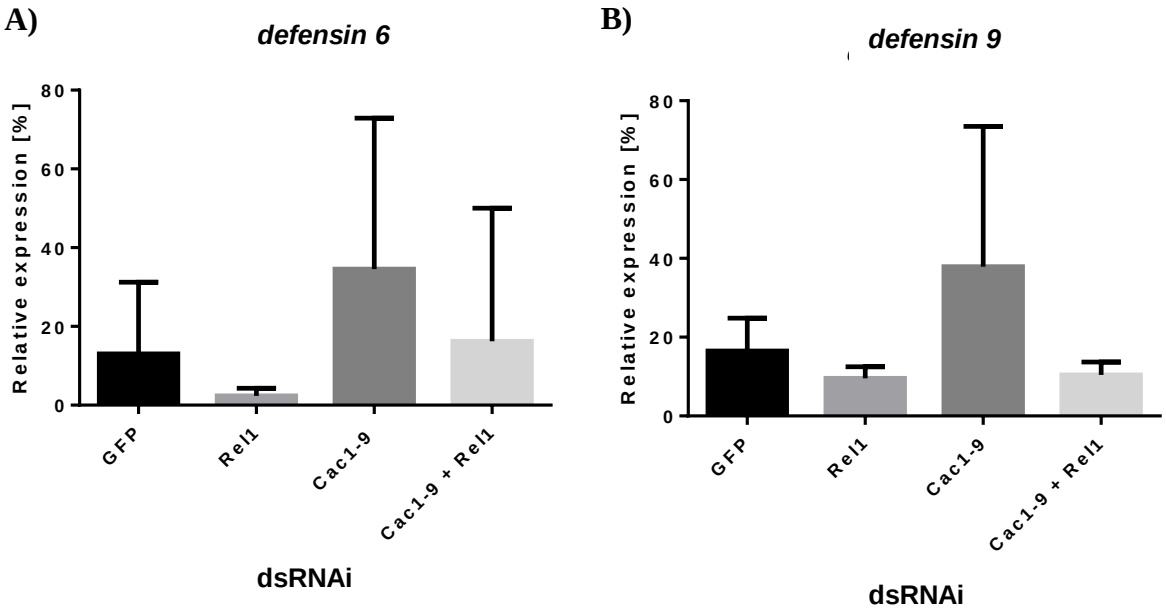


Figure 9: Expression levels after KD in male ticks analyzed by the relative qRT-PCR. The successful gene silencing for **A)** *rel1* and **B)** *cac2*. The gene silencing efficacy was compared to the control group of male ticks injected with GFP dsRNAi. The results are related to the housekeeping gene *elongation factor*. The error bars represent standard errors from five replicates. In each graph, cDNA with the highest expression was set as 100% (relative expression).

4.6. Comparison of the tick immune genes expression levels

To identify read-out genes over-expressed after stimulation the NF-κB pathway we performed a gene-specific qRT-PCR using the prepared cDNA of *I. ricinus*. The relative quantification qRT-PCR was used to determine gene expression of the tick immune genes- *def 6* (IR21+IR22), *def 9* (IR696+IR697), *c3-1* (IR139+IR140), *c3-2* (IR141+IR142), *c3-3* (IR872+IR873) and *factor b* (IR7+IR8). The expression was measured in male nymphs. The primers are shown in Table III of the Supplement. The expression levels of immune genes are shown in Figure 10.

The expression of *c3*-1 after Cactus KD was significantly different from the GFP control group. The expression can be rescued by co-injection of *rel1*. Thus, *c3*-1 could be potentially used as a read-out gene of the Toll pathway. The expression of other genes (including *defensins*) was not significantly different from the controls meaning that their expression was not regulated by the NF-κB pathway.



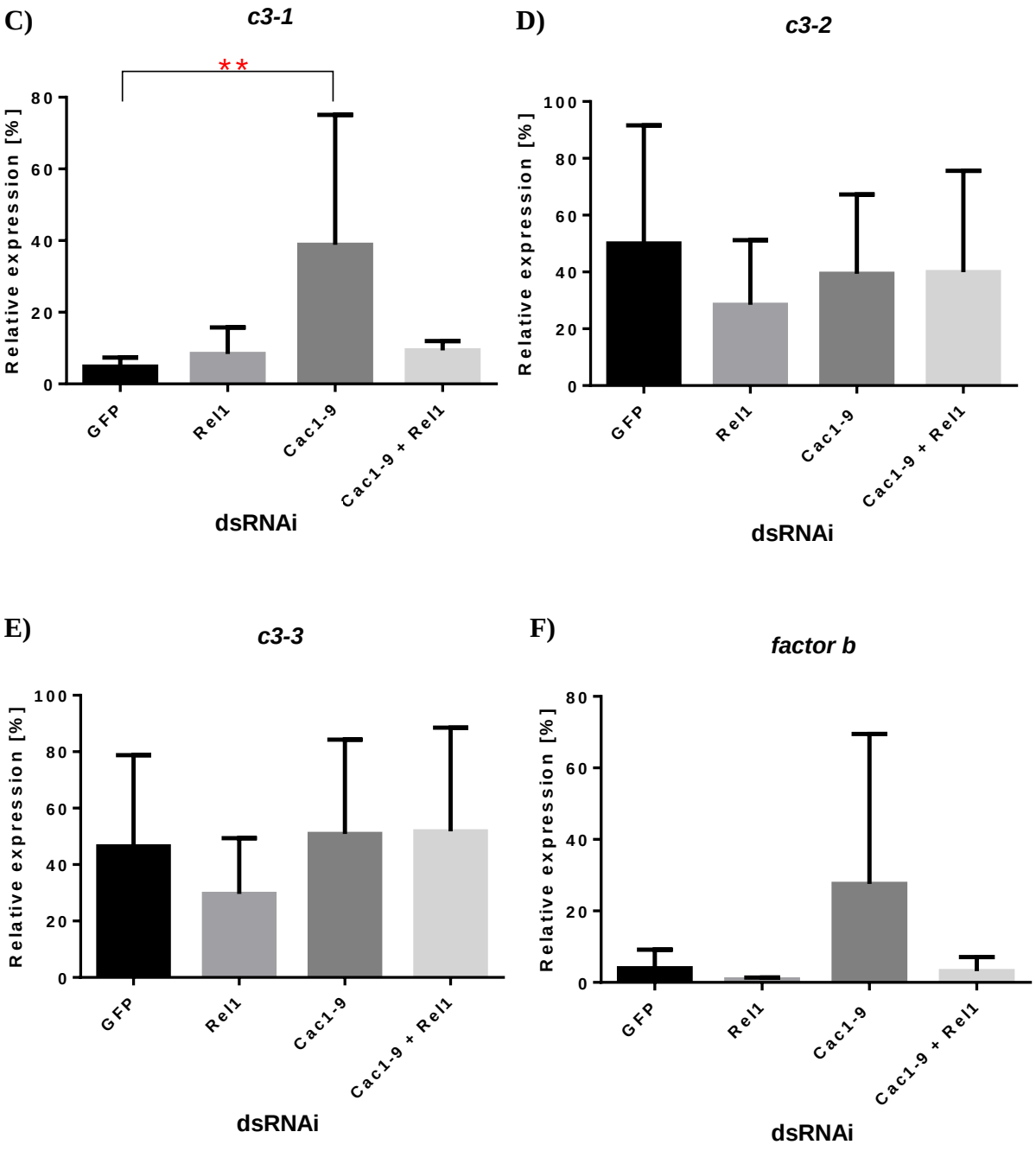


Figure 10: Relative expression of immune genes (*def 6, def 9, c3-1, c3-2, c3-3 and factor b*) after RNAi silencing of the NF-κB pathway in male ticks. The relative expression of immune genes in male ticks was calculated based on the expression of housekeeping gene *elongation factor*. GFP dsRNA served as a control. The immune gene **C)** *c3-1* was significantly up-regulated after RNAi silencing in male ticks compared to GFP. The expression was rescued by co-injection of *rel1*. The other genes **A)** *def 6*, **B)** *def 9*, **D)** *c3-2*, **E)** *c3-3*, and **F)** *factor b* were expressed, but their expression was not significantly different compared to the GFP control.

The error bars represent standard errors from five independent replicates. In each graph, cDNA with the highest expression was set as 100% (relative expression). The statistical analysis was performed by the One-way ANOVA test, where ** indicated $P<0.01$.

5. Discussion

The tick immune system, as reported for invertebrates or other arthropods, is the foremost defence against pathogens and largely impacts the ability of tick to be a competent vector for pathogens (Chávez et al., 2017). To date, the tick innate immunity is not fully described. However we know, that the tick immune system has evolved mechanisms to distinguish self from non-self and produce effectors molecules against the pathogens (Palmer & Jiggins, 2015). The genome sequencing data demonstrate that ticks possess many immune molecules such as components of invertebrate immunity- TEPs and produce antimicrobial peptides such as *defensins* (Urbanová, et al. 2015). *Defensins* fight primarily against the Gram-positive bacterial infection due to the cysteine-stabilized α-helix and β-sheet structure. Because of this structure motif, they are pivotal effector elements of the innate immunity (Wang & Zhu, 2011).

Considering that Hoffmann´s publication in 2003 with description of Toll pathway in the insect model organism *D. melanogaster* (Hoffmann, 2003) we also get closer to the recognition of the tick immune system. *D. melanogaster* transcription factor Dorsal/Dif of the Toll pathway, in ticks known as Rel1, regulates expression of defensin genes and other AMPs after Cactus degradation (Hoffmann, 2003). So, by using RNAi silencing as a powerful scientific tool, it could provide better understanding of the tick gene functions.

Purpose of this thesis was to find read-out (effector) genes of the tick immune system related to the putative Toll pathway and to determinate their expression after stimulation or inhibition of this pathway by silencing of the pathway components, Cactus and Rel1 by RNAi.

In the first part of my thesis we showed, that the tick immunity system employs 9 defensin genes found in the *I. scapularis* genome. By counting their upstream NF-κB binding sites we found different number of NF-κB BS in available upstream lengths of the *I. scapularis* genome. This finding indicates that various numbers of NF-κB BS could somehow affect the expression of defensin genes driven by the Toll pathway. After that, we performed phylogenetic analysis, which showed division of the *defensins* into three groups. After successful primer design we selected two defensin genes – *defensin 6* and *defensin 9* for the expression studies in fully-fed male nymphs after NF-κB silencing. However, these defensin genes did not show significant expression deviances after the knockdown of transcription factor Cactus.

Due to find read-out genes of the tick immune system related to the putative Toll pathway we chose for the measurement of the expression C3 family proteins and Factor B. The recent researches showed the significant expression of TEPs and Factor B after the injection of microbial models. The TEPs were up-regulated after the injection of *Escherichia coli*, *Micrococcus luteusand* and *Candida albicans*, representing Gram-negative, Gram-positive bacteria and yeast into the hemocoel of adult unfed tick females of *I. ricinus.* In this case, the effect of RNAi silencing of individual TEPs showed, that the greatest gene expression induction was observed for one of three α2-macroglobulins (*a2m1*) and one of three molecules related to C3-complement component (*c3-1*) after the injection of *C. albican* (Urbanová et al., 2015).

In this research, we measured the expression of C3 family proteins after the injection of dsRNA for Cactus KD into the hemocoel of fully-fed male nymphs of *I. ricinus.* A quantitative real-time PCR expression analysis proved the significant gene expression for *c3-1*. The result presents C3-1 as one of the effector molecule of the putative tick Toll pathway. This discovery of the C3-1 molecule could significantly improve further understanding of the tick immune system.

Another measurement of the expression after Cactus KD was performed for the immune molecule Factor B (IrC2/Bf). In the recent research, for Factor B was proven the significant up-regulation after the injection of the yeast *C. albicans* and some *Borrelia* spirochetes. strains into unfed *I. ricinus* females (Urbanová et al., 2018). Here, for Factor B was not observed any significant expression after KD of transcription factor Cactus in fully-fed male nymphs of *I. ricinus.*

It was reported, that immune molecule Factor B is mainly synthesized in cells of the tick fat body associated with the trachea trunks, referred to as the tracheal-fat body complex (TRA+FB) (Urbanová et al., 2018). In this tissue we can also find expression of some TEP genes (Urbanová et al., 2015). With this in mind, we could further do another measurement of their expression after Cactus knockdown not using tick whole body, but only certain tissue such as TRA+FB complex. After performing this step, we could hopefully find significantly different expression of some immune molecules playing an important immune role in ticks.

With this continuation we could identify other immune molecules in tick which could lead to the better understanding of the tick immune system and discovery of novel strategies for efficient control of ticks and tick-transmitted diseases.

6. Conclusion

We showed that the genome of *Ixodes scapularis* contains 9 defensin genes clustered into three groups. However, the selected *defensin 6* and *defensin 9* genes did not show any significant expression change after knockdown of the Toll pathway transcription factor Cactus in the nymphal developmental stage. The expression was also measured for C3 family thioester-containing proteins. Here, the gene *c3-1* was found to be significantly up-regulated after Cactus KD. The gene expression of *factor b* did not show any significant change. Hereby, we may say that we found the first effector gene (*c3-1*) of the putative tick Toll immune pathway. This finding paves the way for further studies of the tick immune system and its interactions with the tick-transmitted pathogens.

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8. Supplement

Table III: List of primers used in this work.

Gene ID	Name	Sequence	Product size
defensin 2	IR-692F	ACATCATTCGCTCAGAATGAAG	101 bp
	IR-693R	TGACGGTGGCATTTATCTTG	
defensin 8	IR-694F	TGGTCTGATCAGCACATCG	114bp
	IR-695R	TGATTGTGACATATATCTTGCCG	
defensin 9	IR-696F	TCACCGTACGTGATTCAACC	103 bp
	IR-697R	CTCCGCTGTGTGTGTTGTTT	
defensin 6	IR-21F	CTGGTTGCTGGAGCGTTTAT	62 bp
	IR-22R	ACGTGAGCCACTTGGTTTTC	
elongation factor	IR-524F	ACGAGGCTCTGACGGAAG	81 bp
	IR-525R	CACGACGCAACTCCTTCAC	
factor b	IR-7R	CAAAGTATGCTGAAGCCAAGG	71 bp
	IR-8F	GACGACGCCCTGCATTAG	
c3-1	IR-139F	TGGATGTGGGTCTCTTGACA	99 bp
	IR-140R	TCCGACGTGTCAGTTCGTAG	
c3-2	IR-141F	GGCTCTCGTAAGCAACCTCA	67 bp
	IR-142R	TACTGTCAAGTCGGCCATGT	
c3-3	IR-872F	ACGAGCGCATTAACGTGAC	105 bp
	IR-873R	TGAGAACCTGGAATGCCTCT	
rel1	IR-13F	GACGTGCACCTCCTCTTGA	90 bp
	IR-14R	CTCCGGATCCCTCTCGTT	
cac2	IR-35F	GCAGGAGCACCTAGTCAAGC	70 bp
	IR-36R	GTGGCCCGCATAGTCTGT	

Table IV: List of sequences used for the phylogenetic tree construction.

Gene ID	Sequence
defensin 1, ISCW024015	ATGAAGGTCCTTGCCGTCTCGCTTGCCTTTTTGCTGATCACTGGTCTGATCAGT ACATCGCTGGCTGAAAATGACGAAGGAGGAGAAAAAGAGCTTGTTCGAGTTCGC CGCACTAGTTACAACTGCCCATTCCAGAAACATAAATGCCATCGTCACTGCAAG AGCATTGGGCACATAGCAGGCTACTGCGGCGGTTTTCGGAACAGGACCTGCATC TGTGTCAAGAAATAG
defensin 2, ISCW016747	ATGAAGGTCCTTGCCGTCTCACTGGCCTTTTTGCTGATCGCTGGTCTGATCAGC ACATCATTCGCTCAGAATGAAGAGGGAGGAGAAAAAGAGCTTGTTCGAGTTCGT CGCGGTGGTTACTACTGCCCATTTTTTCAAGATAAATGCCACCGTCACTGCCTA AGCATTGGGCGAAGAGCAGGCTACTGCGGTGGTTTTCTGAAAAAGACCTGCATC TGTGTCATGAAATAG
defensin 3, ISCW024381	ATGAAGGTCCTTGCCGTCTCGCTTGCCTTTTTGCTGATCATTGGTCTGATCAGT ACATCGCTGGCTGAAAATAACAAAGAAGGGGAAAAAGACCTTGTTCGAGTTCGT AATGGTTACTACTGCCCATTCCAGCAAGACAAATGCCACCTTCACTGCATAAGC ATTGGGCGAAAAGCAGGCTACTGCGGCAATTTTCTGAAAAGAACCTGCATCTGC GTCATGAAATAG
defensin 4, ISCW005926	ATGGACACCACGGCCACCTTTAAGTTGAGCTCAACGAAGAACACAGTCCAAGTA GAACACCGCATCATCAACCTTGAAATCATGAAGGTCATTGCTGTTGCCTTGATC GCCCTTCTCGTTGCTGGAGCGTTTATGACCTCCAGCGCACAAGAAGAAGAGGAC CAAGTGGCTCACGTTCGAGTTCGACGTGGTTTTGGATGCCCCTTCGACCAAGGG GCGTGTCACAGGCACTGCCAGAGCATCGGACGACGCGGAGGTTACTGCGCGGGA ATTATCAAGCAGACGTGCACATGCTACCACAACTAA
defensin 5, ISCW005927	ATGAAGGCCGTTGCTATCGCTCTCGTCGTCATGATGATTGCCGGTCTTATCAGC ACTTCCTGCTCACAAGAAGATGATAGCCAGGTGGCTCATGTCAGAGTTCGTCGT GGGTTCGGCTGTCCTCTCAACCAGGGGGCATGTCACAACCACTGCAGAAGCATC AAGCGCCGGGGTGGTTACTGCTCGGGAATCATCAAGCAAACCTGCACCTGCTAC CGGAAGTGA
defensin 6, ISCW005928	ATGAGGGTCATTGCTGTTACCTTGATCGCCCTTCTGGTTGCTGGAGCGTTTATG ACTTCCAGCGCACAAGAGGAAGAAAACCAAGTGGCTCACGTTCGAGTTCGACGT GGTTTTGGATGTCCCTTCGACCAAGGGGCGTGTCACAGGCACTGCCAGAGCATC GGACGTCGCGGAGGTTACTGCGCGGGATTTATCAAGCAGACGTGCACATGCTAC CACAACTAG
defensin 7, ISCW011162	ATGAAGGTCGTTGGAATTGCTCTTGTGGTTCGCCTTTTCAGCTTTTCGTGCTCT CAAGGAGTCCATAGCCAGGTGCCTCACGTCAGAGTTCGTCGCGCGTTCGGCTGT CCATTCGACCAAGGAACCTGCCACAGTCACTGCAGAAGCATCAGACGCCGGGGT GAACGCTGTTCAGGATTCGCGAAACGAACTTGCACCTGTTACCAGAAATAA
defensin 8, ISCW022594	ATGAAGGTTCTTGCCGTCCCGCTTGCCTTTTTGCTGGTCGTTGGTCTGATCAGC ACATCGCTGGCCCAAAATGGGGAAGGAGGAGAAAAAGAACTCGTTCGAGTTCGT CGCGGTGGCTACTACTGCCCATTCCGGCAAGATATATGTCACAATCACTGCAGG AGCTTCGGGCGAAAAGCAGGCTACTGCGGCGGTTTTCTGAAAAAGACCTGCATC TGCGTTATGAAATAG
defensin 9, ISCW022102	ATGAATACGTCTTCATTATTCACGGTGGCGTTGATTGCCTTCGCTGGGTTCATG ACGATTCAGCTTGTTTCACCGTACGTGATTCAACCTTTCTTTGACATAGGTTTC GGTTGTCCGAAGTCTGCCCTGTCCTGCTCCCAGCAATGTAGAGAAAACAACACA CACAGCGGAGGATACTGCAACGGACCCTTCAATATTGTCTGTAGCTGCTACTGA

Table V: Weights of fully-fed nymphs and the length of feeding for GFP.

GFP		
Nymph ID	**Weight (mg)**	**Days**
1.1	4.62	3
1.2	4.44	3
1.3	4.37	3
1.4	5.00	3
1.5	4.32	3
1.6	2.78	3
1.7	2.70	3
1.8	2.71	3
1.9	2.96	3
1.10	2.51	3
1.11	2.87	3
1.12	2.90	3
1.13	3.47	3
1.14	2.77	3
1.15	2.96	3
1.16	2.74	3
1.17	5.97	4
1.18	5.25	4
1.19	5.81	4
1.20	3.51	4

Table VI: Weights of fully-fed nymphs and the length of feeding for Rel1.

Rel1		
Nymph ID	**Weight (mg)**	**Days**
2.1	4.98	3
2.2	4.39	3
2.3	6.27	3
2.4	5.38	3
2.5	2.20	3
2.6	2.95	3
2.7	3.12	3
2.8	3.02	3
2.9	3.47	3
2.10	3.06	3
2.11	3.57	3
2.12	2.72	3
2.13	2.45	3
2.14	3.50	3
2.15	2.89	3
2.16	6.22	4
2.17	5.53	4
2.18	4.84	5
2.19	4.96	5

Table VII: Weights of fully-fed nymphs and the length of feeding for Cactus 1-9.

Cactus 1-9		
Nymph ID	**Weight (mg)**	**Days**
3.1	4.61	3
3.2	5.15	3
3.3	4.28	3
3.4	4.17	3
3.5	2.14	3
3.6	2.93	3
3.7	3.11	3
3.8	2.57	3
3.9	2.48	3
3.10	3.00	3
3.11	2.25	3
3.12	2.73	3
3.13	4.73	4
3.14	4.27	4
3.15	4.75	4
3.16	4.22	5
3.17	2.94	5
3.18	3.46	5

Table VIII: Weights of fully-fed nymphs and the length of feeding for Cactus 1-9 + Rel1.

Cactus 1-9 + Rel1		
Nymph ID	**Weight (mg)**	**Days**
4.1	6.26	3
4.2	5.33	3
4.3	4.73	3
4.4	5.66	3
4.5	5.01	3
4.6	2.60	3
4.7	2.62	3
4.8	3.10	3
4.9	2.63	3
4.10	2.46	3
4.11	2.63	3
4.12	2.95	3
4.13	3.24	3
4.14	2.91	3
4.15	2.49	3
4.16	2.88	3
4.17	3.46	3
4.18	3.44	3
4.19	2.97	3
4.20	4.71	4