The transcription factor Relish controls *Anaplasma marginale* infection in the bovine tick *Rhipicephalus microplus*

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Abstract

Rhipicephalus microplus is an important biological vector of *Anaplasma marginale*, the etiological agent of bovine anaplasmosis. The knowledge of tick immune responses to control bacterial infections remains limited. In this study, we demonstrate that transcription factor Relish from the IMD signaling pathway has an important role in the control of *A. marginale* infection in ticks. We found that RNA-mediated silencing of Relish caused a significant increase in the number of *A. marginale* in the midgut and salivary glands of *R. microplus*. In addition, the IMD pathway regulates the expression of the gene that encodes the antimicrobial peptide (AMP) microplusin. Moreover, microplusin expression was up-regulated in the midgut (2x) and salivary glands (8x) of *A. marginale*-infected *R. microplus*. Therefore, it is plausible to hypothesize that microplusin may be involved in the *A. marginale* control. This study provides the first evidence of IMD signaling pathway participation on the *A. marginale* control in *R. microplus*.

Keywords: Immunity; Signaling pathways; Toll; IMD; Jak/Stat; Tick

1 Introduction

Ticks are obligate ectoparasites that feed on the blood of a broad number of vertebrate hosts. As result of the hematophagy behavior, ticks acquire a wide variety of microorganisms and transmit them to vertebrate hosts, making them important vectors of several pathogens, including protozoa, viruses, and bacteria(Sonenshine and Roe, 2014). *Rhipicephalusmicroplus* primarily anectoparasite of cattle and responsible for considerable economic losses for the livestock industry in tropical and subtropical regions. *R. microplus* is the vector of the obligate intracellular Gram-negative bacteria *Anaplasma marginale*, the etiologic agent of bovine anaplasmosis(Kocan et al., 2010). This disease causes major economic losses due to temporary infertility, abortion, increased mortality, and high treatment costs (Kocan et al., 2010). *R. microplus* acquires *A. marginale* via intake of infected blood in the acute and persistent phases of the disease (Futse et al., 2003) and become reservoirs (Kocan et al., 2010). Although transovarial transmission of *A. marginale* has not been confirmed(Esteves et al., 2015), transstadial and intrastadial transmission has been demonstrated(Aguirre et al., 1994; Samish et al., 1993). In this context, adult maleticks have a greater epidemiological importance in the transmission of *A. marginale* because of their mobility between infected and uninfected animals, allowing transmission of pathogens(Kocan et al., 2010).

As reported for invertebrate, ticks, as well as other arthropods,need to defend themselves against pathogens(Sonenshine and Hynes, 2008). For this purpose, immune mechanisms have evolved to distinguish self from non-self and produce effectors molecules against pathogens (Palmer and Jiggins, 2015). As observed in

other invertebrates, the immune system of *Drosophila melanogaster* is activated by the recognition of pathogen-associated molecular patterns (PAMPs), including for instance peptidoglycan and lipopolysaccharide, that are sensed by pattern recognition receptors (PRR) (Imler et al., 2000; Royet and Dziarski, 2007). However, the knowledge of the system PAMP-PRR in ticks is very limited (Smith and Pal 2014; Palmer and Jiggins, 2015). After pathogen recognition, distinct immune responses may occur, including phagocytosis by hemocytes. This response is likely mediated by a complement-like system involving lectins, that contain fibrinogen-related proteins (FREPS), thioester-containing proteins (TEPs), and convertase-like factors. Other tick defense mechanisms against pathogens involve the production of reactive oxygen species, proteases, protease inhibitors, and antimicrobial peptides (AMPs) (Hajdusek et al., 2013; Kopacek et al., 2010; Smith and Pal, 2014; Sonenshine and Hynes, 2008).

In the fruit fly, *Drosophila melanogaster*, transcription of AMP genes are regulated by intracellular signaling pathways such as Toll, Immune deficiency (IMD), and Janus kinase/signaling transducer activator of transcription (Jak/Stat). The Toll pathway is activated by Gram-positive bacteria and fungi, culminating in the production of the AMPs defensin, drosomycin, and metchnikowin whereas the IMD pathway primarily responds to Gram-negative bacteria infection and controls the expression of other AMPs, including cecropin, attacin, drosocin, and diptericin (Hoffmann, 2003). The Jak/Stat pathway is important in the anti-viral defense of *Drosophila* (Myllymaki and Ramet, 2014) and *Aedes aegypti* (Souza-Neto et al., 2009) and is involved in the control of the protozoan *Plasmodium* by the *Anopheles aquasalis* (Bahia et al., 2011).

There is a lack of information regarding the immune system of ticks compared with the data from other arthropods (Hajdusek et al., 2013; Kopacek et al., 2010; Smith and Pal, 2014; Sonenshine and Hynes, 2008). However, the assembly of a genomic database of *Ixodes scapularis* (Gulia-Nuss et al., 2016; Megy et al., 2012), the availability of a transcriptome database of *R. microplus* (Guerrero et al., 2005; NCBI (Biosample SAMN02463642 and Bioproject PRJNA232001)), and nucleotide sequences of other tick species deposited in Gene Bank have increased the number of tick immune signaling pathway studies. Liu et al. (2012) showed the Jak/Stat pathway in *I. scapularis* ticks has a crucial role in the control of *Anaplasma phagocytophilum*. This pathway is involved in the expression regulation of a salivary glands gene family that encodes 5.3 kDa antimicrobial peptides (Liu et al., 2012). The Jak/Stat pathway of *I. scapularis* is also involved in the expression of another AMP, Dae2, which limits the proliferation of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. Further, it was shown that the intake of cytokine IFN- γ from the host blood activates the Jak/Stat pathway, which in turn induces expression of anti-*Borrelia* AMP Dae2 (Smith et al., 2016). In addition, the Jak/Stat pathway in *I. scapularis* regulates the expression of the glycoprotein peritrofin-1, which forms the peritrophic matrix in arthropods and separates the lumen from the midgut epithelium. Silencing of Stat decreased *B. burgdorferi* colonization of *I. scapularis* probably by decreasing the level of peritrofin-1, indicating that colonization of tick midgut with this pathogen depends on integrity of the peritrophic membrane (Narasimhan et al., 2014). Recently, Shaw et al. 2017 showed that the IMD pathway controls *B. burgdorferi*, *A. phagocytophilum* and *A. marginale* colonization in *I. scapularis* and *Dermacentor andersoni* ticks. Moreover, lipids derived

from these bacteria elicit IMD pathway. However, the effectors responsible for the protection against colonization have not been identified(Shaw et al., 2017). However, only a few studies have investigated the immune defenses of *R. microplus*. Our group has purified and characterized several AMPs, including hemocidins(Belmonte et al., 2012; Cruz et al., 2010; Fogaca et al., 1999), microplusin, defensin(Fogaca et al., 2004), and ixodidin(Fogaca et al., 2006) from *R. microplus*. Moreover, we have studied the mode of action of microplusin, which occurs through its capacity to chelate copper ions needed for bacterial respiration, a copper dependent process (Silva et al., 2009). We have also shown that the hemocidin Hb33-61, different from microplusin, causes bacterial membrane permeabilization(Cruz et al., 2010). *In silico* search for sequences of the Toll, IMD, Jnk and Jak/Stat signaling pathways in *R. microplus* has recently been completed. Several components of these pathways were identified, indicating the high degree of homology of Toll, Jnk, and Jak/Stat pathways among arthropods. However, some pathway components upstream of IMD, including IMD adapter and the associated proteins Fadd, Dredd, Pirk, and Dnr1, were not found in tick databases (Rosa et al., 2016).In addition, *A. marginale* infection in BME26 cells (derived from *R. microplus* embryos) has been shown to promote a down-regulation of immune-related genes of Toll, IMD, JNK and Jak/Stat pathways (Rosa et al., 2016).

In this study, we demonstrated that transcription factor Relish from IMD pathway controls *A. marginale* infection of the midgut and salivary glands of *R. microplus*. In addition, Relish is involved in AMP microplusin regulation, which might be responsible for controlling *A. marginale* infection. In this context, understanding the tick'immune system will help to elucidate the vector-pathogen interface and provide

valuable data that will serve as the basis for other studies involving novel strategies for the control of bovine anaplasmosis.

2 Materials and Methods

2.1 Ethics Statement

All experiments involving animals were approved by the Institutional Animal Care and Use Committee and the Biosafety Committee (IACUC: 2013-66, Biosafety: B-010-13) of the University of Idaho in accordance with institutional guidelines based on the Guide for the Care and Use of Laboratory Animals developed by the U.S. National Institutes of Health (NIH).

2.2 Cattle, pathogen and tick vector

To obtain adult *R. microplus* (La Minita strain), two grams of larvae were placed into a cloth patch on the dorsal region of a spleen-intact naive Holstein calf aged 5–7 months (C48498). Prior to infestation, the calf was confirmed to be negative for *A. marginale* by nested PCR (Scoles et al., 2007) and competitive inhibition enzyme-linked immunosorbent assay (Knowles et al., 1996). After 14 days, engorged nymphs were manually removed from the calf and held in an incubator at 26° C and 92% relative humidity to molt to the adult stage. Unfed adults were sexed and males used for experiments. Four additional calves presenting the same characteristics described above were used in this study: An *A. marginale*-infected calf (C47981): this animal was inoculated with $\sim 10^8$ *A. marginale*-infected erythrocytes (St. Maries strain) as previously

described (Futse et al., 2003); and three uninfected calves (C1440, C48481, and C1439).

2.3 Double strand RNA (dsRNA) production

The genes Dorsal (GenBank: KF828755), Relish (GenBank: KF828760), and Stat (GenBank: KF828772) were selected for knockdown studies using RNA-mediated interference (RNAi). Primer sets for each gene were designed (Supplementary Table 1) using Primer3 software (Untergasser et al., 2012). The target fragments of DNA were amplified using complementary DNA (cDNA) produced from *R. microplus* midgut RNA and primers containing the T7 polymerase promoter sequence. The amplified DNA fragments were purified using a PCR product purification kit (Thermo Fisher Scientific, USA). dsRNAs were synthesized using the T7 Ribomax Express RNAi System (Promega) according to the manufacturer's instructions and cDNA as template. The purified dsRNA molecules were suspended in 0.1 mM EDTA, pH 8.0 (Thermo Fisher Scientific) (dsRNA buffer), quantified by spectrophotometry, analyzed by gel electrophoresis, and stored at -80° C.

2.4 Injection of ticks with dsRNA and *Anaplasma marginale* acquisition

Four groups of 100 freshly molted male *R. microplus* ticks were injected with 10^{11} dsRNA molecules. Dorsal (dsD), Relish (dsR) or Stat (dsS) were injected in the coxal membrane at the base of the fourth leg on the right ventral side using a 36-gauge needle and a Hamilton syringe coupled to a microprocessor-controlled UMP3 injection pump apparatus (World Precision Instruments, San Antonio, TX, USA). The control

group was injected with dsRNA buffer. After injection, male ticks were immediately transferred to patches placed on an infected calf (C47981). After 7 days of acquisition feeding during peak bacteremia, ticks were removed and transferred to an uninfected calf (C1440), where they were allowed to feed for 5 days for bacterial replication in tick salivary glands. Another group of 100 freshly molted and non-injected (n.i.) males were allowed to feed on two uninfected calves (C48481 and C1439) following the same procedure described above. The survival rate was calculated by the number of attached ticks divided by the total number of ticks placed on the calves. Tick midguts and salivary glands were dissected after 12 days of blood feeding. Each biological replicate corresponds to organs from two males. The samples were kept at -80° C.

2.5 RNA and DNA extraction and cDNA synthesis

Total RNA and genomic DNA were extracted using TRIzol[®] reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA were treated with DNase I (Ambion) to eliminate contaminating genomic DNA. RNA concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using ~ 350 ng of purified total RNA as template in a 20 µL reaction volume containing 2.5 µM oligo(dT)₁₂₋₁₈, 0.5 mM of dNTPs, 5 mM of DTT, 2 units of RNaseOUT ribonuclease, and 10 units of SuperScript III reverse transcriptase, according to the manufacturer's instructions (Thermo Fisher Scientific).

2.6 Quantitative PCR (qPCR)

Gene expression was quantified by qPCR on a CFX96 Real-Time PCR Detection System (Bio-Rad). The amplification reaction was performed in two technical replicates in a final volume of 15 µl using 10 mM of specific primers (Supplementary Table 1), 1 µl of cDNA as template and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The qPCR conditions consisted of an initial cycle at 95° C for 10 min for denaturation, followed by 55 cycles at 95° C for 15 s for denaturation, 60° C for 30 s for annealing, and 72° C for 30 s for extension. The specificity of the qPCR amplification products was determined via melting curve analysis by measuring the levels of fluorescence between 60°C and 95°C, with increments of 0.5°C. Gene expression data were analyzed by the CFX Manager Software (Bio-Rad) to obtain quantification cycle (Cq) values (Bustin et al., 2009). The 40S ribosomal protein S3a (Supplementary Table 1) was used as the endogenous reference gene. Expression data were normalized using the mean Cq values of the reference gene using the $2^{(-\Delta\Delta C(t))}$ method, as described by (Livak and Schmittgen, 2001). The results were presented as changes in relative gene expression in organs of experimental in relations to control groups.

The total number of *A. marginale* in tick midguts and salivary glands was quantified by qPCR using genomic DNA as template, specific primers (Supplementary Table 1), and a TaqMan probe that targeted *msp5*, as detailed by (Bifano et al., 2014).

2.7 Statistical Analysis

The statistical significance of the differences in the levels of gene expression between the experimental and control groups was evaluated using IBM SPSS software version 2.0. Extreme values (points that were distant more than 3 interquartile ranges

below the first quartile and above the third quartile) were identified and excluded from the analysis. The Kruskal-Wallis test was used to assess similarities between the groups evaluated, and the Mann-Whitney U test with Bonferroni correction was used to compare the median values. A p-value of less than 0.05 ($P < 0.05$) was considered statistically significant.

3 Results

3.1 *A. marginale* modulates gene expression of transcription factors and antimicrobial peptides

The innate immune response is the first line of defense against microbial infections in animals. Toll, IMD, and Jak/Stat signaling pathways represent the major signaling pathways involved in innate immunity in arthropods and participate in the activation of the expression of several immune effectors, including AMPs (Palmer and Jiggins, 2015). In the presence of the bacterium *A. marginale*, the expression of Dorsal, a Toll pathway transcription factor, is down-regulated in tick midgut and salivary glands whereas the expression of Relish and Stat was not modulated (Figure 1A and B).

The relative expression of microplusin increased in the midgut (2X) (Figure 1C) and salivary glands (8X) (Figure 1D) after infection with *A. marginale*. The genes that encode defensin and lysozyme were down-regulated in the midgut, but not in salivary glands (Figure 1C). The expression of the ixodidin gene was not modulated by *A. marginale* infection in either midgut or salivary glands (Figure 1C and D).

3.2 Relish is involved in the control of infection with *A. marginale*

In this study, we assessed whether the disruption of Toll, IMD, and Jak/Stat signaling pathways in male ticks would interfere with *A. marginale* infection in the midgut and salivary glands. For this, their respective transcription factors, Dorsal (D), Relish (R), and Stat (S) were used in RNAi experiments. The injection of dsD, dsR, or dsS promoted a significant increase in the number of *A. marginale* in both midgut and salivary glands (Figure 2A and B).

The efficiency of gene silencing of ticks injected with dsD, dsR, or dsS is shown in Figure 3. Interestingly, we observed that gene silencing was not specific for treatment with dsD and dsS, in contrast to the silencing achieved with dsR. The expression of Relish significantly decreased in the midgut and salivary glands of ticks injected with dsR (Figures 3B), and the level of silencing reached 91%. The relative expression of Relish also decreased in the midgut and salivary glands of ticks injected with dsD (48%) and dsS (45% and 69%, respectively). The fold change in expression of Dorsal reduced in midgut (45%) and salivary glands (67%) of ticks injected with dsD, although in midgut was not significant (Figure 3A). Finally, the relative expression of Stat in ticks injected with dsStat reduced in both midgut (79%) and salivary glands (93%) (Figure 3C).

The rate of tick survival of the five *A. marginale*-infected tick groups which were injected with dsRNA or dsRNA buffer after first feeding varied between 31% and 52%, whereas the survival rate in the non-injected (n.i) group was approximately 60% (Table 1). The surviving ticks were allowed to feed on a second naïve calf for more than five days. At the end of the second feeding, the survival rates were higher than after the first feeding for all groups (85-95% for n.i. group and 82-90% for injected ticks). The tick death observed in the first feeding could be due to the fragility of freshly molted adult

ticks. In addition, the injection procedure was probably the reason for the lower rate observed in the injected ticks compared to n.i. group in the first feeding.

3.3 Regulation of AMPs by Relish and Stat

The expression of microplusin was down-regulated in tick midgut and salivary glands after treatment with dsR and dsS (Figure 4A). In these two groups, Relish expression was reduced (Figure 3B) suggesting that Relish regulates the expression of microplusin (Figure 4A). In the dsD group, although Relish expression was reduced in salivary glands (Figure 3B), the expression of microplusin was not modulated (Figure 4A).

The relative expression of other antimicrobial peptides: ixodidin, defensin, and lysozyme was not affected by Relish silencing. However, as their expression was downregulated in the salivary glands of ticks treated with dsStat (Figures 4B-D), we hypothesized that the Jak/Stat pathway may regulate the expression of ixodidin, defensin, and lysozyme.

4 Discussion

The survival, development, and multiplication of *A. marginale* in the midgut cells and salivary glands of *R. microplus* depend on the molecular interactions between this bacterium and its biological vector (Zivkovic et al., 2010). These two organs are barriers against the development of *A. marginale* within the tick (Ueti et al., 2007). The midgut is the initial site of interaction between ticks and pathogens and is probably the most important organ for pathogen survival and proliferation (Hajdusek et al., 2013), whereas the salivary glands are the primary site of transmission of pathogens to the vertebrate

295 host (Reuben Kaufman, 2010). We observed that *A. marginale* induced the gene
296 expression of microplusin in these two organs (Figure 1C and D). The up-regulation of
297 microplusin was also observed in *Amblyomma sculptum* and *A. aureolatum* ticks infected
298 with *Rickettsia rickettsii* (Fogaça A. C., personal communication). In contrast, transcript
299 levels of two other AMPs, lysozyme and defensin, decreased in the midgut of infected
300 *R. microplus* ticks (Figure 1C). Furthermore, the transcription factor Dorsal was down-
301 regulated in both midgut and salivary glands (Figure 1A and B). The modulation of
302 immune-related signaling pathways and the consequent synthesis of AMPs could be
303 due to the response of ticks to control the bacterial infection or a response of the
304 bacterium to evade the vector immune defenses. Our research group has observed a
305 significant decrease in the expression of genes that compose the immune pathway in
306 the *R. microplus* cell line BME26 infected with *A. marginale*. In contrast, the challenge
307 with other pathogens, including *R. rickettsii*, *Micrococcus luteus*, *Enterobacter cloacae*,
308 and *Saccharomyces cerevisiae* induced several immune pathway components (Rosa et
309 al., 2016).

310 Understanding how Toll, IMD, and Jak/Stat signaling pathways respond to infection
311 is essential to elucidate the immune response of vectors and the interaction between
312 ticks and pathogens. Our results showed that Relish dsRNA specifically silenced its
313 target gene. As result of Relish knockdown, a higher number of *A. marginale* was
314 observed within the midgut and salivary glands (Figures 2A and B). The relative
315 expression of Relish was also significantly decreased in the experimental group injected
316 with dsD and dsS (Figure 3B) which may explain the higher bacterial load observed in
317 these groups. Because treatment with dsD and dsS did not specifically silence their

respective genes, we could not infer the importance of Toll and Jak/Stat pathways in the control of *A. marginale* by *R. microplus*.

To the best of our knowledge, the non-specific silencing of genes that encode the transcription factors involved in signaling pathways has not been previously reported in arthropod immune system literature. We raise the following hypotheses to explain our results: i. off-target effect; and ii. crosstalk of signaling pathways.

The off-target effect is a limitation of the RNA interference technique and corresponds to the recognition of random mRNAs with partial similarity with target mRNAs by small interfering RNAs (siRNAs) (Naito et al., 2005). Although we did not detect a significant similarity among Dorsal, Relish, and Stat genes using BLAST (data not shown), we cannot discard the possibility of off-target effects in the silencing experiments. To evaluate the off-target effect, Naito et al., (2005) created the dsCheck software (<http://dscheck.rnai.jp/>), which performs a scan for each siRNA formed in the RNAi process in specific genomic banks to find off-targets candidates. Unfortunately, only a few genomic banks are available and *R. microplus* is not one of them, making analysis of off-target effects for silencing assays more difficult. However, The *D. melanogaster* dsRNA sequence homologs to ones from *R. microplus* used in the RNAi assays were screened in the dsCheck software (Naito et al., 2005) against *D. melanogaster* bank and no cross-matching of the sequences related to the transcription factors Dorsal, Relish and Stat were found in off-target searches (data not shown). In the future, the availability of a *R. microplus* genomic bank will facilitate the screening of potential off-target effects.

The other raised hypothesis is the crosstalk of signaling pathways. Crosstalk is the ability of components of a particular signaling pathway to affect the components of another pathway, or a “cross-activation”, with actions on separate targets, but in the end, producing an identical action with a single effector (Calil et al., 2007). In mammalian cells, crosstalk may occur in various ways. One of them is the recognition of the same PAMP by two or more distinct receptors from independent pathways (Franchi et al., 2006; Hayashi et al., 2001). The other type of crosstalk involves the expression of the specific effector gene by different signaling pathways (Thaiss et al., 2016). Other example of crosstalk was demonstrated by Basak and colleagues (2008). These authors showed the existence of cross-regulation mechanisms of transcription factors NF- κ B in mammals during the activation of NF- κ B through the canonical or noncanonical pathways. Interestingly, the expression of RelB (noncanonical) is regulated by itself and RelA (canonical) (Basak et al., 2008).

Crosstalk between IMD and Toll pathways has also been reported in *D. melanogaster*. The IMD transcription factor Relish can be heterodimerized with Dorsal or Dif from the Toll pathway, potentiating the immune response against microorganisms (Tanji et al., 2007).

As observed in mammals and *D. melanogaster*, it is plausible to assume the crosstalk of signaling pathways in ticks, since this mechanism would be important to enhance the immune response. Besides, the absence of some components of the IMD signaling pathway strengthens the possibility of crosstalk. No sequence homologous to the intracellular protein IMD or proteins that interact with it (Fadd, Pirk, Dnr1, and caspase Dredd) were found in ticks (Palmer and Jiggins, 2015; Rosa et al., 2016).

Therefore, it is possible that Relish is activated by molecules of the Toll and Stat pathways. Recently, Shaw et al. (2017) proposed the presence of two functionally distinct IMD pathways, one in insects and another in ticks. They showed that infection-derived lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyldiacylglycerol (PODAG)) stimulate the IMD pathway of ticks, even in the absence of IMD and its adapter proteins. In this way, an active IMD pathway with missing upstream elements was demonstrated for the first time in ticks (Shaw et al., 2017).

The specific silencing of Relish by dsR suggests that microplusin is regulated by this transcription factor (Figure 4A). However, we cannot discard the possibility that microplusin is also regulated by the Jak/Stat pathway because the relative expression of microplusin decreased in ticks treated with dsS (Figure 4A), although this treatment silenced both Relish and Stat (Figure 3B and C). It was previously shown that both Dorsal and Relish can independently induce the expression of cecropin, defensin, and gambicin in *Anopheles* sp. (Garver et al., 2009; Luna et al., 2006). Therefore, it is possible that *R. microplus* male ticks control *A. marginale* infection in the midgut and salivary glands via expression of microplusin, which is regulated by IMD pathway. However, the importance of microplusin on the *Anaplasma* infection just will be corroborated upon the silencing gene.

Future studies to assess the effect of AMPs on the bacterial infection are warranted. The role played by immune components on the control of other pathogen transmitted by *R. microplus*, including *Babesia* *bovis* and *B. bigemina*, the etiological agents of

bovine babesiosis, is also warranted and may provide novel strategies for the control ticks and their pathogens.

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6 References

Aguirre, D.H., Gaido, A.B., Vinabal, A.E., De Echaide, S.T., Guglielmone, A.A., 1994. Transmission of *Anaplasma marginale* with adult *Boophilus microplus* ticks fed as nymphs on calves with different levels of rickettsaemia. *Parasite* 1, 405-407.

Bahia, A.C., Kubota, M.S., Tempone, A.J., Araujo, H.R., Guedes, B.A., Orfano, A.S., Tadei, W.P., Rios-Velasquez, C.M., Han, Y.S., Secundino, N.F., Barillas-Mury, C., Pimenta, P.F., Traub-Cseko, Y.M., 2011. The JAK-STAT pathway controls *Plasmodium vivax* load in early stages of *Anopheles aquasalis* infection. *PLoS neglected tropical diseases* 5, e1317.

Basak, S., Shih, V.F., Hoffmann, A., 2008. Generation and activation of multiple dimeric transcription factors within the NF-kappaB signaling system. *Molecular and cellular biology* 28, 3139-3150.

406 Belmonte, R., Cruz, C.E., Pires, J.R., Daffre, S., 2012. Purification and
 407 characterization of Hb 98-114: a novel hemoglobin-derived antimicrobial peptide from
 408 the midgut of *Rhipicephalus (Boophilus) microplus*. *Peptides* 37, 120-127.

409 Bifano, T.D., Ueti, M.W., Esteves, E., Reif, K.E., Braz, G.R., Scoles, G.A., Bastos,
 410 R.G., White, S.N., Daffre, S., 2014. Knockdown of the *Rhipicephalus microplus*
 411 cytochrome c oxidase subunit III gene is associated with a failure of *Anaplasma*
 412 marginale transmission. *PloS one* 9, e98614.

413 Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M.,
 414 Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T.,
 415 2009. The MIQE guidelines: minimum information for publication of quantitative real-
 416 time PCR experiments. *Clinical chemistry* 55, 611-622.

417 Calil, I., Tineli, R.A., Vicente, W.V.A., Rodrigues, A.J., Evora, P.R.B., 2007. The
 418 Concept of Crosstalk and its Implications for Cardiovascular Function and Disease. *Arq*
 419 *Bras Cardiol* 88, e25-30.

420 Cruz, C.E., Fogaca, A.C., Nakayasu, E.S., Angeli, C.B., Belmonte, R., Almeida, I.C.,
 421 Miranda, A., Miranda, M.T., Tanaka, A.S., Braz, G.R., Craik, C.S., Schneider, E.,
 422 Caffrey, C.R., Daffre, S., 2010. Characterization of proteinases from the midgut of
 423 *Rhipicephalus (Boophilus) microplus* involved in the generation of antimicrobial
 424 peptides. *Parasites & vectors* 3, 63.

425 Esteves, E., Pohl, P.C., Klafke, G.M., Reck, J., Fogaca, A.C., Martins, J.R., Daffre,
 426 S., 2015. Low temperature affects cattle tick reproduction but does not lead to
 427 transovarial transmission of *Anaplasma marginale*. *Veterinary parasitology* 214, 322-
 428 326.

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

432 Fogaca, A.C., da Silva, P.I., Jr., Miranda, M.T., Bianchi, A.G., Miranda, A., Ribolla,
 433 P.E., Daffre, S., 1999. Antimicrobial activity of a bovine hemoglobin fragment in the tick
 434 *Boophilus microplus*. *The Journal of biological chemistry* 274, 25330-25334.

435 Fogaca, A.C., Lorenzini, D.M., Kaku, L.M., Esteves, E., Bulet, P., Daffre, S., 2004.
 436 Cysteine-rich antimicrobial peptides of the cattle tick *Boophilus microplus*: isolation,
 437 structural characterization and tissue expression profile. *Developmental and*
 438 *comparative immunology* 28, 191-200.

439 Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.D., Ozoren, N., Jagirdar, R.,
 440 Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E.P., Nunez, G., 2006.
 441 Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in
 442 salmonella-infected macrophages. *Nature immunology* 7, 576-582.

443 Futse, J.E., Ueti, M.W., Knowles, D.P., Jr., Palmer, G.H., 2003. Transmission of
 444 *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the
 445 absence of vector-pathogen interaction. *Journal of clinical microbiology* 41, 3829-3834.

446 Garver, L.S., Dong, Y., Dimopoulos, G., 2009. Caspar controls resistance to
 447 *Plasmodium falciparum* in diverse anopheline species. *PLoS pathogens* 5, e1000335.

448 Guerrero, F.D., Miller, R.J., Rousseau, M.E., Sunkara, S., Quackenbush, J., Lee,
 449 Y., Nene, V., 2005. BmiGI: a database of cDNAs expressed in *Boophilus microplus*, the
 450 tropical/southern cattle tick. *Insect biochemistry and molecular biology* 35, 585-595.

Gulia-Nuss, M., Nuss, A.B., Meyer, J.M., Sonenshine, D.E., Roe, R.M., Waterhouse, R.M., Sattelle, D.B., de la Fuente, J., Ribeiro, J.M., Megy, K., Thimmapuram, J., Miller, J.R., Walenz, B.P., Koren, S., Hostetler, J.B., Thiagarajan, M., Joardar, V.S., Hannick, L.I., Bidwell, S., Hammond, M.P., Young, S., Zeng, Q., Abrudan, J.L., Almeida, F.C., Ayllon, N., Bhide, K., Bissinger, B.W., Bonzon-Kulichenko, E., Buckingham, S.D., Caffrey, D.R., Caimano, M.J., Croset, V., Driscoll, T., Gilbert, D., Gillespie, J.J., Giraldo-Calderon, G.I., Grabowski, J.M., Jiang, D., Khalil, S.M., Kim, D., Kocan, K.M., Koci, J., Kuhn, R.J., Kurtti, T.J., Lees, K., Lang, E.G., Kennedy, R.C., Kwon, H., Perera, R., Qi, Y., Radolf, J.D., Sakamoto, J.M., Sanchez-Gracia, A., Severo, M.S., Silverman, N., Simo, L., Tojo, M., Tornador, C., Van Zee, J.P., Vazquez, J., Vieira, F.G., Villar, M., Wespiser, A.R., Yang, Y., Zhu, J., Arensburger, P., Pietrantonio, P.V., Barker, S.C., Shao, R., Zdobnov, E.M., Hauser, F., Grimmekhuijzen, C.J., Park, Y., Rozas, J., Benton, R., Pedra, J.H., Nelson, D.R., Unger, M.F., Tubio, J.M., Tu, Z., Robertson, H.M., Shumway, M., Sutton, G., Wortman, J.R., Lawson, D., Wikel, S.K., Nene, V.M., Fraser, C.M., Collins, F.H., Birren, B., Nelson, K.E., Caler, E., Hill, C.A., 2016. Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease. *Nature communications* 7, 10507.

Hajdusek, O., Sima, R., Ayllon, N., Jalovecka, M., Perner, J., de la Fuente, J., Kopacek, P., 2013. Interaction of the tick immune system with transmitted pathogens. *Frontiers in cellular and infection microbiology* 3, 26.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., Aderem, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099-1103.

474 Hoffmann, J.A., 2003. The immune response of *Drosophila*. *Nature* 426, 33-38.

475 Imler, J.L., Tauszig, S., Jouanguy, E., Forestier, C., Hoffmann, J.A., 2000. LPS-
 476 induced immune response in *Drosophila*. *Journal of endotoxin research* 6, 459-462.

477 Knowles, D., Torioni de Echaide, S., Palmer, G., McGuire, T., Stiller, D., McElwain,
 478 T., 1996. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and
 479 erythrocyte stages identifies persistently infected cattle. *Journal of clinical microbiology*
 480 34, 2225-2230.

481 Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The
 482 natural history of *Anaplasma marginale*. *Veterinary parasitology* 167, 95-107.

483 Kopacek, P., Hajdusek, O., Buresova, V., Daffre, S., 2010. Tick innate immunity.
 484 *Advances in experimental medicine and biology* 708, 137-162.

485 Liu, L., Dai, J., Zhao, Y.O., Narasimhan, S., Yang, Y., Zhang, L., Fikrig, E., 2012.
 486 *Ixodes scapularis* JAK-STAT pathway regulates tick antimicrobial peptides, thereby
 487 controlling the agent of human granulocytic anaplasmosis. *The Journal of infectious*
 488 *diseases* 206, 1233-1241.

489 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using
 490 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.

491 Luna, C., Hoa, N.T., Lin, H., Zhang, L., Nguyen, H.L., Kanzok, S.M., Zheng, L.,
 492 2006. Expression of immune responsive genes in cell lines from two different
 493 Anopheline species. *Insect molecular biology* 15, 721-729.

494 Megy, K., Emrich, S.J., Lawson, D., Campbell, D., Dialynas, E., Hughes, D.S.,
 495 Koscielny, G., Louis, C., Maccallum, R.M., Redmond, S.N., Sheehan, A., Topalis, P.,

496 Wilson, D., VectorBase, C., 2012. VectorBase: improvements to a bioinformatics
 497 resource for invertebrate vector genomics. *Nucleic acids research* 40, D729-734.

498 Myllymaki, H., Ramet, M., 2014. JAK/STAT pathway in *Drosophila* immunity.
 499 *Scandinavian journal of immunology* 79, 377-385.

500 Naito, Y., Yamada, T., Matsumiya, T., Ui-Tei, K., Saigo, K., Morishita, S., 2005.
 501 dsCheck: highly sensitive off-target search software for double-stranded RNA-mediated
 502 RNA interference. *Nucleic acids research* 33, W589-591.

503 Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-
 504 Epstein, R., Deponete, K., Fish, D., Fikrig, E., 2014. Gut microbiota of the tick vector
 505 *Ixodes scapularis* modulate colonization of the Lyme disease spirochete. *Cell host &*
 506 *microbe* 15, 58-71.

507 Palmer, W.J., Jiggins, F.M., 2015. Comparative Genomics Reveals the Origins and
 508 Diversity of Arthropod Immune Systems. *Molecular biology and evolution* 32, 2111-
 509 2129.

510 Reuben Kaufman, W., 2010. Ticks: physiological aspects with implications for
 511 pathogen transmission. *Ticks and tick-borne diseases* 1, 11-22.

512 Rosa, R.D., Capelli-Peixoto, J., Mesquita, R.D., Kalil, S.P., Pohl, P.C., Braz, G.R.,
 513 Fogaca, A.C., Daffre, S., 2016. Exploring the immune signalling pathway-related genes
 514 of the cattle tick *Rhipicephalus microplus*: From molecular characterization to
 515 transcriptional profile upon microbial challenge. *Developmental and comparative*
 516 *immunology* 59, 1-14.

517 Royet, J., Dziarski, R., 2007. Peptidoglycan recognition proteins: pleiotropic sensors
 518 and effectors of antimicrobial defences. *Nature reviews. Microbiology* 5, 264-277.

519 Samish, M., Pipano, E., Hadani, A., 1993. Intrastadial and interstadial transmission
520 of *Anaplasma marginale* by *Boophilus annulatus* ticks in cattle. American journal of
521 veterinary research 54, 411-414.

522 Scoles, G.A., Ueti, M.W., Noh, S.M., Knowles, D.P., Palmer, G.H., 2007.
523 Conservation of transmission phenotype of *Anaplasma marginale* (Rickettsiales:
524 Anaplasmataceae) strains among *Dermacentor* and *Rhipicephalus* ticks (Acari:
525 Ixodidae). Journal of medical entomology 44, 484-491.

526 Shaw, D.K., Wang, X., Brown, L.J., Chavez, A.S., Reif, K.E., Smith, A.A., Scott,
527 A.J., McClure, E.E., Boradia, V.M., Hammond, H.L., Sundberg, E.J., Snyder, G.A., Liu,
528 L., DePonte, K., Villar, M., Ueti, M.W., de la Fuente, J., Ernst, R.K., Pal, U., Fikrig, E.,
529 Pedra, J.H., 2017. Infection-derived lipids elicit an immune deficiency circuit in
530 arthropods. Nature communications 8, 14401.

531 Silva, F.D., Rezende, C.A., Rossi, D.C., Esteves, E., Dyszy, F.H., Schreier, S.,
532 Gueiros-Filho, F., Campos, C.B., Pires, J.R., Daffre, S., 2009. Structure and mode of
533 action of microplusin, a copper II-chelating antimicrobial peptide from the cattle tick
534 *Rhipicephalus* (*Boophilus*) *microplus*. The Journal of biological chemistry 284, 34735-
535 34746.

536 Smith, A.A., Navasa, N., Yang, X., Wilder, C.N., Buyuktanir, O., Marques, A.,
537 Anguita, J., Pal, U., 2016. Cross-Species Interferon Signaling Boosts Microbicidal
538 Activity within the Tick Vector. Cell host & microbe 20, 91-98.

539 Smith, A.A., Pal, U., 2014. Immunity-related genes in *Ixodes scapularis*--
540 perspectives from genome information. Frontiers in cellular and infection microbiology 4,
541 116.

542 Sonenshine, D.E., Hynes, W.L., 2008. Molecular characterization and related
 543 aspects of the innate immune response in ticks. *Frontiers in bioscience : a journal and*
 544 *virtual library* 13, 7046-7063.

545 Sonenshine, D.E., Roe, R.M., 2014. Overview: Ticks, People, and Animals, in:
 546 Sonenshine, D.E., Roe, R.M. (Eds.), *Biology of Ticks*. Oxford University Press, New
 547 York, pp. 3-16.

548 Souza-Neto, J.A., Sim, S., Dimopoulos, G., 2009. An evolutionary conserved
 549 function of the JAK-STAT pathway in anti-dengue defense. *Proceedings of the National*
 550 *Academy of Sciences of the United States of America* 106, 17841-17846.

551 Tanji, T., Hu, X., Weber, A.N., Ip, Y.T., 2007. Toll and IMD pathways synergistically
 552 activate an innate immune response in *Drosophila melanogaster*. *Molecular and cellular*
 553 *biology* 27, 4578-4588.

554 Thaiss, C.A., Levy, M., Itav, S., Elinav, E., 2016. Integration of Innate Immune
 555 Signaling. *Trends in immunology* 37, 84-101.

556 Ueti, M.W., Reagan, J.O., Jr., Knowles, D.P., Jr., Scoles, G.A., Shkap, V., Palmer,
 557 G.H., 2007. Identification of midgut and salivary glands as specific and distinct barriers
 558 to efficient tick-borne transmission of *Anaplasma marginale*. *Infection and immunity* 75,
 559 2959-2964.

560 Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M.,
 561 Rozen, S.G., 2012. Primer3--new capabilities and interfaces. *Nucleic acids research* 40,
 562 e115.

563 Zivkovic, Z., Esteves, E., Almazan, C., Daffre, S., Nijhof, A.M., Kocan, K.M.,
 564 Jongejan, F., de la Fuente, J., 2010. Differential expression of genes in salivary glands

of male *Rhipicephalus (Boophilus) microplus* in response to infection with *Anaplasma marginale*. BMC genomics 11, 186.

7 Figure legends

Fig 1. Tick gene expression modulation by *Anaplasma marginale* infection. The gene expression of the transcription factors Dorsal, Relish, and Stat in the (A) midgut and (B) salivary glands and the AMPs microplusin, ixodidin, defensin, and lysozyme in the (C) midgut and (D) salivary glands from males fed for 12 days on either in uninfected or *A. marginale*-infected calves was assessed by RT-qPCR. Threshold values were normalized according to the Cq of the reference gene (S3a ribosomal). The data represent the median of 16 – 26 pools of two midguts or salivary glands pairs. * P <0.05, ** P <0.01 and *** P <0.001.

Fig 2. *Anaplasma marginale* loads on dsRNA-injected tick in the (A) midgut and (B) salivary glands. Bacteria number was determined by qPCR using specific primers and a TaqMan probe for msp5 gene. Data represent the median of 6 – 13 pools of 2 midguts or salivary glands pairs. * P <0.05 and ** P <0.01. dsD: dsRNA Dorsal, dsR: dsRNA Relish, dsS: dsRNA Stat and Mock: ticks injected with dsRNA buffer.

Fig 3. Gene knockdown efficiency. The gene expression of the transcription factors (A) Dorsal, (B) Relish and (C) Stat genes in midgut and salivary glands from dsRNA-injected ticks (dsD, dsR, and dsS) and dsRNA buffer-injected ticks (Mock) was evaluated by RT-qPCR. Threshold values were normalized according to the Cq of the reference gene

(S3a ribosomal). The data represent the median of 11 – 22 pools of two midguts or salivary glands.* P <0.05, ** P <0.01 and *** P <0.001. dsD: dsRNA Dorsal, dsR: dsRNA Relish, dsS: dsRNA Stat.

Fig 4. Gene expression of the AMPs in ticks injected with dsRNA from transcription factors. The gene expression of the AMPs (A) microplusin, (B) ixodidin, (C) defensin, and (D) lysozyme in the midgut and salivary glands from dsRNA-injected ticks (dsD, dsR, and dsS) and dsRNA buffer-injected ticks (Mock) was evaluated by RT-qPCR. Threshold values were normalized according to the Cq of the reference gene (S3a ribosomal). The data represent the median of 10 – 22 pools of two midguts or salivary glands. * P <0.05, ** P <0.01 and *** P <0.001. dsD: dsRNA Dorsal, dsR: dsRNA Relish, dsS: dsRNA Stat.

602 Figure 1

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608 Figure 3

Table 1: Survival rate of ticks.

Injected ticks		
dsRNA	First feeding	Second feeding
dsD	31.0% (31 ^a /100 ^b)	83.8% (26 ^a /31 ^b)
dsR	33.0% (33 ^a /100 ^b)	81.8% (27 ^a /33 ^b)
dsS	52.0% (52 ^a /100 ^b)	90.4% (47 ^a /52 ^b)
dsRNA buffer (Mock)	51.0% (51 ^a /100 ^b)	86.2% (44 ^a /51 ^b)
Non-injected ticks		
Uninfected ticks	60.0% (60 ^a /100 ^b)	85.0% (51 ^a /60 ^b)
<i>A.marginale</i> -infected ticks	59.0% (59 ^a /100 ^b)	94.9% (56 ^a /59 ^b)

Survival rates number of attached ticks (a) /number of ticks placed in calves (b).
dsD: dsRNA Dorsal, dsR: dsRNA Relish, dsS: dsRNA Stat.